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THE EFFECT OF TWO MODALITIES OF SLEEP DISRUPTION ON IMMUNITY IN HEALTHY YOUNG FEMALE PARTICIPANTS

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Zuha Ajlan

A Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree Masters of Science in Medicine

Johannesburg, South Africa, 2023

DECLARATION

I, Zuha Ajlan, declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Masters of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.____  ____

(Signature of candidate) 28th day of July 2023 in Johannesburg

PRESENTATIONS EMERGING FROM THIS STUDY

Brain Function Research Group Research Day, Johannesburg, South Africa, 14 September 2022.
Oral presentation: "Protectors of the night: A story of sleep disturbances"

9th Annual Conference "Cutting edge advancements in research" hosted by Manchester Medical Research Students Society, Manchester, United Kingdom, 29th April 2023.
Poster presentation: "The effect of sleep disruption on the immune system in health young women" [3rd place abstract and poster presentation]

Society of Neuroscientists in Africa Conference, Johannesburg, South Africa, 11th – 14th July 2023.
Poster presentation: "The effect of sleep disruption on the immune system in health young women"

14th Cross-Faculty Postgraduate Symposium, University of the Witwatersrand, Johannesburg, South Africa, 6th – 8th September 2023.
Oral presentation: "The effect of two modalities of sleep disruption on the immune system in health young women" [1st Place Masters oral presentation]

Neurology World Conference, Miami, United States of America, September 2023.
Oral presentation: "The effect of two modalities of sleep disruption on the immune system in health young women"

ABSTRACT

Studies have shown that sleep deprivation leads to an inappropriate immune response by elevating pro-inflammatory markers, including interleukin (IL-)1, IL-6, and tumour necrosis factor (TNF-)α. This inappropriate immune activation increases the risk of developing autoimmune disorders. Despite women representing 80% of patients with autoimmune disorders and having a greater prevalence of poor sleep quality and sleep disorders, most experimental human studies investigating sleep and immunity focused on men. Therefore, this study assessed the effect of sleep fragmentation vs. sleep restriction on sleep parameters. I then compared the immune response after the two types of sleep disruptions relative to a normal sleep episode and I investigated the association between sleep architecture and immune markers in healthy young women in the follicular phase of their menstrual cycle.

Fourteen healthy females underwent a randomised-crossover controlled study consisting of one adaptation night and three randomised, non-consecutive sleep conditions, namely: baseline night (BN, uninterrupted 8 hours of sleep); restriction night (RN, sleep was limited to the first 4 hours of their habitual sleep episode); fragmentation night (FN, eight randomised forced awakenings through an 8-hour sleep opportunity night). Polysomnographic (PSG) sleep recordings were obtained for each condition, and plasma was collected 2.5 hours after their habitual waketime following each condition. A multiplex Luminex assay was used to measure the concentration of nine cytokines. I compared PSG-extracted sleep variables between the three experimental nights. I ran mixed models analyses testing cytokine levels in each sleep condition (RN vs. FN vs. BN) in unadjusted analyses and then adjusting for order of the condition (first vs. second vs. third experimental night), day of follicular phase of the menstrual cycle and age. I also used an unadjusted mixed model analysis to test the association between cytokine levels and each sleep variable.

Total sleep time, non-rapid eye movement (NREM) and rapid eye movement (REM) were reduced in FN and RN but were lowest during RN ($p<0.001$). I found an effect of sleep condition on IL-8 ($F = 3.40$, $P = 0.05$) with IL-8 being lower in RN vs FN or BN. There was no effect of condition on the other cytokines in unadjusted or adjusted analyses. Lower wake after sleep onset (WASO) and higher NREM were associated with higher IL-8 concentration regardless of the sleep condition. Lower stage 2 (N2) ($F = 6.28$, $\beta = -0.001$, $P = 0.02$) and higher stage 3 (N3) ($F = 7.01$, $\beta = 0.004$, $P = 0.01$) was associated with a higher TNF-α regardless of the sleep condition.

In conclusion, the study shows that acute sleep disruption alters sleep architecture and leads to an inappropriate immune activity in young healthy women. Future studies should try and investigate chronic sleep fragmentation vs chronic sleep restriction on the immune system.
Keywords: Sleep restriction, sleep fragmentation, disrupted sleep, cytokines, inflammation, women's health

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"*To the stars that listen – the dreams that are answered*" – A court of Mist and Fury, Sarah J Maas

It starts with dreamers who dream, hope, pray and manifest a future they want for themselves. But no journey is as simple as we hope; after all a book without conflict is never an interesting read. This study has been an emotional journey, full of ups and downs, but I am immensely grateful for the support of the incredible people who have been there for me every step of the way. I want to take a moment to express my sincerest thanks and gratitude to those who have made this possible.

To my mom and dad, thank you for believing in me, for your love and support, for dropping me off at Wits Medical School in the evenings and picking up a zombie the following day. I know the wait to complete this had driven you up the wall, but I am afraid you raised a perfectionist who ensured she refined this to the best of her ability.

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LIST OF ABBREVIATIONS AND SYMBOLS

α	Alpha
β	Beta
˚C	Degrees Celsius
γ	Gamma
ANA	Antinuclear antibodies
BMI	Body mass index
BN	Baseline night
CC	Caged control
CRP	C-reactive protein
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffer saline
ECG	Electrocardiogram
EEG	Electroencephalography
ELISA	Enzyme linked immunoassay
EMG	Electromyogram
EOG	Electrooculogram
ESS	Epworth sleepiness scale
FCS	Foetal calf serum
5PL	Five parametric logistic
FN	Fragmentation night
GHQ	General health questionnaire
IBS	Irritable bowel syndrome
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRLSSS	International restless leg syndrome severity scale
i.p.	Intraperitoneal
ISI	Insomnia sleep index
LLOQ	Lower limit of quantification
LOD	Limit of detection
LPS	Lipopolysaccharides
M1	Type 1 macrophages
M2	Type 2 macrophages
MFI	Median fluorescence intensities

MS	Multiple sclerosis
NK cells	Natural killer cells
NREM	Non-rapid eye movement
N1	Stage 1 of sleep
N2	Stage 2 of sleep
N3	Stage 3 of sleep
NS	Normal saline
OSA	Obstructive sleep apnoea
PBMC	Peripheral blood mononuclear cells
PGE2	Prostaglandin E_2
PSD	Partial sleep deprivation
PSG	Polysomnography
PSQI	Pittsburgh sleep quality index
qPCR	Quantitative real-time polymerase chain reaction
RA	Rheumatoid arthritis
REDCap	Research electronic data capture
REM	Rapid eye movement
RN	Restriction night
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT	Room temperature
SCN	Suprachiasmatic nucleus
SE	Sleep efficiency
SLE	Systemic lupus erythematosus
sTNFr	Soluble tumour necrosis factor receptor
STOP BANG	**Snoring** history, **tired** during the day, **observed** stop of breathing while sleeping, high blood **pressure**, **body** mass index ≥ 35 kg/m^2, **age** > 50 years, **neck** circumference > 40 cm and male **gender**
Th	Helper T-cells
Th1	Type 1 helper T-cells
Th2	Type 2 helper T-cells
Th17	Type 17 helper T-cells
TIB	Time in bed
TNF	Tumour necrosis factor
TNFRF	Tumour necrosis factor receptor fragments

TSD	Total sleep deprivation
TST	Total sleep time
WASO	Wake after sleep onset
VAS	Visual analogue scale

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CHAPTER 1: INTRODUCTION

In a fast-paced world where having poor and insufficient sleep has become the norm, we find ourselves grappling with a silent epidemic (Naiman, 2017). In a 2020 National Health Institute Survey, it was reported that 14.5% of adults in the United States of America experienced difficulty falling asleep and 17.8% struggled with maintaining sleep (Adjaye-Gbewonyo *et al.,* 2022). They further reported that women were 1.4 times more likely to complain of sleep difficulties compared to men. Another two recent, independent studies in South Africa reported a high prevalence of poor sleep health, poor sleep quality, frequent arousals during sleep, and emerging sleep disorders like insomnia, particularly among women, in both young-middle aged adults (18–40 years) and the older (aged between 40–72 years old) South African population (Davy *et al.,* 2021; Gómez-Olivé *et al.,* 2018). However, this experience of poor sleep by women is not isolated to the American and South African population; similar experiences have been reported in numerous countries across the globe including Australia (Blunden *et al.,* 2022), China (Lu *et al.,* 2019), Egypt (Abou-Khadra *et al.,* 2021; El Hafeez *et al.,* 2022), Germany (Schlarb *et al.,* 2017), Luxemburg (Schlarb *et al.,* 2017), Pakistan (Umar *et al.,* 2022) and Turkey (Duran and Erkin, 2021). These alarming figures reflect a global concern that demands our attention.

Sleep, often overlooked, is a fundamental biological process ubiquitous in many living species needed for regulatory and restorative functions (Benington and Craig Heller, 1995; Ibarra-Coronado *et al.,* 2015). While the regulatory role of sleep on immunity, cognition, and cardiometabolic function has extensively been studied (Dinges *et al.,* 1995; Krueger, 2008), the specific relationship between sleep and immune function remains a significant area of investigation.

Recent studies have reported a diurnal immune activation pattern in healthy individuals, whereby pro-inflammatory cytokines were elevated during night-time sleep, and anti-inflammatory cytokines were higher during daytime wakefulness (Krueger *et al.,* 2011; Opp, 2005a) However, an increasing number of studies investigating the effect of experimental sleep deprivation on immune function have reported greater states of daytime pro-inflammatory cytokines following acute and chronic sleep deprivation (Borbély *et al.,* 1981; Friess *et al.,* 1995; Grønli *et al.,* 2014; Hsiao *et al.,* 2015; Irwin *et al.,* 2016; Zauggli and Douglass, 1995). These studies also suggest that those states of chronic inflammation led to

an inappropriate immune response, highlighting a potential link in the development of sleep and autoimmune disorders (Dinges *et al.*, 1995; Krueger, 2008).

Women are more susceptible to disturbed sleep and sleep disorders (Lee, 1992; Lee and Kryger, 2008; Lu *et al.*, 2019; Mallampalli and Carter, 2014). This is due to both external factors such as social, environmental, fulfilling primary caregiving roles and shift work like in healthcare, and internal circadian factors whereby women have a shorter duration of circadian melatonin release and an earlier core temperature trough, compared to men (Baker *et al.*, 2001; Duffy *et al.*, 2011). This makes women more susceptible to disturbed sleep and developing sleep disorders. Alarmingly, women also face a higher risk of developing autoimmune disorders such as multiple sclerosis and systemic lupus erythematosus, which are often accompanied by lower sleep quality (Angum *et al.*, 2020; Costa *et al.*, 2005; Desai and Brinton, 2019; Hsiao *et al.*, 2015; Kivity and Ehrenfeld, 2010). However, the underlying factors contributing to the exacerbation of sleep and autoimmune symptoms in these patients remain unclear.

The present dissertation aims to address this knowledge gap by exploring the relationship between sleep and immunity. While recognizing the trilateral relationship among sleep, immune activation, and pain in autoimmune disorders, this study will focus specifically on the basic relationship between sleep and immune function on healthy young women. By investigating this association in an experimental setting, my findings will contribute to a better understanding of sleep-related inappropriate immune activation and potentially contribute to the literature that will ultimately inform the development of targeted interventions.

1. BACKGROUND

1.1. A Brief History of Sleep Research

Humans are curious creatures. Since the dawn of time, we have been intrigued by the metaphysical realm and those that we cannot see. Philosophers, poets, physicians, and scientists have all been curious about love, emotions, death, and dreams. Many had assumed that sleep is a metaphysical state between the realm of the living and the dead. In many cultural lore such as in Egypt, there have been accounts of astral projection – a dreamlike

state where one leaves their physical body – during sleep and visits different realms, people, and places.

Dreaming, and ultimately sleep itself, has been a behaviour of interest since the beginning of science. Aristotle was sceptical of the claims of dreams being the bridge between the physical world and the metaphysical realm. Instead, Aristotle had claimed that dreams are a state of mental activity during sleep (Gregoric and Fink, 2022). He further stated that the beginning of an illness may be detected during sleep before it manifests as physical symptoms (Gregoric and Fink, 2022).

However, it was not until the 20th century following the discovery of neural activity, and rhythmic generation and propagation of electric activity in the brain, that Aristotle's claim that dreaming was a state of mental activity was proven to be true (Kryger *et al.*, 2017). In 1928, German psychiatrist Hans Berger was the first man to record the electrical activity of the brains and demonstrate changes in electrical activity during wakefulness and sleep (Kryger *et al.*, 2017). This invention of the electroencephalogram (EEG) had revolutionised the scientific investigation into sleep research.

1.2. Definition of Sleep, Functions and Techniques for Sleep Measurement

Since then, sleep is recognised as a necessary period of rest within the 24-hour day. Despite the lack of physical activity during an individual's sleeping state, sleep is an active state. In many species, sleep is a period of decreased physical and metabolic activity, with a parasympathetic dominance leading to the slowing down of basic functions such as feeding, cardiovascular function, and breathing (Kryger *et al.*, 2017; Zielinski *et al.*, 2016). However, it is also a period of intense brain activity (Kryger *et al.*, 2017).

Sleep plays a major role in regulating the immune system and cardiometabolic health, releasing growth hormone, and maintaining insulin levels among other functions (Kryger, Roth & Dement, 2005). However, research has shown that poor or insufficient sleep increases the risk of developing lieu of disorders such as autoimmune disorders (example: systemic lupus erythematosus) (Angum *et al.*, 2020; Costa *et al.*, 2005; Desai and Brinton, 2019; Hsiao *et al.*, 2015; Kivity and Ehrenfeld, 2010), hypertension (Li and Shang, 2021), diabetes mellitus (Koren and Taveras, 2018; Reutrakul and Van Cauter, 2018), and schizophrenia (Ashton and Jagannath, 2020; Dule *et al.*, 2020; Kaskie *et al.*, 2017).

Thus, the complex relationship of sleep with other physiological and biological functions in the body posed further questions on sleep's role. This led to the development of several techniques to measure sleep health. Sleep health is characterised by a multitude of variables including sleep quality, sleep duration, sleep regularity (determined by wake- and bedtimes), and daytime sleepiness. In this following section, I will first examine the different ways of measuring sleep and highlight their advantages, starting with subjective sleep measures using questionnaires and moving to measurements of period of rest versus activity using actigraphy, and finally a short discussion on the gold standard for measuring objective sleep, polysomnography.

1.2.1. Questionnaires

Questionnaires have been developed to measure various sleep variables to understand an individual's perception of sleep. These subjective data provide an indication of quality of sleep and are useful to gather information of sleep for specific populations. Commonly used questionnaires include: the Epworth Sleepiness Scale (ESS) (Johns, 1998) to measure daytime sleepiness, and the Pittsburgh Sleep Quality Index (PSQI) (Buysse *et al.,* 1989) to measure the quality of sleep over a month. In both questionnaires, individuals with a score greater than 5 are reported to have higher daytime sleepiness and poor sleep quality, respectively (Buysse *et al.,* 1989; Johns, 1998).

Depending on the investigation and aims of the study, questionnaires can also be used to gather subjective information, or to screen for sleep disorders. Such commonly used validated and standardized questionnaires include Insomnia Sleep Index (ISI) for insomnia, International Restless Leg Syndrome Severity Scale (IRLSSS) for restless leg syndrome (Walters *et al.,* 2003), the STOP BANG (Oshita *et al.,* 2020) and the Berlin questionnaire (Khaledi-Paveh *et al.,* 2016) for obstructive sleep apnoea (OSA). These questionnaires are an excellent and reliable tool to provide an overview of an individual's subjective sleep health.

1.2.2. Actigraphy

Actigraphy is a non-invasive procedure whereby individuals are asked to wear an actigraphy device on their wrist (Cole *et al.,* 1992). Depending on the protocol or purpose of the investigation, the individuals are allowed to go about their day. The actigraphy device will then collect their daily movement, exposure to light, sleep and wake activity – determined by

the amount of movement, throughout their day. It also notifies the period where the actigraphy watch was not worn. This data allows to assess the measure of activity and determine an individual's sleep/wake pattern, sleep onset, sleep offset, total sleep duration, sleep onset latency (if they keep a sleep diary), awakenings, wake after sleep onset (WASO), and sleep efficiency (see Table 1 in Methods for definitions) (Cole *et al.,* 1992). However, it is limited to the extent of activity and lifestyle of an individual, it cannot distinguish different sleep stages, and sometimes the battery might die.

1.2.3. Polysomnography

Polysomnography (PSG) is considered as the gold standard method for measuring sleep. Polysomnographic recordings use a combination of EEG, electromyography (EMG) and electrooculography (EOG) to determine the different stages of sleep. To obtain PSG, electrodes are placed on specific areas of the scalp, face, and chin, in accordance with the American Society of Sleep Medicine (Iber, Chesson and Quan, 2007), and sleep is measured in the form of electrical activity. A detailed description of performing PSG is outlined in the Method section of this dissertation.

The EEG displays the electrical activity of the brain by means of electrode placement on the scalp, and changes in wave frequency and amplitude are used to identify sleep stages. EEG visualizes a spectrum of wave frequencies that form during the sleep-wake cycle: Delta waves (0.5–4 Hz), theta waves (4–8 Hz), alpha waves (8–12 Hz), beta waves (13–35 Hz), and gamma waves (<35 Hz) (Abhang *et al.,* 2016). These changes in electrical activity, couple with information from the EOG and EMG, categorise sleep into two main sleep stages: rapid eye movement (REM) sleep and non-rapid eye movement (NREM), which is further divided in light stage 1 sleep (N1), stage 2 sleep (N2), and deep stage 3 sleep (N3) (Dement and Kleitman, 1957).

1.3. Normal Sleep Architecture, The 2-Process Model of Sleep Regulation and Effects of Sleep Deprivation on Sleep Architecture

1.3.1. Normal sleep architecture as measured by PSG

A normal 8-hour nocturnal sleep opportunity in a healthy young individual is typically characterised by four to six 90-minute cycles that transition between the three NREM sleep stages to REM sleep (Bahammam *et al.,* 2016; Dement and Kleitman, 1957; Kryger *et al.,*

2017; Patel *et al.*, 2021) (Figure 1). NREM is a heterogenous state of sleep characterised by high amplitude, low-frequency delta waves (0.5–4 Hz) and spindles (7–15 Hz) with low postural muscle tone on the EMG (Eban-Rothschild *et al.*, 2018; Ohayon *et al.*, 2004). In a typical night for healthy young adults, total sleep time (TST) is distributed as 2% to 5% of N1; 45% to 55% of N2; 15% to 20% of N3; and 20% to 25% of REM (Figure 1) (Ebrahimi *et al.*, 2008; Patel *et al.*, 2021). The next paragraph describes each sleep stage in more detail.

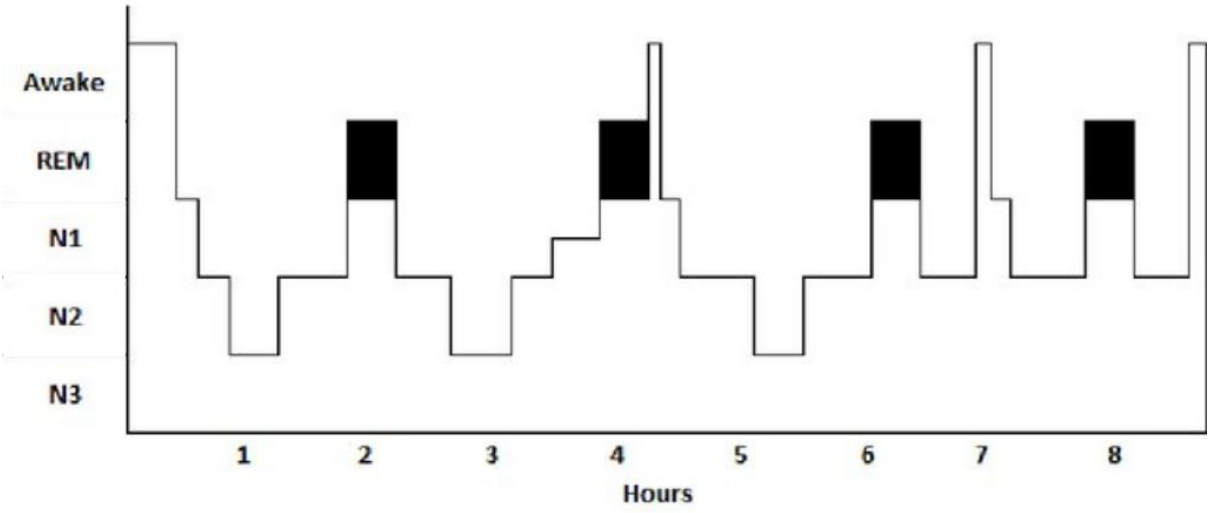


Figure 1: A hypnogram illustrating the succession of sleep stages in a healthy individual through a normal sleep episode of 8 hours (Adapted from (Bahammam *et al.*, 2016)). Non-rapid eye movement (NREM), Stage 1 of sleep (N1), stage 2 of sleep (N2), stage 3 of sleep (N3), Rapid eye movement (REM) sleep. The black region on the graph depicts REM.

N1 sleep has the lowest threshold for arousal i.e., it is the lightest stage of sleep. The EEG shows a transition from clear alpha waves (wake stage) to a low-voltage mixed frequency pattern in the occipital region during N1 (Carskadon and Dement, 1994; Kryger, Avidan and Berry, 2014; Kryger, Roth and Dement, 2017; Patel, Reddy and Araujo, 2021). As individuals' transition into a deeper N2 sleep, sleep spindles form and the duration of N2 increases with each sleep cycle (Carskadon and Dement, 1994; Patel, Reddy and Araujo, 2021). Next, individuals' transition either into N3 or REM sleep. N3, also known as slow-wave sleep (SWS), has the highest arousal threshold. It is considered the deepest state of sleep and slow delta waves (0–4Hz) are present. SWS are predominantly present in the first third of the night (Carskadon and Dement, 1994; Kryger, Avidan and Berry, 2014; Kryger, Roth and Dement, 2017; Patel, Reddy and Araujo, 2021). Conversely, REM sleep is dominant in the last third of the night (Carskadon and Dement, 2005; Eban-Rothschild, Appelbaum and de Lecea, 2018) to prepare the body for waking. Vivid dreaming occurs during REM sleep; it is dominated by theta and gamma waves with the body being atonic except for ocular movements (Eban-Rothschild *et al.,* 2018).

Furthermore, studies have reported changes in sleep architecture with age and between sexes (Amarala *et al.*, 2016; Ancoli-Israel, 2005; Baker *et al.*, 2001; Calhoun *et al.*, 2014; Duffy *et al.*, 2015, 2011; Ohayon *et al.*, 2004; Yoon *et al.*, 2003; Zhang and Wing, 2006). In a meta-analysis study by Ohayon *et al.* (2004), it was reported that sleep onset latency, N1 and N2 sleep increase with age. It has been shown that the duration of sleep or TST, decreases in adolescents from 11 to 18 years of age (see Table 1 of Methods for definitions) (Amarala *et al.,* 2016). It has been further reported that REM sleep increases in children from age 5 to early adolescence but decreases from young adulthood to middle age (Ohayon *et al.,* 2004). Moreover, older individuals (<59 years of age) tend to sleep earlier and wake up earlier than younger adults (Li *et al.,* 2018) This is because older adults reach peak melatonin concentration 37 minutes earlier during sleep than young individuals (18–32 years old) (Yoon *et al.,* 2003).

Women are reported to have a shorter circadian rhythm than their male counterparts (Baker *et al.,* 2001; Duffy *et al.,* 2011). Gender differences have been observed in sleep patterns and sleep disorders. Young boys (4–8 years) are at higher risk of being diagnosed with pathological insomnia than girls (Bruni *et al.,* 1996). Inversely, pubescent girls (12–16 years) are more likely to be diagnosed with pathological insomnia symptoms in comparison to age-

matched male counterparts (Calhoun *et al.,* 2014; Zhang and Wing, 2006). This may be due to the hypothalamic-pituitary activation and release of gonadal hormones including oestrogen and progesterone, that are responsible for reducing and delaying melatonin release which results in a reduction of SWS (Duffy *et al.,* 2011; Luboshitzky *et al.,* 2002; Okatani *et al.,* 2000). Overall, during adulthood, women have been reported to have more insomnia than men (Kryger *et al.,* 2014; Mellinger, 1985).

1.3.2. The two-process model of sleep regulation

In the Borbély model of sleep regulation (1982), sleep is regulated by two processes: Process S and Process C (Figure 2). Briefly: Process S, or the homeostatic process, describes the gradual accumulation of sleep pressure with time awake (Figure 2) (Borbély, 1982). This sleep pressure is subsequently relieved by sleep, particularly SWS. Sleep deprivation is followed by a SWS rebound in the recovery nights. On the other hand, Process C – also known as the circadian process – is controlled by the master biological clock situated in the suprachiasmatic nucleus (SCN) of the hypothalamus (Figure 2) (Borbély, 1982). In a normally entrained individual (i.e., someone with a ~16 hour wake episode and ~8 hour sleep episode, with regular wake times and bedtimes, and whose sleep episode is timed to happen when the homeostatic pressure is high and the SCN is no longer giving a signal for wakefulness), the SCN increases the signals for wakefulness a few hours before wake time, following a trough in the core body temperature (Borbély, 1982). This wake signal culminates a few hours before bedtime, thus balancing the increased sleep pressure built during wake in a normally entrained individual (Figure 2) (Borbély, 1982). The circadian system follows an endogenous rhythm caused by the firing of SCN neurons. This rhythm is roughly 24 hours long (circa-dia) and its timing (phase) can shift with a number of environmental cues or zeitgebers, with light exposure being the most influential on the phase response curve (Khalsa *et al.,* 2003). Light exposure at sensitive parts of the phase response curve can lead to phase delay shifts when the timing of the light exposure is centred before the timing of the 24-hour core body temperature trough, or phase advance shifts when light is centred after the core body temperature trough (Khalsa *et al.,* 2003). This effect of light is mediated by intrinsically photosensitive retinal ganglion cells containing melanopsin, a photopigment with maximum sensitivity to light of 480 nm (blue spectrum) (Brainard *et al.,* 2001). Rapid eye movement sleep has been shown to have a circadian regulation with most REM centred on core body temperature trough(Czeisler *et al.,* 1980).

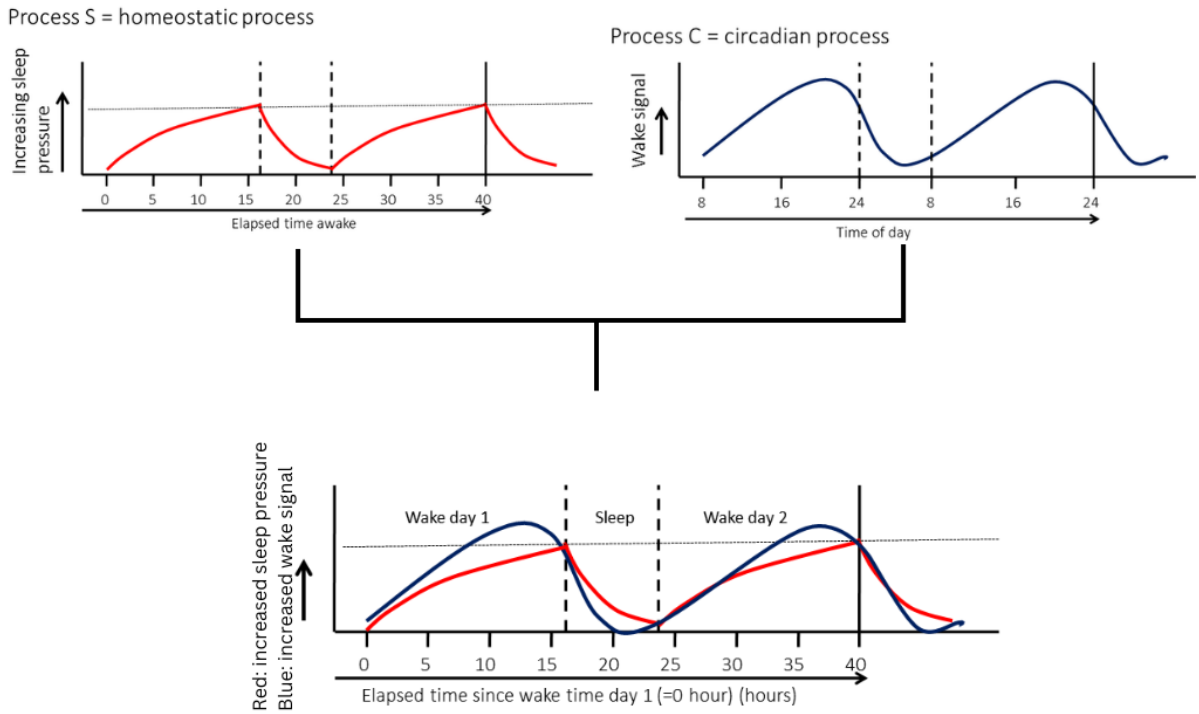


Figure 2: Graphic representation of the 2-process model of sleep regulation.

The elapsed time awake shows the number of hours awake since wake-time. In Process S (homeostatic process), the sleep pressure builds up as the time an individual is awake increases. The time of day shown for Process C (circadian process) is shown over 2 days. There is an increase in wake signalling during the day. However, the wake signalling starts to decrease during the night and completely drops once individuals are asleep (Adapted from Borbély, 1982).

1.3.3. Sleep deprivation and effects on sleep architecture

The length of nocturnal sleep varies from person to person; however, various intrinsic and extrinsic factors influence the longevity of sleep an individual experiences (Carskadon and Dement, 2005). Observational and epidemiological studies have determined that adequate sleep of 7–8 hours is associated with good health (Ong *et al.,* 2016). Conversely, deviations from the optimal range with more, or less sleep hours are associated with higher rates of comorbidities and poorer health outcomes (Bruce Alberts *et al.,* 2002; Gangwisch *et al.,* 2007, 2006; Hall *et al.,* 2008; Tamakoshi *et al.,* 2004).

Ample literature has investigated the effects of total and partial sleep deprivation on adolescents and grown men. These studies reported a significant reduction in N1, N2 and REM while significantly N3 increasing in proportion during the nights following total or partial sleep deprivation compared to baseline night of uninterrupted sleep (Ong *et al.,* 2016; Parry *et al.,* 1999; Plante *et al.,* 2016). This is known as SWS rebound (Brillante *et al.,* 2012).Ong *et al.* (2016) reported that restricting sleep to the first 4 hours of the night in adolescents led to a 4-hour sleep episode with lower amount of REM (minutes) sleep and higher percentage of SWS (minutes) than that observed in a normal 8-hour sleep episode. This is not surprising since SWS dominates in the first half of the time, whist REM sleep dominates the second half of the night (Figure 1). In turn, SWS (minutes) increased above baseline on the third night of sleep restriction, as expected from the SWS rebound following acute sleep deprivation (Brillante *et al.,* 2012). The duration of N2 (minutes) and REM (minutes) sleep remained elevated after three nights of 9-hour recovery sleep (Ong *et al.,* 2016).

