Effects of Intensified Training on Immune and Endocrine Biomarkers: Identifying Biomarkers to Highlight the Negative States of Overtraining

by

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Abstract

Intensified training coupled with sufficient recovery is required to improve athletic performance; however, a stress-recovery imbalance can lead to the negative states of overtraining (non-functional overreaching (NFOR) or the overtraining syndrome (OTS)). Blunted cortisol and testosterone responses to a 30-minute, high intensity cycling test consisting of alternating blocks of 1 minute at 55% maximum workload (W_{max}) and 4 minutes at 80% W_{max} (known as the 55/80) has previously been identified suggesting that these exercise-induced hormones are possible biomarkers of NFOR/OTS in males. However, it remains unknown whether the same phenomenon occurs in females. These hormonal alterations associated with overtraining may impair the immune response. Cortisol, for example, promotes the maturation and migration of dendritic cells (DCs); key antigen presenting cells which subsequently stimulate a T cell response, yet very little is currently known about their response to intensified training. Specifically, there are currently no clear reliable immune biomarkers to highlight when athletes are entering states of NFOR/OTS. Therefore, the aims of this thesis were:

1. To examine the salivary and plasma cortisol and testosterone, and plasma progesterone responses in females to the previously developed 30-minute cycling stress test (55/80).
2. To assess the current literature examining the effects of intensified training periods on lymphocyte and DC number and function.
3. To establish the reproducibility of T cell and DC count responses to a newly developed 30-minute stress test utilising submaximal physiological thresholds to prescribe intensity (20/50).
4. To investigate the endocrine (salivary and plasma cortisol and testosterone) and immune (DC toll-like receptor (TLR) and cytokine) alterations that may occur with a 9-day intensified training period.

The first experimental Chapter, Chapter 4, investigated the plasma and salivary cortisol, testosterone and plasma progesterone response to the 55/80 cycling test in physically fit, healthy females. The effects of oral contraceptives on the hormonal responses to the 55/80 were also examined. Chapter 4 concluded that the 55/80 induced hormonal elevations in females, similar in magnitude to males, however, these exercise-induced elevations were attenuated in oral contraceptive users. These findings indicate that the 55/80 is a valuable tool to highlight exercise-induced hormone alterations associated with NFOR/OTS in naturally menstruating females, but not females prescribed oral contraceptive pills. Given the known interaction between the endocrine and immune systems, Chapter 5 assessed the existing literature examining the effects of intensified exercise training on DC and lymphocyte counts and function, to identify potential gaps for investigation. The systematic review and meta-analysis concluded that although some immune biomarkers alter after a period of intensified training, definitive immune biomarkers are limited. Specifically, due to low study numbers, further investigation into the DC response in human models was required. Given the importance that DCs play in orchestrating the immune response, yet the limited literature investigating their response to heavy training periods, Chapter 6 developed an exercise stress test capable of eliciting robust and reproducible elevations in DC and T cell counts (known as the 20/50). The 30-minute 20/50 was developed to address weaknesses with the design of the 55/80 such that exercise intensity was prescribed

utilising submaximal anchors (ventilatory threshold; VT_1), rather W_{max} to ensure physiological consistency. Chapter 6 concluded that the 20/50 induced robust and reproducible elevations in DC and T cell counts, assessed via coefficient of variations (CV), Bland-Altman plots, smallest real difference (SRD) and intraclass correlation coefficients (ICC). The 20/50 can therefore be used before and after a period of intensified training to highlight any exercise-induced DC and T cell alterations that may occur. Chapter 7 draws together the work of all previous Chapters to examine the resting and exercise-induced immune (DC and T cell count and function) and hormonal (plasma and salivary cortisol and testosterone) responses before and after a 9-day period of intensified training. Although the 20/50 led to elevated immune cell counts, stimulated DC TLR 7 and 9 expression and the percentage of interleukin (IL)-10 producing T cells, and reductions in stimulated plasmacytoid DC Interferon (IFN)-α secretion, no alterations were seen after the intensified training period compared with before the training period. Blunted exercise-induced plasma and salivary testosterone and plasma cortisol were observed in response to the 20/50 after the 9-day intensified training period, highlighting their usefulness as biomarkers of NFOR/OTS. The results indicate that the participants were able to withstand an ~80% intensification of their habitual training load without experiencing maladaptive immune responses, indicating that the immune system may be more robust than the endocrine system during periods of heavy training.

In conclusion, the studies in this thesis demonstrate that salivary testosterone, and plasma cortisol and testosterone, with the addition of plasma progesterone in females, are potential diagnostic biomarkers for detecting overreaching and possible OTS (Chapters 4 and 7). In contrast, immune biomarkers related to DC and T cell count and function appear less sensitive indicators of these states. However, the immune system in healthy, recreationally active males and females proved robust, tolerating ~80% increases in habitual training load over 9-days without significant declines in T cell or DC immunity. These findings support the view that heavy exercise may not be inherently immunosuppressive (Chapter 7), with the mobilisation and redistribution of effector cells potentially enhancing immunosurveillance at tissue sites (Chapter 6). Chapters 6 and 7 also contribute valuable insight into DC responses following intensified training; an area of limited research despite DCs' central role in immune regulation (Chapter 5). Finally, the 20/50 stress test effectively detects endocrine alterations associated with intensified training, supporting its practical use in monitoring athlete training loads during periods of heavy training, such as training camps.

List of Key Abbreviations

55/80	A continuous 30-minute cycle consisting of alternating blocks of 1 minute at 55% W_{max} and 4 minutes at 80% W_{max}
20/50	A continuous 30-minute cycle consisting of alternating blocks of 1 minute at 20% below VT_1 and 4 minutes at 50% between VT_1 and $\dot{V}O_{2max}$
ACSM	American College of Sports Medicine
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
CD	Cluster of differentiation
CRH	Corticotrophin releasing hormone
DAMPs	Damage associated molecular patterns
DC	Dendritic cell
ECSS	European College of Sport Science
ELISA	Enzyme linked immunosorbent assay
FOR	Functional overreaching
FSH	Follicle stimulating hormone
g	Gravitational force
GnRH	Gonadotrophin-releasing hormone
HPA	Hypothalamic pituitary adrenal
HPG	Hypothalamic pituitary gonadal
HR	Heart rate
HR_{max}	Maximum heart rate
IL	Interleukin
km	Kilometre
L	Litre
LH	Luteinising hormone
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
mL	Millilitre
NFOR	Non-functional overreaching
OTS	Overtraining syndrome
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cell
PRRs	Pattern recognition receptors

RPE	Ratings of perceived exertion
SD	Standard deviation
SNS	Sympathetic nervous system
Tc	Cytotoxic T cell ($CD8^+$)
TCR	T cell receptor
Th	Helper T cell ($CD4^+$)
TLR	Toll-like receptor
TRIMP	Training impulse
URI	Upper respiratory illness
$\dot{V}O_{2max}$	Maximum oxygen uptake
$\dot{V}O_{2peak}$	Peak oxygen uptake
VT_1	Ventilatory threshold
W	Watt
W_{max}	Maximum power output

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Preface

The work presented in this thesis is that of the authors, with reference made to all published resources used, and has not been previously submitted for another degree award to Nottingham Trent University, or any other University.

Some of the work in this thesis has been published in peer-reviewed journals and presented at conferences:

Published Original Investigations:

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Baker, C., Piasecki, J., Hunt, J, A., & Hough, J. (2024). The reproducibility of dendritic cell and T cell counts to a 30-min high-intensity cycling protocol as a tool to highlight overtraining. Experimental Physiology, 109, 380–392. https://doi.org/10.1113/EP091326

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Baker, C., Piasecki, J., Hunt, J, A., & Hough, J. (2024). The reproducibility of dendritic cell and T cell counts to a 30-min high-intensity cycling protocol as a tool to highlight overtraining. *The Physiological Society, Physiology 2023,* Harrogate (Poster Presentation).

Baker, C., Piasecki, J., Hunt, J. A., Foulds, G., & Hough, J. (2024). Plasma and salivary hormone responses to a 30-min exercise stress test in young, healthy, physically active females. *Physical Activity and the Endocrine System 2023,* Nottingham (Oral Presentation)
*1st Place Prize for oral communications

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Baker, C., Piasecki, J., Hunt, J. A., Foulds, G., & Hough, J. (*2025*). The effects of intensified training on dendritic cell toll-like receptor function and the endocrine stress response in healthy males and females. *Physical Activity and Endocrine System 2024,* Nottingham (Oral Presentation).

Baker, C., Piasecki, J., Hunt, J. A., Foulds, G., & Hough, J. (*2025*). The effects of intensified training on dendritic cell toll-like receptor function and the endocrine stress response in healthy males and females. *Royal Society of Chemistry Analytical Biosciences Group Early Career Researcher Meeting 2024,* Nottingham (Oral Presentation).

Baker, C., Piasecki, J., Hunt, J. A., Foulds, G., & Hough, J. (*2025*). The effects of intensified training on dendritic cell toll-like receptor function and the endocrine stress response in healthy males and females. *Isokinetic Medical Conference 2025,* Madrid (Oral Presentation).

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1 Chapter 1: Introduction and aims

Successful training programs involve overloading the body to stimulate positive training adaptation, whilst also preventing inadequate recovery (Meeusen et al., 2010 & 2013; Le Meur et al., 2014). Whilst athletes often complete rigorous training sessions with the aim of performance optimisation, a short-term performance decrement, named overreaching, may arise in as little as 7 days if insufficient recovery occurs (Halson et al., 2002). Overreached athletes experience temporary performance impairment, but following sufficient recovery (days- to- weeks) a performance "super-compensatory" effect may occur, referred to as functional overreaching (FOR) (Meeusen et al., 2013). If FOR is not recognised and the training/recovery imbalance continues then a "non-functional overreaching" (NFOR) state can occur (taking weeks- to- months to recover) (Meeusen et al., 2013). If NFOR is not recognised and the training/recovery imbalance continues further, then there is an increased risk of suffering from the overtraining syndrome (OTS), which may take many months or even years for full recovery to occur (Meeusen et al., 2013). Symptoms of maladaptation occur in both athletes from individual and team sports (Matos et al., 2011), with the incidence of experiencing NFOR/OTS at least once in an athletes' career being as high as 35% in young swimmers (Raglin et al., 2000), 37% in Swedish Olympic sport athletes (Kenttä et al., 2001), and 67% in US men elite distance runners (Morgan et al., 1987). Aside from performance decrements, other negative symptoms of overreaching include an increase in upper respiratory illnesses (URI) (Bishop & Gleeson, 2009; Gleeson et al., 2012), mood and sleep disturbances (Morgan et al., 1987; Raglin et al., 2000) and persistent fatigue (Meeusen et al., 2013). Nevertheless, little progress has been made on uncovering objective biomarkers that focus on identifying underlying mechanisms of NFOR/OTS (Armstrong & VanHeest, 2002; Wyatt et al., 2013). A recent systematic review was conducted to establish and detail the physiological and psychological changes that occur as a result of OTS in athletes, with the aim to identify the current state of overtraining research (Weakley et al., 2022). The authors concluded that, although OTS can be observed in field settings, there is limited data on how its physiological and psychological symptoms present. They suggest the likely causes are ambiguous terminology, challenges in long-term monitoring, and the need for prospective testing. Although the American College of Sports Medicine (ACSM) and the European College of Sport Science (ECSS) describe the Overtraining Syndrome (OTS) as being as debilitating as an orthopaedic injury, a testing protocol that is both comprehensive and practical for routine implementation has yet to be established (Meeusen et al., 2013). As a result, it has been concluded that there is currently a lack of high-quality scientific evidence to fully understand OTS in athletes (Weakley et al., 2022).

Both endocrine and immune biomarkers have been proposed as capable indicators of the negative states of overtraining. Cortisol and testosterone are two commonly measured hormones within the overtraining literature due to their ability to indicate the body's catabolic/anabolic state, respectively. At rest, basal levels of hormones such as cortisol and testosterone are often unaltered in overtrained athletes (Cadegiani & Kater, 2019). However, consistent blunting in the endocrine response to exercise have been shown following periods of intensified training (Meeusen et al., 2004; Hough et al., 2013; Hough et al., 2015; Leal et al., 2021a); a phenomenon highlighted in the most recent overtraining guidelines (Meeusen et al., 2013). Meeusen et al.

(2004) used two maximal cycle tests separated by 4 hours in well-trained athletes to show that the exercise-induced responses of cortisol and adrenocorticotropic hormone (ACTH; a precursor hormone to cortisol) to the second maximal cycle were reduced by ∼118% and ∼73%, respectively, after a 10-day training period where training volume was increased by ~58%. However, the two exercise bout protocol and lengthy recovery time used may make this an impractical tool for athletes.

Hough et al. (2011) therefore developed a 30-minute high intensity cycling protocol (55/80), consisting of alternating blocks of 1 minute at 55% W_{max} and 4 minutes at 80% $\dot{V}O_{2max}$. The 55/80 was shown to induce robust elevations in both salivary and plasma cortisol (~7nmol/L and 150 nmol/L from pre to post 55/80, respectively) and salivary testosterone (~400 pmol/L from pre to post 55/80) in healthy males (Hough et al., 2011). When implemented before and after an 11-day intensified training period, the 55/80 highlighted a ~116% (cortisol) and ~21% (testosterone) reduction in the peak salivary hormonal responses after the training period (Hough et al., 2013). This was further highlighted in a group of male elite triathletes (Hough et al., 2015).

To ensure the salivary hormonal changes identified with training in the aforementioned studies were not due to inconsistent cortisol and testosterone responses to the 55/80, Hough et al. (2021) investigated their reproducibility to the 55/80. The results indicated that the salivary cortisol and testosterone response to the 55/80 were not different between the three times completed and produced an ICC for pre to peak post exercise as good (0.89) for cortisol and moderate (0.53) for testosterone. This repeatability study included male participants only, so it is not known whether females also display reproducible hormone responses to the 55/80. Additionally, this study only investigated salivary hormones, not plasma hormones, which would provide further insight into the hormonal perturbations induced by the 55/80 stress test. To include females in future overtraining research of this nature, it is therefore of interest to further this work by assessing the reproducibility in females, with the added insights provided by measuring plasma hormones.

The 55/80 stress test is based solely on the percentage of W_{max}, identified from work rate achieved at $\dot{V}O_{2max}$. It has been argued that using a percentage of maximum to prescribe exercise intensity assumes that all participants will experience the same homeostatic perturbations to the same relative intensity, not taking into account submaximal physiological thresholds (Jaminick et al., 2020). However, large differences in homeostatic perturbations, that is, oxygen uptake kinetics and blood lactate responses, have been reported across multiple studies using exercise within the 'moderate intensity' zone (60–80% $\dot{V}O_{2max}$) (Jamnick et al., 2020). As such, the use of submaximal anchors, such as VT_1, is recommended to prescribe exercise intensity (Mann et al., 2013). Therefore, the development of an adjusted version of 55/80 stress test that prescribes intensity based on the VT_1 is required.

The impact of heavy periods of training on the immune system remains unclear, with some evidence suggesting a decline in immunity, and increased risk of URIs after repeated arduous exercise bouts (Walsh, 2019; Nieman, 1994). As discussed in detail in Chapter 5, whether exercise suppresses immune function and

leads to increased susceptibility to infection was thrown into question with the publication of a recent 'debate' paper, whereby evidence both supporting and disputing this notion is presented (Simpson et al., 2020). It is however known that cortisol plays an important role in the anti-inflammatory response of the immune system to exercise by increasing the phagocytic potential of neutrophils and monocytes (Blannin et al., 1996; Ortega et al., 1996), supressing pro-inflammatory mediators such as reactive oxygen species (ROS) (Franchimont, 2004), dampening the secretion of pro-inflammatory cytokines (Petrovsky et al., 1998), inducing lymphocytopenia (Okutsu et al., 2005) and regulating the maturation and migration of DCs towards lymph nodes (Liberman et al., 2018). Testosterone also possesses anti-inflammatory effects on the immune system by modulating TLR expression (Chen et al., 2018), dampening pro-inflammatory cytokine release such as IL-1β and tumour necrosis factor (TNF)-α (Chrysohoou et al., 2013; Kalinchenko et al., 2010) and inhibiting macrophage inflammatory protein 1-α and 1-β (Bobjer et al., 2013). Therefore, temporarily dysfunctional cortisol and testosterone secretion caused by a period of intensified training may lead, in part, to an impaired immune response during intensified exercise.

