Impact of Physical Activity on Immune Function and Inflammation in the Elderly

by

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Abstract

Physiological ageing is accompanied with an increase in systemic inflammatory mediators (inflammageing), a functional decline of the immune system (immunesenescence), altered endocrine function (adrenopause) and reduced physical activity which predisposes the elderly to increased risk of disease. Little is known about the interplay between physical activity, inflammageing, adrenopause and immunesenescence and what impact interventions may have in the elderly. This thesis identified the consequences of inflammageing and its association with immunesenescence and the impact physical activity plays on limiting the severity of inflammageing.

Cytomegalovirus drives immunesenescence but was not associated with inflammageing. Instead inflammageing was associated with reduced physical activity and increased body fat. Furthermore, inflammageing and adrenopause was associated with increased frailty and mortality risk over a ten-year period.

Accelerometer defined physical activity levels in healthy elders revealed a reduced inflammatory profile and improved neutrophil migration towards interleukin-8. Acute exercise revealed an enhanced inflammatory profile indicative of positive tissue adaptation. Furthermore there was a reduced ratio of cortisol to dehydroepiandrosterone sulphate which was accompanied by enhanced neutrophil and monocyte bactericidal function. Subsequently ten-weeks of high-intensity interval training, which was more than half the time commitment of regular aerobic training, revealed similar reduced inflammation and improved neutrophil and monocyte bactericidal capacity.

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To my wife Kim and my son Ruaridh

I love you both

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Chapter 1: Introduction

1.1 The Ageing Population

Life expectancy in the developed world is increasing and has done so over the past 150 years [1]. Advances in sanitation, medical care and socio-economic circumstances mean more people are now living longer than at any time in history. Current predictions suggest that by 2035 nearly one-quarter of the UK population will be aged sixty-five years or over and within those the number of over 85 year olds will have doubled [2]. On a global scale it is predicted that those over sixty years old will account for twenty-one percent of the population and equate to around two billion people [3].

Recently it has been recognised that although lifespan is increasing there are clear disparities between this and time spent towards the end of life in a healthy condition (Healthspan) [4]. Currently in the UK, women and men can expect to spend the last eleven and eight years, respectively, in poor health. The impact of these unhealthy years is obvious in terms of a significant increase in health care costs as well as reduced quality of life in the ageing population [5]. Subsequently the need to improve healthspan by identification of risks to health in old age earlier and using economic, personalised non-invasive interventions is paramount.

A common biological definition of ageing is: "*Increasing frailty of an organism with time that reduces the ability to deal with stress, resulting in increased chance of disease and death*". Frailty is a syndrome associated with the elderly and confers a reduced capacity of a number of integral psychological, sociological, medical and physiological components [6-8]. Notably the clinical definition of frailty includes decrements in musculoskeletal function assessed by limitations to walking speed, ability to rise from a chair unaided and balance control, with compromised cognitive

capacity affecting the ability to carry out activities of daily living [7, 9, 10] . Broader definitions of age-related frailty extend to loss of cardiovascular and immune function [11, 12].

This reduced functional capacity ultimately leads to the increased prevalence of adverse health outcomes due to an inability to effectively deal with or provide resistance against cumulative stressors and ensuing loss of homeostasis [8]. As individuals become frail it is apparent that they may present no life-threatening illnesses, thus frailty does not always include frank disease [6]. Furthermore, apparently healthy elders may not register as frail, yet have underlying significant health conditions which would suggest they are not in a healthy state. Subsequently the 'Healthy Ageing Phenotype' (HAP) has recently been proposed in order to bring together the multiple disciplines of gerontology and incorporate frailty and disease in a comprehensive definition of healthy ageing, Figure 1.1 [13]. Together, definitions of healthy ageing and frailty are becoming important tools for gerontological science and medicine which allows for better understanding of how to treat poor healthspan and intervene before health deteriorates beyond recovery.

Although ageing research includes extending lifespan this tends to be in animal models [14, 15]. Human ageing research instead is focused on improving the health status of the elderly and improving their healthspan. In order to accomplish this it is necessary to gain insight into the risk and progression of disease as well as which factors can influence disease and promote healthspan. The HAP suggests that the underlying cause of age-related chronic disease states is due to a combination of genetic predispositions and lifestyle influences such as stress, smoking, alcohol and physical activity [13]. Importantly, there are factors associated with ageing and chronic disease which may be susceptible to interventions. Low grade chronic

systemic inflammation is one such factor which not only provides relatively accurate predictive measurements of disease but is also directly associated with behavioural lifestyle choices which are modifiable [16].

In light of this the general aims of this thesis are two-fold. Firstly; assess the causes and consequences of systemic inflammation in elderly individuals. This will entail focussing on such things as lifestyle choices indicative of physical activity levels. Furthermore, focus will include assessment of latent viral infections which are capable of driving the immune system towards a dysregulated functional state, which increases risk of morbidity and mortality. Secondly; this thesis aims to assess the effects of physical activity in older individuals on both immune function and systemic inflammation.

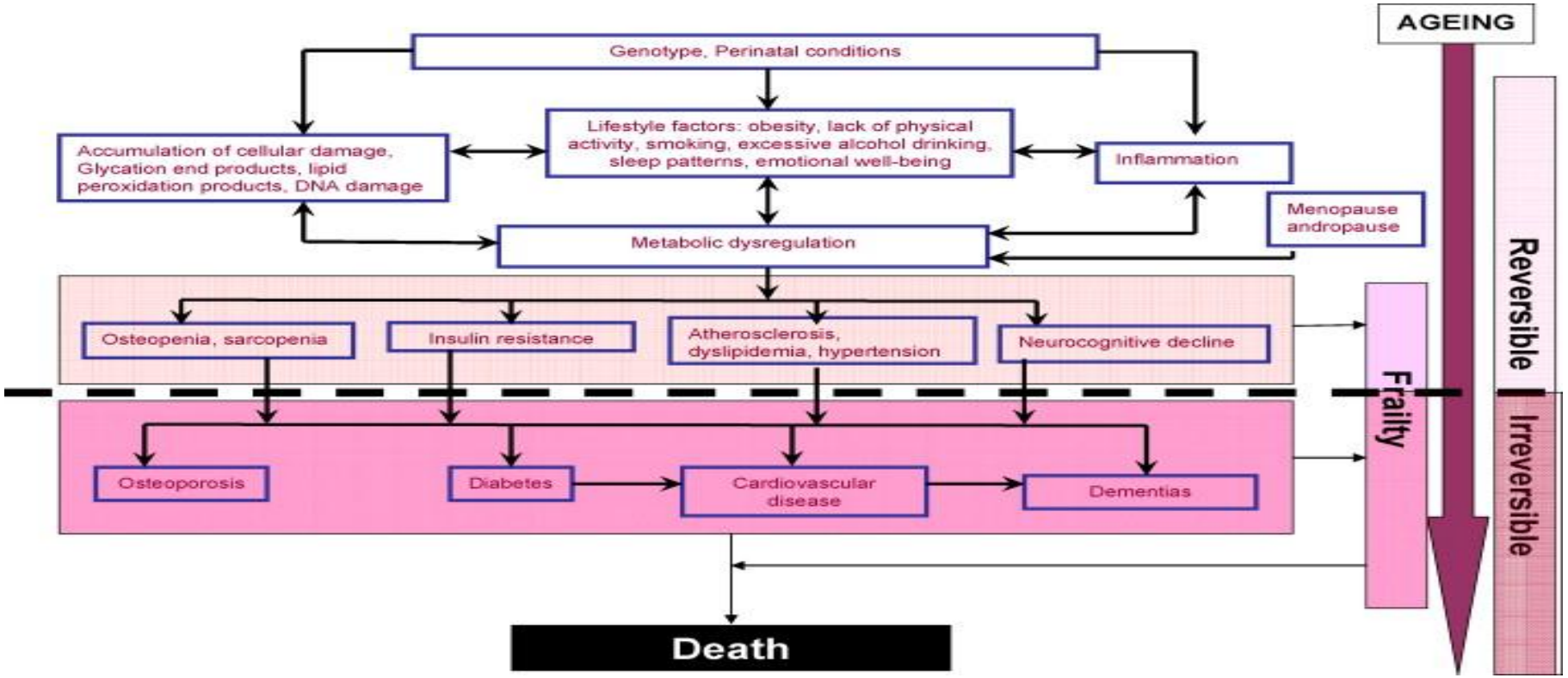

Fig. 1.1: The Healthy Ageing Phenotype. This figure highlights the common underlying pathways of the major chronic conditions which are lifestyle and age dependent. In particular highlighting the vicious circle of pathways which leads first to conditions that may be reversible by modifying lifestyle factors before irreversible damage is done where management of these conditions is the only option. This irreversible situation is then associated with increased frailty and mortality in the elderly. Taken directly from Franco et al. [13].

1.2 Low Grade Chronic Systemic Inflammation (Inflammageing)

1.2.1 Inflammageing and health

Physiological ageing is associated by a chronic sub-clinical systemic inflammatory state, termed inflammageing [17], characterised by elevated levels of serum pro-inflammatory mediators such as interleukin (IL) 6, IL-1β and TNFα and acute phase proteins such as C-reactive protein (CRP). Further, the levels of mediators that counteract the inflammatory state, such as IL-10, are reduced with age compounding the inability to maintain immune homoeostasis [18]. Importantly, chronic low-grade inflammation is now accepted as a key pathogenic factor in the development or worsening of several age-related pathologies including cardiovascular disease [19], Alzheimer's disease, sarcopenia, dementia and type-2 diabetes [20]. Furthermore inflammageing has been recognised as an independent risk factor for frailty and all-cause mortality [16]. Table 1.1 highlights the key age-related inflammatory mediators, their cellular sources and relations with disease. Interestingly, studies in centenarians show that these extremely long-lived individuals maintain the cytokine profile of younger adults and do not develop inflammageing [21].

Epidemiological and experimental studies have identified a number of biological markers which are associated with inflammation which predict both morbidity and mortality in the elderly . Increased concentrations of circulating inflammatory cytokines such as TNFα, IL-6, IL-1 and their cognate receptors function in a complex network where the role of one cytokine may be modified by another. It has become clear recently hyper-production of these cytokines are strong independent risk factors of infection, disease and mortality. Furthermore increased cytokine

production results in the increase of other inflammatory biological mediators such as chemokines like MCP-1, IL-8 and MIP-1α and acute phase proteins such as CRP.

What is clear is that with age the functional balance of inflammatory versus anti-inflammatory mediators is weighted towards an inflammatory state. IL-10 is the typical anti-inflammatory type 2 cytokine which acts to dampen and resolve inflammation by directing its actions against potent type 1 (Th1) cytokines such as TNFα. Although some have suggested IL-10 may not be reduced in the elderly the ratio of TNFα to IL-10 is often markedly increased. Mooradian and colleagues assessed the impact of TNFα concentrations of elderly nursing home residents (average age 89 years) and showed it was associated with significant mortality after only 4 months [22]. In this study the comparison group showed no detectable TNFα, highlighting the difficulty in measuring these cytokines which are usually only present with underlying disease.

TNFα however is linearly associated with both IL-6 and CRP concentrations, both contributors to the inflammatory state [23]. IL-6 stimulates the hepatic production of CRP, an acute phase protein released primarily in response to infection and aids the immune response by binding to pathogens for immune recognition [24]. Ageing has been shown to increase CRP concentrations and has furthermore been associated with increased risk of metabolic disorders such as insulin resistance and diabetes [25, 26]. Additionally when in combination with IL-6 and IL-1 receptor antagonist (RA), CRP is associated with low strength and poor physical performance. Cesari and colleagues assessed 1020 individuals from Italy as part of the InCHIANTI study and found after adjustment for covariates IL-6 and CRP levels associated with low

grip strength and self-reported physical performance [27], both elements of the frailty index. Similar findings have been found by several groups and highlight the association with inflammatory imbalance and frailty. Chronic low-grade IL-6 production has been suggested to inhibit the production of insulin-like growth factor 1 (IGF-1), a potent anabolic growth factor even in the elderly, and can cause reduced muscle mass and lead to poor physical functioning [28, 29]. Not only has increased IL-6 and reduced IGF-1 been associated with reduced muscle function and mobility but in combination with TNFα was associated with increased mortality [30].

Inflammatory cytokine production is accompanied by an anti-inflammatory response which is designed to aid resolution of inflammation. The association of IL-10 with frailty was not significant in the InCHIANTI study, but this cytokine is now being included in more longitudinal studies and may turn out to be an influence on ageing.
The predictive value of inflammatory/anti-inflammatory biomarkers compared to traditional risk factors such as cholesterol and blood pressure is gaining more acceptance in gerontological research and medicine. It is recognised that there is a reduced validity with increasing age of such traditional physiological biomarkers, especially in cardiovascular disease assessment. Therefore the combination of assessing inflammatory and anti-inflammatory biomarkers with traditional clinical checks will enhance the early detection of frailty and disease risk in the elderly. Furthermore understanding the causes of chronic systemic inflammation could lead to mechanisms which can be targeted and treated before long term damage to health occurs.

Table 1.1: Inflammatory mediators of the elderly. Typical cytokines, chemokines and growth factors measured in the elderly, their source and place of activity, adapted from De Martinis *et al. [31]*.

Inflammatory Biomarker	Cellular Source	Target	Actions	Effect of Age on Production	Associations with age-related disease
IL-1β	Macrophages Dendritic Cells Endothelia Epithelia Stromal	Endothelium, hepatocytes, hypothalamus	Enhances immune response, fever, sickness, acute phase protein release	Increased	Frailty, autoimmune disease, emphysema
IL-2	T-cells	T-cell, B-cell, NK-cell	Activation, proliferation	Decreased	Autoimmune disorders
IL-6	Macrophages Dendritic Cells T-cells Endothelia Adipocytes Stromal	Liver, B-cells	Acute phase protein release, cellular proliferation	Increased	Insulin resistance, Alzheimers, Cerebrovascular Disease
IL-8	Monocytes, Macrophages, Stroma, Endothelia	Neutrophils, T-cells	Activation , chemotaxis	Increased	Atherosclerosis, Cancer
IL-10	Macrophages, T-cells	T-cells, mast cells, B-cells	Inhibits cytokine release, costimulator for proliferation	Decreased	Cancer, autoimmune disorders
IL-17	T-cells	Nuetrophils, monocytes, stroma, endothelia	Enhances immune responses, promotes a pro-inflammatory environment	Increased	Autoimmune disorders
TNFα	Macrophages, T-cells, Monocytes, Neutrophils, NK-cells	Most cells	Celll Death, Activation	Increased	Sarcopenia, Anaemia, Osteoperosis
CRP	Hepatocytes	Most cells	Enhances immune responses, promotes a pro-inflammatory environment	Increased	Cardiovascular disease
IFNγ	T-cells, NK-cells	All cells except erythrocytes	Enhances immune responses, antiviral, promotes a pro-inflammatory environment	Increased	Autoimmune and Autoinflammatory disorders
GM-CSF	T-cells, Macrophages, Endothelia, Stroma	All myeloid cells and endothelia	Growth factor, chemoattractant	Decreased	Rheumatoid Arthritis
IGF-1	Stroma, Endothelia	Adipocytes, muscle cells, epithelia, stroma	Growth Factor, Proliferation, Mitogenic	Decreased	Frailty, Sarcopenia, Osteoperosis, Cancer

IL (interleukin), TNFα (Tumour necrosis factor – α), CRP (C - reactive protein), IFN-γ (Interferon-γ), GM-CSF (Granulocyte/monocyte – colony stimulating factor), IGF-1 (Insulin like growth factor-1), NK (natural killer).

1.2.2 Causes of Inflammageing

The exact causes of inflammageing are variable between individuals and result from a combination of factors. In particular accumulation of adipose tissue and a dysfunctional immune system are suggested to be two primary and interlinked mediators of inflammageing [32]. Furthermore, the interactions with a dysregulated endocrine function, immunesenescence and obesity have led to suggestions that the endocrine system may play an integral role in the severity of systemic inflammation. Senescent cells are also pro-inflammatory and may make a contribution [33].

1.2.2.1 Adipose Tissue Derived Inflammation

Obesity and its associated poor health conditions have had an enormous impact on the health of the global population. Along with ageing, obesity is now considered a major worldwide health issue and is positively correlated with increasing age [34]. As with ageing, obesity predisposes individuals to increased risk of a number of diseases including type-2 diabetes, atherosclerosis, liver disease and certain types of cancer. Recently, obesity and many of its negative effects have been attributed to a close link between metabolism and the immune system [35]. Advances in our understanding of obesity have revealed a complex network of cells within the fatty tissue which secrete a range of inflammatory mediators and direct many localised and systemic cellular interactions. Adipocyte tissue is thus no longer considered simply as an energy storage tissue but a complex endocrine organ [36].

Adipose tissue derived bioactive mediators have been characterised and are known as adipokines (Table 1.2) [36, 37]. The anti-inflammatory adiponectin and pro-

inflammatory leptin are the most abundant of adipokines produced directly from adipocytes and their ratio is associated with the occurrence of insulin resistance [38]. Along with increased production of cytokines (IL-6 and TNFα), chemokines (MCP-1 and CXCL5), and mediators of fibrinolysis (PAI-1), obesity is associated with a persistent low-grade chronic inflammation [39].

Adipose tissue consists of a number of cell types which together contribute to increased systemic inflammation. Lean adipose tissue consists primarily of small volume adipocytes, anti-inflammatory or alternatively activated M2 macrophages and $CD4^+$ T-cells [40]. Obesity progression is characterised by increased uptake and storage of triglycerides in the adipocyte causing significant hypertrophy. There is also a progressive shift in the phenotype of resident immune cells as adipose infiltration of pro-inflammatory M1 macrophages and cytotoxic $CD8^+$ T-cells increases [40, 41]. This combination causes an increase in soluble pro-inflammatory mediators released from the various cells. As obesity progresses the adipocyte volume further increases, M1 to M2 macrophages shifts further towards a predominance of M1 and inflammation and hypoxia mediated vascular dysfunction occurs causing adipocyte necrosis [42]. At this stage the metabolic control of the adipose tissue is severely impaired and the pro-inflammatory state can further increase dramatically. Critically the adipose tissues sensitivity towards insulin and its production of the adipokines adiponectin and leptin are altered [43].

1.2.2.2 Adiponectin

Adiponectin is the most abundant adipokine released from adipocytes circulating in excess of 5 $mg \cdot ml^{-1}$ and exhibits mainly anti-inflammatory effects [44]. Adiponectin

binds to two cellular receptors (ADIPOR1 and ADIPOR2) causing activation of AMP-activated protein kinase (AMPK) and peroxisome-proliferator-activated receptor-α (PPARα) [45] . AMPK activation can increase fatty acid oxidation and glucose transporter type-4 (GLUT4) translocation and ultimately reduces insulin resistance through a non-insulin dependent uptake of glucose [46]. Furthermore, by blocking the actions of nuclear factor-κB (NF-κB) adiponectin can reduce the synthesis of pro-inflammatory cytokines such as TNFα and IFN-γ in macrophages whilst increasing production of anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist (IL-1RA) from monocytes, macrophages and dendritic cells [47, 48]. Recently adiponectin has been shown to reduce neutrophil apoptosis and production of IL-8 via an AMPK dependent manner [49]. Furthermore, due to the structure of adiponectin which is homologous to complement factor C1q, adiponectin can associate to form multimeric oligomers and bind to apoptotic cells and facilitate their removal by macrophages [50]. Early uptake of apoptotic cells promotes an M2 phenotype in macrophages and can protect against inflammation [36].

Adiponectin expression is inhibited by the actions of TNFα, IL-6, hypoxia and oxidative stress which could explain why obese individuals have lower plasma levels than non-obese [51-53]. As ageing is accompanied by systemic increases in IL-6 and TNFα, even in the absence of obesity, this could have major consequences for adiponectin derived protection in the elderly. Subsequently low adiponectin levels in the elderly may contribute to the dysregulation of the production of pro-inflammatory cytokines leading to elevated systemic inflammation as well as immune dysfunction [54].

1.2.2.3 Leptin

Leptin has the primary function of regulating feeding behaviour through its actions on the central nervous system. Leptin concentrations are increased in order to stop the desire and requirement to feed; therefore leptin resistance has been associated with both obesity and insulin resistance. Indeed leptin concentrations directly correlate with adipose mass and can be assessed as a measure of stored energy. Leptin has directly opposite effects from adiponectin being generally pro-inflammatory. Leptin shares structural similarities to the pro-inflammatory cytokines IL-2, IL-6, IL-12 and granulocyte colony stimulating factor (G-CSF) [37]. Subsequently, leptin can directly stimulate monocytes and macrophages to increase production of IL-6 and IL-12 as well as chemokines such as MCP-1 [55]. Leptin can stimulate proliferation of monocytes via the IL-2 receptor and also activates neutrophils enhancing their superoxide production [56, 57]. Leptin also has regulatory effects on NK-cells and T-cells through mediating proliferation, differentiation and cytotoxic functions [55, 58]. Therefore leptin has many beneficial effects especially during acute infection by mediating the inflammatory response towards pathogens. However, as obesity increases elevated production of leptin and reduced adiponectin contribute to an uncontrolled inflammatory overload [59]. This imbalance leads to heightened adipose driven systemic inflammation through both immune and metabolic perturbations.

1.2.2.3 The Obesity Paradox

Although it is clear that obesity is a major driving force behind systemic inflammation and that inflammation can act to reduce production of anti-inflammatory mediators from adipocytes there is a paradox suggesting otherwise [60]. Recent studies have suggested that metabolically healthy obese individuals have reduced inflammatory profiles similar to non-obese individuals. In their recent study Phillips and Perry measured metabolic health as defined by a number of criteria including blood pressure, insulin sensitivity, cholesterol and triglycerides [61]. They found that from 2047 men and women aged 45 - 74, individuals who were metabolically healthy and obese had reduced levels of IL-6, CRP, PAI-1 and increased adiponectin compared to metabolically unhealthy obese individuals. Furthermore these values were comparable to non-obese individuals even after accounting for age, gender, medication and other confounding factors. Although this study could not confirm causality it has been recently estimated that the prevalence of metabolically healthy obesity is as much as thirty five percent [61]. Therefore obesity driven inflammation may be overestimated in the general population and only account for a small percentage of systemic inflammation observed in the elderly.

Table 1.2: Adipose tissue derived biomarkers, their cellular source and primary function. Adapted from Ouchi *et al.* [36]

Adipokine	Primary Cellular Source	Primary Function
Leptin	Adipocytes	Appetite control
Adiponectin	Adipocytes	Insulin sensitizer and anti-inflammatory
Resistin	PBMCs	Promotes macrophage production of IL-6 and TNFα causing insulin resistance
RBP4	Liver, adipocytes, macrophages	Implicated in sytstemic insulin resistance
Lipocalin 2	Adipocytes, macrophages	Promotes adipocyte production of TNF causing insulin resistance
ANGPTL2	Adipocytes	Local and vascular inflammation
TNFα	Stromal vascular cells, adipocytes	Inflammation, antagonsim of insulin signalling
IL-6	Adipocytes, stromal vascular cells, liver, muscle	Changes with source and target tissue
IL-18	Stromal vascular cells	Broad-spectrum inflammation
CCL2	Adipocytes, stromal vascular cells	Monocyte recruitment
CXCL5	Macrophages	Antagonsim of insulin signalling via JAK-STAT pathway interference
MCP-1	Adipocytes, macrophages	Immune cell recruitment
PAI-1	Adipocyte, endothelia	Inhibits fibrinolysis
NAMPT	Adipocytes, macrophages	Monocyte chemotactic activity
SFRP5	Adipocytes	Suppression of pro-inflammatory WNT signalling

RBP4 (Retinol binding protein-4), ANGPTL2 (Angiopoietin-related protein-2), TNFα (Tumour necrosis factor alpha), IL (Interleukin), CCL2 (Chemokine (c-c motif) ligand 2), CXCL5 (C-X-C motif chemokine 5), MCP-1 (Monocyte chemotactic protein-1), PAI-1 (Plasminogen activator inhibitor-1), NAMPT (Nicotinamide phosphoribosyltransferase), SFRP5 (Secreted frizzled-related protein 5).

1.2.2.4 Endocrine Dysregulation with Age

There is a functional modification of the endocrine system with age that can influence the severity of inflammation [62]. The hypothalamic-pituitary-adrenal (HPA) axis serves as the primary neuroendocrine unit and is one of the most studied endocrine components affected by ageing. The HPA axis consists of the hypothalamus, pituitary gland and adrenal glands and is responsible for regulating the stress response in humans. Physiological and psychological stress stimulates the secretion of corticotrophin releasing hormone (CRH) which directs its actions on the anterior pituitary gland to produce adrenocorticotropic hormone (ACTH). ACTH exerts its actions on the zona fasciculata to produce cortisol and the zona reticularis to produce dehydroepiandrosterone (DHEA) which are then released into the blood stream. The HPA axis has a negative feedback mechanism involving cortisol mediated reduction in ACTH which in turn reduces cortisol secretion [63]. DHEA on the other hand does not have this mechanism and is subsequently the most abundant hormone in the body [64]. Following synthesis DHEA is distinctively sulphated (DHEAs) and released into the blood where it is converted back to DHEA and its steroidal metabolites in the tissue.

Ageing is associated with a severe decline in the synthesis and release of DHEAs which peaks around 20 – 30 years old before reducing to around 10% of original concentrations after the age of 60 years [65]. Cortisol changes with age are less dramatic with some studies showing increases, others showing no change and some decreasing [66-68]. Deuschle and colleagues suggested that there is an increase in nocturnal cortisol production with age which results in an overall increase in 24 hour cortisol levels [66]. As cortisol is a diurnally controlled hormone which peaks during the night and is at its lowest following waking in the morning methodological

differences explain much of the variance observed in cortisol concentrations. Regardless of cortisol changes there is an age-associated increase in the ratio of cortisol to DHEAs which has profound effects for health [69]. With respect to immunity cortisol is immune suppressive, whereas DHEAs is immune enhancing [70]. As the elderly have a dysregulated immune function which increases their susceptibility to infection it is thought the increased ratio of cortisol to DHEAs may contribute to immunesenescence [71, 72]. Neuroendocrine dysfunction has also been associated with sarcopenia, osteoporosis, mental wellbeing and prevalence of frailty in the elderly [73, 74] .

Exposure to physiological and psychological stress increases with age and is accompanied by hyperactivation of the HPA-axis resulting in elevated cortisol production. Cortisol is typically an anti-inflammatory hormone and increased cortisol levels have been associated with reduced T-cell function during healthy ageing [75]. However it appears that with time repeated exposure of T-cells to glucocorticoids (GC) causes an age associated resistance which would result in a heightened inflammatory response and may explain the paradox of inflammageing in the presence of a raised cortisol:DHEAs ratio [76, 77]. A recent study in young individuals infected with rhinovirus revealed those who had increased resistance to GC were more likely to develop an infection and had increased pro-inflammatory cytokine production [78]. It is unclear however whether GC resistance is present in innate immune cells of the elderly.

DHEAs is the natural endogenous antagonist of cortisol yet its production is severely reduced with age [70]. DHEAs is considered immune enhancing and recently it has been shown that DHEA supplementation improved natural killer cell cytotoxic

function and increased IL-2 production leading to improved proliferation whilst reducing IL-6 concentrations [79]. DHEAs is also able to increase neutrophil superoxide generation *in vitro* [80]. DHEA is associated with improved immune responses and reduced systemic inflammation and is now considered a primary candidate as a supplement to improve immunity in elderly people [70, 81, 82].

1.2.2.5 Chronic Antigenic Challenge

Ageing is accompanied by increased exposure to clinical and sub-clinical infections as well as continuous exposure to latent viral infections due to immunesenescence, discussed in chapter 1.2.4. Repeated exposure to infection stimulates the growth of memory and effector immune cells which occupy a significant proportion of the available immune space. Repeated antigenic challenge is the primary reason for exhaustion of the immune system and driving it towards a state of dysregulation and dysfunction, known as immunesenescence [83]. Undoubtedly increased antigenic challenge can increase chronic systemic inflammation in the elderly and one of the dominant theories of inflammageing is that it is driven in large part by the sustained efforts of the immune system to control infections, primarily chronic viral infections [17].

1.2.2.6 Cytomegalovirus

Persistent latent viral infections are believed to be a primary cause of both immunesenescence and inflammageing, most notably the herpes virus cytomegalovirus (CMV) [84]. CMV is a highly prevalent virus of the herpes family which also includes Epstein-Barr virus (EBV) and varicella-zoster virus (VZV). CMV

is one of the most studied of all herpes viruses and infects 60–85% of the Western population and approximately 80–90% of those over 65 years [85]. CMV infects cells primarily of the myeloid lineage. Monocytes are one of the main cells infected by CMV which alters their phenotype to increase adhesion and migration potential [86]. Upon migration to a suitable tissue the virus can replicate and seed in the local endothelial and stromal environment. It is here the virus remains latent due to continual immune surveillance until local and/or systemic immunity is compromised and reactivation occurs [87].

Infection with CMV has been shown to dramatically alter the T-cell phenotype and function in both young and older adults [88]. In particular there is a CMV associated reduction in the diversity of the T-cell receptor (TCR) repertoire which is accompanied by increased proportions of pro-inflammatory effector memory cells [89]. Comparisons between age and health matched seropositive (CMV+) and seronegative (CMV-) individuals have shown that there is an expansion of the CD8+ cells and to some extent the CD4+ memory T cell compartment in CMV+ individuals [89]. Phenotypic alterations in T cells from CMV+ individuals demonstrate a reduced ability for antigen presenting cell co-stimulation and activation due to reduced expression of the cognate receptor for CD80/CD86, CD28 [90, 91]. Furthermore CMV stimulation has been shown to upregulate the production of IL-10, the classic anti-inflammatory cytokine, which acts directly on reducing program death ligand on dendritic cells allowing for viral evasion from the immune response [92]. These mechanisms may act to preserve the viral lifespan by evading the immune response developed to combat reactivation. Interestingly stimulation of T cells from CMV+ and CMV- individuals with CMV protein epitopes reveals that there is an elevated

production of IL-6 and TNFα not only from T cells but also NK cells and macrophages from CMV+ individuals [93]. Hyper-production of inflammatory cytokines by CMV may act to prolong the viral lifespan. Recently it has been shown that elevated levels of TNFα in elderly individuals can attenuate the IL-2 proliferative response of T cells which would confirm this [94].

Ageing is associated with both an increased prevalence of CMV infection, reactivation and its consequent immune alterations [95]. Furthermore, CMV infection has been suggested to increase the risk of all-cause mortality in the elderly and is a component of the Immune Risk Profile (IRP), a set of immune markers associated with increased mortality on older adults [96]. In light of this it is reasonable to assume that CMV infection promotes chronic systemic inflammation through remodelling of the immune system and increasing risk of mortality from novel or more severe infectious episodes. As the elderly are exposed to signs of immune dysfunction the failure to control CMV infection is increased, however the exact mechanism of age-related CMV associated mortality has not been elucidated. Recently it has been shown that CMV may infect monocytes and utilise their mobility to seed in tissues such as vascular cells, liver and lungs [87]. CMV infected monocytes can alter the phenotype of monocytes and adopt a pro-inflammatory phenotype which causes them to be increase cellular adhesion. It has also been speculated that CMV infection may lead to atheroma plaque formation by adhesive monocyte infiltration in the vascular endothelial bed resulting in foam cell generation [97, 98].

1.2.2.7 Cellular Senescence

One of the most noticeable consequences of latent viral infection is its ability to drive oligoclonal expansion and subsequently the prevalence of senescent T-cells specific to the virus [99, 100]. Not limited to immune cells, cellular senescence is the state of irreversible growth arrest following multiple rounds of clonal expansion believed to be induced by oncogenic insults [101-103]. Since the discovery of replicative senescence some fifty two years ago it is now thought that senescence occurs in order to limit the risk of cancer development [104-106]. Therefore it is of no surprise that with ageing the frequency of senescent cells increases in all tissues and in mouse models the elimination of senescent cells delays age associated dysfunction highlighting this intricate relationship [107, 108]. Although it has been shown that increased cellular senescence is associated with heightened risk of age-related diseases and physical dysfunctions it remains unclear the specific mechanisms.

Senescence is believed to be induced by a number of causes including repeated cell division, telomere shortening, DNA damage responses (DDR) inducing double strand breaks, protein aggregation, strong mitogenic signals and increased reactive oxygen species production [33, 109]. These senescence inducers create a phenotype which is characterised by such things as cell cycle arrest, increased activity of senescence-associated β-glactosidase activity and synthesis and secretion of pro-inflammatory mediators. Activation of the p53 and the $p16^{INK4a}$ tumour suppressor pathways are believed to be the primary, but not exclusive, pathways responsible for inducing a senescent response. Subsequently activation of the NF-κB pathway occurs and results in increased pro-inflammatory mediator production [110]. Therefore as inflammageing is associated with an absence of

detectable pathogens it is likely that inflammageing is in part driven by the accumulation of senescent cells [33]. Consequently, the senescence-associated secretory phenotype (SASP) has been coined detailing this phenomenon [111, 112].

The SASP has been shown to produce a number of inflammatory mediators capable of modifying cells and tissue function, including MMP3, TNFα, IL-6, IL-8, MCP-1, IGF binding proteins and PAI-1 [113, 114]. What is clear is that the SASP is not limited to one cell type or tissue and can be found at varying degrees in tissues such as adipose and immune [115]. Although cell senescence can be viewed as an example of antagonistic pleiotropy by promoting protection in younger age, the accumulation that occurs is thought to promote an increased incidence of cancer and other age related disorders [116]. Interestingly the SASP may not only be limited to chronological age accumulation but has been recently suggested to promote neighbouring cells to become senescent and adopt the SASP [117, 118]. Therefore geriatric and medical sciences are striving to implement interventions which can specifically target cell senescence and the SASP.

1.2.3 Consequences of Inflammageing

As described in section 1.2.1 inflammageing is associated with increased mortality and age-related frailty. The increase in mortality is due to a broad range of effects of increased inflammation on physiology.

1.2.3.1 Severity of Infections and Disease in the Elderly

Inflammageing can influence the severity and prevalence of infections and disease in older adults [119]. Respiratory diseases and influenza infection are the fourth major contributors to mortality in the elderly. Pneumonia in the elderly can account for as much as thirty percent of hospitalised elders whilst ninety percent of influenza deaths are in the over 65 year olds. Mortality data of elderly individuals compared to young individuals suggests that fifty percent more will die from sepsis infection whilst there is a 400 fold increase in elderly deaths from gastrointestinal infections [120]. Furthermore, one in seven deaths of over 85 year olds can be attributed to bacterial diseases, highlighting the progressive age associated inability to mount an immune response and control inflammation [121].

The increased incidence of these infections is due in large part to the ageing of the immune system, termed immunesenescence, which includes reduced functioning of innate immune cells such as neutrophils and NK cells [122-124]. These cells act as the front line defence against bacteria and viruses respectively and the age-related reduction in the microbicidal function influences the increased susceptibility to infection in the elderly. The basal increase in production of cytokines by monocyte and macrophages is paradoxically combined with a reduced inflammatory response to pathogens, compromising the innate response to infection [125]. The reduced

ability to generate an anti-inflammatory response, notably IL-10 production, means that inflammation is not resolved promptly. This contributes to the increased frailty and mortality seen in elders after infections such as influenza [126].

1.2.3.2 Inflammageing and chronic age-related diseases

Inflammageing has also been suggested to be an underlying mechanism behind many of the major age-related chronic diseases [127]. Not only have inflammatory biomarkers such as CRP and IL-6 been associated with increased risk of metabolic dysfunction, including insulin resistance, but they and TNFα are independently associated with cardiovascular disease (CVD), Alzheimers disease and type-2 diabetes amongst others [31]. Chronic diseases are becoming increasingly prevalent in the elderly with approximately thirty percent of all deaths attributable to CVD including coronary heart disease and stroke. Furthermore, fifty percent of people with diabetes will die from CVD [128]and over 800,000 elderly individuals suffer from Alzheimers disease in the UK [129].

1.2.3.3 Insulin Resistance

Many of the tissues which are affected by ageing are also affected by obesity, vascular, heart, skeletal muscle, liver etc. Insulin is a pancreas derived hormone which regulates the uptake, oxidation and storage of metabolic fuels in tissues such as the liver, skeletal muscle and adipose tissue. Recently it has been shown that immune cells such as adipose resident macrophages also require the use of insulin [41, 130]. Over time resistance to the actions of insulin develop which leads to increased inflammation and vascular endothelial dysfunction. With ageing, there is

often an increase in circulating concentrations of insulin (hyperinsulinaemia) which is a result of both increased glucose concentrations due to poor diet, and the inability to uptake glucose as a fuel due to the prevalence of insulin resistance [131]. Insulin resistance has been shown to have a clear link with increased TNFα production. TNFα binding to cells can disrupt insulin receptor (IR) activity by activation of NF-κB and c-Jun amino-terminal kinase (JNK) which causes phosphorylation of the IR [37]. Yuan and colleagues suggested that over-expression of inhibitor of nuclear factor kapp-B kinase subunit beta (IKKβ) can attenuate TNFα induced insulin resistance by reducing the expression of NF-κB [132]. Therefore hyper-production of TNFα as with ageing results in insulin resistance and a lack of glucose uptake by cells. This can have detrimental effects as the cell switches energy metabolism in order to survive and produces elevated free radicals in the process [133]. Insulin resistant cells may become necrotic requiring an inflammatory mediated clearance. In summary systemic insulin resistance can perpetuate heightened inflammation through altered metabolic function leading to cell death.

1.2.3.4 Frailty and Physical Dysfunction

Frailty can be viewed as the stage where complete homeostasis may be lost or compromised and progression towards disease is rapid leading to death [31]. Therefore predicting those who are becoming frail has become a major health care challenge of recent times. Defining frailty is complex due to the methods utilised to measure it, however a few criteria have been validated and are clinically relevant. The Fried Frailty Criteria is the most widely implemented objective approach to characterising and classifying frailty [134]. Fried and colleagues defined frailty on the basis of muscle weakness, exhaustion, slow walking speed, weight loss and low

activity status [7]. These measures are easy to assess in clinic and at home and can be converted into objective quantifiable scores and frailty is defined as having 3 out of 5 of these conditions. The Fried Frailty Score is also relatively robust in that if some of the measures are not able to be assessed, e.g. weight loss in a specific time, alternative measurement criteria can be adopted. Prevalence of frailty has been recently estimated at around four percent for men and nine percent for women among community dwelling elders (64 – 74 years) in the UK [135]. This percentage and total number of the population is expected to significantly increase due to our ageing population.

Frailty has an underlying inflammatory phenotype with cross sectional studies associating CRP, IL-6, IGF-1, DHEAs and white blood cell subtypes with progression and severity of frailty [136-138]. Cross sectional analysis does not determine causality and only a few studies have assessed the longitudinal associations between inflammation and frailty [139]. Therefore identification of inflammatory biomarkers to predict frailty in the elderly is critical to providing interventions that prevent frailty.

1.2.4 Immunesenescence and Inflammation

It is clear that the increased susceptibility to disease and severity of infection is associated primarily with a dysfunctional immune response. Immunesenescence is the age-related changes of the immune system which can be defined by modifications in immune tissue development, innate and adaptive cell number, phenotype and function [85, 125]. The intimate relationship with immunesenescence and chronic systemic inflammation in the elderly and are now considered a major cause of age-related disease driven morbidity and mortality.

1.2.4.1 Adaptive Immunesenescence

Consisting primarily of naive and antigen specific B and T lymphocytes the adaptive arm is responsible for elimination of pathogen infected cells and conveying lifelong protection (memory) against re-infection. However due to increased antigenic exposure with age and as the adaptive arm has limited self-renewal capabilities, it eventually becomes dysfunctional leading to increased risk of infection and immune-related disease [140-142]. Table 1.3 outlines some of the common adaptive immune changes with age.

Ageing is accompanied by a severe involution of the thymus which is characterised by a gradual replacement of functional stromal cells with fat and fibrous tissue [143]. Although thymic involution can result in functional mass being 10% of that of a young person the overall size of the peripheral T cell pool is unchanged with age [85]. Maintenance of the T-cell pool number is achieved by expansion of circulating cells, including naive and antigen experienced memory T cells. Contributing to this expansion is the activation of memory T cells by previously encountered antigens

and latent viruses such as CMV. In the case of CMV this can lead to up to 15% of peripheral CD8+ T cells being CMV specific, reducing the ability to respond to new pathogens or vaccines [141]. The reduced output of naive T cells and a concomitant increase in memory T cells results in a profound contraction of the diversity of the TCR repertoire.

Although the adaptive immune system shows a broad range of changes with age (Table 1.4) it is the innate immune system changes with age which are believed to exert the greatest impact on systemic inflammation [144].

Table 1.3: Adaptive immune changes associated with increased age

	Alteration with age
T-cell	Thymic Involution Reduced T cell repertoire Impaired TCR sensitivity Reduced Naïve cell numbers Increased memory and effector memory numbers Inverted CD4: CD8 ratio Loss of costimulatory molecules CD27 and CD28 Increase of negative regulatory receptors KLRG, CD57 Increased inflammatory cytokine production
B-Cell	Reduced numbers of B cells Increased monoclonal production Increased somatic hypermutation
Telomere	Shorter telomeres and suppressed telomerase
Cellular Senescence	Increased expression of $p16^{ink4a}$
Stem Cell Progenitors	Reduced lymphoid lineage

TCR (T-cell receptor), CD (Cluster of differentiation), KLRG (Killer cell lectin-like receptor subfamily G1).

1.2.4.2 Innate Immunesenescence

The innate immune system is the immediate non-specific first line of defence against infection which is responsible for recognition and clearance of pathogens and consists of both humoral and cellular components. Few studies have shown defects in the humoral arm, though complement proteins such as C1q are increased with age [145].

The cellular components of innate immunity consist of a diverse group of cells including neutrophils, monocytes, macrophages, dendritic cells and NK-cells. These cells are responsible for control of pathogenic invasion via a number of direct cytotoxic properties and resolution through secretion of a number of regulatory cytokines and chemokines which can direct the adaptive immune response.

Therefore a dysfunctional innate immune response impacts on both immediate responses and immune memory. Furthermore, there is a growing consensus that activation of the cellular innate immune system with age significantly contributes to the dysregulated inflammatory state [146]. Thus dysregulated inflammation is also a consequence of increased levels of basal inflammatory products and impaired immune responses. The age-related changes to innate immunity are shown in Table 1.4.

Table 1.4: Innate immune changes associated with increased age

	Alteration with Age
Neutrophils	↔ Numbers, adherence & TLR expression
	↓Chemotaxis
	↓Phagocytosis
	↓Superoxide production
	↓Apoptosis
	↓Lipid raft formation
	↓NET production
Monocyte/Macrophages	↔ Numbers
	↓Chemotaxis
	↓Phagocytosis
	↓Superoxide production
	↓Apoptosis
	↓Lipid raft formation
	↓MHC-II expression
	↓Stimulated cytokine production
	↓TLR expression
	↑Prostaglandin synthesis
	↑Basal cytokine production
	↑Pro-inflammatory phenotype
Dendritic Cells	↓Antigen presentation
	↓TLR signalling
	↓Chemotaxis
	↓Decreased IFN production
NK-cells	↑Cytotoxic CD56dim population
	↓Cytotoxicity
	↓Proliferation
	↓Chemokine production

NET (Neutrophil extracellular traps), MHC (Major histocompatibility complex), TLR (Toll-like receptor), CD (Cluster of differentiation).

1.2.4.2.1 NK cells

NK cells represent 10-15% of peripheral blood lymphocytes and are responsible for defence against tumour and virally infected cells [147]. NK cells constitutively express activating (e.g. NKp30 and NKp46) and inhibitory (e.g. KIR and CD94) receptors and can be distinguished by their surface density of CD56 and lack of expression of CD3. $CD56^{bright}$ NK cells are termed immunoregulatory and produce a range of cytokines whilst the $CD56^{dim}$ population which accounts for 90% of the NK cells are cytotoxic effector cells [148]. Ageing is associated with a reduced percentage of $CD56^{bright}$ cells and increased cytotoxic $CD56^{dim}$ cells [149]. This shift in phenotype towards a predominantly mature state is associated with altered expression of NK receptors but reduced cytotoxic capacity with age. Recently it has been shown that NK dysfunction with age is due to the reduced release and binding of the pore-forming molecule perforin [149]. Furthermore, NK cells from the elderly have been consistently shown to have reduced chemokines and cytokine production which will elongate the infection resolution process and inflammation [125, 146].

1.2.4.2.2 Dendritic Cells

Dendritic cells (DCs) are professional antigen presenting cells, sitting at a crucial interface between innate and adaptive immunity and are responsible for priming the T-cell mediated immune response. DCs can be categorised into two distinct types; plasmacytoid DCs (pDCs) and myeloid DCs (mDCs). pDC's are critical to host defence against pathogenic invasion, particularly viral, by producing type I and III interferon via TLR ligation in order to direct the immune response. They are capable of initiating an NK-cell response whilst also activating T-cells via antigen

presentation. mDCs on the other hand are responsible for cytokine production that initiates a Th1 cytotoxic response against pathogenic challenges.

mDCs from the elderly have reduced primary functions such as endocytosis, chemotaxis and activation of T-cells [150, 151]. Basal levels of pro-inflammatory cytokines are increased in mDCs from the elderly due to aberrant reduced expression of PI3K which can stimulate NF-κB dependent production of IL-6 and TNFα [150]. Studies have shown with stimulation that elderly mDC pro-inflammatory cytokine production to be increased, decreased or unchanged [150, 152, 153]. pDCs from the elderly appear to have reduced production of inflammatory cytokines following activation via TLR7 and 9 with viral DNA [152]. This reduced TLR function has been attributed to a reduced expression and aberrant signalling. Furthermore, the dysfunctional TLR responses of pDCs can be directly associated with reduced priming of T-cells and production of IFN [154]. Therefore dysfunctional TLR responses of pDC's will elongate the infectious episode and stimulate heightened inflammation in the long run.

1.2.4.2.3 Monocytes and Macrophages

Although monocytes represent a relatively small proportion of circulating leukocyte numbers (approximately 5 - 10%) they are key mediators of the immune response to infection by responding to inflammatory signals and differentiating into antigen presenting cells such as macrophages or dendritic cells. Monocytes are capable of providing direct cytotoxicity through phagocytosis and oxidative killing of pathogens as well as processing and presenting them to the adaptive immune arm via human leukocyte antigens (HLA). Monocytes are heterogeneous and can be identified by

the differential expression of CD14 and CD16, with 2 distinct subsets identified, classical $CD14^{++}/CD16^{-}$ and pro-inflammatory (non-classical) $CD14^{+}/CD16^{+}$ [78]. These cells differ in their expression of toll-like receptors (TLRs), adhesion molecules and chemokine receptors with CD16 expressing monocytes having greater surface density than the CD16 negative monocytes [155]. Recently, the pro-inflammatory population has been subdivided into 2 groups, $CD14^{+dim}/CD16^{++bright}$ and $CD14^{++bright}/CD16^{+dim}$ and are characterised by pro-inflammatory capacity and elevated expression of PRRs such as TLR2 and TLR4 [156, 157]. Agonists against TLRs cause NF-κB activation resulting in pro-inflammatory cytokine production.

Inflammatory cytokine production from monocytes in the elderly is either increased or decreased depending on the particular TLR which is stimulated . Both TLR1 and TLR2 expression and stimulation of TLR2 results in reduced cytokine production in the elderly, though TLR4 responses are increased [125, 158]. Additionally adherent monocytes from the elderly which are predominantly $CD16^{+}$ show an increase in TLR5 (bacterial flagellin components) stimulated production of IL-6 [159]. Therefore monocytes from the elderly appear to have reduced inflammatory potential which negates adaptive immune response whilst constitutively producing more TNFα from $CD16^{+}$ monocytes.

Monocytes migrate from the peripheral blood towards sites of infection in a similar manner to neutrophils except for the detection of chemokines which is controlled by other chemokines. Migration in the elderly is impaired and in chronic inflammatory conditions such as atherosclerosis there is an elevated concentration of MCP-1, the ligand for the monocyte chemokines receptor CCR2 and to a lesser extent CCR4 [160]. Both systemic MCP-1 and CCR2 expression on monocytes are increased with age suggesting that dysfunctional migration may be due to downstream signals

associated with MCP-1/CCR2 activation. Migration to sites of inflammation results in macrophage differentiation and resolution of infection, however aged monocytes have reduced CX3CR1 expression which is necessary for tissue transmigration [161].

Because of their critical role in macrophage and DC homeostasis and inflammation, monocyte changes with ageing may have a significant impact on immune dysregulation and heightened inflammation. Recent studies have identified an age-related shift from a classical to non-classical phenotype and suggest a systemic activation status [162, 163]. Ageing has been consistently shown to increase both numbers and percentages of non-classical monocytes at the expense of classical monocytes. Furthermore, adhesion molecule expression is decreased for CD62L but not CD50, the receptor for lymphocyte function-associated antigen 1 (LFA-1), although CD50 surface density is increased [162]. Additionally the expression of human leukocyte antigen DR (HLA-DR), the human homolog to MHC-II, is decreased and ultimately reduces antigen presentation to CD4+ T-cells [163].

Age associated changes of the monocyte are thus indicative of promoting systemic aberrant inflammation through basal output of cytokines and an inability to maintain immune surveillance.

1.2.4.2.4 Neutrophils

Neutrophils account for 50-60% of all leukocytes and are primarily responsible for the clearance and control of opportunistic bacteria, yeast and fungi as well as regulation of innate and adaptive immune interactions [123, 125]. Neutrophils are one of the first cells to engage foreign pathogens and attempt to resolve infection. To

do this they have an anti-microbial arsenal which includes phagocytosis, reactive oxygen species (ROS) production, extracellular DNA trap formation (NETS) and secretion of antimicrobial molecules and peptides such as the protease elastase and the defensin human neutrophil peptide-1 [164]. Furthermore, neutrophils are able to mediate the immune response via production of cytokines, chemokines and growth factors including TNFα, IL-8, CCL3, IP-10, MIP-1α, IL-12, VEGF and others [164, 165].

Reduced neutrophil functions have the potential to contribute to chronic systemic inflammation through each of the above dysregulated functions. During acute infection the neutrophilia observed is similar between young and elderly individuals suggesting that granulopoiesis is unaltered with age [123]. However, recent evidence suggests that elevated numbers of neutrophils is associated with all-cause mortality in the elderly [166]. The process of migration towards the site of infection is complex. Neutrophils are required to adhere to activated endothelium, a process mediated by the interactions of endothelial ligands such as intercellular adhesion molecule 1 (ICAM-1) and their cognate receptors on the surface of the neutrophil. The expression of the integrins CD11a, CD11b and CD18 appear to be unchanged between healthy elders and young individuals [167]. Adhesion to the endothelium leads to detection of chemokines produced by the infected site and ultimately transmigration across the vascular endothelium. Receptors specific for chemotactic agents are unchanged with age yet *in vitro* analysis has revealed that the elderly have reduced chemotaxis [146, 168, 169]. Age associated reduced chemotactic ability is a consequence of constitutive activation of signalling pathways involving phosphoinositide 3 kinase (PI3K) [168]. Dysfunctional chemotaxis by neutrophils

may promote systemic inflammation due to collateral tissue damage caused by aberrant migration. In order to move through tissue to the site of infection neutrophils release the protease elastase which breaks down elastin and collagen in connective tissue. This causes elevated tissue damage and promotes an inflammatory response that may be above and beyond the initial inflammatory response from the infection. Elderly individuals have been recently shown to have increased systemic elastase, although it is not clear the exact source in this study [170]. Furthermore in recent animal studies assessing neutrophil function following burn-induced lung injury it was shown that there were elevated inflammatory responses in the old mice compared to the young [171]. This effect was mediated by increased expression of ICAM-1 in the lung and reduced expression of CXCR2 on the neutrophil.

Neutrophils are professional phagocytes and therefore express a plethora of receptors responsible for the detection of, adhesion to and uptake of pathogenic microbes. Neutrophil phagocytic capacity in the elderly has been consistently shown to be impaired, although only when opsonised components are used [172, 173]. Phagocytosis incorporates two main variables affected by ageing, the percentage of cells able to phagcytose and the numbers of microbes ingested on a per cell basis. It has been suggested that these impairments are due to signalling defects and/or reduced expression of receptors such as CD16 on the neutrophil cell surface [172]. TLR agonist stimulation on neutrophils has been shown to increase a number of functions including cytokine release and superoxide generation whilst reducing migratory capacity. Although the expression of TLR4 on neutrophils appears unchanged whether there is an age related effect for TLR function remains to be determined.

Following phagocytosis pathogens are contained within phagosomes which combine with lysosomes to form the phagolysosome. It is here pathogens are exposed to microbicidal killing by an oxidative burst consisting of superoxide (O^{2-}). Depending on the pathogenic challenge neutrophils from the elderly show either a reduced [*Staphyloccocus aureus* (*S. Aureus*)] or unchanged [*Escherichia coli* (*E. Coli*)] production of O^{2-} [174]. This may in part be due to the mechanisms recruited to deal with pathogens, such as complement and Fc receptor-mediated responses for gram-positive bacteria, LPS binding by CD14/TLR4 or fMLP ligation with its receptor which is further increased on exposure to TNFα. Signal transduction of a number of effector functions have been shown to be impaired. These include pro-survival responses to GM-CSF via the Janus kinase signalling pathway and reduced expression of suppressor of cytokine signalling 1 (SOCS1) and SOCS3 [175].The latter signalling pathway mediates the production of cytokines, chemokines and reactive oxygen species (ROS). Neutrophil function in the elderly is thus impaired primarily due to modifications of signalling pathways that reduce microbicidal activity whilst elongating lifespan and increasing inflammatory cytokine production. It is unclear whether these modifications happen in the bone marrow during development or occur in the peripheral tissue in response to micro-environmental changes.

One factor that complicates interpretation of neutrophil data is the short-lifespan and subsequent release from the bone marrow (BM) of ‘new’ neutrophils, which results in a mixed population of potentially functionally different cells. As neutrophils are known to lose their function as they age [176], it is plausible that these cells are residing in the circulation for longer in older adults. To date, no study has been able to

determine the percentage of newly released neutrophils and neutrophils close to the end of their life in the peripheral blood. *In vitro* studies culturing isolated neutrophils have identified phenotypic differences in expression of CXCR2 and CXCR4 [177, 178]. CXCR4 functions as the receptor to keep newly matured neutrophils in the BM and return of aged neutrophils back to BM. As ageing is associated with increased low-grade systemic inflammation, which lengthens neutrophil survival time, neutrophil composition may be significantly different between young and old.

1.2.5 Interventions to attenuate Inflammation and Immunesenescence

1.2.5.1 Therapeutics

As inflammageing is associated with increased morbidities it represents a target that could reduce morbidity in elders. Metformin is prescribed to reduce hepatic glucose production and reduce cardiovascular disease risk by improving insulin sensitivity [179]. This drug works by stimulating a range of cell processes via activation of AMP kinase, including autophagy [180]. Recently metformin has been associated with reduced inflammation by inhibiting NF-κB activation and reducing CRP levels and has improved survival of patients with type-2 diabetes [181]. Furthermore, metformin has been shown to alter T-cell biology by potentially improving cell generation and protective immunity in cancer patients [182].

Recently it has been proposed that all elderly people be placed on statin therapy regardless of health status [183]. Statins are typically prescribed to combat hypercholesterolemia but have also been shown to have anti-inflammatory and anti-hypertensive actions [179]. Indeed epidemiological studies suggest that the benefits of statin therapy may be mediated primarily through its anti-inflammatory actions [184]. Recent unpublished data from our group suggests that statins can repair the migratory and functional defects of neutrophils from the elderly (E. Sapey personal communication) suggesting not only anti-inflammatory benefits but also immune enhancing effects.

However, the cost of prescribing statins and metformin to all elderly individuals is considerable and a small proportion of individuals have negative side effects to statin

therapy [185]. Thus there is a need for a cost-effective, safe and efficacious intervention to reduce inflammation driven morbidity.

1.2.5.2 Exercise and Health

Maintenance of regular physical activity throughout life is associated with a number of major health benefits including, reduced risk of cardiovascular disease, diabetes and stroke as well as reduced physical disability (sarcopenia) and mortality [186]. However, after the age of 45 years there is a progressive and sharp decline in both time and intensity of physical activity participation which has been attributed primarily to a lack of available time [187]. Reduced physical activity, especially in the elderly, is known to lead to sarcopenia, reduced endurance capacity and a concomitant increase in visceral adiposity, with the latter contributing to inflammation [188, 189]. The anti-inflammatory and immune enhancing effects of exercise have been extensively researched in young healthy and non-healthy individuals but until recently few studies have assessed the impact in the elderly.