In another independent crossover study investigating the effect of a single night of early partial sleep deprivation (sleep opportunity from 3:00 AM–7:00 AM) and late sleep deprivation (sleep opportunity from 9:00 PM–1:00 AM) on women with premenstrual dysmorphic syndrome compared to healthy, age-matched controls reported that both healthy controls and premenstrual dysmorphic syndrome patients had a shorter total sleep time, a decrease in sleep latency and REM latency, decreased N2 (%), and greater sleep efficiency and REM (%) during early sleep deprivation compared to late sleep deprivation. Both conditions showed N1 (%), and N2 (%) decreased, however, REM sleep was lower during late sleep deprivation, compared to a night of uninterrupted sleep and early sleep deprivation (Parry *et al.,* 1999). They further showed that REM (%) was greater during early, and late

sleep deprivation in healthy controls compared to women with premenstrual dysmorphic disorder (Parry *et al.*, 1999).

Similarly, in a cohort study healthy 64 participants (n = 41 females) were divided into three groups: control group (n = 24) who received uninterrupted sleep, a partial sleep deprivation (PSD) group (n = 21) who was kept awake for the first 4 hours of the night and were allowed to sleep for the remaining 4 hours, and a fragmentation group (n = 17) who underwent 7 forced arousals of 20 minutes and 1 forced arousal of 1 hour (Finan *et al.,* 2015). All participants underwent a single baseline followed by three experimental nights depending on the group (control, PSD, and fragmentation). They reported there was an increase in N1 and REM latency in participants who underwent chronic fragmented sleep and SWS had decreased during chronic fragmentation compared to control and PSD (Finan *et al.,* 2015).

These studies give a glimpse of the effect of sleep deprivation on sleep architecture regardless of the method of sleep deprivation. This illustrates the sensitive nature of sleep which is altered after sleep deprivation and sleep may not return to normal in some studies following sleep recovery. However, I have mentioned that sleep plays a regulatory role in many homeostatic functions including immunity so it can be speculated that if sleep is dysregulated then the immune system may have an inappropriate response. In the next section, I will explore the role of the immune system in a healthy individual followed by some evidence of the relationship between sleep and immunity.

1.4. Basic Description and Measurements of Immune Function

1.4.1. The immune system

The immune system orchestrates a defence against pathogens in general, however, when not tightly controlled, can lead to allergies and autoimmune disorders (Alberts *et al.,* 2002). The immune system is composed of immune cells, immune proteins (such as the complement and inflammatory proteins, but also immunoglobulins or antibodies) and immune signalling molecules such as cytokines and chemokines (B Alberts *et al.,* 2002). The immune cells make up the white blood cells in the blood (monocytes, which become macrophages in tissues, granulocytes including neutrophils, eosinophils, and basophils; and lymphocytes which include the natural killer, T and B lymphocytes) but also comprise immune cells that are resident in tissues (example: macrophages and dendritic cells) (Alberts *et al.,* 2002). Cytokines are produced by immune cells and are involved in cell signalling (this signalling

includes activation of immune cells for pro-inflammatory cytokines and suppression of the immune response by anti-inflammatory cytokines (Alberts *et al.,* 2002). However, this cell signalling also targets non-immune related cells, such as endothelial cells, the myocardium and endometrial lining) (Alberts *et al.,* 2002). Chemokines are a cytokine subtype and help direct the migration of white blood cells to target tissues (Alberts *et al.,* 2002). The immune response has been categorized into innate and adaptive immunity (Alberts *et al.,* 2002), which we will cover in more detail in the following sections.

1.4.1.1. Innate immune cells and cytokines

The innate immune response, which includes macrophages, neutrophils, natural killer cells, eosinophils, basophils, and mast cells, is stimulated by the recognition of foreign surface proteins found on pathogens (Alberts *et al.,* 2002). Those proteins engage with the toll-like receptors present on phagocytic cells (macrophages and neutrophils), resulting in their activation which leads to the clearing of pathogens (Alberts *et al.,* 2002). Those foreign surface proteins also trigger the complement cascade (Alberts *et al.,* 2002), which also contributes to clearing the pathogen. Natural killer cells are non-specific (i.e., not specific to a specific pathogen) cytotoxic cells which clear intracellular pathogens by clearing the cells infected by it. The natural killer (NK) cells produce interferon (IFN)-γ that stimulates macrophages (Dale *et al.,* 2008). Macrophage function is further enhanced by activated Effector $CD4^+$ Helper T cells (as described in the cell-mediated immunity section later). The macrophages are further sub-divided into pro-inflammatory macrophages (M1) and anti-inflammatory macrophages (M2) (Murray, 2017). M1 secrete pro-inflammatory cytokines when activated: IL-1β, IL-6, IL-8, TNF-α, and IFN-γ (Figure 3).

These cytokines are of particular interest because they are elevated in immunological disorders such as common autoimmune disorders like rheumatoid arthritis (Moulton, 2018; Sangle *et al.,* 2015; Xu *et al.,* 2019).

IL-1β are released by M1 macrophages to recruit, activate and mobilises T lymphocytes and macrophages to the site of injury (Figure 3) (Murphy *et al.,* 2008; Garlan and Jailan, 2016; Broderick and Hoeffman; 2022). They make the vascular endothelium more permeable to allow further recruitment of immune cells to the target site. Furthermore, IL-1β stimulates the release of TNF-α and IL-6 by macrophages, monocytes, neutrophils, and T-cells (Figure 3) (Murphy *et al.,* 2008; Garlan and Jailan, 2016; Broderick and Hoeffman; 2022).

In addition to IL-1, TNF-α also promotes the permeability of endothelial cell permeability which promotes the recruitment of inflammatory protein and immune cells to the target site (Murphy *et al.*, 2007). It further promotes the recruitment and activation of phagocytes like neutrophils, induce apoptosis (cell-death) and inflammation (Figure 3). Depending on the pathway it activates, it can promote tissue regeneration, promote host defence, and cell survival (Murphy *et al.*, 2007).

IL-8 is a chemoattractant cytokine produced by monocytes and neutrophils that recruits and activates neutrophils, lymphocytes, basophils, and T-cells at the site of inflammation or infection (Zeng *et al.*, 2012) (Figure 3).

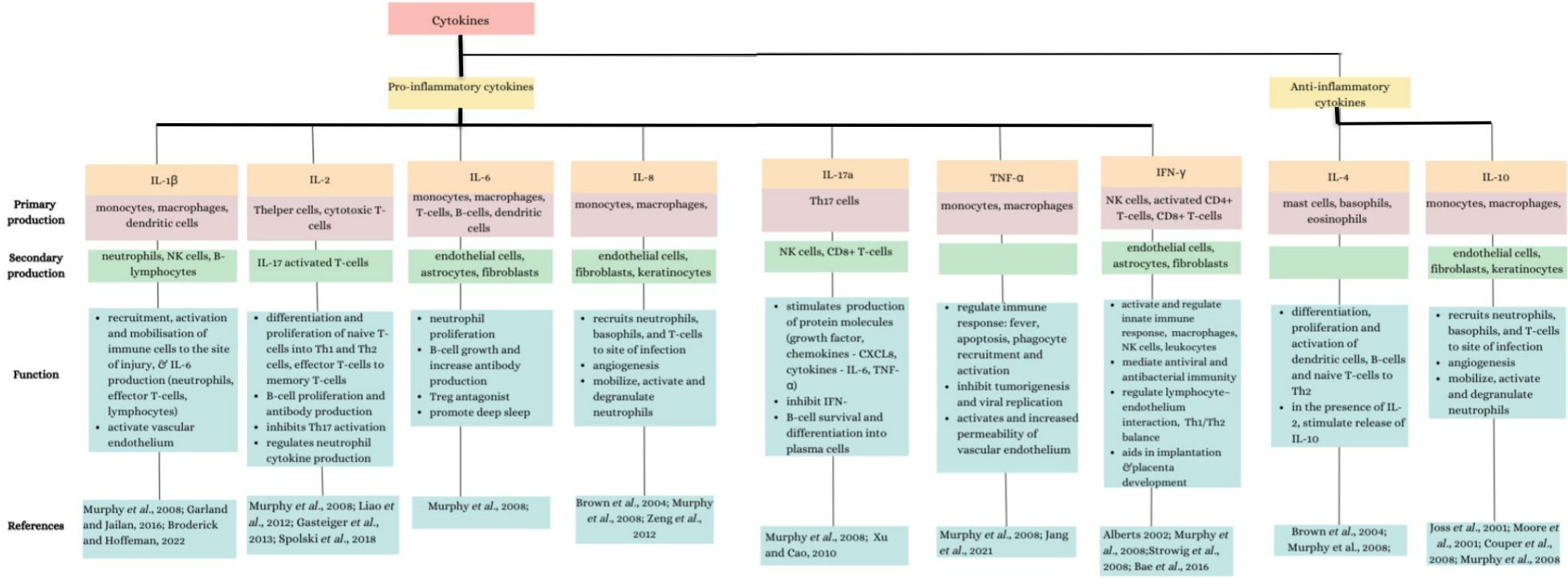


Figure 3: Figure 3: Summary of the site of production and main functions of nine cytokines: IL-1, IL-2, Il-4, IL-6, IL-8, IL-10, IL-17a, TNF-α, and IFN-γ

M2 macrophages are involved in an anti-inflammatory response and are activated by cytokines released by the M1 macrophages. In the immune response, M1 and M2 are thus usually triggered simultaneously to maintain the immune homeostatic balance (i.e., the M2 macrophages will help prevent an overblown immune response) (Kaplan *et al.,* 2018). M2 macrophages release amongst others, the anti-inflammatory cytokine IL-10 (Kaplan *et al.,* 2018). IL-10 plays an important immunomodulatory role by suppressing the monocytes and macrophage (downregulates the expression co-stimulatory molecules on macrophages and inhibits secretion of the pro-inflammatory cytokines by macrophages and granulocytes) (Figure 3), which in turn limits the secretion of pro-inflammatory cytokines (in particular TNF-α, IL-1β, and IFN-γ), and activation of cytotoxic T-cells (Couper *et al.,* 2008; Joss *et al.,* 2000; Moore *et al.,* 2001). IL-10 also dampens the adaptive immune response: the lower secretion of pro-inflammatory cytokines prevents the activation of cytotoxic $CD8^+$ T-cells. IL-10 directly downregulates the CD4 Helper T cell response Th1 (which forms part of the adaptive immune response) cells (Couper *et al.,* 2008; Joss *et al.,* 2000; Moore *et al.,* 2001).

IL-4 has a ubiquitous function. IL-4 is secreted by eosinophils, basophils, mast cells and Th2 cells (Alberts *et al.,* 2002). This secretion of IL-4 in turn stimulates the activation and proliferation of B-cells to produce antibodies – especially immunoglobulin E (IgE) (Alberts *et al.,* 2002). IL-4 promotes the differentiation and proliferation of naïve T-cells into Th2 cells which in turn secrete IL-4 that contribute to the positive feedback loop. On the other side, it has specific anti-inflammatory effects: IL-4 suppresses Type 17 $CD4^+$ helper T cells (Th17) (Zhou *et al.,* 2021). IL-4 is associated with the synthesis of IgE, which are involved in immune response against parasites and worms, but also key agents in the development of allergies.

1.4.1.2. Adaptive immune system

In contrast to the innate immune system which is non-specific and does not lead to the formation of specific memory immune cells, the adaptive immune response is pathogen-specific and develops a pathogen-specific memory, which allows for a swifter and more efficient response once the memory cells have been established. The adaptive immune response consists of the T-cell-regulated immune response and the B-cell-mediated humoral response (Murphy *et al.,* 2007).

i. B Lymphocytes or humoral immunity

B-cells (lymphocytes) produce antibodies such as immunoglobulin (Ig)G, IgA and IgM that bind to and mark foreign antigens found on the surface of pathogens, thus inhibiting the pathogen from binding to the host as well as enhancing the chemotaxis of phagocytes (B Alberts *et al.,* 2002; Murphy *et al.,* 2007). Before meeting their specific antigen, B lymphocytes circulate in a naïve state and need coactivation by a $CD4^+$ Helper T-cell to trigger their first activation. That first activation then leads to the formation of memory B cells specific of the pathogen. Those memory B cells do not need a co-activation by a $CD4^+$ Helper T-cell to get activated after re-exposure to the same antigen. This property is used in the development of vaccines (discussed later).

i. T lymphocytes or cell-mediated immunity

On the other hand, $CD4^+$ $CD8^+$ (double positive) T-cells mature in the thymus into either helper T-cells (expressing solely the membrane receptor CD4) or cytotoxic T-cells (expressing solely the membrane receptor CD8) (Murphy *et al.,* 2007).

a. $CD4^+$ T cells

$CD4^+$ helper T cells, when activated, can perform several functions. Those are divided into Type 1 helper T cells (Th1), Type 2 helper T cells (Th2), Type 17 Helper T cells (Th17), and regulatory T cells (Tregs) (Murphy *et al.,* 2007).

Type 1 Helper T (Th1) cells secrete IL-2 and IFN-γ. IFN-γ is critical for activating the innate and adaptive immune response. It is predominantly produced by NK cells (innate immunity), $CD4^+$ Th1 cells and $CD8^+$ cytotoxic T cells (Alberts *et al.,* 2002; Murphy, Travers and Walport, 2007; Strowig, Brilot and Münz, 2008). It induces the expression of major histocompatibility Type II proteins on the surface of infected cells and is one of first activators of monocytes and macrophages upon infection. IFN-γ promotes the differentiation and proliferation of M1 macrophages, and aids in the transition from innate to adaptive immunity by inducing macrophage (M1) to release IL-2 (Bae *et al.,* 2016). IFN-γ further upregulates the proliferation and activation of $CD8^+$ cytotoxic T-cells, and the differentiation of naive T-cells into Th1 cells that further release IFN-γ (Figure 3) (Bae *et al.,* 2016; Curtsinger *et al.,* 2012). IL-2 proliferates and activates natural killer (NK) cells and cytotoxic T cells(Henney *et al.,* 1981; Wang *et al.,* 2000). In turn, regulatory T cells (Tregs) suppress

IL-2 production to inhibit immune activation to maintain immunomodulatory homeostasis (Figure 3) (Gasteiger *et al.,* 2013).

Th2 synthesizes IL-4, IL-6, and IL-10 to stimulate B-cells to produce antibodies, in response to a pathogen (Alberts *et al.,* 2002; Vazquez, Catalan-Dibene and Zlotnik, 2015).

Type 17 helper T cells synthesize IL-17 (Murphy *et al.,* 2007; Zenobia and Hajishengallis, 2015). IL-17 takes several isoforms, of which IL-17a is the most prominently expressed form of IL-17 (Murphy *et al.,* 2007; Zenobia and Hajishengallis, 2015). IL-17a is secreted by helper T-17 cells, macrophages, neutrophils, and NK cells (Murphy *et al.,* 2007; Zenobia and Hajishengallis, 2015). Once secreted IL-17a recruits and activates neutrophils and monocytes and is speculated to assist in the transition from innate to acquired immune activity (Zenobia and Hajishengallis, 2015). Moreover, IL-17a promotes neuroinflammation by promoting microglial activation, which further leads to the secretion of pro-inflammatory cytokines including IL-8 (Figure 3) (Shi *et al.,* 2018; Yu *et al.,* 2016). Chronic overexpression of IL-17a is hypothesized to be responsible for the pathogenesis of inflammatory and autoimmune disorders including RA, MS, psoriasis, Crohn's disease, and SLE (Hsiao *et al.,* 2015; Shi *et al.,* 2018; Yu *et al.,* 2016).

Regulatory T cells (Tregs) represent a small proportion of circulating $CD4^+$ Helper T cells. They have immunoregulatory functions by suppressing activated Th1 production of IL-2 through IL-10 (Zhou *et al.,* 2021). Tregs are activated by IL-2 and IL-4 to inhibit immune activation to maintain immunomodulatory homeostasis (Gasteiger *et al.,* 2013). The regulatory T-cells (Tregs) (which form part of helper T cells, as described above) also decrease cytotoxic activation (Murphy *et al.,* 2007). In total, Tregs decrease the immune response by decreasing cytotoxic T-cell activation and preventing excess B-cell and macrophage activity by decreasing Effector CD4 Helper T-cell activation (Corthay, 2009; Duffy *et al.,* 2018; Murphy *et al.,* 2007; Sakaguchi *et al.,* 2008).

b. CD8+ cytotoxic T cells

Cytotoxic T cells directly attack cells of the self, infected by intracellular pathogens, by recognizing a specific antigen presented within major histocompatibility Type I molecules present on the surface of all nucleated cells in the body. Cytotoxic T-cells are activated and proliferated by IL-2. After the initial binding, cytotoxic T-cells further secrete IL-2 until a certain concentration is reached which results in the cytotoxic T-cells entering a refractory

state where they cannot secrete any new IL-2. Instead, these cytotoxic T-cells rely on the circulating IL-2 to remain activated (Cox *et al.*, 2011).

Cumulatively, the immune system is an interconnected network of cells, proteins and cell signalling molecules that are activated in response to a foreign stimulus and maintain homeostasis. Due to a multitude of factors that can affect immunity, multiple techniques have been developed. In the following section, I will briefly describe some of the main techniques used to describe the immune system clinically and in research studies.

1.4.2. Techniques to measure immune function

It is essential to understand the immune response for clinical, biological, and basic sciences studies investigating possible mechanisms, therapeutics, and interventions. Thus, numerous methods have been established and verified for measuring immune function in various biological samples including blood, plasma, urine, stool, and cerebrospinal fluid. Many studies investigating immune response commonly use cytokine measurements among others.

1.4.2.1. Cytokine measurement

Cytokines such as IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-γ and IL-17a are small proteins that play an important role in cellular signalling and the regulation of the immune system, as reviewed in the previous section. They can be measured through flow cytometry on the cells which express them (for example, IL-6 on the surface of monocytes and dendritic cells). The levels of cytokine produced by immune cells (and an indirect measurement of their activation) can also be quantified in body fluids, including plasma, mucous, urine and the cerebrospinal fluid. Techniques such as enzyme-linked immunosorbent assays (ELISA) and multiplex assays allow the quantification of cytokines. Those can also be run in tissue culture supernatants to assay their function (Khalifian *et al.*, 2015; Mountjoy, 2021). This provides valuable information about the immune system against infectious responses, including the balance of pro-inflammatory and anti-inflammatory cytokines throughout the body including activation.

These techniques – among others – have been used to measure immune activity and determine their relationship with sleep.

1.5. Diurnal Rhythms of Immune Function and Bidirectional Relationships Between Sleep and Immune Function

1.5.1. Diurnal rhythms of immune function

Diurnal rhythms of immune markers pose the question of whether they are under a pure circadian influence (i.e., under the regulation of the master circadian clock, the SCN), or under a pure sleep influence (i.e., disappearing once sleep does not occur during the biological night) or a combination of both. In the following paragraph we will show how studies were able to tease apart these two main influences on diurnal rhythms. Technically a third influence of posture (i.e., during the biological day we are sitting or upright, vs during the biological night, we are typically in a supine posture) would also need to be considered but the studies reviewed here mainly focused on sleep vs circadian influences.

The immune system exhibits a diurnal release of pro-inflammatory cytokines including IL-1, IL-6, IL-8, and TNF-α (Besedovsky *et al.*, 2012). In healthy young men, these cytokines are highest during the night and decline as the night ends and are lower during the day compared to nighttime (Born *et al.*, 1997; Dimitrov *et al.*, 2007; Lange, Dimitrov and Born 2010; Besedovsky, Lange and Haack 2019). This supports the claim that sleep favours immune defence against microbial infection.

The rhythmicity of cytokine release during the night aligns with the rhythmicity of the cells expressing those cytokines on their surface. Some of those diurnal rhythms are attributed to endogenous circadian rhythms while others are related to sleep itself. For example, using PBMC techniques, certain monocytes, such as IL-6^+, and IL-10^+ monocytes, continue to exhibit circadian release even in individuals who are totally sleep deprived for 24 hours (Lange *et al*, 2010). Interestingly, in that study, IL-6^+ monocytes rose to similar levels as when the individuals were not sleep deprived, while IL-10^+ monocytes showed a biphasic release pattern during the biological night, albeit at a lower concentration, during sleep deprivation compared to normal sleep (Lange *et al.*, 2010). On the other hand, the circulation of TNF-α^+ monocytes, IL-12^+ monocytes, and IL-12^+ dendritic cells decreased significantly during the night of total sleep deprivation vs. during the 8-hour sleep episode, thus suggesting that those were more under the influence of sleep itself (Lange *et al.*, 2010). These cell populations show a decrease in the first half of the night when individuals are sleep deprived, whereas they increase in individuals who have had a full night's sleep. These findings

contrast with the elevated daytime pro-inflammatory cytokine levels observed in sleep-deprived individuals, which will be discussed later in this section. Additionally, studies have shown that IL-6 is higher during the biological night when sleep occurs than during the biological day when wake occurs. In addition, they found higher serum IL-6 levels during N1, N2, and REM sleep, and lower levels during SWS sleep (Redwine *et al.*, 2000; Vgontzas *et al.*, 2005; see Appendix A). After an acute partial sleep deprivation (participant kept awake the first 4 hours of their habitual sleep episode), IL-6 concentration during the day was higher than after a normal night's sleep. During the acute PSD itself, when participants were kept awake, IL-6 concentration stayed low and when participants were allowed to fall asleep, IL-6 rose to about the same levels as the highest level observed on a normal night's sleep (Redwine *et al.*, 2000; Vgontzas *et al.*, 2005; see Appendix A). These findings emphasize the intricate interplay between circadian rhythms, sleep, and the immune system.

Overall, it seems that either due to circadian rhythmicity or sleep itself, there is a higher circulation of activated immune cells and expression of pro-inflammatory cytokines during the sleep episode than during wake in individuals who had undisturbed 8 hours of sleep. However, this balance has been shown to be modified by different types of sleep deprivation. Conversely, immune activation has been shown to disrupt sleep architecture. The two following paragraphs will examine this bidirectional relationship between sleep and the immune system.

I will first show evidence of how immune activation disrupts sleep. I will then show how different paradigms of sleep disruption may be associated with changes in immune markers.

1.5.2. Bidirectional relationship between sleep and immunity

There is a bidirectional relationship between the immune system and sleep whereby normal sleep is associated with a pro-inflammatory state during the biological night and some markers of immune activity modify sleep architecture.

1.5.2.1. Immune activation disrupts sleep.

The effect of immune activation on the sleep-wake pathway was first observed in animals expressing sickness behaviour during infections. Indeed, during sickness behaviour, animals display depression, lethargy, fever, reduced appetite, and long periods of rest/sleep to name a few (Hart and Hart, 2019). The observation of similar sickness behaviours between animals

and humans led to numerous human and animal studies investigating the interaction between the immune system and sleep.

i. Evidence from controlled human studies supporting higher acute immune activation is associated with longer NREM and decreased REM with no change in sleep duration.

In a randomized placebo-controlled and balanced study, young, healthy male participants were acutely injected once at 11:00 PM with a bacterial endotoxin (Lipopolysaccharide, LPS) of either 0.2 ng/kg, 04 ng/kg or 0.8 ng/kg or a placebo, to explore the impact of infection and subsequent immune activation on sleep behaviour (Mullington *et al.,* 2000; see Appendix A). The researchers reported a dose-dependent increase in TNF-α, IL-1a, and IL-6 whereby 0.2 ng/kg of LPS had the lowest increase in cytokines and 0.8 ng/kg had the highest increase in pro-inflammatory cytokine concentration (Mullington *et al.,* 2000). Moreover, they reported that 0.8 ng/kg of LPS led to the development of a fever (temperature > 38 °C) from 2:00 AM to 6:00 AM (Mullington *et al.,* 2000). No effects were observed in this study on either intervention on total sleep duration, but there was an increase in the amount of NREM in the participants after receiving 0.4 and 0.8 ng/kg of LPS (Mullington *et al.,* 2000). In addition, in the night after 0.8 mg/kg LPS injection, there was a decrease in the duration of REM, along with an increased latency period before REM sleep onset (Mullington *et al.,* 2000). Furthermore, the administration 0.4 and 0.8 ng/kg of LPS at 11:00 PM initially caused a temporary decrease in white blood cell count (leukopenia) ~ 4 hours after LPS administration, followed by a subsequent increase (leucocytosis). The administration of LPS also resulted in an increase in cortisol, and higher circulating levels of TNF-α and IL-6 sampled hourly during the sleep episode after the 0.4 and 0.8 ng/kg LPS injection compared to 0.2 ng/kg of LPS and placebo (Mullington *et al.,* 2000).

This experimental study coupled with other human studies highlights the impact of infection and immune activation on sleep behaviour in healthy humans. Mechanisms by which IL-1β, IL-6, and TNF-α modify sleep architecture have been associated with how they upregulate the hypothalamic sleep-active neurons while down-regulating the physiological levels of wake-active neurons to maintain sleep (Krueger, 2008; Zielinski *et al.,* 2016).

ii. Evidence that chronic immune activation disrupts sleep in patient populations: high chronic activation is associated with more disrupted sleep.

Patients with Guillain Barré Syndrome (excluding patients with chronic respiratory or cardiac issues, chronic pain, paralyzed, or had advanced metabolic disorders), an acute autoimmune neurological disorder leading to root and peripheral nerve demyelination, were reported to have reduced total sleep time and sleep efficiency, decreased REM sleep time, arousal index, and increased wake time after sleep onset (Bahnasy *et al.,* 2018). After a month of immunotherapy to alleviate Guillain Barre symptoms, polysomnography still showed similar abnormal sleep architecture as during the acute episode (Bahnasky *et al.*, 2018).

Furthermore, several studies on cancer patients have consistently reported a higher prevalence of poor sleep quality, difficulty initiating sleep, and higher number of arousals after sleep onset, even when adjusting for pain (Wang *et al.,* 2010) whereas some studies did not account for pain (Clevenger *et al.,* 2012; Tucker *et al.,* 2021). Studies have reported a significant association between high IL-6 levels and poor sleep quality, and sleep disorders before undergoing either chemotherapy or surgery (Wang *et al.*, 2010; Clevenger *et al.,* 2012; Tucker *et al.,* 2021). In the studies that did not account for pain, we cannot be certain if pain contributed to poor sleep alone, or whether the immune response during cancer contributed to poor sleep or whether both contributed to poor sleep. In adjusted models, lower sleep quality, as measured by the global PSQI score, was independently associated with higher daytime circulating IL-6 levels (Clevenger *et al.,* 2012). However, these studies also revealed that one year after successful treatment, daytime IL-6 levels significantly decreased, and patients reported improvements in their sleep quality. In turn, increased immune activation (i.e., IL-6) in cancer patients contributes to poor sleep. Successful completion of cancer therapy has shown a positive effect of successful treatment on both IL-6 levels and sleep quality.

There is therefore evidence, from human experimental and clinical studies that immune activation have an effect on sleep composition. Indeed, there is also evidence that sleep has an effect on immune activation. Hence a reciprocal relationship exists between the immune system and sleep. In the following, by describing the results of experimental sleep deprivation studies (also summarized in Appendix A), I will show the evidence that sleep affects immune activation.

1.5.2.2. Effects of sleep deprivation on immune function

Experimental human sleep studies assessing the levels of pro-inflammatory cytokines after either acute or chronic (>24 hour) partial or total sleep deprivation have extensively demonstrated that nighttime levels of IL-1, IL-6 and TNF-α are decreased in the plasma or serum compared to a baseline night of uninterrupted sleep (Vgontzas *et al.,* 1999; Irwin *et al.,* 2006; Haack *et al.,* 2007; van Leeuwen *et al.,* 2009; Axelsson *et al.,* 2013; Hunt *et al.,* 2021, see details in Appendix A). The levels of these cytokines are reported to be significantly increased the following morning compared to a night of uninterrupted sleep (Vgontzas *et al.,* 1999; Irwin *et al.,* 2006; Haack *et al.,* 2007; van Leeuwen *et al.,* 2009; Axelsson *et al.,* 2013; Hunt *et al.,* 2021, see details in Appendix A). In two experimental studies, C-reactive protein (CRP), a marker of the monocyte-macrophage pathway activation was significantly higher in participants undergoing 88 hours of total sleep deprivation, or 4.2 hours of partial sleep deprivation for 10 days when compared to controls who slept for 8.2 hours each night for 10 days (Meier-Ewert *et al.,* 2004; van Leeuwen *et al.,* 2009; Appendix A). Similarly, Haack *et al.,* (2007) and van Leeuwen *et al*. (2009) also reported elevated IL-6, CRP, and monocyte/macrophage activity (as measured through increased secretion of IL-1β, IL-6 and CRP) (Appendix A). These human studies did not focus on changes in sleep architecture triggered by sleep deprivation protocols. However, mouse models have given some insights as to the role of REM vs NREM in immune function. In those mouse models, researchers have shown that 72-hour REM sleep deprivation (obtained by putting rats on a platform surrounded with water, which they would fall into when they became atonic during REM sleep (see methods of Hakki *et al.,* 2001)) led to an elevation in pro-inflammatory cytokines from the monocyte/macrophage pathway (IL-1α, IL-1β, IL-6, TNF-α) and hyperthermia (Yehuda *et al.,* 2009; Table 2). They further reported that after a week-long sleep recovery period, TNF-α, and body temperature remained elevated in the mice (Yehuda *et al.,* 2009).

IL-2 plays an essential role in activating and regulating Tregs. Experimental sleep studies have reported that partially depriving healthy young adults of sleep for 3 to 5 days results in decreased daytime IL-2 activity (Irwin *et al.,* 1994; Axelson *et al.,* 2013; see Appendix A). The effect of acute sleep deprivation is seen in animal models that have demonstrated that acute sleep deprivation may lead to CD25 (IL-2 activation marker) gene deletion which

contributes to poor activation of Tregs, which may promote the development of autoimmunity in murine models (Willeford *et al.,* 1995; Xu *et al.,* 2019).

Unlike the pro-inflammatory cytokines discussed previously, the effect of sleep deprivation on IL-8 is less studied. As reviewed in section 1.2.1.1., IL-8 is an immune cell chemoattractant cytokine produced by monocytes and neutrophils (Zeng, Huang and Yao, 2012). Rats who develop spontaneous oral ulcers were sleep deprived for 72 hours. The investigators reported aggravated oral ulcer lesions, and elevated IL-1β, IL-6, IL-8 and TNF-α (Chen *et al.,* 2019) after the 72-h sleep deprivation. They also reported that IL-6 and IL-8 remained elevated in serum after 3 days of sleep recovery (Chen *et al.,* 2019; Table 2).

Human studies have shown that IL-8 is significantly elevated in the plasma of patients with obstructive sleep apnoea (OSA) compared to healthy controls (Li *et al.,* 2021). In another study assessing the effect of 14-hour (6:30 PM – 8:30 AM) vs. 24-hour (8:30 AM – 8:30 AM) shifts on urine IL-8 concentration in emergency room physicians, IL-8 levels had doubled after the 24-hour shifts and did not return to baseline levels after a single night of sleep recovery (Dutheil *et al.,* 2013). Thus, there is a possible elevation in daytime IL-8 concentration following states of sleep deprivation as seen in shiftwork, and in sleep disorders like OSA.

