THE EFFECTS OF SLEEP DYSREGULATION AND ADIPONECTIN ON IMMUNITY IN OLDER ADULTS

by

Alessandra Rossi

A thesis submitted to
the University of Birmingham
for the degree of
DOCTOR OF PHILOSOPHY

School of Immunity and Infection
College of Medical and Dental Sciences
University of Birmingham

May 2014

ABSTRACT

Short sleep duration and poor sleep continuity have been related to adverse health outcomes. Sleep disturbances are more frequent among older people, who also experience a reduction in immune function (immunosenescence). This thesis tested the hypothesis that sleep disruption in old age contributes to immunosenescence.

93 healthy older subjects had their sleep recorded by actigraphy and immunological parameters were assessed. The data indicated that sleep continuity and duration in older adults does not influence innate immune function but was associated with changes in blood cell numbers, in particular an increase in the granulocyte:lymphocyte ratio. Differences in serum IL-4 and adiponectin were associated with long sleep duration and poor sleep continuity was also associated with raised serum cortisol. However, preliminary data obtained from a small pilot study of partial sleep deprivation in young and old adults did not show similar changes therefore causality was not confirmed.

In vitro experiments were performed to evaluate whether adiponectin, whose levels change with age, affected neutrophil apoptosis and phagocytosis. Adiponectin extended lifespan of neutrophils and inhibited bacterial phagocytosis.

The findings suggest that sleep dysregulation does not contribute to immunosenescence and *in vitro* studies add weight to the literature showing immunomodulatory roles for adiponectin.

A mia madre.

ACKNOWLEDGEMENTS

During my PhD I have been surrounded by many friendly and supportive people, without them these years would have been much harder, therefore I am thankful to all of them.

First of all I would like to thank my supervisor, Prof. Janet Lord, for giving me the opportunity to accomplish this PhD in her lab. In particular I am grateful for her encouragement, patience and advices I also thank Dr Anna Phillips for helping me with statistics.

A particular acknowledgement goes to all the volunteers who gave blood, especially the ones who participated in the partial sleep deprivation study, this was not an easy task to carry out and therefore I thank them very much for their outstanding effort.

I thank Dr Peter Hampson for introducing me to the main techniques of the lab and for his help in the sleep projects. I am grateful to Mrs Hema Chahal not just for her technical assistance but for taking good care of me and the whole group. I would like to thank Dr Jon Hazeldine for his answers to all my silly questions, David Bartlett for his time spent elucidating statistical issues, Niharika Arora Duggal for her help throughout the PhD and all the members of the Lord lab for their friendship and feedback. I am also grateful to Mrs Gianina Luca and Prof. Mehdi Tafti for introducing me to the sleep techniques during my exchange in Lausanne, and for their useful suggestions about my project. Last but not least, I thank Ana Vitlic for all the times she helped me in recruiting volunteers and for bleeding them for me. Most importantly, I thank Ana for her friendship and for having shared the joys and sorrows of this PhD with me.

Together with Ana, I acknowledge my best flatmates Emmanuelle, Mathilde, Claire and Charlotte for the good times spent together at home with our lovely Simba. I must thank Martina and all my Birmingham friends, with whom I had really great times. I must also

mention my athletic teams and coaches for giving me the opportunity to train with them as I really enjoyed my sessions at the track.

I am grateful to my Italian friends who continued to keep in touch during these years. A person who has constantly been at my side despite the distance is my boyfriend Tommaso, he has always believed in me and has been a source of continuous love, patience and support, without him I would have not been able to face all the difficulties. Finally, I thank my family for their unconditional love despite my absence from home. I know you are proud of me.

TABLE OF CONTENTS

Page No.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
AICAR	5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CD	Cluster of differentiation
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HCl	Hydrogen chloride
HOCl	Hypochlorous acid
Mg^{2+}	Magnesium
mRNA	Messenger ribonucleic acid
Na_3VO_4	Sodium orthovanadate
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaF	Sodium fluoride
$O_2^{\cdot -}$	Superoxide anion

CHAPTER 1

INTRODUCTION

1.1 SLEEP

Sleep is a constitutive behavior of animals indispensable for wellbeing, health and survival. In other words, it represents a physiological, cyclical state of rest for both the body and the mind during which body movements and consciousness are minimised.

The average sleep duration experienced by adults has declined over the last decades [1-3], and disruptions in sleep parameters have been related to a variety of negative health outcomes, both in animals and humans. For instance, rats subjected to total sleep deprivation exhibited global deterioration finally resulting in death (maximal survival up to 32 days) [4] and in humans epidemiological studies have indicated that both short and long sleep durations are significantly associated with increased morbidity and all-cause mortality [5-10]. The general belief that the ideal sleep duration is between 7 and 8 hours was confirmed by the majority of studies [5-7, 9], however Kripke et al. found that the risk of mortality was lowest for people sleeping between 5 and 6.5 hours per night as assessed by actigraphy [11].

1.1.1 PHYSIOLOGY OF SLEEP

During sleep the brain performs a characteristic cycle of electrical activity which can be measured by electroencephalography (EEG). On the basis of the differences in the electric wave amplitudes and frequencies, two main kinds of sleep have been identified: non-rapid-eye-movement (NREM) sleep and rapid-eye-movement (REM) sleep. NREM sleep can be divided into three progressively deeper stages: N1, N2 and N3. Stages N1 and N2 are characterized by low amplitude, high frequency (3-7 Hertz) EEG waves (theta waves), whereas stage N3, also named slow wave sleep (SWS) has high amplitude (>75 μV), low frequency (<4.5 Hertz) EEG waves (delta waves) [12, 13]. REM sleep consists of low amplitude, high frequency EEG activity similar to those of N1 and N2. During REM sleep alpha waves (20-60 μV, 6-13 Hertz), typical of wakefulness, may occur [14, 15]. The presence

of bursts of rapid eye movement, which give this stage of sleep its name, allows the identification of REM sleep (Figure 1.1) [15, 16].

These sleep stages differ from each other not only by the frequency and amplitude of EEG waves, but are also associated with distinct functions: whilst stage N1 represents the transition between wakefulness and sleep, and stage N2 represents the onset of sleep, SWS is thought to be the deepest and most revitalizing phase of sleep. In fact, SWS is the stage the most related to sleep need; it is accompanied by a gradual decrease in body temperature, breathing frequency, blood pressure and heart rate [17], favouring the renewal of body energy [18]. In contrast, REM sleep entails absent muscle activity, manifestation of dreams and processing of information [15, 16].

NREM and REM sleep occur cyclically during the night: healthy individuals usually obtain 4 to 6 cycles per night with each cycle lasting approximately 90 minutes [19]. Though sleep cycles maintain the same length, the individual stages of sleep are subjected to modifications during the night: longer periods of SWS are typical of the onset of sleep and they shorten progressively in the night to allow longer REM sleep phases, which predominate towards the end of the period of sleep [20, 21] (Figure 1.2).

1.1.2 MEASUREMENT OF SLEEP PARAMETERS

The gold standard for measuring the physiological body changes that occur during sleep is polysomnography, a multi-parameter test which monitors many functions including brain electrical activity through EEG, eye movements through electrooculography (EOG), muscle activity through electromyography (EMG) and heart rhythm through electrocardiography (ECG). Polysomnography is used to diagnose a variety of sleep disorders and conditions, including restless leg syndrome, obstructive sleep apnea syndrome (OSAS) and narcolepsy.

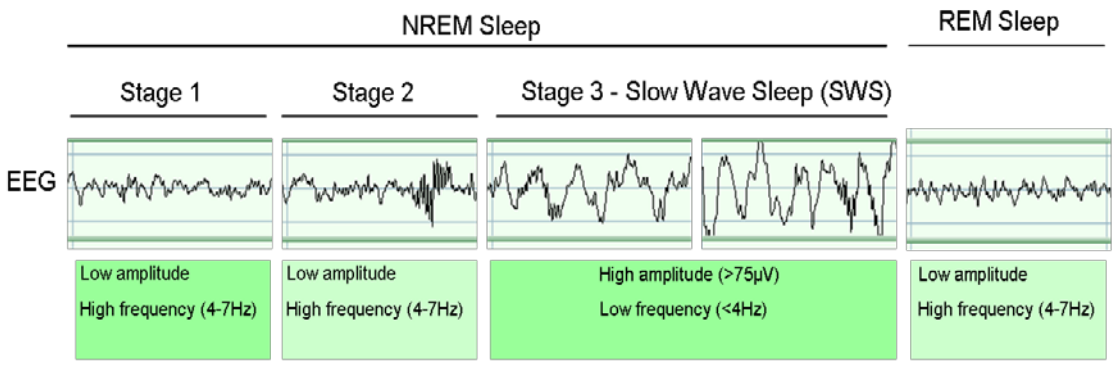


Figure 1.1 The different stages of sleep as measured by EEG. NREM and REM sleep are characterized by the different frequencies and amplitudes of the EEG waves (Figure adapted from Bryant et al., 2004 [18]).

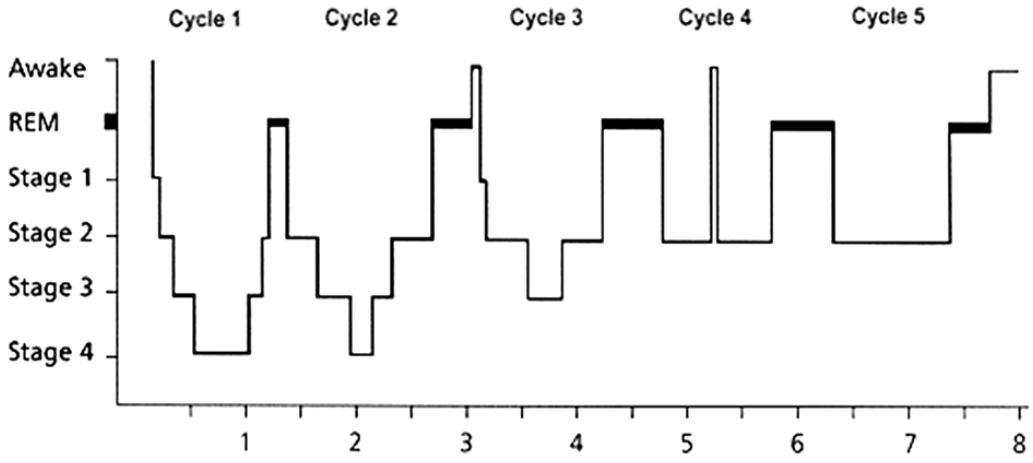


Figure 1.2 Cycles and stages of sleep during the night. Subsequent cycles are featured by a progressive shorter SWS phase and longer REM sleep (Figure adapted from Neubauer et al. [20]).

Despite the wide range of information that it provides, this powerful technique has the disadvantage of being invasive and even disruptive to physiological sleep. Because of its invasiveness and cost, polysomnography is often substituted with actigraphy, a non-invasive and cheap method to study the sleep-wake behaviour and circadian rhythms [22]. The actigraph consists of an electrode-free, watch-shaped device which is worn on the wrist of the non-dominant arm or on the ankle. The parameters that the actigraph can register are:

- the total sleep time,
- sleep latency (the time spent awake while in bed before falling asleep),
- sleep efficiency (the percentage of time spent asleep over the total time spent in bed),
- wake after onset sleep (WASO, an indicator of disrupted sleep),
- average sleep and wake bouts (indicators of the physiological fragmentation of sleep)
- movements performed by the wearer (index of physical activity).

Usually, the actigraph is continuously worn for at least three consecutive days, allowing the achievement of objective measures [23]. Its main limitation is the impossibility to distinguish the different stages of sleep. Importantly, actigraphy has been validated against polysomnography: actigraphic and polysomnographic measures were recorded in healthy individuals [24-26], in insomniacs [27] and OSAS patients, [28] and they were found to correlate in all cases. In particular, several studies reported that actigraphy accurately measures sleep duration and efficiency [24, 29, 30], while actigraphic WASO and nocturnal waking episodes often show a lower correlation when compared to polysomnographic recordings [26, 27, 30]. Specifically, the largest discrepancy between actigraphy and polysomnography has been found in subjects displaying a high level of WASO [27].

The most practical and cost-effective method to assess sleep quantity and continuity in large epidemiological studies remains the use of sleep questionnaires, in which subjects are asked to provide information about their sleep habits. Several sleep questionnaires and diaries have been validated: among them, the Pittsburgh Sleep Quality Index (PSQI) is one of the most

common questionnaires used to determine a number of parameters including sleep duration, sleep latency, sleep efficiency, subjective sleep quality, daytime dysfunction and use of sleep medication [31]. However, self-reported, subjective data provided by questionnaires and sleep diaries do not always reproduce objective measures of sleep such as actigraphic recordings [32, 33].

1.1.3 NEUROCHEMICAL REGULATION OF SLEEP

Sleep depends on two processes, called Process S and Process C. Process S is involved in the maintaining of sleep homeostasis and it is related to sleep debt, while Process C, which stands for "circadian", determines the natural rhythmicity of sleep and wakefulness, in which maximal sleepiness is experienced towards the evening and is minimal in the morning [34]. A wide range of neuropeptides and steroids have been found to have a role in the regulation of these two processes. In humans the circadian rhythm of sleep is entrained to a 24 hour period and it is finely regulated by a specific region in the brain, called the suprachiasmatic nucleus (SCN). The SCN is a bilateral structure situated in the anterior part of the hypothalamus which serves as a central circadian oscillator or "body clock" [35, 36]; it does not only control the sleep-wake cycle but the organization of the whole organism at cellular and tissue level. This is evidenced by the fact that lesions of the SCN in rodents extinguished endocrine and behaviour circadian rhythms [37-39], moreover, SCN-specific neurons display their typical circadian rhythm in neural firing not only *in vivo* but also when transplanted *in vitro* [40].

To orchestrate the body's circadian rhythms, the SCN has to synchronise various internal biological rhythms with the environment through both internal and external cues. One of the most important external cues is light, which is caught and processed by the retinal visual system and is transmitted to the SCN where it regulates its function [41]. The internal cues are represented by several systemic factors which can influence further the sleep-wake behaviour, the duration and the continuity of sleep.

1.1.3.1 Melatonin

The major internal cue maintaining the circadian rhythm of sleep is the hormone melatonin, secreted by the pineal gland under the control of the paraventricular nucleus (PVN), which itself is controlled by the SCN, and para/sympathetic innervations. Melatonin expression and release is suppressed by light and stimulated by dark through the retinal-hypothalamic tract [42, 43] and its secretion occurs in parallel to the individual's habitual bedtime and induces evening sleepiness [44]. Its endocrine role is to synchronize the circadian rhythms to the peripheral organs and tissues: in particular, melatonin can entrain both daily and seasonal rhythms [42, 45]. Apart from this function, melatonin has also been suggested to have a somnogenic role, as its administration in humans has been found to increase total sleep duration, improving efficiency and latency [46, 47]. Melatonin is thought to exert this effect by inhibiting the firing of the neurons located in the SCN through a negative feedback process, as demonstrated *in vitro* [48, 49], and possibly through interaction with γ-aminobutyric acid (GABA)-ergic neurons [50].

1.1.3.2 Regulation of sleep by local neurotransmitters

Studies have shown that sleep and wakefulness are modulated by multiple neuronal systems releasing different neurotransmitters such as orexins, neuropeptide Y (NPY), GABA, galanin [51], adenosine, serotonin, acetylcholine, dopamine and histamine [52, 53]. Cholinergic and monoaminergic neurons are believed to maintain wakefulness as their activity is higher during wake time and decreases during NREM sleep [53]. Orexins, also called hypocretins, are excitatory neuropeptides synthetized by the lateral hypothalamus [54] whose main functions are to enhance food intake [54, 55] , stabilize the sleep-wake pattern and maintain wakefulness. In fact, dysfunctions in the orexigenic neurotransmission are typical of narcolepsy, a sleep disorder characterized by daytime sleepiness, sleep fragmentation, cataplexy and frequent REM sleep episodes [56-59].

Studies performed in animals and humans indicate that another peptide, NPY, expressed mainly by the PVN, the arcuate nucleus and the SCN [60], increases food intake [61] and exerts sleep-promoting properties, decreasing sleep latency and REM sleep, increasing stage N2 and the total sleep time [62]. NPY exerts those functions by acting as a physiological antagonist of corticotropin-releasing hormone (CRH), diminishing corticotropin (ACTH) and cortisol levels [62], whose roles in regulating sleep are discussed in detail below (section 1.1.3.3).

GABA is the major inhibitory neurotransmitter in the mammalian central nervous system. It acts by binding to specific transmembrane receptors: $GABA_A$ and $GABA_B$ receptors, causing the hyperpolarization of the target neuron. It is well known that GABA and all the GABA receptor agonists induce and maintain the sleep process: benzodiazepines and the other GABA receptor agonists reduce sleep latency and fragmentation and promote NREM sleep. However, benzodiazepines and other drugs that enhance GABA neurotransmission have been also shown to increase high frequency EEG activity during NREM sleep in different species [63].

1.1.3.3 Hypothalamo-pituitary-somatotropic and hypothalamo-pituitary-adrenocortical axes

In addition to the neural mechanisms described above, other humoral factors, generally termed sleep regulatory substances (SRS) [64], can regulate sleep. Among the SRS are hormones belonging to the hypothalamo-pituitary-somatotrophic (HPS) and the hypothalamo-pituitary-adrenocortical (HPA) systems. The molecules at the top of the HPS and HPA axes are growth hormone-releasing hormone (GHRH) and CRH respectively. Both of these hormones are secreted by many hypothalamic regions, such as the arcuate nucleus and the PVN [65, 66], and they act on their receptors expressed by the anterior pituitary gland (or adenohypophysis) causing the release of growth hormone (GH) and ACTH. GH induces the liver to secrete insulin-like growth factor 1 (IGF-1) [67] and ACTH signals the adrenal gland to produce cortisol [65]. Somatostatin is another molecule secreted by the PVN; its function is to inhibit the release of GH by the adenohypophysis [68]. Apart from somatostatin, there are no

other physiological inhibitors for these hormones, whose production is mainly regulated by negative feedback [65, 69] (Figure 1.3).

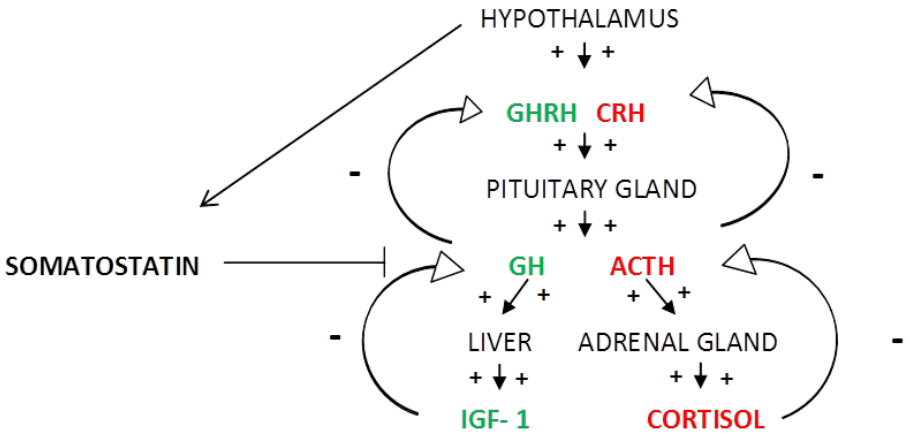


Figure 1.3 Diagram representing the hormonal cascade characterising the HPS and HPA axes. The hormones belonging to the HPS system and HPA systems are respectively shown in green and red. The expression of these factors is regulated by reciprocal negative feedback.

All these factors are expressed in a pulsatile and circadian manner. Hypothalamic expression of GHRH has been shown to be low in the morning, increase in the afternoon and again decrease at night in rats [70]. As a consequence the maximal pulses of circulating GH have been observed during the first half of the night in humans, in temporal association with SWS, progressively decreasing late in the night and in the morning [71, 72]. With regard to the HPA axis, CRH mRNA expression has been investigated in the PVN of rats with the maximal level observed at midnight while the minimal expression was recorded in the early afternoon [73]. The rhythmic variation in the hypothalamic expression of CRH explains the circadian rhythm of serum ACTH and cortisol also observed in humans, as these hormones rise in the early hours of the morning before awakening and their nadir is reached during the first half of the

night [74]. Figure 1.4 shows the fluctuations of cortisol and GH in adults during the night in relation with the EEG recordings.

A physiological sharp increase in cortisol level occurs in the morning, specifically within 30-45 minutes after awakening. This event is termed cortisol awakening response (CAR) [75, 76] and is mainly modulated by the time of awakening, psychological and physical factors [75-77]. Moreover, the HPA system is activated in response to stress, during which the release of cortisol is enhanced independently of its circadian rhythm [65, 78].

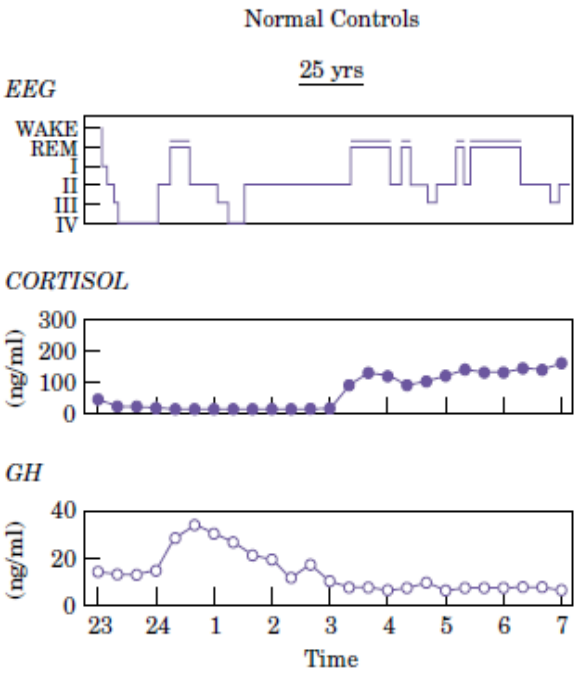


Figure 1.4 Sleep EEG, cortisol and GH secretion in representative young adults. (Figure adapted from Steiger A., 2002 [79]).

As mentioned before, sleep is influenced by the HPS and HPA axes. Several lines of evidence have demonstrated a bidirectional communication between sleep and these endocrine systems. For instance, GHRH can enhance SWS and REM sleep in different animal species after intravenous (i.v.) or intracerebroventricular (i.c.v.) administration, even in hypophysectomized rats [80-82]. The pro-somnogenic function of GHRH was confirmed in

males after i.v. [83-85] and intranasal [86] administration of GHRH, although the effect measured was weaker in elderly subjects [87]. However, administration of a GHRH antagonist did not affect SWS sleep [88]. With regard to GH, administration of antiserum to GH in rats impaired NREM sleep [89] but administration of high dosages of GH [90] or IGF-1 [91] decreased NREM sleep as a result of GHRH negative feedback inhibition. In addition, acute or prolonged infusion of GH in men was not associated with sleep changes [92].

Several studies have also investigated the effect of CRH on sleep. Contrarily to GHRH, CRH administration induced a decrease in SWS and NREM sleep in rats and rabbits [80, 93]. The same effects were also observed in young men subjected to repeated CRH i.v. injections, which caused enhanced cortisol levels and decreased GH surge, reinforcing the idea of a reciprocal interaction between HPA and HPS activities [51, 94]. Importantly, when CRH was administered during SWS, but not during other sleep stages, the expected increase in ACTH and cortisol levels was blunted, supporting the idea that sleep itself can also influence the function of the HPA axis [95]. Continuous nocturnal infusion [96] and pulsatile i.v. administration of cortisol have been found to increase SWS and decrease REM sleep in both young and older adults, with a parallel increase in the levels of GH [97, 98]. Therefore, elevated cortisol levels could mediate a negative feedback inhibition of endogenous CRH and reduce the CRH:GHRH ratio in favour of the latter [97].

1.1.3.4 Ghrelin

Beyond the factors described above, several other hormones are believed to be important for the regulation of sleep. Ghrelin is an appetite-promoting peptide [99] initially isolated from the stomach [100], and subsequently discovered to be expressed by other tissues such as the pituitary gland [101] and the hypothalamus [102], where it can bind the receptor for GH secretagogues (GHS-R) [103]. The presence of both ghrelin and GHS-Rs in the hypothalamic-pituitary system, together with the ability of ghrelin to increase GH release suggests a hypophysiotropic role for this peptide [104-106]. A study conducted by Weikel and colleagues

showed that ghrelin enhances SWS in humans: hourly administration of ghrelin in healthy young males during the night enhanced SWS and decreased REM sleep, increasing also the levels of GH, prolactin, ACTH and cortisol [107]. In addition, ghrelin serum concentration shows a daily rhythm, its peak occurring at the sleep onset and its levels decreasing during the night, as assessed in young males [108]. Intriguingly, physiological short sleep duration has been associated with higher morning ghrelin levels [109].

1.1.4 SLEEP AND HEALTH

Sleep behaviour deeply impacts upon human health. Epidemiological studies have elucidated the higher risk of mortality of long (>8 hours per night) and short (<7 hours per night) sleepers [5-9, 11], although the optimal sleep duration has recently been reported to be between 5 and 6.5 hours per night [11]. Moreover, variation in sleep circadian timing, duration and continuity have also been associated with a number of adverse outcomes, including the development of insulin resistance [110, 111], hypertension [112, 113], obesity [109, 114], type 2 diabetes mellitus [115-117], cardiovascular diseases [118, 119], depression [120], cognitive decline [121, 122], increased risk of different types of cancer [123-125] and increased risk to common infections [126], particularly pneumonia [127] and colds [128] as well as a decreased response to vaccination [129]. It is important to note that one of the common features shared by the majority of these conditions is a pro-inflammatory state.

To assess whether inflammation can be influenced by sleep duration, several inflammatory markers such as C reactive protein (CRP) and cytokines have been measured in individuals after protocols of total/acute sleep deprivation and partial/chronic sleep deprivation. Short periods of total sleep deprivation have been associated both with increased [130] and decreased levels of CRP [131], while individuals subjected to longer periods of restricted sleep duration showed increased [130, 132] or unaltered [133] CRP levels. The effects of sleep deprivation on cytokine levels are discussed in section 1.2.7.

1.1.4.1 Sleep and obesity

Several large population studies have identified a U-shaped relationship between sleep duration and excessive body weight across all age groups and in several ethnic groups [109, 134, 135]. Although both short and long sleepers tend to become overweight, obesity appears to be mostly linked with short sleep duration [136-138]. Consequently, sleep duration has also been found to be associated with increased risk of metabolic syndrome [139-141] and type II diabetes [115-117]. On the basis of these findings, a number of groups have tried to elucidate the connection between sleep and metabolic functions. First of all, alteration of the circadian rhythm itself induces metabolic impairments independently of sleep duration: feeding mice at a different circadian time resulted in a gain of weight of the animals [142]; similarly, shift workers are characterized by impaired glucose and lipid metabolism [143, 144]. Interestingly, the metabolism of glucose varies during at 24 hour period, as glucose concentration is higher in the evening compared to the morning in response to the same meal [145].

In addition to these studies, physiological sleep duration has also been found to be associated with changes in metabolism. Women sleeping less than 5 hours or longer than 8 hours per night displayed higher serum triglyceride levels and lower high-density lipoprotein (HDL) cholesterol, whereas among males, only longer sleepers showed reduced levels of low-density lipoprotein (LDL) [146]. Sleep deprivation protocols have further strengthened the link between sleep and metabolism. A single night of total sleep deprivation is sufficient to cause a reduction in insulin sensitivity in response to oral glucose administration [147], whereas protocols of partial sleep deprivation decreased glucose tolerance [148, 149], and higher total and LDL cholesterol levels in post-menopausal women [150]. Interestingly, Nedeltcheva and colleagues confirmed that lack of sleep modulates energy intake and expenditure, as partially sleep-deprived subjects experienced a reduced amount of fat loss compared to the control group in response to the same protocol of caloric restriction [151]. In addition, food intake also influences the sleep behaviour, as Michalsen et al. showed that a fasting period of 7 days

prompted a decrease in sleep arousals and an increase in the self-reported quality of sleep in non-obese individuals [152].

In light of these results, it could be argued that the sleep-regulating centres in the brain directly contribute to the modulation of metabolism. This hypothesis is supported by the fact that the neurons in the SCN project to other areas of the hypothalamus communicating with orexigenic and anorexigenic neurons, involved in increasing and inhibiting appetite and food intake respectively [153]. Additionally, energy expenditure and food intake are known to be affected by the hormones of the HPS and HPA axes [154]. Finally, the white adipose tissue (WAT) is known to secrete a number of cytokines and other molecules (adipokines) known to modulate appetite, energy expenditure and metabolism [155, 156], which might also affect the sleep regulatory centres in the brain.

1.1.4.2 Sleep and leptin

Leptin is one of the most studied adipokines. Its expression increases after food intake and insulin administration [157-159]; like insulin, leptin acts as a satiety signal, decreasing caloric intake thus having weight-reducing effects [160, 161]. Despite this anti-appetite function, obese people are characterized by high serum levels of leptin: this apparent contradiction can be explained by the condition of leptin-resistance which usually develops in parallel with obesity [162-164].

Many reports have shown that leptin levels are associated with sleep duration, though results are often contradictory. For instance Taheri et al. carried out a large population-based longitudinal study where a U-shaped relationship between body mass index (BMI) and self-reported sleep duration was observed [109]. In the same study leptin levels were lower in short sleepers and significantly increased in association with longer sleep duration. No differences were detected for adiponectin levels whereas morning serum levels of ghrelin negatively associated with sleep duration and efficiency [109]. The same positive correlation between

leptin levels and sleep duration was found in another epidemiological study by Chaput and colleagues [165], but it was not determined in a smaller cohort of adolescents [166]. Furthermore, Spiegel et al. analyzed the levels of leptin and ghrelin in a smaller group of healthy male volunteers allowed to sleep for 4 hours per night for two consecutive nights and compared with a group subjected to extended sleep duration. In this study the authors reported leptin levels to be markedly decreased and ghrelin levels increased in the sleep-deprived group [167].

The data obtained from these studies suggest that short sleep duration increase appetite and promote obesity through modulation of leptin levels. However, more recently Hayes et al. reported that each hour reduction in physiological sleep duration was associated with increased morning level of leptin in a group of healthy adults [168]. In agreement with the latter study, it has also been shown that leptin levels significantly raised in sleep-restricted participants after 5 nights [169] and a single night of partial sleep deprivation [170]. Similarly, an increment in leptin concentration was observed following one night of total sleep loss [171].

Despite these contradictions in the literature, leptin may represent an important element involved in the regulation of sleep. In fact, serum leptin levels show a clear circadian rhythm, increasing in the night and reaching the maximal peak around 3 a.m. in humans [172]. Moreover, both *ob-ob* mice (carrying a loss-of-function mutation in the leptin gene) and *db-db* mice (carrying a loss-of-function mutation in the leptin receptor gene) are characterized by impaired sleep, displaying an increase in the overall sleep time, in particular in NREM sleep, as well as increased sleep fragmentation, with altered EEG waveforms and sleep rhythms [173, 174].

Leptin receptor is expressed in the arcuate nucleus of the hypothalamus and in other nuclei, including the PVN [175, 176]. Active transcription of leptin mRNA has been demonstrated in the hypothalamus, cerebral cortex and pituitary [177, 178]. It is also well established that leptin can cross the blood-barrier brain (BBB) likely through a saturable transport system and has been

detected in human cerebrospinal fluid, its levels correlating with plasma levels and with BMI [179, 180]. Thus, systemic leptin can reach the brain and is believed to regulate neurons of the arcuate nucleus by inhibiting the release of products known to be involved in regulation of food intake and also sleep, such as NPY [181, 182]. In addition, Luheshi and colleagues found that i.c.v. administration of leptin increased the levels of interleukin (IL)-1β in the rat hypothalamus, and the effects of leptin on food intake and body temperature were partially suppressed by the co-administration of IL-1 receptor antagonist (IL-1RA) and were absent in IL-1 receptor-deficient mice [183]. This study highlights a potential mechanism of leptin action in the hypothalamus mediated by IL-1β. Such a mechanism could also explain the link between leptin and sleep duration, as IL-1β is also a pro-somnogenic cytokine (discussed below).

1.1.4.3 Sleep and adiponectin

Adiponectin is another very abundant adipokine which is considered a strong anti-diabetic, anti-atherogenic and anti-inflammatory molecule which enhances insulin sensitivity and decreases glucose production by the liver [184, 185]. Lack of sleep has not always been associated with changes in adiponectin serum levels, for instance Taheri and colleagues did not observe any difference in adiponectin levels in relation with sleep [109]. Similar observations were made in other studies, investigating adiponectin levels in adolescents [166] and in police officers working primarily on the day shift and characterized by different sleep durations [186]. However, a recent study has investigated adiponectin levels in sleep-deprived volunteers allowed to sleep 4 hours for five consecutive nights. In this condition the authors found a decline in adiponectin serum levels only in sleep-restricted caucasian women, while in African women adiponectin appeared to increase and in males no differences were found [187]. In addition, a study conducted on 109 Japanese healthy males showed a positive association between self-reported sleep duration and adiponectin serum levels [188]. In agreement with the latter, in a group of Saudi teen-aged girls, adiponectin concentration was

found to be higher in lean girls compared to obese ones, and increased in proportion to hours of sleep [189].

Although a correlation between physiological sleep duration and adiponectin values has not always been found, adiponectin serum concentration is strongly diminished in patients suffering from OSAS, as several studies have reported the same result independently of obesity and gender and in different ethnic groups [190-192] . OSAS is a sleep condition characterized by interrupted breathing during sleep, provoked by obstruction of the upper respiratory tract, and a high pro-inflammatory status, as patients are characterized by higher levels of tumor necrosis factor (TNF)-α, CRP and IL-6 [193-195]. Therefore, the lower levels of adiponectin in OSAS patients could be due to the incremented levels of these factors, which have been shown to decrease adiponectin expression in adipocytes [196-199].

A few groups reported that adiponectin serum levels show a circadian rhythm, declining in the night and increasing during the day with a peak reached in the late morning [200, 201], while other studies failed to find any daily variation [202]. A matter of debate is also the presence of adiponectin in the brain. Adiponectin is expressed by the pituitary gland but not by the brain [203-206], moreover it has been suggested that adiponectin cannot cross the BBB [207, 208]. In contrast, other studies have shown that adiponectin is present in human cerebrospinal fluid (CSF) at a very low levels (0.1% compared to the serum concentration) and mainly the low molecular weight (LMW) isoform [206, 209, 210]. In fact, adiponectin increases in the CSF after i.v. administration in mice [206, 211] and it can activate AMP-activated protein kinase (AMPK) in the hypothalamus [211]. The action of adiponectin in the central nervous system (CNS) is further indicated by the high expression of adiponectin receptors in different hypothalamic regions [205, 206].

1.2 THE IMMUNE SYSTEM

The immune system is represented by a heterogeneous group of cells and tissues which are responsible for defending the body against infectious agents and aberrant cells recognized as “non-self” and therefore potentially harmful for the organism. The immune system can be broadly divided into the innate and adaptive immune systems, though in reality they are interconnected. The innate immune system is characterized by a rapid, non-specific identification of invading elements providing a swift response to avoid the spread of rapidly dividing pathogens. The role of adaptive immunity is to amplify and improve the innate response by building specificity and memory against a particular agent in order to fight it more efficiently on second exposure. Therefore, the adaptive immune response is considered “acquired” and is slower but more specific than the innate response.

The components of innate immunity are represented by:

- anatomical barriers (epithelial cells, mucus);
- soluble components: complement system, circulating anti-microbial proteins, inflammatory mediators, including chemical factors (histamine, bradykinin, serotonin, leukotrienes, and prostaglandins) and cytokines;
- basophils/mast cells: involved in allergy-related inflammation;
- neutrophils: specialised in recognition and killing of rapidly divinding bacteria, yeast and fungi;
- eosinophils: deal with parasitic infections;
- monocytes-macrophages: kill microbes especially intracellular bacteria and also link innate and adaptive immunity by presenting antigen to lymphocytes and releasing proinflammatory cyotkines;
- natural killer (NK) cells: recognize and clear tumors and virus-infected cells;
- dendritic cells: ingest and process antigens and present them to lymphocytes.

The components of adaptive immunity include:

- T lymphocytes, divided into cytotoxic CD8+ T cells, helper CD4+ T cells and the less common γδ T cells. Further subpopulations can be distinguished according to the phenotype and the cytokines secreted (discussed in section 1.2.5);
- B lymphocytes which are responsible for the secretion of the five types of antibodies IgA, IgD, IgE, IgG, and IgM.

1.2.1 NEUTROPHILS

Neutrophils, also called polymorphonuclear cells because of their characteristic multilobed nucleus, are the largest immune population (10^9-10^{10} cells/litre of blood), which provide the first protective response against bacteria and fungi. Neutrophils are post-mitotic, short-lived cells that undergo spontaneous apoptosis in the absence of infection. In humans, neutrophil half-life was initially estimated to be 7-10 hours in the blood [212, 213], although a more recent work by Pillay and colleagues shows that circulating human neutrophils can survive in the circulation for up to 5.4 days [214]. Consequently, neutrophil turnover is rapid: approximately 10^9 cells per kg of body weight are produced daily by the bone marrow in humans [215]. During infection, neutrophils are recruited to the inflamed tissue where they activate their anti-microbial functions in order to clear the pathogen. Their survival is also prolonged by the presence of cytokines, microbial factors [216] and hypoxia [217-219].

1.2.1.1 Neutrophil migration to the site of infection

When the body is challenged by the entry of a microorganism, neutrophils transmigrate from the blood to the infected tissues. Neutrophil transendothelial migration consists of three distinct stages:

1) rolling: once activated, the vascular endothelium increases the surface expression of P- and E-selectins [220, 221], allowing the binding of neutrophils through their ligand sialyl-Lewis^x [222]. The affinity of this carbohydrate-selectin interaction is weak, therefore neutrophils continue to move and “roll” on the endothelium.

2) tight adhesion: during the "rolling" stage, neutrophils are exposed to pro-inflammatory cytokines and chemokines which further modify the expression of neutrophil surface receptors. For instance, IL-8 leads to increased expression and conformational activation of the integrins Mac-1 (CD11b/CD18) and lymphocyte function-associated antigen (LFA)-1 (CD11a/CD18) [223, 224]: once rearranged, these integrins can bind to ICAM-1 on endothelial cells with high affinity, thereby arresting the "rolling" and promoting the firm adhesion of neutrophils to the blood vessel wall [224, 225] .

3) diapedesis: at this point, neutrophils are ready to transit into the infected tissues. The transendothelial migration is mediated by several surface proteins, such as CD31 and junctional adhesion molecules (JAMs) [226, 227].

Once transmigrated, neutrophils follow the gradient of chemotactic factors including IL-8 [228], the bacterial product N-formylmethionyl-leucyl-phenylalanine (fMLP) [229] and complement factors, such as C5a [230] to reach the site of infection. These chemoattractants interact with their respective receptors expressed by neutrophils, causing a morphological polarization of the cell along the gradient and the formation of pseudopods and lamellipodia [231].

In addition, in the circulation neutrophils are in a resting state and are not fully committed to perform a complete microbicidal activity. In the infected tissues, neutrophils are exposed to the gradient of chemokines as well as microbial factors and a range of cytokines produced locally. These molecules, for example TNF-α, represent priming agents, as they can boost neutrophil anti-microbial function: neutrophil sensitivity to microbial stimuli such as fMLP is enhanced and their full killing abilities are achieved [232].

1.2.1.2 Phagocytosis

The process of phagocytosis allows neutrophils to recognize, uptake and ingest the invading microbes. Pathogens are identified and captured by two different mechanisms:

1) pathogens express specific surface molecules, also called pathogen-associated molecular patterns (PAMPs), which are directly recognized by specific neutrophil receptors, the pattern recognition receptors (PRRs). An example of a PAMP is lipopolysaccharide (LPS) and a family of receptors called the Toll-like receptors (TLRs) are the main PRRs. For example TLR4 recognizes LPS [233];

2) microorganisms are opsonized by serum proteins: complement factors C3b and iC3b and antibodies cover their membrane. Neutrophils express receptors for immunoglobulins as well as for complement factors, therefore they bind to opsonized pathogens. In particular, neutrophils express three classes of the Fcγ receptor: the most relevant ones are FcγRI (CD64), FcγRIIa (CD32) and FcγRIIIb (CD16), which recognise the Fc portion of immunoglobulins [234]. In addition, neutrophils display the complement receptors CR1 (CD35) and CR3, (the dimer CD11b/CD18, alternatively called Mac-1) which bind respectively to the complement components C3b and its cleavage product iC3b [235, 236]. After contact, neutrophils undergo specific cytoskeletal rearrangements and the microbes are then surrounded by extended pseudopods and enclosed into the maturing phagosomes where they are killed. From the signalling perspective, neutrophil phagocytosis is accompanied by activation of phosphoinositide 3-kinase (PI3K) [237, 238] and the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase (ERK) 1/2 and p38 [239, 240].

1.2.1.3 Oxygen-independent bactericidal processes

Neutrophil anti-microbial mechanisms following pathogen uptake are classified as either oxygen-dependent or oxygen-independent. Among the oxygen-independent processes are the phagosome acidification and the fusion of the phagosome with the cytoplasmic granules to form the phagolysosome, where the microbial destruction takes place [241]. The four main cytoplasmic granules (azurophilic, specific, gelatinase granules and lysosomes) contain antimicrobial proteins such as myeloperoxidase (MPO), elastase, cathepsin G, lysozymes,

lactoferrin, acid hydrolases and defensins, creating an environment able to eliminate the pathogens [242].

1.2.1.4 Oxidative burst

The oxygen-dependent system by which neutrophils kill microbes includes the generation of $O_2^{\cdot -}$ and other reactive oxygen species (ROS). $O_2^{\cdot -}$ is produced by the enzymatic complex nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, consisting of a Rho GTPase and five phagocytic oxidases (gp91, p22, p40, p47, p67), which are disassembled in resting neutrophils and assembled on plasma and phagosome membranes upon neutrophil stimulation [242]. $O_2^{\cdot -}$ can be reduced to H_2O_2 which is converted into HOCl by the enzyme MPO in the phagolysosomes [243]. Several different phagolysosome residing enzymes produce other radical species having microbicidal properties [242].

1.2.1.5 Neutrophil extracellular traps (NETS)

In 2004 another neutrophil mechanism of defense was discovered: neutrophils can produce NETs. NETs consist of extracellular fibers of DNA coated in histones and other proteins derived from azurophilic and specific granules, such as elastase, MPO, cathepsin G and lactoferrin [244]. Once NETs are released from the cells, which happens in response to a range of stimuli (IL-8, LPS, both Gram-positive and Gram-negative bacteria), they contact and kill extracellular pathogens [244].

1.2.2 NEUTROPHIL APOPTOSIS

Apoptosis is defined as a programmed, active and ordered form of cell death whose features distinguish it from necrosis. Necrotic cells are characterized by loss of membrane integrity and cell content, thus they can lead to *in vivo* inflammation as a result of the leakage of cytoplasmic content. On the contrary, apoptotic cells undergo cell shrinkage, membrane blebbing and chromatin condensation followed by collapse of the nucleus into apoptotic bodies, small vescicles containing parts of the fragmented nucleus which are cleared by local

phagocytes [245, 246]. Importantly, the membrane and organelle integrity are maintained during the formation of the apoptotic bodies, avoiding the release of cellular content and consequent tissue inflammation [245, 246]. The other peculiarity of apoptotic cell death is the activation of a group of proteins, the caspases, which are cysteine-containing aspartate-specific proteases which mediate the changes within the apoptotic cell ultimately causing its death [247].

The process of apoptosis can be initiated either from the intracellular space or from the extracellular environment: the internal signals, which are generated in response to stress and DNA damage, stimulate the intrinsic, mitochondrial apoptotic pathway whereas the external stimuli trigger the death receptor apoptotic pathway. The cellular stressors which directly damage the DNA, such as radiations and oxidative stress, induce the internal apoptotic pathways [248-251], whereas the extrinsic apoptosis is triggered by extracellular signals such as TNF-α, Fas ligand (FasL), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) which interact with and activate the respective death receptor (DR) [252, 253]. Both of these two apoptotic programs converge on activation of caspases [252].

As mentioned above, neutrophil lifespan is very short: circulating neutrophils are thought to die as a consequence of cytokine deprivation-induced apoptosis, a homeostatic mechanism to maintain the appropriate neutrophil number under physiological condition as well as to limit uncontrolled inflammatory processes [216, 254]. Ageing neutrophils disappear from the circulation and accumulate in organs such as the spleen, the liver and the bone marrow, where they have been shown to undergo apoptosis as evidenced by murine studies [255-257]. Apoptotic neutrophils are then recognized and phagocytosed by local macrophages and dendritic cells; this process is accompanied by macrophage secretion of anti-inflammatory mediators such as IL-10 and transforming growth factor (TGF)-β [254, 258]. The prompt removal of dying neutrophils represents a process to avoid self-injury, as late apoptotic cells are subjected to secondary necrosis releasing the vescicular toxic content in the extracellular space [254] causing tissue damage also in sterile and non-inflammatory conditions.

In infection, infiltrating neutrophils have an extended lifespan: bacterial products (LPS) [216], pro-inflammatory cytokines [216] (such as granulocyte macrophage-colony stimulating factor (GM-CSF)) [259] and hypoxia [217-219] contribute to delay the spontaneous apoptotic process, thus neutrophils have more time to clear the pathogen.

Nevertheless, neutrophils are eventually induced to apoptosis once their anti-microbial functions are activated: phagocytosis [260, 261], ROS [260, 262, 263] and NETs production [264] promote neutrophil death independently of the effective killing of the microorganism. Especially in this setting, the removal of apoptotic neutrophils by macrophages is essential to resolve the acute inflammation [265].

Neutrophil spontaneous apoptosis strongly depends on the balance of the pro- and anti-apoptotic proteins belonging to the Bcl-2 family: these cells highly express the two pro-apoptotic proteins, Bax and Bak [266, 267] as well as the BH3-only members Bad, Bim, Bid, Bik [266]. Among the anti-apoptotic proteins, Mcl-1 is highly expressed in fresh neutrophils [266, 268] and appears to be the most relevant in dictating neutrophil apoptosis, as Bcl-2 is weakly expressed [266, 269] and Bcl-X_L expression is controversial [266, 270]. In addition Mcl-1 levels positively correlate with neutrophil survival [271, 272] and suppression of endogenous Mcl-1 expression confirmed the essential role of this protein in delaying neutrophil spontaneous apoptotsis [272, 273].

PI3K is a family of protein kinases responsible for the production of phosphatidilynositol 3,4 bisphosphate (PtdIns $(3,4)P_2$) and phosphatidilynositol 3,4,5 triphosphate (PtdIns $(3,4,5)P_3$) [274], which in turn induce the binding of protein kinase B (PKB) and its phosphorylation [275]. The activity of these kinases, particularly PI3Kγ, participate in the regulation of neutrophil spontaneous apoptosis, as both the levels of PtdIns $(3,4,5)P_3$ and the activation of PKB are diminished during neutrophil apoptosis, in parallel with an accumulation of intracellular ROS [276]. Anti-apoptotic stimuli such as GM-CSF and LPS have also been shown to exert their

effect through activation of PI3K, which acts by enhancing Bad phosphorylation [277, 278] and expression [279].

With regard to the MAPK family, there are contradictory reports relating to their role in neutrophil spontaneous apoptosis. Pongracz and colleagues showed that the MEK-1/ERK 1/2 inhibitor PD98059 and the p38 inhibitor SB202190 do not affect the apoptotic rate, and the phosphorylation of ERK 1/2 and p38 do not increase during the apoptotic process, suggesting that the modulation of these single pathways is not sufficient to influence spontaneous apoptosis [280]. However, some groups have demonstrated that p38 activation accelerates neutrophil apoptosis [281] and antagonizes LPS-delayed neutrophil apoptosis [282]. In contrast, an anti-apoptotic role for p38 has been proposed in other studies [283, 284]. Despite the fact that ERK 1/2 does not have a role in regulating neutrophil spontaneous apoptosis, its activation is necessary for the anti-apoptotic role exerted by several stimuli, such as LPS [282, 285] and GM-CSF [277].

The pro-inflammatory transcriptional factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) is also involved in prolonging neutrophil lifespan [286]. In resting cells, NF-kB is located into the cytoplasm where it forms a complex with inhibitor of kB (IkB): once cells are activated by pro-inflammatory stimuli, IkB is degraded and NF-kB can translocate into the nucleus and begin the transcription of inflammatory genes [287]. Activation of NF-kB by PI3K and MAPK signalling pathways has been shown to mediate the anti-apoptotic effect exerted by LPS [288], type-1 interferons (IFNs) [289] and hypoxia [217]. However, the target genes promoting the anti-apoptotic function of NF-kB in neutrophils are still largely unknown.

Neutrophils express high levels of Fas receptor (CD95) and they are highly susceptible to apoptosis when stimulated with activating anti-Fas antibodies [290]. Upon stimulation, CD95 aggregates and undergoes clustering, [291] triggering the death signal through recruitment of

Fas-associated protein with death domain (FADD) and pro-caspase-8, forming the so-called death-inducing signaling complex (DISC) [263]. However, neutrophil constitutive apoptosis is not affected by the use of antagonist antibodies against CD95 and other death receptors and clustering of CD95 has been shown to occur spontaneously, inducing the formation of the DISC complex and initiating the extrinsic apoptotic pathway [263]. In particular, accumulation of ceramide and generation of ceramide-rich lipid rafts within the membrane is necessary to mediate CD95 clustering [291, 292]. In neutrophils, increased levels of intracellular ceramide are associated with an increased rate of apoptosis [293], moreover disruption of ceramide-rich lipid rafts delays apoptosis [263].

Ceramides are a family of sphingolipids that are generated through different mechanisms. They can be synthetized "de novo", or through the hydrolysis of sphingomyelin by activation of sphingomyelinases or through the "salvage pathway" [293, 294]. Interestingly, ceramide production in neutrophils is enhanced by the production of ROS which accumulate during spontaneous apoptosis [262, 263]. ROS are already known to trigger neutrophil apoptosis following neutrophil activation [262, 295], hence the modulation of ceramide represents a mechanism regulating neutrophil apoptosis.

Another important sphingolipid involved in apoptotic regulation is sphingosine-1-phosphate (S1P), a product of ceramide metabolism [294]. In contrast to its precursor, S1P shows distinct anti-apoptotic function in different cell types [296, 297] including LPS-treated neutrophils [298].

1.2.3 NK CELLS

NK cells are cytotoxic and cytokine-producing lymphocytes belonging to the innate immune system and representing 10-15% of circulating lymphocytes [299]. In addition, they are also present in peripheral tissues such as the liver [300], lung [301] and peritoneal cavity [302]. Their main function is to recognise and kill tumoral and virus-infected cells. NK cells are identified by a range of surface receptors, notably expression of CD56 and CD16 and the absence of

CD3 [299]. Two major subsets of NK cells can be identified according to CD56 and CD16 expression: $CD56^{dim}$ $CD16^{high}$ NK cells represent 90% of all the circulating NK cells and show high cytotoxicity using both granule exocytosis and death receptor ligation pathways to kill target cells. $CD56^{bright}$ NK cells constitute the remaining 5-10% of the circulating NK cell pool and produce cytokines such as IL-10, IL-13, GM-CSF, TNF-α and IFN-γ [303], therefore their major role is to boost the function of other immune cells present in the environment [299].

NK cell cytotoxicity against target cells is achieved through two different mechanisms, granule exocytosis and death receptor-mediated killing. Granule exocytosis is the main process by which NK cells kill target cells [304]. NK cells bind to the target cell forming an immunological contact allowing NK cells to secrete the cytotoxic content of intracellular vesicles thus promoting the apoptosis of the target cell. The effector molecules released by activated NK cells are perforin and the granzyme proteases. Perforin is a protein which forms a complex on the target membrane and is necessary for the passage of granzymes into the cytoplasm. Granzymes are a family of serine proteases that are primarily responsible for inducing target cell death through the granule exocytosis pathway [304, 305]. Of the five granzymes expressed by human NK cells, granzyme B has been the most studied as is indispensable for triggering both caspase-dependent and independent apoptosis [305, 306].

NK cells can also kill target cells through engagement of death receptors. Either when stimulated with cytokines [307] or activatory ligands [308], NK cells increase the surface expression of FasL and TNF-related apoptotic inducing ligand (TRAIL), both ligands of the tumour necrosis factor family. Upon ligation with their cognate receptors (Fas and TRAIL-R1/TRAIL-R2 respectively), they cause the death of target cells through caspase activation [304].

NK cell cytotoxicity is strictly regulated. Starting from the process of NK cell differentiation, which takes place in the bone marrow, only NK cells capable of distinguishing self major

histocompatibility complex (MHC) class I molecules achieve an efficient killing ability, meaning that the binding between self MHC class I molecules and inhibitory receptors on the surface of NK cells is necessary for the acquisition of NK cell full effector function [309]. In addition, mature circulating NK cells express a wide range of activatory and inhibitory receptors, through which they collect signals from the environment. Only when the activatory signals overcome the inhibitory signals, NK cells can gain their full cytotoxic ability [310].

1.2.4 T and B LYMPHOCYTES

T lymphocytes are cells responsible for mounting an adaptive, specific immune response towards viral, bacterial, parasitic microorganisms and malignant cells. The antigen specificity showed by T cells is due to the high variability of the T cell receptor (TCR) expressed on their surface, obtained through genetic rearrangement during their thymic development [311]. Once they have reached maturation in the thymus, naïve T lymphocytes (characterized by high surface expression of C-C chemokine receptor (CCR)7 and CD45 receptor antagonist (CD45RA) [312]) leave the thymus and circulate through the blood and lymphoid systems, where they can encounter antigen presented on MHC by the antigen-presenting cells (macrophages, dendritic cells and B lymphocytes) and are activated through the TCR [313]. Upon activation, T cells proliferate and a proportion of them enter the memory pool, which accumulates and remains over the individual's lifespan, assuring a faster, stronger and more specific response in successive exposures with the same antigen [313]. Memory T cells are divided into subpopulations according to their function and phenotype: central memory (T_{CM}) cells, effector memory (T_{EM}) cells [314] and revertant effector memory (T_{EMRA}) cells [315, 316] (Table 1.1).

MEMORY T CELL	PHENOTYPE	FUNCTION
T_{CM}	CCR7+, CD45RA-	Proliferation Differentiation into T_{EM} cells
T_{EM}	CCR7-, CD45RA-	Rapid production of effector cytokines
T_{EMRA}	CCR7-, CD45RA+	Terminally differentiated Similar function to T_{EM} cells

Table 1.1 List of memory T cell subsets, their phenotypes and function.

The partition between naïve and memory T cells exists for both CD4+ and CD8+ T cells. In particular, activated CD8+ T cells, known as cytotoxic T lymphocytes (CTL) display a major cytotoxic role whereas CD4+ T cells mainly secrete cytokines to coordinate the response of the various immune populations, thus they are named T helper cells. According to the type of cytokines released and the receptors for chemokines expressed, CD4+ T helper cells can be further distinguished into T helper (Th) 1, Th 2 and Th 17 [313, 314, 317, 318]. Finally, another subset of T cells has been identified as suppressor or regulatory T cells (Tregs): these cells are involved in maintaining lymphocyte homeostasis and avoiding autoimmune diseases. They are phenotypically identified by expression of CD4, CD25 and FOXP3, a transcriptional factor essential for their inhibitory role. Their immune suppressive role is achieved by contact dependent and independent methods, the latter including secretion of anti-inflammatory cytokines such as IL-10 [319].

B lymphocytes mature in the bone marrow and also circulate in the blood and lymph. Once activated by the uptake of antigen and contact with CD4+ T cells [320], they mature towards antibody producing cells, termed plasma cells. Like T cells, B cells also are long lived cells which provide immune memory. More recently it has been shown that a subset of regulatory

B cells (Bregs), identified by expression of CD24 and CD34, also has regulatory function secreting IL-10 [321].

1.2.5 CYTOKINES

Cytokines are immunomodulatory molecules, secreted mostly, but not only, by immune cells. Generally, they can be divided into three functional categories: hematopoietic growth factors, innate immunity modulators and adaptive immunity modulators. A more detailed functional classification is shown in table 1.2. Similar to these cytokines there are other soluble factors called chemokines with the specific function of inducing cell migration (i.e. IL-8, monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α).

FUNCTIONAL CLASS	PRIMARY ROLE	OTHER EFFECTS	EXAMPLES
lymphocyte growth factors	clonal expansion	Th1/Th2/Th17 polarization	IL-2, IL-4, IL-7, IL-17, IL-15
Th1 cytokines	↑ Th1 response	clonal expansion of CTL	IFN-γ, IL-2, IL-12, IL-18
Th2 cytokines	↑ Th2 responses	↑ antibody production	IL-4, IL-5, IL-18, IL-25, IL-33
Th17 cytokines	↑ Th17 responses, ↑ IFNγ	autoimmune responses	IL-17, IL-23, IFN-γ
pro-inflammatory cytokines	↑ inflammatory mediators	↑ innate immune responses	IL-1α, IL-1β, TNF-α, IL-12, IL-18, IL-23, IL-32, IL-33, CD40L
anti-inflammatory cytokines	↓ inflammatory genes	↓ cytokine-mediated lethality	IL-10, IL-13, TGF-β, IL-22, IL-1Ra, IFN-α,β
colony stimulating factors	hematopoiesis	pro and anti-inflammatory	IL-3, IL-7, G-CSF, GM-CSF, M-CSF
type I interferons	↑ class I MHC, anti-viral	anti-inflammatory, anti-angiogenic	IFN-α, IFN-β
type II interferons	macrophage activation	increase in class II MHC	IFN-γ

Table 1.2 Functional classification of cytokines (table adapted from Dinarello C.A., 2007 [322]).

1.2.6 SLEEP AND THE IMMUNE SYSTEM

A large body of literature supports the existence of a bidirectional regulation between sleep and immunity, as long term lack of sleep can induce sickness and immune responses influence sleep physiology [18]; however the mechanisms at the basis of this interrelation have not been fully elucidated.

The connection between sleep and the immune system is primarily suggested by the fact that the number of immune cells shows a daily circadian rhythm. In humans, the number of white blood cells (WBC) shows a circadian fluctuation, being higher late in the day and minimal early in the morning [323]. The levels of monocytes and neutrophils reach minimal values towards the night [324] whereas total T and B cells and both the CD4+ and CD8+ subsets have been consistently found to be more abundant during the night [323-326]. The most marked rhythm is showed by naïve T cells [326]. On the contrary, NK cells decrease during the night [323, 324, 327].

Apart from changes in immune cell number, also immune functions display a circadian rhythm. In humans, neutrophil phagocytosis is maximal in the dark period [328], whereas NK cell cytotoxicity appears to be minimal during the night [329]. In addition, the concentration of several cytokines varies during the 24-hour period. In humans, serum levels of IL-1 peak at the onset of SWS [330] and other pro-inflammatory cytokines, such as IL-2, IFN-ɣ and IL-6 reach their maximal level later in the night [331, 332], although contrasting findings have also been reported [333]. The *in vitro* generation of IFN-γ and IL-10 also show a circadian rhythm, with the ratio between IFN-γ:IL-10 peaking in the night, implying a shift towards higher Th1:Th2 cytokine balance [334, 335]. In light of those data, it has been postulated that the rest period represents a pro-inflammatory status [336]. Importantly, cytokines appear to play a role in sleep regulation. In general the pro-inflammatory cytokines, especially TNF-α and IL-1β, exert a somnogenic effect by encouraging the SWS stage. Anti-inflammatory cytokines, such as IL-4 and IL-10, are supposed to inhibit spontaneous sleep [18].

The circadian rhythmicity observed in these various aspects of the immune system is likely to be related to circadian hormonal variations. For instance, strong inverse correlations were found between the total circulating lymphocytes, as well as the CD4+ and CD8+ subsets, and the circadian rhythms of cortisol. The peak of lymphocytes occurs in the night when the level of cortisol reaches its nadir [325, 337, 338]. Indeed, cortisol is known to have powerful immune suppressive properties, decreasing mitogen-induced lymphocyte proliferation [339], antagonizing the actions of IL-1β [340, 341] and suppressing cytokine production by leukocytes as well as inhibiting NK cell activity [342, 343]. In particular, naive CD4+ T cells were found to be negatively correlated with cortisol rhythms and to upregulate the CXC chemokine receptor type 4 (CXCR4) after exposure to cortisol, suggesting that this steroid acts to redirect naive T cells to bone marrow [326]. Cortisol circadian pattern was also found to be positively associated with the number of circulating neutrophils [325], probably because of its anti-apoptotic role exerted specifically on neutrophils [344, 345].

Catecholamines (epinephrine and norepinephrine) are mainly secreted by the adrenal gland and the sympathetic nervous system. Similarly to cortisol, their concentration raises in condition of stress [346] and show a distinct circadian rhythm, their nadir being at night [326, 347]. These hormones contribute to increase the number of circulating granulocytes and NK cells [348] and exert immunomodulatory roles by predominantly suppressing systemic immune functions [349]. Interestingly, catecholamines promote the mobilization of effector CD8+ T cells to the circulation [326].

Melatonin has been discovered to act upon the immune system by increasing monocyte activation, production of IL-1, IL-6, TNF-α and oxidative burst [350, 351]. On the contrary, it has been shown to inhibit IL-8 release by LPS-stimulated neutrophils [352]. With respect to lymphocytes, melatonin enhances peripheral blood mononuclear cells (PBMCs) proliferation [353], it promotes the release of the pro- inflammatory cytokines IL-2, IL-6, IFN-γ [354] and it is associated with increased Th1 response [355].

Also GH has been demonstrated to have a role in immune regulation. It can increase IFN-γ-producing CD4+ T cells [356] and stimulate B cell immunoglobulin synthesis [357] and enhance hematopoiesis by promoting proliferations of progenitor cells and T lymphopoiesis [358]. Ghrelin, which stimulates GH release [103, 106], decreases the production of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6) by human T lymphocytes and monocytes. In addition, ghrelin also promotes thymopoiesis and T cell development [359, 360].

It is therefore possible that these hormones represent the main cause of diurnal variations in cytokines levels and immune cell numbers.

1.2.6.1 How cytokines affect sleep

The first data showing that cytokines could influence sleep architecture come from studies conducted on infected animals. Different animal hosts have been challenged with viral, fungal and bacterial pathogens or single microbial components and the results systematically indicate that such infections increase total sleep and NREM sleep duration [361-366]. Studies in humans were also performed. In particular, volunteers injected with evening low doses of LPS displayed prolonged and potentiated NREM sleep and decreased REM sleep, as result of increased levels of endogenous pro-inflammatory cytokines and their soluble receptors [367-369], however these results were not reproduced when subjects were administered with higher doses of LPS [367] or when LPS was administered in the morning [370].

A number of cytokines and chemokines have been studied for their ability to regulate sleep in either animals or humans; amongst them only two, TNF-α and IL-1β, have been widely investigated to ascertain their role in promoting spontaneous sleep [371-373]. Systemic or central injection of either TNF-α or IL-1β has been shown to enhance the duration of NREM sleep, especially SWS duration in every species tested so far (rats, mice, rabbits, monkeys, cats and sheep) [371, 373, 374]. In confirmation of these findings, blockage of endogenous TNF-α [375, 376] and IL-1β [377, 378] decreases NREM sleep duration, and mice lacking TNF- α receptor [379], IL-

1 type 1 receptor [380] or both [381] spend less time asleep. OSA patients who were subjected to treatment with Etanercept, a TNF-α antagonist, experienced a significant decrease of sleepiness and increased sleep latency, although no change in other sleep parameters was registered [382].

TNF-α and IL-1β activate NF-κB, which promotes the production of TNF-α and IL-1β in a positive-feedback loop, thus resulting in an amplification of their somnogenic effect. The involvement of NF-kB in regulating spontaneous sleep has been confirmed by use of its inhibitory peptide, which caused reduction of NREM sleep in rats and rabbits [383]. Consequently, factors that can inhibit NF-κB activation, such as IL-4 and IL-10, inhibit sleep [383-385].

The processes by which the CNS detects the concentration of systemic cytokines are at least three:

1) cytokines send signals to the brain at the level of the choroid plexus and the circumventricular organs: they activate endothelial cells which produce second messengers that can enter the brain (humoral pathway);

2) cytokines can stimulate the primary afferent nerves, such as vagal nerves, which in turn transport the information to the brain (neural pathway);

3) cytokines can also be actively transported into the CNS (cellular pathway) [373, 386, 387].

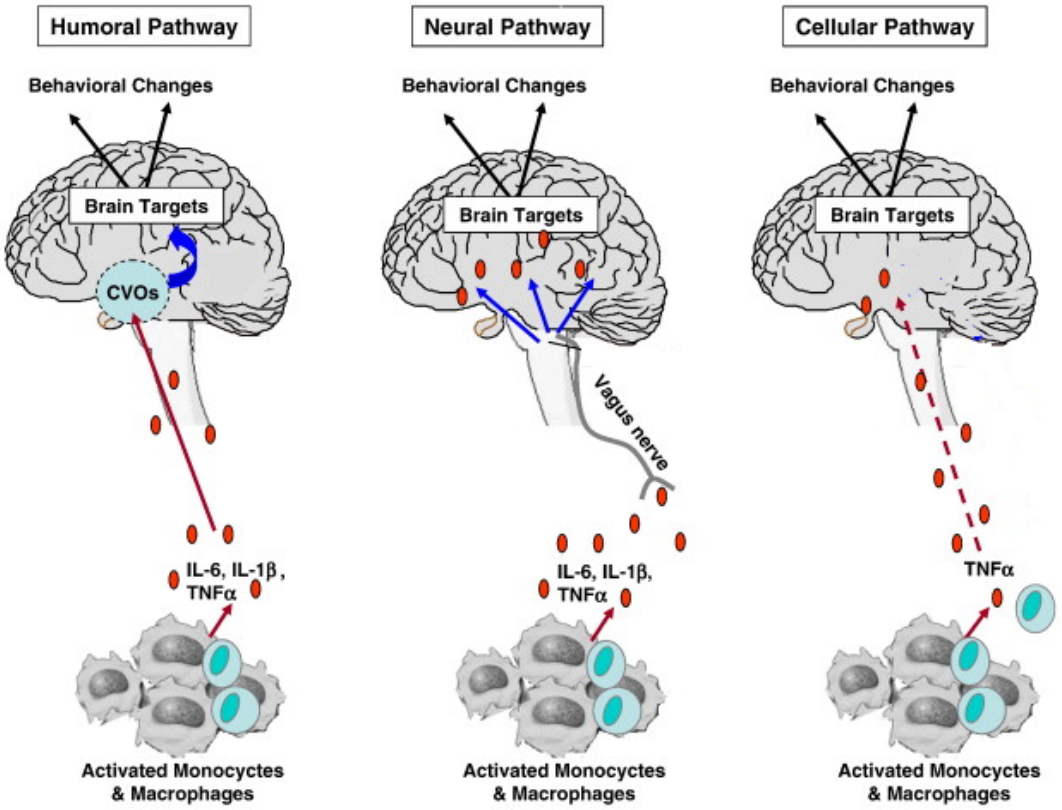


Figure 1.5 Mechanisms by which peripheral cytokine communicate with the brain. The three (humoral, neural and cellular) processes through which cytokines influence the CNS (figure adapted from Capuron and Miller, 2011 [387]).

Numerous reports have shown that cytokines and their receptors are expressed *in loco* and released in the CNS by neurons and microglia [374, 388, 389]. In addition, hypothalamic neurons can also express IL-1 and TNF-α [390, 391]. Notably, Beynon and Coogan found endogenous expression of IL-1β and IL-1 Receptor 1 (IL-1R1) in the SCN of both young and old mice, observing a different circadian rhythm in the expression of IL-1R1 between young and old animals. Although no differences were seen in the circadian rhythm of IL-1β, this cytokine was secreted at a lower level in the old mice. IL-1β and IL-1R1 were also expressed in the PVN, where they showed a different circadian expression between old and young mice [392]. Collectively, these data indicate that TNF-α and IL-1β are involved in physiological sleep regulation. IL-6 is another cytokine possibly involved in sleep regulation, as one study

conducted on 17 healthy men showed that evening intranasal administration of IL-6 increased the duration of SWS sleep in the second half of the night [393], although its injection in rabbits did not influence the sleep pattern [394].

More work is necessary to better elucidate the relationship between the network of cytokines and the sleep-regulating brain regions.

1.2.7 THE EFFECTS OF SLEEP DEPRIVATION ON THE IMMUNE SYSTEM

In order to understand the impact that sleep has on the immune system, the effects of sleep deprivation on immune cell numbers and functions have been examined. However, the existing literature varies considerably in its conclusions, probably because of the differences in the duration of sleep deprivation used in the different studies. The acute sleep deprivation protocols usually consist of periods ranging from a few hours to 126 hours of total sleep deprivation. Alternatively, partial sleep deprivation protocols allow sleep for a restricted duration, usually few hours per night for multiple nights [395]. The most consistent result across all the studies appears to be the rise in neutrophils following both protocols of acute and partial sleep deprivation. The majority of studies also report either increased or unchanged serum pro-inflammatory cytokines, while data on lymphocyte counts and functions are inconsistent. Interestingly, acute and partial sleep deprivation seem to have an opposite effect on NK cell cytotoxicity, the former enhancing and the latter inhibiting the ability of NK cells to kill target cells (tables 1.3 and 1.4).

1.2.7.1 Acute sleep deprivation

The studies conducted on totally sleep-deprived individuals indicated that such a loss of sleep caused an increase in the number of WBC in the blood, particularly neutrophils and monocytes [323, 396, 397]. Apart from these studies, the literature shows a range of contrasting results from studies investigating several aspects of the immune system. Some of these sleep deprivation protocols have been performed in combination with additional stressors, such as

military activities [398]. Moreover the length of total sleep deprivation, the methodologies used, the age and the gender of the participants and the use of a control groups varied among the studies. Therefore these differences could explain the inconsistencies found. The data obtained from these acute sleep deprivation studies are summarized in table 1.3.

1.2.7.2 Partial sleep deprivation

Studies of partial sleep deprivation, which mimic a situation of chronic lack of sleep, are even more difficult to interpret because of the vast range in the period of restricted sleep (from one single night [399-401] up to 12 nights [133] of reduced sleep time) and because of the presence of potential confounders, such as strenuous exercise and decreased caloric intake included in some study [402]. Again, differences in the experimental protocols and methodologies used, and the age and gender of the participants could also explain the disparity in findings among the studies. Gender seems to play an important role, as Irwin and colleagues observed dissimilar responses to partial sleep deprivation between males and females [401]. In general such protocols cause a decrease in immune function, such as NK cell cytotoxicity. However, this condition has often also been associated with increased production of cytokines following cell stimulation, as measured *in vitro* (summarized in table 1.4). Accordingly, Irwin and colleagues also found increased activity of the transcriptional factor NF-kB in PBMCs obtained from donors subjected to one night of partial sleep deprivation [403].

IMMUNE VARIABLE	EFFECT OF ACUTE SLEEP DEPRIVATION
IMMUNE CELL NUMBER	↑ WBC [323, 396, 397] ↑ = neutrophils [323, 397, 404] ↑ monocytes [323, 396] ↑ = lymphocytes [323, 396, 397, 404] ↑ = CD19+ B lymphocytes [323, 396, 397] ↑ = CD3+ T lymphocytes [323, 397] ↑↓ CD4+ T lymphocytes [323, 396, 397] ↑ = CD8+ T lymphocytes [323, 396, 397] ↑↓ NK cells [323, 396, 404]
IMMUNE CELL FUNCTION	↓ neutrophil phagocytosis [398] ↑ neutrophil degranulation [405] = neutrophil adherence [406] ↑ NK cell cytotoxicity [329, 396] ↓ = lymphocyte proliferation [396, 406] ↑ whole blood production of IL-1β [323] ↑ whole blood production of TNF-α [323] = whole blood production of IL-6 [323]
SERUM LEVEL OF CYTOKINES	↑ = IL-1β [131, 397] ↑ = TNF-α [397, 407] ↑↓ = IL-6 [131, 407, 408]
OTHER HUMORAL FACTORS	↑↓= CRP [130, 131, 407] ↑↓= cortisol [407, 409-411] ↑ = Igs, complement factors [397, 412]
VACCINE RESPONSE	↓= Igs [413, 414]

Table 1.3 Effects of acute sleep deprivation on the immune system. Summary of data from studies showing an increase, decrease or no change (=) in immune parameters.