The anti-inflammatory effects of exercise have received significant attention in the last few years and are summarised in Table 1.5 [190]. The mechanism by which exercise acts appears to be primarily through modifications of the main contributors to inflammageing; altered adiposity and modified immune function. Exercise has known thermogenic properties which increases energy expenditure and can reduce adipose tissue by utilising the abundant energy contained within. As part of this process exercise alters the blood lipid profile by decreasing low-density lipoprotein (LDL) particles and increasing high-density lipoprotein (HDL) particles [191]. Therefore exercise reduces the risk of obesity by utilising triglycerides as an

abundant energy source and offering protection from CVD by improving the cholesterol clearance transport mechanism (LDL: HDL) [192].

Increased adipose tissue results in elevated concentrations of pro-inflammatory adipokines leading to persistent low-grade systemic inflammation [130]. Regular exercise has been shown to consistently reduce the amount of both abdominal and visceral fat regardless of age and gender [193]. Consequently there is a shift in the ratio of pro to anti-inflammatory adipokine secretion characterised by elevated adiponectin and reduced leptin as well as IL-6 and TNFα. Therefore a reduction in adipocyte mass will directly reduce the pro-inflammatory production of adipokines whilst increasing the anti-inflammatory phenotype. However, as much as 30% of circulating IL-6 is known to be produced from adipose tissue with only 10% of this being directly from the adipocyte [194]. The remaining IL-6 is produced primarily from resident macrophages suggesting that exercise itself has an impact on macrophage adipose infiltration and differentiation.

IL-6 release is intrinsically and transiently linked with exercise duration and intensity and is specifically expressed at higher levels of intensity [195]. However in this context it acts as an anti-inflammatory stimulator which can reduce production of systemic TNFα. Muscle synthesis of IL-6 in response to exercise has been well documented and accounts for a large portion of systemic concentrations. Muscle derived IL-6 has been shown to increase production of the anti-inflammatory cytokines IL-10 and IL-1RA as well as the anti-inflammatory glucocorticoid cortisol. Thus it appears that muscle derived IL-6 may act as an anti-inflammatory effector [196].

1.2.5.3 The Myokine Paradigm

Recently skeletal muscle has been identified as a major endocrine organ capable of producing a large amount of pro-inflammatory cytokines, chemokines and growth factors [197]. Until recently the production of such inflammatory mediators has led many to suggest that certain intensities, durations and modes of exercise may in fact be chronically damaging and not suitable for patients with inflammatory mediated disease. This would seem counterintuitive as exercise is associated with a plethora of health benefits. Collectively termed as myokines, their release from contracting skeletal muscle may in fact play a role in exercise induced protection from chronic disease and morbidity [198]. Typical pro-inflammatory cytokines and chemokines are now being considered as anti-inflammatory and tissue protective following exercise-induced release from skeletal muscle.

Although IL-6 was the first myokine identified as being synthesised and secreted by contracting skeletal muscle recent data suggests muscle can secrete around forty different cytokines, growth factors and metalloproteinases [190]. IL-6 is generally and persistently produced from a number of cells including immune, adipocyte and endothelial, however during exercise the majority of IL-6 found systemically is produced by the skeletal muscle [199]. Importantly the magnitude of the muscle derived IL-6 response, and other cytokines, is highly dependent on intensity and duration of exercise. One of the major differences between exercise induced and infection induced IL-6 responses is the dynamics of the preceding and following cytokine release. In response to infection, TNFα is one of the first cytokines released which magnifies the inflammatory response by increasing systemic temperature and

directing both immune and humoral responses to the infection. Following TNFα release the rest of the inflammatory milieu including IL-1, IL-6, IL-8 and IL-10 are secreted. In contrast the response to exercise does not induce a TNFα response (unless exercise is extremely severe) in the muscle or systemically, instead IL-6 is one of the first cytokines consistently produced followed by the anti-inflammatory IL-1RA and IL-10 and the chemokine IL-8 [197].

Classified typically as a pro-inflammatory cytokine having a severe impact on progression and severity of chronic diseases such as cardiovascular disease and diabetes, IL-6 is often cited as the 'geriatric cytokine' due to its presence in most age related diseases [200, 201]. From a number of observations, IL-6 is suggested to have a causal role in developing insulin resistance and promoting metabolic disease in the elderly [31]. Indeed chronic IL-6 production can disrupt insulin binding and signalling via increased expression of intracellular SOCS3. SOCS3 expression is also increased upon leptin binding and as leptin is homologous to IL-6, at elevated levels both disrupt the insulin receptor substrate-1 (IRS-1) pathway following insulin binding [202]. However, during exercise IL-6 is released from the muscle and does not activate intracellular SOCS3, instead bypassing it directly to AMPK activation and subsequent increased expression of GLUT-4 [203]. This exercise-mediated increased expression of GLUT-4 is independent of insulin signalling, however due to the reduced IRS-1 inhibition, insulin also stimulates GLUT-4 translocation to the cell membrane and allows efficient uptake of glucose.

Furthermore, IL-6 can act to dampen the inflammatory effects of other pro-inflammatory cytokines. As mentioned previously TNFα can directly reduce insulin

sensitivity through inhibiting tyrosine phosphorylation of IRS-1 via impaired Akt signalling. IL-6 infusion has been shown to reduce the endotoxin mediated TNFα response *in vivo* and although immune cell function was not assessed it does highlight systemic reductions in TNFα [204]. Importantly the IL-6 infusions in this study had comparable TNFα responses to those individuals who had undertaken an exercise session. This study highlights that it is most likely not the IL-6 cytokine itself which is different between exercise and basal conditions but more the ratio of IL-6 to TNFα. Therefore in the case of elderly individuals who may have significantly more systemic TNFα present, IL-6 at varying concentrations can act to reduce a TNFα mediated response. This mechanism is most probably through reducing TNFα induced insulin resistance. In light of this the current hypothesis suggests that exercise-induced IL-6 production is in fact an energy sensing mechanism that allows the muscle to replenish glycogen stores [190, 199].

In addition to IL-6 another typically pro-inflammatory mediator IL-8 is acutely responsive to exercise [205, 206]. IL-8 is typically a pro-inflammatory chemokine produced by macrophages and endothelial cells during infection to attract neutrophils and other immune cells to the site of inflammation. IL-8 mRNA expression and protein in muscle has been shown to increase in response to exercise [205]. However IL-8 may be influenced to a greater degree by the intensity, duration and mode of exercise than IL-6. Typically, increased IL-8 is observed following exhaustive eccentric or muscle damaging exercise such as running and not moderate concentric exercise like cycling. However others have shown small but significant increases in IL-8 following concentric exercise bouts suggesting that IL-8 release has a dual role in exercise induced adaptations [207]. During muscle

damaging eccentric or high intensity exercise IL-8 has been suggested to be released as an inflammatory mediator attracting immune cells to mediate the damage response and promote repair [208]. In non-damaging exercise sessions however immune cell infiltration in muscle is not observed suggesting an alternative role. Indeed in recent studies it has been shown that exercise increases expression of the IL-8 receptor CXCR2 on microvascular endothelial cells promoting IL-8 induced angiogenesis [209, 210]. Therefore exercise induced IL-8 can act to promote damaged muscle cell healing as well as stimulating vascular growth and increasing blood and nutrient supply to the muscle [208].

Clearly muscle acts as an endocrine organ enhancing its own protection and adaptation. Over time and with a lack of stimulation these actions may be impaired but with the introduction of even small amounts of exercise, the muscle can provide the potential to reduce systemic inflammation and improve quality of life in elderly individuals.

1.2.5.4 Exercise Mediated Immune Changes

Exercise induced effects on the immune system have received considerable attention over the last 25 years and are summarised in Table 1.5. Ever since it was recognised that physical activity load was associated with altered immune function there has been a goal to determine the beneficial effects of exercise on immunity. Individuals who perform moderate bouts of structured exercise appear to be at a reduced risk of infectious episodes suggesting that exercise can mediate the immune response to infection [211, 212]. Acute exercise has consistently been shown to alter the circulating numbers and percentages of subtypes of immune cells

[213-216]. Chronic exercise training does not always confirm these findings suggesting that functional alterations may be an epigenetic effect. Much of the work assessing exercise and immune function has been carried out in young healthy individuals; however a growing number of studies are assessing the impact of exercise in the elderly and those with chronic disease, particularly inflammatory related diseases.

1.2.5.4.1 Adaptive Immunity

The majority of studies assessing immune function alterations in response to exercise have been conducted on the adaptive arm of the immune system. These studies have focused on such things as the phenotype changes, proliferative capacity, co-stimulatory potential and cytokine shift of T-cells [217]. Acute exercise has been shown to alter the phenotype of T-cells circulating in both young and elderly individuals as well as improving their proliferative capacity [218, 219]. Unfortunately this effect appears to be transient and is not observed following longitudinal exercise interventions in both young and old volunteers [211, 220, 221]. Active elderly individuals have been shown to have reduced IL-2 receptor expression which may relate to functional changes [222]. Secretion of cytokines by T-cells in response to exercise has shown some promise and it has been suggested that exercise may shift T-cells in the elderly towards a Th2 anti-inflammatory/humoral response rather than the pro-inflammatory Th1, although the mechanism and consequences of this remain elusive [223-225].

1.2.5.4.2 Innate Immunity

Innate immunity and the effect chronic and acute exercise has received considerably less attention than the adaptive arm. The majority of studies have assessed the effects on NK-cells and monocytes.

Like most other immune cells there is an intensity and duration dependent redistribution of NK-cell numbers, specifically increased proportions of the $CD56^{bright}$ regulatory NK-cell population. Exercise induced functional changes have yielded inconsistent findings [219, 226]. NK-cell cytotoxicity has been shown to be increased in response to 6 months of aerobic training in elderly individuals but another study showed no differences in NK-cell function after aerobic training in elderly women [227, 228]. Infection with CMV has been shown to alter the phenotype of NK-cells by increasing the expression of CD57 on the cell surface [229]. Acute exercise has been shown to mobilise NK-cells to a tremendous degree yet CMV infected individuals NK-cells were less responsive [230]

Dendritic cells from the elderly have been suggested to have reduced TLR-induced cytokine production but increased basal inflammatory state and production of TNFα and IL-6 [152]. The effect of exercise on elderly dendritic cell function is not well researched. Studies have shown that acute exercise can mobilise myeloid and plasmacytoid DC's in relation to catecholamine production, and prolonged exercise can alter the balance of DC subtypes [231]. However as DC function is conducted in tissue understanding the relevance of this in humans is difficult. Recently 5 weeks of aerobic training in rats was shown to increase antigen presentation capability via the HLA-DR homolog MHC-IA/IE, leukocyte activation and IL-12 production of bone marrow isolated DC's [232]. Although this study was conducted in young adult rats using *in vitro* differentiated DC's the alterations suggest heightened immune functioning and potential anti-tumour activity via IL-12 production from aerobic training. Replication of these findings in aged humans would yield an incredible insight into immune self-tolerance deficiencies and tumour recognition which could both be restored by exercise.

Migration of peripheral blood monocytes and the subsequent differentiation towards adipose tissue is critical to the development of sustained inflammation. Whether exercise can moderate this process and reduce inflammation in the elderly is unknown. As exercise reduces the size of adipocytes it has been suggested that the production of chemokines (MIP1α and MCP-1) responsible for macrophage infiltration is reduced [233]. Vascular adhesion molecules such as ICAM-1 are increased with ageing and chronic disease but have been shown to be reduced with 6 months of aerobic exercise [234]. Therefore it is plausible that exercise reduces the migratory capacity of monocytes into adipose tissue. Macrophages with the inflammatory phenotype (M1) are preferentially associated with inflamed adipose tissue [235]. The effects of exercise on macrophage phenotype in humans remains to be determined, however in mice it has been recently shown that exercise induces a phenotypic switch of M1 to M2 macrophages in the adipose tissue as well as reducing M1 infiltration [236].

As the monocyte and its differentiated forms are intrinsically linked with increased systemic inflammation the impact of exercise on monocyte function may be critical to reduced inflammation. In response to acute bouts of exercise monocyte numbers are increased with the $CD16^+$ population accounting for the largest percentage change [156, 157]. Although following acute exercise the numbers and percentages of subtype's return to normal base levels there is a marked reduction in CD16 expressing monocytes after exercise training interventions [156]. Timmerman showed that elderly active individuals had lower percentages of $CD16^+$ monocytes than inactive age and sex matched individuals [237]. Furthermore, 12 weeks of aerobic training resulted in both reduced percentages of $CD16^+$ monocytes as well as LPS stimulated TNFα monocyte production in the inactive group. Although this

study showed no changes in TLR4 expression others have suggested that TLR2 and TLR4 expression are reduced by chronic exercise training in the elderly [238, 239]. Indeed following prolonged acute exercise TLR expression is reduced for several hours. Mechanisms behind the exercise induced reduction in TLR expression are unknown however it has been suggested that anti-inflammatory cytokines and/or glucocorticoid production may have an impact [240]. Additionally, 12 weeks of both strength and aerobic training resulted in an increase in the monocyte expression of CD80 [241]. Reduced expression of CD80 on monocytes has been shown to relate to poor vaccine responses in the elderly due to reduced activation potential of T-cells and antigen presentation capabilities [242]. Phagocytosis of pathogens is reduced with ageing however the effects of exercise have received little attention. To date the only study to assess phagocytosis was in middle aged men and showed no changes following 12 weeks of training [243].

The majority of neutrophil functions are impaired with age and can contribute to systemic inflammation through secondary mechanisms such as reduced pathogen clearance and aberrant migration and collateral tissue damage. Studies in overweight women (53 ± 9.8 years) have shown reduced neutrophil numbers following six weeks training which was associated with reduced BMI and insulin sensitivity however it is unclear if these are directly linked [244]. The effects of neutrophil numbers in response to acute exercise are dependent on the intensity and duration of the bout. High intensity exercise has been shown to cause increased numbers of neutrophils and this is further increased over several hours, thought to be controlled by increased glucocorticoid production [245]. Lower intensity exercise shows a typical leukocyte biphasic response with an initial increase followed by a return to baseline in the immediate hours following cessation. Some studies have

shown no differences between active and inactive elders for neutrophil numbers which suggests differences may be due to underlying inflammatory morbidities in the inactive groups [226].

Even fewer studies in the elderly have assessed neutrophil function in response to exercise. Yan and colleagues showed that although lower than the young, neutrophil phagocytosis was maintained better in active elderly men [226]. Oxidative killing of bacteria in response to exercise has to date not been assessed. Migratory capacity of neutrophils is critical to resolution of infection, 4 weeks of moderate aerobic exercise was shown to improve and maintain migration towards infection in young healthy individuals [246].

Table 1.5: Inflammatory and Immune findings from cross-sectional and randomised control trials examining associations between exercise training statuses in elderly adults.

Reference	Participants	Training Status	Findings (against inactive)
Colbert *et al.* (2004)	Males and females aged 70-79 years (*n* = 3075) Exercise Duration (mins/week)	Self-reported exercise duration and energy expenditure 0 (*n* = 1836), >0 to <180 (*n* = 657), >180 (*n* = 471)	↓CRP & ↓IL-6 concentrations
Stewart *et al.* (2007)	Physically active elderly males & females [71 ± 4 years (*n* =17)] Physically inactive elderly males & females [71 ± 4 years (*n* =14)]	Self reported physical activity levels + Active group >35ml/kg/min estimated VO_{2max} Inactive group <26ml/kg/min estimated VO_{2max} Inactive = 12-weeks resistance/aerobic training	↔ TNFα, IL-6, IL-1β, CRP at baseline ↔ TNFα, IL-6, IL-1β ↓CRP in inactive elders
Cesari *et al.* (2004)	Males and females aged 75.4 ± 0.2 years (*n* =1020)	Physical performance defined by: walking speed, chair-standing test, standing balance test, hand-grip strength	↓CRP, IL-6 & IL-1RA with increased physical performance
Nicklas *et al.* (2008)	Elderly non-disabled, community-dwelling males and females at risk of physical disability[70-89 years (*n* =369)	Self reported physical activity levels + Short Physical Performace Battery & 400-m walk test 12 -months of aerobic, strength & flexibility exercise (PA) or health education intervention (SA)	↓IL-6 & ↔CRP
Reuben *et al.* (2003)	Healthy elderly males and females [70-79 years (*n* =870)]	Self reported physical activity levels assigned to caloric values	↓IL-6 & ↓CRP
Spielmann *et al.* (2011)	Healthy males aged 18-61 years (*n* = 102)	Grouped into low, moderate high active according to age-adjusted VO_{2max} scores	↑Proportion of naive CD8+ T-cells; ↓proportions of senescent/exhausted CD4+ and CD8+ T-cells
Yan *et al.* (2001)	Untrained middle-aged males [51.6 ± 4.3 years (*n* =33)] Untrained elderly males [66.3 ± 3.2 years (*n* =20)] Trained middle-aged males [50.7 ± 8.2 years (*n* =18)] Trained elderly males [65.1 ± 4.5 years (*n* =28)]	Untrained: Daily exercise activity of 1-3 MET Trained: Daily exercise activity of 4-6 MET	↑neutrophil phagocytosis; ↓proportion NK-cells; ↓CD4:CD8 ratio; ↔NK-cell activity
Nieman *et al.* (1993)	Trained women [72.5 ± 1.8 (*n* =12)] Untrained women [73.5 ± 1.2 (n=30)]	Trained: VO_{2max} of 31.3 ± 0.9 ml/kg/min Untrained: VO_{2max} of 18.7 ± 0.9 ml/kg/min	↑NK-cell tumour killing ↑T-cell proliferation
McFarlin *et al.* (2006)	Physically active males and females (*n* =23) Physically inactive males and females (n=21)	Self reported physical activity levels + Active group >35ml/kg/min estimated VO_{2max} Inactive group <26ml/kg/min estimated VO_{2max}	↓LPS-stimulated IL-6, IL-1β & TNFα ↓CRP and TLR4
Shinkai *et al.* (1995)	Elderly male runners [63.8 ± 3.5 years (*n* =17)] Elderly sedentary males [65.8 ± 3.5 years (*n* =19)]	Trained: VO_{2max} of 38.8 ± 1.3 ml/kg/min Untrained: VO_{2max} of 29.2 ± 0.9 ml/kg/min	↑IL-2, ↑IL-4, ↑IFNγ ↑T-cell proliferation
Ogawa *et al.* (2003)	Trained women [63 ± 1 (*n* =9)] Untrained women [63 ± 1 (n=12)]	Trained: VO_{2max} of 32.3 ± 1.0 ml/kg/min Untrained: VO_{2max} of 27.8 ± 0.9 ml/kg/min	↑IL-2 and ↑IL-4 positive $CD8^+$ T-cells ↑IL-4 positive $CD4^+$ T-cells
Timmerman *et al.* (2008)	Physical active males & females [70.9 ± 4.6 years (*n* =15)] Physical inactive males & females [71 ± 5.7 years (*n* =15)]	Self reported physical activity levels + Active group: Males >35ml/kg/min, females >28ml/kg/min Inactive group: Males <26ml/kg/min, females <23ml/kg/min Inactive = 12-weeks resistance/aerobic training	↓CRP & ↓LPS-stimulated TNFα ↓Inflammatory monocyte % and ↓LPS-stimulated TNFα

1.2.5.5 Exercise and cortisol and DHEAs

Due to the associations between the cortisol to DHEAs ratio, immunesenescence and inflammation, in the elderly research has focused on whether there is exercise induced protection of adrenal function in the elderly. Ravaglia and colleagues [247] showed that older men (~67 years) had reduced DHEAs concentrations than middle-aged men (~58 years). However when the two age groups were split for physical activity levels, both middle-aged and elderly active men had increased DHEAs concentrations compared to inactive elderly men. Furthermore the active elderly men were comparable to the inactive middle-aged men. Similarly aerobic capacity has been associated with increased DHEAs in women and men. Heaney and colleagues [248] recently showed that although there was no difference in concentrations of cortisol, DHEAs or their ratio with increasing levels of physical activity in elderly individuals, a short exercise bout caused significant reductions in cortisol but not DHEAs.

1.3 Summary and Aims of Thesis

Elderly individuals have an increased incidence and severity of infection and chronic disease than the young. This is accompanied by multi-system functional declines including an increased incidence of sarcopenia, osteoarthritis, cardiovascular function and dementia. An underlying factor contributing to this phenomenon is the increase in low-grade chronic systemic inflammation (inflammageing) characterised by elevated levels of pro-inflammatory and reduced anti-inflammatory mediators. Factors contributing to inflammageing are complex and not completely understood; however the dominant mechanisms are believed to be lifelong antigenic challenges

resulting in immune dysfunction (immunesenescence), reduced levels of physical activity and increased prevalence of obesity, age-related loss of anti-inflammatory sex steroids and hyperactivation of the HPA axis. Taken together obesity, sedentary behaviour and immune mediated inflammation contributes to the majority of morbidities and mortality observed in the elderly.

Physical activity and exercise offers a mechanism by which immune dysfunction, obesity and subsequently inflammation can be attenuated in the elderly. Exercise both acute and chronic is energy demanding and subsequently utilises adipose tissue as an energy source reducing the risk of obesity. Similarly exercise can modify the number and function of circulating immune cells to reduce the risk of infection and modify cytokine output by muscle and adipose tissue. However, until recently few studies have assessed the impact of physical activity on inflammation and immune function in the elderly.

In light of this, the aims of this thesis were to:

1. Identify the consequences and causes of inflammageing, focussing on lifestyle and CMV serostatus, in healthy elderly individuals.

2. Assess the effects of physical activity as an intervention on inflammation and immunesenescence.

Chapter 2: General Materials and Methods

As each chapter has different participant groups and slightly different approaches to methodology, the general methods will be described below whilst specific study methods included before the pertinent results. Therefore, this section will include primarily the analytical analyses conducted on blood serum and plasma to determine inflammatory mediator responses. Furthermore, all immune function components will be included here.

2.1 Blood cell counts and preparation of serum and plasma

Blood was taken from healthy volunteers by venepuncture. All studies were approved by the relevant local research ethics committee and all participants gave written informed consent. All blood samples were processed immediately. Plasma was obtained by centrifugation of EDTA treated blood at 3000 rpm (5804 Eppendorf centrifuge, Eppendorf UK Ltd., Stevenage, UK) for 10 minutes at 4°C. Serum was obtained by the same centrifugation of clotting factor treated blood following 30 minutes standing upright to clot. Plasma and serum were snap frozen and stored at -80°C for later analysis.

Complete blood differentials, including leukocyte counts were completed using EDTA treated whole blood immediately after sampling on a fully automated Coulter^{TM} ACT^{diff} haematology analyser (Beckman-Coulter, High Wycombe, UK). All samples were analysed in triplicate.

2.2 Analysis of serum and plasma biomarkers

2.2.1 Luminex based assays

Detection of serum free cytokines was measured using commercially available XMap multiplex Luminex kits (Bio-Rad, Hemel Hempstead, UK) according to manufacturer's instructions. One kit was specific for type 1 cytokines which contained combinations of the following cytokines (IL-1b, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17A, TNFα, GM-CSF, MCP-1 and VEGF) whilst the second kit measured ICAM-1, VCAM-1 and MIF. Briefly, antibody coupled magnetic beads were added to a 96-well greiner NUNC plate. Serum was first centrifuged at 10,000 x g to remove platelets and cell debris and then diluted 1:4 with assay specific buffer before addition of 50 µl of sample and standards to each well. Plates were then sealed, foil covered and incubated for 30 minutes on an orbital shaker at 350 rpm. Following incubation, washing was performed 3 times before addition of the biotinylated detection antibody and incubated as before. Following incubation, washing was performed and streptavidin-RPE, which binds the detection antibody, was added and incubated as before but for exactly 10 minutes. Following washing and re-suspension plates were read on a Luminex^{200} instrument (Luminex Corp, Austin, TX, USA). The system identifies and quantitates cytokines based on bead colour and RPE fluorescence. The concentration of the cytokines is determined and automatically calculated by the Bio-Plex Manager^{TM} (Bio-Rad, Hemel Hempstead, UK) software using a standard curve derived from the recombinant cytokine standard.

2.2.2 ELISA

All ELISA's were performed using commercially available pre-validated kits and according to manufacturer's guidelines except for CMV which was an in house pre-validated gift from Prof. Paul Moss (School of Cancer Sciences). All standards, controls and test samples were assessed in duplicate. Test sample concentrations were determined from the standard curve produced from the recombinant standards provided. All ELISAs were quantified on a Synergy-2 microplate reader (BioTek, Bedfordshire, UK) within 10-minutes of adding stop solution. Standard curves and value extrapolation were conducted using GraphPad Prism® software v5 (GraphPad Software, La Jolla, USA). Intra-assay coefficient of variation was less than 5% for each ELISA.

2.2.2.1 CRP*:* Detection of serum free C-reactive protein (CRP) was determined by a commercially available high-sensitivity ELISA (IBL International, Hamburg, Germany). Serum samples were centrifuged at 2,500 x*g* for 10 minutes to remove platelets before being diluted 1:1000 in kit dilution buffer. Wells pre-coated with monoclonal anti-CRP antibodies were incubated with 100 µl of either test serum or standards (0 – 10 $mg{\cdot}L^{-1}$). Plates were covered with adhesive film and incubated for 30 minutes. Following incubation, plates were washed 3 times using kit wash buffer. After patting excess fluid from the plate 100 µl of CRP conjugated to horseradish peroxidise (HRP) was added and incubated for 30 minutes at RT. Wells were washed as before and 100 µl of chromogen solution containing 3,3',5,5'-tetramethylbenzidine (TMB) was added and incubated for 10 minutes at RT in the dark. Following incubation 50 µl of the stop solution containing 0.5M sulphuric acid (H_2SO_4) was added to each well.

2.2.2.2 Cortisol and DHEAs*:* Detection of serum cortisol and DHEAs was completed independently using commercial ELISA kits (IBL International, Hamburg, Germany). Briefly, wells pre-coated with anti-cortisol or anti-DHEAs rabbit polyclonal antibodies were incubated with duplicates of 20 µl (cortisol) or 25 µl (DHEAs) of either test serum, standards (cortisol: 0 – 2208 $nmol{\cdot}L^{-1}$ and DHEAs: 0 – 26000 $nmol{\cdot}L^{-1}$) or high and low controls in the presence of 200 µl of cortisol or DHEAs conjugated to HRP. Plates were covered with adhesive film and incubated for 60 minutes. Following incubation, plates were washed 4 times using the kit wash buffer. After patting excess fluid from the plate 100 µl of substrate solution containing TMB was added and incubated for 15 minutes at RT in the dark. Following incubation 100 µl of the stop solution containing 0.5M H_2SO_4 was added to each well.

2.2.2.3 Adiponectin, Leptin and IFNγ*:* Detection of plasma adiponectin/Acrp30 and leptin and serum IFN-γ were completed independently using sandwich ELISA kits (R&D Systems, Abingdon, UK). Briefly, mouse anti-human adiponectin, leptin or IFN-γ capture antibodies were diluted in sterile PBS to working concentrations of 2, 4 and 4 $\mu g{\cdot}ml^{-1}$ respectively. Wells were incubated overnight at RT with 100 µl capture antibodies. Following incubation wells were washed 3 times in 400 µl wash buffer (PBS/0.05% Tween® 20). Non-specific binding was reduced by blocking the wells with 300 µl of reagent diluent [PBS + 1% protease free BSA (Fisher Scientific, Loughborough, UK)] for 1 hour at RT. Following blocking plates were washed as before 3 times. Leptin (0 – 2000 $pg{\cdot}ml^{-1}$) and adiponectin (0 – 4000 $pg{\cdot}ml^{-1}$) standards and test plasma were diluted in reagent diluent before incubating duplicates of 100 µl for 2 hours at RT. Plates were then washed as before 3 times. Biotinylated mouse anti-human adiponectin, IFNγ or leptin detection antibodies were diluted in reagent diluent and 100 µl per well incubated for a further 2 hours at RT.

Following incubation the washing was repeated before incubating wells with 100 µl of HRP conjugated Streptavidin for 20 minutes at RT in the dark. Following incubation the washing was repeated before addition of 100 µl TMB substrate solution and incubated for 20 minutes at RT in the dark. Reactions were stopped by addition of 50 µl 2N H_2SO_4 (Sigma-Aldrich).

2.2.2.4 PAI-1*:* Detection of plasma plasminogen activator inhibitor-1 (PAI-1) was completed using a sandwich ELISA kit (Life Technologies). Briefly, wells pre-coated with anti-PAI-1 antibodies were incubated with 100 µl of standards and samples diluted 1:2 in the appropriate buffer provided, covered and incubated for 2 hours at RT. Following incubation, plates were washed 4 times using the kit wash buffer. After patting excess fluid from the plate 100 µl of biotinylated human PAI-1 biotin conjugate solution was added to each well (except the blank) and incubated for a further 2 hours as before. Plates were washed again before 100 µl of streptavidin-HRP working solution was added to each well and plates again covered and incubated at RT for 30 minutes. Following a similar wash, wells were incubated with 100 µl of chromagen solution in the dark for 30 minutes at RT before stopping the reaction with 100 µl of stop buffer.

2.2.2.5 Insulin*:* Detection of plasma insulin was completed using a sandwich ELISA kit (Life Technologies). Briefly, wells pre-coated with anti-insulin antibodies were incubated with 50 µl of standards, controls and samples as well as 50 µl of anti-insulin-HRP conjugate for 30 minutes at RT. Following incubation, plates were washed 3 times using the kit wash buffer. Following washing 100 µl of TMB chromagen solution was added to each well and incubated for 15 minutes at RT in the dark. Following incubation the reaction was stopped by adding 100 µl of acidic stop solution.

2.2.2.6 MIF and Endothelin-1*:* Detection of serum macrophage migration inhibitory factor (MIF) and plasma Endothelin-1 were completed independently using sandwich ELISA kits (Abcam®, Cambridge, UK). Briefly, wells pre-coated with anti-MIF or Endothelin-1 antibodies were incubated with 100 µl of standards and samples diluted 1:2 in the appropriate buffer provided, covered and incubated for 1 hour at RT. Following incubation, plates were washed 5 times using the kit wash buffer. After patting excess fluid from the plate 100 µl of HRP conjugated MIF or Endothelin-1 working solution was added to each well and plates again covered and incubated at RT for 30 minutes. Following a similar wash, wells were incubated with 100 µl of chromagen solution in the dark for 30 minutes at RT before stopping the reaction with 100 µl of stop buffer.

2.2.2.7 CMV*:* Detection of serum CMV specific IgG antibodies was completed using an in-house validated indirect ELISA (gratefully received from Prof. Paul Moss, School of Cancer Sciences; University of Birmingham, UK). Briefly, MRC-5 fibroblasts were infected with human CMV strains AD169 (G. Wilkinson, Cardiff, UK) or Towne1125 at a multiplicity of infection (MOI) of 4:1 for 4 hours. Mock infections were also conducted to eliminate non-specific IgG binding. Supernatants were harvested and centrifuged to pellet the virus before quantification by stimulation of PBMC's from CMV positive donors. Viral or mock lysate (50 µl) diluted to 1:4000 from stock in carbonate-bicarbonate buffer (Sigma-Aldrich) were then bound to an ELISA NUNC plate overnight at 4°C. Plates were washed 3 times in 200 µl of wash buffer (PBS/0.05% Tween® 20) on an automated plate wash. Following this 25 µl of donor serum samples [pre-diluted 1:150 in dilution buffer (PBS/0.05% Tween® 20 + 1% BSA)] and 50 µl of standards (0 – 1000 Au) were added in duplicate to both viral and mock lysate wells alongside 75 µl and 50 µl of dilution buffer respectively.

Following a 1 hour incubation at RT, plates were washed as before and then incubated for an additional 1 hour at RT with 100 µl of goat anti-human IgG HRP conjugated secondary antibody (pre-diluted 1:8000 in dilution buffer; Southern Biotech, Alabama, USA). Following incubation, plates were washed as before 3 times before addition of 100 µl TMB substrate solution and incubated for 10 minutes at RT in the dark. Reactions were stopped by addition of 100 µl 2N H_2SO_4 (Sigma-Aldrich) and OD recorded at 450 nm and 570 nm within 10 minutes on a Synergy-2 microplate reader (BioTek). Analysis was completed by subtracting the average mock lysate readings from the average viral lysate readings. Values greater than 10 Au were considered to be CMV positive.

2.2.2.8 Vitamin D*:* Plasma samples (500 µl) were sent to the University Hospitals Birmingham clinical analysis laboratory for determination of vitamin D concentrations. Samples were analysed in duplicate for concentrations of vitamin D2 and D3 using tandem mass spectrometry under clinical settings.

2.3 Measurement of Metabolic Biomarkers

Serum and plasma fasting glucose, glycerol, total triglycerides, total cholesterol and HDL-cholesterol (Instrumentation Laboratories Ltd., Warrington, UK) and non-esterified free fatty acids (Randox, UK) were assessed independently using an ILab-600 semi-automated clinical spectrophotometric analyser (Instrumentation Laboratories Ltd.). Analyis was conducted in collaboration with UoB department of Sport and Exercise Sciences. Briefly, the analyser was quality control checked for each metabolite to be measured before 300 µl of either serum or plasma was added to an individual spectrophotometric vial. Samples were placed in the machine and

assayed independently in duplicate. Results were calculated automatically by the analyser using iLAB-600 software (Instrumentation Laboratories Ltd.).

2.4 Assays of Immune cell function

2.4.1 Neutrophil Isolation

Neutrophils were isolated from heparin treated whole blood by discontinuous centrifugation. Blood was transferred into a sterile 50 ml Falcon™ and diluted 6:1 with 5% (w/v) dextran (Amersham Biosciences, Uppsala, Sweden) reconstituted in 0.9% saline and erythrocytes allowed to sediment for 30-45 minutes at room temperature (RT). The leukocyte rich buffy coat was carefully removed and layered on top of a discontinuous gradient in a 15 ml Falcon™ consisting of 5 ml 56% Percoll® (Sigma-Aldrich, Dorset, UK) and 2.5 ml 80% Percoll®. Gradients were prepared by diluting 1x Percoll® (45 ml) with 5 ml of sterile 9% (w/v) sodium chloride (NaCl: Sigma-Aldrich) before dilution of 40 ml of this working stock with 10 ml sterile 0.9% (w/v) NaCl to create an 80% Percoll® stock. The 56% Percoll® stock was created by dilution of 28 ml working stock with 22 ml of sterile 0.9% (w/v) NaCl and the 56% Percoll® carefully layered on the 80% Percoll®.

Gradients were centrifuged at 110 *x g* for 20 minutes at RT with no brake in a benchtop centrifuge. Following centrifugation, neutrophils suspended at the interface of the 80-56% Percoll® were carefully removed and placed into a sterile 50 ml Falcon™ and washed once at 400 *xg* for 10 minutes in RPMI-1640 media supplemented with 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (GPS: all Sigma-Aldrich). The supernatant was discarded and cells resuspended in RPMI-1640 supplemented with GPS and 0.15% (volume/volume (v/v)) bovine serum albumin (Sigma-Aldrich) to a final concentration of $2x10^{6.}ml^{-1}$. Purity was determined

by cytospin and differential staining using a commercially available Geimsa stain (Diff-Qik: Gentaur Europe, Brussels, Belgium). Neutrophils consistently comprised ≥98% of the cell population.

2.4.2 Neutrophil Chemotaxis

Neutrophil migration was assessed using an Insall Chamber (Weber Scientific International Ltd, Teddington, UK) and has been described previously [249]. Briefly, glass coverslips were coated with 7.5 % endotoxin free bovine serum albumin (Sigma) and isolated neutrophils incubated for 20 minutes at RT to allow cells to adhere. Coverslips were then inverted onto the Insall chamber and the chamber filled with either the RPMI as a control or RPMI containing 100nM IL-8. Neutrophil migration was then immediately recorded using a Zeiss Axiovert-100 microscope and a FAST Mono-12bit QICAM digital camera. Recording lasted 12 minutes with images taken every 20 seconds using Improvision OpenLab software. After recording, ten randomly selected cells were tracked and analysed using Image-J software (Wayne Rasband, NIH, MD, USA). All videos were analysed in duplicate by two independent researchers blinded to participant characteristics.

Neutrophil migration was assessed using 3 parameters. Average cell speed (chemokinesis) of movement ($\mu m \cdot min^{-1}$) was measured from the distance travelled between frames in any given direction over time. Average cell velocity (chemotaxis), defined as the speed in a constant direction was also measured in $\mu m \cdot min^{-1}$. As the Insall chamber allows the formation of a stable chemoattractant gradient with a defined constant direction on the y-axis only distance travelled on this axis was calculated for chemotaxis. Finally accuracy (chemotactic index) was calculated by the cosine of the angle between the cells direction and the orientation of the chemoattractant gradient at each time frame. This formed a vector analysis of

movement which was expressed on a comparative scale (CS) ranging from -1 to +1 where +1 represents movement directly towards the chemoattractant source.

2.4.3 Monocyte Isolation

PBMCs were isolated from heparin treated whole blood by density gradient centrifugation. Blood was transferred into a sterile 50 ml Falcon™ and diluted 1:1 with sterile phosphate buffered saline (PBS). Blood mixtures were layered over Ficoll-Paque™ PLUS (GE Healthcare, Uppsala, Sweden) at blood: Ficoll ratio of 4:3 ml and centrifuged at 400 *x g* for 30 minutes at RT with no brake. Following centrifugation, mononuclear cells suspended at the interface of the Ficoll and plasma were removed and washed twice, 400 *x g* for 10 minutes, in PBS. Cells were counted and viability assessed by trypan blue exclusion before being suspended in 1ml of PBS for magnetic isolation of monocytes. PBS was prepared by dissolving one PBS tablet (Sigma-Aldrich) per 200ml of distilled water and sterilised by autoclaving.

$CD14^+$ monocytes were isolated from PBMCs using the EasySep™ Human Enrichment Kit (Stem Cell Technologies, Grenoble, France). PBMCs ($15x10^{6}\cdot ml^{-1}$). PBMCs were placed in a 5 ml (12 x 75 mm) polystyrene tube on ice and washed, 250 *x g* for 10 minutes at 4°C, in 2 ml cell sorting buffer (PBS containing 1mM Ethylenediamine-Tetraacetic Acid (EDTA) and 2% bovine serum albumin (BSA)). Cell pellets were resuspended in 0.5 ml sorting buffer before addition of 15 µl of the EasySep™ monocyte enrichment cocktail and incubated at 4°C for 10 minutes. Following incubation, 15 µl of EasySep™ magnetic particles were added and incubated at 4°C for 5 minutes. Cell suspensions were then made to a total volume of 2.5 ml with sorting buffer and placed inside a Purple EasySep™ Magnet for 2.5 minutes at RT. Following incubation, the cell suspension containing $CD14^+$ and

$CD16^+$ monocytes was poured into a 5 ml polystyrene tube and washed twice (250 *xg* for 10 minutes) in Hank's balanced salt solution (HBSS) supplemented with calcium and magnesium (Gibco®, Life Technologies, Paisley, UK). Cell pellets were resuspended in RPMI supplemented with GPS at a final concentration of $2x10^{6}.ml^{-1}$ ready for migration analysis.

2.4.4 Monocyte Migration

Monocyte migration was measured using 5µm transwell chambers (Corning Costar, Amsterdam, Netherlands) towards monocyte chemoattractant protein-1 (MCP-1: R&D systems, UK) or RPMI supplemented with GPS as a control in sterile conditions. Chamber membranes were pre-wet with RPMI supplemented with GPS and placed into the 24-well plate containing either 900 µl of RPMI supplemented with GPS or 900 µl of RPMI supplemented with GPS containing 10nM MCP-1. Plates were incubated at 37°C in a sterile humidified 5% CO_2 atmosphere for 1 hour. Following incubations chambers were removed and immersed 3 times in PBS before fixing with 3.7% (w/v) formaldehyde for 2 minutes at RT. Chambers were immersed 3 times again in PBS and permeabilised in 100% methanol for 20 minutes before immersion in PBS again. Differential staining using a commercially available Giemsa stain (Diff-Qik: Gentaur Europe) was conducted by incubation of chambers in the dark for 15 minutes. Giemsa staining was washed off with PBS and inside chambers gently cleaned with cotton wool buds to remove any non-migrated cells and chambers left to dry for a minimum of 30 minutes. Once dry, chambers were viewed under a light microscope and adherent monocytes counted in five fields of vision at 20x magnification.

To determine if any monocytes had detached from the chamber membrane and passed into the chemoattractant each 900 µl sample was removed into a 5 ml

polystyrene tube. Wells were then incubated for 10 minutes at 37°C in 100 µl of 2% (w/v) trypsin (Sigma-Aldrich) before addition of 400 µl cold PBS supplemented with 1% BSA and transferred to a 5 ml polystyrene tube. Cells in both samples were counted by flow cytometry. Using an Accuri™ C6 flow cytometer (BD Biosciences), 50 µl of each sample was extracted and cells counted in the leukocyte gating applied on the forward v side scatter. Cell numbers were then added to the appropriate membrane counts and total monocyte migration calculated by subtracting the average number of monocytes per field plus flow cytometry counts in the control from the MCP-1 sample.

2.4.5 Determination of Monocyte Production of Pro-Inflammatory Cytokines

Monocyte cytokine production was evaluated by exposing heparin treated whole blood to lipopolysaccharide (LPS). Blood was diluted 1:1 in 5 ml polystyrene tubes with Iscove's Modified Dulbecco's Medium (IMDM) supplemented with GPS. LPS (Sigma-Aldrich) was added at a final concentration of 10 $ng \cdot ml^{-1}$, or the same volume of IMDM as a negative control, in the presence of 3 $\mu g \cdot ml^{-1}$ of Brefeldin A and incubated at 37°C in a sterile humidified 5% CO_2 atmosphere for 4 hours. Following incubation samples were placed on ice and washed twice, 250 *x g* for 10 minutes at 4°C, with 2 ml cold PBS supplemented with 1% BSA. Samples were then resuspended in 100 µl of PBS supplemented with 1% BSA and incubated on ice with appropriate surface antibodies or their concentration-matched isotype control (Table 2.1) for 30 minutes in the dark. Samples were then washed as before in PBS supplemented with 1% BSA before resuspending the pellet in 2 ml of 1x BD FACS™ lysis buffer (reconstituted in distilled water) for 10 minutes at RT in the dark. Samples were then washed twice as before fixing and permeabilising cells using a commercially available fix and perm kit (Life Technologies). Fixation was completed

by incubating the cell pellet in 100 µl Reagent A for 15 minutes at RT in the dark. Following a single wash the cell pellet was permeabilised by incubating in 100 µl Reagent B and addition of the appropriate intracellular cytokine antibody or their concentration-matched isotype control for 30 minutes in the dark on ice. Samples were then washed twice as before and resuspended in PBS supplemented with 1% BSA and stored in the dark on ice until flow cytometric determination of monocyte cytokine production.

Table 2.1: Anti-human primary antibodies used for monocyte analysis

Antigen	Fluorochrome	Isotype	Dilution	Clone	Supplier
CD14	PcB	IgG2a	1:100	M5E2	BD Bioscience
CD16	FITC	IgG1	1:25	3G8	BD Bioscience
CCR2	APC	IgG2b	1:10	48607	R&D Systems
CCR4	APC	IgG2b	1:10	205410	R&D Systems
TLR2	APC	IgG2a	1:20	TL2.1	BD Bioscience
TLR4	APC	IgG2a	1:20	HTA125	eBioscience
HLA-DR	PE-CF594	IgG2a	1:50	G46-6	BD Bioscience
CD11b	APC	IgG1	1:20	ICRF44	BD Bioscience
CD18	PE	IgG1	1:20	6.7	BD Bioscience
TNFα	PE	IgG1	1:100	MAb11	eBioscience
IL-6	PE	IgG1	1:25	MQ2-13A5	eBioscience

2.4.6 Flow Cytometry for Immune Cell Phenotyping

Neutrophils were analysed from isolated cells whilst monocyte phenotype was assessed from isolated PBMCs. Briefly, 200 µl of isolated cells ($1x10^{6}.ml^{-1}$) were dispensed into 5 ml polystyrene tubes and washed twice (250 x g for 5 minutes at 4°C) in PBS supplemented with 1% BSA before supernatants discarded and cells resuspended in 100 µl PBS supplemented with 1% BSA containing surface specific antibodies or their concentration-matched isotype control (Table 2.2). Following 30-minute incubation in the dark on ice, cells were washed twice as before and

resuspended in 300 µl PBS supplemented with 1% BSA for flow cytometric analysis. All flow cytometry was performed using a CyAn^{ADP} bench top flow cytometer (DAKO). Neutrophil and monocyte proportions were determined by gating of 10,000 and 5,000 cells, respectively, on the forward scatter (FS) versus side scatter (SS) flow cytometry dot-plot. Cells were then excluded for doublets before appropriate gating strategies applied to determine the percentage and surface density of each of the measured antigens.

Table 2.2: Anti-human primary antibodies used for neutrophil analysis

Antigen	Fluorochrome	Isotype	Dilution	Clone	Supplier
CD16	FITC	IgG1	1:25	3G8	BD Bioscience
CD16	APC	IgG1	1:100	CB16	eBioscience
CXCR1	FITC	IgG2b	1:50	8F1-1-4	eBioscience
CXCR2	PE	IgG1	1:50	5E8-C7-F10	eBioscience
CXCR4	APC	IgG2a	1:50	12G5	eBioscience
CD11b	APC	IgG1	1:20	ICRF44	BD Bioscience
CD18	PE	IgG1	1:20	6.7	BD Bioscience
TLR2	APC	IgG2a	1:20	TL2.1	BD Bioscience
TLR4	APC	IgG2a	1:20	HTA125	eBioscience

2.4.7 Neutrophil and Monocyte Phagocytosis and Superoxide Generation

Neutrophil and monocyte phagocytosis and superoxide generation were assessed in whole blood immediately after sampling using commercially available kits according to manufacturer's instructions (BD Biosciences, Oxford, UK). For all samples, the volume of blood tested was adjusted to ensure that neutrophil and monocyte concentrations in respect of bacteria were consistent across the three time-points.

Briefly, phagocytosis was assessed in heparin treated whole blood which had been cooled on ice for 10 minutes. Samples were then incubated for 10 minutes on ice

(control) or in a 37°C (test) water bath with 20 µl of pre-opsonised FITC-labelled *E.Coli*. Phagocytosis was halted by the addition of 2 ml ice cold phosphate buffered saline (PBS; Sigma-Aldrich, UK) whilst cell surface bound FITC was quenched by adding 100 µl of the kit trypan blue solution. Unbound free bacteria were removed by washing twice in 2 ml PBS at 250 *x g* for 5 minutes at 4°C. The supernatant was gently removed and erythrocytes lysed and leukocytes fixed by adding 2 ml of 1x erythrocyte lysis buffer, containing 1% paraformaldehyde, and incubated for 20 minutes in the dark at RT. Samples were washed twice as before in PBS before staining leukocyte DNA by addition of 200 µl propidium iodide (PI) provided in the kit for 10 minutes in the dark at RT before flow cytometry analysis was performed.

Superoxide generation was assessed in heparin treated whole blood which had been cooled in a 5 ml polystyrene tube on ice for 10 minutes. Samples were then incubated for 10 minutes at 37°C with 20 µl of opsonised *E.Coli* (test) or PBS (control) in a water bath. 20µl of solution containing dihyrdorodamine-123 (DHR-123), which is converted to rodamine-123 (R-123) in the presence of reactive oxidants, was incubated for another 10 minutes at 37°C. Superoxide activity was halted by removing samples from the 37°C, placing on ice and adding 2 ml of 1x erythrocyte lysis buffer, containing 1% paraformaldehyde, and incubated for 20 minutes in the dark at RT. Samples were washed twice at 250 *x g* for 5 minutes at 4°C in 2ml PBS before staining leukocyte DNA as before for 10 minutes in the dark at RT before flow cytometry analysis was performed.

Phagocytosis and superoxide analysis was determined on a Cyan ADP^{430} flow cytometer (Beckman-Coulter, High Wycombe, UK) equipped with 3 solid-state lasers emitting light at 405nm (Argon), 488nm (Violet) and 642nm (Red). FITC and R-123 were detected in FL1 whilst PI was detected in FL2 using the Argon laser. To ensure

only leukocytes were analysed, samples were first gated on their PI positive profile. This gate was then applied to the forward/side scatter profile to enhance typical neutrophil and monocyte identification. 10,000 neutrophils and 5,000 monocytes were acquired for analysis. Phagocytic and superoxide capacity was determined by the relative increase in percentage and median fluorescent intensity (MFI) in FL1 compared to negative controls. Gating strategy is depicted in Figure 2.1. Data were analysed using Summit v4.3 (DAKO, Cambridgeshire, UK).

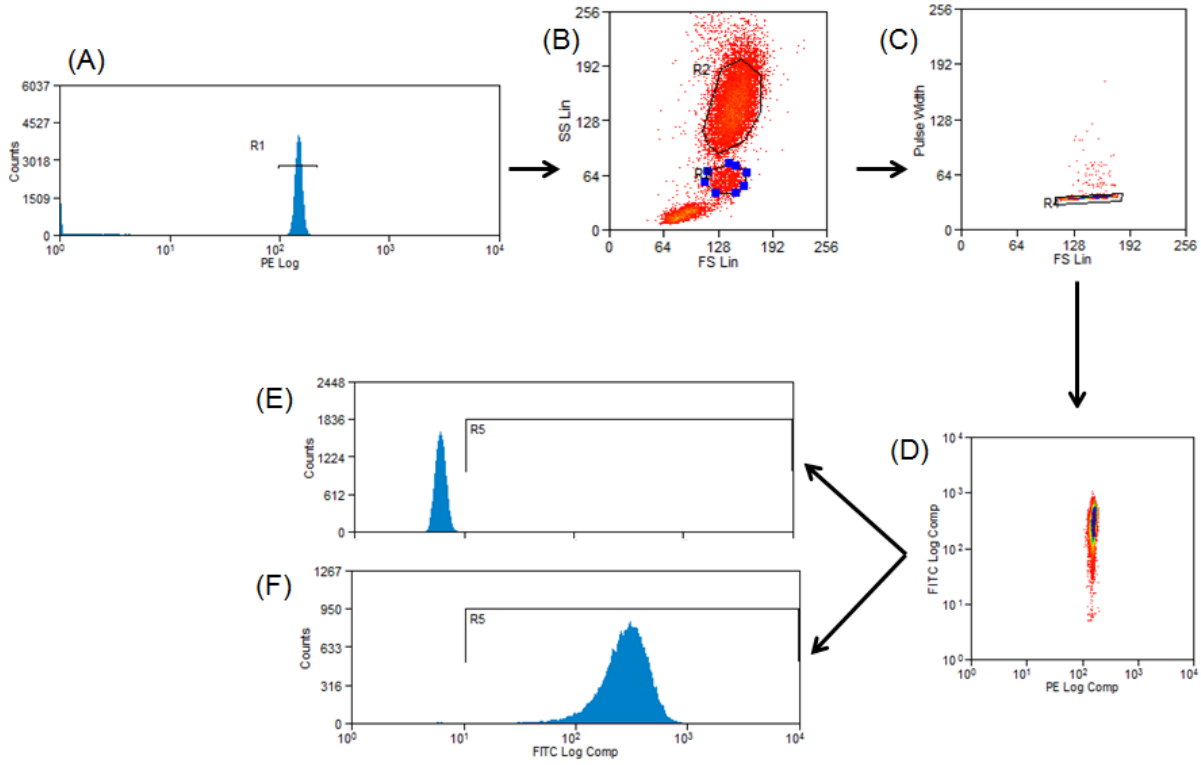

Fig. 2.1: Gating strategy to identify bactericidal function. DNA dye identifies leukocytes (A) before applying this gate to the forward/side scatter which enables clear identification of the monocyte and neutrophil populations (B). Individual populations are then gated on before identifying and eliminating cells which have joined together using size v width (C). This population of single cells is then applied to a dot plot of DNA (PE) versus phagocytosis/superoxide (FITC) in which electronic compensation is applied to ensure no overlap of the two fluorochromes (D). Histograms are then generated which represent the negative control (E) and the positive test (F) allowing for quantification of cell function.

Chapter 3: Systemic Inflammation in the Elderly: Causes and Consequences

3.0 Introduction

A number of studies have suggested that increased low-grade chronic systemic inflammation (inflammageing) occurs in the elderly and is associated with negative health outcomes such as depression, reduced physical mobility, cardiovascular disease, type-2 diabetes and Alzheimer's disease [31]. The consequences of inflammageing are therefore profound in respect to being a causative mechanism of poor health, frailty and mortality in the elderly [82, 134]. It remains unclear the exact causal mechanisms of inflammageing although possible factors include increased adiposity, reduced physical activity, alterations to the HPA axis, notable the increased cortisol:DHEAs ratio, and reduced production of sex steroids [32, 190, 250]. However one of the dominant theories is that inflammageing is driven by the sustained efforts of the immune system to control infections [251].

Ageing is associated with increased incidence and severity of infectious episodes, due in large part to the age-related decline in immunity, termed immunesenescence [252]. The changes in the immune system with age are not simply a decline in function; instead the response is remodelled, with innate cells such as macrophages producing pro-inflammatory cytokines in the resting state but showing a compromised response when exposed to pathogens. Therefore with age the immune system adopts a heightened pro-inflammatory status but reduced functional responses upon challenge leading to reduced pathogen clearance. Several age-related changes to the immune system have been shown to be associated with increased mortality, termed the immune risk profile (IRP) [96]. Individuals who are infected with latent viral infections of the herpes family, specifically Cytomegalovirus

(CMV) are more likely to show accelerated immunesenescence and other elements of the IRP [89].

CMV is a prevalent β-herpes virus infecting 60-90% of the population and undergoes intermittent reactivation requiring the adaptive immune system to contain and control it [95]. Although in healthy individuals CMV infection is asymptomatic it can lead to severe disease and death in those who are immune compromised. Furthermore, CMV has been shown to stimulate the production of pro-inflammatory cytokines from the immune system [93]. These observations have led to the suggestion that CMV infection is a primary driver of inflammageing and that this effect can contribute to increased morbidity including reduced physical functioning and mortality.

In light of this, the aims of this chapter were to:

1. Use a longitudinal cohort analysis to assess the contribution of systemic CMV infection on the progression of inflammation and adrenopause.
2. Investigate the relationship between systemic inflammation, adrenopause and ageing over a ten year period.
3. Determine lifestyle factors which influence systemic inflammation and adrenopause.
4. Determine the consequences of systemic inflammation and adrenopause on frailty and mortality.

3.1 Study protocol: The Hertfordshire Ageing Study

3.1.1 Participants

The Hertfordshire Ageing Study (HAS) has been described previously and the protocol for recruitment of participants is presented in Figure 2.1 [253]. Briefly, 6803 birth certificates dated between 1920 and 1930 were recovered and following investigation by the National Health Service Central Register 2621 subjects were found to be alive in the UK, of which 1428 were still residing in Hertfordshire. Home interviews and clinical assessment for a wide range of ageing characteristics and peripheral blood samples were obtained on 717 participants in 1993/94. Ten years later (2003/5) home interviews and clinical assessment including peripheral blood samples were conducted on 294 of the original 717 participants. Mortality was ascertained between these assessments and revealed 17% of participants were deceased. Table 3.1 shows the data available and measurements taken by researchers and trained research nurses during both clinical assessment and home interviews.

Intravenous blood samples were collected and processed by research technicians for whole blood differentials, erythrocyte sedimentation rate (ESR), albumin, thyroid stimulating hormone (TSH), free T4 (T4), testosterone, sex hormone-binding globulin (SHBG), and peripheral blood mononuclear cell (PBMC) DNA isolation. Serum was snap frozen and stored at -80°C until analysis was conducted. This project was given access by the MRC Human Epidemiology Unit, Southampton University, to the stored serum for analysis of various components as described above and the associated lifestyle data and physiological measurements made on the cohort.