IL-17a is secreted mainly by Th17 cells, but can also be secreted at times by macrophages, neutrophils, and NK cells, as described in section 1.2.1.1. Once secreted IL-17a recruits and activates neutrophils and monocytes and is speculated to assist in the transition from innate to acquired immune activity (Zenobia and Hajishengalis, 2000). Although understudied in experimental sleep research, IL-17a is a target for research in drug development and its role in inflammation (Zenobia and Hajishengalis, 2000). The relationship between inflammation and sleep may have led to researchers investigate the role of IL-17a in sleep. Acute REM sleep deprivation in murine models and experimental partial sleep deprivation in human studies has been associated with significantly higher levels of daytime IL-17a in the blood (Mueller *et al.,* 2008; van Leeuwen *et al.,* 2009; Yehuda *et al.,* 2009; Cui *et al.,* 2019; Appendix A). They further report that despite an opportunity of uninterrupted sleep recovery, IL-17a remained elevated (Mueller *et al.,* 2008; van Leeuwen *et al.,* 2009; Yehuda *et al.,* 2009; Cui and Wan, 2019; Appendix A). This suggests that IL-17a may be sensitive to sleep deprivation, and daytime IL-17a would be elevated and not return to baseline levels after an opportunity of sleep recovery.

Under normal circumstance, there is a diurnal release of anti-inflammatory cytokines, which are low during the first half of nocturnal sleep, and gradually rise as an individual approaches wakefulness (Lange *et al.,* 2010). A study reported that healthy young men had higher levels of IFN-ɣ than IL-4 during the first half of the night when given an uninterrupted 8-hour sleep opportunity (Dimitrov *et al.,* 2004; see Appendix A). IL-4 levels rose during the second half of the night as the men drew closer to wake (Dimitrov *et al.,* 2004; see Appendix A). However, they reported that when the men were totally sleep deprived for 24 hours, they had lower production of both IFN-ɣ and IL-4 (Dimitrov *et al.,* 2004; see Appendix A). This finding contrasts with insomnia patients who are reported to have higher levels of nighttime anti-inflammatory cytokines IL-4 and IL-10 compared to pro-inflammatory cytokine, IFN-ɣ (Sakami *et al.,* 2002). Conversely, pro-inflammatory cytokines are higher during the night and lower during the day in healthy normal sleepers (Appendix A). These findings suggest that sleep deprivation, poor sleep quality and difficulty initiating sleep, creates a shift from nighttime pro-inflammatory to anti-inflammatory cytokines, ultimately perpetuates a cycle of sleep disruption and immune dysregulation (Appendix A).

This role of sleep regulating the immune system plays a pivotal role in fighting infections and acquiring immunity via vaccination. Studies have shown that individuals who get ≤ 6 hours of sleep are more susceptible to infections like the common cold and pneumonia and have a higher morbidity and mortality rate compared to individuals who get 7 to 8 hours of sleep (Cappuccio *et al.,* 2010; Patel *et al.,* 2012; Prather *et al.,* 2015; Taylor *et al.,* 2017). This suggests that sleep deprivation leads to a dampened immune response and may increase the pathogenesis and exacerbate the symptoms of the disease. For instance, in a rat model with oral ulcers, the rats had elevated daytime pro-inflammatory cytokines and aggravated oral ulcer lesions following REM sleep deprivation (Chen *et al.,* 2019).

This lower immunity is also seen during acquired immunity. In a study by Taylor and colleagues (2017), healthy college students underwent rigorous screening by clinical psychologists certified in sleep medicine and behavioural sleep medicine for either having normal sleep (n = 68) or insomnia (as defined by the DSM-V) (n = 65) (60% women in each group) through questionnaires (PSQI, ESS, Morning/Evening Sleepiness Questionnaire, GHQ, ISI, and Perceived Stress Scale) and interviews. Participants from both groups, normal sleepers (control) and insomnia, were vaccinated against influenza A at the same time of day (12:00 — 2:00 PM). Pre and post—four weeks vaccination showed that students with

insomnia had an overall lower antibody count against influenza virus A compared to the healthy controls. Moreover, it has been shown that adequate sleep before and immediately after vaccination improves antibody production and provides greater clinical protection against infection (Spiegel *et al.*, 2002; Lange *et al.*, 2003; Lange *et al.*, 2011; Prather *et al.*, 2012). This suggests that overall, poor sleep is associated with a lower appropriate immune response to an immune challenge.

Collectively, we have shown the bidirectional relationship between sleep and immunity. Insufficient sleep results in suppress immune activation that increases the susceptibility to infections and developing sleep and autoimmune disorders. It also results in an increased pro-inflammatory state during daytime. However, it should be noted (and is seen in Appendix A) most studies assessing the effect of sleep and immune system are conducted on men or a disproportionate number of men to women, despite women having a higher prevalence of developing sleep and autoimmune disorders. In the following paragraphs, I will present the relationship between sex, sleep quality and autoimmune manifestations. I will first describe the role of sleep on autoimmunity, then the role of female hormonal regulation on the immune system. This will lead us to our gaps in the literature, in particular that there are very few studies examining the relationship between sleep and the immune system in women.

1.6. Menstrual cycle, sleep and immune activity

1.6.1. Sleep and the menstrual cycle

The female sex hormones, oestrogen, and progesterone, fluctuate for an average of 28 days due to the menstrual cycle. This hormonal fluctuation affects the recruitment of immune cells to the endometrium for possible implantation. In two separate studies, women (aged 18 > years) were reported to have a higher REM latency during the high oestrogen, low progesterone – follicular phase. Inversely, they had lower REM latency during the high progesterone, and low oestrogen period – luteal phase of the menstrual cycle, while the NREM phases were unaffected (Driver *et al.*, 1996; Lee *et al.*, 1990; Okatani *et al.*, 2000). Indeed, studies have shown a greater incidence of sleep disturbances experienced by women during the luteal phase of their menstrual cycle (Bixler, 2009; Bixler *et al.*, 2009; Driver *et al.*, 1996).

1.6.2. Immune response and the menstrual cycle

Women have a greater cell-mediated and humoral response and have been shown to clear infections faster than men (high oestrogen, low progesterone) (Gay *et al.,* 2004, 2021; Kivity and Ehrenfeld, 2010; Walters *et al.,* 2003). Oestrogen, the predominant sex hormone in women, has a complex relationship with inflammation. Oestrogen is responsible for the growth and repair of the endometrial lining, development of the ova (egg cell) and release of the ova during ovulation. States of high oestrogen, namely follicular or proliferative phase, favours anti-inflammatory Th2, and humoral immune activation. Conversely, high progesterone state – luteal phase – favours pro-inflammatory Th1 cells which regulate cell-mediated immunity and activate monocytes (Berbic and Fraser, 2013; Gay *et al.,* 2021; Lélu *et al.,* 2011; Robinson *et al.,* 2014).

The uterine immune cell population changes throughout the menstrual cycle with the rise and fall of oestrogen and progesterone (Berbic and Fraser, 2013; Collins *et al.,* 2022). During the follicular phase of the menstrual cycle, oestrogen levels rise (Berbic and Fraser, 2013; Collins *et al.,* 2022). The rise in oestrogen increases the level of Treg cells in the uterus that suppress cytotoxic T-cell activity (Berbic and Fraser, 2013; Collins *et al.,* 2022). However, during the luteal phase, oestrogen decreases; this results in elevated leukocyte recruitment to facilitate the endometrial breakdown and clearance of debris from the uterine cavity when no fertilization occurs or prepare the endometrium for implantation of the fertilized ovum (Berbic and Fraser, 2013; Gay *et al.,* 2021). Furthermore, during pregnancy there is an up regulation of Th2 cells and a suppression of Th1 and Th17 cells resulting in an anti-inflammatory effect to allow for the foetus to grow without being attacked by the pregnant parent's immune system (Collins *et al.,* 2022). The body then transitions to a pro-inflammatory state towards the end of pregnancy to prepare the body for parturition (childbirth) (Mor *et al.,* 2011).

Lastly, menopause is characterised by low oestrogen levels. It has been observed to be associated with higher pro-inflammatory cytokines, increase monocyte and macrophage activity, and an elevated risk of T-cell mediated autoimmune disorders (Bixler, 2009; Driver *et al.,* 1996).

1.6.1.3. Women and autoimmune disorders

Approximately 4% of the world's population has an autoimmune disorder, of which 80% of the patients are women (Gleicher and Barad, 2007; Invernizzi *et al.,* 2009; Kivity and

Ehrenfeld, 2010; Straub, 2007; Whitacre, 2001). The onset of autoimmune disorders in females may commence as early as 15 to 20 years old during their reproductive age (Angum *et al.,* 2020; Desai and Brinton, 2019). This sex bias may be due to differences in immune activation, sex hormones, and even sleep patterns between the sexes. Although the ratio of predominance between the sexes (females: males) may vary between different autoimmune disorders from 9:1 in SLE, to 3:1 in MS, 1:2 in RA before the age of 50 but > 2 in women above 60 years of age (Desai and Brinton, 2019; Whitacre, 2001).

In a prospective cohort study in 1990 in France, Salliot and colleagues (2021) recruited 98 995 women between the ages of 40–65 years. The women answered questionnaires based on their lifestyle choices, reproductive state, and health conditions every two years, with twelve questionnaires answered in total. Women with incomplete questionnaires, self-reported or non-validated RA, and no menstruation had been excluded from the study. Salliot and colleagues (2021) reported an association between early age at first pregnancy (< 22 years) and early age of menopause (≤ 45 years) with increased risk of RA in a study population of 71 616 women. Salliot *et al.,* (2021) found no association between oral contraceptives and RA; they found an inverse relationship between perimenopausal progestin intake and RA. This suggests that a decline in sex hormone production increases the risk of RA in women and supplementing declining hormones during perimenopause may reduce the risk of autoimmunity.

This effect of reproductive hormones is not isolated to RA. In fact, many autoimmune disorders such as SLE, type-1 diabetes, MS and even psoriasis, can manifest, improve, or worsen during different states of the menstrual cycle (Desai and Brinton, 2019; Kim *et al.*, 2022; Whitacre, 2001; Yang *et al.,* 2023; Ysrraelit and Correale, 2019). For instance, states of high progesterone like the luteal phase or pregnancy can suppress RA and MS symptoms but worsen three to four months postpartum when progesterone drops. Conversely, high progesterone worsens SLE symptoms (Desai and Brinton, 2019).

1.6.1.4. Women, sleep, and autoimmune disorders

In epidemiological studies, poor sleep is associated with an increased risk of developing autoimmune diseases, suggesting a direct role of sleep in maintaining immune homeostasis. Individuals with sleep disorders such as insomnia and OSA and other types of sleep disturbances are shown to be at a greater risk of developing autoimmune diseases such as

SLE and RA (Hsiao *et al.,* 2015; Kang and Lin, 2012; Valencia-Flores *et al.,* 1999). Palma and Tufik (2010) reported that sleep deprivation elevated the production of antinuclear antibodies that target self-cells and reduced self-tolerance thus, accelerating the onset of SLE in their rat model.

In a longitudinal study following 84 996 Taiwanese with non-sleep apnoea sleep disorders (included acute and chronic insomnia, sleep disturbances, unspecified sleep disturbances in a 24-hour sleep-wake cycle, and dysfunction in sleep stages or sleep arousals) and 84 996 healthy age-matched controls (Hsiao *et al.,* 2015). In both groups, 60.4% were women over the age of 20 years that were recently diagnosed with sleep disorder but did not have a prior history of autoimmune disorders. They reported 7 731 new autoimmune disorder diagnosis in non-sleep apnoea sleep disorder patients, which was almost 1.5 times greater than the development of autoimmune disorders in healthy controls without sleep disorders (Hsiao *et al.,* 2015). This supports the claims that poor sleep – and subsequently sleep disorders – may increase the odds of developing autoimmune disorders.

Thus, women combined have a higher risk of having spontaneous lower sleep quality and a higher risk of developing an autoimmune disorder. Despite women's greater susceptibility to sleep and autoimmune disorders, insufficient studies have been conducted that either focused primarily on women or used a proportionate sex population in the study (Appendix A) to combat this imbalance.

Experimental studies have focused on the effect of restricted sleep (partial or acute) on immune markers. However, there is still no information on the role of the different sleep stages in immune homeostasis; is there a change in sleep architecture during acute sleep disruption? If so, is there an effect of change in sleep architecture on the immune. Another gap in the literature pertains to comparing different types of sleep disruption. Indeed, a much more natural form of disruption, instead of sleep restriction, is sleep fragmentation, which is found in sleep disorders such as OSA, periodic limb movement disorder or insomnia, autoimmune disorder patients such as SLE, MS, and RA as well as in both healthy and unhealthy aging (Alzheimer's and Parkinson's) individuals. As far as literature search has revealed there are no experimental studies focused on assessing the effect of sleep fragmentation (continuous disruption of sleep) on immune function. Lastly, there is an absence of research investigating the effect of sleep deprivation on young women despite women having a greater prevalence of sleep disorders and autoimmune disorders. Therefore,

this study aims to determine the effect of sleep restriction and sleep fragmentation on immune markers in otherwise healthy and young women.

1.7. Study Objectives

The study aims to determine and compare the effects of a single night of sleep restriction (restricting sleep to the first 4 hours of the night) compared with a single night of sleep fragmentation on immune function in healthy, young female participants during the follicular phase of the menstrual cycle.

Objective 1: The study investigates the effects of sleep restriction vs sleep fragmentation, compared with baseline sleep (8 hours of uninterrupted sleep) on:

- sleep parameters (N1, N2, N3, REM, amount of wake after sleep onset, total sleep time, sleep efficiency, sleep latency and REM latency).

Objective 2: The study investigates the effects of sleep restriction vs sleep fragmentation, compared with baseline sleep (8 hours of uninterrupted sleep) on:

- specific pro-inflammatory and anti-inflammatory cytokines (using a multiplex cytokine assay): IL-1β, IL-2, IFN-γ, IL-4, IL-6, IL-8, IL-10, IL-17a and TNF-α.

Objective 3: The study investigates the relationship between each sleep stage (N1, N2, N3, REM and amount of wake after sleep onset) on immune function as determined with the same markers as above.

CHAPTER 2: METHODS

2.1. Ethics

All screening and experimental procedures of this study were approved by the Human Research Ethics Committee of the University of the Witwatersrand (Clearance number: M201127), in accordance with the Declaration of Helsinki (Appendix B). All participants were explained screening and experimental procedures and gave written consent before starting the study.

2.2. Participant Screening and Enrolment

2.2.1. Inclusion and exclusion criteria

Figure 4 shows the overall flow of the study, from screening to the experimental sleep study conditions. Participants were recruited either through word of mouth or an email advertising the study to the undergraduate and postgraduate students registered at the University of the Witwatersrand, with the permission of the Faculty of Health Sciences registrar office (Appendix B and C). All eligible female participants were required to have a healthy body mass index (BMI: 18–26 kg/m^2), who are non-smoking, physically and mentally healthy aged 18–30 years, and not taking any chronic medications except oral contraceptives. Moreover, all eligible females were required to have a regular menstrual cycle, had regular, subjective good quality 8-hour habitual sleep with regular bedtimes and wake times and did not take regular naps. To ensure all volunteers met the eligibility criteria, each interested individual received a standardized pre-screening email before they were invited for further screening to ensure volunteers met the minimum eligibility requirements (Appendix D). Once they had cleared the pre-screening stage, they were screened using standardised questionnaires: the General Health Questionnaires (GHQ) (Williams and Goldberg, 1988) and the Pittsburgh Sleep Quality Index (Buysse *et al.*, 1989) where volunteers had to score less than 6 to be eligible for the study (section 2.2.2.).

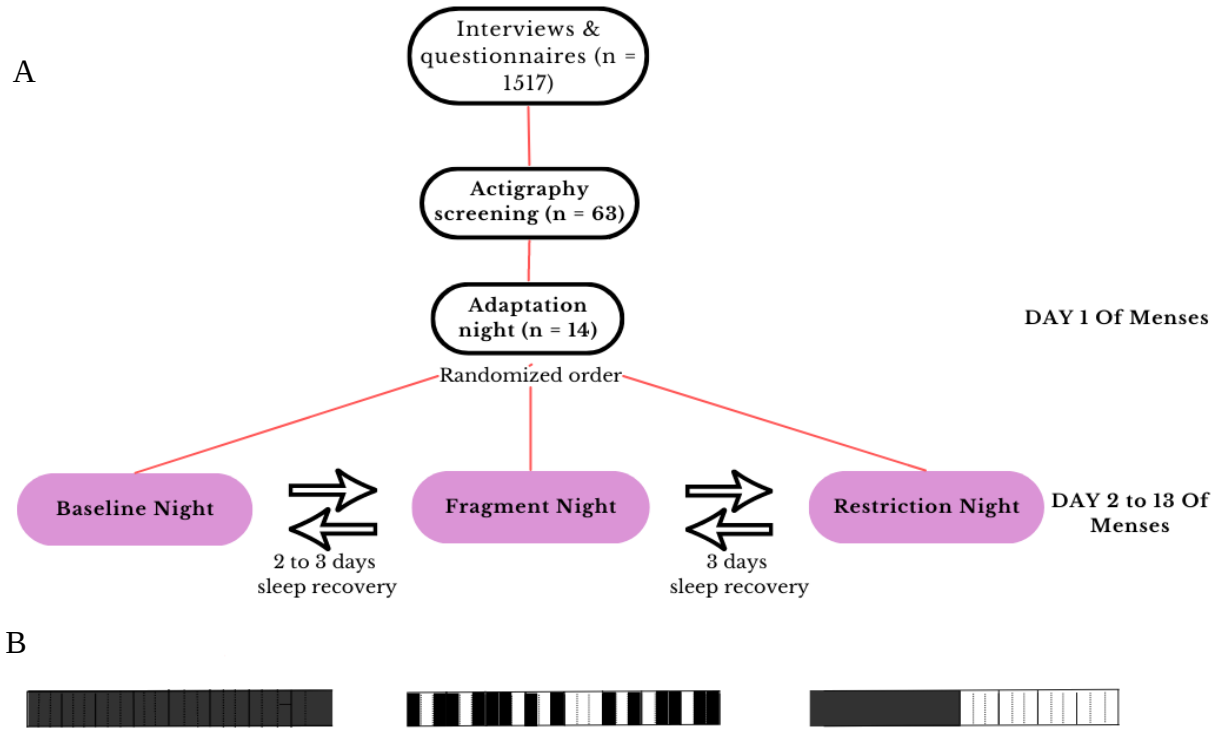


Figure 4: (A) Illustration of the study design. N = 1 517 interested volunteers were screened to participate in the study by being invited for interviews and questionnaires at the Wits Sleep Laboratory. N = 63 participants cleared the interviews and questionnaires and underwent a week-long screening phase consisting of actigraphy recordings and completion of a daily sleep diary. Only 14 participants met all the study requirements after screening and were invited to partake in in experimental phase, which began with spending the night at the Wits Sleep Laboratory for an "adaptation night" on the first day of menses.
(B) The three experimental nights occurred in a randomised order: Baseline, Restriction and Fragmentation nights. Each 8-hour night was divided into eight blocks with thick black lines showing 1-hour (60 minutes) intervals. Each hour block was further subdivided into three blocks of 20 minutes periods. The black blocks depict periods of allocated sleep time, and the white blocks represent periods of allocated wake time. During baseline nights (left) the participants were allowed a full, uninterrupted 8-hour (480 minutes) sleep opportunity. On the fragmentation nights (middle), in a randomised order, the participants were allocated a full 60-minutes block of forced awakening, and in the remaining seven hour-long blocks, the participants were woken up for a randomly selected 20 min period and were allowed to sleep for the remaining 40 min in each hour, according to Smith *et al.*,'s protocol (2007). On the restriction nights (right), participants were allowed to sleep for the first 4 hours (240 minutes) of the night and were kept awake for the remaining 4 hours of the night. Participants were given a 2-day sleep recovery period following baseline night, and 3 to 4 days of sleep recovery following fragmentation and restriction night.

2.2.2. Questionnaire screening

The screening and experimental procedures were explained to participants, who gave written informed consent before the beginning of the study. The volunteers were screened for physical and psychological medical conditions using customised (Appendix E) and standardised (Appendix F and G) questionnaires. The 30-item GHQ (Williams and Goldberg, 1988) was used to assess the psychological health of volunteers and needed to score less than 6 to be eligible for the study (Appendix F). A score of less than or equal to 5 indicated that the participants were not anxious or depressed and had an overall positive mental health (Williams and Goldberg, 1988). However, a score greater than 5 indicated that an individual is anxious or depressed (Williams and Goldberg, 1988).

Moreover, participants were required to have approximately 8 h of sleep per night with good subjective sleep quality, regular habitual bedtimes, and wake times, and did not engage in habitual daytime napping. The validated PSQI was used to assess the quality of sleep of the volunteers, and to eliminate potential sleep disturbances (Appendix G). The PSQI assessed seven domains (subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication, and daytime dysfunction) in participants which could be analysed separately. Volunteers needed to score less than 6 on the PSQI to be eligible which indicated participants had a good sleep quality with no nighttime disturbances (Buysse *et al.*, 1989). Next, eligible volunteers who had cleared the questionnaires and met the minimum requirements for the study had their weight and height measured using a weight scale and a wall height scale to calculate the BMI in the Wits Sleep Laboratory, 6th floor, Wits Faculty of Health Sciences campus. The BMI was calculated using the formula: weight (kg)/[height (m)]^2. Volunteers who met all requirements proceeded to undergo a week-long screening phase for daily activity recordings using an actigraphy device and sleep diary data by means of completing an electronic sleep diary every morning using the electronic database, Research Electronic Data Capture (REDCap).

2.2.3. Habitual sleep wake cycle screening phase

During the screening phase, volunteers were required to wear an Actiwatch Spectrum or Actiwatch 2 device (Philips Respironics, Murrysville, PA) on the wrist of their non-dominant hand to record their daily activity, and to complete an electronic sleep diary for all seven days of this screening phase. The participants were asked to record when they took off the

Actiwatch in the sleep diary which was completed every morning, shortly after waking. The customized sleep diary (Appendix H) contained information on the times the volunteers fell asleep, time of wake, self-reported sleep quality, morning vigilance, time and duration the Actiwatch was taken off (Appendix H). Sleep quality and morning vigilance were assessed on 100 mm visual analogue scales (VAS) anchored from "no sleep" to "best sleep I ever had", and from "not alert and fresh" to "most alert and fresh I felt", respectively (Appendix H). This week-long assessment of their sleep-wake cycle and daily routine was to ascertain that the volunteers had an 8-hour sleep-wake cycle with good quality sleep, regular bed- and waketimes, and did not habitually engage in daytime naps. The bedtime and wake times that were consistent for five out of seven days during this screening phase was used to determine the bedtime and wake time for participants during their experimental sleep nights.

2.3. Experimental Phase

After the screening, all eligible participants proceeded to the experimental phase of the study which entailed four nights in the Wits Sleep Laboratory. All experimental nights took place during the follicular phase (Days 1–13) of each participant's menstrual cycle. This was to rule out any effect the menstrual cycle (ovarian hormones) may have on sleep and the immune system described 1.6.2 (Chaireti *et al.*, 2016). All participants were instructed to refrain from caffeine and alcohol consumption, as well as acute medication use 24 h before each experimental night.

The first night for all participants was an "adaptation" night (Figure 4) which took place on the first day of the menstrual cycle (Day 1). After the adaptation night, in a randomised order, the participants underwent three experimental nights, namely: (i) baseline night (BN) – 8 hours of uninterrupted sleep; (ii) fragmentation night (FN) – participants experienced eight randomised intervals of forced awakenings through an 8 hour sleep opportunity; (iii) restriction night (RN) – sleep was restricted to the first 4 hours of the night and remained awake for the remaining 4 hours of the night. Following the BN participants had two nights of sleep recovery (sleeping at home) while a minimum of three to four nights of sleep recovery after both sleep disruption nights (FN and RN) to ensure that there were no effects of the experimental sleep nights when participants follow-up on their next experimental sleep night. However, there were three instances when participants returned to the lab exceeded the 2 to 4 days sleep recovery following an experimental sleep night: (i) If the sleep recovery period ended on either Friday or Saturday, and blood samples needed to be collected on

Saturday or Sunday morning, respectively. In this situation, participants were scheduled for a Sunday night and blood sample was taken on Monday morning as the clinical associate is unavailable on the weekend to collect blood samples. (ii) If participants encountered scheduling conflicts during their sleep recovery period, they would request to reschedule their next visit. (iii) In an average 28-day cycle, if a participant ovulated (Day 14) during the sleep recovery period, their study participation would be deferred to the following month; participants would return to the Wits Sleep Laboratory on Day 1 of the menstrual cycle to continue the experimental sleep nights in a new menstrual cycle.

2.3.1. Sleep adaptation night

The sleep adaptation night serves to create a familiarity between the participants and their new surrounding (sleep laboratory), and the experimental procedures (e.g., sleeping with the electrodes on their face and head for the PSG recordings). The purpose is to prevent any changes in sleep due to the procedures and new sleep environment that may affect the outcome of the study. The next morning, the electrodes were removed, and they were allowed to have an individualised breakfast of their choice. During the adaptation night, no additional procedures were conducted i.e., blood was not collected as was done following experimental sleep nights.

The three experimental nights were randomised for order using block randomization. To achieve this, I wrote down the six possible sequences for the experimental nights on separate pieces of paper placed inside a beaker: (i) BN FN RN (ii) BN RN FN (iii) RN BN FN (iv) RN FN BN (v) FN RN BN (vi) FN BN RN. The participants blindly picked a sequence from the beaker before leaving the laboratory which meant the choice of sequences decreased to 5, 4, 3, 2 and 1 after the first, second, third, fourth and fifth participant, respectively. When the six possible sequences had been picked, we would then start the roll out of the six sequences again. This was to ensure a balanced representation of the order of the experimental nights. The participants were not aware of the order, they only found out on the day.

2.3.2. Baseline night (BN)

Similar to adaptation night, participants were fitted with scalp, ocular, and chin electrodes for standardized PSG recordings. Participants were then allowed to sleep for 8 h at their habitual sleep time without any sleep interruptions. Upon awakening (at habitual wake times), all electrodes were removed from the participants. Participants had an individualized breakfast

consisting of a standard selection of breakfast items (bread, cereals, jam, butter, juice, and milk). Though breakfast was not identical between participants, there was a narrow range of breakfast choices, and we did not believe that having bread vs oat-based cereal may affect the cytokine measurements. Participants waited for 2.5 h to allow for sleep inertia (defined as a transitionary state between sleep and wakefulness where individual's performance is impaired and have a higher desire for sleep; Trotti, 2017) to dissipate, and 6 ml of blood were drawn by a certified clinical associate. Thereafter, participants were free to take a shower, and allowed to leave the Wits Sleep Laboratory to go about their day. They were instructed to return after two days of sleep recovery at home for their next experimental sleep night.

2.3.3. Fragmentation night (FN)

Following the same PSG electrode placement protocol, the participants went to bedrooms at their habitual bedtimes (lights-out; Table 1 in section 2.4.1). During FN, I followed the protocol described by Smith *et al.*, (2007) (Figure 3 (B)). Briefly: the 8-hour sleep opportunity was subdivided into eight 1 h blocks. Each hour was further subdivided into three blocks of 20 minutes. For seven out of eight 1-hour blocks, participants were randomly allocated 40 minutes sessions of sleep and 20 min of forced wake. For the remaining 1-hour period, each participant was forced to remain awake for the whole hour. During the eight intervals of forced awakening, the participants were to remain awake but were not permitted to look at screens (e.g., laptops and mobile phones) as studies have shown that light-emitting e-Readers alter the circadian rhythm of individuals and acutely enhance wakefulness (Chang *et al.,* 2015). Instead, participants were allowed to engage in activities such as studying, reading a book, talking, or playing with playdough to provide enough stimulation to remain awake. During each forced awakening session, I started a countdown timer of either 20 or 60 min once the participants were sitting upright in bed. I stayed in the room with each participant for the entire forced awakening session to ensure wakefulness. Once the time was up for the forced wakefulness session, the participants were allowed to return to sleep. Similarly, I started the timer when the participant first entered N2 to show the start of the sleep session. The participants were allowed to sleep undisturbed until the countdown timer ended. This process repeated until lights-on (Table 1 in section 2.4.1).

The participant's habitual wake time (lights-on) marked the end of the fragmentation protocol. The electrodes were removed, and they were given an individualised breakfast. Thereafter, 6 ml of blood were drawn about 2.5 hours after wake time (to allow for sleep

inertia to dissipate) and participants were allowed to leave the laboratory. The participants returned to the lab after 3 to 4 days of sleep recovery at home for their next experimental night.

2.3.4. Restriction night (RN)

Following the standard PSG electrode placement protocol (described in section 2.4.1), PSG electrodes were placed on the face and head of each participant. Once electrodes were placed, participants were allowed to sleep at their habitual bedtime (lights off; Table 1). For RN, participants were only allowed to sleep for the first 4 hours of an 8-hour sleep night. Once the participant was awake (4 hours after lights off) the study investigators were present in the room to ensure participants were sitting upright, awake and avoiding falling asleep. I initiated a 4-hour countdown to ensure participants stayed awake for the required duration (second 4 hours of the night). Similar to FN, participants were not allowed to use the blue-light emitting devices, however, they were allowed to do paper-based work such as studying, reading, drawing, or using play dough. At the end of the 4-hour wake period (at habitual wake time – lights-on), all electrodes were removed. Participants had an individualized breakfast (as per the other experimental nights), and 2.5 hours after lights-on, 6 ml of blood was drawn by a certified clinical associate. The participants were asked to return to the lab after four days of sleep recovery for their next experimental night.

2.4. Details of Experimental Procedures Conducted:

2.4.1. Polysomnography overnight recording

To obtain objective data on sleep, all participants were fitted with scalp, ocular, and chin electrodes for PSG readings on all four of their sleep nights (adaptation, baseline, fragmentation, and restriction night). The PSG recordings included electrode placements for EEG, EOG and EMG recordings. The electrode placements for the EEG included electrodes C3, C4, F1, F2, O1 and O2 referenced to A1 and A2, according to the international 10–20 system as per the American Society of Sleep Medicine (ASSM) (Iber, Chesson and Quan, 2007). More specifically, six PSG electrodes were placed on the scalp, one on the forehead (ground), and one electrode behind each ear on the temporomandibular joint (A1 and A2) as reference electrodes for the EEG recordings (Figure 5). The EOG recordings were done by placing one electrode 1cm to the right and 1 cm up for the right ocular electrode, 1 cm left and 1cm down for the left ocular electrode. Lastly, two electrodes were placed on the left and

right digastric muscles under the chin for the EMG recordings. EEG signals were run at a sampling rate of 200Hz, with a high-pass filter at 0.3 Hz and a low-pass filter at 3 Hz. Recordings were stored on a computerized EEG system (Cadwell Easy III, version 4.4.5.0/5.2.0.69, Cadwell Laboratories Inc, Kennewick WA). These data were used to determine total sleep time (TST), the time spent awake (to confirm that the sleep disruption procedures were successful), and all other sleep variables (see Table 1 for a full list).

After all electrodes were placed, participants conducted several exercises which included blinking their eyes, moving their eyes up and down, and left and right while their eyes were closed, grinding their teeth and yawning, to ensure that all aspects of the PSG recording were functional. Next, participants were sent to bed at their habitual sleep times (lights off) and the procedure for the respective night as described in section 2.3 was followed. The electrode placement was done by two investigator who alternated taking turns placing the electrodes each night. The investigators were satisfied with the electrode placement if the PSG reported impedance less than 5Ω – showing that there was minimal interference in recording.