IMMUNE VARIABLE	EFFECT OF PARTIAL SLEEP DEPRIVATION
IMMUNE CELL NUMBER	↑ = WBC [150, 400, 415] ↑↓ neutrophils [150, 400, 402, 415, 416] ↑ = monocytes [150, 400, 402, 416] ↑↓= lymphocytes [150, 400, 402] ↑↓ CD19+ B lymphocytes [132, 402] ↑↓= CD3+ T lymphocytes [132, 402] ↑↓= CD4+ T lymphocytes [132, 402, 417] ↑↓= CD8+ T lymphocytes [132, 402, 417] ↓ NK cells [132, 400, 402] ↑ CD8+ T cells producing TNF-α [335]
IMMUNE CELL FUNCTION	↑ neutrophil chemotaxis [402] ↓ NK cell cytotoxicity [399, 400] ↓ PBMC production of IL-2 [400] ↓ PBMC production of TNF-α [132] ↑ PBMC transcription of IL-1β, IL-6 [132] ↑↓ PBMC proliferation [132, 417] ↑ monocyte production of IL-6, TNF-α [401]
SERUM LEVEL OF CYTOKINES	↑ = TNF-α [418, 419] = IL-1β [402] ↑↓= IL-6 [133, 402, 418-420] = IL-8 [416]
OTHER HUMORAL FACTORS	↑ = CRP [130, 132, 133, 416] = dehydroepiandrosterone (DHEA) [421] ↑↓ cortisol [148, 418, 422, 423] ↑ Igs [402]

Table 1.4 Effects of partial sleep deprivation on the immune system. Summary of data from studies showing an increase, decrease or no change (=) in immune parameters.

1.3 ADIPONECTIN

A significant portion of this thesis focused on the effects of the adipokine adiponectin on innate immune function, notably neutrophil function and survival. Adiponectin is an abundant serum adipokine with levels in serum ranging between 5-10 μg/ml [424-426] , mainly produced by the WAT as well as cardiac myocytes [427], muscle cells [428] and PBMCs [429]. This protein is characterised by an N-terminal collagen-like region and a C-terminal complement factor C1q-like globular domain [430-432]. It circulates as a full-length protein which can oligomerise to form trimers (known as LMW adiponectin) and hexamers (medium molecular weight (MMW) adiponectin) which can then further oligomerise to form a polymer, the high molecular weight (HMW) isoform of adiponectin [424]. Adiponectin also exists as a proteolytic cleavage fragment, called globular adiponectin, consisting of the globular C-terminal domain which is cleaved by a leukocyte elastase secreted by activated monocytics and neutrophils [433]. Trimers and oligomers of adiponectin are the main form present in the circulation [434] with the globular fragment reported at very low concentrations only by one group [435].

Adiponectin serum levels are diminished in diseases such as type 2 diabetes, cardiovascular disease and obesity, while weight loss results in a significant increase in adiponectin production [436, 437]. Its concentration is negatively affected by insulin [438], sex hormones [439] and pro-inflammatory cytokines, mainly TNF-α [196], while the activation of peroxisome-proliferator-activated receptor (PPAR)-γ by its ligands thiazolidinediones, which are used in the treatment of type 2 diabetes, induces its expression [198]. Although adiponectin is decreased in these chronic low grade inflammatory conditions, elevated levels of adiponectin are present in chronic autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and type 1 diabetes mellitus [440]. Circulating adiponectin levels also differ by gender and age, being higher in women [441] and in older adults [442].

Two adiponectin receptors have been identified, AdipoR1 and AdipoR2: they contain seven transmembrane domains but are structurally and functionally distinct from G-protein-coupled receptors [443]. Globular adiponectin predominantly activates AdipoR1, whereas AdipoR2 engages mainly with the full-length variant of adiponectin [444]. In addition, T-cadherin acts as a receptor which sequesters MMW and HMW adiponectin but not the trimeric and globular isoforms [445, 446]. The expression of AdipoR1 and AdipoR2 is ubiquitous in the majority of organs with AdipoR1 expressed at high level in skeletal muscle and AdipoR2 present at high level in the liver [444, 447]. With regard to the immune system, Pang and Narendran analyzed the surface expression of AdipoR1 and AdipoR2 on human PBMCs by flow cytometry, finding that both of these receptors are expressed by approximately 1% of T cells, 93% of monocytes, 47% of B cells, and 21% of NK cells [448], mainly the $CD56^{dim}$ subset [449]. T cells seem to retain AdipoR1 and AdipoR2 into clathrin-coated vescicles and to upload them at the surface upon stimulation [450]. Concerning neutrophils, one study showed that these cells express the mRNA for both receptors [429] while another study failed to detect AdipoR2 mRNA and protein expression in these cells [451]. Intriguingly, both adiponectin receptors have been found to be downregulated in monocytes derived from type 1 diabetes patients [452].

Through these two receptors adiponectin triggers the activation of AMPK and other signaling molecules, such as p38 MAPK, AMPK and PPAR-α and γ [424, 453]. Recently, adaptor protein containing PH domain, PTB domain and Leucine zipper motif (APPL)1 has been identified as an AdipoR1 and AdipoR2 binding protein. The association between AdipoR1 and APPL1 is enhanced by adiponectin and this complex induces the downstream signalling pathways [453]. Recently it has been shown that the AdipoR1-APPL1 complex associates with protein phosphatase 2A (PP2A), which is activated and in turn inactivates protein kinase C (PKC) ζ, allowing liver kinase B (LKB)1 to translocate into the cytosol and activate AMPK [454]. AMPK can also be activated by adiponectin in an APPL1-independent and Ca^{2+}-dependent pathway involving activation of Ca^{2+}/calmodulin-dependent kinase kinase II [455]. In fact,

adiponectin promotes an increase in the intracellular Ca^{2+} levels through activation of phospholipase C (PLC) [456]. Adiponectin has also been shown to activate other signalling molecules: for instance it enhances MEK-1/ERK 1/2 signalling in human vascular smooth muscle cells, vascular endothelial cells [457], lung epithelial cell lines [458], pancreatic β cells [459] and primary human hepatocytes [460]. However, suppression of ERK 1/2 by adiponectin has also been demonstrated in smooth muscle cells when stimulated with IGF-1 [461] and IL-6 [462]. Contradictory results also exist concerning adiponectin-mediated activation of the PI3K/PKB pathway: PKB appears to be phosphorylated by adiponectin treatment in pancreatic β cells [459], prostate cancer cell lines [463], endothelial cell lines [464] and lung epithelial cell line [458], but it leads to suppression of PKB phosphorylation in breast cancer cell lines [465] and in human macrophages when transformed to foam cells [466]. Importantly, Mao et al. demonstrated that APPL1 mediates adiponectin phosphorylation of p38 and PKB, in C2C12 [467] whereas contrasting results have been shown regarding APPL1-mediated activation of ERK 1/2 [457, 467]. This is supported by the fact that APPL1 interacts with both the two PI3K subunits and PKB [468, 469].

1.3.1 ADIPONECTIN AND THE IMMUNE SYSTEM

As discussed above, adiponectin receptors are expressed by immune cells and their surface expression is modulated in certain conditions. Therefore a number of studies have investigated the effect played by adiponectin in the regulation of immune functions, demonstrating an immunomodulatory role for this adipokine.

1.3.1.1 Adiponectin effects on innate immunity

Adiponectin has been demonstrated to inhibit the differentiation of myelomonocytic progenitors, arresting the formation of colony-forming units-granulocyte-macrophage (CFU-GM) from human bone marrow mononuclear cells and human hematopoietic CD34+ stem cells [470]. In particular, it has been suggested that lymphocyte-secreted adiponectin could play a major role in suppressing granulopoiesis [429]. In agreement with these studies, adiponectin

influences hematopoietic stem cells (HSCs) by increasing their proliferation and maintaining the cells in a functionally immature state through p38 MAPK activation in mice [471].

Full-length adiponectin decreases neutrophil ROS production stimulated by phorbol myristate acetate (PMA) [472] and fMLP [451]. A recent study has shown that full-length and globular adiponectin isoforms differentially modulate phagocyte oxidative burst: the full-length isoform has a negative effect whereas globular adiponectin increases the oxidative burst by enhancing NADPH oxidase activity through $p47^{phox}$ phosphorylation via p38 MAPK and ERK 1/2 activation [451]. Adiponectin can also decrease LPS-induced neutrophil IL-8 production [473].

Adiponectin also negatively regulates monocytes and macrophages by inhibiting IL-8, IL-6, TNF-α and IFN-ɣ secretion and increasing the production of the anti-inflammatory IL-10 following stimulation [474-476]. This anti-inflammatory role of adiponectin on monocytes has also been confirmed by Lovren and colleagues, who recently demonstrated that adiponectin enhances the anti-inflammatory M2 macrophage phenotype induced by IL-4, these effects being mediated by AMPK, PPARα and ɣ activation [477]. In contrast, Cheng et al. reported that adiponectin primes unstimulated human macrophages towards a pro-inflammatory M1 phenotype, enhancing the production of TNF-α and IL-6 [478]. Moreover, another study found that HMW, but not LMW adiponectin enhances IL-6 production in primary human monocytes [479]. In agreement with the pro-inflammatory role of adiponectin shown in the latter studies, adiponectin has also been found to stimulate the release of C-C chemokine ligand (CCL)2 CCL3 CCL4 and CCL5 in unstimulated human monocytes, and induction in cells obtained from overweight subjects is impaired, while the surface abundance of CCR2 and CCR5 results decreased [480].

Similarly, the effect of adiponectin on NK cell cytotoxicity is controversial. Adiponectin was shown to increase the ability of NK cells to kill target cells in lean subjects but not in obese

subjects [481], whereas Kim et al demonstrated that adiponectin suppressed the IL-2-enhanced cytotoxic activity of a human NK cell line, without affecting basal NK cell cytotoxicity but suppressing the production of IFN-γ [482]. The latter group extended the finding *in vivo* by using knock out (KO) mice for adiponectin: adiponectin KO mice injected with a lymphoma cell line displayed lower tumoral growth, lower number of myeloid-derived suppressor cells, increased NK cell population and cytotoxicity. The increased NK cell killing ability was explained at least in part by higher expression of the activatory receptor Ly49D on the surface of NK cells obtained from adiponectin KO mice [483]. On the contrary Wilk and colleagues found that this adipokine does not affect human NK cell cytotoxicity *in vitro* [449].

1.3.1.2 Adiponectin effects on adaptive immunity

Studies investigating the effect of adiponectin on adaptive immunity have also yielded contradictory results. Adiponectin indirectly inhibits B lymphopoiesis by activating cyclooxygenase in stromal cells [484]. With regard to adaptive functions, this adipokine has been reported to inhibit the generation of antigen-activated CD137+ T lymphocytes by inducing apoptosis and decreasing their proliferation. Adiponectin also diminishes the secretion of the pro-inflammatory cytokines IFN-ɣ, TNF-α and IL-2 by activated CD137+ T lymphocytes [450]. Moreover, co-culturing adiponectin-treated dendritic cells and T cells, Tsang and colleagues observed a decrease in T cell proliferation, a reduction of IL-2 production, and an increase in the percentage of the anti-inflammatory subset of regulatory T cells (Tregs) [485]. Palmer and colleagues obtained slightly different results, showing that adiponectin caused higher production of IFN-γ, but lower production of IL-6 by human PBMCs, particularly by CD4+ and CD8+ T cells, upon stimulation with hepatitis C virus (HCV) [486]. In support of a pro-inflammatory function of adiponectin on T cells, more recent work demonstrated that the addition of adiponectin to resting CD4+ T cells promoted their differentiation into Th1 cells, increasing the expression of the pro-inflammatory cytokines

IFN-γ, IL-6 and decreasing the expression of the anti-inflammatory IL-4, typical of Th2 T lymphocytes [478].

The somehow inconsistent findings regarding adiponectin signalling and the effects exerted on innate and adaptive immune cell populations presented in this section are likely to be due to the various sources of adiponectin used, commercial or not, containing unspecified mixture of the three HMW, MMW and LMW isoforms and often contaminated with LPS [487]. In addition, the activation status of the cells treated with adiponectin may have differently affected the results [479].

1.3.1.3 In vivo studies

In order to examine the effects of adiponectin in the context of inflammatory disorders, adiponectin has been administered to different animal models of inflammatory diseases. Adiponectin administration prompted beneficial effects in a murine model of experimental colitis [488], and it protected against the development of LPS-induced acute lung injury (ALI) in mice [489], both the animal models being adiponectin-deficient. Moreover, adiponectin treatment resulted in improved hypertension in mice models of metabolic syndrome [490].

With regard to human studies, the concentration of circulating adiponectin has been extensively investigated and it appears to be lower in a number of inflammatory disorders, such as metabolic syndrome, type 2 diabetes mellitus, [437], essential hypertension [491] and OSAS [190-192]. In subjects affected by these diseases, adiponectin often shows a strong, negative association with the inflammatory markers TNF-α, IL-6 and CRP [492-494]. These associations are not surprising as TNF-α and IL-6 are suppressors of adiponectin expression [196, 197, 495] but also adiponectin could inhibit TNF-α and IL-6 expression, as demonstrated in studies cited above [450, 474]. Moreover, a reciprocal negative regulation has been evidenced between CRP and adiponectin [199, 496], reinforcing the associations found *in vivo*.

However, adiponectin often exhibits higher serum concentrations in other diseases, particularly autoimmune diseases [497], including systemic lupus erythematosus (SLE) [498], Behcet's disease [499], rheumatoid arthritis (RA) [500] and chronic obstructive pulmonary disease (COPD) [501] despite their pro-inflammatory status. The causes for its upregulation as well as the role played by adiponectin in these disorders are not clear. One hypothesis could be that a rise in adiponectin level serves as a compensatory mechanism. A second possibility is the emergence of "adiponectin resistance" in which the increase in adiponectin is the result of a failure in adiponectin signaling [502]. Nevertheless, adiponectin can act as a pro-inflammatory adipokine and enhance the inflammatory status rather than contrasting it. Ehling and colleagues pointed out a potential pro-inflammatory role of adiponectin in arthritis, as it enhanced inflammation and matrix degradation in human synovial fibroblasts by increasing IL-6 and matrix metalloproteinase (MMP)1 via p38 activation [503].

1.4 AGEING

Ageing is a multifaceted mechanism; it includes all the modifications occurring with time and it concerns all the organisms. More specifically, it can be defined as "a continuous process that starts at conception and continues until death", characterized by accumulation of physiological changes in the body systems, causing functional decay of organs and tissues and ultimately leading to the death of the organism [504].

1.4.1 LONGEVITY AND HEALTHY LIFE EXPECTANCY

Life expectancy has increased since the mid-19th century and the aged population is predicted to continue growing over the coming years. However, longer life is not always accompanied by good health, on the contrary the susceptibility towards infection [505], cancers [506] chronic inflammation [507] and autoimmune diseases [508, 509] increases during old age. These data indicate that life expectancy, without a similar increase in healthy life expectancy, negatively impact upon quality of life in old age. For this reason much of the research on ageing now

focuses on understanding how to delay the functional decline of the body often through improving lifestyle.

1.4.2 SLEEP CHANGES IN AGEING

Numerous studies in older adults suggest that sleep disturbances negatively impact upon health. In a large epidemiological study, Dew et al. showed that individuals with sleep latency (the time taken to fall asleep once in bed) greater than 30 minutes or with sleep efficiency lower than 80% had more than a 2 times greater risk of death at follow up [510]. Sleep complaints are frequent among older individuals, since more than 50% of the elderly (> 65 years) reported at least one sleep problem on a regular basis [511]. As shown in figure 1.6, ageing is accompanied by sleep alterations, mainly:

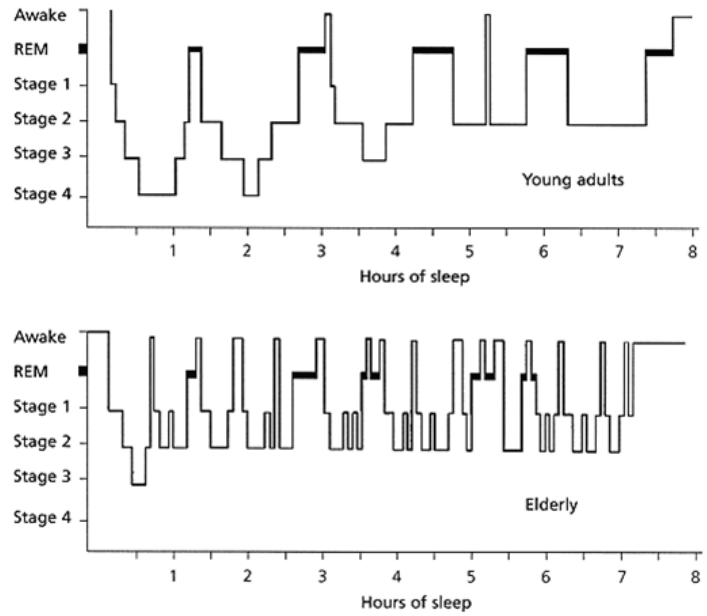
1. Decreased nocturnal sleep duration and efficiency and longer sleep latency. Both stages 1 and 2 NREM sleep increase but SWS sleep and REM sleep decrease [512-516];
2. Increased sleep fragmentation due to increased WASO and number of awakenings [512, 515, 516];
3. Advanced sleep-wake phase (altered circadian rhythm) [517-519] .

The causes underlying sleep changes in the elderly are many, including: general poor health condition [511], chronic pain due to diseases typical of old age (arthritis, depression, Alzheimer's disease), medication, a more sedentary lifestyle, use of stimulants (caffeine, smoking) and poor nutrition [520]. However, studies in healthy older adults suggest that the homeostatic and circadian mechanisms which regulate sleep are naturally altered during ageing. For this reason, sleep disruption occurring in aged subjects has to be considered physiological [512, 513].

Older adults also experience increased daytime napping compared to young individuals: regular napping (4-7 times per week) is reported by 10% of people aged between 55 and 64 years of age and by 25% of people being between 75-84 years of age [521, 522]. The prevalence

of this habit with age could be considered the main consequence of the age-dependent disturbances in the sleep pattern during the night and circadian rhythm [521].

A



B

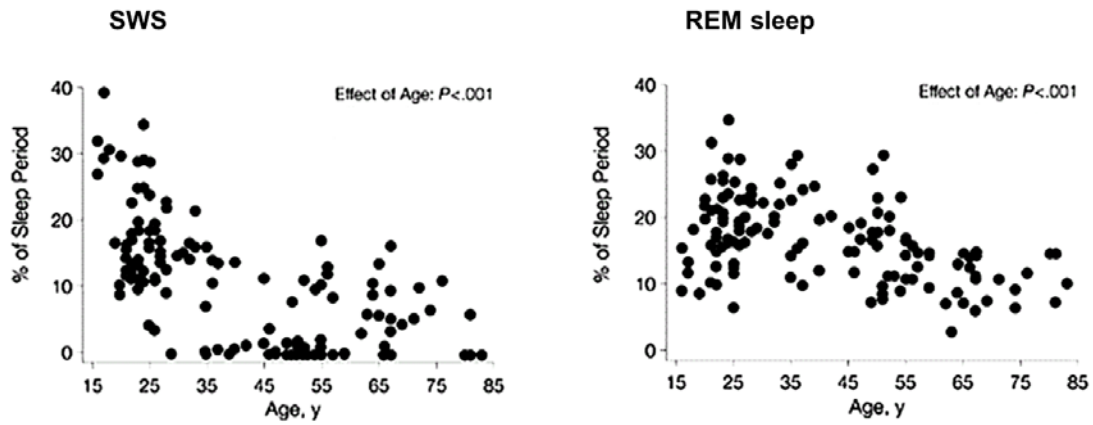


Figure 1.6 Age-related changes in sleep architecture. A. Typical hypnographs of a young and an elderly subject (Figure adapted from Neubauer et al., 1999 [20]). B. The decrease in SWS and REM sleep over the age (Figure adapted from Van Cauter et al., 2000 [513]).

The reasons for these physiological sleep disturbances could be found in the aged-related endocrine changes. Nocturnal levels of melatonin drop dramatically from childhood to adulthood and continue to decline progressively as a result of the degeneration of the pineal gland. This decline in melatonin secretion is thought to be the main cause of sleep disruption in older adults and leads to the phase advance of the sleep-wake cycle. In addition, during normal ageing, GH production is lower [523, 524] and the HPA axis tends to be hyperactivated, thus triggering the rise of circulating CRH, ACTH and cortisol [525-528]. Therefore, the change of the ratio between GHRH:CRH in favor of CRH is believed to contribute to sleep disturbances during ageing [529, 530].

There have been few studies investigating the relationship between cortisol and sleep in the aged population. Prinz and colleagues showed that 24-hour urinary free cortisol was positively associated with earlier habitual rising in the morning and decreased REM sleep in the elderly [531, 532], whereas 6 months melatonin administration in elderly women did not affect cortisol levels despite a marked improvement in sleep continuity [533].

The process of adrenopause may also modulate sleep with ageing: the adrenal hormone DHEA, a precursor of sex hormones, and its sulfate isoform (DHEAS) decline in the circulation with age. Its peak appears to be in the third decade of life; thereafter its level decreases progressively to 10-20% of the maximal values by age 70 [534]. Importantly, it is considered the main antagonist of cortisol: the higher the ratio between cortisol and DHEAS is the greater the catabolic status and frailty are [535]. Moreover, DHEA acts as immune enhancing factor which contrasts the immunosuppressive effects of cortisol [536], thus the rise in cortisol to DHEAS ratio in old age is believed to contribute to the development of immunosenescence (discussed extensively in the section 1.4.3) [537].

With regard to sleep, the role of DHEA has not been extensively investigated: its administration led to increased REM sleep in young individuals [538], and its endogenous

levels increased after melatonin treatment in elderly women [533], therefore its physiological decline seems to contribute to the sleep impairment during ageing. However, its effect on sleep pattern could be indirect and mediated by the altered production of other hormones, particularly testosterone and estradiol [539].

1.4.3 SENESCENCE OF THE IMMUNE SYSTEM

During the process of ageing there is a functional deterioration of the immune system termed immunosenescence. The effects of immunosenescence affect both the innate and the adaptive arms of the immune system and the consequences include increased incidence of infectious diseases [540, 541], autoimmune diseases [542], malignancies [543, 544], and reduced response to vaccination [545, 546]. Another feature of immunosenescence is the development of a subclinical pro-inflammatory status, named “inflammaging” which contributes to age-associated frailty, morbidity, and mortality [507].

1.4.3.1 Ageing of the hematopoietic stem cell compartment

With ageing the numbers of HSCs increases [547], but they are characterized by accumulated DNA damage, suggesting a lower DNA repair efficiency. In addition they have a lower mobilization ability, increased intracellular ROS levels [548] and their differentiation potential is skewed towards myeloid instead of lymphoid progenitors [547, 548]. Indeed, aged HSCs display higher transcription of genes involved in cell cycle regulation and myeloid differentiation [547], as well as epigenetic disregulation [549]. In addition to these intrinsic mechanisms, ageing of HSCs seems to be driven by cell-extrinsic mechanisms related to the marrow niche, such as increased bone formation and adipogenesis, altered extracellular matrix components and cytokines [548].

1.4.3.2 Innate immunosenescence

Ageing is not accompanied by a reduction in circulating neutrophil numbers, or the ability to produce a neutrophilia in response to infection [550] and constitutive apoptosis is unaltered

during ageing, though aged neutrophils are less sensitive to the anti-apoptotic action of pro-inflammatory factors [551]. The expression of surface molecules, such as the integrin dimers LFA-1 (CD11b/CD18) and Mac-1 (CD11a/CD18) does not differ between neutrophils from young and old donors [550, 552] suggesting that age does not impair neutrophil ability to adhere to the blood vessel and extravasate into the tissues. However, a number of groups have shown that several aspects of neutrophil function are reduced with ageing. With regard to chemotaxis, the majority of studies have reported impaired migration of neutrophils isolated from elderly donors [552, 553]. Ageing does not influence the ability of neutrophils to phagocytose non-opsonized pathogens [554], whereas a distinct decrease in the ingestion of opsonised yeast and bacteria by neutrophils isolated from aged donors has been observed by a number of groups [555-557]. In particular, Butcher et al. attributed this decline to an age-dependent reduction in the expression of CD16, a low affinity Fc receptor [556]. The effect of age on ROS production is more controversial. Whilst some studies have demonstrated a decline in superoxide production by aged neutrophils in response to stimulation with fMLP and *Staphylococcus aureus* (*S. aureus*) [557, 558], others have observed similar levels of ROS production in response to *Escherichia coli* (*E. coli*) and PMA in cells from young and aged donors [559, 560].

Neutrophil functional decline is associated with a modification in receptor distribution and signalling pathways. Reduced membrane fluidity and formation of lipid rafts are observed in aged neutrophils from rodents [561] and could explain the impaired intracellular signalling of PI3K, MAPKs and Src homology region 2 domain-containing phosphatase-1 (SHP-1) [562]. Moreover, an increase in intracellular levels of Ca^{2+} has also been detected in aged resting neutrophils [557], underlying an inability to further increase Ca^{2+} concentration in response to stimulation [563].

Human ageing is accompanied by a decrease in the ability of monocytes to produce IL-6 and TNF-α [564] and to mount an effective oxidative burst upon stimulation [565]. However, higher

levels of TNF-α and lower levels of TGF-β are secreted by resting monocytes [566], in line with the theory of "inflammaging" (discussed in section 1.4.3.4). The number of macrophage in the bone marrow is lower in the elderly [567]. In addition, murine and human studies revealed that macrophages also display lower ROS production and phagocytosis, reduced secretion of IL-6, TNF-α, IL-2 and IL-1β upon LPS stimulation [568-570], possibly caused by impaired TLR signaling through p38 MAPK and NF-kB. Also, antigen presentation by macrophages is negatively affected by age, in this case because of a decrease in the expression of class II MHC molecule [569].

With regard to the NK cell population, there is a significant increase in NK cell number with age [571], specifically the $CD56^{dim}$ subset increases whereas the $CD56^{bright}$ population decreases [572, 573] and produces lower amounts of cytokines and chemokines, in particular IFN-γ, MIP-1α, regulated on activation normal T cells expressed and secreted (RANTES) and IL-8 production in response to IL-2 [574]. Despite the overall increase in their number, NK cells from aged subjects exhibit decreased cytotoxicity [575, 576], which is caused by a reduction in the secretion and binding of perforin to the target cells [577].

Dendritic cells are also negatively affected by age. The expression of class II MHC and the co-stimulatory molecules CD86 and CD40 is decreased with age [578] together with the ability to uptake antigens [579]. In addition, the phagocytosis of self-antigens and apoptotic cells by myeloid dendritic cells also declines with age, increasing the risk of developing autoimmune diseases, whereas cytokine production and T cell-induced activation have been variously reported to increase and to decrease with ageing [580].

1.4.3.3 Adaptive immunosenescence

The thymus reaches its maximal size in the early years of life and gradually reduces in size with age at a rate of 3% per year. This process, termed thymic involution, is accompanied by a reduced output of naive T cells, and reduced TCR diversity [581, 582]. As the number of

lymphocytes is kept constant the peripheral pool expands and an altered distribution of naïve and memory T cells is seen, with a distinct increase in the number of memory and effector T cells, particularly the CD8+ cytotoxic subset [583]. Furthermore, persistent subclinical infections, specifically Cytomegalovirus (CMV) infection, contribute to increased CD8+ memory T cell counts This condition can lead to an increase in the CD8+:CD4+ T cell ratio in the oldest old (>85 years) [583-585], which is associated with increased mortality [586-588]. Ageing also seems to be accompanied by unbalanced Th1 and Th2 responses, with amplification of the Th2 activity [589]. In addition, T cells from older subjects also show a reduction in proliferation in response to both non-specific mitogens and to antigens [590], due largely to shortening of telomeres. In addition, the highly differentiated T cells lose expression of the co-stimulatory molecule CD28 and acquire CD57 [591] and receptors associated with NK cells, such as NKG2D [592]. These changes contribute to the increased susceptibility to autoimmunity with age.

The B cell population is also negatively affected by age. The percentage of B lymphocytes amongst PBMCs is reduced with age [593] and B cells from old individuals display a reduced B Cell Receptor (BCR) repertoire [594]. With regards to B cell responses, the main effect of ageing seems to be a decrease in the humoral response [595, 596], despite the fact that an increase in serum IgG and IgA occurs *in vivo* with age [597]. Most importantly, it is well established that the response to vaccination is diminished during ageing, with antibody titres developed against influenza lower in the elderly compared to young individuals [598]. More than a lack in B cell function, this might be the consequence of a lack of T cell help [598] and reduced macrophage responses, leading to poor activation of dendritic cells and reduced antigen presentation [599].

1.4.3.4 Inflammaging

The term “inflammaging” was used for the first time by Franceschi et al, who noted a chronic, low grade pro-inflammatory status that develops and persists during human ageing, as a

consequence of the higher basal production of pro-inflammatory cytokines (IL-1β, IL-6, IFN-α, IFN-β, TNF-α) over the anti-inflammatory mediators (IL-10) [600, 601]. Importantly, inflammaging is believed to promote frailty and chronic age-associated diseases [507, 602]. Sedentary lifestyle, decreased secretion of sex steroids and increased WAT, which is a source of pro-inflammatory cytokines, are the main mechanisms underlying the development of inflammaging during ageing. However, the existence of an anti-inflammaging process has also been postulated and it is mediated by the hyperactivation of the HPA axis during ageing [525-527]. In fact, cortisol and the upstream HPA hormones have clear anti-inflammatory effects upon immune cell function, including the production of pro-inflammatory cytokines [603-605], suggesting that the hyperactive HPA axis counteracts the development of inflammaging. It has been suggested that the hyperactivation of the HPA axis can also be considered the result of inflammaging, as several cytokines have been shown to increase the HPA function [606, 607]. Interestingly, the anti-inflammaging action of the HPA axis and particularly cortisol may also explain the apparent paradox of coexisting age-related chronic inflammation and decline of immune function.

1.4.4 ADIPOKINE CHANGES IN AGEING

As discussed above, clear immune regulatory effects have been demonstrated for the adipokines leptin, adiponectin, resistin and visfatin. Therefore, changes in their levels could contribute to immunosenescence. In the recent literature, more studies have been performed to try and determine whether their serum concentrations differ with age and not just in relation to BMI.

It is well established that leptin serum levels are higher in women than in men [608, 609]; apart from gender, leptin levels have been reported to change during ageing, although results are contradictory. One study reported that leptin concentration does not differ with age in men, while pre-menopausal women display lower serum leptin levels than post-menopausal

women [609]. However, another study showed that leptin serum levels significantly decreased in females during ageing, while in men there was a non-significant decline in its levels [610]. Adiponectin levels tend to increase with age [442, 611, 612], while resistin levels do not appear to differ between young and old people [613]. Serum visfatin levels in the context of ageing have still to be properly investigated.

1.5 AIMS OF THE THESIS

This thesis set out to determine the relationship between changes in sleep duration and continuity with ageing and immunosenescence, aiming to uncover also the mechanisms underlying any associations found with a particular focus on the role of adipokines.

The specific objectives were:

1. To determine the effects of age on sleep duration and continuity in a cohort of healthy aged humans;
2. To determine associations of short and long sleep duration and sleep continuity with a range of parameters of innate and adaptive cell phenotype and function;
3. To determine if there is a relation between sleep duration and continuity and soluble factors, notably cytokines and adipokines;
4. To determine the effect of adipokines, specifically adiponectin, on neutrophil function.

1.6 HYPOTHESES TO BE TESTED

Given that ageing is accompanied by negative changes in both sleep and the immune system, it was hypothesised that lack of sleep could be associated with a decline in immune function in the aged population, thus contributing to the process of immunosenescence. In particular, we proposed that sleep dysregulation would:

- be associated with decreased innate and adaptive immune functions;
- promote a pro-inflammatory status (inflammaging).

We also hypothesised that changes to the serum levels of adrenal hormones and adipokines, could mediate changes seen in both sleep and immune functions. In particular, adiponectin is a potent anti-inflammatory adipokine whose effect on neutrophils has not been extensively investigated. It was therefore decided to study the effect of adiponectin on neutrophil apoptosis and phagocytosis, predicting that this adipokine would have promoted neutrophil apoptosis and inhibit phagocytosis.

CHAPTER 2

METHODS

2.1 SUBJECTS AND BLOOD COLLECTION

93 healthy older adults (> 60 years old) were recruited from the Birmingham 1000 Elders group to investigate the relationship between physiological sleep and immune functions. An additional 10 young (> 35 years old) and 10 elderly (> 60 years old) volunteers were recruited for participating in a pilot study exploring the effects of partial sleep deprivation on immune functions. For analysis of the role played by the adipokine adiponectin in modulation of neutrophil functions the majority of the experiments involved young blood donors aged under 35 year old and recruited from staff and students at the university. The sleep and ageing study was approved by the North Staffordshire local research ethics committee and the studies of adiponectin function and the effects of partial sleep deprivation on immunity were approved by the University of Birmingham research ethics committee. All participants provided written informed consent. Participants were asked to fill in a health screening questionnaire in which they provided information about their recent illnesses and infections, chronic diseases and medication (see Appendix I). Height and weight measurements were also recorded to calculate their BMI, calculated as the mass (kg) divided by the height squared (m^2).

Fasted subjects attended the University Clinical Research Facility (CRF) in the morning (time between 9 and 11) and a sample of peripheral blood was collected for measurement of immune parameters. 5ml of blood was collected into a vacutainer containing ethylenediaminetetraacetic acid (EDTA) tube, in order to perform an immune cell count using a Coulter A^C T diff analyser (Beckman Coulter Inc., Brea, CA), and 5 ml of blood was collected into an anti-coagulant free vacutainer tube, which was centrifuged at 3000 rpm (JOUAN, MR211*i*) for 5 minutes to obtain the serum sample. 30 ml of blood was drawn in to heparin tubes to allow isolation of cells and to perform the functional immune assays.

2.2 ACTIGRAPHY

Actigraphy is a non-invasive method of monitoring human rest/activity cycles. A small watch-like actigraph unit (Respironics Inc., Murrysville, PA) was worn on the non-dominant wrist by volunteers for 1 week. The actigraph measures gross motor activity, light and dark exposure in order to determine the time spent asleep and a number of specific parameters evaluating different aspects of the continuity of sleep (Table 2.1). Data were analyzed using Respironics Actiware software (Respironics Inc.). An example of an actigraph output is shown in Figure 2.1.

Volunteers were also asked to fill in a 7 day sleep diary, in which they reported self-assessed sleep duration plus the episodes of napping during the daytime.

SLEEP PARAMETER	DEFINITION
Time spent asleep	Time spent asleep during the night
Sleep latency	The interval of time taken for an individual to fall asleep after going to bed
Sleep efficiency	The percentage of time spent asleep over the time spent in bed
Wake After Sleep Onset (WASO)	The amount of time spent awake after the beginning of sleep
Average sleep bout (avg sleep bout)	The average length of time spent asleep between two awakenings during the night

Table 2.1 Sleep parameters measured by actigraphy.

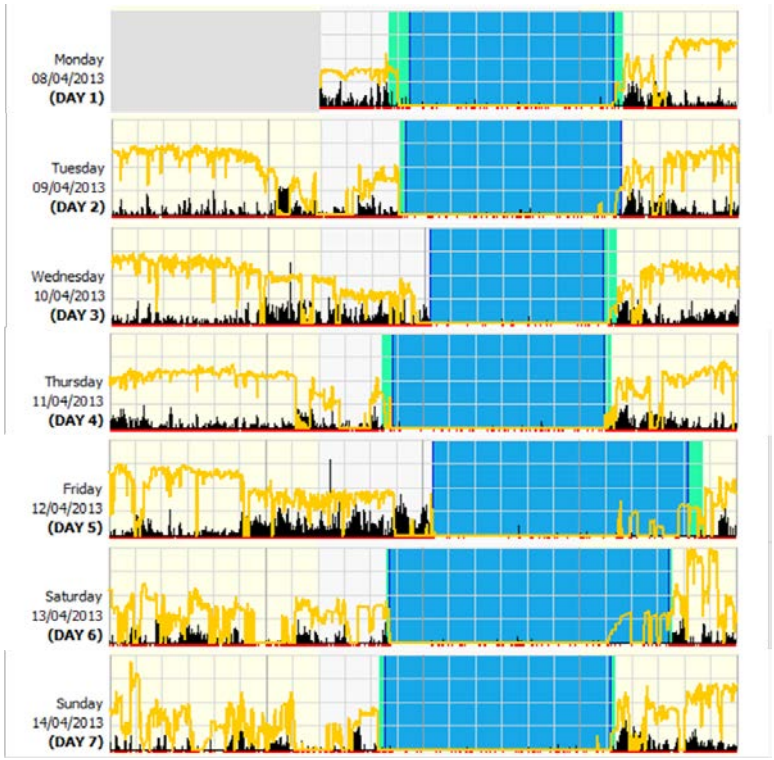


Figure 2.1 An example of actigraph recordings. The individual's movements and light exposure are recorded 24 hours a day for a seven day period. The yellow line represents the light intensity; the black bars indicate the strength of movements performed; the red line at the bottom suggests the time spent awake whilst the blue area is the period at which the actigraph considers the wearer to be asleep.

2.3 TISSUE CULTURE

All cell culture experiments were performed in class 2 type A/B3 biological safety cabinets employing sterile equipment and reagents. For cell culture the standard media was RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (GPS) (all purchased from Sigma-Aldrich, Poole, UK) and with 10% heat-inactivated (HI) fetal calf serum (FCS; Sera Laboratories International Ltd, Haywards Heath, UK), hereafter referred to as complete medium (CM). To prepare HI-FCS, FCS was incubated for 30 minutes in a water bath at 56^{o}C. Phosphate buffered saline (PBS) was prepared by dissolving one PBS tablet (Sigma-Aldrich) in 200 ml distilled water, and was autoclaved prior to use. Sterile pipettes were purchased from Corning Life Sciences (New York, USA)**,** 15 ml and 50 ml

polypropylene Falcon™ tubes from BD Biosciences, fluorescence-activated cell sorting (FACS) tubes were purchased from Becton Dickinson, 96-well round bottomed plates were from Sarstedt and flexible 96-well round bottomed plates were from BD Falcon™ (New Jersey, USA).

2.4 PBMCs AND NK CELL ISOLATION, PHENOTYPE AND FUNCTIONAL ASSAYS

2.4.1 ISOLATION OF PBMCs FROM WHOLE BLOOD

PBMCs were isolated from peripheral blood by Ficoll density gradient centrifugation. Peripheral blood was mixed 1:1 with RPMI-1640 medium supplemented with 1% GPS in 50 ml Falcon™ tubes and overlaid onto 6 ml of Ficoll-Hypaque™ PLUS (GE Healthcare, Little Chalfont,UK) and gradients centrifuged at 1200 rpm for 30 minutes at room temperature (RT) with no brake (JOUAN, MR211*i*). Post centrifugation, mononuclear cells, which reside at the interface between the plasma and the Ficoll layer, were harvested, washed with PBS and pelleted by centrifugation at 1200 rpm (JOUAN, MR211*i*) for 10 minutes at RT. After washing, cells were re-suspended in 10 ml PBS and counted using a hemocytometer. At least $1x10^7$ cells were used to isolate NK cells, $1x10^6$ PBMCs were stained for phenotypic analysis and the rest were re-suspended in freezing medium consisting of HI-FCS supplemented with 10% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and gradually frozen at a temperature of -80°C by means of 5100 Cryo 1°C Freezing Container (Nalgene).

2.4.2 ISOLATION OF NK CELLS FROM PBMCs

NK cells were isolated from PBMCs by negative selection using MACS® technology (Human NK cell isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, PBMCs were washed twice in MACS® buffer at 300 x g for 10 minutes at 4^oC and the subsequent pellet re-suspended in 40 µl of MACS® buffer per 10^7 cells. 10 µl of NK cell biotin-antibody cocktail was added and the sample incubated for 15 minutes at 4^oC with occasional mixing. Post incubation, 30 µl of MACS® buffer and 20 µl of NK cell microbead cocktail were added and the sample was incubated for 15 minutes at 4^oC.

Cells were washed in MACS® buffer (2 ml per 10^7 cells) at 300 x g for 10 minutes at 4°C and the pellet re-suspended in 500 µl of MACS® buffer. For magnetic separation, an LS column (Miltenyi Biotec) was placed in the magnetic field of a QuadroMACS separator (Miltenyi Biotec) and washed with 3 ml of MACS® buffer. The PBMCs were then applied to the column, followed by 9 ml of MACS® buffer. The eluted cells represented the purified NK cell population. NK cells were centrifuged at 300 x g for 10 minutes at 4°C and re-suspended to a final concentration of $1x10^6$ /ml in CM. NK cell purity was assessed by flow cytometry by staining with antibodies against CD3 and CD56. NK cell purity was routinely greater than 95% of the isolated cell population.

2.4.2.1 NK cell cytotoxicity assay

The NK cell cytotoxic assay used the MHC class I-deficient erythroleukaemic cell line K562 as the target cell and a two-colour flow cytometry assay based on the protocol of Godoy-Ramirez et al. [614]. K562 cells were purchased from the American Type Culture Collection (ATCC, Middlesex, UK) (ATCC number CCL-243) and were cultured in CM at 37°C in a humidified 5% CO^2 atmosphere. Cells were fed and split on the day prior to experimentation. The cultures of K562 cells were replaced on a monthly basis.

For the cytotoxicity assay NK cells were serially diluted in a 96-well round bottomed plate to which 100 µl of K562 cells ($1x10^5$ /ml) were added in order to give various effector:target cell ratios, specifically 10:1, 5:1 and 2.5:1. The plate was then centrifuged at 50 x g for 1 minute to increase cell contact before being incubated for 2 hours at 37°C. To account for the spontaneous death of K562 cells, 100 µl of these cells were incubated during this period in absence of NK cells. Post incubation, cells were pelleted by centrifugation at 250 x g for 5 minutes and stained with phycoerytrin (PE)-conjugated mouse anti-human CD56 (0.3 µg/ml) (clone C5.9) (Dako Ltd, Cambridge, UK) for 10 minutes on ice to identify NK cells. Cells were then washed once in PBS and the resulting pellet re-suspended in 100 µl of the cell

impermeable DNA binding dye sytox® blue dead cell stain (Invitrogen, Carlsbad, CA), diluted 1:8000. The well containing K562 cells alone was stained with sytox® blue to asses spontaneous cell death. Cells were rapidly transferred to FACS tubes and analyzed by flow cytometry using a CyAn™ ADP flow cytometer. The emission of the two fluorochromes PE and Violet-1 (respectively for CD56 and sytox®) was recorded, spectral overlap was compensated by running NK cell samples stained separately with each dye.

Data were analyzed using Summit version 4.3. NK and K562 populations were identified by gating on PE and side scatter (Figure 2.2 A). The K562 population, which shows greater side scatter, was gated and Violet 1 fluorescence was detected in a separate FACS plot (Figure 2.3 B). 2000 events were recorded and the percentage of dead K562 was calculated as follows:
% of sytox® positive cells in test sample - % of sytox® positive cells in spontaneous sample.

2.4.3 NK AND T CELL PHENOTYPING BY IMMUNOSTAINING

PBMCs were re-suspended at $2x10^6$/ml in PBS and 50 µl dispensed into a 96-well round bottomed plate. For colour compensation, each antibody was added separately to each well, but all the antibodies were added together in the test well. The plate was incubated for 15 minutes in the dark, after which samples were washed in PBS (250 x g for 5 minutes at 4^oC), re-suspended in PBS and transferred to FACS tubes for flow cytometric analysis on a CyAn™ ADP bench top cytometer (Dako).

The percentage of NK cells within the PBMC population was determined by gating 15,000 PBMCs and evaluating the number of CD3-CD56+ cells counted. The percentage of T lymphocytes within the PBMC population was determined by gating 15,000 PBMCs (Figure 2.3A) and evaluating the number of CD3+ cells counted (Figure 2.3B). The percentage of CD4+ and CD8+ T cells amongst CD3+ T lymphocytes was determined by gating the CD3+ T cell population (Figure 2.3C). The percentage of naïve T lymphocytes was evaluated in the CD3+CD4+ (Figure 2.3D) and CD3+CD8+ populations, with cells double positive for both

CD45RA and CCR7 considered naïve T (Figure 2.3E). The antibodies used and the final concentrations are listed in table 2.2.

A B

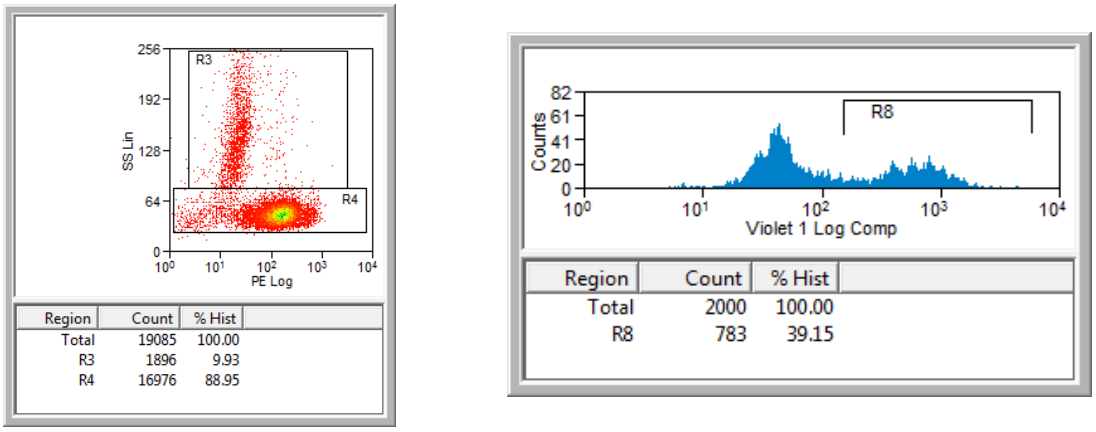


Figure 2.2 Flow cytometric analysis of NK cell cytotoxicity. A. Immunostaining of NK cells and K562 co-cultures with PE-conjugated mouse anti-human CD56 and sytox®. K562 can be identified and gated in Region 3 (R3), and NK cells in Region 4 (R4) thus allowing to determine the effector:target cell ratio (K562 represent the 9.93% of the mixed population). **B.** Histogram showing the percentage of dead K562, measured as the percentage of sytox® positive cells in Region 8 (R8) within 2000 events recorded; the percentage of spontaneous K562 death will be subtracted from this value to obtain the percentage of K562 specifically killed by NK cells.

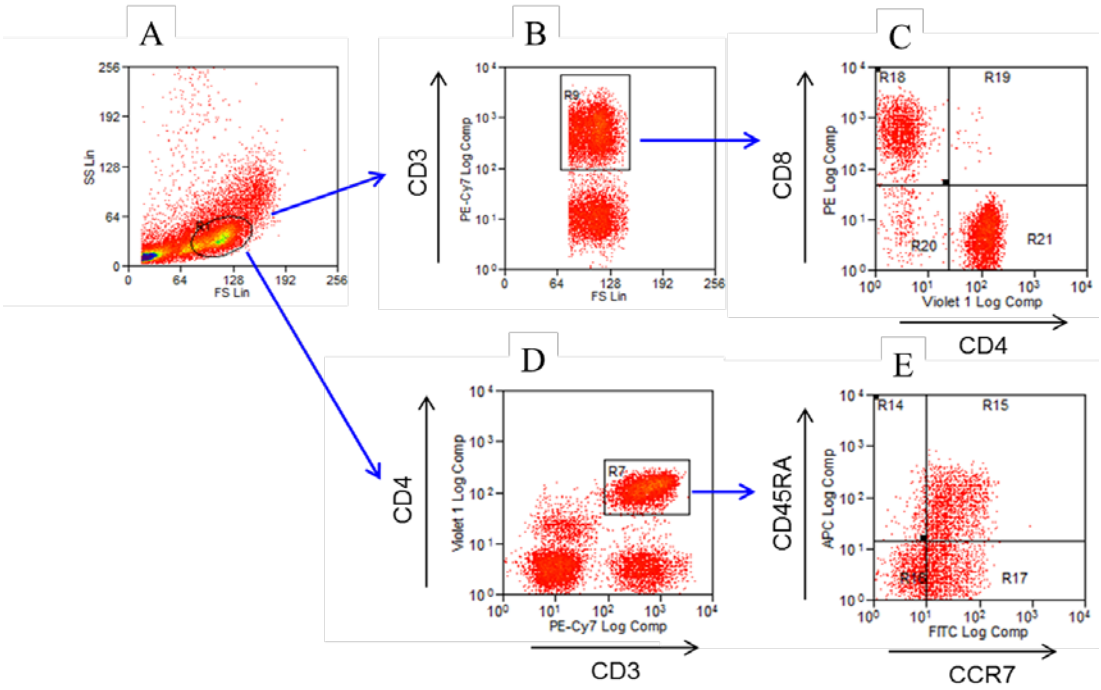


Figure 2.3 Plots and gating strategy used to measure lymphocyte subsets. A. Forward scatter (FS) vs side scatter (SS) plot in which PBMCs are gated in R1. Blue arrows indicate the setting of the gates. **B.** FS vs PE-cyanine 7 (PE-Cy7) (CD3). CD3+ T cells are gated in R9 **C.** Violet 1 (CD4) vs PE (CD8) plot from which the percentages of CD4+ and CD8 + T cells are measured. **D.** Violet 1 (CD4) vs PE-Cy7 (CD3) plot; the cells double positive for both CD3 and CD4 are gated in R7. **E.** Fluorescein isothiocyanate (FITC) (CCR7) vs allophycocyanin (APC) (CD45RA) plot; the cells double positive for both CCR7 and CD45RA represent the naive CD4+T lymphocyte subset.

SURFACE MARKER	QUANTITY	COMPANY
Anti-human CD3 PE-Cy7 (clone UCHT1)	5 µg/ml	eBiosciences
Anti-human CD4 eFluor® 450 (Violet 1) (clone OKT4)	4 µg/ml	eBiosciences
Anti-human CD8 PE (clone UCHT4)	20 µl/ml	Immunotools
Anti-human CCR7 FITC (clone 150503)	2.5 µg/ml	R&D system
Anti-human CD45RA APC (clone HI100)	0.01 µg/ml	Biolegend
Anti-human CD56 PE (clone C5.9)	0.3 µg/ml	Dako
ISOTYPE CONTROLS	**FINAL CONCENTRATION**	**COMPANY**
Anti-Mouse IgG1 PE-Cy7	5 µg/ml	eBiosciences
Anti-Mouse IgG2b eFluor® 450	4 µg/ml	eBiosciences
Anti-mouse IgG2a PE	1:50	Dako
Anti-mouse IgG2a FITC	2.5 µg/ml	Dako
Anti-mouse IgG2b APC	0.01 µg/ml	Dako
Anti-mouse IgG2b PE	0.3 µg/ml	Dako

Table 2.2 Antibodies and concentration used to stain T cell subsets and NK cells.

2.5 MEASUREMENT OF NEUTROPHIL FUNCTIONS IN WHOLE BLOOD

To assess neutrophil ROS production and phagocytosis in whole blood, two commercially available kits were used, respectively Phagoburst™ and Phagotest™ (Glycotope-Biotechnology GmbH, Heidelberg, Germany). All the reagents were provided by the kits and the experiments were performed according to the manufacturer's instructions. For the sleep studies flow cytometric analyses were conducted using a Cyan™ ADP flow cytometer (software Summit version 4.3), for the experiments regarding adiponectin effects on neutrophil functions flow cytometric analyses were conducted using a BD Accuri C flow cytometer (Accuri Cytometers Inc, Ann Arbor, MI) (software CFlow Plus 1.0.227.4).

2.5.1 OXIDATIVE BURST

100 µl peripheral blood was incubated either with 20 µl of *E. coli* or with washing buffer (negative control) for 10 minutes at 37°C. Following incubation, 20 µl of the fluorogenic substrate dihydrorhodamine (DHR) 123 was added and the blood incubated for a further 10 minutes at 37°C. After 10 minutes, red cells were lysed and the remaining cells were fixed in lysing solution for 20 minutes at RT and immediately washed twice with washing solution by centrifugation at 250 x g for 5 minutes. Cells were re-suspended in 200 ul of DNA staining solution and incubated for 10 minutes on ice before flow cytometric analysis using a CyAn^{TM} ADP flow cytometer. The production of ROS was measured by the mean fluorescence intensity (MFI) of FITC positive neutrophils from which the MFI of the negative control was subtracted.

2.5.2 PHAGOCYTOSIS

100 µl of peripheral blood was incubated with 20 µl of FITC-labelled *E. coli* for 10 minutes either at 37°C or at 0°C (negative control). Following incubation, phagocytosis was stopped by addition of ice cold quenching solution and the cells were immediately washed twice with washing solution (250 x g for 5 minutes). Following washing, red cells were lysed and the remaining cells were fixed in lysing solution for 20 minutes at RT followed by two further washing steps. Cells were resuspended in 200 µl of DNA staining solution and incubated for 10 minutes on ice before flow cytometric analysis. The FS vs SS plot (Figure 2.4A) allows the recognition of neutrophils, characterized by greater size and granularity compared to the other blood cell populations. Neutrophils were gated and the FITC fluorescence intensity measured on a separate histogram (Figure 2.4B). The phagocytic index was used as a measurement of the phagocytic capability of the cells and was calculated as the percentage of neutrophils which had ingested bacteria (the percentage of FITC positive cells) multiplied by the MFI of the FITC positive population. This value was then divided by 100 to give the phagocytic index.

A B C

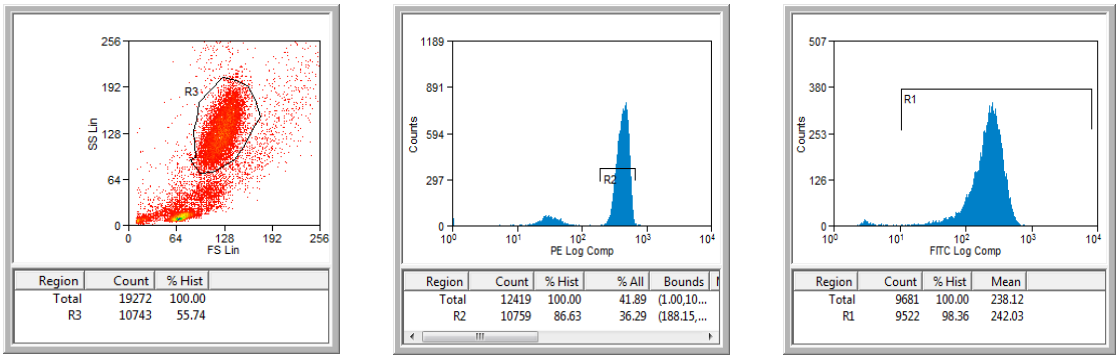


Figure 2.4 Plots and gating strategy used to measure neutrophil phagocytosis and oxidative burst. A. FS vs SS FACS plot in which neutrophils are distinguished and gated in Region 3 (R3). Arrows indicate the setting of the gates. **B.** Histogram showing the PE fluorescence, which indicates the content of DNA among cells gated in R3: the major peak gated in R2 represents neutrophil DNA, whereas the minor peak preceding R2 represents the DNA from free, non-ingested bacteria. **C.** Histogram showing the FITC fluorescence among the neutrophil population gated in R2: Region 1 (R1) indicates the FITC positive cells.

2.6 NEUTROPHIL ISOLATION, FUNCTIONAL ASSAYS AND PROTEIN EXPRESSION

2.6.1 ISOLATION OF NEUTROPHILS FROM WHOLE BLOOD

Neutrophils were isolated by density centrifugation using Percoll® (Sigma-Aldrich) as previously described [615]. Briefly, 2% Dextran 500 (Amersham Bioscience, Uppsala, Sweden) made up in 0.9% NaCl (Sigma-Aldrich) solution, was added to peripheral blood at a 1 in 6 ratio to sediment erythrocytes. The leukocyte layer was removed and overlaid onto a Percoll® two step gradient comprising 2.5 ml of 80% Percoll® and 5 ml of 56% Percoll® in a sterile 15 ml Falcon™ tube. The 80% and 56% Percoll solutions were obtained from a stock 90% Percoll® solution which was prepared with 45 ml of Percoll® and 5 ml of 9% NaCl solution. From this, further dilutions were made with 0.9% NaCl solution.

The Percoll® gradient was then centrifuged at 1100 rpm for 20 minutes (JOUAN, MR211i) with no brake. After centrifugation, the PBMCs, which equilibrate at the plasma-56% Percoll interface, are removed using a sterile, fine tip Pasteur pipette and discarded. The neutrophil

enriched layer at the 56%-80% Percoll interface is removed and washed in RPMI-1640 + GPS by centrifugation at 1600 rpm (JOUAN, MR211i) for 10 minutes. Neutrophils were then counted, re-suspended in the appropriated medium and at the appropriated concentration (1-5x10^6/ml). The purity of isolated neutrophils was determined by differential staining using a commercial May-Grunwald Giemsa stain (Diff-Quick, Baxter Healthcare, UK) and light microscopy, and was routinely greater than 97%.

2.6.2 MEASUREMENT OF NEUTROPHIL FUNCTION ON ISOLATED CELLS

Levels of ROS generated by isolated neutrophils were measured using a luminol-based chemiluminescent assay [616]. Freshly isolated neutrophils were resuspended at 2x10^6 cells/ml in Hanks Balanced Salt Solution (HBSS) containing Ca^{2+} and Mg^{2+} (Sigma-Aldrich). Neutrophils were transferred to opaque 96 well plates (Greiner Bio-One GmbH, Stonehouse, UK) and luminol was added at a final concentration of 100 μM. Chemiluminescence in response to the stimulus fMLP (2.5 μM) (Sigma-Aldrich) was then measured at one minute intervals for a period of 30 minutes using a luminometer (Centro LB 960, Berthold Technologies, Bad Wildbad, Germany). The area under the curve (AUC) was calculated using GraphPad Prism® software version 4 (GraphPad Software Ltd, La Jolla, CA).

Using reagents provided by the Phagotest™ kit phagocytosis by isolated neutrophils was also examined. FITC labeled *E. coli* were opsonized using 10% autologous plasma in HBSS and incubated in 0.5 ml eppendorf tubes at 37°C for 30 minutes. PBS was added to bacteria and eppendorf tubes were spun for 1 minute at 13,000 rpm (MSE microfuge). Opsonized *E. coli* were resuspended in RPMI-1640 + GPS at the same initial volume. Isolated neutrophils were also resuspended in RPMI-1640 + GPS at 5x10^6/ml. 50 μl of neutrophils were dispensed into a 96-well round bottomed plate and 20 μl of *E. coli* were added to each well (1:40 ratio between neutrophils and bacteria). The suspensions were incubated for 90 minutes (unless otherwise specified) at 37°C, after which phagocytosis was stopped by addition of quenching solution (100 μl) to extinguish the FITC fluorescence of surface bound bacteria. For every

experiment, a suspension of neutrophils and bacteria was incubated at 0^{o}C to act as negative control. The samples were transferred to FACS tubes; 10,000 cells were gated and fluorescence was analyzed by flow cytometry (BD Accuri C6 flow cytometer) and the phagocytic index was determined (as explained in section 2.7.2). Cytospins of the suspensions were also obtained and visualized using a LEICA DMI 6000 B microscope x63 objective (Leica Microsystems, Wetzlar, Germany).

The binding of bacteria to the neutrophil membrane was also evaluated. Suspensions were incubated at 4^{o}C for 30, 60 and 90 minutes after which quenching solution was not added; samples were directly transferred to FACS tubes and analyzed by flow cytometry. 10,000 neutrophils were gated, FITC negative cells were considered unbound.

2.6.3 ASSESSMENT OF NEUTROPHIL APOPTOSIS

2.6.3.1 Membrane exposure of phosphatidylserine

Apoptotic cells are characterized by exposure of phosphatidylserine on the outer membrane. To detect extracellular phosphatidylserine and to distinguish apoptotic cells from late apoptotic/necrotic cells, Annexin V/ propidium iodide (PI) staining was used. Neutrophils (1 x 10^{5}) were suspended in 100 µl of Annexin V buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$) to which was added 1 µl of AnnexinV-FITC (BD Biosciences, San Jose, CA) and cells were incubated in the dark at RT for 10 minutes. After this incubation, PI was added (5 µg/ml). Cells were then transferred into FACS tubes and fluorescence was analyzed by flow cytometry (BD Accuri C6 Flow Cytometer, Accuri Cytometers Inc.). 10,000 neutrophils were gated on a FS vs SS plot; neutrophils that were positive for Annexin V but negative for PI fluorescence were considered apoptotic [617].

2.6.3.2 Caspase 3 activation

Cleavage and activation of caspases, particularly caspase 3, is a hallmark of apoptotic cell death [247], including neutrophil apoptosis. To measure the amount of activated caspase 3

neutrophils were fixed in 50 µl of medium A (Fix and Perm® kit, Life technologies, Carlsbad, CA) for 20 minutes at RT, then washed in PBS and permeabilized in 50 µl of medium B for 20 minutes at RT. Neutrophils were washed in PBS and resuspended in 100 µl of PBS, then incubated with FITC-conjugated anti-active caspase-3 (clone C92-605; BD Biosciences, San Jose, CA) or isotype matched antibody (FITC-conjugated rabbit anti-mouse IgG, Southern Biotech) were added in the respective wells (2.5 µg/ml) and cells were incubated for 30 minutes at RT. Neutrophils were washed in PBS, resuspended in PBS and transferred into FACS tubes. 10,000 neutrophils were gated and fluorescence was analyzed by flow cytometry (BD Accuri C6 Flow Cytometer, Accuri Cytometers Inc.).

2.6.3.3 Count of apoptotic nuclei

Apoptotic neutrophils are characterized by loss of their characteristic multi-lobed nuclei and acquisition of single rounded nuclei. Cytospin preparations were differentially stained using a commercial May-Grunwald Giemsa stain and apoptotic nuclear morphology was observed by light microscopy (Olympus IX71 microscope, Olympus America Inc., Center Valley, PA). Apoptotic nuclei were counted amongst 200 cells. Pictures were taken at an original magnification of x40.

2.6.4 ANALYSIS OF SURFACE PROTEIN EXPRESSION AND CERAMIDE ACCUMULATION

Surface expression of receptors and extracellular ceramide accumulation were measured by direct or indirect immunofluorescence staining. 100 µl of cells (10^6/ml) were plated into 96-well plates and incubated with the primary antibody for 20 minutes at 4°C (the incubation time for ceramide detection was 30 minutes). Following incubation, cells were washed in PBS. For directly conjugated antibodies, cells were re-suspended in 200 µl PBS, transferred into FACS tubes and analyzed by flow cytometry (BD Accuri C6 Flow Cytometer). For antibodies requiring staining with a secondary antibody, cells were re-suspended in 100 µl PBS and 3 µl of goat serum was added for 5 minutes to prevent non-specific binding. The

relevant secondary antibody was added and cells were incubated at 4°C for 20 minutes. Samples were washed in PBS (250 x g for 5 minutes), re-suspended in PBS, transferred into FACS tubes and analyzed by flow cytometry. Concentration-matched isotype control antibodies or relevant secondary antibodies were used as negative controls. The antibodies used and their concentrations are listed in Table 2.3.

PRIMARY ANTIBODIES	**QUANTITY**
Rabbit anti-human AdipoR1 (Phoenix Pharmaceuticals, Inc., Burlingame, CA)	5 µg/ml
Rabbit anti-human AdipoR2 (Phoenix Pharmaceuticals, Inc.)	5 µg/ml
Mouse anti-human CD11b (clone 2LPM19c, Dako, Ely, UK)	20 µl
FITC-conjugated mouse anti-human CD16 (clone DJ130c, Dako)	4 µg/ml
FITC-conjugated mouse anti-human Mac-1 (clone CBRM1/5, eBioscience, San Diego, CA)	20 µl
Isotype FITC-conjugated mouse IgG1k (Dako)	as the primary antibody
Mouse anti-human ceramide (clone MID 15B4) (Enzo Life Sciences)	10 µg/ml
Isotype mouse IgM (Dako)	as the primary antibody
SECONDARY ANTIBODIES	**QUANTITY**
FITC-conjugated goat anti-rabbit (Southern Biotech, Birmingham, AL)	2.5 µg/ml
FITC-conjugated goat anti-mouse (Southern Biotech)	10 µg/ml

Table 2.3 Antibodies and the concentrations used in immunostaining of neutrophils.

2.6.5 MEASUREMENT OF F-ACTIN

Neutrophils were either unstimulated or stimulated with unlabeled, opsonized *E. coli* for 5 minutes, after which they were fixed and permeabilized as for caspase 3 staining. To measure differences in actin polymerization in response to bacterial stimulation, F-actin was analyzed using FITC labelled phalloidin (Sigma-Aldrich), a high affinity probe for F-actin. Neutrophils were incubated with phalloidin (1 µg/ml) at 4°C for 30 minutes. After washing in PBS, samples were analyzed by flow cytometry and 10,000 neutrophils were measured. Cells stained for F-actin were also observed by fluorescence microscopy: cytospins of the suspensions were obtained, nuclei were counterstained with DAPI (Life Technologies) and fluorescence was visualized using a LEICA DMI 6000 B microscope x63 objective (Leica Microsystems, UK).

2.6.6 ANALYSIS OF PROTEIN EXPRESSION BY WESTERN BLOT

Neutrophils were spun at 4000 rpm for 4 minutes (MSE microcentrifuge) prior to resuspension in lysis buffer (20 mM 3-morpholinopropanesulfonic acid phosphate buffered (MOPS), 50 mM NaF, 50 mM β-glycerophosphate , 50 mM Na_3VO_4, 1% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM AEBSF and 1% proteases and phosphatase inhibitor cocktails, all purchased from Sigma-Aldrich). Lysis of neutrophils was performed on ice for 30 minutes with occasional vortexing. Lysates were centrifuged at 13,000 rpm for 1 minute (MSE microcentrifuge), supernatants collected and combined with an equal volume of 2X sodium dodecyl sulfate (SDS) sample buffer (125 mM HCl pH 6.8, 5% glycerol, 2% SDS, 1% β-mercaptoethanol, 0.003% bromophenol blue) and boiled for 10 minutes.

Samples were loaded onto a 10% SDS- polyacrylamide gel together with a pre-stained protein standard marker (Bio-Rad, Hercules, CA). Gels were run for 2 hours at 130 V and then transferred onto methanol-activated 0.45 micron polyvinylidene difluoride (PVDF) membrane (Scientific Laboratory Supplies Ltd, Hessle, UK) in a wet blotter and proteins transferred at 0.5 A for 1.5 hours. After proteins were transferred, the membrane was placed on an orbital

shaker and non-specific protein binding was blocked using either 5% bovine serum albumin (BSA) (Sigma-Aldrich) for the phosphospecific antibodies, or 5% non-fat milk in Tris Buffer Saline Tween20 (TBST) (20 mM Tris HCl pH 7,5, 150 mM NaCl, 0.1% Tween20, Sigma-Aldrich) for one hour at RT. Membranes were then incubated with the primary antibody (listed in table 2.4) overnight at 4°C, then washed for 3x10 minutes in TBST and incubated with the appropriate secondary antibody (Horseradish-peroxidase (HRP)-linked anti-rabbit or anti-mouse IgG; GE Healthcare, Uppsala, Sweden) for 1 hour at RT and then washed again 3x10 minutes in TBST. The membrane was immersed in enhanced chemiluminesence (ECL) solution (GeneFlow, Lichfield, UK) for 3 minutes, to visualise proteins either by autoradiography against X-ray film (GE Healthcare) or in a BioRad chemi-doc technology (BioRad).

To confirm that proteins were equally loaded, membranes were subjected to mild stripping by incubating in stripping buffer (200 mM glycine, 0,1% SDS, 1% Tween 20) for 2x 10 minutes, followed by 2x10 minute washes in PBS and 2x5 minutes washes in TBST, with all the incubations performed at RT. Membranes were then blocked again in either 5% BSA or 5% non-fat milk in TBST for one hour and then probed again overnight at 4°C with the appropriate antibody.

Densitometric analyses were performed using National Institute of Health ImageJ software package (http://rsbweb.nih.gov/ij/).

ANTIBODY	DILUTION
P-AMPK (Thr172) (Cell Signaling Technology)	1:1000
AMPK (Cell Signaling Technology)	1:1000
P-PKB (Ser473) (Cell Signaling Technology)	1:1000
PKB (Cell Signaling Technology)	1:1000
P-ERK 1/2 (Thr202/Tyr204) (Cell Signaling Technology)	1:1000
ERK 1/2 (Cell Signaling Technology)	1:1000
P-p38 (Thr180/Tyr182) (Cell Signaling Technology)	1:1000
p38 (Santa Cruz Biotechnology, Santa Cruz, CA)	1:500
Mcl-1 (clone S-19) (Santa Cruz Biotechnology)	1:500
β-actin (Sigma-Aldrich)	1:5000

Table 2.4 Antibodies and the dilutions used for Western blots.

2.7 MEASUREMENT OF SERUM FACTORS

2.7.1 MEASUREMENT OF CORTISOL AND DHEAS SERUM LEVELS

Serum concentrations of the adrenal hormones cortisol and DHEAS were assessed by enzyme-linked immunosorbent assay (ELISA) using commercial kits purchased from IBL International (Hamburg, Germany) and the assays were performed according to manufacturer's instruction. Briefly, the standard curve and the quality controls supplied by the kits, and the serum samples (20 µl for cortisol ELISA and 25 µl for DHEAS ELISA) were dispensed into the plate provided by the kit, whose wells are already coated with anti-cortisol or anti-DHEAS antibodies. 200 µl of enzyme conjugate solution were added to each well and the plate was incubated for one hour at RT. After washing the wells, 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) solution provided by the kit was dispensed into each well and

the plate was incubated for 15 minutes at RT in the dark. To stop the colorimetric reaction, 100 µl of stop solution supplied by the kit was added and the plate was was read using a microplate reader (EL808, BioTek®, Potton, UK) at 450 nm.

2.7.2 MEASUREMENT OF SERUM ADIPOKINE LEVELS

Serum concentrations of leptin and adiponectin were measured by ELISA. Leptin and adiponectin ELISA kits were purchased from R&D Systems (Minneapolis, MN) and the assays were performed according to manufacturer's instructions. Briefly, 96 well-plates (NUNC™, eBiosciences, San Diego, CA) were coated with the relevant capture antibody over night at RT. ELISA plates were then washed three times with washing solution (0.05% Tween 20 in PBS) using an automated microplate washer (ASYS, Atlantis). Plates were then blocked for 1 hour at RT using 300 µl of reagent diluent. Following three washes, 100 µl of diluted samples and standards were placed into the appropriate wells and incubated for 2 hours at RT. Plates were then washed three times before incubation with biotinylated detection antibody for 1 hour at RT. Following three more washes, streptavidin conjugated with horseradish peroxidase (HRP) and the chromogen substrate TMB (Sigma-Aldrich) were added in sequence. After 10 minutes, the colorimetric reaction was stopped by addition of 50µl H_2SO_4. The plate was read using a microplate reader (EL808, BioTek®) at 450 nm and corrected by subtracting the reading at 550 nm.

2.7.3 MEASUREMENT OF SERUM CYTOKINE LEVELS

Serum levels of the cytokines: GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ, TNF-α were detected using multiplex technology and a Human Cytokine 10-Plex Panel (Luminex® assay, Invitrogen, Carlsbad, CA). The kit provides polystyrene beads which are internally dyed with red and infrared fluorophores of different intensities, allowing differentiation of one bead from another. Beads of defined spectral properties are conjugated to cytokine-specific capture antibodies. Briefly, the wells of a microplate were coated with

mixed beads for 30 minutes. Following coating, the wells were washed and the standards or test samples were added and incubated for 2 hours at RT. After washing, cytokine-specific biotinylated detector antibodies were added and incubated for one hour allowing them to bind to the appropriate immobilized cytokines. After washing to remove the excess biotinylated antibody, PE-streptavidin was added and incubated for 30 minutes to allow the formation of a four-member solid phase sandwich. The excess PE-streptavidin was washed and the beads were analyzed using a Luminex detection system (Luminex 100, Bio-Rad). By monitoring the spectral properties of the beads and the amount of associated PE fluorescence, the concentration of cytokines was determined.