DCs are professional antigen presenting cells that take up antigen in the innate immune system and present it on their surface membrane to cells of the adaptive system, allowing the coordination of a specific immune response (Yin et al., 2021). The chief mechanism for pathogen recognition in DCs is via their TLRs (Janeway, 1989). TLRs are a family of pattern recognition receptors (PRRs) that are located both on the cell membranes of DCs and intracellularly in endosomes (Yamamoto & Takeda, 2010). Each TLR recognises a specific type of microbe, which once bound to the TLR, causes the DC to mature and upregulate the pro-inflammatory cytokines required to initiate the appropriate pro-inflammatory response from T cells (Lancaster et al., 2005). Glucocorticoids, such as cortisol, and androgens such as testosterone, have been shown to regulate TLR transcriptional pathways and TLR induced pro-inflammatory cytokine production in both humans (Rozkova et al., 2006) and rodents (Chen et al., 2018). Specifically, testosterone levels have been inversely correlated with a number of inflammatory mediators such as IL-1β and TNF-α in humans (Chrysohoou et al., 2013; Kalinchenko et al., 2010), and cortisol has been shown to interfere with TLR signalling by acting through glucocorticoid receptors to activate or repress target gene transcription, increase expression of the natural inhibitors of TLR pathways, and utilise cofactors essential for both glucocorticoid receptors and TLR signalling (Chinenov & Rogatsky, 2007). Rozkova et al. (2006) treated monocyte-derived DCs from humans with glucocorticoids and examined their expression of TLR2, 3 and 4, providing evidence that glucocorticoids skewed DC differentiation to a distinct population incapable of inducing an immune response; reduced DC IL-12 and TNF-α secretion and T cell stimulatory function. To extend their in vitro findings, they also analysed the distribution of DC subsets in the blood of patients treated with hight-dose corticosteroids, ultimately evidencing that administration of high-dose corticosteroids to patients with systemic autoimmunity induces a decrease of circulating mDC and pDC (Rozkova et al., 2006).

Despite their vital role in orchestrating the immune response, very little is known about how DCs respond to intensified training periods, such as those undertaken by elite athletes, as highlighted by the systematic search of the literature In Chapter 5 (Baker et al., 2022). Therefore, investigating changes in DC function in

response to an intensified training period is of interest. In addition, to examine their usefulness as a biomarker of overtraining, it is important to firstly establish that their response to the stress test used to highlight endocrine alterations with intensified training, also induces robust and reproducible immune alterations. This will ensure any alterations seen after the training period are due to the intensified training itself, and not due to daily variation in the markers, in response to the exercise stress test.

Therefore, the main aims of this thesis were:

1. To examine the salivary and plasma cortisol and testosterone, and plasma progesterone responses in females to the previously developed 30-minute cycling stress test (55/80).
2. To assess the current literature examining the effects of intensified training periods on lymphocyte and DC number and function.
3. To establish the reproducibility of T cell and DC count responses to a newly developed 30-minute stress test utilising submaximal physiological thresholds to prescribe intensity (20/50).
4. To investigate the endocrine (salivary and plasma cortisol and testosterone) and immune (DC TLR and cytokine) alterations that may occur with a 9-day intensified training period.

The hypotheses of this thesis were:

1. That the 55/80 would induce robust elevations in plasma and salivary cortisol and progesterone in females, but not salivary and plasma testosterone.
2. That the 20/50 would be stressful enough to elicit robust elevations in T cells and DC counts, and these elevations would be reliable.
3. That the 9-day intensified training period would lead to blunted salivary and plasma cortisol and testosterone responses to the 20/50. It was also hypothesised that resting T cell and DC counts would be lower post training, coupled with an attenuation in their exercise-induced response to the 20/50 post training.

2 Chapter 2: Literature review

2.1 Overreaching/OTS

An athlete's training programme can be divided into structured blocks of training cycles; a process called periodisation (Mujika, 2009). The blocks are made up of macrocycles; the longest training cycle and is generally referred to as a single competitive season (Naclerio et al., 2013). One macrocycle involves a number of mesocycles which are typically 6-8 weeks in duration (Whyte, 2006), which in turn contain 2-6 interrelated microcycles. The microcycles serve as a recurring unit over the length of a mesocycle and typically last 7 days (Turner, 2011).

To enhance exercise performance, athletes can strategically increase their training volume and intensity during certain points of their competitive season (Slivka et al., 2010). In accordance with basic principles of biological adaptation, such as the general adaptation syndrome, adaptation only occurs if "an organism is exposed to a stimulus to the quality or intensity of which it is not adapted" (Selye, 1938 p. 758), for example, athletes undergoing periods of intensified training. Across the course of a training cycle, the body is repeatedly exposed to stressful stimuli, such as exercise stress or psychological stress, often coupled with limited recovery. This is the action of *overtraining* and can result in short-term declines in performance, termed *overreaching,* which can occur in as little at 7 days (Urhausen & Kindermann, 2002; Halson et al., 2002). As described in Chapter 1, overreaching can be used by athletes during training cycles to enhance exercise or sporting performance, because a *super-compensatory* improvement in exercise performance above baseline levels can occur if sufficient recovery is subsequently implemented, this is called FOR (Birrer et al., 2013; Meeusen et al., 2013). If sufficient recovery is not implemented during this short-term performance decrement, athletes may enter a state of NFOR*,* which will lead to a stagnation or decrease in performance that can take weeks- to- months for full recovery to occur (Meeusen et al., 2013). If NFOR is left undiagnosed and the training/recovery imbalance continues, athletes experience a heightened risk of suffering from the OTS, which can take months- to- years to fully recover from (Meeusen et al., 2013) (Figure 2.1). Therefore, for successful adaptations to training to occur, training cycles must carefully balance sufficient exercise stimuli and recovery (Whyte, 2006).

As briefly described in Chapter 1, symptoms of NFOR/OTS most commonly occur in endurance events such as swimming, cycling or running (Cardoos, 2015), including both individual (37%) and team sport (17%) athletes (Matos et al., 2011), likely due to the high training volumes required for peak performance in these types of events. Symptoms associated with overtraining include poor sleep, increased risk of URI, low mood and performance declines (Meeusen et al., 2013). Despite the incidence of NFOR/OTS across an athlete's career spanning as much as 30–60% (Birrer et al., 2013; Morgan et al., 1987) and the lack of prospective studies investigating OTS, little progress has been made on uncovering objective and reliable biomarkers that focus on identifying the occurrence of NFOR/OTS and the underlying mechanisms leading to the associated symptoms (Armstrong & Vanheest, 2002; Schwellnus et al., 2016; Meeusen et al., 2013).

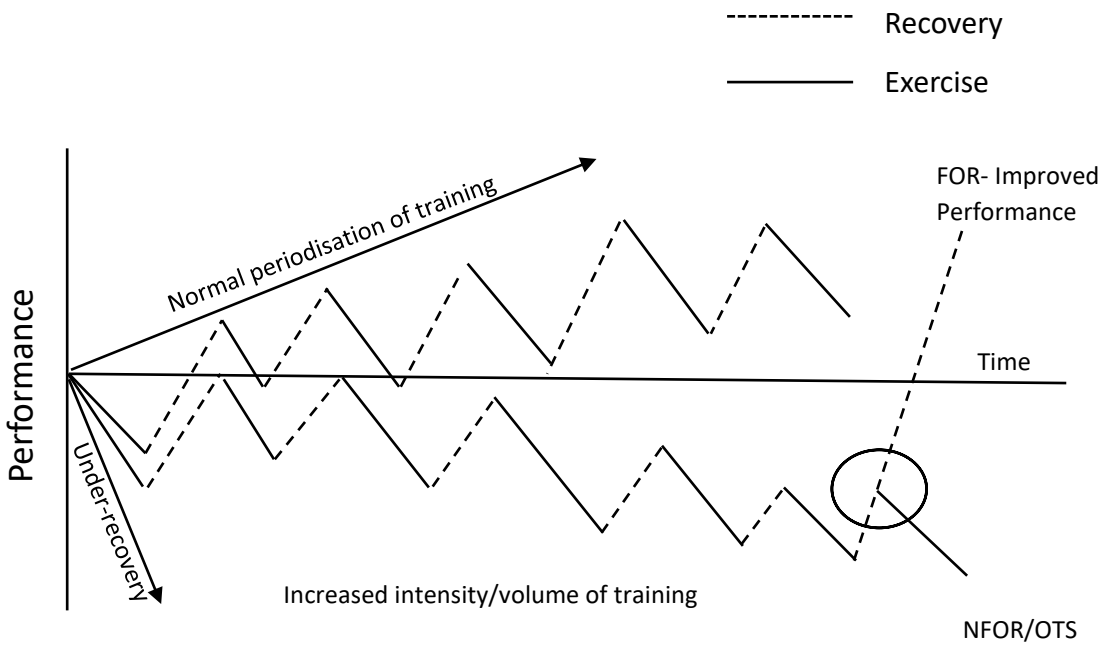


Figure 2.1. schematic of performance outcomes during the normal periodisation of training (top arrow), the super-compensatory increase in performance when sufficient recovery is implemented during functional overreaching (FOR) i.e. a period of increased stress with reduced recovery, and the performance decrements that occur with non-functional overreaching (NFOR) and the overtraining syndrome (OTS). NFOR/OTS occur when sufficient recovery is not implemented in a timely manner, thus experiencing a long lasting decline in performance, rather than the super compensatory response in performance as seen with FOR.

2.2 Hormones as biomarkers of overtraining

As previously mentioned, the negative states of overtraining (NFOR/OTS) are often retrospectively diagnosed based on the time taken for recovery, and currently, there are no clear biomarkers to establish when they occur (Meeusen et al., 2013). Current criteria for diagnosing OTS in athletes suggest an unexplained drop in performance (i.e. >10% decline in max performance), persistent fatigue lasting > 4 weeks, no underlying diseases leading to performance declines and some additional confounders supporting the evidence i.e. significant increases in training load (Meeusen et al., 2013). However, finding a biological marker of overtraining would allow for the continual monitoring of athletes and support the reduction in the incidence of these negative overtraining states.

The hormones cortisol and testosterone have been suggested as possible biomarkers of overtraining as when measured together, they provide an indication of the body's state of stress by showing the body's catabolic/anabolic balance. As mentioned in Chapter 1, both hormones have shown potential in their use as a biomarker of overtraining (Meeusen et al., 2004; Hough et al., 2013; Hough et al., 2015). A review of the

literature investigating these hormones as possible biomarkers of overtraining has been provided later in this Chapter.

2.3 Hormones

Hormones are signalling molecules synthesised and secreted by endocrine glands located in various locations of the human body depending on their function i.e. the adrenal glands are located on top of the kidneys, and the hypothalamus and pituitary glands are in the brain (Hiller-Sturmhöfel & Bartke, 1998). Each gland synthesises and secrete a number of hormones which are secreted directly into the circulatory system for action (Capen, 1983). Additionally, some glands secrete their hormones into specialised networks of blood vessels called portal systems for efficient transport. For example, the hypothalamus secretes its releasing or inhibiting hormones into a hypophyseal portal system that directly connects the hypothalamus to the anterior pituitary gland. In turn, the pituitary gland secretes hormones into the secondary capillary plexus; a network of capillaries formed by the division of portal veins, which drains into the systemic circulation (described in more detail in Section 2.6). As chemical messengers, hormones are responsible for maintaining body homeostasis and controlling metabolic and physiological functions (Barrett et al., 2005).

Hormones are classified based on their molecular structure, which directly affects the way in which they are stored, secreted and transported in the circulatory system, and their mechanism of action (Hiller-Sturmhöfel & Bartke, 1998). Steroid hormones have a molecular structure similar to cholesterol whereas peptides/protein hormones are formed of amino acid chains connected via peptide bonds (Nussey & Whitehead, 2001). This thesis will focus primarily on steroid hormones; specifically, glucocorticoids (cortisol), progestogens (progesterone) and androgens (testosterone), and touch theoretically upon their peptide/protein precursors.

2.4 Hormone action on target cells

Target cells express specific hormone receptor proteins, such that circulating hormones act selectively. Hormones arrive at their target cell via the blood stream and once unbound from any protein carrier (i.e. in a free state), can bind to their target cell's specific protein receptor either on the cell membrane or within the cytoplasm. This hormone-receptor interaction triggers a cascade of biochemical reactions inside the target cell that ultimately leads to the desired functional outcome.

Peptide hormones are water soluble meaning they are readily dissolved in plasma and bind to receptors on the plasma membrane of their target cells. Steroid hormones, however, are lipid soluble and bind to receptors located intracellularly in the cytoplasm of the cell (Litwack, 2022).

2.4.1 Peptide/protein hormones

In their free form, the peptide/protein hormones bind with their specific receptor on the plasma membrane of a target cell, forming a hormone-receptor complex. G protein-coupled receptors are the most common type of cell surface receptor within the endocrine system (Hinson et al., 2010). The formation of this complex activates G protein, which activates the enzyme adenylate cyclase in the plasma membrane which subsequently catalyses the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). This cAMP serves as a second messenger to activate protein kinases which go on to phosphorylate specific proteins required to cause a physiological response i.e. a change in metabolism (Litwack, 2022) (Figure 2.2).

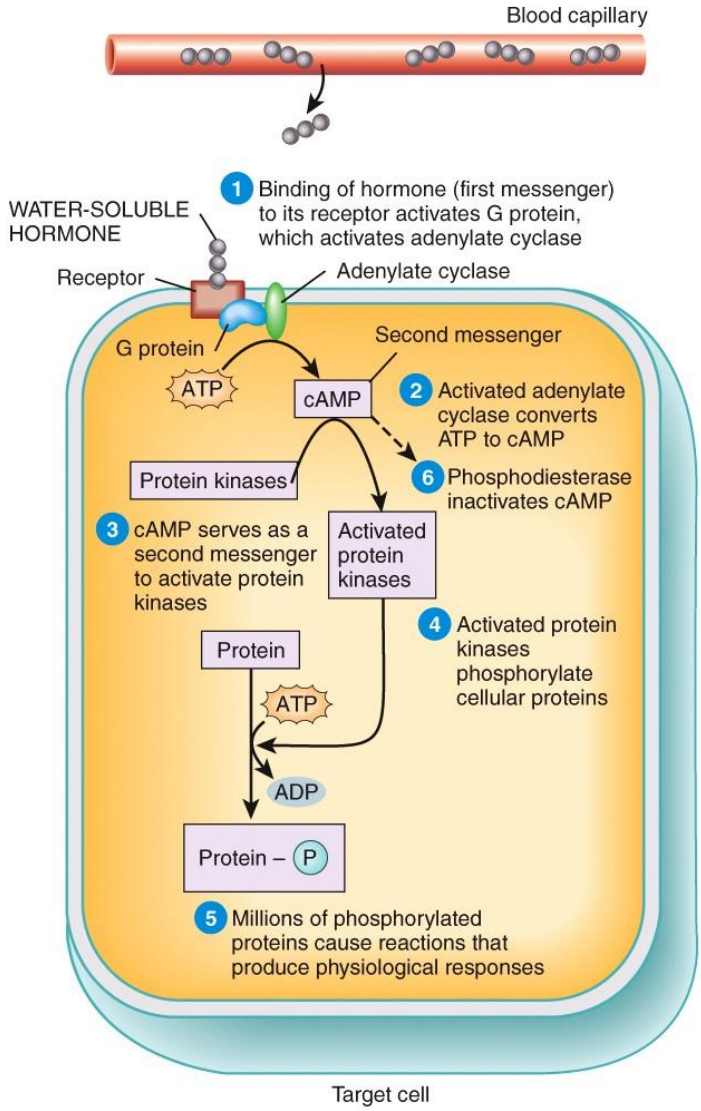


Figure 2.2. Mechanism of action of the water-soluble hormones (peptides and proteins). Water soluble hormones cannot pass through cell membranes of target cells. Instead, they cause action by binding to a surface hormone receptor (first messenger), which actives G-protein and int urn, cyclic AMP (the second messenger). This leads to a downstream of events ultimately leading to the desired physiological response. Taken from Tortora and Derrickson, (2023). Principles of Anatomy and Physiology, 16th ed, Page 654.

2.4.2 Steroid hormones

As steroid hormones are lipid-soluble, they can pass through the cell membrane and connect with their specific receptors in the cytoplasm of the cell (Hinson et al., 2010). The activated receptor-hormone complex enters the nucleus of the cell and alters DNA gene expression, forming new messenger RNA (mRNA) that is tasked to re-enter the cytoplasm and direct synthesis of specific proteins on ribosomes. These newly formed proteins thus act to alter the physiological activity of the cell (Chen & Farese, 1999) (Figure 2.3).