At the end of the sleep study, the PSG recordings were scored in accordance with the American Society of Sleep Medicine guidelines (Iber, Chesson and Quan, 2007) by two different investigators. An 80% agreement between the two was required, and in cases where there was less than 80% agreement, a third investigator with sleep scoring experience was called upon to assist. The hypnograms of the experimental sleep nights are presented in Appendix I.

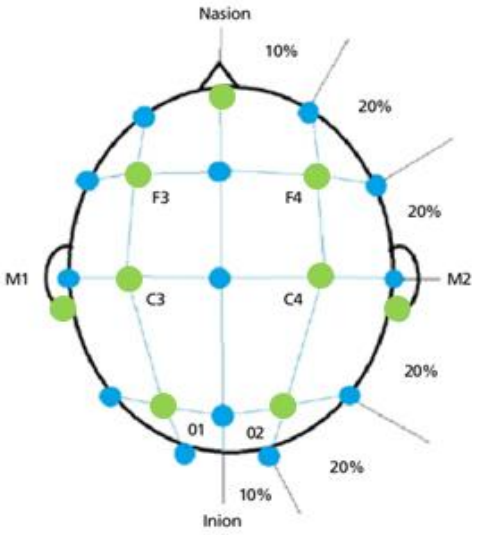


Figure 5: Electroencephalogram placements for electrodes depicted in green using the International 10-20 electrode placement system as per the American Society of Sleep Medicine (Iber, Chesson and Quan, 2007).
All the green placements were used. The blue dots are part of the ASSM but were not used in this study.

Table 1: Definitions of sleep parameters (Kryger *et al.*, 2014)

Sleep parameter	**Definition**
Arousal	An episode of wake lasting less than 15 seconds.
Lights off	The habitual bedtimes of participants when they would lay in bed and the investigator would turn off the lights.
Lights on	Habitual wake times of participants when the investigator would turn on the lights.
Non-rapid eye movement (NREM)	The total time or percentage spent in non-rapid eye movement sleep (Sum of N1, N2 and N3) sleep.
Rapid eye movement (REM) latency	The time taken from sleep onset to REM in a single epoch.
Sleep efficiency (SE)	The ratio between the time spent sleeping over the total time in bed.
Sleep onset latency	The time it takes to transition from wake to N1.
Sleep onset	The transition from wakefulness to sleep.
Time in bed (TIB)	The total time spent from lights off and lights on regardless of sleep and wake.
Total sleep time (TST)	The total time spent sleeping during a sleep recording from lights off to lights on.
Total wake time (wake)	The total duration a person was awake after sleep onset. It includes arousals, wake after sleep onset, and wake period for more than a single 30-second epoch.
Wake after sleep onset (WASO)	The total time spent awake after having initially fallen sleep – excluding arousals.

2.4.2. Blood sample collection and processing

A trained clinical associate collected 6 ml of blood from each participant about 2.5 hours after habitual waketime in an Ethylenediaminetetraacetic acid (EDTA) tube. I used the blood to isolate plasma to measure the pro-inflammatory and anti-inflammatory cytokines, and the buffy coat to isolate PBMC. A disposable 3 ml pipette was used to add 4.5 ml of Ficoll-Paque Plus (endotoxin tested, sterile solution, GE Healthcare Bio-Sciences AB, Sweden) into a 15 ml Falcon tube covered with aluminium foil to prevent the photosensitive histopaque from being exposed to light. Holding the Falcon tube at a 45° angle, blood was layered on top of the histopaque using a disposable 3 ml pipette. The layered blood was then centrifuged at 277 g in an Avanti J-26S XP centrifuge for 30 minutes with the brakes off. Once the blood had been centrifuged, the was isolated into a 5 ml glass tubes. The plasma was centrifuged again at 7871 g for 15 minutes at 4 ºC in a Biocen 22 R (Orto Alresa) centrifuge to completely remove the platelets and constituents from the plasma. The centrifuged plasma was then pipetted into two 2 ml clear, homopolymer, boil-proof microtube cryovials (PAXGMCT-200-C, Lasec) and then stored in a -80 ºC freezer at the School of Physiology.

2.4.3. Luminex assay

Dr Monica Gomes assisted in analysing the following cytokines were using a ProcartaPlex human cytokine kit (ThermoFisher Invitrogen Catalogue #PPX-09-MXRWFZ4; Bender MedSystems GmbH, Vienna, Austria): IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17a, TNF-α and IFN-γ. The 96-well kit's instructions and method were followed in duplicate. Briefly, the capture beads fluid was vortexed for 30 seconds before adding 50 µL of the capture beads fluid into each well. The plate was then washed with the washing buffer. Universal Assay Buffer (UAB) (25 µl) was subsequently added to each well. Standard and/or plasma samples (25 µl) were added to allocated wells in the plate. Background wells contained 50 µL UAB. The plate was sealed and incubated with shaking at room temperature (RT) for 2 hours. The plate was then washed twice, followed by incubation with shaking for 30 minutes at room temperature with the biotinylated detection antibodies. The plate was washed again followed by the addition of 50 µL streptavidin-phycoerythrin detection solution. The plate was incubated with shaking for 30 minutes at room temperature. The plate was washed again after which, a reading buffer was added to each well. The plate was incubated with shaking for 5 minutes at room temperature and then read using the Luminex 200TM instrument (Luminex

Corporation, Austin, TX, USA). The Median Fluorescence Intensities (MFI) with a bead count greater than 30 were used. The MFI for the standards were averaged then the average background was subtracted to read the final MFI. We calculated the limits of detection (LOD) and lower limit of quantification (LLOQ) for each cytokine (Table 2), which showed that IL-6 and IFN-γ was below the Luminex kit's detection. Fortunately, we had an ELISA kit available to measure IL-6, however, we did not have the necessary kit to measure IFN-γ. The final MFI values from the Luminex were used to generate a standard curve using a 5-parameter logistics regression. Cytokine concentrations for participants were interpolated from the standard curve using GraphPad Prism 9 (GraphPad Prism version 9.0.0 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

Table 2: Limit of detection (LOD) and lower limit of quantification (LLOQ) for each of the cytokines using the Luminex.

Cytokines	LOD	LLOQ (pg / ml)
IL-1	3.99	2.76
IL-2	4.10	8.79
IL-4	3.32	13.00
IL-6[a]	0.00	13.00
IL-8	16.49	2.54
IL-10	7.09	1.68
IL-17a	2.84	2.98
TNF-α	1.77	6.15
IFN- γ	3.78	13.00

IL = Interleukin IFN = Interferon TNF = Tumour necrosis factor

[a] measured using an ELISA.

Lower limit of detection (LOD): is the lowest analyte concentration at which one can distinguish from the concentration in the blank and the analytes can be detected.
Formula: Average MFI + (2 x standard deviation)

Lower limit of quantification (LLOQ): is the smallest concentration an analyte can be reliably detected and used for the study.
Used the values given as per manufacturer's guidelines.

2.4.4. Enzyme linked immunoassay (ELISA)

Dr Monica Gomes measured IL-6 concentration using an Elabscience Human IL-6 ELISA kit (Elabscience, Catalogue # E-EL-H6156) in duplicate as per the manufacturer's instructions. The 96-well plate was precoated with human IL-6 specific antibodies. The standards and plasma samples were added to the allocated wells of the plate and were incubated. The plasma contents were removed and biotinylated detection antibody specific for human IL-6 were added to the wells. The plates were once again incubated and washed. Next, avidin-horseradish peroxidase (HRP) conjugate was added to each well, incubated and then washed. A substrate solution was added to the wells which allowed for the wells containing human IL-6, biotinylated detection antibody and avidin-HRP conjugate to appear blue. A stop solution was then added which ended the enzyme-substrate reaction and changed the colour to yellow. Finally, the optical density was measured you spectrophometrically at a wavelength of 450 nm. I calculated the average wavelength for each standard and subtracted it from the blank. I calculated the LLOQ as described in 2.4.3. The final wavelength values from the ELISA were used to generate a standard curve using a 5-parameter logistics regression. Cytokine concentrations for participants were interpolated from the standard curve using GraphPad Prism 9 (GraphPad Prism version 9.0.0 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

Detection range: 6.25–400ng/mL; Sensitivity: 2.92 ng/mL

LOD: 8.73 ng/mL

LLOQ: 2.92 ng/mL

2.5 Statistical Analysis

I tested for normality of all sleep variables and cytokine concentrations using the Shapiro-Wilk test on R-studio (R-studio Team, 2020). Based on the test for normality and the objective, I either ran a univariate analysis of variance (ANOVA) or Friedman test between the independent variable: the experimental sleep nights (BN vs FN vs RN). If the univariate results showed significance (P-value < 0.05), a multivariate analysis was conducted next where I adjusted for order of the experimental sleep nights, day of the follicular phase of the menstrual cycle, and the age of participants (Table 3).

Table 3: Independent variables used in the study.

Independent variable	Type of variable	Level
Experimental sleep conditions	Nominal categorical	Baseline night (BN), Fragmentation night (FN), Restriction night (RN)
Order of experimental sleep conditions	Nominal categorical	First order night, second order night, third order night. E.g., If the randomized order is BN, FN, RN then BN is order night 1, FN is order night 2 is and RN is order night 3
Day of the follicular phase of the menstrual cycle	Linear	Day 1–13
Age (years)	Linear	18–30

Objective 1: The PSG recordings from the experimental sleep conditions for each participant were scored by two scorers who had 85% agreement between them. A comprehensive sleep report was generated on Cadwell Easy III which quantified the duration (minutes) and percentage of each sleep stage (N1, N2, N3 and REM), TIB, TST, SE, WASO, REM latency, and sleep onset latency (see Table 1 in section 2.4.1). Next, I used the Shapiro-Wilk test to test each sleep variable for normality. For descriptive statistics, I calculated the mean and standard deviation (SD) or the median [IQR] for each sleep variable in each experimental sleep condition. Based on results of normality testing on sleep continuous variables, a one-way repeated measures ANOVA (normally distributed outcome variables) or a Friedman test (non-normally distributed outcome variables) was run to determine the effect of the experimental sleep conditions, order of the experimental sleep conditions, day of the menstrual cycle, and age of participants (independent variables) on sleep architecture (dependent variable) using R-studio (R-studio Team, 2020). This was followed by a Tukey or Dunn post-hoc test.

Objective 2: The cytokine concentrations were presented with the median and interquartile range except for TNF-α which was represented with Median [Minimum; Maximum]. A mixed models analysis (Proc Mixed in SAS 9.4, using a random intercept, specifying an unstructured covariance matrix) was used to analyse the relationship between pro-inflammatory cytokines (IL-1β, IL-2, IL-6, IL-8, IL-17a, TNF-α and IFN-γ) as well as anti-inflammatory cytokines (IL-4 and IL-10) (dependent variables), and experimental sleep conditions, in an univariate analysis first, then in a multivariate analysis with the effect of experimental sleep condition on cytokine levels being adjusted for order of experimental sleep conditions, day of the follicular phase of the menstrual cycle and age (independent variables).

Objective 3: A mixed models analysis (Proc Mixed in SAS 9.4, using a random intercept and specifying an unstructured covariance matrix) to test the association between immune markers and sleep architecture variables obtained from the PSG: TST, WASO (Table 1 on section 2.4.1) over the 8-hour sleep opportunity (i.e., when the normal sleep episode would have occurred), total global NREM, amount of N2, N3, REM, and the number of arousals.

CHAPTER 3: RESULTS

3.1. Participants Demographics

Figure 6 shows the screening steps which led to the final participant enrolment number. A total of 1 517 volunteers showed interest in the study via email responses. During the email screening, volunteers were excluded if they did not meet the eligibility criteria described in section 2.1. Of the volunteers, 620 met the minimum requirements and were emailed custom links for screening questionnaires, which included the GHQ (Williams and Goldberg, 1988), and PSQI (Buysse *et al.*, 1989), through an online database system, REDCap (Harris *et al.*, 2009). Volunteers were required to score less than 6 on both the GHQ and the PSQI (Buysse *et al.*, 1989; Williams and Goldberg, 1988). This screening reduced the numbers to 63 eligible volunteers who were invited to further complete a week-long screening phase where actigraphy and daily sleep diaries were administered to ensure that volunteers had regular sleep-wake cycles. During the week-long screening phase, an additional 42 volunteers were excluded. Of the 21 eligible volunteers who remained: four dropped out before the study commenced, and three were unable to complete all their experimental sleep nights. In the end, a total of 14 female participants completed all the experimental conditions (Figure 6), and their demographic and basic screening characteristics are displayed in Table 4.

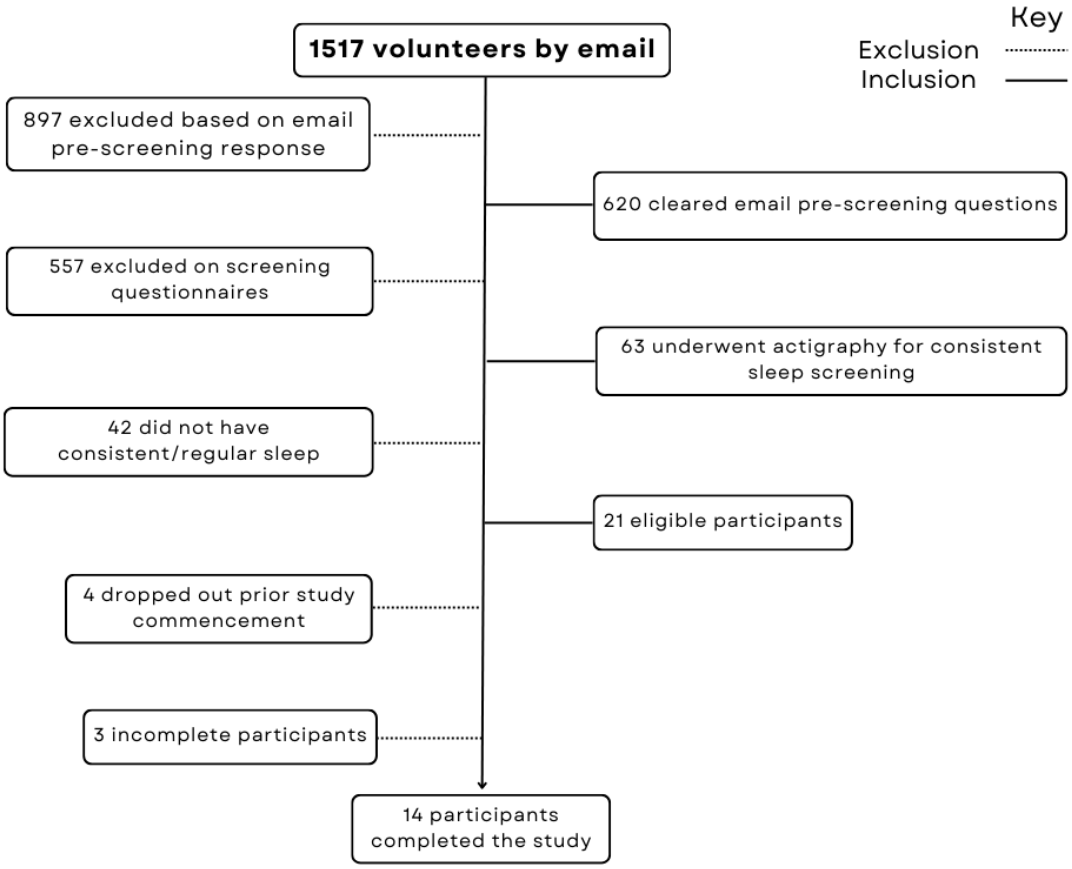


Figure 6: Flow diagram illustrates the volunteer recruitment process, eligibility, and final number of participants.

Table 4: Characteristics of the 14 healthy female participants who completed all sleep conditions presented.

Feature	Mean (SD)
Age (years)	21 (2)
Body mass index (kg/m^2)	22 (3)
Total GHQ score	1 (1) [0—4][1]
Total PSQI score	3 (2) [0—5][1]
Average Sleep Quality VAS (mm)[2]	83 (7)
Average Morning Vigilance VAS (mm)[3]	82 (8)

VAS: Visual analogue scale

[1]The range [minimum—maximum] was used to show the distribution of questionnaire results.

[2]The 100 mm VAS scale was anchored at 0 = "Worst sleep I have ever had" and 100 = "Best sleep I ever had"

[3]The 100 mm VAS scale was anchored at 0 = "Not alert and fresh" and 100 = "Most alert and fresh I felt"

3.2. Effect of Experimental Sleep Nights on Sleep Parameters

Data for sleep latency, N1 (minutes), N2 (% TST), N3 (minutes) and N3 (% TST) were normally distributed, while the remaining sleep parameters were not normally distributed. As such, one-way repeated measures ANOVAs were used to compare the normally distributed sleep parameters over the three experimental sleep nights (sleep conditions, i.e., BN, FN, and RN). I used a non-parametric Friedman Test to test the association between non-normally distributed sleep parameters and experimental sleep conditions. Table 5 and Figures 7 and 8 show the changes in sleep parameters across the three sleep conditions. As shown in Table 5, there were no significant differences across the experimental sleep conditions in NREM (% TST), N1 (minutes), N2 (% TST), N3 (minutes), REM latency (minutes) and sleep onset latency (minutes).

Statistically significant differences ($P < 0.001$) were found between the three sleep conditions for various variables, namely: WASO (minutes), SE (%), TST (minutes), NREM (minutes), N1 (% TST), N2 (minutes), REM (minutes), REM (% TST), and total time awake (minutes) (Table 5), and ($P < 0.05$) between experimental sleep condition and N3 (% TST). WASO and total time awake (as defined in Table 1 of section 2.4.1) did not differ between the two experimental sleep disruption nights, i.e., FN and RN, however, the duration of WASO and total time awake had significantly increased compared to an uninterrupted BN. As expected from the experimental sleep disruption protocol, the duration was approximately 4 hours: specifically, almost 4 hours for FN and above 4 hours for RN (χ^2 (2) = 21.60; $P < 0.001$), as displayed in Table 5 and Figure 7. In contrast, during an uninterrupted BN, WASO and total time awake were significantly shorter; only 20 and 32 minutes, respectively (Table 5, Figure 7).

SE and TST were lowest during RN, and FN having a slightly higher SE and TST (49% vs 54%, 245 vs 281 minutes, respectively) (SE: $\chi^2(2) = 23.20$; $P < 0.001$; TST: $\chi^2(2) = 26.10$, $P < 0.001$). There was no difference between the duration of N1 (minutes) across the three experimental sleep nights, the proportion of N1 (%TST) had doubled during FN (9%) and RN (8%) compared to BN (4%). Conversely, the duration of N2 had halved from BN (308 minutes) to FN (159 minutes) and RN (143 minutes) (χ^2 (2) = 21.60; $P < 0.001$) but N2 (%) did not change across the three experimental sleep nights. Moreover, RN had the highest N3 (%TST) compared to BN (χ^2 (2) = 13.90; $P = 0.03$) but N3 (%TST) did not differ between FN and BN. Furthermore, the duration of NREM sleep (in minutes) exhibited a significant

decrease from baseline (BN) to FN and RN. Notably, RN participants demonstrated the shortest NREM sleep duration (225 minutes), whereas BN participants exhibited the longest NREM sleep duration (410 minutes), followed by FN participants (243 minutes). Despite these variations in NREM sleep duration, the percentage of NREM sleep (%) distribution during sleep remained consistent across the three experimental sleep conditions.

Lastly, the duration of REM halved from BN (76 minutes) to FN (37 minutes) and was a third of BN during RN (21 minutes) (χ^2 (2) = 13.90; $P < 0.001$) (Table 5). Overall, the findings highlight the impact of different experimental sleep conditions on various stages of sleep and sleep-related variables (Table 5, Figure 7).

I tested for the unadjusted effect of the order of randomization of the experimental sleep nights, days of the follicular phase of the menstrual cycle, and age on sleep parameters to check for possible confounding factors and collinear variables. However, there were no statistically significant effects of any of these factors on sleep parameters hence, I have opted to omit these results from my dissertation.

Table 5: The effect of three sleep conditions on the characteristics of sleep architecture parameters

Characteristics	**Baseline (BN)**	**Fragmentation (FN)**	**Restriction (RN)**	**Statistics**
Time in bed (minutes)	518 (36)	523 (49)	501 (13)	$\chi^2(2) = 5.81$ $P = 0.054$
Sleep onset latency (minutes)	8 (8)	26 (24)	13(15)	$F = 2.79$ $P = 0.08$
REM latency (minutes)	143 (111)	222 (126)	97 (60)	$\chi^2(2) = 5.15$ $P = 0.06$
Wake after sleep onset (minutes)	20 (21)	232 (40)^{A}	244 (18) ^{A}	$\chi^2(2) = 21.60$ **$P < 0.001$***
Sleep efficiency (%)	95 (5)	54 (6)^{A}	49 (3) ^{AB}	$\chi^2(2) = 23.20$ **$P < 0.001$****
Total sleep time (minutes)	486 (40)	281 (21)^{A}	245 (17) ^{AB}	$\chi^2(2) = 26.10$ **$P < 0.001$****
NREM (minutes)	410 (53)	243 (24)^{A}	225 (14) ^{AB}	$\chi^2(2) = 23.30$ **$P < 0.001$****
NREM (%)	84 (9)	87 (7)	92 (4)	$\chi^2(2) = 5.14$ $P = 0.07$
N1 (minutes)	20 (10)	24 (8)	20 (11)	$F = 0.25$ $P = 0.77$
N1 (%)	4 (2)	9 (3)^{A}	8 (4)^{A}	$\chi^2(2) = 12.50$ **$P < 0.001$****
N2 (minutes)	308 (58)	159 (33)^{A}	143 (19)^{A}	$\chi^2(2) = 21.60$ **$P < 0.001$****
N2 (%)	63 (10)	57 (12)	58 (7)	$F = 1.64$ $P = 0.21$
N3 (minutes)	81 (26)	60 (34)	62 (17)	$F = 2.98$ $P = 0.06$
N3 (%)	17 (6)	21 (11)	25 (8) ^{A}	$\chi^2(2) = 13.90$ **$P = 0.03$***
REM (minutes)	76 (44)	37 (20)^{A}	21 (11) ^{AB}	$\chi^2(2) = 13.90$ **$P < 0.001$****
REM (%)	16 (9)	13 (7)	8 (4)	$\chi^2(2) = 5.14$

				P* < 0.001*
Total time awake (minutes)	32 (23)	245 (43)[A]	258 (20) [A]	$\chi^2(2) = 21.10$ ***P* < 0.001****

Data are expressed as mean (SD).

The percentage (%) of NREM, N1, N2, N3 and REM are % of total sleep time.

A Friedman test was used to compare non-parametric sleep parameters (WASO, sleep efficiency, total sleep time, NREM, N2 (minutes), N3 (%), REM (minutes) and total time awake (minutes)) between the three sleep conditions: Baseline, Fragmentation and Restriction night.

Asterisks (*) are used to present significance.

*Significant difference $p<0.05$. ** Significant difference $p<0.001$.

Dunn's post-hoc analysis was used to compare the effect across the experimental sleep conditions.

A Significant difference compared to baseline night.

B Significant difference compared to fragmentation night.

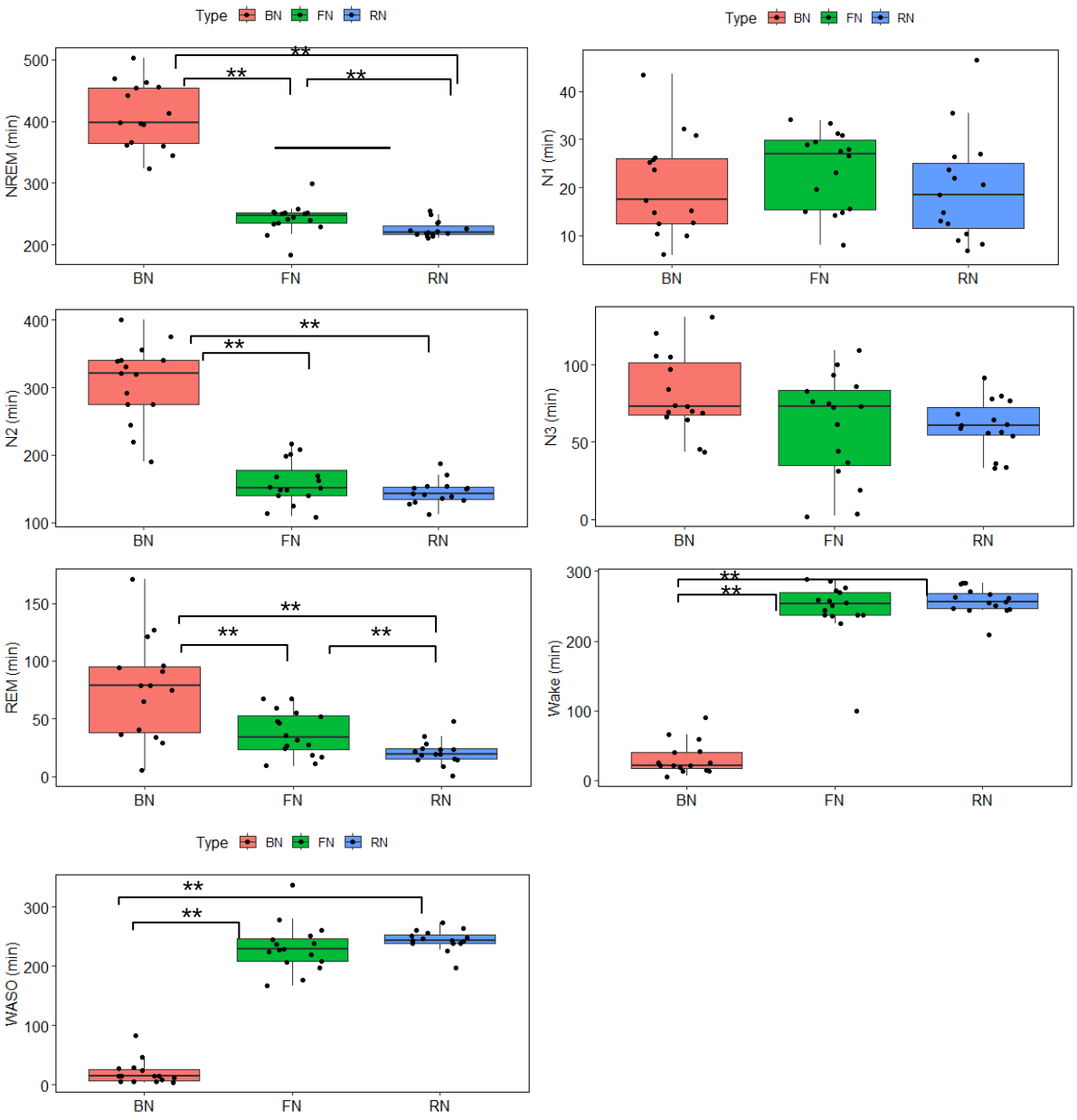


Figure 7: Distribution of time spent in minutes for the various sleep parameters (NREM, N1, N2, N3, REM, total time awake and wake after sleep onset (WASO)) across the three experimental sleep conditions: baseline night (BN), fragmentation night (FN) and restriction night (RN). A Tukey-post hoc was done for N2 (minutes) whereas a Dunn post-hoc was run for N1, N3, REM, TST and WASO. N = 14 for each experimental sleep condition (BN, FN, RN)

***P*-value < 0.05 ** *P*-value < 0.001

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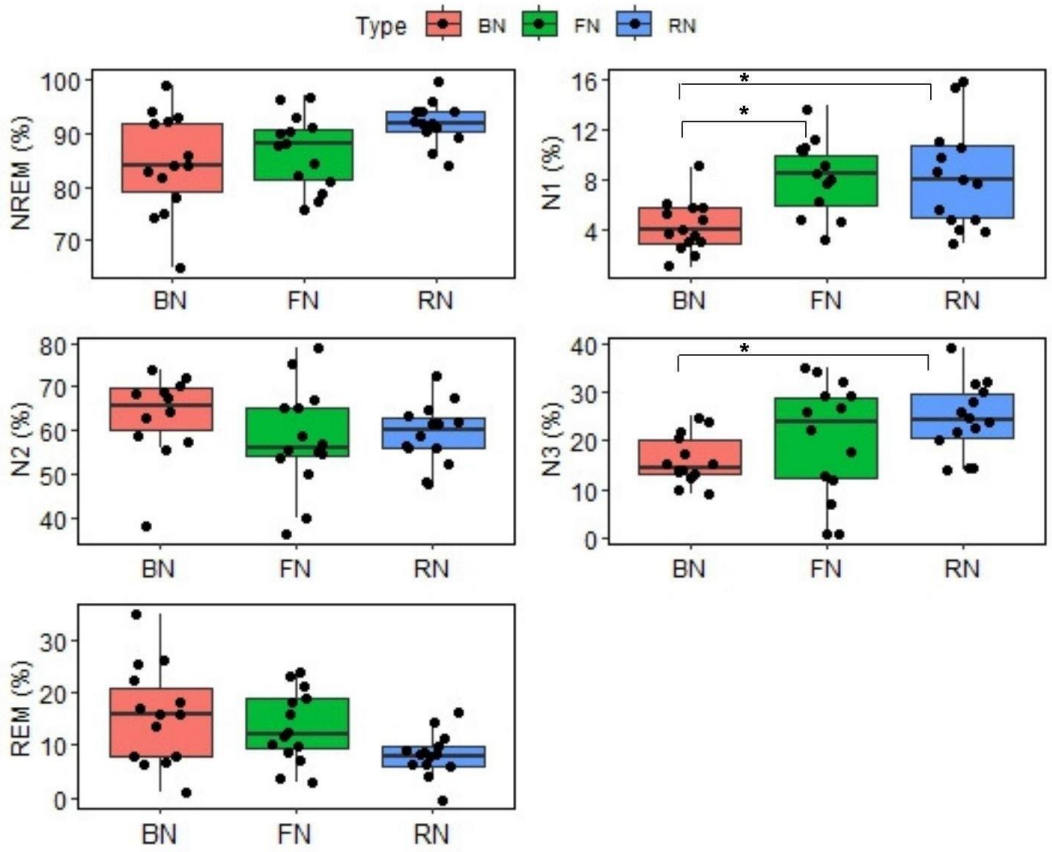


Figure 8: The percentage distribution for the various sleep stages (NREM, N1, N2, N3, REM) out of total sleep time across the three sleep conditions: baseline night (BN), fragmentation night (FN) and restriction night (RN). A Tukey-post hoc was done for N1 (%) whereas a Dunn post-hoc was run for NREM, N2, N3 and REM

N = 14 for each experimental sleep condition

*****P-value < 0.05.

3.3. Effect of Experimental Sleep Nights on Immunity

To determine the effect of the experimental sleep deprivation on immunity (through measurements of various plasma cytokine concentration: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17a, TNF-α, and IFN-ɣ), I used a 5-parameter logistic (5PL) regression to create the standard curve and measure the concentration of the cytokines based on the MFI obtained from the Luminex. Due to the lower sensitivity of the multiplex Luminex to IL-6 and IFN-ɣ, no values were obtained for them using Luminex. Fortunately, I was able to obtain IL-6 concentration data, but not IFN-ɣ, using an ELISA (Table 6). In the assay, 34% of IL-1β, 18% of IL-2, 64% of IL-4, 26% of IL-8, 42% of IL-10, 23% of IL-17a, 85% of TNF-α and 100% of IFN-ɣ, were calculated as zero pg/ml concentrations (or undetectable), which makes me doubt the sensitivity of the kit especially for TNF-α and IFN-ɣ (Table 6) as it is not possible for there to be no cytokines present even in a healthy individual (Liu *et al.,* 2021). I have illustrated the distribution of the cytokines across the three experimental sleep conditions without zeroes i.e., excluded the undetectable values in Figure 10. Moreover, a Chi-squared test compared each of the measurable cytokines for 14 participants for each experimental sleep conditions and reported that there was no significant difference between the detectable cytokine concentration for experimental sleep condition (Table 6). Thus I opted to include the zero pg/ml concentration in my statistical analysis (Figure 10 and Table 7). A mixed-model analysis was conducted where I assessed the unadjusted and adjusted effects of experimental sleep conditions, order of randomization, days of the follicular phase of the menstrual cycles, and age, on the eight cytokines: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17a, and TNF-α (Table 6, Figure 9).