2.8 STATISTICAL ANALYSES

In the sleep studies data were analyzed using IBM SPSS statistics version 20 (New York, NY). To check if data followed a normal distribution, the Kolmogorov-Smirnov test was used. Non-normally distributed data were either log (ln) or square root (sqrt) transformed to confer a normal distribution. To identify potential confounder variables (for example age, BMI), bivariate correlations or t-test as appropriate were performed between social demographics and anthropometric variables and each of the sleep, immune and hormone variables. Significant confounding variables were then entered at Block 1 in linear regressions with the predicting variable added at Block 2 in the model. Change in R square (ΔR^2) is reported as a measure of the effect size. When independent samples were split into more than two categories (i.e. sleep variables split into tertiles), Univariate General Linear Model with least significant difference (LSD) post hoc tests was used; analyses were controlled for significant confounding variables. Groups of matched samples were analyzed by Repeated Measures General Linear Model with the Greenhouse–Geisser correction. Analyses were run both unadjusted and adjusted for gender and BMI. Results were reported as mean ± standard deviation (SD) in tables and graphed as mean value ± standard error mean (SEM) using either SPSS or SigmaPlot 12.0.

Power analysis was conducted using G*Power version 3.1.9.2 (Dusseldorf, Germany).

For analysis of in vitro results data were analyzed using GraphPad Prism® software, version 4. Two-tailed paired and unpaired Student's t-test were used to compare respectively two groups of paired and independent samples. Repeated measures ANOVA was used to analyze more than two groups of matched samples, followed by Tukey's multiple comparison test.

A p value of less than 0.05 was accepted as significant in all analyses.

CHAPTER 3

THE EFFECTS OF SLEEP ON THE IMMUNE SYSTEM

3.1 INTRODUCTION

Sleep is a physiological behaviour, essential for maintaining human health and wellness. As shown by several epidemiological studies, sleep duration influences the risk of morbidity, with the strongest association being between short sleep duration and obesity-related diseases [115, 118, 139-141]. In addition the risk of mortality increases with both very short and very long sleep [5-11] and in subjects reporting poor sleep continuity [510, 618]. As sleep duration and continuity decline with age [512-519, 619, 620], it is possible that this contributes to some of the detrimental effects of ageing upon the body's major systems and to age-related pathology.

To understand how sleep can influence tissue functions, particularly of the immune and endocrine systems, studies of sleep deprivation have been performed on small groups of individuals. The data achieved from these experimental protocols confirm the link between sleep behaviour and risk of metabolic diseases, as glucose tolerance, insulin response and cholesterol levels were altered following the period of partial sleep deprivation [148, 150]. Levels of adipokines were also found to be altered after a period of partial sleep deprivation, although contradictory results have been reported especially regarding the change in serum leptin levels [167-169]. In addition, other works investigated the response of the immune system to acute and partial sleep deprivation, focusing on changes in cell numbers, function and level of inflammatory markers. Again, discrepant outcomes are present in the literature, probably due to different protocols of sleep deprivation used, however the majority of studies support the hypothesis that forced loss of sleep tends to lower immune functions [398-400] and increase inflammation [131, 323, 418].

Old age is physiologically accompanied by changes in sleep architecture, including a reduction in total sleep duration, an increase in sleep latency and fragmentation and a phase-advance in the sleep-wake cycle [20, 512-514, 517-519, 619]. Ageing is also accompanied by remodelling of the immune system, immunosenescence, and an increase in systemic

inflammation, inflammaging, contributing to the higher frequency of infections [540], and a range of age-related diseases including cancer [621], autoimmune diseases [508] and chronic inflammatory disease. What is currently poorly understood is whether there is a link between age-related changes to sleep duration and continuity, immunosenescence and inflammaging.

3.1.1 HYPOTHESIS AND AIMS

The main hypothesis tested in this chapter is that a physiological lack of sleep occurring during ageing contributes to the development of immunosenescence. In addition, it is proposed that changes in sleep will be associated with and mediated by age-related changes in soluble factors, such as cytokines, adipokines and adrenal hormones.

These hypotheses were addressed in the following aims:

- To determine sleep duration and architecture in a cohort of healthy older subjects;
- To determine if immunosenescence and/or inflammaging was more frequent in aged individuals with short sleep duration and/or impaired sleep continuity;
- To determine associations between serum cytokine, endocrine hormone and adipokine levels and sleep parameters;
- To determine causality by testing if 3 days of partial sleep deprivation can induce features of immunosenescence or increase inflammaging in young subjects or exacerbate these in older subjects.

3.2 METHODS

The methods used for the various functional assays are described in chapter 2. 100 healthy elderly adults (>60 years old) were initially recruited from the Birmingham 1000 Elders group and gave written informed consent to participate in the study. Because of issues in blood taking and breaking of the actigraphs, the final cohort recruited from whom blood and sleep measurements were obtained consisted of 93 healthy older adults. The study was approved by the South Staffs Local Research Ethics Committee. For sleep measurements, an actigraph was

worn by volunteers for one week following blood sampling and participants kept a sleep diary in the same week.

Additionally 10 healthy young (<35 years old) and 10 elderly volunteers (>60 years old) were recruited to participate in a partial sleep deprivation study consisting of sleeping for four hours during three consecutive nights from 1 a.m. to 5 a.m.

The exclusion criteria for participation in both studies were: diagnosis of sleep disorders (and thus use of sleep medication), use of any medication known to modify immune function (such as steroids) and diabetes (both type 1 and 2). For the partial sleep deprivation study participants showing a baseline sleep duration of 4 hours or less were also excluded.

Fasted volunteers came to the University CRF between 9 a.m. and 11 a.m. the morning before the study began, again after 3 nights of partial sleep deprivation and again after a single day of sleep recovery; on each of these occasions they gave a blood sample. Volunteers wore an actigraph for the week before beginning the partial sleep deprivation to estimate their baseline sleep duration and they continued wearing it for the duration of the experimental protocol to ascertain adherence to the protocol and during the following day of sleep recovery to evaluate the amount of sleep recovery. All participants gave written consent.

Blood was collected to assess immune cell function as described in chapter 2.

3.3 RESULTS

3.3.1 SUBJECT DEMOGRAPHICS

The 93 healthy older adults (mean age: 75.06 ± 6.75; 42 men; mean BMI: 26.2 ± 4.42, 92 Caucasians, 1 Asian) recruited considered themselves to be generally healthy, were not taking any sleep medication, had not travelled to a different time zone in the month preceding the actigraphic measurement, had not been diagnosed with a sleep disorder or a mental illness and had not worked shifts in the year preceding the study, as reported in the healthy screening questionnaire. However, over 60 of the group (64%) declared they were taking a regular

medication, most frequently anti-hypertensive drugs (40%) or statins to lower cholesterol levels (28%).

3.3.1.1 Sleep characteristics

The actigraph recorded nocturnal sleep duration, efficiency, latency, WASO and average sleep bout for one week and the average values across the seven nights were used for the analyses. The sleep diary contained information about sleep duration and the time spent napping. Table 3.1 shows the objective and subjective sleep characteristics of the cohort.

Within the cohort age and BMI did not correlate with any of the sleep parameters, whereas sleep latency ($t(91)= -2.055$, $p=0.043$) and average sleep bout ($t(91)= -2.512$, $p=0.014$) were longer in females. As there were differences between genders as well as a different number of men and women participating in the study, all the statistical analyses performed were adjusted for gender and if necessary additional covariates. Sleep parameters were also analysed with regard to the use of statins and anti-hypertensive drugs but there was no effect of the use of these medications (data not shown).

SLEEP PARAMETER	MEAN (± SD) (n)
Time spent asleep (minutes per night) (actigraphy)	417 (± 55) (93)
Sleep efficiency (% per night) (actigraphy)	85.64 (± 5.03) (93)
Sleep latency (minutes per night) (actigraphy)	20.47 (± 8.04) (93)
WASO (minutes per night) (actigraphy)	46.34 (± 21.01) (93)
Avg sleep bout (minutes per night) (actigraphy)	23.13 (± 10.80) (93)
Time spent asleep (hours per night) (sleep diary)	6.38 (± 1.09) (88)
Napping (minutes per week) (sleep diary)	67.42 (± 101.17) (88)

Table 3.1 Sleep characteristics of the volunteers recruited obtained by actigraphy and sleep diary.

Actigraphic sleep duration was compared to subjective sleep duration reported in the sleep diary and a strong correlation was found between the two measures of sleep duration (R^2=0.464, β=0.680, p<0.001). The time spent napping per week reported in the sleep questionnaire was also negatively associated with both subjective (R^2=0.185, β= -0.430, p<0.001) and objective (R^2=0.153, β= -0.391, p<0.001) time spent asleep per night, potentially representing a compensatory mechanism for nocturnal short sleep duration. Several correlations were discovered also amongst sleep variables measured by actigraphy (Table 3.2). In particular, sleep duration positively correlated with sleep efficiency and average sleep bout.

Data from sleep diaries were analysed against immune and hormonal variables but they did not offer significant results (data not shown), thus in this thesis they are not further considered and only analyses conducted on actigraphic sleep recordings are presented.

Groups of tertiles were created for each actigraphic sleep variable (Table 3.3). Tertile groups were preferred to groups of hours of sleep as they retain an equal number of volunteers. Such

tertiles have been used for following analyses complementing the information obtained from the linear regression analyses.

	Time spent asleep		Efficiency (ln)		Latency (sqrt)		WASO (ln)		Avg sleep bout (ln)	
	β	**p**	**β**	**p**	**β**	**p**	**β**	**p**	**β**	**p**
Time spent asleep			0.393	**<0.001**	0.285	0.112	-0.097	0.353	0.345	**0.001**
Efficiency (ln)	0.393	**<0.001**			-0.253	**0.014**	-0.736	**<0.001**	0.641	**<0.001**
Latency (sqrt)	0.285	0.112	-0.253	**0.014**			0.284	0.112	-0.086	0.413
WASO (ln)	-0.097	0.353	-0.736	**<0.001**	0.284	0.112			-0.718	**<0.001**
Avg sleep bout (ln)	0.345	**0.001**	0.641	**<0.001**	-0.086	0.413	-0.718	**<0.001**		

Table 3.2 Bivariate correlations between sleep variables. β coefficient and p values are reported; p values of statistically significant associations are highlighted in red.

	1ST TERTILE (min-max)	2ND TERTILE (min-max)	3RD TERTILE (min-max)
Time spent asleep (minutes)	296 – 395	396 – 448	449 – 580
Efficiency (%)	71.64 – 85.15	85.16 – 88.20	88.24 – 93.71
Latency (minutes)	10.00 – 16.17	16.42 – 22.51	23.52 – 43.26
WASO (minutes)	14.34 – 36.34	37.00 – 51.00	51.08 – 148.52
Avg sleep bout (minutes)	8.57 – 17.38	17.51 – 25.13	25.16 – 68.55

Table 3.3 Sleep variables divided into groups of tertiles (n=31 each). The minimum and the maximum values of every sleep variables are reported for each tertile.

3.3.2 RELATIONSHIP BETWEEN SLEEP AND IMMUNE PARAMETERS

3.3.2.1 Sleep influences the number of circulating immune cells

The circulating pool of total WBC, granulocytes, monocytes and lymphocytes were assessed using a coulter cell counter, whereas the NK cell population (CD56+ CD3-) was measured by immunostaining and flow cytometry. The granulocyte to lymphocyte ratio (G:L) was also calculated as this parameter is considered a marker of inflammation [622, 623] and predicts mortality in several conditions [624-626]. All the participants had WBC and granulocyte counts in the normal range (data not shown), excluding the presence of infectious diseases.

Linear regressions were performed for cell counts with the sleep parameters (time spent asleep, efficiency, latency, WASO and average sleep bout) adjusting for gender only (Table 3.4). Both sleep duration and sleep efficiency were negatively correlated with the number of WBC, granulocytes, monocytes and the G:L ratio. WASO and average sleep bout, two complementary indicators for sleep fragmentation, were also associated with the G:L ratio in a positive and negative manner respectively. Average sleep bout also negatively correlated

with the number of monocytes. Latency did not show any significant relationship with any of the immune cell populations. Additionally, immune cells were compared amongst tertiles of sleep parameters. The results obtained from a Univariate General Linear Model (controlled for gender) reproduced the significant changes observed with the linear regressions (data not shown). Although sleep duration did not predict the number of lymphocytes according to the linear regression performed before, the analysis of tertiles revealed that the group of long sleepers was characterised by a significant decrease in the number of lymphocytes ($F(2,68)=3.988$, $p=0.023$, $\eta^2=0.105$) (Figure 3.1). These results indicate an influence of sleep on the distribution of the circulating immune cell populations in the elderly.

PREDICTOR	WBC			Granulocytes (ln)			Monocytes (sqrt)			Lymphocytes (sqrt)			G:L (sqrt)			NK cell number		
	ΔR^2	β	p	ΔR^2	β	p	ΔR^2	β	p	ΔR^2	β	p	ΔR^2	β	p	ΔR^2	β	p
Time spent asleep	0.122	-0.350	**0.002**	0.124	-0.352	**0.002**	0.070	-0.265	**0.022**	0.010	-0.102	0.387	0.070	-0.265	**0.022**	0.012	0.110	0.307
Efficiency (ln)	0.101	-0.317	**0.006**	0.106	-0.326	**0.005**	0.115	-0.339	**0.003**	0.006	0.078	0.508	0.137	-0.371	**0.001**	0.014	-0.047	0.666
Latency (sqrt)	0.015	0.129	0.294	0.002	0.047	0.701	0.018	0.138	0.257	0.033	0.187	0.124	0.010	-0.103	0.395	0.011	-0.107	0.327
WASO (ln)	0.016	0.128	0.283	0.018	0.136	0.250	0.023	0.153	0.1949	0.036	-0.190	0.107	0.083	0.289	**0.012**	0.007	0.084	0.438
Avg sleep bout (ln)	0.012	-0.111	0.365	0.017	-0.113	0.273	0.057	-0.246	**0.040**	0.039	0.203	0.093	0.080	-0.291	**0.014**	0.002	-0.050	0.654

Table 3.4 Linear regressions performed between sleep parameters and blood cell counts. ΔR^2, β coefficient and p value are reported. The p values of statistically significant regressions are highlighted in red.

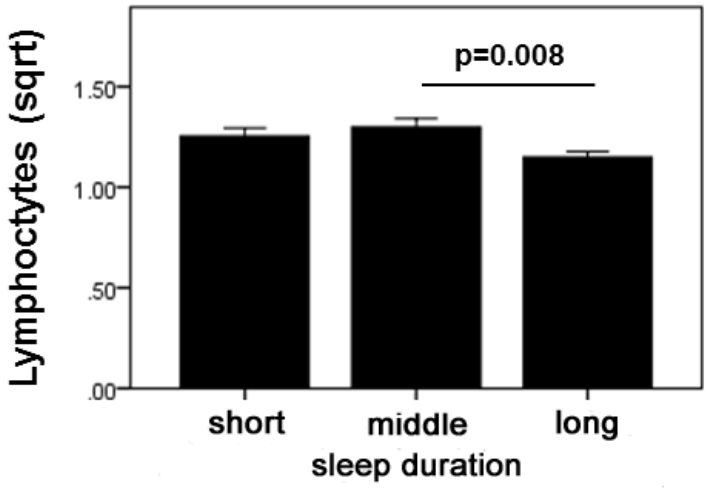


Figure 3.1 Lymphocyte cell count amongst tertiles of sleep duration. The cohort was divided into tertiles of short (n=23), middle (n=26) or long (n=25) sleep duration and lymphocyte count determined and expressed as the square root (sqrt) of the value. Blood cell counts were not obtained from the whole cohort, only from 74 individuals. Data are mean ± SEM.

The use of anti-hypertensive drugs was associated with increased numbers of WBC (t(68)= -2.374, p=0.020), granulocytes (t(68)= -3.364, p=0.001) and the G:L ratio (t(68)= -2.306, p=0.024), whereas the use of statins was associated with increased numbers of WBC (t(68)= -2.085, p=0.041) and granulocytes (t(68)= -2.958, p=0.004). Therefore, the regressions were repeated with further adjustment for the use of these medications. After controlling for these variables, the significant associations between WBC, granulocytes and G:L ratio with sleep duration and efficiency persisted, and G:L ratio remained significantly associated with both WASO and average sleep bout (data not shown).

3.3.2.2 Sleep affects neutrophil ROS production

Neutrophil phagocytosis and ROS production were assessed in whole blood and NK cell cytotoxicity was measured by challenging isolated NK cells with the erythroleukaemic target cell line K562.

Gender and BMI were not associated with changes in neutrophil function and NK cell cytotoxicity, whereas age significantly correlated with neutrophil ROS production (R^2=0.073, β= -0.283, p=0.010) and NK cell cytotoxicity (R^2=0.091, β=0.271, p=0.020). Innate immune functions were not affected by the use of both anti-hypertensive drugs and statins, as assessed by parametric t-test (data not shown). Linear regressions were performed between sleep parameters and innate immune cell functions. Analyses for neutrophil ROS production and NK cell cytotoxicity were adjusted for age and gender, whereas the phagocytic index was controlled just for gender. No significant associations were found between sleep parameters and immune functions, suggesting that altered sleep does not accelerate the development of innate immunosenescence (Table 3.5).

Neutrophil and NK cell functions were also analysed amongst tertiles of sleep variables. The results showed significant differences in neutrophil ROS production amongst the tertiles of average sleep bout, with highest function in the middle tertile (F(2,76)=3.232, p=0.045,

η^2=0.078) and thus lower ROS production mainly showed by the tertile of short average sleep bout. Moreover, the middle tertile of sleep duration was characterised by a trend towards higher neutrophil phagocytic ability than long or short sleepers although the differences did not reach statistical significance (F(2,78)=2.124, p=0.126, η^2=0.052) (Figure 3.2). No significant differences were observed with other sleep parameters. NK cell data were still not significantly altered by sleep if tertiles of sleep parameters were used for analysis (data not shown).

These innate immune functions were not affected by use of medications (data not shown), therefore analyses were not further adjusted.

PREDICTOR	**Neutrophil phagocytosis**			**Neutrophil ROS production (ln)**			**NK cell cytotoxicity (sqrt)**		
	ΔR^2	**β**	**p**	**ΔR^2**	**β**	**p**	**ΔR^2**	**β**	**p**
Time spent asleep	0.002	-0.040	0.722	0.004	0.065	0.550	0.021	-0.144	0.212
Efficiency (ln)	0.002	0.039	0.726	0.004	0.085	0.556	0.000	-0.015	0.897
Latency (sqrt)	0.002	-0.040	0.724	0.000	0.063	0.884	0.016	0.131	0.274
WASO (ln)	0.001	-0.023	0.836	0.006	-0.082	0.452	0.001	0.033	0.780
Avg sleep bout (ln)	0.012	0.112	0.332	0.038	0.208	0.064	0.005	-0.070	0.559

Table 3.5 Linear regressions performed between sleep parameters and innate immune functions. ΔR^2, β coefficient and p value are shown.

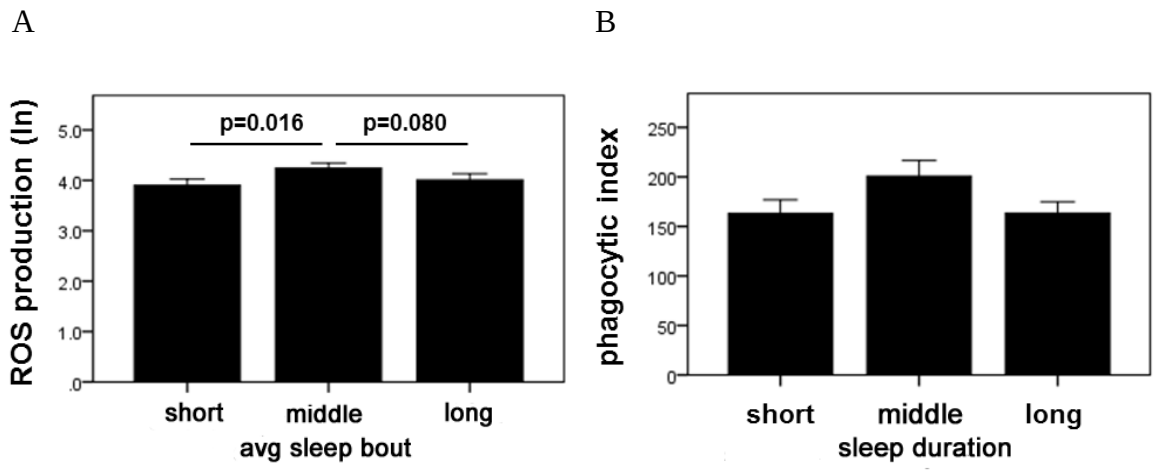


Figure 3.2 Neutrophil functions amongst tertiles of sleep parameters. The cohort was divided into tertiles of short (n=27), middle (n=26) or long (n=30) average sleep bout for analysis of neutrophil ROS production (**A**) and short (n=26), middle (n=30) and long (n=28) sleep duration for analysis of neutrophil phagocytosis (**B**). Data are mean ± SEM.

3.3.2.3 The distribution of T cell subsets is affected by sleep

The number of circulating naïve T lymphocytes is negatively affected by age, whereas memory T cells increase [582, 583, 585]. In parallel, the ratio between CD8+ and CD4+ T lymphocytes is often found to be increased with advanced age, possibly as a consequence of subclinical infections [584]. To explore whether sleep could enhance immunosenescence of T cell subsets, PBMCs were stained with antibodies against specific markers and T cells subpopulations were identified.

In our cohort of volunteers females had significantly higher percentages of CD4+ naïve T cells (t(88)=,-2.163 p=0.033), CD8+ naïve T cells (t(88)=-1.028, p=0.034) as well as CD4+ and CD8+ naïve:memory T cell ratios (t(88)=-2.042, p=0.044) (t(88)=-2.076, p=0.041). Age correlated with the percentages of CD4+ T cells (R^2=0.048, β=0.219, p=0.038), CD8+ T cells (R^2=0.086, β=-0.294, p=0.005) and the CD4+:CD8+ T cell ratio (R^2=0.050, β=0.224, p=0.034), whereas BMI had no effect on the distribution of these subpopulations. T cell subsets were not affected by the use of either anti-hypertensive drugs or statins (data not shown).

Linear regressions were performed between sleep parameters and T cell subsets adjusting for gender only (CD4+ naïve T cells, CD8+ naïve T cells, CD4+ and CD8+ naïve:memory T cell ratios) or both age and gender (CD4+ T cells, CD8+ T cells, CD4+:CD8+ T cell ratio). Results are shown in table 3.6. There were significant negative associations between both CD4+ T cells and the CD4+:CD8+ T cell ratio with sleep efficiency and positive correlations between both CD4+ T cells and the CD4+:CD8+ T cell ratio with WASO. Surprisingly, it was found that naïve CD4+ and CD8+ T cells as well as CD4+ naïve:memory T cell ratio were negatively associated with sleep duration, indicating that long sleep duration is associated with a decrease in the pool of naïve T cells. Analysis of tertiles of sleep variables reproduced the results obtained from the linear regressions (data not shown).

PREDICTOR	CD3+ T cells			CD4+ T cells			CD8+ T cells (sqrt)			CD4+:CD8+ T cells (sqrt)		
	ΔR^2	β	p	ΔR^2	β	p	ΔR^2	β	p	ΔR^2	β	p
Time spent asleep	0.004	-0.062	0.561	0.028	-0.169	0.104	0.005	-0.068	0.508	0.006	-0.079	0.450
Efficiency (ln)	0.005	0.068	0.528	0.086	-0.293	**0.004**	0.018	-0.133	0.197	0.048	-0.203	**0.033**
Latency (ln)	0.028	0.172	0.113	0.000	-0.006	0.953	0.001	-0.026	0.807	0.000	0.005	0.959
WASO (ln)	0.013	-0.116	0.280	0.050	0.227	**0.031**	0.001	0.034	0.747	0.044	0.215	**0.040**
Avg sleep bout (ln)	0.023	0.157	0.152	0.014	-0.123	0.258	0.000	0.021	0.843	0.009	-0.098	0.364
PREDICTOR	**CD4+ naive T cells**			**CD8+ naïve T cells (sqrt)**			**CD4+ naïve:memory T cells (sqrt)**			**CD8+ naïve:memory T cells (sqrt)**		
	ΔR^2	β	p	ΔR^2	β	p	ΔR^2	β	p	ΔR^2	β	p
Time spent asleep	0.055	-0.236	**0.023**	0.050	-0.224	**0.031**	0.055	-0.235	**0.023**	0.028	-0.168	0.108
Efficiency (ln)	0.017	-0.129	0.215	0.025	-0.159	0.128	0.025	-0.157	0.132	0.011	-0.103	0.325
Latency (sqrt)	0.000	-0.010	0.928	0.016	-0.131	0.220	0.000	0.013	0.906	0.022	-0.152	0.155
WASO (ln)	0.028	-0.169	0.108	0.005	-0.073	0.486	0.027	-0.166	0.114	0.009	-0.097	0.359
Avg sleep bout (ln)	0.014	0.123	0.252	0.000	-0.016	0.879	0.011	0.108	0.315	0.000	0.009	0.934

Table 3.6 Linear regressions performed between sleep parameters and T cell subsets. ΔR^2, β coefficient and p value are reported. The p values of statistically significant regressions are highlighted in red.

3.3.2.4 Sleep is associated with changes in the levels of some cytokines

Ageing is accompanied by a chronic low grade inflammatory status, termed inflammaging, resulting from the increased concentration of pro-inflammatory cytokines [600]. 10 cytokines (IL-1 β, IL-2, IL-4, IL-5, IL6, IL-8, IL-10, GM-CSF, IFN-γ, TNF-α,) were measured by Luminex® technology in the serum of elderly volunteers. The concentration of some of these cytokines was often too low to be detected, therefore the concentration ascribed was 0 pg/ml. In addition, some values were very high and exceeded the healthy value [627, 628] thus these data were excluded from the analysis.

With regard to age, gender and BMI, only GM-CSF was positively correlated with BMI (R^2=0.065, β=0.256, p=0.037). Cytokines did not associate with age and did not differ between genders (data not shown). Linear regressions were thus adjusted for gender and analysis for GM-CSF was adjusted for gender and BMI. IL-10 and IL-6 were negatively associated with sleep duration and latency respectively, whereas IL-8 negatively correlated with both sleep duration and latency (Table 3.7).

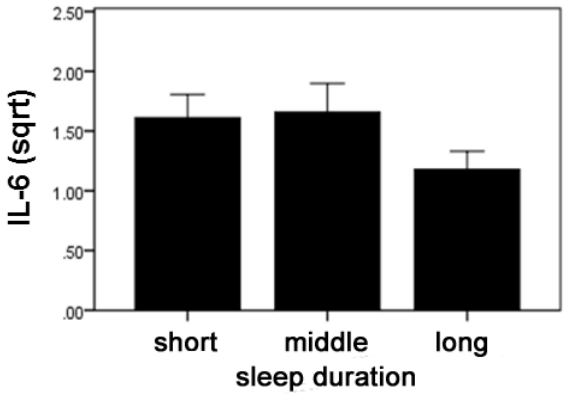
With regard to the use of medications, IL-4 was significantly upregulated in subjects using anti-hypertensive drugs (t(70)=-2.540, p=0.013) and IL-8 was lower in subjects taking statins (t(84)=2.656, p=0.010). After adjustments for these medications, IL-8 lost its association with sleep duration (ΔR^2=0.020, β=-0.142, p=0.187) but still correlated with sleep latency (ΔR^2=0.082, β=-0.297, p=0.006). Analysis of tertiles of sleep parameters did not reveal any additional significant differences. IL-6 showed a trend towards being reduced in long sleepers but this did not reach significance (F(2,85)=1.381, p=0.257, η^2=0.031) (Figure 3.3A) and IL-4 was significantly different amongst tertiles of sleep duration (F(2,60)=3.704, p=0.030, η^2=0.110) (Figure 3.3B).

In summary, these data indicate that sleep duration and latency affects the inflammatory status in elderly volunteers, although the effect was limited to just a few cytokines notably IL-4 after adjustment for the use of medications.

PREDICTOR	IL-1β (sqrt)			IL-2 (sqrt)			IL-4 (sqrt)			IL-5 (sqrt)			IL-6 (sqrt)		
	ΔR^2	β	p	ΔR^2	β	p	ΔR^2	β	p	ΔR^2	β	p	ΔR^2	β	p
Time spent asleep	0.000	0.013	0.982	0.008	0.092	0.415	0.022	0.148	0.202	0.009	0.094	0.399	0.024	-0.154	0.145
Efficiency (ln)	0.011	0.106	0.367	0.000	0.010	0.929	0.022	0.147	0.204	0.009	0.094	0.400	0.008	0.084	0.426
Latency (sqrt)	0.002	0.042	0.728	0.002	-0.043	0.714	0.003	0.053	0.653	0.001	-0.036	0.755	0.066	-0.263	**0.014**
WASO (ln)	0.003	-0.056	0.636	0.002	-0.048	0.674	0.000	0.003	0.979	0.000	-0.016	0.884	0.000	-0.007	0.950
Avg sleep bout (ln)	0.024	0.163	0.182	0.004	0.069	0.559	0.024	0.161	0.176	0.001	-0.033	0.777	0.002	0.044	0.690
PREDICTOR	**IL-8 (sqrt)**			**IL-10 (sqrt)**			**GM-CSF (sqrt)**			**IFN-γ (sqrt)**			**TNF-α (sqrt)**		
	ΔR^2	β	p	ΔR^2	β	p	ΔR^2	β	p	ΔR^2	β	p	ΔR^2	β	p
Time spent asleep	0.053	-0.231	**0.027**	0.043	-0.209	**0.050**	0.007	-0.083	0.505	0.015	-0.124	0.269	0.005	0.070	0.546
Efficiency (ln)	0.022	0.149	0.157	0.001	0.028	0.793	0.005	0.072	0.562	0.003	0.058	0.604	0.003	0.051	0.661
Latency (sqrt)	0.103	-0.326	**0.002**	0.026	-0.163	0.132	0.016	0.130	0.294	0.002	-0.040	0.728	0.002	0.040	0.737
WASO (ln)	0.015	-0.122	0.249	0.001	0.030	0.784	0.030	-0.179	0.151	0.017	-0.131	0.245	0.005	-0.071	0.543
Avg sleep bout (ln)	0.003	0.053	0.630	0.006	-0.079	0.472	0.013	0.124	0.349	0.001	0.025	0.833	0.027	0.169	0.155

Table 3.7 Linear regressions performed between sleep parameters and cytokines. ΔR^2, β coefficient and p value are reported. The p values of statistically significant regressions are highlighted in red.

A



B

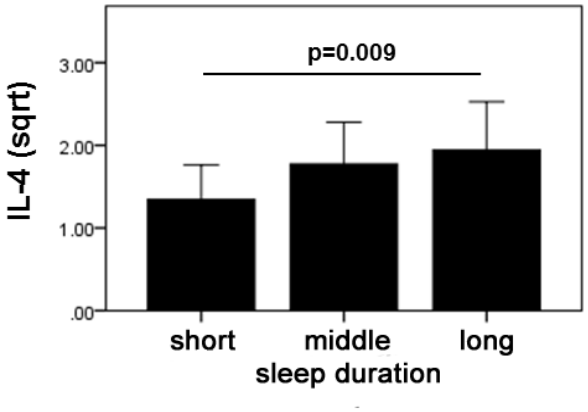


Figure 3.3 Serum concentrations of IL-6 and IL-4 amongst tertiles of sleep duration.
The cohort was divided into tertiles of sleep duration: short sleep n=30, middle sleep n=30, long sleep n=31 for IL-6 (**A**); short sleep n=24, middle sleep n=25, long sleep n=23 for IL-4 (**B**). Cytokine levels were converted to the square root value (sqrt) and data are mean ± SEM.

3.3.3 SLEEP, BMI, ADIPOKINES AND ADRENAL HORMONES

3.3.3.1 Serum concentration of adiponectin is affected by sleep

BMI has been shown to have a U-shaped relationship with sleep duration in young adults and elderly subjects [109, 134, 135]. Leptin, the main adipokine which promotes satiety, positively correlates with sleep duration [109] although few studies on sleep-deprived subjects showed opposite results in the variation of levels of leptin [169, 171]. In the majority of studies adiponectin did not associate with sleep pattern [109, 166], though some groups reported this adipokine to be positively associated with sleep duration [188, 189].

As previous studies reported a U-shaped relationship between sleep duration and BMI, this relationship was assessed firstly by splitting sleep duration into tertiles and evaluating the differences amongst groups. Age (R^2=0.000, β=-0.009, p=0.933) and gender (t(85)=0.811, p=0.420) did not associate with BMI. No significant differences for BMI were observed amongst the three groups of sleep duration (F(2,81)=1.613, p=0.206, η^2=0.038). Analyses adjusted for gender only revealed that there were no significant differences also amongst tertiles of the other sleep variables (efficiency: F(2,81)=0.113, p=0.893, η^2=0.003; latency: F(2,81)=2.355, p=0.101, η^2=0.055; WASO: F(2,81)=0.290, p=0.749, η^2=0.007; average sleep bout: F(2,81)=2.488, p=0.089, η^2=0.088). Linear regressions did not show any significant result either (sleep duration: ΔR^2=0.002, β=-0.046, p=0.676; efficiency: ΔR^2=0.007, β=-0.087, p=0.428; latency: ΔR^2=0.008, β=0.093, p=0.399; WASO: ΔR^2=0.000, β=0.000, p=0.998; average sleep bout: ΔR^2=0.001, β=0.037, p=0.743).

BMI was significantly higher in subjects taking statins (t(81)= -2.374, p=0.020) but not anti-hypertensive drugs (t(81)=-1.086, p=0.281). Therefore, the Univariate General Linear Model analyses were repeated controlling for use of statins, and in this case BMI was significantly different amongst tertiles of sleep duration (F(2,71)=6.042, p=0.004, η^2=0.145), with lower BMI values displayed by the tertile of middle sleep duration (Figure 3.4).

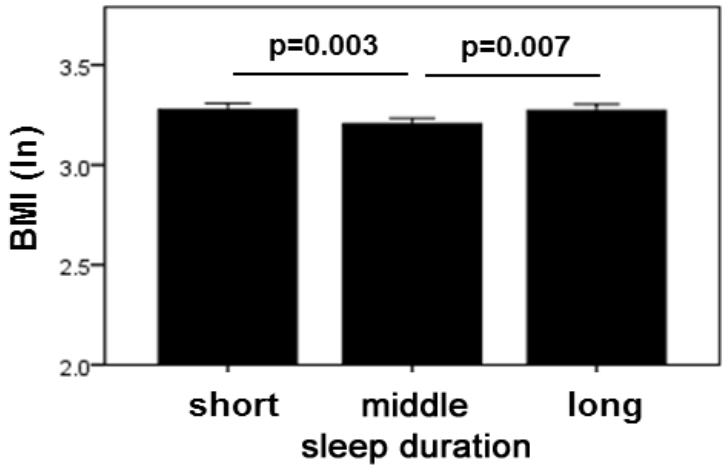


Figure 3.4 Differences in the BMI amongst tertiles of sleep duration. The cohort was divided into tertiles of short (n=28), middle (n=30) or long (n=25) sleep duration and related to BMI expressed as the log value. BMI data were obtained from 83 individuals. Data are mean ± SEM.

Serum concentrations of leptin were strongly affected by gender ($t(91)= -3.491$, $p=0.001$) and BMI ($R^2=0.385$, $\beta=0.621$, $p<0.001$), but not age ($R^2=0.000$, $\beta= -0.018$, $p=0.865$), whereas adiponectin levels were higher in females ($t(91)= -2.965$, $p=0.004$) and positively correlated with age ($R^2=0.106$, $\beta=0.325$, $p=0.001$) but were not associated with BMI ($R^2=0.018$, $\beta= -0.133$, $p=0.219$). Again, both linear regressions and Univariate General Linear Models were used to analyse these adipokines with regard to sleep parameters. Analyses for leptin were controlled for gender and BMI whereas analyses for adiponectin were controlled for gender and age. Linear regressions did not reveal any significant correlation (Table 3.8), whereas adiponectin concentration was significantly different amongst tertiles of sleep duration and WASO (Figure 3.5). Long sleepers had higher levels of adiponectin than middle ($p=0.021$) or short sleepers ($p=0.017$) ($F(2,86)=3.810$, $p=0.026$, $\eta^2=0.081$) and levels of adiponectin were lower in those with long WASO ($p=0.019$) and short WASO ($p<0.001$) than those within the middle tertile of WASO ($F(2,86)=7.012$, $p=0.002$, $\eta^2=0.140$) (Figure 3.5).

Adiponectin levels were not affected by use of either anti-hypertensive drugs ($t(86)=1.078$, $p=0.284$) or statins ($t(86)=1.551$, $p=0.125$), whereas concentrations of leptin were significantly higher in subjects using statins ($t(86)= -2.504$, $p=0.017$) but not anti-hypertensive drugs ($t(86)= -1.061$, $p=0.293$). Therefore, linear regressions and Univariate General Linear Model analyses were repeated for levels of leptin with adjustment for use of statins, but no other significant results were found (data not shown).

PREDICTOR	Leptin (sqrt)			Adiponectin		
	ΔR^2	**β**	**p**	**ΔR^2**	**β**	**p**
Time spent asleep	0.001	-0.025	0.733	0.030	0.174	0.065
Efficiency (ln)	0.006	-0.079	0.290	0.017	0.131	0.165
Latency (sqrt)	0.016	0.128	0.089	0.009	-0.099	0.307
WASO (ln)	0.003	0.051	0.499	0.000	0.005	0.961
Avg sleep bout (ln)	0.009	-0.098	0.205	0.001	0.027	0.783

Table 3.8 Linear regressions performed between sleep parameters and the adipokines leptin and adiponectin. ΔR^2, β coefficient and p value are shown.

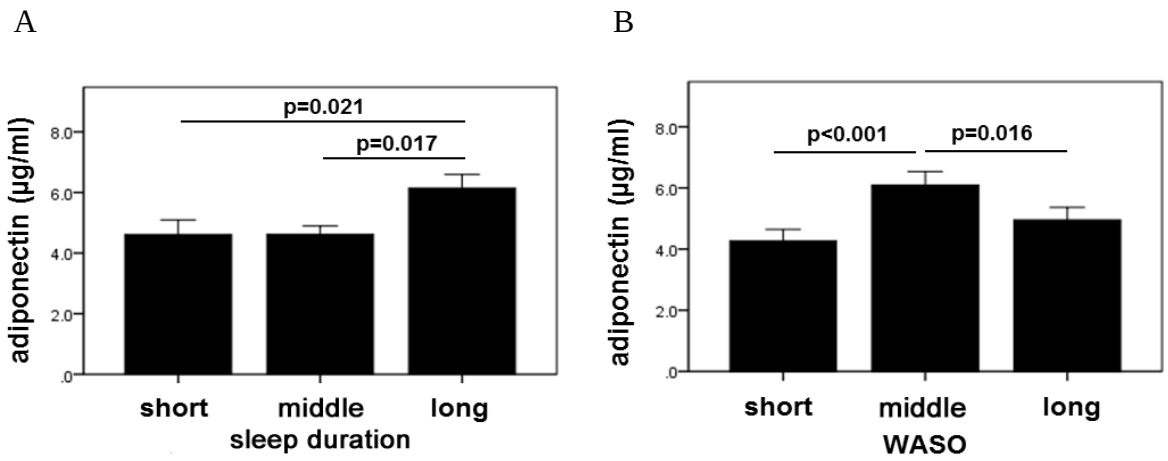


Figure 3.5 Serum concentration of adiponectin amongst tertiles of sleep duration and WASO. The cohort was divided into tertiles of short (n=31), middle (n=31) and long (n=31) sleep duration (**A**) and WASO (**B**) and serum adiponectin was determined. Data are mean ± SEM.

3.3.3.2 Cortisol, but not DHEAS, associates with parameters of sleep fragmentation

The secretion of cortisol from the adrenal gland shows a diurnal rhythm dependent on the release of ACTH which in turn is regulated by the hypothalamic, anti somnogenic factor CRH [51, 65, 74]. Cortisol concentration increases in response to stress [65] and its levels are slightly increased with age [525, 526, 528] contributing to frailty [535] and immunosenescence [537]. On the contrary, DHEA and its sulphate ester decrease with age [534]. A high ratio between cortisol and DHEAS (C:D) is considered an indicator of frailty [535]. Moreover these two hormones have opposite immunomodulatory roles, cortisol is a potent immune suppressor whereas DHEAS exerts immune enhancing functions [536].

In our volunteers, total secretion of cortisol was measured at one single time point in the morning. Cortisol, DHEAS and the C:D ratio were all analysed both with linear regressions and amongst tertiles of sleep variables. Cortisol was associated with age (R^2=0.188, β=0.434, p<0.001), but did not correlate with BMI (R^2=0.015, β= -0.121, p=0.278) or differ between genders (t(84)=1.270, p=0.208). DHEAS did not significantly correlate with BMI (R^2=0.004, β= -0.018, p=0.551), age (R^2=0.039, β= -0.198, p=0.061) but it was significantly elevated in males (t(88)=2.505, p=0.014); the C:D ratio did not differ between genders (t(82)= -1.895, p=0.062) and did not associate with either age (R^2=0.011, β=0.104, p=0.345) and BMI (R^2=0.002, β= -0.041, p=0.720). Thus, analyses were adjusted either for both gender and age or gender only. Linear regressions revealed a positive association between cortisol and WASO and a negative association with average sleep bout. In contrast, DHEAS was not influenced by sleep parameters (Table 3.9). Analysis of tertiles of sleep parameters did not reveal any further significant differences (data not shown).

PREDICTOR	Cortisol (ln)			DHEAS (ln)			C:D (sqrt)		
	ΔR^2	**β**	**p**	**ΔR^2**	**β**	**p**	**ΔR^2**	**β**	**p**
Time spent asleep	0.007	-0.084	0.443	0.010	0.102	0.325	0.019	-0.147	0.180
Efficiency (ln)	0.002	-0.040	0.712	0.000	-0.008	0.937	0.001	-0.034	0.757
Latency (sqrt)	0.004	0.061	0.583	0.000	-0.016	0.880	0.000	0.012	0.916
WASO (ln)	0.042	0.210	**0.035**	0.001	-0.034	0.744	0.012	0.108	0.328
Avg sleep bout (ln)	0.073	-0.280	**0.005**	0.009	-0.096	0.368	0.018	-0.137	0.221

Table 3.9 Linear regressions performed between sleep parameters, cortisol, DHEAS and C:D. ΔR^2, β coefficient and p values are shown. The p values of statistically significant regressions are highlighted in red.

With regard to use of medications, levels of cortisol were significantly higher in subjects using anti-hypertensive drugs ($t(79)=-2.033$, $p=0.045$), but not DHEAS levels ($t(83)=0.805$, $p=0.423$) and C:D ratio ($t(75)= -1.998$, $p=0.052$). Use of statins also did not affect the concentration of these hormones (cortisol: $t(79)= -0.515$, $p=0.608$, DHEAS: $t(83)=0.019$, $p=0.985$, C:D: $t(75)= -0.809$, $p=0.421$). Therefore, cortisol analyses were repeated with adjustment for use of anti-hypertensive drugs.

The linear regression between cortisol and WASO lost significance ($\Delta R^2=0.033$, $\beta=0.188$, $p=0.075$) whereas the association between cortisol and average sleep bout was confirmed after adjustment ($\Delta R^2= 0.069$, $\beta= -0.277$, $p=0.009$).

3.3.4 THE EFFECT OF PARTIAL SLEEP DEPRIVATION ON IMMUNE FUNCTIONS

Associations had been found between sleep duration or average sleep bout and specific immune parameters, such as the G:C ratio, neutrophil ROS production and levels of cytokines and adipokines. It was thus necessary to try and determine causality and thus a partial sleep deprivation study was carried out to determine direct effects of shortened sleep duration and

whether there were any age-related differences in effects seen. 10 healthy young individuals (mean age: 27.4 ± 2.4, 5 males, mean BMI: 22.37 ± 2.05, 9 Caucasians, 1 Asian) and 10 healthy older individuals (mean age: 74.2 ± 6.1, 7 males, mean BMI: 24.93 ± 3.46, 9 Caucasians, 1 Asian) were recruited to participate in the protocol of partial sleep deprivation. In the health screening questionnaire all the participants reported themselves as generally healthy, were not taking any sleep medication, had not travelled to a different time zone in the month preceding the study and had not worked shifts in the year preceding the study. None of the young volunteers recruited reported taking medications, whereas 6 elderly participants declared the regular use of medication, namely statins (n=3) and anti-hypertensive drugs (n=4). A schematic representation of the partial sleep deprivation protocol is shown in figure 3.6.

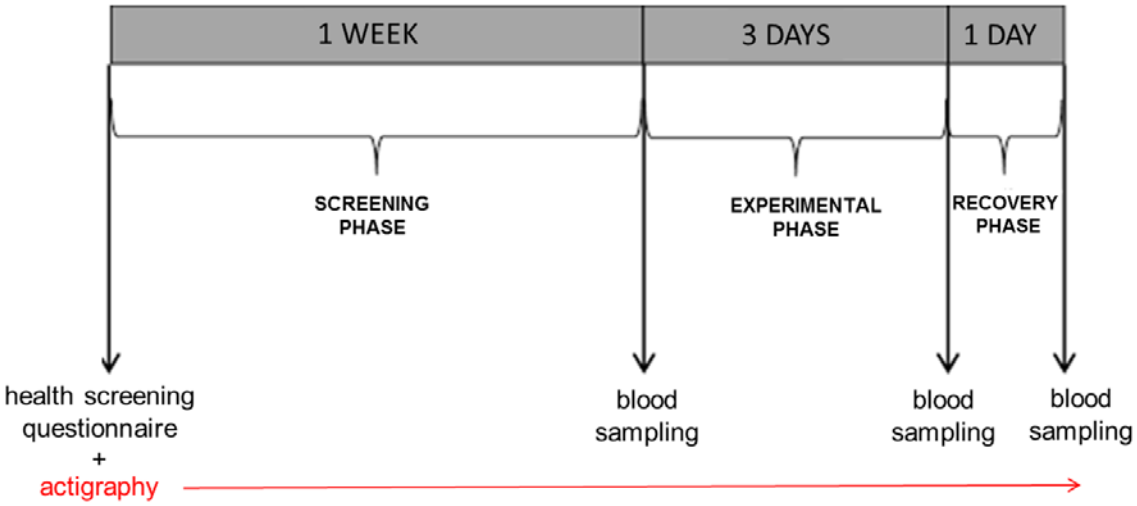


Figure 3.6 Schematic representation of the partial sleep deprivation protocol. Participants filled in the health screening questionnaire and were given the actigraph which was worn for the following two weeks. During the first week (screening phase) the baseline sleep characteristics were measured. After the screening phase, volunteers donated a sample of blood on three occasions: before and after the three nights of partial sleep deprivation (experimental phase), and after a single night of sleep recovery (recovery phase).

3.3.4.1 Sleep characteristics

The baseline sleep duration and the other parameters measured in the first screening week by actigraphy were averaged across the seven nights and are shown in table 3.10. No significant differences were found in the baseline sleep parameters between young and elderly. The volunteers affirmed that they did not nap during the three days of experimental protocol and no significant napping periods were recorded by actigraphy. Actigraphy also confirmed that all the participants followed the partial sleep deprivation protocol and their level of sleep during the recovery day was determined (Table 3.10). Sleep latency and WASO were significantly decreased during the partial sleep deprivation nights compared to baseline nights (data not shown). There was no difference in the sleep recovery period between the young and elderly subjects.

SLEEP PARAMETER (n=10)	YOUNG mean (± SD)	ELDERLY mean (± SD)	P value
Baseline time spent asleep (minutes per night)	406 (± 46)	375 (± 62)	0.227
Sleep efficiency (% per night)	84.05 (± 7.71)	83.83 (± 4.73)	0.938
Sleep latency (minutes per night)	20.01 (± 10.01)	20.59 (± 9.03)	0.826
WASO (minutes per night)	57.38 (± 27.93)	51.20 (± 17.76)	0.554
Avg sleep bout (minutes per night)	17.29 (± 7.61)	18.59 (± 9.51)	0.747
Partial sleep deprivation (minutes per night)	221 (± 15)	217 (± 25)	0.722
Sleep recovery (minutes per night)	450 (± 66)	400 (± 82)	0.175

Table 3.10 Sleep parameters recorded by actigraphy before, during and after the partial sleep deprivation protocol.

3.3.4.2 Partial sleep deprivation does not alter the number of circulating immune cells

Several studies reported that protocols of both acute and partial sleep deprivation induce an increase in WBC count, especially neutrophils [150, 397, 402, 416]. In addition, NK cells have been reported to decrease in sleep deprived subjects [132, 400]. Here the volunteers subjected to the protocol of partial sleep deprivation, both young and elderly, did not display significant changes in the number of immune cells in the circulation as analysed by Repeated Measures General Linear Model (both unadjusted analyses and adjusted for gender and BMI). Only in the young individuals there was a trend towards changes in the number of NK cells in unadjusted analyses (Table 3.11 and figure 3.7).

	YOUNG						ELDERLY					
	unadjusted			adjusted			unadjusted			adjusted		
	F	p	η^2	F	p	η^2	F	p	η^2	F	p	η^2
WBC	1.252	0.313	0.152	0.644	0.513	0.114	0.433	0.547	0.046	0.003	0.965	0.000
Granulocytes	0.524	0.544	0.070	0.936	0.405	0.158	0.446	0.530	0.047	0.017	0.904	0.002
Monocytes	1.793	0.213	0.204	0.041	0.928	0.008	1.820	0.206	0.168	0.661	0.478	0.086
Lymphocytes	2.109	0.188	0.232	0.110	0.760	0.022	1.375	0.278	0.133	1.044	0.367	0.130
G:L	0.827	0.450	0.106	0.051	0.940	0.010	1.157	0.325	0.114	0.025	0.934	0.004
NK cells	3.469	0.059	0.278	1.281	0.306	0.155	0.342	0.678	0.037	0.534	0.549	0.071

Table 3.11 The effect of partial sleep deprivation and recovery on immune cell numbers in the groups of young and elderly volunteers. F, p and η^2 are shown for unadjusted analyses and adjusted for gender and BMI. WBC, granulocytes, monocytes and lymphocytes are measured as number of cells $x10^9$/L, NK cells are measured as % of PBMCs.

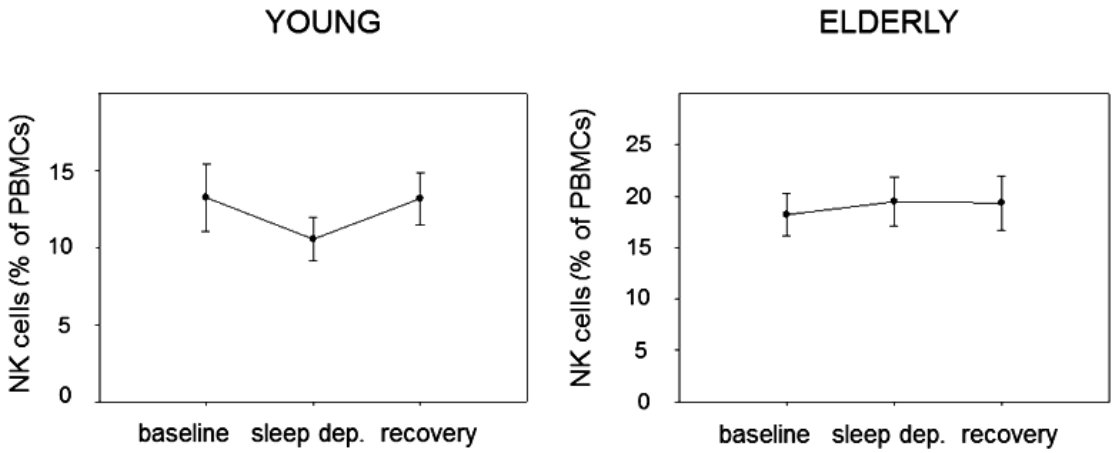


Figure 3.7 The effect of partial sleep deprivation and recovery on the percentage of NK cells. The percentage of NK cells in the PBMC fraction was measured at baseline, after 3 days of partial sleep deprivation and one day after the study in young (n=10) and old (n=10) subjects. Data are mean ± SEM. Two tailed paired T test analyses revealed no statistical differences in NK cell percentage between baseline and partial sleep deprivation in both age groups (young: t(9)=2.074, p=0.068, elderly: t(9)=-0.635, p=0.541). A significant increase in NK cell percentage between partial sleep deprivation and sleep recovery was found in the young group (young: t(9): -2.700, p=0.024, elderly: t(9)=-0.068, p=0.947).

3.3.4.3 Partial sleep deprivation increases neutrophil ROS production in the young

Few studies have looked at the effect of partial sleep deprivation on innate immune cell functions. In this study and in unadjusted analyses the young group showed an increase in neutrophil ROS production after the partial sleep deprivation (p=0.012), which was returned to baseline after the sleep recovery period. The elderly volunteers also showed a trend towards higher neutrophil ROS production after partial sleep deprivation and recovery, although the differences did not reach statistical significance (Table 3.12). Neutrophil phagocytosis was unchanged in both the groups. NK cell cytotoxicity was not significantly affected by the partial sleep deprivation in the young group, whilst it appeared to be persistently reduced after both partial sleep deprivation and recovery in the elderly (unadjusted analyses), although the changes did not reach significance (Table 3.12 and figure 3.8).

3.3.4.4 Partial sleep deprivation affects T cell subset distribution in young but not in elderly individuals

The effect of partial sleep deprivation on T cell subpopulations is poorly fully understood, with the literature reporting this either to be unchanged or increased after sleep disruption [132, 402, 417]. In this study the young volunteers displayed a significant decrease in CD4+ and CD8+ naïve T cell subsets after the day of sleep recovery compared to the value directly after the partial sleep deprivation (unadjusted results). However, the same change was not seen in elderly individuals (Table 3.13, figure 3.9).

	YOUNG						ELDERLY					
	unadjusted			adjusted			unadjusted			adjusted		
	F	p	η^2	F	p	η^2	F	p	η^2	F	p	η^2
Neutrophil ROS production	6.615	**0.012**	0.424	1.620	0.240	0.188	3.690	0.066	0.291	0.369	0.624	0.050
Neutrophil phagocytosis	1.641	0.229	0.154	0.316	0.661	0.043	1.102	0.351	0.109	1.205	0.324	0.147
NK cell cytotoxicity	1.641	0.229	0.154	0.316	0.661	0.043	3.332	0.065	0.994	0.316	0.392	0.124

Table 3.12 The effect of partial sleep deprivation and recovery on innate immune functions. F, p and η^2 are reported for unadjusted analyses and adjusted for gender and BMI. Neutrophil ROS production is reported as MFI, neutrophil phagocytosis is calculated as phagocytic index and NK cell cytotoxicity is measured as the percentage of K562 killed by NK cells.

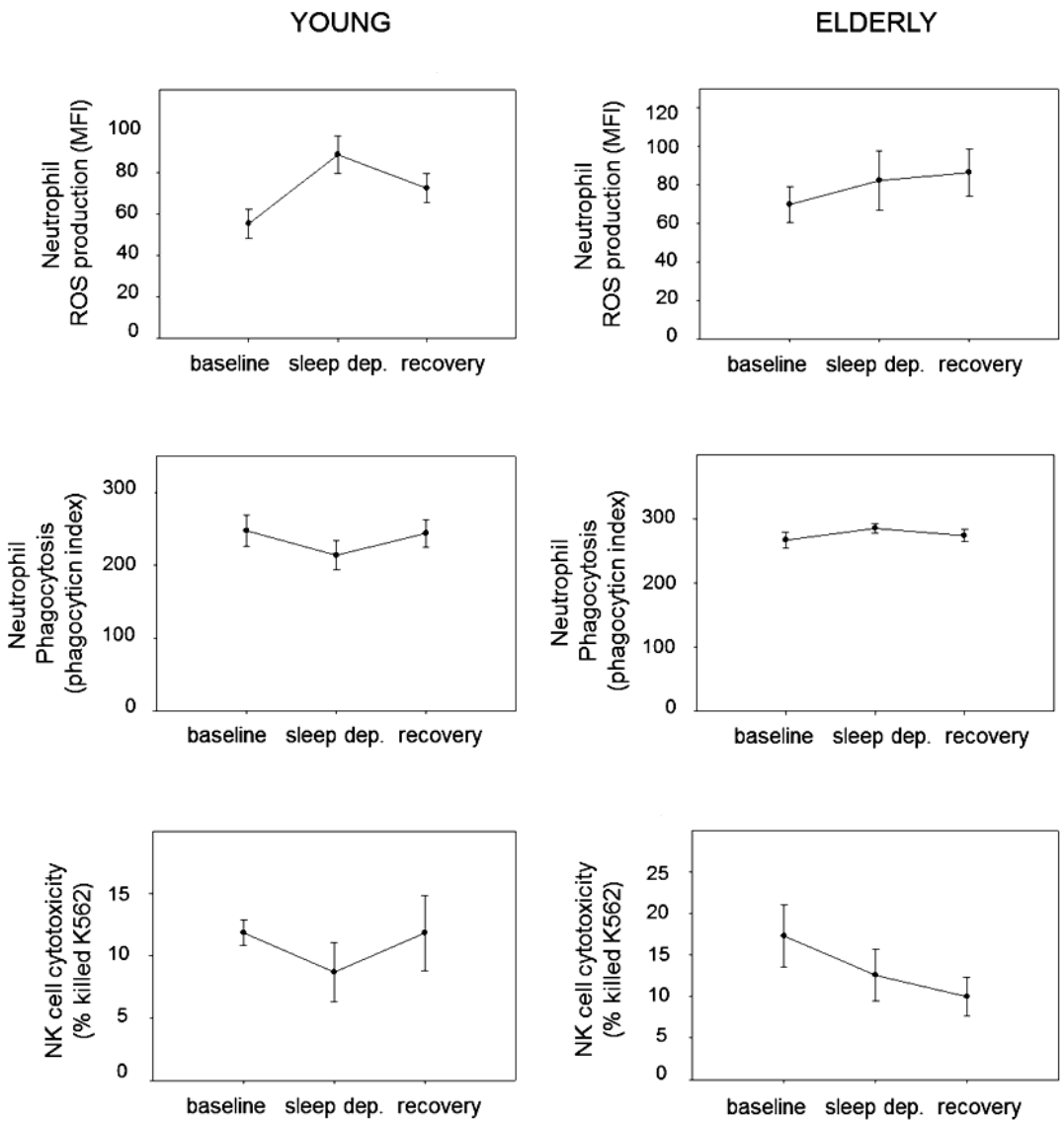


Figure 3.8 The effect of partial sleep deprivation and recovery on innate immune functions. Neutrophil ROS production in response to FITC labelled *E coli* (expressed as MFI), neutrophil phagocytosis of *E coli* expressed as phagocytic index, and NK cell cytotoxicity expressed as % lysis of target cells in young (n=10) and elderly (n=10) subjects at baseline, after 3 days of partial sleep deprivation and one day of recovery. Data are mean ± SEM. Two tailed paired T test analyses confirmed a significant increase in neutrophil ROS production between baseline and partial sleep deprivation in the young, but not in the elderly group (young: t(9)=-3.067, p=0.013, elderly: t(9)=-1.533, p=0.160). No significant differences were observed between partial sleep deprivation and sleep recovery (two tailed paired T test, young: t(9)=1.781, p=0.109, elderly: t(9)=-0.806, p=0.441). A lack of significant differences was also observed for NK cell cytotoxicity (between baseline and partial sleep deprivation: young: t(9)=1.323, p=0.218, elderly: t(9)=1.945, p=0.084) (between partial sleep deprivation and sleep recovery, young: t(9)=-1.985, p=0.078, elderly: t(9)=0.892, p=0.396).

	YOUNG						ELDERLY					
	unadjusted			adjusted			unadjusted			adjusted		
	F	p	η^2	F	p	η^2	F	p	η^2	F	p	η^2
CD3+ T cells	2.245	0.160	0.200	0.921	0.388	0.116	1.457	0.262	0.139	0.382	0.644	0.052
CD4+ T cells	4.746	**0.043**	0.345	0.148	0.761	0.021	1.558	0.244	0.148	0.736	0.432	0.095
CD8+ T cells	1.375	0.278	0.133	1.045	0.372	0.130	3.289	0.094	0.268	2.548	0.152	0.267
CD4+:CD8+ T cells	2.790	0.103	0.237	1.026	0.375	0.128	2.462	0.130	0.215	0.658	0.486	0.086
CD4+ naive T cells	0.504	0.606	0.053	0.974	0.401	0.122	0.940	0.396	0.095	3.900	0.073	0.358
CD8+ naive T cells	7.395	**0.006**	0.451	0.185	0.812	0.026	2.860	0.088	0.241	2.384	0.135	0.254
CD4+ naïve:memory T cells	0.206	0.742	0.022	4.003	0.051	0.364	2.245	0.160	0.200	0.921	0.388	0.116
CD8+ naïve:memory T cells	2.977	0.092	0.249	0.650	0.498	0.085	1.433	0.265	0.137	4.087	0.057	0.369

Table 3.13 The effect of partial sleep deprivation and recovery on the distribution of T cell subsets. F, p and η^2 are shown for unadjusted analyses and adjusted for gender and BMI. The T cell subpopulations values are percentage of PBMCs.

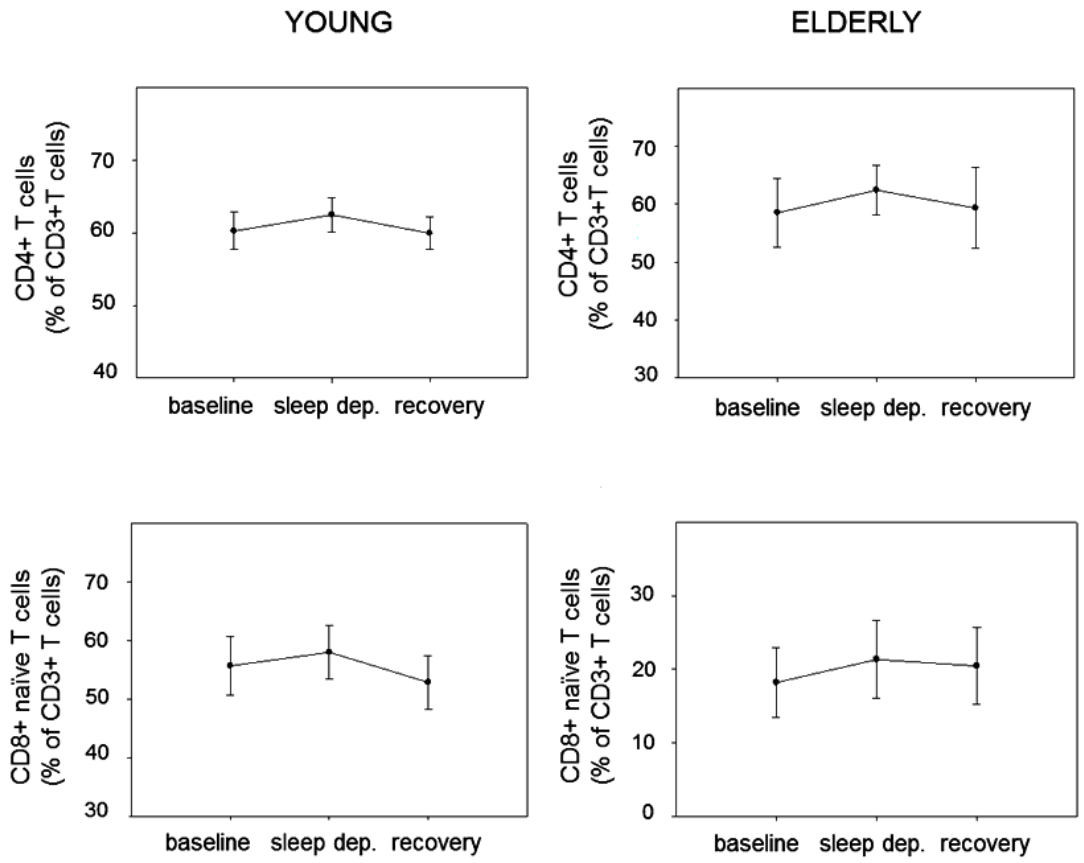


Figure 3.9 The effect of partial sleep deprivation and recovery on CD4+ and CD8+ naïve T cells. Percentages of CD4+ and naïve CD8+ T cells were determined in young and elderly subjects at baseline, after 3 days of partial sleep deprivation and one after sleep recovery. Data are expressed as % of CD3 T cells and are mean ± SEM (n=10). Two tailed paired T test revealed no significant differences in the percentage of CD4+ T cells between baseline and partial sleep deprivation in both the young and the elderly groups (young: $t(9)=-1.879$, $p=0.093$, elderly: $t(9)=-1.890$, $p=0.091$). A significant decrease was observed between baseline and sleep recovery just in the young (young: $t(9)=3.709$, $p=0.005$, elderly: $t(9)=-0.976$, $p=0.354$). No significant differences were observed for the percentage of naïve CD8+ T cells between baseline and partial sleep deprivation in both groups (young: $t(9)=-1.619$, $p=0.140$, elderly: $t(9)=-2.059$, $p=0.070$), while the young showed a significant decrease of this T cell subset after the night of sleep recovery (young: $t(9)=3.513$, $p=0.007$, elderly: $t(9)=0.690$, $p=0.507$).

3.3.4.5 Partial sleep deprivation does not affect serum cytokines

Protocols of acute and partial sleep deprivation have often been shown to cause a rise in circulating levels of pro-inflammatory cytokines, however again the results are often discrepant [133, 402, 416, 418]. Here no significant changes in the levels of any cytokines were observed in either of the groups, with both adjusted and unadjusted analyses (Table 3.14). Analysing the results in more detail, it was noticed that the pro-inflammatory cytokines IL-6 and IL-8 were transiently raised in young individuals after the partial sleep deprivation period, but such a transient increase did not happen in the elderly (Figure 3.10) In addition, the anti-inflammatory cytokine IL-4 was increased in the volunteers after the sleep recovery period (Figure 3.10).

3.3.5 THE EFFECT OF PARTIAL SLEEP DEPRIVATION ON ADIPOKINES AND ADRENAL HORMONES

3.3.5.1 Partial sleep deprivation does not affect levels of leptin and adiponectin

Serum concentrations of adipokines, especially leptin, have been found to be altered by sleep deprivation protocols [167, 169-171, 187]. Thus, leptin and adiponectin were measured but no significant effect was seen of partial sleep deprivation on either adiponectin or leptin levels (Table 3.15 and figure 3.11).

	YOUNG						ELDERLY					
	unadjusted			adjusted			unadjusted			adjusted		
	F	p	η^2	F	p	η^2	F	p	η^2	F	p	η^2
IL-1β (sqrt)	0.365	0.643	0.039	0.756	0.464	0.097	1.642	0.226	0.170	0.099	0.895	0.016
IL-2 (sqrt)	0.503	0.602	0.053	0.760	0.469	0.098	0.517	0.563	0.061	0.158	0.786	0.026
IL-4	0.770	0.414	0.079	0.099	0.773	0.014	0.776	0.405	0.088	2.364	0.174	0.283
IL-5 (sqrt)	0.289	0.648	0.031	0.185	0.732	0.026	0.919	0.386	0.103	0.372	0.620	0.058
IL-6 (sqrt)	1.321	0.291	0.128	1.101	0.351	0.136	0.328	0.681	0.039	0.063	0.902	0.010
IL-8	0.548	0.562	0.057	1.599	0.244	0.186	0.195	0.754	0.027	0.315	0.689	0.059
IL-10 (sqrt)	0.634	0.500	0.073	0.735	0.462	0.109	0.060	0.888	0.008	0.002	0.989	0.000
GM-CSF (sqrt)	0.032	0.968	0.004	0.369	0.693	0.050	0.180	0.780	0.025	0.177	0.703	0.034
IFN-γ (sqrt)	0.676	0.502	0.070	0.428	0.627	0.058	0.125	0.810	0.015	0.914	0.394	0.132
TNF-α (sqrt)	0.459	0.353	0.049	0.594	0.477	0.078	0.692	0.434	0.080	0.299	0.611	0.048

Table 3.14 The effect of partial sleep deprivation and recovery on serum concentration of cytokines. F, p and η^2 are shown for unadjusted analyses and adjusted for gender and BMI. Concentrations of all cytokines are in pg/ml.

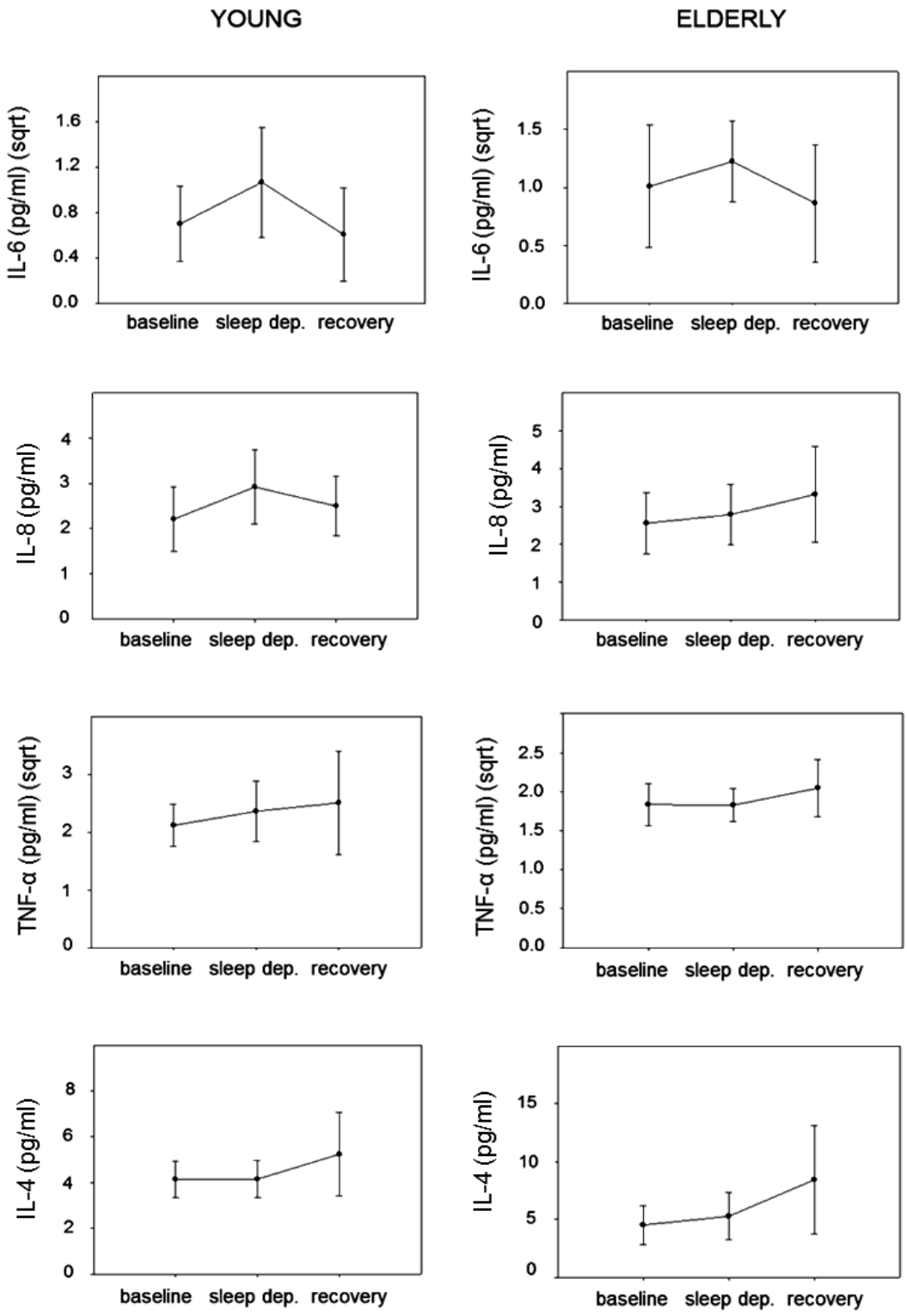


Figure 3.10 The effect of partial sleep deprivation and recovery on serum IL-4, IL-6, IL-8 and TNF-α levels. Serum IL-4, IL-6, IL-8 and TNF-α were determined by multiplex in young and elderly subjects at baseline, after 3 days of partial sleep deprivation and one after sleep recovery. Data are mean ± SEM (n=10). Two tailed paired T test revealed no significant differences between baseline and partial sleep deprivation and between partial sleep deprivation and sleep recovery (data not shown).