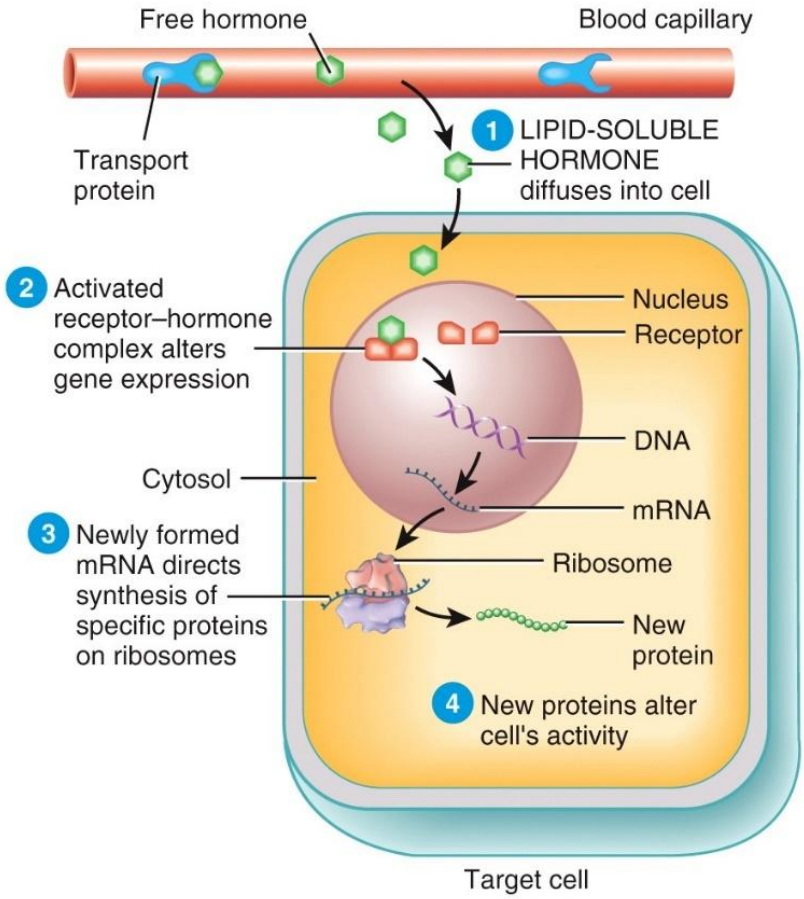


Figure 2.3. Mechanism of action of the lipid-soluble steroid hormones. Lipid soluble hormones can pass through cell membranes whereby they bind to the receptor in the cytoplasm of the cell. This alters gene expression and leads to the formation of new mRNA, which drives synthesis of specific proteins. Taken from Tortora and Derrickson, (2023). Principles of Anatomy and Physiology, 16th ed, Page 653.

2.5 Salivary cortisol and testosterone

Cortisol and testosterone can be measured in saliva, as well as plasma. These hormones enter the saliva via diffusion through acinar cells in the saliva glands; therefore, their concentrations are independent of salivary flow rate (El-Farhan et al., 2017; Vining et al., 1983). The salivary concentrations of unconjugated steroid hormones, such as cortisol and testosterone are thought to reflect the concentration of free, bioavailable steroid hormones in plasma i.e. unbound to proteins (Vining et al., 1983).

Significant positive associations have been shown between serum and salivary free testosterone in both males and females at rest (de Wit et al., 2017). Studies investigating the association between salivary and serum testosterone have reported correlation coefficients ranging from *r* = 0.74 to ~1.00 in males (Aarregger et al., 2007; Cardoso et al., 2011). Strong positive correlations have also been reported for cortisol with studies reporting correlation coefficients of *r* = ~0.84 (Crewther et al., 2010).

These associations have also been validated in response to exercise. Short high intensity cycling exercise in the form of a Wingate test elicited a moderate correlation between salivary and serum testosterone (*r* = 0.57) and a strong correlation with cortisol (*r* = ~0.81) (Crewther et al., 2010). Additionally, 30 minutes of cycling at 60% and 80% $\dot{V}O_{2max}$ both displayed strong positive correlations between salivary and serum testosterone (*r* = ~0.72) (Lane & Hackney, 2015). Significant positive correlations in salivary and serum cortisol have also been shown in response to a ~25-minute resistance exercise bout (*r* = ~0.62) (Cadore et al., 2008). The validation of saliva as a biological fluid for the measurement of free biologically active cortisol and testosterone enables a non-invasive method of hormone collection and may represent the pattern of free hormones in the circulation.

2.6 Hypothalamic pituitary adrenal axis

Cortisol is synthesised and secreted though activation of the hypothalamic pituitary adrenal axis (HPA). The HPA pathways starts with the secretion of corticotropin-releasing hormone (CRH) from the hypothalamus in response to stress i.e. exercise and psychological stress (Herman et al., 2016; McMurrray & Hackney, 2000). Specifically, stress activates the sympathetic nervous system (SNS), increasing levels of noradrenaline in the brain stimulating the release of CRH. CRH then travels through the hypothalamic-hypophyseal portal system, as described in Section 2.3, which carries CRH directly to the pituitary gland. The exposure of CRH to the anterior pituitary gland induces the production of the steroid hormone ACTH which enters the circulation via the secondary plexus capillaries (Papadimitiou & Priftis, 2009) (Figure 2.4). Once released into the circulation either bound to transcortin (~75%), loosely bound to plasma albumin (~15%) or in free form (~10%), ACTH travels to the adrenal glands located on top of each kidney (Norman and Litwack, 1997).

The adrenal glands are made up of the adrenal cortex and the adrenal medulla. The adrenal cortex is formed of three sections: the zona glomerulosa, zona fasciculata and zona reticularis. ACTH binds to receptors on the membrane of the zona fasciculata and zona reticularis inducing a cascade of downstream events ultimately leading to the production of cortisol. The HPA axis is operated via a negative feedback system, and is governed by a circadian rhythm, whereby peak concentrations occur early in the morning upon waking, plateau a few hours after waking and nadir between midnight and 03:00 (Crofford et al., 1997; Papadimitriou et al., 2009).

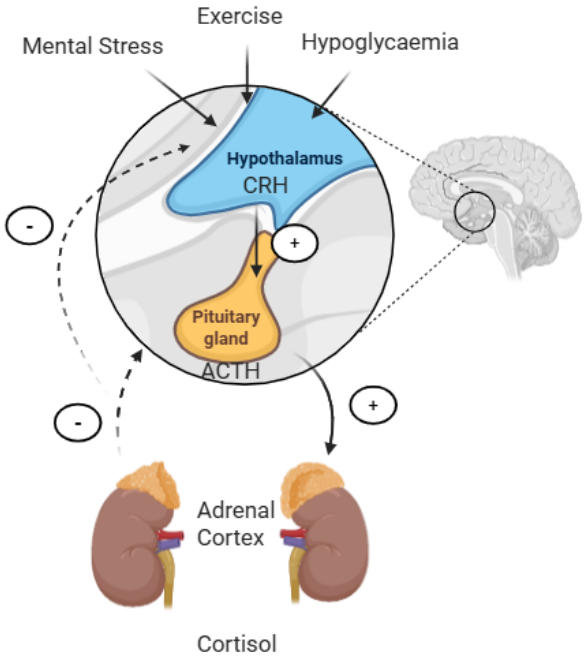


Figure 2.4. The negative feedback control of the hypothalamic pituitary adrenal axis production of cortisol. Corticotropin-releasing hormone is released from the hypothalamus following a stress stimulus. This in turn stimulates the pituitary glands to secrete adrenocorticotropic hormone, which ultimately stimulates the adrenal cortex to secrete cortisol. Created using Bio Render.

2.7 Hypothalamic pituitary gonadal axis

The hypothalamic pituitary gonadal (HPG) axis comprises of the hypothalamus, pituitary gland and gonads (testes in males and ovaries in females). It is responsible for the production of testosterone and progesterone, thus both hormones follow a similar pathway of synthesis.

The hypothalamus releases gonadotropin-releasing hormone (GnRH) which travels to the pituitary gland via the hypothalamic-hypophyseal portal system and stimulates the secretion of the gonadotropins; luteinising hormone (LH) and follicle-stimulating hormone (FSH). In turn, LH and FSH stimulate the gonadal release of steroid sex hormones such as testosterone from the testes in males and ovaries in females, and progesterone (Sharma et al., 2022) (Figure 2.5). In females, the majority of testosterone is produced by the ovaries (25%–50%) and adrenal glands (25%), with the remaining testosterone synthesised by the conversion of androstenedione to testosterone (Parish et al., 2021). In males the majority of testosterone is synthesised and secreted from the Leydig cells of the testes, with the remaining 5% from the adrenal cortex (Lee & Laycock, 1978; Williams, 1962). Similarly to cortisol, testosterone also follows a circadian rhythm, whereby peak

concentrations occur early in the morning upon waking, and plateau a few hours after waking (Walton et al., 2007).

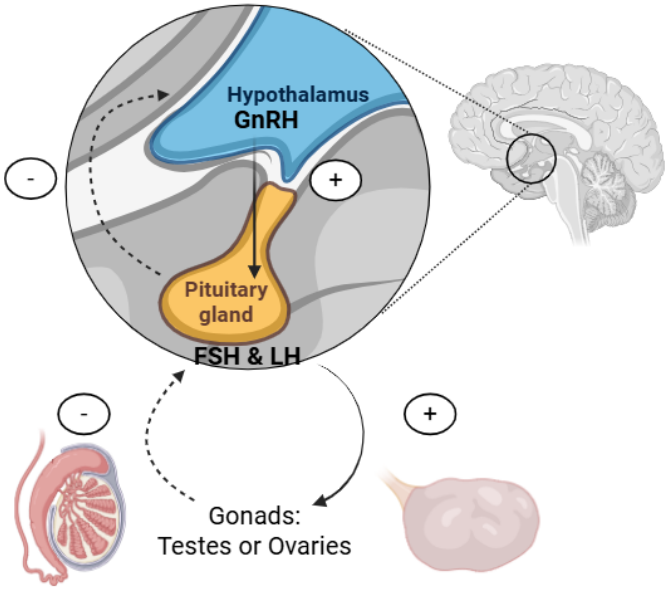


Figure 2.5. The negative feedback control of the hypothalamic pituitary gonadal axis production of testosterone. Gonadotropin-releasing releasing hormone is released from the which stimulates the pituitary glands to secrete the gonadotropins; follicle stimulating hormone and luteinizing hormone. This ultimately stimulates the gonads (testes or ovaries) to secrete testosterone. Created using Bio Render. Created using Bio Render.

2.8 Cortisol response to acute exercise

Generally, acute exposure to endurance and resistance exercise results in elevated plasma and salivary cortisol concentrations. The magnitude of these elevations is largely determined by the intensity and duration of the exercise, and the training status of the athlete (McMurray & Hackney, 2000). A threshold intensity of ~60% $\dot{V}O_{2max}$ has been proposed to induce cortisol increases to exercise, with higher exercise intensities leading to larger elevations (Davies & Few, 1973). Treadmill running for 60 minutes (Davies & Few, 1973) and 30 minutes of cycling exercise (Hill et al., 2008) at 60% $\dot{V}O_{2max}$ in moderately trained males were both shown to elevate plasma cortisol, with larger elevations seen when cycling at 80% $\dot{V}O_{2max}$ (Hill et al., 2008).

However, in highly endurance trained males, salivary and serum cortisol elevations were only shown when cycling for 30 minutes at 80% $\dot{V}O_{2max}$, but not at 60% $\dot{V}O_{2max}$ (VanBruggen et al., 2011). Similarly, Sato et al. (2016) exercised trained endurance runners and untrained controls for 15 minutes at a low intensity, 15 minutes at a moderate intensity and 15 minutes at a high intensity (90% $\dot{V}O_{2max}$). Whilst the untrained controls showed elevated cortisol at the end of each intensity level, endurance trained athletes only displayed significant increases after the high intensity exercise session. Popovic et al. (2019) also showed more pronounced cortisol responses to a graded exercise test in untrained individuals compared to elite athletes.

This indicates the modulatory effects of training status on exercise-induced cortisol responses with largest differences seen between sedentary and highly trained individuals (Hackney & Walz, 2013; Tremblay et al., 2004). A summary of the acute responses of cortisol to exercise is outlined in Table 2.1.

Studies included in Table 2.1 are a representative selection of studies showing a range of exercise durations, modes, participant training statuses and hormonal outcomes. This is the same for Table 2.2, 2.3 and 2.4.

Table 2.1. Acute exercise effects on salivary and plasma cortisol.

Reference	Participant Population	Maximal Oxygen Uptake ($\dot{V}O_{2max}$) Values (ml/kg/min)	Mode And Intensity of Exercise	Duration of Exercise	Response	Percentage Change
Hew-Butler et al. (2008)	Trained endurance athletes (males)	55.0 ± 5.0	Running $\dot{V}O_{2max}$ test	<15 min	Increase	*37%
Hew-Butler et al. (2008)	Trained endurance athletes (males)	55.0 ± 5.0	Treadmill run at 60% peak treadmill speed	60 min	No change	*NA
Hill et al. (2008)	Moderately trained males	65.5 ± 7.1	Cycling at 40% $\dot{V}O_{2max}$	30 min	No change	*NA
Hill et al. (2008)	Moderately trained males	65.5 ± 7.1	Cycling at 60 and 80% $\dot{V}O_{2max}$	30 min	Increase	60% $\dot{V}O_{2max}$: *40% 80% $\dot{V}O_{2max}$: *83%
Tremblay et al. (2005)	Trained endurance athletes (males)	67.1 ± 8.1	Running 55% $\dot{V}O_{2max}$	120 min	Increase	*22%
Tremblay et al. (2005)	Trained endurance athletes (males)	67.1 ± 8.1	Running 55% $\dot{V}O_{2max}$	40 and 80 min	Decrease	40 min: *-31% 80 min: *-27%
Hough et al. (2011)	Physically active males	50.9 ± 8.8	Cycle at ~75% W_{max}	30 min	Increase	*80% **120%
Hough et al. (2013)	Physically active males	52.0 ± 5.0	Cycle at ~75% W_{max}	30 min	Increase	**210%
Baker et al. (2024)	Physically active females	41.0 ± 5.7	Cycle 1 min at 55% and 4 min at 80% $\dot{V}O_{2max}$	30 min	Increase	*86% **140%

VanBruggen et al. (2011)	Endurance trained males	58.2 ± 6.4	Cycle at 60% $\dot{V}O_{2max}$	30 min	No change	NA
VanBruggen et al. (2011)	Endurance trained males	58.2 ± 6.4	Cycle at 80% $\dot{V}O_{2max}$	30 min	Increase	*40% **171%

*plasma, ** saliva. $\dot{V}O_{2max}$ values are mean ± standard deviation.

2.9 Cortisol responses after intensified training periods

It has been hypothesised that repeated arduous training bouts that can lead to NFOR/OTS cause hypothalamus-pituitary mediated hormone dysregulation (Meeusen et al., 2013). Alterations in the resting concentrations of cortisol after periods of intensified training have been shown to increase (Purge et al., 2006; Svendsen et al., 2016), not change (Hough et al., 2013; Slivka et al., 2010; Bresciani et al., 2011) and decrease (Lucia et al., 2001), highlighting inconsistency in the literature.

The main reasons for differences between studies relates to the participant training level and study design. The most obvious reason is that both Purge et al. (2006) and Svendsen et al. (2016) measured plasma cortisol, whereas the studies showing no alterations measured salivary cortisol (Hough et al., 2013; Slivka et al., 2010; Bresciani et al., 2011). Therefore, basal salivary cortisol may not be as sensitive to intensified training periods as basal plasma cortisol. Secondly, the two studies who found resting cortisol levels increased (Svendsen et al., 2016; Purge et al., 2006) used elite athletes with a superior $\dot{V}O_{2max}$ (69.2 –72.0 ml/kg/min) compared to the studies (Hough et al., 2013; Slivka et al., 2010; Bresciani et al., 2011) who found no cortisol alterations (45.2 – 63.4 ml/kg/min). Studies investigating long term basal cortisol concentrations i.e. via hair cortisol, suggest that elite level endurance athletes show higher basal cortisol compared to non-athlete controls, indicating a possible modulation of training status on basal cortisol levels (Skoluda et al., 2012).