Table 6: N out of 14 with measurable cytokines for each experimental sleep condition.

Cytokine	BN	FN	RN	*P*-value
IL-1β	11	13	13	$P = 0.446$
IL-2	11	13	13	$P = 0.446$
IL-4	5	10	9	$P = 0.158$
IL-6	13	13	13	$P = 0.478$
IL-8	11	13	13	$P = 0.446$
IL-10	11	13	13	$P = 0.446$
IL-17a	7	10	12	$P = 0.159$
TNF- α	11	13	13	$P = 0.446$

The mixed model analysis revealed there was no association between experimental sleep conditions, order of randomisation of the experimental sleep conditions, days of the follicular phase of the menstrual cycle, and age of participants (independent variables), and IL-1β, IL-6, IL-17a, TNF-α, and IL-10 (dependent variables) (Table 7, Figure 9). The results also highlighted a univariate association between the experimental sleep condition and IL-8 concentrations (F = 3.40, P = 0.05), order of the experimental sleep conditions and IL-2 (F = 3.70, P = 0.04), and the days of the follicular phase of the menstrual cycle and IL-4 (F = 5.07, β = 0.08, P = 0.04). Due to the narrow age range of my participants (19 – 23 years), Table 7 also presents the normal cytokine levels in the plasma of 10 healthy young adults over the age of 18 (Jackman *et al.*, 2011).

Table 7: The median and interquartile ranges of the concentration for eight cytokines and the mixed model statistical effect of condition, order, day of the follicular phase of the menstrual cycle, and age on all the cytokines.

Cytokine	Concentration (pg/ml) – according to sleep condition	Effect of condition[1]		Effect of order[1]		Effect of follicular phase days		Effect of age		Normal values in plas *et al.*, 2011) (pg/ml)
		Unadjusted	Adjusted[ǂ]	Unadjusted	Adjusted[ǂ]	Unadjusted	Adjusted[ǂ]	Unadjusted	Adjusted[ǂ]	
IL-1β	**BN:** 7.51 [0.00; 7.64] **FN:** 7.64 [7.31; 7.78] **RN:** 7.51 [0.00; 7.71]	*F* = 0.67 *P* = 0.52	*F* = 0.45 *P* = 0.64	*F* = 0.43 *P* = 0.65	*F* = 0.03 *P* = 0.97	*F* = 0.53 *P* = 0.47	*F* = 0.06 *P* = 0.80	*F* = 0.10 *P* = 0.74	*F* = 0.09 *P* = 0.76	1.2(1.5)
IL-2	**BN:** 18.72 [18.62; 19.23] **FN:** 18.72 [18.30; 19.13] **RN:** 18.30 [0.00; 18.72]	*F* = 1.00 *P* = 0.38	*F* = 2.23 *P* = 0.13	*F* = 3.70 *P* = **0.04***	*F* = 2.93 *P* = 0.07	*F* = 3.75 *β* = 0.55 *P* = 0.06	*F* = 1.22 *P* = 0.28	*F* = 1.49 *P* = 0.23	*F* = 1.70 *P* = 0.20	4.35 (11.90)
IL-6	**BN:** 3.70 [3.32; 5.40] **FN:** 4.37 [3.281;6.41] **RN:** 4.39 [3.35; 5.65]	*F* = 0.36 *P* = 0.70	*F* = 0.22 *P* = 0.80	*F* = 3.04 *P* = 0.06	*F* = 0.87 *P* = 0.43	*F* = 0.44 *P* = 0.51	*F* = 1.43 *P* = 0.24	*F* = 0.49 *P* = 0.48	*F* = 0.55 *P* = 0.46	22.00(8.60)
IL-8	**BN:** 2.76 [2.60; 3.04] **FN:** 2.79 [2.60; 2.90] **RN:** 2.65 [0.00; 2.67]	*F* = 3.40 *P* = **0.05**	*F* = 2.12 *P* = 0.14	*F* = 0.81 *P* = 0.45	*F* = 1.41 *P* = 0.26	*F* = 0.00 *P* = 0.95	*F* = 1.60 *P* = 0.22	*F* = 3.20 *P* = 0.08	*F* = 2.51 *P* = 0.12	9.40(3.70)
IL-17a	**BN:** 0.60 [0.42; 0.60] **FN:** 0.80 [0.42; 6.29] **RN:** 0.42 [0.42; 1.34]	*F* = 0.29 *P* = 0.75	*F* = 0.44 *P* = 0.65	*F* = 3.21 *P* = 0.07	*F* = 2.24 *P* = 0.15	*F* = 2.09 *P* = 0.16	*F* = 0.92 *P* = 0.35	*F* = 0.89 *P* = 0.35	*F* = 1.16 *P* = 0.30	33.60 (2.86)
TNF-α	**BN:** 0.82—2.72 **FN:** 0.00—9.28 **RN:** 0.00—9.81	*F* = 0.63 *P* = 0.54	*F* = 0.19 *P* = 0.83	*F* = 1.73 *P* = 0.20	*F* = 2.08 *P* = 0.15	*F* = 1.38 *P* = 0.25	*F* = 2.77 *P* = 0.11	*F* = 2.15 *P* = 0.15	*F* = 2.89 *P* = 0.10	5.85 (0.39)
IL-10	**BN:** 0.00 [0.00; 3.75] **FN:** 3.67 [0.00: 3.74] **RN:** 3.67 [3.67; 3.74]	*F* = 1.31 *P* = 0.28	*F* = 1.36 *P* = 0.28	*F* = 0.58 *P* = 0.56	*F* = 0.39 *P* = 0.68	*F* = 1.91 *P* = 0.28	*F* = 0.00 *P* = 0.94	*F* = 1.41 *P* = 0.24	*F* = 1.24 *P* = 0.27	37.60(2.11)
IL-4	**BN:** 0.00 [0.00; 15.07] **FN:** 3.63 [0.00; 21.68] **RN:** 0.00 [0.00; 15.07]	*F* = 0.71 *P* = 0.51	*F* = 0.37 *P* = 0.70	*F* = 1.85 *P* = 0.20	*F* = 0.10 *P* = 0.85	*F* = 5.07 *β* = 0.08 ***P* = 0.04***	*F* = 0.78 *P* = 0.40	*F* = 0.65 *P* = 0.43	*F* = 2.09 *P* = 0.19	225(51.7)

Concentration of cytokine data are shown as Median [Quartile 1; Quartile 3] for each sleep condition. For TNF-α, the data are represented as Median [Min; Max] (see discussion on TNF-α)

The unadjusted values present the univariate analysis of the effect of experimental conditions on the pro-inflammatory cytokines whereas the adjusted presents the multivariate analysis that accounts for the order, day of the follicular phase of the menstrual cycle and age of the participants.

IL-4, IL-6, IL-8, IL-17a and TNF-α were log-transformed for the mixed models analysis, Beta (SE) are for the log transformed values. **P*-value <0.05 is significant. [1] The post-hoc test for significant values is shown in the text. The numerator and denominator of the degrees of freedom were 2 and 26 for IL-6 respectively, 2 and 22 for IL-1, IL-2, IL-8, IL-10, TNF-α, 2 and 10 for IL-4, and 2 and 14 for IL-17a. ǂ Adjusted models included all main effects (sleep condition, order of sleep condition, follicular phase days, and age).

Mean (Standard error) presented for the normal values of cytokines in the plasma of healthy young adults (Jackman *et al.*, 2011)

IL-2: There was no association between the experimental sleep conditions, the follicular phase of the menstrual cycle, and age on IL-2. However, there was a significant unadjusted association between order (F = 3.70, P = 0.04) and IL-2. The first order night was associated with the lowest IL-2 concentration (β = -5.16, P = 0.02) and highest IL-2 concentration on the third order night. Thus, it can be inferred that IL-2 concentrations increased with the order of experimental sleep nights (Table 7). Although there was no association between experimental sleep condition and IL-2, when assessing the combined association between experimental sleep conditions, the order of the experimental sleep conditions and IL-2 concentration, the significance of the P-value for experimental sleep conditions and order decreased compared to the unadjusted P-values (adjusted experimental sleep conditions: F = 1.98, P = 0.16 vs unadjusted experimental sleep conditions: F = 1.00, P = 0.38; adjusted order of the experimental sleep conditions: F = 4.74, P = 0.02 vs unadjusted order: F = 3.70, P = 0.04, respectively) (Table 7). This suggests a positive collinear association of the order of experimental sleep conditions. Lastly, there was no significant interaction between the experimental sleep conditions and the order of experimental sleep conditions on IL-2 concentrations.

IL-8: Similarly, there was no association between order of experimental sleep conditions, day of the follicular phase of the menstrual cycle and age, and IL-8 concentrations in the plasma (Table 7). However, unadjusted experimental sleep conditions (F = 3.40, P = 0.05) (Figure 9 and Table 7) was associated with changes in daytime IL-8 concentrations. IL-8 concentration was associated to decrease during RN (Median [IQR]: 2.65 pg/ml [2.51 pg/ml—2.67 pg/ml]) compared to BN (2.76 pg/ml [2.60 pg/ml—3.04 pg/ml]). There was no difference in IL-8 concentration between FN (2.79 pg/ml [2.60 pg/ml—2.90 pg/ml]) and BN (Figure 9).

IL-4: There was no association between experimental sleep condition, order of sleep condition and age on IL-4 concentration (Table 7). However, there was an unadjusted association between the days of the follicular phase of the menstrual cycle and IL-4 concentration (β = 0.08, F = 5.07, P = 0.04). Daytime IL-4 concentration was associated to increase each day of the follicular phase of the menstrual cycle.

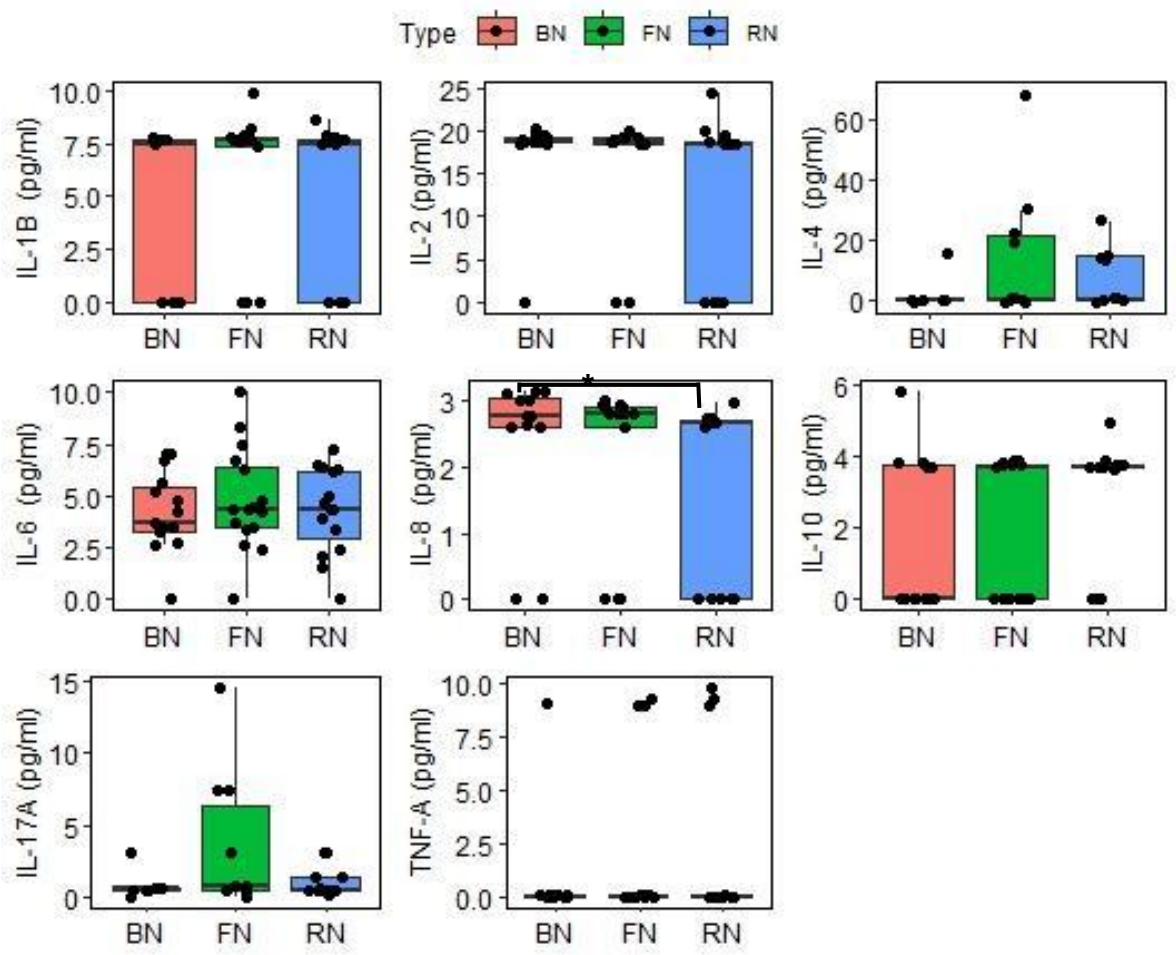


Figure 9: Distribution of cytokine concentrations (pg/ml) during the three sleep conditions: baseline night (BN), fragmentation night (FN), and restriction night (RN).

The mean fluorescence intensity (MFI) of IL-1β, IL-2, IL-4, IL8, IL-10, IL-17a and TNF-α obtained from Luminex assay and IL-6 from ELISA was used to generate a standard curve for each cytokine on GraphPad Prism 6. The cytokine concentrations were measured from the MFI curve. However, some cytokine assays had negative MFI i.e., they were less than the blank (control) which I interpreted as there being zero pg/ml of cytokines. The statistical analysis of the study included zero pg/ml in our analysis. The graphs show the distribution of the cytokine concentration when zero pg/ml were included in the analysis.

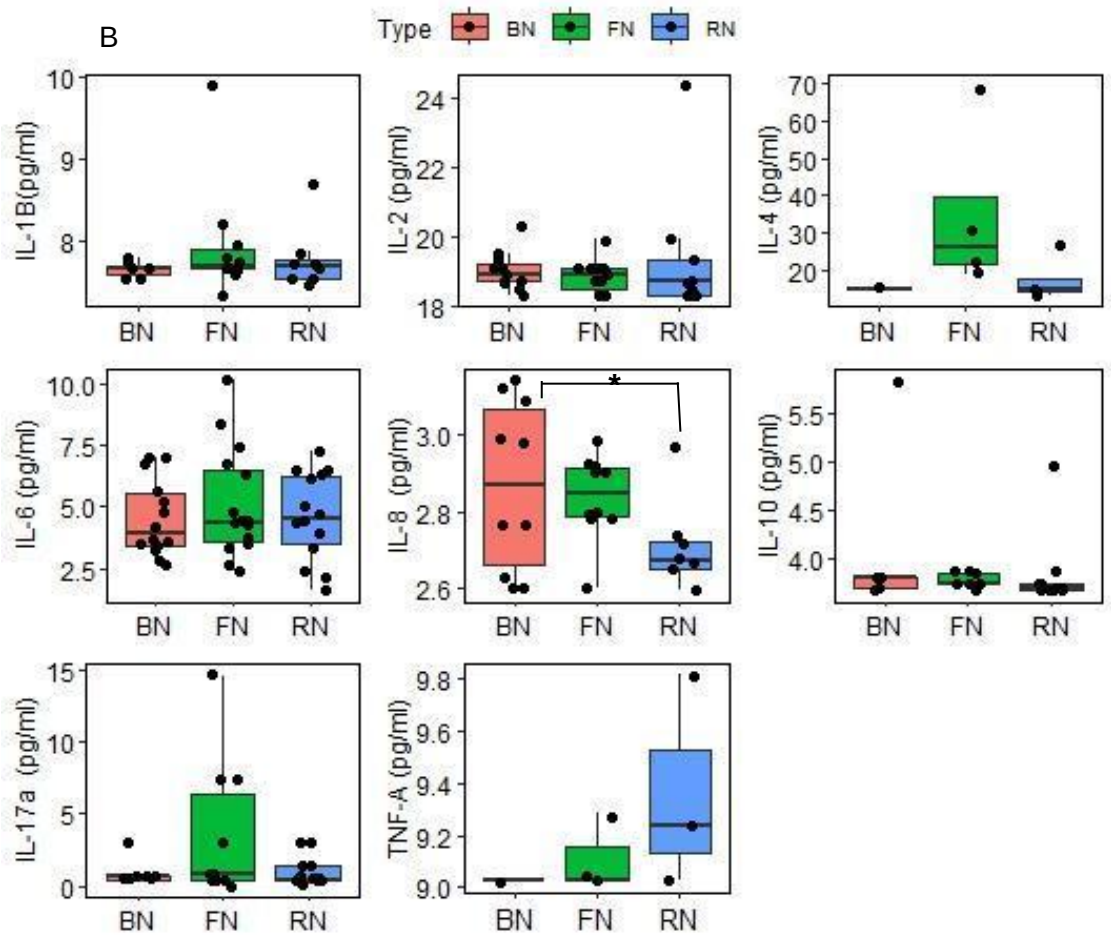


Figure 10: Distribution of cytokine concentrations (pg/ml) during the three sleep conditions: baseline night (BN), fragmentation night (FN), and restriction night (RN).

The mean fluorescence intensity (MFI) of IL-1β, IL-2, IL-4, IL8, IL-10, IL-17a and TNF-α obtained from Luminex assay and IL-6 from ELISA was used to generate a standard curve for each cytokine on GraphPad Prism 6. The cytokine concentrations were measured from the MFI curve. However, some cytokine assays had negative MFI i.e., they were less than the blank (control) which I interpreted as being undetectable. The statistical analysis of the study including zero pg/ml in our analysis. These graphs exclude the zero pg/ml values and only depict concentrations above zero pg/ml.

3.4. Association Between Sleep Architecture and Cytokines

The third objective of my study was to go beyond the simple effects of experimental sleep conditions, which resulted in different sleep architecture compositions, as seen in Section 3.1., and investigate the association between sleep architecture parameters and cytokine concentration levels directly (Table 8), rather than through the prism of sleep conditions.

As such, an unadjusted mixed model regression analysis was performed to investigate the association between individual sleep architecture parameters (TST, WASO, NREM, N2, N3, and REM – all in minutes, all tested separately in unadjusted models) and the absolute concentrations of cytokines. As shown in Table 7, longer WASO (minutes) was associated with lower IL-8 concentration (β = -0.0003, F = 4.39, P = 0.04). Shorter N2 (minutes) (β = -0.001, F = 4.50, P = 0.04) and longer N3 (minutes) (β = 0.004, F = 4.65, P = 0.04) were associated with higher TNF-α concentration.

Table 8: Relationship between sleep parameters and cytokine concentrations

Cytokines	**TST (minutes)**	**WASO (minutes)**	**NREM (minutes)**	**N1 (minutes)**	**N2 (minutes)**	**N3 (minutes)**	**REM (minutes)**
IL-1β	β = -0.00 *F* = 0.82 *P* = 0.37	β = 0.00 *F* = 0.55 *P* = 0.46	β = -0.00 *F* = 0.68 *P* = 0.41	β = -0.05 *F* = 0.68 *P* = 0.41	β = -0.00 *F* = 1.61 *P* = 0.27	β = 0.03 *F* = 2.31 *P* = 0.14	β = -0.01 *F* = 0.51 *P* = 0.48
IL-2	β = 0.00 *F* = 1.05 *P* = 0.31	β = -0.01 *F* = 1.63 *P* = 0.21	β = 0.01 *F* = 0.93 *P* = 0.34	β = -0.03 *F* = 0.07 *P* = 0.79	β = 0.00 *F* = 0.55 *P* = 0.46	β = 0.04 *F* = 1.29 *P* = 0.26	β = 0.02 *F* = 0.76 *P* = 0.39
IL-6	β = -0.00 *F* = 0.00 *P* = 0.97	β = 0.00 *F* = 0.00 *P* = 0.99	β = 0.00 *F* = 0.11 *P* = 0.91	β = 0.00 *F* = 0.02 *P* = 0.88	β = -0.00 *F* = 0.01 *P* = 0.93	β = 0.00 *F* = 0.33 *P* = 0.56	β = -0.00 *F* = 0.17 *P* = 0.68
IL-8	β = 0.00 *F* = 3.43 *P* = 0.07	β = -0.0003 *F* = 4.39 ***P* = 0.04***	β = 0.00 *F* = 3.25 *P* = 0.08	β = 0.00 *F* = 0.02 *P* = 0.89	β = 0.00 *F* = 3.70 *P* = 0.06	β = 0.00 *F* = 0.01 *P* = 0.91	β = 0.00 *F* = 1.86 *P* = 0.18
IL-17a	β = -0.00 *F* = 0.05 *P* = 0.82	β = 0.00 *F* = 0.10 *P* = 0.75	β = -0.00 *F* = 0.33 *P* = 0.57	β = -0.01 *F* = 1.83 *P* = 0.19	β = -0.00 *F* = 0.01 *P* = 0.92	β = -0.00 *F* =1.18 *P* = 0.29	β = 0.00 *F* = 0.48 *P* = 0.49
TNF-α	β = -0.00 *F* = 1.58 *P* = 0.22	β = 0.00 *F* = 1.60 *P* = 0.21	β = -0.00 *F* = 2.47 *P* = 0.12	β = -0.01 *F* = 3.16 *P* = 0.08	β = -0.001 *F* = 4.50 ***P* = 0.04***	β = 0.004 *F* = 4.65 ***P* = 0.04***	β = -0.00 *F* = 0.03 *P* = 0.85
IL-4	β = -0.00 *F* = 1.42 *P* = 0.25	β= 0.00 *F* = 1.01 *P* = 0.33	β = -0.00 *F* = 2.10 *P* = 0.17	β = -0.01 *F* = 1.57 *P* = 0.23	β = -0.00 *F* = 3.91 *P* = 0.07	β = 0.00 *F* = 1.84 *P* = 0.20	β = -0.00 *F* = 0.09 *P* = 0.77
IL-10	β = -0.00 *F* = 2.99 *P* = 0.09	*B* = 0.00 *F* = 1.55 *P* = 0.22	β = -0.00 *F* = 2.76 *P* = 0.11	β = -0.00 *F* = 0.07 *P* = 0.78	β = -0.00 *F* = 2.57 *P* = 0.12	β = -0.00 *F* = 0.25 *P* = 0.62	β = -0.00 *F* = 1.37 *P* = 0.25

*$P < 0.05$, β = Estimate. The numerator and denominator of the degrees of freedom were 1 and 27 for IL-6 respectively, 1 and 23 for IL-1, IL-2, IL-8, IL-10, TNF-α, 1 and 11 for IL-4, and 1 and 15 for IL-17a.

The analysis revealed there was no significant association between any of the sleep parameters (total time spent asleep, time spent in any sleep stage, time spent awake) with IL-1β, IL-2, IL-4, IL-6, IL-10 and IL-17a.

There was a significant association between WASO and IL-8 (β = -0.0003, F = 4.39, P = 0.04) whereby an increase in WASO resulted in a lower daytime IL-8 concentrations (Table 8). Lastly, TNF-α was not associated with TST, NREM and REM time. However, there was a significant association between minutes spent in N2 (β = -0.001, F = 6.28, P = 0.02) and N3 (β = 0.004, F = 7.01, P = 0.01) and daytime TNF-α concentration (Table 8).

CHAPTER 4: DISCUSSION

Sleep plays a pivotal role in regulating immune activation as seen by the literature. Existing data show that sleep deprivation results in dysregulation and pathogenesis of sleep and autoimmune disorders. They have further reported that many individuals with sleep disorders and sleep disturbances have symptoms and develop autoimmune disorders including RA, MS and SLE (Åkerstedt *et al.,* 2023; Desai and Brinton, 2019; Kotb *et al.,* 2013; Young *et al.,* 2018). Despite women comprising as majority of the sleep and autoimmune disorder patient population, studies assessing the relationship between sleep and the immune system in a female population are limited. Thus, it was imperative for me to investigate the effects of acute sleep disruption on sleep architecture, and pro- and anti-inflammatory cytokines in healthy young women, compared with a night of uninterrupted sleep.

Firstly, this study showed there was a relationship between the duration of TST (minutes), WASO (minutes), NREM (minutes), N1 (% TST), N2 (minutes), N3 (% TST), REM (minutes), REM (% TST), sleep efficiency (%), and total time awake (minutes), and acute experimental sleep disruption nights – FN and RN. This was partially anticipated as participants had a 4-hour sleep opportunity during a RN and a 4-hour 40 minutes opportunity during FN. This difference in sleep opportunities is due to the fragmentation protocol which led to multiple sleep onset latencies from the multiple forced awakenings. Both sleep disruption nights were associated with a decrease in NREM (minutes) and REM (minutes) duration, however, RN was associated with lowest NREM (minutes) and REM (minutes) duration. My results showed that experimental sleep nights were associated with lower daytime IL-8 concentrations particularly during RN. I further reported that IL-2 concentration was associated to increase with order of experimental sleep nights whereas IL-4 concentration was associated to increase with the days of the follicular phase of the menstrual cycle. Lastly, my study reported that a longer WASO was associated with a decrease in IL-8 concentration. Shorter N2 (minutes) and longer N3 (minutes) duration was associated with an increase in daytime TNF-α concentration.

4.1. The Effect of Sleep Conditions on Sleep Parameters

Sleep architecture was significantly different across the three experimental sleep nights. Due to the study design, participants experienced significantly longer time awake and poorer sleep efficiency during RN and FN compared to a night of uninterrupted sleep, BN.

Light N1 is typically the shortest sleep stage experienced by healthy adults (Kryger *et al.,* 2017, 2014). In my study, N1 (%TST) doubled during both RN and FN compared to BN. However, the total amount of N1 (minutes) was not different between all three experimental sleep conditions. There was no change in the duration of N1 (minutes) during FN in spite of the multiple forced awakenings. This is probably due to the build-up of homeostatic sleep pressure, leading to a shorter time to get back to sleep and transitioning nearly immediately to deeper stages of sleep after the forced awakenings (Hilditch and McHill, 2019; Trotti, 2017). This is in accordance with the 2-process sleep model and the accumulation of Process S during sleep deprivation (Borbély, 2022). Frequent arousals resulted in a build-up of sleep pressure experienced by participants during a fragmentation night as their sleep duration halved. The sleep pressure created by reduced sleep duration may have resulted in greater sleepiness, micro-sleeping, and falling asleep thus, in spite of the multiple awakenings during FN, the fragmentation protocol did not result in higher N1.

Conversely, the time spent in N2 (minutes) significantly halved during FN and RN compared to BN but did not differ between the experimental sleep disruptions (i.e., FN and RN). Similarly, N2 (% TST) and N3 (minutes) did not differ between the experimental sleep conditions. Furthermore, acute sleep deprivation resulted in a significant increase in N3 (%TST) during RN, accompanied by a corresponding decrease in REM (%TST). The duration of REM (minutes) halved during FN whereas RN was a third of BN. This would be expected as REM is mainly present in the second half of a sleep episode in normally entrained conditions. During RN, I allowed the participants sleep the first 4 hours of their normal sleep episode but kept them awake afterwards, while during FN, sleep was interrupted regularly throughout the sleep episode, allowing for a more equal distribution of the sleep stage curtailment. This further implies that during acute sleep deprivation, the brain prioritizes N3 sleep over REM sleep, particularly during restrictive sleep patterns.

Most partial sleep deprivation studies have shown the effect of sleep duration reduced to 4–6 hours either acutely or over a number of days. These studies have found a similar changes in sleep composition namely significant reductions in N1, N2 and REM with increased N3 (Parry *et al.,* 1999; Donga *et al.,* 2010; Philip *et al.,* 2012; Lu *et al.,* 2014). In contrast, chronic sleep deprivation may lead to an increase in REM sleep duration (Plante *et al.,* 2016).

4.2. Effect of Sleep Conditions on Cytokines

My study explored the effect of randomized acute experimental sleep deprivation, order of the experimental nights, day of the follicular phase of the menstrual cycle, and age on pro- and anti-inflammatory cytokines concentrations.

This study did not find an effect of a single night of experimental sleep conditions, order of experimental sleep conditions, day of the follicular phase of the menstrual cycle, and age on IL-1β, IL-6, IL-10, and TNF-α. However, existing studies had reported elevated IL-1β, IL-2, IL-6 and TNF-α in young men following partial and total sleep deprivation (See Appendix A; Vgontzas *et al.*, 1999, 2005; Redwine *et al.*, 2000; Shearer *et al.*, 2001; Haack, Pollmächer and Mullington, 2004; Meier-Ewert *et al.*, 2004; Haack, Sanchez and Mullington, 2007).

Experimental sleep conditions were not associated with changes in daytime IL-2 concentration following sleep deprivation. However, the order of randomisation of the experimental sleep conditions showed was associated with a change in daytime IL-2 concentration. Daytime IL-2 concentration in the bloodstream increased by 0.52 pg/ml on the second-order night and then by 5.33 pg/ml on the third-order night compared to the first-order night. Previous studies have shown a decrease in nocturnal and increased daytime IL-2 levels following sleep deprivation (Irwin *et al.*, 1996; Moldofsky *et al.*, 1986). They also reported low IL-2 production to be associated with low nocturnal NK cell count and activation after a night of PSD (Moldofsky *et al.*, 1986; Irwin *et al.*, 1996; Irwin, 2002). Although I did not measure nocturnal IL-2 concentration, daytime IL-2 increased following experimental sleep disruption compared to a night of uninterrupted sleep especially if the sleep disruption nights were either on their second or third order night.

The lack of changes in daytime IL-2 concentration following experimental sleep conditions contradicts existing studies on men. Irwin *et al.*, (1996) reported that depriving young men of sleep from 10:00 PM — 3:00 AM reported a decrease in IL-2 production. This may be due to the difference in sleep restriction protocol where I had deprived participants of sleep during the second half of the night whereas Irwin *et al.*, (1996) had deprived them of sleep during the first half of the night. Another possible reason for this is that nocturnal IL-2 may increase during the first half of the night and decrease during the second half as seen in other pro-inflammatory cytokines (Lange *et al.*, 2010); when female participants were woken up in the second half of the night, the IL-2 levels had begun to decrease to return to normal.