	YOUNG						ELDERLY					
	unadjusted			adjusted			unadjusted			adjusted		
	F	**p**	**η^2**	**F**	**p**	**η^2**	**F**	**p**	**η^2**	**F**	**p**	**η^2**
Leptin	1.133	0.344	0.112	0.112	0.864	0.016	1.023	0.369	0.102	0.143	0.829	0.020
Adiponectin	1.101	0.342	0.109	3.955	0.060	0.361	0.865	0.416	0.088	0.256	0.729	0.035

Table 3.15 The effect of partial sleep deprivation and recovery on serum concentration of leptin and adiponectin. F, p and η^2 are shown for unadjusted analyses and adjusted for gender and BMI. Concentrations of leptin and adiponectin are ng/ml and µg/ml respectively.

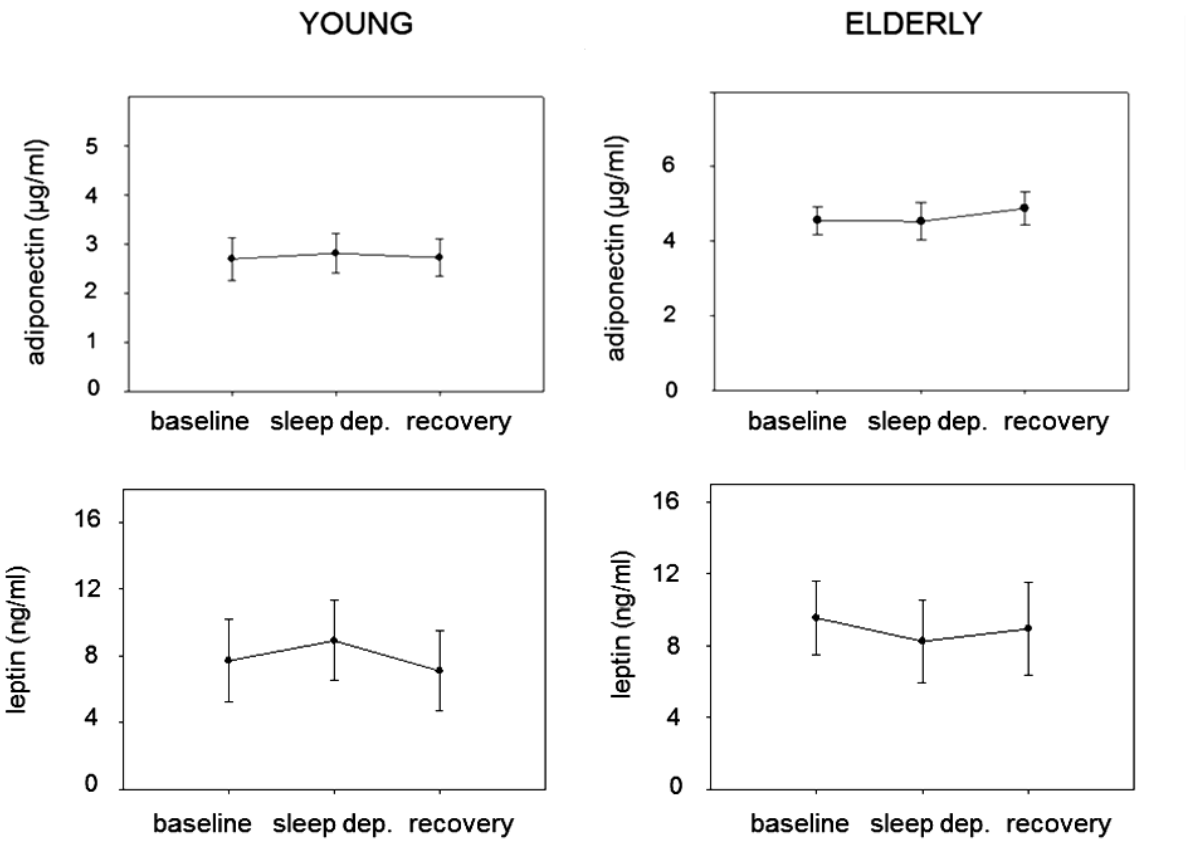


Figure 3.11 The effect of partial sleep deprivation and recovery on adiponectin and leptin. Serum levels of adiponectin and leptin were determined by ELISA in young and elderly subjects at baseline, after 3 days of partial sleep deprivation and one after sleep recovery. Data are mean ± SEM (n=10). Two tailed paired T test revealed no significant differences between baseline and partial sleep deprivation and between partial sleep deprivation and sleep recovery (data not shown).

3.3.5.2 Sleep recovery decreases the levels of cortisol

Studies of partial sleep deprivation have reported that cortisol is usually decreased in the morning following the experimental protocol [170, 418, 422], whereas in the evening of the same day it appears elevated, suggesting a decreased functionality of the negative-feedback mechanism driven by this hormone [148, 423]. In this study both young and elderly volunteers showed a trend towards lower morning cortisol level after partial sleep deprivation though this did not reach significance (Table 3.16 and figure 3.12). When young and elderly data were analysed together as one single group, the unadjusted analysis became significant for reduced cortisol (unadjusted analysis: $F=3.805$, $p=0.038$, $\eta^2=0.167$; analysis adjusted for age, gender and BMI: $F=0.620$, $p=0.533$, $\eta^2=0.037$). Serum DHEAS and the C:D ratio were not significantly affected by the partial sleep deprivation protocol (Table 3.16).

	YOUNG						ELDERLY					
	unadjusted			adjusted			unadjusted			adjusted		
	F	**p**	**η^2**	**F**	**p**	**η^2**	**F**	**p**	**η^2**	**F**	**p**	**η^2**
Cortisol	3.192	0.073	0.262	0.192	0.807	0.027	1.593	0.237	0.150	1.791	0.219	0.204
DHEAS	0.003	0.988	0.000	0.332	0.662	0.045	0.097	0.827	0.011	3.237	0.101	0.316
C:D	0.919	0.363	0.093	3.413	0.107	0.328	0.853	0.383	0.087	0.371	0.571	0.050

Table 3.16 The effect of partial sleep deprivation and recovery on serum concentration of cortisol, DHEAS and the ratio C:D. F, p and η^2 are shown for unadjusted analyses and adjusted for gender and BMI. Concentrations of cortisol and DHEAS are measured as ng/ml.

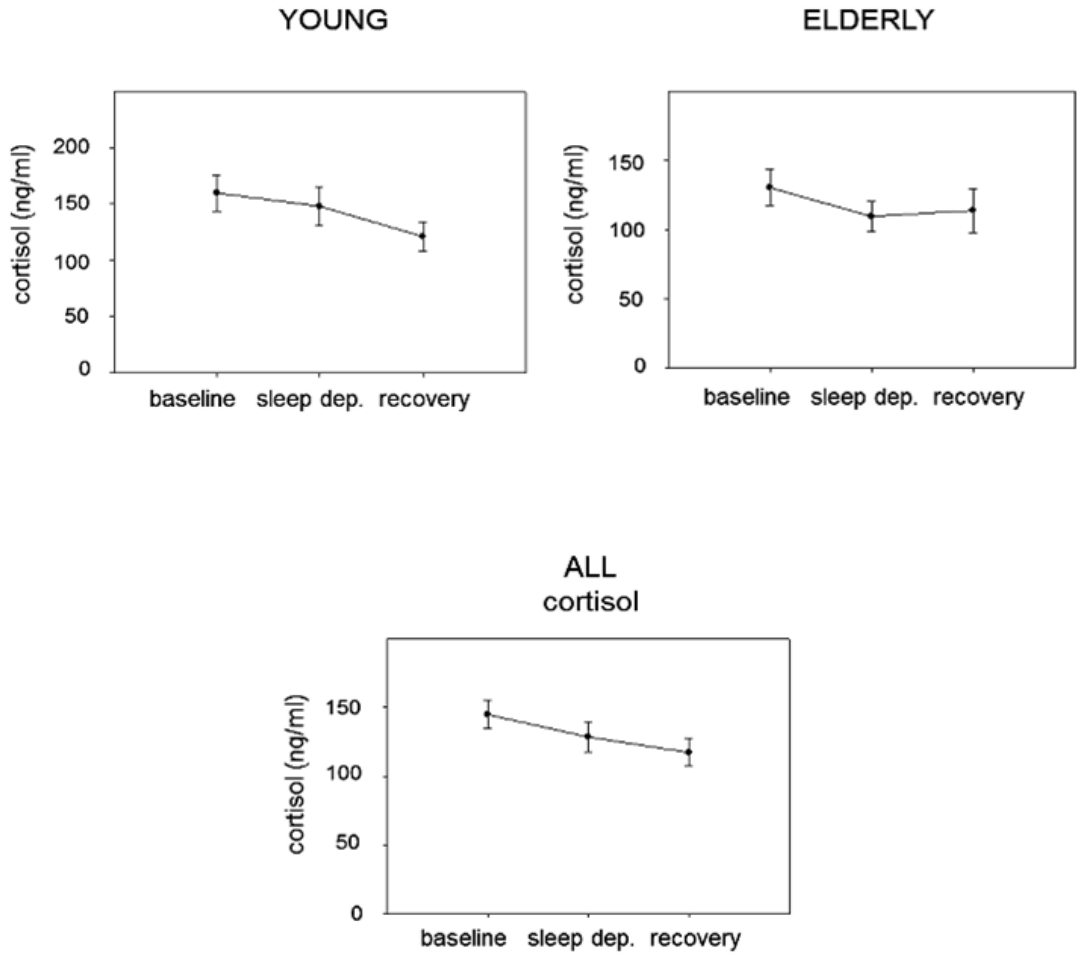


Figure 3.12 The effect of partial sleep deprivation and recovery on serum cortisol. Serum cortisol was determined by ELISA in young and elderly subjects at baseline, after 3 days of partial sleep deprivation and one after sleep recovery. Data are mean ± SEM (n=10). Two tailed paired T test analyses revealed a significant drop in cortisol level between baseline and partial sleep deprivation in the elderly but not in the young group (young: $t(9)=0.714$, $p=0.493$, elderly: $t(9)=2.303$, $p=0.047$). No significant difference between the partial sleep deprivation and sleep recovery was observed (young: $(t)=1.536$, $p=0.159$, elderly: $(t)=-0.290$, $p=0.779$). Interestingly, there was a significant decrease in cortisol level between baseline and sleep recovery in the young group (young: $(t)=3.070$, $p=0.013$, elderly: $(t)=1.376$, $p=0.202$). Similarly, when the whole group was considered, the significant decrease in cortisol between baseline and sleep recovery was reproduced ($t(19)=3.122$, $p=0.006$).

3.4 DISCUSSION

3.4.1 SLEEP AND THE IMMUNE SYSTEM

Ageing is characterised by a physiological decline in the quantity and continuity of sleep, with a reduction in total time spent asleep, specifically SWS and REM sleep duration, an increment in fragmentation and an advance in the sleep-wake cycle [512-514, 516-519, 619, 620]. During ageing the immune system also undergoes physiological changes, namely a general impairment in immune functions (immunosenescence) which affects both innate and adaptive arms of immunity [559, 582], and a chronic, persistent increase in the levels of inflammatory cytokines (inflammaging) [507]. To understand whether sleep disruption could negatively impact upon the immune system in old age, several immune variables were evaluated in relation to sleep duration and continuity in a cohort of healthy elderly subjects, as well as in subjects undergoing a session of partial sleep deprivation. Despite significant associations observed in a few immune and endocrine parameters, our elderly volunteers did not show major changes in relation to both physiological and forced sleep disruption. Therefore, sleep dysregulation does not appear to be linked in any major way to immunosenescence.

With regard to the cohort of elderly participants recruited, the age range was sufficiently broad (from 62 to 93 years old) and average age sufficiently old (40% of the participants were between 70 and 80) for the study of immunosenescence, as the decline of immune functions is observed as early as the age of 60 [629]. However, the sleep parameters displayed by the cohort recruited were very uniform. In particular, the sleep duration observed within the middle tertile ranged from 6.36 to 7.27 hours per night and the majority of the participants (n=35) had an average nocturnal sleep duration between 6.30 and 7.30 hours. Only two volunteers had an average sleep duration slightly below five hours (minimum: 4.56 hours per night) and amongst the long sleepers, only two participants slept more than 8.30 hours and just one displayed more than 9 hours of sleep duration. With such a narrow variability it is more difficult to find significant associations by use of linear regressions as well as to detect

significant differences when tertiles of sleep duration are analysed. The variables showing the highest variability were the parameters of sleep fragmentation WASO and average sleep bout.

The average sleep duration showed by our participants is in line with other studies [515, 516, 630, 631] but when the baseline sleep parameters were compared between the 10 older and 10 younger volunteers before undergoing the session of partial sleep deprivation, no significant differences in any of the sleep parameters considered were found. This suggests that both sleep duration and continuity were not disturbed by the ageing process in the older adults recruited. The overall preserved continuity of sleep presented by this cohort could explain the lack of association between sleep and immunosenescence.

3.4.1.1 Sleep and immune cell numbers

In this study physiological sleep duration and efficiency were found to be negatively associated with the number of WBC, granulocytes, monocytes and the G:L ratio. Two parameters of sleep fragmentation, WASO and average sleep bout, were also associated with the G:L ratio. Moreover, the group of long sleepers had significantly lower numbers of lymphocytes. This is the first study finding such a relationship between blood cell counts and physiological sleep, one other study reported that individuals having the habit of going to sleep earlier had lower numbers of granulocytes and higher numbers of lymphocytes compared to individuals habitually going to sleep later, but no associations were found between cell counts and sleep duration [632].

Elevated counts of circulating WBC, granulocytes and a raised G:L ratio are distinct characteristics of a pro-inflammatory status [622-624]. These biomarkers are also associated with development of chronic inflammatory conditions such as cardiovascular and metabolic diseases [622, 633-635] and with increased risk of mortality [624, 625, 636]. Therefore, from the data obtained it could be proposed that short sleep duration and poor sleep continuity may contribute to inflammaging. However, in our elderly cohort none of the pro-inflammatory

cytokines was raised in the short sleepers, or associated with sleep duration in adjusted analyses. The level of the anti-inflammatory cytokine IL-4 was significantly lower in short sleepers compared to long sleepers when analyses were adjusted for use of anti-hypertensive medications, whereas IL-6 was not raised in short sleepers but also showed a trend towards lower values only in long sleepers. However, the level of the anti-inflammatory cytokine IL-10 also negatively correlated with the time spent asleep and it is possible that this cytokine contributes to the trend towards lower IL-6 in the long sleepers. From these data it cannot be inferred that the pro-inflammatory status is more pronounced in short sleepers and more likely that longer sleep is anti-inflammatory.

In parallel we determined that long sleepers have lower numbers of all the immune cell types considered, which could contribute to the higher risk of mortality observed in long sleepers [5-11]. These associations between sleep and immune cell counts could be caused by changes in the hematopoietic process, specifically with regard to myeloid and lymphoid differentiation. The hematopoietic compartment in bone marrow is known to be affected by ageing, with a characteristic skewing towards myelopoiesis [547, 548]. How sleep duration could affect such changes has not been investigated here, but corticosteroids are known to modulate hematopoiesis [637], though the single and modest association between cortisol and average sleep bout would not support this hypothesis.

The majority of studies of partial sleep deprivation reported that short sleep duration induces an increase in WBC, especially neutrophils [150, 402, 415, 416], except for one study by Irwin et al., which showed the percentage, but not the number of granulocytes to be decreased following a single night of partial sleep deprivation [400]. Here sleep-deprived participants did not show any significant changes in the number of circulating immune cells in both young and elderly volunteers subjected to 3 nights of partial sleep deprivation. This could be due to both the small number of volunteers recruited and the individual response towards this stressful condition. Indeed for many of the parameters tested data approached but did not reach

significance suggesting that the study was inadequately powered. This study was in fact very much a pilot study and before any firm conclusions can be made the study needs to increase its sample size, with a power calculation made using the variance figures established in this study. In fact, the post-hoc power analyses conducted showed a low power value of less than 0.4. Further analysis indicated a required sample size of 34 individuals in total in order to achieve a medium effect size of 0.5 using t test with power at 0.80 and alpha at 0.05.

3.4.1.2 Sleep and innate immune functions

As sleep influences the risk of morbidity and mortality, this study aimed to determine whether poor sleep quantity and continuity could affect innate immune functions therefore contributing to immunosenescence. Neutrophil functions (ROS production and phagocytosis) and NK cell cytotoxicity were assessed. No changes have been found apart from lower neutrophil ROS production in subjects with shorter average sleep bout. Intriguingly, the tertile of middle sleep duration showed a trend towards higher neutrophil phagocytosis compared to both short and long sleepers, potentially indicating a healthier condition of this group, although differences were not statistically significant. The overall results indicate that physiological sleep does not have a substantial influence on neutrophil and NK cell functions therefore it does not contribute significantly to innate immunosenescence in the elderly.

Few studies have looked at the effect of sleep deprivation on neutrophil functions. One study found that acute sleep deprivation decreased neutrophil phagocytosis [406] while another study showed that individuals subjected to continuous exercise and partial sleep deprivation showed an increment in neutrophil chemotaxis [402]. Here the protocol of partial sleep deprivation did not induce substantial changes in the immune functions considered. The unadjusted analyses suggested that neutrophil ROS production was significantly increased after the partial sleep deprivation and it decreased after the day of sleep recovery in the young group, whilst in the elderly the increase in this function was not significant. These data could be interpreted as a consequence of the process of immunosenescence, resulting in neutrophil ROS production in

the elderly which is refractory to the increase induced in the young after exposure to a stressor.

NK cell cytotoxicity appears to vary according to the protocol of sleep deprivation used: it increases in individuals subjected to acute sleep deprivation [329, 396] whereas it decreases after a protocol of partial sleep deprivation [399, 400]. NK cell cytotoxicity was not affected by sleep continuity or duration in the healthy elderly but showed a strong trend towards a decrease after the partial sleep deprivation and interestingly there was no recovery after one night of normal sleep. In particular this result may indicate that NK cell function is susceptible to partial sleep deprivation in the elderly and is less flexible and cannot recover as quickly. Again the data did not quite reach significance but an increased sample size could confirm this potentially important finding.

3.4.1.3 Sleep and T lymphocytes

Adaptive immunosenescence is characterized by a reduction in the thymic output of naïve T cells [581, 582]. Moreover, a decrease in the ratio between helper CD4+ and cytotoxic CD8+ T cells is often reported in old age, possibly due to persistent subclinical viral infections [583, 584]. Importantly, a low CD4:CD8 ratio is associated with higher risk of mortality [586, 587]. In this study the percentage of CD4+ cells and the CD4+:CD8+ T cell ratio negatively correlated with sleep efficiency and was positively associated with WASO. In addition, both CD4+ and CD8+ naïve T cells and the naïve:memory CD4+ T cell ratio significantly decreased with increased time spent alseep. These results were unexpected as inadequate sleep continuity (poor sleep efficiency and high sleep WASO) was associated with an increased circulating pool of CD4+T cells and the CD4+:CD8+ T cell ratio. In addition, short sleep duration was associated with increased numbers of circulating naïve CD4+ and CD8+ T cells, as well as a higher CD4+ naïve:memory T cell ratio. Therefore, with regard to adaptive immunity a physiological lack of sleep seems to be beneficial and would not contribute to the development of immunosenescence.

However, in the elderly partially deprived of sleep no such changes were observed, though the adaptive system of elderly individuals might be less sensitive to temporary changes in sleep duration. In the literature few studies have reported that partial sleep deprivation caused an increase in the percentage of both CD4+ and CD8+ T cells [417], although other studies did not show any changes [132]. The differences in the protocols administered, the age and gender of the volunteers recruited might all contribute to the discrepancies between our study and the literature.

3.4.1.4 Sleep and cytokines

Cytokines are inflammatory mediators whose role in regulating sleep has been recently determined. In general, pro-inflammatory cytokines (mainly TNF-α and IL-1β) are believed to enhance sleepiness, increase sleep time and specifically SWS duration [371-373]. Importantly, elderly individuals are characterised by a persistent, low grade pro-inflammatory status (inflammaging), with a raised level of pro-inflammatory cytokines and a decrease in anti-inflammatory cytokines [600, 601]. In this study the level of the pro-inflammatory cytokine IL-8 was negatively associated with sleep duration, though this disappeared when analysis was adjusted for use of statins. Importantly, we found that long sleepers were characterised by higher levels of the anti-inflammatory cytokine IL-4 after adjustment for medications. As discussed above, the remaining cytokines showed no association with sleep duration and a case cannot be made for altered sleep contributing to inflammaging in this cohort of elderly subjects. In the literature a few epidemiological studies conducted on elderly adults (>50 years old) showed contrasting results: in a longitudinal study, Ferrie et al. found that sleep duration between 7 and 8 hours was associated with lower levels of IL-6 and CRP compared to <5 or >9 hours of sleep [638], whereas Dowd and colleagues reported that individuals sleeping over 8 hours per night had increased concentration of IL-6 [639]. These analyses were based on self-reported sleep duration. Interestingly, in a younger cohort of individuals long self-reported sleep duration was associated with increased levels of CRP and IL-6, however in

the same individuals polysomnographic short sleep duration significantly correlated with higher levels of TNF-α with no associations with IL-6 and CRP [640]. These inconsistent findings are roughly in line with the lack of association with cytokines revealed by our analyses. They also strengthen the importance and novelty of the few associations found here, as there are no studies evaluating levels of inflammatory markers with physiological sleep measured by actigraphy in the elderly. Intriguingly, serum levels of both IL-8 and IL-6 negatively correlated with sleep latency. This could mean that long sleep latency decreases inflammation, however this is more likely to indicate that these cytokines promote sleepiness therefore they reduce the onset latency of sleep. In the literature there are data to support a pro-somnogenic role of both IL-6 [393] and IL-8 [641] as their administration enhanced NREM/SWS duration in humans (IL-6) and rabbits (IL-8). However, IL-6 has also been shown to negatively correlate with SWS and sleep efficiency in humans [642-644] and no data are available regarding the relationship between these cytokines and sleep latency. Thus, the interpretation of these results is premature and more studies are needed to clarify the role of these cytokines on the regulation of sleep.

Studies of partial sleep deprivation have reported conflicting results regarding the levels of cytokines [133, 402, 416, 418, 419]. Due to the low number of participants and the inter-individual variability in response to partial sleep deprivation, no significant differences were seen in the levels of any of the 10 cytokines measured, including TNF-α which has a well documented effect on sleep. Nevertheless, young individuals were characterised by a trend towards increased IL-6 and IL-8 after partial sleep deprivation, whereas these cytokines remained unchanged in the elderly. This might also explain the transient increase in neutrophil ROS production in sleep-deprived young subjects, as both IL-6 and IL-8 prime neutrophils for enhanced oxidative burst [645, 646].

3.4.2 SLEEP, METABOLISM AND ADRENAL HORMONES

3.4.2.1 Relationship between sleep, BMI and adipokines

A U-shaped relationship between BMI and sleep duration has been reported in both the general [109] and the elderly population [134, 135]. In the cohort of elderly subjects studied here there were no significant differences in the BMI amongst tertiles of sleep duration, however after controlling for use of statins, BMI was significantly lower in the middle tertile of sleep duration, in agreement with the previous studies [109, 134, 135].

Levels of leptin, which correlated with BMI and also varied with the use of statins, did not change in relation to any of the sleep parameters considered, contrary to epidemiological studies showing lower levels of leptin in self-reported short sleepers [109, 165]. With regard to the anti-inflammatory adipokine adiponectin, its concentration was significantly higher in long sleepers. This finding is in agreement with Kotani et al., who found a positive association between adiponectin levels and self-reported sleep duration in men [188], but such an association was not observed in the large cohort investigated by Taheri and colleagues [109]. Interestingly serum adiponectin concentration has been reported to be negatively associated with levels of several pro-inflammatory cytokines *in vivo* (such as TNF-α and IL-6) in obese people as well as in subjects suffering from other inflammatory diseases [190, 196, 494]. In addition, IL-6 and TNF-α are known to inhibit adiponectin expression by adipocytes [196-198]. Therefore, the higher levels of adiponectin found in long sleepers are in line with the finding of a trend towards a lower IL-6 level in long sleepers.

Leptin serum concentration has been reported both to be decreased [167]and increased in partially sleep deprived individuals [169]. With regard to adiponectin, a single study showed that a protocol of partial sleep deprivation caused a decrease in adiponectin concentration in Caucasian but not African women [187]. In this study, despite the different levels of adiponectin in elderly long sleepers, partial sleep deprivation did not cause any change in its concentration in both young and elderly groups. Similarly, leptin levels were not affected by

the experimental protocol. This might suggest that a longer period of partial sleep deprivation is required in order to alter the levels of adiponectin or leptin.

3.4.2.2 Relationship between sleep, cortisol and DHEAS

Cortisol is released in a pulsatile and diurnal manner by the adrenal gland [65, 74]. In particular cortisol level shows a transitory, elevated peak early in the morning after awakening [74-76]. Its concentration also increases in response to stress [65] and it is believed to contribute to frailty and immunosenescence in the elderly, processes which are antagonised by the other adrenal steroid DHEAS which acts as an immune enhancer [536].

It is well established that the hormones belonging to the HPA axis regulate physiological sleep [51] with a marked anti-somnogenic role for CRH [51, 94] which induces peripheral production of cortisol through ACTH secretion [65]. However disturbed sleep could represent itself a source of stress which might influence the production of cortisol, or the altered HPA axis which occurs with age leading to a raised C:D ratio [647] could contribute to altered sleep. In this study cortisol did not associate with the time spent asleep. Similarly, a lack of association between awakening salivary cortisol levels and actigraphic sleep duration was previously reported in middle-aged subjects [648]. This could suggest either that the age-related changes in HPA axis activation are not sufficient to affect sleep duration, or that physiologically short or long sleep duration is not a sufficiently stressful condition to raise the levels of this stress hormone. However, higher WASO and shorter average sleep bout were associated with increased levels of cortisol, although only a significant correlation between cortisol and average sleep bout persisted after correction for use of anti-hypertensive drugs. These data could imply that highly fragmented sleep represents a condition of stress able to enhance cortisol levels, or that the increased HPA axis in older adults results in sleep fragmentation.

The protocol of partial sleep deprivation induced only minimal changes in the levels of cortisol in both young and elderly individuals, which did not reach significance until the two data sets were combined. However, cortisol levels decreased progressively after the partial sleep deprivation and recovery of sleep rather than increased. These results were not completely unexpected, as morning levels of cortisol have been reported to be decreased both in serum [422] and saliva [170] following sessions of partial sleep deprivation. As the blood was taken at the same time in the morning (between 9 a.m. and 11 a.m.) and the volunteers woke up earlier after the partial sleep deprivation (5 a.m.) compared to the baseline day, the decrease in cortisol levels following the partial sleep deprivation could be the consequence of the different time between awakening and blood taking, cortisol levels being dependent on the time of awakening [75, 77]. In fact, previous works showed that cortisol level peaked earlier after partial sleep deprivation protocols, but the value was lower compared to the baseline value [418, 420]. This decrease in cortisol level could be considered a beneficial effect for immune functions; however we did not measure the concentration of this hormone at other times during the day. Other groups reported cortisol levels to be significantly elevated in the evening following the partial sleep deprivation period [148, 423], suggesting a dysregulation of the HPA system and its circadian rhythm.

Moreover, cortisol levels decreased further in response to sleep recovery in the volunteers. This result is in agreement with Pejovic et al. [420] who found a reduction in the 24-hour profile of cortisol after sleep recovery compared to baseline, but not with the study by Wu et al. in which morning cortisol concentration returned to baseline following a day of sleep recovery [422].

3.5 LIMITATIONS OF THE STUDIES

To investigate the relationship between sleep behaviour and the immune system in old age, a cohort of 93 individuals was recruited. The major limitations characterising this study are

related to the small number of volunteers taking part, compared to other epidemiological studies, and the amount of occasional missing data, such as information missing for BMI, use of medications, blood cell counts and results from immunological and hormonal assays (i.e. neutrophil and NK cell functions, concentration of hormones and cytokines). Because of the lack of these data, the analyses could not be fully controlled for the whole cohort. In addition, the analyses performed were not controlled for the existence of multicolinearity between sleep variables, which may have further affected the results.

With regard to the protocol of partial sleep deprivation, the major limitation was represented by the low number of volunteers recruited in both groups. Importantly, participants were not kept in the same controlled standard conditions (i.e.: in hospital) during the partial sleep deprivation session, as they were allowed to conduct their every-day life as normal. This means that they were free to eat what they preferred, they could perform more or less physical activity according to their needs, and all these variables could have influenced the outcomes of this study. Moreover, some of the elderly volunteers reported to use anti-hypertensive drugs and/or statins: statistical analyses were not adjusted for use of medications due to the small sample size and the pilot nature of the study.

Finally, in both studies it would have been useful to measure the chronotype of the subjects (i.e. diurnal or nocturnal preference) and the daily intake of caffeine, as these variables might have influenced the results. In addition, measuring the level of other circulating factors, such as GH, ghrelin and catecholamines, could have helped to give a better interpretation of the results, as these hormones have effects on the regulation of sleep, response to stress and influence immune functions.

3.6 CONCLUSIONS

In summary, it can be concluded that physiological sleep duration and continuity exert an influence on the immune system though this is not major. There were associations with the

distribution of circulating immune populations, the G:L ratio and T cell subsets, as well as inflammatory status (main effect seen for IL-4 levels), though this was most marked for the anti-inflammatory adipokine adiponectin rather than classical cytokines. There were no major effects on innate immune functions, other than neutrophil ROS production. Sleep fragmentation was associated with activation of HPA axis as determined by increased cortisol levels. Therefore, physiological sleep does not appear to significantly contribute to immunosenescence but it could contribute to promote inflammaging. The data obtained from the partial sleep deprivation study were not conclusive due to small sample size and thus causality for any of these parameters has not been established.

CHAPTER 4

THE EFFECT OF ADIPONECTIN ON NEUTROPHIL APOPTOSIS AND PHAGOCYTOSIS

4.1 INTRODUCTION

Neutrophils are the most abundant immune cell in the blood, representing the first line of defence against microbial pathogens. Thanks to their chemotactic ability, these cells move towards the site of infection where they contribute to the removal and the killing of pathogens through the processes of phagocytosis, degranulation, the production of ROS and the generation of NETs [242, 244]. As reported for other immune cells, neutrophils are negatively affected by ageing, with impairments described in many of their defensive strategies such as: phagocytosis [556], chemotaxis and ROS production [551, 557, 558].

Neutrophils are post mitotic cells characterized by a very short lifespan, undergoing constitutive apoptosis approximately 5 days after leaving the bone marrow [214]. However, their lifespan is increased at sites of infection by a range of factors including the bacterial product LPS [216], pro-inflammatory cytokines [259] and hypoxia [217]. The tight regulation of apoptosis and the prompt removal of apoptotic neutrophils are central to the resolution of the inflammatory response and prevention of tissue damage and chronic inflammation [258]. In contrast neutrophil apoptosis does not seem to be altered during ageing [551].

Adiponectin is the most abundant adipokine present in the blood, its serum concentration is between 5 and 10 μg/ml [426]. The full-length isoform has aroused increasing interest because of its insulin-sensitising [649], anti-atherosclerotic [650] and anti-inflammatory properties [424]. In particular adiponectin prevents LPS-induced ALI in mice [489], protects against LPS-induced liver injury in obese mice models [651] and inhibits neutrophil oxidative burst *in vitro* [451]. However, some groups have reported pro-inflammatory functions of adiponectin on dendritic cells [652] and macrophages [478]. Adiponectin appears to exert many of these actions through activation of AMPK, with phosphorylation of AMPK shown to increase following treatment with adiponectin in several cell types including phagocytes [451].

As adiponectin has immune regulatory effects changes in its levels with age could contribute to immunosenescence, including reduced neutrophil function. As described in chapter 1, adiponectin levels have been reported to increase with age [442, 611, 612].

4.1.1 HYPOTHESIS AND AIMS

Contradictory results exist in relation to the effect that adiponectin has on apoptosis [450, 653-657]. Importantly, an association between low serum adiponectin concentration and high levels of apoptotic markers in the blood was recently reported [473]. The effect of adiponectin on neutrophil phagocytosis and apoptosis has not been investigated, though any modulatory effect could contribute to a pro- or anti-inflammatory role for this adipokine. Thus, the aim of this chapter was to assess whether neutrophil apoptosis or phagocytosis are influenced *in vitro* by adiponectin and to define the mechanism through which adiponectin acts. Since adiponectin is mainly considered an anti-inflammatory adipokine and has already been demonstrated to inhibit the oxidative burst in neutrophils, it was hypothesized that it would decrease neutrophil phagocytosis and increase their constitutive apoptosis.

4.2 METHODS

The methods used for the various assays, assessment of neutrophil apoptosis and analysis of adiponectin effects on signalling pathways are described in chapter 2. The other protocol details specific to studies in this chapter are described here.

4.2.1 SUBJECTS

Heparinized and anticoagulant-free peripheral blood was obtained from healthy young (< 35 years old) and elderly (> 60 years old) human donors and all volunteers gave written informed consent prior to their participation. Neutrophils were isolated as previously described (Chapter 2) and the effects of adiponectin on their function and survival determined.

4.2.2 NEUTROPHIL TREATMENTS

To exclude unwanted effects from contamination of adiponectin with LPS, cells were treated with Polymyxin B (10 μg/ml) (Millipore, Billerica, MA) 30 minutes before addition of adiponectin (Enzo Life Sciences, Farmingdale, NY) at the physiological concentration of 10 μg/ml [425, 426], unless otherwise specified.

To study phagocytosis, neutrophils were re-suspended in RPMI-1640 + GPS in the absence of serum to exclude possible interference by adiponectin in the serum. As the study of neutrophil apoptosis requires longer incubation times, for these experiments neutrophils were re-suspended in CM. Serum-free medium was also used for comparison (Corning® SF Medium, Cellgro®, Mediatech Inc., Manassas, VA).

Pharmacological protein kinase inhibitors and activators were employed to determine the signalling pathways triggered by adiponectin and their role in adiponectin mediated regulation of phagocytosis and apoptosis. Specific inhibitors were added to neutrophil culture 30 minutes before the addition of adiponectin. The equivalent volume of diluent (water or DMSO) was added to control wells. In table 4.1 the drugs and the respective concentrations used are listed.

COMPOUND	FINAL CONCENTRATION
AICAR (Enzo Life Sciences)	1 mM
Compound C (Sigma-Aldrich)	10 μM
LY294002 (Millipore)	10 μM
PD98059 (Cell Signaling Technology, Beverly, MA)	10 μM
SB202190 (Sigma-Aldrich)	10 μM
Cycloheximide (Sigma-Aldrich)	5 μM

Table 4.1 List of compounds and concentrations used to inhibit or activate specific pathways.

4.3 RESULTS

4.3.1 ELDERLY SUBJECTS SHOW HIGHER SERUM LEVEL OF ADIPONECTIN

Serum adiponectin levels have been reported to increase with age [611, 612, 658]. To confirm this finding we compared the concentration of this adipokine in serum samples obtained from young and elderly volunteers. In agreement with the literature, the results show a marked increase of adiponectin serum levels in elderly subjects compared to the young ($p<0.001$) (Figure 4.1).

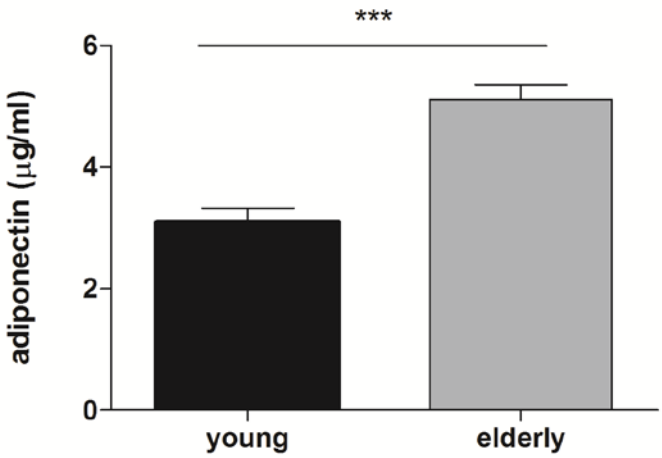


Figure 4.1 Elderly subjects display high serum levels of adiponectin. Concentration of adiponectin was assessed by ELISA in serum samples obtained from young (n=57) and elderly (n=98) individuals. Data are expressed as mean ± SEM. *** indicates $p<0.001$.

4.3.2 NEUTROPHIL SURFACE EXPRESSION OF ADIPONECTIN RECEPTORS

The expression of AdipoR1 and AdipoR2 by neutrophils is still a matter of debate. One study found a high expression of both AdipoR1 and AdipoR2 mRNA in neutrophils [429], whereas another study failed to detect AdipoR2 at both the protein and mRNA level [451]. Therefore, the protein expression of AdipoR1 and AdipoR2 was measured on neutrophil membranes by indirect immunostaining and flow cytometry (Figure 4.2). The two receptors were found to be highly expressed both in young and elderly subjects and modest but significant differences were found in the percentage of neutrophils expressing AdipoR2 with age (84.1% ± 2.5 for young vs 93.6% ± 1.1 for elderly, p=0.003) and between young males and females (78.7% ±2.9 for young males versus 89.4 ± 2.3 for young females, p=0.020) (Table 4.2).

4.3.3 ADIPONECTIN DELAYS NEUTROPHIL APOPTOSIS

Recently it has been demonstrated that adiponectin plays a significant role as a hematopoietic factor, not only in the maintenance of the pool of hematopoietic stem cells but also in the inhibition of myelopoiesis and granulopoiesis [429, 470]. As the impact of adiponectin on the lifespan of mature neutrophils is unexplored, it was determined whether it could modulate neutrophil apoptosis. In the following experiments adiponectin was added to cultures of neutrophils obtained from young donors, as the rate of spontaneous apoptosis is unaffected by ageing [551].

The results (Figure 4.3A and B) show that addition of adiponectin at physiological concentrations (1 and 10 μg/ml) decreased neutrophil apoptosis in a dose dependent manner, after both 6 and 20 hours incubation. The apoptotic cells were taken as those that were Annexin V positive and PI negative (Figure 4.3C). The most effective concentration of 10 μg/ml adiponectin was used in the remaining experiments.

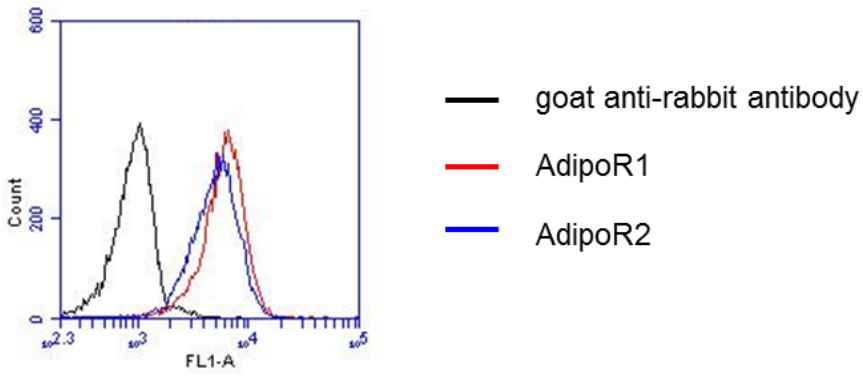


Figure 4.2 Neutrophils express adiponectin receptors. Isolated neutrophils were immunostained for the adiponectin receptors Adipo R1 and Adipo R2. Representative FACS plots are shown.

		AdipoR1		AdipoR2	
young	males	82.2% ± 5.6	83.5% ± 3.4	78.7% ± 2.9	84.1% ± 2.5
	females	84.8% ± 4.4		89.4% ± 2.3*	
elderly	males	87.4% ± 4.0	88.7% ± 2.5	92.9% ± 1.9	93.6% ± 1.1**
	females	90.0% ± 3.5		94.3% ± 1.1	

Table 4.2 Gender and age difference in the percentage of neutrophils expressing AdipoR1 and AdipoR2 on the surface. The percentage of neutrophils expressing AdipoR1 and AdipoR2 is shown for the young and the elderly donors, and for the gender categories in each age group (n=10 in each age group, n=5 in each gender category). * indicates $p<0.05$ and ** indicates $p<0.01$.

To exclude any interference of the FCS present in the medium in which neutrophils were cultured, apoptosis was also evaluated in neutrophils cultured in serum-free media. Adiponectin decreased the number of apoptotic cells after 20 hours of incubation ($p=0.005$) (Figure 4.3D).

Activation of caspase 3 is essential for induction of apoptotic cell death [247]. To assess whether adiponectin inhibited the cleavage of caspase 3, neutrophils were stained with an antibody against its cleaved active form. As shown in figure 4.4A adiponectin significantly decreased the percentage of cells expressing active caspase 3 after 20 hours of incubation ($p=0.027$). To further confirm the anti-apoptotic effect of adiponectin, the morphology of nuclei was examined after 20 hours of culture. As shown in figure 4.4B and C, there was a higher number of neutrophils displaying mono-lobed nuclei (indicated with arrows) in the control sample compared to adiponectin-treated cells, the majority of which still had the multi-lobed nuclear morphology of healthy neutrophils ($p<0.001$) (Figure 4.4B and C).

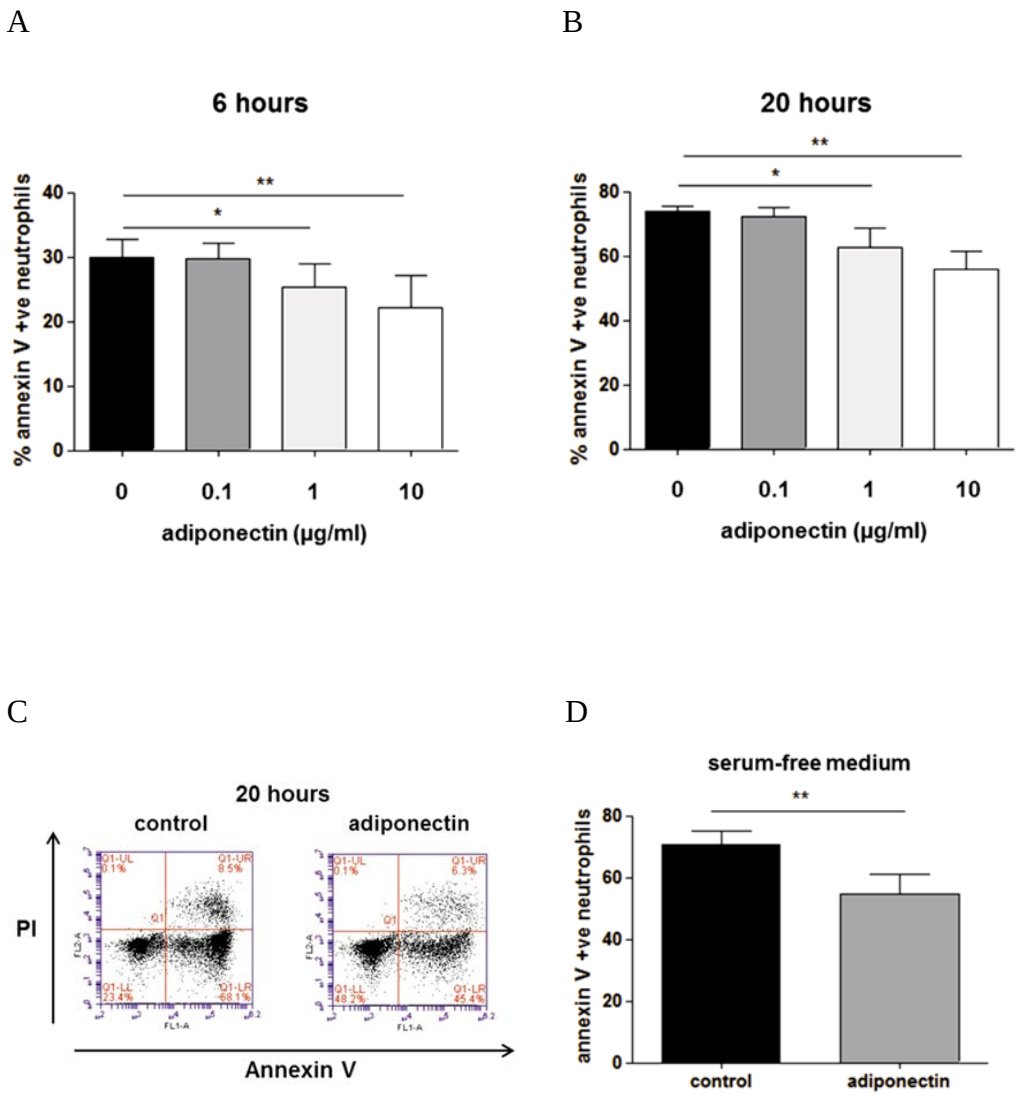


Figure 4.3 Adiponectin inhibits neutrophil spontaneous apoptosis assessed by Annexin V/PI staining. Human neutrophils were treated with adiponectin at the concentrations shown and the percentage of apoptotic cells was evaluated at (**A**) 6 hours and (**B**) 20 hours incubation ($n \geq 6$ experiments). **C.** Representative FACS plots showing the decrease in the percentage of Annexin V positive cells induced by adiponectin (10 µg/ml) after 20 hours incubation. **D.** Apoptosis was also assessed on neutrophils incubated in serum-free media with and without of adiponectin after 20 hours incubation (10 µg/ml). Data are expressed as mean ± SEM. * indicates $p<0.05$, ** $p<0.01$.

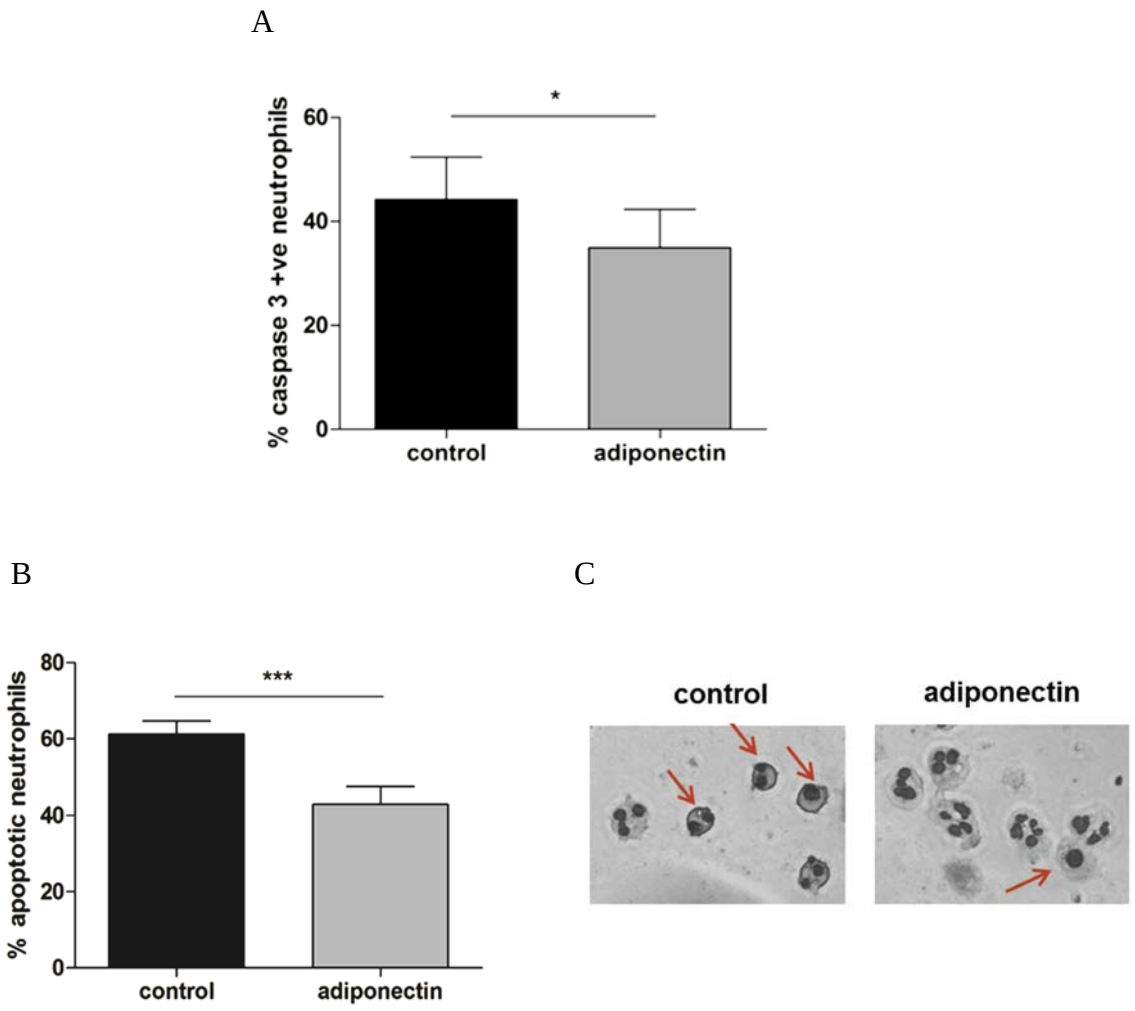


Figure 4.4 Adiponectin inhibits neutrophil spontaneous apoptosis as assessed by caspase-3 cleavage and nuclear morphology. A. The presence of active caspase 3 was measured by immunostaining after 20 hours of incubation with and without 10 μg/ml of adiponectin (n=5). **B.** Nuclear morphology was examined and apoptotic nuclei were counted (n=5 experiments). **C.** Representative pictures are shown: arrows indicate late apoptotic neutrophils. Data are expressed as mean ± SEM. * indicates $p<0.05$, ** $p<0.01$.

4.3.4 ADIPONECTIN INHIBITS LOSS OF MCL-1 EXPRESSION

The anti-apoptotic protein Mcl-1 is known to be the main regulator of apoptosis in neutrophils [273] and a number of anti-apoptotic agents contribute to increase its expression by inhibiting its turnover [268, 659]. Thus, Mcl-1 protein levels were assessed in adiponectin-treated neutrophils and compared with controls. As shown in Figure 4.5A and B, adiponectin maintained the expression of Mcl-1 in neutrophils after 6 and 20 hours of incubation.

To understand whether adiponectin increased Mcl-1 levels by enhancing its stability, neutrophils were pre-treated with cycloheximide (5 μg/ml), an inhibitor of protein synthesis, and Mcl-1 levels were evaluated after 6 hours of incubation. As previously shown [659], cycloheximide on its own decreased Mcl-1 protein expression, however addition of adiponectin in cycloheximide-treated cells induced a significant increase in Mcl-1 levels ($p=0.027$) (Figure 4.5C).

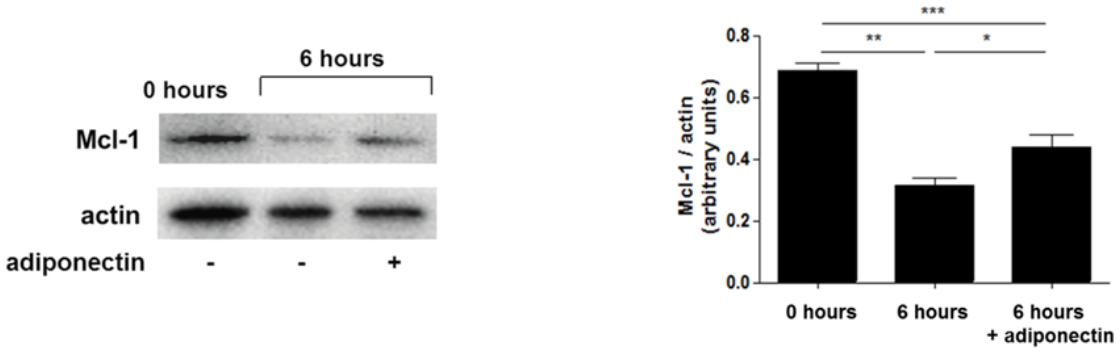
In summary, these findings show that adiponectin exerts an anti-apoptotic effect on neutrophils, by decreasing caspase 3 activation and Mcl-1 rate of turnover.

4.3.5 ADIPONECTIN ACTIVATES AMPK, PKB, ERK 1/2 AND p38 MAPKs

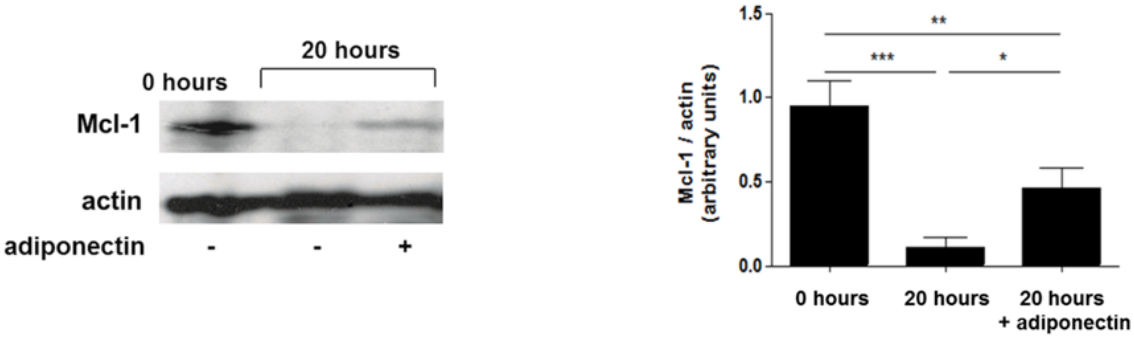
Adiponectin has been reported to activate AMPK and p38 MAPK in monocytes and other cell types [451, 478]. Therefore the phosphorylation of these and other kinases involved in regulating neutrophil apoptosis, the PI3K substrate PKB and ERK 1/2 [278, 282, 660], was examined in response to adiponectin treatment.

Adiponectin triggered a transient activation of AMPK for up to 15 minutes and phosphorylation of PKB, ERK 1/2 and p38 MAP kinases which persisted for up to 30 minutes as revealed by time course experiments (Figure 4.6).

A



B



C

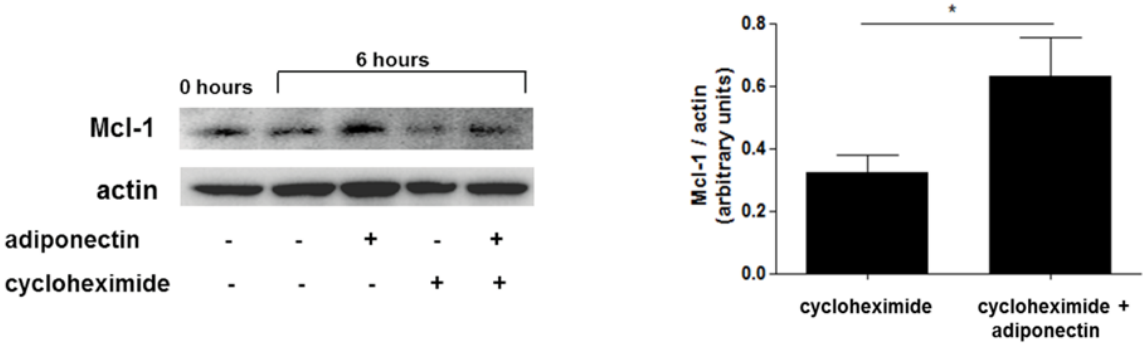


Figure 4.5 Adiponectin upregulates Mcl-1 levels by increasing its stability. A. Representative Western blots and densitometric analyses showing Mcl-1 protein expression in freshly isolated, untreated and adiponectin-treated neutrophils after 6 hours and (**B**) 20 hours of incubation. **C.** Representative Western blots and densitometric analysis showing the effect of cycloheximide (5 µg/ml) on Mcl-1 upregulation mediated by adiponectin. Densitometric analyses are expressed as the ratio of Mcl-1 to β-actin (n≥3). Data are expressed as mean ± SEM. * indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$.

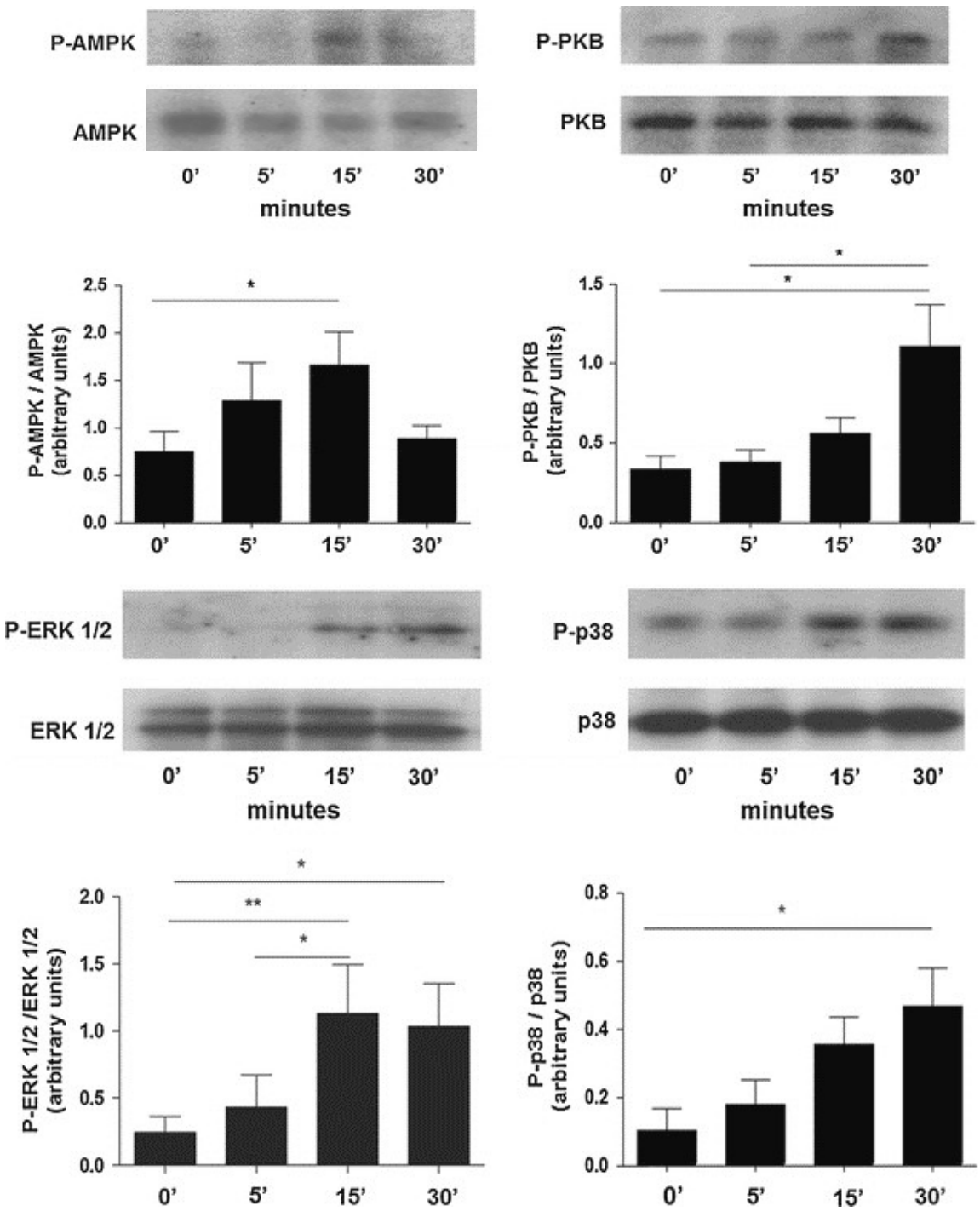


Figure 4.6 Adiponectin triggers phosphorylation of AMPK, PKB, ERK 1/2 and p38 MAPKs. Representative Western blots and densitometric analyses showing time courses for AMPK, PKB, ERK 1/2 and p38 phosphorylation mediated by adiponectin. Densitometric analyses are expressed as the ratio of phosphorylated to unphosphorylated forms of the proteins (n=4). Data are expressed as mean ± SEM. * indicates $p<0.05$, ** $p<0.01$.

4.3.6 ADIPONECTIN EXERTS ITS ANTI-APOPTOTIC EFFECT VIA AMPK AND PKB

As it was found that adiponectin activated multiple intracellular pathways, it was necessary to determine whether they all contributed to adiponectin's anti-apoptotic effect. Thus, neutrophils were treated with specific pharmacological inhibitors against AMPK (Compound C), PI3K (LY294002), MEK1/ERK 1/2 (PD98059) and p38 (SB202190) for 30 minutes prior to the addition of adiponectin and apoptosis was measured by Annexin V/ PI staining after 20 hours incubation. The results show that the anti-apoptotic action of adiponectin was significantly reduced by pharmacological inhibition of AMPK, PKB and ERK 1/2, but not p38 MAPK (Figure 4.7).

Phosphorylation of AMPK appeared to decline before the maximal phosphorylation of PKB, ERK 1/2 and p38, therefore the next question posed was whether AMPK was responsible for the activation of the other kinases. To address this question, neutrophils were pre-treated with the AMPK inhibitor compound C for 30 minutes prior to addition of adiponectin for a further 30 minutes. After these incubations, proteins were extracted for assessment of the phosphorylation of PKB, ERK 1/2 and p38 MAPK. As revealed in Figure 4.8, compound C itself triggered the phosphorylation of PKB, ERK 1/2 and p38, although only the phosphorylation of p38 reached statistical significance. Compound C also blocked the phosphorylation of PKB mediated by adiponectin, with no significant effect on ERK 1/2 or p38 MAP kinases, indicating that activation of AMPK could contribute to adiponectin-mediated phosphorylation of PKB (Figure 4.8A). To test this hypothesis, neutrophils were incubated with the pharmacological activator of AMPK, AICAR (1 mM) for 30 minutes, but AICAR failed to increase the phosphorylation of PKB, as well as the other kinases ERK 1/2 and p38 (Figure 4.8B). From these results it was concluded that activation of AMPK is not involved in adiponectin-mediated phosphorylation of PKB, ERK 1/2 and p38.

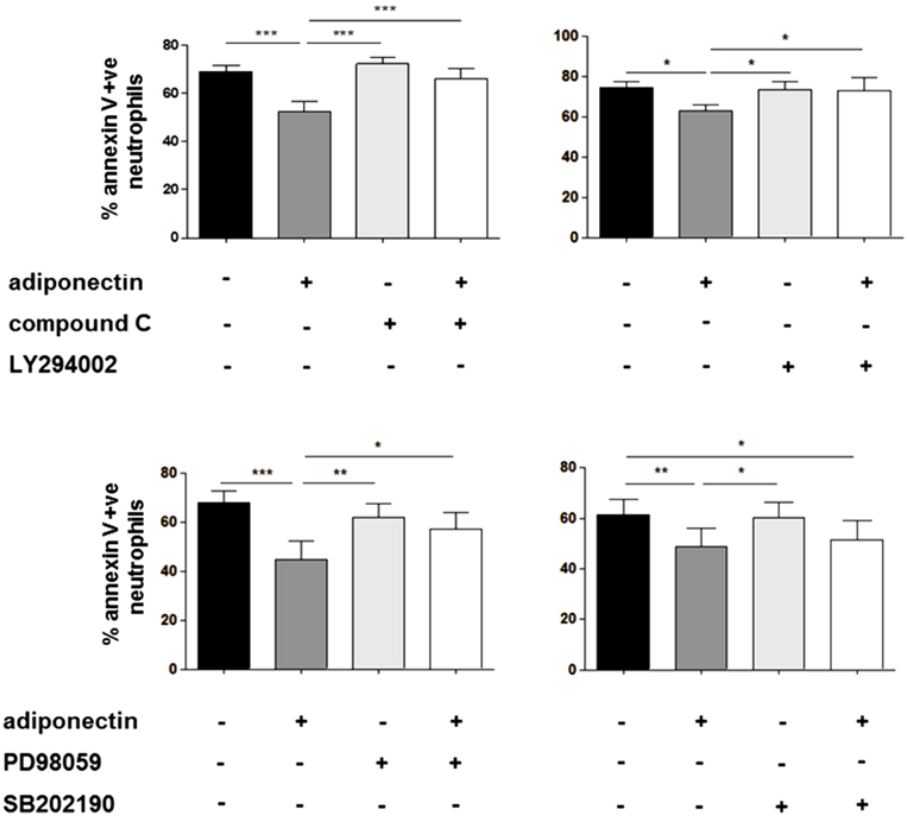
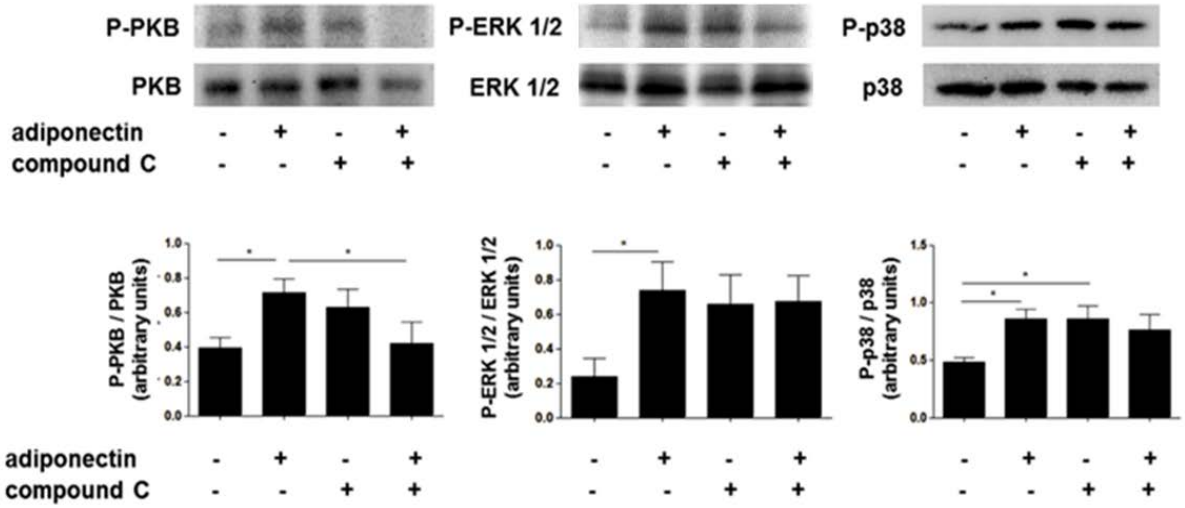


Figure 4.7 AMPK, PKB and ERK 1/2 mediate the anti-apoptotic role of adiponectin. Neutrophils were incubated with compound C (AMPK inhibitor) (10 µM), LY294002 (PI3K inhibitor) (10 µM), PD98059 (MEK-1/ERK 1/2 inhibitor) (10 µM), or SB202190 (p38 inhibitor) (10 µM) for 30 minutes prior to treatment with adiponectin (10 µg/ml). Apoptosis was measured by Annexin-V / PI staining after 20 hours of incubation (n=7). Data are mean ± SEM. * indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$.

A



B

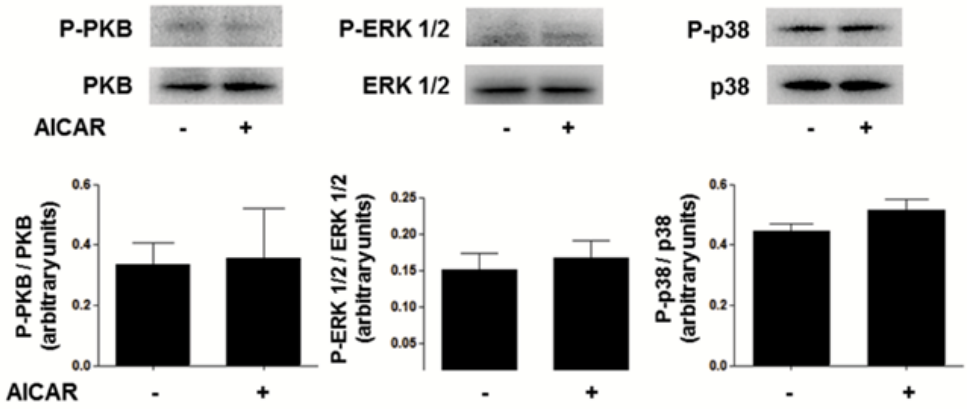


Figure 4.8 Adiponectin does not appear to activate PKB, ERK 1/2 and p38 via AMPK. Representative Western blots and densitometric analyses showing (**A**) phosphorylation of PKB, ERK 1/2 and p38 in response to adiponectin (10 µg/ml) in the absence or presence of compound C (10 µM) (n=6) and (**B**) effect of AICAR (1 mM) on PKB, ERK 1/2 and p38 phosphorylation (n≥3). Densitometric analyses are expressed as the ratio of the phosphorylated to unphosphorylated forms of the proteins. Data are expressed as mean ± SEM. * indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.3.7 ACTIVATION OF AMPK INHIBITS NEUTROPHIL APOPTOSIS

AMPK is activated by a low intracellular ATP:AMP ratio [661] and its activation is associated with increased apoptosis in cancer cells [662]. However, activation of AMPK also results in increased survival in post mitotic cells, such as neurons subjected to a transient starvation [663]. To understand the role of AMPK in the context of neutrophil apoptosis, cells were treated with AICAR and compound C, activators and inhibitors of AMPK respectively, and apoptosis assessed. AICAR reduced the percentage of apoptotic neutrophils as measured by Annexin V staining ($p=0.002$) and caspase 3 activation ($p=0.006$) after 20 hours incubation (Figure 4.9A and B). Compound C slightly increased neutrophil apoptosis as measured by Annexin V staining, although this difference did not reach statistical significance after 20 hours of incubation ($p=0.092$) (Figure 4.7). However, compound C significantly increased activation of caspase 3 at the same time point ($p=0.016$) (Figure 4.9C). Compound C on its own did not affect the levels of Mcl-1 but it decreased Mcl-1 expression in adiponectin-treated cells after 6 hours incubation (Figure 4.10A). In line with these data, Mcl-1 levels were unchanged after 20 hours of treatment with AICAR (Figure 4.10B). AICAR and compound C efficacy in modulating AMPK activation were evaluated and confirmed by western blot (Figure 4.10C).