Additionally, confounding variables that can impact cortisol responses to training may also cause variation in study findings. For example, Svendsen et al. (2016) supplemented their participants with a low dose (2%) carbohydrate drink before, during and after training sessions. Although, they did conclude that the carbohydrate supplement did not ameliorate cortisol alterations, so is unlikely to play a huge role in the disparities. Particularly because carbohydrate intake during intensified training periods is known to reduce cortisol responses to exercise training, rather than increase it (Costa et al., 2005). In contrast, Slivka et al. (2010) report that during the time spent cycling (169 km/day bike tour) *ad libitum* energy was provided with as many energy bars and sports drinks as desired. This could have contributed to their lack of salivary cortisol changes.

Variations in the training load can also be held to account for disparities. Although the durations of the intensified training periods do not differ to a great degree between studies finding varied resting cortisol responses, the characteristics of the actual training sessions do. The two studies finding increases in resting plasma cortisol used high volume training i.e. two sessions per day of high intensity training, lasting two times the usual training session durations (>82% Heart rate maximum) (Svendsen et al., 2016), with Purge et al. (2006) reporting a significant moderate correlation (r = 0.527, P= 0.001) between weekly training volume and plasma cortisol concentrations. In contrast, Bresciani et al. (2011) used a low volume, 3 days/week running training regime of 30–40-minute sessions and Hough et al. (2013) used 1.5 hour/day cycling at 75% $\dot{V}O_{2max}$.

Although limited differences between “healthy” and “overtrained” athletes occur at rest, studies have shown that in response to a stress stimulus, cortisol is blunted, implicating it as a possible biomarker of the negative states of overtraining. Hough et al. (2013) showed a ~166% blunting in the salivary cortisol response to a 30-minute cycle after an 11-day intensified training period in recreationally active males. Similarly, Meeusen et al. (2004) showed a ~118% blunting in the exercise induced plasma cortisol response to a maximal cycling test after a 10-day training period compared to before in well-trained male cyclists. It is therefore suggested that hormone levels behave more homogenously upon stimulation compared to an “at rest” measure between “healthy” and “overtrained” cohorts (Carrard et al., 2022).

A summary of studies investigating alterations in resting and exercise induced cortisol levels after a period of intensified training are shown in Table 2.2.

Table 2.2. Effect of exercise training periods on salivary and plasma cortisol.

Reference	Participant Population	Maximal Oxygen Uptake ($\dot{V}O_{2max}$) Values (ml/kg/min)	Duration of Training	Resting or Exercised Sample	Response	Percentage Change
Purge et al. (2006)	Elite male rowers	69.2 ± 3.1	6 months	Rest	Increase	*42%
Svendsen et al. (2016)	Trained male cyclists	72.0 ± 5.0	8 days	Rest	Increase	*67%
Slivka et al. (2010)	Well trained male cyclists	63.4 ± 1.4	21 days	Rest	No change	**NA
Bresciani et al. (2011)	Recreationally active males	45.2 ± 2.3	9 weeks	Rest	No change	**NA
Hough et al. (2013)	Recreationally active males	52.0 ± 5.0	11 days	Exercise	Blunted response	**166%
Meeusen et al. (2004)	Well-trained male cyclists	66.0 ± 1.8	10 days	Exercise	Blunted response	*118%
Hough et al. (2015)	Elite male triathletes	67.6 ± 4.5	10 days	Exercise	No change	**NA
Lucia et al. (2001)	Elite male cyclists	75.3 ± 2.3	4 weeks	Rest	Decrease	*40%
Verde et al. (1992)	Trained male distance runners	65.3 ± 4.9	3 weeks	Exercise	No change	*NA

*plasma, ** saliva. $\dot{V}O_{2max}$ values are mean ± standard deviation.

2.10 Testosterone response to acute exercise

Plasma and salivary testosterone elevate in response to acute exercise (Hough et al., 2011; Tremblay et al., 2005; Lehmann et al., 1993; Baker et al., 2024; Jensen et al., 1991; O'Leary et al., 2013; Gonzalez-Bono et al., 2002). These testosterone elevations appear to be more responsive to higher intensity exercise of longer durations (Hough et al., 2011; Tremblay et al., 2005; Lehmann et al., 1993). Specifically, an intensity dependant increase in plasma and salivary testosterone was shown in response to 30 minutes cycling at low (40% $\dot{V}O_{2max}$), moderate (60% $\dot{V}O_{2max}$) and high (80% $\dot{V}O_{2max}$) intensities in well-trained cyclists (Lane and Hackney, 2014). A similar intensity dependant response has also been found with treadmill running in well trained males running at 60, 75, 90 and 100% of $\dot{V}O_{2max}$, such that plasma testosterone only elevated in response to 5 mins running at 90% and 100% $\dot{V}O_{2max}$ (Kraemer et al., 2003).

In terms of the temporal responses of testosterone to acute exercise bouts, studies report that testosterone levels return to baseline 15 minutes to 1 hour after exercise depending on exercise intensity. Kraemer et al. (2003) reported that plasma testosterone retuned to baseline 15 minutes after graded exercise to exhaustion, whereas Galbo et al. (1997) reported that graded and prolonged exhaustive treadmill running lead to plasma testosterone gradually returning to baseline 30 minutes post recovery. In contrast, Lane and Hackney (2014) found that both salivary and plasma testosterone peaked immediately after the exercise bout and started declining 30 minutes post exercise, but remained elevated above baseline (Lane and Hackney, 2014). Interestingly, Hough et al. (2011) also reported a ~10-minute time lag between the peak salivary and plasma testosterone increases in response to 30 minutes of interval cycling for 1minute at 55% W_{max} and 4 minutes cycling at 80% W_{max}.

Resistance exercise has also been shown to elicit elevations in circulating testosterone levels (Jensen et al., 1991), attributed to the robust influence of the anaerobic glycolysis pathways in triggering acute hormonal surges following physical exertion (Kraemer & Ratamess, 2005). Whilst heavy resistance bouts have been shown to increase plasma testosterone immediately, returning to baseline ~30 minutes post exercise (Kvorning et al., 2006), a major determinant for plasma and salivary testosterone increases is the muscle mass used (Riachy et al., 2020). For example, participation in resistance exercise involving a limited muscle mass, even at high intensity, does not appear to elevate plasma testosterone concentrations above resting levels (Migiano et al., 2010). In a study of young, untrained men, unilateral biceps curl exercise alone failed to elicit a significant post-exercise increase in plasma testosterone (Hansen et al., 2001). In contrast, when bilateral knee extensions and leg press exercises were incorporated into the protocol, a significant elevation in testosterone concentration was observed (Hansen et al., 2001).

A summary of studies reporting the acute responses of testosterone to exercise is outlined in Table 2.3.

Table 2.3. Acute exercise effects on salivary and plasma testosterone.

Reference	Participant Population	Maximal Oxygen Uptake ($\dot{V}O_{2max}$) (ml/kg/min)	Type of exercise	Duration of Exercise	Response	Percentage Change
Hough et al. (2011)	Physically active males	50.9 ± 8.8	Cycle at ~75% W_{max}	30 min	Increase	**58%
Hough et al. (2011)	Physically active males	50.9 ± 8.8	Cycle at ~75% W_{max}	30 min	No change	*NA
Tremblay et al. (2005)	Endurance trained males	67.1 ± 8.1	Treadmill running at 55% $\dot{V}O_{2max}$	80 min	Increase	*20%
Baker et al. (2024)	Recreationally active females	41.0 ± 5.7	Cycle 1 min at 55% and 4 min at 80% $\dot{V}O_{2max}$	30 min	Increase	**93%
Baker et al. (2024)	Recreationally active females	41.0 ± 5.7	Cycle 1 min at 55% and 4 min at 80% $\dot{V}O_{2max}$	30 min	No change	**NA%
Jensen et al. (1991)	Elite endurance and strength trained males	66.1 ± 5.7	Cross country running at ~70% $\dot{V}O_{2max}$	90 min	Increase	*37%
Jensen et al. (1991)	Elite endurance and strength trained males	66.1 ± 5.7	80% 1RM 8 reps x 3 sets	90 min	Increase	*27%

			of 9 strength exercises			
Lehmann et al. (1993)	Recreationally active males	52.0 ± 5.2	Incremental cycling to exhaustion	~30 min	Increase	*23%
O'Leary et al. (2013)	Recreationally active females	50.7 ± 9.0	Treadmill running at 65-70% $\dot{V}O_{2max}$	60 min	Increase	*92%
Gonzalez-Bono et al. (2002)	National-level Basketball players	41.9 ± 1.2	Incremental cycling to exhaustion	~20 min	Increase	**31%

*plasma, ** saliva, 1RM: 1 repetition maximum. values are mean ± standard deviation

2.11 Testosterone responses after intensified training periods

Similarly to the effects of intensified training periods on cortisol, resting testosterone concentrations have also been shown to decrease (Lucia et al., 2001), not change (Urhasusen et al., 1998; Flynn et al., 1994, Bresciani et al., 2011) and increase (Grandys et al., 2009; Alghadir et al., 2015) after periods of intensified training.

As outlined in Section 2.10, the variability observed across studies may be attributed to several factors, including differences in exercise modality (e.g., endurance vs. resistance training), training intensity, recovery duration, participant characteristics (e.g., recreationally active vs. elite athletes), and the timing of testosterone measurement (e.g., immediately post-exercise vs. several minutes or hours afterward) (Riachy et al., 2020).

Studies showing increases in resting plasma (Grandys et al., 2009) and salivary (Alghadir et al., 2015) testosterone tend to use previously untrained, sedentary individuals. It has been shown that testosterone perturbations to exercise are higher in previously sedentary/untrained individuals compared to well-trained individuals (Hackney, 2001). Specifically, testosterone levels in exercise-hypogonadal males have been shown to be 50-85% of the levels found in aged matched sedentary controls (Gulledge and Hackney, 1996).

However, in response to acute exercise stress, both salivary and plasma testosterone elevations are blunted after periods of intensified training. A ~44% exercise induced blunting in salivary testosterone has been shown in response to 10 days of intensified training in elite triathletes whereby swim distances were increased by 45%, running training hours were increased by 25% and cycling training hours were increased by 229% (Hough et al., 2015). Similarly, 11 days of daily cycling for 1.5 hours at 75% $\dot{V}O_{2peak}$ in recreationally active males blunted the exercise induced salivary testosterone response by ~21% (Hough et al., 2013). Although less consistent, plasma testosterone has also been shown to elicit the same exercise induced blunting as salivary testosterone (Leal et al., 2021a). As such, exercise induced alterations in salivary and plasma testosterone may be a more reliable biomarker of the negative states of overreaching than resting measurements.

A summary of studies investigating alterations in resting and exercise induced testosterone levels after a period of intensified training are shown in Table 2.4.

Table 2.4. Effect of exercise training periods on salivary and plasma testosterone.

Reference	Participant Population	Maximal Oxygen Uptake ($\dot{V}O_{2max}$) (ml/kg/min)	Duration of Training	Rest or Exercised Sample	Response	Percentage Change
Alghadir et al. (2015)	Recreationally active males	Not available	4 weeks	Rest	Increase	**~55%
Urhasusen et al. (1998)	Male cyclists and triathletes	61.2 ± 1.8	19 months	Rest	No change	*NA
Flynn et al. (1994)	Male long distance runners	65.2 ± 1.3	6 weeks	Rest	No change	*NA
Bresciani et al. (2011)	Recreationally active males	45.2 ± 2.3	9 weeks	Rest	No change	**NA
Hough et al. (2013)	Recreationally active males	52.7 ± 5.7	11 days	Exercise	Blunted response	**21%
Hough et al. (2015)	Elite male triathletes	67.6 ± 4.5	10 days	Exercise	Blunted response	**44%
Lucia et al. (2001)	Elite male cyclists	75.3 ± 2.3	4 weeks	Rest	Decrease	*40%
Leal et al. (2021a)	Recreationally active males	59.0 ± 6.0	12 days	Exercise	Blunted	*24% **31%
Grandys et al. (2009)	Recreationally active males	46.0 ± 3.7	5 weeks	Rest	Increase	*17%

*plasma, ** saliva. $\dot{V}O_{2max}$ values are mean ± standard deviation.

2.12 Immunology

Immunology is the scientific study of the body's immune system and its' defences against infection and disease to maintain internal immune homeostasis (Abbas & Janeway, 2000). It encompasses all bodily mechanisms and responses to defend against foreign substances; a function essential to body homeostasis (Beisel, 1999). Various factors have been shown to positively and/or negatively influence the function of the immune system, for example, exercise (Walsh, 2019) and hormonal perturbations (Dhabhar, 2014). This thesis will primarily focus on the effects of exercise and the subsequent endocrine alterations on the functioning of both the cellular and soluble elements that comprise the immune system.

2.13 The immune system

All blood cells originate in the bone marrow from hematopoietic stem cells, which differentiate into erythrocytes (red blood cells), megakaryocytes (platelet precursors), and leukocytes (white blood cells essential for immune function) (Stuart & Weir, 2012). A healthy adult has ~4.5-11 x 10^9 leukocytes/L of blood (Ware, 2020), with 60-70% of those circulating leukocytes comprising of granulocytes, 5-15% of monocytes, 20-25% of lymphocytes and less than 1% of DCs (Gleeson et al., 2013; Haller Hasskamp et al., 2005). As previously mentioned, this thesis will focus on T lymphocytes (T cells) and DCs.

The immune system is divided into the innate and adaptive arms, which work synergistically. The innate system acts as the first line of defence, providing a rapid, non-specific response without requiring antigen presentation (McComb et al., 2019). Key innate immune cells include granulocytes (eosinophils, basophils, neutrophils), monocytes/macrophages, natural killer (NK) cells, and DCs (Nicholson, 2016). These fast responders possess a battery of defence mechanisms such as phagocytosis, the process of engulfing and destroying of pathogens (Dempsey et al., 2013).

If the innate immune system fails to eliminate an immune threat, cytokine (danger) signals from innate cells activate the adaptive immune system (Iwasaki & Medhitov, 2015). Also called the acquired immune system, it develops after pathogen exposure or vaccination. Unlike the non-specific innate immune system, the adaptive system requires antigen presentation and recognition, leading to a slower but antigen-specific response (Stuart & Weir, 2012). With its ability to acquire memory, it enables rapid reinfection responses, often preventing symptoms and conferring immunity (Ahuja, 2008). The adaptive system includes lymphocytes: T cells and B cells (Gleeson et al., 2013). DCs are specialised antigen-presenting cells that connect the innate and adaptive immune systems through antigen presentation, detailed later in this Chapter.

2.14 Cellular components of the immune system: T- lymphocytes and dendritic cells

2.14.1 T-Lymphocytes (CD3+ T cells)

Immature lymphocytes circulate in the blood and mature in the thymus, where they become T cells, classifying the thymus as a primary lymphoid organ (Sun et al., 2023). Within the thymus, developing T cells undergo T cell receptor (TCR) gene recombination, generating a diverse repertoire capable of recognising a wide range of antigens. They then undergo positive and negative selection, ensuring that only cells with appropriate affinity for self- major histocompatibility complex (MHC) molecules survive. This process also determines whether they commit to the $CD4^+$ or $CD8^+$ lineage. T cells that interact with MHC class II become $CD4^+$ helper T cells (Th), while those that recognise MHC class I become $CD8^+$ cytotoxic T cells (Tc). Once matured, naïve T cells exit the thymus and migrate to secondary lymphoid organs, such as the spleen and lymph nodes, via the bloodstream (Gleeson et al., 2013).

As T cells are highly skilled migrators, they can scan almost all parts of the body for foreign material, thus playing crucial roles in immune surveillance and mounting adaptive immunity against infection and cancer (Raskov et al., 2020). Cluster of differentiation (CD) markers are unique surface proteins displayed on cell membranes used to identify immune cell phenotypes. Upon activation in peripheral tissues via antigen presentation by DCs, $CD4^+$ T cells and $CD8^+$ T cells further differentiate into specialised effector subsets. Under normal circumstances, a 2:1 ratio of $CD4^+$ T cells to $CD8^+$ T cells cells are in circulation (Ahuja, 2008). Their differentiation and phenotypes are tightly regulated by cytokines, transcription factors, chemokines, and metabolic signals (Raskov et al., 2020).

Once activated, $CD4^+$ T cells produce large quantities of the cytokine Interleukin (IL)-2 and proliferate vigorously (Tak et al., 2014). Following the original antigenic stimulation (after 48-72 hours) the newly proliferated $CD4^+$ T cells terminally differentiate in various subsets of effector $CD4^+$ T cells depending on the stimulus i.e. Th1, Th2, Th17, Th22, Th9 and regulatory T cells (Tak et al., 2014). The two most common subtypes are type 1 (Th1), which play a major role in protecting hosts against intracellular bacteria and viruses (Yin et al., 2021), and type 2 (Th2) which work to protect the host against parasitic infections and allergies (Nakayama et al., 2017).