Unfortunately, there are no recent studies investigating the relationship between sleep deprivation and IL-2 in healthy people. However, there have been studies that investigated the effect of OSA or chronic insomnia on IL-2 (Ren *et al.*, 2021; Sarinc Ulasli *et al.*, 2015; Savard *et al.*, 2003). These studies have reported elevated IL-1, IL-2, IL-6 and TNF-α levels in a predominantly female insomniacs (Ren *et al.*, 2021) suggesting that chronic sleep fragmentation (as experienced by OSA patients) and restriction (insomnia) can develop a pro-inflammatory profile.

Experimental sleep conditions were significantly associated with IL-8 concentration. Specifically, RN was associated with a decrease in IL-8 concentration by 0.12 pg/ml compared to an uninterrupted BN. My findings contradict Faraut *et al.*, (2011)'s study that did not find a change in daytime IL-8 concentration in healthy young men following a single night of sleep deprivation between 2:00 AM – 4:00 AM and were allowed to sleep from 11:00 PM – 2:00 AM and then from 4:00 AM – 7:00 AM. This contradiction in findings may be attributed to different protocols in sleep deprivation where Faraust *et al.*, (2011) had allowed male participants to return to sleep whereas my female participants were not allowed to return to sleep during RN. Furthermore, my study did not report a significant change in IL-8 concentration following FN. Unfortunately, there are no current studies investigating effect of fragmented sleep on IL-8 concentration in healthy individuals, but IL-8 has been measured in patients with autoimmune and sleep disorders. Many studies investigating the effect of sleep disorders like OSA and autoimmune disorders like SLE on IL-8 concentrations reported that these patients experience fragmented sleep and increased serum IL-8 concentration (Wang *et al.*, 2020; Li *et al.*, 2021; Zeng *et al.*, 2021). It can be speculated that chronic fragmented sleep may significantly increase the levels of systemic IL-8 which may contribute to the pathogenesis of disorders like SLE and OSA and can be a possible therapeutic target.

Similarly, experimental sleep conditions, order of experimental sleep conditions, day of the follicular phase of the menstrual cycle, and age were not associated with changes in IL-17a concentration. This contradicts existing literature which has reported elevated IL-17a concentration following sleep fragmentation and restriction protocols in both murine models (Palma *et al.*, 2006; Palma and Tufik, 2010; Yehuda *et al.*, 2009) and human studies (van Leeuwen *et al.*, 2009; Raymond, Ostli Eilertsen, *et al.*, 2017; Ge, Huang and Yao, 2020a; Fahmawi *et al.*, 2023; see Appendix A). These murine models have reported that pro-inflammatory cytokines including IL-17a elevate following sleep fragmentation, and remains elevated after sleep recovery (Palma *et al.*, 2006; Yehuda *et al.*, 2009; Palma and Tufik,

2010). However, in a murine study implementing an acute sleep restriction protocol only reported an increase in IL-17a concentration after a month of acute sleep restriction (Fahmawi *et al.,* 2023) whereas Yehuda *et al.,* (2009) found significant elevation in IL-17a after 72 hours of REM sleep deprivation which remained elevated after a week of sleep recovery. Similarly, experimental chronic sleep restriction studies (sleep opportunity from 3:00 AM – 7:00 AM) in healthy male participants showed elevated daytime IL-17a concentration after 5 nights of sleep restriction (van Leeuwen *et al.,* 2009). They also reported IL-17a remained elevated following three nights of sleep recovery (van Leeuwen *et al.,* 2009). Clinical studies in patients with sleep disorders like OSA, or autoimmune disorders like SLE who experience fragmented sleep and chronic low sleep duration, have also reported a greater daytime IL-17a concentration (Huang *et al.,* 2016; Raymond *et al.,* 2017).

It is possible that IL-17a initially decreases in humans following a single night of sleep restriction but increases as sleep deprivation becomes chronic, and a build-up of sleep pressure results in elevated daytime IL-17a following sleep deprivation. Further studies investigating the mechanism of sleep, sleep restriction and fragmentation on IL-17a are needed.

Experimental sleep conditions, order of experimental sleep conditions and age were not associated with IL-4. However, this study reported an association between the days of the follicular phase of the menstrual cycle and IL-4. IL-4 is understudied in human sleep studies. Existing animal studies have shown that pro-inflammatory cytokines increase and anti-inflammatory cytokines like IL-4 significantly decrease in the hippocampus after total sleep deprivation (Arvin *et al.,* 2023; Wadhwa *et al.,* 2018). This contradicts my findings of lack of change in IL-4 concentration following the acute experimental sleep conditions in the plasma. This may be due to a few reasons: firstly, IL-4 may not change after a single night of sleep deprivation in healthy female participants. Secondly, 64% of our IL-4 concentrations were measured as zero pg/mL; this may be related to the relative lack of sensitivity of our assay for IL-4. Lastly, our sample size of 14 young women may have been too small to accurately present changes in IL-4 following sleep disruption.

Sequentially in a physiologically healthy person (Figure 11): immune cells such as macrophages and Th1 cells release TNF-α and IL-2 respectively (Parameswaran and Patil, 2010; Ross and Cantrell, 2018). TNF-α stimulates the differentiation of naive T cells into either Th1 or Th17 which release IL-6 and IL-17a, respectively. IL-17a further promotes the

release of IL-2 from Th1 cells (Ge *et al.,* 2020b; McGeachy *et al.,* 2019). IL-2 stimulates the release of the anti-inflammatory cytokine, IL-4 (Zhou *et al.,* 2021). The presence of both IL-2 and IL-4 stimulates the release of IL-10 (Zhou *et al.,* 2021). These anti-inflammatory cytokines create negative feedback where IL-10 inhibits TNF-α, while IL-10 and IL-4 both inhibit IL-17a, thus maintaining homeostasis. However, in the context of sleep deprived participants, despite the absence of a relationship between IL-4 and variables of interest: sleep conditions, order, and age, I found that IL-4 increased by 0.08 pg/mL as the follicular days of the menstrual cycle progressed. Interestingly, the pro-inflammatory cytokine, IL-2, is the only other cytokine in this study that was observed to increase with the follicular days of the menstrual cycle by 0.55 pg/ml. As mentioned in section 4.2.1., daytime IL-2 increased with the order of experimental sleep conditions. IL-2 stimulates the expression of IL-4 receptors which differentiate Th2 to synthesise IL-4 (Zhou *et al.,* 2021). It is thus possible that higher IL-2 in the menstrual cycle could then induce also higher IL-4 levels. IL-2 and IL-4 promote the production of IL-10, this fluctuation in IL-2/IL-4 may have subsequently affected the non-significant changes in IL-10 reported in my study.

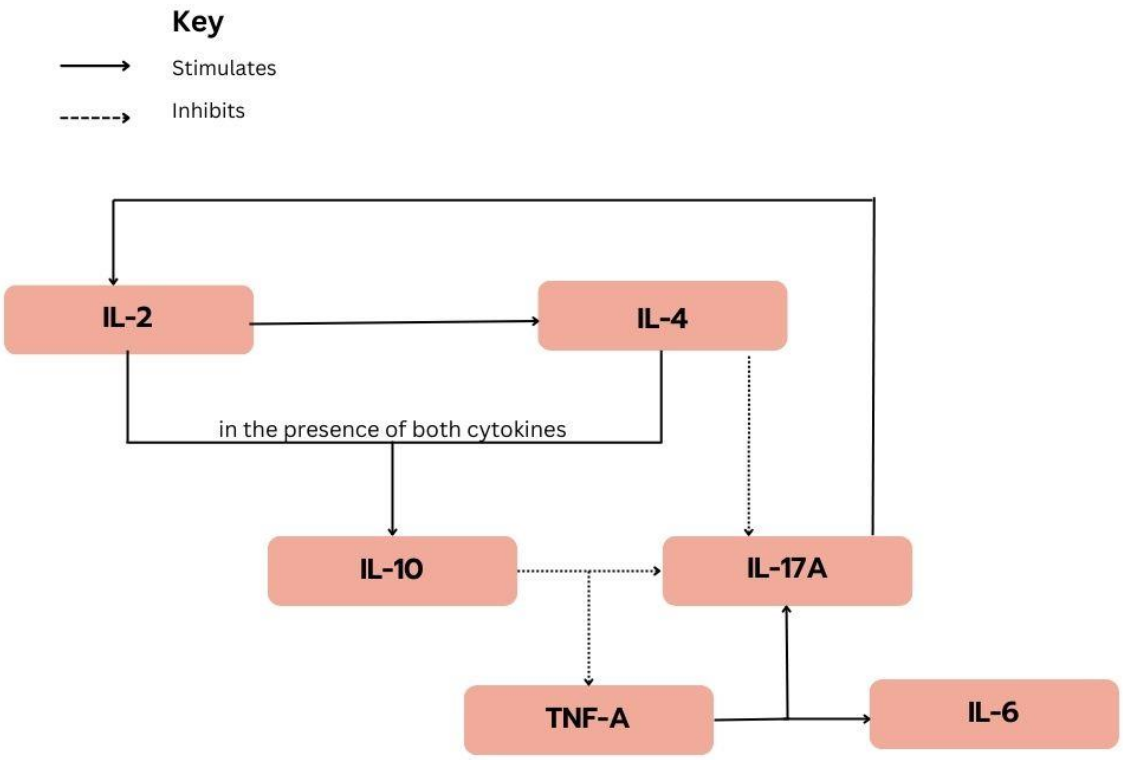


Figure 11: The homeostatic stimulation and inhibition of cytokines in healthy individuals. (This figure was generated from the information gathered from: (B Alberts *et al.*, 2002; Bae *et al.*, 2016; Brown *et al.*, 2004; Garlanda and Jaillon, 2016; Ge *et al.*, 2020b; Liao *et al.*, 2011; McGeachy *et al.*, 2019; Murphy *et al.*, 2007; Spolski *et al.*, 2018; Strowig *et al.*, 2008; Xu and Cao, 2010; Zeng *et al.*, 2021; Zhou *et al.*, 2021)

4.3. The effect of sleep conditions on cytokines and sleep parameters

Lastly, I explored the association between sleep architecture and systemic cytokines following acute sleep disruption in healthy young female participants. There was no significant association between the sleep parameters and IL-1β, IL-2, IL-4, IL-6, IL-10 and IL-17a. This study found a negative association between WASO and IL-8 whereby longer WASO (minutes) was associated with lower IL-8 concentration. However, there was no association between the duration of TST (minutes), global NREM (minutes), individual NREM stages (i.e., N1, N2 and N3) and REM (minutes) with IL-8 concentration.

TNF-α was not affected by experimental sleep condition, order, day of the follicular phase of the menstrual cycle and age. However, there was a significant unadjusted association between N2 (minutes) and N3 (minutes), and TNF-α. My study found that a shorter duration in N2 (minutes) and longer N3 (minutes) was associated with increased daytime TNF-α concentration in participants. However, only the association between N3 (minutes) and TNF-α concentration was more robust after adjusting for experimental sleep conditions. There was no interaction between N2 and N3 on TNF-α concentration. This aligns with existing murine studies where exogenous intraventricular or intraperitoneal administration of TNF-α concentration showed to increase NREM and SWS duration (Haack *et al.,* 2004; Kubota *et al.,* 2001; Opp, 2005a). Although, human and animals have shown that sleep deprivation leads to elevated daytime TNF-α concentration (Baune *et al.,* 2008; Haack *et al.,* 2004; Idriss and Naismith, 2000; Kaushal *et al.,* 2012; Opp, 2005b), as far as I know, they have not assessed for the effect of sleep stages on TNF-α. It is possible that during sleep disruption, high levels of N3 may favour TNF-α over N2 and REM as a defence mechanism. However, more research is needed.

Existing literature on partial sleep deprivation and total sleep deprivation shows that participants have significantly greater levels of pro-inflammatory cytokines – IL-1β, IL-2, IL-6 and TNF-α – after partial or total sleep deprivation (See Appendix A: Vgontzas *et al.,* 1999; Shearer *et al.,* 2001; Redwine *et al.,* 2003; Haack, Pollmächer and Mullington, 2004; Baune *et al.,* 2008; Elmenhorst *et al.,* 2008). Similarly, IL-8 and IL-17a have been studied in sleep disorders such as OSA (Sarinc Ulasli *et al.,* 2015) and insomnia or autoimmune disorders like SLE (Mao *et al.,* 2018; Nielepkowicz-Goździńska *et al.,* 2014; Palma *et al.,* 2006; Raymond *et al.,* 2017b, 2017a) and RA (Gaffen, 2009; Gremese *et al.,* 2023; Hwang *et al.,* 2004) have shown significantly elevated levels of IL-8 and IL-17a in these patients. There is a circadian

release of pro-inflammatory cytokines including IL-1β, IL-2, IL-6 and TNF-α to regulate the immune response during sleep (Lange *et al.,* 2003). In cases of acute sleep deprivation, there is a possible disruption in the circadian release of these cytokines which may cause a daytime build-up of the pro-inflammatory cytokines in the bloodstream (Lange *et al.,* 2010). This build-up results in sleep inertia and a greater need for sleep, however, due to controlled experimental conditions the participants are not able to sleep (Hilditch and McHill, 2019; Trotti, 2017). My study therefore highlights three key points: women, unlike men, may have a more robust immune system as they did not experience a greater inflammatory response as seen by only IL-8 being elevated. Next, the study demonstrated the innate drive to maintain homeostasis by regulating the sleep stages – whereby participants experienced a greater SWS and reduced REM sleep during sleep disruption to minimise possible immune dysregulation. Lastly, the study presented the sensitive relationship between sleep stages – namely N2 and N3 – and pro-inflammatory cytokine, TNF-α.

I need to acknowledge a few limitations of this study. I had initially aimed to assess the effect of a single night of sleep disruption on the immune system and sleep parameters of 20 young women, however, due to time constraints and participants dropping out, we were only able to complete 14 female participants. This reduction in sample size may have weakened the statistical power of the analysis. In addition, during the course of data collection, the isolated plasma was cryopreserved in a -80 ˚C freezer. Unforeseen circumstances resulted in the -80 ˚C freezer having broken down twice during the course of the data collection which resulted in premature, uncontrolled thawing of most of the plasma and PBMC samples. This may have altered the cytokine levels measured in participants and possibly contributed to the zero pg/ml concentrations reported. Moreover, the Luminex kit I used to measure cytokine concentrations was not as sensitive to IL-6 and IFN-ϒ thus I was not able to measure IFN-ϒ concentrations. However, I did have an IL-6 ELISA kit and was able to acquire measurements. Moreover, upon further inspection, I found that it would have been better to use the BIOCOM Luminex kit instead ThermoFischer kit as the BIOCOM Luminex kit was more sensitive to my cytokines of interest. For instance, the BIOCOM kit can detect TNF-α as low as 0.98 pg/ml vs ThermoFischer that detects at 8.64 pg/ml. Similarly, due to the fragile nature of PBMC, I did not run PBMC through flow cytometry after two uncontrolled thawing of PBMC as I was worried that the PBMC had undergone apoptosis.

CHAPTER 5: CONCLUSION

Our society has normalized voluntarily sleep deprivation due to work/school commitments and working long nightshifts, or behaviour which alter sleep such as social media use at night and binge-watching Netflix. This makes us guilty of disregarding the detrimental effect of sleep deprivation on health. This study set to investigate how two different types of acute sleep deprivation, sleep restriction to the first four hours of the night or sleep fragmentation resulting in a similar decrease in sleep duration may affect the immune system. By comprehensively exploring the effects of sleep deprivation on individual cytokines, we may be able to guide the direction of future investigations into the complex interaction between sleep and autoimmune illnesses. Ultimately, with better understanding of the underlying mechanisms it may be possible to create tailored treatments for better patient outcomes, and even better, inform potential simple prophylactic means to limit the risk of crippling illnesses.

This study creates room for venues of further research which include but are not limited to: repeating this study with a larger sample size, more sensitive cytokine analysis kit, and taking blood samples before the experimental sleep condition, during the condition, and within an hour after the end of the condition. Studies can also take daily blood samples of women during their menstrual cycle to establish their baseline, before women undergo experimental sleep deprivation conditions to compare the effect of sleep deprivation on the cytokine concentration in the blood stream throughout the menstrual cycle and not limit it to the follicular phase of the menstrual cycle. Lastly, future studies can compare the effect of uninterrupted sleep and interrupted sleep on the sleep architecture and immune response of healthy participants and patients with sleep and/or autoimmune disorders. This may allow researchers to identify changes in sleep architecture and potential biomarkers as therapeutic targets for these patients.

Despite the limitations of this study, it is the first to examine the effect of two controlled forms of sleep deprivation on the immune response, by measuring cytokine concentrations, in healthy female participants. I was able to show that 1) a single night of sleep deprivation alters specific cytokines levels in the morning and 2) time spent in various sleep stages are associated with but do not alter systemic cytokine levels. My data reports that experimental sleep conditions (i.e., BN, FN and RN) were not associated with changes in daytime IL-1β, IL-2, IL-4, IL-6, IL-10, IL-17a and TNF-α concentrations. However, RN was associated with lower IL-8 concentration. This is surprising as previous studies have shown that sleep deprivation does lead to changes in cytokine concentration including IL-1 β, IL-6 and TNF-α

in the blood stream. This leads to my hypothesis that the immune system in healthy young women may be more resilient to physiological disruption induced by acute partial sleep deprivation and that a single night of sleep restriction or sleep fragmentation may not significantly disrupt the immune system. My study further reported that a lower N2 (minutes) and higher N3 (minutes) was associated with higher TNF-α concentration.

Finally, this study highlights an integral homeostatic modulatory role of sleep on the immune response in healthy young women. This study also presents a potential resilience of the immune system to acute partial sleep deprivation. However, I caution individuals against poor and insufficient sleep which may lead to an inappropriate immune response. Healthcare practitioners, researchers, drug developers and policy makers should consider sleep when assessing patients, evaluating side effects of medication, and developing policies for work hours.

CHAPTER 6: REFERENCES

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CHAPTER 7: APPENDICES

Appendix A: Table of sleep deprivation in human studies

Table 9: The effect of different forms of sleep deprivation on immune function: brief summary of the literature (human studies).

	Reference	Study population	Type of sleep disruption	Effect on immune system
Innate immunity	Dinges *et al.*, 1994	Healthy young participants (N = 20, **7 females** and 13 males) aged 21–30 years	A week-long laboratory study: 2 days of baseline, uninterrupted regular sleep. 64 hours of TSD. 2 days of recovery sleep. Blood samples were taken between 10:00 PM – 10:30 PM each night	64-hour TSD compared to pre-TSD: • Granulocytes and monocyte count gradually **increased** with each night of sleep deprivation. • NK cell markers CD 56 and CD 57 **continued to increase** post-recovery. • NK cells count fluctuated. It **initially increased** from baseline to the first night of TSD then **decreased** on the **second night**, and **sharply increased** by the **final night** of TSD. • NK cell activity increased after 63 hours of TSD but returned to normal after recovery.
	Irwin *et al.*, 1994	Healthy male participants (N = 24) aged 22–61 years	4 consecutive days of lab study: Day 1 of adaptation Day 2 of baseline Day 3 of PSD (remain awake for the second half of the night). Day 4 of sleep recovery. Blood samples taken between 7:00 AM – 9:00 AM after each night.	• **Decreased granulocytes (%)** and **NK cell activity**, and **increased lymphocytes (% and count)** in the morning following PSD. • **IL-2** significantly **decreased** and remained low after recovery.

Vgontzas *et al.*, 1999	Healthy young adult males (N = 8) aged 20–29 years	7-day lab study: 1 adaptation night 3 baseline night Followed by 24 h TSD; then 2 nights of sleep recovery. 24-hour blood plasma analysis	At baseline: • **Biphasic** release of IL-6 (Nadirs at 8:00 AM – 9:00 PM; zeniths at 7:00 PM and 5:00 AM). Post sleep deprivation: • **Increased slow wave sleep** and **daytime sleepiness.** • **Increased IL-6** levels during the day compared to baseline. • **Decreased nighttime IL-6** levels compared to baseline.
Mullington *et al.*, 2000	Healthy male participants aged 21—34 years old (N = 19)	Single-blind placebo control design was used. The participants had 7 days between the placebo, and experimental study. The participants received an intravenous, bolus injection of either a placebo or lipopolysaccharides endotoxin from *Salmonella abortus equi*, an hour before lights off. PSG was used to measure sleep. Blood samples were taken every 30 minutes from 06:00 PM — 12:00 AM the following day.	Endotoxin administration resulted in: • an **increase** in NREM • a **decrease** in REM • an **increase** in REM latency • an **increase** in circulating levels of cytokines (TNF-α, IL-1ra), and cortisol. • an initial decrease in leukocytes followed by an increase.
Shearer *et al.*, 2001	Healthy adult males (N = 42) aged 21–47 years	10-day lab study: 1 Adaptation day 2 Baseline days (11:30 PM — 7:30 AM) Subjects were randomly assigned 4 days of PSD (with two 2 h nap opportunities per day) or 4 days of TSD. Within these two groups (PSD or TSD) subjects were further	• Average 24 h cortisol levels were not significantly different during the 88-h period of sleep deprivation between TSD and PSD conditions. After TSD: • Linear **increase** of IL-6 observed from day 2 through day 5.

		assigned to a placebo or low dose of caffeine (9 TSD plus placebo; 12 TSD plus caffeine; 10 PSD plus placebo; and 11 PSD plus caffeine). Blood samples taken every 1.5 hours every day of the protocol	
Dimitrov *et al.*, 2004	Healthy males (N = 14) aged 21–31 years	Within participant crossover design. All participants underwent 1 night of normal 8 h sleep and 1 night of TSD. Blood sampled every 2 hours between 9:00 PM — 7:00 AM for each night	• IFN-γ/IL-4 ratio of $CD4^+$ cells increased during the early part of the night while participants slept compared to TSD but decreased during the latter part of the night. • $CD8^+$ producing increased during the early night and then decreased compared to TSD.
Haack, Pollmächer and Mullington, 2004	Healthy males (N = 22) aged 21–34 years	Participants underwent two sleep conditions that were 7–14 days apart. Initially, participants were randomly assorted into sleep or sleep deprivation conditions. Both groups start with an adaptation night. Sleep condition: Sleep was permitted from 11:00 PM — 07:00 AM. Sleep deprivation condition: 40 h of TSD. Recovery sleep occurs from 11:00 PM after TSD however length of recovery is not specified. Blood was taken every 30 minutes during the night and every 60 minutes during the day	• sTNF-receptor p55 slightly increased values at the onset of the normal nocturnal sleep period time in both conditions. • Time-of-day effects were significant for sTNF-receptor p75. • Diurnal variations in plasma levels of both soluble TNF receptors p55 and p75 in healthy men. They peaked at 6:00 AM, an hour before wake. Sleep deprivation condition: • Nocturnal increase of sTNF-receptor p75 levels. • Cortisol levels were initially low and gradually increased through the night when

			participants should be sleeping
Meier-Ewert *et al.*, 2004	Experiment 1: Healthy male volunteers (N = 10) aged 22–37 years. Subjects had regular sleep-wake times and slept for 6–9 h Experiment 2: Healthy subjects (N = 10, 6 males and **4 females**) aged 26–38 years. 5 controls and 5 PSD. Subjects had regular sleep-wake times and slept for 6–9 h	Experiment 1*:* All Subjects spent 10 nights at the lab. Three baseline nights followed by 88 h TSD. Three nights of sleep recovery. CRP measured 3-hourly for the 5 days of total sleep deprivation. Experiment 2*:* Subjects spent 13 nights in a controlled laboratory. 2 baseline nights of 8.2 h of sleep. The PSD group was allowed 4.2 h of sleep for the next 10 days (Slept 2 h later than bedtime & woke up 2 h earlier than normal). Followed by one night of 14 h sleep recovery. Controls had regular sleep for 10 days. CRP measured hourly on day 1 and day 10 over 24 hours. In both experiments, only daily averages of log CRP were reported, not the 3-hourly or hourly measurements.	Experiment 1: • CRP levels **increased** over the TSD period and remained elevated after sleep recovery. Experiment 2: • CRP **increased** four-fold. • Higher systolic blood pressure associated with higher CRP.
Irwin *et al.*, 2006	Healthy volunteers (N = 30, 17 males and 13 **females**) aged 25–59 years with no physical or mental illness, and non-smokers. Regular sleep-wake times with ±8 h	Four consecutive nights in a controlled sleep laboratory. 3 baseline nights of uninterrupted sleep. 1 PSD night: allowed to sleep the first half of the night (4-hours).	• **Increased** monocyte production of IL-6 and TNF-α after PSD compared to baseline.
Haack, Sanchez and	Healthy participants (N = 18, **6 females**	16 days and 15 nights. Day 1: Adaptation day.	4-hour sleep conditions: • IL-6 and CRP levels

Mullington, 2007	and 12 males) between the ages 21–40 years.	Day 2: Baseline day. Subjects were randomised to an 8-hour sleep (23:00 to 07:00; N = 8) per night or 4-hour sleep (23:00 to 03:00; N = 10) per night for 12 days. In the 4-hour sleep condition, subjects stayed awake in bed for the second half of the night (4-hour).	increased. 8-h sleep conditions compared to 4-hour sleep: • Decrease of IL-6 and CRP levels.
van Leeuwen *et al.*, 2009	Healthy male participants (N = 19, 6 control and 13 experimental groups) aged 19 to 29 with regular sleep-wake hours	Control group: Ten days of regular 8 h of sleep Experimental group: 2 baselines with regular 8 hours of sleep. 5 Night of 4-hour PSD, stayed awake for the second half of the night. 3 night of sleep recovery Blood taken in the morning at 7:00 AM	• **NK cell count (at 7:00 am) decreased** to 65% of baseline during PSD but increased to 85% of baseline after sleep recovery. • T-helper cells and cytotoxic T-cells remained **unchanged**. • **B-cells increased** after PSD but decreased after sleep recovery. • **CRP** levels continued to **increase** during PSD and after sleep recovery. • **IL-1β, IL-6 and IL-17 were elevated** after five nights of PSD and remained elevated after recovery.
Wolkow *et al.*, 2015	Healthy firefighters (N = 35) aged 23–55 years were divided into control groups (N = 18; **females** = 3, males = 15) and PSD (N = 17; **females** = 3; males = 14)	1 adaptation night 3 experimental nights: Both groups performed simulated firefighting activity before bed. Control group was allowed full 8 hours of sleep. PSD group had a delayed bedtime and were only allowed to sleep in the last 4 hours of the night.	• During full 8-hour sleep, there was an association between a **decrease** in evening cortisol and **increase** in morning IL-6 • PSD: **increased morning IL-6** was associated with **increased** evening cortisol

			1 night of sleep recovery. Fingertip capillary blood was measured four times (morning, afternoon, evening, and night).	
	Hunt *et al.*, 2021	Healthy participants (N = 79; **females = 43**, males =36), aged 27.8±6.4 years	Two groups: First group: 2 nights of uninterrupted 8h sleep. 2 weeks of recovery followed by 2 nights of fragmented sleep (Smith *et al.*, 2009). Second group: 2 nights of fragmented sleep. 2 weeks of recovery followed by 2 days of uninterrupted sleep.	• **Increased IL-6 and TNF-α** during fragmented sleep compared to uninterrupted sleep.
	Tucker *et al.*, 2021	Cervical cancer survivors (N = 71)	Regular sleep measured using an actigraphy watch and self-reported questionnaires, Medical Outcome Study (MOS)-Sleep.	• **Inverse relationship** between **sleep duration,** and **IL-6, IL-2, IL-1β, and TNF-α** i.e., increased sleep duration resulted in lower levels of these cytokines. • **Self-reported sleep problems** are inversely correlated to **IL-5, IL-10.** Patients with sleep problems had lower levels of anti-inflammatory cytokines.
Adaptive immunity	Born *et al.*, 1997	Healthy male participants (N = 10) aged 21–29 years	Two 51-hour sessions with a 10-day gap between each session. Regular sleep condition: Lights were turned off for nocturnal sleep at 11:00 PM. First night: subjects were required to get up at 0700 AM. On the second night, they were allowed to sleep ad libitum, and stayed in bed until the end of the session at 11:00 PM.	24 h TSD compared to regular sleep: • **Increased neutrophils, eosinophils, basophils, lymphocytes, B-cell (CD 9^+), $CD3^+$ T cells, $CD4^+$ T cells.**

		Sleep deprivation condition: 24 hours of TSD followed by recovery sleep with a regular wake-sleep cycle.	
Click or tap here to enter text.			
Aho *et al.*, 2013	Healthy male participants (Exp = 8, control = 4) aged 19–29	2 baseline nights, 5 consecutive PSD (Sleep for first 4 hours of the night) then two nights of sleep recovery	After PSD: • **Upregulation** of stress and immune-related gene transcripts, **leukocyte, and B-cell activation, and IL-8** production. • **Downregulation** of **NK-cell function transcripts, Th1 cells and IFN-ɣ**.
Axelsson *et al.*, 2013	Healthy male participants (N = 9) aged 23–28 years	1 Adaptation night with regular sleep followed by 4 days at home sleep. Then 10 consecutive nights in the sleep laboratory: 2 baseline nights (8-hour sleep), 5 PSD (sleep last 4 hours of night), 3 sleep recovery nights. Blood samples taken at regular intervals on Day 2 baseline sleep and Day 5 PSD.	After 5 nights of PSD: • **Elevated TNF-α** and **cortisol**. • **Reduced IL-1, -2** and **TNF-α responses**. • **Shift** to **Th2 cell activation**.

TSD = total sleep deprivation PSD = Partial sleep deprivation CRP = C-reactive protein IL = interleukin TNF = Tumour necrosis factor

Appendix B: Amended ethics application submitted to the Human Research Ethics Committee and the final approved medical ethics clearance.



HREC (MEDICAL) Application form for 2020

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

APPLICATION TO THE HUMAN RESEARCH ETHICS COMMITTEE: (MEDICAL) FOR CLEARANCE OF RESEARCH

Please read the APPENDIX section at the end of this application form before completing this application form. Also please note that no data may be collected before issue of an ethics clearance.

Category 1 and 2 applications (see below) are subject to monthly closing dates, which may be viewed at "https://www.wits.ac.za/media/wits-university/research/documents/002.Ethics%20Meeting%20and%20Submission%20Dates.pdf " Categories 1 and 2 are submitted online at https://www.witsethics.co.za/Login.aspx and one hard copy of the application and all attachments must be submitted to the Ethics Office on the 3rd floor of the PV Tobias Building, preferably on the same day as the online application.

Categories 3,4 and 5 below are not yet online and two completed hard copies of this form may be submitted to the Ethics Office at any time

Category 7 is not yet online and two completed hard copies of the form referenced below may be submitted to the Ethics Office at any time

Please complete ALL sections of this application form; the Committee needs the information to make a decision. If this is not done, clearance is unlikely - you may have to resubmit a fully completed application.