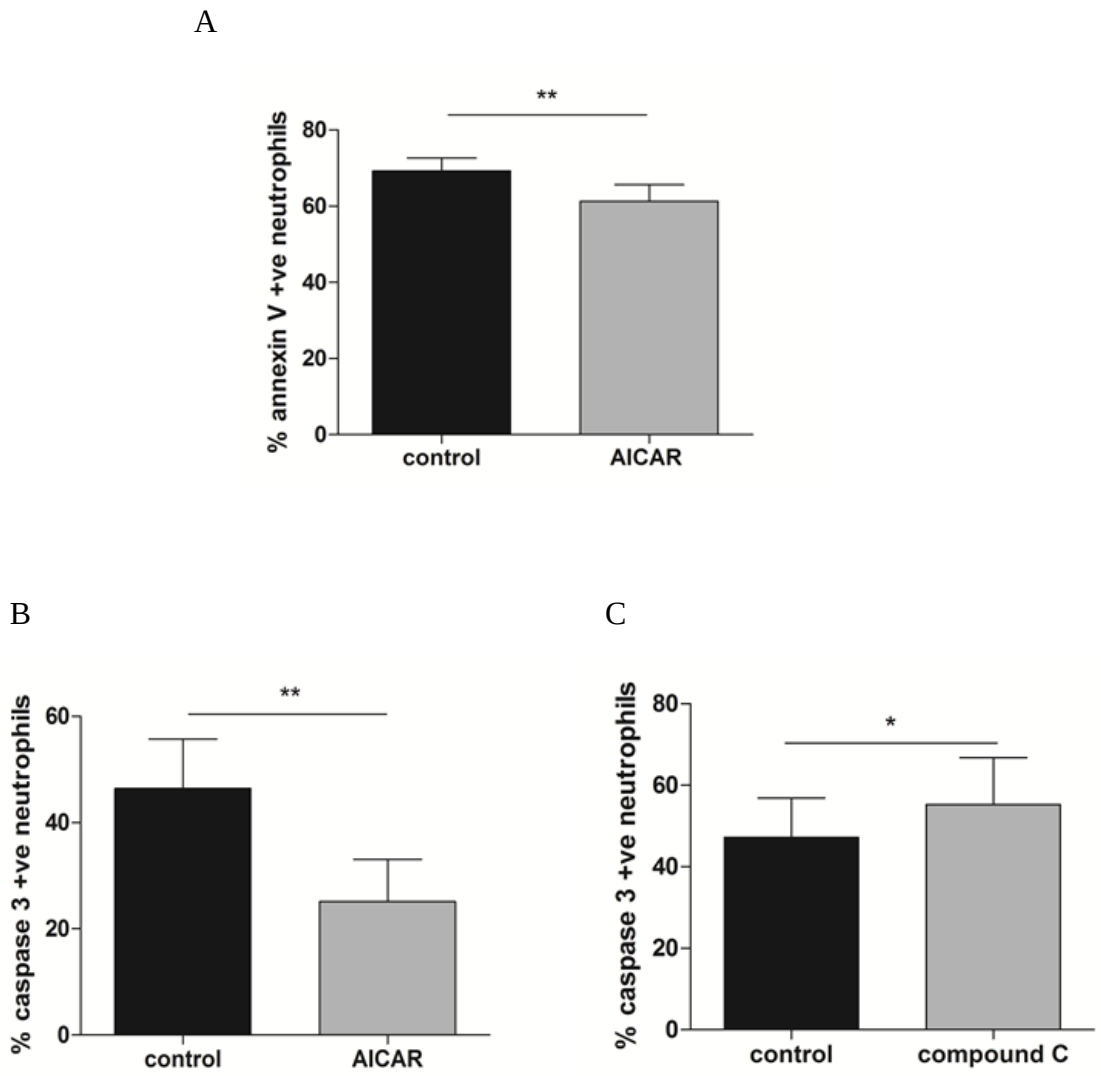
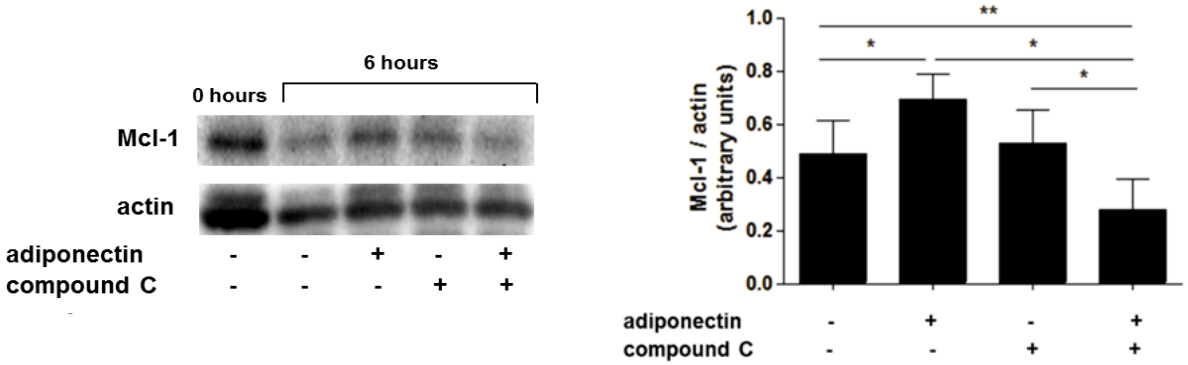
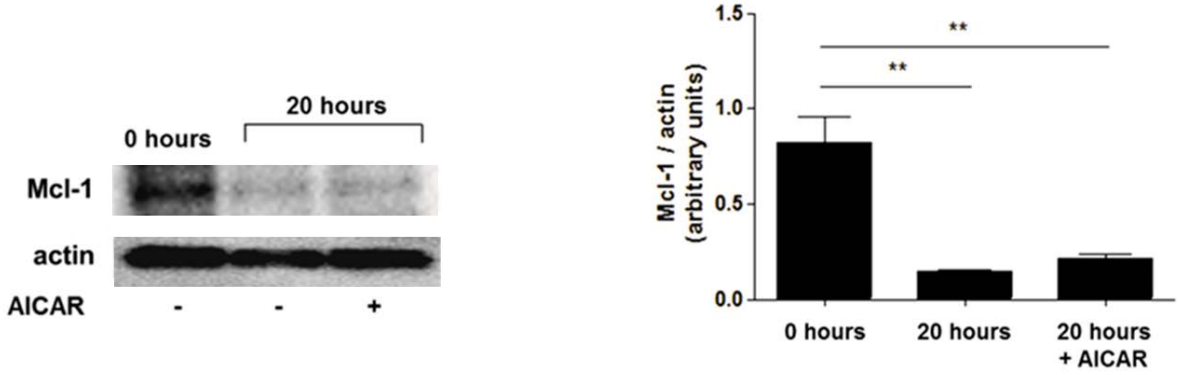


Figure 4.9 AMPK regulates neutrophil apoptosis. Neutrophils were incubated with the AMPK activator AICAR (1 mM) or inhibitor compound C (10 µM). Apoptosis was measured by (**A**) Annexin V staining and (**B** and **C**) caspase 3 activation after 20 hours incubation (n=7). Data are expressed as mean ± SEM. * indicates $p<0.05$, ** $p<0.01$.

A



B



C

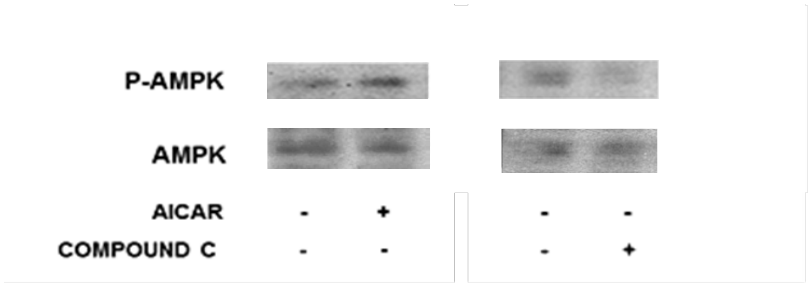
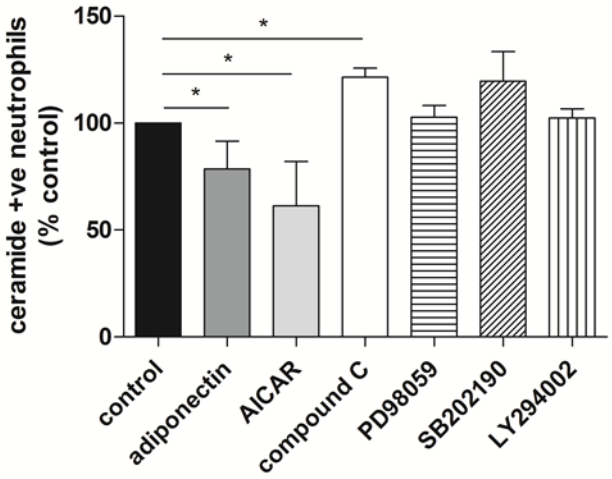


Figure 4.10 AMPK modulates neutrophil apoptosis without affecting Mcl-1 levels. Representative Western blots and densitometric analysis showing the effect of (**A**) compound C and adiponectin on Mcl-1 levels after 6 hours treatment and (**B**) the effect of AICAR on Mcl-1 after 20 hours treatment. Densitometric analyses are expressed as the ratio of Mcl-1 to β-actin (n=6). Data are expressed as mean ± SEM. * indicates $p<0.05$, ** $p<0.01$. **C.** Representative Western blot showing the efficacy of AICAR (1 mM) and compound C (10 μM) in modulating the phosphorylation of AMPK in neutrophils. Neutrophils were incubated with these compounds for 30 minutes after which proteins were extracted and analysed by Western blotting.

4.3.8 ADIPONECTIN AND AMPK REGULATE THE ACCUMULATION OF CERAMIDE IN NEUTROPHIL MEMBRANES

Ceramide is a pro-apototic sphingolipid physiologically generated by cells including neutrophils. During the neutrophil lifespan ceramide accumulates in the membrane forming ceramide-rich lipid rafts; these promote death receptor clustering and ultimately induce cell death [263, 293], AICAR [664], compound C [665] and adiponectin itself [666] have been shown to modulate the generation of ceramide, thus the accumulation of ceramide on neutrophil membranes was assessed after 20 hours treatment with these compounds. In addition, the level of ceramide was also evaluated after treatment with inhibitors of PI3K, ERK 1/2 and p38 MAPKs (Figure 4.11). AICAR and compound C respectively decreased and increased accumulation of ceramide on neutrophil membranes, suggesting a potential mechanism through which modulation of AMPK regulates neutrophil apoptosis. Adiponectin also significantly reduced the content of ceramide, although the decrease was less pronounced than AICAR treatment (Figure 4.11). PI3K, ERK 1/2 and p38 inhibition did not alter ceramide accumulation. Therefore, amongst the signalling pathways activated by adiponectin, only AMPK appears to be involved in adiponectin-mediated decrease of ceramide.

A



B

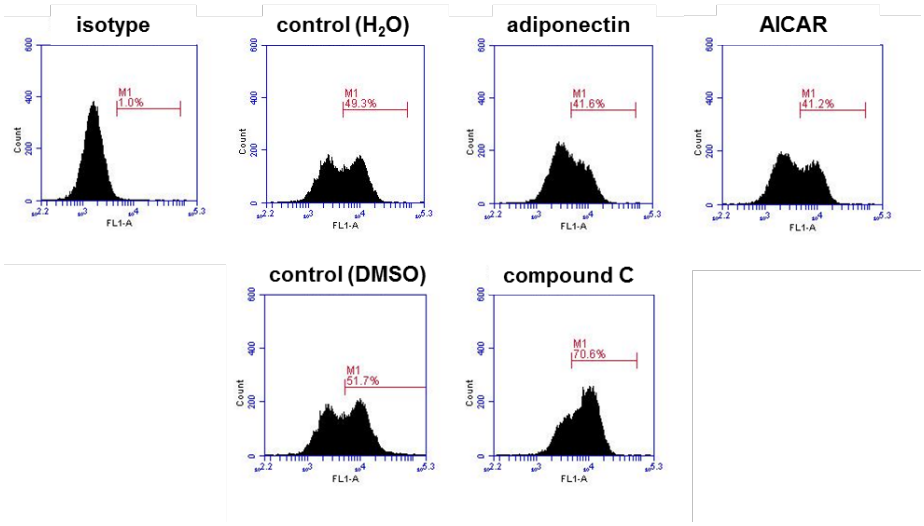


Figure 4.11 Adiponectin and pharmacological modulators of AMPK activity regulate the accumulation of ceramide in neutrophil membrane. Neutrophils were incubated for 20 hours with adiponectin (10 µg/ml), AICAR (1 mM), compound C (10 µM), PD98059 (10 µM), SB202190 (10 µM) and LY294002 (10 µM) ($n \geq 4$) and the surface level of ceramide was determined. Data are expressed as mean percentage relative to control ± SEM. * indicates $p<0.05$. **B.** Representative FACS plots showing the modulation of ceramide content in neutrophil membranes by adiponectin, AICAR and compound C.

4.3.9 EFFECT OF ADIPONECTIN ON NEUTROPHIL FUNCTION IN WHOLE BLOOD

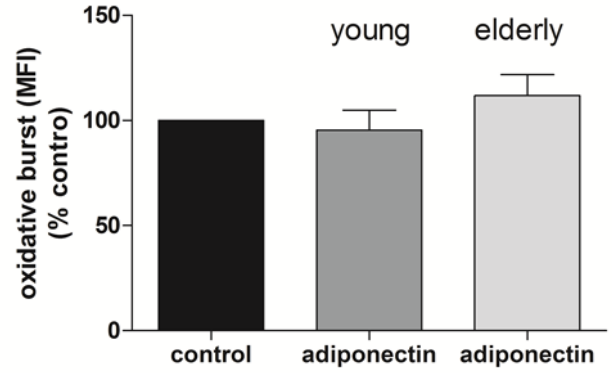
Both neutrophil phagocytosis and superoxide generation have been shown to be reduced with age [556, 557], though for superoxide generation the literature is conflicting and suggest that the decline is seen mainly in response to gram positive bacteria [557]. To examine whether adiponectin could influence neutrophil phagocytosis and oxidative burst and to determine whether this differed with age, 100 µl of whole blood was pre-treated with Polymyxin B for 30 minutes and then incubated with 10 µg/ml of adiponectin for one hour. After this incubation the neutrophil functional assays were performed. Data were normalized to the percentage increase above the control untreated value.

Adiponectin did not significantly affect neutrophil oxidative burst in whole blood from young or elderly subjects (Figure 4.12A), but it did significantly decrease the phagocytic index and the percentage of phagocytic cells in both the young ($p=0.023$ and $p=0.019$) and elderly group ($p=0.006$ and $p=0.031$) (Figure 4.12B and C).

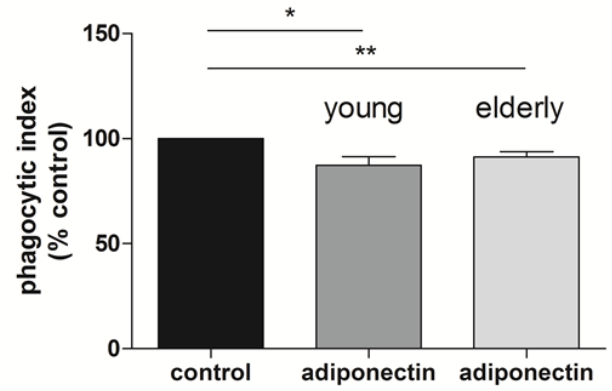
4.3.10 ADIPONECTIN DECREASES ROS PRODUCTION FROM ISOLATED NEUTROPHILS

Full length adiponectin is known to be a powerful inhibitor of ROS production on isolated neutrophils [451, 472]. Although adiponectin did not alter neutrophil oxidative burst in whole blood, a significant decrease in fMLP-stimulated ROS production was observed in isolated neutrophils pre-incubated with adiponectin (10 µg/ml) (Figure 4.13).

A



B



C

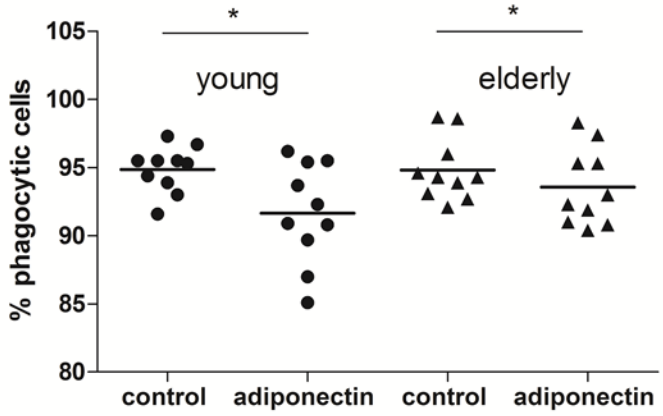


Figure 4.12 Adiponectin decreases neutrophil phagocytosis but not oxidative burst in whole blood. (**A**) Neutrophil ROS production, or (**B**) phagocytic index measured in untreated (control) and adiponectin treated cells from young or old donors (n=10), expressed as percentage of control. Data are mean ± SEM. (**C**) Percentage of phagocytic cells both in young and old volunteers (n=10). Horizontal bar shows the mean values. * indicates $p<0.05$ and ** indicates $p<0.001$.

A B

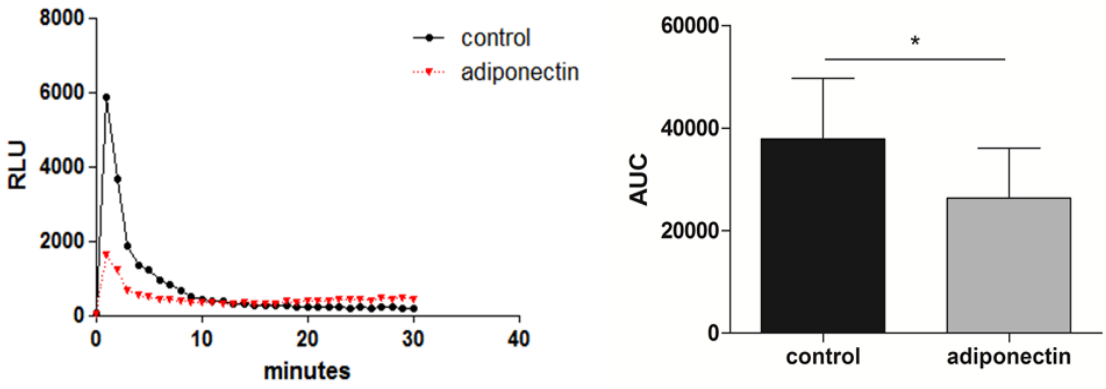


Figure 4.13 Adiponectin inhibits ROS production by isolated neutrophils. A. Isolated neutrophils were stimulated with fMLP, the luminescence was recorded for 30 minutes and the values plotted. Representative plots obtained from untreated and adiponectin-treated neutrophils stimulated with fMLP are shown. **B.** ROS production expressed as AUC calculated from the plots (n=5). Data are mean ± SEM. * indicates $p<0.05$ and ** indicates $p<0.001$.

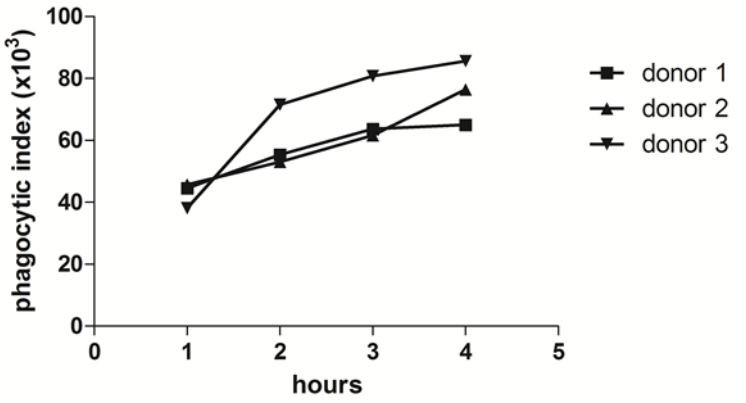
4.3.11 MEASUREMENT OF PHAGOCYTOSIS USING ISOLATED NEUTROPHILS

Neutrophil phagocytosis was measured in the whole blood using the commercial kit for flow cytometry Phagotest™. The same reagents provided by the kit were employed to optimize a protocol for studying the phagocytosis of isolated neutrophils in absence of serum. Neutrophils isolated from three different donors were incubated with a fixed amount of bacteria (20 µl of volume, 40:1 ratio between *E. coli* and neutrophils) and a time course was performed to measure the phagocytosis after 1, 2, 3, and 4 hours. Both the phagocytic index and the percentage of phagocytic neutrophils are shown in Figure 4.14A and B. From these experiments a high variability was seen in the phagocytic index. In general, the percentage of phagocytic cells reached a plateau after 2 hours, whereas the phagocytic index was more variable with a more pronounced increase between 1 and 2 hours. Hence, an incubation time of 1.5 hours was chosen to assess the differences in neutrophil phagocytosis induced by adiponectin on isolated neutrophils.

To assess whether adiponectin can directly modulate neutrophil phagocytosis, isolated neutrophils suspended in serum-free media were pre-treated with adiponectin for one hour and then challenged with opsonized *E. coli* (40:1 ratio between *E. coli* to neutrophils). As shown in figure 4.15A, adiponectin decreased phagocytosis of isolated neutrophils in a dose-dependent manner. Intriguingly, the reduction in the phagocytic index induced by adiponectin on isolated neutrophils was greater than that observed in whole blood, possibly because of the presence of endogenous adiponectin in the blood.

The inhibitory effect of adiponectin was also time-dependent. During the time course shown in figure 4.15B, the maximal reduction of phagocytosis was observed after 90 minutes of incubation with the bacteria. Different bacteria to neutrophil ratios (5:1, 20:1 and 40:1) (Figure 4.15C) and microscopic evaluation (data not shown) have been tested and the decrease was reproducible.

A



B

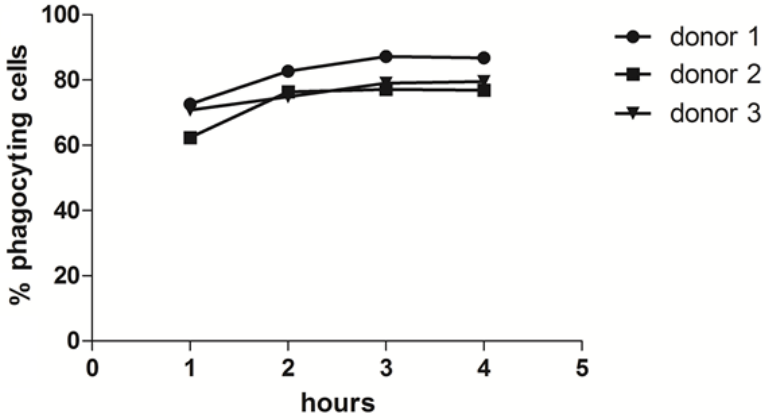
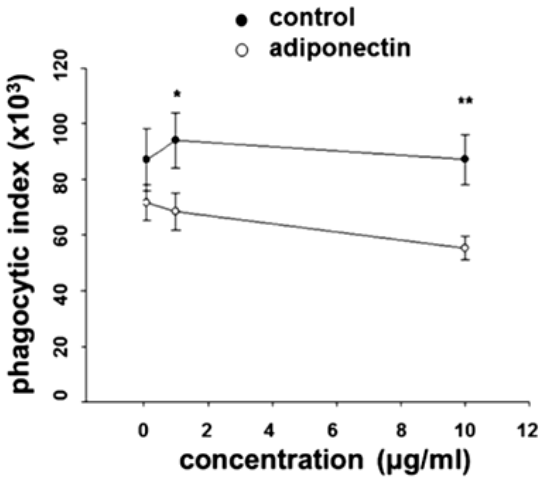
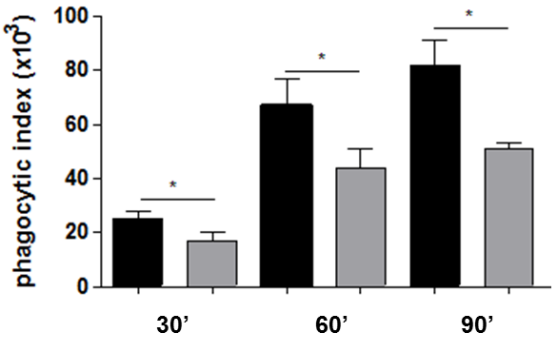


Figure 4.14 Time course of neutrophil phagocytosis with plasma-opsonized *E. Coli*. Time course of the phagocytic index (**A**) and the percentage of phagocytosing cells (**B**) measured at 1, 2, 3 and 4 hours after the addition of bacteria, in three different samples.

A



B



C

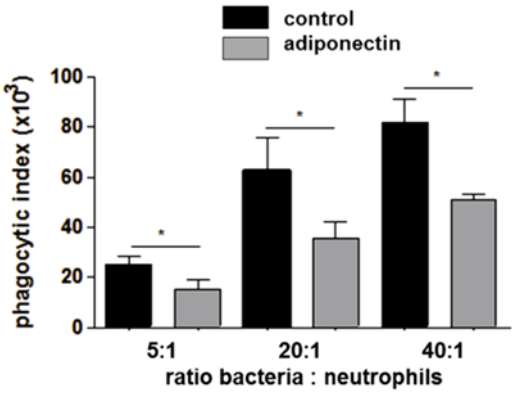


Figure 4.15 Adiponectin inhibits phagocytosis by isolated neutrophils. A. Isolated human neutrophils (n=6) were treated with adiponectin at different concentrations (0.1, 1 and 10 µg/ml) for one hour prior to addition of opsonized FITC labelled *E. coli*; after 90 minutes the phagocytic index was assessed by flow cytometry. **B.** Time course of neutrophil phagocytosis (30, 60, 90 minutes) with and without addition of adiponectin (10 µg/ml) (n=6). **C.** Neutrophil phagocytosis measured at different bacteria to neutrophil ratios with and without addition of adiponectin (n=6). Data are expressed as mean ± SEM. * indicates $p<0.05$, ** indicates $p<0.01$.

4.3.12 EFFECT OF ADIPONECTIN ON EXPRESSION OF PHAGOCYTIC RECEPTORS

Phagocytosis of opsonized pathogens is initiated and promoted by a range of receptors expressed on neutrophils. To evaluate whether adiponectin could reduce the phagocytosis of *E. coli* through regulation of phagocytic receptors, surface expression of CD16, CD11b and TLR4 on unstimulated neutrophils was assessed after one hour treatment with adiponectin. CD11b, CD16 and TLR4 expression was unaffected by treatment with adiponectin (Figure 4.16A, B and C).

The dimer CD11b/CD18, also known as complement receptor 3 (CR3), or Mac-1, is a receptor involved in phagocytosis of iC3b-opsonized pathogens. It is activated by conformational change when neutrophils are stimulated with pro-inflammatory agents [667]. Mac-1 activation was assessed in neutrophils pre-incubated with adiponectin and stimulated with opsonized unlabelled *E. coli* for 90 minutes using an antibody against the activation epitope. As shown by Figure 4.17A and B, Mac-1 activation (expressed as MFI) and the percentage of cells expressing active Mac-1 in response to *E. coli* stimulation were significantly decreased when adiponectin was added to the culture. As the conformational change that Mac-1 undergoes during its activation increases its affinity towards ligands [667], whether the binding of bacteria to neutrophils was also affected by adiponectin was determined. The binding of FITC labelled *E. coli* to the neutrophil membrane was measured at 4^{o}C after pre-incubation with adiponectin at different time points (30, 60 and 90 minutes). A significant increase in the percentage of unbound neutrophils (FITC negative cells) was found in samples treated with adiponectin (Figure 4.17C).

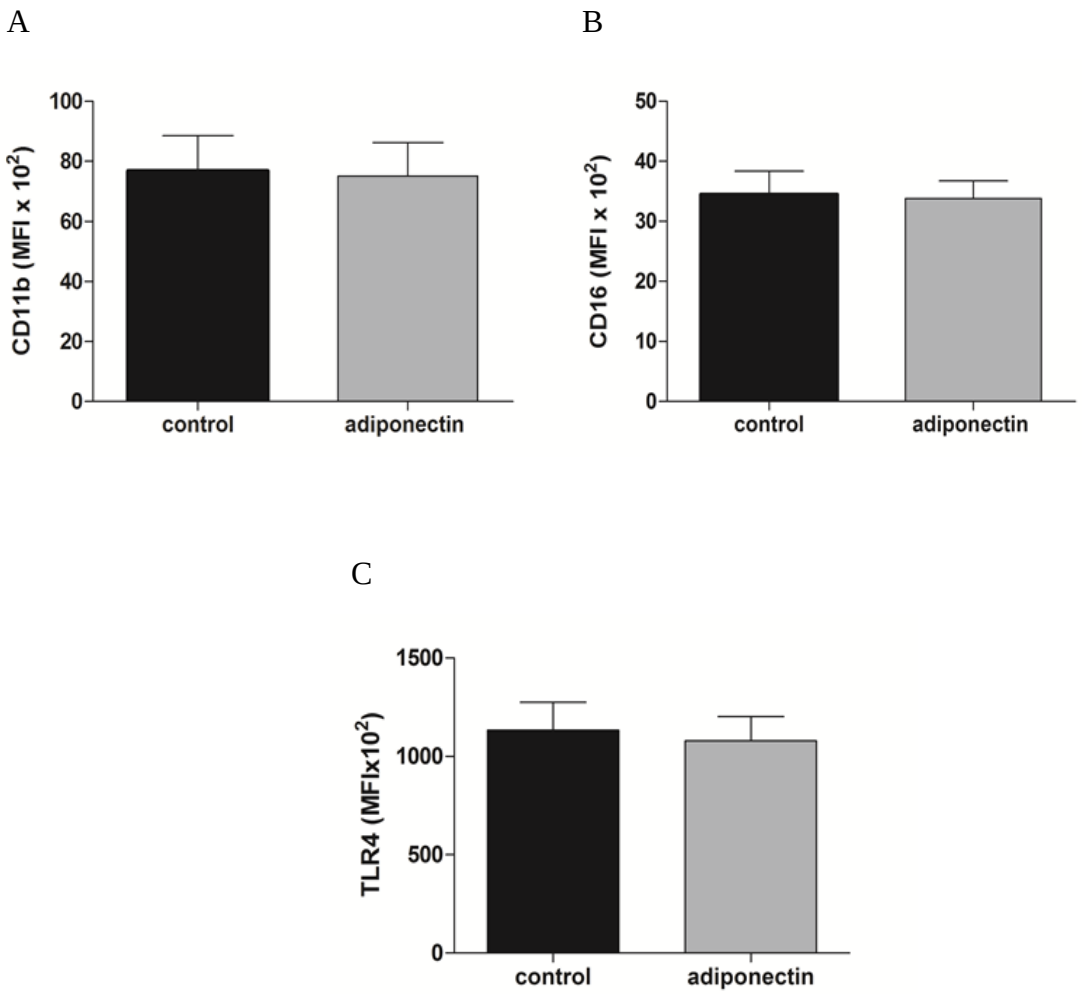


Figure 4.16 Adiponectin treatment does not alter neutrophil surface expression of the phagocytic receptors CD11b, CD16 and TLR4. Isolated neutrophils treated with adiponectin for 1 hour were immunostained for expression of (**A**) CD11b, (**B**) CD16 and (**C**) TLR4. Data are expressed as mean ± SEM (n=5).

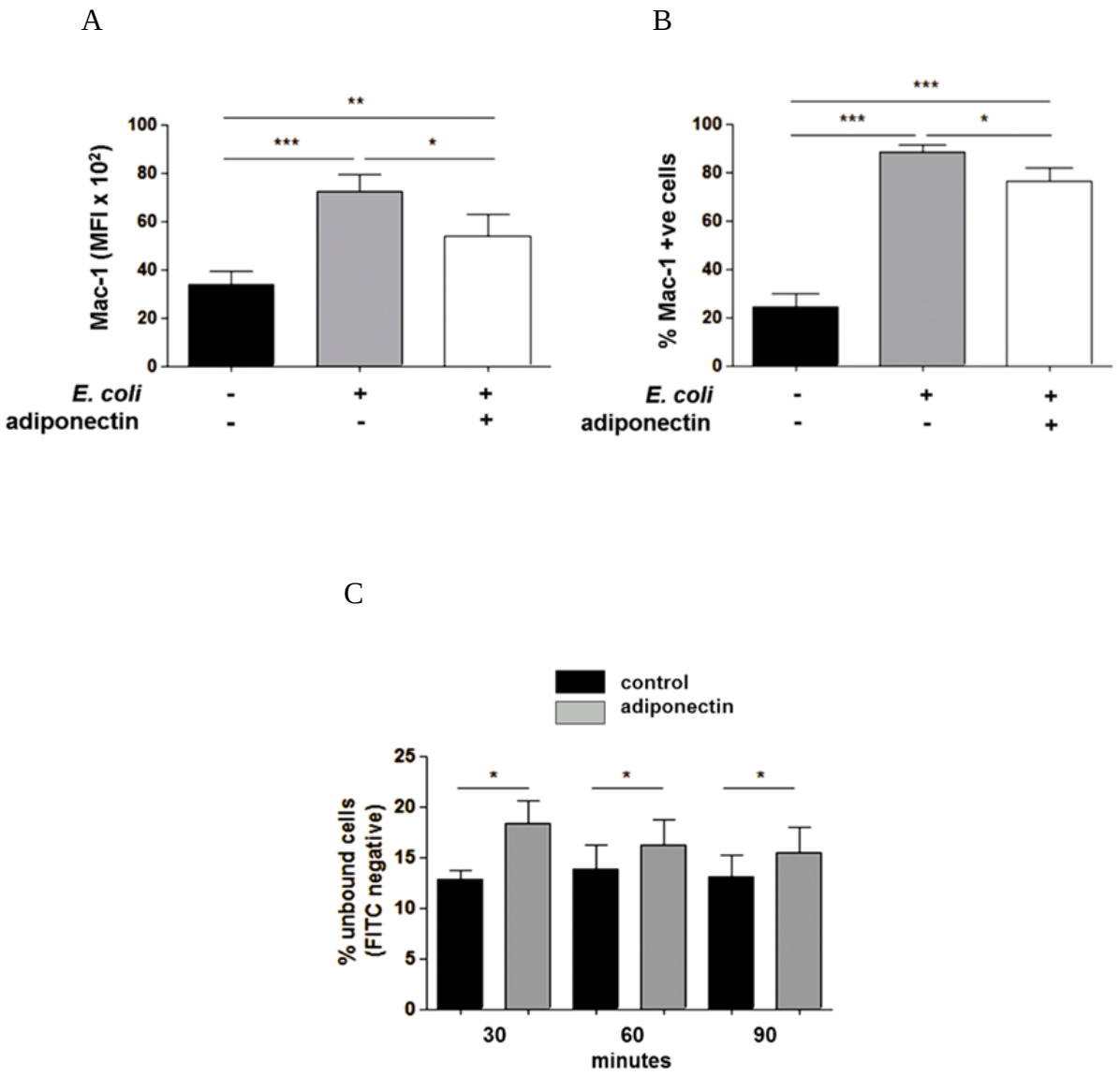


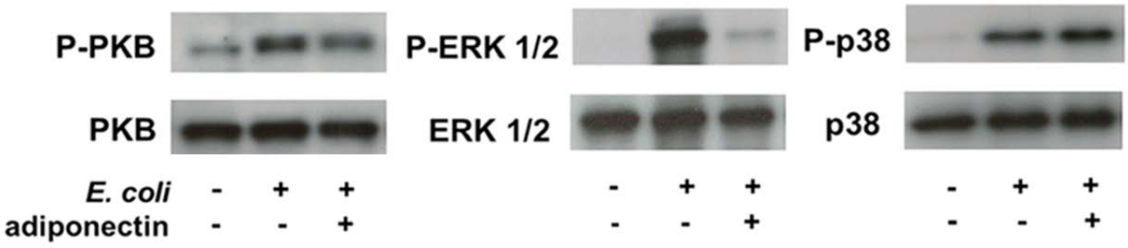
Figure 4.17 Adiponectin inhibits the activation of Mac-1 and the binding of *E. coli* on neutrophil cell wall. Isolated neutrophils treated with adiponectin for 1 hour and stimulated with unlabelled opsonized *E. coli* were immunostained for activated Mac-1. **A.** Adiponectin decreases Mac-1 expression (MFI) and (**B**) the percentage of neutrophils bearing active Mac-1. Data are mean ± SEM (n = 7). **C**. After one hour treatment with adiponectin, the binding of bacteria to the neutrophil surface was measured by flow cytometry at 30, 60 and 90 minutes of incubation with opsonized FITC labeled *E. coli* at 4°C. FITC negative cells were considered unbound. Data are expressed as the percentage of cells with no *E.coli* bound and are mean ± SEM (n≥7). * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

4.3.13 ADIPONECTIN DECREASES NEUTROPHIL PHAGOCYTOSIS VIA INHIBITION OF PKB AND ERK 1/2

Neutrophil phagocytosis is sustained by activation of PI3K/PKB and MAPK signalling pathways, thus the effect of adiponectin on phosphorylation of PKB, ERK 1/2 and p38 MAPK in *E. coli*-stimulated neutrophils was examined by Western blotting. Pre-treatment with adiponectin was associated with significantly lower phosphorylation of both PKB and ERK 1/2 compared to untreated stimulated neutrophils, though the effect on ERK 1/2 was more pronounced (Figure 4.18A and B), whereas phosphorylation of p38 was unaffected by the presence of adiponectin.

The involvement of the two signalling pathways PI3K/PKB and ERK 1/2 signalling for complete neutrophil phagocytosis by using the pharmacological inhibitors LY294002 (PI3K inhibitor) and PD98059 (MEK1/ERK 1/2 inhibitor). These drugs were added 30 minutes before the addition of bacteria and beginning of the assay, and both of them decreased phagocytosis of *E. coli* in a dose-dependent manner (Fig. 4.19A and B). The efficacy of the two inhibitors was confirmed by Western blot (4.19C).

A



B

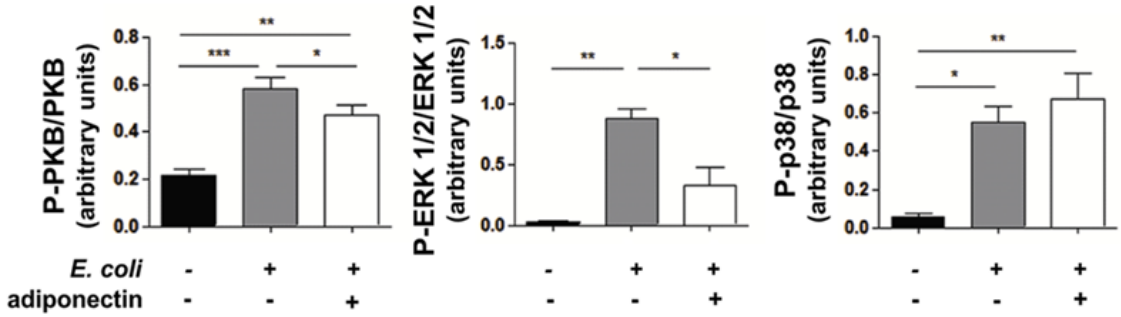


Figure 4.18 Adiponectin decreases the phosphorylation of PKB and ERK 1/2 but not p38 in response to *E. coli*. Neutrophils pre-incubated with adiponectin were challenged with unlabelled opsonised *E. coli* for 10 minutes after which proteins were extracted and analysed by Western blotting. **A**. Representative Western blots showing the effect of adiponectin on phosphorylation of PKB, ERK 1/2 and p38 MAPK in response to *E. coli* stimulation. **B**. Densitometric analysis of three separate experiments for the effect of adiponectin on phosphorylation of PKB, ERK 1/2 and p38 MAPK, expressed as the ratio between the phosphorylated and unphosphorylated proteins. Data are expressed as mean ± SEM (n = 5). * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$ for treated versus control cells.

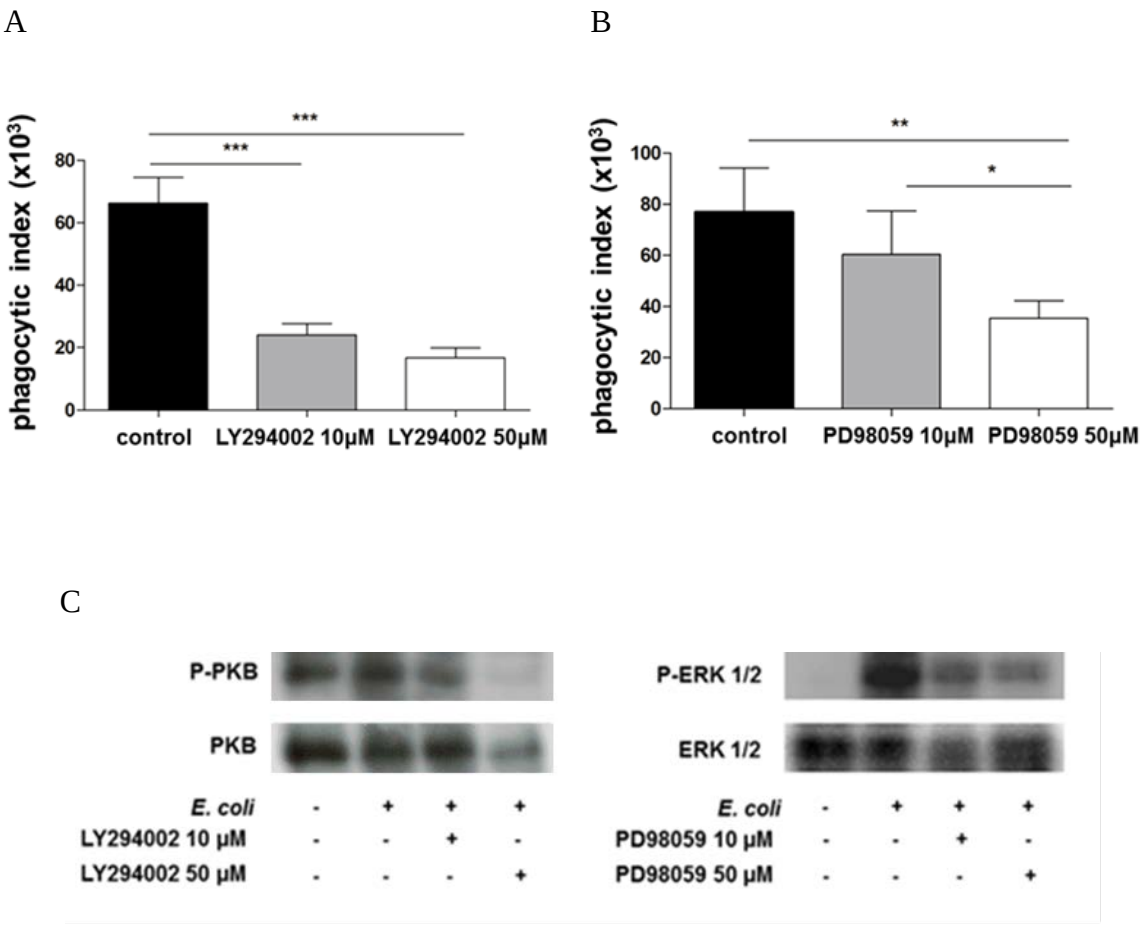


Figure 4.19 Inhibition of PI3K/PKB and MEK1/ERK1/2 pathways results in reduced neutrophil phagocytosis. The PI3K inhibitor LY294002 (**A**) and MEK-1/ERK 1/2 inhibitor PD98059 (**B**) were added to neutrophils at the two concentrations of 10 µM and 50 µM for 30 minutes prior to addition of opsonised FITC labeled *E. coli* and the phagocytic index was measured by flow cytometry. Control samples were treated with the highest concentration of carrier (DMSO) used. Data are expressed as mean ± SEM (n≥7). * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$. **C.** Neutrophils were pre-incubated with LY294002 and PD98059 (50 µM and 10 µM) for 30 minutes, stimulated with unlabelled opsonised *E. coli* for 10 minutes after which proteins were extracted and analysed by western blotting. Representative Western blots showing the efficacy of LY294002 and PD98059 in inhibiting the phosphorylation of PKB and ERK 1/2 in response to *E. coli* stimulation.

4.3.14 ADIPONECTIN EFFECT ON PHAGOCYTOSIS IS NOT MEDIATED BY AMPK

As adiponectin induces activation of AMPK in phagocytes, it was next decided to determine whether this kinase could be involved in adiponectin-mediated reduction of neutrophil phagocytosis. Neutrophils were pre-treated with the AMPK activator AICAR (1 mM) for 30 minutes prior to the addition of *E. coli* but AICAR did not significantly decrease phagocytosis (Figure 4.20). Thus, AMPK activation does not contribute to inhibition of phagocytosis in adiponectin-treated neutrophils.

4.3.15 PI3K, BUT NOT ERK 1/2, REGULATE MAC-1 ACTIVATION

The results show that adiponectin inhibited the activation of PI3K-PKB and ERK signalling and also Mac-1 activation in neutrophils challenged with *E. coli*. It was then asked whether these two signalling pathways could regulate activation of Mac-1. Using the specific inhibitors LY294002 and PD98059, it was possible to determine that PI3K, but not ERK 1/2, were partially involved in the conformational change of Mac-1 after neutrophils were stimulated with bacteria (Figure 4.21).

4.3.16 ACTIN POLYMERIZATION IS INHIBITED BY ADIPONECTIN

Actin polymerization is necessary to sustain neutrophil phagocytosis [668]. Activation of PI3K and generation of PtdIns(3,4,5)P_3 is required for extension of pseudopods [237, 238] and cytoskeletal rearrangements also depend on ERK 1/2 activation [669]. As adiponectin decreased PKB and ERK 1/2 phosphorylation in bacteria stimulated neutrophils (Figure 4.18A), it was hypothesized that the decrease in the activation of PI3K and ERK 1/2 could negatively influence F-actin generation in response to *E. coli*. FITC phalloidin staining confirmed the hypothesis, as adiponectin treatment reduced the amount of polymerized actin after 5 minutes of stimulation with unlabelled *E. coli* (Figure 4.22). This lack of F-actin formation could be responsible for a lack of pseudopod maturation and phagosome formation.

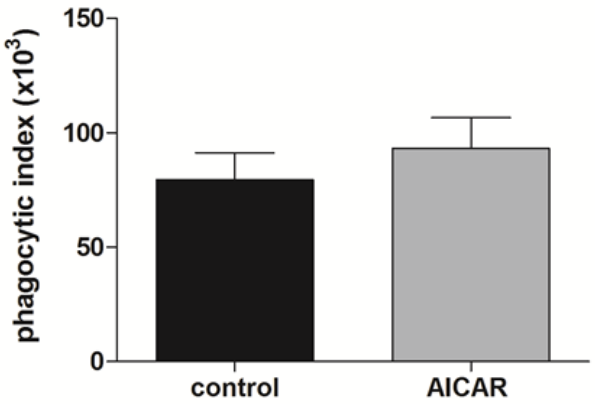
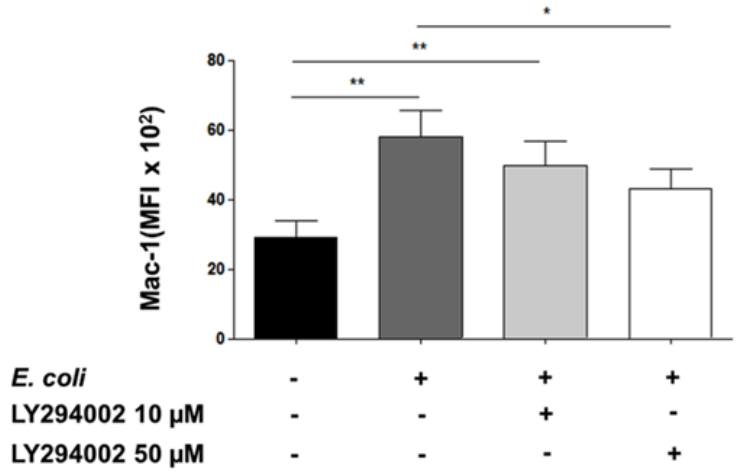


Figure 4.20 AMPK activation does not affect neutrophil phagocytosis. A. Neutrophils were incubated with AICAR (1 mM) for 30 minutes prior to addition of FITC labelled *E. coli* and measurement of phagocytic index. Data are expressed as mean ± SEM (n=6).

A



B

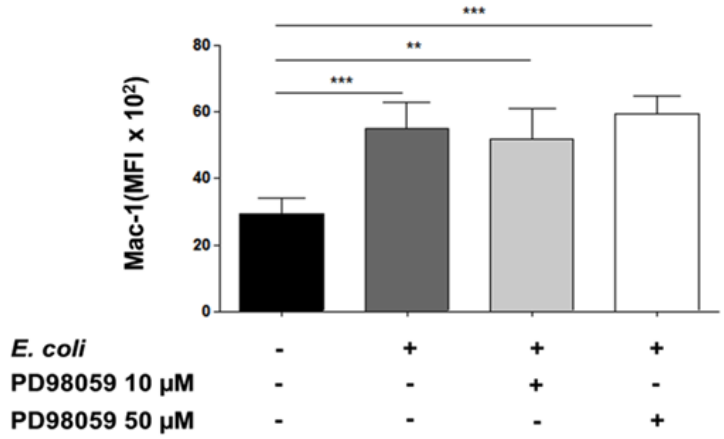
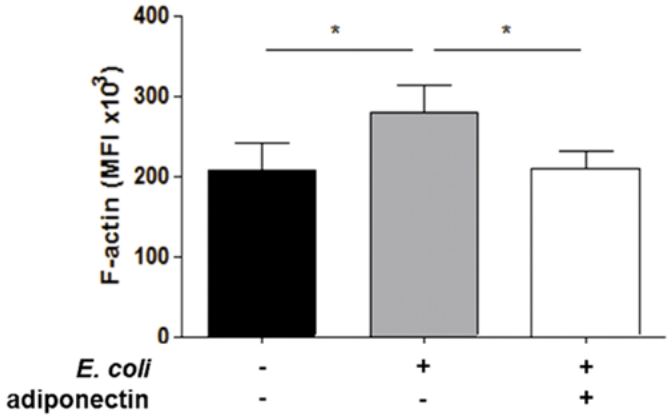


Figure 4.21 Inhibition of PI3K decreases Mac-1 activation. A. The PI3K inhibitor LY294002 and (**B**) the MEK1 inhibitor PD98059 (10 and 50 µM) were added to neutrophils for 30 minutes before stimulating the cells with unlabelled opsonised *E. coli.* After 90 minutes the activation of Mac-1 was measured by flow cytometry. Unstimulated and control *E. coli* stimulated samples were incubated with the highest concentration of carrier (DMSO) used. Data are expressed as mean ± SEM (n=6). * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

A



B

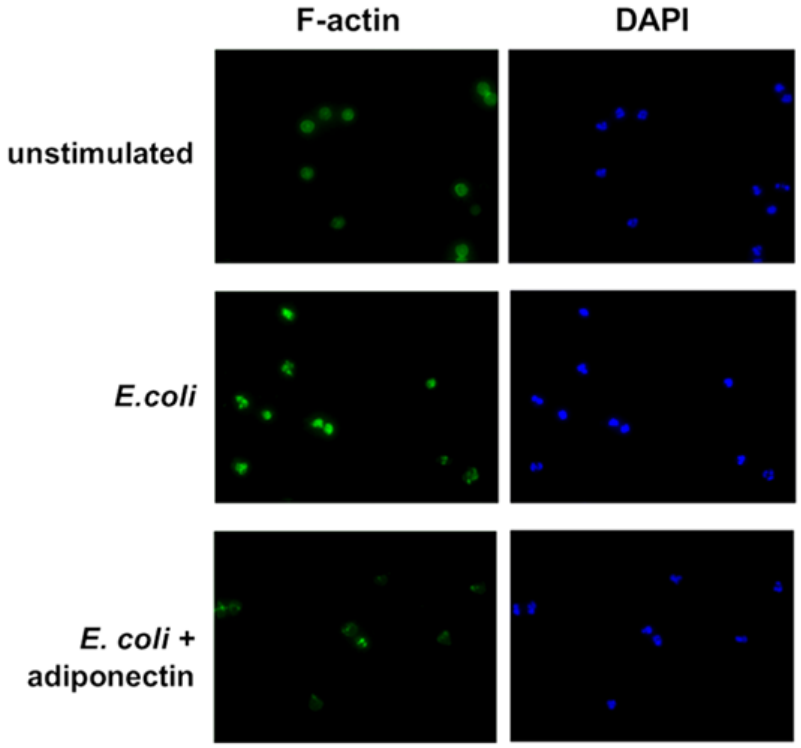


Figure 4.22 Adiponectin inhibits actin polymerization in response to *E. coli* stimulation. **A**. Neutrophils were pre-incubated with adiponectin and stimulated with unlabelled opsonized *E. coli* for 5 minutes, after which intracellular F-actin was stained with FITC phalloidin and measured by flow cytometry. Data are expressed as mean ± SEM (n=7). * indicates $p<0.05$. **B**. Representative images of F-actin in unstimulated and *E. coli* stimulated neutrophils with and without pre-treatment with adiponectin.

4.3.17 COMPLEMENT FACTOR C1Q DOES NOT RESCUE PHAGOCYTOSIS OF NEUTROPHILS TREATED WITH ADIPONECTIN

The C-terminal domain of adiponectin shares structural homology with the complement protein C1q. Interestingly, the reduction of macrophage phagocytosis by adiponectin was completely rescued by the use of a C1qRp antibody [470]. Therefore whether addition of complement protein C1q protein to the phagocytosis assay would be able to inhibit the effect of adiponectin was determined. Neutrophils were pre-incubated with human serum C1q (at the physiological serum concentration of 100 μg/ml [670] prior to the addition of adiponectin. However, C1q did not block the negative effect of adiponectin on neutrophil phagocytosis (Figure 4.23).

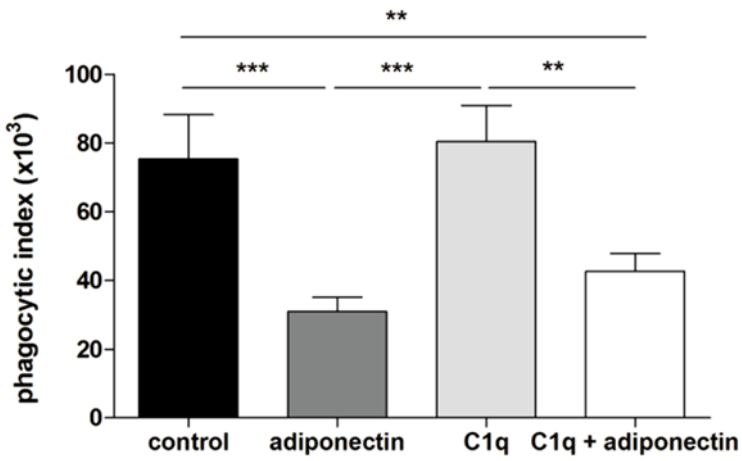


Figure 4.23 Inhibition of neutrophil phagocytosis by adiponectin is not prevented by addition of complement factor C1q. C1q (100 μg/ml) was added to neutrophils 15 minutes before the addition of adiponectin to saturate C1q receptors. Phagocytosis of opsonized FITC labelled *E. coli* was then measured by flow cytometry. Data are expressed as mean ± SEM (n=6). ** indicates $p<0.01$, *** indicates $p<0.001$.

4.4 DISCUSSION

4.4.1 ADIPONECTIN REGULATION OF NEUTROPHIL APOPTOSIS

Neutrophil lifespan is regulated by several factors which modulate pro- and anti-apoptotic signalling pathways, accelerating or inhibiting the process of spontaneous apoptosis [292]. Here, adiponectin was shown to inhibit neutrophil spontaneous apoptosis *in vitro*. Adiponectin prolonged neutrophil lifespan in a dose dependent manner at both 6 and 20 hours of treatment and the same effect was observed when neutrophils were incubated in serum free medium.

This result was unexpected as this adipokine has been extensively studied with regard to its anti-inflammatory role [424] and you would predict that extending neutrophil lifespan would be pro-inflammatory. However, contradictory results have been reported with regard to its role on inflammation and also on apoptosis. For instance, whilst adiponectin enhances apoptosis in several tumor cell lines [470, 653-655], as well as activated T lymphocytes [450], it has been shown to protect post-mitotic cells, such as neurons [656] and endothelial cells [657] from apoptosis when these cells were stimulated with pro-apoptotic factors. In addition, low serum adiponectin was recently found to be associated with high levels of apoptotic markers in the blood in healthy obese subjects [473]. In light of the existing literature, the anti-apoptotic effect reported here for adiponectin on unstimulated neutrophils, which are short-lived, post-mitotic cells, is in agreement with previous findings.

Adiponectin enhanced neutrophil survival by decreasing the cleavage of caspase-3 and reducing Mcl-1 degradation as demonstrated by addition of the protein synthesis inhibitor cycloheximide B [671]. Mcl-1 is the main anti-apoptotic protein belonging to the Bcl-2 family expressed by neutrophils [268] and its importance in the regulation of neutrophil apoptosis is well established [271-273], with most anti-apoptotic factors acting through the maintenance of expression of this protein. Adiponectin thus acts by similar mechanisms to other known neutrophil pro-survival factors.

The data reported here also showed that the main anti-apoptotic signalling pathways activated by adiponectin were AMPK, PI3K/PKB and the MAPK ERK 1/2 [671]. p38 phosphorylation was also increased by adiponectin, though pharmacological blockade of this kinase did not prevent the anti-apoptotic function of adiponectin. The exact role of p38 in the context of neutrophil apoptosis is controversial as it has been reported both to be anti-apoptotic [283, 284] and pro-apoptotic [281, 672] and may depend on the activation or priming status of the cell.

The activation of AMPK and p38 by adiponectin has been reported by others [424] and PKB and ERK 1/2 phosphorylation in response to adiponectin has been shown in several cell types [457-460, 463, 464]. Both adiponectin receptors (AdipoR1 and AdipoR2) have been shown to contribute to adiponectin-mediated ERK 1/2 phosphorylation, specifically through Src-mediated Ras activation, and partially through protein kinase A (PKA) activation, but independently of PKC activation [457]. Activation of Src and Ras by adiponectin could also explain the increased phosphorylation of AMPK, PKB and p38. In fact, c-Src has been shown to induce AMPK phosphorylation during hypoxia-reoxygenation in bovine aortic endothelial cells [673] and cancer cell lines [674], whilst Ras can bind and activate PI3K in fibroblastic cell lines [675] and the Src-Ras axis activates p38 in cardiac myocytes [676].

APPL1 is an adaptor protein that interacts with the intracellular tails of adiponectin receptors, and this event is enhanced by the binding of adiponectin to AdipoR1 and AdipoR2 [453]. APPL1 is likely to participate in activating the downstream pathways cited above. Indeed it has already been shown to mediate adiponectin and insulin-induced phosphorylation of AMPK, PKB and p38 [467], whereas contrasting results exist concerning its involvement in the activation of ERK 1/2 [457, 467]. No data currently exist with regards to Src or Ras activation by APPL1. Future studies in neutrophils could determine if APPL1 is expressed and plays a role in regulation of apoptosis by adiponectin.

Although it has not been investigated in this thesis, NF-kB could be another anti-apoptotic factor activated by adiponectin, through PKB [677] and ERK 1/2 [678]. NF-kB activation can potently delay neutrophil apoptosis [286] and it was found to be activated by adiponectin by other groups, in unstimulated monocytic and endothelial cell lines [679, 680]. The PI3K/PKB pathway negatively regulates neutrophil apoptosis [276] and mediates the inhibition of apoptosis conferred by several pro-survival factors [277-279, 659]. Although ERK 1/2 activation does not appear to affect the lifespan of resting neutrophils, this MAPK contributes to inhibition of apoptosis in neutrophils stimulated with LPS [282, 285], GM-CSF [277, 659] and when neutrophils are cultured at high concentration [681]. Importantly, both PI3K/PKB [278, 659] and ERK 1/2 [659] increase Mcl-1 stabilization in stimulated neutrophils, therefore their activation could explain the increase in Mcl-1 levels mediated by adiponectin.

To further investigate the role of AMPK activity in the context of neutrophil apoptosis the activating agent AICAR was used and it reduced neutrophil apoptosis without increasing Mcl-1 stabilization, whereas the AMPK inhibitor compound C significantly increased neutrophil apoptosis as measured by caspase-3 cleavage. AMPK is physiologically activated by decreased levels of intracellular ATP [661] and by hypoxia [682]. Although AMPK phosphorylation has not been assessed in hypoxic neutrophils, it is known that neutrophils subjected to hypoxia have a longer lifespan [217], thus AMPK could potentially participate in delaying apoptosis in that context. Moreover, transient activation of AMPK enhances survival in other post mitotic cells, such as neurons [663]. How AMPK activation could inhibit neutrophil apoptosis was also investigated here. Both AICAR and compound C have been reported to affect the generation of ceramide [664, 665]and this was confirmed here in neutrophils as AMPK modulators altered ceramide accumulation measured within the cell membrane. The change in ceramide content represents a plausible mechanism by which AICAR and compound C could regulate neutrophil apoptosis as previous studies from our laboratory have shown that spontaneous neutrophil apoptosis is triggered by the accumulation

of ceramide in the cell membrane. The latter causes ligand-independent clustering of death receptors, activation of death receptor signalling and caspase dependent cell death [263]. Surprisingly, Mcl-1 protein levels were not affected by AICAR or compound C treatments. However in the literature there is no direct evidence for an increase in ceramide content being associated with a decline in Mcl-1 levels.

Crucially, adiponectin also decreased the content of ceramide in the neutrophil membrane. This result was in agreement with previous reports showing that adiponectin exerted its anti-apoptotic action on pancreatic beta cells and cardiomyocytes by decreasing the ratio between ceramide and its anti-apoptotic metabolite S1P [666]. However, in the latter study, the action of adiponectin on ceramide and S1P levels was independent of AMPK.

In agreement with Chedid et al., it was found that adiponectin also reduced ROS production by isolated neutrophils stimulated with fMLP. This finding is consistent with the decrease observed in the level of ceramide, as ROS are responsible for enhancing the activation of sphingomyelinase, the enzyme responsible for the synthesis of ceramide [263]. Generation of ceramide-enriched membrane rafts can mediate the extracellular apoptotic pathway initiated by clustering of CD95 [263] and adiponectin could inhibit the extrinsic pathway of apoptosis by blocking the aggregation of FADD and caspase-8 following CD95 clustering.

The activation of AMPK by adiponectin was transient and preceded the maximal phosphorylation of PKB, ERK 1/2 and p38. Experiments performed to elucidate the interconnection between these pathways indicated that AMPK activation was not directly responsible for the phosphorylation of PKB, ERK 1/2 and p38, as the pharmacological AMPK activator AICAR did not induce activation of these pathways [671]. This result is in agreement with a previous study showing that AICAR did not alter the phosphorylation of the MAPKs ERK 1/2 and p38 in neutrophils [683].

In contrast, the addition of compound C to neutrophils induced an increase in p38 phosphorylation, indicating that compound C may have off target effects that activate this kinase. In fact, compound C has been found by others to exert certain actions independently of AMPK activation [684-686]. Intriguingly, compound C significantly reduced the effect of adiponectin on the phosphorylation of PKB, however activation of PKB, as well as ERK 1/2 and p38 does not appear to occur downstream of AMPK activation as shown by treatment with AICAR. In addition, the decrease of PKB phosphorylation in neutrophils treated with both compound C and adiponectin could explain the decrease in adiponectin-mediated Mcl-1 stabilization in the presence of compound C.

4.4.2. ADIPONECTIN EFFECTS ON NEUTROPHIL FUNCTION

Neutrophils are the first innate immune cell to be recruited to site of infection where upon arrival they assist in eliminating invading pathogens. Among the several defensive strategies employed by neutrophils, the processes of phagocytosis and superoxide generation are the best characterized. Adiponectin decreases the killing ability of neutrophils by impairing the generation of ROS through inhibition of $p47^{phox}$ phosphorylation [451]. This observation was confirmed here on isolated neutrophils. However, addition of adiponectin to whole blood did not decrease the oxidative burst produced in response to *E. coli* [687]. This could be due to the presence of factors in the serum combating the effects of adiponectin, other cell populations present in the whole blood, as well as the different stimulus used (*E. coli* was used to stimulate the oxidative burst in the blood whereas isolated neutrophils were stimulated with fMLP). In contrast, the reduction in neutrophil phagocytosis was observed both in the whole blood and on isolated neutrophils incubated with adiponectin. No substantial differences were observed between neutrophils obtained from young and elderly donors.

The inhibitory function of adiponectin on isolated neutrophils was dose and time dependent and was much greater compared to the whole blood. This difference could be possibly due to

the presence of adiponectin in the blood, though we cannot exclude the possibility that in whole blood the effects of adiponectin are modified by other serum factors or immune cell populations. Another issue to consider is the time of incubation with *E. coli*. The Phagotest™ kit involves incubating whole blood with bacteria for 10 minutes, whereas the shortest incubation time used here with isolated neutrophils was 30 minutes. In the time course performed on isolated neutrophils an additive inhibitory effect of adiponectin over time was noted. Thus, the lower inhibitory effect of adiponectin observed in whole blood stimulations may be a consequence of the shorter incubation time.

Adiponectin did not alter the expression of the phagocytic receptors CD11b, CD16 and TLR4 on unstimulated neutrophils, but it decreased the activation of the complement receptor Mac-1 in response to *E. coli* stimulation. Mac-1 is one of the main integrins expressed by neutrophils. Due to its large range of ligands, which include extracellular matrix proteins (i.e. fibronectin), blood proteins (i.e. fibrinogen), complement factors (i.e. iC3b) [688], adhesion molecules (i.e. intercellular adhesion molecule-1, ICAM-1) [667] and microbial components [689], it is involved in several processes, such as phagocytosis, adherence to the endothelium and chemotaxis [690, 691]. Mac-1 is expressed in a low-affinity conformational state on resting neutrophils. When cells are challenged with activatory stimuli the integrin undergoes clustering (to increase receptor avidity) and conformational changes in its extracellular portion to increase the receptor affinity for its ligands [667, 692]. The factors known to increase Mac-1 affinity and avidity are chemotactic molecules, cytokines, PMA, cations and physiological ligands [667, 690]. In this thesis, opsonized *E. coli* also resulted in increased activation of Mac-1 and adiponectin also reduced the binding of bacteria to the neutrophil surface.

It is important to highlight that Mac-1 is not just involved in the binding of bacteria to the phagocyte surface but also in their ingestion [693]. In addition, Mac-1 can cooperate with

FcγRs enhancing FcγR-mediated phagocytosis, as blocking Mac-1 function downregulated the uptake of Ig-coated erythrocytes and yeast [694, 695]. Given the complexity and the importance of Mac-1 function in the different stages of phagocytosis, the reduced activation of Mac-1 caused by adiponectin could also have contributed to decrease the uptake of opsonised *E. coli*.

In contrast to the findings in resting neutrophils, the PKB and ERK 1/2 signalling pathways were negatively affected by adiponectin when neutrophils were stimulated with bacteria. PKB phosphorylation results from activation of PI3K, which is involved in the process of phagocytosis [237, 238, 696]. ERK 1/2, whose activation can occur both downstream and independently of PI3K [238], also appears to be essential for optimal phagocytosis [240, 697]. The role of PKB and ERK 1/2 in neutrophil phagocytosis was confirmed here by the use of the specific inhibitors LY29004 and PD98059, which decreased neutrophil phagocytosis in a dose-dependent manner. With the use of these inhibitors, it was also possible to determine that the conformational change in Mac-1 following adiponectin treatment was dependent on PI3K but not ERK 1/2 activation. This was expected as previous studies have shown that Mac-1 activation is mediated by cytoskeletal rearrangements [698] and the PI3K product PtdIns-(3,4,5)P_3 might activate integrins through regulation of the cytoplasmatic domain by adaptor proteins [692].

The PI3K inhibitor LY29004 inhibited neutrophil phagocytosis to a greater extent compared to inhibition of Mac-1 activation, indicating that PI3K participates in multiple aspects of the phagocytic mechanism. In fact PI3K, its substrate PKB and its product PtdIns-(3,4,5)P_3 are indispensable for cell polarization and elongation [699], pseudopodia extension [237, 238], F-actin polarization [700, 701] and phagosome closure [702].

ERK 1/2 has been reported to be necessary for macrophage phagocytosis of *Francisella tularensis*, being activated by the tyrosine kinase Syk [697]. In addition, ERK 1/2

phosphorylates cortactin, a protein that promotes actin polymerization [669] and ERK 1/2 inhibition in macrophages resulted in reduced cell elongation in response to mycobacteria [699]. Thus ERK 1/2 presumably regulates the ingestion of bacteria by facilitating the formation of pseudopods through cytoskeletal rearrangements, though to a lesser extent than PI3K. Therefore, the reduction in neutrophil content of F-actin observed in response to bacterial stimulation could the result of PKB and ERK 1/2 inhibition by adiponectin.

Apart from the pathways explored here, the modulation of other protein kinases could lead to reduced phagocytic ability in response to adiponectin treatment. One of these kinases could be PKC ζ, whose activity has been found to be decreased by adiponectin [454]. PKC ζ activation is necessary for complete phagocytosis of bacteria *Helicobacter pilori* by monocytes [703], it is activated by PI3K and contributes to F-actin accumulation [701] and possibly to activation of Mac-1 [704]. Therefore, its activation in response to *E. coli* could have been dampened by pre-incubation by adiponectin, though this would need further research to confirm this proposal.

As AMPK is activated by adiponectin in phagocytes [451] its role in neutrophil phagocytosis was determined, but activation by AICAR did not decrease neutrophil phagocytosis, thus AMPK does not participate in the adiponectin inhibitory effect. A previous work has shown that AICAR increased neutrophil phagocytosis [705], though this was not seen in this thesis.

Adiponectin could also have inhibited neutrophil phagocytosis through its C-terminal C1q-homology domain, which has been found to exert an antagonistic action on macrophages [470], therefore impairing macrophage phagocytosis. The main receptor for C1q is C1qRp, but C1q is believed to bind to other phagocytic receptors expressed by neutrophils, particularly CR1 [706]. To determine whether adiponectin impaired neutrophil phagocytosis through its C1q-homologous domain, cells were pre-incubated with C1q at a physiological concentration before the addition of adiponectin, but neutrophil phagocytosis was not rescued by C1q treatment. Importantly, C1q has been reported to enhance the uptake of apoptotic bodies in

human macrophages [707] and monocytic cell lines [707, 708], suggesting that this complement factor regulates the phagocytic ability in a cell-specific manner.

4.4.3 PRO- AND ANTI-INFLAMMATORY ACTION OF ADIPONECTIN ON NEUTROPHILS

Full length adiponectin appears to have a marked effect on neutrophil biology. Previous work has reported that this adipokine inhibits superoxide generation [451], and in this thesis it was shown that it also delayed neutrophil apoptosis and inhibited phagocytosis. At first sight, these findings appear contradictory, as adiponectin exerted both a pro-inflammatory function, by increasing neutrophil lifespan, and an anti-inflammatory effect by dampening neutrophil phagocytic ability. The main difference between the two conditions is the activation status of cells. Adiponectin decreased apoptosis in resting neutrophils by increasing the phosphorylation of the pro-survival kinases AMPK, PKB and ERK 1/2 and decreased neutrophil phagocytosis when neutrophils were incubated with *E. coli* through a reduction in the phosphorylation of PKB and ERK 1/2. Intriguingly, phosphorylation of p38 was enhanced by adiponectin in resting neutrophils but it was not decreased in neutrophils exposed to *E. coli*. It is possible to argue that extending neutrophil lifespan but reducing the cells phagocytic ability, would overall be pro-inflammatory.

This ambiguous effect of adiponectin is not entirely novel. As discussed in the introduction (section 1.3), a number of studies have reported pro-inflammatory effects of adiponectin through induction of PKB and ERK 1/2 phosphorylation in unstimulated cells, i.e.muscle, endothelial cells and hepatocytes [457-460], whilst others have reported the exact opposite effect on PKB and ERK 1/2 phosphorylation in cells stimulated with different factors [461, 462, 464-466]. Opposite effects of adiponectin were also found regarding the expression of pro-inflammatory cytokines (IL-6 and TNF-α) in human macrophages, depending on whether adiponectin was administered in resting cells [478] or cells subjected to further stimulations [474].

Supporting these findings, two groups independently investigated the activity of NF-kB on resting and stimulated monocytic and endothelial cell lines in response to adiponectin and reported that adiponectin enhanced the transcriptional activity of NF-kB in unstimulated cells, whereas it decreased NF-kB activity when cells were pre-treated with adiponectin and then stimulated with pro-inflammatory stimuli (i.e. LPS, TNF-α), [679, 680]. Among the different isoforms of adiponectin, the only one able to influence NF-kB activity was the HMW [679].

Apart from adiponectin, the pro-inflammatory cytokine IFN-γ also exerts differential effects according to the state of the cell: Frausto-Del-Rio et al. showed that despite IFN-γ enhancing F-actin polymerization through PI3K activation on resting monocyte-derived macrophages, it decreased F-actin content when cells were challenged with IgG-coated sheep red blood cells and CCL5 stimulation, thereby reducing the phagocytosis of IgG-SRBC and non-opsonized *E. coli* [709].

Despite adiponectin exerting opposite functions, common pathways may contribute to the reduction of both neutrophil apoptosis and phagocytosis. For instance, the decline in the content of ceramide in the neutrophil membrane not only represents an anti-apoptotic mechanism but it could also mediate the decrease in neutrophil phagocytosis caused by adiponectin, as ceramide-rich lipid rafts are required for receptor clustering [710] and ingestion of various bacteria [711]. Moreover, as mentioned before, the inhibitory effect that adiponectin exerts on ROS production is consistent with reduced apoptosis.