$CD8^+$ T cells are major killers of intracellular pathogens and tumour cells by secreting large amounts of pro-inflammatory cytokines (i.e. IFN-γ and TNF-α), perforin and protease granzyme B (Raskov et al., 2020; Paul & Ohashi, 2020). Perforins and protease granzyme B are injected into infected cells to initiate apoptosis by cell lysis (Janeway et al., 2001; Tak et al 2014).

2.14.2 Dendritic cells

Since their discovery in mice approximately 50 years ago, DCs have been a major focus of immunological research (Steinman & Cohn, 1973). Their ability to act as sentinels of the immune system, whereby they sample, store, and process both soluble and cell-associated antigens make them important regulators of the adaptive immune response (Yin et al., 2021).

Located in peripheral and lymphatic tissue, DCs can express high levels of MHC II (HLA-DR$^+$), and lack expression of other lineage markers expressed on monocytes, T cells, B cells and NK cells; thus, are referred to as being Lineage$^-$ HLA-DR$^+$ (Brown et al., 2018; Ueda et al., 2003). In the peripheral blood, two major subtypes of DC exist: plasmacytoid DCs (pDCs) and myeloid DCs (mDCs). Upon antigenic stimulation, immature DCs mature and upregulate their expression of MHC II, co-stimulatory molecules and the chemokine receptor CCR7 (Merad et al., 2023). When CCR7 interacts with its ligands, CCL19 and CCL21, it gains the ability to traffic towards lymph nodes via afferent lymphatics (mDCs) and high endothelial venules (pDCs), where they interact with naïve T cells (Liu et al., 2021; Forster et al., 1999; Jang et al., 2006).

mDCs are the major DC subtype, characterised by classical dendritic morphology and high expression of MHC II (Liu et al., 2021). They are specialised to act against infections and tumours by promoting CD4$^+$ T cell differentiation towards a Th1 phenotype and cross-presenting antigen to CD8$^+$ T cells via MHC I (Haniffa et al., 2013; Jongbloed et al., 2010). pDCs constitute a smaller proportion of peripheral blood DCs than mDCs and are characterised by relatively low expression of MHC I and II, high levels of CD123 (IL-3R a-chain) and type 1 IFN production (Ye et al., 2020). In fact, pDCs express 100-1000-fold more IFN-α than other blood cells following viral infection, establishing their importance in mounting anti-viral and anti-bacterial responses (Haniffa et al. 2013; Siegal et al., 1999).

2.15 Pathogen recognition

Invading microbes contain structural motifs that can be readily identified as non-self, called pathogen-associated molecular patterns (PAMPs) (Dempsey et al., 2003). Damage-associated molecular patterns (DAMPS) are endogenous danger molecules originating from within the host which are released from stressed cells and damaged tissue (Roh et al., 2018). Both PAMPs and DAMPs are recognised by germ-line encoded receptors called PRRs expressed on innate immune cells, triggering an innate immune response (Palm & Medzhitov, 2009).

DCs sense the presence of potential pathogens via a system of PRRs called TLRs (Janeway, 1989). TLRs are type I transmembrane glycoproteins located both on the cell surface (TLR 1, 2, 4, 5, 6 and 10) and in intracellular endosomes (TLR 3, 7, 8 and 9) of immune cells (Yamamoto & Takeda, 2010). DC maturation occurs when its TLR recognises a microbe, upregulating the key signals required to induce T cell signalling (described later in this Chapter). To date, there are 10 functional TLRs known in humans, each possessing

specificity for molecular patterns present on bacteria, fungi, viruses and parasites (Mazzoni & Segal, 2004). This thesis will focus on the intracellularly expressed TLR7 (pDC), 8 (mDC) and 9 (pDC).

2.16 Dendritic cell-T cell interaction

2.16.1 Antigen uptake

DCs internalise exogenous antigens via three main pathways: phagocytosis, receptor-mediated endocytosis, and macropinocytosis (Figure 2.6). In phagocytosis, opsonised particles bind to specific surface receptors and are engulfed into membrane-derived phagosomes, which transport them to late endosomal/lysosomal antigen processing compartments (EAPCs) for MHC II loading (Hoffman et al., 2012; Liu & Roche, 2015). Macropinocytosis involves non-specific uptake of soluble antigens into macropinosomes, which fuse with early endosomes and multivesicular EAPCs for degradation and MHC II loading (Norbury et al., 1997; Donaldson, 2018). In receptor-mediated endocytosis, small antigens bind to specific surface receptors and are internalised in clatharin-coated vesicles. After clatharin is shed, antigens are transported to early endosomes and then EAPCs for processing, degradation, and MHC II loading (Garrett et al., 2000; Mak & Saunder, 2006; Platt et al., 2010). The loaded MHC II complexes then migrate to the DC plasma membrane for antigen presentation.

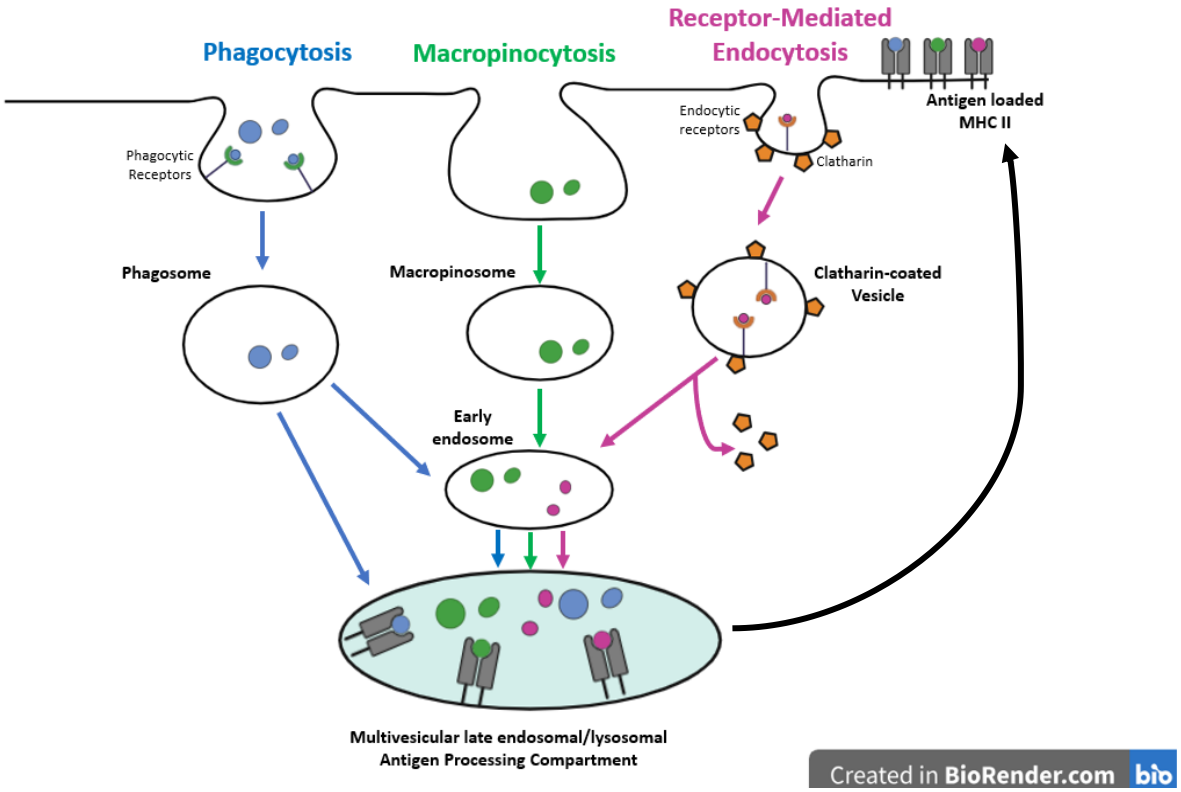


Figure 2.6. Pathways of dendritic cell exogenous antigen uptake. Phagocytosis is the specific uptake of opsonised particles which are engulfed into membrane-derived phagosomes and transported to endosomal antigen processing compartments for MHC loading. Macropinocytosis is the non-specific uptake of soluble antigens into macropinosomes, which ultimately fuse with endosomal antigen processing compartments for MHC loading. Receptor-mediated endocytosis is the binding of small antigens to surface receptors which are internalised into clatharin coated vesicles. When the clatharin in shed, antigens are transported for MHC loading. Figure adapted from: Liu et al. (2015). Macropinocytosis in phagocytes: regulation of MHC class II restricted antigen presentation in dendritic cells.

2.16.2 Antigen presentation

Activation of naïve T cells requires a coordinated series of three signals: a) antigen loaded MHC I/II molecules, b) co-stimulatory molecules (CD80/86) and c) instructive cytokine signals (Yin et al., 2021; Wehr et al., 2019) (Figure 2.7). As previously described, DCs present antigen peptide fragments via either MHC II to the TCR of $CD4^+$ T cells, termed 'direct antigen presentation' or shuttled through a specialised pathway to MHC I to engage with the TCRs of $CD8^+$ T cells, termed 'antigen cross-presentation' (Zhang et al., 2021; Gutierrez-Martinex et al., 2015). Simultaneously, the costimulatory molecules CD80/86 ligate to CD28 on the T cell to amplify the initial T cell activation signals provided by the antigen loaded MHC (Magee et al., 2012). Finally, cytokines drive the differentiation and polarisation of the T cells, instructing them to proliferate. Importantly, DCs are highly adaptable and can readily alter their transcriptional profile in a condition specific manner to support the appropriate $CD4^+$ T cell response (Hilligan & Ronchese, 2020).

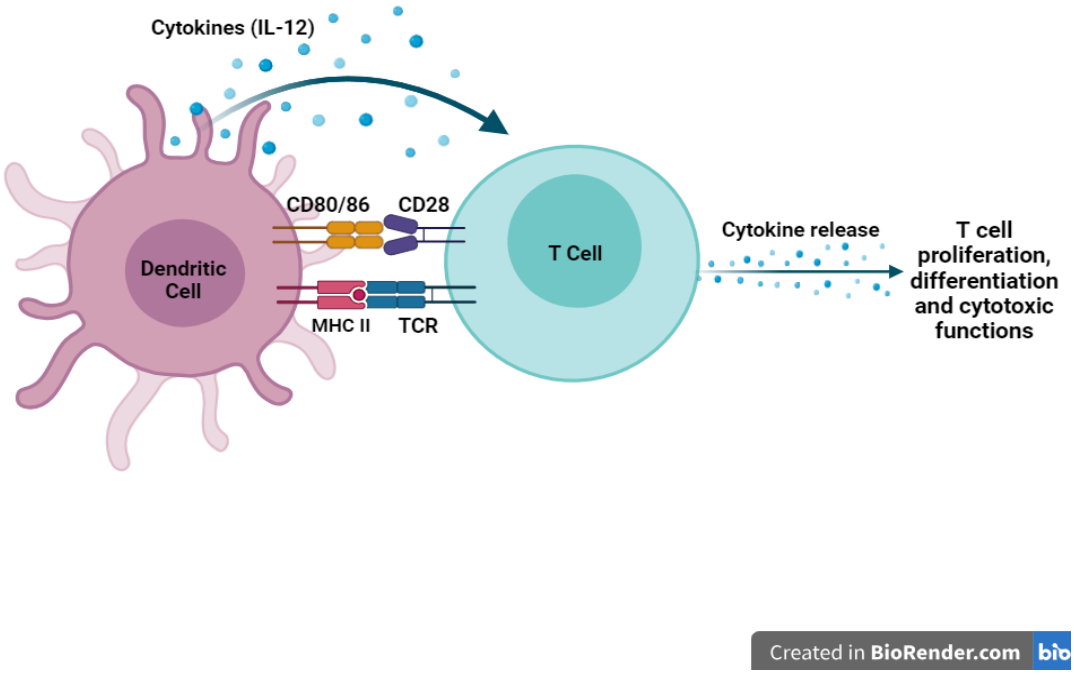


Figure 2.7. The dendritic cell- T cell interaction for antigen presentation. Three signals are required for T cell stimulation: 1) Major histocompatibility complex (MHC) loaded with antigen must bind to the T cell receptor, 2) Co-stimulatory molecules CD80/86 must bind to CD28 in order to amplify the signal and 3) Cytokines drive the T cell response.

2.17 Exercise and infection risk

While high physical fitness and regular moderate intensity exercise may protect the immune system (Gleeson et al., 2011; Duggal et al., 2019), repeated arduous exercise bouts, as practiced by athletes, may increase infection risk (Ekblom et al., 2006; Spence et al., 2007). Specifically, some exercise immunologists argue that short bouts (≤ 45 minutes) of moderate intensity exercise are immunoenhancing, whereas repeated long-lasting (> 2 hours) intense exercise bouts can be immunosuppressive (Simpson et al., 2020; Walsh et al., 2015).

URI is the second most common reason athletes seek medical attention during training or competition at the summer or winter Olympic Games (Engebretsen et al., 2012; Soligard et al., 2014), accounting for 35–65% of non-injury sports medicine cases (Fricker, 1997). Over three years, 70% of illnesses in 322 Olympic athletes led to complete training/competition absence, while 30% resulted in reduced training volume/intensity (Palmer-Green et al., 2013). A study of 32 elite and 31 recreational triathletes and cyclists over five months showed that elite athletes undergoing intense training experienced significantly more URIs than recreational athletes, with some facing recurrent episodes at higher rates than the general population (Spence et al., 2007; Fricker et al., 2000). Recurrent URIs are linked to persistent fatigue (Reid et al., 2004), impaired inflammatory regulation (Cox et al., 2007), and a reversible defect in $CD4^+$ T cell IFN-γ secretion, a cytokine influencing illness severity and duration (Clancy et al., 2006).

This increased URI susceptibly with intense training is not exclusive to endurance athletes, but has also been shown in swimming (Gleeson et al., 2000), football (Bury et al., 1998; Tiernan et al., 2022), tennis (Novas et al., 2003), American football (Fahlman & Engels, 2005), rugby union (Cunniffe et al., 2011), futsal (Moreira et al., 2013) and military training (Carins & Booth, 2002; Linenger et al., 1993; Martinez-Lopez et al., 1993).

Two theoretical frameworks have long been used to explain how exercise can exert both immune enhancing and suppression effects and alter susceptibility to illness: The J-Curve (Neiman et al., 1994) and the 'Open Window' theory (Pedersen & Ullum, 1994).

2.17.1 J-Curve model of URI

The J-curve model suggests that taking part in some regular moderate physical activity decreases the relative risk of URI below that of sedentary individuals (Nieman et al., 1993), however prolonged periods of strenuous training or intense acute bouts of exercise is associated with an above average risk of URI (Heath et al., 1991; Nieman et al., 1994) (Figure 2.8). In the context of the J-curve model (Figure 2.8), 'low' training loads refer to sedentary individuals, 'moderate' training loads refer to moderate physical activity within the recommended guidelines, and 'high' training loads refer to strenuous training regimes or intense bouts of exercise.

Early evidence of a J-shaped relationship between exercise and URI risk came from studies on marathon training and races. In a sample of 140 athletes from the 1982 Two Oceans Marathon (56 km), 33% reported URI symptoms within two weeks post-race, compared to 15% of age-matched non-runners (Peters & Bateman, 1982). Those finishing the race faster (< 4 hours) had a higher prevalence of symptoms than those taking > 4 hours, suggesting a link between exercise intensity and URI risk (Peters & Bateman, 1982).

Neiman et al. (1990) reported similar findings in the 1987 Los Angeles Marathon, where 12.9% of 2000 runners experienced URI symptoms in the week after the race, compared to 2.2% of non-runners. Notably, 40% of runners reported URI symptoms in the two months prior, and those training > 96 km per week during this period were twice as likely to report illness as those training < 32 km. Recurrence of recent infections may account for a significant proportion of reported URIs at endurance events; 33% of runners with URI symptoms in the three weeks before the 2000 Stockholm Marathon reported post-race episodes (Ekblom et al., 2006).