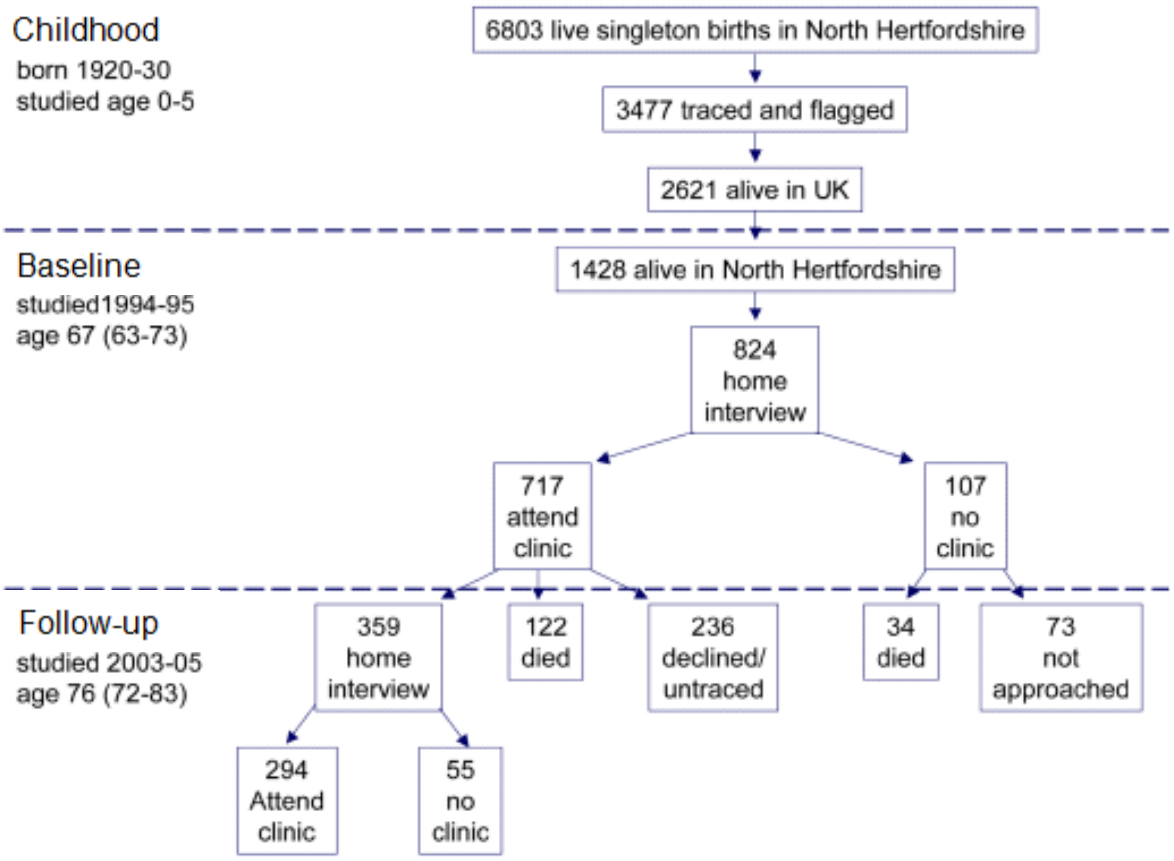

Fig. 3.1: Recruitment of participants into the HAS study. Adapted from Sydall *et al* [253] .

Table 3.1: Data available from the HAS. Adapted from Syddall *et al.* [253].

	Childhood	Baseline	Follow-Up
Year	1920-30	1994-95	2003-05
Age (years)	0-5	63-73	72-83
n	2621	824/717	359/294
Early Life Measures			
Weight at Birth	✓		
Method of Infant Feeding	✓		
Weight at 1 Year Old	✓		
Illnesses and Development to Age 5	✓		
Ageing Markers			
Grip Strength		✓	✓
Cognitive Function			
AH4 IQ and Mill Hill vocabulary tests		✓	✓
Abbreviated mental test and MMSE			✓
Skin Thickness		✓	
Eye Examination			
Visual Acuity		✓	✓
Intraoccular Pressure, Macular Degenerations, Lens Opacity		✓	
Audiometry		✓	✓
Falls in the Past Year			✓
Frailty			✓
Medical Characteristics			
Anthropometry			
Height, Weight, Waist and Hip Circumference		✓	✓
Skinfolds (triceps, biceps, subscapular, suprailiac)			✓
Prescribed Medication		✓	✓
Blood Pressure		✓	✓
Blood Samples		✓	✓
Stored for Posterity		✓	✓
Extracted DNA		✓	✓
Cardiovascular Symptoms		✓	✓
Lung Function: FEV and FVC			✓
Respiratory Symptoms		✓	✓
Urine Samples			✓
Hand Examination for Osteoarthritis			✓
Reproductive History		✓	✓
SF36 Self Assessed Health			✓
Lifestyle Characteristics			
Social Class		✓	✓
Diet Questionnaire			✓
Physical Performance			
Questionnaires		✓	✓
Battery of Tests		✓	✓
Occupational History		✓	✓

AH4 (Alice Heims-4), MMSE (Mini mental state examination), FEV (Forced expiratory volume), FVC (Forced vital capacity), SF36 (Short-Form 36).

3.1.2 Statistical Analysis

All analyses were conducted using either Stata 11 (Stata Statistical Software, StataCorp 2009) or PASW version 18.0 (Chicago, IL, USA) and all data are presented as mean ± SD unless otherwise stated. Normality was assessed using Kolmogrov-Smirnov analysis; natural log transformation of distributed variables violating normality was completed. Independent sample T-tests were conducted to assess differences of variables between men and women at baseline and follow-up. Three categorical groups were created based on CMV infection over time and represented as either being negative (CMV-/-), positive (CMV+/+) or having converted from negative to positive (CMV-/+) over time. Data were then analysed using repeated measures ANOVA to assess the effect of time on inflammation and then its interaction with CMV status and gender. Following this, BMI and smoking status were included in analyses as covariates to adjust for potential confounding variables. The assumption of sphericity was tested using Mauchley's method and any violations corrected for using the Greenhouse-Geisser method. Partial η^2 is reported as a measure of effect size. Post-hoc pair wise analysis with Bonferroni correction was conducted to assess inflammatory variable differences over time in each CMV group. Linear regression analysis of the log-normalised data was used to test for significant associations between lifestyle factors either baseline and follow-up and inflammatory mediator's. Individual lifestyle components were entered individually as independent variables (model 1); following this, any significant related lifestyle factors were entered together (model 2) to assess their mutually adjusted associations with cytokine levels. Follow-up time was calculated as the time elapsed in decimal years between the 1994/5 baseline and 2003/5 follow-up clinics. Pearson's correlation coefficients and principal components analysis were used to

explore the interrelationships between biomarkers. Cox's proportional hazards models and logistic regression models were used to analyse associations between the 1994/5 baseline panel of biomarkers and all-cause mortality and between data collection points and Fried frailty at the follow-up, respectively. Analyses were conducted with and without adjustment for the potential confounding effects of gender, follow-up duration (for logistic models for frailty only), and 1994/5 baseline age, height, BMI, smoking status, alcohol consumption, social class in adulthood, walking speed and number of systems medicated. Receiver operating characteristic (ROC) curves were used to explore the ability of the baseline biomarkers to discriminate between frailty at follow-up. Data were principally analysed for men and women combined, but analyses were repeated for men and women separately. A 5% significance level was principally used to identify statistically significant associations, but a Bonferroni correction was also applied to enable identification of significant associations after allowance for multiple comparisons. Statistical significance was accepted at $p<0.05$.

3.2 Results

3.2.1 Participant Characteristics

Participant characteristics are presented in Table 3.2 for the 411 men and 306 women recruited into the study at the baseline (1994/5) clinic. The average age was 67.5 years for both men and women. Typical differences were evident between men and women for height (p<.001) and weight (p<.001) but not BMI (p=.703). Significant gender differences were evident for lifestyle choices. Men smoked more (p=.035), drunk more alcohol (p<.001), travelled more often in cars (p<.001) and carried more heavy loads (p<.001) while women watched more hours of TV (p=.004). Clinical measurements were all within the normal ranges however women had higher ESR, SHBG, TSH and lower free thyroxine (T4) values (all p<.001) than men (Table 3.2). Inflammatory profiles were all within normal ranges and showed men having higher IL-10, TNFα and lower IL-1β (all p<.001) than women. Endocrine profiles revealed a typical profile of men having higher DHEAs concentrations (p<.001) than women which resulted in a lower cortisol:DHEAs ratio (p<.001). Of the participants at baseline, men were at a greater mortality risk [HR: 1.51 (1.03 -2.21), p=.03] than females which was evident by 81 men (19.7%) and 40 women (13.1%) dying before the 2003/5 follow-up clinic.

As the total white cell count (WCC) is made up from the differentials of predominantly neutrophils, monocytes and lymphocytes there were strong inter-correlations ranging from 0.62 to 0.95 between WCC and each of the cell types (all p<.001). Neutrophils and monocytes correlated with each other (r=0.61, p<.001) whilst CRP correlated with WCC, neutrophil, monocytes and ESR (r range = 0.33 to 0.37, p<.001 for all). Cortisol and DHEAs were correlated with each other (r=.091,

p=.016) and the ratio. Cortisol and DHEAs did not correlate with any of the other biomarkers measured (range: *r*=0.01 to 0.18, *p*>.05 for all). Principal component analysis of the measured biomarkers confirmed these associations with the first component accounting for 21% of the variation in the data and was a weighted average of WCC, neutrophils, monocytes, lymphocytes and CRP. The second component accounted for 13% of the variation in the data and was a contrast between cortisol and DHEAs.

Of those individuals measured at baseline, 254 returned (153 men) for assessment at the 2003/5 follow-up and were measured for frailty status (Table 3.3). At follow-up women were more likely to be frail (*p*=.05) with a frailty prevalence of 11.9% compared to 5.2% for men. The average age of men was 66.9 years whilst women were 67.3 years old and were followed up 10.4 and 9.9 years respectively. Similar social, clinical, inflammatory and endocrine characteristics were evident between males and females from this group as those in the baseline assessment (Table 3.2). At follow-up 34% and 29% of men and women were respectively negative for CMV infection while 10% of men and 5% of women had converted to being positive for CMV during the time between measurements.

Inter-correlations between biomarkers were similar in these participants except for cortisol and DHEAs which were independent of each other (*r*=.0.01, *p*=.90) but not the ratio as this is a component of the two. Cortisol and DHEAs did not correlate with any of the other biomarkers measured (range: *r*=0.02 to 0.15, *p*>.05 for all). Principal component analysis of the measured biomarkers confirmed these associations with the first component accounting for 19% of the variation in the data and was a weighted average of WCC, neutrophils, monocytes, lymphocytes and CRP. The

second component accounted for 12% of the variation in the data and was a contrast between cortisol and DHEAs.

Table 3.2: Characteristics of men and women assessed at baseline clinic

	Men (*n* =411)	Women (*n* =306)
Anthropometry		
Age (years)	67.5 (2.4)	67.5 (2.2)
Height (cm)	172.1 (6.6)	159.3 (5.8)***
Weight (kg)	79.8 (12.6)	68.7 (11.7)***
Body Mass Index (kg/m^2)	26.9 (3.6)	27.0 (4.3)
Waist: Hip Ratio	0.94 (0.05)	0.80 (0.05)***
Social Characteristics		
Current Smoker[a]	55 (14)	33 (12)*
Weekly Moderate or High Alcohol Intake[a,b]	90 (25)	21 (7)***
Alcohol (Units per week)[d]	3.0 (0.3, 11.0)	0.0 (0.0, 3.0)***
Non-manual Social Class[a,c]	147 (41)	120 (43)
Fairly Brisk of Fast Walking Pace[a]	99 (28)	60 (21)
Weekly Walking Total (Hrs)[d]	75.0 (45, 120)	72.5 (45, 120)
Daily TV Watching (Hrs)[d]	3.0 (2.0, 4.0)	3.0 (2.0, 4.0)**
Climbing Stairs Daily or Several[a, e]	302 (85)	224 (80)
Regular Car Travel[a, f]	219 (55)	85 (28)***
Carrying Heavy Loads Daily or Several[a,g]	68 (19)	15 (5)***
Immune Composition		
White Cell Count (x10^9.L^{-1})[d]	5.6 (4.7, 6.6)	5.6 (4.6, 6.5)
Neutrophils (x10^9.L^{-1})[d]	3.4 (2.8, 4.3)	3.4 (2.7, 4.3)
Monocytes (x10^9.L^{-1})[d]	0.4 (0.3, 0.5)	0.4 (0.3, 0.4)
Lymphocytes (x109.L-1)[d]	1.6 (1.3, 2.0)	1.8 (1.4, 2.0)
Clinical		
ESR (mm.hr^{-1})[d]	6 (4, 10)	12 (6, 20)***
Albumin (g.L^{-1})	42.4 (2.0)	42.4 (2.0)
SHBG (nmol.L^{-1})[d]	39.0 (29.1, 50.2)	52.6 (38.4, 79.3)***
Testosterone (nmol.L^{-1})[d]	16.0 (11.8, 20.3)	no data
Haemoglobin (g.L^{-1})	14.5 (1.0)	13.9 (0.9)
TSH (µU.L^{-1})[d]	1.6 (1.1, 2.3)	2.0 (1.4, 3.1)***
T4 (pmol.L^{-1})[d]	14.6 (13.5, 16.0)	13.8 (12.6, 15.5)***
Number of Systems Medicated[d]	1 (0, 2)	1 (0, 2)
Inflammation		
IL-1ß (pg.ml^{-1})[d]	11.3 (5.6, 19.6)	16.3 (10.9, 24.0)***
IL-6 (pg.ml^{-1})[d]	1.2 (0.3)	0.9 (0.4, 1.9)
IL-10 (pg.ml^{-1})[d]	2.9 (2.5, 4.7)	1.9 (0.3, 2.3)***
TNFα (pg.ml^{-1})[d]	0.8 (0.3, 1.0)	0.5 (0.3, 1.4)***
IFNγ (pg.ml^{-1})[d]	2.3 (0.0, 8.8)	3.9 (0.0, 11.3)
CRP (mg.L^{-1})[d]	2.2 (1.1, 4.2)	2.6 (1.0, 5.0)
Endocrine		
Cortisol (nmol.L^{-1})[d]	300 (232, 372)	278 (219, 355)
DHEAs (nmol.L^{-1})[d]	2231 (1737, 2895)	1418 (825, 1418)***
Cortisol: DHEAs ratio[d]	0.12 (0.09, 0.18)	0.19 (0.11, 0.35)***
CMV Status		
Seronegative[a]	151 (38)	100 (34)
Seropositive[a]	242 (62)	198 (66)
Seroconverted[a]	N/A	N/A

Data are mean (SD) unless indicated. [a]Number and percentage; [b]Defined as weekly alcohol consumption of ≥ 11 units for men and ≥ 8 units for women; [c]Defined as classes I, II, IIINM of the 1990 OPCS coding of social class; [d]Variable was positively skewed; median and inter-quartile range shown; [e]Defined as those who climb stairs daily or several times per day as opposed to never, occasionally or once a week; [f]Assessed as those who regularly travel in a car; [g]Defined as those who carry heavy loads either daily or several times per day; [h]Assessed as either being negative or positive at both baseline and follow-up or having converted from negative to positive from baseline to follow-up. *p<.05, **p<.01, ***p<.001 different from males

Table 3.3: Baseline characteristics of participants assessed at follow-up clinic

	Men (*n* =153)	Women (n=101)
Anthropometry		
Age (years)	66.9 (2.2)	67.3 (2.1)
Height (cm)	172.6(6.3)	159.8 (5.1)***
Weight (kg)	80.4 (2.2)	69.2 (10.6)***
Body Mass Index (kg/m^2)	26.9 (3.5)	27.0 (3.9)
Waist: Hip Ratio	0.93 (0.05)	0.80 (0.05)***
Social Characteristics		
Current Smoker[a]	19 (12.4)	9 (8.9)*
Weekly Moderate or High Alcohol Intake[a,b]	40 (26.1)	9 (8.9)***
Alcohol (Units per week)[d]	3.0 (0.2, 10.2)	0.3 (0.0, 3.0)***
Non-manual Social Class[a,c]	73 (48.3)	43 (43.4)
Fairly Brisk of Fast Walking Pace[a]	42 (27.5)	26 (25.7)
Weekly Walking Total (Hrs)[d]	72.5 (45, 120)	72.5 (40, 110)
Daily TV Watching (Hrs)[d]	3.0 (2.0, 4.0)	3.5 (2.0, 5.0)***
Climbing Stairs Daily or Several[a, e]	133 (86)	92 (87)
Regular Car Travel[a, f]	85 (55)	26 (25)***
Carrying Heavy Loads Daily or Several[a,g]	34 (22)	3 (3)***
Follow-Up Time (years)[d]	10.4 (10.2, 10.5)	9.9 (9.8, 10.1)
Fried Frailty[a,h]	8 (5.2)	12 (11.9)*
Immune Composition		
White Cell Count (x10^9.L^{-1})[d]	5.5 (4.8, 6.6)	5.5 (4.7, 6.5)
Neutrophils (x10^9.L^{-1})[d]	3.4 (2.8, 4.0)	3.2 (2.5, 4.0)
Monocytes (x10^9.L^{-1})[d]	0.4 (0.3, 0.5)	0.3 (0.3, 0.4)
Lymphocytes (x109.L-1)[d]	1.6 (1.3, 2.0)	1.7 (1.4, 2.1)
Clinical		
ESR (mm.hr^{-1})[d]	6 (4, 10)	14.0 (8, 20)***
Albumin (g.L^{-1})	42.7 (2.0)	42.3 (2.0)
SHBG (nmol.L^{-1})[d]	36.3 (27.9, 48.3)	51.2 (34.2, 79.2)**
Testosterone (nmol.L^{-1})[d]	16.3 (11.1, 20.3)	no data
Haemoglobin (g.L^{-1})	14.5 (1.0)	13.5 (0.9)
TSH (µU.L^{-1})[d]	1.7 (1.2, 2.3)	2.1 (1.5, 3.3)**
T4 (pmol.L^{-1})[d]	14.2 (13.2, 15.6)	13.7 (12.6, 15.3)
Number of Systems Medicated[d]	1 (0, 2)	1 (0, 2)
Inflammation		
IL-1β (pg.ml^{-1})[d]	11.3 (5.6, 17.7)	18.3 (11.7, 26.2)***
IL-6 (pg.ml^{-1})[d]	1.2 (0.2, 2.0)	0.9 (0.4, 2.0)
IL-10 (pg.ml^{-1})[d]	2.3 (2.5, 4.7)	1.9 (0.3, 2.1)***
TNFα (pg.ml^{-1})[d]	0.25 (1.3, 4.7)	0.82 (1.3, 3.7)**
IFNγ (pg.ml^{-1})[d]	2.3 (1.7, 110)	5.9 (2.6, 171)
CRP (mg.L^{-1})[d]	1.9 (0.9, 4.0)	3.1 (1.1, 5.6)**
Endocrine		
Cortisol (nmol.L^{-1})[d]	308 (235, 385)	274 (210, 349)*
DHEAs (nmol.L^{-1})[d]	2181 (1734, 2888)	1495 (797, 2261)***
Cortisol: DHEAs ratio[d]	0.12 (0.09, 0.17)	0.19 (0.10, 0.38)***
CMV Status		
Seronegative[a,h]	50 (34)	31 (29)
Seropositive[a,h]	83 (56)	65 (64)
Seroconverted[a,h]	15 (10)	5 (5)

Values are mean (SD) unless indicated. [a]Number and percentage. [b]Defined as weekly alcohol consumption of ≥ 11 units for men and ≥ 8 units for women. [c]Defined as classes I, II, IIINM of the 1990 OPCS coding of social class. [d]Variable was positively skewed; median (25th and 75th percentile). [e]Defined as those who climb stairs either daily or several times per day as opposed to never, occasionally or once a week. [f]Assessed as those who regularly travel in a car as opposed to not regularly travelling by car. [g]Defined as those who carry heavy loads either daily or several times per day. [h]Assessed at the follow-up clinic. *p<.05, **p<.01, ***p<.001 different from males.

3.2.2 Evidence for Inflammageing

Analysis of serum CRP and pro- and anti-inflammatory cytokines revealed a profile of increased systemic inflammation during ageing in line with previous observations [254]. There were significant main effects of ageing for IL-6, TNFα, CRP, IL-10 and IL-1β (Fig. 3.2). IL-6 (Fig. 3.2A) concentrations increased 2.3-fold over time [$F_{(1,154)}$ = 117.3, p<.001; η^2=.432], whilst TNFα (Fig. 3.2B) showed a 4.3-fold increase over the 10-year period [$F_{(1,104)}$ = 126.5, p<.001; η^2=.549]. CRP (Fig. 3.2C) levels increased modestly 1.2-fold from baseline to follow-up [$F_{(1,240)}$ = 5.0, p=.026; η^2=.021] and IL-1β (Fig. 3.2F) decreased modestly by from baseline to follow-up [$F_{(1,147)}$ = 10.46, p=.002; η^2=.066]. IFNγ (Fig. 3.2D) did not change over time [$F_{(1,199)}$ = 1.7, p=.188; η^2=.009]. In addition levels of the anti-inflammatory cytokine IL-10 (Fig. 3.2E) were reduced by 65% over the 10-year period [$F_{(1,53)}$ = 42.8, p<.001; η^2=.447].

3.2.3 Impact of Cytomegalovirus Infection on Inflammageing

The analysis was repeated for the three different CMV groupings: those who were CMV seronegative at both time points (CMV -/-), those who were seronegative at baseline and converted during the 10 year period (CMV -/+) and those who were seropositive at both time points (CMV +/+). Similar increases were found in both IL-6 (Fig. 3.3A) and TNFα (Fig. 3.3B) between baseline and follow-up in all three groups. IL-6 showed a 2.4-, 2.2- and 2.4-fold increase in the CMV-/+ (p=.03), CMV-/- (p<.001) and CMV+/+ (p<.001) groups, respectively. There was a corresponding 6.0, 4.2- and 4.1-fold increase in TNFα in the CMV-/+, CMV-/- and CMV+/+ groups respectively (all p<.001). CRP (Fig. 3.3C) concentrations did not change over time in the CMV-/+ (p=.541) or the CMV-/- (p=.736) but did increase significantly in the CMV+/+ (p=.004). In contrast IL-1β (Fig. 3.3F) concentrations did not change over time in the CMV-/+ (p=.975) or the CMV-/- (p=.517) but did decrease significantly in

the CMV+/+ (p<.001). IL-10 (Fig. 3.3E) concentrations were reduced 0.8- and 0.5-fold in the CMV-/- (p=.001) and the CMV+/+ (p=.01) groups respectively, and a trend towards a reduction in the CMV-/+ (p=.07) was seen. Concentrations of IFNγ (Fig. 3.3D) did not change over the 10-year period in the group as a whole (p=.188) and no change was seen in any of the three CMV groupings, CMV-/+ (p=.928), CMV-/- (p=.401) and CMV+/+ (p=.474).

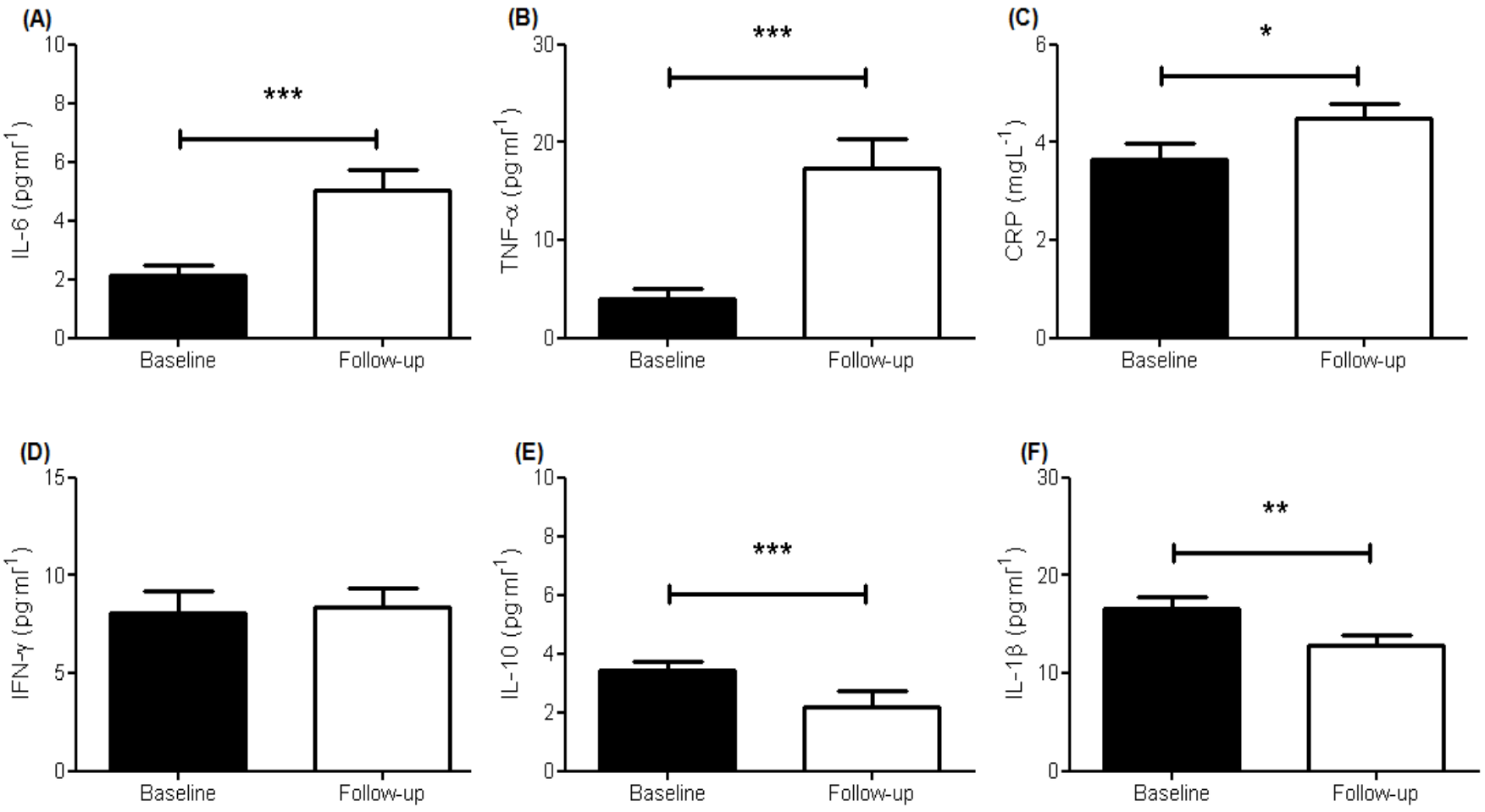

Fig. 3.2: Evidence of Inflammageing. Cytokines IL-6 (A), TNFα (B), IFNγ (D), IL-10 (E) and IL-1β (F) and CRP (C) in serum at baseline and 10 year follow-up in participants from the Hertfordshire Ageing Study. Data are mean ± S.E.M. analysed by mixed model repeated measures ANOVA. *p<.05, **p<.01, ***p<.001 for Baseline compared with Follow-up.

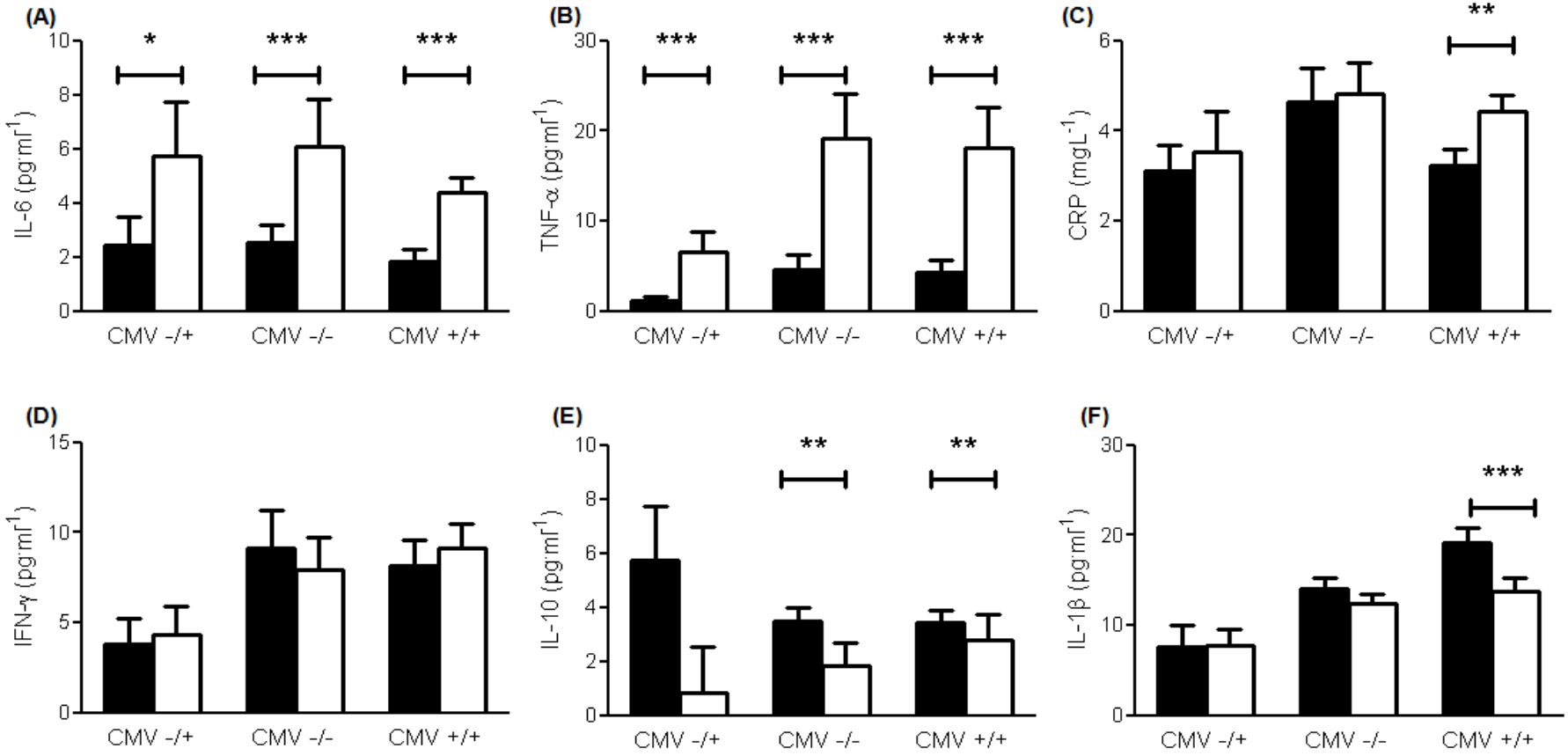

Fig. 3.3: CMV and Inflammageing. Impact of CMV serostatus and ageing on serum cytokine and CRP concentrations. At baseline (Black Bars) and 10-year follow-up (White Bars) participants were grouped by CMV serostatus as either negative at baseline and positive at follow-up (CMV-/+), negative at both time points (CMV-/-) or positive at both time points (CMV+/+). Serum IL-6 (A), TNFα (B), CRP (C), IFNγ (D) , IL-10 (E) and IL-1β (F), were measured at baseline and 10 year follow up. Data are mean ± S.E.M. analysed by mixed model repeated measures ANOVA with Bonferroni corrected pair wise comparisons. **p*<.05, ***p*<.01, ****p*<.001 for Baseline compared with Follow-up.

3.2.4 Evidence for Adrenopause with Ageing

Endocrine dysfunction with age is associated with an imbalance of anti-inflammatory cortisol and the immune enhancing androgen DHEAs. The ratio of cortisol to DHEAs has been previously associated with morbidity and mortality in the elderly and therefore may play a role in systemic inflammation. As CMV infection was found not to be driving systemic inflammation its relationship with cortisol and DHEAs was determined. Interestingly, CMV is known to infect functional adrenocortical cells causing an increased and persistent production of cortisol. It is suggested that CMV increases cortisol in order to reduce the immune response against it and also to propagate replication.

Analysis of serum cortisol and DHEAs revealed a profile of altered adrenal cortex function during ageing in line with previous observations (Fig. 3.4). There were significant main effects of ageing for concentrations of cortisol and DHEAs and the ratio of cortisol:DHEAs. Cortisol (Fig. 3.4A) concentrations increased by 29% over time [$F_{(1,263)}$ = 48.08, p<.001; η^2=.155] whilst DHEAs (Fig. 3.4B) concentrations decreased by 21% over time [$F_{(1,259)}$ = 14.80, p<.001; η^2=.054]. At both time points males had higher DHEAs concentrations than females (p<.001) and subsequently the reduced DHEAs concentrations were driven by male participants. Because of these opposite effects over time the ratio of cortisol:DHEAs (Fig. 3.4C) increased by 179% over the 10-year time period [$F_{(1,259)}$ = 7.80, p=.006; η^2=.029].

3.2.4 Impact of Cytomegalovirus Infection on Adrenopause

When the analysis was repeated for the three CMV groupings similar patterns of change for cortisol (Fig. 3.4D), DHEAs (Fig. 3.4E) and the ratio of cortisol:DHEAs (Fig. 3.4F) were observed. Cortisol showed a 30%, 37% and 24% increase in the CMV-/+, CMV-/- and CMV+/+ groups respectively (all p<.001). There was a

corresponding 32%, 32% and 13% decrease in DHEAs concentrations in the CMV-/+, CMV-/- and CMV+/+ groups respectively (all $p<.001$). Consequently, cortisol:DHEAs ratios increased by 81%, 150% and 202% for the CMV-/+ ($p=.009$), CMV-/- ($p<.001$) and CMV+/+ ($p<.001$) groups respectively.

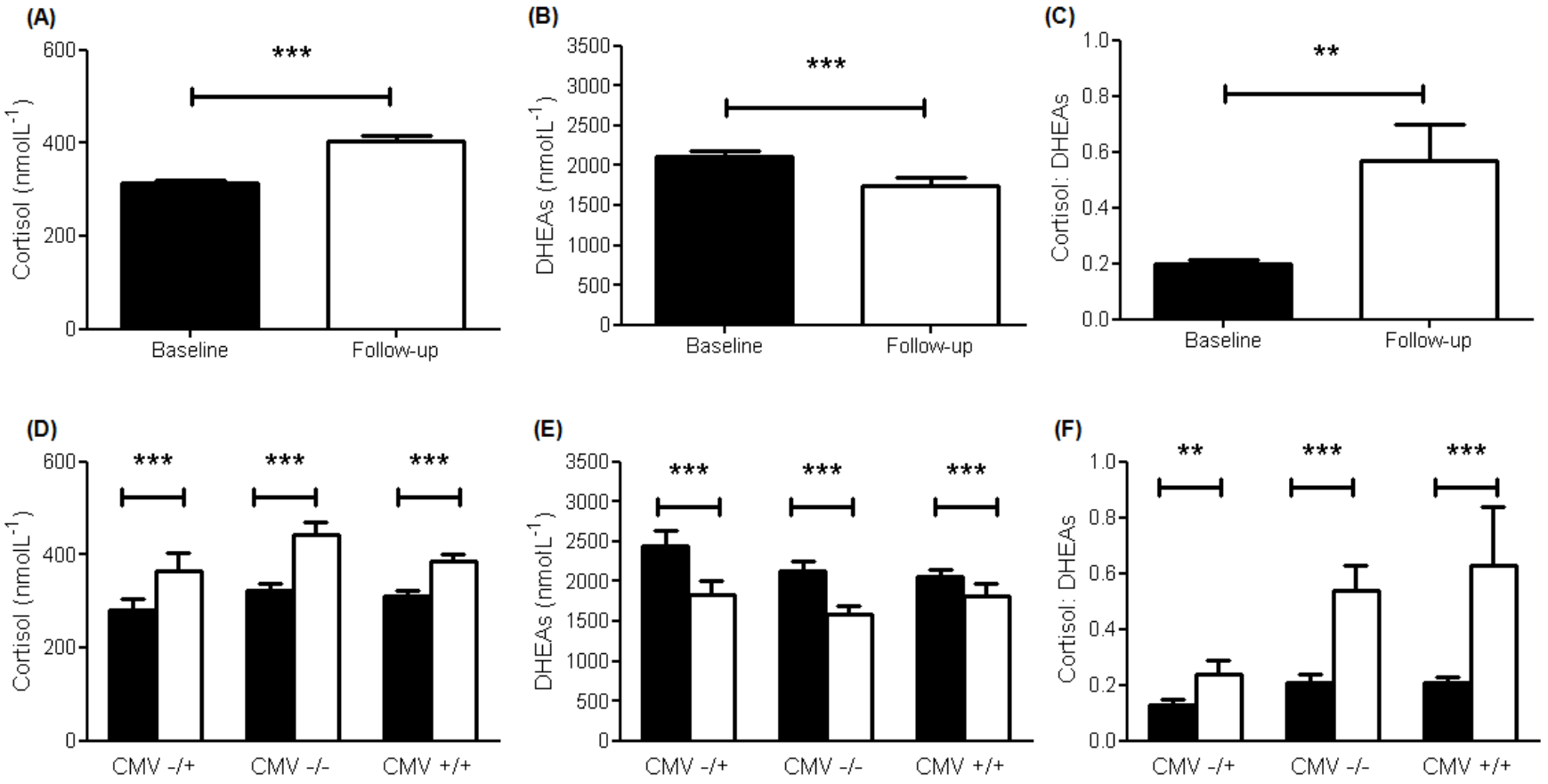

Fig. 3.4: Adrenopause and CMV Infection. Impact of ageing and CMV serostatus on serum cortisol and DHEAs concentrations. Serum concentrations of cortisol (A), DHEAs (B) and the cortisol:DHEAs ratio (C) were determined at baseline (Black Bars) and 10-year follow-up (White Bars). Participants were grouped by CMV serostatus as either negative at baseline and positive at follow-up (CMV-/+), negative at both time points (CMV-/-) or positive at both time points (CMV+/+) for Cortisol (D), DHEAs (E), or the ratio of cortisol to DHEAs (F). Data are mean ± S.E.M. analysed by mixed model repeated measures ANOVA with Bonferroni corrected pair wise comparisons. **p<.01, ***p<.001 for Baseline compared with Follow-up.

3.2.5 Impact of Behavioural Lifestyle Choices on Inflammageing

As CMV did not appear to be driving systemic inflammation or adrenal alterations over time in the elderly, behavioural lifestyle choices were assessed for associations with observed changes in inflammation and adrenal function. Table 3.4 highlights the relationship between baseline lifestyle choices, social status, body composition and baseline serum IL-6, CRP, TNFα, IL-1β, IL-10 and the cortisol:DHEAs ratio. No associations were evident for IFN-γ (data not shown). Of all the independent variables assessed only social class did not influence any of the biological markers assessed suggesting that unlike the prevalence of a number of diseases, socioeconomic status does not have an influence on inflammageing. The data suggest that increased CRP levels were associated with lifestyle factors consistent with sedentary behaviours such as more hours watching TV (p<.001) and less walking distance (p<.001), as well as an unhealthy lifestyle consisting of smoking (p=.025) and being overweight [increased BMI and waist to hip ratio (both p<.001)]. Following mutually adjusted analyses all of these lifestyle factors remained significant except for waist:hip ratio. Around 13% of the increase in CRP concentrations can be attributed to being overweight, reduced walking speed and increased hours watching TV. These data suggest that both body composition and physical activity levels account for raised CRP concentrations in elderly individuals.

CRP is synthesised in response to IL-6 production, in this model IL-6 concentrations were positively associated with BMI (p=.004) highlighting its relationship with body composition and CRP production. TNFα concentrations were associated with more hours watching TV (p=.016) and a lack of climbing stairs regularly (p=.032), which after mutual adjustment remained significant. These data suggest that increased TNFα concentrations in healthy elderly are mainly associated with physical inactivity

and less so with body composition. IL-1β had an unexpected association which implied that drinking (p=.016) may protect against increased concentrations whilst having an increased waist:hip ratio (p<.001) was also associated with reduced concentrations. However, physical inactivity was associated with increased concentrations of IL-1β indicated by more travelling in cars and increased hours watching TV (both p<.001). Following mutual adjustment both physical inactivity associations remained highly significant whilst waist:hip ratio was severely attenuated (p=.043) and alcohol drinking was completely attenuated.

Interestingly unadjusted analysis of IL-10 concentrations revealed that waist:hip ratio (p<.001), alcohol consumption (p=.001) and carrying heavy loads (p=.003) were positively associated with increased concentrations whilst watching more TV was negatively associated. These data would suggest that IL-10 concentrations would be reduced in physically inactive individuals but be increased if they were overweight. Following mutual adjustment, physical inactivity measures were attenuated and were not significant (p=.838 and p=.811 respectively) whilst waist:hip ratio (p<.001) remained significant suggesting IL-10 is influenced more by body composition in the elderly than physical activity levels.

The ratio of cortisol:DHEAs has been suggested to be a good predictor of morbidity in both young and elderly individuals. The cortisol:DHEAs ratio was influenced by both body composition and physical activity levels. Increased ratios were associated with those that watched more TV (p=.039) and carried few heavy loads (p<.001), whilst lower ratios were observed in those who smoked (p=.001), drank more alcohol (p=.007) and had increased waist:hip ratios (p<.001). Increased alcohol intake (p=.791) and watching TV (p=.094) were attenuated with mutual adjustment analysis and lost significance, but the relationship with cortisol:DHEAs ratio remained

significant for carrying loads (p=.025), smoking (p=.008) and waist: hip ratio (p<.001).

Taken together it is clear that at an average age of 67 ± 2 years leading a sedentary lifestyle accounts for heightened systemic inflammation and reduced anti-inflammatory potential. The R^2 values in Table 3.2 show that mutually associated sedentary lifestyle choices and body composition contributed to around 2%, 13%, 2% and 7% of elevations in inflammatory IL-6, CRP, TNFα and IL-1β respectively. Almost 20% of reduced anti-inflammatory potential (IL-10) was associated with sedentary lifestyles while also accounting for 2% of the elevations in the cortisol:DHEAs ratio.

Table 3.5 highlights the relationship between baseline behavioural lifestyle choices, social status, body composition and the 10-year follow-up serum concentrations of IL-6, CRP, TNFα, IL-1β, IL-10 and the cortisol:DHEAs ratio. As previously shown ageing was associated with increased levels of IL-6, CRP, TNFα and cortisol:DHEAs ratio, whilst IL-10 and IL-1β were reduced in those who were assessed at both time points. Therefore, baseline biomarker values were included in the model to eliminate the bias that having higher concentrations at baseline resulted in higher concentrations at the 10-year follow-up.

No associations were evident for IFN-γ (data not shown). CRP was elevated 10-years after baseline if participants had a higher BMI (p=.007) and drunk more alcohol (p=.008). Following mutually adjusted analysis it was revealed that CRP increases in later life were mainly driven by body composition and is most likely a reflection of elderly individuals becoming more inactive. Interestingly IL-6 had a similar pattern of association as before, relating to BMI (p=.018) along with CRP, however those that

carried more heavy loads were likely to have reduced IL-6 concentrations (p=.032). Associations remained following mutual adjustment suggesting that being active in younger life can lower the risk of elevated IL-6 in later life. The fact there was no association with CRP and load carrying suggests that the reduced IL-6 levels associated with load carrying is enough to impact the IL-6 stimulation of CRP.

No associations were observed for TNFα however there was a trend for individuals who drank more alcohol to have higher TNFα (p=.073) whilst IL-1β was positively associated with smoking (p=.041). Although at baseline individuals who had higher waist:hip ratios were likely to have elevated IL-10 concentrations this association was lost at follow-up (p=.771), instead those who travelled more in cars (p=.006) were more likely to have reduced IL-10 concentrations. The cortisol:DHEAs ratio showed no association of baseline lifestyle factors affecting the future concentrations.

Although these data could not assess whether those who completed the follow-up assessment maintained their physical activity parameters during the ten year period it is clear that behavioural lifestyle choices can significantly influence systemic inflammation ten years later. The R^2 values in Table 3.5 show that mutually associated sedentary lifestyle choices and body composition at baseline contributed to around 3%, 4% and 2% of future elevated IL-6, CRP and IL-1β concentrations respectively. Almost 10% of reduced anti-inflammatory potential (IL-10) at follow-up was associated with travelling more in a car 10-years earlier. Taken together the data in Tables 3.4 and 3.5 suggest that leading a sedentary lifestyle can account for considerable proportions of future heightened systemic inflammation and reduced anti-inflammatory potential.

Table 3.4: Lifestyle factors and inflammation. Relationship between lifestyle factors at baseline and the level of serum inflammatory, anti-inflammatory and endocrine biomarkers at baseline for the 717 HAS participants.

		CRP				IL-6				TNF-α				IL-1β				IL-10				Cortisol: DHEAs			
Model	Predictor	β	t	*p*	R^2	β	t	*p*	R^2	β	t	*p*	R^2	β	t	*p*	R^2	β	t	*p*	R^2	β	t	*p*	R^2
1	Body Mass Index (kg/m$^{2)}$	**.295**	**7.662**	**<.001**	**.085**	**.146**	**2.920**	**.004**	**.019**	.006	.136	.892	-.002	.029	.619	.536	-.001	-.085	-1.885	.060	.005	.039	.981	.327	.000
	Waist: Hip Ratio	**.091**	**2.260**	**.024**	**.007**	.053	1.054	.292	.000	-.035	-.731	.461	-.001	**-.164**	**-3.576**	**<.001**	**.025**	**.443**	**10.883**	**<.001**	**.194**	**-.224**	**-5.773**	**<.001**	**.049**
	Alcohol (grouped)	.027	.672	.502	-.001	-.064	-1.279	.202	-.002	-.039	-.827	.409	-.001	**-.111**	**-2.416**	**.016**	**.010**	**.145**	**3.260**	**.001**	**.019**	**-.106**	**-2.703**	**.007**	**.010**
	Smoking	**.090**	**2.255**	**.025**	**.007**	.000	-.004	.997	-.003	.000	.002	.998	-.002	.005	.116	.907	-.002	.072	1.589	.113	.003	**-.132**	**-3.342**	**.001**	**.016**
	Social Class	.074	1.838	.067	.004	-.022	-.435	.663	-.002	.001	.020	.984	-.002	-.022	-.462	.644	.002	-.012	-.265	.791	-.002	-.034	-.852	.395	.000
	Total Walking	**-.126**	**-3.169**	**.002**	**.014**	-.034	-.678	.498	-.001	.019	.407	.684	-.002	.047	1.012	.312	.000	.040	.877	.381	.000	-.014	-.341	.733	.001
	Car Travel	-.028	.687	.492	-.001	-.093	-1.840	.066	.006	.032	.673	.501	-.001	**.176**	**3.884**	**<.001**	**.029**	**-.149**	**-3.337**	**.001**	**.020**	-.060	1.512	.131	.002
	Hours Watching TV	**.155**	**3.915**	**<.001**	**.023**	.067	1.338	.182	-.002	**.114**	**2.419**	**.016**	**.011**	**.162**	**3.543**	**<.001**	**.024**	-.081	-1.800	.072	.005	**.082**	**2.066**	**.039**	**.005**
	Climbing Stairs	.031	.777	.437	-.001	-.028	-.554	.580	-.002	**.101**	**2.145**	**.032**	**.008**	.048	1.033	.302	.000	-.035	-.773	.440	-.001	.016	-.415	.678	.001
	Carrying Heavy Loads	.019	.472	.637	-.001	.009	.177	.860	-.002	.002	.041	.967	-.002	.001	.011	.991	-.002	**.136**	**3.025**	**.003**	**.016**	**-.146**	**-.3716**	**<.001**	**.020**
2	Body Mass Index (kg/m$^{2)}$	.285	7.129	<.001																					
	Waist: Hip Ratio	.027	.659	.510										-.101	-2.026	.043		.445	9.856	<.001		-.171	-3.956	<.001	
	Alcohol (grouped)													-.033	-.676	.500		-.015	.217	.828		.018	.265	.791	
	Smoking	.096	2.418	.016																		-.108	-2.673	.008	
	Social Class																								
	Total Walking	-.102	-.2680	.008																					
	Car Travel													.134	2.853	.005		.008	.205	.838					
	Hours Watching TV	.113	2.914	.004	.126					.113	2.420	.016		.137	2.994	.003	.066					.067	1.677	.094	
	Climbing Stairs									.101	2.147	.032	.023												
	Carrying Heavy Loads																	.010	.240	.811	.196	-.091	-2.240	.025	.071

Linear regression analysis of the log-normalised data was used to test for significant associations between lifestyle factors at baseline sampling and the level of baseline inflammatory, anti-inflammatory and endocrine biomarkers. Individual lifestyle components were entered individually as independent variables (model 1); following this, any significant related lifestyle factors were entered together (model 2) to assess their mutually adjusted associations with cytokine levels. Values which are highlighted and bold represent significant associations.

Table 3.5: Lifestyle factors and future inflammation. Relationship between lifestyle factors at baseline and the level of serum inflammatory, anti-inflammatory and endocrine biomarkers at follow-up for the 254 HAS participants returning.

		CRP				IL-6				TNF-α				IL-1β				IL-10				Cortisol: DHEAs			
Model	Predictor	β	t	*p*	R^2	β	t	*p*	R^2	β	t	*p*	R^2	β	t	*p*	R^2	β	t	*p*	R^2	β	t	*p*	R^2
1	Body Mass Index (kg/m$^{2)}$	**.167**	**2.724**	**.007**	**.028**	**.147**	**2.382**	**.018**	**.022**	-.048	-.578	.564	.002	.066	.922	.357	.004	.024	.208	.836	.001	.042	.671	.499	.002
	Waist: Hip Ratio	.046	.736	.462	.002	.007	.117	.907	.000	.007	.088	.930	.000	.051	.715	.476	.003	.034	.292	.771	.001	-.051	-.814	.417	.003
	Alcohol (grouped)	**.165**	**2.692**	**.008**	**.027**	-.001	-.021	.984	.000	.149	1.805	.073	.022	.011	.152	.879	.000	.018	.154	.878	.000	-.019	.301	.763	.000
	Smoking	.076	1.230	.220	.006	.081	1.312	.191	.007	.069	.825	.411	.005	**.146**	**2.061**	**.041**	**.021**	-.082	-.709	.480	.007	-.056	-.907	.365	.003
	Social Class	.025	.392	.695	.001	.026	.417	.677	.001	-.079	-.950	.344	.006	-.016	-.215	.830	.000	-.138	-1.192	.237	.019	-.043	-.682	.496	.002
	Total Walking	.008	.133	.895	.000	.067	1.071	.285	.004	-.022	-.262	.794	.000	-.081	-1.141	.255	.007	.015	.133	.895	.000	-.040	-.644	.520	.002
	Car Travel	-.027	-.439	.661	.001	.055	.879	.380	.003	-.039	-.472	.638	.002	.028	.387	.699	.001	**-.313**	**-2.834**	**.006**	**.098**	-.021	-.334	.739	.000
	Hours Watching TV	.082	1.327	.186	.007	.102	1.646	.101	.010	.027	.325	.745	.001	.102	1.437	.152	.010	.168	1.466	.147	.028	.092	1.477	.141	.008
	Climbing Stairs	.014	.222	.825	.000	.036	.571	.569	.001	.123	1.483	.140	.015	.011	.155	.877	.000	-.005	-.039	.969	.000	.081	1.300	.195	.007
	Carrying Heavy Loads	-.051	-.817	.415	.003	**-.133**	**-2.158**	**.032**	**.018**	-.054	-.649	.518	.003	-.050	-.696	.487	.002	.128	1.110	.271	.016	.017	.270	.787	.000
2	Body Mass Index (kg/m$^{2)}$	.163	2.673	.008		.144	2.353	.019																	
	Waist: Hip Ratio																								
	Alcohol (grouped)	.176	1.731	.085	.043																				
	Smoking																								
	Social Class																								
	Total Walking																								
	Car Travel																								
	Hours Watching TV																								
	Climbing Stairs																								
	Carrying Heavy Loads					-.130	-2.128	.034	.031																

Linear regression analysis of the log-normalised data was used to test for significant associations between lifestyle factors at baseline sampling and the level of follow-up inflammatory, anti-inflammatory and endocrine biomarkers. Lifestyle components were entered individually as independent variables (model 1); following this, any significant related lifestyle factors were entered together (model 2) to assess their mutually adjusted associations with cytokine levels. Values which are highlighted and bold represent significant associations.

3.2.6 Associations with Inflammation, Adrenopause and Frailty

To assess the consequences of systemic inflammation and adrenopause on the health of the elderly participants the baseline inflammatory associations with prevalence of frailty and all-cause mortality were assessed for the cohort. Determination of frailty in this cohort has been described previously [134].

The associations between the biological markers at baseline and frailty status at follow-up are outlined in Table 3.6. Unadjusted analyses of the increase [OR (95% CI) and *p* value] for WCC [2.51 (1.49, 4.24), *p*=.001], ESR [2.23 (1.28, 3.88), *p*=.005], neutrophils [2.17 (1.29, 3.65), *p*=.004], monocytes [3.22 (1.80, 5.74), *p*<.001], lymphocytes [2.00 (1.23, 3.25), *p*=.005], T4 [1.86 (1.11, 3.13), *p*=.002] and the cortisol:DHEAs ratio [1.78 (1.17, 2.72), *p*=.007] as well as a decrease in DHEAs [2.04 (1.30, 3.23), *p*=.002] levels were all associated with increased risk of being frail 10-years later. CMV titre has been previously associated with progression to frailty; however there was no association here. Following Bonferroni correction (all *p*<.005) increases in WCC, monocytes and the reduction in DHEAs remained associated with frailty. Results were unaltered by adjustment for gender, baseline age and time between measurements. Results for ESR (*p*=.054) and T4 (*p*=.07) were attenuated by adjusting for height, weight, BMI, smoking, alcohol, social class and walking speed. Although results for neutrophils (*p*=.068) were attenuated by adjustment for co-morbidities, white cell count (*p*=.019), monocytes (*p*=.001), lymphocytes (*p*=.016), DHEAs (*p*=.023) and cortisol:DHEAs (*p*=.037) remained significantly associated with frailty 10-years after baseline clinical assessment.

Figure 3.5 shows the associations of white cell count (Fig. 3.5A), neutrophils (Fig. 3.5B), monocytes (Fig. 3.5C), lymphocytes (Fig. 3.5D), DHEAs (Fig. 3.5E) and

cortisol:DHEAs (Fig. 3.5F) when split into tertiles and assessed against the prevalence of frailty, revealing the clear associations with elevated biological blood markers and frailty in older age.

Table 3.6: Inflammation and frailty. Associations between Baseline biomarkers and Fried frailty status at Follow-Up for the 254 HAS participants returning.

Biomarker	OR[a]	95% CI	*p*	OR[b]	95% CI	*p*	OR[c]	95% CI	*p*	OR[d]	95% CI	*p*
Immune Composition												
White Cell Count ($x10^9 \cdot L^{-1}$)	2.51	(1.49, 4.24)	.001	2.85	(1.60, 5.07)	<.001	2.36	(1.24, 4.51)	.009	2.22	(1.14, 4.33)	.019
Neutrophils ($x10^9 \cdot L^{-1}$)	2.17	(1.29, 3.65)	.004	2.42	(1.37, 4.25)	.002	1.92	(1.05, 3.52)	.035	1.83	(0.96, 3.52)	.068
Monocytes ($x10^9 \cdot L^{-1}$)	3.22	(1.80, 5.74)	<.001	4.11	(2.11, 7.99)	<.001	4.23	(1.94, 9.22)	<.001	3.88	(1.73, 8.69)	.001
Lymphocytes (x109.L-1)	2.00	(1.23, 3.25)	.005	2.07	(1.26, 3.40)	.004	2.00	(1.14, 3.53)	.016	2.09	(1.15, 3.79)	.016
Clinical												
CMV Titre (Au)	1.10	(0.69, 1.75)	.702	0.99	(0.61, 1.59)	.956	0.96	(0.59, 1.58)	.891	0.92	(0.57, 1.56)	.910
ESR ($mm \cdot hr^{-1}$)	2.23	(1.28, 3.88)	.005	2.36	(1.29, 4.30)	.005	2.04	(0.99, 4.21)	.054	1.77	(0.85, 3.65)	.125
Albumin ($g \cdot L^{-1}$)	0.70	(0.44, 1.10)	.123	0.66	(0.41, 1.06)	.085	0.59	(0.34, 1.02)	.058	0.48	(0.26, 0.91)	.024
SHBG ($nmol \cdot L^{-1}$)	0.98	(0.63, 1.52)	.936	0.90	(0.57, 1.40)	.631	0.89	(0.51, 1.55)	.678	0.88	(0.48, 1.61)	.688
Testosterone ($nmol \cdot L^{-1}$)[e]	1.54	(0.70, 3.42)	.285	1.06	(0.44, 2.56)	.892	4.06	(0.81, 20.39)	.089	5.42	(0.95, 30.87)	.057
Haemoglobin ($g \cdot L^{-1}$)	0.84	(0.52, 1.37)	.495	0.84	(0.50, 1.42)	.522	0.69	(0.39, 1.22)	.203	0.82	(0.46, 1.48)	.515
TSH ($\mu U \cdot L^{-1}$)	0.75	(0.48, 1.17)	.202	0.72	(0.45, 1.16)	.178	0.73	(0.42, 1.26)	.254	0.68	(0.39, 1.20)	.186
T4 ($pmol \cdot L^{-1}$)	1.86	(1.11, 3.13)	.019	2.08	(1.16, 3.72)	.014	1.78	(0.95, 3.32)	.070	1.41	(0.74, 2.67)	.296
Inflammation												
IL-1β ($pg \cdot ml^{-1}$)	0.92	(0.51, 1.63)	.764	0.94	(0.51, 1.72)	.839	0.81	(0.43, 1.54)	.525	0.76	(0.40, 1.44)	.400
IL-6 ($pg \cdot ml^{-1}$)	1.75	(0.87, 3.54)	.116	1.93	(0.90, 4.16)	.092	1.65	(0.73, 3.76)	.231	1.64	(0.71, 3.76)	.243
IL-10 ($pg \cdot ml^{-1}$)	0.58	(0.29, 1.14)	.116	0.55	(0.26, 1.18)	.124	0.47	(0.17, 1.29)	.142	0.63	(0.22, 1.83)	.400
TNFα ($pg \cdot ml^{-1}$)	0.98	(0.61, 1.72)	.545	0.99	(0.61, 1.74)	.613	0.89	(0.48, 1.61)	.435	0.81	(0.43, 1.55)	.444
CRP ($mg \cdot L^{-1}$)	1.49	(0.88, 2.51)	.138	1.59	(0.88, 2.86)	.123	1.18	(0.58, 2.42)	.649	1.04	(0.49, 2.18)	.926
Endocrine												
Cortisol ($nmol \cdot L^{-1}$)	1.04	(0.63, 1.74)	.869	1.08	(0.63, 1.84)	.789	1.07	(0.59, 1.94)	.824	1.14	(0.60, 2.16)	.699
DHEAs ($nmol \cdot L^{-1}$)	0.49	(0.31, 0.77)	.002	0.45	(0.27, 0.74)	.002	0.42	(0.23, 0.74)	.003	0.50	(0.27, 0.91)	.023
Cortisol: DHEAs ratio	1.78	(1.17, 2.72)	.007	2.03	(1.25, 3.30)	.004	2.02	(1.20, 3.41)	.008	1.79	(1.03, 3.10)	.037

OR: Odds Ratio, *CI*: Confidence Interval, *p*: probability value. [a] Unadjusted OR for frailty per standard deviation increase in biomarker. [b] OR adjusted for gender and baseline age. [c] OR adjusted as in footnote b as well as height, weight, BMI, smoking, alcohol, social class and walk speed. [d] OR adjusted as in footnote c as well as number of systems medicated as a marker of co-morbidity. [e] Based on data for males only.