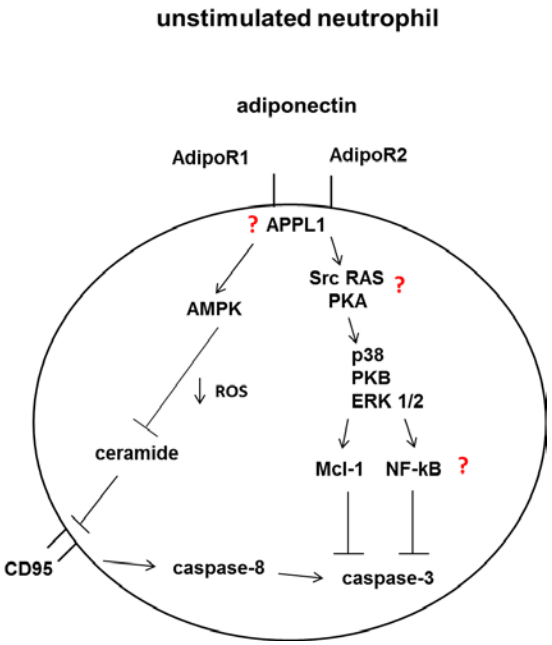
It is evident that some molecules activated by adiponectin receptors are responsible for the differential modulation of the downstream signal pathways in resting and stimulated cells. The first factor to be recruited by adiponectin receptors is the adaptor molecule APPL1: this could represent the key protein modulating adiponectin signalling. Although APPL1 has been shown to participate in the activation of PI3K [469] and PKB [467, 712], as well as AMPK, ERK 1/2 and p38 [467, 712, 713], Bohdanowicz et al. demonstrated that overexpression of APPL1

decreased phosphorylation of PKB in macrophages in response to IgG-coated beads. In particular, they showed that APPL1 moved to phagosomes recruiting two inositol-5 phosphatases which are responsible for dephosphorylation of both PtdIns(3,4,5)P_3 and PtdIns(3,4,5)P_2, which serve as platforms for PKB activation, thereby inhibiting its phosphorylation [714]. In the case of neutrophils, APPL1 could have been recruited to the plasma membrane by adiponectin receptors, and it would have localized to forming phagosomes before their closure, thus recruiting these phosphatases and dampening PKB phosphorylation in response to stimulation. This hypothesis could partially explain also the inhibition of ERK 1/2 phosphorylation, as this is both dependent and independent on PI3K [238]. Importantly, the same group found that APPL1 does not inhibit p38 phosphorylation in response to IgG-coated beads, which is coherent with results shown here as no change in p38 phosphorylation was seen with adiponectin in *E. coli* stimulated neutrophils.

4.4.4 CONCLUSION AND FUTURE PERSPECTIVES

In summary, the work presented here demonstrated that adiponectin affects neutrophil biology by exerting an anti-apoptotic effect and by reducing the phagocytic ability of these cells (Figure 4.24). Supplemental work is necessary to further elucidate the reason underlying the apparent difference in effects on signalling pathways in resting and activated cells. As already discussed, APPL1 could be the main reason for this, and therefore its role should be investigated in more detail with regards to PI3K/ PKB and ERK 1/2 activation in different contexts. In addition, it would be useful to determine which signalling pathways are induced by the two adiponectin receptors in neutrophils, and consequently, it might be of interest to assess whether incubation with adiponectin and/or with pro-inflammatory stimuli alters the expression of the two receptors on neutrophil surface.

A



B

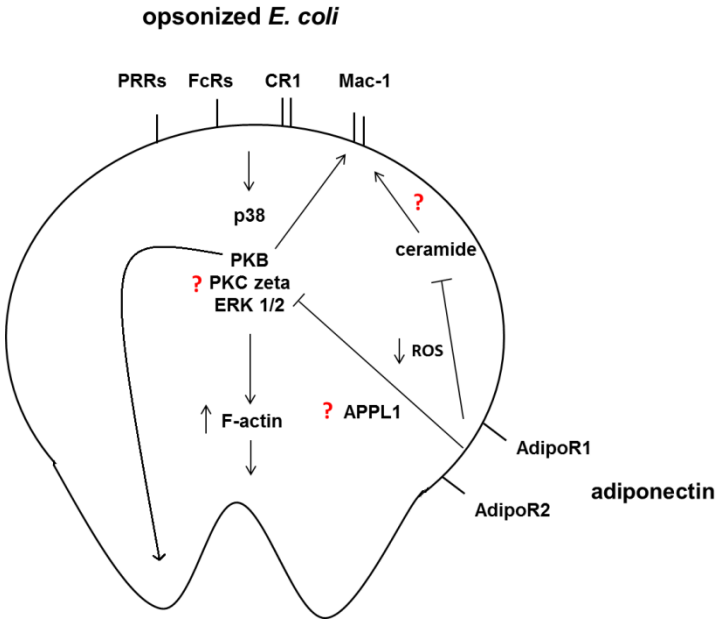


Figure 4.24 Proposed model for action of adiponectin on unstimulated (A) and *E. coli* stimulated neutrophils (B). Red question marks pathways induced by adiponectin that have still to be confirmed in neutrophils.

CHAPTER 5

GENERAL DISCUSSION

5.1 THE INFLUENCE OF SLEEP ON THE IMMUNE SYSTEM

The world population has experienced a decline in sleep duration over the past century [1-3] and both short and long sleep duration have been associated with increased risk of mortality [5-11]. Lack of sleep has also been linked to development of several morbidities, particularly metabolic [115-117, 139-141] and cardiovascular diseases [118, 119], and all of these are characterized by a high inflammatory status. Moreover, short sleep duration decreases the efficacy of vaccinations [129] and increases the risk of infections [127, 128]. Therefore, understanding how sleep acts upon the immune system has aroused increasing interest in the scientific community, though less so in respect to the elderly.

Several lines of evidence have demonstrated the somnogenic effect exerted by pro-inflammatory cytokines [371-373], however no study has extensively investigated how immune functions and inflammatory processes are governed by sleep. The majority of work conducted so far examined whether a few immunological parameters, especially the levels of cytokines and the number of circulating immune cells, could be altered following a session of acute or partial sleep deprivation. Moreover, sleep duration has often been the only parameter of sleep considered. Thus, the novelty of this project is in the large number of immune and sleep variables measured, allowing a comprehensive understanding of how both physiological sleep duration and continuity might impact on the immune system. Moreover the focus has been on this relationship in the context of ageing, which is not well researched.

Healthy elderly individuals were recruited as the aged population has been suggested to suffer from physiological disturbances of sleep and these could negatively affect immune functions. In parallel, the effects of partial sleep deprivation on both young and elderly individuals were also investigated to assess whether this protocol could have differential outcomes on immunity in the two age groups.

The 93 elderly subjects recruited were characterised by homogeneous sleep duration, the majority of subjects displaying average sleep duration between 6.30 and 7.30 hours per night and very few subjects sleeping less than 5 hours or longer than 8.30 hours per night, despite their age range being quite broad. Surprisingly in the volunteers undergoing the partial sleep deprivation protocol no significant differences were detected in the baseline sleep parameters between young and elderly subjects. This lack of differences in sleep duration in our older volunteers may reflect the fact that they were all in very good health. In this thesis the initial hypothesis that sleep could affect the process of immunosenescence was not confirmed. Nevertheless, the results obtained showed that physiological sleep, both duration and qualitative parameters, can modulate the immune system in the elderly, particularly with regard to the number of circulating immune cell populations and T lymphocyte subsets. In general, short sleep duration as well as sleep disruption (low efficiency and average sleep bout, high WASO) were associated with a higher number of WBC, granulocytes, monocytes and a higher G:L ratio. Long sleepers were characterised by reduced number of lymphocytes and percentages of naïve T cells.

To understand which factors could mediate or be modified by such changes, the serum concentration of pro- and anti-inflammatory cytokines, the adipokines leptin and adiponectin, and the adrenal stress hormones cortisol and DHEAS were measured. Serum adiponectin and lymphocyte numbers appear to be respectively increased and decreased in the tertile of long sleepers. In the literature adiponectin has been reported to indirectly inhibit B lymphopoiesis in bone marrow cultures [484], thus the reduction observed in the total population of lymphocytes could be caused specifically by decreased differentiation of B cells by this adipokine as B and T cell numbers are usually altered similarly as they have a common stem cell progenitor.

Adiponectin also decreases myelopoiesis and granulopoiesis both in mice and humans [429, 470, 471]. Although a tendency and not a significant association between adiponectin and sleep

duration (p=0.065) was seen, it is possible that this adipokine could contribute to the reduction in the number of circulating granulocytes and monocytes seen in long sleepers. However, its levels did not significantly correlate with sleep efficiency either, whereas the number of granulocytes and monocytes showed a significant reduction with this sleep parameter.

Both the expression of adiponectin from the WAT [196-198] and hematopoiesis [322] are regulated by cytokines. The results shown here indicate that long sleep is associated with lower levels of IL-8 (analyses unadjusted for medications) and higher levels of IL-4. These two cytokines are known to be produced mainly by monocytes (IL-8) [715] and Th2 lymphocytes (IL-4) [322] and, together with adiponectin concentration, the differences in their serum levels support the idea that sleep modestly influences the pro-inflammatory status of elderly individuals, with long sleep duration associated with decreased systemic inflammation. IL-8 is a potent chemotactic factor [716] and it can stimulate neutrophil mobilisation from the bone marrow [717, 718], hence this cytokine could contribute to increased number of circulating granulocytes in short sleepers, though the association of IL-8 with short sleep was not significant.

In this study significant associations between the G:L ratio and the parameters of sleep fragmentation, WASO and average sleep bout were found. These two sleep variables were also correlated with cortisol levels, although the significant relationship between cortisol and WASO was lost when adjusted for use of anti-hypertensive drugs. Importantly, glucocorticoids delay apoptosis in neutrophils [344, 345] but they promote apoptosis in T lymphocytes [719]. Specifically, cortisol decreases the pool of naïve T cells in the circulation [326] and its levels have been shown to positively correlate with the neutrophil to lymphocyte ratio [720]. Although in our cohort cortisol was not significantly associated with the G:L ratio (R^2=0.024, β=0.155, p=0.189), we cannot exclude its involvement in increasing G:L ratio in conditions of highly fragmented sleep.

In addition to these factors, it is possible that other circulating molecules, such as catecholamines, could also have determined those changes. In fact, Zhang et al. revealed that the 24-hour urinary norephinephrine and epinephrine were negatively associated with sleep duration and efficiency, and positively correlated with latency as measured by actigraphy in a cohort of middle-aged individuals [648]. Moreover, it has been shown that catecholamines can rise in response to acute stress, causing leucocytosis through mobilisation of both lymphocytes and granulocytes [348]. Finally, it is also possible that the altered number of peripheral immune cells is subsequent to modifications happening in the bone marrow environment, where hematopoiesis takes place. Fluctuations in the local levels of cytokines and adipokines, which might be not well be represented by the levels measured in the serum, could have determined these differences.

As neutrophil and NK cell functions were not linked to sleep behaviour, it can be concluded that physiological sleep disruption does not contribute to accelerate innate immunosenescence. When adaptive immunosenescence is considered, this appears to be even reduced with disrupted sleep, as the percentage of naïve T cells were negatively correlated with sleep duration, and CD4+ T cells as well as the CD4+:CD8+ T cell ratio were increased in association with higher sleep fragmentation. However, to confirm such relationships it would have been helpful to screen our volunteers for other factors thought to be influencing these parameters, notably CMV infection. CMV is thought to be mainly responsible for inversion of the CD4+:CD8+ T cell ratio in old age and for accumulation of terminally differentiated CD8+ memory T cells [584, 585]. Thus, if aged long sleepers have higher frequency of CMV infection, sleep would not be the primary cause driving these changes.

Finally, as few markers of inflammation were significantly related to sleep duration, it is not possible to affirm that sleep may have an influence in accelerating the process of inflammaging in elderly individuals.

Despite the impact of physiological sleep shown on these aspects of immunity, the study of partial sleep deprivation did not corroborate these findings or confirm causality. In fact, the partial sleep deprivation protocol induced modest effects on the immune parameters considered, especially in young volunteers. Recruitment of a higher number of volunteers is necessary in order to detect significant changes prompted by forced lack of sleep and different responses displayed by young and elderly individuals.

5.2 ADIPONECTIN AND NEUTROPHILS: IN VITRO STUDIES AND IN VIVO IMPLICATIONS

Adiponectin is the adipokine mostly secreted by the WAT [424-426], it has elicited increasing interest as it protects from the development of insulin resistance, type II diabetes and cardiovascular diseases [184, 185]. Adiponectin appears to have a bidirectional communication with the immune system, as it acts as a potent modulator of immune functions and its expression by the WAT is regulated by cytokines [196-198]. With regard to neutrophils, before the publication of the results in this thesis [671, 687], adiponectin was only known to reduce the oxidative burst in response to fMLP and PMA stimulation [451, 472]. It was decided to further investigate the role of adiponectin on neutrophil apoptosis and phagocytosis, as these are other mechanisms essential to the resolution of systemic inflammation (apoptosis) and infection (phagocytosis). The *in vitro* findings show a dual pro- and anti-inflammatory role exerted by adiponectin in promoting neutrophil survival and inhibiting neutrophil phagocytic ability respectively. As discussed in chapter 4, these apparent contradictions in the actions exerted by adiponectin have already been observed in relation to NF-kB activity in endothelial and monocytic cells [679, 680] and in other studies exploring the modulation of pro-inflammatory programs in macrophages [474, 478]. Such opposite effects were dependent on whether adiponectin was administered in unstimulated cells or cells subjected to further stimulations.

The main issue that could not be addressed in this thesis is whether these results achieved in an *in vitro* setting would be reproducible *in vivo*. To answer this question it would have been useful to carry out experiments on animal models, by evaluating neutrophil functions in a KO model for adiponectin and/or in wild type (wt) animals after systemic injection of this adipokine. From the sleep study performed with elderly volunteers it was observed that increased levels of adiponectin were associated with lower number of granulocytes in long sleepers, suggesting that adiponectin's inhibitory effect on granulopoiesis could be greater than its anti-apoptotic action *in vivo*. Nevertheless, adiponectin levels are higher in certain inflammatory diseases, such as COPD [501], in which reduced levels of neutrophil apoptosis are well documented [721, 722]. In both cases, we cannot exclude the participation of other circulating factors which regulate both neutrophil lifespan and adiponectin expression; if this is the case, adiponectin would not directly modulate the number of circulating neutrophils *in vivo*. Whether adiponectin can delay neutrophil apoptosis also in a pro-inflammatory environment, i.e. when cells are activated, remains to be elucidated. As we [687] and others [451, 470, 474] noted a distinct anti-inflammatory effect of adiponectin on activated cells, it is conceivable that, in a pro-inflammatory environment, adiponectin could promote neutrophil death.

Although the exact role played by adiponectin on neutrophil apoptosis *in vivo* remains to be established, the inhibitory action exerted by adiponectin on neutrophil phagocytosis is more likely to persist *in vivo*, as pre-treatment of whole blood with adiponectin decreased the bacterial uptake by neutrophils. Moreover, several *in vivo* studies indicated an anti-inflammatory role for this adipokine [488, 489, 723]. As confirmed in this thesis and shown by others [442, 611, 612], old age is characterised by higher levels of adiponectin, thus this finding could also help to explain the decreased phagocytic ability and the increased rate of infections typical of elderly individuals [505]. Therefore, although adiponectin exhibits many beneficial effects, raised levels of this adipokine could be harmful by increasing the risk of infection.

5.3 CONCLUSION AND PERSPECTIVES

Although the results shown here demonstrate that physiological sleep disruption during ageing does not enhance immunosenescence, our data highlight that long sleep duration is associated with lower pro-inflammatory status and the number of myeloid cells, higher levels of adiponectin, decreased lymphocyte count and naïve T cell subsets. Conversely, short sleep duration and poor sleep continuity associate with a higher number of several immune cell populations and increased level of cortisol. Therefore, it is proposed that a duration of sleep between 6.30-7.30 hours per night (as displayed by the middle tertile of sleep duration) and an improvement in sleep continuity, i.e. efficiency, latency and fragmentation, could induce a beneficial effect on the overall health status of the elderly population by controlling the immune and hormonal variables mentioned before. This, in turn, would also help to contrast the development of obesity, cardiovascular and metabolic diseases, for which inflammation is a crucial common factor [724-726].

The results in this thesis reinforce previous evidence pointing to the existence of a complex network of communications between the CNS and peripheral organs, particularly the immune system, the adrenal gland and the WAT. Physiological sleep duration and continuity were associated with changes in the number of circulating immune cells and serum concentration of cytokines, particularly IL-6 and IL-8, adipokines and cortisol in elderly subjects. All these factors have been shown to participate in the regulation of vital functions such as thermoregulation [727, 728], appetite, metabolism [729, 730] and sleep [98, 174, 371-373]. Therefore, the data in this thesis and the literature allow the proposal of a model for the interaction of sleep with the immune system, the adipose tissue and the HPA axis in old age (Figure 5.1).

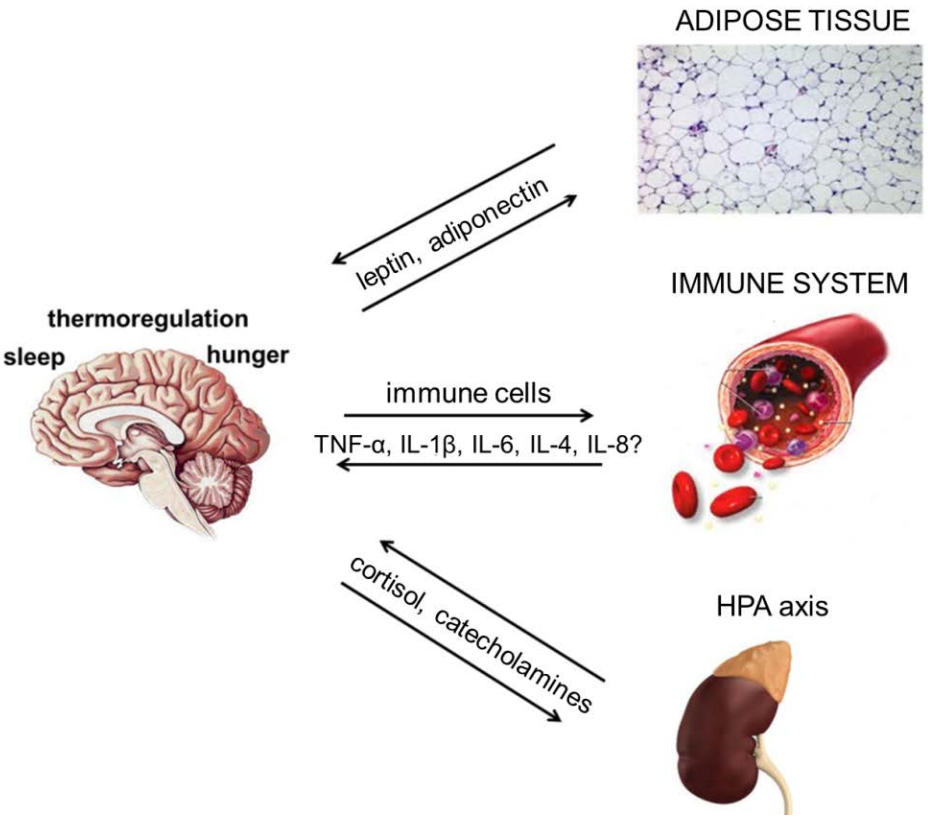


Figure 5.1 Proposed model of bidirectional communications between the CNS and peripheral organs through soluble factors, as determined by findings from this thesis and previous studies. Circulating immune cells and soluble factors mediate the bi-directional communication lying between the CNS and the immune system: immune cell number is influenced by sleep duration and continuity as assessed in this thesis, while cytokines regulate sleep [371-373] and body temperature [727] and their serum levels are also influenced by sleep duration [640]. The adipose tissue communicates with the CNS through adipokines, mainly leptin, which regulates food intake [729], and adiponectin, which has been found to be upregulated in long sleepers in this thesis. Moreover, the adrenal gland secretes cortisol and catecholamines, whose levels fluctuate under psychological stress [65, 346] and whose role in regulating sleep [79, 347] and hunger [731] has also been proposed.

REFERENCES

1. Jean-Louis, G., D.F. Kripke, S. Ancoli-Israel, M.R. Klauber and R.S. Sepulveda, *Sleep duration, illumination, and activity patterns in a population sample: effects of gender and ethnicity.* Biological psychiatry, 2000. **47**(10): p. 921-7.
2. Bixler, E., *Sleep and society: an epidemiological perspective.* Sleep medicine, 2009. **10 Suppl 1**: p. S3-6.
3. Kripke, D.F., R.N. Simons, L. Garfinkel and E.C. Hammond, *Short and long sleep and sleeping pills. Is increased mortality associated?* Archives of general psychiatry, 1979. **36**(1): p. 103-16.
4. Rechtschaffen, A., B.M. Bergmann, C.A. Everson, C.A. Kushida and M.A. Gilliland, *Sleep deprivation in the rat: X. Integration and discussion of the findings.* Sleep, 1989. **12**(1): p. 68-87.
5. Tamakoshi, A. and Y. Ohno, *Self-reported sleep duration as a predictor of all-cause mortality: results from the JACC study, Japan.* Sleep, 2004. **27**(1): p. 51-4.
6. Kripke, D.F., L. Garfinkel, D.L. Wingard, M.R. Klauber and M.R. Marler, *Mortality associated with sleep duration and insomnia.* Archives of general psychiatry, 2002. **59**(2): p. 131-6.
7. Chien, K.L., P.C. Chen, H.C. Hsu, T.C. Su, F.C. Sung, M.F. Chen and Y.T. Lee, *Habitual sleep duration and insomnia and the risk of cardiovascular events and all-cause death: report from a community-based cohort.* Sleep, 2010. **33**(2): p. 177-84.
8. Cappuccio, F.P., L. D'Elia, P. Strazzullo and M.A. Miller, *Sleep duration and all-cause mortality: a systematic review and meta-analysis of prospective studies.* Sleep, 2010. **33**(5): p. 585-92.
9. Kakizaki, M., S. Kuriyama, N. Nakaya, T. Sone, M. Nagai, Y. Sugawara, A. Hozawa, S. Fukudo and I. Tsuji, *Long sleep duration and cause-specific mortality according to physical function and self-rated health: the Ohsaki Cohort Study.* Journal of sleep research, 2013. **22**(2): p. 209-16.
10. Gallicchio, L. and B. Kalesan, *Sleep duration and mortality: a systematic review and meta-analysis.* Journal of sleep research, 2009. **18**(2): p. 148-58.
11. Kripke, D.F., R.D. Langer, J.A. Elliott, M.R. Klauber and K.M. Rex, *Mortality related to actigraphic long and short sleep.* Sleep medicine, 2011. **12**(1): p. 28-33.
12. Hori, T., Y. Sugita, E. Koga, S. Shirakawa, K. Inoue, S. Uchida, H. Kuwahara, M. Kousaka, T. Kobayashi, Y. Tsuji, M. Terashima, K. Fukuda and N. Fukuda, *Proposed supplements and amendments to 'A Manual of Standardized Terminology, Techniques and Scoring System for Sleep Stages of Human Subjects', the Rechtschaffen & Kales (1968) standard.* Psychiatry and clinical neurosciences, 2001. **55**(3): p. 305-10.
13. Finelli, L.A., H. Baumann, A.A. Borbely and P. Achermann, *Dual electroencephalogram markers of human sleep homeostasis: correlation between theta activity in waking and slow-wave activity in sleep.* Neuroscience, 2000. **101**(3): p. 523-9.
14. Tan, X., I.G. Campbell and I. Feinberg, *Internight reliability and benchmark values for computer analyses of non-rapid eye movement (NREM) and REM EEG in normal young adult and elderly subjects.* Clinical neurophysiology : official journal of the International Federation of Clinical Neurophysiology, 2001. **112**(8): p. 1540-52.
15. Nardone, R., S. Golaszewski, Y. Holler, M. Christova, E. Trinka and F. Brigo, *Neurophysiological insights into the pathophysiology of REM sleep behavior disorders: a review*. Neuroscience research, 2013. **76**(3): p. 106-12.
16. Aserinsky, E. and N. Kleitman, *Regularly occurring periods of eye motility, and concomitant phenomena, during sleep.* Science (New York, N Y), 1953. **118**(3062): p. 273-4.

17. Somers, V.K., M.E. Dyken, A.L. Mark and F.M. Abboud, *Sympathetic-nerve activity during sleep in normal subjects.* The New England journal of medicine, 1993. **328**(5): p. 303-7.
18. Bryant, P.A., J. Trinder and N. Curtis, *Sick and tired: Does sleep have a vital role in the immune system?* Nature reviews. Immunology, 2004. **4**(6): p. 457-67.
19. Brezinova, V., *Sleep cycle content and sleep cycle duration.* Electroencephalography and clinical neurophysiology, 1974. **36**(3): p. 275-82.
20. Neubauer, D.N., *Sleep problems in the elderly.* American family physician, 1999. **59**(9): p. 2551-8, 2559-60.
21. Pracka, D. and T. Pracki, *Spectral analysis in cyclic changes of human sleep evaluation.* Acta neurobiologiae experimentalis, 1996. **56**(1): p. 255-8.
22. Ancoli-Israel, S., R. Cole, C. Alessi, M. Chambers, W. Moorcroft and C.P. Pollak, *The role of actigraphy in the study of sleep and circadian rhythms.* Sleep, 2003. **26**(3): p. 342-92.
23. Littner, M., C.A. Kushida, W.M. Anderson, D. Bailey, R.B. Berry, D.G. Davila, M. Hirshkowitz, S. Kapen, M. Kramer, D. Loube, M. Wise and S.F. Johnson, *Practice parameters for the role of actigraphy in the study of sleep and circadian rhythms: an update for 2002.* Sleep, 2003. **26**(3): p. 337-41.
24. Mullaney, D.J., D.F. Kripke and S. Messin, *Wrist-actigraphic estimation of sleep time.* Sleep, 1980. **3**(1): p. 83-92.
25. Cole, R.J., D.F. Kripke, W. Gruen, D.J. Mullaney and J.C. Gillin, *Automatic sleep/wake identification from wrist activity.* Sleep, 1992. **15**(5): p. 461-9.
26. de Souza, L., A.A. Benedito-Silva, M.L. Pires, D. Poyares, S. Tufik and H.M. Calil, *Further validation of actigraphy for sleep studies.* Sleep, 2003. **26**(1): p. 81-5.
27. Lichstein, K.L., K.C. Stone, J. Donaldson, S.D. Nau, J.P. Soeffing, D. Murray, K.W. Lester and R.N. Aguillard, *Actigraphy validation with insomnia.* Sleep, 2006. **29**(2): p. 232-9.
28. Elbaz, M., G.M. Roue, F. Lofaso and M.A. Quera Salva, *Utility of actigraphy in the diagnosis of obstructive sleep apnea.* Sleep, 2002. **25**(5): p. 527-31.
29. Sadeh, A., P.J. Hauri, D.F. Kripke and P. Lavie, *The role of actigraphy in the evaluation of sleep disorders.* Sleep, 1995. **18**(4): p. 288-302.
30. Kushida, C.A., A. Chang, C. Gadkary, C. Guilleminault, O. Carrillo and W.C. Dement, *Comparison of actigraphic, polysomnographic, and subjective assessment of sleep parameters in sleep-disordered patients.* Sleep medicine, 2001. **2**(5): p. 389-96.
31. Buysse, D.J., C.F. Reynolds, 3rd, T.H. Monk, S.R. Berman and D.J. Kupfer, *The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research.* Psychiatry research, 1989. **28**(2): p. 193-213.
32. Girschik, J., L. Fritschi, J. Heyworth and F. Waters, *Validation of self-reported sleep against actigraphy.* Journal of epidemiology / Japan Epidemiological Association, 2012. **22**(5): p. 462-8.
33. van den Berg, J.F., H.M. Miedema, J.H. Tulen, A. Hofman, A.K. Neven and H. Tiemeier, *Sex differences in subjective and actigraphic sleep measures: a population-based study of elderly persons.* Sleep, 2009. **32**(10): p. 1367-75.
34. Borbely, A.A., *A two process model of sleep regulation.* Human neurobiology, 1982. **1**(3): p. 195-204.
35. Reppert, S.M. and D.R. Weaver, *Molecular analysis of mammalian circadian rhythms.* Annual review of physiology, 2001. **63**: p. 647-76.
36. Rusak, B. and I. Zucker, *Neural regulation of circadian rhythms.* Physiological reviews, 1979. **59**(3): p. 449-526.
37. Abe, K., J. Kroning, M.A. Greer and V. Critchlow, *Effects of destruction of the suprachiasmatic nuclei on the circadian rhythms in plasma corticosterone, body*

temperature, feeding and plasma thyrotropin. Neuroendocrinology, 1979. **29**(2): p. 119-31.
38. Satinoff, E. and R.A. Prosser, *Suprachiasmatic nuclear lesions eliminate circadian rhythms of drinking and activity, but not of body temperature, in male rats.* Journal of biological rhythms, 1988. **3**(1): p. 1-22.
39. Moore, R.Y. and V.B. Eichler, *Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat.* Brain research, 1972. **42**(1): p. 201-6.
40. Green, D.J. and R. Gillette, *Circadian rhythm of firing rate recorded from single cells in the rat suprachiasmatic brain slice.* Brain research, 1982. **245**(1): p. 198-200.
41. Golombek, D.A. and R.E. Rosenstein, *Physiology of circadian entrainment.* Physiological reviews, 2010. **90**(3): p. 1063-102.
42. Macchi, M.M. and J.N. Bruce, *Human pineal physiology and functional significance of melatonin.* Frontiers in neuroendocrinology, 2004. **25**(3-4): p. 177-95.
43. Perreau-Lenz, S., A. Kalsbeek, M.L. Garidou, J. Wortel, J. van der Vliet, C. van Heijningen, V. Simonneaux, P. Pevet and R.M. Buijs, *Suprachiasmatic control of melatonin synthesis in rats: inhibitory and stimulatory mechanisms.* The European journal of neuroscience, 2003. **17**(2): p. 221-8.
44. Cajochen, C., S.B. Khalsa, J.K. Wyatt, C.A. Czeisler and D.J. Dijk, *EEG and ocular correlates of circadian melatonin phase and human performance decrements during sleep loss.* The American journal of physiology, 1999. **277**(3 Pt 2): p. R640-9.
45. Vanecek, J., *Cellular mechanisms of melatonin action.* Physiological reviews, 1998. **78**(3): p. 687-721.
46. Brzezinski, A., M.G. Vangel, R.J. Wurtman, G. Norrie, I. Zhdanova, A. Ben-Shushan and I. Ford, *Effects of exogenous melatonin on sleep: a meta-analysis.* Sleep medicine reviews, 2005. **9**(1): p. 41-50.
47. Cajochen, C., K. Krauchi and A. Wirz-Justice, *Role of melatonin in the regulation of human circadian rhythms and sleep.* Journal of neuroendocrinology, 2003. **15**(4): p. 432-7.
48. Shibata, S., V.M. Cassone and R.Y. Moore, *Effects of melatonin on neuronal activity in the rat suprachiasmatic nucleus in vitro.* Neuroscience letters, 1989. **97**(1-2): p. 140-4.
49. Mason, R. and B. Rusak, *Neurophysiological responses to melatonin in the SCN of short-day sensitive and refractory hamsters.* Brain research, 1990. **533**(1): p. 15-9.
50. Wang, F., J. Li, C. Wu, J. Yang, F. Xu and Q. Zhao, *The GABA(A) receptor mediates the hypnotic activity of melatonin in rats.* Pharmacology, biochemistry, and behavior, 2003. **74**(3): p. 573-8.
51. Steiger, A., *Neurochemical regulation of sleep.* Journal of psychiatric research, 2007. **41**(7): p. 537-52.
52. Schwartz, J.R. and T. Roth, *Neurophysiology of sleep and wakefulness: basic science and clinical implications.* Current neuropharmacology, 2008. **6**(4): p. 367-78.
53. Espana, R.A. and T.E. Scammell, *Sleep neurobiology for the clinician.* Sleep, 2004. **27**(4): p. 811-20.
54. Sakurai, T., A. Amemiya, M. Ishii, I. Matsuzaki, R.M. Chemelli, H. Tanaka, S.C. Williams, J.A. Richardson, G.P. Kozlowski, S. Wilson, J.R. Arch, R.E. Buckingham, A.C. Haynes, S.A. Carr, R.S. Annan, D.E. McNulty, W.S. Liu, J.A. Terrett, N.A. Elshourbagy, D.J. Bergsma and M. Yanagisawa, *Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior.* Cell, 1998. **92**(4): p. 573-85.
55. Edwards, C.M., S. Abusnana, D. Sunter, K.G. Murphy, M.A. Ghatei and S.R. Bloom, *The effect of the orexins on food intake: comparison with neuropeptide Y, melanin-concentrating hormone and galanin.* The Journal of endocrinology, 1999. **160**(3): p. R7-12.

56. Lin, L., J. Faraco, R. Li, H. Kadotani, W. Rogers, X. Lin, X. Qiu, P.J. de Jong, S. Nishino and E. Mignot, *The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene.* Cell, 1999. **98**(3): p. 365-76.
57. Nishino, S., B. Ripley, S. Overeem, G.J. Lammers and E. Mignot, *Hypocretin (orexin) deficiency in human narcolepsy.* Lancet, 2000. **355**(9197): p. 39-40.
58. Chemelli, R.M., J.T. Willie, C.M. Sinton, J.K. Elmquist, T. Scammell, C. Lee, J.A. Richardson, S.C. Williams, Y. Xiong, Y. Kisanuki, T.E. Fitch, M. Nakazato, R.E. Hammer, C.B. Saper and M. Yanagisawa, *Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation.* Cell, 1999. **98**(4): p. 437-51.
59. Thannickal, T.C., R.Y. Moore, R. Nienhuis, L. Ramanathan, S. Gulyani, M. Aldrich, M. Cornford and J.M. Siegel, *Reduced number of hypocretin neurons in human narcolepsy.* Neuron, 2000. **27**(3): p. 469-74.
60. Chronwall, B.M., D.A. DiMaggio, V.J. Massari, V.M. Pickel, D.A. Ruggiero and T.L. O'Donohue, *The anatomy of neuropeptide-Y-containing neurons in rat brain.* Neuroscience, 1985. **15**(4): p. 1159-81.
61. Stanley, B.G. and S.F. Leibowitz, *Neuropeptide Y injected in the paraventricular hypothalamus: a powerful stimulant of feeding behavior.* Proceedings of the National Academy of Sciences of the United States of America, 1985. **82**(11): p. 3940-3.
62. Antonijevic, I.A., H. Murck, S. Bohlhalter, R.M. Frieboes, F. Holsboer and A. Steiger, *Neuropeptide Y promotes sleep and inhibits ACTH and cortisol release in young men.* Neuropharmacology, 2000. **39**(8): p. 1474-81.
63. Lancel, M., *Role of GABAA receptors in the regulation of sleep: initial sleep responses to peripherally administered modulators and agonists.* Sleep, 1999. **22**(1): p. 33-42.
64. Krueger, J.M., J. Fang, M.K. Hansen, J. Zhang and F. Obal, Jr., *Humoral Regulation of Sleep.* News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society, 1998. **13**: p. 189-194.
65. Tsigos, C. and G.P. Chrousos, *Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress.* Journal of psychosomatic research, 2002. **53**(4): p. 865-71.
66. Bruhn, T.O., E.L. Anthony, P. Wu and I.M. Jackson, *GRF immunoreactive neurons in the paraventricular nucleus of the rat: an immunohistochemical study with monoclonal and polyclonal antibodies.* Brain research, 1987. **424**(2): p. 290-8.
67. Schneider, H.J., U. Pagotto and G.K. Stalla, *Central effects of the somatotropic system.* European journal of endocrinology / European Federation of Endocrine Societies, 2003. **149**(5): p. 377-92.
68. Brazeau, P., W. Vale, R. Burgus, N. Ling, M. Butcher, J. Rivier and R. Guillemin, *Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone.* Science (New York, N Y), 1973. **179**(4068): p. 77-9.
69. Miki, N., M. Ono, H. Miyoshi, T. Tsushima and K. Shizume, *Hypothalamic growth hormone-releasing factor (GRF) participates in the negative feedback regulation of growth hormone secretion.* Life sciences, 1989. **44**(7): p. 469-76.
70. Gardi, J., F. Obal, Jr., J. Fang, J. Zhang and J.M. Krueger, *Diurnal variations and sleep deprivation-induced changes in rat hypothalamic GHRH and somatostatin contents.* The American journal of physiology, 1999. **277**(5 Pt 2): p. R1339-44.
71. Holl, R.W., M.L. Hartman, J.D. Veldhuis, W.M. Taylor and M.O. Thorner, *Thirty-second sampling of plasma growth hormone in man: correlation with sleep stages.* The Journal of clinical endocrinology and metabolism, 1991. **72**(4): p. 854-61.
72. Van Cauter, E., M. Kerkhofs, A. Caufriez, A. Van Onderbergen, M.O. Thorner and G. Copinschi, *A quantitative estimation of growth hormone secretion in normal man: reproducibility and relation to sleep and time of day.* The Journal of clinical endocrinology and metabolism, 1992. **74**(6): p. 1441-50.

73. Watts, A.G., S. Tanimura and G. Sanchez-Watts, *Corticotropin-releasing hormone and arginine vasopressin gene transcription in the hypothalamic paraventricular nucleus of unstressed rats: daily rhythms and their interactions with corticosterone.* Endocrinology, 2004. **145**(2): p. 529-40.
74. Weitzman, E.D., *Circadian rhythms and episodic hormone secretion in man.* Annual review of medicine, 1976. **27**: p. 225-43.
75. Fries, E., L. Dettenborn and C. Kirschbaum, *The cortisol awakening response (CAR): facts and future directions.* International journal of psychophysiology : official journal of the International Organization of Psychophysiology, 2009. **72**(1): p. 67-73.
76. Elder, G.J., M.A. Wetherell, N.L. Barclay and J.G. Ellis, *The cortisol awakening response - Applications and implications for sleep medicine.* Sleep medicine reviews, 2013.
77. Kudielka, B.M. and C. Kirschbaum, *Awakening cortisol responses are influenced by health status and awakening time but not by menstrual cycle phase.* Psychoneuroendocrinology, 2003. **28**(1): p. 35-47.
78. Young, E.A., J. Abelson and S.L. Lightman, *Cortisol pulsatility and its role in stress regulation and health.* Frontiers in neuroendocrinology, 2004. **25**(2): p. 69-76.
79. Steiger, A., *Sleep and the hypothalamo-pituitary-adrenocortical system.* Sleep medicine reviews, 2002. **6**(2): p. 125-38.
80. Ehlers, C.L., T.K. Reed and S.J. Henriksen, *Effects of corticotropin-releasing factor and growth hormone-releasing factor on sleep and activity in rats.* Neuroendocrinology, 1986. **42**(6): p. 467-74.
81. Obal, F., Jr., P. Alfoldi, A.B. Cady, L. Johannsen, G. Sary and J.M. Krueger, *Growth hormone-releasing factor enhances sleep in rats and rabbits.* The American journal of physiology, 1988. **255**(2 Pt 2): p. R310-6.
82. Obal, F., Jr., R. Floyd, L. Kapas, B. Bodosi and J.M. Krueger, *Effects of systemic GHRH on sleep in intact and hypophysectomized rats.* The American journal of physiology, 1996. **270**(2 Pt 1): p. E230-7.
83. Kerkhofs, M., E. Van Cauter, A. Van Onderbergen, A. Caufriez, M.O. Thorner and G. Copinschi, *Sleep-promoting effects of growth hormone-releasing hormone in normal men.* The American journal of physiology, 1993. **264**(4 Pt 1): p. E594-8.
84. Marshall, L., I. Derad, C.J. Strasburger, H.L. Fehm and J. Born, *A determinant factor in the efficacy of GHRH administration in promoting sleep: high peak concentration versus recurrent increasing slopes.* Psychoneuroendocrinology, 1999. **24**(3): p. 363-70.
85. Steiger, A., J. Guldner, U. Hemmeter, B. Rothe, K. Wiedemann and F. Holsboer, *Effects of growth hormone-releasing hormone and somatostatin on sleep EEG and nocturnal hormone secretion in male controls.* Neuroendocrinology, 1992. **56**(4): p. 566-73.
86. Perras, B., L. Marshall, G. Kohler, J. Born and H.L. Fehm, *Sleep and endocrine changes after intranasal administration of growth hormone-releasing hormone in young and aged humans.* Psychoneuroendocrinology, 1999. **24**(7): p. 743-57.
87. Guldner, J., T. Schier, E. Friess, M. Colla, F. Holsboer and A. Steiger, *Reduced efficacy of growth hormone-releasing hormone in modulating sleep endocrine activity in the elderly.* Neurobiology of aging, 1997. **18**(5): p. 491-5.
88. Jessup, S.K., B.A. Malow, K.V. Symons and A.L. Barkan, *Blockade of endogenous growth hormone-releasing hormone receptors dissociates nocturnal growth hormone secretion and slow-wave sleep.* European journal of endocrinology / European Federation of Endocrine Societies, 2004. **151**(5): p. 561-6.
89. Obal, F., Jr., B. Bodosi, A. Szilagyi, B. Kacsoh and J.M. Krueger, *Antiserum to growth hormone decreases sleep in the rat.* Neuroendocrinology, 1997. **66**(1): p. 9-16.

90. Mendelson, W.B., S. Slater, P. Gold and J.C. Gillin, *The effect of growth hormone administration on human sleep: a dose-response study.* Biological psychiatry, 1980. **15**(4): p. 613-8.
91. Obal, F., Jr., L. Kapas, J. Gardi, P. Taishi, B. Bodosi and J.M. Krueger, *Insulin-like growth factor-1 (IGF-1)-induced inhibition of growth hormone secretion is associated with sleep suppression.* Brain research, 1999. **818**(2): p. 267-74.
92. Kern, W., R. Halder, S. al-Reda, E. Spath-Schwalbe, H.L. Fehm and J. Born, *Systemic growth hormone does not affect human sleep.* The Journal of clinical endocrinology and metabolism, 1993. **76**(6): p. 1428-32.
93. Opp, M., F. Obal, Jr. and J.M. Krueger, *Corticotropin-releasing factor attenuates interleukin 1-induced sleep and fever in rabbits.* The American journal of physiology, 1989. **257**(3 Pt 2): p. R528-35.
94. Holsboer, F., U. von Bardeleben and A. Steiger, *Effects of intravenous corticotropin-releasing hormone upon sleep-related growth hormone surge and sleep EEG in man.* Neuroendocrinology, 1988. **48**(1): p. 32-8.
95. Spath-Schwalbe, E., D. Uthgenannt, G. Voget, W. Kern, J. Born and H.L. Fehm, *Corticotropin-releasing hormone-induced adrenocorticotropin and cortisol secretion depends on sleep and wakefulness.* The Journal of clinical endocrinology and metabolism, 1993. **77**(5): p. 1170-3.
96. Born, J., E. Spath-Schwalbe, H. Schwakenhofer, W. Kern and H.L. Fehm, *Influences of corticotropin-releasing hormone, adrenocorticotropin, and cortisol on sleep in normal man.* The Journal of clinical endocrinology and metabolism, 1989. **68**(5): p. 904-11.
97. Friess, E., H. Tagaya, C. Grethe, L. Trachsel and F. Holsboer, *Acute cortisol administration promotes sleep intensity in man.* Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 2004. **29**(3): p. 598-604.
98. Bohlhalter, S., H. Murck, F. Holsboer and A. Steiger, *Cortisol enhances non-REM sleep and growth hormone secretion in elderly subjects.* Neurobiology of aging, 1997. **18**(4): p. 423-9.
99. Wren, A.M., L.J. Seal, M.A. Cohen, A.E. Brynes, G.S. Frost, K.G. Murphy, W.S. Dhillo, M.A. Ghatei and S.R. Bloom, *Ghrelin enhances appetite and increases food intake in humans.* The Journal of clinical endocrinology and metabolism, 2001. **86**(12): p. 5992.
100. Kojima, M., H. Hosoda, Y. Date, M. Nakazato, H. Matsuo and K. Kangawa, *Ghrelin is a growth-hormone-releasing acylated peptide from stomach.* Nature, 1999. **402**(6762): p. 656-60.
101. Korbonits, M., M. Kojima, K. Kangawa and A.B. Grossman, *Presence of ghrelin in normal and adenomatous human pituitary.* Endocrine, 2001. **14**(1): p. 101-4.
102. Lu, S., J.L. Guan, Q.P. Wang, K. Uehara, S. Yamada, N. Goto, Y. Date, M. Nakazato, M. Kojima, K. Kangawa and S. Shioda, *Immunocytochemical observation of ghrelin-containing neurons in the rat arcuate nucleus.* Neuroscience letters, 2002. **321**(3): p. 157-60.
103. Arvat, E., L. Di Vito, F. Broglio, M. Papotti, G. Muccioli, C. Dieguez, F.F. Casanueva, R. Deghenghi, F. Camanni and E. Ghigo, *Preliminary evidence that Ghrelin, the natural GH secretagogue (GHS)-receptor ligand, strongly stimulates GH secretion in humans.* Journal of endocrinological investigation, 2000. **23**(8): p. 493-5.
104. Howard, A.D., S.D. Feighner, D.F. Cully, J.P. Arena, P.A. Liberator, C.I. Rosenblum, M. Hamelin, D.L. Hreniuk, O.C. Palyha, J. Anderson, P.S. Paress, C. Diaz, M. Chou, K.K. Liu, K.K. McKee, S.S. Pong, L.Y. Chaung, A. Elbrecht, M. Dashkevicz, R. Heavens, M. Rigby, D.J. Sirinathsinghji, D.C. Dean, D.G. Melillo, A.A. Patchett, R. Nargund, P.R. Griffin, J.A. DeMartino, S.K. Gupta, J.M. Schaeffer, R.G. Smith and

L.H. Van der Ploeg, *A receptor in pituitary and hypothalamus that functions in growth hormone release.* Science (New York, N Y), 1996. **273**(5277): p. 974-7.
105. Ong, H., N. McNicoll, E. Escher, R. Collu, R. Deghenghi, V. Locatelli, E. Ghigo, G. Muccioli, M. Boghen and M. Nilsson, *Identification of a pituitary growth hormone-releasing peptide (GHRP) receptor subtype by photoaffinity labeling.* Endocrinology, 1998. **139**(1): p. 432-5.
106. Takaya, K., H. Ariyasu, N. Kanamoto, H. Iwakura, A. Yoshimoto, M. Harada, K. Mori, Y. Komatsu, T. Usui, A. Shimatsu, Y. Ogawa, K. Hosoda, T. Akamizu, M. Kojima, K. Kangawa and K. Nakao, *Ghrelin strongly stimulates growth hormone release in humans.* The Journal of clinical endocrinology and metabolism, 2000. **85**(12): p. 4908-11.
107. Weikel, J.C., A. Wichniak, M. Ising, H. Brunner, E. Friess, K. Held, S. Mathias, D.A. Schmid, M. Uhr and A. Steiger, *Ghrelin promotes slow-wave sleep in humans.* American journal of physiology. Endocrinology and metabolism, 2003. **284**(2): p. E407-15.
108. Dzaja, A., M.A. Dalal, H. Himmerich, M. Uhr, T. Pollmacher and A. Schuld, *Sleep enhances nocturnal plasma ghrelin levels in healthy subjects.* American journal of physiology. Endocrinology and metabolism, 2004. **286**(6): p. E963-7.
109. Taheri, S., L. Lin, D. Austin, T. Young and E. Mignot, *Short sleep duration is associated with reduced leptin, elevated ghrelin, and increased body mass index.* PLoS medicine, 2004. **1**(3): p. e62.
110. Spiegel, K., K. Knutson, R. Leproult, E. Tasali and E. Van Cauter, *Sleep loss: a novel risk factor for insulin resistance and Type 2 diabetes.* Journal of applied physiology, 2005. **99**(5): p. 2008-19.
111. Matthews, K.A., R.E. Dahl, J.F. Owens, L. Lee and M. Hall, *Sleep duration and insulin resistance in healthy black and white adolescents.* Sleep, 2012. **35**(10): p. 1353-8.
112. Gangwisch, J.E., S.B. Heymsfield, B. Boden-Albala, R.M. Buijs, F. Kreier, T.G. Pickering, A.G. Rundle, G.K. Zammit and D. Malaspina, *Short sleep duration as a risk factor for hypertension: analyses of the first National Health and Nutrition Examination Survey.* Hypertension, 2006. **47**(5): p. 833-9.
113. Tochikubo, O., A. Ikeda, E. Miyajima and M. Ishii, *Effects of insufficient sleep on blood pressure monitored by a new multibiomedical recorder.* Hypertension, 1996. **27**(6): p. 1318-24.
114. Cappuccio, F.P., F.M. Taggart, N.B. Kandala, A. Currie, E. Peile, S. Stranges and M.A. Miller, *Meta-analysis of short sleep duration and obesity in children and adults.* Sleep, 2008. **31**(5): p. 619-26.
115. Beihl, D.A., A.D. Liese and S.M. Haffner, *Sleep duration as a risk factor for incident type 2 diabetes in a multiethnic cohort.* Annals of epidemiology, 2009. **19**(5): p. 351-7.
116. Gangwisch, J.E., S.B. Heymsfield, B. Boden-Albala, R.M. Buijs, F. Kreier, T.G. Pickering, A.G. Rundle, G.K. Zammit and D. Malaspina, *Sleep duration as a risk factor for diabetes incidence in a large U.S. sample.* Sleep, 2007. **30**(12): p. 1667-73.
117. Yaggi, H.K., A.B. Araujo and J.B. McKinlay, *Sleep duration as a risk factor for the development of type 2 diabetes.* Diabetes care, 2006. **29**(3): p. 657-61.
118. Sabanayagam, C. and A. Shankar, *Sleep duration and cardiovascular disease: results from the National Health Interview Survey.* Sleep, 2010. **33**(8): p. 1037-42.
119. Cappuccio, F.P., D. Cooper, L. D'Elia, P. Strazzullo and M.A. Miller, *Sleep duration predicts cardiovascular outcomes: a systematic review and meta-analysis of prospective studies.* European heart journal, 2011. **32**(12): p. 1484-92.
120. Riemann, D. and U. Voderholzer, *Primary insomnia: a risk factor to develop depression?* J Affect Disord, 2003. **76**(1-3): p. 255-9.

121. Benito-Leon, J., E.D. Louis and F. Bermejo-Pareja, *Cognitive decline in short and long sleepers: A prospective population-based study (NEDICES).* Journal of psychiatric research, 2013. **47**(12): p. 1998-2003.
122. Auyeung, T.W., J.S. Lee, J. Leung, T. Kwok, P.C. Leung, J. Woo and Y.K. Wing, *Cognitive deficit is associated with phase advance of sleep-wake rhythm, daily napping, and prolonged sleep duration--a cross-sectional study in 2,947 community-dwelling older adults.* Age, 2013. **35**(2): p. 479-86.
123. Schernhammer, E.S., F. Laden, F.E. Speizer, W.C. Willett, D.J. Hunter, I. Kawachi, C.S. Fuchs and G.A. Colditz, *Night-shift work and risk of colorectal cancer in the nurses' health study.* Journal of the National Cancer Institute, 2003. **95**(11): p. 825-8.
124. Lehrer, S., S. Green, L. Ramanathan and K.E. Rosenzweig, *Obesity and deranged sleep are independently associated with increased cancer mortality in 50 US states and the District of Columbia.* Sleep & breathing = Schlaf & Atmung, 2013.
125. Zhang, X., E.L. Giovannucci, K. Wu, X. Gao, F. Hu, S. Ogino, E.S. Schernhammer, C.S. Fuchs, S. Redline, W.C. Willett and J. Ma, *Associations of self-reported sleep duration and snoring with colorectal cancer risk in men and women.* Sleep, 2013. **36**(5): p. 681-8.
126. Mohren, D.C., N.W. Jansen, I.J. Kant, J. Galama, P.A. van den Brandt and G.M. Swaen, *Prevalence of common infections among employees in different work schedules.* Journal of occupational and environmental medicine / American College of Occupational and Environmental Medicine, 2002. **44**(11): p. 1003-11.
127. Patel, S.R., A. Malhotra, X. Gao, F.B. Hu, M.I. Neuman and W.W. Fawzi, *A prospective study of sleep duration and pneumonia risk in women.* Sleep, 2012. **35**(1): p. 97-101.
128. Cohen, S., W.J. Doyle, C.M. Alper, D. Janicki-Deverts and R.B. Turner, *Sleep habits and susceptibility to the common cold.* Archives of internal medicine, 2009. **169**(1): p. 62-7.
129. Prather, A.A., M. Hall, J.M. Fury, D.C. Ross, M.F. Muldoon, S. Cohen and A.L. Marsland, *Sleep and antibody response to hepatitis B vaccination.* Sleep, 2012. **35**(8): p. 1063-9.
130. Meier-Ewert, H.K., P.M. Ridker, N. Rifai, M.M. Regan, N.J. Price, D.F. Dinges and J.M. Mullington, *Effect of sleep loss on C-reactive protein, an inflammatory marker of cardiovascular risk.* Journal of the American College of Cardiology, 2004. **43**(4): p. 678-83.
131. Frey, D.J., M. Fleshner and K.P. Wright, Jr., *The effects of 40 hours of total sleep deprivation on inflammatory markers in healthy young adults.* Brain, behavior, and immunity, 2007. **21**(8): p. 1050-7.
132. van Leeuwen, W.M., M. Lehto, P. Karisola, H. Lindholm, R. Luukkonen, M. Sallinen, M. Harma, T. Porkka-Heiskanen and H. Alenius, *Sleep restriction increases the risk of developing cardiovascular diseases by augmenting proinflammatory responses through IL-17 and CRP.* PloS one, 2009. **4**(2): p. e4589.
133. Haack, M., E. Sanchez and J.M. Mullington, *Elevated inflammatory markers in response to prolonged sleep restriction are associated with increased pain experience in healthy volunteers.* Sleep, 2007. **30**(9): p. 1145-52.
134. van den Berg, J.F., A. Knvistingh Neven, J.H. Tulen, A. Hofman, J.C. Witteman, H.M. Miedema and H. Tiemeier, *Actigraphic sleep duration and fragmentation are related to obesity in the elderly: the Rotterdam Study.* International journal of obesity, 2008. **32**(7): p. 1083-90.
135. Lopez-Garcia, E., R. Faubel, L. Leon-Munoz, M.C. Zuluaga, J.R. Banegas and F. Rodriguez-Artalejo, *Sleep duration, general and abdominal obesity, and weight change among the older adult population of Spain.* Am J Clin Nutr, 2008. **87**(2): p. 310-6.

136. Anic, G.M., L. Titus-Ernstoff, P.A. Newcomb, A. Trentham-Dietz and K.M. Egan, *Sleep duration and obesity in a population-based study.* Sleep medicine, 2010. **11**(5): p. 447-51.
137. Vorona, R.D., M.P. Winn, T.W. Babineau, B.P. Eng, H.R. Feldman and J.C. Ware, *Overweight and obese patients in a primary care population report less sleep than patients with a normal body mass index.* Archives of internal medicine, 2005. **165**(1): p. 25-30.
138. Hasler, G., D.J. Buysse, R. Klaghofer, A. Gamma, V. Ajdacic, D. Eich, W. Rossler and J. Angst, *The association between short sleep duration and obesity in young adults: a 13-year prospective study.* Sleep, 2004. **27**(4): p. 661-6.
139. Hall, M.H., M.F. Muldoon, J.R. Jennings, D.J. Buysse, J.D. Flory and S.B. Manuck, *Self-reported sleep duration is associated with the metabolic syndrome in midlife adults.* Sleep, 2008. **31**(5): p. 635-43.
140. Ju, S.Y. and W.S. Choi, *Sleep duration and metabolic syndrome in adult populations: a meta-analysis of observational studies.* Nutrition & diabetes, 2013. **3**: p. e65.
141. Choi, J.K., M.Y. Kim, J.K. Kim, J.K. Park, S.S. Oh, S.B. Koh and A. Eom, *Association between short sleep duration and high incidence of metabolic syndrome in midlife women.* The Tohoku journal of experimental medicine, 2011. **225**(3): p. 187-93.
142. Arble, D.M., J. Bass, A.D. Laposky, M.H. Vitaterna and F.W. Turek, *Circadian timing of food intake contributes to weight gain.* Obesity, 2009. **17**(11): p. 2100-2.
143. Ribeiro, D.C., S.M. Hampton, L. Morgan, S. Deacon and J. Arendt, *Altered postprandial hormone and metabolic responses in a simulated shift work environment.* The Journal of endocrinology, 1998. **158**(3): p. 305-10.
144. Hampton, S.M., L.M. Morgan, N. Lawrence, T. Anastasiadou, F. Norris, S. Deacon, D. Ribeiro and J. Arendt, *Postprandial hormone and metabolic responses in simulated shift work.* The Journal of endocrinology, 1996. **151**(2): p. 259-67.
145. Van Cauter, E., K.S. Polonsky and A.J. Scheen, *Roles of circadian rhythmicity and sleep in human glucose regulation.* Endocrine reviews, 1997. **18**(5): p. 716-38.
146. Kaneita, Y., M. Uchiyama, N. Yoshiike and T. Ohida, *Associations of usual sleep duration with serum lipid and lipoprotein levels.* Sleep, 2008. **31**(5): p. 645-52.
147. VanHelder, T., J.D. Symons and M.W. Radomski, *Effects of sleep deprivation and exercise on glucose tolerance.* Aviation, space, and environmental medicine, 1993. **64**(6): p. 487-92.
148. Spiegel, K., R. Leproult and E. Van Cauter, *Impact of sleep debt on metabolic and endocrine function.* Lancet, 1999. **354**(9188): p. 1435-9.
149. Spiegel, K., R. Leproult, M. L'Hermite-Baleriaux, G. Copinschi, P.D. Penev and E. Van Cauter, *Leptin levels are dependent on sleep duration: relationships with sympathovagal balance, carbohydrate regulation, cortisol, and thyrotropin.* The Journal of clinical endocrinology and metabolism, 2004. **89**(11): p. 5762-71.
150. Kerkhofs, M., K.Z. Boudjeltia, P. Stenuit, D. Brohee, P. Cauchie and M. Vanhaeverbeek, *Sleep restriction increases blood neutrophils, total cholesterol and low density lipoprotein cholesterol in postmenopausal women: A preliminary study.* Maturitas, 2007. **56**(2): p. 212-5.
151. Nedeltcheva, A.V., J.M. Kilkus, J. Imperial, D.A. Schoeller and P.D. Penev, *Insufficient sleep undermines dietary efforts to reduce adiposity.* Annals of internal medicine, 2010. **153**(7): p. 435-41.
152. Michalsen, A., F. Schlegel, A. Rodenbeck, R. Ludtke, G. Huether, H. Teschler and G.J. Dobos, *Effects of short-term modified fasting on sleep patterns and daytime vigilance in non-obese subjects: results of a pilot study.* Annals of nutrition & metabolism, 2003. **47**(5): p. 194-200.

153. Huang, W., K.M. Ramsey, B. Marcheva and J. Bass, *Circadian rhythms, sleep, and metabolism.* The Journal of clinical investigation, 2011. **121**(6): p. 2133-41.
154. Yuen, K.C., L.E. Chong and M.C. Riddle, *Influence of glucocorticoids and growth hormone on insulin sensitivity in humans.* Diabetic medicine : a journal of the British Diabetic Association, 2013. **30**(6): p. 651-63.
155. Wozniak, S.E., L.L. Gee, M.S. Wachtel and E.E. Frezza, *Adipose tissue: the new endocrine organ? A review article.* Digestive diseases and sciences, 2009. **54**(9): p. 1847-56.
156. Lago, F., R. Gomez, J.J. Gomez-Reino, C. Dieguez and O. Gualillo, *Adipokines as novel modulators of lipid metabolism.* Trends in biochemical sciences, 2009. **34**(10): p. 500-10.
157. Saladin, R., P. De Vos, M. Guerre-Millo, A. Leturque, J. Girard, B. Staels and J. Auwerx, *Transient increase in obese gene expression after food intake or insulin administration.* Nature, 1995. **377**(6549): p. 527-9.
158. Kolaczynski, J.W., J.P. Ohannesian, R.V. Considine, C.C. Marco and J.F. Caro, *Response of leptin to short-term and prolonged overfeeding in humans.* The Journal of clinical endocrinology and metabolism, 1996. **81**(11): p. 4162-5.
159. Saad, M.F., A. Khan, A. Sharma, R. Michael, M.G. Riad-Gabriel, R. Boyadjian, S.D. Jinagouda, G.M. Steil and V. Kamdar, *Physiological insulinemia acutely modulates plasma leptin.* Diabetes, 1998. **47**(4): p. 544-9.
160. Halaas, J.L., K.S. Gajiwala, M. Maffei, S.L. Cohen, B.T. Chait, D. Rabinowitz, R.L. Lallone, S.K. Burley and J.M. Friedman, *Weight-reducing effects of the plasma protein encoded by the obese gene.* Science (New York, N Y), 1995. **269**(5223): p. 543-6.
161. Larsson, H., S. Elmstahl, G. Berglund and B. Ahren, *Evidence for leptin regulation of food intake in humans.* The Journal of clinical endocrinology and metabolism, 1998. **83**(12): p. 4382-5.
162. Considine, R.V., M.K. Sinha, M.L. Heiman, A. Kriauciunas, T.W. Stephens, M.R. Nyce, J.P. Ohannesian, C.C. Marco, L.J. McKee, T.L. Bauer and et al., *Serum immunoreactive-leptin concentrations in normal-weight and obese humans.* The New England journal of medicine, 1996. **334**(5): p. 292-5.
163. Frederich, R.C., A. Hamann, S. Anderson, B. Lollmann, B.B. Lowell and J.S. Flier, *Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action.* Nature medicine, 1995. **1**(12): p. 1311-4.
164. Myers, M.G., Jr., R.L. Leibel, R.J. Seeley and M.W. Schwartz, *Obesity and leptin resistance: distinguishing cause from effect.* Trends in endocrinology and metabolism: TEM, 2010. **21**(11): p. 643-51.
165. Chaput, J.P., J.P. Despres, C. Bouchard and A. Tremblay, *Short sleep duration is associated with reduced leptin levels and increased adiposity: Results from the Quebec family study.* Obesity, 2007. **15**(1): p. 253-61.
166. Martinez-Gomez, D., J.C. Eisenmann, S. Gomez-Martinez, E.E. Hill, B. Zapatera, O.L. Veiga and A. Marcos, *Sleep duration and emerging cardiometabolic risk markers in adolescents. The AFINOS study.* Sleep medicine, 2011. **12**(10): p. 997-1002.
167. Spiegel, K., E. Tasali, P. Penev and E. Van Cauter, *Brief communication: Sleep curtailment in healthy young men is associated with decreased leptin levels, elevated ghrelin levels, and increased hunger and appetite.* Annals of internal medicine, 2004. **141**(11): p. 846-50.
168. Hayes, A.L., F. Xu, D. Babineau and S.R. Patel, *Sleep duration and circulating adipokine levels.* Sleep, 2011. **34**(2): p. 147-52.

169. Simpson, N.S., S. Banks and D.F. Dinges, *Sleep restriction is associated with increased morning plasma leptin concentrations, especially in women.* Biological research for nursing, 2010. **12**(1): p. 47-53.
170. Omisade, A., O.M. Buxton and B. Rusak, *Impact of acute sleep restriction on cortisol and leptin levels in young women.* Physiology & behavior, 2010. **99**(5): p. 651-6.
171. Pejovic, S., A.N. Vgontzas, M. Basta, M. Tsaoussoglou, E. Zoumakis, A. Vgontzas, E.O. Bixler and G.P. Chrousos, *Leptin and hunger levels in young healthy adults after one night of sleep loss.* Journal of sleep research, 2010. **19**(4): p. 552-8.
172. Sinha, M.K., J.P. Ohannesian, M.L. Heiman, A. Kriauciunas, T.W. Stephens, S. Magosin, C. Marco and J.F. Caro, *Nocturnal rise of leptin in lean, obese, and non-insulin-dependent diabetes mellitus subjects.* The Journal of clinical investigation, 1996. **97**(5): p. 1344-7.
173. Laposky, A.D., M.A. Bradley, D.L. Williams, J. Bass and F.W. Turek, *Sleep-wake regulation is altered in leptin-resistant (db/db) genetically obese and diabetic mice.* American journal of physiology. Regulatory, integrative and comparative physiology, 2008. **295**(6): p. R2059-66.
174. Laposky, A.D., J. Shelton, J. Bass, C. Dugovic, N. Perrino and F.W. Turek, *Altered sleep regulation in leptin-deficient mice.* American journal of physiology. Regulatory, integrative and comparative physiology, 2006. **290**(4): p. R894-903.
175. Elmquist, J.K., C. Bjorbaek, R.S. Ahima, J.S. Flier and C.B. Saper, *Distributions of leptin receptor mRNA isoforms in the rat brain.* The Journal of comparative neurology, 1998. **395**(4): p. 535-47.
176. Mercer, J.G., N. Hoggard, L.M. Williams, C.B. Lawrence, L.T. Hannah and P. Trayhurn, *Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization.* FEBS letters, 1996. **387**(2-3): p. 113-6.
177. Morash, B., A. Li, P.R. Murphy, M. Wilkinson and E. Ur, *Leptin gene expression in the brain and pituitary gland.* Endocrinology, 1999. **140**(12): p. 5995-8.
178. Morash, B., D. Wilkinson, P. Murphy, E. Ur and M. Wilkinson, *Developmental regulation of leptin gene expression in rat brain and pituitary.* Molecular and cellular endocrinology, 2001. **185**(1-2): p. 151-9.
179. Schwartz, M.W., E. Peskind, M. Raskind, E.J. Boyko and D. Porte, Jr., *Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans.* Nature medicine, 1996. **2**(5): p. 589-93.
180. Banks, W.A., A.J. Kastin, W. Huang, J.B. Jaspan and L.M. Maness, *Leptin enters the brain by a saturable system independent of insulin.* Peptides, 1996. **17**(2): p. 305-11.
181. Stephens, T.W., M. Basinski, P.K. Bristow, J.M. Bue-Valleskey, S.G. Burgett, L. Craft, J. Hale, J. Hoffmann, H.M. Hsiung, A. Kriauciunas and et al., *The role of neuropeptide Y in the antiobesity action of the obese gene product.* Nature, 1995. **377**(6549): p. 530-2.
182. Dyzma, M., K.Z. Boudjeltia, B. Faraut and M. Kerkhofs, *Neuropeptide Y and sleep.* Sleep medicine reviews, 2010. **14**(3): p. 161-5.
183. Luheshi, G.N., J.D. Gardner, D.A. Rushforth, A.S. Loudon and N.J. Rothwell, *Leptin actions on food intake and body temperature are mediated by IL-1.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(12): p. 7047-52.
184. Trujillo, M.E. and P.E. Scherer, *Adiponectin--journey from an adipocyte secretory protein to biomarker of the metabolic syndrome.* Journal of internal medicine, 2005. **257**(2): p. 167-75.
185. Guerre-Millo, M., *Adiponectin: an update.* Diabetes & metabolism, 2008. **34**(1): p. 12-8.

186. Charles, L.E., J.K. Gu, M.E. Andrew, J.M. Violanti, D. Fekedulegn and C.M. Burchfiel, *Sleep duration and biomarkers of metabolic function among police officers.* Journal of occupational and environmental medicine / American College of Occupational and Environmental Medicine, 2011. **53**(8): p. 831-7.
187. Simpson, N.S., S. Banks, S. Arroyo and D.F. Dinges, *Effects of sleep restriction on adiponectin levels in healthy men and women.* Physiology & behavior, 2010. **101**(5): p. 693-8.
188. Kotani, K., N. Sakane, K. Saiga, M. Kato, K. Ishida, Y. Kato and Y. Kurozawa, *Serum adiponectin levels and lifestyle factors in Japanese men.* Heart and vessels, 2007. **22**(5): p. 291-6.
189. Al-Disi, D., N. Al-Daghri, L. Khanam, A. Al-Othman, M. Al-Saif, S. Sabico and G. Chrousos, *Subjective sleep duration and quality influence diet composition and circulating adipocytokines and ghrelin levels in teen-age girls.* Endocrine journal, 2010. **57**(10): p. 915-23.
190. Masserini, B., P.S. Morpurgo, F. Donadio, C. Baldessari, R. Bossi, P. Beck-Peccoz and E. Orsi, *Reduced levels of adiponectin in sleep apnea syndrome.* Journal of endocrinological investigation, 2006. **29**(8): p. 700-5.
191. Zhang, X.L., K.S. Yin, H. Mao, H. Wang and Y. Yang, *Serum adiponectin level in patients with obstructive sleep apnea hypopnea syndrome.* Chinese medical journal, 2004. **117**(11): p. 1603-6.
192. Kelly, A., S. Dougherty, A. Cucchiara, C.L. Marcus and L.J. Brooks, *Catecholamines, adiponectin, and insulin resistance as measured by HOMA in children with obstructive sleep apnea.* Sleep, 2010. **33**(9): p. 1185-91.
193. Alberti, A., P. Sarchielli, E. Gallinella, A. Floridi, G. Mazzotta and V. Gallai, *Plasma cytokine levels in patients with obstructive sleep apnea syndrome: a preliminary study.* Journal of sleep research, 2003. **12**(4): p. 305-11.
194. Ryan, S., C.T. Taylor and W.T. McNicholas, *Systemic inflammation: a key factor in the pathogenesis of cardiovascular complications in obstructive sleep apnoea syndrome?* Postgraduate medical journal, 2009. **85**(1010): p. 693-8.
195. Yokoe, T., K. Minoguchi, H. Matsuo, N. Oda, H. Minoguchi, G. Yoshino, T. Hirano and M. Adachi, *Elevated levels of C-reactive protein and interleukin-6 in patients with obstructive sleep apnea syndrome are decreased by nasal continuous positive airway pressure.* Circulation, 2003. **107**(8): p. 1129-34.
196. Bruun, J.M., A.S. Lihn, C. Verdich, S.B. Pedersen, S. Toubro, A. Astrup and B. Richelsen, *Regulation of adiponectin by adipose tissue-derived cytokines: in vivo and in vitro investigations in humans.* American journal of physiology. Endocrinology and metabolism, 2003. **285**(3): p. E527-33.
197. Fasshauer, M., S. Kralisch, M. Klier, U. Lossner, M. Bluher, J. Klein and R. Paschke, *Adiponectin gene expression and secretion is inhibited by interleukin-6 in 3T3-L1 adipocytes.* Biochemical and biophysical research communications, 2003. **301**(4): p. 1045-50.
198. Maeda, N., M. Takahashi, T. Funahashi, S. Kihara, H. Nishizawa, K. Kishida, H. Nagaretani, M. Matsuda, R. Komuro, N. Ouchi, H. Kuriyama, K. Hotta, T. Nakamura, I. Shimomura and Y. Matsuzawa, *PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein.* Diabetes, 2001. **50**(9): p. 2094-9.
199. Yuan, G., X. Chen, Q. Ma, J. Qiao, R. Li, X. Li, S. Li, J. Tang, L. Zhou, H. Song and M. Chen, *C-reactive protein inhibits adiponectin gene expression and secretion in 3T3-L1 adipocytes.* The Journal of endocrinology, 2007. **194**(2): p. 275-81.
200. Calvani, M., A. Scarfone, L. Granato, E.V. Mora, G. Nanni, M. Castagneto, A.V. Greco, M. Manco and G. Mingrone, *Restoration of adiponectin pulsatility in severely obese subjects after weight loss.* Diabetes, 2004. **53**(4): p. 939-47.