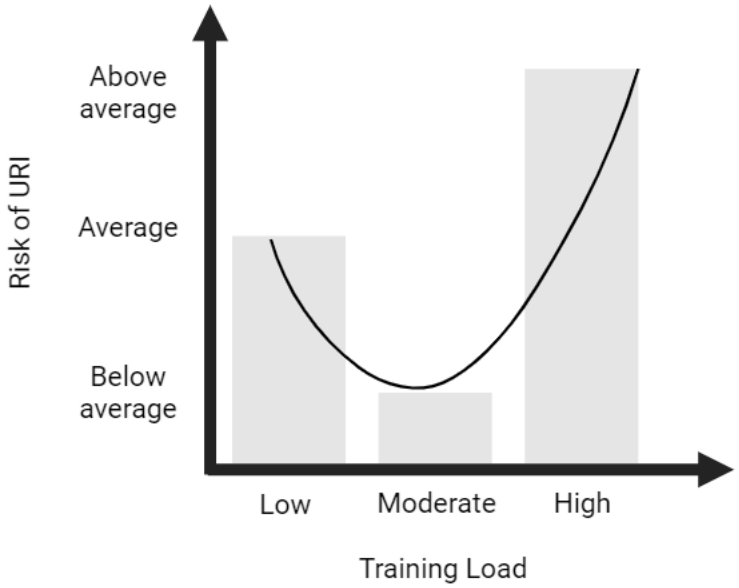


Figure 2.8. The proposed J-curve of the relationship between training load and risk of upper respiratory illness. Individuals that take part in some regular moderate physical activity decrease the relative risk of URI below that of sedentary individuals. However prolonged periods of strenuous training or intense acute bouts of exercise is associated with an above average risk of URI. Adapted from Neiman (1994).

The J-curve theory has been disputed however, with critics highlighting the incompatibility between high incidences of URI and training requirements for elite athlete performance (Gleeson et al., 2013). Malm et al. (2006) noted that much research focuses on sub-elite athletes, while truly elite athletes show lower illness rates, forming an S-shaped curve (Figure 2.9). A 5-year study of elite Australian track and field athletes found those completing 80% of planned training weeks were seven times more likely to meet performance goals, with each missed week reducing success likelihood by 26% (Raysmith & Drew, 2016). Similarly, Martensson et al. (2014) found a negative correlation between annual training hours and sickness days, suggesting elite endurance athletes achieve high training volumes by minimising illness. This S-curve perspective suggests that resilience to infections during intense training is a prerequisite for elite performance (Malm, 2006; Gleeson et al., 2013). Alternatively, elite athletes may adopt lifestyle practices that effectively reduce their exposure to pathogens or enhance immunity. For example, dietary supplements such as vitamin D (He et al., 2013) and probiotics (Cox et al., 2010) have been shown to reduce URI incidence, symptoms duration and symptom severity in elite endurance athletes. Additionally, reducing direct contact exposure, especially in

change rooms, frequent hand washing and avoiding poorly ventilated indoor environments are also effective methods at reducing infection risk that could be adopted by elite athletes (Nguyen et al., 2005).

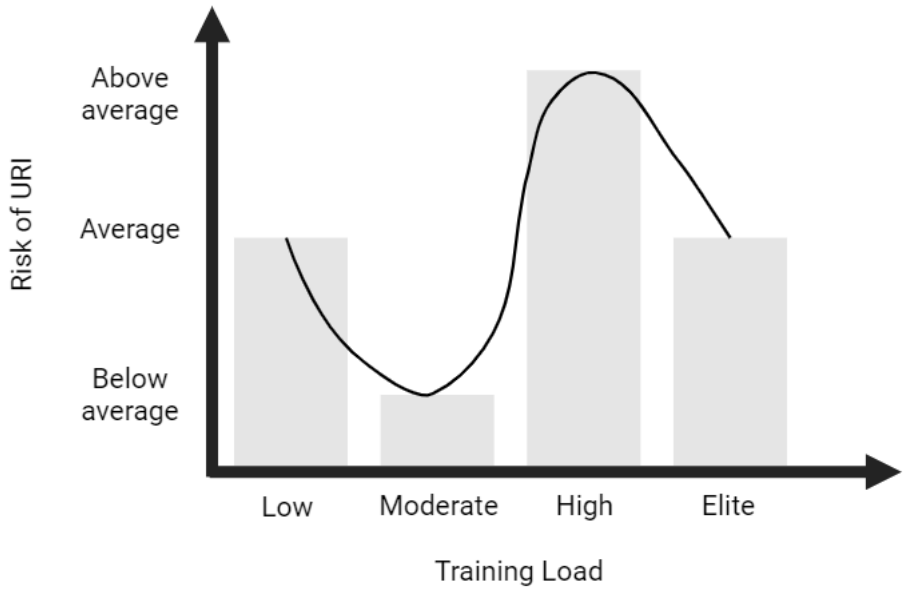


Figure 2.9. The proposed S-shaped curve showing the relationship between training load and the risk of upper respiratory illness. Individuals that take part in some regular moderate physical activity decrease the relative risk of URI below that of sedentary individuals, but prolonged periods of strenuous training or intense acute bouts of exercise is associated with an above average risk of URI. Athletes who are truly elite have an average risk of URI, suggesting that resilience to infections during intense training is a prerequisite for elite performance. Adapted from Malm (2006).

2.17.2 The 'Open Window' theory

The 'Open Window' theory suggests that strenuous exercise bouts or periods of intensified training may impair aspects of the immune system, by creating a window of increased infection risk lasting 3–72 hours post-exercise (Pedersen & Ullum, 1994). It was argued that whilst a single bout of exercise is associated with an initial enhancement in immune function, it is quickly followed by a transient period of immune depression (the 'Open Window') possibly leaving the host susceptible to infection (Pedersen & Ullum, 1994). The performance of a second exercise bout within this window with inadequate recovery may blunt the exercise induced immune-enhancement and prolong the post exercise decline in immunity, further increasing the athlete's susceptibility to infection (Simpson et al., 2015). It is suggested that if this pattern continues over prolonged periods, chronic immunosuppression may occur which could pose a serious issue considering many athletes use this model of overreaching to gain positive training adaptation (Figure 2.10).

Specifically, it was argued that the post-exercise decline in circulating lymphocytes (lymphocytopenia), especially those with high effector functions such as $CD8^+$ T cells, drives this window of opportunity for

infection (Pedersen & Ullum, 1994; Steensberg et al., 2001). Repeated exposure to this acute lymphocytopenia over time is thought to bare additive negative consequences to infections risk (Pedersen & Ullum, 1994). However, the 'Open-Window' theory is not accepted by all, with suggestions that reported reductions in immune cell function post-exercise could reflect the lowered number of immune cells in circulation after exercise, which are redistributed to tissues for enhanced immunosurveillance at sites of infection risk (Simpson et al., 2020).

This exercise induced redistribution of effector T cells has been demonstrated in rodents with fluorescent cell tracking (Kruger et al., 2008) and in humans by the proportional reduction of cells expressing homing receptors for tissue and organ sites (Campbell et al., 2009), and is thought to enhance identification and eradication of tumour cells. In line with this theory, it has been shown that cancer cells incubated with exercised serum form fewer tumours when inoculated into mice (Hojman et al., 2018), and 4 weeks of voluntary wheel running prior to tumour cell inoculation reduced tumour growth by 61%, attributed to the increased infiltration of effector immune cells to tumour sites (Pedersen et al., 2016). This phenomenon is discussed in more detail in Chapter 5.

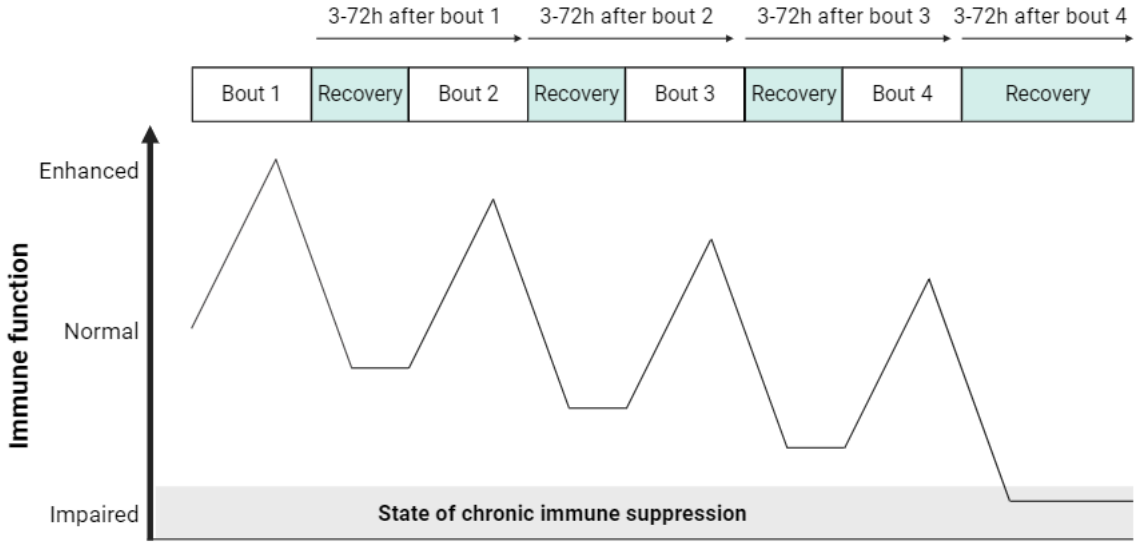


Figure 2.10. The 'Open-Window' theory. Strenuous exercise bouts or periods of intensified training impairs aspects of the immune system, creating a window of increased infection risk lasting 3–72 hours post-exercise. Whilst a single bout of exercise is associated with an initial enhancement in immune function, it is quickly followed by a transient period of immune depression (the 'Open Window') leaving the host susceptible to infection. The performance of a second exercise bout within this window with inadequate recovery blunts the exercise induced immune-enhancement and prolongs the post exercise decline in immunity, further increasing the athlete's susceptibility to infection. If this pattern continues over prolonged periods, chronic immunosuppression may occur which could pose a serious issue considering many athletes use this model of overreaching to gain positive training adaptation. Adapted from the model proposed by Pedersen and Ullum (1994).

2.18 Exercise and immune cell counts

Acute exercise temporarily alters circulating leukocyte numbers and composition, returning to baseline within 6–24 hours post-exercise (Simpson et al., 2015; Robson et al., 1999). While total leukocyte counts are similar between athletes and healthy controls, those athletes participating in endurance sports, particularly long-distance running, may exhibit lower resting lymphocyte levels (Simpson et al., 2015; Baker et al., 2022). Longitudinal studies show declines in circulating NK cells and Tc cells, after 12 weeks and 6 months of intensive training in swimmers and cyclists, respectively (Baj et al., 1994; Gleeson et al., 2000).

Leukocyte counts can increase two- to three-fold after brief exercise and up to five-fold after prolonged endurance exercise (0.5–3.0 hours) (leukocytosis), driven by catecholamines and shear stress from increased cardiac output, indicating immune cell changes are intensity-dependent (Simpson et al., 2013; Walsh et al., 2011; Robson et al., 1999). Lymphocytes constitute a large proportion of the exercise induced leukocytosis, with $CD8^+$ T cells and NK cells preferentially mobilised due to their abundance of β_2-adrenoreceptors and high effector functions (Anane et al., 2009; Baker et al., 2023). Within as little as 3 minutes of exercise recovery (Rooney et al., 2018), a glucocorticoid driven rapid reduction in blood lymphocyte count (lymphocytopenia) occurs which, as previously described, is interpreted as an open window for infection (Pedersen & Ullum, 1994) or a redistribution of effector cells for enhanced tissue immunosurveillance (Simpson et al., 2020). However, evidence in lymphocytes also suggests that the decrease in the circulating lymphocyte pool, driven by corticosteroids may reflect a reduction of cells moving into the circulation from the lymphoid organs, rather than an increase of cells leaving the blood (Bloemena et al., 1990). This reduced efflux from the organs into the blood results in a lower 'net' circulating value, manifesting as lymphocytopenia. Specifically, fluorescently labelled lymphocytes were intravenously injected into rabbits either treated with prednisolone, a corticosteroid, or untreated (control) and their distribution in peripheral blood was measured (Bloemena et al., 1990). In contrast to sole glucocorticoid infusion, exercise promotes the real ingress of immune cells through muscle-derived myokines and by changing adhesion/endothelial activation, which together alter tissue-targeting behaviour (Kurz et al., 2022; Bettariga et al., 2024). The endocrine regulation of leukocyte mobilisation is discussed in more detail later in this Chapter.

This well-established biphasic lymphocyte response to exercise has been shown in response to various exercise durations and intensities including 60 minutes cycling at 60% $\dot{V}O_{2max}$ (Shinkai et al., 1992), 75 minutes cycling at 75% $\dot{V}O_{2max}$ (Ronsen et al., 2001), 45 minutes treadmill running at 80% $\dot{V}O_{2max}$ (Neiman et al., 1994) and 120 minutes treadmill running at 60% $\dot{V}O_{2max}$ (Shek et al., 1995), with higher intensity exercise inducing the largest lymphocytopenia (Walsh et al., 2011; Simpson et al., 2007).

DC numbers elevate by ~100-150% in as little as 15-30 minutes of acute exercise and return to baseline within 30 minutes post-exercise (Brown et al., 2018; Baker et al., 2023). A preferential mobilisation of pDCs over mDCs in response to exercise, such as 60 minutes of ice hockey and a 20-minute cycle at 80% $\dot{V}O_{2max}$ has been reported (Suchaneck et al., 2010; Brown et al., 2018) but is not consistently seen after a marathon race

(Nickel et al., 2011) or 30 minutes high intensity interval cycling (Baker et al., 2023). The degree of DC mobilisation is positively correlated with the concentration of catecholamine release into the blood during exercise (Suchanek et al., 2010), driven by the density of adrenergic receptors on the surface of DCs (Maestroni, 2000).

Although the density (cells/g) of T cells and DCs in organs of the lymphatic system (lymph nodes, spleen, thymus, tonsils) is higher than in blood, the Chapters in this thesis will measure circulating blood cells. Specifically, the density of T cells in the lymphoid tissues is ~10^9 cells/g, compared to ~10^6 cells/g in the blood. For DCs, the density in lymphoid tissues is ~10^8 cells/g, compared to ~10^4 cells/g in the blood (Sender et al., 2023). Despite T cells and DCs being more numerous in lymphoid tissues, measuring these immune cells in the blood is a less invasive and more accessible method of PBMC harvesting. Additionally, as mentioned above, circulating DCs and T cells are very responsive to exercise stress which makes them great candidates for investigating exercise induced alterations (Brown et al., 2018; Simpson et al., 2007).

2.19 The effect of acute exercise on immune cell function

2.19.1 T Lymphocytes

Studies show decreased mitogen-stimulated T cell proliferation during and after exercise (Moyna et al., 1996; Shinkai et al., 1992; Simpson et al., 2015; Green et al., 2002; Nieman et al., 1994), though many are limited by increased NK cell contamination in lymphocyte populations. Additionally, the migratory and homing capacity of $CD4^+$ T cells and $CD8^+$ T cells towards supernatants from rhinovirus-infected epithelial cells decreased 60 minutes after running for 120 minutes at 60% $\dot{V}O_{2max}$ (Bishop et al., 2009). This reduced migratory capacity could transiently limit tissue immune surveillance. However, these studies fail to account for the antigen-specific properties of memory T cells, as in vitro mitogens differ from clinically relevant antigens. As such, when stimulated with viral peptides against common cold antigens, T-cell activation and proliferation was enhanced after 30 minutes of steady-state exercise (Simpson et al., 2014).

The cytokine profile of circulating T cells is also altered by acute exercise. Cycling to exhaustion at ~74% $\dot{V}O_{2max}$ reduced the number of circulating IFN-γ producing type 1 $CD4^+$ T cells, and their subsequent stimulated IFN-γ release post exercise, but did not affect IL-4 producing type 2 $CD4^+$ T cells i.e. those that promote a humoral immune response (Lancaster et al., 2004). Likewise, 60 minutes cycling at 75% $\dot{V}O_{2max}$ significantly decreased IL-2 production from stimulated T cells compared to resting values (Tvede et al., 1993), but 18 minutes of continuous incremental cycling exercise (6 minutes at 55%, 70% and 85% $\dot{V}O_{2max}$) failed to alter the stimulated T cell IL-4 response (Moyna et al., 1996). Thus, acute exercise may alter the balance of T cell cytokine production away from type 1 (pro-inflammatory/anti-viral) immunity and towards type 2 (anti-inflammatory/host-protective) immunity (Moyna et al., 1996; Lancaster et al., 2004). Whilst this skewed cytokine balance may increase susceptibility to acute infection, it may be beneficial in reducing low grade

inflammation, contributing to the well-known positive anti-inflammatory effects of acute exercise (Dinarello, 1997).