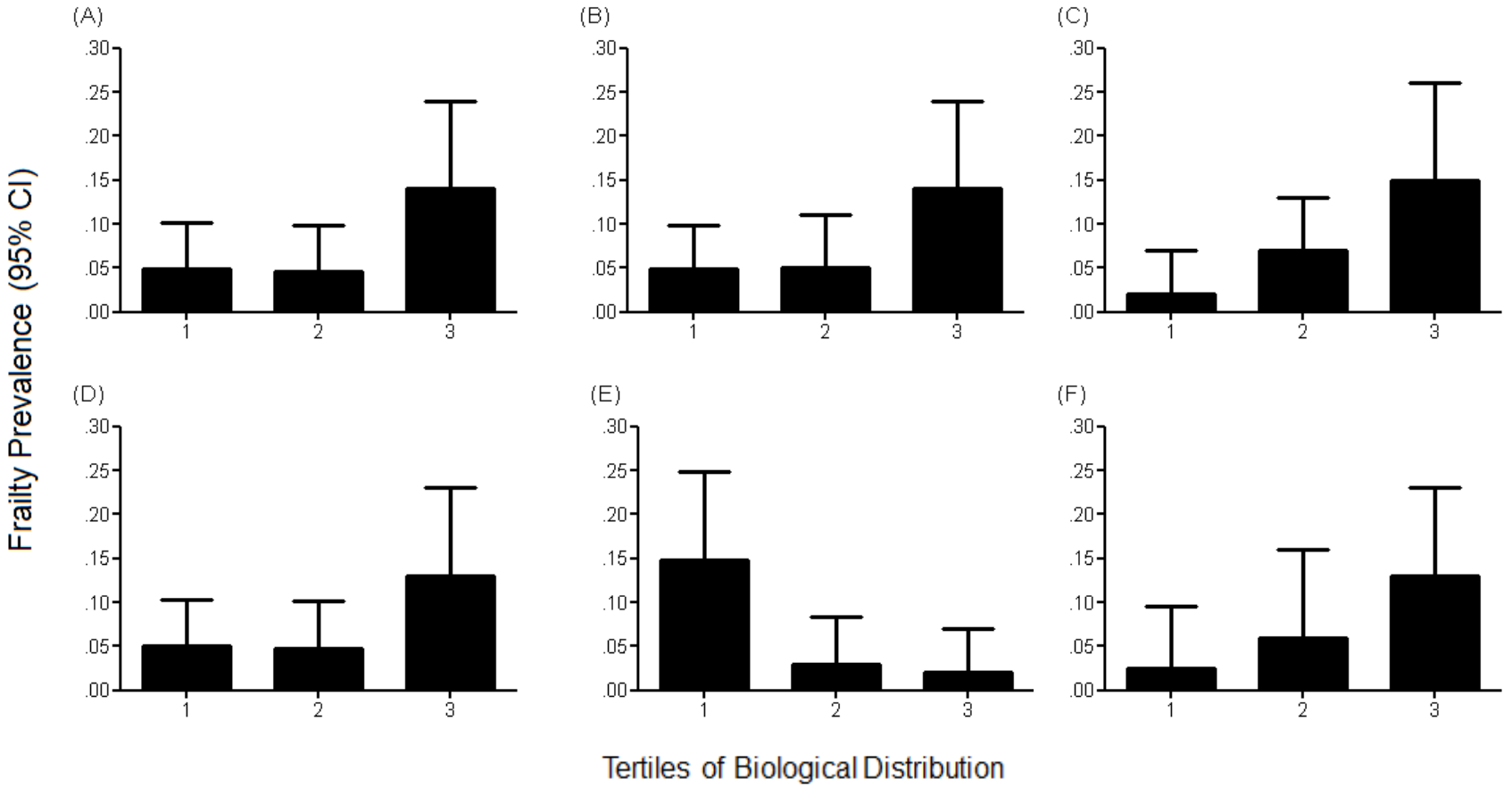

Fig. 3.5: Frailty and Inflammation. Prevalence of frailty measured by the Fried frailty index at Follow-Up for individuals according to the baseline distribution of White Cell Count (A), neutrophils (B), monocytes (C), lymphocytes (D), DHEAs (E) and cortisol:DHEAs (F) when data were split in to tertiles.

3.2.6 Associations with Inflammation, Adrenopause and Mortality

Frailty has been previously associated with increased all-cause mortality [134, 255], therefore association between the same biological markers and mortality was assessed to understand the relationship between frailty, inflammation and mortality in this cohort. Table 3.7 shows the associations between measured biological markers for the 717 participants at the baseline and all-cause mortality during the period between baseline and follow-up clinics. Unadjusted analyses of increase [HR (95% CI) and *p* value] in levels of ESR [1.33 (1.11, 1.58), *p*=.002], neutrophils [1.33 (1.11, 1.59), *p*=.002], monocytes [1.19 (1.00, 1.43), *p*=.005] and IL-1β [1.17 (1.00, 1.36), *p*=.004] were associated with increased all-cause mortality. Following Bonferroni correction (all *p*<.005) both increased ESR and neutrophils remain associated with mortality. Results were unaltered by adjustment for gender and baseline age however the associations between monocytes and mortality and IL-1β and mortality were attenuated after adjustment for height, smoking, alcohol, social class, walk speed and co-morbidities at baseline.

Table 3.7: Inflammation and mortality. Associations between Baseline biomarkers and all-cause mortality in the years prior to Follow-Up for the 122 HAS participants who died.

Biomarker	HR[a]	95% CI	*p*	HR[b]	95% CI	*p*	HR[c]	95% CI	*p*	HR[d]	95% CI	*p*
Immune Composition												
White Cell Count ($x10^{9} \cdot L^{-1}$)	1.18	(0.98, 1.42)	.081	1.17	(0.98, 1.41)	.089	1.15	(0.96, 1.39)	.133	1.15	(0.98, 1.41)	.155
Neutrophils ($x10^{9} \cdot L^{-1}$)	1.33	(1.11, 1.59)	.002	1.31	(1.09, 1.57)	.003	1.21	(1.00, 1.47)	.046	1.21	(1.09, 1.57)	.054
Monocytes ($x10^{9} \cdot L^{-1}$)	1.19	(1.00, 1.43)	.054	1.19	(0.99, 1.42)	.064	1.13	(0.93, 1.36)	.214	1.12	(0.99, 1.42)	.235
Lymphocytes (x109.L-1)	1.10	(0.91, 1.32)	.319	1.11	(0.93, 1.33)	.261	1.06	(0.88, 1.28)	.560	1.07	(0.93, 1.33)	.490
Clinical												
CMV Titre (Au)	0.97	(0.77, 1.22)	.799	0.97	(0.77, 1.22)	.713	0.96	(0.79, 1.22)	.754	0.96	(0.78, 1.25)	.789
ESR ($mm \cdot hr^{-1}$)	1.33	(1.11, 1.58)	.002	1.31	(1.10, 1.56)	.002	1.22	(1.01, 1.48)	.037	1.22	(1.10, 1.56)	.041
Albumin ($g \cdot L^{-1}$)	0.93	(0.77, 1.11)	.401	0.92	(0.77, 1.11)	.386	0.94	(0.78, 1.12)	.480	0.94	(0.77, 1.11)	.488
SHBG ($nmol \cdot L^{-1}$)	0.93	(0.77, 1.11)	.411	0.93	(0.78, 1.11)	.433	0.95	(0.79, 1.14)	.587	0.98	(0.78, 1.11)	.856
Testosterone ($nmol \cdot L^{-1}$)[e]	1.18	(0.98, 1.42)	.075	1.16	(0.97, 1.40)	.108	1.15	(0.94, 1.40)	.172	1.14	(0.97, 1.40)	.193
Haemoglobin ($g \cdot L^{-1}$)	0.98	(0.78, 1.24)	.894	0.98	(0.78, 1.24)	.888	0.99	(0.76, 1.28)	.937	1.00	(0.78, 1.24)	.989
TSH ($\mu U \cdot L^{-1}$)	0.94	(0.78, 1.13)	.521	0.96	(0.80, 1.15)	.655	0.96	(0.80, 1.15)	.643	0.97	(0.80, 1.15)	.783
T4 ($pmol \cdot L^{-1}$)	0.95	(0.79, 1.14)	.609	0.96	(0.80, 1.15)	.664	1.00	(0.83, 1.20)	.979	1.00	(0.80, 1.15)	.987
Inflammation												
IL-1β ($pg \cdot ml^{-1}$)	1.17	(1.00, 1.36)	.044	1.18	(1.00, 1.38)	.045	1.16	(0.98, 1.37)	.085	1.14	(1.00, 1.38)	.149
IL-6 ($pg \cdot ml^{-1}$)	0.97	(0.78, 1.20)	.760	0.98	(0.79, 1.22)	.857	0.96	(0.77, 1.19)	.703	0.96	(0.79, 1.22)	.706
IL-10 ($pg \cdot ml^{-1}$)	1.09	(0.87, 1.37)	.431	1.09	(0.87, 1.37)	.459	1.02	(0.81, 1.29)	.846	1.02	(0.87, 1.37)	.882
TNFα ($pg \cdot ml^{-1}$)	1.01	(0.99, 1.03)	.072	1.01	(1.00, 1.03)	.067	1.01	(1.00, 1.03)	.060	0.89	(0.69, 1.14)	.346
CRP ($mg \cdot L^{-1}$)	0.92	(0.78, 1.17)	.655	0.95	(0.78, 1.17)	.647	0.98	(0.80, 1.20)	.834	0.98	(0.78, 1.17)	.875
Endocrine												
Cortisol ($nmol \cdot L^{-1}$)	1.08	(0.90, 1.31)	.409	1.08	(0.89, 1.31)	.426	1.09	(0.90, 1.32)	.395	1.08	(0.89, 1.31)	.430
DHEAs ($nmol \cdot L^{-1}$)	1.18	(0.97, 1.43)	.091	1.19	(0.98, 1.44)	.083	1.12	(0.92, 1.37)	.262	1.10	(0.98, 1.44)	.354
Cortisol: DHEAs ratio	1.04	(0.86, 1.26)	.668	1.04	(0.86, 1.25)	.710	1.02	(0.84, 1.24)	.814	1.00	(0.86, 1.25)	.978

HR: Hazard Ratio, *CI*: Confidence Interval, *p*: probability value. [a] Unadjusted HR for mortality per standard deviation increase in biomarker. [b] HR adjusted for gender and baseline age. [c] HR adjusted as in footnote b as well as height, weight, BMI, smoking, alcohol, social class and walk speed. [d] HR adjusted as in footnote c as well as number of systems medicated as a marker of co-morbidity. [e] Based on data for males only

3.3 Discussion

A well-controlled inflammatory response to infection and maintenance of protective inflammatory status is critical to the homeostasis of the individual. However with increased age there is an increase in low-grade chronic systemic inflammation, termed ‘inflammageing’ in the apparent absence of overt infection [256]. Systemic inflammation is associated with increased risk of chronic disease and infectious episodes in the elderly. Furthermore, systemic inflammation has been suggested to be a risk factor for frailty and all-cause mortality in elderly individuals [134, 251]. The cause of inflammageing remains unknown however there are strong associations with increased adiposity, physical inactivity and immunesenescence. The majority of studies investigating inflammageing and its causes have assessed these relationships in cross-sectional analyses with respect to differences between young and old individuals. These studies have limited power as in effect they are a ‘snap-shot’ of two distinct and independent groups of individuals.

A number of longitudinal ageing studies have been and are being conducted, each with their own set of limitations. Probably one of the best known is the Baltimore Longitudinal Study of Aging (BLSA) which has collected over 50 years of ‘normal’ ageing research [257]. The BLSA is a comprehensive assessment of over 3000 individuals as they age in an attempt to understand the interplay between ageing and health. It is one of the most successful of gerontological studies being conducted but like all longitudinal research suffers two inherent limitations. One is the people who take part are often healthier than the majority of a population and therefore less likely to suffer disease, plus the participants are from one small geographical area. This is

an important point especially with studies such as the OCTO and NONA studies as well as Japanese studies [258, 259]. These participants appear to be from areas in the world where advanced age is more frequent most likely due to diet and lifestyle choices. Regardless of these limitations each study does have the extremely valuable power of following a single group of individuals over time and therefore it is important for more studies to adopt this strategy. As each study population is made of demographically different and various environmental exposures the aim of longitudinal studies is finding a common pathway of outcome variables, in this case inflammageing, frailty and mortality.

This study is the first to show that in a cohort of elderly individuals inflammageing is occurs independently of cytomegalovirus (CMV) infection [256] and thus does not support previous suggestions that the two are intrinsically linked. Behavioural lifestyle choices that are indicative of a sedentary nature appear to influence the progression of inflammageing to a greater degree. Furthermore, systemic inflammation and adrenal function and not CMV infection were predictive of frailty and mortality at the 10-year follow-up [134].

3.3.1 Cytomegalovirus Infection and Inflammageing

Confirming findings from other groups comparing inflammatory differences between young and old individuals and between old individuals over time, ageing causes an increase in low grade systemic inflammation which is accompanied by a reduced anti-inflammatory potential [251]. It was shown here that levels of IL-6, TNFα and CRP were increased over a ten year period in relatively healthy elderly participants [256].

The longitudinal OCTO-Immune and NONA-Immune studies followed small cohorts of the very elderly (>85 years) for up to 6 years and correlated a number of immune markers with mortality [260, 261]. These studies were the first to define a set of parameters termed the “immune risk profile” (IRP) which was associated with increased mortality during follow up. Interestingly, these parameters included CMV sero-positivity and a profile of raised pro-inflammatory cytokines. However, these studies did not compare the inflammatory status of individuals who remained CMV-sero-negative or who sero-converted in the 6 year study period. Similarly, Roberts *et al* conducted a prospective analysis of anti-CMV antibody titres and found that increased titre was associated with increased TNFα, IL-6 and raised mortality but did not consider longitudinal differences between CMV sero-negative and sero-positive individuals [262].

CMV infection has the potential to increase the inflammatory milieu in a number of ways. Although it is predominantly dormant undergoing intermittent reactivation there is the underlying theory that the reactivation only occurs due to impaired immune control [263]. It has been shown that in times of physiological stress or during alternate infection requiring immune intervention CMV DNA can be readily detected in serum and urine [264, 265]. Indeed CMV is predominantly associated with mortality in immune suppressed individuals such as those undergoing stem cell therapy or those infected with HIV [266]. Therefore as the elderly are known to have impaired immune function it is reasonable to assume that CMV reactivation occurs more frequently. Indeed recent reports have suggested this is the case [267]. Thus CMV reactivation leads to persistent immune activation resulting in not only elevated

systemic inflammation but also immune exhaustion of the adaptive immune system, particularly CD8+ T-cells [267]. Furthermore, uncontrolled or reduced surveillance of CMV infection leads to localised tissue damage as the virus attempts to propagate itself whilst the immune system attempts to eliminate it. Therefore the current theory behind the association between CMV and increased mortality is that it drives a vicious circle reducing immunity in elderly individuals and increasing their risk of other infections whilst contributing to immune related tissue damage through dysregulated immune responses [84, 261]. To date no study has assessed the impact of CMV in the elderly on increased systemic inflammation over time accounting for individuals who are negative, positive and have converted from negative to positive during the time. This study is thus the first to address the impact of CMV serostatus on the increase in inflammatory markers during ageing and reveals that increased chronic systemic inflammation over a ten year period occurs independently of CMV infection [256]. These findings suggest that mortality associated with inflammation and CMV infection may in fact be independent of each other. Thus CMV infection does not appear to determine the age-related increase in serological markers of inflammation.

In light of these findings other factors are likely responsible for systemic inflammation. Several genetic and environmental factors have been suggested to contribute to inflammageing. Although the current study was unable to assess genetic differences other studies have suggested associations. IL-6 polymorphisms (G174C) are associated with elevated IL-6 levels and increase in prevalence in the over 65 year olds [268], possibly reflecting an evolutionary benefit of a robust pro-inflammatory response in early life which is not conducive to a healthy old age.

Indeed raised inflammatory cytokines such as IL-6 are not seen in centenarians or members of families with extended longevity, supporting the notion that an anti-inflammatory genotype is beneficial in reaching extreme old age [269].

Amongst lifestyle factors that could influence inflammageing are the age-related increases in adiposity, reduced physical activity, the loss of sex hormones during menopause and adrenopause which are all suggested to increase systemic inflammation [7, 82, 270]. Indeed, additional analyses of our data suggested that lifestyle factors such as levels of inactivity (hours watching TV, distance walked daily, level of car travel) and a higher BMI were contributing to elevated levels of circulating pro-inflammatory cytokines.

3.3.2 Behavioural Lifestyle Choices and Inflammageing

The elderly have been shown to reduce the amount of structured exercise; however there are many elders who augment this with increased levels of habitual physical activity such as DIY, walking and gardening [271]. Therefore the idea that reduced structured exercise results in reduced physical activity in the elderly is a misnomer and in fact some remain active throughout later life. However there remains good evidence that less than 1 in 5 older adults takes enough exercise to meet the Chief Medical Officers recommendation of 150 minutes per week aerobic exercise and 2-4 bouts of resistance exercise [272].

A number of cross-sectional studies have assessed the relationship between physical activity and systemic inflammation in young, middle-aged and elderly cohorts [195, 273, 274]. Although each study assesses a different population with

varying degrees of inclusion criteria the general consensus is that physical activity attenuates systemic inflammation that can be explained in part by reduced adiposity [190]. In this study the data show the systemic effects of physical activity and some of the determinants which can affect these. The results show that behavioural choices which result in a more sedentary lifestyle are associated with increased systemic inflammation and also that sedentary behaviours are associated with chronic systemic inflammation some ten years later. In particular, increased levels of CRP, IL-6, TNFα and IL-1β were associated with being overweight, slow walking speed, travelling often by car, increased hours of watching TV and not carrying heavy loads.

Structured exercise sessions have been shown to stimulate cytokines, chemokines and growth factor production from muscle known as myokines, even in the elderly, whilst reducing the size and function of adipose tissue [275]. Although these inflammatory mediators appear pro-inflammatory their actions are now considered tissue protective and ultimately anti-inflammatory [198]. This is down to the complex interactions of the dynamics of cytokine production. The classic exercise induced 'myokine', IL-6 has been recently shown to not only stimulate glucose production in the liver but also improves the actions and sensitivity of insulin allowing energy utilisation to increase [203, 276]. Furthermore, exercise induced IL-6 promotes lipolysis within adipose tissue and ultimately reducing its size and pro-inflammatory macrophage content [203]. This is accompanied by reduction in TNFα and IL-1β production which is further enhanced by IL-6 reducing endotoxin production of TNFα [204]. Not only does exercise induced IL-6 promote reduced inflammatory cytokines but exercise also stimulates an anti-inflammatory environment consisting of IL-1ra

and IL-10 amongst others [277]. This so-called anti-inflammatory effect is the muscles way of protecting itself, repairing and adapting to the stresses involved with exercise.

The fact that insulin sensitivity is improved in exercised muscle even in insulin sensitive individuals shows how robust this organ is in utilising energy. Additionally, habitual activity has been shown to increase adiponectin and reduce leptin, two adipose derived hormones suggesting that the function of the adipose tissue is also modified [278]. Although here there was no effect of physically active lifestyles on IL-6 production walking and reduced car travel were associated with reduced TNFα and IL-1β whilst a similar effect was observed with CRP. Therefore although IL-6 was not directly affected by increased levels of activity it's systemic effects were most likely influencing the production of other cytokines.

Due to its abundance in the blood CRP is one of the most common biological markers measured and associated with various health conditions [279]. Subsequently reduced physical activity levels leading to a sedentary lifestyle are consistently associated with increased CRP concentrations [280, 281]. Longitudinal studies in younger individuals have suggested that physical activity induced reductions in CRP are independent of baseline CRP concentrations and body composition suggesting a dose dependent response of physical activity and CRP [239]. Here CRP concentrations in the elderly were associated with both activity status and body composition independent of each other but over time body composition influenced CRP to a greater extent. This is in contrast to a number of other studies which suggest that CRP production is dependent exclusively on body

fat content in the elderly [273]. However recently it has been shown that resistance type exercises which do not reduce body fat but increase muscle mass can stimulate large reductions in circulating CRP [281]. It was not possible to determine whether the individuals in this study were undertaking any form of resistance exercise.

Physical activity has been shown to modulate and improve immune function leading to reduced pro-inflammatory potential. Studies have revealed a synergistic improvement in immune function and reduced inflammation with aerobic exercise and resistance exercise interventions [282]. It was not possible to determine immune function in this study and therefore assess the contribution it played in reducing systemic inflammation. Although it was shown that being active promotes a reduction in cortisol and the ratio of cortisol to DHEAs. Cortisol is typically an immune suppressing mediator whilst DHEAs is a precursor to sex hormones, improving muscle and other tissue functions but is also immune enhancing [70]. The ratio of these two endocrine products is associated with a number of adverse health conditions including frailty and mortality in the elderly which will be discussed in the following sections [134]. Therefore it is likely that the improved ratio of DHEAs to cortisol could infer enhanced immune function.

It is clear that maintaining a reasonable physical activity status in later life can promote health benefits by reducing systemic inflammation and promoting endocrine function. Although it is not clear from this study what the exact mechanisms are, our observations are indicative of a number of different actions, particularly reduced adipose tissue induced inflammation and increased myokine production. Subsequently further epidemiological assessment is warranted to include such

factors as immune function and muscle mass in order to determine the dynamics of inflammatory biological mediator reduction in the elderly.

3.3.3 Consequences of Inflammageing

Here higher baseline levels of total WCC, neutrophils, monocytes, lymphocytes, ESR and free T4, and lower levels of DHEAS as well as higher ratios of cortisol to DHEAS were all associated with an increased likelihood of being frail at ten year follow-up in a cohort of community dwelling older people. Furthermore higher baseline levels of ESR, neutrophils, monocytes and IL-1β were associated with increased likelihood of all-cause mortality across the ten years of the study. These findings complement and add to cross-sectional studies by Leng and colleagues [283] who showed that high neutrophil and monocyte counts were associated with frailty in disabled older women and Voznesensky and colleagues [284] demonstrated associations of frailty with DHEAS in community dwelling older people. These data also confirm findings from O'Hartaigh and colleagues [166] who suggested that increased neutrophil counts in particular were highly predictive of cardiovascular mortality in over three thousand elderly individuals.

This is the first study to show longitudinal associations with frailty across both the immune and endocrine axis, suggesting that the age associated changes to the cellular immune system and hypothalamic-pituitary-adrenal (HPA) axis are central to the rate of development of age associated diseases and, as a consequence, to frailty and mortality. White blood cells are important co-ordinators of inflammation and the increase in their numbers could contribute to inflammageing and thereby increase frailty and mortality. Associations between interleukins or CRP and frailty have not

been shown in this study; this may reflect the complexities of inflammation which involves an inflammatory milieu of multiple proteins rather than a specific inflammatory protein. This would explain the positive associations between ESR and frailty and mortality in this study. ESR is a non-specific marker of inflammation which is characterised by increased rate of sedimentation of red blood cells [285]. During periods of heightened inflammation fibrinogen products are found in the blood which cause erythrocytes to clump together and subsequently sediment quicker. ESR is limited by the inability to define exactly what types of inflammatory proteins are present and subsequently where the inflammation may be originating. However it is perhaps a more powerful useful marker of general inflammatory status [286]. It may be that a single general marker of inflammatory status such as white cell count can be used to help identify people with a greater risk of frailty and mortality and to guide interventions.

Both cortisol and DHEAS are products of the HPA axis; with ageing there is an imbalance between the two hormone levels, due to reducing DHEAS levels from age thirty years onwards, and relatively stable or slightly increased cortisol levels [120]. Here cortisol was increased whilst DHEAs was reduced over a ten-year period. The data suggest that the increase in cortisol could be due to CMV infection as it has been shown that CMV can infect cortisol producing cells promoting cortisol production in order to dampen the immune response towards it [287]. Raised levels of thyroid hormone are thought to further modulate the HPA axis through central changes in sensitivity to corticotrophin releasing hormone [288]. Furthermore, cortisol and DHEAs have direct effects on frailty via interaction with anabolic and catabolic pathways within myocytes [289]. Our own lab has recently shown that

following injury elderly individuals fair worse when they have increased cortisol to DHEAs ratio and have poorer physical function 6 months after their injury [290, 291]. Importantly, this ratio can be manipulated via pharmacological supplementation with DHEAs and potentially exercise interventions. A recent randomised controlled trail by Kenny and colleagues demonstrated that DHEAs supplementation reduced sarcopenia in already frail women involved in gentle exercise when compared to placebo [292].

Frailty is a dynamic process characterised by frequent transitions between frailty states over time. For individuals who are frail, the probability of transitioning to a pre-frail state decreases over time and the probability of dying increases. Early identification is essential and there is ample opportunity for prevention and treatment with medical, nutritional and exercise interventions; pharmacological treatment is on the horizon. In addition to augmenting our understanding of frailty this study suggests a role for the use of biomarkers in screening populations to identify people with a greater likelihood of becoming frail before the syndrome has developed in order to facilitate targeted intervention. Furthermore, these biomarkers are simple, inexpensive and routinely used in clinical practice. This has exciting implications in-terms of healthcare planning and policy making and high potential of significant social, economic and well-being benefits at both individual and global population levels.

3.4 Study limitations

This study has some limitations. Firstly, it cannot completely exclude the effects of co-existing sub-clinical infections at the time of cytokine and endocrine analysis. However, participants were presumed fit and able to attend clinic appointments for data collection and results were screened prior to analysis for patterns suggestive of acute infection or haematological malignancy and four results removed from the data set. Secondly, consideration of the impact of other latent viral infections such as Epstein Barr Virus and Varicella Zoster Virus, which might possibly contribute to inflammation in the CMV negative participants. Also, due to the relatively low seroconversion rate for CMV during the study period the size of the CMV-/+ group is quite small and the impact of seroconversion on serum markers should be addressed in a larger study.

Study participants were lost to follow-up between the 1993/5 baseline and 2003/5 follow-up clinics due to a variety of reasons (including 121 to mortality, loss to follow-up, refusal to participate) and it has been previously shown that a healthy participant effect is evident in HAS. In the current study, the 153 men who went on to have CMV status and frailty classified at the 2003/5 HAS follow-up were significantly ($p<0•05$) younger, less likely to be current smokers, were of higher social class, and had lower white cell count, ESR, neutrophil, monocytes, T4, IL-6 and CRP levels at the baseline clinic than the 258 men who only participated in the baseline study. Selection effects were less evident for women; the 101 women who were sampled at both time points were significantly ($p<0•05$) less likely to be current smokers and had lower neutrophil levels at the 1994/5 clinic than the 205 women who only participated

in the 1994/5 study, but were otherwise similar. These selection effects have the potential to bias results. However, the analyses were internal to the HAS sample; bias would only be introduced if the associations between CMV status, inflammageing, biomarkers and frailty were systematically different among those who participated in our study, and those who did not; this seems unlikely. Finally, there are numerous candidate biomarkers within the immune-endocrine axis that were not analysed and there is scope to widen this biomarker battery. However this may add unnecessary complexity and specifically chose readily available biomarkers to facilitate easy translation into the clinical environment.

3.5 Future Work

Future work is limited by the samples available and the data collected over the clinical assessments. Funding is being sought to bring back surviving participants and complete a third battery of tests. This would be of particular interest as it is thought that individuals who reach the age of eighty five or more may be genetically predisposed to having increased tolerance of inflammation and subsequently reduced inflammation. Therefore having three ten year assessments would allow us to reanalyse data and split them on genetic variation also. Additionally it would be of interest to assess the metabolic profile of each of the participants. Recent evidence suggests that systemic inflammation is attenuated in obese individuals who are metabolically healthy. Although BMI was controlled for in the longitudinal analyses this was a statistical control and may not reflect the actual metabolic health of the individual.

Furthermore, it is clear there are intrinsic links between inflammageing and immunesenescence. This was evident in this study with inflammatory biomarkers being associated with rudimentary components of frailty and immunesenescence markers associated with frailty and mortality. Although CMV infection was not found to be driving inflammageing, future work should consider more than just one latent herpes virus in order to rule out such a link.

3.6 Conclusions

In conclusion, ageing is associated with an increased level of systemic pro-inflammatory mediators consistent with the theory of inflammageing. CMV infection does not appear to drive the age-related increase in serum markers of inflammation. This suggests that interventions to control CMV infection incidence in the population would have little or no impact on the inflammatory profile of ageing individuals. However, it is well established that the cellular immune response against CMV is extremely immune-dominant and increases further in older adults, occupying valuable “immune space” and potentially impairing the response to other pathogens and to vaccinations in old age. However, we cannot rule out the impact CMV may have in conjunction with other latent viral infections such as EBV and VZV. Reactivation and immunodominance of these latent herpes infections are much less and therefore less likely to impact our findings to a great extent. Further epidemiological analyses of the impact of CMV infection on health status in the elderly thus remain warranted.

Lifestyle choices were associated with increased systemic inflammation at baseline and influenced inflammatory status ten years later. This suggests that behavioural modification through increasing physical activity levels from a younger age than 63 years may attenuate inflammation at a later age. Importantly modifications over the age of 63 years when systemic inflammation is present may also attenuate increases in later life, although it is unclear to what extent these modifications will be and how exactly to change behaviour lifestyle choices.

Chapter 4: Habitual Physical Activity, Inflammation and Innate Immune Function

4.0 Introduction

The previous chapter outlined the concept that systemic inflammation can be modulated by behavioural lifestyle choices associated with increased physical activity and reduced body fat. Although inflammation was predicted by body composition and activity levels independently of each other it is well known that physical activity can reduce body fat. Studies in obese individuals who have had fat removal surgery show altered inflammatory metabolic phenotypes with reduced serum leptin and IL-6 concentrations [293]. Therefore adipose tissue is a significant source of pro-inflammatory cytokines and reducing its mass will reduce inflammation. However muscle is also a major producer of cytokines such as IL-6 and termed a myokine. Their impact is context dependent but following exercise IL-6 levels increase and down regulate other pro-inflammatory cytokines such as TNFα, thus having an overall anti-inflammatory effect. This suggests that physical activity and fat mass modulate inflammation on parallel paths rather than the same path.

Furthermore in the previous chapter systemic inflammation and endocrine dysfunction were found to be predictive of both frailty and mortality irrespective of body composition. Frailty is the concept of reduced physical functioning in the elderly and is measured by a composite score of physiological parameters. Many of the parameters used to assess frailty are related to physical functioning which can be modulated by physical activity. Strength measurement is assessed as a determinant of muscle loss/weakness but even in the elderly muscle maintenance is possible through regular exercise. Therefore maintenance of physical activity can attenuate the age-related increase in inflammation and reduce its impact on tissue damage and frailty.

Mortality due to a respiratory infection such as pneumonia is the fourth major cause of death in older adults, indicating a loss of immunity with age. In the previous chapter frailty and mortality were both predicted by immune components associated with the innate immune system. However if physical functioning was predictive of inflammatory cytokines it is likely that the more active individuals have a better innate immune response. Indeed previous studies in the elderly have shown that physical activity can improve innate immune function [294]. In particular monocytes and neutrophils which are key intermediaries in the inflammatory response have most often been shown to have improved function following physical activity assessment.

In light of this literature, physical activity has the potential to modulate inflammation by reducing adipose mass and improving immune function thereby reducing aberrant systemic inflammation. The majority of studies assessing inflammatory status in relation to physical activity in the elderly have assessed either systemic inflammatory cytokines or immune function independent of each other. This does not allow assessment of the larger picture showing the relationship with immunity and inflammation and the impact physical activity can have. Furthermore it is unclear the level of physical activity elderly individuals need to attain to gain inflammatory benefits. The majority of studies have assessed physical activity as a questionnaire or in distinct groups which may not be reflective of the majority of the population.

Therefore the aims of this chapter were:

1. To assess comprehensively the inflammatory phenotype and innate immune function in healthy elderly individuals who were habitually active or inactive.
2. Determine whether habitual physical activity was predictive of frailty and to what extent.

3. Assess the impact of physical activity on innate immune function and its relationship with inflammation.

4.1 The Physical Activity and Healthy Ageing Study Methods

4.1.1 Participants

Participants were recruited via GPs from 54 primary care trusts (PCT's) in and around the Birmingham and The Black Country area (Fig. 4.1). Search patterns revealed 3470 suitable candidates with GP's assessing each candidate and signed as suitable according to the inclusion/exclusion criteria (Appendix I). Surgeries then sent each candidate a request to take part in research at the University of Birmingham from which 473 (13.6%) interested candidates returned pre-paid envelopes with their contact details. From this 202 (42.7%) candidates were recruited with another 15 recruited from an ageing conference held at the University. Recruits were booked into the Wellcome Trust Clinical Research Facility (WTCRF) at the Queen Elizabeth Hospital between 8:30 and 9:30am having fasted overnight. Inclusion/Exclusion criteria are shown in appendix 1**.** Ethical approval was obtained from Birmingham and Black Country LREC.

Following consent participants had their blood glucose (Accu-check Performa) and 'office' blood pressure (GH Pro2000 dinamap, USA) assessed to ensure there were no undiagnosed pathologies which would exclude them from the study. Six participants were excluded due to blood glucose concentrations of over 7mmol·L^{-1} which was deemed to be at risk of undiagnosed diabetes.

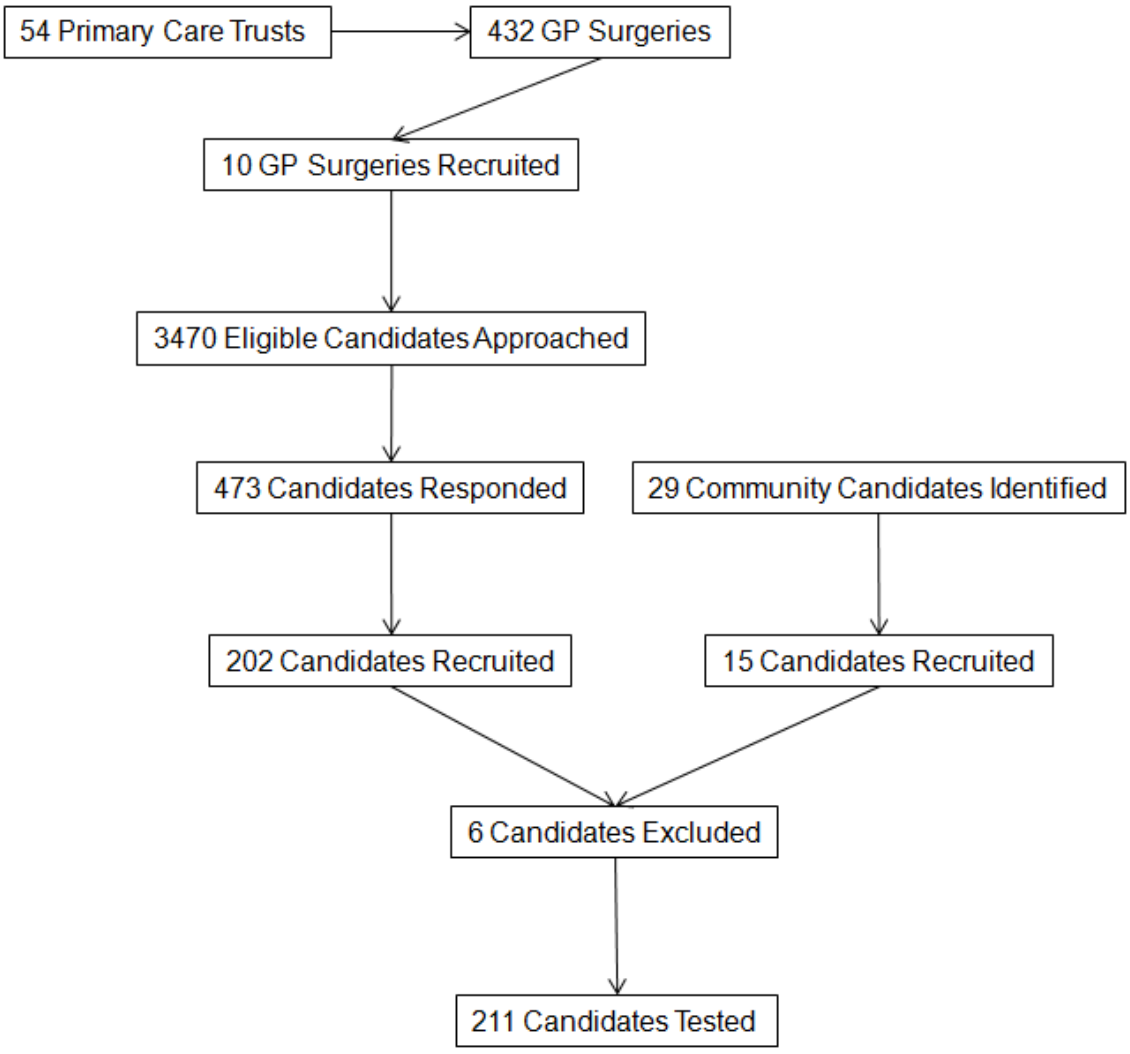

Fig. 4.1: PAHA recruitment. Recruitment protocol of the 217 individuals enrolled in the study.

Upon satisfactory blood glucose and blood pressure results participants had their weight, height, waist and hip circumference measured. Following this blood samples were acquired by trained phlebotomists from a vein in the antecubital fossa. Peripheral blood (40 ml) was collected into vacutainers (Becton-Dickinson, Oxford, UK) containing lithium heparin or EDTA anticoagulants or clotting factor for serum isolation. Following this each participant received a small breakfast before having the questionnaires and accelerometer device explained to them in order to assess quantitative physical activity levels.

4.1.2 Accelerometry

Physical activity (PA) status was determined from seven continuous days of accelerometer (GT3X, Actigraph, FL, USA) wear. Participants wore the GT3X around the waist above the non-dominant leg continuously, except for when bathing (including water based activities). The GT3X was programmed to record data in three orthogonal planes (vertical (VT), antero-posterior (AP) and medio-lateral (ML)) every 60-seconds. Tri-axial vector magnitude (VM) was calculated from the total counts of each of the movement planes using the formula $VM=\sqrt{(VT^2 + AP^2 + ML^2)}$ and total steps and counts calculated. Total time and percentage of time spent in sedentary, light, moderate, vigorous and very vigorous activity levels was assessed using cut-offs previously described [295]. Other variables collected and computed by the software include energy expenditure and mean metabolic equivalents (METs). Sleep quality (SQ) was determined from seven continuous nights of GT3X wear. Participants were asked to remove the device from the waist each night when preparing for bed and position it snugly around the ankle. Participants recorded the times they were attempting to begin evening sleep and the time they fully awaken in the morning. Using these data and the ActiLife software actogram, individual 60-

second epochs were used to determine sleep onset, latency, total sleep time (TST), wake after sleep onset (WASO), number of awakenings and efficiency from the Cole-Kripke algorithm [296].

4.1.3 Body Composition

Body fat percentage, muscle mass, bone mineral density and estimated visceral fat mass were measured and analysed by trained NHS clinical research radiographers using dual energy x-ray absorptiometry (DEXA).

4.1.4 Physical Functioning

Physical functioning was assessed using a number of methods. Grip Strength was determined in both hands using the best score from three attempts using a Takei Grip-D TKK-5401 dynamometer. Timed-Up-and-Go was determined by the time taken to stand up from seated, walk six metres and return to seating. Participants were instructed to walk at their regular walking pace. Balance was assessed using the Berg Balance Score as previously described where a score out of 56 is associated with progress towards frailty [297]. Leg strength was assessed by trained NHS clinical research radiographers using a Leonardo^{TM} GRFP jump mat with the best score from three two-legged jumps calculated. Sleep quality was assessed by the Pittsburgh Sleep Quality Index questionnaire as previously described. Anxiety and depression was assessed using the Hospital Anxiety and Depression (HADS) questionnaire as preciously described.

Progression towards frailty was defined from composite scoring of quintiles of the following measurements: Arm and leg muscle density and grip strength adjusted for age, gender and BMI; Time to walk 6m adjusted for age, height and gender and energy expenditure adjusted for age and gender. All quintiles were scored so that a

score of 1 resulted in the best score and 5 the worst score. Together with the self-reported functional limitations score (1-5) measurements were added together to give a continuous functioning score. Following this, scores were split into groups of three with the classifications as low functioning, high functioning and moderate functioning.

4.1.5 Recall Analysis

Following completion of the 211 participants; accelerometer data was analysed and split into quintiles of physical activity levels stratified for age and gender. Participants in the lowest activity quintile were then matched for gender, age and medication with participants from the highest activity quintile and invited back for further testing. Participants returned to the WTCRF at 8am having fasted overnight where they were consented again by a trained research nurse and similar blood glucose and blood pressure assessments conducted as well as anthropometric measurements. Participants were given the accelerometer to wear again for 7 continuous days to confirm they had not significantly changed their activity patterns since the first clinical assessment. Peripheral blood was taken as before with serum and plasma isolation processing described in section 2.1.

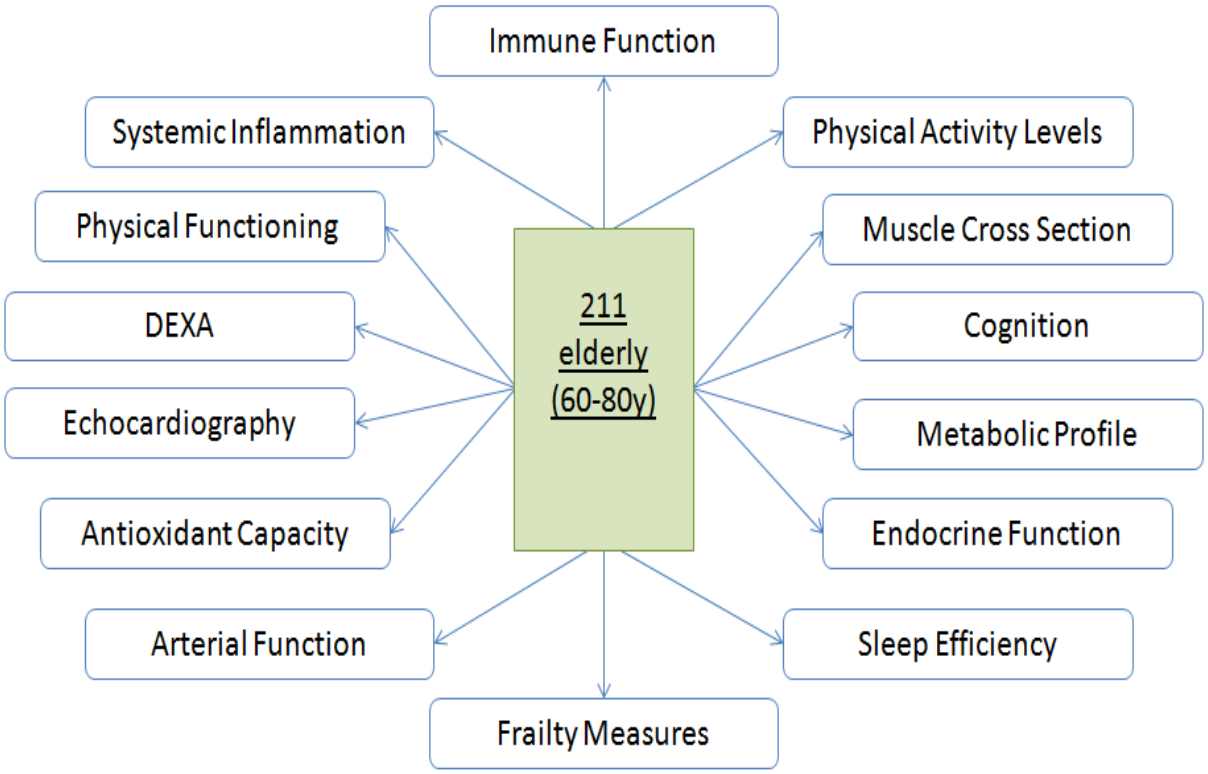

Fig. 4.2: Physiological functions examined during the PAHA study.

4.2 Statistics

Statistical analysis was performed similar to Chapter 3. Physical capability was defined from composite scoring of physical functions (grip strength, walk speed, berg balance score and) and split into tertiles to assess progression towards being frail with 1: High Functioning 2: Moderate Functioning and 3: Low Functioning. Multivariate ANOVA was then conducted to assess the association of age and physical activity scores on progression towards frailty. Linear regression analysis was then conducted to assess biomarker associations with physical capability. Analyses were conducted with and without adjustment for the potential confounding effects of age, gender, smoking, drinking and medications. Neutrophil migratory dynamic calculations were intrinsically linked to each other and therefore multivariate

ANOVA was conducted to assess the variation of physical activity status with migratory dynamics. Univariate ANOVA's adjusting for potential confounding factors were then completed on all measured immune and inflammatory variables to assess differences between physical activity groups. Statistical significance was accepted at $p<.05$.

4.3 Results from the Whole Cohort

4.3.1 Participant Characteristics

Participant characteristics for the 100 men and 111 women who took part in the study are presented in Table 4.1. The average age was 67 for men and 65 for women. No differences were observed for BMI, however DEXA analysis revealed that women had significantly greater percentage of body fat but less estimated visceral adipose mass, bone mineral density (BMD) and waist: hip ratio than men (all $p<.001$). Although these values were different, BMI, body fat and BMD were all considered normal with the majority of participants considered overweight and not obese. No differences were observed for resting blood pressure, however women had a slightly higher resting heart rate than men ($p=.002$) but still within the normal range of 60 -84 bpm. Men drank more alcohol ($p=.030$), were stronger according to jump power ($p=.019$) and grip strength ($p<.001$) which remained after adjustment for arm muscle density.

No effects for gender were evident for physical activity levels or potential confounding factors such as smoking and alcohol consumption or medication. Furthermore, health status of these participants was unusually high with below average percentages for smoking (5%) and alcohol consumption. Furthermore, medication use was on average 57% for this cohort compared to recent studies suggesting medication use was 81% for over 60 year olds [298].

Cortisol and DHEAs were lower in women but they had a slightly higher cortisol: DHEAs ratio (all $p<.001$), whilst IL-8 ($p=.010$) and MIF ($p=.037$) were the only biological biomarkers to be different between males and females. The metabolic

biomarkers leptin, adiponectin, cholesterol, free fatty acids and glycerol were all elevated in women compared to men (all $p<.001$). However, inflammatory phenotypes suggested good health with a number of lower than typically observed values for biological markers. In particular the cortisol: DHEAs ratio has been suggested to be closer to 1.0 in the elderly compared to a median value of 0.14 seen here [69]. Furthermore, levels of typical pro-inflammatory cytokines TNFα, IL-6 and IL-1β were virtually undetectable whilst CRP concentrations were mostly below the CVD risk value of 3.0 $mg{\cdot}L^{-1}$. In summary inflammatory phenotype was not considered clinically significant in any participants.

Metabolic data was also indicative of good health. As diabetes and any metabolic disorders were considered exclusion criteria it was unsurprising to observe healthy values for glucose, insulin, leptin, adiponectin and cholesterol. Furthermore, physical functioning in the participants was excellent with balance scores and walking tests considerably better than the general population. Although women tended to have worse sleep than men ($p=.037$) and be slightly more anxious ($p=.006$) these values were not considered clinically significant.

Table 4.1: Participant characteristics for men and women

	Male (*n* =100)	Female (*n* =111)
Social Characteristics		
Age (years)[a]	67 (64, 71)	65 (63, 69)
BMI ($kg \cdot m^2$)[a]	25.6 (23.4, 27.4)	25.0 (22.7, 27.8)
Waist: Hip[a]	0.93 (0.90, 0.97)	0.82 (0.78, 0.88)***
Body Fat (%)[b]	28.4 (6.0)	38.8 (6.6)***
Visceral Fat Mass (g)[a]	1245 (741, 1904)	468 (315, 994)***
Bone Mineral Density ($g \cdot cm^2$)[b]	1.2 (0.1)	1.1 (0.1)***
Systolic BP (mmHg)[b]	137 (16)	134 (17)
Diastolic BP (mmHg)[b]	80 (10)	78 (11)
MAP (mmHg)[b]	99 (10)	97 (12)
Resting HR (bpm)[a]	63 (57, 73)	68 (63, 76)**
Current Smoker[d]	6 (6)	5 (5)
Drink Alcohol[d]	94 (94)	94 (85)
Alcohol Consumption[b,e]	6.3 (2.7)	5.1 (2.7)*
Activity Score		
7-Day GT3X Accelerometer Wear		
(Counts·hr^{-1})[b,c]	61388 (18719)	65018 (18326)
(Vector·hr^{-1})[b,c]	36089 (10746)	38448 (10660)
(Steps·hr^{-1})[b,c]	500 (188)	490 (154)
Physical Activity Score (IPAQ)[a]	22.8 (12.8, 40.8)	26.5 (13.2, 52.9)
Clinical		
Medicated[d,f]	55 (55)	66 (59)
Statin Use[d,g]	18 (32)	15 (23)
Seasonal Vaccinated[d,h]	34 (41)	30 (32)
White Blood Cells ($x10^9 \cdot L^{-1}$)[a]	5.2 (4.2, 5.9)	4.9 (4.1, 6.3)
Neutrophils ($x10^9 \cdot L^{-1}$)[a]	3.2 (2.6, 3.9)	2.9 (2.4, 3.8)
Monocytes ($x10^9 \cdot L^{-1}$)[a]	0.35 (0.30, 0.50)	0.40 (0.30, 0.50)
Lymphocytes ($x10^9 \cdot L^{-1}$)[a]	1.4 (1.1, 1.9)	1.5 (1.3, 1.9)
CMV Positive[d]	57 (58)	71 (65)
CMV Titre (AU)[a]	43.2 (3.7, 882.9)	58.0 (4.2, 1034.9)
Total Vitamin D ($nmol \cdot L^{-1}$)[a]	54.0 (36.0, 70.5)	55.0 (40.0, 73.0)
Vitamin D2 ($nmol \cdot L^{-1}$)[b]	0 (0, 0)	0 (0, 0)
Vitamin D3 ($nmol \cdot L^{-1}$)[a]	53.0 (34.0, 69.5)	52.0 (39.0, 72.0)
Physical Functioning		
Grip Strength (kg)[b]	38 (8)	23 (4)***
Two-Leg Jump Power (Watts)[b]	33.7 (6.3)	26.0 (4.7)*
Berg Balance Score[a]	56 (55, 56)	56 (55, 56)
Timed-Up-and-Go (sec)[b]	8.1 (1.6)	8.0 (1.6)
Pittsburgh Sleep Quality Index[a]	5 (4, 7)	6 (5, 7)*
Hospital Anxiety Depression Score[a]	0.29 (0.14, 0.50)	0.43 (0.21, 0.57)*
Anxiety Score[a]	0.43 (0.14, 0.71)	0.57 (0.29, 0.86)**
Depression Score[a]	0.14 (0.00, 0.43)	0.14 (0.00, 0.43)

Continued overleaf

Table 4.1 continued

	Male (*n* =100)	Female (*n* =111)
Endocrine		
Cortisol (nmol·L^{-1})[a]	346 (301, 402)	292 (248, 358)***
DHEAs (nmol·L^{-1})[a]	2743 (1848, 3811)	1782 (1141, 2455)***
Cortisol: DHEAs[a]	0.13 (0.08, 0.19)	0.17 (0.11, 0.28)***
Inflammatory		
CRP (mg·L^{-1})[a]	0.89 (0.55, 1.68)	1.00 (0.51, 1.96)
TNFα (pg·ml^{-1})[a]	0.00 (0.00, 0.28)	0.00 (0.00, 0.33)
IL-1β (pg·ml^{-1})[a]	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
IL-6 (pg·ml^{-1})[a]	0.00 (0.00, 0.47)	0.00 (0.00, 0.54)
IL-8 (pg·ml^{-1})[a]	4.95 (3.24, 6.89)	6.02 (4.20, 7.59)*
IL-17 (pg·ml^{-1})[a]	0.00 (0.00, 2.04)	0.00 (0.00, 0.00)
MIF (ng·ml^{-1})[a]	0.62 (0.45, 2.20)	0.51 (0.32, 1.97)*
GM-CSF (pg·ml^{-1})[a]	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
MCP-1 (pg·ml^{-1})[a]	24.01 (17.73, 30.08)	22.48 (17.73, 30.77)
VEGF (pg·ml^{-1})[a]	49.20 (29.85, 86.10)	58.65 (31.80, 106.70)
VCAM-1 (ng·ml^{-1})[a]	334 (211, 475)	291 (202, 496)
ICAM-1 (ng·ml^{-1})[a]	283 (159, 403)	247 (163, 438)
PAI-1 (pg·ml^{-1})[a]	886 (528, 1708)	907 (502, 1573)
Endothelin-1 (pg·ml^{-1})[a]	0.43 (0.31, 0.94)	0.49 (0.30, 1.08)
Anti-Inflammatory		
IL-4 (pg·ml^{-1})[a]	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
IL-10 (pg·ml^{-1})[a]	0.00 (0.00, 0.00)	0.00 (0.00, 0.44)
IL-13 (pg·ml^{-1})[a]	0.52 (0.22, 1.33)	0.76 (0.31, 1.54)
Metabolic		
Fasting Blood Glucose (mmol·L^{-1})[a]	5.6 (5.3, 6.1)	5.5 (5.1, 5.8)
Fasting Plasma Glucose (mmol·L^{-1})[b]	5.3 (0.5)	5.2 (0.5)*
Fasting Plasma Insulin (μlU·ml^{-1})[a]	14.0 (11.1, 19.2)	14.2 (10.2, 18.2)
HOMA-IR [a,i]	1.8 (1.4, 2.5)	1.8 (1.3, 2.3)
HOMA-β [a,j]	131.7 (107.3, 159.3)	133.9 (115.7, 153.3)
Leptin (ng·ml^{-1})[a]	5.8 (3.8, 9.9)	17.2 (11.1, 25.0)***
Adiponectin (μg·ml^{-1})[a]	2.9 (2.2, 3.9)	4.2 (3.4, 5.4)***
Leptin: Adiponectin[a]	1.8 (1.1, 3.2)	4.4 (2.5, 7.1)***
Triglycerides (mmol·L^{-1})[a]	1.05 (0.77, 1.36)	1.10 (0.87, 1.31)
Total Cholesterol (mmol·L^{-1})[a]	5.1 (4.3, 5.8)	6.0 (5.4, 6.7)***
HDL (mmol·L^{-1})[a]	1.4 (1.3, 1.7)	1.8 (1.5, 2.1)***
LDL (mmol·L^{-1})[a]	3.7 (3.0, 4.4)	4.2 (3.6, 4.9)***
VLDL (mmol·L^{-1})[a]	0.21 (0.15, 0.27)	0.22 (0.17, 0.26)
NEFA (mmol·L^{-1})[a]	0.43 (0.34, 0.55)	0.53 (0.42, 0.69)***
Glycerol (μmol·L^{-1})[a]	44.0 (35.0, 55.3)	64.0 (52.5, 84.5)***

BMI (Body Mass Index), BP (Blood Pressure), MAP (Mean Arterial Pressure), IPAQ (International Physical Activity Questionnaire), CMV (Cytomegalovirus), CRP (C-Reactive Protein), TNFα (Tumour Necrosis Factor α), IL (Interleukin), MIF (Macrophage Migratory Inhibitory Factor), GM-CSF (Granulocyte-Macrophage Colony Stimulating Factor), MCP-1 (Monocyte Chemotactic Protein 1), VEGF

(Vascular Endothelial Growth Factor), VCAM-1 (Vascular Cell Adhesion Molecule 1)ICAM-1 (Intracellular Adhesion Molecule 1), PAI-1 (Plasminogen Activator Inhibtor-1), HDL (High Density Lipoprotein), LDL (Low Density Lipoprotein), VLDL (Very Low Density Lipoprotein), NEFA (Non-Esterified Fatty Acid). HOMA (Homeostatic Model Assessment).