201. Gavrila, A., C.K. Peng, J.L. Chan, J.E. Mietus, A.L. Goldberger and C.S. Mantzoros, *Diurnal and ultradian dynamics of serum adiponectin in healthy men: comparison with leptin, circulating soluble leptin receptor, and cortisol patterns.* The Journal of clinical endocrinology and metabolism, 2003. **88**(6): p. 2838-43.
202. Shea, S.A., M.F. Hilton, C. Orlova, R.T. Ayers and C.S. Mantzoros, *Independent circadian and sleep/wake regulation of adipokines and glucose in humans.* The Journal of clinical endocrinology and metabolism, 2005. **90**(5): p. 2537-44.
203. Wilkinson, M., R. Brown, S.A. Imran and E. Ur, *Adipokine gene expression in brain and pituitary gland.* Neuroendocrinology, 2007. **86**(3): p. 191-209.
204. Rodriguez-Pacheco, F., A.J. Martinez-Fuentes, S. Tovar, L. Pinilla, M. Tena-Sempere, C. Dieguez, J.P. Castano and M.M. Malagon, *Regulation of pituitary cell function by adiponectin.* Endocrinology, 2007. **148**(1): p. 401-10.
205. Thundyil, J., D. Pavlovski, C.G. Sobey and T.V. Arumugam, *Adiponectin receptor signalling in the brain.* British journal of pharmacology, 2012. **165**(2): p. 313-27.
206. Neumeier, M., J. Weigert, R. Buettner, J. Wanninger, A. Schaffler, A.M. Muller, S. Killian, S. Sauerbruch, F. Schlachetzki, A. Steinbrecher, C. Aslanidis, J. Scholmerich and C. Buechler, *Detection of adiponectin in cerebrospinal fluid in humans.* American journal of physiology. Endocrinology and metabolism, 2007. **293**(4): p. E965-9.
207. Pan, W., H. Tu and A.J. Kastin, *Differential BBB interactions of three ingestive peptides: obestatin, ghrelin, and adiponectin.* Peptides, 2006. **27**(4): p. 911-6.
208. Spranger, J., S. Verma, I. Gohring, T. Bobbert, J. Seifert, A.L. Sindler, A. Pfeiffer, S.M. Hileman, M. Tschop and W.A. Banks, *Adiponectin does not cross the blood-brain barrier but modifies cytokine expression of brain endothelial cells.* Diabetes, 2006. **55**(1): p. 141-7.
209. Ebinuma, H., T. Miida, T. Yamauchi, Y. Hada, K. Hara, N. Kubota and T. Kadowaki, *Improved ELISA for selective measurement of adiponectin multimers and identification of adiponectin in human cerebrospinal fluid.* Clinical chemistry, 2007. **53**(8): p. 1541-4.
210. Kos, K., A.L. Harte, N.F. da Silva, A. Tonchev, G. Chaldakov, S. James, D.R. Snead, B. Hoggart, J.P. O'Hare, P.G. McTernan and S. Kumar, *Adiponectin and resistin in human cerebrospinal fluid and expression of adiponectin receptors in the human hypothalamus.* The Journal of clinical endocrinology and metabolism, 2007. **92**(3): p. 1129-36.
211. Kubota, N., W. Yano, T. Kubota, T. Yamauchi, S. Itoh, H. Kumagai, H. Kozono, I. Takamoto, S. Okamoto, T. Shiuchi, R. Suzuki, H. Satoh, A. Tsuchida, M. Moroi, K. Sugi, T. Noda, H. Ebinuma, Y. Ueta, T. Kondo, E. Araki, O. Ezaki, R. Nagai, K. Tobe, Y. Terauchi, K. Ueki, Y. Minokoshi and T. Kadowaki, *Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake.* Cell metabolism, 2007. **6**(1): p. 55-68.
212. Price, T.H., G.S. Chatta and D.C. Dale, *Effect of recombinant granulocyte colony-stimulating factor on neutrophil kinetics in normal young and elderly humans.* Blood, 1996. **88**(1): p. 335-40.
213. Cartwright, G.E., J.W. Athens and M.M. Wintrobe, *The Kinetics of Granulopoiesis in Normal Man.* Blood, 1964. **24**: p. 780-803.
214. Pillay, J., I. den Braber, N. Vrisekoop, L.M. Kwast, R.J. de Boer, J.A. Borghans, K. Tesselaar and L. Koenderman, *In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days.* Blood, 2010. **116**(4): p. 625-7.
215. Dancey, J.T., K.A. Deubelbeiss, L.A. Harker and C.A. Finch, *Neutrophil kinetics in man.* The Journal of clinical investigation, 1976. **58**(3): p. 705-15.
216. Colotta, F., F. Re, N. Polentarutti, S. Sozzani and A. Mantovani, *Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products.* Blood, 1992. **80**(8): p. 2012-20.

217. Walmsley, S.R., C. Print, N. Farahi, C. Peyssonnaux, R.S. Johnson, T. Cramer, A. Sobolewski, A.M. Condliffe, A.S. Cowburn, N. Johnson and E.R. Chilvers, *Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity.* The Journal of experimental medicine, 2005. **201**(1): p. 105-15.
218. Hannah, S., K. Mecklenburgh, I. Rahman, G.J. Bellingan, A. Greening, C. Haslett and E.R. Chilvers, *Hypoxia prolongs neutrophil survival in vitro.* FEBS letters, 1995. **372**(2-3): p. 233-7.
219. Leuenroth, S.J., P.S. Grutkoski, A. Ayala and H.H. Simms, *Suppression of PMN apoptosis by hypoxia is dependent on Mcl-1 and MAPK activity.* Surgery, 2000. **128**(2): p. 171-7.
220. Jung, U. and K. Ley, *Regulation of E-selectin, P-selectin, and intercellular adhesion molecule 1 expression in mouse cremaster muscle vasculature.* Microcirculation, 1997. **4**(2): p. 311-9.
221. Szmitko, P.E., C.H. Wang, R.D. Weisel, J.R. de Almeida, T.J. Anderson and S. Verma, *New markers of inflammation and endothelial cell activation: Part I.* Circulation, 2003. **108**(16): p. 1917-23.
222. McEver, R.P., K.L. Moore and R.D. Cummings, *Leukocyte trafficking mediated by selectin-carbohydrate interactions.* The Journal of biological chemistry, 1995. **270**(19): p. 11025-8.
223. Takami, M., V. Terry and L. Petruzzelli, *Signaling pathways involved in IL-8-dependent activation of adhesion through Mac-1.* Journal of immunology, 2002. **168**(9): p. 4559-66.
224. Seo, S.M., L.V. McIntire and C.W. Smith, *Effects of IL-8, Gro-alpha, and LTB(4) on the adhesive kinetics of LFA-1 and Mac-1 on human neutrophils.* American journal of physiology. Cell physiology, 2001. **281**(5): p. C1568-78.
225. McDowall, A., B. Leitinger, P. Stanley, P.A. Bates, A.M. Randi and N. Hogg, *The I domain of integrin leukocyte function-associated antigen-1 is involved in a conformational change leading to high affinity binding to ligand intercellular adhesion molecule 1 (ICAM-1).* The Journal of biological chemistry, 1998. **273**(42): p. 27396-403.
226. Luu, N.T., G.E. Rainger, C.D. Buckley and G.B. Nash, *CD31 regulates direction and rate of neutrophil migration over and under endothelial cells.* Journal of vascular research, 2003. **40**(5): p. 467-79.
227. Woodfin, A., M.B. Voisin and S. Nourshargh, *Recent developments and complexities in neutrophil transmigration.* Current opinion in hematology, 2010. **17**(1): p. 9-17.
228. Baggiolini, M., B. Dewald and B. Moser, *Human chemokines: an update.* Annual review of immunology, 1997. **15**: p. 675-705.
229. Maher, J., J.V. Martell, B.A. Brantley, E.B. Cox, J.E. Niedel and W.F. Rosse, *The response of human neutrophils to a chemotactic tripeptide (N-formyl-methionyl-leucyl-phenylalanine) studied by microcinematography.* Blood, 1984. **64**(1): p. 221-8.
230. Ward, P.A. and L.J. Newman, *A neutrophil chemotactic factor from human C'5.* Journal of immunology, 1969. **102**(1): p. 93-9.
231. Stephens, L., L. Milne and P. Hawkins, *Moving towards a better understanding of chemotaxis.* Current biology : CB, 2008. **18**(11): p. R485-94.
232. Dewas, C., P.M. Dang, M.A. Gougerot-Pocidalo and J. El-Benna, *TNF-alpha induces phosphorylation of p47(phox) in human neutrophils: partial phosphorylation of p47phox is a common event of priming of human neutrophils by TNF-alpha and granulocyte-macrophage colony-stimulating factor.* Journal of immunology, 2003. **171**(8): p. 4392-8.
233. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-20.

234. Selvaraj, P., N. Fifadara, S. Nagarajan, A. Cimino and G. Wang, *Functional regulation of human neutrophil Fc gamma receptors.* Immunologic research, 2004. **29**(1-3): p. 219-30.
235. Gordon, D.L., G.M. Johnson and M.K. Hostetter, *Characteristics of iC3b binding to human polymorphonuclear leucocytes.* Immunology, 1987. **60**(4): p. 553-8.
236. Fearon, D.T., *Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leukocyte, B lymphocyte, and monocyte.* The Journal of experimental medicine, 1980. **152**(1): p. 20-30.
237. Cox, D., C.C. Tseng, G. Bjekic and S. Greenberg, *A requirement for phosphatidylinositol 3-kinase in pseudopod extension.* The Journal of biological chemistry, 1999. **274**(3): p. 1240-7.
238. Garcia-Garcia, E. and C. Rosales, *Signal transduction during Fc receptor-mediated phagocytosis.* Journal of leukocyte biology, 2002. **72**(6): p. 1092-108.
239. McLeish, K.R., J.B. Klein, P.Y. Coxon, K.Z. Head and R.A. Ward, *Bacterial phagocytosis activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase cascades in human neutrophils.* Journal of leukocyte biology, 1998. **64**(6): p. 835-44.
240. Giraldo, E., L. Martin-Cordero, M.D. Hinchado, J.J. Garcia and E. Ortega, *Role of phosphatidylinositol-3-kinase (PI3K), extracellular signal-regulated kinase (ERK) and nuclear transcription factor kappa beta (NF-k beta) on neutrophil phagocytic process of Candida albicans.* Mol Cell Biochem, 2010. **333**(1-2): p. 115-20.
241. Lee, W.L., R.E. Harrison and S. Grinstein, *Phagocytosis by neutrophils.* Microbes and infection / Institut Pasteur, 2003. **5**(14): p. 1299-306.
242. Segal, A.W., *How neutrophils kill microbes.* Annual review of immunology, 2005. **23**: p. 197-223.
243. Klebanoff, S.J., *Myeloperoxidase: friend and foe.* Journal of leukocyte biology, 2005. **77**(5): p. 598-625.
244. Brinkmann, V., U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D.S. Weiss, Y. Weinrauch and A. Zychlinsky, *Neutrophil extracellular traps kill bacteria.* Science (New York, N Y), 2004. **303**(5663): p. 1532-5.
245. Fink, S.L. and B.T. Cookson, *Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells.* Infection and immunity, 2005. **73**(4): p. 1907-16.
246. Kerr, J.F., A.H. Wyllie and A.R. Currie, *Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.* British journal of cancer, 1972. **26**(4): p. 239-57.
247. Earnshaw, W.C., L.M. Martins and S.H. Kaufmann, *Mammalian caspases: structure, activation, substrates, and functions during apoptosis.* Annual review of biochemistry, 1999. **68**: p. 383-424.
248. Zhang, Y., D. Xing and L. Liu, *PUMA promotes Bax translocation by both directly interacting with Bax and by competitive binding to Bcl-X L during UV-induced apoptosis.* Molecular biology of the cell, 2009. **20**(13): p. 3077-87.
249. Gentile, M., L. Latonen and M. Laiho, *Cell cycle arrest and apoptosis provoked by UV radiation-induced DNA damage are transcriptionally highly divergent responses.* Nucleic acids research, 2003. **31**(16): p. 4779-90.
250. Stevenson, M.A., S.S. Pollock, C.N. Coleman and S.K. Calderwood, *X-irradiation, phorbol esters, and H2O2 stimulate mitogen-activated protein kinase activity in NIH-3T3 cells through the formation of reactive oxygen intermediates.* Cancer research, 1994. **54**(1): p. 12-5.
251. Kroemer, G., L. Galluzzi and C. Brenner, *Mitochondrial membrane permeabilization in cell death.* Physiological reviews, 2007. **87**(1): p. 99-163.

252. Mahmood, Z. and Y. Shukla, *Death receptors: targets for cancer therapy.* Experimental cell research, 2010. **316**(6): p. 887-99.
253. Papenfuss, K., S.M. Cordier and H. Walczak, *Death receptors as targets for anti-cancer therapy.* Journal of cellular and molecular medicine, 2008. **12**(6B): p. 2566-85.
254. Fox, S., A.E. Leitch, R. Duffin, C. Haslett and A.G. Rossi, *Neutrophil apoptosis: relevance to the innate immune response and inflammatory disease.* Journal of innate immunity, 2010. **2**(3): p. 216-27.
255. Suratt, B.T., S.K. Young, J. Lieber, J.A. Nick, P.M. Henson and G.S. Worthen, *Neutrophil maturation and activation determine anatomic site of clearance from circulation.* American journal of physiology. Lung cellular and molecular physiology, 2001. **281**(4): p. L913-21.
256. Furze, R.C. and S.M. Rankin, *The role of the bone marrow in neutrophil clearance under homeostatic conditions in the mouse.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2008. **22**(9): p. 3111-9.
257. Rankin, S.M., *The bone marrow: a site of neutrophil clearance.* Journal of leukocyte biology, 2010. **88**(2): p. 241-51.
258. Savill, J., I. Dransfield, C. Gregory and C. Haslett, *A blast from the past: clearance of apoptotic cells regulates immune responses.* Nature reviews. Immunology, 2002. **2**(12): p. 965-75.
259. Brach, M.A., S. deVos, H.J. Gruss and F. Herrmann, *Prolongation of survival of human polymorphonuclear neutrophils by granulocyte-macrophage colony-stimulating factor is caused by inhibition of programmed cell death.* Blood, 1992. **80**(11): p. 2920-4.
260. Zhang, B., J. Hirahashi, X. Cullere and T.N. Mayadas, *Elucidation of molecular events leading to neutrophil apoptosis following phagocytosis: cross-talk between caspase 8, reactive oxygen species, and MAPK/ERK activation.* The Journal of biological chemistry, 2003. **278**(31): p. 28443-54.
261. Watson, R.W., H.P. Redmond, J.H. Wang, C. Condron and D. Bouchier-Hayes, *Neutrophils undergo apoptosis following ingestion of Escherichia coli.* Journal of immunology, 1996. **156**(10): p. 3986-92.
262. Kasahara, Y., K. Iwai, A. Yachie, K. Ohta, A. Konno, H. Seki, T. Miyawaki and N. Taniguchi, *Involvement of reactive oxygen intermediates in spontaneous and CD95 (Fas/APO-1)-mediated apoptosis of neutrophils.* Blood, 1997. **89**(5): p. 1748-53.
263. Scheel-Toellner, D., K. Wang, R. Craddock, P.R. Webb, H.M. McGettrick, L.K. Assi, N. Parkes, L.E. Clough, E. Gulbins, M. Salmon and J.M. Lord, *Reactive oxygen species limit neutrophil life span by activating death receptor signaling.* Blood, 2004. **104**(8): p. 2557-64.
264. Remijsen, Q., T.W. Kuijpers, E. Wirawan, S. Lippens, P. Vandenabeele and T. Vanden Berghe, *Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality.* Cell death and differentiation, 2011. **18**(4): p. 581-8.
265. Serhan, C.N., S.D. Brain, C.D. Buckley, D.W. Gilroy, C. Haslett, L.A. O'Neill, M. Perretti, A.G. Rossi and J.L. Wallace, *Resolution of inflammation: state of the art, definitions and terms.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2007. **21**(2): p. 325-32.
266. Moulding, D.A., C. Akgul, M. Derouet, M.R. White and S.W. Edwards, *BCL-2 family expression in human neutrophils during delayed and accelerated apoptosis.* Journal of leukocyte biology, 2001. **70**(5): p. 783-92.
267. Dibbert, B., M. Weber, W.H. Nikolaizik, P. Vogt, M.H. Schoni, K. Blaser and H.U. Simon, *Cytokine-mediated Bax deficiency and consequent delayed neutrophil apoptosis: a general mechanism to accumulate effector cells in inflammation.*

Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(23): p. 13330-5.
268. Moulding, D.A., J.A. Quayle, C.A. Hart and S.W. Edwards, *Mcl-1 expression in human neutrophils: regulation by cytokines and correlation with cell survival.* Blood, 1998. **92**(7): p. 2495-502.
269. Iwai, K., T. Miyawaki, T. Takizawa, A. Konno, K. Ohta, A. Yachie, H. Seki and N. Taniguchi, *Differential expression of bcl-2 and susceptibility to anti-Fas-mediated cell death in peripheral blood lymphocytes, monocytes, and neutrophils.* Blood, 1994. **84**(4): p. 1201-8.
270. Weinmann, P., P. Gaehtgens and B. Walzog, *Bcl-Xl- and Bax-alpha-mediated regulation of apoptosis of human neutrophils via caspase-3.* Blood, 1999. **93**(9): p. 3106-15.
271. Edwards, S.W., M. Derouet, M. Howse and R.J. Moots, *Regulation of neutrophil apoptosis by Mcl-1.* Biochemical Society transactions, 2004. **32**(Pt3): p. 489-92.
272. Leuenroth, S.J., P.S. Grutkoski, A. Ayala and H.H. Simms, *The loss of Mcl-1 expression in human polymorphonuclear leukocytes promotes apoptosis.* Journal of leukocyte biology, 2000. **68**(1): p. 158-66.
273. Dzhagalov, I., A. St John and Y.W. He, *The antiapoptotic protein Mcl-1 is essential for the survival of neutrophils but not macrophages.* Blood, 2007. **109**(4): p. 1620-6.
274. Fruman, D.A., R.E. Meyers and L.C. Cantley, *Phosphoinositide kinases.* Annual review of biochemistry, 1998. **67**: p. 481-507.
275. Osaki, M., M. Oshimura and H. Ito, *PI3K-Akt pathway: Its functions and alterations in human cancer.* Apoptosis, 2004. **9**(6): p. 667-676.
276. Xu, Y., F. Loison and H.R. Luo, *Neutrophil spontaneous death is mediated by down-regulation of autocrine signaling through GPCR, PI3Kgamma, ROS, and actin.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(7): p. 2950-5.
277. Klein, J.B., M.J. Rane, J.A. Scherzer, P.Y. Coxon, R. Kettritz, J.M. Mathiesen, A. Buridi and K.R. McLeish, *Granulocyte-macrophage colony-stimulating factor delays neutrophil constitutive apoptosis through phosphoinositide 3-kinase and extracellular signal-regulated kinase pathways.* Journal of immunology, 2000. **164**(8): p. 4286-91.
278. Francois, S., J. El Benna, P.M. Dang, E. Pedruzzi, M.A. Gougerot-Pocidalo and C. Elbim, *Inhibition of neutrophil apoptosis by TLR agonists in whole blood: involvement of the phosphoinositide 3-kinase/Akt and NF-kappaB signaling pathways, leading to increased levels of Mcl-1, A1, and phosphorylated Bad.* Journal of immunology, 2005. **174**(6): p. 3633-42.
279. Cowburn, A.S., K.A. Cadwallader, B.J. Reed, N. Farahi and E.R. Chilvers, *Role of PI3-kinase-dependent Bad phosphorylation and altered transcription in cytokine-mediated neutrophil survival.* Blood, 2002. **100**(7): p. 2607-16.
280. Pongracz, J., P. Webb, K. Wang, E. Deacon, O.J. Lunn and J.M. Lord, *Spontaneous neutrophil apoptosis involves caspase 3-mediated activation of protein kinase C-delta.* The Journal of biological chemistry, 1999. **274**(52): p. 37329-34.
281. Aoshiba, K., S. Yasui, M. Hayashi, J. Tamaoki and A. Nagai, *Role of p38-mitogen-activated protein kinase in spontaneous apoptosis of human neutrophils.* Journal of immunology, 1999. **162**(3): p. 1692-700.
282. Nolan, B., A. Duffy, L. Paquin, M. De, H. Collette, C.M. Graziano and P. Bankey, *Mitogen-activated protein kinases signal inhibition of apoptosis in lipopolysaccharide-stimulated neutrophils.* Surgery, 1999. **126**(2): p. 406-12.
283. Alvarado-Kristensson, M. and T. Andersson, *Protein phosphatase 2A regulates apoptosis in neutrophils by dephosphorylating both p38 MAPK and its substrate caspase 3.* The Journal of biological chemistry, 2005. **280**(7): p. 6238-44.

284. Alvarado-Kristensson, M., F. Melander, K. Leandersson, L. Ronnstrand, C. Wernstedt and T. Andersson, *p38-MAPK signals survival by phosphorylation of caspase-8 and caspase-3 in human neutrophils.* The Journal of experimental medicine, 2004. **199**(4): p. 449-58.
285. Klein, J.B., A. Buridi, P.Y. Coxon, M.J. Rane, T. Manning, R. Kettritz and K.R. McLeish, *Role of extracellular signal-regulated kinase and phosphatidylinositol-3 kinase in chemoattractant and LPS delay of constitutive neutrophil apoptosis.* Cellular signalling, 2001. **13**(5): p. 335-43.
286. Ward, C., E.R. Chilvers, M.F. Lawson, J.G. Pryde, S. Fujihara, S.N. Farrow, C. Haslett and A.G. Rossi, *NF-kappaB activation is a critical regulator of human granulocyte apoptosis in vitro.* The Journal of biological chemistry, 1999. **274**(7): p. 4309-18.
287. McDonald, P.P., A. Bald and M.A. Cassatella, *Activation of the NF-kappaB pathway by inflammatory stimuli in human neutrophils.* Blood, 1997. **89**(9): p. 3421-33.
288. Choi, M., S. Rolle, M. Wellner, M.C. Cardoso, C. Scheidereit, F.C. Luft and R. Kettritz, *Inhibition of NF-kappaB by a TAT-NEMO-binding domain peptide accelerates constitutive apoptosis and abrogates LPS-delayed neutrophil apoptosis.* Blood, 2003. **102**(6): p. 2259-67.
289. Wang, K., D. Scheel-Toellner, S.H. Wong, R. Craddock, J. Caamano, A.N. Akbar, M. Salmon and J.M. Lord, *Inhibition of neutrophil apoptosis by type 1 IFN depends on cross-talk between phosphoinositol 3-kinase, protein kinase C-delta, and NF-kappa B signaling pathways.* Journal of immunology, 2003. **171**(2): p. 1035-41.
290. Liles, W.C., P.A. Kiener, J.A. Ledbetter, A. Aruffo and S.J. Klebanoff, *Differential expression of Fas (CD95) and Fas ligand on normal human phagocytes: implications for the regulation of apoptosis in neutrophils.* The Journal of experimental medicine, 1996. **184**(2): p. 429-40.
291. Grassme, H., A. Jekle, A. Riehle, H. Schwarz, J. Berger, K. Sandhoff, R. Kolesnick and E. Gulbins, *CD95 signaling via ceramide-rich membrane rafts.* The Journal of biological chemistry, 2001. **276**(23): p. 20589-96.
292. Grassme, H., A. Cremesti, R. Kolesnick and E. Gulbins, *Ceramide-mediated clustering is required for CD95-DISC formation.* Oncogene, 2003. **22**(35): p. 5457-70.
293. Seumois, G., M. Fillet, L. Gillet, C. Faccinetto, C. Desmet, C. Francois, B. Dewals, C. Oury, A. Vanderplasschen, P. Lekeux and F. Bureau, *De novo C16- and C24-ceramide generation contributes to spontaneous neutrophil apoptosis.* Journal of leukocyte biology, 2007. **81**(6): p. 1477-86.
294. Hannun, Y.A. and L.M. Obeid, *Principles of bioactive lipid signalling: lessons from sphingolipids.* Nature reviews. Molecular cell biology, 2008. **9**(2): p. 139-50.
295. Ruiz, L.M., G. Bedoya, J. Salazar, O.D. Garcia de and P.J. Patino, *Dexamethasone inhibits apoptosis of human neutrophils induced by reactive oxygen species.* Inflammation, 2002. **26**(5): p. 215-22.
296. Cuvillier, O., G. Pirianov, B. Kleuser, P.G. Vanek, O.A. Coso, S. Gutkind and S. Spiegel, *Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate.* Nature, 1996. **381**(6585): p. 800-3.
297. Young, M.M., M. Kester and H.G. Wang, *Sphingolipids: regulators of crosstalk between apoptosis and autophagy.* Journal of lipid research, 2013. **54**(1): p. 5-19.
298. Lin, W.C., C.F. Lin, C.L. Chen, C.W. Chen and Y.S. Lin, *Inhibition of neutrophil apoptosis via sphingolipid signaling in acute lung injury.* The Journal of pharmacology and experimental therapeutics, 2011. **339**(1): p. 45-53.
299. Cooper, M.A., T.A. Fehniger and M.A. Caligiuri, *The biology of human natural killer-cell subsets.* Trends in immunology, 2001. **22**(11): p. 633-40.

300. Kim, S., K. Iizuka, H.S. Kang, A. Dokun, A.R. French, S. Greco and W.M. Yokoyama, *In vivo developmental stages in murine natural killer cell maturation.* Nature immunology, 2002. **3**(6): p. 523-8.
301. Hayakawa, Y. and M.J. Smyth, *CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity.* Journal of immunology, 2006. **176**(3): p. 1517-24.
302. Gonzaga, R., P. Matzinger and A. Perez-Diez, *Resident peritoneal NK cells.* Journal of immunology, 2011. **187**(12): p. 6235-42.
303. Cooper, M.A., T.A. Fehniger, S.C. Turner, K.S. Chen, B.A. Ghaheri, T. Ghayur, W.E. Carson and M.A. Caligiuri, *Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset.* Blood, 2001. **97**(10): p. 3146-51.
304. Smyth, M.J., E. Cretney, J.M. Kelly, J.A. Westwood, S.E. Street, H. Yagita, K. Takeda, S.L. van Dommelen, M.A. Degli-Esposti and Y. Hayakawa, *Activation of NK cell cytotoxicity.* Molecular immunology, 2005. **42**(4): p. 501-10.
305. Afonina, I.S., S.P. Cullen and S.J. Martin, *Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B.* Immunological reviews, 2010. **235**(1): p. 105-16.
306. Shresta, S., D.M. MacIvor, J.W. Heusel, J.H. Russell and T.J. Ley, *Natural killer and lymphokine-activated killer cells require granzyme B for the rapid induction of apoptosis in susceptible target cells.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(12): p. 5679-83.
307. Sato, K., S. Hida, H. Takayanagi, T. Yokochi, N. Kayagaki, K. Takeda, H. Yagita, K. Okumura, N. Tanaka, T. Taniguchi and K. Ogasawara, *Antiviral response by natural killer cells through TRAIL gene induction by IFN-alpha/beta.* European journal of immunology, 2001. **31**(11): p. 3138-46.
308. Wallin, R.P., V. Screpanti, J. Michaelsson, A. Grandien and H.G. Ljunggren, *Regulation of perforin-independent NK cell-mediated cytotoxicity.* European journal of immunology, 2003. **33**(10): p. 2727-35.
309. Hoglund, P. and P. Brodin, *Current perspectives of natural killer cell education by MHC class I molecules.* Nature reviews. Immunology, 2010. **10**(10): p. 724-34.
310. Lanier, L.L., *NK cell receptors.* Annual review of immunology, 1998. **16**: p. 359-93.
311. Germain, R.N., *T-cell development and the CD4-CD8 lineage decision.* Nature reviews. Immunology, 2002. **2**(5): p. 309-22.
312. Mackay, C.R., *Dual personality of memory T cells.* Nature, 1999. **401**(6754): p. 659-60.
313. Lanzavecchia, A. and F. Sallusto, *Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells.* Science (New York, N Y), 2000. **290**(5489): p. 92-7.
314. Sallusto, F., D. Lenig, R. Forster, M. Lipp and A. Lanzavecchia, *Two subsets of memory T lymphocytes with distinct homing potentials and effector functions.* Nature, 1999. **401**(6754): p. 708-12.
315. Sallusto, F., J. Geginat and A. Lanzavecchia, *Central memory and effector memory T cell subsets: function, generation, and maintenance.* Annual review of immunology, 2004. **22**: p. 745-63.
316. Di Mitri, D., R.I. Azevedo, S.M. Henson, V. Libri, N.E. Riddell, R. Macaulay, D. Kipling, M.V. Soares, L. Battistini and A.N. Akbar, *Reversible senescence in human CD4+CD45RA+CD27- memory T cells.* Journal of immunology, 2011. **187**(5): p. 2093-100.
317. Hamann, D., P.A. Baars, M.H. Rep, B. Hooibrink, S.R. Kerkhof-Garde, M.R. Klein and R.A. van Lier, *Phenotypic and functional separation of memory and effector human CD8+ T cells.* The Journal of experimental medicine, 1997. **186**(9): p. 1407-18.

318. Appay, V., R.A. van Lier, F. Sallusto and M. Roederer, *Phenotype and function of human T lymphocyte subsets: consensus and issues.* Cytometry. Part A : the journal of the International Society for Analytical Cytology, 2008. **73**(11): p. 975-83.
319. Vignali, D.A., L.W. Collison and C.J. Workman, *How regulatory T cells work.* Nature reviews. Immunology, 2008. **8**(7): p. 523-32.
320. Shapiro-Shelef, M. and K. Calame, *Regulation of plasma-cell development.* Nature reviews. Immunology, 2005. **5**(3): p. 230-42.
321. Iwata, Y., T. Matsushita, M. Horikawa, D.J. Dilillo, K. Yanaba, G.M. Venturi, P.M. Szabolcs, S.H. Bernstein, C.M. Magro, A.D. Williams, R.P. Hall, E.W. St Clair and T.F. Tedder, *Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells.* Blood, 2011. **117**(2): p. 530-41.
322. Dinarello, C.A., *Historical insights into cytokines.* European journal of immunology, 2007. **37 Suppl 1**: p. S34-45.
323. Born, J., T. Lange, K. Hansen, M. Molle and H.L. Fehm, *Effects of sleep and circadian rhythm on human circulating immune cells.* Journal of immunology, 1997. **158**(9): p. 4454-64.
324. Suzuki, S., S. Toyabe, T. Moroda, T. Tada, A. Tsukahara, T. Iiai, M. Minagawa, S. Maruyama, K. Hatakeyama, K. Endoh and T. Abo, *Circadian rhythm of leucocytes and lymphocytes subsets and its possible correlation with the function of the autonomic nervous system.* Clinical and experimental immunology, 1997. **110**(3): p. 500-8.
325. Kronfol, Z., M. Nair, Q. Zhang, E.E. Hill and M.B. Brown, *Circadian immune measures in healthy volunteers: relationship to hypothalamic-pituitary-adrenal axis hormones and sympathetic neurotransmitters.* Psychosomatic medicine, 1997. **59**(1): p. 42-50.
326. Dimitrov, S., C. Benedict, D. Heutling, J. Westermann, J. Born and T. Lange, *Cortisol and epinephrine control opposing circadian rhythms in T cell subsets.* Blood, 2009. **113**(21): p. 5134-43.
327. Bourin, P., I. Mansour, C. Doinel, R. Roue, P. Rouger and F. Levi, *Circadian rhythms of circulating NK cells in healthy and human immunodeficiency virus-infected men.* Chronobiology international, 1993. **10**(4): p. 298-305.
328. Melchart, D., P. Martin, M. Hallek, M. Holzmann, X. Jurcic and H. Wagner, *Circadian variation of the phagocytic activity of polymorphonuclear leukocytes and of various other parameters in 13 healthy male adults.* Chronobiology international, 1992. **9**(1): p. 35-45.
329. Matsumoto, Y., K. Mishima, K. Satoh, T. Tozawa, Y. Mishima, T. Shimizu and Y. Hishikawa, *Total sleep deprivation induces an acute and transient increase in NK cell activity in healthy young volunteers.* Sleep, 2001. **24**(7): p. 804-9.
330. Moldofsky, H., F.A. Lue, J. Eisen, E. Keystone and R.M. Gorczynski, *The relationship of interleukin-1 and immune functions to sleep in humans.* Psychosomatic medicine, 1986. **48**(5): p. 309-18.
331. Lissoni, P., F. Rovelli, F. Brivio, O. Brivio and L. Fumagalli, *Circadian secretions of IL-2, IL-12, IL-6 and IL-10 in relation to the light/dark rhythm of the pineal hormone melatonin in healthy humans.* Natural immunity, 1998. **16**(1): p. 1-5.
332. Straub, R.H. and M. Cutolo, *Circadian rhythms in rheumatoid arthritis: implications for pathophysiology and therapeutic management.* Arthritis and rheumatism, 2007. **56**(2): p. 399-408.
333. Guan, Z., A.N. Vgontzas, T. Omori, X. Peng, E.O. Bixler and J. Fang, *Interleukin-6 levels fluctuate with the light-dark cycle in the brain and peripheral tissues in rats.* Brain, behavior, and immunity, 2005. **19**(6): p. 526-9.

334. Petrovsky, N. and L.C. Harrison, *Diurnal rhythmicity of human cytokine production: a dynamic disequilibrium in T helper cell type 1/T helper cell type 2 balance?* Journal of immunology, 1997. **158**(11): p. 5163-8.
335. Dimitrov, S., T. Lange, S. Tieken, H.L. Fehm and J. Born, *Sleep associated regulation of T helper 1/T helper 2 cytokine balance in humans.* Brain, behavior, and immunity, 2004. **18**(4): p. 341-8.
336. Besedovsky, L., T. Lange and J. Born, *Sleep and immune function.* Pflugers Archiv-European Journal of Physiology, 2012. **463**(1): p. 121-137.
337. Miller, A.H., R.L. Spencer, J. hassett, C. Kim, R. Rhee, D. Ciurea, F. Dhabhar, B. McEwen and M. Stein, *Effects of selective type I and II adrenal steroid agonists on immune cell distribution.* Endocrinology, 1994. **135**(5): p. 1934-44.
338. Mazzoccoli, G., A. De Cata, A. Greco, M. Damato, N. Marzulli, M.P. Dagostino, S. Carughi, F. Perfetto and R. Tarquini, *Aging related changes of circadian rhythmicity of cytotoxic lymphocyte subpopulations.* Journal of circadian rhythms, 2010. **8**: p. 6.
339. Faict, D., J.L. Ceuppens and P. De Moor, *Transcortin modulates the effect of cortisol on mitogen-induced lymphocyte proliferation and immunoglobulin production.* Journal of steroid biochemistry, 1985. **23**(5A): p. 553-5.
340. Opp, M.R. and L. Imeri, *Rat strains that differ in corticotropin-releasing hormone production exhibit different sleep-wake responses to interleukin 1.* Neuroendocrinology, 2001. **73**(4): p. 272-84.
341. Cupps, T.R., T.L. Gerrard, R.J. Falkoff, G. Whalen and A.S. Fauci, *Effects of in vitro corticosteroids on B cell activation, proliferation, and differentiation.* The Journal of clinical investigation, 1985. **75**(2): p. 754-61.
342. Callewaert, D.M., V.K. Moudgil, G. Radcliff and R. Waite, *Hormone specific regulation of natural killer cells by cortisol. Direct inactivation of the cytotoxic function of cloned human NK cells without an effect on cellular proliferation.* FEBS letters, 1991. **285**(1): p. 108-10.
343. Zhou, J., S. Olsen, J. Moldovan, X. Fu, F.H. Sarkar, V.K. Moudgil and D.M. Callewaert, *Glucocorticoid regulation of natural cytotoxicity: effects of cortisol on the phenotype and function of a cloned human natural killer cell line.* Cellular immunology, 1997. **178**(2): p. 108-16.
344. Liles, W.C., D.C. Dale and S.J. Klebanoff, *Glucocorticoids inhibit apoptosis of human neutrophils.* Blood, 1995. **86**(8): p. 3181-8.
345. Cox, G. and R.C. Austin, *Dexamethasone-induced suppression of apoptosis in human neutrophils requires continuous stimulation of new protein synthesis.* Journal of leukocyte biology, 1997. **61**(2): p. 224-30.
346. Goldstein, D.S., *Catecholamines and stress.* Endocrine regulations, 2003. **37**(2): p. 69-80.
347. Dodt, C., U. Breckling, I. Derad, H.L. Fehm and J. Born, *Plasma epinephrine and norepinephrine concentrations of healthy humans associated with nighttime sleep and morning arousal.* Hypertension, 1997. **30**(1 Pt 1): p. 71-6.
348. Benschop, R.J., M. Rodriguez-Feuerhahn and M. Schedlowski, *Catecholamine-induced leukocytosis: early observations, current research, and future directions.* Brain, behavior, and immunity, 1996. **10**(2): p. 77-91.
349. Elenkov, I.J. and G.P. Chrousos, *Stress hormones, proinflammatory and antiinflammatory cytokines, and autoimmunity.* Annals of the New York Academy of Sciences, 2002. **966**: p. 290-303.
350. Morrey, K.M., J.A. McLachlan, C.D. Serkin and O. Bakouche, *Activation of human monocytes by the pineal hormone melatonin.* Journal of immunology, 1994. **153**(6): p. 2671-80.

351. Barjavel, M.J., Z. Mamdouh, N. Raghbate and O. Bakouche, *Differential expression of the melatonin receptor in human monocytes.* Journal of immunology, 1998. **160**(3): p. 1191-7.
352. Silva, S.O., M.R. Rodrigues, V.F. Ximenes, A.E. Bueno-da-Silva, G.P. Amarante-Mendes and A. Campa, *Neutrophils as a specific target for melatonin and kynuramines: effects on cytokine release.* Journal of neuroimmunology, 2004. **156**(1-2): p. 146-52.
353. Kuhlwein, E. and M. Irwin, *Melatonin modulation of lymphocyte proliferation and Th1/Th2 cytokine expression.* Journal of neuroimmunology, 2001. **117**(1-2): p. 51-7.
354. Garcia-Maurino, S., M.G. Gonzalez-Haba, J.R. Calvo, M. Rafii-El-Idrissi, V. Sanchez-Margalet, R. Goberna and J.M. Guerrero, *Melatonin enhances IL-2, IL-6, and IFN-gamma production by human circulating CD4+ cells: a possible nuclear receptor-mediated mechanism involving T helper type 1 lymphocytes and monocytes.* Journal of immunology, 1997. **159**(2): p. 574-81.
355. Miller, S.C., S.R. Pandi-Perumal, A.I. Esquifino, D.P. Cardinali and G.J. Maestroni, *The role of melatonin in immuno-enhancement: potential application in cancer.* International journal of experimental pathology, 2006. **87**(2): p. 81-7.
356. Dimitrov, S., T. Lange, H.L. Fehm and J. Born, *A regulatory role of prolactin, growth hormone, and corticosteroids for human T-cell production of cytokines.* Brain, behavior, and immunity, 2004. **18**(4): p. 368-74.
357. Yoshida, A., C. Ishioka, H. Kimata and H. Mikawa, *Recombinant human growth hormone stimulates B cell immunoglobulin synthesis and proliferation in serum-free medium.* Acta endocrinologica, 1992. **126**(6): p. 524-9.
358. Hanley, M.B., L.A. Napolitano and J.M. McCune, *Growth hormone-induced stimulation of multilineage human hematopoiesis.* Stem cells, 2005. **23**(8): p. 1170-9.
359. Hattori, N., *Expression, regulation and biological actions of growth hormone (GH) and ghrelin in the immune system.* Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society, 2009. **19**(3): p. 187-97.
360. Dixit, V.D., E.M. Schaffer, R.S. Pyle, G.D. Collins, S.K. Sakthivel, R. Palaniappan, J.W. Lillard, Jr. and D.D. Taub, *Ghrelin inhibits leptin- and activation-induced proinflammatory cytokine expression by human monocytes and T cells.* The Journal of clinical investigation, 2004. **114**(1): p. 57-66.
361. Toth, L.A. and J.M. Krueger, *Alteration of sleep in rabbits by Staphylococcus aureus infection.* Infection and immunity, 1988. **56**(7): p. 1785-91.
362. Toth, L.A., J.E. Rehg and R.G. Webster, *Strain differences in sleep and other pathophysiological sequelae of influenza virus infection in naive and immunized mice.* Journal of neuroimmunology, 1995. **58**(1): p. 89-99.
363. Toth, L.A., *Immune-modulatory drugs alter Candida albicans-induced sleep patterns in rabbits.* Pharmacology, biochemistry, and behavior, 1995. **51**(4): p. 877-84.
364. Toth, L.A., E.A. Tolley, R. Broady, B. Blakely and J.M. Krueger, *Sleep during experimental trypanosomiasis in rabbits.* Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine, 1994. **205**(2): p. 174-81.
365. Lancel, M., J. Cronlein, P. Muller-Preuss and F. Holsboer, *Lipopolysaccharide increases EEG delta activity within non-REM sleep and disrupts sleep continuity in rats.* The American journal of physiology, 1995. **268**(5 Pt 2): p. R1310-8.
366. Shoham, S., R.A. Ahokas, C.M. Blatteis and J.M. Krueger, *Effects of muramyl dipeptide on sleep, body temperature and plasma copper after intracerebral ventricular administration.* Brain research, 1987. **419**(1-2): p. 223-8.
367. Mullington, J., C. Korth, D.M. Hermann, A. Orth, C. Galanos, F. Holsboer and T. Pollmacher, *Dose-dependent effects of endotoxin on human sleep.* American journal of

physiology. Regulatory, integrative and comparative physiology, 2000. **278**(4): p. R947-55.
368. Pollmacher, T., W. Schreiber, S. Gudewill, H. Vedder, K. Fassbender, K. Wiedemann, L. Trachsel, C. Galanos and F. Holsboer, *Influence of endotoxin on nocturnal sleep in humans.* The American journal of physiology, 1993. **264**(6 Pt 2): p. R1077-83.
369. Trachsel, L., W. Schreiber, F. Holsboer and T. Pollmacher, *Endotoxin enhances EEG alpha and beta power in human sleep.* Sleep, 1994. **17**(2): p. 132-9.
370. Korth, C., J. Mullington, W. Schreiber and T. Pollmacher, *Influence of endotoxin on daytime sleep in humans.* Infection and immunity, 1996. **64**(4): p. 1110-15.
371. Opp, M.R., *Cytokines and sleep.* Sleep medicine reviews, 2005. **9**(5): p. 355-64.
372. Krueger, J.M., F.J. Obal, J. Fang, T. Kubota and P. Taishi, *The role of cytokines in physiological sleep regulation.* Annals of the New York Academy of Sciences, 2001. **933**: p. 211-21.
373. Imeri, L. and M.R. Opp, *How (and why) the immune system makes us sleep.* Nature reviews. Neuroscience, 2009. **10**(3): p. 199-210.
374. Krueger, J.M., *The role of cytokines in sleep regulation.* Current pharmaceutical design, 2008. **14**(32): p. 3408-16.
375. Takahashi, S., L. Kapas, J. Fang and J.M. Krueger, *An anti-tumor necrosis factor antibody suppresses sleep in rats and rabbits.* Brain research, 1995. **690**(2): p. 241-4.
376. Takahashi, S., D.D. Tooley, L. Kapas, J. Fang, J.M. Seyer and J.M. Krueger, *Inhibition of tumor necrosis factor in the brain suppresses rabbit sleep.* Pflugers Arch, 1995. **431**(2): p. 155-60.
377. Opp, M.R. and J.M. Krueger, *Interleukin 1-receptor antagonist blocks interleukin 1-induced sleep and fever.* The American journal of physiology, 1991. **260**(2 Pt 2): p. R453-7.
378. Opp, M.R. and J.M. Krueger, *Anti-interleukin-1 beta reduces sleep and sleep rebound after sleep deprivation in rats.* The American journal of physiology, 1994. **266**(3 Pt 2): p. R688-95.
379. Fang, J., Y. Wang and J.M. Krueger, *Mice lacking the TNF 55 kDa receptor fail to sleep more after TNFalpha treatment.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 1997. **17**(15): p. 5949-55.
380. Fang, J., Y. Wang and J.M. Krueger, *Effects of interleukin-1 beta on sleep are mediated by the type I receptor.* The American journal of physiology, 1998. **274**(3 Pt 2): p. R655-60.
381. Baracchi, F. and M.R. Opp, *Sleep-wake behavior and responses to sleep deprivation of mice lacking both interleukin-1 beta receptor 1 and tumor necrosis factor-alpha receptor 1.* Brain, behavior, and immunity, 2008. **22**(6): p. 982-93.
382. Vgontzas, A.N., E. Zoumakis, H.M. Lin, E.O. Bixler, G. Trakada and G.P. Chrousos, *Marked decrease in sleepiness in patients with sleep apnea by etanercept, a tumor necrosis factor-alpha antagonist.* The Journal of clinical endocrinology and metabolism, 2004. **89**(9): p. 4409-13.
383. Kubota, T., T. Kushikata, J. Fang and J.M. Krueger, *Nuclear factor-kappaB inhibitor peptide inhibits spontaneous and interleukin-1beta-induced sleep.* American journal of physiology. Regulatory, integrative and comparative physiology, 2000. **279**(2): p. R404-13.
384. Kushikata, T., J. Fang, Y. Wang and J.M. Krueger, *Interleukin-4 inhibits spontaneous sleep in rabbits.* The American journal of physiology, 1998. **275**(4 Pt 2): p. R1185-91.
385. Kushikata, T., J. Fang and J.M. Krueger, *Interleukin-10 inhibits spontaneous sleep in rabbits.* Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research, 1999. **19**(9): p. 1025-30.

386. Dantzer, R., J.C. O'Connor, G.G. Freund, R.W. Johnson and K.W. Kelley, *From inflammation to sickness and depression: when the immune system subjugates the brain.* Nature reviews. Neuroscience, 2008. **9**(1): p. 46-56.
387. Capuron, L. and A.H. Miller, *Immune system to brain signaling: neuropsychopharmacological implications.* Pharmacology & therapeutics, 2011. **130**(2): p. 226-38.
388. Gosselin, D. and S. Rivest, *Role of IL-1 and TNF in the brain: twenty years of progress on a Dr. Jekyll/Mr. Hyde duality of the innate immune system.* Brain, behavior, and immunity, 2007. **21**(3): p. 281-9.
389. Garden, G.A. and T. Moller, *Microglia biology in health and disease.* Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology, 2006. **1**(2): p. 127-37.
390. Breder, C.D., C.A. Dinarello and C.B. Saper, *Interleukin-1 immunoreactive innervation of the human hypothalamus.* Science (New York, N Y), 1988. **240**(4850): p. 321-4.
391. Ignatowski, T.A., B.K. Noble, J.R. Wright, J.L. Gorfien, R.R. Heffner and R.N. Spengler, *Neuronal-associated tumor necrosis factor (TNF alpha): its role in noradrenergic functioning and modification of its expression following antidepressant drug administration.* Journal of neuroimmunology, 1997. **79**(1): p. 84-90.
392. Beynon, A.L. and A.N. Coogan, *Diurnal, age, and immune regulation of interleukin-1beta and interleukin-1 type 1 receptor in the mouse suprachiasmatic nucleus.* Chronobiology international, 2010. **27**(8): p. 1546-63.
393. Benedict, C., J. Scheller, S. Rose-John, J. Born and L. Marshall, *Enhancing influence of intranasal interleukin-6 on slow-wave activity and memory consolidation during sleep.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2009. **23**(10): p. 3629-36.
394. Opp, M., F. Obal, Jr., A.B. Cady, L. Johannsen and J.M. Krueger, *Interleukin-6 is pyrogenic but not somnogenic.* Physiology & behavior, 1989. **45**(5): p. 1069-72.
395. Mullington, J.M., N.S. Simpson, H.K. Meier-Ewert and M. Haack, *Sleep loss and inflammation.* Best practice & research. Clinical endocrinology & metabolism, 2010. **24**(5): p. 775-84.
396. Dinges, D.F., S.D. Douglas, L. Zaugg, D.E. Campbell, J.M. McMann, W.G. Whitehouse, E.C. Orne, S.C. Kapoor, E. Icaza and M.T. Orne, *Leukocytosis and natural killer cell function parallel neurobehavioral fatigue induced by 64 hours of sleep deprivation.* The Journal of clinical investigation, 1994. **93**(5): p. 1930-9.
397. Ruiz, F.S., M.L. Andersen, R.C. Martins, A. Zager, J.D. Lopes and S. Tufik, *Immune alterations after selective rapid eye movement or total sleep deprivation in healthy male volunteers.* Innate immunity, 2012. **18**(1): p. 44-54.
398. Palmblad, J., K. Cantell, H. Strander, J. Froberg, C.G. Karlsson, L. Levi, M. Granstrom and P. Unger, *Stressor exposure and immunological response in man: interferon-producing capacity and phagocytosis.* Journal of psychosomatic research, 1976. **20**(3): p. 193-9.
399. Irwin, M., A. Mascovich, J.C. Gillin, R. Willoughby, J. Pike and T.L. Smith, *Partial sleep deprivation reduces natural killer cell activity in humans.* Psychosomatic medicine, 1994. **56**(6): p. 493-8.
400. Irwin, M., J. McClintick, C. Costlow, M. Fortner, J. White and J.C. Gillin, *Partial night sleep deprivation reduces natural killer and cellular immune responses in humans.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 1996. **10**(5): p. 643-53.
401. Irwin, M.R., C. Carrillo and R. Olmstead, *Sleep loss activates cellular markers of inflammation: sex differences.* Brain, behavior, and immunity, 2010. **24**(1): p. 54-7.

402. Boyum, A., P. Wiik, E. Gustavsson, O.P. Veiby, J. Reseland, A.H. Haugen and P.K. Opstad, *The effect of strenuous exercise, calorie deficiency and sleep deprivation on white blood cells, plasma immunoglobulins and cytokines.* Scandinavian journal of immunology, 1996. **43**(2): p. 228-35.
403. Irwin, M.R., M. Wang, D. Ribeiro, H.J. Cho, R. Olmstead, E.C. Breen, O. Martinez-Maza and S. Cole, *Sleep loss activates cellular inflammatory signaling.* Biological psychiatry, 2008. **64**(6): p. 538-40.
404. Heiser, P., B. Dickhaus, W. Schreiber, H.W. Clement, C. Hasse, J. Hennig, H. Remschmidt, J.C. Krieg, W. Wesemann and C. Opper, *White blood cells and cortisol after sleep deprivation and recovery sleep in humans.* European archives of psychiatry and clinical neuroscience, 2000. **250**(1): p. 16-23.
405. Costa, R.J., A.H. Smith, S.J. Oliver, R. Walters, N. Maassen, J.L. Bilzon and N.P. Walsh, *The effects of two nights of sleep deprivation with or without energy restriction on immune indices at rest and in response to cold exposure.* European journal of applied physiology, 2010. **109**(3): p. 417-28.
406. Palmblad, J., B. Petrini, J. Wasserman and T. Akerstedt, *Lymphocyte and granulocyte reactions during sleep deprivation.* Psychosomatic medicine, 1979. **41**(4): p. 273-8.
407. Chennaoui, M., F. Sauvet, C. Drogou, P. Van Beers, C. Langrume, M. Guillard, B. Gourby, C. Bourrilhon, G. Florence and D. Gomez-Merino, *Effect of one night of sleep loss on changes in tumor necrosis factor alpha (TNF-alpha) levels in healthy men.* Cytokine, 2011. **56**(2): p. 318-24.
408. Shearer, W.T., J.M. Reuben, J.M. Mullington, N.J. Price, B.N. Lee, E.O. Smith, M.P. Szuba, H.P. Van Dongen and D.F. Dinges, *Soluble TNF-alpha receptor 1 and IL-6 plasma levels in humans subjected to the sleep deprivation model of spaceflight.* The Journal of allergy and clinical immunology, 2001. **107**(1): p. 165-70.
409. Ackermann, K., R. Plomp, O. Lao, B. Middleton, V.L. Revell, D.J. Skene and M. Kayser, *Effect of sleep deprivation on rhythms of clock gene expression and melatonin in humans.* Chronobiology international, 2013. **30**(7): p. 901-9.
410. von Treuer, K., T.R. Norman and S.M. Armstrong, *Overnight human plasma melatonin, cortisol, prolactin, TSH, under conditions of normal sleep, sleep deprivation, and sleep recovery.* Journal of pineal research, 1996. **20**(1): p. 7-14.
411. Vgontzas, A.N., G. Mastorakos, E.O. Bixler, A. Kales, P.W. Gold and G.P. Chrousos, *Sleep deprivation effects on the activity of the hypothalamic-pituitary-adrenal and growth axes: potential clinical implications.* Clinical endocrinology, 1999. **51**(2): p. 205-15.
412. Hui, L., F. Hua, H. Diandong and Y. Hong, *Effects of sleep and sleep deprivation on immunoglobulins and complement in humans.* Brain, behavior, and immunity, 2007. **21**(3): p. 308-10.
413. Lange, T., B. Perras, H.L. Fehm and J. Born, *Sleep enhances the human antibody response to hepatitis A vaccination.* Psychosomatic medicine, 2003. **65**(5): p. 831-5.
414. Benedict, C., M. Brytting, A. Markstrom, J.E. Broman and H.B. Schioth, *Acute sleep deprivation has no lasting effects on the human antibody titer response following a novel influenza A H1N1 virus vaccination.* BMC immunology, 2012. **13**: p. 1.
415. Boudjeltia, K.Z., B. Faraut, P. Stenuit, M.J. Esposito, M. Dyzma, D. Brohee, J. Ducobu, M. Vanhaeverbeek and M. Kerkhofs, *Sleep restriction increases white blood cells, mainly neutrophil count, in young healthy men: a pilot study.* Vascular health and risk management, 2008. **4**(6): p. 1467-70.
416. Faraut, B., K.Z. Boudjeltia, M. Dyzma, A. Rousseau, E. David, P. Stenuit, T. Franck, P. Van Antwerpen, M. Vanhaeverbeek and M. Kerkhofs, *Benefits of napping and an extended duration of recovery sleep on alertness and immune cells after acute sleep restriction.* Brain, behavior, and immunity, 2011. **25**(1): p. 16-24.

417. Wilder-Smith, A., F.B. Mustafa, A. Earnest, L. Gen and P.A. Macary, *Impact of partial sleep deprivation on immune markers.* Sleep medicine, 2013. **14**(10): p. 1031-4.
418. Vgontzas, A.N., E. Zoumakis, E.O. Bixler, H.M. Lin, H. Follett, A. Kales and G.P. Chrousos, *Adverse effects of modest sleep restriction on sleepiness, performance, and inflammatory cytokines.* The Journal of clinical endocrinology and metabolism, 2004. **89**(5): p. 2119-26.
419. Lekander, M., A.N. Andreasson, G. Kecklund, R. Ekman, M. Ingre, T. Akerstedt and J. Axelsson, *Subjective health perception in healthy young men changes in response to experimentally restricted sleep and subsequent recovery sleep.* Brain, behavior, and immunity, 2013.
420. Pejovic, S., M. Basta, A.N. Vgontzas, I. Kritikou, M.L. Shaffer, M. Tsaoussoglou, D. Stiffler, Z. Stefanakis, E.O. Bixler and G.P. Chrousos, *Effects of recovery sleep after one work week of mild sleep restriction on interleukin-6 and cortisol secretion and daytime sleepiness and performance.* American journal of physiology. Endocrinology and metabolism, 2013. **305**(7): p. E890-6.
421. Schule, C., F. Di Michele, T. Baghai, E. Romeo, G. Bernardi, P. Zwanzger, F. Padberg, A. Pasini and R. Rupprecht, *Neuroactive steroids in responders and nonresponders to sleep deprivation.* Annals of the New York Academy of Sciences, 2004. **1032**: p. 216-23.
422. Wu, H., Z. Zhao, W.S. Stone, L. Huang, J. Zhuang, B. He, P. Zhang and Y. Li, *Effects of sleep restriction periods on serum cortisol levels in healthy men.* Brain research bulletin, 2008. **77**(5): p. 241-5.
423. Leproult, R., G. Copinschi, O. Buxton and E. Van Cauter, *Sleep loss results in an elevation of cortisol levels the next evening.* Sleep, 1997. **20**(10): p. 865-70.
424. Tilg, H. and A.R. Moschen, *Adipocytokines: mediators linking adipose tissue, inflammation and immunity.* Nature reviews. Immunology, 2006. **6**(10): p. 772-83.
425. Arita, Y., S. Kihara, N. Ouchi, M. Takahashi, K. Maeda, J. Miyagawa, K. Hotta, I. Shimomura, T. Nakamura, K. Miyaoka, H. Kuriyama, M. Nishida, S. Yamashita, K. Okubo, K. Matsubara, M. Muraguchi, Y. Ohmoto, T. Funahashi and Y. Matsuzawa, *Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity.* Biochemical and biophysical research communications, 1999. **257**(1): p. 79-83.
426. Hoffstedt, J., E. Arvidsson, E. Sjolin, K. Wahlen and P. Arner, *Adipose tissue adiponectin production and adiponectin serum concentration in human obesity and insulin resistance.* The Journal of clinical endocrinology and metabolism, 2004. **89**(3): p. 1391-6.
427. Pineiro, R., M.J. Iglesias, R. Gallego, K. Raghay, S. Eiras, J. Rubio, C. Dieguez, O. Gualillo, J.R. Gonzalez-Juanatey and F. Lago, *Adiponectin is synthesized and secreted by human and murine cardiomyocytes.* FEBS letters, 2005. **579**(23): p. 5163-9.
428. Delaigle, A.M., J.C. Jonas, I.B. Bauche, O. Cornu and S.M. Brichard, *Induction of adiponectin in skeletal muscle by inflammatory cytokines: in vivo and in vitro studies.* Endocrinology, 2004. **145**(12): p. 5589-97.
429. Crawford, L.J., R. Peake, S. Price, T.C. Morris and A.E. Irvine, *Adiponectin is produced by lymphocytes and is a negative regulator of granulopoiesis.* Journal of leukocyte biology, 2010. **88**(4): p. 807-11.
430. Hu, E., P. Liang and B.M. Spiegelman, *AdipoQ is a novel adipose-specific gene dysregulated in obesity.* The Journal of biological chemistry, 1996. **271**(18): p. 10697-703.
431. Scherer, P.E., S. Williams, M. Fogliano, G. Baldini and H.F. Lodish, *A novel serum protein similar to C1q, produced exclusively in adipocytes.* The Journal of biological chemistry, 1995. **270**(45): p. 26746-9.

432. Pajvani, U.B., X. Du, T.P. Combs, A.H. Berg, M.W. Rajala, T. Schulthess, J. Engel, M. Brownlee and P.E. Scherer, *Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications fpr metabolic regulation and bioactivity.* The Journal of biological chemistry, 2003. **278**(11): p. 9073-85.
433. Waki, H., T. Yamauchi, J. Kamon, S. Kita, Y. Ito, Y. Hada, S. Uchida, A. Tsuchida, S. Takekawa and T. Kadowaki, *Generation of globular fragment of adiponectin by leukocyte elastase secreted by monocytic cell line THP-1.* Endocrinology, 2005. **146**(2): p. 790-6.
434. Waki, H., T. Yamauchi, J. Kamon, Y. Ito, S. Uchida, S. Kita, K. Hara, Y. Hada, F. Vasseur, P. Froguel, S. Kimura, R. Nagai and T. Kadowaki, *Impaired multimerization of human adiponectin mutants associated with diabetes. Molecular structure and multimer formation of adiponectin.* The Journal of biological chemistry, 2003. **278**(41): p. 40352-63.
435. Fruebis, J., T.S. Tsao, S. Javorschi, D. Ebbets-Reed, M.R. Erickson, F.T. Yen, B.E. Bihain and H.F. Lodish, *Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(4): p. 2005-10.
436. Stofkova, A., *Leptin and adiponectin: from energy and metabolic dysbalance to inflammation and autoimmunity.* Endocrine regulations, 2009. **43**(4): p. 157-68.
437. Inadera, H., *The usefulness of circulating adipokine levels for the assessment of obesity-related health problems.* International journal of medical sciences, 2008. **5**(5): p. 248-62.
438. Mohlig, M., U. Wegewitz, M. Osterhoff, F. Isken, M. Ristow, A.F. Pfeiffer and J. Spranger, *Insulin decreases human adiponectin plasma levels.* Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme, 2002. **34**(11-12): p. 655-8.
439. Nishizawa, H., I. Shimomura, K. Kishida, N. Maeda, H. Kuriyama, H. Nagaretani, M. Matsuda, H. Kondo, N. Furuyama, S. Kihara, T. Nakamura, Y. Tochino, T. Funahashi and Y. Matsuzawa, *Androgens decrease plasma adiponectin, an insulin-sensitizing adipocyte-derived protein.* Diabetes, 2002. **51**(9): p. 2734-41.
440. Fantuzzi, G., *Adiponectin and inflammation: consensus and controversy.* The Journal of allergy and clinical immunology, 2008. **121**(2): p. 326-30.
441. Saltevo, J., H. Kautiainen and M. Vanhala, *Gender differences in adiponectin and low-grade inflammation among individuals with normal glucose tolerance, prediabetes, and type 2 diabetes.* Gender medicine, 2009. **6**(3): p. 463-70.
442. Schautz, B., W. Later, M. Heller, A. Peters, M.J. Muller and A. Bosy-Westphal, *Impact of age on leptin and adiponectin independent of adiposity.* The British journal of nutrition, 2012. **108**(2): p. 363-70.
443. Yamauchi, T., J. Kamon, Y. Ito, A. Tsuchida, T. Yokomizo, S. Kita, T. Sugiyama, M. Miyagishi, K. Hara, M. Tsunoda, K. Murakami, T. Ohteki, S. Uchida, S. Takekawa, H. Waki, N.H. Tsuno, Y. Shibata, Y. Terauchi, P. Froguel, K. Tobe, S. Koyasu, K. Taira, T. Kitamura, T. Shimizu, R. Nagai and T. Kadowaki, *Cloning of adiponectin receptors that mediate antidiabetic metabolic effects.* Nature, 2003. **423**(6941): p. 762-9.
444. Kadowaki, T. and T. Yamauchi, *Adiponectin and adiponectin receptors.* Endocrine reviews, 2005. **26**(3): p. 439-51.
445. Hug, C., J. Wang, N.S. Ahmad, J.S. Bogan, T.S. Tsao and H.F. Lodish, *T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(28): p. 10308-13.

446. Denzel, M.S., M.C. Scimia, P.M. Zumstein, K. Walsh, P. Ruiz-Lozano and B. Ranscht, *T-cadherin is critical for adiponectin-mediated cardioprotection in mice.* The Journal of clinical investigation, 2010. **120**(12): p. 4342-52.
447. Shimada, K., T. Miyazaki and H. Daida, *Adiponectin and atherosclerotic disease.* Clinica chimica acta; international journal of clinical chemistry, 2004. **344**(1-2): p. 1-12.
448. Pang, T.T. and P. Narendran, *The distribution of adiponectin receptors on human peripheral blood mononuclear cells.* Annals of the New York Academy of Sciences, 2008. **1150**: p. 143-5.
449. Wilk, S., A. Jenke, J. Stehr, C.A. Yang, S. Bauer, K. Goldner, K. Kotsch, H.D. Volk, W. Poller, H.P. Schultheiss, C. Skurk and C. Scheibenbogen, *Adiponectin modulates NK-cell function.* European journal of immunology, 2013. **43**(4): p. 1024-33.
450. Wilk, S., C. Scheibenbogen, S. Bauer, A. Jenke, M. Rother, M. Guerreiro, R. Kudernatsch, N. Goerner, W. Poller, D. Elligsen-Merkel, N. Utku, J. Magrane, H.D. Volk and C. Skurk, *Adiponectin is a negative regulator of antigen-activated T cells.* European journal of immunology, 2011. **41**(8): p. 2323-32.
451. Chedid, P., M. Hurtado-Nedelec, B. Marion-Gaber, O. Bournier, G. Hayem, M.A. Gougerot-Pocidalo, J. Frystyk, A. Flyvbjerg, J. El Benna and J.C. Marie, *Adiponectin and its globular fragment differentially modulate the oxidative burst of primary human phagocytes.* The American journal of pathology, 2012. **180**(2): p. 682-92.
452. Pang, T.T., M. Chimen, E. Goble, N. Dixon, A. Benbow, S.E. Eldershaw, D. Thompson, S.C. Gough and P. Narendran, *Inhibition of islet immunoreactivity by adiponectin is attenuated in human type 1 diabetes.* The Journal of clinical endocrinology and metabolism, 2013. **98**(3): p. E418-28.
453. Deepa, S.S. and L.Q. Dong, *APPL1: role in adiponectin signaling and beyond.* American journal of physiology. Endocrinology and metabolism, 2009. **296**(1): p. E22-36.
454. Deepa, S.S., L. Zhou, J. Ryu, C. Wang, X. Mao, C. Li, N. Zhang, N. Musi, R.A. DeFronzo, F. Liu and L.Q. Dong, *APPL1 mediates adiponectin-induced LKB1 cytosolic localization through the PP2A-PKCzeta signaling pathway.* Molecular endocrinology, 2011. **25**(10): p. 1773-85.
455. Zhou, L., S.S. Deepa, J.C. Etzler, J. Ryu, X. Mao, Q. Fang, D.D. Liu, J.M. Torres, W. Jia, J.D. Lechleiter, F. Liu and L.Q. Dong, *Adiponectin activates AMP-activated protein kinase in muscle cells via APPL1/LKB1-dependent and phospholipase C/Ca2+/Ca2+/calmodulin-dependent protein kinase kinase-dependent pathways.* The Journal of biological chemistry, 2009. **284**(33): p. 22426-35.
456. Buechler, C., J. Wanninger and M. Neumeier, *Adiponectin receptor binding proteins--recent advances in elucidating adiponectin signalling pathways.* FEBS letters, 2010. **584**(20): p. 4280-6.
457. Lee, M.H., R.L. Klein, H.M. El-Shewy, D.K. Luttrell and L.M. Luttrell, *The adiponectin receptors AdipoR1 and AdipoR2 activate ERK1/2 through a Src/Ras-dependent pathway and stimulate cell growth.* Biochemistry, 2008. **47**(44): p. 11682-92.
458. Nigro, E., O. Scudiero, D. Sarnataro, G. Mazzarella, M. Sofia, A. Bianco and A. Daniele, *Adiponectin affects lung epithelial A549 cell viability counteracting TNFalpha and IL-1ss toxicity through AdipoR1.* The international journal of biochemistry & cell biology, 2013. **45**(6): p. 1145-53.
459. Wijesekara, N., M. Krishnamurthy, A. Bhattacharjee, A. Suhail, G. Sweeney and M.B. Wheeler, *Adiponectin-induced ERK and Akt phosphorylation protects against pancreatic beta cell apoptosis and increases insulin gene expression and secretion.* The Journal of biological chemistry, 2010. **285**(44): p. 33623-31.

460. Wanninger, J., M. Neumeier, J. Weigert, S. Bauer, T.S. Weiss, A. Schaffler, C. Krempl, C. Bleyl, C. Aslanidis, J. Scholmerich and C. Buechler, *Adiponectin-stimulated CXCL8 release in primary human hepatocytes is regulated by ERK1/ERK2, p38 MAPK, NF-kappaB, and STAT3 signaling pathways.* American journal of physiology. Gastrointestinal and liver physiology, 2009. **297**(3): p. G611-8.
461. Motobayashi, Y., Y. Izawa-Ishizawa, K. Ishizawa, S. Orino, K. Yamaguchi, K. Kawazoe, S. Hamano, K. Tsuchiya, S. Tomita and T. Tamaki, *Adiponectin inhibits insulin-like growth factor-1-induced cell migration by the suppression of extracellular signal-regulated kinase 1/2 activation, but not Akt in vascular smooth muscle cells.* Hypertension research : official journal of the Japanese Society of Hypertension, 2009. **32**(3): p. 188-93.
462. Li, L., K. Zhang, X.J. Cai, M. Feng, Y. Zhang and M. Zhang, *Adiponectin upregulates prolyl-4-hydroxylase alpha1 expression in interleukin 6-stimulated human aortic smooth muscle cells by regulating ERK 1/2 and Sp1.* PloS one, 2011. **6**(7): p. e22819.
463. Barb, D., A. Neuwirth, C.S. Mantzoros and S.P. Balk, *Adiponectin signals in prostate cancer cells through Akt to activate the mammalian target of rapamycin pathway.* Endocrine-related cancer, 2007. **14**(4): p. 995-1005.
464. Kobashi, C., M. Urakaze, M. Kishida, E. Kibayashi, H. Kobayashi, S. Kihara, T. Funahashi, M. Takata, R. Temaru, A. Sato, K. Yamazaki, N. Nakamura and M. Kobayashi, *Adiponectin inhibits endothelial synthesis of interleukin-8.* Circulation research, 2005. **97**(12): p. 1245-52.
465. Kim, K.Y., A. Baek, J.E. Hwang, Y.A. Choi, J. Jeong, M.S. Lee, D.H. Cho, J.S. Lim, K.I. Kim and Y. Yang, *Adiponectin-activated AMPK stimulates dephosphorylation of AKT through protein phosphatase 2A activation.* Cancer research, 2009. **69**(9): p. 4018-26.
466. Tian, L., N. Luo, X. Zhu, B.H. Chung, W.T. Garvey and Y. Fu, *Adiponectin-AdipoR1/2-APPL1 signaling axis suppresses human foam cell formation: differential ability of AdipoR1 and AdipoR2 to regulate inflammatory cytokine responses.* Atherosclerosis, 2012. **221**(1): p. 66-75.
467. Mao, X., C.K. Kikani, R.A. Riojas, P. Langlais, L. Wang, F.J. Ramos, Q. Fang, C.Y. Christ-Roberts, J.Y. Hong, R.Y. Kim, F. Liu and L.Q. Dong, *APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function.* Nature cell biology, 2006. **8**(5): p. 516-23.
468. Mitsuuchi, Y., S.W. Johnson, G. Sonoda, S. Tanno, E.A. Golemis and J.R. Testa, *Identification of a chromosome 3p14.3-21.1 gene, APPL, encoding an adaptor molecule that interacts with the oncoprotein-serine/threonine kinase AKT2.* Oncogene, 1999. **18**(35): p. 4891-4898.
469. Yang, L., H.K. Lin, S. Altuwaijri, S. Xie, L. Wang and C. Chang, *APPL suppresses androgen receptor transactivation via potentiating Akt activity.* The Journal of biological chemistry, 2003. **278**(19): p. 16820-7.
470. Yokota, T., K. Oritani, I. Takahashi, J. Ishikawa, A. Matsuyama, N. Ouchi, S. Kihara, T. Funahashi, A.J. Tenner, Y. Tomiyama and Y. Matsuzawa, *Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages.* Blood, 2000. **96**(5): p. 1723-32.
471. DiMascio, L., C. Voermans, M. Uqoezwa, A. Duncan, D. Lu, J. Wu, U. Sankar and T. Reya, *Identification of adiponectin as a novel hemopoietic stem cell growth factor.* Journal of immunology, 2007. **178**(6): p. 3511-20.
472. Magalang, U.J., R. Rajappan, M.G. Hunter, V.K. Kutala, P. Kuppusamy, M.D. Wewers, C.B. Marsh and N.L. Parinandi, *Adiponectin inhibits superoxide generation by human neutrophils.* Antioxidants & redox signaling, 2006. **8**(11-12): p. 2179-86.