2.19.2 Dendritic cells

Whilst DC count alterations in response to exercise is becoming well established, limited research investigating the effects of exercise on DC function exists. In male recreational marathon runners, a reduction in the mRNA and protein expression of TLR7 in DCs 24 hours post marathon has been shown (Nickel et al., 2012). TLR7 is an important intracellular TLR responsible for recognising viral DNA and initiating an anti-viral T cell response (Iwasaki & Medzhitov, 2015). Similarly, a combined endurance/resistance exercise bout reduced DC TLR responsiveness, evidenced by the less pronounced upregulation of activation markers; MHC II on pDCs and CD86 on mDCs (Deckx et al., 2015). Increased DC migratory markers were also found suggesting that one bout of exercise increases the migratory capacity of DCs less prone to driving inflammatory responses, potentially as a mechanism to counteract exercise-induced tissue damage and inflammation (Deckx et al., 2015). Conversely, no significant upregulation of the co-stimulatory molecules CD80/86 were found after a 60-minute intense ice hockey training session (Suchanek et al., 2010).

2.20 The effect of overreaching on immune cell function

As described at the beginning of this Chapter, athletes often use *'overtraining',* characterised by short term intensified training periods incorporating limited recovery periods, to achieve positive training adaptations. However, studies have shown that several aspects of T cell and DC function are sensitive to increases in training load in already well-trained athletes (Gleeson et al., 2013; Verde et al., 1992; Robson et al., 1999). A comprehensive review of the lymphocyte and DC response to periods of intensified training in both rodents and humans can be found in Chapter 5 (Baker et al., 2022).

6 days of intensified cycling training reduced stimulated lymphocyte proliferation, the ability of $CD8^+$ T cells cells to egress from the blood into tissues, and the number of circulating IFN-γ producing $CD4^+$ T cells (Lancaster et al., 2004 & 2003; Witard et al., 2012). A ~38% increase in normal cycling training load over 3 weeks resulted in reduced stimulated T cell proliferation immediately after a 30-minute treadmill run at 80% $\dot{V}O_{2max}$ but an increase in stimulated proliferation at rest (Verde et al., 1992). Baj et al. (1994) also reported an increase in stimulated lymphocyte proliferation at rest after a 6-month training block, involving cycling ~500 km per week in elite male cyclists. This suggests that whilst at rest the immune systems of endurance trained athletes may be able to tolerate the stress of heavy training, the superimposition of an acute bout of exercise on the chronic training stress results in immunosuppression (Verde et al., 1992).

Very little is currently known about the effects of intensified training on DC function in humans, and evidence currently available is conflicting (Baker et al., 2022). Groups investigating rodent DCs found increases in DC MHC II expression to 5 weeks progressive treadmill running (Chiang et al., 2007; Liao et al., 2006) and 8 weeks

of swimming for 45 minutes per day (Abdalla et al., 2017), but lowered expression after 4 weeks of treadmill running (MacKenzie et al., 2016). Either no change (Liao et al., 2006; Fernandes et al., 2019) or an upregulation (Abdalla et al., 2017; Mackenzie et al., 2016) in the co-stimulatory molecules CD80/86 expression have been found, and an upregulation of DC IL-12 (Chiang et al., 2007) and IFN-γ (Abdalla et al., 2017) secretion.

Differences in the stimulants used between studies to induce DC maturation could be the cause of disparities in results. For example, although Chiang et al. (2007), Abdalla et al. (2017) and Mackenzie et al. (2016) all used similar exercise protocols, measured bone marrow derived DCs and cultured them in granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4, different stimulants were used to induce DC maturation. Abdalla et al. (2017) and Chiang et al. (2007) used lipopolysaccharide (LPS), whereas Mackenzie et al. (2016) used ovalbumin (OVA). It is known that LPS induces a stronger upregulation of MHC II and co-stimulatory molecules (CD80/86) expression than OVA stimulation in rodents (Lutz et al., 1999; Kukutsch et al., 2000). Additionally, as shown in Chapter 5, low study numbers and large heterogeneity exists between studies investigating DC function in response to intensified training, likely also contributing to the lack of consistency in the literature.

Conflicting findings relating to the effects of intensified training on DC TLR function also currently exists in the literature. The ability of DCs to secrete cytokines such as IL-12, TNF-α and IL-1β upon TLR stimulation was significantly reduced by a 24-week period of intense training in competitive swimmers (Morgado et al., 2011). Similarly, 12 weeks of a combined endurance and resistance training program was shown to reduce stimulated TLR4 and 7 induced TNF-α secretion (Deckx et al., 2016). Conversely, Baker et al. (in press; Chapter 7) found no alterations in DC TLR expression or their subsequent cytokine expression after a 9-day intensified cycling training period, yet 10 weeks of intensified endurance training in recreational marathon runners was shown to upregulate DC TLR4 and 7 mRNA and protein expression (Nickel et al., 2011).

2.21 Immune-endocrine crosstalk

The endocrine system is heavily involved in the regulation of the immune system. Glucocorticoids such as cortisol, and androgens such as testosterone are known to possess anti-inflammatory effects on the immune system by downregulating the $CD8^+$ T cell anti-viral response (Bonneau et al., 1998), supressing pro-inflammatory mediators such as ROS (Franchimont, 2004) and inducing lymphocytopenia (Okutsu et al., 2005).

2.21.1 Glucocorticoids

Chronic exposure to hormones associated with stress, such as cortisol, have been shown to bare negative effects on health including increased infection risk (Cohen, 2005), delayed wound healing (Walburn et al., 2009; Ebrecht et al., 2004), impaired vaccination responses (Regan et al., 2021; Burns, 2012) and the progression of some cancers (Larsson et al., 2021; Antoni et al., 2006).

Cortisol has been shown to downregulate the expression of DC costimulatory molecules CD80/86 and dampen pro-inflammatory cytokine production, such as IL-12 and TNF-α, which subsequently reduces their

ability to prime naïve $CD8^+$ T cells (Elftman et al., 2007; Piemonti et al., 1999). In vivo studies show that DC migration towards lymph nodes is inhibited by cortisol, largely due to a downregulation of the homing receptor, CCR7 (Vizzardelli et al., 2006; Cumberbatch et al., 2006). Physiologically relevant concentrations of cortisol have been linked to reduced antigen processing by impairing the generation of antigenic peptides for MHC loading in virus-infected rodent DCs, and enhanced IL-10 expression (Truckenmiller et al., 2005 & 2006; Elftman et al., 2007; Rutella et al., 2006). Most of the cortisol effects are mediated through their interaction with cytoplasmic glucocorticoid receptors and modulation of the subsequent transcriptional levels (Rozkova et al., 2006; Riccardi et al., 2002).

Cortisol has also been shown to modulate TLR transcriptional pathways and TLR induced pro-inflammatory cytokine release of DCs. Rozkova et al. (2006) found increased expression of TLR2, 3 and 4 on DCs treated with cortisol, however their subsequent ability to stimulate T cells from TLR-derived signals was lessened. The same group further evidenced that patients treated with high dose corticosteroids displayed significantly reduced DC IL-12 and TNF-α secretion, impaired T cell stimulatory function and skewed DC differentiation favouring a distinct population incapable of inducing an efficient immune response (Rozkova et al., 2006). Therefore, the accumulation of acute cortisol increases in response to repeated intense bouts of exercise may therefore lead to maladaptive T cell and DC function.

As briefly mentioned earlier, the well-established biphasic response of lymphocyte mobilisation to an acute bout of exercise is largely regulated by catecholamines and glucocorticoids (Simpson et al., 2013). The release of catecholamines i.e. adrenaline, induces the mobilisation of effector cells such as $CD8^+$ T cells, both directly, via the action of adrenaline on lymphocyte β2-adrenoreceptors (Graff et al., 2018) and expression of adhesion molecules (Shephard, 2003), and indirectly, via increased cardiac output and shear stress mobilising lymphocytes from endothelial walls (Shephard, 2003). Whilst catecholamines drive the lymphocytosis of $CD8^+$ T cells during exercise, glucocorticoids such as cortisol influence their egress out of the circulation (lymphocytopenia) and into the tissues and organs for immunosurveillance and eradication of tumour cells (Okutsu et al., 2005; Simpson et al., 2020) (Figure 2.11). As mentioned in section 2.18, evidence in lymphocytes also suggests that the decrease in the circulating lymphocyte pool, driven by glucocorticoids may instead reflect a reduced efflux of cells from the organs into the circulating blood system, rather than an increased influx of cells from the blood into the organs, resulting in a net lymphocytopenia (Bloemena et al., 1990).

As previously discussed, when experiencing the negative states of overtraining i.e. NFOR/OTS, athletes may experience glucocorticoid resistance, resulting in HPA axis dysfunction and blunted cortisol responses to stress (Sorrells et al., 2009). Therefore, in contrast to the well characterised cortisol-mediated egress of lymphocytes during normal exercise recovery, the blunted cortisol response to exercise stress in overtrained athletes may impair the normal redistribution of lymphocytes into tissues, thus reducing the post-exercise lymphocytopenia and consequently immunosurveillance (Witard et al., 2012). For example, 7 days of intensified cycling training led to a ~15% decrease in exercise-induced plasma cortisol, coinciding with a ~44% reduction post exercise lymphocytopenia of $CD8^+$ T cells, compared to 7 days of normal training (Witard et

al., 2012). The reduced capacity for $CD8^+$ T cells to leave the blood after exercise with the 7-day intensified training period was largely driven by a reduced egress of naïve and highly cytotoxic $CD8^+$ T cells (Witard et al., 2012). Secondly, as cortisol is a known anti-inflammatory agent, an inability to secrete cortisol upon HPA stimulation may lead to persisting immune activation, which could cause decreasing immune cell metabolic capacity and function, and a shift in cytokine profiles to become persistently more pro-inflammatory, posing a risk to systemic homeostasis (Baskerville et al., 2024).

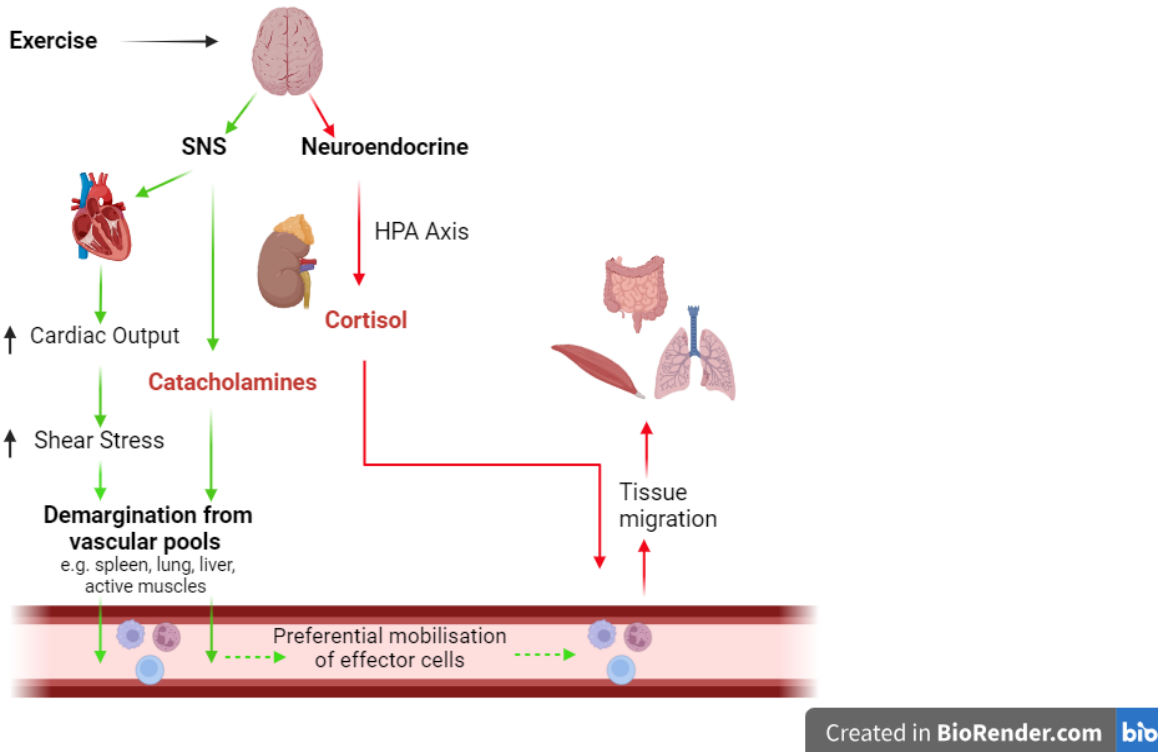


Figure 2.11. The physiological mechanisms behind the exercise induced redistribution of effector immune cells. The release of catecholamines i.e. adrenaline, induces the mobilisation of effector cells such as Tc cells, both directly, via the action of adrenaline on lymphocyte β2-adrenoreceptors and expression of adhesion molecules, and indirectly, via increased cardiac output and shear stress mobilising lymphocytes from endothelial walls (green arrows). Whilst catecholamines drive the lymphocytosis of Tc cells during exercise, glucocorticoids such as cortisol influence their egress out of the circulation (lymphocytopenia) and into the tissues and organs for immunosurveillance and eradication of tumour cells (red arrows).

The most notable effect of glucocorticoids on T cell function is the cortisol induced shift from a type 1 towards a type 2 immune profile. Specifically, glucocorticoids have been shown to favour the generation of type 2 $CD4^+$ T cells and production of anti-inflammatory cytokines, thus their widespread use in treating inflammatory diseases (Witard et al., 2012; Barnes, 2010). Carbohydrate intake during exercise limited the exercise induced rise in cortisol, which often coincides with an attenuated reduction in IFN-γ secreting $CD4^+$ T cells and $CD8^+$ T cells after 2 hours cycling at 65% $\dot{V}O_{2max}$ in moderately to well-trained males (Lancaster et

al., 2005). Additionally, in patients with glucocorticoid-resistance asthma, there is a failure of $CD4^+$ T cells to secrete IL-10 (Xystrakis et al., 2006).

2.21.2 Androgens

Androgens such as testosterone have been shown to supress the expression of TLR4, 8 and 9 in DCs (Corrales et al., 2012; Ainola et al., 2018) through interactions with DC cytoplasmic androgen receptors (Buendia-Gonzalez & Legorreta-Herrera, 2022). Additionally, DCs from males tend to secrete a lower concentration of IFN-α than females during viral infections, further suggesting a modulatory effect of androgens (Meier et al., 2009; Buendia-Gonzalez & Legorreta-Herrera, 2022). In accordance, it was found that lowering the androgen concentration in mice by castration promoted the maturation of DCs (Koh et al., 2009; Buendia-Gonzalez & Legorreta-Herrera, 2022). Additionally, DCs from men with hypogonadism show increased DC activation, inversely related to testosterone concentration, further providing evidence of the anti-inflammatory and potentially immunosuppressive nature of testosterone (Corrales et al., 2006; Buendia-Gonzalez & Legorreta-Herrera, 2022).

Testosterone is also implicated in the anti-inflammatory regulation of T cells. Decreasing testosterone concentrations were shown to increase the expression of transcription factors responsible for promoting the differentiation of $CD4^+$ T cells towards pro-inflammatory type 1 $CD4^+$ T cells (Arredouani et al., 2014). In accordance, testosterone administration in mice inhibits IL-12 signalling in T cells (Kissick et al.,2014), reduces $CD8^+$ T cells activity (Buendia-Gonzalez & Legorreta-Herrera, 2022) and upregulates the anti-inflammatory IL-10 secretion of $CD4^+$ T cells, promoting a type 2 response (Liva et al., 2001).

3 Chapter 3: General Methods

3.1 Ethical approval

All studies presented in this thesis were granted ethical approval by the Nottingham Trent University Human Invasive Research Ethics Committee. All studies conformed to the standards set by the Declaration of Helsinki. Prior to study participation, written informed consent was obtained from all participants (Appendix 10.1), and health questionnaires (Appendix 10.2) were completed and reviewed by the lead researcher.

3.2 Peak oxygen uptake assessment ($\dot{V}O_{2peak}$)

3.2.1 Step protocol (Chapter 4)

Peak oxygen uptake ($\dot{V}O_{2peak}$) was assessed on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands), using a continuous step protocol, starting at 60 W increasing by 35 W every 3 minutes until volitational fatigue. Expired air was assessed throughout the test for oxygen consumption and carbon dioxide production using breath-by-breath analysis (Metalyzer 3B, Cortex Medical, Germany). The highest 30 second rolling average of the second-by-second data was taken to be $\dot{V}O_{2peak}$. Heart rate (HR) was assessed using a HR monitor (Polar F2, Polar Electro Oy, Kempele, Finland) and ratings of perceived exertion (RPE) recorded using the Borg scale (Borg, 1982) 30 seconds before the end of each step.