[a] Variable positively skewed, median (25^{th} and 75^{th} percentile). [b] Mean (SD). [c] Defined as total activity per hour of non-bedtime wear. [d] Population Number (% of population). [e] Composite score of alcohol consumption per week. [f] Defined as taking any prescribed medication for co-morbidity. [g] Those who take medication also take statins. [h] Having had the seasonal vaccination immediately prior to clinic. [i] Insulin resistance as defined by the Homeostatic Model Assessment . [j] Pancreatic β-cell function as defined by the Homeostatic Model Assessment. *p<.05, **p<.01, ***p<.001 for women differing from men

4.3.2 Age Associated Systemic Inflammation

In the previous chapter it was suggested that ageing is associated with increased systemic inflammation, this was re-examined herein this cross-sectional study of a very healthy and well characterised group of older adults. Within the group increasing age was associated with increased concentrations of cortisol (r =.264, *p*<.001), IL-6 (r = .172, *p*=.007) and a trend for raised CRP (r=.094, *p*=.091) and significantly reduced concentrations of DHEAs (r = -.169, *p*=.015) and IL-13 (r = -.148, *p*=.017). These results are similar to the previous chapter suggesting that ageing is associated with elevated systemic inflammation.

4.3.3 CMV Infection and Inflammatory Status

CMV infection was present in 58% of men and 65% of women, however CMV titres were not associated with gender (*p*=.307) or age (*r*=0.07, *p*=.92) and there was no difference in age between CMV positive or negative groups (*p*=.653). Table 4.2 highlights immune, inflammatory, anti-inflammatory and endocrine biomarker differences between CMV positive and negative individuals. CMV positive individuals had higher WCC (*p*=.022) than negative individuals which was driven by higher lymphocyte concentrations (*p*=.013), which is indicative of the T-cell response observed in CMV positive individuals. Of all the other measured biomarkers, only the growth factor VEGF (*p*=.014) was significantly higher in those infected with CMV. These findings are in agreement with the previous chapter confirming that CMV infection is not a driving force behind systemic inflammation in the elderly.

4.3.4 Metabolic Health and Inflammation

In light of recent suggestions that adipose tissue accounts for only 30% of circulating inflammatory mediators and that systemic inflammation can be driven by metabolic health (MH) regardless of obesity it was necessary to assess MH in this study [299].

The Karelis and Meigs criteria were used to assess MH in this cohort (Table 4.3). Nine participants (4%) were identified as being metabolically unhealthy for both criteria but showed no differences between any of the inflammatory biomarkers measured (data not shown). There was a trend for the metabolically unhealthy individuals to have reduced physical activity levels (p = .085) than the healthy. These data highlight the homogeneity of the cohort in regard to health status and suggest that this cohort regardless of physical activity status and medication were very closely related in terms of overall health.

Table 4.2: CMV and inflammation. Median (IQR) of immune, inflammatory biomarkers, cortisol and DHEAs differences between CMV positive (n=128) and negative (n=83) participants.

	CMV Status	
	Negative	Positive
Immune		
White Blood Cells ($x10^{9}\cdot L^{-1}$)	4.6 (4.1, 5.6)	5.2 (4.2, 6.3)*
Neutrophils ($x10^{9}\cdot L^{-1}$)	2.8 (2.4, 3.8)	3.2 (2.6, 4.1)
Monocytes ($x10^{9}\cdot L^{-1}$)	0.35 (0.30, 0.45)	0.40 (0.30, 0.50)
Lymphocytes ($x10^{9}\cdot L^{-1}$)	1.4 (1.2, 1.7)	1.5 (1.3, 2.1)*
Endocrine		
Cortisol ($nmol\cdot L^{-1}$)	312 (256, 387)	330 (277, 380)
DHEAs ($nmol\cdot L^{-1}$)	2340 (1540, 3340)	2109 (1428, 3146)
Cortisol: DHEAs	0.14 (0.09, 0.23)	0.15 (0.10, 0.24)
Inflammatory		
CRP ($mg\cdot L^{-1}$)	0.91 (0.46, 1.68)	1.00 (0.54, 1.87)
TNFα ($pg\cdot ml^{-1}$)	0.00 (0.00, 0.16)	0.00 (0.00, 0.41)
IL-1β ($pg\cdot ml^{-1}$)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
IL-6 ($pg\cdot ml^{-1}$)	0.00 (0.00, 0.54)	0.00 (0.00, 0.47)
IL-8 ($pg\cdot ml^{-1}$)	5.82 (3.32, 7.74)	5.32 (3.32, 6.89)
IL-17 ($pg\cdot ml^{-1}$)	0.00 (0.00, 0.00)	0.00 (0.00, 0.48)
MIF ($ng\cdot ml^{-1}$)	0.54 (0.39, 1.81)	0.62 (0.36, 2.35)
GM-CSF ($pg\cdot ml^{-1}$)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
MCP-1 ($pg\cdot ml^{-1}$)	23.55 (18.70, 30.50)	22.79 (17.32, 30.23)
VEGF ($pg\cdot ml^{-1}$)	46.89 (23.76, 83.17)	56.53 (36.90, 104.42)*
VCAM-1 ($ng\cdot ml^{-1}$)	292 (219, 453)	365 (202, 500)
ICAM-1 ($ng\cdot ml^{-1}$)	275 (158, 399)	295 (167, 453)
PAI-1 ($pg\cdot ml^{-1}$)	705 (465, 1412)	911 (544, 1718)
Endothelin-1 ($pg\cdot ml^{-1}$)	0.44 (0.31, 0.88)	0.47 (0.33, 1.03)
Anti-Inflammatory		
IL-4 ($pg\cdot ml^{-1}$)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
IL-10 ($pg\cdot ml^{-1}$)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
IL-13 ($pg\cdot ml^{-1}$)	0.60 (0.22, 1.59)	0.67 (0.30, 1.33)

*p<.05 different from CMV negative participants

Table 4.3: Metabolic Health Criteria

	Karelis	Meigs
Blood Pressure (mmHg)		SBP ≥ 130 or DBP ≤ 85 or treatment
Triglycerides ($mmol \cdot L^{-1}$)	≤ 1.70	≥ 1.70
HDL ($mmol \cdot L^{-1}$)	≥ 1.30 & no treatment	< 1.04 (M); < 1.30 (F)
LDL ($mmol \cdot L^{-1}$)	≤ 2.60 & no treatment	
Total Cholesterol ($mmol \cdot L^{-1}$)	≥ 5.20	
Fasting Plasma Glucose ($mmol \cdot L^{-1}$)		≥ 5.60 or treatment
HOMA-IR	≤ 1.95	
Others		Wasit > 102cm (M) or >88cm (F)
Criteria	≥ 4 of above	< 3 of above

4.3.5 Behavioural Lifestyle Choices and Inflammation

In order to assess the impact of behavioural lifestyle choices on inflammatory biomarkers, linear regression analysis was conducted similar to the previous chapter (Table 4.4). The data revealed that a more inactive lifestyle was associated with increased systemic inflammation. Indeed having low physical activity levels was associated with significant increases in CRP (p=.005), TNFα (p=.002), PAI-1 (p=.001), MCP-1 (p=.033) and a trend for increased soluble ICAM-1 (p=.07). Whilst having excess adipose tissue as measured by fat mass, BMI and percentage fat revealed associations with increased levels of CRP (p<.001), IL-8 (p=.015), IL-10 (p=.046), IL-13 (p=.050), GM-CSF (p=.023), PAI-1 (p<.001), VEGF (p=.006), soluble ICAM-1 (p=.036) and cortisol: DHEAS ratio (p=.045). Mutual adjustment was conducted when both body composition and activity levels were influencing the inflammatory marker. In both instances body composition and not physical activity independently influenced the increased production of CRP (p=.007) and PAI-1 (p<.001). These findings suggest that being physically inactive and/or having excess adipose tissue contribute significantly towards systemic inflammation. Interestingly

the majority of inflammatory mediators were better predicted by measures of body fat.

4.3.6 Relationships with Inflammation and Physical Function

Analysis of physical functioning and thus progression towards frailty revealed effects for age and physical activity (Fig. 4.3) and were highly inter-correlated (r= -.216, p=.001), therefore multivariate ANOVA was conducted. There was a significant multivariate effect for age and activity on the prevalence of low physical functioning [$F_{(4, 150)}$ = 35.94, p<.001; η^2=.324]. Univariate analysis revealed that increasing age (Fig. 4.1A) was associated with heightened prevalence of poor physical functioning [$F_{(2, 150)}$ = 98.61, p<.001; η^2=.568] as those individuals who were high functioning were significantly younger than those with low or moderate physical function (both p<.001). In contrast, increased activity levels (Fig. 4.1B) were associated with reduced prevalence of low physical function [$F_{(2, 150)}$ = 11.94, p<.001; η^2=.137] with those individuals who were least frail being more active than the other two groups (both p<.001). Consequently, in this cohort low physical functioning was overall predicted best by age (β = .434, t(6.50), p<.001) and then physical activity levels (β = -.336, t(-5.03), p<.001). From the R^2 value these independent variables accounted for 35.7% of the variance in physical function scores.

Table 4.4: Lifestyle and inflammation. Relationship between lifestyle factors and the level of serum inflammatory, anti-inflammatory and endocrine biomarkers in 211 healthy elderly participants

		Body Composition and Physical Activity						Body Composition																		Physical Activity					
		CRP			PAI-1			IL-8			GM-CSF			ICAM-1			IL-10			IL-13			VEGF			Cortisol: DHEAs			MCP-1			TNFα		
Model	Predictor	β	*p*	R^2	β	*p*	R^2	β	*p*	R^2	β	*p*	R^2	β	*p*	R^2	β	*p*	R^2	β	*p*	R^2	β	*p*	R^2	β	*p*	R^2	β	*p*	R^2	β	*p*	R^2
1	BMI (kg/m^2)	.295	<.001	.087	.370	<.001	.137	-.022	.756	.000	.112	.112	.013	.133	.056	.018	.143	.046	.020	-.001	.987	.000	.102	.143	.010	-.128	.065	.016	-.080	.253	.006	-.065	.355	.004
	Body Fat (%)	.280	<.001	.078	.250	<.001	.062	.170	.015	.029	.017	.810	.000	.145	.036	.021	.081	.258	.001	.137	.050	.019	.192	.006	.037	.139	.045	.019	-.040	.571	.002	-.045	.524	.002
	Visceral Fat (g)	.265	.001	.070	.484	<.001	.234	.057	.491	.003	.188	.023	.035	.089	.276	.008	.090	.286	.008	.059	.478	.003	.087	.295	.008	-.149	.068	.022	-.017	.841	.000	-.125	.131	.016
	Waist: Hip Ratio	.018	.803	.000	.220	.001	.048	-.188	.007	.035	-.010	.886	.000	-.090	.197	.008	.042	.558	.002	-.148	.033	.022	-.104	.136	.011	-.133	.071	.021	-.029	.677	.001	-.020	.775	.000
	Resting Heart Rate (bpm)	.134	.054	.018	.100	.150	.010	.010	.892	.000	-.058	.410	.003	.109	.166	.012	-.094	.189	.009	.023	.739	.001	.157	.024	.025	.091	.192	.008	-.043	.541	.002	-.155	.027	.024
	Smoking	.066	.354	.004	.010	.886	.000	-.048	.499	.002	-.070	.329	.005	-.061	.386	.004	.081	.266	.006	-.093	.190	.009	.030	.670	.001	-.136	.053	.018	.077	.278	.006	.020	.782	.000
	Alcohol Consumption	-.127	.069	.016	.001	.992	.000	-.019	.782	.000	.041	.563	.002	-.050	.471	.003	-.137	.055	.019	-.070	.319	.005	-.070	.315	.005	-.165	.017	.027	.035	.620	.001	-.047	.500	.002
	Quantitative Physical Activity[a]	-.199	.005	.040	-.233	.001	.054	-.068	.342	.000	-.105	.145	.011	-.129	.070	.017	-.089	.227	.008	-.038	.594	.001	-.075	.296	.006	-.062	.383	.004	.082	.250	.007	-.222	.002	.049
	Qualitative Physical Activity[b]	-.194	.061	.038	-.132	.196	.018	-.117	.255	.014	.013	.898	.000	-.079	.444	.006	-.027	.799	.001	-.133	.192	.018	-.198	.052	.039	-.025	.804	.001	-.217	.033	.047	-.120	.246	.014
2	Body Composition	.244	.001		.457	<.001																											
	Physical Activity	-.132	.067	.095	-.087	.279	.238																											

Linear regression analysis of the log-normalised data was used to test for significant associations between lifestyle factors and the level of inflammatory, anti-inflammatory and endocrine biomarkers. Individual lifestyle components were entered individually as independent variables (model 1); following this, any significant related lifestyle factors were entered together (model 2) to assess their mutually adjusted associations with cytokine levels.

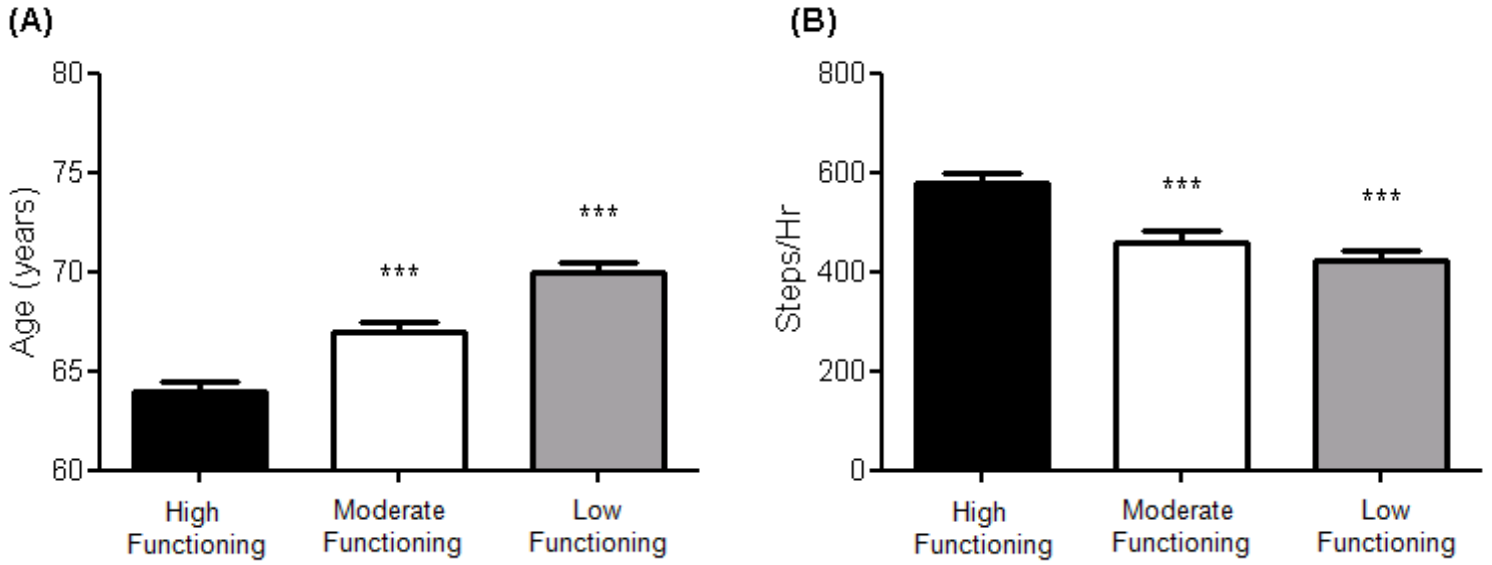

Fig. 4.3: Physical Function, age and activity levels. Associations of age (A) and accelerometer derived steps per hour (B) on physical function. ****p*<.001 different from the High Functioning individuals.

Linear regression (Table 4.5) was used to assess the associations of biological parameters including immune cell numbers, inflammatory cytokines and proteins, endocrine hormones and vitamin-D on composite physical function scores. Unadjusted analysis showed that increased white cell count (p=.048), neutrophils (p=.008), cortisol: DHEAs ratio (p=.017) and reduced Vitamin D3 concentrations (p=.006) were all associated with lower physical function scores. Associations between white cell count (p=.119) and cortisol: DHEAs (p=.603) were attenuated following adjustment for age and gender (Model 2). Following further adjustment (Model 3) for smoking, drinking and medication associations with neutrophils (p=.067) were also attenuated leaving Vitamin D3 concentrations (p=.002) as the only biomarker associated with reduced physical function and thus potential for progression to frailty. It should be noted that there was an unadjusted association of reduced TNFα (p=.032) and reduced physical function which was attenuated following adjustment for age and gender (p=.069). Although these values may indicate TNFα as a poor predictor of poor health the concentrations within this cohort were extremely low and may not represent physiologically meaningful findings.

Table 4.5: Inflammation and physical function. Biomarker association with physical functioning capability

Biomarker	Model 1 β	Model 1 t	Model 1 *p*	Model 2 β	Model 2 t	Model 2 *p*	Model 3 β	Model 3 t	Model 3 *p*
Immune Composition									
White Cell Count ($x10^9 \cdot L^{-1}$)	.166	1.995	.048	.116	1.570	.119	.091	1.195	.234
Neutrophils ($x10^9 \cdot L^{-1}$)	.223	2.706	.008	.167	2.261	.025	.140	1.845	.067
Monocytes ($x10^9 \cdot L^{-1}$)	.078	.923	.358	.020	.271	.787	-.007	-.089	.930
Lymphocytes ($x10^9 \cdot L^{-1}$)	-.032	-.375	.708	-.028	-.377	.707	-.035	-.464	.643
Inflammatory									
CRP ($mg \cdot L^{-1}$)	.115	1.406	.162	.106	1.486	.139	.088	1.195	.234
TNFα ($pg \cdot ml^{-1}$)	-.176	-2.171	.032	-.130	-1.829	.069	-.117	-1.616	.108
IL-1β ($pg \cdot ml^{-1}$)	-.059	-.722	.471	-.042	-.590	.556	-.037	-.495	.621
IL-6 ($pg \cdot ml^{-1}$)	-.139	-1.711	.089	-.121	-.1.690	.093	-.129	-1.797	.075
IL-8 ($pg \cdot ml^{-1}$)	.031	.379	.705	.029	.398	.691	.025	.346	.730
IL-17 ($pg \cdot ml^{-1}$)	-.053	-.648	.518	.016	.224	.823	.020	.275	.783
MIF ($ng \cdot ml^{-1}$)	-.044	-.544	.587	-.046	-.646	.519	-.044	-.607	.545
GM-CSF ($pg \cdot ml^{-1}$)	.023	.277	.782	.076	1.045	.298	.063	.850	.397
MCP-1 ($pg \cdot ml^{-1}$)	.075	.923	.357	.076	1.064	.289	.072	.993	.322
VEGF ($pg \cdot ml^{-1}$)	.079	.968	.335	.097	1.353	.178	.103	1.426	.156
VCAM-1 ($ng \cdot ml^{-1}$)	.089	1.094	.276	.050	.706	.481	.030	.418	.676
ICAM-1 ($ng \cdot ml^{-1}$)	.094	1.156	.250	.061	.858	.392	.040	.554	.581
PAI-1 ($pg \cdot ml^{-1}$)	-.004	-.055	.956	.055	.774	.440	.014	.183	.855
Endothelin-1 ($pg \cdot ml^{-1}$)	.038	.468	.640	-.025	-.355	.723	-.031	-.426	.670
Anti-Inflammatory									
IL-4 ($pg \cdot ml^{-1}$)	.076	.931	.353	.148	2.061	.041	.160	2.226	.028
IL-10 ($pg \cdot ml^{-1}$)	.047	.565	.573	.065	.899	.370	.081	1.104	.272
IL-13 ($pg \cdot ml^{-1}$)	.092	1.133	.259	.136	1.913	.058	.138	1.927	.056
Endocrine									
Cortisol ($nmol \cdot L^{-1}$)	.060	.740	.461	-.039	-.518	.605	-.044	-.584	.560
DHEAs ($nmol \cdot L^{-1}$)	-.186	-2.328	.021	-.072	-.869	.386	-.066	-.785	.434
Cortisol: DHEAs	.192	2.405	.017	.041	.522	.603	.034	.423	.673
Clinical									
Total Vitamin D ($nmol \cdot L^{-1}$)	-.206	-2.558	.012	-.178	-2.504	.013	-.198	-2.781	.006
Vitamin D2 ($nmol \cdot L^{-1}$)	-.223	-2.769	.006	.041	.574	.567	.049	.673	.502
Vitamin D3 ($nmol \cdot L^{-1}$)	.044	.537	.592	-.197	-2.789	.006	-.218	-3.082	.002

Model 1: Unadjusted Model

Model 2: Adjusted for Age

Model 3: Adjusted for Age, Tobacco, Drinking, Medicated

4.4 Results from the Recall Analysis

4.4.1 Participant Characteristics

These observations suggest heightened systemic inflammation is associated with a sedentary lifestyle and being overweight (Table 4.3). Although the participants in this study were not frail as defined by the 'Fried Frailty Scale' the progression towards frailty was associated with inflammatory markers as seen in the previous chapter, particularly elevated neutrophil concentrations, endocrine dysfunction and in this study, Vitamin D3 concentrations (Table 4.4). Additionally the most significant association with progression towards frailty was observed in those who were least physically active and as expected older.

Therefore as heightened inflammation and frailty are associated with immunesenescence leading to increased risk of infection and morbidity it was necessary to determine the associations with physical activity status and immune function in this cohort. The purpose of this section was to assess neutrophil and monocyte function in relation to physical activity status. Table 4.6 shows the participant characteristics and biomarker concentrations between those who had low and high physical activity levels (PAL). Ten males and ten females were recalled from each group. The high PAL group were on average 72% more active than the low PAL group when assessed by accelerometry and 120% more active from self reporting questionnaires. As expected the high PAL group had a lower BMI (p=.003), body fat percentage (p=.050) and visceral fat mass (p<.001). White blood cell counts (p=.009), driven by lymphocyte concentrations (p=.004) were lower in the high PAL group whilst Vitamin D3 (p=.027) concentrations were higher. The inflammatory cytokines IL-6 (p=.043) and IL-8 (p=.037) as well as the atherothrombotic risk factor

PAI-1 (p=.019) were all lower in the high PAL group. The anti-inflammatory cytokine IL-10 (p=.040) was higher in the low PAL active group. As shown previously these differences can be driven by body fat mass and upon re-assessment accounting for body fat percentage no differences were observed.

The low PAL group had significantly worse metabolic profiles than the high PAL group as evidenced by increased plasma glucose (p=.021) and insulin (p=.011) concentrations resulting in increased insulin resistance (p=.013) and reduced pancreatic β-cell function (p=.040). Additionally the adipokine balance between leptin and adiponectin (p=.004) was elevated in the low PAL group as a consequence of increased leptin concentrations (p=.017) and a trend for reduced adiponectin concentrations (p=.070). Again, following adjustment for fat content these results were attenuated except for insulin (p=.022) and insulin resistance (p=.026) suggesting that physical activity independently improves insulin sensitivity.

Table 4.6: Characteristics of participants recalled for further analysis.

	Physical Activity Status	
	Low	High
Social Characteristics		
Gender (Males/Females)	10/10	10/10
Age (years)[a]	65 (64, 69)	66 (64, 70)
BMI ($kg \cdot m^2$)[a]	25.5 (23.5, 29.1)	23.0 (21.4, 24.2)**
Waist: Hip[a]	0.89 (0.82, 0.96)	0.86 (0.81, 0.93)
Body Fat (%)[b]	36.4 (6.7)	32.0 (7.6)*
Visceral Fat Mass (g)[a]	1301 (875, 2246)	588 (376, 834)***
Bone Mineral Density ($g \cdot cm^2$)[b]	1.17 (0.16)	1.13 (0.15)
Systolic BP (mmHg)[b]	127 (13)	128 (16)
Diastolic BP (mmHg)[b]	77 (7)	77 (6)
MAP (mmHg)[b]	94 (9)	94 (9)
Resting HR (bpm)[a]	61 (57, 71)	66 (62, 70)
Current Smoker[d]	2 (10)	0 (0)***
Drink Alcohol[d]	18 (90)	19 (95)
Alcohol Consumption[b,e]	4.6 (2.6)	5.9 (2.8)
Activity Score		
7-Day GT3X Accelerometer Wear		
(Counts·hr^{-1})[b,c]	43047 (11412)	74060 (15183)***
(Vector·hr^{-1})[b,c]	25277 (6582)	43415 (9144)***
(Steps·hr^{-1})[b,c]	345 (113)	599 (154)***
Physical Activity Score (IPAQ)[a]	13.4 (11.3)	29.4 (16.9)*
Clinical		
Medicated[d,f]	11 (55)	7 (35)
Statin Use[d,g]	4 (36)	1 (14)
Seasonal Vaccinated[d,h]	9 (45)	11 (55)
White Blood Cells ($x10^9 \cdot L^{-1}$)[a]	5.3 (4.8, 6.4)	4.5 (4.0, 5.2)**
Neutrophils ($x10^9 \cdot L^{-1}$)[a]	3.2 (2.7, 3.9)	2.8 (2.7, 3.7)
Monocytes ($x10^9 \cdot L^{-1}$)[a]	0.30 (0.20, 0.40)	0.25 (0.20, 0.30)
Lymphocytes ($x10^9 \cdot L^{-1}$)[a]	1.6 (1.4. 2.1)	1.3 (1.1, 1.5)**
CMV Positive[d]	8 (40)	11 (55)
CMV Titre (AU)[a]	6.2 (2.1, 439.8)	35.3 (3.8, 2045.2)
Total Vitamin D ($nmol \cdot L^{-1}$)[a]	39.5 (28.5, 48.0)	58.0 (39.3, 80.5)*
Vitamin D2 ($nmol \cdot L^{-1}$)[b]	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
Vitamin D3 ($nmol \cdot L^{-1}$)[a]	39.5 (28.5, 48.0)	58.0 (39.3, 80.5)*
Physical Functioning		
Grip Strength (kg)[b]	30 (25, 40)	27 (22, 36)
Two-Leg Jump Power (Watts)[b]	31.6 (25.5, 36.7)	32.1 (26.9, 36.4)
Berg Balance Score[a]	55 (1)	55 (1)
Timed-Up-and-Go (sec)[b]	8.5 (7.3, 9.3)	7.9 (7.0, 9.0)
Pittsburgh Sleep Quality Index[a]	6 (1)	6 (2)
Hospital Anxiety Depression Score[a]	0.29 (0.14, 0.50)	0.29 (0.14, 0.54)
Anxiety Score[a]	0.36 (0.36, 0.57)	0.43 (0.18, 0.86)
Depression Score[a]	0.29 (0.04, 0.40)	0.14 (0.00, 0.36)

Continued overleaf

Table 4.6 continued: Characteristics of participants recalled for further analysis

	Physical Activity Status	
	Low	High
Endocrine		
Cortisol ($nmol \cdot L^{-1}$)	340 (288, 391)	354 (291, 468)
DHEAs ($nmol \cdot L^{-1}$)	1777 (1128, 3450)	1793 (1128, 2887)
Cortisol: DHEAs	0.26 (0.25)	0.30 (0.30)
Inflammatory		
CRP ($mg \cdot L^{-1}$)	1.32 (0.70, 2.42)	1.57 (0.57, 2.84)
TNFα ($pg \cdot ml^{-1}$)	0.00 (0.00, 1.37)	0.00 (0.00, 0.00)
IL-1β ($pg \cdot ml^{-1}$)	0.30 (0.19, 052)	0.19 (0.16, 0.29)
IL-6 ($pg \cdot ml^{-1}$)	1.72 (1.02, 2.33)	1.35 (0.63, 2.67)*
IL-8 ($pg \cdot ml^{-1}$)	7.57 (2.15)	6.49 (1.44)*
IL-17 ($pg \cdot ml^{-1}$)	0.00 (0.00, 18.82)	0.00 (0.00, 1.90)
MIF ($ng \cdot ml^{-1}$)	0.69 (0.39, 2.10)	0.49 (0.28, 1.26)
GM-CSF ($pg \cdot ml^{-1}$)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
MCP-1 ($pg \cdot ml^{-1}$)	42.38 (15.94)	42.62 (18.41)
VEGF ($pg \cdot ml^{-1}$)	68.68 (25.94, 121.14)	47.67 (38.53, 74.56)
VCAM-1 ($ng \cdot ml^{-1}$)	311 (201, 474)	292 (172, 555)
ICAM-1 ($ng \cdot ml^{-1}$)	291 (143, 418)	310 (143, 412)
PAI-1 ($pg \cdot ml^{-1}$)	1733 (761, 2300)	581 (448, 891)*
Endothelin-1 ($pg \cdot ml^{-1}$)	0.49 (0.28, 1.26)	0.39 (0.31, 1.34)
Anti-Inflammatory		
IL-4 ($pg \cdot ml^{-1}$)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
IL-10 ($pg \cdot ml^{-1}$)	17.01 (6.36, 23.69)	10.22 (5.19, 16.51)*
IL-13 ($pg \cdot ml^{-1}$)	7.59 (4.93)	5.64 (3.69)
Metabolic		
Fasting Blood Glucose ($mmol \cdot L^{-1}$)[a]	5.4 (0.5)	5.2 (0.6)
Fasting Plasma Glucose ($mmol \cdot L^{-1}$)[b]	5.4 (0.5)	5.0 (0.4)*
Fasting Plasma Insulin ($\mu IU \cdot ml^{-1}$)[a]	21.2 (10.5, 27.3)	11.4 (9.5, 13.0)*
HOMA-IR [a,i]	2.7 (1.4, 3.6)	1.5 (1.3, 1.7)*
HOMA-β [a,j]	162.1 (120.0, 194.8)	129.8 (114.4, 142.2)*
Leptin ($ng \cdot ml^{-1}$)[a]	15.0 (6.8, 24.1)	8.0 (3.5, 13.7)*
Adiponectin ($\mu g \cdot ml^{-1}$)[a]	3.0 (2.3, 3.9)	3.5 (2.7, 5.5)
Leptin: Adiponectin[a]	4.9 (2.5, 7.9)	2.3 (1.0, 3.1)*
Triglycerides ($mmol \cdot L^{-1}$)[a]	1.05 (0.82, 1.41)	0.91 (0.69, 1.35)
Total Cholesterol ($mmol \cdot L^{-1}$)[a]	5.3 (4.3, 6.0)	5.6 (5.3, 5.8)
HDL ($mmol \cdot L^{-1}$)[a]	1.3 (1.3, 1.6)	1.6 (1.4, 2.0)*
LDL ($mmol \cdot L^{-1}$)[a]	3.9 (3.1, 4.4)	3.9 (3.3, 4.4)
VLDL ($mmol \cdot L^{-1}$)[a]	0.21 (0.16, 0.28)	0.18 (0.14, 0.27)
NEFA ($mmol \cdot L^{-1}$)[a]	0.48 (0.24, 0.56)	0.40 (0.31, 0.61)
Glycerol ($\mu mol \cdot L^{-1}$)[a]	47.5 (38.3, 61.5)	44.0 (29.0, 76.0)

4.4.2 Neutrophil Functional Dynamics and Physical Activity Status

Neutrophil migration towards IL-8 was assessed in low and high PAL groups using an Insall chamber and time-lapse microscopy and Image-J to assess directional movement and speed [168]. Fig. 4.4 highlights the migration paths of neutrophils from one representative participant in each group; for the purpose of visualisation all cells are centred at a single co-ordinate and tracked according to their individual movements.

Neutrophil migratory dynamics were highly correlated and intrinsically linked with each other, therefore multivariate ANOVA was utilised to assess the variance between the two activity groups. Homogeneity of covariance matrices of migratory dynamics was confirmed by Box's *M* test (p=.735). There was a significant multivariate effect for physical activity status on neutrophil migration [$F_{(4,\ 38)}$=5.50, p=.002; η^2=.386]. Univariate analysis revealed that the chemotactic index (Fig. 4.5A) and chemotaxis (Fig. 4.5B) were greater in the highly active elders [$F_{(1,\ 38)}$=11.80, p=.001; η^2=.237] and [$F_{(1,\ 38)}$=5.03, p=.031; η^2=.117], respectively. Neither chemokinesis (Fig. 4.5C) [$F_{(1,\ 38)}$=2.54, p=.120; η^2=.063] or persistence (Fig. 4.5D) of migration [$F_{(1,\ 38)}$=1.31, p=.260; η^2=.033] showed differences towards IL-8. These results suggest that neutrophil migratory dynamics are maintained in physically active elderly individuals. Indeed to assess whether migration was comparable to that of the young, neutrophils from ten young (23 ± 4 years) participants were assessed [mean (SEM); p]. Young participants had similar values for chemotactic index [0.41 (0.04); p=.985] and chemotaxis [1.68 (0.21); p=.891] to the physically active elders whilst being significantly greater than inactive elders, p=.001 and p=.021 respectively. Furthermore, to assess whether the body fat induced differences in inflammation were accounting for differences the individual

components IL-6, IL-8, PAI-1 and body fat percentage were entered as covariates. MANCOVA results revealed that neutrophil chemotactic differences were driven by physical activity status and not body fat or inflammatory mediators.

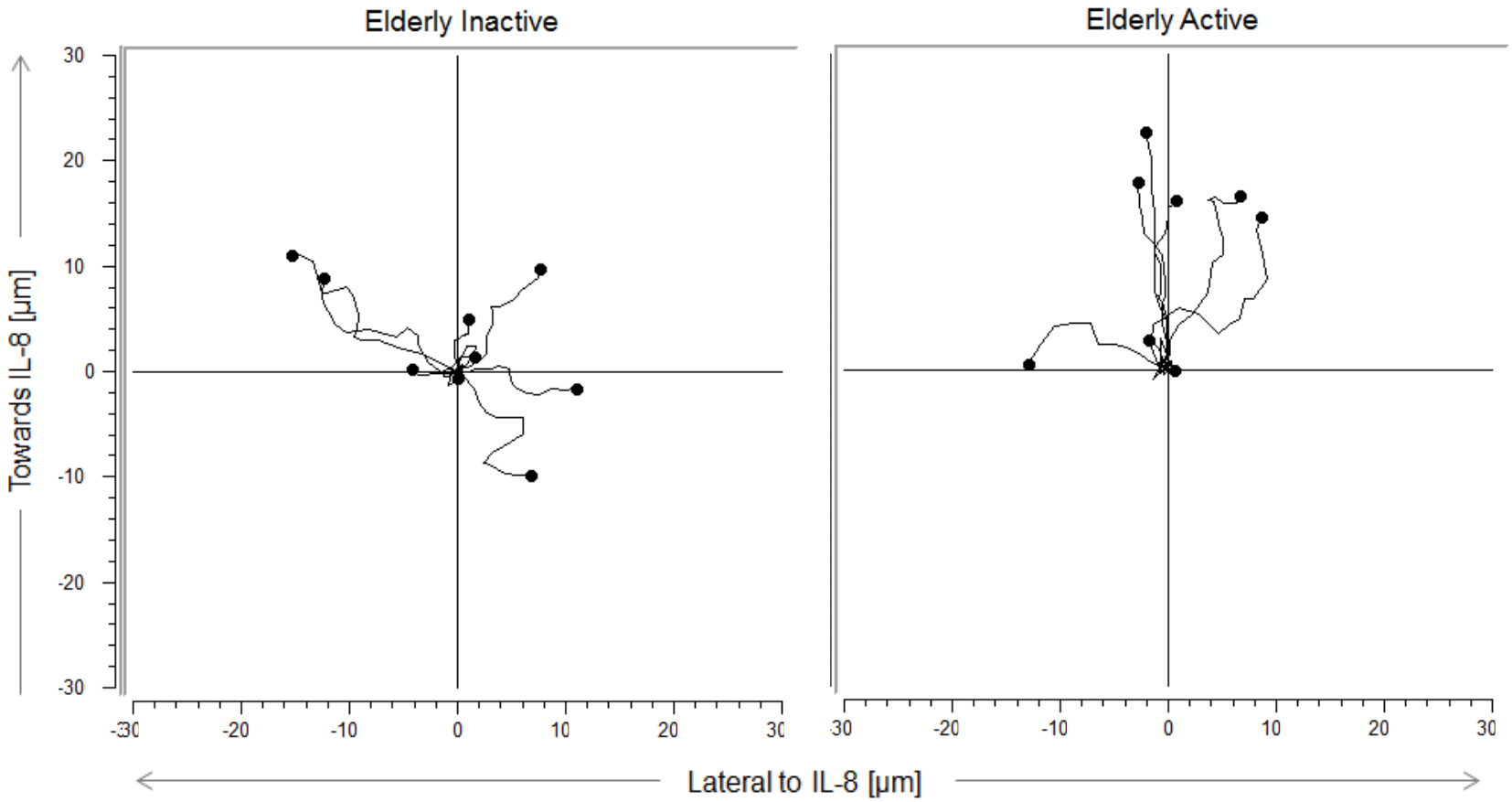

Fig. 4.4: Neutrophil migration and physical activity. Migration pathways of neutrophils from a representative low and high active elderly individual. Figures represent migration paths of single cells following centralisation to point 0, 0 on the axis. Migratory dynamics towards IL-8 (y-axis) are presented.

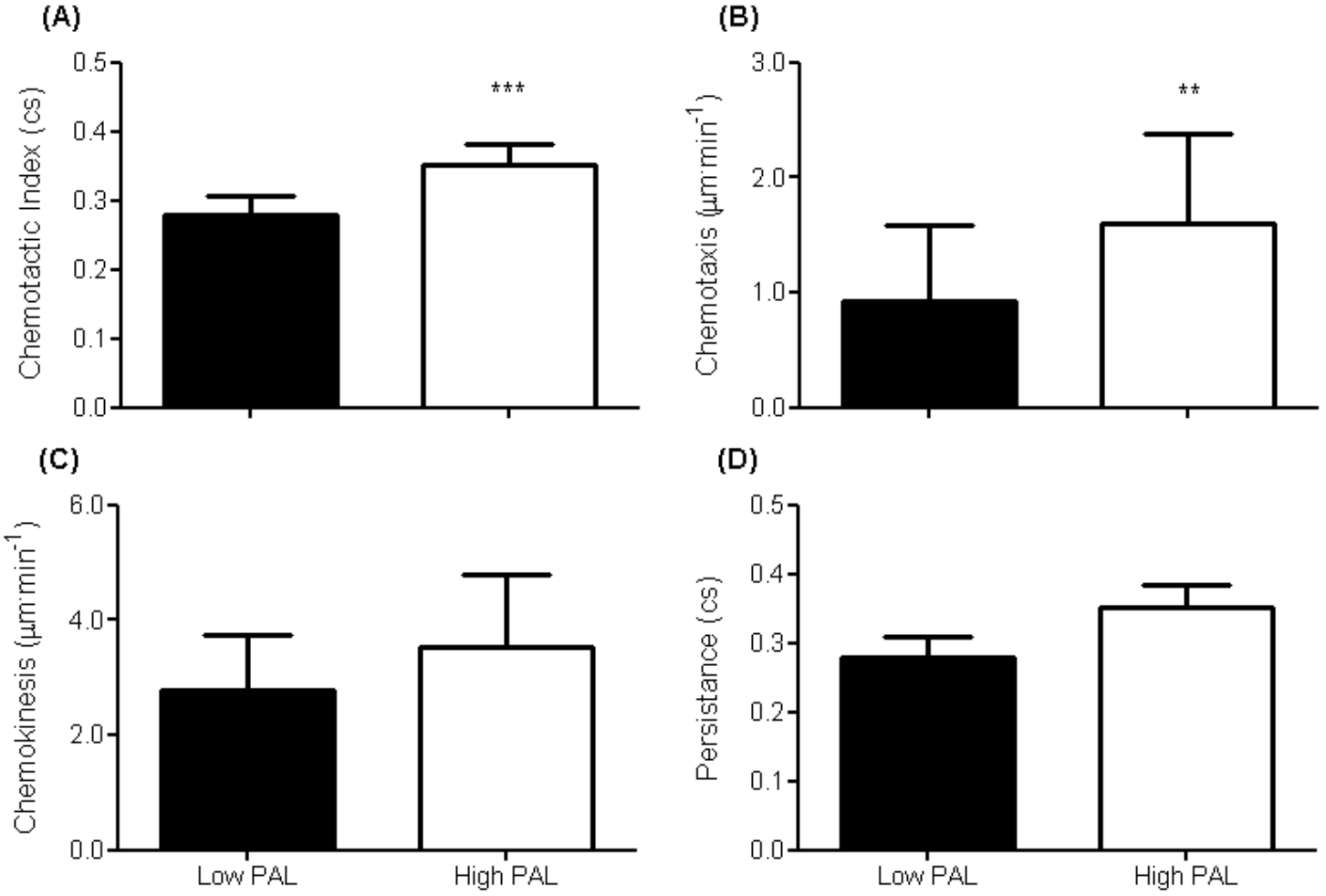

Fig. 4.5: Neutrophil migratory characteristics and physical activity. Neutrophil migration parameters towards IL-8 from low active (black bars) and highly active (white bars) elders. Data are mean ± SEM. ***p*<.01, ****p*<.001 compared to low PAL group

4.4.3 Neutrophil Receptor Expression Influencing Migratory Dynamics

4.4.3.1 Neutrophil Chemokine Receptor Expression

To determine whether the differences in migration were due to expression of the neutrophil IL-8 receptors CXCR1 (Fig. 4.6A) and CXCR2 (Fig. 4.6B), flow cytometric analysis of $CD16^{+}$ neutrophils was conducted. No differences were observed for CXCR1 [$F_{(1, 39)} = .012$, $p=.913$; $\eta^2=.000$] or CXCR2 [$F_{(1, 39)} = 1.03$, $p=.316$; $\eta^2=.029$] expression.

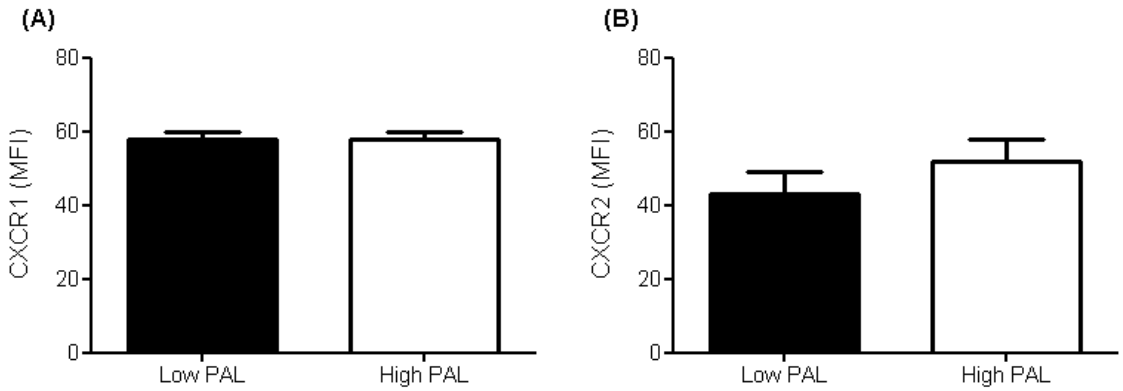

Fig. 4.6: Neutrophil surface expression of the IL-8 receptors CXCR1 (A) and CXCR2 (B) for low active (black bars) and highly active (white bars) elders. Data are mean ± SEM.

4.4.3.2 Neutrophil Adhesion

Neutrophils were migrated on glass cover slips coated in 7.5% BSA which can mimic the properties of ICAM-1 [300]. Therefore to determine whether migration could be affected by the surface expression of the ICAM-1 receptors, CD11b and CD18 were measured. Fig. 4.7A shows a 62% increased expression of CD11b [$F_{(1, 39)}$ = 3.57, p=.035; η^2=.109] on neutrophils from the high PAL group whilst no differences were observed for CD18 [$F_{(1, 39)}$ = .304, p=.585; η^2=.010; Fig. 4.7B]. Although CD11b expression differed between groups there were no significant associations with CD11b expression and migratory parameters suggesting CD11b expression may not be the primary contributor to migratory variances.

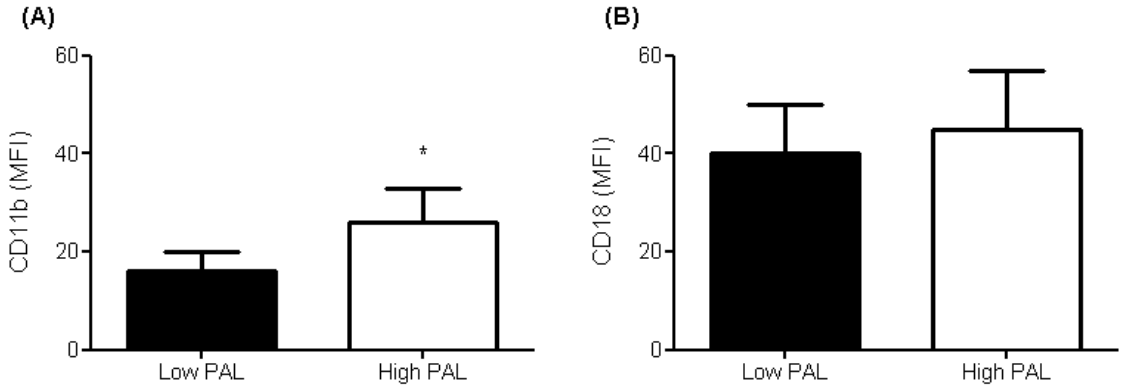

Fig. 4.7: Neutrophil surface expression of adhesion molecules CD11b (A) and CD18 (B) for low active (black bars) and highly active (white bars) elders. Data are mean ± SEM. *p<.05 different from low PAL group.

4.4.4 Neutrophil Pathogen Recognition Receptors

Upon arrival at the site of infection neutrophils are required to recognise bacterial components in order to begin resolution of infection. In order to determine whether physical activity status might affect the recognition of gram-negative and gram-positive bacterial components, expression of TLR2 (Fig. 4.8A) and TLR4 (Fig. 4.8B) on $CD16^{+}$ neutrophils was assessed. No differences were observed between groups for the expression of TLR2 [$F_{(1, 39)} = 1.44$, p=.238; η^2=.039] or TLR4 [$F_{(1, 39)} = .401$, p=.531; η^2=.012] suggesting both groups are equally capable of recognition of bacterial cell components.

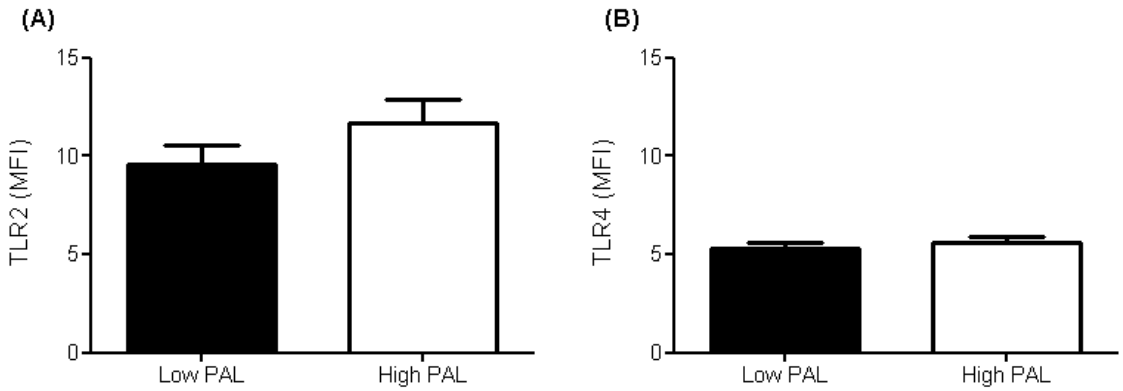

Fig. 4.8: Neutrophil expression of bacterial recognition receptors TLR2 (A) and TLR4 (B) revealed no differences between low active (black bars) and highly active (white bars) elders. Data are mean ± SEM.

4.4.5 Neutrophil Bactericidal Activity

In order to assess whether physical activity had an effect on neutrophil bacterial killing, assessment of phagocytosis of opsonised *E.Coli* (Fig. 4.9A) and superoxide production (Fig. 4.9B) in response to *E.Coli* was determined. Physical activity did not affect the ability of circulating neutrophils to uptake bacteria [$F_{(1, 39)}$ = .421, p=.521; η^2=.012] however there was a small but significant reduction in the superoxide produced per cell in the highly active elders [$F_{(1, 39)}$ = 3.11, p=.044; η^2=.088]. As superoxide production has previously been reported to be reduced in the elderly, and subsequently may be an indicator of neutrophil senescence, the same analysis was conducted on 10 younger individuals. Mean superoxide production for the young (23 ± 4 years) participants was 104 ± 8 (SEM) which was significantly less than the least active elders (p=.032) but not the highest active elders (p=.509). Therefore it could be that the superoxide production towards *E.Coli* was hyper-responsive in the least active elders leading to abnormal production of superoxide radicals.

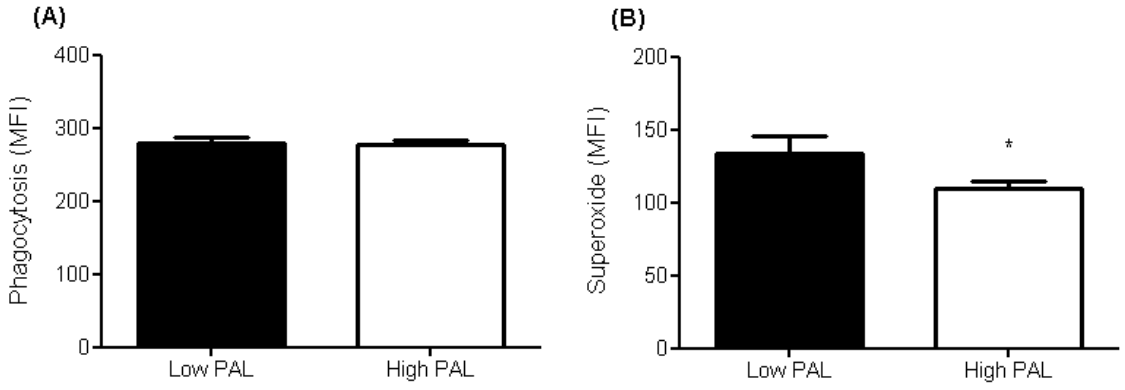

Fig. 4.9: Neutrophil bactericidal activity. Neutrophil phagocytosis (A) of *E.Coli* and superoxide generation (B) towards *E.Coli* for low active (black bars) and highly active (white bars) elders. Data are mean ± SEM. *p<.05 different from low PAL group.

4.4.6 Monocyte Phenotype

CD14+ monocytes consist of 3 distinct subtypes which are characterised by their expression of CD16 and are functionally distinct from each other. Although there was a trend for the highly active elders to have lower circulating numbers of monocytes (*p*=.056) there were no differences between the groups for the total numbers or percentages of the subtypes, Table 4.7.

Table 4.7: Monocyte Subtypes and PAL.
Median (IQR) monocyte subtype numbers and percentages in low and high active elders

	Physical Activity Status	
	Low	High
Monocytes ($x10^{9}.L^{-1}$)		
Classical	0.27 (0.18, 0.34)	0.18 (0.17, 0.26)
Intermediate	0.02 (0.01, 0.03)	0.02 (0.01, 0.02)
Non-Classical	0.03 (0.01, 0.04)	0.02 (0.01, 0.03)
Monocytes (%)		
Classical	86.0 (84.1, 89.5)	86.7 (82.1, 89.7)
Intermediate	5.4 (4.1, 7.1)	5.4 (3.9, 6.6)
Non-Classical	7.8 (5.1, 9.4)	7.2 (6.2, 9.4)

4.4.7 Monocyte Chemotaxis

No differences were observed between the activity groups for monocyte migration (*p*=.494) towards MCP-1 (Fig. 4.10A) using the transwell method. There was a trend towards total monocyte expression of CCR2 (Fig. 4.10B) being higher in the active elders [$F_{(1,\ 36)}$=3.48, *p*=.070; η^2=.088]. CCR2 expression on the classical [$F_{(1,\ 36)}$=4.51, *p*=.041; η^2=.111] and intermediate [$F_{(1,\ 36)}$=4.92, *p*=.033; η^2=.120] monocytes was on average 33% (for both) greater in the high active group compared to the least active group. The secondary receptor for MCP-1 on monocytes, CCR4 (Fig. 4.10C) was not different between groups and showed no differences for the monocyte subtypes. No differences were observed between activity groups for total

monocyte expression of the adhesion markers CD11b (Fig. 4.10D) or CD18 (Fig. 4.10E).

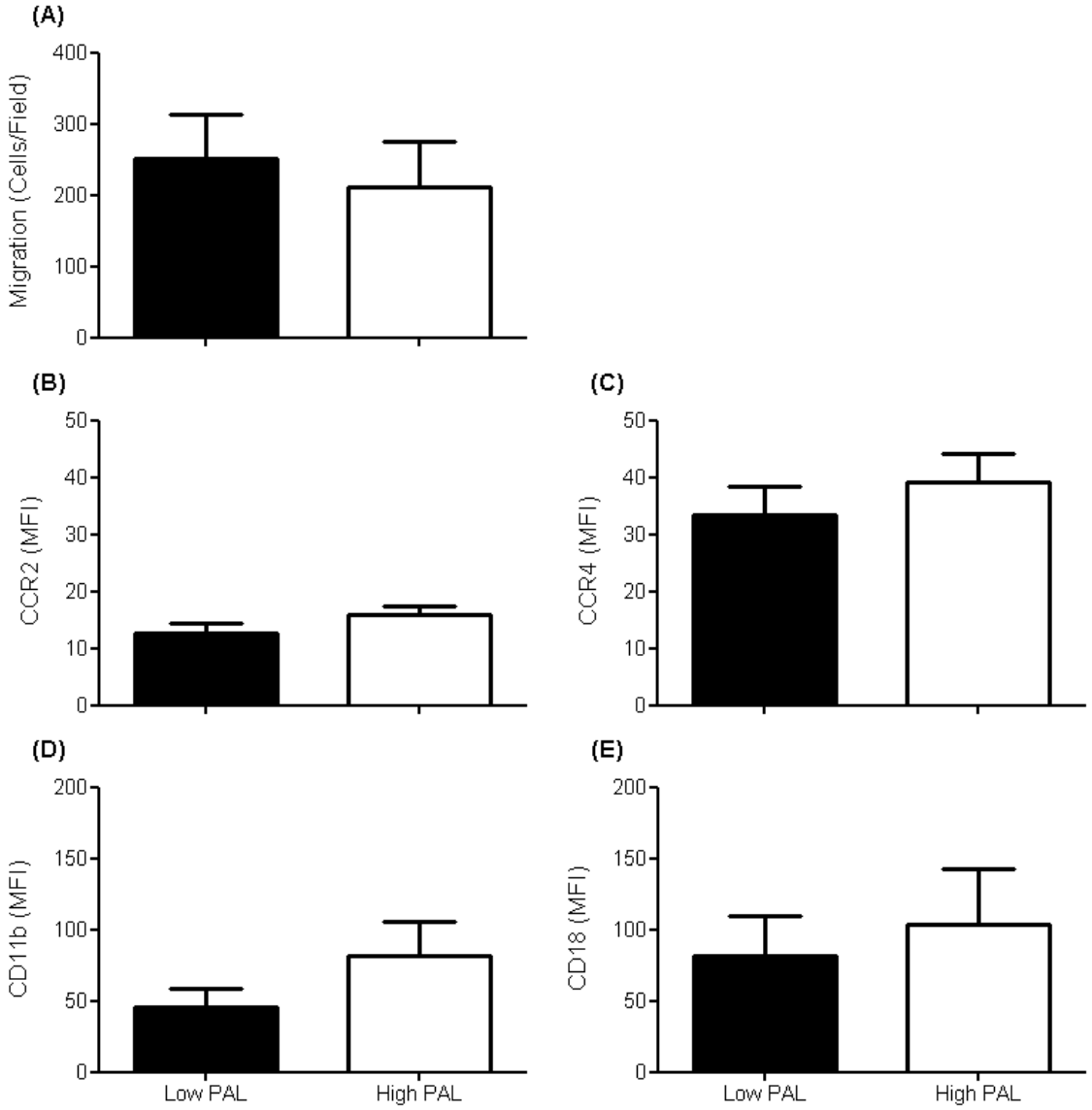

Fig. 4.10: Monocyte function. Monocyte migration towards MCP-1 (A) and the expression of the MCP-1 receptors CCR2 (B) and CCR4 (C) and the adhesion molecules CD11b (D) and CD18 (E) on monocytes isolated from physically inactive (black bars) and active (white bars) elders. Data are mean ± SEM.