Categories:

New Application:
Resubmission – state protocol no.
Case Report
Sub-study
Lab study
Application to amendment already approved study, e.g with phased projects
Waivers – not this form. Please refer to https://www.wits.ac.za/media/wits-university/research/documents/Ethics%20Waiver%20form%202019.doc

SECTION 1
PRINCIPAL INVESTIGATOR/S

TITLE: Dr NAME: Stella SURNAME: Iacovides

WITS STAFF NUMBER: A0003888 WITS STUDENT NUMBER:

PROFESSIONAL STATUS OR STUDENT YEAR OF STUDY AND DEGREE Senior Lecturer

UNIVERSITY DEPARTMENT / DIVISION: School of Physiology

NON-WITS SITE / INSTITUTION:

DETAILS OF WHERE STUDY WILL BE DONE HOSPITAL/INSTITUTION WHERE EMPLOYED:

WHERE WILL THE RESEARCH BE CARRIED OUT:

FULL-TIME OR PART-TIME EMPLOYEE: Full-time **HPCSA NO**: N/A

SECTION 2

CONTACT PERSON DETAILS:

TITLE: Dr **NAME**: Stella

SURNAME: Iacovides **TEL NO:** 0117172265

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SUPERVISOR DETAILS:

Title Name Surname Tel No Cell No Email

CO-INVESTIGATOR DETAILS:

Title	Full Name	Surname
Prof	Peter	Kamerman
Miss	Felicia	Siboza
Dr	Karine	Scheuermaier
Miss	Zuha	Ajlan

TRAINING:

ETHICS TRAINING:

Ethics training is a compulsory requirement for consideration of this application. NB: If ethics training has not been done as yet, you may submit the application and later on submit the ethics training certificate – Refer to the DOH 2015 guidelines for online ethics training sites at http://nhrec.health.gov.za/index.php/grids-preview, DOH 2015 Ethics, page 56

Full Name Ethics Course Name Date of Ethics course

ONLY FOR INVESTIGATOR-INITIATED CLINICAL TRIALS:

GOOD CLINICAL PRACTICE (GCP) TRAINING DETAILS FOR ALL INVESTIGATORS

(Please note that investigators' meetings do not qualify as GCP training)

Full Name Ethics Course Name Date of Ethics course

3.1 PURPOSE OF THE RESEARCH:

The Effect of Sleep Disruption on Pain and Central Pain Modulation in Healthy Female Participants

Postgraduate: Degree / diploma : No

Undergraduate: Degree / diploma : No

Not for Degree Purposes: Yes

OBJECTIVES OF THE RESEARCH (please list): *(Do not say see attached!)*

To compare the effects of a single night of sleep restriction (restricting sleep to the first 4 hours of the night) compared with a single night of sleep fragmentation on the perception of experimentally induced pain, and on pain modulation in healthy, young female participants.

More specifically, we wish to investigate whether sleep restriction is different from sleep fragmentation in its effects on:
the perception of deep-tissue pain (experimentally induced ischaemia),
the perception of cutaneous pain (pin-prick sensitivity),
subjective spontaneous pain,

central pain modulation (using the cold pressor task as a conditioning stimulus, and noxious heat as a test stimulus),
inflammatory blood markers (Interleukin-6 and cortisol).

3.3 SUMMARY OF THE RESEARCH (give a brief outline of the research plan such that reviewers understand what will be done. (*Do not say see attached!*):

Twenty healthy females, between 18 and 30 years of age, will be asked to volunteer for this randomised crossover-controlled study. Participants will be recruited using posters placed around the Wits Medical School and Main campus, and also on various social media platforms. Using standardised and customised questionnaires, volunteers will first be screened in an interview to ensure that they fit the study criteria; they will be required to be generally healthy, non-smoking, non-obese (BMI < 25kg/m2) individuals free of pain, free of chronic medical conditions (including psychiatric conditions), and not using any chronic medications. Volunteers will also be required to have regular sleep-wake cycles, with good sleep quality, and regular sleep of an average of 8 hours per night. Furthermore, the Pittsburgh Sleep Quality Index (PSQI) (attached) will be used to screen for sleep disorders/sleep disturbances, and volunteers will need to score less than 5 on the global PSQI score (indicating good sleep quality) [1]. Psychological health will be assessed using the 30-item General Health Questionnaire (GHQ) (attached), and volunteers will have to score less than 6, indicating a healthy psychological status [2]. All study procedures will take place at the School of Physiology's Sleep Laboratory, housed at the University if the Witwatersrand's Medical School. ***Screening Phase:*** Volunteers will be further screened for a week to confirm that they have regular sleep-wake cycles. During this screening week, volunteers will be required to complete a customised sleep diary every morning shortly after waking. Included in the sleep diary are questions on the time they went to sleep, the time they woke up, and how long they felt it took them to fall asleep (see Sleep diary attached). Also included in the sleep diary are assessments of sleep quality and morning vigilance, which will be quantified using 100mm visual analogue scales (VAS), anchored at "no sleep" to "best sleep I have ever had" for sleep quality, and "not at all alert and fresh" to "most alert and fresh I have ever felt" for morning vigilance. Volunteers will also wear an Actiwatch Spectrum device (Philips Respironics, Murrysville, PA) on the wrist of their non-dominant arm to provide a record of daily activity during the screening week. Together, the sleep diary and activity data will be used to confirm that volunteers have regular sleep-wake schedules, and that participants do not engage in daytime napping. Also, habitual wake- and sleep-times will be determined from the screening phase, and these will be used during the experimental phase. ***Experimental Phase:*** After screening, eligible volunteers will need to spend four nights in the sleep laboratory. For all participants, the first night in the laboratory will be an adaptation night to familiarise them with the environment and procedures. The adaptation night will take place on day 1 of their menstrual cycle (the first day of menstruation is represented as day 1). Two nights later, in a randomised order, participants will undergo the experimental phase: a "baseline" night (normal, uninterrupted sleep), a "sleep restriction" night, and a "sleep fragmentation" night. For each night, an eight-hour block for time in bed will be scheduled using the average lights-off to lights-on times from the sleep diaries. Then following both the sleep fragmentation and the sleep restriction nights, the participants will be allowed at least 4 nights of sleep recovery at home before returning to the laboratory. Following the baseline night, participants will be allowed 1 to 2 nights at home before returning to the laboratory. All experimental nights will be during each participant's follicular phase of the menstrual cycle (between day 1 and day 13 of the menstrual cycle), to avoid any effect of reproductive hormones on pain perception [3-6]. Participants will be asked to refrain from consuming alcohol, caffeine, and any medications for 24-hours before each experimental night. ***Adaptation Night:*** Participants will be fitted with scalp electrodes for polysomnographic (PSG) recordings. Participants will sleep for approximately 8 hours, according to their habitual sleep- and wake times. After waking, the electrodes will be removed, participants will be given a standardised breakfast, and the leave. They will be asked to arrive back at the sleep laboratory two-

three nights after the adaptation night for either their first sleep disruption night or their baseline night (order will be randomised). To ensure participants maintain their regular sleep schedules, they will need to wear an Actiwatch and to complete a sleep diary from the time they leave the laboratory until they return to the laboratory for their next night. ***Baseline Night:*** To record sleep, PSG electrodes will be placed on the participants. They will then complete the Profile of Mood States (POMS) questionnaire [7] to assess mood, and the Pennebaker Inventory of Limbic Languidness (PILL) [8] to assess spontaneous pain, and their sensitivity to touch will be assessed via non-painful somatosensory assessment using von Frey monofilaments [9] (all edescribed in detail under "procedures"). Thereafter, participants will sleep uninterrupted, for approximately 8 hours- according to their habitual sleep-wake cycles. After waking, electrodes will be removed, and participants will undergo wake-time procedures (as described in detail below). Then, they will be free to leave the sleep laboratory. They will need to complete a sleep diary and wear an Actiwatch until they return to the sleep laboratory for their next night to confirm whether they maintained a regular sleep-wake cycle and did not have any daytime naps. ***Sleep Restriction Night:*** *PSG*lectrodes will be placed on the participants, and mood, spontaneous pain and sensitivity to touch will be assessed. Lights-off will occur at each participants habitual sleep-time, however they will only sleep for the first 4hours of the night. Upon awaking, participants will have to stay in bed but be seated upright and fully responsive for the second half of the night. At their habitual wake time (four hours after they are awoken), participants will leave the bedroom, electrodes will be removed, and they will complete the wake-time procedures before leave the sleep laboratory. They will need fill out their sleep diary and to wear an Actiwatch until they return to the sleep laboratory for their next night. ***Sleep***
Fragmentation Night: PSG electrodes will be placed on the participants, and mood, spontaneous pain and sensitivity to touch will be assessed. Participants will go to bed at their usual bedtime, but we will use a sleep fragmentation protocol [10] that requires participants to be awake for one randomly selected full hour, and for 20-minutes each hour for the remaining seven hours. At their habitual wake time, electrodes will be removed, participants complete the wake-time procedures, and then be free to depart from the laboratory. They will need to fill out a sleep diary and wear an Actiwatch until they return to the sleep laboratory for their next night. ***Wake- Time Procedures (after each night):****Mood, spontaneous pain, and touch sensitivity will be reassessed. Sleep quality and morning vigilance will be assessed. Then, an anaesthetic cream will be applied to the area on the forearm of the non-dominant arm where the hypertonic saline will be injected, electrodes will be removed, and after breakfast, all pain assessments will all be done in a systematic order, once the pain of the previous experiment subsides completely.*

The following protocol will be used with participants in times of distress during the data collection process.

If a participant indicates they are experiencing a high level of stress or emotional distress OR if a participant exhibits behaviours suggestive that the discussion/interview is too stressful such as uncontrolled crying, shaking etc. The PI will respond by stopping the discussion/interview/experimental procedures, and will offer immediate support. The PI will then assess mental status by asking questions such as: "Tell me what you are feeling right now?" "Do you feel you are able to go on about your day?"
"Do you feel safe?" In cases where the participant feels safe and they are able to carry on, the PI will resume the interview/discussion/experimental procedures. In cases where a participant is unable to carry on, the response will be as follows:
The interview/experimental procedures will be discontinued.
The participant will be referred to certified coach (Dr Antonia Wadley) or a medical doctor (Dr Karine Scheuermaier) onsite, for a first evaluation (on-site counselling), or,
The PI will offer to refer the participant to an organisation that specifically deals with the identified issue, (e.g. OSS or campus health), or
With participant consent, contact a member of the health care team listed on the resource list to secure an appointment for the participant.

Further, we will Follow-up *with the following:*
Follow participant up with courtesy call in 48hours (if participant consents) and
Encourage the participant to call, if he/she experiences increased distress in the hours/days following the interview, for support services.

3.4 SCIENTIFIC APPROVAL

Has the protocol been approved a scientific committee, *e.g.* Wits Post Graduate Assessors' Committee? Yes

3.5 SELECT STUDY DESIGN

Randomized Control

3.6 WILL THE STUDY INVOLVE HUMAN MATERIALS

Yes

Human Data
Human Material Blood

3.7 IS THE STUDY INTENDED TO INFORM IMPROVED CLINICAL OR THERAPEUTIC PRACTICE

No

SECTION 4

4,1 DOCUMENTATION REQUIREMENTS

Is this project a secondary analysis of data in an established database?
No
Note: If "yes", written consent to access a database from the database gatekeeper plus a list of the data to be recorded (see Procedures) must accompany this application.

Is this a sub-study, using data from an already-approved primary study
No
Note:If "yes", written consent to access a database from the database gatekeeper plus a list of the data to be recorded (see Procedures) must accompany this application.

Is this documentation attached? No

Is this project a retrospective patient record review? No
Note: for a retrospective review the date of the ethics committee meeting at which the application is considered sets the final date for the patient records. If the initial and / or final date is after the meeting date, the study is prospective. Patient records include ALL DATA collected on patients including blood results and radiographs etc.

What is the initial date for the patient records? What is the final date for the patient records? Is this documentation attached? NA
Note: the following must accompany this application – written proof of application for permission from the hospital or clinic CEO to do the study, written permission from the clinical entity in which the patients in which patients records are based, how the patients will be selected, what type of records will be examined, a list of the variables to be extracted (see 6.1).

Is this documentation attached? NA

Note: the following must accompany this application

Written proof of application for permission from the hospital or clinic CEO to do the study:
NA
Written permission from the clinical entity in which the patients in which patients records are based:

NA
How the patients will be selected :

What type of records will be examined :

List of the variables to be extracted (see Procedures) :
NA
Have patients consented for the anonymous use of their data?
NA

Is this project a prospective patient record review? No
Patient records include ALL DATA collected on patients including blood results and radiographs etc.

What is the initial date for the patient records? What is the final date for the patient records?
Note: the following must accompany this application – written proof of application for permission from the hospital or clinic CEO to do the study, written permission from the clinical entity in which the patients in which patients records are based, how the patients will be selected, what type of records will be examined, a list of the variables to be extracted (see 6.1).

Note: the following must accompany this application

Written proof of application for permission from the hospital or clinic CEO to do the study :
NA
Written permission from the clinical entity in which the patients in which patients records are based :
NA
How the patients will be selected :

What type of records will be examined :

List of the variables to be extracted (see Procedures) :
NA
Have patients consented for the anonymous use of their data?
NA

Is this project a prospective patient record review? No
Patient records include ALL DATA collected on patients including blood results and radiographs etc.

What is the initial date for the patient records? What is the final date for the patient records?
Note: the following must accompany this application – written proof of application for permission from the hospital or clinic CEO to do the study, written permission from the clinical entity in which the patients in which patients records are based, how the patients will be selected, what type of records will be examined, a list of the variables to be extracted (see 6.1).

Note: the following must accompany this application

Written proof of application for permission from the hospital or clinic CEO to do the study:
NA
Written permission from the clinical entity in which the patients in which patients records are based:
NA
How the patients will be selected :

What type of records will be examined :

List of the variables to be extracted (see Procedures) :
NA

If this project involves prospective studies with drugs at a teaching hospital associated with this University, approval must first be obtained from the Hospital's relevant Committee.

Has application been made?(If not, this application cannot be considered) *NA*

If radiation or isotopes are to be used in prospective studies , written approval must be obtained from the Director, Radiation and Health Physics Unit (james.larkin@wits.ac.za / 011-717 6931).
Note: for patients these are radiation dosages over and above those for standard diagnosis / therapy

Is this attached? *NA*

Is a Participant Information Sheet attached?(For written consent)
(see guidance at https://www.wits.ac.za/media/wits-university/research/documents/InfoSheet.doc)
Yes
Informed Consent Form is attached.(For written consent) Yes.

If informed consent will be verbal or If informed consent is not considered necessary – a written motivation and justification needs to be attached

If a questionnaire or interview is to be used in the research, it must be attached.

Is it attached? Yes

SECTION 5

5 STUDY POPULATION
5.1 If patients/patient records are being studied, state where and how they are selected: N/A

5.2 Where the participants are not patients, Will they be invited to volunteer? Yes
5.3 Will they be selected? Yes
5.4 State who is invited to volunteer or how the participants are selected:

Healthy young Females, between the ages of 18 - 30 years. Only females will be chosen for the study a) for the safety the female investigator, and b) because youung male participants have also shown to be less compliant within similar studies conducted in out School in the past.

5.5 Are the participants subordinate to the person doing the recruiting? No
If yes, justify the selection of subordinate participants:
5.6 Will control patients/participants be used? No
If yes, explain who they are and how they will be recruited: N/A
5.7 What is the age range of participants in the study? Need to be able to select >1 group 18 - 30 years olds
5.8 If participants are minors (under 18 years), from whom will consent be obtained?
If participants are minors, is an Assent Document provided? NA

5.9 Gender: Female

5.10 Number of patients/participants 20 controls 0

5.11 Will the research benefit the patients/participants in any direct way? No
If yes, explain in what way:

5.12 Will participants receive any remuneration? Yes
If yes, explain what the remuneration is for and how much will be paid
Participants will be remunerated for their time and transport costs. Remuneration will be R350 per session.

5.13 Will participation, non-participation or withdrawal from the study disadvantage patients/participants in any way? No
If yes, explain in what way:
SECTION 6
PROCEDURES (Throughout this section, " Procedures" refers to study, not clinical, procedures.)

6.1 Mark research procedure(s) that will be used and attached what is required: Record review: NA
Interview form / questionnaire: Yes

Self-administered questionnaire: Yes

Focus group: (questions to be used must be attached. Note: there is no confidentiality in a focus group, participants must be told this) No

Examination: No
Nature:

Drug or other substance administration: No
Nature:

Radiographs: No

Isotope administration: No
Nature:

Blood sampling;
(State amount to be collected and the frequency of sampling)

Age Group	Amount	Frequency	Blood Type
18-79	5ml	3 times	Venous

Will a biobank be used in the study No (***See page 7 regarding biobanks)***
Biopsy: No
Explain:

Other procedures: No
Explain:

Use this space to elaborate on procedures marked above:

Questionnaires: (all attached): ***Screening Questionnaire*** – to ensure suitability for the study. ***General Health Questionnaire*** – to ensure they are generally psychologically healthy individuals. ***Pittsburgh Sleep Quality Index***– to ensure that the volunteers do not suffer from a sleep disorder or have poor sleep quality.

Sleep Diary - to assess bed- and wake-times, general sleep questions, and also includes the *Visual Al1alogue Scale (VAS)* - to assess the subjects' sleep quality and morning vigilance. *Pe11-11e bakerb we11to try of L im bic L a11g uid.1ess*- use d to assess spontaneous p ain, where participants are required to ra te the experience of a symptom using a scale ranging from 0 to 4, with 0 being "'not at all' and 4 being• very much". *Profile of mood states* - to assess mood each evening during the experimental nights.

Touch sensitivity will be assessed using von Frey monofilaments (Optihair2-Set, Marstock Nervtest, Germany) that exert forces between 0.25 and 512mN graded by a factor of 2. The « method of limits " method will be use d to assess touch threshold in blind-folded participants, by way of a series of ascending and descending stimulus intensities.

Pain Assessments: *Hypertonic Saline (deep -muscle chemical pain):* Muscle pain will be induced by a 0.5ml intra-muscular injection of 5% sterile hypertonic saline. This injection produces a mild-moderate, short-duration (less than 10 minutes) muscle pain that has no long- term side effects in healthy individuals. A sterile 25 Gauge, 16mm needle will be use d for the intra-muscular injection of the forearm and a 23 Gauge, 25mm needle will be use d for the intra-muscular injection into the lower back. Both these needles will be connected to a 1ml syringe and injections will be administered as a bolus over a maximum of 10 seconds. A medical doctor or registered nurse will perform all injections. One hour before all injections, a topical anaesthetic cream (lignocaine 2.5%, prilocaine 2.5%) will be app lied to the skin at the site of the injection.

The site of the injection will be into the muscle mass of the extensor muscles of the forearm. With the palm downward, we will measure the mid-point between the elbow and wrist, and locate a lower and upper forearm area. The upper part of the forearm will then be divided into an equal upper and lower part, and the injection will be given into the muscle mass of the upper part. This is a muscular site with no risk to any nerves or blood vessels.

Sub-J faximal effort tourniquet *te st (deep-muscle ischaemia):* The sub-maximal effort tourniquet [11]is a standard procedure that has been previously described. A torniquet cuff is positioned on the participant's arm, above the elbow, and the arm is placed on a pillow to the side. Prior to inflating the cuff to 200 mmHg, the participant' s arm is elevated and supported in a vertical position for 30 seconds to promote venous drainage. The cuff is then inflated before the arm is lowered and returned to the side.

After inflation of the cuff, to promote forearm ischaemia, the subjects engage in 20 handgrip exercises using a handgrip dynamometer at 30% of their maximum force. The duration of each squeeze is 2 seconds with an inter-squeeze interval of 2 seconds. The experimenter will then start a stopwatch for 10 minutes, after which the cuff will be deflated. (previous publications using the sub -maximal effort tourniquet test have kept the cuff inflated for 20 minutes and no tissue damage at all) . ***Pin Prick*** *(Meclra11ical t /rre sltolds) :* Punctate mechanical pain thresholds will be

assessed using weighted pin-prick stimulators (PinPrick Stimulators, MRC Systems, Germany), that deliver calibrated forces between 8 and 512 mN. Participants will be blindfolded, and the pin-pricks (starting from the smallest force) will be applied perpendicularly to the anterior glabrous skin sw-face of the randomly chosen hand. Participants will be instructed to indicate when the stimulus first becomes pricking or painful; this force will be record ed as their mechanical

pain threshold. *Co11ditio11ed P ai.,,. J fodula tio 11[12]:* As a pre-test, participants will be familiarised with the equipment and the test stimulus intensity determined. This determination will be do ne by placing a 25x50mm Peltier thermode (MSA thermal stimulator, Somedic, Sweden) on the distal volar aspect of the randomly chosen forearm then increasing the temperature gradually from 32 °C with 1°C/second to maximum 52 °C. Participants will click on the trigger when they start feeling pain (heat pain threshold) and when the pain becomes intolerable (heat pain tolerance level). This will be repeated three times to obtain the average of the threshold/tolerance, which will then be used to calculate the test temperature using the equation: HPTL (heat pain tolerance level) - HPT (heat pain threshold) / 2 + HPT (heat pain threshold). Follo wing this, a series of 3, 5-second duration stimulations, with 10 second intervals between stimulations. will occur. Participants will rate the pain on the 0-10 numerical pain rating scale.

(NRS). The first stimulation will be set at 1°C below the calculated temperature and increased by 1°C for each stimulation. The heat stimulation that is rated closest to 6/10 on the NRS during the test will be used as the test stimulus temperature.The test begins with placement of the 25x50mm Peltier thermode on the proximal volar aspect of the forearm opposite to the arm that was used in the pre-test. Thermode temperature will be raised from baseline (32°C) at 2°C per second to the test stimulus temperature determined in the pre-test and be kept at that temperature for 120 seconds. Following this, the temperature will be lowered to baseline at 8°C/second. The pain intensity will be rated continuously using a 100mm VAS anchored at "no pain" to "worst pain imaginable". The average pain score of the test-stimulus will be calculated for the total time and for the duration of test-stimulus (0–30, 31–60, 61–90, and 91–120 seconds) [12]. Five minutes after concluding the test stimulus only part of the conditioned pain modulation protocol, the procedure will be repeated, but this time the hand of the opposite arm is placed in 7°C water (a painful cold stimulus). Participants will be asked to immerse their hand into a container of circulating water up to the wrist.While the hand is immersed, the test-stimulus commences with the other hand (following the exact protocol described above). The test stimulus ends when the conditioning stimulus also ends or until the pain caused by the water immersion causes the participant to withdraw the hand.

Blood samples: The 5 mL EDTA tube with blood will be centrifuged, and the plasma will be pipetted out of the tube and put in vials to be frozen at -70 °C. Once all data have been collected, the frozen plasma samples will be assessed for interleukin-6 by an enzyme-linked immunosorbent assay (ELISA), and cortisol by radioimmunoassay.

Any left-over blood will be destroyed using standard procedures for the elimination of biological samples.

6.2 Is/are procedure/(s) routine for:
diagnosis/management? No
specific to this research? No
Identify which of the procedures above are routine for diagnosis and management of patients: and identify those procedures specific to the research;

None of these procedures are routine for diagnosis. We are studying the effects of different types of sleep deprivation on pain perception and pain modulation in healthy young female adults.

All procedures described above are therefore specific to the research.

Who will carry out the procedure(s)? Felicia Siboza (MSc Physiology student, BSc (Hons)), Stella Iacovides (Senior Lecturer, School of Physiology, Bsc (Hons), PhD), Peter Kamerman (Professor, School of Physiology, Bsc (Hons), PhD), Dee Muller (Registered nurse, PhD), Delene Nciweni (Registered nurse), an honours student in 2021, and another MD or registered nurse may be asked to administer the hypertonic saline injections when Dee Muller and Delene Nciweni are not available. We also wish to add another MSc Student (Zuha Ajlan), who will also collect data and Dr Karine Scheuermaier (MD).
When will the research project commence after obtaining ethics clearance, and over what approximate time period will the research be done? Data collection will begin once ethical clearance has been obtained, and if all goes well, the research should end within 2-3years. DATA COLLECTION START DATE: 04-Jan-2021 ESTIMATED STUDY END DATE: 31-Jul-2023
For studies being done outside the Gauteng Academic Hospitals, please list the number of studies currently being done by the Principal Investigator, the number of patients per study and where they are being done:
N/a
For applications outside the Gauteng Academic Hospitals. Is the Application Outside of Gauteng? N/s

Is the investigator involved in a clinical: Part-Time / Full-Time capacity at the study site? If so, explain in what way: N/s

SECTION 7

RISKS OF THE STUDY PROCEDURE(S):

Please consult the risk table at https://www.wits.ac.za/media/wits-university/research/documents/RiskTable%28Med%292019.docx and indicate the level of risk to:

Patients/Participants: None

Research team members: None

All other persons: None

Please indicate whether the patients/participants will be exposed to any levels of: Physical discomfort: Yes

Pain: Yes

Possible complications: No

Side effects from agents used: No **Breach of confidentiality:** No **Possible stigmatisation:** No **Psychological stress:** No

If you have checked any of the above except "No risk" please provide details:

A mild-moderate pain will be felt in the forearm after the 5% hypertonic saline injection. Mild pain or physical discomfort could also be experienced in the arm while performing the sub-maximal effort tourniquet test. Although these procedures do cause pain/physical discomfort, they do not damage the tissues.

SECTION 8
GENERAL

For any study, has permission from relevant authority/ies been obtained to do the study? Yes
State name of authority/ies and provide written proof of application for permission(s): Faculty of Health Sciences

Has this study been submitted to other Ethics Committees? No
If yes, what is the status of the application?

How will confidentiality be maintained so that participants are not identifiable to persons not involved in the research? Please answer the questions below:

Will data be anonymous? Yes, all records will be coded and kept confidential to ensure anonymity.
How?

Will identifiable data be coded and the 'links' kept separate? Yes, links will be kept separate in different spreadsheets. The names of the participants will not be revealed in the final report or in any publication or presentation. Participants will be given a reference number during the study which will only be known to the investigator (separate spreadsheet). All records collected during the study are kept in the research area, under the supervision of the responsible investigator, and individual will only be made available to the researchers and individual participants. **How?**

Who will have access to the raw data? Only the investigators. In the event that the journal (which will publish the results) requires access, we will make the anonymous (coded) data available to them.

To whom will results be made available? (*e.g.* participants, supervisor, hospital management,etc) The School of Physiology, the scientific community, and the participants themselves.

How will the results be disseminated?(*e.g.* journal article, conference presentation, departmental, seminar, etc) Data will be presented at appropriate local and international pain-and/or sleep-physiology conferences, and the results will be written-up as at least 2 separate research articles to be submitted for publication in appropriate international peer-reviewed journals in the fields of pain and/or sleep.

Will there be financial costs to:

Participants: No
Hospital/Institution: No
Other: No

Explain any box marked "Yes": N/A

If no protocol is attached please indicate: Budget? How will the research be funded?
Please give details of the source of funds
NRF Thuthuka funding granted to Stella Iacovides, Brain Function Research Group (BFRG) and the Physiology Sleep Laboratory, Wits University.

8.8 Any other information, which may help the Committee to evaluate this application, may be provided here:

The procedure of injecting hypertonic saline intramuscularly, and all other painful procedures have previously been granted by the CRHS: clearance no. M040529, and HREC (medical): clearance nos. M080627, M150211, M170283.

Signatures:

~~HEAD /~~ RESEARCH COORDINATOR OF DEPARTMENT / ENTITY IN WHICH STUDY WILL BE CONDUCTED (Where applicable) (Wits Students Academic HOD must sign)

Name: **Frederic Michel** **Department / Entity** **Physiology**

Tel No: **0117172353** **Email:** **Frederick.michel@wits.ac.za**

Signature:  **Date:** **10/02/2012**

APPENDIX

Please note that it is the responsibility of the Principal Investigator in an application to ensure that he/she has received the final HREC (Medical) Clearance before commending any research. This is signified by, and only by, the issuing of a Clearance Certificate, which will be headed as such.
Please indicate clearly, where correspondence should be sent; failure to do this may cause delays.
Please provide the supervisor's email address (where applicable) for sending copies of correspondence.
It is required that a protocol detailing the background to the research, the design of the investigation and all procedures, is submitted with the application.
If any doubt exists please contact Ms Zanele Ndlovu, Ms Mapula Ramaila, or Mr Rhulani Mkansi, Faculty of Health Sciences, Phillip Tobias Building, 3rd Floor, Offices 301 and 304, 3rd Floor, 29 Princess of Wales Terrace, Parktown, 2193, Email: Zanele.Ndlovu@wits.ac.za Mapula.Ramaila@wits.ac.za' Rhulani.Mkansi@wits.ac.za ,Tel 011 717 1234/2656/2700.
Please note that written clearances will not normally be available until 14-28 working days after a Committee meeting .
STORAGE OF BLOOD AND/OR TISSUE SAMPLES:
The policy of the ethics committee is:
If, blood or tissue specimens are to be stored for future analysis and / or it is planned that analysis may be done outside Wits, then the specimens must be stored at Wits with release of sub-samples only once projects have been approved by the local Research Ethics Committee applicable to where the research will be done, as well as by the Wits Human Research Ethics Committee: (Medical);
A separate information sheet and consent form for this is required. Please see the Standard Operating procedure at https://www.witshealth.co.za/Services/Research-Ethics/Operating-Procedures
For information on Biobanks and the Biobank Ethics Committee within the Wits Human Research Ethics Committee (Medical) go to "https://www.wits.ac.za/research/researcher-support/research-ethics/ethics- committees"
Only approved analyses may be done;

Specimens may not be shared with anyone, unless approved by the Wits Human Research Ethics Committee (Medical).
Evaluation of applications from private sites / institutions without any affiliation to Wits may be done but is at the discretion of the Wits Human Research Ethics Committee (Medical). In such instances a processing fee is payable (R10 000.00 +VAT).
Researchers from abroad should obtain ethics clearance BEFORE arriving at Wits, as a tight time schedule is not considered a valid reason for departing from Wits Standard Operating Procedure. A Wits collaborator may help obtain the clearance.
Researchers with syndicates in the Wits Health Consortium – please read the home page at www.witshealth.co.za regarding the requirement that the syndicate must be based in a Wits academic department or recognised research entity
Please note: No late applications will be accepted after the submission date listed at ;
"https://www.wits.ac.za/media/wits-university/research/documents/002.Ethics%20Meeting%20and%20Submission%20Dates.pdf" Applications received after this date will be carried forward to the following meeting; incomplete applications will not be accepted
No data may be collected before a clearance is issued by the HREC (Medical).

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R49 Dr S Iacovides; Profs P Kamerman & K Scheuermaier; Miles F Siboza and Z Ajlan

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M201127

NAME: and Z Ajlan **(Principal Investigator)**	Dr S Iacovides; Profs P Kamerman & K Scheuermaier; Miles F Siboza
DEPARTMENT:	School of Physiology Medical School University
PROJECT TITLE:	*The effect of sleep disruption on pain and the immune systems of heathy female participants*
DATE CONSIDERED:	2020/11/27
DECISION:	Approved unconditionally
CONDITIONS:	Permission of the Registrar noted on 2021/06/02
SUPERVISOR:	Dr S Iacovides; Profs P Kamerman & K Scheuermaier
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2021/05/10

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Philip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **November** and therefore reports and re-certification will be due in the month of **November** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator _____ Date _____

Appendix C: Recruitment email that was sent to The University of the Witwatersrand student population

Good day!

Do you go to bed and wake up at approximately the same time every day?

Do you get about 8 hours of sleep each night?"

Are you between the ages of 18 and 30 years old?

Do you have a regular menstrual cycle?

Are you generally healthy, free from chronic illness and chronic use of medication?