473. Trellakis, S., A. Rydleuskaya, C. Fischer, A. Canbay, S. Tagay, A. Scherag, K. Bruderek, P.J. Schuler and S. Brandau, *Low adiponectin, high levels of apoptosis and increased peripheral blood neutrophil activity in healthy obese subjects.* Obesity facts, 2012. **5**(3): p. 305-18.
474. Folco, E.J., V.Z. Rocha, M. Lopez-Ilasaca and P. Libby, *Adiponectin inhibits pro-inflammatory signaling in human macrophages independent of interleukin-10.* The Journal of biological chemistry, 2009. **284**(38): p. 25569-75.
475. Saijo, S., K. Nagata, Y. Nakano, T. Tobe and Y. Kobayashi, *Inhibition by adiponectin of IL-8 production by human macrophages upon coculturing with late apoptotic cells.* Biochemical and biophysical research communications, 2005. **334**(4): p. 1180-3.
476. Wolf, A.M., D. Wolf, H. Rumpold, B. Enrich and H. Tilg, *Adiponectin induces the anti-inflammatory cytokines IL-10 and IL-1RA in human leukocytes.* Biochemical and biophysical research communications, 2004. **323**(2): p. 630-5.
477. Lovren, F., Y. Pan, A. Quan, P.E. Szmitko, K.K. Singh, P.C. Shukla, M. Gupta, L. Chan, M. Al-Omran, H. Teoh and S. Verma, *Adiponectin primes human monocytes into alternative anti-inflammatory M2 macrophages.* American journal of physiology. Heart and circulatory physiology, 2010. **299**(3): p. H656-63.
478. Cheng, X., E.J. Folco, K. Shimizu and P. Libby, *Adiponectin induces pro-inflammatory programs in human macrophages and CD4+ T cells.* The Journal of biological chemistry, 2012. **287**(44): p. 36896-904.
479. Neumeier, M., J. Weigert, A. Schaffler, G. Wehrwein, U. Muller-Ladner, J. Scholmerich, C. Wrede and C. Buechler, *Different effects of adiponectin isoforms in human monocytic cells.* Journal of leukocyte biology, 2006. **79**(4): p. 803-8.
480. Neumeier, M., S. Bauer, H. Bruhl, K. Eisinger, A. Kopp, S. Abke, R. Walter, A. Schaffler and C. Buechler, *Adiponectin stimulates release of CCL2, -3, -4 and -5 while the surface abundance of CCR2 and -5 is simultaneously reduced in primary human monocytes.* Cytokine, 2011. **56**(3): p. 573-80.
481. O'Shea, D., T.J. Cawood, C. O'Farrelly and L. Lynch, *Natural killer cells in obesity: impaired function and increased susceptibility to the effects of cigarette smoke.* PloS one, 2010. **5**(1): p. e8660.
482. Kim, J., C.H. Lee, C.S. Park, B.G. Kim, S.W. Kim and J.H. Cho, *Plasma levels of MCP-1 and adiponectin in obstructive sleep apnea syndrome.* Archives of otolaryngology--head & neck surgery, 2010. **136**(9): p. 896-9.
483. Han, S., A.L. Jeong, S. Lee, J.S. Park, K.D. Kim, I. Choi, S.R. Yoon, M.S. Lee, J.S. Lim, S.H. Han, Y. Yoon do and Y. Yang, *Adiponectin deficiency suppresses lymphoma growth in mice by modulating NK cells, CD8 T cells, and myeloid-derived suppressor cells.* Journal of immunology, 2013. **190**(9): p. 4877-86.
484. Yokota, T., C.S. Meka, T. Kouro, K.L. Medina, H. Igarashi, M. Takahashi, K. Oritani, T. Funahashi, Y. Tomiyama, Y. Matsuzawa and P.W. Kincade, *Adiponectin, a fat cell product, influences the earliest lymphocyte precursors in bone marrow cultures by activation of the cyclooxygenase-prostaglandin pathway in stromal cells.* Journal of immunology, 2003. **171**(10): p. 5091-9.
485. Tsang, J.Y., D. Li, D. Ho, J. Peng, A. Xu, J. Lamb, Y. Chen and P.K. Tam, *Novel immunomodulatory effects of adiponectin on dendritic cell functions.* International immunopharmacology, 2011. **11**(5): p. 604-9.
486. Palmer, C., T. Hampartzoumian, A. Lloyd and A. Zekry, *A novel role for adiponectin in regulating the immune responses in chronic hepatitis C virus infection.* Hepatology, 2008. **48**(2): p. 374-84.
487. Naot, D., G.A. Williams, J.M. Lin, J. Cornish and A. Grey, *Evidence that contamination by lipopolysaccharide confounds in vitro studies of adiponectin activity in bone.* Endocrinology, 2012. **153**(5): p. 2076-81.

488. Nishihara, T., M. Matsuda, H. Araki, K. Oshima, S. Kihara, T. Funahashi and I. Shimomura, *Effect of adiponectin on murine colitis induced by dextran sulfate sodium.* Gastroenterology, 2006. **131**(3): p. 853-61.
489. Konter, J.M., J.L. Parker, E. Baez, S.Z. Li, B. Ranscht, M. Denzel, F.F. Little, K. Nakamura, N. Ouchi, A. Fine, K. Walsh and R.S. Summer, *Adiponectin attenuates lipopolysaccharide-induced acute lung injury through suppression of endothelial cell activation.* Journal of immunology, 2012. **188**(2): p. 854-63.
490. Ohashi, K., S. Kihara, N. Ouchi, M. Kumada, K. Fujita, A. Hiuge, T. Hibuse, M. Ryo, H. Nishizawa, N. Maeda, K. Maeda, R. Shibata, K. Walsh, T. Funahashi and I. Shimomura, *Adiponectin replenishment ameliorates obesity-related hypertension.* Hypertension, 2006. **47**(6): p. 1108-16.
491. Adamczak, M., A. Wiecek, T. Funahashi, J. Chudek, F. Kokot and Y. Matsuzawa, *Decreased plasma adiponectin concentration in patients with essential hypertension.* American journal of hypertension, 2003. **16**(1): p. 72-5.
492. Hajri, T., H. Tao, J. Wattacheril, P. Marks-Shulman and N.N. Abumrad, *Regulation of adiponectin production by insulin: interactions with tumor necrosis factor-alpha and interleukin-6.* American journal of physiology. Endocrinology and metabolism, 2011. **300**(2): p. E350-60.
493. Ouchi, N., S. Kihara, T. Funahashi, T. Nakamura, M. Nishida, M. Kumada, Y. Okamoto, K. Ohashi, H. Nagaretani, K. Kishida, H. Nishizawa, N. Maeda, H. Kobayashi, H. Hiraoka and Y. Matsuzawa, *Reciprocal association of C-reactive protein with adiponectin in blood stream and adipose tissue.* Circulation, 2003. **107**(5): p. 671-4.
494. Engeli, S., M. Feldpausch, K. Gorzelniak, F. Hartwig, U. Heintze, J. Janke, M. Mohlig, A.F. Pfeiffer, F.C. Luft and A.M. Sharma, *Association between adiponectin and mediators of inflammation in obese women.* Diabetes, 2003. **52**(4): p. 942-7.
495. Wang, B., J.R. Jenkins and P. Trayhurn, *Expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture: integrated response to TNF-alpha.* American journal of physiology. Endocrinology and metabolism, 2005. **288**(4): p. E731-40.
496. Devaraj, S., N. Torok, M.R. Dasu, D. Samols and I. Jialal, *Adiponectin decreases C-reactive protein synthesis and secretion from endothelial cells: evidence for an adipose tissue-vascular loop.* Arteriosclerosis, thrombosis, and vascular biology, 2008. **28**(7): p. 1368-74.
497. Toussirot, E., B. Gaugler, M. Bouhaddi, N.U. Nguyen, P. Saas and G. Dumoulin, *Elevated adiponectin serum levels in women with systemic autoimmune diseases.* Mediators of inflammation, 2010. **2010**: p. 938408.
498. Rovin, B.H., H. Song, L.A. Hebert, T. Nadasdy, G. Nadasdy, D.J. Birmingham, C. Yung Yu and H.N. Nagaraja, *Plasma, urine, and renal expression of adiponectin in human systemic lupus erythematosus.* Kidney international, 2005. **68**(4): p. 1825-33.
499. Ozgen, M., S.S. Koca, N. Dagli, M. Balin, B. Ustundag and A. Isik, *Serum adiponectin and vaspin levels in rheumatoid arthritis.* Archives of medical research, 2010. **41**(6): p. 457-63.
500. Ebina, K., A. Fukuhara, W. Ando, M. Hirao, T. Koga, K. Oshima, M. Matsuda, K. Maeda, T. Nakamura, T. Ochi, I. Shimomura, H. Yoshikawa and J. Hashimoto, *Serum adiponectin concentrations correlate with severity of rheumatoid arthritis evaluated by extent of joint destruction.* Clinical rheumatology, 2009. **28**(4): p. 445-51.
501. Chan, K.H., S.C. Yeung, T.J. Yao, M.S. Ip, A.H. Cheung, M.M. Chan-Yeung and J.C. Mak, *Elevated plasma adiponectin levels in patients with chronic obstructive pulmonary disease.* The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease, 2010. **14**(9): p. 1193-200.

502. Aprahamian, T.R. and F. Sam, *Adiponectin in cardiovascular inflammation and obesity.* International journal of inflammation, 2011. **2011**: p. 376909.
503. Ehling, A., A. Schaffler, H. Herfarth, I.H. Tarner, S. Anders, O. Distler, G. Paul, J. Distler, S. Gay, J. Scholmerich, E. Neumann and U. Muller-Ladner, *The potential of adiponectin in driving arthritis.* Journal of immunology, 2006. **176**(7): p. 4468-78.
504. Balcombe, N.R. and A. Sinclair, *Ageing: definitions, mechanisms and the magnitude of the problem.* Best practice & research. Clinical gastroenterology, 2001. **15**(6): p. 835-49.
505. Gavazzi, G. and K.H. Krause, *Ageing and infection.* The Lancet infectious diseases, 2002. **2**(11): p. 659-66.
506. Berger, N.A., P. Savvides, S.M. Koroukian, E.F. Kahana, G.T. Deimling, J.H. Rose, K.F. Bowman and R.H. Miller, *Cancer in the elderly.* Transactions of the American Clinical and Climatological Association, 2006. **117**: p. 147-55; discussion 155-6.
507. Franceschi, C., *Inflammaging as a major characteristic of old people: can it be prevented or cured?* Nutrition reviews, 2007. **65**(12 Pt 2): p. S173-6.
508. Johnson, S.A. and J.C. Cambier, *Ageing, autoimmunity and arthritis: senescence of the B cell compartment - implications for humoral immunity.* Arthritis research & therapy, 2004. **6**(4): p. 131-9.
509. Goronzy, J.J. and C.M. Weyand, *Immune aging and autoimmunity.* Cellular and molecular life sciences : CMLS, 2012. **69**(10): p. 1615-23.
510. Dew, M.A., C.C. Hoch, D.J. Buysse, T.H. Monk, A.E. Begley, P.R. Houck, M. Hall, D.J. Kupfer and C.F. Reynolds, 3rd, *Healthy older adults' sleep predicts all-cause mortality at 4 to 19 years of follow-up.* Psychosomatic medicine, 2003. **65**(1): p. 63-73.
511. Foley, D.J., A.A. Monjan, S.L. Brown, E.M. Simonsick, R.B. Wallace and D.G. Blazer, *Sleep complaints among elderly persons: an epidemiologic study of three communities.* Sleep, 1995. **18**(6): p. 425-32.
512. Ohayon, M.M., M.A. Carskadon, C. Guilleminault and M.V. Vitiello, *Meta-analysis of quantitative sleep parameters from childhood to old age in healthy individuals: developing normative sleep values across the human lifespan.* Sleep, 2004. **27**(7): p. 1255-73.
513. Van Cauter, E., R. Leproult and L. Plat, *Age-related changes in slow wave sleep and REM sleep and relationship with growth hormone and cortisol levels in healthy men.* JAMA : the journal of the American Medical Association, 2000. **284**(7): p. 861-8.
514. Neikrug, A.B. and S. Ancoli-Israel, *Sleep disorders in the older adult - a mini-review.* Gerontology, 2010. **56**(2): p. 181-9.
515. Yoon, I.Y., D.F. Kripke, S.D. Youngstedt and J.A. Elliott, *Actigraphy suggests age-related differences in napping and nocturnal sleep.* Journal of sleep research, 2003. **12**(2): p. 87-93.
516. Huang, Y.L., R.Y. Liu, Q.S. Wang, E.J. Van Someren, H. Xu and J.N. Zhou, *Age-associated difference in circadian sleep-wake and rest-activity rhythms.* Physiology & behavior, 2002. **76**(4-5): p. 597-603.
517. Bliwise, D.L., F.P. Ansari, L.B. Straight and K.P. Parker, *Age changes in timing and 24-hour distribution of self-reported sleep.* The American journal of geriatric psychiatry : official journal of the American Association for Geriatric Psychiatry, 2005. **13**(12): p. 1077-82.
518. Haimov, I. and P. Lavie, *Circadian characteristics of sleep propensity function in healthy elderly: a comparison with young adults.* Sleep, 1997. **20**(4): p. 294-300.
519. Buysse, D.J., T.H. Monk, J. Carrier and A. Begley, *Circadian patterns of sleep, sleepiness, and performance in older and younger adults.* Sleep, 2005. **28**(11): p. 1365-76.

520. Vaz Fragoso, C.A. and T.M. Gill, *Sleep complaints in community-living older persons: a multifactorial geriatric syndrome.* Journal of the American Geriatrics Society, 2007. **55**(11): p. 1853-66.
521. Buysse, D.J., K.E. Browman, T.H. Monk, C.F. Reynolds, 3rd, A.L. Fasiczka and D.J. Kupfer, *Napping and 24-hour sleep/wake patterns in healthy elderly and young adults.* Journal of the American Geriatrics Society, 1992. **40**(8): p. 779-86.
522. Foley, D.J., M.V. Vitiello, D.L. Bliwise, S. Ancoli-Israel, A.A. Monjan and J.K. Walsh, *Frequent napping is associated with excessive daytime sleepiness, depression, pain, and nocturia in older adults: findings from the National Sleep Foundation '2003 Sleep in America' Poll.* The American journal of geriatric psychiatry : official journal of the American Association for Geriatric Psychiatry, 2007. **15**(4): p. 344-50.
523. Finkelstein, J.W., H.P. Roffwarg, R.M. Boyar, J. Kream and L. Hellman, *Age-related change in the twenty-four-hour spontaneous secretion of growth hormone.* The Journal of clinical endocrinology and metabolism, 1972. **35**(5): p. 665-70.
524. Zadik, Z., S.A. Chalew, R.J. McCarter, Jr., M. Meistas and A.A. Kowarski, *The influence of age on the 24-hour integrated concentration of growth hormone in normal individuals.* The Journal of clinical endocrinology and metabolism, 1985. **60**(3): p. 513-6.
525. Van Cauter, E., R. Leproult and D.J. Kupfer, *Effects of gender and age on the levels and circadian rhythmicity of plasma cortisol.* The Journal of clinical endocrinology and metabolism, 1996. **81**(7): p. 2468-73.
526. Giordano, R., M. Bo, M. Pellegrino, M. Vezzari, M. Baldi, A. Picu, M. Balbo, L. Bonelli, G. Migliaretti, E. Ghigo and E. Arvat, *Hypothalamus-pituitary-adrenal hyperactivity in human aging is partially refractory to stimulation by mineralocorticoid receptor blockade.* The Journal of clinical endocrinology and metabolism, 2005. **90**(10): p. 5656-62.
527. Dodt, C., K.J. Theine, D. Uthgenannt, J. Born and H.L. Fehm, *Basal secretory activity of the hypothalamo-pituitary-adrenocortical axis is enhanced in healthy elderly. An assessment during undisturbed night-time sleep.* European journal of endocrinology / European Federation of Endocrine Societies, 1994. **131**(5): p. 443-50.
528. Maggio, M., E. Colizzi, A. Fisichella, G. Valenti, G. Ceresini, E. Dall'Aglio, L. Ruffini, F. Lauretani, L. Parrino and G.P. Ceda, *Stress hormones, sleep deprivation and cognition in older adults.* Maturitas, 2013. **76**(1): p. 22-44.
529. Antonijevic, I.A., H. Murck, R.M. Frieboes, J. Barthelmes and A. Steiger, *Sexually dimorphic effects of GHRH on sleep-endocrine activity in patients with depression and normal controls - part I: the sleep eeg.* Sleep research online : SRO, 2000. **3**(1): p. 5-13.
530. Antonijevic, I.A., H. Murck, R.M. Frieboes and A. Steiger, *Sexually dimorphic effects of GHRH on sleep-endocrine activity in patients with depression and normal controls - part II: hormone secretion.* Sleep research online : SRO, 2000. **3**(1): p. 15-21.
531. Prinz, P., S. Bailey, K. Moe, C. Wilkinson and J. Scanlan, *Urinary free cortisol and sleep under baseline and stressed conditions in healthy senior women: effects of estrogen replacement therapy.* Journal of sleep research, 2001. **10**(1): p. 19-26.
532. Prinz, P.N., S.L. Bailey and D.L. Woods, *Sleep impairments in healthy seniors: roles of stress, cortisol, and interleukin-1 beta.* Chronobiology international, 2000. **17**(3): p. 391-404.
533. Pawlikowski, M., M. Kolomecka, A. Wojtczak and M. Karasek, *Effects of six months melatonin treatment on sleep quality and serum concentrations of estradiol, cortisol, dehydroepiandrosterone sulfate, and somatomedin C in elderly women.* Neuro endocrinology letters, 2002. **23 Suppl 1**: p. 17-9.
534. Friess, E., T. Schiffelholz, T. Steckler and A. Steiger, *Dehydroepiandrosterone--a neurosteroid.* European journal of clinical investigation, 2000. **30 Suppl 3**: p. 46-50.

535. Baylis, D., D.B. Bartlett, H.E. Syddall, G. Ntani, C.R. Gale, C. Cooper, J.M. Lord and A.A. Sayer, *Immune-endocrine biomarkers as predictors of frailty and mortality: a 10-year longitudinal study in community-dwelling older people.* Age, 2013. **35**(3): p. 963-71.
536. Hazeldine, J., W. Arlt and J.M. Lord, *Dehydroepiandrosterone as a regulator of immune cell function.* The Journal of steroid biochemistry and molecular biology, 2010. **120**(2-3): p. 127-36.
537. Buford, T.W. and D.S. Willoughby, *Impact of DHEA(S) and cortisol on immune function in aging: a brief review.* Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme, 2008. **33**(3): p. 429-33.
538. Friess, E., L. Trachsel, J. Guldner, T. Schier, A. Steiger and F. Holsboer, *DHEA administration increases rapid eye movement sleep and EEG power in the sigma frequency range.* The American journal of physiology, 1995. **268**(1 Pt 1): p. E107-13.
539. Caufriez, A., R. Leproult, M. L'Hermite-Baleriaux, M. Kerkhofs and G. Copinschi, *Effects of a 3-week dehydroepiandrosterone administration on sleep, sex steroids and multiple 24-h hormonal profiles in postmenopausal women: a pilot study.* Clinical endocrinology, 2013.
540. Dicarlo, A.L., R. Fuldner, J. Kaminski and R. Hodes, *Aging in the context of immunological architecture, function and disease outcomes.* Trends in immunology, 2009. **30**(7): p. 293-4.
541. Emori, T.G., S.N. Banerjee, D.H. Culver, R.P. Gaynes, T.C. Horan, J.R. Edwards, W.R. Jarvis, J.S. Tolson, T.S. Henderson, W.J. Martone and et al., *Nosocomial infections in elderly patients in the United States, 1986-1990. National Nosocomial Infections Surveillance System.* Am J Med, 1991. **91**(3B): p. 289S-293S.
542. Lindstrom, T.M. and W.H. Robinson, *Rheumatoid arthritis: a role for immunosenescence?* Journal of the American Geriatrics Society, 2010. **58**(8): p. 1565-75.
543. Pawelec, G., E. Derhovanessian and A. Larbi, *Immunosenescence and cancer.* Critical reviews in oncology/hematology, 2010. **75**(2): p. 165-72.
544. Fulop, T., R. Kotb, C.F. Fortin, G. Pawelec, F. de Angelis and A. Larbi, *Potential role of immunosenescence in cancer development.* Annals of the New York Academy of Sciences, 2010. **1197**: p. 158-65.
545. Chen, W.H., B.F. Kozlovsky, R.B. Effros, B. Grubeck-Loebenstein, R. Edelman and M.B. Sztein, *Vaccination in the elderly: an immunological perspective.* Trends in immunology, 2009. **30**(7): p. 351-9.
546. Saurwein-Teissl, M., T.L. Lung, F. Marx, C. Gschosser, E. Asch, I. Blasko, W. Parson, G. Bock, D. Schonitzer, E. Trannoy and B. Grubeck-Loebenstein, *Lack of antibody production following immunization in old age: association with CD8(+)CD28(-) T cell clonal expansions and an imbalance in the production of Th1 and Th2 cytokines.* Journal of immunology, 2002. **168**(11): p. 5893-9.
547. Pang, W.W., E.A. Price, D. Sahoo, I. Beerman, W.J. Maloney, D.J. Rossi, S.L. Schrier and I.L. Weissman, *Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(50): p. 20012-7.
548. Geiger, H., G. de Haan and M.C. Florian, *The ageing haematopoietic stem cell compartment.* Nature reviews. Immunology, 2013. **13**(5): p. 376-89.
549. Chambers, S.M., C.A. Shaw, C. Gatza, C.J. Fisk, L.A. Donehower and M.A. Goodell, *Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation.* PLoS biology, 2007. **5**(8): p. e201.
550. Lord, J.M., S. Butcher, V. Killampali, D. Lascelles and M. Salmon, *Neutrophil ageing and immunesenescence.* Mechanisms of ageing and development, 2001. **122**(14): p. 1521-35.

551. Tortorella, C., G. Piazzolla, F. Spaccavento, S. Pece, E. Jirillo and S. Antonaci, *Spontaneous and Fas-induced apoptotic cell death in aged neutrophils.* Journal of clinical immunology, 1998. **18**(5): p. 321-9.
552. Wessels, I., J. Jansen, L. Rink and P. Uciechowski, *Immunosenescence of polymorphonuclear neutrophils.* TheScientificWorldJournal, 2010. **10**: p. 145-60.
553. Fulop, T., A. Larbi, N. Douziech, C. Fortin, K.P. Guerard, O. Lesur, A. Khalil and G. Dupuis, *Signal transduction and functional changes in neutrophils with aging.* Aging cell, 2004. **3**(4): p. 217-26.
554. Emanuelli, G., M. Lanzio, T. Anfossi, S. Romano, G. Anfossi and G. Calcamuggi, *Influence of age on polymorphonuclear leukocytes in vitro: phagocytic activity in healthy human subjects.* Gerontology, 1986. **32**(6): p. 308-16.
555. Mege, J.L., C. Capo, B. Michel, J.L. Gastaut and P. Bongrand, *Phagocytic cell function in aged subjects.* Neurobiology of aging, 1988. **9**(2): p. 217-20.
556. Butcher, S.K., H. Chahal, L. Nayak, A. Sinclair, N.V. Henriquez, E. Sapey, D. O'Mahony and J.M. Lord, *Senescence in innate immune responses: reduced neutrophil phagocytic capacity and CD16 expression in elderly humans.* Journal of leukocyte biology, 2001. **70**(6): p. 881-6.
557. Wenisch, C., S. Patruta, F. Daxbock, R. Krause and W. Horl, *Effect of age on human neutrophil function.* Journal of leukocyte biology, 2000. **67**(1): p. 40-5.
558. Tortorella, C., G. Piazzolla, F. Spaccavento, F. Vella, L. Pace and S. Antonaci, *Regulatory role of extracellular matrix proteins in neutrophil respiratory burst during aging.* Mechanisms of ageing and development, 2000. **119**(1-2): p. 69-82.
559. Panda, A., A. Arjona, E. Sapey, F. Bai, E. Fikrig, R.R. Montgomery, J.M. Lord and A.C. Shaw, *Human innate immunosenescence: causes and consequences for immunity in old age.* Trends in immunology, 2009. **30**(7): p. 325-33.
560. Esparza, B., H. Sanchez, M. Ruiz, M. Barranquero, E. Sabino and F. Merino, *Neutrophil function in elderly persons assessed by flow cytometry.* Immunological investigations, 1996. **25**(3): p. 185-90.
561. Alvarez, E., V. Ruiz-Gutierrez, F. Sobrino and C. Santa-Maria, *Age-related changes in membrane lipid composition, fluidity and respiratory burst in rat peritoneal neutrophils.* Clinical and experimental immunology, 2001. **124**(1): p. 95-102.
562. Shaw, A.C., S. Joshi, H. Greenwood, A. Panda and J.M. Lord, *Aging of the innate immune system.* Current opinion in immunology, 2010. **22**(4): p. 507-13.
563. Fulop, T., Jr., Z. Varga, J. Csongor, G. Foris and A. Leovey, *Age related impairment in phosphatidylinositol breakdown of polymorphonuclear granulocytes.* FEBS letters, 1989. **245**(1-2): p. 249-52.
564. Delpedro, A.D., M.J. Barjavel, Z. Mamdouh, S. Faure and O. Bakouche, *Signal transduction in LPS-activated aged and young monocytes.* Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research, 1998. **18**(6): p. 429-37.
565. Alvarez, E. and C. Santa Maria, *Influence of the age and sex on respiratory burst of human monocytes.* Mechanisms of ageing and development, 1996. **90**(2): p. 157-61.
566. Pinke, K.H., B. Calzavara, P.F. Faria, M.P. do Nascimento, J. Venturini and V.S. Lara, *Proinflammatory profile of in vitro monocytes in the ageing is affected by lymphocytes presence.* Immunity & ageing : I & A, 2013. **10**(1): p. 22.
567. Ogawa, T., M. Kitagawa and K. Hirokawa, *Age-related changes of human bone marrow: a histometric estimation of proliferative cells, apoptotic cells, T cells, B cells and macrophages.* Mechanisms of ageing and development, 2000. **117**(1-3): p. 57-68.
568. Plowden, J., M. Renshaw-Hoelscher, C. Engleman, J. Katz and S. Sambhara, *Innate immunity in aging: impact on macrophage function.* Aging cell, 2004. **3**(4): p. 161-7.
569. Boehmer, E.D., J. Goral, D.E. Faunce and E.J. Kovacs, *Age-dependent decrease in Toll-like receptor 4-mediated proinflammatory cytokine production and mitogen-*

activated protein kinase expression. Journal of leukocyte biology, 2004. **75**(2): p. 342-9.
570. Chelvarajan, R.L., S.M. Collins, J.M. Van Willigen and S. Bondada, *The unresponsiveness of aged mice to polysaccharide antigens is a result of a defect in macrophage function.* Journal of leukocyte biology, 2005. **77**(4): p. 503-12.
571. Borrego, F., M.C. Alonso, M.D. Galiani, J. Carracedo, R. Ramirez, B. Ostos, J. Pena and R. Solana, *NK phenotypic markers and IL2 response in NK cells from elderly people.* Experimental gerontology, 1999. **34**(2): p. 253-65.
572. Krishnaraj, R., *Senescence and cytokines modulate the NK cell expression.* Mechanisms of ageing and development, 1997. **96**(1-3): p. 89-101.
573. Almeida-Oliveira, A., M. Smith-Carvalho, L.C. Porto, J. Cardoso-Oliveira, S. Ribeiro Ados, R.R. Falcao, E. Abdelhay, L.F. Bouzas, L.C. Thuler, M.H. Ornellas and H.R. Diamond, *Age-related changes in natural killer cell receptors from childhood through old age.* Human immunology, 2011. **72**(4): p. 319-29.
574. Krishnaraj, R. and T. Bhooma, *Cytokine sensitivity of human NK cells during immunosenescence. 2. IL2-induced interferon gamma secretion.* Immunology letters, 1996. **50**(1-2): p. 59-63.
575. Facchini, A., E. Mariani, A.R. Mariani, S. Papa, M. Vitale and F.A. Manzoli, *Increased number of circulating Leu 11+ (CD 16) large granular lymphocytes and decreased NK activity during human ageing.* Clinical and experimental immunology, 1987. **68**(2): p. 340-7.
576. Mariani, E., P. Roda, A.R. Mariani, M. Vitale, A. Degrassi, S. Papa and A. Facchini, *Age-associated changes in CD8+ and CD16+ cell reactivity: clonal analysis.* Clinical and experimental immunology, 1990. **81**(3): p. 479-84.
577. Hazeldine, J., P. Hampson and J.M. Lord, *Reduced release and binding of perforin at the immunological synapse underlies the age-related decline in natural killer cell cytotoxicity.* Aging cell, 2012. **11**(5): p. 751-9.
578. Varas, A., R. Sacedon, C. Hernandez-Lopez, E. Jimenez, J. Garcia-Ceca, J. Arias-Diaz, A.G. Zapata and A. Vicente, *Age-dependent changes in thymic macrophages and dendritic cells.* Microscopy research and technique, 2003. **62**(6): p. 501-7.
579. Agrawal, A., S. Agrawal, J.N. Cao, H. Su, K. Osann and S. Gupta, *Altered innate immune functioning of dendritic cells in elderly humans: a role of phosphoinositide 3-kinase-signaling pathway.* Journal of immunology, 2007. **178**(11): p. 6912-22.
580. Agrawal, A. and S. Gupta, *Impact of aging on dendritic cell functions in humans.* Ageing research reviews, 2011. **10**(3): p. 336-45.
581. Lynch, H.E., G.L. Goldberg, A. Chidgey, M.R. Van den Brink, R. Boyd and G.D. Sempowski, *Thymic involution and immune reconstitution.* Trends in immunology, 2009. **30**(7): p. 366-73.
582. Agarwal, S. and P.J. Busse, *Innate and adaptive immunosenescence.* Ann Allergy Asthma Immunol, 2010. **104**(3): p. 183-90; quiz 190-2, 210.
583. Fagnoni, F.F., R. Vescovini, G. Passeri, G. Bologna, M. Pedrazzoni, G. Lavagetto, A. Casti, C. Franceschi, M. Passeri and P. Sansoni, *Shortage of circulating naive CD8(+) T cells provides new insights on immunodeficiency in aging.* Blood, 2000. **95**(9): p. 2860-8.
584. Khan, N., N. Shariff, M. Cobbold, R. Bruton, J.A. Ainsworth, A.J. Sinclair, L. Nayak and P.A. Moss, *Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals.* Journal of immunology, 2002. **169**(4): p. 1984-92.
585. Pawelec, G., A. Akbar, C. Caruso, R. Effros, B. Grubeck-Loebenstein and A. Wikby, *Is immunosenescence infectious?* Trends in immunology, 2004. **25**(8): p. 406-10.
586. Olsson, J., A. Wikby, B. Johansson, S. Lofgren, B.O. Nilsson and F.G. Ferguson, *Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus*

infection in the very old: the Swedish longitudinal OCTO immune study. Mechanisms of ageing and development, 2000. **121**(1-3): p. 187-201.
587. Wikby, A., B. Johansson, J. Olsson, S. Lofgren, B.O. Nilsson and F. Ferguson, *Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study.* Experimental gerontology, 2002. **37**(2-3): p. 445-53.
588. Wikby, A., P. Maxson, J. Olsson, B. Johansson and F.G. Ferguson, *Changes in CD8 and CD4 lymphocyte subsets, T cell proliferation responses and non-survival in the very old: the Swedish longitudinal OCTO-immune study.* Mechanisms of ageing and development, 1998. **102**(2-3): p. 187-98.
589. Rink, L., I. Cakman and H. Kirchner, *Altered cytokine production in the elderly.* Mechanisms of ageing and development, 1998. **102**(2-3): p. 199-209.
590. Haynes, L. and A.C. Maue, *Effects of aging on T cell function.* Current Opinion in Immunology, 2009. **21**(4): p. 414-7.
591. Wood, K.L., H.L. Twigg, 3rd and A.I. Doseff, *Dysregulation of CD8+ lymphocyte apoptosis, chronic disease, and immune regulation.* Frontiers in bioscience, 2009. **14**: p. 3771-81.
592. Alonso-Arias, R., M.A. Moro-Garcia, A. Lopez-Vazquez, L. Rodrigo, J. Baltar, F.M. Garcia, J.J. Jaurrieta and C. Lopez-Larrea, *NKG2D expression in CD4+ T lymphocytes as a marker of senescence in the aged immune system.* Age, 2011. **33**(4): p. 591-605.
593. Breitbart, E., X. Wang, L.S. Leka, G.E. Dallal, S.N. Meydani and B.D. Stollar, *Altered memory B-cell homeostasis in human aging.* The journals of gerontology. Series A, Biological sciences and medical sciences, 2002. **57**(8): p. B304-11.
594. Ademokun, A., Y.C. Wu and D. Dunn-Walters, *The ageing B cell population: composition and function.* Biogerontology, 2010. **11**(2): p. 125-37.
595. Frasca, D., R.L. Riley and B.B. Blomberg, *Aging murine B cells have decreased class switch induced by anti-CD40 or BAFF*. Experimental gerontology, 2007. **42**(3): p. 192-203.
596. Frasca, D., A.M. Landin, S.C. Lechner, J.G. Ryan, R. Schwartz, R.L. Riley and B.B. Blomberg, *Aging down-regulates the transcription factor E2A, activation-induced cytidine deaminase, and Ig class switch in human B cells.* Journal of immunology, 2008. **180**(8): p. 5283-90.
597. Paganelli, R., I. Quinti, U. Fagiolo, A. Cossarizza, C. Ortolani, E. Guerra, P. Sansoni, L.P. Pucillo, E. Scala, E. Cozzi and et al., *Changes in circulating B cells and immunoglobulin classes and subclasses in a healthy aged population.* Clinical and experimental immunology, 1992. **90**(2): p. 351-4.
598. Murasko, D.M., E.D. Bernstein, E.M. Gardner, P. Gross, G. Munk, S. Dran and E. Abrutyn, *Role of humoral and cell-mediated immunity in protection from influenza disease after immunization of healthy elderly.* Experimental gerontology, 2002. **37**(2-3): p. 427-39.
599. Aydar, Y., P. Balogh, J.G. Tew and A.K. Szakal, *Altered regulation of Fc gamma RII on aged follicular dendritic cells correlates with immunoreceptor tyrosine-based inhibition motif signaling in B cells and reduced germinal center formation.* Journal of immunology, 2003. **171**(11): p. 5975-87.
600. Franceschi, C., M. Capri, D. Monti, S. Giunta, F. Olivieri, F. Sevini, M.P. Panourgia, L. Invidia, L. Celani, M. Scurti, E. Cevenini, G.C. Castellani and S. Salvioli, *Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans.* Mechanisms of ageing and development, 2007. **128**(1): p. 92-105.
601. Fagiolo, U., A. Cossarizza, E. Scala, E. Fanales-Belasio, C. Ortolani, E. Cozzi, D. Monti, C. Franceschi and R. Paganelli, *Increased cytokine production in mononuclear*

cells of healthy elderly people. European journal of immunology, 1993. **23**(9): p. 2375-8.
602. Giunta, S., *Exploring the complex relations between inflammation and aging (inflamm-aging): anti-inflamm-aging remodelling of inflamm- aging, from robustness to frailty.* Inflammation research : official journal of the European Histamine Research Society ... [et al.], 2008. **57**(12): p. 558-63.
603. Butcher, S.K., V. Killampalli, D. Lascelles, K. Wang, E.K. Alpar and J.M. Lord, *Raised cortisol:DHEAS ratios in the elderly after injury: potential impact upon neutrophil function and immunity.* Aging cell, 2005. **4**(6): p. 319-24.
604. Chrousos, G.P., *Stressors, stress, and neuroendocrine integration of the adaptive response. The 1997 Hans Selye Memorial Lecture.* Annals of the New York Academy of Sciences, 1998. **851**: p. 311-35.
605. Flammer, J.R. and I. Rogatsky, *Minireview: Glucocorticoids in autoimmunity: unexpected targets and mechanisms.* Molecular endocrinology, 2011. **25**(7): p. 1075-86.
606. Besedovsky, H., E. Sorkin, D. Felix and H. Haas, *Hypothalamic changes during the immune response.* European journal of immunology, 1977. **7**(5): p. 323-5.
607. Turnbull, A.V. and C.L. Rivier, *Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action.* Physiological reviews, 1999. **79**(1): p. 1-71.
608. Rosenbaum, M., M. Nicolson, J. Hirsch, S.B. Heymsfield, D. Gallagher, F. Chu and R.L. Leibel, *Effects of gender, body composition, and menopause on plasma concentrations of leptin.* The Journal of clinical endocrinology and metabolism, 1996. **81**(9): p. 3424-7.
609. Cicero, A.F., P. Magni, P. Lentini, M. Ruscica, E. Dozio, F. Strollo and C. Borghi, *Sex hormones and adipokines in healthy pre-menopausal, post-menopausal and elderly women, and in age-matched men: data from the Brisighella Heart study.* Journal of endocrinological investigation, 2011. **34**(7): p. e158-62.
610. Isidori, A.M., F. Strollo, M. More, M. Caprio, A. Aversa, C. Moretti, G. Frajese, G. Riondino and A. Fabbri, *Leptin and aging: correlation with endocrine changes in male and female healthy adult populations of different body weights.* The Journal of clinical endocrinology and metabolism, 2000. **85**(5): p. 1954-62.
611. Cnop, M., P.J. Havel, K.M. Utzschneider, D.B. Carr, M.K. Sinha, E.J. Boyko, B.M. Retzlaff, R.H. Knopp, J.D. Brunzell and S.E. Kahn, *Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex.* Diabetologia, 2003. **46**(4): p. 459-69.
612. Koh, S.J., Y.J. Hyun, S.Y. Choi, J.S. Chae, J.Y. Kim, S. Park, C.M. Ahn, Y. Jang and J.H. Lee, *Influence of age and visceral fat area on plasma adiponectin concentrations in women with normal glucose tolerance.* Clinica chimica acta; international journal of clinical chemistry, 2008. **389**(1-2): p. 45-50.
613. Vilarrasa, N., J. Vendrell, J. Maravall, M. Broch, A. Estepa, A. Megia, J. Soler, I. Simon, C. Richart and J.M. Gomez, *Distribution and determinants of adiponectin, resistin and ghrelin in a randomly selected healthy population.* Clinical endocrinology, 2005. **63**(3): p. 329-35.
614. Godoy-Ramirez, K., K. Franck and H. Gaines, *A novel method for the simultaneous assessment of natural killer cell conjugate formation and cytotoxicity at the single-cell level by multi-parameter flow cytometry.* Journal of immunological methods, 2000. **239**(1-2): p. 35-44.
615. Jepsen, L.V. and T. Skottun, *A rapid one-step method for the isolation of human granulocytes from whole blood.* Scandinavian journal of clinical and laboratory investigation, 1982. **42**(3): p. 235-8.

616. Gyllenhammar, H., *Lucigenin chemiluminescence in the assessment of neutrophil superoxide production.* Journal of immunological methods, 1987. **97**(2): p. 209-13.
617. Dive, C., C.D. Gregory, D.J. Phipps, D.L. Evans, A.E. Milner and A.H. Wyllie, *Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry.* Biochimica et biophysica acta, 1992. **1133**(3): p. 275-85.
618. Martin, J.L., L. Fiorentino, S. Jouldjian, M. Mitchell, K.R. Josephson and C.A. Alessi, *Poor self-reported sleep quality predicts mortality within one year of inpatient post-acute rehabilitation among older adults.* Sleep, 2011. **34**(12): p. 1715-21.
619. Van Cauter, E., L. Plat, R. Leproult and G. Copinschi, *Alterations of circadian rhythmicity and sleep in aging: endocrine consequences.* Hormone research, 1998. **49**(3-4): p. 147-52.
620. Yoon, I.Y., D.F. Kripke, J.A. Elliott, S.D. Youngstedt, K.M. Rex and R.L. Hauger, *Age-related changes of circadian rhythms and sleep-wake cycles.* Journal of the American Geriatrics Society, 2003. **51**(8): p. 1085-91.
621. Malaguarnera, L., E. Cristaldi and M. Malaguarnera, *The role of immunity in elderly cancer.* Critical reviews in oncology/hematology, 2010. **74**(1): p. 40-60.
622. Buyukkaya, E., M.F. Karakas, E. Karakas, A.B. Akcay, M. Kurt, I.H. Tanboga and N. Sen, *Correlation of Neutrophil to Lymphocyte Ratio With the Presence and Severity of Metabolic Syndrome.* Clinical and applied thrombosis/hemostasis : official journal of the International Academy of Clinical and Applied Thrombosis/Hemostasis, 2012.
623. Imtiaz, F., K. Shafique, S.S. Mirza, Z. Ayoob, P. Vart and S. Rao, *Neutrophil lymphocyte ratio as a measure of systemic inflammation in prevalent chronic diseases in Asian population.* International archives of medicine, 2012. **5**(1): p. 2.
624. Papa, A., M. Emdin, C. Passino, C. Michelassi, D. Battaglia and F. Cocci, *Predictive value of elevated neutrophil-lymphocyte ratio on cardiac mortality in patients with stable coronary artery disease.* Clinica chimica acta; international journal of clinical chemistry, 2008. **395**(1-2): p. 27-31.
625. Tokgoz, S., M. Kayrak, Z. Akpinar, A. Seyithanoglu, F. Guney and B. Yuruten, *Neutrophil lymphocyte ratio as a predictor of stroke.* Journal of stroke and cerebrovascular diseases : the official journal of National Stroke Association, 2013. **22**(7): p. 1169-74.
626. Azab, B., N. Shah, J. Radbel, P. Tan, V. Bhatt, S. Vonfrolio, A. Habeshy, A. Picon and S. Bloom, *Pretreatment neutrophil/lymphocyte ratio is superior to platelet/lymphocyte ratio as a predictor of long-term mortality in breast cancer patients.* Medical oncology, 2013. **30**(1): p. 432.
627. Kim, H.O., H.S. Kim, J.C. Youn, E.C. Shin and S. Park, *Serum cytokine profiles in healthy young and elderly population assessed using multiplexed bead-based immunoassays.* Journal of translational medicine, 2011. **9**: p. 113.
628. Tavakkol Afshari, J., R. Farid Hosseini, S. Hosseini Farahabadi, F. Heydarian, M.H. Boskabady, R. Khoshnavaz, A. Razavi, E. Ghayoor Karimiani and G. Ghasemi, *Association of the expression of IL-4 and IL-13 genes, IL-4 and IgE serum levels with allergic asthma.* Iranian journal of allergy, asthma, and immunology, 2007. **6**(2): p. 67-72.
629. Sapey, E., H. Greenwood, G. Walton, E. Mann, A. Love, N. Aaronson, R.H. Insall, R.A. Stockley and J.M. Lord, *Phosphoinositide 3-kinase inhibition restores neutrophil accuracy in the elderly: toward targeted treatments for immunosenescence.* Blood, 2014. **123**(2): p. 239-48.
630. Carskadon, M.A., E.D. Brown and W.C. Dement, *Sleep fragmentation in the elderly: relationship to daytime sleep tendency.* Neurobiology of aging, 1982. **3**(4): p. 321-7.
631. Van Den Berg, J.F., F.J. Van Rooij, H. Vos, J.H. Tulen, A. Hofman, H.M. Miedema, A.K. Neven and H. Tiemeier, *Disagreement between subjective and actigraphic*

measures of sleep duration in a population-based study of elderly persons. Journal of sleep research, 2008. **17**(3): p. 295-302.
632. Adachi, K., K. Nishijo and T. Abo, *Those with the habit of going to sleep early show a higher ratio of lymphocytes while those with the habit of staying up late show a higher ratio of granulocytes.* Biomed Res, 2010. **31**(2): p. 143-9.
633. Grzybowski, M., R.D. Welch, L. Parsons, C.E. Ndumele, E. Chen, R. Zalenski and H.V. Barron, *The association between white blood cell count and acute myocardial infarction in-hospital mortality: findings from the National Registry of Myocardial Infarction.* Acad Emerg Med, 2004. **11**(10): p. 1049-60.
634. Grau, A.J., A.W. Boddy, D.A. Dukovic, F. Buggle, C. Lichy, T. Brandt and W. Hacke, *Leukocyte count as an independent predictor of recurrent ischemic events.* Stroke, 2004. **35**(5): p. 1147-52.
635. Babio, N., N. Ibarrola-Jurado, M. Bullo, M.A. Martinez-Gonzalez, J. Warnberg, I. Salaverria, M. Ortega-Calvo, R. Estruch, L. Serra-Majem, M.I. Covas, J.V. Sorli and J. Salas-Salvado, *White blood cell counts as risk markers of developing metabolic syndrome and its components in the PREDIMED study.* PloS one, 2013. **8**(3): p. e58354.
636. Azab, B., M.A. Shariff, R. Bachir, J.P. Nabagiez and J.T. McGinn, Jr., *Elevated preoperative neutrophil/lymphocyte ratio as a predictor of increased long-term survival in minimal invasive coronary artery bypass surgery compared to sternotomy.* J Cardiothorac Surg, 2013. **8**: p. 193.
637. Rinehart, J., L. Keville, S. Clayton and J.A. Figueroa, *Corticosteroids alter hematopoiesis in vitro by enhancing human monocyte secretion of granulocyte colony-stimulating factor.* Exp Hematol, 1997. **25**(5): p. 405-12.
638. Ferrie, J.E., M. Kivimaki, T.N. Akbaraly, A. Singh-Manoux, M.A. Miller, D. Gimeno, M. Kumari, G. Davey Smith and M.J. Shipley, *Associations between change in sleep duration and inflammation: findings on C-reactive protein and interleukin 6 in the Whitehall II Study.* Am J Epidemiol, 2013. **178**(6): p. 956-61.
639. Dowd, J.B., N. Goldman and M. Weinstein, *Sleep duration, sleep quality, and biomarkers of inflammation in a Taiwanese population.* Annals of epidemiology, 2011. **21**(11): p. 799-806.
640. Patel, S.R., X. Zhu, A. Storfer-Isser, R. Mehra, N.S. Jenny, R. Tracy and S. Redline, *Sleep duration and biomarkers of inflammation.* Sleep, 2009. **32**(2): p. 200-4.
641. Garcia-Garcia, F., H. Yoshida and J.M. Krueger, *Interleukin-8 promotes non-rapid eye movement sleep in rabbits and rats.* Journal of sleep research, 2004. **13**(1): p. 55-61.
642. Vgontzas, A.N., D.A. Papanicolaou, E.O. Bixler, A. Lotsikas, K. Zachman, A. Kales, P. Prolo, M.L. Wong, J. Licinio, P.W. Gold, R.C. Hermida, G. Mastorakos and G.P. Chrousos, *Circadian interleukin-6 secretion and quantity and depth of sleep.* The Journal of clinical endocrinology and metabolism, 1999. **84**(8): p. 2603-7.
643. Hong, S., P.J. Mills, J.S. Loredo, K.A. Adler and J.E. Dimsdale, *The association between interleukin-6, sleep, and demographic characteristics.* Brain, behavior, and immunity, 2005. **19**(2): p. 165-72.
644. Friedman, E.M., M.S. Hayney, G.D. Love, H.L. Urry, M.A. Rosenkranz, R.J. Davidson, B.H. Singer and C.D. Ryff, *Social relationships, sleep quality, and interleukin-6 in aging women.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(51): p. 18757-62.
645. Kharazmi, A., H. Nielsen, C. Rechnitzer and K. Bendtzen, *Interleukin 6 primes human neutrophil and monocyte oxidative burst response.* Immunology letters, 1989. **21**(2): p. 177-84.
646. Guichard, C., E. Pedruzzi, C. Dewas, M. Fay, C. Pouzet, M. Bens, A. Vandewalle, E. Ogier-Denis, M.A. Gougerot-Pocidalo and C. Elbim, *Interleukin-8-induced priming of neutrophil oxidative burst requires sequential recruitment of NADPH oxidase*

components into lipid rafts. The Journal of biological chemistry, 2005. **280**(44): p. 37021-32.
647. Orentreich, N., J.L. Brind, R.L. Rizer and J.H. Vogelman, *Age changes and sex differences in serum dehydroepiandrosterone sulfate concentrations throughout adulthood.* The Journal of clinical endocrinology and metabolism, 1984. **59**(3): p. 551-5.
648. Zhang, J., R.C. Ma, A.P. Kong, W.Y. So, A.M. Li, S.P. Lam, S.X. Li, M.W. Yu, C.S. Ho, M.H. Chan, B. Zhang and Y.K. Wing, *Relationship of sleep quantity and quality with 24-hour urinary catecholamines and salivary awakening cortisol in healthy middle-aged adults.* Sleep, 2011. **34**(2): p. 225-33.
649. Berg, A.H., T.P. Combs and P.E. Scherer, *ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism.* Trends in endocrinology and metabolism: TEM, 2002. **13**(2): p. 84-9.
650. Han, S.H., I. Sakuma, E.K. Shin and K.K. Koh, *Antiatherosclerotic and anti-insulin resistance effects of adiponectin: basic and clinical studies.* Progress in cardiovascular diseases, 2009. **52**(2): p. 126-40.
651. Masaki, T., S. Chiba, H. Tatsukawa, T. Yasuda, H. Noguchi, M. Seike and H. Yoshimatsu, *Adiponectin protects LPS-induced liver injury through modulation of TNF-alpha in KK-Ay obese mice.* Hepatology, 2004. **40**(1): p. 177-84.
652. Jung, M.Y., H.S. Kim, H.J. Hong, B.S. Youn and T.S. Kim, *Adiponectin induces dendritic cell activation via PLCgamma/JNK/NF-kappaB pathways, leading to Th1 and Th17 polarization.* Journal of immunology, 2012. **188**(6): p. 2592-601.
653. Cong, L., J. Gasser, J. Zhao, B. Yang, F. Li and A.Z. Zhao, *Human adiponectin inhibits cell growth and induces apoptosis in human endometrial carcinoma cells, HEC-1-A and RL95 2.* Endocrine-related cancer, 2007. **14**(3): p. 713-20.
654. Dieudonne, M.N., M. Bussiere, E. Dos Santos, M.C. Leneveu, Y. Giudicelli and R. Pecquery, *Adiponectin mediates antiproliferative and apoptotic responses in human MCF7 breast cancer cells.* Biochemical and biophysical research communications, 2006. **345**(1): p. 271-9.
655. Brakenhielm, E., N. Veitonmaki, R. Cao, S. Kihara, Y. Matsuzawa, B. Zhivotovsky, T. Funahashi and Y. Cao, *Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(8): p. 2476-81.
656. Qiu, G., R. Wan, J. Hu, M.P. Mattson, E. Spangler, S. Liu, S.Y. Yau, T.M. Lee, M. Gleichmann, D.K. Ingram, K.F. So and S. Zou, *Adiponectin protects rat hippocampal neurons against excitotoxicity.* Age, 2011. **33**(2): p. 155-65.
657. Chandrasekar, B., W.H. Boylston, K. Venkatachalam, N.J. Webster, S.D. Prabhu and A.J. Valente, *Adiponectin blocks interleukin-18-mediated endothelial cell death via APPL1-dependent AMP-activated protein kinase (AMPK) activation and IKK/NF-kappaB/PTEN suppression.* The Journal of biological chemistry, 2008. **283**(36): p. 24889-98.
658. Adamczak, M., E. Rzepka, J. Chudek and A. Wiecek, *Ageing and plasma adiponectin concentration in apparently healthy males and females.* Clinical endocrinology, 2005. **62**(1): p. 114-8.
659. Derouet, M., L. Thomas, A. Cross, R.J. Moots and S.W. Edwards, *Granulocyte macrophage colony-stimulating factor signaling and proteasome inhibition delay neutrophil apoptosis by increasing the stability of Mcl-1.* The Journal of biological chemistry, 2004. **279**(26): p. 26915-21.
660. Yasui, K., Y. Sekiguchi, M. Ichikawa, H. Nagumo, T. Yamazaki, A. Komiyama and H. Suzuki, *Granulocyte macrophage-colony stimulating factor delays neutrophil*

apoptosis and primes its function through Ia-type phosphoinositide 3-kinase. Journal of leukocyte biology, 2002. **72**(5): p. 1020-6.
661. Corton, J.M., J.G. Gillespie and D.G. Hardie, *Role of the AMP-activated protein kinase in the cellular stress response.* Current biology : CB, 1994. **4**(4): p. 315-24.
662. Perrier, S. and T. Jarde, *Adiponectin, an anti-carcinogenic hormone? A systematic review on breast, colorectal, liver and prostate cancer.* Current medicinal chemistry, 2012. **19**(32): p. 5501-12.
663. Weisova, P., D. Davila, L.P. Tuffy, M.W. Ward, C.G. Concannon and J.H. Prehn, *Role of 5'-adenosine monophosphate-activated protein kinase in cell survival and death responses in neurons.* Antioxidants & redox signaling, 2011. **14**(10): p. 1863-76.
664. Erickson, K.A., M.E. Smith, T.S. Anthonymuthu, M.J. Evanson, E.S. Brassfield, A.E. Hodson, M.A. Bressler, B.J. Tucker, M.O. Thatcher, J.T. Prince, C.R. Hancock and B.T. Bikman, *AICAR inhibits ceramide biosynthesis in skeletal muscle.* Diabetology & metabolic syndrome, 2012. **4**(1): p. 45.
665. Jin, J., T.D. Mullen, Q. Hou, J. Bielawski, A. Bielawska, X. Zhang, L.M. Obeid, Y.A. Hannun and Y.T. Hsu, *AMPK inhibitor Compound C stimulates ceramide production and promotes Bax redistribution and apoptosis in MCF7 breast carcinoma cells.* Journal of lipid research, 2009. **50**(12): p. 2389-97.
666. Holland, W.L., R.A. Miller, Z.V. Wang, K. Sun, B.M. Barth, H.H. Bui, K.E. Davis, B.T. Bikman, N. Halberg, J.M. Rutkowski, M.R. Wade, V.M. Tenorio, M.S. Kuo, J.T. Brozinick, B.B. Zhang, M.J. Birnbaum, S.A. Summers and P.E. Scherer, *Receptor-mediated activation of ceramidase activity initiates the pleiotropic actions of adiponectin.* Nature medicine, 2011. **17**(1): p. 55-63.
667. Diamond, M.S. and T.A. Springer, *The dynamic regulation of integrin adhesiveness.* Current biology : CB, 1994. **4**(6): p. 506-17.
668. Herant, M., V. Heinrich and M. Dembo, *Mechanics of neutrophil phagocytosis: experiments and quantitative models.* J Cell Sci, 2006. **119**(Pt 9): p. 1903-13.
669. Ammer, A.G. and S.A. Weed, *Cortactin branches out: roles in regulating protrusive actin dynamics.* Cell Motil Cytoskeleton, 2008. **65**(9): p. 687-707.
670. Dillon, S.P., A. D'Souza, B.T. Kurien and R.H. Scofield, *Systemic lupus erythematosus and C1q: A quantitative ELISA for determining C1q levels in serum.* Biotechnol J, 2009. **4**(8): p. 1210-4.
671. Rossi, A. and J.M. Lord, *Adiponectin inhibits neutrophil apoptosis via activation of AMP kinase, PKB and ERK 1/2 MAP kinase.* Apoptosis, 2013. **18**(12): p. 1469-80.
672. Frasch, S.C., J.A. Nick, V.A. Fadok, D.L. Bratton, G.S. Worthen and P.M. Henson, *p38 mitogen-activated protein kinase-dependent and -independent intracellular signal transduction pathways leading to apoptosis in human neutrophils.* The Journal of biological chemistry, 1998. **273**(14): p. 8389-97.
673. Zou, M.H., X.Y. Hou, C.M. Shi, S. Kirkpatick, F. Liu, M.H. Goldman and R.A. Cohen, *Activation of 5'-AMP-activated kinase is mediated through c-Src and phosphoinositide 3-kinase activity during hypoxia-reoxygenation of bovine aortic endothelial cells. Role of peroxynitrite.* The Journal of biological chemistry, 2003. **278**(36): p. 34003-10.
674. Mizrachy-Schwartz, S., N. Cohen, S. Klein, N. Kravchenko-Balasha and A. Levitzki, *Up-regulation of AMP-activated protein kinase in cancer cell lines is mediated through c-Src activation.* The Journal of biological chemistry, 2011. **286**(17): p. 15268-77.
675. Rodriguez-Viciana, P., P.H. Warne, A. Khwaja, B.M. Marte, D. Pappin, P. Das, M.D. Waterfield, A. Ridley and J. Downward, *Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras.* Cell, 1997. **89**(3): p. 457-67.

676. Aikawa, R., T. Nagai, S. Kudoh, Y. Zou, M. Tanaka, M. Tamura, H. Akazawa, H. Takano, R. Nagai and I. Komuro, *Integrins play a critical role in mechanical stress-induced p38 MAPK activation.* Hypertension, 2002. **39**(2): p. 233-8.
677. Kane, L.P., V.S. Shapiro, D. Stokoe and A. Weiss, *Induction of NF-kappaB by the Akt/PKB kinase.* Current biology : CB, 1999. **9**(11): p. 601-4.
678. Jiang, B., S. Xu, X. Hou, D.R. Pimentel, P. Brecher and R.A. Cohen, *Temporal control of NF-kappaB activation by ERK differentially regulates interleukin-1beta-induced gene expression.* The Journal of biological chemistry, 2004. **279**(2): p. 1323-9.
679. Haugen, F. and C.A. Drevon, *Activation of nuclear factor-kappaB by high molecular weight and globular adiponectin.* Endocrinology, 2007. **148**(11): p. 5478-86.
680. Tomizawa, A., Y. Hattori, K. Kasai and Y. Nakano, *Adiponectin induces NF-kappaB activation that leads to suppression of cytokine-induced NF-kappaB activation in vascular endothelial cells: globular adiponectin vs. high molecular weight adiponectin.* Diabetes & vascular disease research : official journal of the International Society of Diabetes and Vascular Disease, 2008. **5**(2): p. 123-7.
681. Atallah, M., A. Krispin, U. Trahtemberg, S. Ben-Hamron, A. Grau, I. Verbovetski and D. Mevorach, *Constitutive neutrophil apoptosis: regulation by cell concentration via S100 A8/9 and the MEK-ERK pathway.* PloS one, 2012. **7**(2): p. e29333.
682. Mungai, P.T., G.B. Waypa, A. Jairaman, M. Prakriya, D. Dokic, M.K. Ball and P.T. Schumacker, *Hypoxia triggers AMPK activation through reactive oxygen species-mediated activation of calcium release-activated calcium channels.* Mol Cell Biol, 2011. **31**(17): p. 3531-45.
683. Alba, G., R. El Bekay, M. Alvarez-Maqueda, P. Chacon, A. Vega, J. Monteseirin, C. Santa Maria, E. Pintado, F.J. Bedoya, R. Bartrons and F. Sobrino, *Stimulators of AMP-activated protein kinase inhibit the respiratory burst in human neutrophils.* FEBS letters, 2004. **573**(1-3): p. 219-25.
684. Saito, S., A. Furuno, J. Sakurai, H.R. Park, K. Shin-ya and A. Tomida, *Compound C prevents the unfolded protein response during glucose deprivation through a mechanism independent of AMPK and BMP signaling.* PloS one, 2012. **7**(9): p. e45845.
685. Emerling, B.M., B. Viollet, K.V. Tormos and N.S. Chandel, *Compound C inhibits hypoxic activation of HIF-1 independent of AMPK.* FEBS letters, 2007. **581**(29): p. 5727-31.
686. Nam, M., W.H. Lee, E.J. Bae and S.G. Kim, *Compound C inhibits clonal expansion of preadipocytes by increasing p21 level irrespectively of AMPK inhibition.* Arch Biochem Biophys, 2008. **479**(1): p. 74-81.
687. Rossi, A. and J. Lord, *Adiponectin inhibits neutrophil phagocytosis of Escherichia coli by inhibition of PKB and ERK 1/2 MAPK signalling and Mac-1 activation.* PloS one, 2013. **8**(7): p. e69108.
688. Jongstra-Bilen, J., R. Harrison and S. Grinstein, *Fcgamma-receptors induce Mac-1 (CD11b/CD18) mobilization and accumulation in the phagocytic cup for optimal phagocytosis.* The Journal of biological chemistry, 2003. **278**(46): p. 45720-9.
689. Thornton, B.P., V. Vetvicka, M. Pitman, R.C. Goldman and G.D. Ross, *Analysis of the sugar specificity and molecular location of the beta-glucan-binding lectin site of complement receptor type 3 (CD11b/CD18).* Journal of immunology, 1996. **156**(3): p. 1235-46.
690. Orchekowski, R.P., J. Plescia, D.C. Altieri and M.L. Bajt, *AlphaMbeta2 (CD11b/CD18, Mac-1) integrin activation by a unique monoclonal antibody to alphaM I domain that is divalent cation-sensitive.* Journal of leukocyte biology, 2000. **68**(5): p. 641-9.

691. Heit, B., P. Colarusso and P. Kubes, *Fundamentally different roles for LFA-1, Mac-1 and alpha4-integrin in neutrophil chemotaxis.* J Cell Sci, 2005. **118**(Pt 22): p. 5205-20.
692. Hughes, P.E. and M. Pfaff, *Integrin affinity modulation.* Trends Cell Biol, 1998. **8**(9): p. 359-64.
693. Rooyakkers, A.W. and R.W. Stokes, *Absence of complement receptor 3 results in reduced binding and ingestion of Mycobacterium tuberculosis but has no significant effect on the induction of reactive oxygen and nitrogen intermediates or on the survival of the bacteria in resident and interferon-gamma activated macrophages.* Microb Pathog, 2005. **39**(3): p. 57-67.
694. Graham, I.L., H.D. Gresham and E.J. Brown, *An immobile subset of plasma membrane CD11b/CD18 (Mac-1) is involved in phagocytosis of targets recognized by multiple receptors.* Journal of immunology, 1989. **142**(7): p. 2352-8.
695. Taborda, C.P. and A. Casadevall, *CR3 (CD11b/CD18) and CR4 (CD11c/CD18) are involved in complement-independent antibody-mediated phagocytosis of Cryptococcus neoformans.* Immunity, 2002. **16**(6): p. 791-802.
696. Wrann, C.D., N.A. Tabriz, T. Barkhausen, A. Klos, M. van Griensven, H.C. Pape, D.O. Kendoff, R. Guo, P.A. Ward, C. Krettek and N.C. Riedemann, *The phosphatidylinositol 3-kinase signaling pathway exerts protective effects during sepsis by controlling C5a-mediated activation of innate immune functions.* Journal of immunology, 2007. **178**(9): p. 5940-8.
697. Parsa, K.V., J.P. Butchar, M.V. Rajaram, T.J. Cremer and S. Tridandapani, *The tyrosine kinase Syk promotes phagocytosis of Francisella through the activation of Erk.* Molecular immunology, 2008. **45**(10): p. 3012-21.
698. Anderson, S.I., N.A. Hotchin and G.B. Nash, *Role of the cytoskeleton in rapid activation of CD11b/CD18 function and its subsequent downregulation in neutrophils.* J Cell Sci, 2000. **113 (Pt 15)**: p. 2737-45.
699. Lasunskaia, E.B., M.N. Campos, M.R. de Andrade, R.A. Damatta, T.L. Kipnis, M. Einicker-Lamas and W.D. Da Silva, *Mycobacteria directly induce cytoskeletal rearrangements for macrophage spreading and polarization through TLR2-dependent PI3K signaling.* Journal of leukocyte biology, 2006. **80**(6): p. 1480-90.
700. Chung, C.Y., G. Potikyan and R.A. Firtel, *Control of cell polarity and chemotaxis by Akt/PKB and PI3 kinase through the regulation of PAKa.* Mol Cell, 2001. **7**(5): p. 937-47.
701. Chodniewicz, D. and D.V. Zhelev, *Chemoattractant receptor-stimulated F-actin polymerization in the human neutrophil is signaled by 2 distinct pathways.* Blood, 2003. **101**(3): p. 1181-4.
702. Stephens, L., C. Ellson and P. Hawkins, *Roles of PI3Ks in leukocyte chemotaxis and phagocytosis.* Curr Opin Cell Biol, 2002. **14**(2): p. 203-13.
703. Allen, L.A. and J.A. Allgood, *Atypical protein kinase C-zeta is essential for delayed phagocytosis of Helicobacter pylori.* Current biology : CB, 2002. **12**(20): p. 1762-6.
704. Lutz, M.A. and P.H. Correll, *Activation of CR3-mediated phagocytosis by MSP requires the RON receptor, tyrosine kinase activity, phosphatidylinositol 3-kinase, and protein kinase C zeta.* Journal of leukocyte biology, 2003. **73**(6): p. 802-14.
705. Bae, H.B., J.W. Zmijewski, J.S. Deshane, J.M. Tadie, D.D. Chaplin, S. Takashima and E. Abraham, *AMP-activated protein kinase enhances the phagocytic ability of macrophages and neutrophils.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2011. **25**(12): p. 4358-68.
706. Eggleton, P., K.B. Reid and A.J. Tenner, *C1q--how many functions? How many receptors?* Trends Cell Biol, 1998. **8**(11): p. 428-31.
707. Takemura, Y., N. Ouchi, R. Shibata, T. Aprahamian, M.T. Kirber, R.S. Summer, S. Kihara and K. Walsh, *Adiponectin modulates inflammatory reactions via calreticulin*

receptor-dependent clearance of early apoptotic bodies. The Journal of clinical investigation, 2007. **117**(2): p. 375-86.
708. Ma, W., V. Rai, B.I. Hudson, F. Song, A.M. Schmidt and G.R. Barile, *RAGE binds C1q and enhances C1q-mediated phagocytosis.* Cellular immunology, 2012. **274**(1-2): p. 72-82.
709. Frausto-Del-Rio, D., I. Soto-Cruz, C. Garay-Canales, X. Ambriz, G. Soldevila, J. Carretero-Ortega, J. Vazquez-Prado and E. Ortega, *Interferon gamma induces actin polymerization, Rac1 activation and down regulates phagocytosis in human monocytic cells.* Cytokine, 2012. **57**(1): p. 158-68.
710. Gulbins, E. and P.L. Li, *Physiological and pathophysiological aspects of ceramide.* American journal of physiology. Regulatory, integrative and comparative physiology, 2006. **290**(1): p. R11-26.
711. Gulbins, E., S. Dreschers, B. Wilker and H. Grassme, *Ceramide, membrane rafts and infections.* J Mol Med (Berl), 2004. **82**(6): p. 357-63.
712. Lin, D.C., C. Quevedo, N.E. Brewer, A. Bell, J.R. Testa, M.L. Grimes, F.D. Miller and D.R. Kaplan, *APPL1 associates with TrkA and GIPC1 and is required for nerve growth factor-mediated signal transduction.* Mol Cell Biol, 2006. **26**(23): p. 8928-41.
713. Varsano, T., M.Q. Dong, I. Niesman, H. Gacula, X. Lou, T. Ma, J.R. Testa, J.R. Yates, 3rd and M.G. Farquhar, *GIPC is recruited by APPL to peripheral TrkA endosomes and regulates TrkA trafficking and signaling.* Mol Cell Biol, 2006. **26**(23): p. 8942-52.
714. Bohdanowicz, M., D.M. Balkin, P. De Camilli and S. Grinstein, *Recruitment of OCRL and Inpp5B to phagosomes by Rab5 and APPL1 depletes phosphoinositides and attenuates Akt signaling.* Molecular biology of the cell, 2012. **23**(1): p. 176-87.
715. Browning, D.D., W.C. Diehl, M.H. Hsu, I.U. Schraufstatter and R.D. Ye, *Autocrine regulation of interleukin-8 production in human monocytes.* American journal of physiology. Lung cellular and molecular physiology, 2000. **279**(6): p. L1129-36.
716. Hammond, M.E., G.R. Lapointe, P.H. Feucht, S. Hilt, C.A. Gallegos, C.A. Gordon, M.A. Giedlin, G. Mullenbach and P. Tekamp-Olson, *IL-8 induces neutrophil chemotaxis predominantly via type I IL-8 receptors.* Journal of immunology, 1995. **155**(3): p. 1428-33.
717. Jagels, M.A. and T.E. Hugli, *Neutrophil chemotactic factors promote leukocytosis. A common mechanism for cellular recruitment from bone marrow.* Journal of immunology, 1992. **148**(4): p. 1119-28.
718. Terashima, T., D. English, J.C. Hogg and S.F. van Eeden, *Release of polymorphonuclear leukocytes from the bone marrow by interleukin-8.* Blood, 1998. **92**(3): p. 1062-9.
719. Brunetti, M., N. Martelli, A. Colasante, M. Piantelli, P. Musiani and F.B. Aiello, *Spontaneous and glucocorticoid-induced apoptosis in human mature T lymphocytes.* Blood, 1995. **86**(11): p. 4199-205.
720. Cohen, S., D. Janicki-Deverts, W.J. Doyle, G.E. Miller, E. Frank, B.S. Rabin and R.B. Turner, *Chronic stress, glucocorticoid receptor resistance, inflammation, and disease risk.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(16): p. 5995-9.
721. Pletz, M.W., M. Ioanas, A. de Roux, O. Burkhardt and H. Lode, *Reduced spontaneous apoptosis in peripheral blood neutrophils during exacerbation of COPD.* The European respiratory journal, 2004. **23**(4): p. 532-7.
722. Zhang, J., J. He, J. Xia, Z. Chen and X. Chen, *Delayed apoptosis by neutrophils from COPD patients is associated with altered Bak, Bcl-xl, and Mcl-1 mRNA expression.* Diagnostic pathology, 2012. **7**: p. 65.
723. Piccio, L., C. Cantoni, J.G. Henderson, D. Hawiger, M. Ramsbottom, R. Mikesell, J. Ryu, C.S. Hsieh, V. Cremasco, W. Haynes, L.Q. Dong, L. Chan, D. Galimberti and

A.H. Cross, *Lack of adiponectin leads to increased lymphocyte activation and increased disease severity in a mouse model of multiple sclerosis.* European journal of immunology, 2013. **43**(8): p. 2089-100.
724. Lumeng, C.N. and A.R. Saltiel, *Inflammatory links between obesity and metabolic disease.* The Journal of clinical investigation, 2011. **121**(6): p. 2111-7.
725. Libby, P., *Inflammation and cardiovascular disease mechanisms.* Am J Clin Nutr, 2006. **83**(2): p. 456S-460S.
726. Gregor, M.F. and G.S. Hotamisligil, *Inflammatory mechanisms in obesity.* Annual review of immunology, 2011. **29**: p. 415-45.
727. Netea, M.G., B.J. Kullberg and J.W. Van der Meer, *Circulating cytokines as mediators of fever.* Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 2000. **31 Suppl 5**: p. S178-84.
728. Doring, H., K. Schwarzer, B. Nuesslein-Hildesheim and I. Schmidt, *Leptin selectively increases energy expenditure of food-restricted lean mice.* International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity, 1998. **22**(2): p. 83-8.
729. Wynne, K., S. Stanley, B. McGowan and S. Bloom, *Appetite control.* The Journal of endocrinology, 2005. **184**(2): p. 291-318.
730. Plata-Salaman, C.R., *Immunoregulators in the nervous system.* Neuroscience and biobehavioral reviews, 1991. **15**(2): p. 185-215.
731. Adam, T.C. and E.S. Epel, *Stress, eating and the reward system.* Physiology & behavior, 2007. **91**(4): p. 449-58.

APPENDIX I: HEALTH SCREENING QUESTIONNAIRE

This screening questionnaire should be completed honestly and accurately as it will determine if you are eligible for the research study.

Name: _____ Date: _____

1. Do you consider yourself to be healthy? Y/N

2. Are you male or female? M/F

3. How old are you (in years)? ……..

4. Do you currently smoke or use elicit substances? Y/N

5. Have you been ill in the last 4 weeks? Y/N

If yes, provide further details:

6. Do you suffer with any chronic illness? Y/N

If yes, provide further details:

7. Do you take regular medication for any illness? Y/N

If yes, please provide further details and dosage:

8. Do you take sleep modifying medications (over the counter or prescribed?) Y/N

9. Have you travelled to a different time zone in the past 4 weeks? Y/N

10. Have you worked shifts in the past year? Y/N

If yes, please provide details:

11. Do you have a diagnosed sleep disorder? Y/N

12. Do you have a diagnosed mental illness? Y/N

13. Do you drink alcohol? Y/N

If the answer is yes, please indicate approximately how many units of alcohol you consumer per week.

Units per week: …

APPENDIX II: PAPERS AND BOOK CHAPTERS PUBLISHED DURING THE COMPLETION OF THE PHD

1. **Rossi, A.** and J.M. Lord, *Adiponectin inhibits neutrophil apoptosis via activation of AMP kinase, PKB and ERK 1/2 MAP kinase.* Apoptosis, 2013. 18(12): p. 1469-80.

2. **Rossi, A.** and J. Lord, *Adiponectin inhibits neutrophil phagocytosis of Escherichia coli by inhibition of PKB and ERK 1/2 MAPK signalling and Mac-1 activation.* PloS one, 2013. **8**(7): p. e69108.

3. Hampson, P., **A. Rossi,** T. Arora, J.M. Lord and S. Taheri, *Sleep and Immunity in Older Age.* "Immunosenescence, Psychosocial and behavioral determinants", Springer 2013, p. 201-19.