4.4.8 Monocyte Bactericidal function

No differences were observed between groups for phagocytosis (Fig. 4.11A) of *E.Coli* or superoxide (Fig. 4.11B) production in response to *E.Coli*, (*p*=.814 and *p*=.421 respectively). There were no differences for TLR-2 (Fig. 4.11C) or TLR-4 (Fig. 4.11D) expression between activity groups or monocyte subtypes.

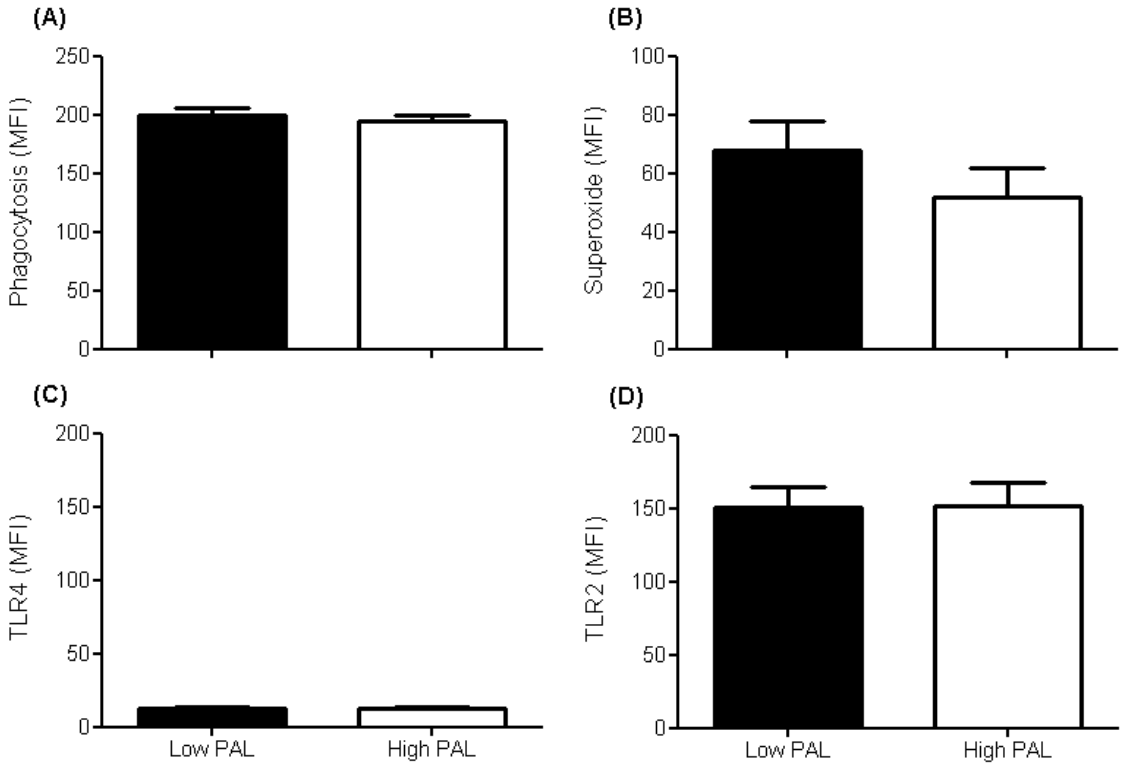

Fig. 4.11: Monocyte bactericidal activity. Monocyte phagocytosis of FITC-labelled *E.Coli* (A) and superoxide generation towards *E.Coli* after phagocytosis (B) as well as the bacterial recognition receptors TLR4 (C) and TLR2 (D) in cells isolated from physically inactive (black bars) and active (white bars) elders. Data are mean ± SEM.

4.4.9 Monocyte cytokine production

It is possible that over expression of inflammatory cytokines from stimulated monocytes may contribute to general systemic inflammation. To assess the inflammatory response to bacterial infection, peripheral blood monocytes were stimulated with 10ng·ml^{-1} of LPS and assessed for the expression of the pro-inflammatory cytokines IL-6 and TNFα. There was a trend (p=.070) for the highly active elders to produce less TNFα upon stimulation with LPS (Fig. 4.12A) but not for IL-6 (p=.290) production (Fig. 4.12B).

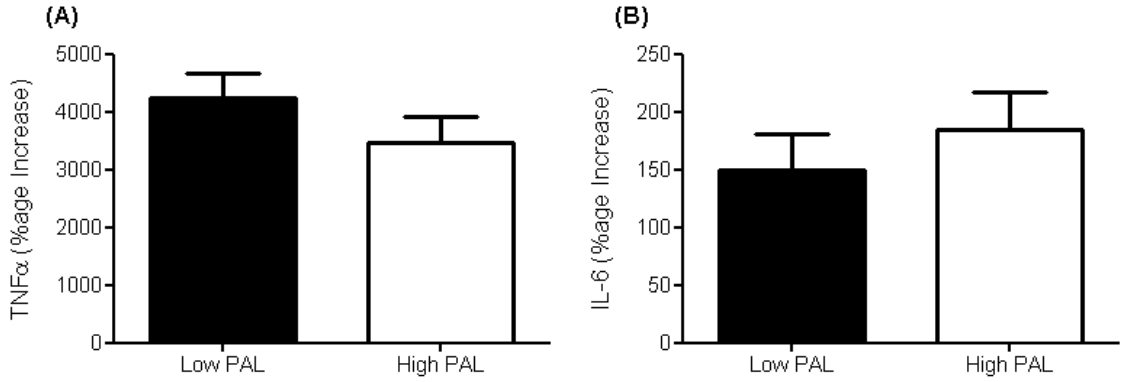

Fig. 4.12: Monocyte cytokine production. Percentage increase from unstimulated to LPS stimulated total monocyte expression of TNFα (A) and IL-6 (B) in cells isolated from physically inactive (black bars) and active (white bars) elders. Data are mean ± SEM.

4.4.10 Monocyte Antigen Presentation Capacity

Elderly individuals have previously been shown to have reduced antigen presentation capabilities. Therefore the expression of HLA-DR, the human analogue of MHC-II, was assessed on monocyte subtypes between groups. Analysis revealed there was a trend for highly active elders to have higher HLA-DR expression on classical monocytes (p=.080) but not intermediate (p=.275) or non-classical (p=.121) compared to less active elders, data not shown.

4.5 Discussion

The previous chapter revealed that increased chronic systemic inflammation occurs over a number of years in healthy older adults and is directly associated with prevalence of frailty and risk of mortality. Furthermore inflammation was not dependent on adaptive immune responses to latent CMV infection as previously thought [256]. These results suggested that inflammageing may be determined to a greater extent by behavioural lifestyle choices such as being sedentary. However we were unable to assess immune function associations with inflammation or with lifestyle choices in the Hertfordshire Ageing Study as immune cells were not available.

Innate immune dysfunction with age results in a number of severe modifications that can result in significantly impaired responses, but also basal activation of cells such as monocytes [162]. Impaired innate function can result in elongated and increased occurrence of infectious episodes. Aberrant responses such as reduced chemotaxis and increased reactive oxygen species production can contribute to this [168, 170].

This chapter aimed to confirm the associations presented in chapter 3 such as the lack of relationship between CMV infection and inflammation, the association of a sedentary lifestyle and inflammation and the impact of systemic inflammation on progression towards physical impairment in older adults. In confirmation of the previous chapter the data showed that age was associated with increased inflammatory mediators and impaired endocrine function, in addition being positive for CMV in old age did not account for elevated inflammation. However accounting for age; body composition and physical inactivity were associated with elevated inflammatory status and inflammatory mediator levels were predictive of lower

physical functioning. As discussed in the previous chapter these findings are comparable to both the previous study and those of others.

In addition metabolic biological variables including leptin, adiponectin, cholesterol, triglycerides and insulin sensitivity were assessed. Increased glucose and insulin content in conjunction with systemic inflammation were associated with insulin sensitivity which can lead to tissue damage resulting in increased risk of type-2 diabetes [301]. Furthermore, cholesterol and lipid by-products are associated with a number of health conditions such as atherosclerosis [130]. Supporting other reports in the literature the data showed that men and women differ significantly in their metabolic profiles such as leptin and adiponectin as well as cholesterol [302]. Similarly physical activity is associated with a number of metabolic differences including improved insulin sensitivity and approximated β-islet function, reduced leptin and increased HDL cholesterol [303]. However the metabolic health of all of the participants as assessed using the Karelis and the Meigs criteria showed that only nine were metabolically unhealthy and none of these were included in the recall analysis [299, 304].

As immune function is reduced with age and is intrinsically linked with increased systemic inflammation it is likely that the effects of physical activity on inflammation and endocrine dysfunction may parallel immune function [294]. Therefore this chapter aimed to assess the relationship of physical activity with innate immune cell function in this cohort and its relationship with systemic inflammation. To do this the top and bottom 20% of each physical activity group was age, gender and clinically matched as best as possible and recalled for assessment of neutrophil and monocyte functional capacity.

Neutrophil dysfunction is well documented with ageing particularly with respect to bactericidal capacity and migration [168, 172]. The consequences of this are profound and increase the risk of infection in the elderly, especially bacterial infection which is one of the leading causes of mortality in the elderly [172]. Our group recently showed that neutrophils from the elderly migrated less accurately than those from young subjects [168]. Here it is confirmed that neutrophils from the elderly have reduced chemotactic ability, however it is clear that the elderly are not a homogenous group but consist of individuals who in effect appear younger than their age. Thus isolated neutrophils from physically active elderly individuals migrated more accurately than physically inactive elders.

Systemic inflammation with ageing is intertwined with immunesenescence with each factor potentially contributing to the severity of the other. Elevated inflammatory mediators, both locally and systemically have been suggested to reduce immune cell function. However, Sapey and colleagues assessed isolated peripheral neutrophil function from the elderly following incubation with plasma from young and old donors [168]. They determined that removing neutrophils from an old environment and placing it into a new one does not restore function. Therefore differences in the elderly neutrophils are likely to be cell intrinsic and not necessarily due to systemic inflammatory exposure, although it cannot be ruled out that the bone marrow inflammatory environment is not driving these cell changes [305]. To address this a very healthy cohort of elderly active and inactive individuals were studied here and the data show that any differences in inflammation were attenuated by adjusting for body fat. However neutrophil migration remained statistically different between physical activity groups adjusting for both inflammatory state and fat mass, suggesting that systemic inflammation may not be exclusively driving neutrophil

defects such as altered chemotaxis. Instead low physical activity appears to be the primary determinant of reduced neutrophil migration which may lead to poorer infection control and subsequently increased mortality often associated with sedentary behaviour [306].

These findings are comparable to animal studies suggesting neutrophil migration towards sites of infection is improved following exercise [307]. Few studies have assessed the impact of exercise on neutrophil migration in humans. Wolach and colleagues suggested neutrophil migration was impaired 24 hours after an acute bout of exercise in young healthy adults suggesting an exercise induced defect [308]. Although the exact mechanism of this impairment remains elusive it was observed that chemotaxis was restored 48 hours after exercise and highlights that exercise sessions are able to transiently alter neutrophil function. Additionally Syu and colleagues showed an increased chemotactic ability immediately after an acute bout of exercise [246]. Furthermore, it was shown that neutrophil chemotaxis was improved following 4 months of moderate exercise sessions in young adults. These findings are compatible with the findings in this chapter that regular bouts of moderate activity have the potential to improve neutrophil migratory dynamics.

This study is the first to assess neutrophil migration using an accurate tool in healthy active and inactive elders. The data suggest that neutrophils from physically active elders have the potential to reach sights of infection both quicker and with improved accuracy (chemotactic index). The consequences for health of this are two-fold; first the neutrophils in theory can engage and deal with infection quicker causing improved resolution. Secondly the accuracy of migration can reduce non-specific tissue damage caused by neutrophils migrating inaccurately through tissue and causing excessive protease mediated damage.

In order to assess whether the effects of physical activity on migration were achieved by chemokine receptor expression, CXCR1 and CXCR2 was assessed. Sapey and colleagues suggested there were no differences for CXCR1 and CXCR2 expression in neutrophils from young and old donors or following stimulation [168]. Furthermore in their study, Sapey and colleagues suggest that neutrophil migratory defects are associated with increased intracellular PI3K expression [168]. Therefore it was not surprising that no differences in chemokine receptor expression on quiescent neutrophils were seen in our active and inactive elders. However, the data did show that the physically active participants had increased basal expression of CD11b but not CD18 suggesting that MAC-1 formation overall was unaltered. This is in agreement with a number of other studies which have suggested MAC-1, one of the ligands for ICAM-1, is unaffected by either age or physical activity status [309]. It is unclear whether increased CD11b was associated with improved adherence to the glass cover slip or indicative of increased activation. Interestingly neutrophil expression of CD11b has been shown to be increased in response to muscle damaging exercise and may reflect an inherent activation status in order to repair muscle [310]. However CD11b has been shown to be unaffected following aerobic exercise sessions [311]. Additionally no differences have been observed between basal CD11b expression in young and elderly individuals[172]. Therefore the role of elevated CD11b expression in the physically active elders remains unclear. Future work should attempt to replicate this and assess *in vitro* what the relation is with migratory function.

Neutrophil bactericidal activity has been shown to be consistently impaired in the elderly and so if neutrophil migration was maintained in the physically active elders it was possible that the same may apply for bacterial killing. Bacterial killing was

assessed by incubating whole blood with opsonised *E.coli* and assessing the percentage of cells capable of ingesting fluorescently labelled bacteria and the relative amount each cell was capable of ingesting. Additionally one of the killing mechanisms, superoxide production, was assessed in a similar manner this time superoxide production was determined fluorescently following ingestion of opsonised *E.coli*. No differences were observed between the activity groups for each of the bactericidal functions suggesting that regular physical activity is unable to modify these responses. Butcher and colleagues suggested that phagocytosis is reduced in the elderly due in part to a reduced expression of CD16 on the cell surface [172]. There were no differences in CD16 expression between the activity groups suggesting that phagocytosis would be unaltered. Furthermore there were no differences in bacterial recognition receptors TLR4 or the gram-positive bacteria specific TLR2. However this is in contrast to others who have shown that older adults are able to improve bacterial killing following acute exercise as well as following longer term exercise interventions [226, 246]. These studies were unable to determine the mechanism by which physical activity can improve bactericidal functions. One difference between this study and others in the literature is perhaps the degree of difference in activity levels between the participants. Yan and colleagues assessed elderly participants with over a 5 fold increase in activity compared to the inactive group and showed only a moderate effect on immune function [226]. Therefore as our active participants had barely double the activity levels of the inactive group it is likely that bactericidal activity is more sensitive to physical activity than migration and if we had more disparate levels of activity the same differences could have been observed.

In light of the neutrophil data it is likely that the lack of effect for physical activity status on monocyte function was also due to the two groups not being different enough in their activity level. Monocyte function in response to physical activity has received a great deal more attention than neutrophils recently and is often associated with improved chemotaxis and inflammatory phenotype [312]. Monocyte migration to the chemokine MCP-1 and the expression of the MCP-1 receptors CCR2 and CCR4 were assessed here, but no differences were found. This is in contrast to some studies reported for younger adults and exercise [313]. In these studies monocyte migration was assessed in younger adults but using pooled peripheral blood mononuclear cells (PBMCs) containing T, B and NK-cells, whereas in this chapter isolated monocytes were used. Any effects seen in young adults could therefore be indirect and mediated by other cells in the PBMC fraction.

Furthermore, inflammatory phenotype has been reported to be improved in elderly individuals participating in physical activity interventions [237]. Reduced CD16+ expression and reduced TLR expression have been associated with improved monocyte inflammatory status due to reduced LPS-induced TNFα production [238]. However it remains unclear whether these are beneficial to the host due to the necessity for the monocyte to direct immune responses by its inflammatory actions. CD16+ monocytes on the other hand are capable of basally producing more inflammatory cytokines such as TNFα and as the elderly have a greater proportion of CD16+ cells reduced numbers may lower systemic inflammation. In contrast to studies which show a reduced CD16 expression following training or in active elders here no differences were seen in receptor expression between activity groups [238, 314].

Monocyte and macrophage phagocytosis is suggested to be impaired in the elderly due in part to cellular dysregulation such as increased inflammatory state and reduced telomere length [158]. Here no difference was seen in TLR or CD16 expression and so it was not surprising that monocyte bactericidal functions were not different. To date the only other study to assess monocyte phagocytosis in the elderly in response to any form of exercise revealed no effect for exercise interventions [243]. In addition, there were no differences in basal or LPS stimulated TNFα or IL-6 production from monocytes in the active and inactive elders, ruling out an effect on monocyte function as a mediator of reduced inflammation. Therefore it can be concluded that in the group studied here physical activity did not have a significant impact on monocyte function.

4.6 Limitations and Future Work

There were some major limitations to this study which are inherent in all cross-sectional investigations. Firstly this study adopted very rigorous inclusion criteria that were similar to the Seniuer Protocol and in doing so there may have been recruitment bias [315]. As each of the participants had to be free from a number of age-associated chronic conditions and be able to walk to the clinic for assessment it was not possible to include truly sedentary individuals in the analysis. However had the study recruited such individuals it would have been impossible to determine whether measured differences were due to underlying morbidities. Therefore although it is admirable that effects for physical activity were seen, future studies

should attempt to screen activity levels even further to determine the lowest healthy limit of activity and assess similar measures.

In respect to biological measures the study was limited by what could be assessed on the blood available and therefore it may have missed intrinsically different variables. This was evident in the assessment of neutrophil migration. Although Sapey and colleagues determined neutrophil migration to be associated with dysregulated PI3K activity it was not possible to assess this here. Therefore it remains unclear whether physical activity is altering the PI3K as a means to improve migration or whether there is another pathway responsible. Future studies assessing neutrophil migration should consider intrinsic cellular pathway analysis as a means to determine the mechanisms of action and identify potential novel therapeutic targets. In addition to this it would be beneficial to assess migration in the groups to a number of other neutrophil targets such as gram-positive *S.aureus* to ensure that the migratory differences are not limited to one chemokine.

4.7 Conclusions

In summary the findings from the previous chapter that ageing is associated with increased systemic inflammation which is not being driven by latent viral CMV infection were confirmed. Physical activity lifestyle choices and body composition were associated with elevated inflammation and endocrine dysfunction and these increases were associated with greater physical impairment. Therefore being physically active in later life may offer protection against many age associated

inflammatory conditions such as Alzheimers and type 2 diabetes and protect from physical frailty.

Neutrophil function, specifically chemotaxis, was enhanced in physically active individuals suggesting improved infection control and resolution and reducing the risk of morbidity and mortality associated by age induced immune dysfunction. Although neutrophils could kill bacteria equally well there is no doubt that improving migration to the site of infection could ultimately limit the infectious episode. There were no differences in monocyte function between active and inactive elders and this may be due primarily due to the lack of truly sedentary participants.

In conclusion, inflammation, endocrine and immune function show modest improvements in physically active elderly individuals and may reduce the risk of morbidity and mortality in later life.

Chapter 5: Inflammatory and Immune Responses to Acute Exercise in Physically Active and Inactive Elders

5.0 Introduction

The interactions of immunesenescence and systemic inflammation drive the elderly towards morbidity and mortality. With an ageing population already currently accounting for 30% of the NHS budget despite making up 16% of the population, there is a pressing need to investigate economical, non-invasive methods to counter the impact of immunesenescence and inflammageing in the elderly. Participation in regular periods of moderate-intensity exercise has been associated with a number of health benefits including reduced risk of cardiovascular disease, increased longevity and a reduced risk of infectious episodes [316]. Furthermore, exercise offers a cost-effective and easy to implement tool with often little need for equipment and can be effectively performed in the home or clinic [294].

Chronic sub-clinical systemic inflammation is associated with increased fat mass, genetic predisposition and immunesenescence. Both chronic and acute exercise sessions have been shown to modulate the inflammatory phenotype of the individual. In particular circulating CRP has been consistently shown to be lower in physically trained individuals [317], those who have undertaken a moderate intensity exercise intervention, non-obese individuals and the young. Subsequently as exercise alters the metabolic profile and adipocyte mass, there is a reduced inflammatory milieu. In addition to alterations in inflammation there is an exercise induced change in adipose resident macrophages from inflammatory M1 to anti-inflammatory M2 [236].

A major consideration of acute exercise is the susceptibility of the participant to infection following exercise if exercise is excessive [211]. Known as the ‘open window’ hypothesis of infection there is an increased susceptibility to contracting

infection due to the lymphopenia and reduced function of T-cells and NK-cells in the hours following exercise [318]. These effects are often associated with the intensity of exercise and nutritional status of the participant [319, 320]. As the elderly are at an increased risk of infection due to immunesenescence it is pertinent to ensure that the intensity of exercise will reduce infection susceptibility whilst improving immune function and inflammatory status in this population.

With increased age, the immune system is remodelled with reduced specific responses to pathogens, increased basal inflammation and reduced immune tolerance. Therefore understanding the effects of acute exercise on innate immunity in the aged is highly pertinent. Few studies have assessed the impact of acute exercise sessions on the innate immune system, and of them monocyte analysis is a majority. However both neutrophils and monocytes represent innate cells that are capable of modulating the immune response and severity of the inflammatory response.

Greater neutrophil phagocytosis has been observed in middle aged participants who regularly exercised compared to non-exercisers [226]. Although few studies have shown reduced absolute circulating numbers of neutrophils in response to exercise, those that have assessed elderly patients with chronic low-grade inflammation suggest a link with inflammation, neutrophil function and exercise [244]. Studies assessing acute exercise effects on neutrophils are few and predominantly in the young. Young to middle aged trained (38 ± 4 years) males have been shown to increase neutrophil phagocytic capacity following 45 minutes cycling compared to sedentary males [245]. In young individuals (20-24 years) a short maximal ergometer test resulted in increased chemotaxis but not percentage of phagocytic cells [246].

These data increase the prospect that neutrophil function can be modified with exercise, however the effects remain to be determined in the elderly.

Monocytes are key mediators of infection resolution by not only aiding clearance of bacteria but also communicating and directing the adaptive response via cytokine production and antigen presentation. Monocyte responses to chronic and acute exercise training have received more attention in the elderly than neutrophils. As toll-like receptor (TLR) activation is reduced with age [144] the majority of studies have focused on TLR changes with exercise. TLR activation and monocyte sub-type composition, particularly the CD16+ subsets are associated with increased inflammation potential. Elderly trained individuals have been shown to have reduced TLR4 expression on CD16+ monocytes and reduced stimulated monocyte production of TNFα [321]. Although others have shown no differences in TLR4 expression or phagocytic capacity following 12-weeks training, there was an increase in co-stimulatory ability of T-cells by increased expression of CD80 in one study [322]. These results suggest that exercise has the ability to modify the pro-inflammatory and functional capacity of monocytes dependent on intensity and duration of exercise sessions. The general consensus being that shorter duration, moderate intensity exercise offers physiological benefits whilst reducing the risk of causing immune and inflammatory dysfunction

It is clear that there is a complex relationship between training status, immune function and inflammation which offers an explanation of the beneficial effects of habitual physical activity and exercise on the health of the elderly. To date no studies have comprehensively assessed the combined effects of an acute bout of exercise on immune function and inflammation in elderly men and women who are either physically active or inactive. The purpose of the current study was to assess whether

predicated aerobic capacity as a result of habitual physical activity in older (>60 years) life was associated with altered inflammatory state and phagocyte function. Specifically the aims of this chapter were:

- To determine whether the activity status of the individuals influenced inflammation and neutrophil/monocyte function at rest.
- To assess whether a short moderate bout of walking could alter inflammatory status and immune function in a protective manner, and if levels of habitual physical activity influenced the response.

5.1 Acute Intervention Exercise Study Methods

5.1.1 Participants

Participants were 31 elderly adults who were recruited from the local Birmingham community, aged between 60 and 77 years. Inclusion criteria were: no current immune and no known hormonal disorder, no eating disorder, no psychiatric disorder, no acute illness and should not be taking medication specifically known to alter the immune system or hormone balance. Participants were also recruited on the basis that they were either regularly physically active or physically inactive and could walk continuously for 20 minutes unaided. Participants were either physically inactive (n=15) or physically active (n=16). Inactive participants were recruited locally and not involved in any regular structured physical activity currently, or in the previous 5 years. Physically active participants were recruited from local activity groups such as rambling, keep fit classes and local running clubs. As is typical with 'healthy' elderly participants, 22% suffered conditions such as hypertension and 48% were taking medications such as an antihypertensive and/or gastrointestinal medication. No significant differences were observed for socio-economic status, chronic illness or medications taken between the exercise groups. All participants gave written informed consent prior to commencement of the study, which was approved by the University Research Ethics Committee (West Midlands, UK).

5.1.2 Exercise Diary

Confirmation of participant activity levels was determined from the completion of a 14-day consecutive exercise diary. Variables recorded were activity type, duration and intensity. Exercise intensity was determined using a 0–10 RPE scale with 0 as rest and 10 as maximal effort [323]. Time spent in moderate and vigorous intensity

activities was based on metabolic equivalents (MET) for given activities [324] and RPE. Minutes spent in moderate and vigorous activities were averaged for the 14-day period and from this an exercise score created using the 0–5 categorical scoring system from the Whitehall Study [325]. Combined scores were determined by weight adjusting the categorical score by multiplying by moderate activities by 2 and vigorous activities by 3.

5.1.3 Experimental Protocol

Participants were informed not to drink alcohol and to abstain from exercise in the 24 hours prior to testing and consume only water on the morning of testing. Testing was conducted between 8 and 9 am and upon arrival participants were informed of the testing timeline for the morning. Following anthropometric (height and weight) characterisation participants completed an incremental submaximal treadmill walk test using Douglas Bag (Cranlea, Birmingham, UK) gas analysis to measure aerobic capacity. Participants were fitted with a heart rate (HR) monitor around the chest and HR measured continuously by telemetry (Polar Electro, Kempele, Finland) and recorded every 15 seconds. Familiarisation of the treadmill, Douglas bag mouthpiece and nose-clip was completed prior to testing. Following 15 minutes of seated rest for venous blood collection participants began walking on the treadmill with the speed gradually increased until a self-rated level of ‘brisk walk’ was achieved. Every 4 minutes, gradient increments of 2% and 3.5%, depending on HR, were applied. During the final minute of each stage expired air samples were collected into Douglas bags for oxygen consumption analysis. Ratings of perceived exertion (RPE) were determined during the final minute of each stage. The test was terminated once the participant had reached 75% of their predicted maximum HR or an RPE rating of ‘hard’ in participants who’s HR did not increase proportional to the exercise intensity.

75% of predicted maximum HR was determined by the formula [208 – (age in years x 0.7)] [326]. Following cessation of exercise participants were immediately seated and venous blood samples taken before remaining seated for the final blood sample 1 hour later.

Oxygen (O_2) consumption and carbon dioxide (CO_2) production were assessed from the Douglas bag samples using a 1440-infrared CO_2 analyser and paramagnetic O_2 analyser (Servomex, Crowborough, UK). Expired air volume was measured using a standard dry gas meter (Harvard Apparatus, Edenbridge, UK) and corrected to current temperature and air pressure. Maximal O_2 consumption (VO_{2max}) was predicted by plotting HR and O_2 consumption during the final 3 stages of exercise and regression analysis using age-adjusted maximum HR.

This study was completed with the collaborations of Dr Anna Phillips of Sport and Exercise Sciences who conducted sections 5.1.1 – 5.1.3.

5.1.4 Biological Analyses

Peripheral blood samples were taken from a vein in the antecubital fossa before the commencement of exercise but after 15 minutes of seated rest (Pre), immediately upon cessation of exercise (Post) and 1 hour after cessation of exercise (1 Hour Post). The following biological analysis was completed and described in section 2.2. Inflammatory and anti-inflammatory cytokines were measured by magnetic XMap technology. CRP, cortisol and DHEAs were measured by ELISA. Neutrophil and monocyte bactericidal function was assessed using phagotest and phagoburst kits, monocyte and neutrophil phenotype were assessed by flow cytometry.

5.1.5 Statistical Analysis

Statistical analyses were performed similar to previous chapters. Individuals were grouped into low and high active groups based on their predicted VO_{2max} and Exercise Diary results. Univariate ANOVA was conducted to assess differences of anthropometric characteristics between the two groups taking into account gender. Following this repeated measure ANOVA and ANCOVA were conducted to assess the changes in biological variables over the three time points and whether physical activity status influenced the effects with BMI included in the analysis as a covariate. The assumption of sphericity was tested using Mauchley's method and any violations corrected for using the Greenhouse-Geisser method. Partial η^2 is reported as a measure of effect size. Post-hoc pairwise analysis with Bonferroni correction was conducted to assess biological variable differences over time in each activity group. Statistical significance was accepted at $p<.05$.

5.2 Results

5.2.1 Group Characteristics and Exercise Test

Group characteristics and exercise test data for the activity groups is presented in Table 5.1. BMI [$F(1, 29)=19.48$, $p<.001$, $\eta^2=.402$] and estimated VO_{2max} [$F(1, 29)=34.61$, $p<.001$, $\eta^2=.601$] differed significantly with VO_{2max} withstanding adjustment for age. There were no differences between groups for any of the data collected during the incremental walk test. Mean duration of the incremental walk test was 22.83 ± 0.94 minutes with final HR at the end of the exercise 133 ± 10 bpm which was equivalent to 76.7 ± 5.5 % of their predicted maximal HR. This HR value suggests that the majority if not all of the exercise session was conducted in an aerobic state. Final RPE score at the end of the test was 5.1 ± 1.2 which was equivalent to a rating of 'Hard'.

Table 5.1: Participant characteristics and exercise test data. Data are Mean ± SD.

	Low Active (N=16)	High Active (N=15)
Sex (M/F)	7/9	9/6
Age (years)	68.0 ± 4.7	65.1 ± 4.4
BMI ($kg \cdot m^2$)	26.0 ± 2.8	22.2 ± 2.0***
Estimated VO_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	31.2 ± 6.1	45.6 ± 5.9***
Estimated VO_{2max} ($L \cdot min^{-1}$)	2.3 ± 0.6	3.1 ± 0.6**
Exercise Duration (min)	22.7 ± 1.3	23.0 ± 0.3
Final HR (bpm)	134 ± 11	132 ± 10
Final HR (% of max)	77.9 ± 5.7	75.4 ± 5.2
Final RPE	5.2 ± 1.2	5.0 ± 1.2

**p<.001, ***p<.001 compared to the Low Active group

5.2.2 Inflammatory and Endocrine Responses to Acute Exercise

5.2.2.1 Inflammatory Mediators

Table 5.2 outlines the effect of the exercise protocol, split by basal activity level groups, on circulating pro and anti-inflammatory cytokines, growth factors and acute phase proteins. There was a significant main effect of exercise for the pro-inflammatory cytokines IL-1β [$F(2, 60)=5.95$, $p=.004$, $\eta^2=.166$], IL-6 [$F(2, 60)=5.04$, $p=.009$, $\eta^2=.144$] and IL-8 [$F(2, 60)=8.69$, $p<.001$, $\eta^2=.225$] which were significantly increased from Pre to 1 Hour Post exercise. Similarly there was a significant main effect of exercise for GM-CSF, an inflammatory protein which stimulates growth and differentiation of neutrophils and monocytes [$F(2, 60)=58.63$, $p<.001$, $\eta^2=.701$]. The acute phase protein CRP showed a significant main effect of exercise [$F(2, 60)=11.24$, $p<.001$, $\eta^2=.286$], which was driven by a reduced concentration at 1 Hour Post exercise in the low active group and was the only inflammatory marker which was significantly different at all time-points between groups ($p=.002$ for all). However, after adjustment for BMI the differences between groups for CRP were attenuated ($p>.05$ for all times). Furthermore, correlation analysis revealed that both BMI [$r(29)=.48$, $p=.004$] and estimated relative VO2max [$r(29)= -.40$, $p=.029$] were associated with CRP whilst absolute VO2max [$r(29)= -.22$, $p=.161$] was not. This suggests that body composition and not aerobic fitness is driving differences in CRP concentrations between groups. Of the anti-inflammatory cytokines there was a significant main effect of exercise for IL-13 which increased from Pre to 1 Hour Post [$F(2, 60)=9.02$, $p<.001$, $\eta^2=.231$].

5.2.2.2 Endocrine Responses

There was a significant main effect of exercise for cortisol (Fig. 5.1A), which decreased from Pre to 1 Hour Post [$F(2, 60)=20.67$, $p<.001$, $\eta^2=.408$]. Males had higher cortisol levels than females at all time-points [$F(1, 29)=5.21$, $p=.03$, $\eta^2=.152$]. There was a significant main effect of exercise for DHEAs (Fig. 5.1B), which increased slightly Post exercise [$F(2, 60)=4.27$, $p=.018$, $\eta^2=.125$], however males had higher levels of DHEAs than females at all time-points [$F(1, 29)=4.63$, $p=.04$, $\eta^2=.138$]. Due to the effects of the exercise on cortisol and DHEAs there was a significant main effect of exercise for the cortisol: DHEAs ratio (Fig. 5.1C), which decreased from Pre to 1 Hour Post exercise [$F(2, 60)=12.76$, $p<.001$, $\eta^2=.298$].

Table 5.2: Acute exercise effects on inflammation. Cytokine, growth factor and acute phase protein concentrations in response to the exercise protocol for physical activity groups. Data are Mean ± SEM.

	Low Active			High Active			Exercise Effect	Exercise x Group
Biomarker	Pre	Post	1 Hour Post	Pre	Post	1 Hour Post	(*p*)	(*p*)
Pro-Inflammatory								
IL-1β ($pg \cdot ml^{-1}$)	0.35 ± 0.07	0.25 ± 0.07	0.79 ± 0.35**	0.21 ± 0.05	0.27 ± 0.05	0.44 ± 0.08**	.004	.995
IL-6 ($pg \cdot ml^{-1}$)	2.46 ± 0.30	2.55 ± 0.30	3.30 ± 0.41**	1.87 ± 0.33	2.03 ± 0.44	2.13 ± 0.30**	.009	.296
IL-8 ($pg \cdot ml^{-1}$)	3.85 ± 0.47	3.37 ± 0.54	4.80 ± 0.65**	2.98 ± 0.51	3.02 ± 0.52	4.30 ± 0.65**	<.001	.645
IL-17 ($pg \cdot ml^{-1}$)	4.30 ± 2.04	2.97 ± 1.26	2.81 ± 1.38	2.39 ± 0.92	8.39 ± 5.88	6.75 ± 4.01	.408	.134
GM-CSF ($pg \cdot ml^{-1}$)	4.56 ± 1.08	4.60 ± 0.98	6.16 ± 1.24***	5.90 ± 1.30	5.83 ± 1.99	6.49 ± 1.59***	<.001	.684
TNF-α ($pg \cdot ml^{-1}$)	9.33 ± 1.22	8.08 ± 0.79	9.78 ± 1.59	8.17 ± 1.43	7.39 ± 1.68	7.83 ± 1.59	.467	.739
CRP ($mg \cdot L^{-1}$)	3.44 ± 1.51	3.48 ± 1.56	3.22 ± 1.46*	0.58 ± 0.11	0.62 ± 0.12	0.66 ± 0.15	<.001	.668
Anti-Inflammatory								
IL-4 ($pg \cdot ml^{-1}$)	0.59 ± 0.07	0.66 ± 0.09	0.68 ± 0.11	0.53 ± 0.09	0.51 ± 0.09	0.60 ± 0.11	.130	.660
IL-10 ($pg \cdot ml^{-1}$)	1.08 ± 0.82	1.01 ± 0.93	1.36 ± 1.06	0.36 ± 0.20	0.39 ± 0.19	0.32 ± 0.14	.707	.148
IL-13 ($pg \cdot ml^{-1}$)	0.83 ± 0.23	0.80 ± 0.20	1.28 ± 0.29***	1.18 ± 0.37	1.19 ± 0.46	1.43 ± 0.40***	<.001	.636

* p<.05, **p<.01, ***p<.001 compared to within group Pre exercise values.

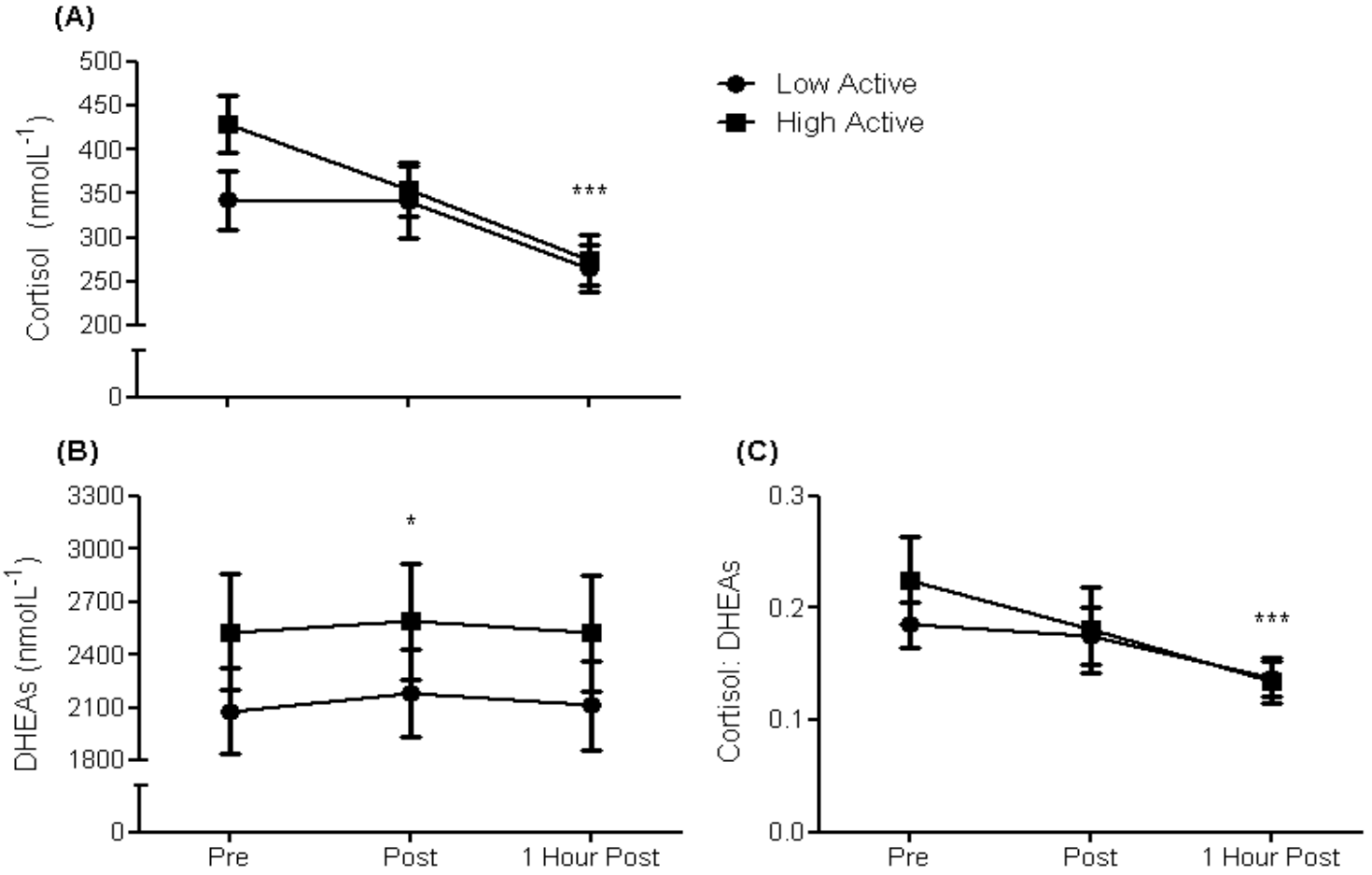

Fig. 5.1: Adrenal response to acute exercise. Cortisol (A), DHEAs (B) and the cortisol: DHEAS (C) response to acute exercise in low and high active elderly individuals. Data are mean ± SEM. *p<.05, ***p<.001 different from Pre exercise values for both groups.

5.2.3 Immune Function Responses to Exercise

5.2.3.1 Immune Cell Composition

The intensity of the exercise test was sufficient to produce a biphasic response in circulating leukocyte numbers (Table 5.3). Exercise caused an increase of 49% in circulating lymphocytes (p<.001), 34% for neutrophils (p<.001) and 28% for total monocytes (p=.001). Within the monocyte population the $CD16^{bright}$ population increased by 44% (p=.005) and 28% in $CD16^{neg}$ (p=.004), no difference was observed at Post exercise for $CD16^{dim}$ monocytes. This highlights the preferential mobilisation of $CD16^{bright}$ monocytes into the peripheral blood compartment following acute exercise. Lymphocytes fell below Pre values 1 Hour Post exercise (p<.001) whilst neutrophils remained above Pre (p=.002) values but below Post (p=.009) at 1 Hour Post exercise. Monocyte numbers were not significantly different from Pre values at 1 Hour Post exercise which was the same for the $CD16^{bright}$ and $CD16^{neg}$ populations. The $CD16^{dim}$ population was reduced from Post to 1 Hour Post exercise (p=.018). There was a trend for the Low Active group to have more $CD16^{+}$ monocytes than the High Active group at Pre exercise (p=.078).

5.2.3.2 Neutrophil Bactericidal Activity

There was a moderate but significant main effect of exercise for the percentage of neutrophils ingesting bacteria (Fig. 5.2A), which increased ~2% from Pre to Post exercise and remained above Pre 1 hour later [$F(2,58)=3.12$, p=.050, η^2=.097]. The amount of bacteria ingested on a per cell basis by neutrophils remained the same throughout (Fig. 5.2B), although there was a trend for the high active group to be able to ingest more bacteria than the low active group at Pre (p=.053), Post (p=.063) and 1 Hour Post (p=.058) exercise as seen by on average 20% greater MFI. There was a significant main effect of exercise for percentage of neutrophils producing

superoxide (Fig. 5.2C) towards ingested bacteria, which increased ~1.5% from Pre to Post exercise and again 1 Hour Post exercise [$F(2, 56)=8.60$, $p=.001$, $\eta^2=.235$]. No effect for exercise was observed for the amount of superoxide being produced by neutrophils towards bacteria (Fig. 5.2D).

Table 5.3: Exercise induced immune cell composition. Immune cell number changes in response to exercise for low and high active elders

	Low Active			High Active			Exercise Effect	Exercise x Group
Cell Number ($x10^{9} \cdot L^{-1}$)	Pre	Post	1 Hour Post	Pre	Post	1 Hour Post	(*p*)	(*p*)
Total Leukocytes	5.26 ± 0.73	7.47 ± 1.83***	5.66 ± 0.81	5.17 ± 1.62	6.90 ± 1.91***	5.66 ± 0.81	<.001	.503
Lymphocytes	1.61 ± 0.23	2.46 ± 0.96***	1.49 ± 0.27***	1.59 ± 0.44	2.31 ± 0.68***	1.37 ± 0.34***	<.001	.802
Neutrophils	3.32 ± 0.72	4.58 ± 1.08***	3.79 ± 0.91**	3.26 ± 1.32	4.23 ± 1.55***	4.01 ± 1.61**	<.001	.159
Monocytes	0.32 ± 0.08	0.43 ± 0.15**	0.37 ± 0.09	0.32 ± 0.13	0.39 ± 0.15**	0.33 ± 0.11	.001	.485
$CD14+/CD16^{neg}$	0.26 ± 0.07	0.37 ± 0.13**	0.32 ± 0.08	0.30 ± 0.11	0.34 ± 0.13**	0.30 ± 0.09	.002	.259
$CD14+/CD16^{dim}$	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01#	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01#	.020	.512
$CD14+/CD16^{bright}$	0.02 ± 0.02	0.03 ± 0.01**	0.02 ± 0.01	0.01 ± 0.01	0.03 ± 0.01**	0.02 ± 0.01	<.001	.183

Data are mean ± SD. **p<.01, ***p<.001 compared to Pre exercise values and # p<.05 less than Post exercise within groups.

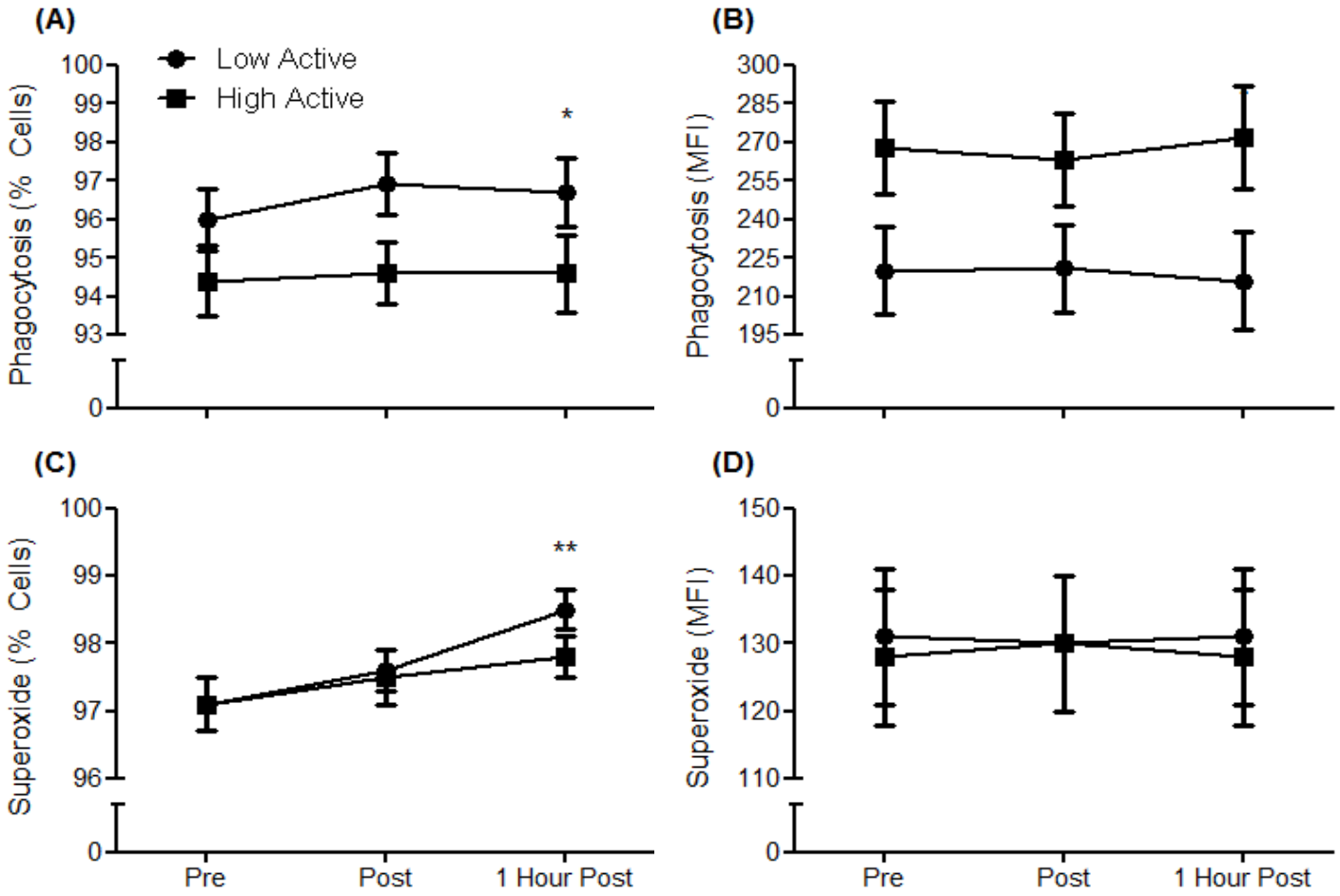

Fig. 5.2: Neutrophil bactericidal activity in response to acute exercise. Neutrophil phagocytosis of FITC-labelled *E.Coli* (A, B) and superoxide generation towards *E.Coli* after phagocytosis (C, D) in active and inactive elders. Data are mean ± SEM. **p*<.05, ***p*<.01 different from Pre exercise for both groups.

5.2.3.3 Neutrophil Phenotype

CD16 is the Fc receptor (FcγRIII) responsible for IgG_1 and IgG_3 binding and previous work from our group has shown that low CD16 expression is related to reduced phagocytosis of *E. coli*[172]. Low CD16 is also a marker of immature neutrophils that may be released prematurely from the bone marrow[327, 328]. There is an on-going debate as to where the exercise induced increased numbers of neutrophils circulating in the blood come from. Some researchers argue that demargination from the endothelium is responsible and others that these cells are released in larger numbers from the bone marrow[329, 330]. CXCR2 is the chemokine receptor for IL-8, however it is also a marker of newly emigrated cells from the bone marrow. Mature neutrophils released from the bone marrow have higher expression of CXCR2 and lower expression of CXCR4 with this relationship reversing as the cell becomes senescent and prepares to be cleared from the system [177].

CD16 expression was unaffected by exercise (Fig. 5.3A). There was a significant main effect of exercise for CXCR2 expression (Fig. 5.3B), with expression decreasing by 17% in response to exercise before returning to Pre exercise values 1 hour later [$F(2, 58)=7.12$, $p=.001$, $\eta^2=.210$]. There was also a small but significant main effect of exercise for CXCR4 expression (Fig. 5.3C) with a 2.5% reduced expression of CXCR4 from Pre to 1 Hour Post exercise [$F(2, 56)=3.81$, $p=.028$, $\eta^2=.120$]. There was a significant main effect of exercise for the CXCR2:CXCR4 ratio [$F(2, 56)=4.41$, $p=.017$, $\eta^2=.136$] which was driven by an increased proportion of CXCR2 and reduced CXCR4 positive neutrophils at the 1 Hour time point. These results suggest that in response to exercise circulating neutrophils consist of a lower proportion of a senescent phenotype. There was a correlation between the

expression of CXCR2 and the percentage of neutrophils ingesting bacteria at the 1 Hour Post exercise [$r(29)=.33$, $p=.038$].

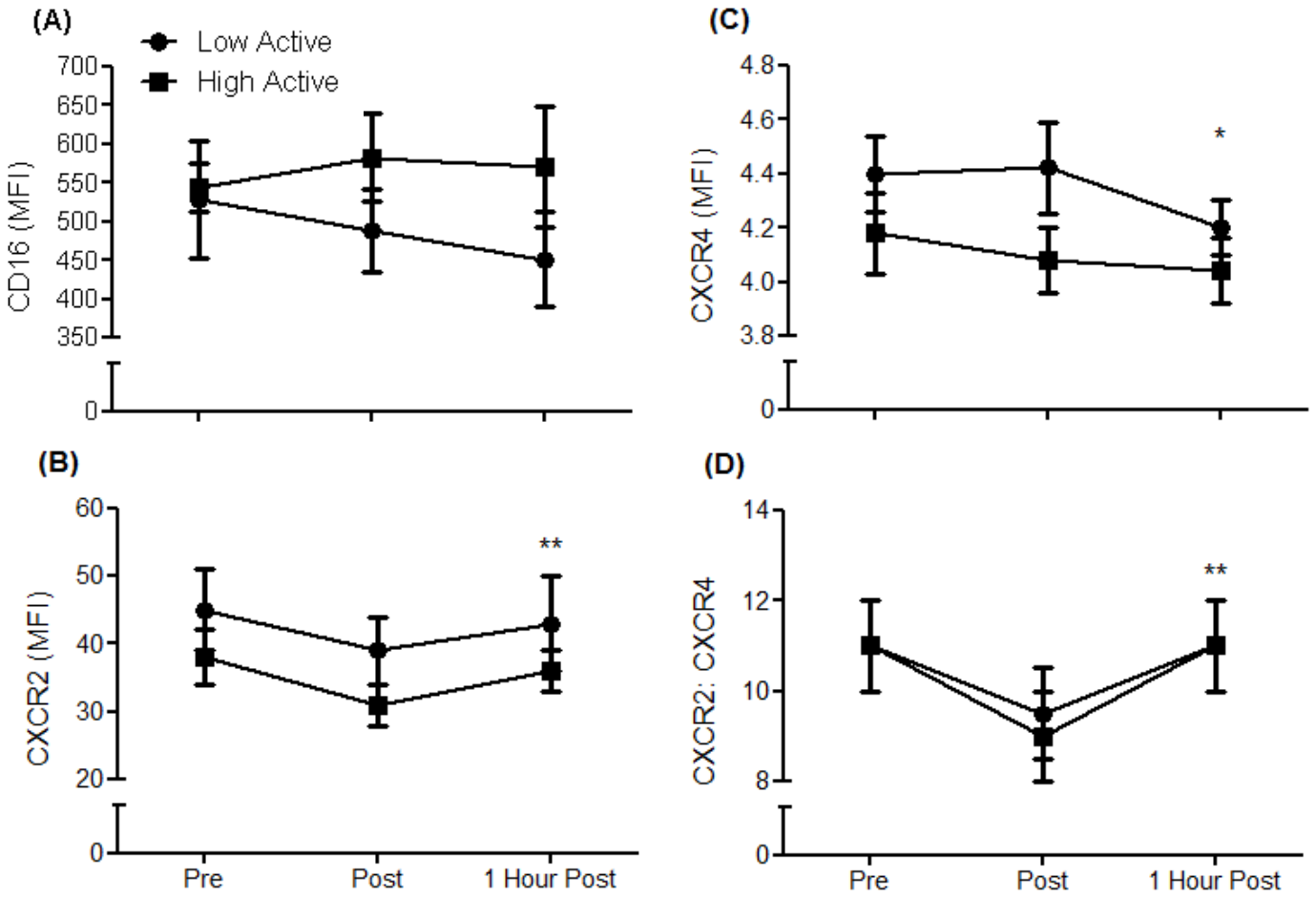

Fig. 5.3: Neutrophil phenotype in response to acute exercise. Neutrophil CD16 (A), CXCR2 (B), CXCR4 (C) and the ratio of CXCR2 to CXCR4 (D) expression in response to acute exercise in active and inactive elders. Data are mean ± SEM. *p<.05, **p<.01 different from Post exercise in both groups.

5.2.3.4 Monocyte Bactericidal Activity

There was a significant main effect of exercise for the percentage of monocytes ingesting bacteria (Fig. 5.4A), which increased 2.5% from Pre to 1 Hour Post exercise [$X^2(2) = 7.55$, p=.023]. The amount of bacterial uptake by monocytes was unchanged over the exercise (Fig. 5.4B). There was a significant main effect of exercise for the percentage of monocytes producing superoxide (Fig. 5.4C) towards ingested bacteria, which increased 5% from Pre to 1 Hour Post exercise [$X^2(2) = 8.87$, p=.012]. There was also a significant main effect of exercise for the superoxide production against bacteria (Fig. 5.4D), which increased 10% from Pre to 1 Hour Post exercise [$X^2(2) = 10.41$, p=.005].

Monocyte populations consist of 3 distinct types which have been identified as functionally distinct. As the exercise bout caused a redistribution of the monocyte subtypes (Table 5.3) the relationships between monocyte CD16 expression and function was assessed. Total number of circulating $CD16^{bright}$ monocytes was associated with increased uptake of *E. coli* at Pre exercise [$r(30) = .299$, p=.05]. Similar to neutrophil function and the exercise induced redistribution of functionally distinct cells; there was a redistribution of monocytes so that they are more functional towards microbial infection 1 hour after cessation of exercise.

5.2.3.5 Monocyte Phenotype

In order to assess the relationship between altered monocyte bactericidal activity and phenotype TLR4 expression was assessed (Fig. 5.5A) in response to exercise. There were no activity group differences for TLR4 expression at any time points and so figures are presented as the whole group. TLR4 expression was on average 21% and 16% (p<.001 for both) higher at all times on the $CD14^{+}/CD16^{dim}$ and

$CD14^+/CD16^{bright}$ populations respectively compared to $CD14^+/CD16^{neg}$. There was a significant main effect of exercise for expression of TLR4 on total $CD14^+$ monocytes [$F(2,56)=26.39$, $p<.001$, $\eta^2=.704$] which was driven by expression of TLR4 on the $CD14^+/CD16^{bright}$ monocytes [$F(2,56)=23.34$, $p<.001$, $\eta^2=.692$]. In both activity groups there was a 4% increased expression Post exercise ($p<.001$) before falling to 4% lower than Pre levels 1 Hour Post exercise ($p<.001$). No significant associations were observed for TLR4 expression on monocyte subtypes and monocyte bactericidal function.