If you answered yes to all of the above questions then we would like to invite you to participate in our study at the Sleep Laboratory at the School of Physiology, University of Witwatersrand.

We are investigating the effects of sleep disruption on pain perception and the immune system in healthy females.

Participation in this study requires spending a total of 4 nights (non-consecutive) in the Wits Sleep Laboratory in specific phases of the menstrual cycle. Sleep will be disrupted for two of the four nights. A series of pain experiments and blood samples will be taken in the morning after each night. All participants will be provided with breakfast and will be reimbursed for time and transportation costs: At the end of the study, participants will receive **R350** per night **(R1400 in total).**

If you are interested in participating, you will undergo a week-long screening period to ensure that you are eligible before commencing the study.

If you are interested in participating or have any questions, please contact Felicia at 1430511@students.wits.ac.za or Zuha at 1602701@students.wits.ac.za

Ethical approval for this study has been granted by the Human Research Ethics Committee (HREC) of the University of Witwatersrand (M201127).

Kind regards,

Felicia Siboza and Zuha Ajlan, MSc candidates

On behalf of our research team, including:

Professor Karine Scheuermaier

Dr Stella Iacovides

Professor Peter Kamerman

Appendix D: Standardised email sent to all individuals who had shown interest in the study to verify they meet the minimum requirements

Good Day!

Thank you so much for your interest in our study! We are not certain when the study will start given the third wave but we hope to start in August.

In the meantime, we are conducting screening which comprises of two phases:

1. Online questionnaires: We will send you a link that contains three questionnaires that will need to be filled out as if you are not in exam season.

2. Activity watch screening: We will arrange a day and time with you where you will be asked to come into the Sleep Lab, 6th Floor, Wits Medical School, to collect an Activity watch and sleep diary that you will need to fill for a week. During this time you will not be in the lab but will go about your daily activities at your place of residence.

Can you please answer the following questions for us, please?

1. How are you feeling?

2. What are you studying?

3. What is your student number?

4. Do you have a regular menstrual cycle?

5. Do you track your menstrual cycle?

6. Do you have a fixed bedtime and wake-up time outside of the exam period?

7. How much sleep do you get outside of exams?

8. Are you taking any medication?

9. Should you be eligible, would you be comfortable in spending 4 non-consecutive nights with us in the Wits Sleep Lab, 6th floors, Wits Medical School?

I have attached an information sheet for your reference, please make sure you read this carefully before you agree to be part of our study.

Please feel free to contact me if you have any questions or concerns.

Sincerely

Appendix E: General information and pain assessment

UNIVERSITY OF THE WITWATERSRAND

SCHOOL OF PHYSIOLOGY

SCREENING QUESTIONNAIRE

Subject profile

Date:.....

Subject name:.....

Subject code:..... (*Not to be completed by subject)*

Date of birth:..... / ..... / ..... Age:.....

Height:.....(m) Body mass:.....(kg)

What is your first language? …….....

What language did you get your education in? ……..

Contact details

Home tel: .....

Work tel: .....

Cell no: .....

E-mail:.....

Subject code:

Other details

1. Do you smoke? **YES / NO**
2. Have you ever been pregnant, and if so, do you have children?

   .....
3. Are you generally a physically healthy person? **YES / NO**
4. Have you suffered from any recent illness (within the last three months), and if so, what was the illness? .....
5. Are you an emotionally and psychologically healthy person? **YES / NO**
6. Do you have an intrauterine device (IUD) fitted? **YES / NO**
7. Are you taking, or have you ever taken, any form of hormonal therapy (e.g. oral contraceptive, injected contraceptive, Diane for skin)? **YES / NO**. If yes, please give details.....

.....

Your menstrual cycle

1. When did you have your very first menstrual period? ..... (year) .....(age)

2. If you have a regular menstrual cycle (ie. you never miss your period AND is it roughly the same length), what is its usual length? …… days

3. If you do have an irregular menstrual cycle, please estimate the maximum and a minimum number of days between your menstrual periods, in a typical year:

   Max: …… days

   Min: ……. days

4. What was the date of the first day of your last period? ..... / ..... / 20.....

5. Usual length of menstruation (bleeding) .....days

6. What should be the approximate date of your next period?..... / ..... /20....

7. Do you suffer from menstrual pain - on the day before menstruation? **YES / NO**

   -during menstruation?

YES / NO

8. When did you start suffering from menstrual pain? ..... (year)

9. Have you always suffered from menstrual pain since your teenage years? **YES / NO**

If NO, when have you not had menstrual pain?

……

……

10. Do you suffer from any complaints, such as irritability, tearfulness or bloatedness, before or during menstruation? **YES / NO**

Please specify:

.....

.....

.....

11. Do you have any complaints, apart from pain, during menstruation e.g. irritability, backache etc.?

……

……

12. Do you ever experience pain at the same place where you experience menstrual pain during the non-bleeding phase of your monthly cycle? **YES / NO**

If yes, please specify:.....

.....

.....

13. Have you ever consulted a medical professional about your period pain? **YES/NO**

..... (year)

14. Have you ever been diagnosed by a doctor with any of the following conditions:

(a)	pelvic inflammatory disease	**YES / NO**
(b)	endometriosis	**YES / NO**
(c)	adenomyosis	**YES / NO**
(d)	uterine polyps	**YES / NO**
(e)	ovarian cysts	**YES / NO**
(f)	ovarian cysts	**YES / NO**
(g)	pelvic congestion syndrome	**YES / NO**

The severity of your menstrual pain

1. How does your menstrual pain affect your working ability?

   Unaffected Rarely affected Moderately affected Severely affected

2. How often do you take painkillers for your menstrual pain?

   Not required Rarely required Often required Always required

3. Do you ever suffer from nausea, headaches, diarrhoea or any other symptoms associated with your period pain?

   Never Seldom Frequently Always

   Explain:.....

4. Mark on the scale below how severe the pain is during menstruation. (Past 6 months)

   No pain at all ⊢——————————————————⊣ **Worst pain I have ever felt**

5. At what time of the day is your period pain the worst?

   Morning Afternoon Evening Night

6. List all the medications you have ever tried for your period pain in the past

……

……

……

7. Is the medication effective at relieving your pain? Explain.

.....

.....

General health

1. Are you aware that you are suffering from any of the following complaints?

Diabetes	**Yes**	**No**
Hypertension	**Yes**	**No**
Muscular disorders	**Yes**	**No**
Bone disorders	**Yes**	**No**
Joint disorders	**Yes**	**No**
Gastrointestinal disorders	**Yes**	**No**
Asthma	**Yes**	**No**
Epilepsy	**Yes**	**No**
Kidney disorders	**Yes**	**No**
Liver disorders	**Yes**	**No**
Autoimmune diseases	**Yes**	**No**
Sensory disorders	**Yes**	**No**
Anxiety or Depression	**Yes**	**No**

2. Are you currently on any regular medication (i.e. that you take at least once a day)?

YES / NO

Name of medication:.....

Taken for what?.....

Since when?.....

3. Do you take anti-inflammatories or painkillers more than once a week?

Yes **No**

4. Do you typically sleep well? **Yes** **No**

If "No", what sort of problems do you have? (tick the problem)

……. Difficulty falling asleep?

……. Waking up in the middle of the night/difficulty falling asleep again?

……. Waking up too early in the morning?

……. Disruptive leg movements/ leg discomfort?

……. Disruptive snoring/ gasping for air?

6. Do you have a regular bedtime and wake up times? **Yes** **No**

If yes; normal bedtime? ……: ……..

normal wake-up time? ……: ……..

7. How many hours do you generally sleep at night?

.....

8. Do you nap; if so, how often?

.....

Previous Pain Intensity

What is the most painful experience you have ever had?

……

……

……

People agree that the following five words represent pain in increasing intensity:

a) Mild
b) Discomforting
c) Distressing
d) Horrible
e) Excruciating

To answer the questions below, write the letter of the most appropriate word in the space provided:

Which word best describes the worst pain you have ever felt?.....

Which word best describes the worst toothache you have ever had?.....

Which word best describes the worst headache you have ever had?.....

Which word best describes the worst stomach-ache you have ever felt?.....

Which word best describes your menstrual pain?.....

Appendix F: General Health Questionnaire

GENERAL HEALTH QUESTIONNAIRE

Subject code: …… Date: ……

Subject name: …… Study day: ……..

We would like to know if you have any medical complaints, and how your health has been in general **over the past few weeks**.
Please answer ALL the questions by simply circling the answer which you think most nearly applies to you.
Remember that we need to know about present and recent complaints, not those that you had in the past.
It is important that you try to answer ALL the questions

Thank you very much for your cooperation

HAVE YOU RECENTLY:

1. – been able to concentrate on whatever you are doing?	Better than usual	Same as usual	Less than usual	Much less than usual
2. – lost much sleep over worry?	Not at all	No more than usual	Rather more than usual	Much more than usual
3. – been having restless, disturbed nights?	Not at all	No more than usual	Rather more than usual	Much more than usual
4. – been managing to keep yourself busy and occupied?	More so than usual	Same as usual	Rather less than usual	Much less than usual
5. – been getting out of the house as much as usual?	More so than usual	Same as usual	Less than usual	Much less than usual
6. – been managing as well as most people would in your shoes?	Better than most	About the same	Rather less well	Much less well
7. – felt on the whole you were doing things well?	Better than most	About the same	Less well than usual	Much less well
8. – been satisfied with the way you've carried out your task?	More satisfied	About same as usual	Less satisfied than usual	Much less satisfied
9. – been able to feel warmth and affection for those near to you?	Better than usual	About same as usual	Less well than usual	Much less well
10. – been finding it easy to get on with other people	Better than usual	About same as usual	Less well than usual	Much less well

11. – spent much time chatting with people	More time than usual	About same as usual	Less well than usual	Much less well
12. – felt that you are playing a useful part in things?	More so than usual	Same as usual	Less well than usual	Much less well
13. – felt capable of making decisions about things?	More so than usual	Same as usual	Less well than usual	Much less well
14. – felt constantly under strain?	Not at all	No more than usual	Rather more than usual	Much more than usual
15. – felt you couldn't overcome your difficulties?	Not at all	No more than usual	Rather more than usual	Much more than usual
16. – been finding life a struggle all the time?	Not at all	No more than usual	Rather more than usual	Much more than usual
17. – been able to enjoy your normal day-to-day activities?	More so than usual	Same as usual	Less so than usual	Much less than usual
18. – been taking things hard?	Not at all	No more than usual	Rather more than usual	Much more than usual
19. – been getting scared or panicky for no good reason?	Not at all	No more than usual	Rather more than usual	Much more than usual
20. – been able to face up to your problems?	More so than usual	Same as usual	Less able than usual	Much less able
21. – found everything getting on top of you?	Not at all	No more than usual	Rather more than usual	Much more than usual
22. – been feeling unhappy and depressed?	Not at all	No more than usual	Rather more than usual	Much more than usual
23. – been losing confidence in yourself?	Not at all	No more than usual	Rather more than usual	Much more than usual
24. – been thinking of yourself as a worthless person?	Not at all	No more than usual	Rather more than usual	Much more than usual
25. – felt that life is entirely helpless?	Not at all	No more than usual	Rather more than usual	Much more than usual
26. – been feeling hopeful about your own future?	More so than usual	About same as usual	Less so than usual	Much less hopeful
27. – been feeling reasonably happy, all things considered?	More so than usual	About same as usual	Less so than usual	Much less than usual
28. – been feeling nervous and strung-up all the time?	Not at all	No more than usual	Rather more than usual	Much more than usual

29. – felt like life isn't worth living?	Not at all	No more than usual	Rather more than usual	Much more than usual
30. – found at times you couldn't do anything because your nerves were too bad?	Not at all	No more than usual	Rather more than usual	Much more than usual

Appendix G: Pittsburgh Sleep Quality Index

PITTSBURGH SLEEP QUALITY INDEX (PSQI)

Name:…… ID No.:……

Date:…… Age:……

Instructions:
The following questions relate to your usual sleep habits during the past month *only*. Your answers should indicate the most accurate reply for the *majority* of days and nights in the past month. Please answer all questions.

1. During the past month, when have you usually gone to bed at night?

   USUAL BED TIME _____

2. During the past month, how long (in minutes) has it usually take you to fall asleep each night?

   NUMBER OF MINUTES _____

3. During the past month, when have you usually gotten up in the morning?

   USUAL GETTING UP TIME _____

4. During the past month, how many hours of *actual sleep* did you get at night? (This may be different than the number of hours you spend in bed).

   HOURS OF SLEEP PER NIGHT _____

For each of the remaining questions, check the one best response. Please answer *all* questions.

5. During the past month, how often have you had trouble sleeping because you

   a) Cannot get to sleep within 30 minutes

Not during the past month ……	Less than once a week ……	Once or twice a week ……	Three or more times a week …..

   b) Wake up in the middle of the night or early morning

Not during the past month ……	Less than once a week ……	Once or twice a week ……	Three or more times a week …..

   c) Have to get up to use the bathroom

Not during the past month ……	Less than once a week ……	Once or twice a week ……	Three or more times a week …..

d) Cannot breathe comfortable

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week .....

e) Cough or snore loudly

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week .....

f) Feel too cold

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week .....

g) Feel too hot

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week .....

h) Had bad dreams

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week .....

i) Have pain

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week .....

j) Other reason(s), please describe:.....

.....

How often during the past month have you had trouble sleeping because of this?

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week .....

6. During the past month, how would you rate your sleep quality overall?

Very good _____
Fairly good _____
Fairly bad _____
Very bad _____

7. During the past month, how often have you taken medicine (prescribed or "over the counter") to help you sleep?

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week .....

8. During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week .....

9. During the past month, how much of a problem has it been for you to keep up enough enthusiasm to get things done?

No problem at all _____
Only a very slight problem _____
Somewhat of a problem _____
A very big problem _____

10. Do you have a bed partner or roommate?

No bed partner or roommate _____
Partner/roommate in other room _____
Partner in same room, but not same bed _____
Partner in same bed _____

Appendix H: Sleep diary, sleep quality and morning vigilance scales

COMPLETE IN THE MORNING

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Date							
Day of the week							
Time you went to bed							
Time you fell asleep							
Time you woke up today							
How long did it take you to fall asleep?							
Did you take anything to	**YES/NO**	**YES/NO**	**YES/NO**	**YES/NO**	**YES/NO**	**YES/NO**	**YES/NO**

help you sleep? (tick the correct one)							
If YES, what did you take?							
How many times did you wake up last night and for how long?							
Number of times							
Number of minutes in total							
Did anything disturb your sleep? (side effects of medication, noise, hot/cold, uncomfortable, etc)							

Did you take a nap yesterday?	**YES/NO**	**YES/NO**	**YES/NO**	**YES/NO**	**YES/NO**	**YES/NO**	**YES/NO**
If YES, what time? **How long?**							
Did you take the Wrist Actigraph off? (shower etc.)	**YES/NO**	**YES/NO**	**YES/NO**	**YES/NO**	**YES/NO**	**YES/NO**	**YES/NO**
If YES, what time? **How long?**							

Instructions for Wearing the Wrist Actiwatch

While you are wearing the watch:

- Please do not remove the watch, unnecessarily. If you must remove it, please carefully record the time you took it off and the time you put it back on (Note down in Sleep Diary).
- The watch is not waterproof and therefore must be removed when bathing or swimming. Please note the time you took it off and put it back on.
- Do not cover the watch with clothing and be careful when putting on and removing clothing.

If you have any questions, you can contact Felicia on 076 351 3318 or Zuha on 081 351 9289 via WhatsApp as well.

Please be sure to return your watch, intact, at the next visit.

Subject Code…….. **Date…….**

Subject Name……. **Day 1……..**

SLEEP QUALITY

Please make a mark on the line to indicate how well you slept last night.

No sleep _____ Best sleep I ever had

MORNING VIGILANCE

Please make a mark on the line to indicate how bright, fresh and alert you feel this morning.

Not at all alert and fresh _____ Most alert and fresh I ever felt

---

Date……. **Day 2……..**

SLEEP QUALITY
Please make a mark on the line to indicate how well you slept last night.

No sleep _____ Best sleep I ever had

MORNING VIGILANCE
Please make a mark on the line to indicate how bright, fresh and alert you feel this morning.

Not at all alert and fresh _____ Most alert and fresh I ever felt

---

Date……. **Day 3……..**

SLEEP QUALITY
Please make a mark on the line to indicate how well you slept last night.

No sleep _____ Best sleep I ever had

MORNING VIGILANCE
Please make a mark on the line to indicate how bright, fresh and alert you feel this morning.

Not at all alert and fresh _____ Most alert and fresh I ever felt

---

Date……. **Day 4……..**

SLEEP QUALITY
Please make a mark on the line to indicate how well you slept last night.

No sleep _____ Best sleep I ever had

MORNING VIGILANCE
Please make a mark on the line to indicate how bright, fresh and alert you feel this morning.

Not at all alert and fresh _____ Most alert and fresh I ever felt

Date……. **Day 5……..**

SLEEP QUALITY
Please make a mark on the line to indicate how well you slept last night.

No sleep _____ Best sleep I ever had

MORNING VIGILANCE
Please make a mark on the line to indicate how bright, fresh and alert you feel this morning.

Not at all alert and fresh _____ Most alert and fresh I ever felt

---

Date……. **Day 6……..**

SLEEP QUALITY
Please make a mark on the line to indicate how well you slept last night.

No sleep _____ Best sleep I ever had

MORNING VIGILANCE
Please make a mark on the line to indicate how bright, fresh and alert you feel this morning.

Not at all alert and fresh _____ Most alert and fresh I ever felt

---

Date……. **Day 7……..**

SLEEP QUALITY
Please make a mark on the line to indicate how well you slept last night.

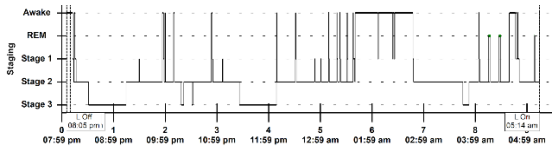
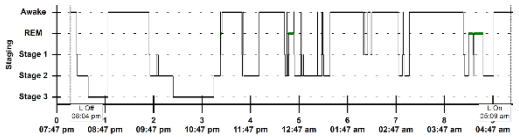
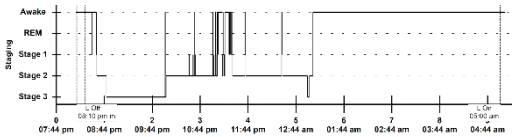
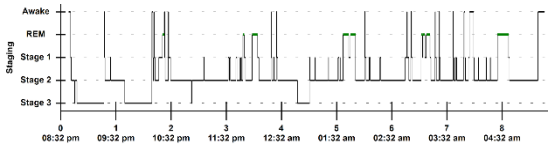
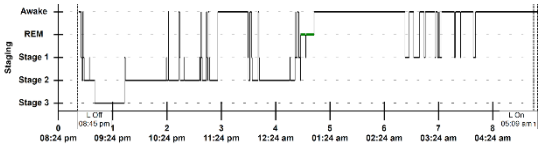
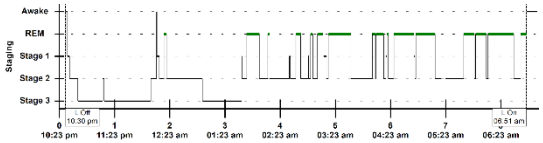
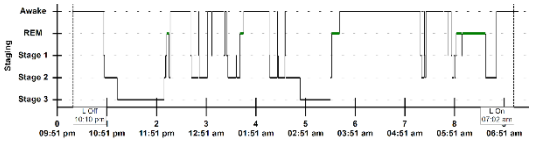
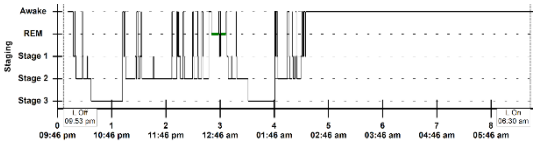
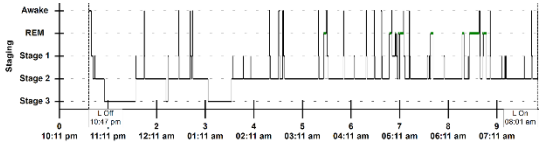
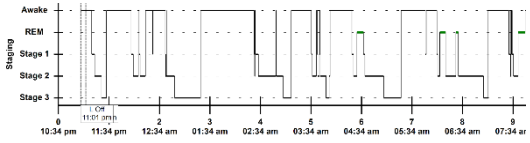
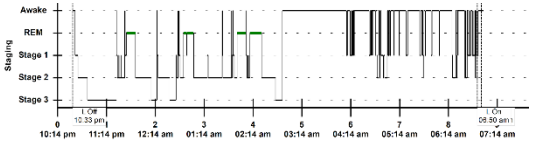
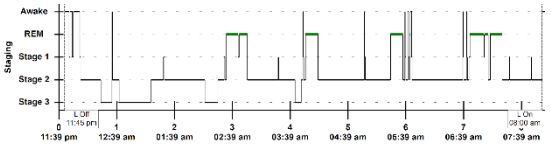
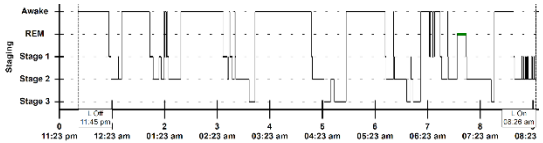
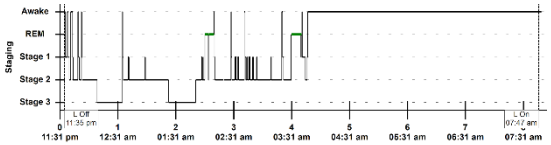
No sleep _____ Best sleep I ever had

MORNING VIGILANCE
Please make a mark on the line to indicate how bright, fresh and alert you feel this morning.

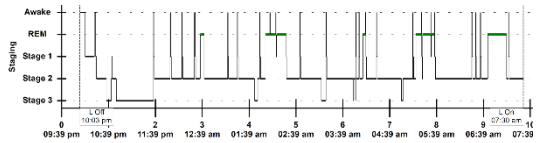
Not at all alert and fresh _____ Most alert and fresh I ever felt

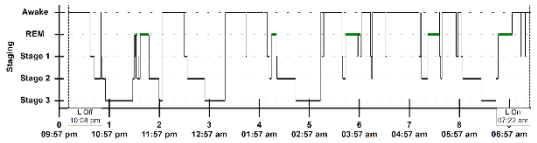
Appendix I: Hypnograms of Experimental Sleep Conditions

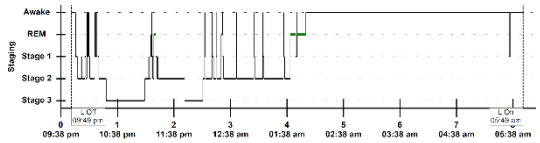
Table 10: Hypnograms are shown for each sleep condition for each participant

Participant	Baseline	Fragmentation	Restriction
1.			
2.			
3.			
4.			
5.			

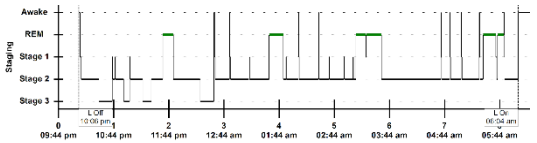
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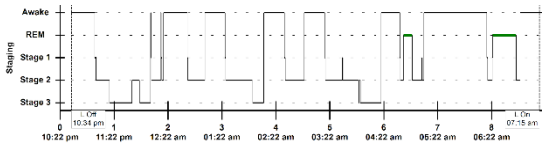

Awake
REM
Stage 1
Stage 2
Stage 3
Staging

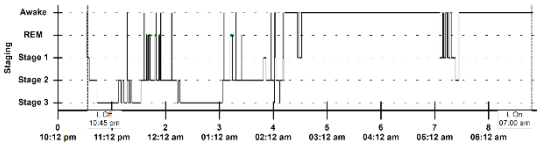

Awake
REM
Stage 1
Stage 2
Stage 3
Staging


Awake
REM
Stage 1
Stage 2
Stage 3
Staging

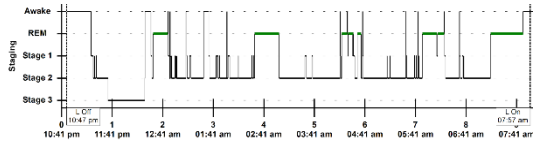
7.

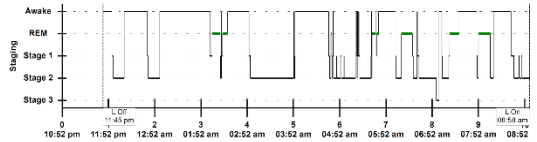

Awake
REM
Stage 1
Stage 2
Stage 3
Staging

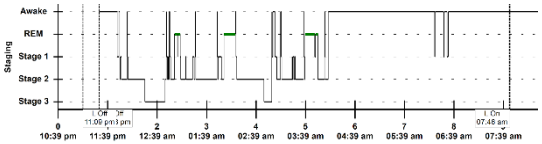

Awake
REM
Stage 1
Stage 2
Stage 3
Staging


Awake
REM
Stage 1
Stage 2
Stage 3
Staging

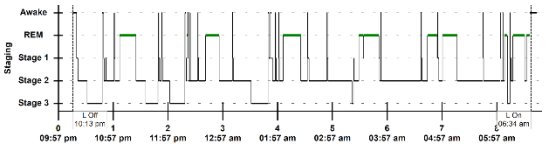
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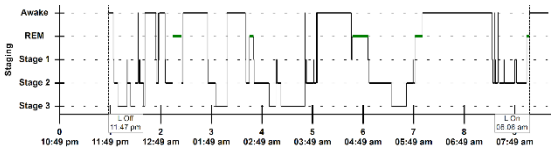

Awake
REM
Stage 1
Stage 2
Stage 3
Staging

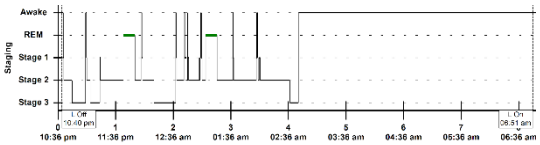

Awake
REM
Stage 1
Stage 2
Stage 3
Staging


Awake
REM
Stage 1
Stage 2
Stage 3
Staging

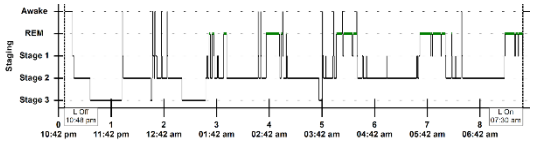
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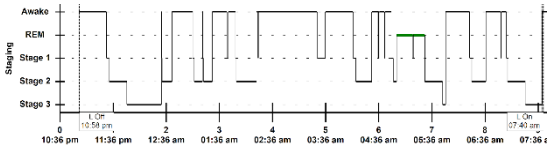

Awake
REM
Stage 1
Stage 2
Stage 3
Staging

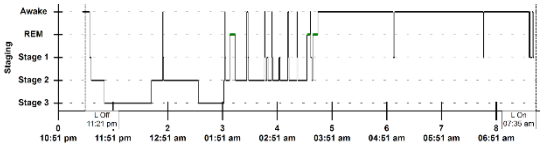

Awake
REM
Stage 1
Stage 2
Stage 3
Staging


Awake
REM
Stage 1
Stage 2
Stage 3
Staging

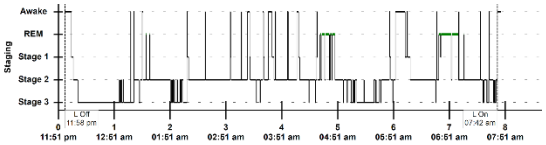
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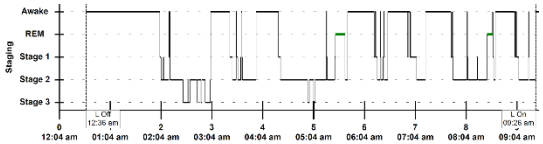

Awake
REM
Stage 1
Stage 2
Stage 3
Staging

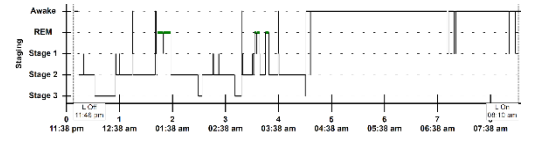

Awake
REM
Stage 1
Stage 2
Stage 3
Staging


Awake
REM
Stage 1
Stage 2
Stage 3
Staging

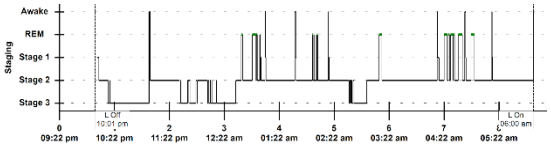
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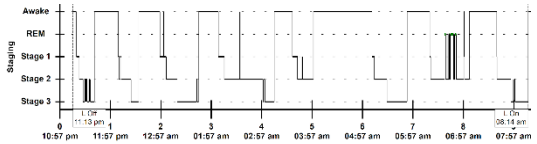

Awake
REM
Stage 1
Stage 2
Stage 3
Staging

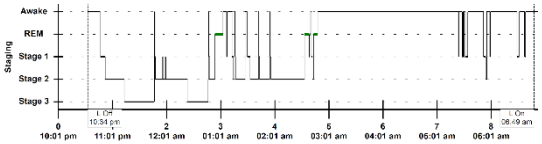

Awake
REM
Stage 1
Stage 2
Stage 3
Staging


Awake
REM
Stage 1
Stage 2
Stage 3
Staging

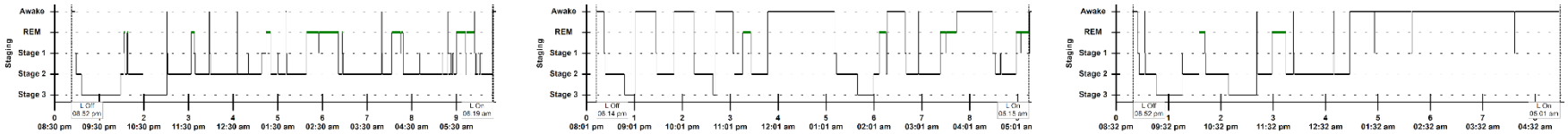
12.


Awake
REM
Stage 1
Stage 2
Stage 3
Staging

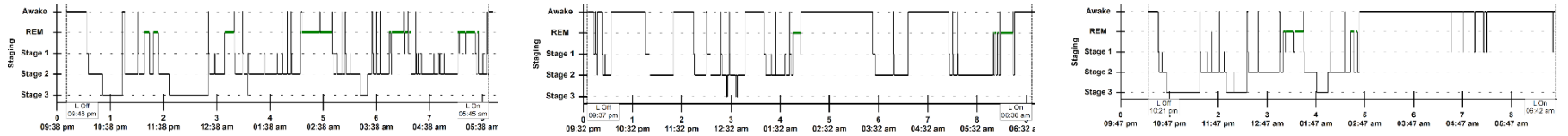

Awake
REM
Stage 1
Stage 2
Stage 3
Staging


Awake
REM
Stage 1
Stage 2
Stage 3
Staging

13.



14.



The Hypnogram for the fragmentation night of Participant 2 was not included due to a technical error that split the report into three sections. We did not have a concatenated hypnogram for Participant 2 to show.