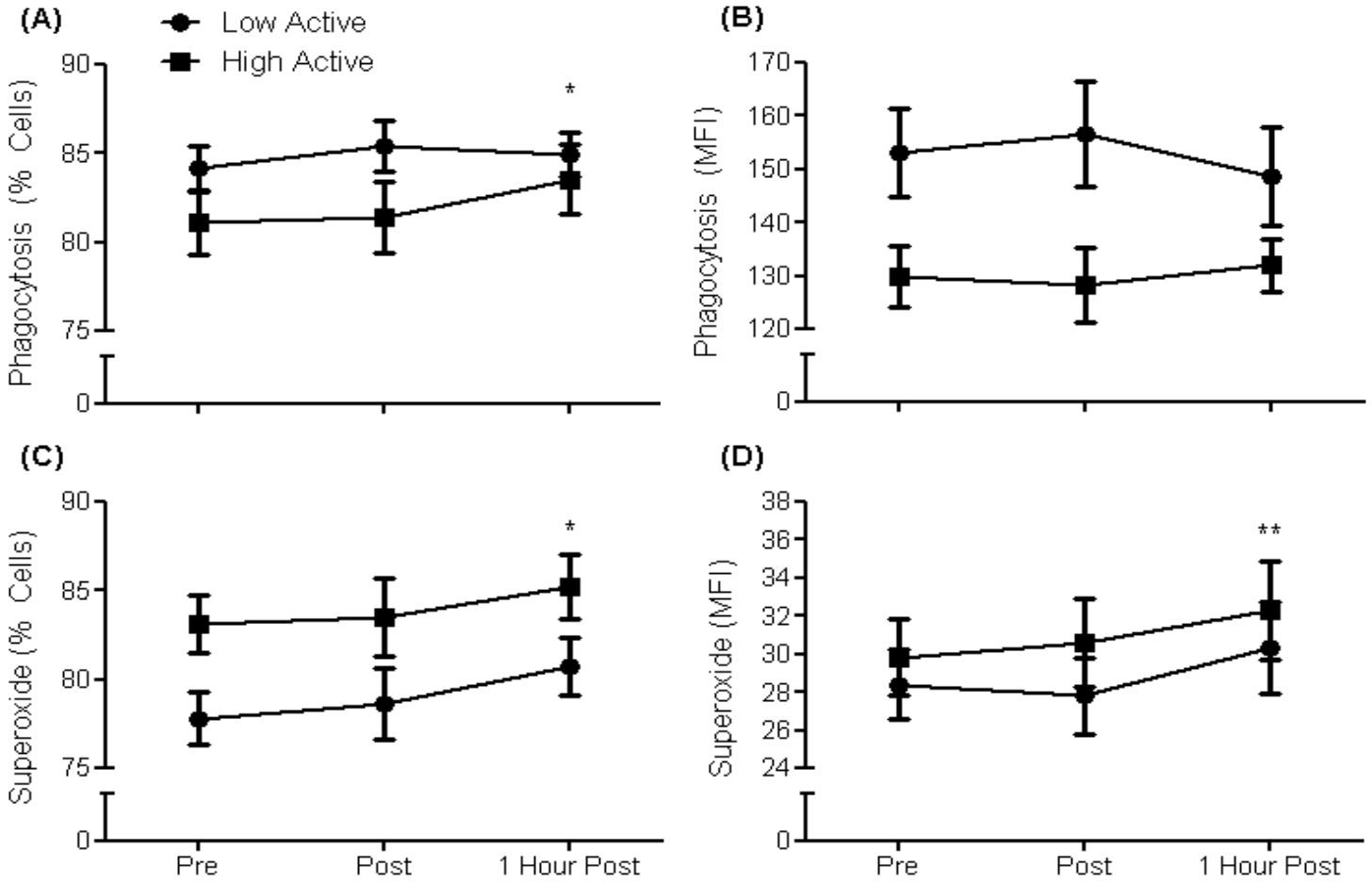

Fig. 5.4: Monocyte bactericidal activity in response to acute exercise. Monocyte phagocytosis of FITC-labelled *E.Coli* (A,B) and superoxide generation towards *E.Coli* after phagocytosis (C,D) in active and inactive elders. Data are mean ± SEM. **p*<.05, ***p*<.01 different from Pre exercise for both groups.

There were no activity group differences for TLR2 expression at any time points and so figures are presented as the whole group (Fig. 5.5B). TLR2 expression was on average 30% higher at all times on $CD14^{+}/CD16^{neg}$ ($p<.001$) and 38% higher on $CD14^{+}/CD16^{dim}$ ($p<.001$) compared to $CD14^{+}/CD16^{bright}$ populations. There was a significant main effect of exercise for expression of TLR2 on total $CD14^{+}$ monocytes [$F(2,56)=122.80$, $p<.001$, $\eta^2=.814$] which was driven by changes in expression of TLR2 on the $CD14^{+}/CD16^{dim}$ monocytes [$F(2,56)=35.48$, $p<.001$, $\eta^2=.559$]. In both groups there was a 4% reduced expression Post exercise ($p<.001$) which again reduced 3% 1 Hour Post exercise ($p<.001$). No significant associations were observed for TLR2 expression on monocyte subtypes and monocyte function. Coupled with the small associations between monocyte CD16 expression and bacterial uptake, it is likely that changes in monocyte function due to exercise are independent of TLR4 and TLR2 expression.

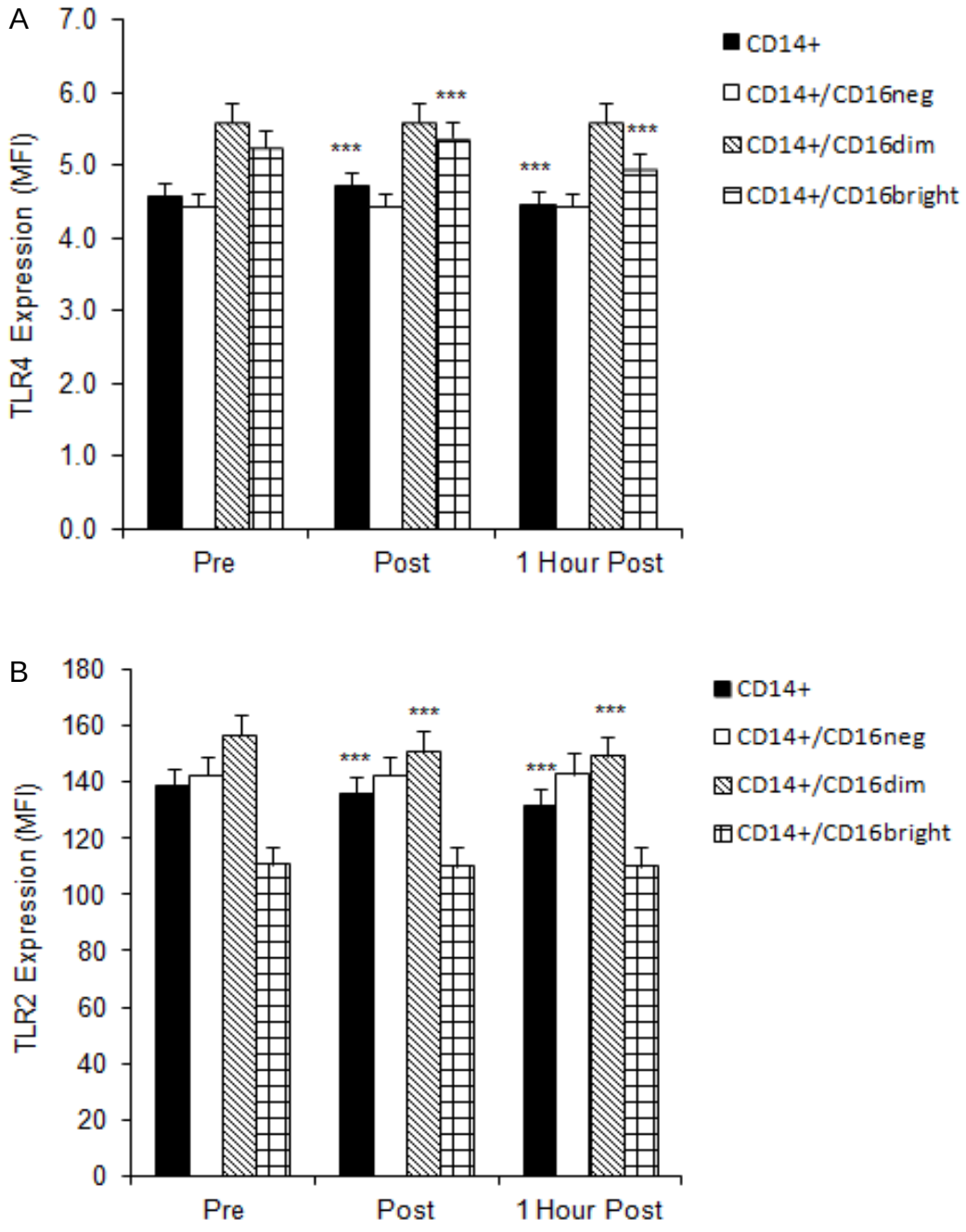

Fig. 5.5: Monocyte phenotype response to acute exercise. Monocyte expression of TLR4 (A) and TLR2 (B) on CD14+ monocyte subtypes in response to acute exercise. Data are mean ± SEM. ***p<.001 compared to Pre exercise levels for that subtype.

5.3 Discussion

5.3.1 Inflammatory Response to Acute Exercise

Transient controlled inflammatory responses are critical to the success of immune responses against infection through recruitment and activation of immune cells. However chronic inappropriate inflammation can lead to diseases such as cardiovascular disease and rheumatoid arthritis [31]. As ageing is associated with a dysfunctional inflammatory status (inflammageing) there is an increased risk of inflammation-related disease in the elderly. Similar to many other studies the results here show that physical inactivity is associated with heightened systemic inflammation and that acute exercise bouts can transiently modify the systemic inflammatory status [120, 273, 317, 331]. Indeed the effects of exercise on inflammation in various conditions in the elderly have received considerable attention in recent years.

CRP is an acute phase protein which is intrinsically linked to heightened risk of cardiovascular disease and in the present study it was significantly reduced in the physically active group before adjustment for BMI. Physical activity has been associated with reduced CRP concentrations in a number of studies following adjustment for BMI. In particular, Henson and colleagues and Pischon and colleagues both observed relationships with sedentary lifestyles and CRP even after adjustment for body fat [332, 333]. However in contrast to these findings, in this chapter following adjustments for BMI the group differences in CRP were attenuated. Furthermore, correlative analysis revealed that body composition was associated with CRP concentration more so than estimated aerobic capacity. This is in contrast to the previous chapter where regression analysis with mutual adjustment for body

composition and quantitative physical activity levels in over 200 participants revealed body fat as the primary driving force behind increased systemic CRP concentrations.

A number of studies have revealed similar patterns of associations with CRP, activity status and BMI in both young and old individuals. However it is difficult to fully separate the effects of BMI and aerobic fitness in smaller cohorts of participants like this and previous studies. Recently Stewart and colleagues revealed a 58% reduction in CRP concentrations following 12-weeks of combined aerobic and resistance training in both young and older participants [282]. Interestingly there were no changes in body fat percentage following training but importantly there were increases in aerobic capacity and muscle mass. This suggests that CRP concentrations may be associated with both muscle and fat composition suggesting an anti-inflammatory effect for muscle. Indeed Petersen and colleagues have suggested the role of muscle as an anti-inflammatory tissue for a number of years and suggest that exercise training should encompass both aerobic and resistance modes [197].

In support of this the acute inflammatory response to exercise can reveal to some extent the capacity of working muscle to reduce systemic inflammation and improve overall health capacity [275]. In this study small but significant increases in circulating concentrations of IL-1β, IL-6, IL-8, GM-CSF and IL-13 were seen following 1 hour of seated rest. Importantly at no point were any of the cytokines produced at a dangerous level indicating infection or damage. These findings are comparable to others who have shown acute transient responses in both pro and anti-inflammatory cytokines [239]. There is no doubt that the intensity, duration and mode of exercise can determine the degree and type of cytokine production as well as the longevity of its presence in the circulation [197, 204, 277]. High intensity long duration exercise is

usually accompanied by systemic increases in a number of pro-inflammatory cytokines including IL-1β, IL-6 and occasionally TNFα [334]. Until recently these increases, especially in at risk populations have been suggested to be detrimental and potentially damaging [335]. However recent studies have shown that the inflammatory response is needed for muscle anabolic responses and muscle protein synthesis after exercise is abolished in the presence of anti-inflammatory drugs [336]. So the pro-inflammatory response effect seen here is in fact a positive one, potentially helping to maintain and build muscle.

Recently it has been shown that cytokine dynamics following acute exercise is indicative of a tissue protective role including improved insulin sensitivity, immune function and muscle vascular angiogenesis [196]. Exercise stimulated muscle derived IL-6 is known to stimulate insulin sensitivity through activation of AMPK rather than SOCS3 at the same time as reducing endotoxin mediated TNFα production [203]. Muscle damage has been shown to increase local and systemic inflammation and although we cannot discount such damage even at relatively low intensities the inflammatory signs of muscle adaptation were present.

The chemokine IL-8 is a chemoattractant but has also been recently shown to enhance muscle microvascular angiogenesis following exercise [209, 337]. Therefore it is likely the increased IL-8 observed here was indicative of adaptive processes required for muscle repair and growth. Additionally following resistance exercise training muscle receptor expression for IL-4 and IL-13 are increased suggesting a potential role in muscle adaptation and hypertrophy as well as an anti-inflammatory response [338]. In confirmation with this we observed an increase in IL-13 production 1 hour after exercise and although the exercise session was not resistance based it is likely that IL-13 was enhancing muscle adaptation.

Furthermore, IL-1β is often cited as a pro-inflammatory cytokine capable of enhancing the inflammatory response and having the potential to cause tissue damage. However recently it has been shown to be a necessary intermediary in the synthesis and production of IL-6 from muscle cells [339, 340]. Therefore as mentioned previously IL-1β stimulated IL-6 can act in a pleiotropic way to repair and adapt muscle in response to stress such as exercise. Although we are unable to assess stimulatory dynamics of IL-1β and IL-6 the concomitant increase observed in both suggests a synergist role which is likely to benefit muscle adaptation via IL-6.

GM-CSF expression is often found in exercising muscle and plasma from athletes and is thought to play a pleiotropic role in muscle repair and adaptation by stimulating growth of neutrophils and monocytes which aide in the response [341]. These cells are integral to cellular and damaged tissue repair therefore it is likely GM-CSF increases observed in this study were aiding tissue adaptation. Taken together our findings are suggestive of a protective and adaptive role for what are typically pro-inflammatory cytokines and growth factors following a short bout of acute exercise in healthy elderly individuals.

To date no study has fully explored the mechanisms of muscle induced cytokine responses acting in a pleiotropic manner in either the young or elderly. However by assessing larger numbers and types of cytokines, chemokines and growth factors the study here has shown the potential for a pleiotropic muscle enhancing effect from a simple walking procedure in the elderly. Future work should include muscle biopsies from the elderly at different time points during exercise to determine the exact dynamics of these typical pro-inflammatory mediators.

5.3.2 Endocrine Response to Acute Exercise

Improving the endocrine status, particularly the ratio of cortisol to DHEAs offers potential therapeutic benefits to the elderly in respect to overall reductions in systemic inflammation and reduced frailty prevalence as cortisol is immune suppressing and catabolic with respect to muscle and bone [134]. The cortisol:DHEAs ratio was reduced in response to an acute bout of walking at a predominantly moderate intensity in elderly individuals. Reductions were mainly driven by reduced concentrations of cortisol rather than increased DHEAs. Cortisol decreased during the exercise bout and further reduced 1 hour after cessation compared to baseline levels. These findings are comparable to Kemmler and colleagues who have shown reduced cortisol in response to exercise during the morning in postmenopausal women [342] and also in men [343]. Circulating cortisol concentrations are dependent on diurnal variations with the highest levels being found during the early hours of the morning during sleep phase [344]. Therefore as with the current study and those of Kremmler it is difficult to assess whether the reductions were independent of exercise and in fact being controlled by diurnal rhythms. In the present study the elderly individuals were assessed around 3 hours after awakening suggesting that diurnal variations were minimal. Also as the intervention was carried out at the same time each day for all participants and lasted only 20 minutes, the diurnal effects therefore should be minimal.

Others have assessed elderly participants with higher intensity exercise bouts in the morning and shown significant increases in cortisol [345]. Relative intensity of exercise has been suggested to be a controlling factor on the cortisol response in young and older individuals with higher intensities (>60% of VO_{2max}) causing activation of the HPA-axis and subsequent increased concentrations [346]. In the

current study the intensity was for the majority of time very low and only reached moderate and hard during the final stages suggesting time spent above 60% VO_{2max} was not sufficient to cause an altered increased HPA response. In light of these data it is likely that intensity of exercise and not time of day modulates cortisol responses greater and the current study cortisol reductions were in response to the exercise bout, although further analysis to confirm this is warranted. Encouragingly the level of exercise was moderate and only lasted for 23 minutes and thus may be easy to implement in the elderly population.

Unlike cortisol, DHEAs concentrations are not diurnally controlled and increased slightly in response to the exercise session; furthermore although not significant there was a clear pattern for the high active elders to have increased DHEAs concentrations at all time-points. DHEAs associations with exercise, especially in the elderly are limited, with the majority of studies assessing the un-sulphated form DHEA [248, 343, 347]. DHEAs is positively associated with muscle mass and is utilised by the body as a precursor to a number of anabolic mediators [348]. Although the present study did not assess overall muscle mass, the differential pattern of DHEAS between active and inactive has been shown previously [349]. Regardless of muscle mass, Li and colleagues recently suggested that a walking session was sufficient to increase DHEAs in middle aged men and Kremmler showed increased DHEAs concentrations in women following exercise [342]. Although others have not shown increases there are methodological variances that may account for no effect. In particular, when assessing strength trained individuals, DHEAs concentrations may be at their peak for that individual and to induce an exercise dependent response would be near impossible [350]. Additionally gender differences are apparent with DHEAs, with males having higher concentrations than

females due to DHEAs utilisation in sex steroid synthesis and muscle metabolism [351, 352]. Therefore exercise induced increases of DHEAs may be more likely in elderly females than men due to the menopause and reduced muscle mass. Although the increases in DHEAs in the present study were seen in females there was a trend towards a difference for males (p=.089). Thus acute exercise induced changes to circulating DHEAs in the elderly may be gender specific and rely on the mode and duration of the exercise bout.

5.3.3 Immune Response to Acute Exercise

The findings of this study suggest the inflammatory and endocrine responses to acute exercise are well placed to direct an enhanced immune response. The increased IL-8 may be contributing to muscle adaptation, but in conjunction with increased GM-CSF can stimulate the production and release of new neutrophils and monocytes from the bone marrow, suggesting an enhanced innate immune response [353]. Increased ratio of DHEAs to cortisol and elevated inflammatory to anti-inflammatory cytokines can prime the immune system for detection of and clearance of pathogens [79]. In the present study a short bout of walking moderately improved neutrophil and monocyte function in both low and highly active elders which was paralleled by immune enhancing inflammatory mediator production and endocrine responses. Specifically the percentage of neutrophils and monocytes able to uptake and produce superoxide to bacteria was increased, particularly during the recovery phase. Although the amount of bacterial uptake by both cell types was unaffected by exercise, monocytes produced significantly more superoxide towards bacteria during the recovery phase.

5.3.4 Neutrophil Response to Acute Exercise

Neutrophil numbers increased in response to the exercise and did not increase any further during the recovery phase. Neutrophil number responses to exercise are well documented with intensity and duration of exercise directing whether numbers keep increasing during recovery (high intensity long duration) or start to fall back to baseline levels (low intensity short duration) [245]. Neutrophil superoxide production and uptake of *E.coli* on a per cell basis was unaffected by acute exercise. In younger sedentary and trained individuals moderate short duration exercise has been shown to cause a transient increase in neutrophil phagocytosis and bactericidal killing [245, 354, 355] while longer higher intensity exercise causes reduced function [356]. As this study is the first to assess an acute walking bout in elderly men and women it is possible that the exercise bout may not have the capacity to alter neutrophil function at the cellular level. However there was a trend (p=.058) for the active elders to have increased uptake of bacteria per cell suggesting habitual training has the ability to modify neutrophil function over a longer period of time. Sedentary men aged below 60 years have been previously shown to have reduced neutrophil function over trained individuals [226, 245, 357] and here we show a trend for similar differences in our elderly cohort, confirming the potential benefit of regular exercise on bacterial uptake by neutrophils. Exercise intensities over 75% of VO_{2max} and 60 minutes duration are often associated with an increase in circulating neutrophil numbers post exercise and again during the recovery phase and subsequently neutrophil dysfunction [294]. Because our exercise protocol was relatively short (~23 minutes) and stopped at 75% of maximum predicted heart rate it is unlikely to have been sufficient to cause transient cellular changes in bactericidal function similar to other studies.

The percentage of circulating neutrophils capable of *E.coli* phagocytosis and production of a superoxide response was increased 1 Hour Post exercise. Although these increases were relatively small, total numbers of circulating cells did not reflect the observed pattern. Instead total circulating neutrophil numbers decreased 1 Hour Post exercise in the present study while percentage of cells increased at the same time point. This was interesting in that the inflammatory and endocrine milieu at this time point was such that it could enhance neutrophil function, particularly by releasing newer potentially more functional or proportionally more functional cells from the bone marrow. Thus these findings suggest that exercise caused a redistribution of functionally and potentially phenotypically distinct neutrophils, similar to what is often seen in lymphocyte subpopulations [214, 358]. Neutrophils are often considered a homogenous terminally differentiated population of professional phagocytes; however it was revealed recently that neutrophil populations have different expression of adhesion molecules causing some to migrate better than others [327]. Therefore, it is plausible that exercise can redistribute functionally distinct neutrophils and benefit the elderly who have reduced neutrophil function and modify the risk of infection.

To investigate this further and to determine whether phenotype could identify functional neutrophils we investigated the acute exercise dependent expression of CXCR2 and CXCR4 on neutrophils for the first time. CXCR2 has previously been reported to be increased, while CXCR4 is non-detectable on newly released mature neutrophils from the bone marrow [359]. Consequently, CXCR2 was reduced while CXCR4 is elevated on older ‘senescent’ neutrophils preparing to be cleared from the system. We observed a significant reduction of CXCR2 and CXCR4 expression immediately and 1 hour after, respectively, exercise cessation in both groups with

CXCR2 returning to basal values 1 Hour Post exercise. CXCR2 expression at 1 Hour Post exercise was also positively correlated with percentage of cells capable of bacterial phagocytosis. Interestingly the ratio of CXCR2 to CXCR4 expressing neutrophil may give an indication of proportions of functional to less functional neutrophils in the circulation [177, 360]. We show here that the ratio with more functional neutrophils is increased at 1 hour post exercise which parallels the observations we see with percentage of neutrophils capable of ingesting bacteria and producing a superoxide killing mechanism. These data are novel and suggest that exercise can redistribute functionally distinct neutrophils with altered phenotypes. Furthermore, the increased concentration of GM-CSF during the recovery has the potential to allow migration of newly matured neutrophils from the bone marrow, which would be consistent with CXCR2 expression changes [341, 361]. Studies in younger individuals have shown increases for neutrophil adhesion and activation molecules CD11b and CD62L in response to exercise [362, 363]. In light of this exercise may not only alter the activation status of neutrophils but also preferentially redistribute newer neutrophils in order to mobilise them in response to stress.

In summary neutrophil function with acute exercise was only slightly modified and although no differences between activity groups was apparent, there was a trend for the highly active individuals to have improved phagocytic capacity compared to inactive elders. Acute low level exercise can redistribute and de-marginalise functionally distinct cells as evident by their phenotype. Whether regular exercise can improve neutrophil function remains to be determined however this is likely considering previous research and the trends observed in this study between active and inactive elders.

5.3.5 Monocyte Response to Acute Exercise

Monocyte dysfunction with age is well documented including reduced chemotaxis, phagocytosis and respiratory burst [364]. To date few studies have assessed monocyte function and phenotype modifications in response to acute bouts of exercise in the elderly. Monocyte bactericidal capacity was improved in response to acute exercise in the elderly, with an increased percentage of monocytes able to uptake bacteria, produce superoxide killing burst and more superoxide generation per monocyte. These findings paralleled those of neutrophils and further suggest the inflammatory milieu may be able to modify immune function. No differences were observed between activity groups for any of the bactericidal functions. This is in agreement with the only other study to assess monocyte phagocytosis in later life. Schaun and colleagues [243] showed no improvements in bactericidal activity in a group of middle aged men (mean age of 54 years old) following 12 weeks of exercise training. Although they did not assess differences between habitual activity levels of their subjects as there were no improvements following training it may be that monocyte phagocytosis is less responsive to exercise induced modifications in the elderly. Furthermore, the responses to an acute exercise bout have not been reported previously.

In young individuals phagocytosis has been shown to increase following exercise whilst superoxide killing is often reduced. LaVoy and colleagues [365] recently showed an increase in phagocytosis following a strenuous 75 km cycle time trial in young trained athletes . These findings are in contrast to this study and suggest intensity and duration may play a role in differences observed between young and elderly. Furthermore the shift towards a myeloid lineage in the elderly may account

for differences between young and old, although further work is required to assess this.

Ageing is associated with an increased proportion of pro-inflammatory CD16+ monocytes yet the production of cytokines following pathogen stimulation is reduced suggesting a reduced immune surveillance capacity. Here a preferential mobilisation of CD16+ monocytes into the peripheral blood was seen following acute exercise before a return to baseline 1 hour after cessation. Although not statistically significant there was a trend (p=.079) towards the low active group having more CD16 on the surface of their monocytes. Monocytes in the sedentary elderly have been previously shown to express more CD16 on their surface than monocytes from active individuals [163, 366]. CD16 expression on monocytes has been associated with increased risk of atherosclerosis due to their adhesive and pro-inflammatory nature [367]. The data shown here are in concurrence with a number of others studies in younger individuals following acute bouts of exercise. It is unclear where the cells go following cessation of exercise however it is possible that the pro-inflammatory monocytes are preferentially removed from the peripheral blood, perhaps to other immune compartments such as the spleen for homeostatic clearance [368]. This has the potential to ultimately improve monocyte function, especially in the elderly and reduce the risk of adverse health conditions.

To investigate whether this biphasic redistribution of monocytes affected function, cell surface phenotype in response to exercise was also investigated. Acute resistance exercise in the elderly does not affect the expression of TLR4 on monocytes however it can augment the LPS induced production of TNFα [321]. Other studies have also assessed the expression of surface molecules responsible for pathogen recognition (TLR2 and TLR4) inflammation and antigen presentation

(HLA-DR) to adaptive cells and concluded that acute exercise is capable of altering the immune potential by removing pro-inflammatory monocytes from the circulation during the recovery phase of exercise [156]. Simpson and colleagues suggested the redistribution of monocytes may impact on the anti-inflammatory effects of exercise and immune surveillance [156, 369]. In agreement with this, increases in TLR4 expression immediately post exercise were seen before falling back to baseline levels during the recovery phase, whilst TLR2 was reduced post exercise and reduced further during the recovery phase.

The inflammatory potential of monocytes is essential for pathogen recognition and clearance and subsequently it is essential to assess whether this was impaired in response to the exercise bout. This study shows for the first time that in elderly individuals the percentage of monocytes capable of phagocytosis and producing a superoxide burst plus the amount of superoxide on a per cell basis towards *E. Coli* is increased during the recovery phase after an acute exercise bout. These findings paralleled those of neutrophils and the alterations to immune enhancing cytokine and endocrine constituents. Thus acute exercise in the elderly of a moderate nature has the potential to modify the innate immune response by redistributing phenotypically distinct cells into the periphery. These cells are capable of enhanced bacterial clearance and may indicate why young and elderly individuals who participate in regular bouts of moderate exercise have enhanced immune function over those who are sedentary and do not exercise.

5.4 Limitations and Future Work

This study is not without limitations. First, although we did not follow the participants for longer than one hour after exercise cessation others have followed participants for a number of days and observed exercise induced changes returning to baseline levels. It is therefore likely that our participants' immune parameters also returned to baseline levels. It is this mechanism of small stressors, which can cause tissue modifications if repeated regularly, that is likely responsible for the exercise training improvements in inflammatory mediators and immune function. Second, we did not observe any difference in inflammatory mediators between the low and high habitual activity groups which is in contrast to a number of other studies [197, 198]. These studies consist mostly of either truly sedentary and/or highly active individuals and represent the extremes of suggested health. In the present study, although groups were determined to be either active or inactive the fact that they could attend the laboratory and complete the walking test suggests that the inactive group were not truly sedentary. Indeed their estimated VO_{2max} levels are above that of previously published sedentary levels and guidelines provided by the American College of Sports Medicine. Future studies should therefore assess the cytokine, chemokine and growth factor dynamics during exercise and in at least the 24 hours following cessation.

Third, we associated an immune enhancing inflammatory and endocrine milieu with enhanced innate immune function. These are observations and do not imply cause and effect. Simpson and colleagues recently attempted to address this issue with baseline monocyte incubation with autologous plasma from the varying exercise time

points. They concluded that serological constituents were unable to alter the surface phenotype of the monocytes but did not consider function [369]. Therefore future studies should focus on the functional changes of immune cells in response to autologous serum from varying time points.

5.5 Conclusions

In summary, acute exercise of a low to moderate intensity and relatively short duration can modify inflammation, endocrine status and immune function in a manner which may offer protection from infection as well as promoting beneficial physiological adaptation. Alterations in inflammatory mediators can stimulate not only muscle adaptation but here we suggest also have immune enhancing effects which can offer protection against pathogenic infection. Physical inactivity status was associated with increased basal CRP; however these effects were lost upon adjustment for BMI. Both the inactive and active groups elicited a similar inflammatory mediator response to exercise suggesting that if the severely inactive become more active they will promote an immune enhancing condition and offer improved protection from infection. In conclusion the data suggest that short walking bouts may benefit the elderly by enhancing the immune response to a potential bacterial challenge.

Chapter 6: Inflammation and Immune Function Response to

High Intensity Interval Training (HIT)

6.0 Introduction

It is well established that regularly physical activity is effective in reducing the risk of age-related conditions such as type-2 diabetes and cardiovascular disease. In this respect, leading a physically active lifestyle reduces several risk factors for chronic disease, including a low exercise capacity, greater central adiposity, reduced insulin sensitivity and low-grade systemic inflammation [190]. Nevertheless, the majority of the population remain physically inactive, citing a lack of time as the primary barrier to exercise participation [187]. Therefore, it is of key importance that exercise strategies are developed to maximise the beneficial adaptations of exercise in a time-efficient manner, and that are acceptable to the general population including the elderly.

One emerging approach to combat physical inactivity focuses on the use of high intensity interval training (HIT) [370]. Recent interest in HIT originates from its time-efficient nature, in which short bouts of high intensity exercise (up to 60 seconds) are combined with periods of low intensity recovery or rest (1 to 2 minutes), providing an exercise session lasting around 20 minutes. A number of well-controlled studies provide evidence for the effectiveness of HIT (when performing three sessions per week), reporting improvements in exercise capacity, blood pressure, blood vessel function and insulin sensitivity in young sedentary individuals following six weeks of HIT training [371, 372]. Furthermore, many of the beneficial adaptations to HIT appear to be similar to those induced by more traditional endurance-type exercise interventions that require a much longer time commitment (a minimum of 30 minutes moderate-intensity exercise per day, five times per week). Therefore, evidence is accumulating to suggest that HIT provides a time-efficient and effective alternative to

traditional moderate-intensity exercise-based interventions as a tool to improve health.

However, a major limitation is the lack of assessment of immune, endocrine and inflammatory mediators which are inherently linked to and an underlying cause of insulin resistance, vascular function and disease risk. Furthermore, all but one of the studies to date have assessed the impact of HIT in young healthy cohorts failing to assess the impact of ageing, a major contributor to the severity of the above pathologies [373]. Finally, the majority of studies to date have assessed the effects of HIT in a laboratory-based environment and have not determined whether it is transferrable to the real world, i.e. a gym setting. The primary measure of the effectiveness of any intervention program is the adherence to the prescribed intervention, in this case an exercise training program. Furthermore, unlike laboratory based interventions, group based interventions are regularly cited as having superior overall adherence [374, 375].

Therefore in light of this the aims of this chapter were:

1) Assess the physiological modifications offered by high intensity interval training compared to an endurance training program.
2) Determine inflammatory, endocrine and immune responses to each training intervention.
3) Determine the impact of age on exercise induced changes provided by exercise interventions.
4) Assess the adherence to exercise interventions in a real world instructor led group based environment.

6.1: High Intensity Interval Training Study Methods

6.1.1 Participants

Participants were 90 healthy sedentary individuals recruited from the University of Birmingham, University Hospital Birmingham and localised area, aged 20 – 60 years old. Exclusion criteria were: known metabolic or cardiovascular diseases and engagement in more than 150 minutes of physical activity per week in the preceding year. All participants were subjected to a 12-lead electrocardiogram examination and verification of no underlying cardiovascular abnormalities provided by a research nurse. All participants gave written informed consent prior to commencement of the study, which was approved by the University Research Ethics Committee (West Midlands, UK).

6.1.2 Pre-Training Experimental Procedures

Anthropometric characteristics were taken and included assessment of body composition using a single frequency bioimpedence analyser (Tanita BC 418 MA Segmental Body Composition Analyser, Tanita, Japan). This device uses an algorithm incorporating age, height and impedance values to estimate relative fat and muscle mass across different segments of the body. Participants were fitted with a HR monitor around the chest and HR measured continuously by telemetry (Polar Electro Ltd., Kempele, Finland) and recorded every 15 seconds. VO_{2max} was assessed from a progressive exercise test to volitional exhaustion on an electronically braked cycle ergometer (Lode BV, Groningen, Netherlands). O_2 consumption and CO_2 production was quantified using a breath-by-breath on-line gas collection system (Oxycon Pro, Jaeger, Germany). Following familiarisation protocols participants began cycling at 25 Watts (W) with cadence required to be

above 50 revolutions per minute (rpm), followed by sequential increments of 35 W every 3 minutes until cadence was reduced to less than 50 rpm. VO_{2max} was determined as the average value obtained in the last 30 seconds of the test and validated when HR was greater than 90% of the age-predicted maximal HR (calculated as 220 – age in years) and respiratory exchange ratio (RER) was greater than 1.1.

A subset of the 90 participants (*n*=27) agreed to have blood samples taken immediately before the aerobic capacity test and immediately after for immune analysis. Participants were supine for at least 15 minutes before commencement of the test after which blood was taken from a vein in the antecubital fossa. Peripheral blood (40 ml) was collected into evacuated vaccutainers (Becton-Dickinson, Oxford, UK) containing lithium heparin or EDTA anticoagulants or clotting factor for serum isolation. Upon voluntary cessation of the aerobic capacity test, participants were laid down and the same blood sampling performed.

At least 48 hours following the VO_{2max} test, participants reported to the laboratory after an overnight fast (> 10 h). Participants were instructed to refrain from performing vigorous activity in the preceding 48 hour period, and were asked to avoid consuming caffeine or alcohol in the previous 24 hours. In addition, participants completed a diet diary outlining their food intake in the preceding 24 hour period which was collected upon arrival to the laboratory. Brachial artery blood pressure measurements were first made in triplicate using an automated sphygmomanometer (Omron 7051T, Omron Corp., Kyoto, Japan) following 15 minutes of supine rest. Immediately following these measures a 20G cannula was inserted into a vein in the antecubital fossa of one arm and a 3-way stop cock attached to permit multiple blood sampling. A 20 ml baseline blood sample was

obtained and distributed into vacutainers containing EDTA, sodium fluoride (NaF) or clotting factor, after which participants consumed a beverage containing 75 g glucose (Fisher Scientific, UK) dissolved in water and made up to 300 ml. Further 5 ml blood samples were obtained after 30, 60, 90 and 120 minutes, and collected into vacutainers containing either EDTA or NaF. Isotonic saline (~5 ml) (B Braun, UK) was used to keep the cannula patent every 15 minutes during the 2 h test.

On completion of the pre-training experimental procedures, participants were randomly assigned to either HIT or endurance training (ET), taking gender, age and BMI into consideration. In this way it was expected that the two groups would be well-matched for exercise capacity (VO_{2max}) and serological measures.

6.1.2 Exercise Training

Exercise sessions took place at the University of Birmingham sports centre in the cycle suite in groups of 10-15 participants. Exercise was carried out on mechanically-braked spinning bikes (Star Trac UK Ltd., Buckinghamshire, UK) that permitted the participant to manually adjust the braking resistance, and thereby control the exercise intensity at which they were working. All exercise sessions at the University of Birmingham sports centre were led by an instructor who was experienced in delivering exercise classes. Before starting the training intervention, each participant was provided with an individual heart rate target value (determined from the results of the VO_{2max} test) to achieve during the exercise sessions. Both training groups (HIT or ET) were asked to attend 3 supervised exercise sessions per week for 10-weeks, although 5 sessions (Monday-Friday) were available in order to maximise attendance. A record of subject attendance at the exercise sessions was maintained throughout the 10 weeks by an investigator.

6.1.2.1 High intensity interval training (HIT)

For each HIT training session participants were provided with a HR monitor that transmitted HR to a central unit (Polar Team 2, Polar Electro Ltd., Warwick, UK) and individual HR values were subsequently projected on to a screen at the front of the room. In this way, participants could track their heart rate throughout the exercise session. Each HIT training session began with a short (5 minute) warm up of low intensity cycling, after which participants performed repeated high intensity sprints of between 15 and 60 seconds in duration, interspersed with periods of active recovery (60 to 120 seconds in duration), Table 6.0. For the high intensity sprints, participants were instructed to adjust the resistance of the bike such that they achieved a heart rate greater than that equivalent to 80% of VO_{2max}. After each high intensity sprint this resistance was removed and participants cycled with minimal resistance. Each session concluded with a 5 minute cool down. Participants were instructed to maintain their habitual physical activity levels, but were asked not to engage in additional exercise training throughout the 10 week training period.

6.1.2.2 Moderate intensity endurance training (ET)

All participants randomised to the ET group were provided with a HR monitor (RS400, Polar Electro Ltd., Warwick, UK) for the 10-week training period which was used to record HR throughout each exercise training session. Each ET session began with a short (5 minute) warm up of low intensity cycling, after which participants performed moderate-intensity steady state cycling for 20 to 40 minutes, Table 6.0. During the steady state period, participants were instructed to adjust the resistance of the bike such that they achieved a HR equivalent to 65% of VO_{2max}. Each session concluded with a 5 minute cool down. In addition to attending 3 supervised exercise sessions per week, participants were asked to perform 2

unsupervised moderate-intensity exercise sessions, such that a total of 5 exercise sessions were performed each week. HR monitors were also worn for unsupervised exercise sessions, thereby providing a monitoring system for participation in the additional sessions, together with completion of training diaries.

Table 6.0: Overview of HIT and ET training protocols

	HIT		ET	
Week	*Session*	*Session time (mins:sec)*	*Session*	*Session time (mins:sec)*
1	30 s HIE, 2 min recovery; repeat 4x	20:00	20 min continuous exercise	30:00:00
2	15 s HIE, 45 s recovery; repeat 8x	18:00	20 min continuous exercise	30:00:00
3	30 s HIE, 90 s recovery; repeat 5x	20:00	25 min continuous exercise	30:00:00
4	60 s HIE, 60 s recovery; repeat 5x	20:00	25 min continuous exercise	30:00:00
5	30 s HIE, 60 s recovery; repeat 7x	20:30	30 min continuous exercise	35:00:00
6	15 s HIE, 45 s recovery; repeat 12x	22:00	30 min continuous exercise	35:00:00
7	30 s HIE, 60 s recovery; repeat 9x	23:30	35 min continuous exercise	40:00:00
8	60 s HIE, 60 s recovery; repeat 7x	24:00:00	35 min continuous exercise	40:00:00
9	30 s HIE, 60 s recovery; repeat 6x followed by 15 s	24:00:00	40 min continuous exercise	45:00:00
10	60 s HIE, 60 s recovery; repeat 5x followed by 15 s	25:00:00	40 min continuous exercise	45:00:00

6.1.3 Post-training experimental procedures

Post-training VO_{2max} was assessed at least 48 hours following the last training session. At this visit, participants were provided with the diet diaries that they completed before training, and were asked to replicate their diet in the 24 hours preceding the next visit. Furthermore, participants were required to visit the laboratory at the same time of day for testing as baseline. More than 48 hours following the post-training VO_{2max} test, participants reported to the laboratory after an overnight fast (> 10 h), and underwent identical assessments to those outlined in 'Pre-training experimental procedures'. Body composition was assessed on a separate day following this.

6.1.4 Biological Analysis

The following biological analysis was completed and described in section 2.2. Inflammatory and anti-inflammatory cytokines were measured by magnetic XMap technology. CRP, cortisol and DHEAs were measured by ELISA. Neutrophil and monocyte bactericidal function was assessed using phagotest and phagoburst kits, monocyte and neutrophil phenotype were assessed by flow cytometry.

6.1.5 Statistics

Statistical analyses were performed similar to Chapter 5. Individuals were randomised to either HIT or ET accounting for age and gender to ensure both groups were characteristically similar. Univariate ANOVA was conducted to assess differences of anthropometric characteristics between the two groups taking into account gender. Following this repeated measure ANOVA and ANCOVA were conducted to assess the changes in biological variables over either: 1) The two times of baseline and follow-up or 2) The times between Pre and Post VO_{2max} testing at baseline and follow-up in consideration of the training group. Following this statistical

analysis was repeated to include assessment of whether individuals in each group who were aged below or above 40 years old showed any biological and training effects. Body fat percentage was significantly higher in the over 40 year olds and was subsequently included as a covariate. The assumptions of sphericity were assessed, partial η^2 reported and Bonferroni post-hoc pairwise analysis conducted as in previous chapters. Statistical significance was accepted at $p<.05$.

This study was completed with the collaborations of Dr Chris Shaw of Sport and Exercise Sciences who conducted sections 6.1.1 – 6.1.3.

6.2 Results

6.2.1 Participant Characteristics in Response to Training and Intervention

Table 6.1 outlines the main physiological changes with each of the training interventions. Adherence to the training intervention was on average 22% greater (p <.001) in the HIT group than the ET group. Subsequently despite the lower adherence, the ET group performed significantly (p<.001) more exercise sessions than the HIT group (33 ± 2 versus 25 ± 1). Thus the average weekly total time commitment for the ET group (128 ± 7 minutes) was more than twice that of the HIT group (55 ± 2 minutes; p<.001). Heart rate data revealed that the HIT group had an average heart rate at the end of each bout of high intensity exercise equivalent to 91 ± 2% of their VO_{2max} whilst the average HR for the ET group was equivalent to 64 ± 2% of their VO_{2max}.

HIT caused similar modifications to body composition and aerobic capacity as ET. There were significant main effects of exercise training but not between exercise groups for reduced weight [$F(1, 88)=13.31$, p<.001, η^2=.131] which equated to a similar effect on BMI [$F(1, 88)=12.54$, p=.001, η^2=.125]. Changes in BMI were associated with reduced body fat percentage [$F(1, 88)=14.25$, p<.001, η^2=.143] and not increased muscle mass although there was a trend for the ET group to have a small increase post training (p =.081). Similarly there was a main effect of exercise training for aerobic capacity increases [$F(1, 88)=79.80$, p<.001, η^2=.476].

Table 6.1: Group characteristics. Participant characteristics for the high intensity (HIT) and endurance (ET) training groups before (Pre) and after (Post) 10-weeks of training. Data are mean ± SEM.

	HIT		ET		Training effect (p)	Training x group (p)
	Pre	*Post*	*Pre*	*Post*		
Gender (males/females)	15/31		15/29			
Adherence (%)	83 ± 3		61 ± 4***			
Age (years)	42 ± 2		43 ± 2			
Body composition						
Weight (kg)	78.7 ± 2.7	77.9 ± 2.6	77.5 ± 2.4	76.6 ± 2.4	<.001	.884
BMI ($kg \cdot m^{-2}$)	27.7 ± 0.7	27.3 ± 0.7	27.7 ± 0.7	27.4 ± 0.7	.001	.939
Fat mass (%)	31.7 ± 1.2	30.8 ± 1.1	32.0 ± 1.2	30.9 ± 1.3	<.001	.421
Muscle mass (%)	65.0 ± 1.2	65.6 ± 1.1	64.7 ± 1.1	65.8 ± 1.3	.714	.074
Exercise capacity						
$VO_{2\,max}$ ($ml \cdot min^{-1} \cdot kg^{-1}$)	32.0 ± 1.0	34.8 ± 1.0	31.5 ± 1.0	33.9 ± 1.1	<.001	.899
$VO_{2\,max}$ ($L.min^{-1}$)	2.5 ± 0.1	2.7 ± 0.1	2.4 ± 0.1	2.6 ± 0.1	<.001	.331

***p<.001 compared to HIT group.

6.2.1.1 Inflammatory, Endocrine and Metabolic Alterations in Response to Training

Participation in the HIT intervention was associated with similar endocrine, inflammatory and metabolic modifications as the ET intervention. There were significant main effects of exercise training but not between exercise groups for reduced cortisol [F(1, 88)=129.81, p<.001, η^2=.637] and increased DHEAs [F(1, 88)=34.80, p<.001, η^2=.349] which equated to a reduction in the cortisol:DHEAs ratio [F(1, 88)=46.29, p<.001, η^2=.416]. Similar main effects were observed for reduced concentrations of the pro-inflammatory CRP [F(1, 88)=5.71, p=.019, η^2=.069] , IL-6 [F(1, 88)=3.99, p=.047, η^2=.051], IL-8 [F(1, 88)=22.93, p<.001, η^2=.252] and MCP-1 [F(1, 88)=11.31, p=.001, η^2=.143] and a trend for increased GM-CSF [F(1, 88)=3.96, p=.051, η^2=.055]. Additionally exercise training was associated with an improved metabolic profile through reduced concentrations of leptin [F(1, 88)=4.91, p=.030, η^2=.066], increased adiponectin [F(1, 88)=5.07, p=.027, η^2=.064] and a subsequent reduced ratio of leptin to adiponectin [F(1, 88)=14.91, p<.001, η^2=.176].

6.2.1.2 Neutrophil and Monocyte Bactericidal Activity

Neutrophil and monocyte capacity to ingest bacteria and produce a superoxide killing response were modified by ten weeks of ET and HIT, Table 6.3. There was a significant main effect of training for an increased amount of bacterial uptake per cell (MFI) for neutrophils [F(1, 22)=7.99, p=.009; η^2=.250] and monocytes [F(1, 22)=18.54, , p<.001; η^2=.457]. No effect for training was observed for the percentage of neutrophils and monocytes able to uptake bacteria. Similarly there was a significant main effect of exercise training for an increased amount of superoxide generation per cell (MFI) for neutrophils [F(1, 22)=11.62, p=.003; η^2=.346] and monocytes [F(1, 22)=4.83, , p=.039; η^2=.187]. Additionally the percentage of

neutrophils [F(1, 22)=37.69, , p<.001; η^2=.653] and monocytes [F(1, 22)=11.63, , p=.002; η^2=.336] producing superoxide killing capabilities was increased following ten weeks of ET training. Taken together both HIT and ET can improve neutrophil and monocyte bactericidal capacity.

Table 6.2: Biomarker response to training. Endocrine, inflammatory and metabolic characteristics for the high intensity (HIT: n=46) and endurance (ET: n=44) training groups before (Pre) and after (Post) 10-weeks of training. Data are Mean ± SEM.

	HIT		ET		Training effect	Training x group
	Pre	*Post*	*Pre*	*Post*	(*p*)	(*p*)
Endocrine						
Cortisol ($nmol \cdot L^{-1}$)	390 ± 14	290 ± 13	351 ± 15	274 ± 14	<.001	.128
DHEAs ($nmol \cdot L^{-1}$)	3261 ± 309	3861 ± 331	3615 ± 332	4319 ± 357	<.001	.640
Cortisol: DHEAs	0.16 ± 0.02	0.09 ± 0.01	0.15 ± 0.02	0.09 ± 0.01	<.001	.457
Inflammatory						
CRP ($mg \cdot L^{-1}$)	1.35 ± 0.40	0.97 ± 0.14	1.43 ± 0.31	0.96 ± 0.11	.019	.211
TNFα ($pg \cdot ml^{-1}$)	0.24 ± 0.06	0.18 ± 0.05	0.97 ± 0.08	1.04 ± 0.12	.770	.756
IL-1β ($pg \cdot ml^{-1}$)	0.49 ± 0.02	0.47 ± 0.01	0.72 ± 0.08	0.72 ± 0.09	.473	.989
IL-6 ($pg \cdot ml^{-1}$)	1.02 ± 0.17	0.89 ± 0.12	0.83 ± 0.20	0.47 ± 0.11	.047	.259
IL-8 ($pg \cdot ml^{-1}$)	3.48 ± 0.16	3.10 ± 0.15	4.10 ± 0.22	3.74 ± 0.21	<.001	.654
IL-17 ($pg \cdot ml^{-1}$)	0.37 ± 0.37	0.00 ± 0.00	0.01 ± 0.01	0.02 ± 0.02	1.000	.307
GM-CSF ($pg \cdot ml^{-1}$)	18.79 ± 6.23	23.47 ± 8.03	19.13 ± 4.91	26.97 ± 5.59	.051	.663
MCP-1 ($pg \cdot ml^{-1}$)	22.56 ± 0.94	21.42 ± 1.05	26.01 ± 0.96	24.22 ± 1.08	.001	.460
VEGF ($pg \cdot ml^{-1}$)	2.67 ± 0.47	2.35 ± 0.41	3.31 ± 0.39	3.43 ± 0.46	.686	.936
Anti-Inflammatory						
IL-4 ($pg \cdot ml^{-1}$)	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	.696	.823
IL-10 ($pg \cdot ml^{-1}$)	0.39 ± 0.31	0.88 ± 0.74	0.71 ± 0.64	0.05 ± 0.05	.739	.172
IL-13 ($pg \cdot ml^{-1}$)	0.10 ± 0.04	0.03 ± 0.02	0.11 ± 0.07	0.11 ± 0.05	.951	.767
Metabolic						
Leptin ($ng \cdot ml^{-1}$)	18.83 ± 1.78	16.12 ± 1.68	15.61 ± 1.78	14.59 ± 1.68	.030	.319
Adiponectin ($\mu g \cdot ml^{-1}$)	2.24 ± 0.18	2.73 ± 0.20	2.21 ± 0.19	2.29 ± 0.21	.027	.107
Leptin: Adiponectin	10.13 ± 1.42	6.61 ± 0.91	9.84 ± 1.42	7.89 ± 0.91	<.001	.270

Table 6.3: Immune function response to training. Neutrophil and monocyte bactericidal function for the high intensity (HIT: n=13) and endurance (ET: n=11) training groups before (Pre) and after (Post) 10-weeks of training. Data are Mean ± SEM.

	HIT		ET		Training Effect	Training x Group
	Pre	Post	Pre	Post	(*p*)	(*p*)
Phagocytosis						
Neutrophil (%)	97.3 ± 0.4	97.0 ± 0.4	96.5 ± 0.5	97.3 ± 0.3	.734	.185
Neutrophil (MFI)	130.6 ± 4.4	152.0 ± 7.9	126.2 ± 3.6	145.5 ± 13.0	.009	.890
Monocyte (%)	94.5 ± 0.7	93.3 ± 0.5	93.6 ± 0.7	93.1 ± 0.6	.058	.451
Monocyte (MFI)	99.3 ± 2.9	113.3 ± 3.9	92.9 ± 3.7	110.4 ± 5.8	<.001	.633
Superoxide Production						
Neutrophil (%)	93.1 ± 12.0	98.4 ± 0.2	95.6 ± 0.7	98.5 ± 0.2	<.001	.089
Neutrophil (MFI)	69.0 ± 6.5	84.6 ± 7.0	77.6 ± 5.8	104.8 ± 10.7	.003	.355
Monocyte (%)	74.4 ± 2.7	81.6 ± 1.6	74.4 ± 4.1	85.6 ± 1.9*	.002	.480
Monocyte (MFI)	22.8 ± 0.9	27.5 ± 2.3	27.8 ± 2.0	30.9 ± 3.7	.039	.730

*$p<.05$ significantly different from corresponding HIT time point

6.2.1.3 Monocyte Phenotype

CD16 expression on CD14 positive monocytes is indicative of a pro-inflammatory phenotype. There was a significant main effect of time on the increased percentage of CD14+/CD16^{neg} monocytes [$F(1,25)=11.34$, $p=.004$, $\eta^2=.311$] suggesting a reduced inflammatory phenotype (Table 6.4). This was confirmed by significant reductions in the percentage of the CD16^{dim} ($p=.009$) and CD16^{bright} ($p=.011$) populations. Differences were similar across the two intervention groups.

TLR expression on monocytes is an indication of inflammatory potential. TLR4 (Fig. 6.1A) and TLR2 (Fig 6.1B) were assessed on the three monocyte subsets, classical (CD14+/CD16^{neg}), intermediate (CD14+/CD16^{dim}) and non-classical (CD14+/CD16^{bright}). No differences were observed between the HIT and ET groups for expression of TLR4 or TLR2 on any of the subsets or in response to training. There was a significant main effect of time for expression of TLR4 on total CD14^{+} monocytes [$F(1,25)=18.45$, $p<.001$, $\eta^2=.302$] which was driven by expression of TLR4 on the CD14^{+}/CD16^{bright} monocytes [$F(1,25)=16.54$, $p<.001$, $\eta^2=.257$]. TLR2 expression was higher at all times on CD14^{+}/CD16^{neg} and CD14^{+}/CD16^{dim} compared to CD14^{+}/CD16^{bright} populations ($p<.05$). There was a significant main effect of time for expression of TLR2 on total CD14^{+} monocytes [$F(1,25)=21.40$, $p<.001$, $\eta^2=.257$] which was driven by changes in expression of TLR2 on the CD14^{+}/CD16^{dim} monocytes [$F(1,25)=19.42$, $p<.001$, $\eta^2=.199$]. These data suggest that HIT exercise training is comparable to ET in reducing the pro-inflammatory potential of monocytes by reducing TLR2 and TLR4 expression on CD16 positive monocytes.

Table 6.4: Monocyte response to training. Percentage of CD14+ monocytes expressing CD16 in the high intensity (HIT: n=14) and endurance (ET: n=13) training groups before (Pre) and after (Post) 10-weeks of training. Data are Mean ± SD.

	HIT		ET		Training Effect	Training x Group
	Pre	Post	Pre	Post	(*p*)	(*p*)
Monocyte (%)						
CD14+/CD16^{neg}	87.2 ± 4.7	91.1 ± 2.9	86.9 ± 5.8	89.8 ± 5.2	.004	.449
CD14+/CD16^{dim}	4.4 ± 2.8	3.1 ± 1.7	4.7 ± 2.4	3.8 ± 2.1	.009	.566
CD14+/CD16^{bright}	8.1 ± 4.4	5.8 ± 3.6	8.4 ± 3.1	7.1 ± 4.2	.011	.487

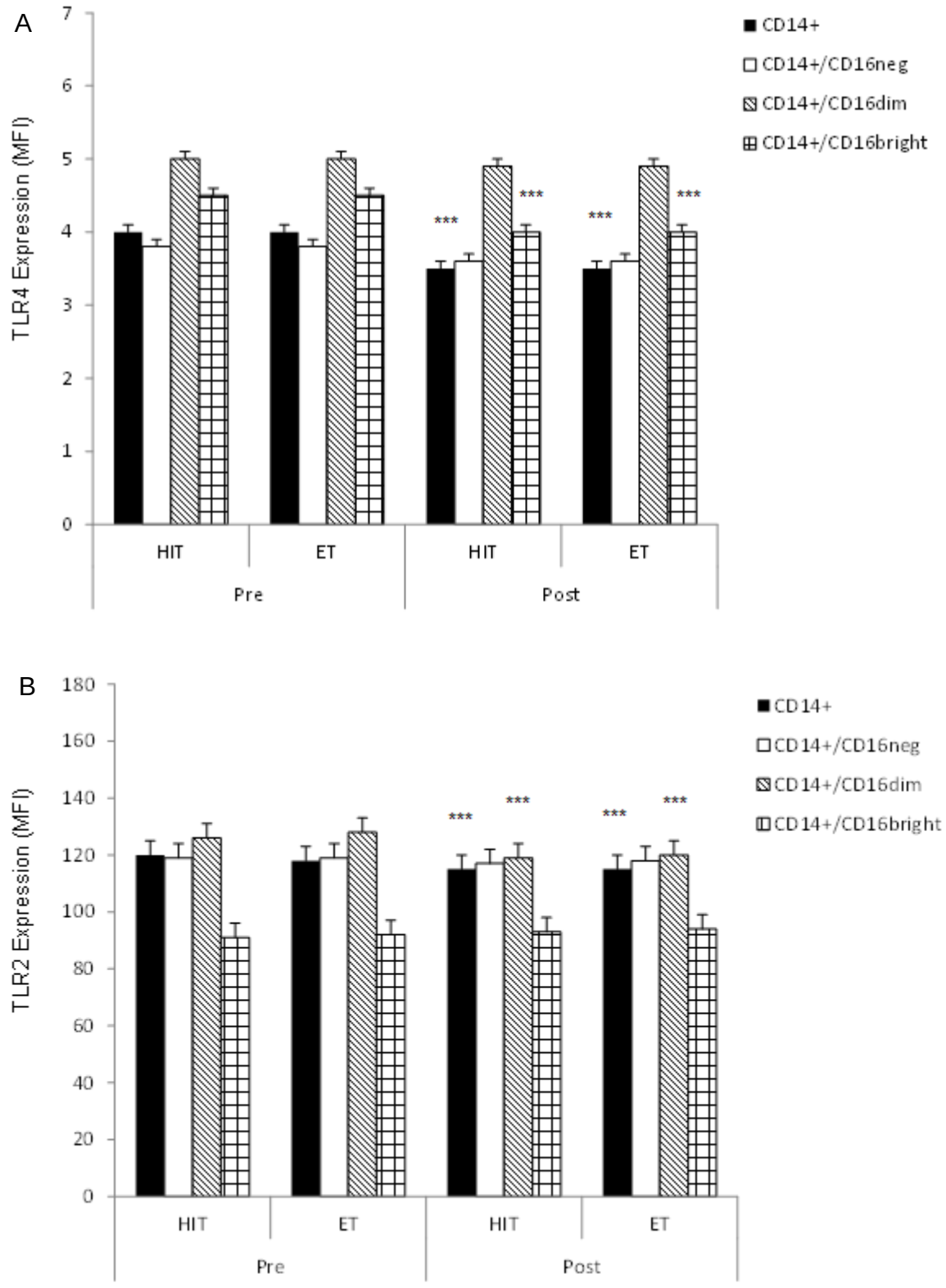

Fig. 6.1: Monocyte phenotype responses to exercise training. Monocyte expression of TLR4 (A) and TLR2 (B) on CD14+ monocyte subtypes in response to 10-weeks of HIT or ET exercise training. Data are mean ± SEM. ***p<.001 compared to Pre baseline measures levels for that subtype.

6.2.2 Participant Characteristics in Response to Training and Age

The cohort tested consisted of an age range spanning 40 years. Therefore as ageing has a significant impact on inflammation, endocrine function and immune function the relationship between age and training was assessed. Participant data was split into two groups, those aged below 40 years (young) and those aged 40 years and above (middle aged). Table 6.5 outlines the similar variables assessed in Table 6.1, split for age. No differences were observed between groups for gender, adherence or the intervention group they were placed in. No differences were observed previously for effects of intervention group and no differences were observed with age groups, therefore all participants in the age groups were analysed together having completed an exercise intervention.

As before, there were main effects of exercise training for reduced weight (p<.001), BMI (p=.001), body fat percentage (p<.001) and VO_{2max} (p<.001). Although no interactions were observed for a training x group effect the middle aged participants had a significantly higher BMI at both Pre (p=.033) and Post (p=.037) training plus body fat percentages at the same times than the young (p=.049 and .031 respectively). Furthermore the middle aged group had lower VO_{2max} values at both Pre and Post training (both p<.001) than the young.

Table 6.5: Group characteristics for ageing. Participant characteristics for the young and middle aged groups before (Pre) and after (Post) 10-weeks of training. Data are mean ± SEM.

	Young		Middle Aged		Training effect	Training x group
	Pre	*Post*	*Pre*	*Post*	(*p*)	(*p*)
Gender (males/females)	17/29		13/31			
Adherence (%)	77 ± 2		74 ± 3			
Age (years)	33 ± 5		52 ± 5***			
Exercise Group (HIT/ET)	24/22		22/22			
Body composition						
Weight (kg)	77.9 ± 2.7	77.0 ± 2.7	78.4 ± 2.4	77.5 ±2.3	<.001	.906
BMI ($kg \cdot m^{-2}$)	26.9 ± 0.7	26.6 ± 0.7	28.5 ± 0.7*	28.2 ± 0.7*	.001	.959
Fat mass (%)	30.1 ± 1.1	29.8 ± 1.1	33.8 ± 1.3*	33.3 ± 1.3*	<.001	.730
Muscle mass (%)	66.3 ± 1.1	66.7 ± 1.1	63.0 ± 1.3	63.1 ± 1.3	.714	.414
Exercise capacity						
$VO_{2\,max}$ ($ml \cdot min^{-1} \cdot kg^{-1}$)	35.1 ± 0.9	38.0 ± 0.9	28.4 ± 0.9***	30.6 ± 0.9***	<.001	.250
$VO_{2\,max}$ ($L.min^{-1}$)	2.8 ± 0.1	2.9 ± 0.1	2.1 ± 0.1***	2.3 ± 0.1***	<.001	.331

*p<.05, ***p<.001 significantly different between the below 40 age group and above 40 age group at the corresponding times

6.2.2.1 Age Associated Inflammatory, Endocrine and Metabolic Alterations in Response to Training

Anti-inflammatory and endocrine effects of exercise were driven by larger overall changes in the older participants than the young (Table 6.6). As before there were main effects of exercise training for reduced cortisol (p<.001), increased DHEAs (p<.001) and a reduced cortisol:DHEAs ratio (p<.001). DHEAs as expected was significantly lower in the middle aged group at both Pre (p=.004) and Post (p=.017) training compared to the young group. Subsequently changes in cortisol were driven to a greater extent in the middle aged participants who had on average a 26% overall reduction compared to 20% in the young group. Similarly DHEAs increased on average by 31% in the middle aged group compared to 24% in the young group. This equated to an average reduction in the cortisol:DHEAs ratio of 38% in the middle aged group compared to 29% in the young.

Similarly exercise training was associated with reduced concentrations of CRP (p=.019), IL-6 (p=.047), IL-8 (p<.001) and MCP-1 (p=.001). Changes in IL-6 were driven to a greater extent in the middle aged group who had on average a 15% change compared to 3% in the young group. Changes in CRP, IL-8 and MCP-1 were similar between ages. Interestingly, at both time points the concentrations of GM-CSF were higher in the middle aged group (both p<.01) than the young group. There was a trend for the young group to have increased GM-CSF concentrations (p=.058) but not the middle aged group (p=.385) following training.

As before leptin (p=.030) was reduced whilst adiponectin (p=.027) was increased in response to training. There were no interactions with age and changes were not

driven by either age group suggesting adipokine levels may be independent of age and dependent solely on adipocyte content.

Table 6.6: Biomarker responses with ageing. Endocrine, inflammatory and metabolic characteristics for the young (n=46) and middle aged (n=44) groups before (Pre) and after (Post) 10-weeks of training. Data are mean ± SEM.

	Young		Middle Aged		Training effect (p)	Training x group (p)
	Pre	*Post*	*Pre*	*Post*		
Endocrine						
Cortisol ($nmol \cdot L^{-1}$)	370 ± 12	295 ± 14	373 ± 17	270 ± 14	<.001	.078
DHEAs ($nmol \cdot L^{-1}$)	4051 ± 359	4636 ± 370	2779 ± 224**	3494 ± 282*	<.001	.556
Cortisol: DHEAs	0.13 ± 0.02	0.08 ± 0.01	0.17 ± 0.02	0.10 ± 0.01	<.001	.180
Inflammatory						
CRP ($mg \cdot L^{-1}$)	1.84 ± 0.50	1.17 ± 0.19	1.03 ± 0.15	0.77 ± 0.11	.019	.310
TNFα ($pg \cdot ml^{-1}$)	0.58 ± 0.09	0.70 ± 0.14	0.56 ± 0.10	0.49 ± 0.08	.770	.193
IL-1β ($pg \cdot ml^{-1}$)	0.61 ± 0.02	0.60 ± 0.02	0.60 ± 0.03	0.58 ± 0.03	.473	.596
IL-6 ($pg \cdot ml^{-1}$)	0.75 ± 0.17	0.69 ± 0.10	1.13 ± 0.23	0.68 ± 0.13	.047	.172
IL-8 ($pg \cdot ml^{-1}$)	3.67 ± 0.22	3.31 ± 0.23	3.88 ± 0.20	3.51 ± 0.15	<.001	.944
IL-17 ($pg \cdot ml^{-1}$)	0.17 ± 0.02	0.17 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	1.000	.307
GM-CSF ($pg \cdot ml^{-1}$)	33.73 ± 7.22	40.73 ± 8.40	7.87 ± 3.65**	9.60 ± 3.71**	.051	.542
MCP-1 ($pg \cdot ml^{-1}$)	23.91 ± 0.80	22.61 ± 0.81	24.56 ± 1.15	22.95 ± 1.31	.001	.734
VEGF ($pg \cdot ml^{-1}$)	3.16 ± 0.47	3.26 ± 0.41	2.76 ± 0.39	2.51 ± 0.43	.686	.613
Anti-Inflammatory						
IL-4 ($pg \cdot ml^{-1}$)	0.01 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	.696	.177
IL-10 ($pg \cdot ml^{-1}$)	0.34 ± 0.31	0.96 ± 0.74	0.89 ± 0.73	0.00 ± 0.00	.739	.083
IL-13 ($pg \cdot ml^{-1}$)	0.05 ± 0.03	0.11 ± 0.05	0.12 ± 0.08	0.03 ± 0.05	.951	.152
Metabolic						
Leptin ($ng \cdot ml^{-1}$)	16.29 ± 1.73	14.67 ± 1.63	18.21 ± 1.85	16.09 ± 1.73	.030	.767
Adiponectin ($\mu g \cdot ml^{-1}$)	2.24 ± 0.21	2.58 ± 0.21	2.20 ± 0.16	2.45 ± 0.19	.027	.743
Leptin: Adiponectin	9.68 ± 1.22	6.82 ± 0.86	10.31 ± 1.61	7.71 ± 0.97	<.001	.858

*p<.05, **p<.01 significantly different between the below 40 age group and above 40 age group at the corresponding times

6.2.2.2 Age-Associated Bactericidal Activity in Response to Exercise

There were significant interactions between exercise training and the age of the participants for monocyte and neutrophil function suggesting that the two groups produced different responses to training (Table 6.7). The main interaction was observed for the amount of bacterial uptake (MFI) by monocytes [$F(1, 22)=5.47$, $p=.029$; $\eta^2=.187$] which was driven by significant increases in values following ten weeks training in the middle aged group ($p=.007$) suggesting an immune enhancing effect of exercise with older age. Similarly there was an interaction for the percentage of neutrophils producing a superoxide killing burst [$F(1, 22)=4.58$, $p=.045$; $\eta^2=.186$] which was driven by a larger magnitude of change in the young ($p<.001$) than in the middle aged group ($p=.020$). Additionally the middle aged group had a small but significantly higher percentage of superoxide producing cells at Pre training ($p=.048$). Taken together, exercise training can improve neutrophil and monocyte bactericidal function in both young and middle aged individuals, however it appears that the middle aged individuals improve to a greater degree. These data would suggest that exercise training can reverse some of the functional immune reductions observed with ageing.

6.2.2.3 Monocyte Phenotype

With regard to monocyte phenotype, increased CD16 expression on CD14 positive monocytes are indicative of a pro-inflammatory phenotype is associated with increased age. There was a significant main effect of time on the increased percentage of CD14+/CD16^{neg} monocytes [$F(1,25)=13.21$, $p=.007$, $\eta^2=.385$] suggesting a reduced inflammatory phenotype (Table 6.8). This was confirmed by significant reductions in the percentage of the CD16^{dim} ($p=.002$) and CD16^{bright} ($p=.024$) populations. Differences were similar across the two age groups.

TLR expression on monocytes in the elderly has been suggested to decrease following exercise training periods. TLR4 (Fig. 6.2A) and TLR2 (Fig 6.2B) expression were assessed on the three monocyte subsets, classical (CD14+/CD16^{neg}), intermediate (CD14+/CD16^{dim}) and non-classical (CD14+/CD16^{bright}). No differences were observed between the age groups for expression of TLR4 or TLR2 on any of the subsets. There was a significant main effect of time for expression of TLR4 on total CD14^{+} monocytes but only in the middle-aged participants [$F(1,25)=38.45$, $p<.001$, $\eta^2=.651$] which was driven by expression of TLR4 on the CD14^{+}/CD16^{bright} monocytes [$F(1,25)=42.35$, $p<.001$, $\eta^2=.711$]. There was a significant main effect of time for expression of TLR2 on total CD14^{+} monocytes from both young and middle-aged participants [$F(1,25)=99.61$, $p<.001$, $\eta^2=.723$] which was driven by changes in expression of TLR2 on the CD14^{+}/CD16^{dim} monocytes [$F(1,25)=102.31$, $p<.001$, $\eta^2=.823$].

Table 6.7: Immune function responses with ageing. Neutrophil and monocyte bactericidal function for the young (n=11) and middle aged (n=13) groups before (Pre) and after (Post) 10-weeks of training. Data are Mean ± SEM.

	Young		Middle-Aged		Training Effect	Training x Group
	Pre	Post	Pre	Post	(*p*)	(*p*)
Phagocytosis						
Neutrophil (%)	96.8 ± 0.4	96.9 ± 0.3	97.3 ± 0.4	97.4 ± 0.5	.734	.973
Neutrophil (MFI)	126.9 ± 4.4	155.2 ± 9.8	130.3 ± 3.9	145.4 ± 9.9	.009	.283
Monocyte (%)	94.89 ± 0.4	93.4 ± 0.4	94.2 ± 0.8	93.1 ± 0.7	.058	.194
Monocyte (MFI)	98.9 ± 4.5	109.5 ± 5.3	96.8 ± 3.1	114.7± 4.1	<.001	.583
Superoxide Production						
Neutrophil (%)	93.1 ± 1.1	98.4 ± 0.2	95.0 ± 0.7*	98.5 ± 0.2	<.001	.045
Neutrophil (MFI)	65.5 ± 5.7	96.0 ± 9.6	75.4 ± 6.8	86.5 ± 8.3	.003	.067
Monocyte (%)	69.9 ± 3.1	83.6 ± 2.0	77.5 ± 2.9*	83.1 ± 1.7	.002	.068
Monocyte (MFI)	25.5 ± 1.5	26.2 ± 2.0	22.1 ± 1.0	30.2 ± 2.9	.039	.029

*$p<.05$ significantly different from corresponding Young time point assessed using repeated measures ANOVA.

Table 6.8: Monocyte responses with ageing. Percentage of CD14+ monocytes expressing or not CD16 for the young (n=14) and middle-aged (n=13) groups before (Pre) and after (Post) 10-weeks of training. Data are mean ± SD.

	Young		Middle-Aged		Training Effect	Training x Group
	Pre	Post	Pre	Post	(*p*)	(*p*)
Monocyte (%)						
CD14+/CD16^{neg}	88.9 ± 4.9	90.3 ± 3.9	85.4 ± 5.1	88.4 ± 4.7	.007	.188
CD14+/CD16^{dim}	4.8 ± 3.1	3.9 ± 2.1	5.7 ± 4.0	4.2 ± 2.8	.002	.418
CD14+/CD16^{bright}	7.6 ± 4.1	5.2 ± 3.2	7.9 ± 3.8	7.1 ± 3.8	.024	.113

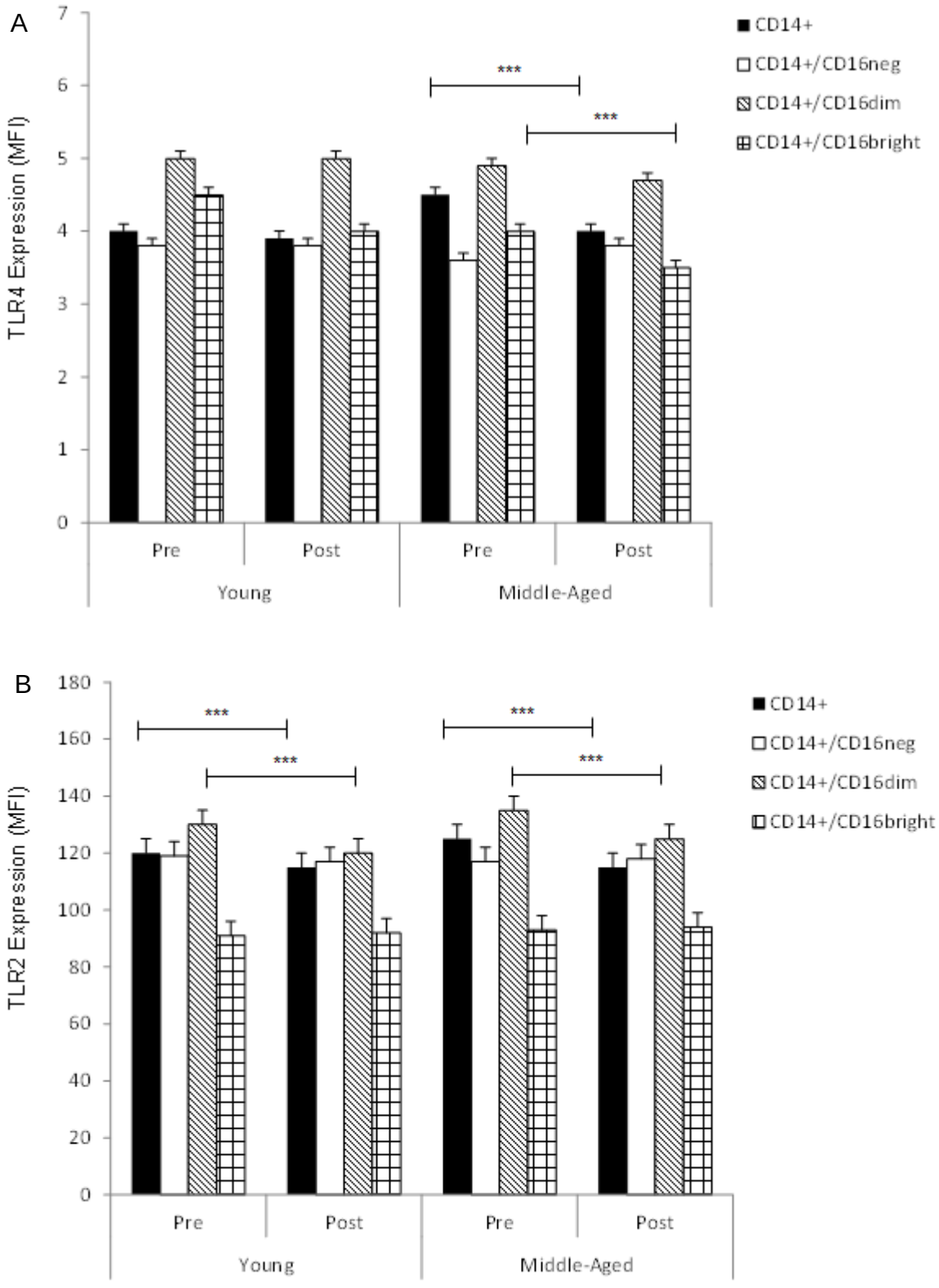

Fig. 6.2: Monocyte phenotype responses to exercise and age. Monocyte expression of TLR4 (A) and TLR2 (B) on CD14+ monocyte subtypes in response to 10-weeks exercise training. ***p<.001 compared to Pre baseline measures for that subtype. Data are mean ± SEM.

6.3 Discussion

Regular bouts of structured exercise sessions are associated with a number of health benefits including reduced risk of chronic diseases mainly through modification of aerobic capacity, muscle mass, adiposity, insulin sensitivity and low grade systemic inflammation. Although it seems obvious that everyone, young and old should participate in some form of regular exercise one of the main cited reasons for not exercising is a lack of available time, especially in those who are middle aged and older [187]. Therefore it is no surprise that the majority of these age groups are physically inactive and unable to adhere to the current health guidelines of at least 150 minutes of moderate exercise per week plus two sessions per week undertaking muscle strengthening exercises [272]. High Intensity Interval Training (HIT) offers an alternative exercise method which incorporates short intense bouts over a reduced period of time which has been shown to elicit similar physiological responses as regular endurance training (ET).

This study compared the effects of ten weeks of high intensity interval training (HIT) at ~91% of VO_{2max} three times per week to that of five weekly sessions of endurance training (ET) at ~64% of VO_{2max} on systemic inflammation, endocrine function and immune function in healthy but sedentary men and women. Ageing has been shown to impair certain adaptations to exercise training, such as the muscle anabolic response, therefore it was important to assess whether HIT could improve adaptation or mimic that of ET in a slightly older cohort [376]. The participants of this study were aged between 20 and 60 years old and as ageing has an impact on all of the above measured variables it was our aim to assess the effects of age.

Not only is this the first study comparing HIT and ET effects on systemic inflammation, endocrine function and immunity, it is also the first to assess the impact of age as well. The main findings of this study were that ten weeks of high intensity interval training elicited comparable reductions in inflammatory and endocrine biomarkers whilst improving innate immune function in a group of healthy but sedentary individuals. Furthermore, HIT was as efficacious as ET in improving the same parameters following stratification of participants into young (20 – 39 year old) and middle aged (40 – 60 years old) individuals.

6.3.1 Fitness improvements and adherence

It is well established that aerobic exercise interventions can improve cardiorespiratory fitness with responses being dependent on duration and intensities of the program [377]. This study confirms findings from other studies that both HIT and ET improved absolute ($L \cdot min^{-1}$) and relative ($ml \cdot min^{-1} \cdot kg^{-1}$) maximal aerobic capacity and reduced body fat percentage in a group of sedentary but healthy individuals [370]. Although in this study the ET group had significantly lower adherence to the training, they actually exercised on average twice as long as the HIT group. Adherence to exercise interventions is problematic in all those who undertake them, especially sedentary individuals. Group based instructor led exercise interventions have been shown to improve adherence and give health benefits especially in at-risk populations such as the elderly and sedentary [378, 379]. Here adherence to HIT was greater than ET even when both were in groups and led by experienced instructors. This opens up the possibility that high-risk groups such as the elderly and diseased may be able to improve health parameters to an even greater extent than previously thought and would adhere to the HIT program.

Typical submaximal aerobic training is characterised by continuous duration activity of a relatively constant intensity whilst HIT is typically interval sessions of varying durations of high and low intensities, usually above or at the anaerobic threshold. Therefore adaptation to HIT involves a number of energy utilisation changes such as fat oxidation and muscle substrate metabolism. Indeed as shown here with an equivalent reduction in body fat we can assume that fat oxidation was increased comparably in both groups. Indeed, Essen and colleagues have previously shown that sedentary individuals used more lipids than stored glycogen following 1 hour of HIT (15 second exercise at maximal capacity followed by 15 second rest) when compared to 1 hour of continuous exercise at 50% maximal capacity [380]. Furthermore, HIT for 6 weeks has been shown to improve intramuscular triglyceride breakdown via cellular alteration of expression of lipolytic proteins as well as glucose uptake proteins [371, 372].

In addition, aerobic capacity is dependent on mitochondrial function allowing energy production in the form of ATP to be increased by oxidative phosphorylation. Unlike ET however, HIT can also increase energy production by upregulation of anaerobic glycolysis as well as aerobic glycolytic enzymes [370]. This does not occur to a significant level in endurance training interventions. Therefore as the duration of total HIT in this study was less than that of ET the comparable aerobic capacity improvements are likely due to improved energy substrate utilisation.

6.3.2 Inflammatory Responses

Alterations in fat oxidation and energy substrate utilisation are associated with protection from chronic diseases with an inflammatory phenotype. Corroborating findings from other studies we show here that ten weeks of exercise training reduces systemic inflammation [334]. CRP and IL-6 are the most commonly measured

inflammatory markers of training interventions [334]. CRP concentrations are consistently shown to be reduced by both aerobic exercise training and resistance training. It remains unclear the mechanism driving exercise induced CRP reductions however it is thought to be primarily due to reduced body fat percentage [25, 280, 381]. Conversely, Stewart and colleagues recently suggested that other mechanisms than fat reduction may drive reduced CRP concentrations as they observed no change in body fat percent but reduced CRP following 12 weeks of training [282]. However, a number of studies have shown that physically active individuals have lower CRP concentrations than physically inactive but also have lower BMI's [239]. In support of this, CRP and body composition were positively related in this study whilst relative but not absolute aerobic fitness was negatively associated suggesting body composition was driving changes in CRP.

IL-6 is the main cytokine responsible for hepatic production of CRP which suggests that changes in circulating inflammatory cytokine concentrations may lead to reduced CRP. Ahmadi and colleagues, Stewart and colleagues and Donges and colleagues amongst others have shown systemic reductions in CRP following training but not IL-6, IL-1β or TNFα [282, 382, 383]. In agreement with this literature, HIT or ET did not show reduced IL-1β or TNFα concentrations in this study, though a small but significant reduction in IL-6 (p=.047) was seen. As mentioned previously IL-6 plays a dual role in inflammation, if produced in response to infection or from adipocytes it is inflammatory by nature whilst if produced by muscle during exercise it is anti-inflammatory and tissue protective [196]. As there was no large change in IL-6 the reduced CRP is likely being driven primarily by reduced body fat percentage.

The cytokine network and indeed the adipokine network are extremely complex and not fully understood. To understand the inflammatory phenotype of adipose tissue

and the response to exercise training adipokine levels were assessed in response to HIT and ET. Leptin and adiponectin production are directly associated with adipose tissue mass and the ratio of the two may therefore be a reliable measure of adipocyte function and adipose tissue induced inflammation [384]. When the ratio of leptin to adiponectin is high it is indicative of a pro-inflammatory phenotype whereby cells produce more TNFα and IL-6 which can inhibit the production of adiponectin. Increased adiponectin is thought to be cardio-protective in that it can increase nitric oxide content allowing improved vascular function as well as improving fat oxidation [32]. A number of studies have shown that both weight loss and exercise can alter the production of leptin and adiponectin [385]. In agreement with this, the data here show that the leptin:adiponectin ratio was significantly reduced following training which was mediated by an increase in adiponectin and a reduction in leptin. In light of this we suggest that reduced IL-6 and CRP following exercise training is primarily due to reduced and altered function of adipocyte mass as evident by altered leptin and adiponectin production.

Few studies comprehensively assess cytokine changes in response to exercise and of those that do the majority are in response to acute exercise but tell us little about the overall impact of the exercise. Therefore in order to determine further health enhancing benefits of the exercise training this study measured a total of 12 different cytokines. Similar to a number of other studies the typical anti-inflammatory cytokines IL-4, IL-10 and IL-13 were unaffected by exercise training [386]. Interestingly we show here that MCP-1 and IL-8 are altered with exercise training. MCP-1 is responsible primarily for attracting monocytes to sites of infection however it is also expressed on vascular endothelial cells and is thought to play a significant role in development of atherosclerosis by tethering monocytes and promoting

differentiation into foam cells and atheroma plaques [387]. Indeed MCP-1 is consistently shown to be elevated in both the peripheral blood and carotid plaques of patients with advanced carotid disease [388]. Both resistance and aerobic training have been shown to reduce circulating MCP-1 suggesting a reduced risk of developing atheroma. Although it is unclear the mechanism by which MCP-1 reductions occur it is thought to be from either reduced immune cell production or reduced endothelial cell production or a combination of both and not likely reduced infectious episodes, although this cannot be ruled out [389]. Importantly, elevated MCP-1 is representative of pro-inflammatory macrophage (M1) differentiation in atherosclerosis suggesting that monocyte differentiation and subsequently function is altered [41, 390]. Therefore reduced MCP-1 concentrations observed here may account for altered macrophage phenotype resident in tissues as well as monocyte phenotype and function.

In addition to this neutrophil immune function may be altered due to changes in immune modulators, in particular GM-CSF was increased in response to ten weeks of training. GM-CSF is typically associated as a pro-inflammatory growth factor stimulating production of neutrophils and monocytes from the bone marrow [391]. Indeed in patients with chronic conditions such as chronic heart failure it is typically elevated and with training there is a significant reduction [392]. However with healthy ageing GM-CSF may be reduced and contribute to impaired neutrophil and monocyte function [393]. Therefore increased GM-CSF after ET and HIT is likely a good factor, improving immune function and reducing risk of disease and infection.

Finally, the data in this thesis has previously suggested that the cortisol:DHEAs ratio may be an important indicator of immune cell function as it acts to suppress when elevated and enhance function when reduced. A number of studies have shown that

sedentary or overweight individuals have a reduced cortisol:DHEAs ratio following exercise training. Izzicupo and colleagues showed that reduced cortisol:DHEAs ratio following 13 weeks training in post-menopausal women reduced their risk of chronic disease [394]. Here 10 weeks of training not only reduced the ratio it did so by reducing cortisol and increasing DHEAs suggesting an extremely favourable alteration to the HPA-axis and ultimately inflammation and immune function.

Taken together, it is likely that exercise induced alterations of inflammatory biomarker levels are indicative of modifications of systemic tissue function. Reduced body fat is contributing to reduced hepatic burden as evidence by reduced IL-6 and CRP levels. This is evident by the altered adipokine concentrations highlighting modified tissue size and function. Reduced MCP-1 and IL-8 are indicative of reduced cardiovascular disease and other chronic disease risks. Furthermore, in combination with increased GM-CSF and reduced cortisol:DHEAs there is potential to improve immune function and further reduce risk of infection and disease.

6.3.3 Immune Function

Both HIT and ET improved neutrophil and monocyte responses to bacteria as well as reducing the inflammatory phenotype of monocytes. The previous chapter suggested that an acute bout of walking can transiently alter neutrophil and monocyte bactericidal capacity as well as cell phenotype and potential inflammatory capacity. This finding suggests that innate immune cell function can be modulated in response to exercise training programs. However few studies have assessed the impact of training interventions on neutrophil function. Cross-sectional studies have revealed that neutrophil phagocytosis is improved in trained compared to untrained individuals across an age range of 20 to 60 years [226, 246]. Furthermore, increased training status in men has been associated with increased neutrophil phagocytosis [245].

More recently, neutrophil chemotaxis and phagocytosis was improved following 2 months of moderate exercise training [246]. In agreement with these studies, this study showed that following 10 weeks of training neutrophil phagocytosis was increased in respect to increased cellular uptake of bacteria and increased cellular production of oxidative killing. These findings were consistent across exercise groups and ages, although improvements appeared to be larger in the young.

Additionally, similar effects on bactericidal activity of monocytes were seen following 10 weeks of training. This is in contrast to Schaun *et al* who observed no change in monocyte bactericidal following 12 weeks of aerobic and strength training [243]. This study did however assess effects in slightly older men than the present study suggesting ageing may attenuate any exercise induced changes. To address the data from this study were stratified in to a young and middle-aged group and this revealed that monocytes produced a greater degree of change for superoxide killing in the middle aged than the younger group. Although the improved bacterial uptake was similar across the two ages it is possible that our conflicting findings from Schaun and colleagues were due to methodological differences. In particular they used isolated monocytes incubated with opsonised zymosan whilst here a whole blood assay was used which reduces handling artefacts.

Monocyte phenotype as previously shown in this thesis (chapter 5) was altered by acute exercise with monocyte expression of CD16, TLR4 and TLR2 all reduced. This would suggest that the monocyte phenotype is shifting from a pro-inflammatory state capable of perpetuating systemic inflammation to a less inflammatory state with improved anti-inflammatory capabilities. In this chapter it is shown that in sedentary

healthy individuals' monocyte phenotype was also altered in response to HIT and ET exercise training. In particular, the percentage of pro-inflammatory CD16 positive monocytes was reduced whilst the expression of TLR2 and TLR4 was slightly reduced on the pro-inflammatory subtypes. This suggests that not only is exercise training improving bactericidal capacity but it is doing so on a developmental component of monocyte biology.

Although we cannot determine the exact mechanism causing these innate cell changes it is possibly due to the alterations in inflammatory status impacting cell differentiation or improved clearance of dysfunctional cells. Incubation of neutrophils and monocytes with various inflammatory or anti-inflammatory mediators has been shown to modulate cell function [395, 396]. Neutrophils isolated from highly inflamed rheumatoid joints exhibit severe dysfunctional properties including elevated and abnormal reactive oxygen species production [397-399]. Furthermore, patients with chronic obstructive pulmonary disorder (COPD) have elevated inflammatory cytokine production and have recently been shown to have impaired neutrophil chemotaxis [170]. Similar patterns are observed in monocytes from chronic inflammatory conditions. Hulsmans and colleagues suggested that individuals with chronic heart conditions have reduced monocyte function but an elevated pro-inflammatory phenotype which contributes to atheroma plaque formation [400]. Therefore as we have shown a reduced inflammatory milieu as well as an immune enhancing endocrine response it is likely that this study not only altered intrinsic cellular function but also maintained correct differentiation in the circulation.

6.3.4 Effects of Age

The one study that has assessed some form of HIT in the elderly and/or middle aged have shown that even when the training is intermittent walking, aerobic capacity, fat oxidation, muscle strength, glucose utilisation and risk factors for cardiovascular disease are attenuated [373]. These findings show that HIT can be adapted for a group of individuals not likely to be able to perform typical exercise sessions such as cycling or running. However, to date no study has assessed whether HIT can improve inflammatory and immune function in an older cohort which are directly associated with disease progression.

Although others have suggested that adaptation to training is impaired with age [376, 401], especially musculoskeletal and cardiorespiratory adaptations, this study did not see any effect for age on physiological improvements following training. Others have shown differences between younger and older cohorts; however the age difference tends to be greater. Here we show that even in middle aged cohorts physiological improvements are evident by both ET and HIT. However, the older group had more body fat and had a lower aerobic capacity, lower levels of DHEAs and GM-CSF and a slightly higher baseline percentage of neutrophils and monocytes producing superoxide responses towards *E.Coli*. In effect both HIT and ET training caused similar alterations regardless of age. In the case of DHEAs which has been suggested to be tissue protective and GM-CSF which can stimulate improved innate immune responses, training initiated a restoration of levels to almost that of younger participants.

As time may play such a significant role in reduced adherence for exercise training, HIT offers a new paradigm which can elicit health improvements in both young and middle-aged individuals. Furthermore, as others have shown physiological

improvements in elderly HIT participants it is likely that HIT can improve inflammation and immune function in those most at risk of chronic disease and infection.

6.4 Limitations & Future Work

This study is not without limitations. It has been assumed that modifications observed in the middle-aged group are translatable to an elderly cohort, however to date no such study assessing inflammation or immune function has been conducted. As the exercise may stress the body to a greater degree than regular training it is possible that the elderly may over-stress and therefore cause heightened damaging inflammation as well as immune suppression. Over training has been previously shown to exhibit such changes; however the work coming from the Japanese groups suggests no adverse effects [373]. Therefore future work should carefully assess the implementation of HIT in the elderly and aim to assess comprehensive inflammatory and immune function.

In addition adaptive immune cell function was not considered. Low aerobic capacity has been associated with increased prevalence of senescent T-cells in the peripheral blood which indicates a functional decrement and may allow infection propagation [402]. Furthermore as exercise can mobilise and alter function of T-cells [294] it is likely that HIT may be able to elicit a similar result. Future work should therefore also assess T-cell phenotype and function in order to semi-quantify such things as thymic function, vaccine response and infection control.

Finally, as was shown in the previous chapter acute exercise transiently alters inflammation and immune function. It would be worthwhile to assess the impact of transient changes in the HIT group to assess a) whether the acute modifications are greater and b) what the dynamics of change are over the training period to attempt and narrow down adaptation dynamics. This would allow for better quantification of the type, duration and intensities of training which would be needed to elicit beneficial improvements and thus personalise exercise prescription.

6.5 Conclusions

In conclusion it is shown here for the first time that high intensity interval training is as effective at modifying inflammation and immune function as regular endurance type training. Furthermore, the effects of HIT are transferable across ages, from young to middle-aged at least. This suggests that with modification and planning, HIT training could be an effective therapeutic intervention to delaying or preventing inflammatory mediated chronic disease and infections in the elderly.

Chapter 7: General Discussion

7.0 General Discussion

Physiological ageing has long been associated with an increased risk of chronic disease and infection. An underlying contributing factor is the increase in low-grade chronic systemic inflammation in the absence of infection (inflammageing) [31, 127]. It remains unclear the exact factors driving inflammageing but include immunesenescence, obesity, loss of anti-inflammatory sex steroids, hyperactivation of the HPA-axis and reduced levels of physical activity [251]. Taken together obesity, sedentary behaviour and immune mediated inflammation contributes to the majority of morbidities and mortality observed in the elderly [31, 403]. Physical activity and exercise offers a mechanism by which immune dysfunction, obesity and subsequently inflammation can be attenuated in the elderly [190, 294]. Exercise both acute and chronic is energy demanding and subsequently utilises adipose tissue as an energy source reducing the risk of obesity. Similarly exercise can modify the number and function of circulating immune cells to reduce the risk of infection and modify cytokine output by muscle and adipose tissue. However, until recently few studies have assessed the impact of physical activity on inflammation and immune function in the elderly. The aims of this thesis were therefore to identify the consequences and causes of inflammageing and assess the effects of physical activity as an intervention on inflammation and immunesenescence in healthy elderly individuals.

7.1 Summary

Chapter 3 investigated the effects of persistent infection by cytomegalovirus (CMV) on inflammageing over a ten year period and the consequences inflammageing presents in later life. Over 700 elderly participants were recruited, had a number of assessments completed including blood taken in 1994 before over 250 returned ten years later for a follow-up analysis. To our surprise, CMV was not driving the increased production of inflammatory mediators in our cohort which was in contrast to suggestions by a number of studies [89, 95, 222]. Furthermore in contrast to other studies CMV infection was not predictive of frailty or mortality over a ten year period [404, 405]. However systemic inflammatory markers including cytokines, endocrine mediators and immune cells were predictive of frailty and mortality suggesting mortality associated with inflammation and CMV infection are independent of each other. Interestingly, measurements of behavioural lifestyle choices reflective of a sedentary behaviour revealed that body fat and physical functioning were influencing inflammageing more than other measures such as CMV infection and social class.

In light of the previous findings, Chapter 4 investigated the impact of physical activity levels on systemic inflammation and immune function in over 200 very healthy elderly participants. Confirming findings from the previous chapter, CMV infection was not predictive of systemic inflammation but physical activity levels and body fat were predictive of inflammation. Therefore as the previous chapter indicated frailty and mortality being associated with immune components it was necessary to assess immune function concurrently. Additionally, CMV is dominated by the adaptive arm and showed no effects. As the immune system with age shifts from a lymphoid to a myeloid dependent state and innate cells are capable of influencing inflammation we

assessed neutrophil and monocyte function in response to physical activity levels. In comparison to findings from others [168] ageing was associated with neutrophil dysfunction, however being physically active in old age attenuated the functional decline. Maintenance of neutrophil function was independent of body fat suggesting that physical activity was influencing intrinsic cell mechanisms. No differences were observed for monocyte function suggesting neutrophils may be more sensitive to physical activity status.

As physical activity was influencing inflammation and innate cell function, Chapter 5 aimed to determine the impact of a short acute bout of exercise in healthy elders. Elderly participants were either grouped as high or low active and completed an incremental walking test. Exercise caused an increase in inflammatory biomarkers and improved endocrine response during the recovery period following exercise cessation. These effects were paralleled with small improvements in neutrophil and monocyte bactericidal function suggesting altered inflammatory mediators can modulate immune function. Interestingly, the low active group had similar inflammatory profiles but also a trend for reduced immune function compared to the active group suggesting regular exercise can improve immune function.

It is clear from previous chapters that physical activity could modify inflammation and immune function in the elderly, however getting them to exercise voluntarily is a significant social issue. Middle-aged and elderly individuals state a lack of time for participating in regular exercise sessions [406]. Therefore the final chapter (Chapter 6) aimed to assess whether a novel high intensity interval training (HIT) intervention with a reduced time commitment could elicit similar results as ‘regular’ exercise interventions. In particular we aimed to determine for the first time whether 10-weeks of HIT could modify inflammatory state and immune function in young and middle-

aged sedentary individuals. HIT was as effective as ‘regular’ training at reducing systemic inflammation, through adipose tissue modification whilst improving neutrophil and monocyte bactericidal function. Interestingly, although the results tended to be more pronounced in the younger participants effects were also observed in the older cohort. Additionally those individuals in the HIT group had a higher adherence to the study than ‘regular’ training. These findings suggest that HIT may provide an effective therapeutic intervention to delaying or preventing inflammatory mediated chronic disease and infections in the elderly.

In light of the findings from this PhD a striking observation can be made. With age there is a pronounced increase in systemic inflammation and marked reduction in immune function. These observations have been attributed previously to chronic repeated infectious episodes [85, 251]. However, ageing is an inherent process and not a pathological condition. Therefore to confidently characterise functional decrements as attributed to ageing they must be independent of pathologies. The findings throughout this thesis suggest that those individuals who maintain a physically active lifestyle have improved immune function and reduced systemic inflammation with no major pathologies. This suggests that as regular exercise is known to reduce the risk of age related pathologies, the ageing process could be better characterised in extremely active elders over time.

Recently master athletes have been suggested as a model of successful ageing, or ‘positive’ control, to study inherent ageing, due to their lack of confounding pathologies [407, 408]. Master athletes are typically defined as being over 50 years old and actively taking part in exercise training [409]. Indeed a 13-year study of elderly competitive runners (>50 years old) versus non-runners suggested that the active group were at a lower risk of disability and mortality [410]. Although the rate of

decline in physiological variables such as VO_{2max} is similar between trained and untrained, the high starting point in master athletes appears to aid protection [409]. Therefore as it is difficult to define minimum exercise requirements to prevent pathological changes with age [411] the master athlete represents an excellent control group for ageing studies. Although master athletes still succumb to infection and inflammatory related disease [412], it is clear that the severity and incidence are less than the general population.

Subsequently, this thesis has shown that differences in immune function and systemic inflammation are evident in elderly individuals who do not have a large gap in physical activity levels. Therefore, future research assessing immune and inflammatory function in the elderly should consider utilising extremely active individuals to fully determine the age related decline in function. 7.5 Elucidating Potential Mechanisms

The exact mechanisms driving sterile chronic systemic inflammation in the elderly remain elusive. During this thesis some of the mechanisms could be elucidated, however one of the key limitations of all of these studies was the apparent good health of our participants. What is clear is that physical activity and body composition are intrinsically linked but not mutually dependent for inflammatory associations.

7.5.1 Inflammation, Immunesenescence and Physical Activity

One of the key observations seen here is that habitual activity leads to reduced systemic inflammation and that acute exercise can promote an inflammatory response most likely in order to promote tissue adaptation. Although accounting for body fat many observations were attenuated however it is unlikely that adipose tissue is the principle source of inflammatory mediators. We were unable to assess

the dynamics of inflammation and the interaction each component has with each other is incompletely understood. Figure 7.1 highlights the cytokine response to exercise and sepsis and shows that disease is in fact a different type of pro-inflammatory situation [196, 197]. This raises the question that perhaps the line between pro and anti-inflammatory is not so clear. In light of the recent findings that IL-6 can block production of TNFα and IL-1β can stimulate cortisol production it is likely that exercise promotes a blurred inflammatory environment which is likely acting against natural ageing with tissue adaptation.

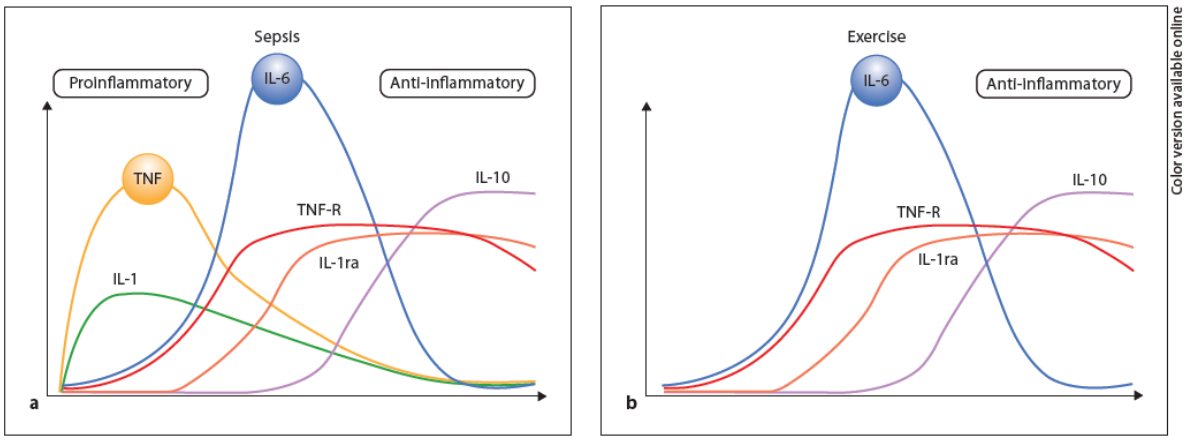

Fig 7.1: Differences between cytokine dynamics during infection and exercise. Taken from Petersen *et al. [197]*

What is clear is that no single factor can account for the exercise induced improvements observed, however systemic inflammation is the likely cause. Improvements are a tightly intertwined network of physiological systems working together, Fig. 7.2. For example we did not account for the potential capacity of exercise to reduce the frequency of senescent cells which promote inflammation. Recent work has shown that physical activity is associated with reduced senescent T-cells therefore it is likely that other senescent cells may be cleared or prevented from accumulating [217, 413]. If this is the case then the inflammatory environment

created by exercise is promoting clearance of dysfunctional cells. This is particularly evident when assessing exercise induced improvements in vaccine efficacy [414]. Not only does systemic inflammation promote efficacy it can also impair it, therefore the impact of exercise on adaptive inflammatory environment most likely aides in vaccine efficacy.

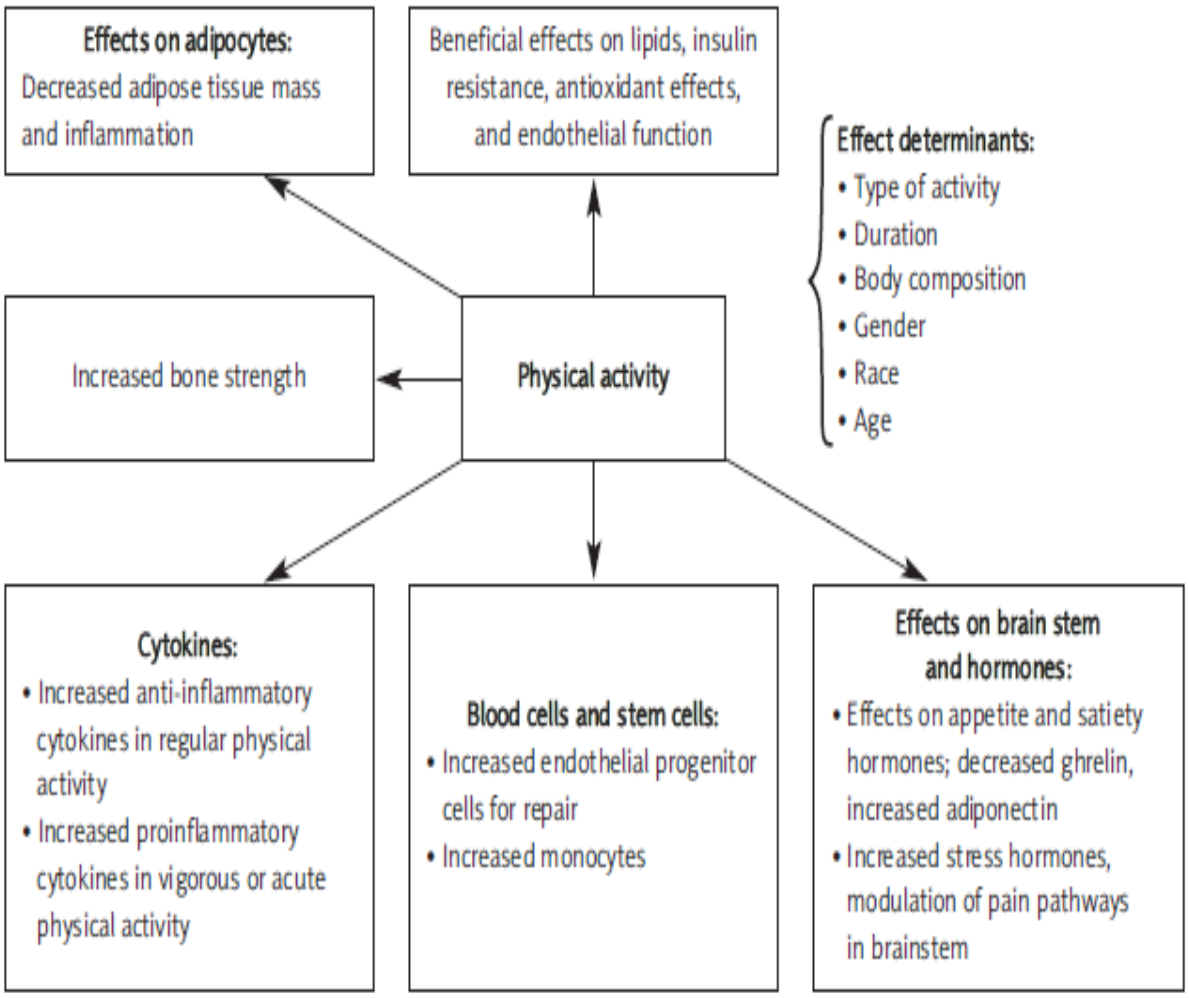

Fig. 7.2: Physical activity and inflammation. Systemic effects of physical activity showing physiological changes which promote an anti-inflammatory environment.

7.5.2 CMV Infection, Inflammation and Mortality

CMV infection has been linked with increased risk of mortality in a number of other studies however, whether CMV infection is directly responsible for mortality remains unclear. The fact we observed no effect on mortality or inflammageing does not rule out the impact this virus has on morbidity and mortality in later life. In our study the cohort were all relatively healthy compared to the general population and were from a considerably more affluent area which is in contrast to others. It has been shown that CMV infection is more prevalent in groups from lower socioeconomic populations and is likely therefore to undergo increased intermittent reactivation. This would undoubtedly lead to premature or quickened immunesenescence leading to increased risk of infection and disease. Subsequently the inflammatory status of these individuals may be different from CMV negative individuals. Additionally, evidence is appearing which suggests low socioeconomic groups participate less in structured and habitual physical activity.

In the acute exercise study (Chapter 5) we observed some interesting trends developing between the high and low active elders who were CMV seropositive. The high active elders appeared to have lower titre's, reduced numbers of monocytes and improved monocyte bactericidal functions. Although these findings were moderate and non-significant it raises the question of whether regular exercise reduces the viral load and subsequently improves the immune capacity of the individual. Recently it was suggested that physical active could reduce the frequency of CMV specific T-cells following findings that physical activity was related to the frequency of senescent T-cells in the peripheral blood compartment [402]. Although it is unlikely that exercise can cause clearance of CMV specific T-cells it is likely that

exercise can limit the movement and seeding of the virus as well as enhancing the immune response against its reactivation. This may not be apparent in young healthy CMV positive individuals but is likely to be more evident in elderly individuals. Recent studies have suggested that CMV utilises innate immune cells in order to migrate to tissue and propagate infection [158]. Monocytes appear to be key mediators of this by promoting differentiation in to pro-inflammatory non-classical monocytes where they migrate to tissue, aided by their adhesive and migratory properties [158, 415]. Whether CMV therefore modifies innate immune function in order to survive remains unknown. Recently LaVoy and colleagues showed no difference in innate bactericidal function in response to exercise or between CMV positive and negative individuals. These participants were all relatively young and may not represent a true reflection of longitudinal CMV infection. Although we saw no direct effect of CMV on systemic inflammation in any of the studies its impact on immunity cannot be ignored. Subsequently it is likely that systemic inflammation may be integral to CMV related mortality as is likely for all underlying morbidities.

7.6 Conclusions and future research into physical activity and inflammation and immune function

In conclusion inflammageing is responsible for the majority of age related tissue dysfunctions including sarcopenia, cardiovascular function and immune function. Thus this thesis reveals that the phenomenon of inflammageing is driven primarily by behavioural sedentary lifestyle choices and less so by immune responses. This was confirmed by the findings that habitual physical activity and a ten-week exercise intervention were associated with reduced inflammatory mediators. These reductions were inherently linked with reduced body fat, a consequence of increased physical fitness suggesting the metabolic alteration benefits of habitual exercise.

Future research will endeavour to assess the most economical and efficacious way of reducing systemic inflammation in the elderly. Our findings from the HIT study offers hope that not only healthy elderly participants can contribute but also those with underlying morbidities. Clearly more research needs to be conducted to tease out the fine interplay between exercise, inflammation and disease in the elderly.

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Appendix I – Inclusion/Exclusion criteria for Chapter 4.

WT Clinical Research Facility Study 0949

Physical Activity and Ageing Study

Name ..... Hospital Number..... Date ...../...../.....

INCLUSION CRITERIA SIGN

1. Age 60 – 80 years
2. Able to walk for 2 minutes or 50 metres without stopping (whichever applies)
3. Patients able and willing to provide written informed consent for the study

EXCLUSION CRITERIA

Subjects presenting with any of the following will not be included in the trial

Significant medical history in the last three years as indicated below

	Y	N
1. General history including : Dementia, Parkinson Disease, Stroke, TIAs, Liver Disease, Cancer		
2. Heart disease. Specifically previous myocardial infarct, cardiomyopathy or valvular disease.		
3. Chest pain (angina pectoris). History recurrent (no more than 1 or 2 episodes per month over the last 6 months) or sudden chest pain typically radiating to the left arm or left side of the neck (unless associated with recent cold, cough or episode leading to bruising		
4. Blood Pressure > 190/120 or tendancy to faint		
5. Pulmonary disease including asthma / COPD leading to significant lung function loss and prescription of corticosteroids. Other medication is permitted.		
6. Rheumatoid or osteoarthritis leading to severe stiffness and exercise intolerance (mild stiffness is allowed if exercise criterium above is met)		
7. Current use of corticosteroids.		
8. Type 1 or 2 diabetes (raised blood sugar when measured on capillary sample is allowed)		
9. History of leg pain on exertion, sufficient to limit walking ability to less than 50 metres or < 2 mins		
10. Any cause to consult the G.P or report of feeling unwell in the previous 10 days		
11. Inability to give informed consent		

This patient is able to exercise as part of the Healthy Ageing Study in the Sports and Exercise Science Building (Birmingham University)

Signature of Nurse.....**Date**.....

Appendix II – Primary papers and review articles published during the completion of this PhD and Undergraduate degree.

1. **Bartlett, D.B.,** Firth, C.M., Phillips, A.C., Moss, P., Baylis, D., Syddall, H., Sayer, A., Cooper, C. and Lord, J.M. (2012). The age-related increase in low-grade systemic inflammation (Inflammaging) is not driven by cytomegalovirus infection. *Aging Cell*
2. Baylis, D., **Bartlett, D.B.**, Syddall, H.E., Ntani, G., Gale, C.R., Cooper, C., Lord, J.M. and Sayer, A.A. (2013). Immune-endocrine biomarkers as predictors of frailty and mortality: a 10-year longitudinal study in community-dwelling older people. *AGE*
3. Baylis, D., **Bartlett, D.B.**, Patel, H.P. & Roberts, H.C. (2013). Understanding how we age: Insights into inflammaging. *Longevity & Healthspan*
4. Greenwood, H. & **Bartlett, D.B**. (2013). Meeting report: British Society for Research on Ageing 2012. *Longevity & Healthspan*
5. Shneerson, C.L., **Bartlett, D.B.**, Lord, J.M and Gale, N. (2014) Supporting Healthy Ageing: a training event for multi-disciplinary healthcare students. *European Journal of Integrative Medicine.*
6. Baylis, D., Ntani, G., Sydall, H.E., **Bartlett, D.B.**, Martin-Ruiz, C., von Zglinicki, T., Kuh, D., Lord, J.M., Sayer, A.A., Cooper, C. (2014). Inflammaging: powering the biological clock of telomere length? A ten year longitudinal study of inflammation, telomere length and physical ageing. *Calcified Tissue International.*
7. Simpson, R.J., Cosgrove, C., Chee, M.N., McFarlin, B.K., **Bartlett, D.B.**, Spielmann, G., O'Connor, D.P., Pircher, H. and Shiels, P.G (2010). Senescent phenotypes and telomere lengths of peripheral blood T-cells mobilized by acute exercise in humans. *Exercise Immunology Review.*