Maximum power output (W_{max}) was determined using the equation:

$$W_{max} = W_{final} + (t/T)W_{inc}$$ (Kreider et al., 1998)

where W_{final} is the power output during the final stage completed, t is the amount of time (seconds) reached in the final uncompleted stage, T is the duration of each stage (180 seconds), and W_{inc} is the workload increment of each stage (35 W). Power outputs equivalent to 55% and 80% were calculated for each participant for use in the 55/80 exercise bout in the main experimental trials.

3.2.2 Ramp protocol (Chapter 6 and 7)

$\dot{V}O_{2peak}$ was assessed on an electronically braked cycle ergometer (Excalibur Sport, Groningen, The Netherlands), using a continuous ramp protocol, starting at 0 W and increasing by 30-45 W per minute until volitational fatigue to complete the test in ~9-12 minutes. The increment chosen was dependent upon

participant cycling experience. Expired air was assessed throughout the test for oxygen consumption and carbon dioxide production using breath-by-breath analysis (Metalyzer 3B, Cortex Medical, Germany) for VT_1 and $\dot{V}O_{2peak}$ to be calculated. HR was assessed using a polar HR monitor (Polar F2, Polar Electro Oy, Kempele, Finland) and RPE recorded using the Borg scale (Borg, 1982) every minute.

The $\dot{V}O_{2peak}$ was defined as the average of the highest exertional oxygen uptake achieved over the last 30 seconds of the Ramp protocol. The VT_1 was determined using the modified V-slope method, confirmed by patterns of change in ventilatory equivalent and end-tidal gas measurements and verified by an independent researcher. To account for the ramp, power outputs at VT_1 and $\dot{V}O_{2peak}$ were adjusted by subtracting 2/3^{rds} of the ramp increment (i.e. power output – 0.6 x ramp increment).

3.3 30-minute cycling stress tests

3.3.1 55/80 (Chapter 4)

Participants completed a 3-minute warmup at 50 W on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). The 55/80 stress test started immediately after the warm-up. The 55/80 stress test was a 30-minute cycle consisting of alternating blocks of 1 minute at 55% W_{max} and 4 minutes at 80% W_{max}, calculated from the power (W) at $\dot{V}O_{2peak}$ during the step protocol described above. HR and RPE were collected at the end of each block.

3.3.2 20/50 (Chapter 6 and 7)

Participants completed a 3-minute warmup at 50 W on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Holland). The 20/50 started immediately after the warm-up. The 20/50 was a 30-minute cycle, split into alternating blocks of 1 minute at a power output 20% below VT_1, and 4 minutes at 50% of the difference between power at VT_1 and $\dot{V}O_{2peak}$. HR and RPE were collected at the end of each block.

3.4 Measurement of training load

Training impulse scores (TRIMP) were calculated to quantify training intensity in Chapter 7 (Banister et al., 1991). The TRIMP scores calculated across the 9-day training period, including the 1.5 hour supervised cycle sessions and all extra training sessions completed, were compared to participants' TRIMP scores calculated from the pre-training 7-day training diary to ensure an increase in normal training load was achieved (Appendix 10.5). To measure HR during the 7-day period prior to commencing the study, plus any extra training sessions outside of the 1.5 hour supervised cycle, participants were given a HR monitor (Polar Beats, Polar Electro Oy, Kempele, Finland). The Polar Beats monitor records the average, minimum and maximum HR during an exercise session and automatically populates the data on the Polar Flow app for researchers to view (Figure 3.1).

TRIMP scores were then calculated using the equation:

t x HR ratio x y (Banister et al., 1991).

Where *t* = the session duration in minutes, *HR ratio* = (Average HR during exercise – Resting HR)/(maximum HR during exercise – Resting HR), *y* = a weighting factor $0.64e^{1.92x}$ for males and $0.86e^{1.67x}$ for females, with e = 2.712 and x = HR ratio.

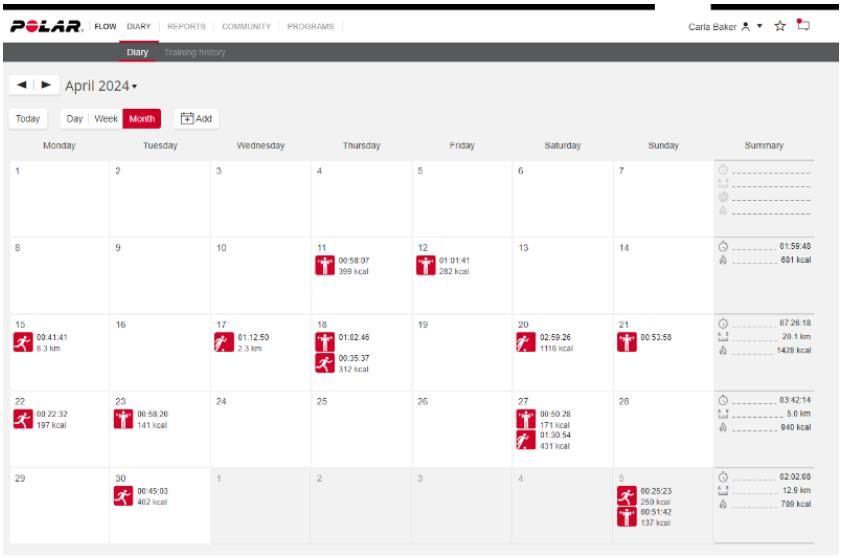


Figure 3.1. Example of the Polar Flow app used to monitor heart rate data outside of the 1.5 hour supervised cycles using a Polar Beats heart rate monitor (Polar Beats, Polar Electro Oy, Kempele, Finland).

3.5 Intensified training period (Chapter 7)

The intensified training period consisted of 9 consecutive weekdays of supervised cycling for 1.5 hours at 75% $\dot{V}O_{2peak}$ on either an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands) or on a Wattbike Atom (Wattbike, Nottingham, UK). Gas samples were collected via Douglas bags and analysed for carbon dioxide production using an O_2/CO_2 analyser (MiniHF 5200, Servomex, Crowborough, UK), and volumes measured via a dry gas meter (Harvard Dry Gas Meter, Harvard Ltd, Edenbridge, UK). The gas samples, along with HR and RPE were collected every 10 minutes for the first 30 minutes and then every 15 minutes thereafter to ensure participants were cycling at the appropriate intensity. If appropriate intensity was not achieved, the resistance was adjusted accordingly to achieve an average of 75% $\dot{V}O_{2peak}$ over the 1.5-hour cycle.

3.6 Urine osmolality

As some analytes being measured in plasma and saliva are affected by hydration status, prior to any trial involving venepuncture blood or salivary sampling, participants provided a urine sample for osmolality analysis. Urine osmolality measures the concentration of particles in the urine as a valid and reliable reflection of hydration status (Armstrong et al., 1998). In accordance with the ACSM position statement, a urine osmolality of <700 mOsmol/kg H_2O was accepted to confirm a euhydrated state (ACSM, 2007). If participants

did not meet this cutoff, they were instructed to consume 500 mL of water. Following 10 minutes of rest another hydration test was completed.

3.7 Blood and saliva collection, handling and hormone analysis

3.7.1 Venepuncture and sample storage

Venepuncture blood sampling was used in Chapters 4, 6 and 7. After a ~15-minute seated rest, 8-12 mL blood was collected via venepuncture from the antecubital fossa using a 23G needle (Vacutainer Safety-Lok, Becton, Dickinson and Co., Berkshire, UK), into two 4-6 mL ethylenediaminetetraacetic acid (EDTA) Vacutainers (Becton, Dickinson and Co., Berkshire, UK). Blood samples were either immediately centrifuged at 2000 X *g* for 15 minutes, and plasma divided into 1.5 mL aliquots for storage at -80˚C in 2mL cryogenic storage vials until analysis (Chapter 4) or subjected to density gradient centrifugation for peripheral blood mononuclear cell (PBMC) isolation prior to plasma aliquoting and storage at -80˚C (Chapters 6 and 7), as described in Section 3.8.1.

3.7.2 Saliva collection and sample storage

Saliva samples were collected via a Salivette (Sarstedt, Leicester, UK), whereby participants lightly chewed the cotton swab for 2 minutes. Saliva samples were immediately centrifuged at 2000 X *g* at 4^{o}C for 15 minutes, and aliquoted into 1.5 mL cryogenic storage vials, frozen and stored at -80^{o}C until analysis via enzyme-linked immunosorbent assay (ELISA).

3.7.3 Determination of hormone concentrations (Chapter 4 and 7)

Plasma cortisol (Chapter 4: R&D systems, Minneapolis, USA, Chapter 7: Tecan, Hamburg, Germany), testosterone (Chapter 4: DRG Instruments, Marburg, Germany, Chapter 7: Tecan, Hamburg, Germany), progesterone and oestrogen (Abcam, Cambridge, UK) were analysed using commercially available ELISA kits. The recommended manufacturer procedures for analyses were followed. On the day of the ELISA analysis, all samples were thawed completely, vortexed and centrifuged at 1500 X *g* for 15 minutes. All samples were exposed to one freeze-thaw cycle only.

Salivary cortisol and testosterone were also analysed using commercially available ELISA kits in accordance with the recommended manufacturer procedures for analyses (Salimetrics, Pennsylvania, USA).

The average sensitivity scores for each ELISA kit are as follows: salivary cortisol (<0.007 ug/dL), salivary testosterone (1 ng/mL), plasma cortisol (4.03 ng/mL), plasma testosterone (0.12 ng/mL), plasma progesterone (0.05 ng/mL) and plasma oestrogen (<9.375 pg/mL). The inter and intra- assay CVs for each plate are displayed in their respective Chapters.

3.8 Immune measures

3.8.1 Isolation of peripheral blood mononuclear cells (PBMCs) (Chapters 6 and 7)

4 mL of EDTA treated whole blood was layered onto 4 mL of Ficoll-Paque PLUS (GE Healthcare, Chicago, Illinois) and centrifuged at 400 X *g* for 25 minutes at 20^{o}C, splitting the whole blood into its separate compartments. The plasma was aspirated and aliquoted into 2 mL cryogenic storage vials and stored at -80^{o}C for hormone analysis as described in Section 3.7.1. The PBMCs were harvested with a Pasteur pipette and dispensed into an empty 15 mL falcon tube, where it was topped up to 14 mL with phosphate buffered saline (PBS) (Thermo Fisher Scientific, Massachusetts, USA), and washed by centrifuging at 400 X *g* for 5 minutes. Protocols then either followed option A (staining only) or B (staining and stimulation).

3.8.2 Monoclonal antibody staining and stimulation

Chapter 6 (Option A)

After washing, the supernatant was poured off, and the PBMCs were resuspended in 4 mL PBS. They were then counted using a haemocytometer before being split into two 2 mL suspensions, washed in PBS for 7 minutes at 500 X *g* and resuspended in 100 uL PBS. Fluorescently conjugated monoclonal antibodies were added to approximately 1 X 10^{6} cells before a 30-minute incubation in the dark at 4°C to identify DC and T cell subpopulations. The monoclonal antibodies used are displayed in Table 3.1 (BD Biosciences, San Diego, USA). After incubation, the PBMCs were washed in PBS for 7 minutes at 500 X *g* and resuspended in 200 uL PBS. Stained and unstained samples were run for 30,000 events on the BD Accuri C6 four-colour flow cytometer (Accuri C6, BD Biosciences, San Diego, USA).

Table 3.1. Fluorescently conjugated monoclonal antibodies used in Chapter 6.

Panel	Antigen	Fluorochrome	Clone#	Manufacturer
Dendritic Cells	Anti-Lineage 2 cocktail (CD3, CD14, CD19, CD20, CD56)	FITC	CD3 Clone # SK7, CD19 Clone # SJ25C1, CD20 Clone # L27, CD14 Clone # MϕP9, CD56 Clone # NCAM16.2	BD Biosciences
	HLA-DR	PerCP-Cy 5.5	G46-6	
	CD11c	APC	S-HCL-3	
	IL-3Rα (CD123)	PE	6H6	
T cells	CD3	FITC	HIT3a	BD Biosciences
	CD4	PE	RPA-T4	
	CD8	APC	RPA-T8	

Chapter 7 (Option B)

After the wash, the supernatant was poured off and PBMCs were resuspended in 500 µL of Gibco RPMI 1640 media (Thermo Fisher Scientific, Massachusetts, USA). 10 µL of PBMCs were added to 90 µL of trypan blue to create a 1 in 10 dilution for live cell counting via a haemocytometer. The cell count was calculated via the equation:

Cell Count (cells/mL) = Count x dilution factor x 10^4

Where the Dilution factor = $\frac{\text{Total volume of dilution}}{\text{Volume of sample in the dilution (10 µL)}}$

1 x10^6 cells were then added to empty 12 x 74 mm round bottom FACS™ tubes with 50 µL fetal bovine serum and topped up to a volume of 500 µL with Gibco RPMI 1640 media (Thermo Fisher Scientific, Massachusetts, USA) for stimulation. Stimulated tubes received 3.5 ug/mL of resiquimod (R848) (Ivivogen, San Diego, USA) and 35.24 ug/mL of ODN CPG 2395 (Ivivogen, San Diego, USA) and incubated for 5 hours at 37°C in a 5% CO_2 humidified atmosphere. After 1 hour of incubation, 5 ug/mL of Brefeldin A (Biolegend, London, UK) was added to block cytokine secretion, then samples returned to the incubator for the final for 4 hours. The unstimulated samples were also placed in the incubator for the same duration but received no stimulants.

Following incubation, cells were washed in 3 mL PBS at 300 X *g* for 5 minutes and resuspended in 50 µL PBS for surface staining. 1 µg/µL of FC block (miltenyi Biotec, Surrey, UK) was added to all tubes and incubated

for 10 minutes at room temperature. The extracellular fluorescently conjugated monoclonal antibodies displayed in Table 3.2 were then added to half of the tubes (stained and unstimulated, and stained and stimulated tubes) before a 30-minute incubation in the dark at 4°C. The PBMCs were then fixed and permeabilised using a no-centrifugation PerFix kit (Beckman Coulter, High Wycombe, UK). To fix the PBMCs, 25 µL of fixative reagent was added to each tube and incubated for 15 minutes at room temperature. The intracellular fluorescently conjugated monoclonal antibodies displayed in Table 3.2 were all added to 300 µL of the permeabilising reagent before being added to each of the stained tubes and incubated for 30 minutes in the dark at room temperature. The PBMCs were then washed by centrifugation in 3 mL of staining buffer at 500 X *g* for 5 minutes and resuspended in 350 µL of staining buffer ready for analysis via spectral flow cytometry (Cytek Auroura, Cytek Biosciences, Chester, UK). FACS™ tubes were run until empty, and the un-mixed data saved for analysis.

Table 3.2 Fluorescently conjugated monoclonal antibodies used in Chapter 7.

Panel	Antigen	Fluorochrome	Clone#	Manufacturer
Extracellular	CD3	Alexa Fluor 700	OKT3	Biolegend
	CD4	APC-Fire 750	RPA-T4	Biolegend
	CD8	BV480	RPA-T8	Fisher Scientific
	HLA-DR	PerCP-Cy5.5	G46-6	BD Biosceinces
	CD14, CD16, CD19, CD20, CD56	Pacific Blue	HCD14, 3G8, HIB19, 2H7, HCD56	Biolegend
	CD11c	PE CY-5	B-ly6	BD Biosciences
	IL-3Rα (CD123)	BV785	6H6	Biolegend
Intracellular	IL-10	Pe-Dazzle594	JES3-19F1	Biolegend
	TNF-α	BV605	Mab11	Biolegend
	IFN-α	APC	LT27:295	Miltenyi Biotec
	TLR7	FITC	S18024F	Biolegend
	TLR8	PE-Cy7	S16018A	Biolegend
	TLR9	PE	S16013D	Biolegend

3.8.3 Flow cytometry analysis

In Chapters 6 and 7, data were analysed using the Kaluza Analysis software (Beckman Coulter, Brea, CA, USA). PBMCs were first gated on the forward scatter vs. side scatter, then gated on the forward scatter height vs. forward scatter area for doublet exclusion. Total DCs were identified as HLA-DR^{+} Lineage^{-} and further

identified as pDCs ($CD123^+$ $CD11c^-$) or mDCs ($CD123^-$ $CD11c^+$). Total T cells were identified as being $CD3^+$ and were further analysed for $CD4^+$ and $CD8^+$ to identify Th ($CD3^+$ $CD4^+$ $CD8^-$) and Tc cells ($CD3^+$ $CD4^-$ $CD8^+$) (Figure 3.2).

In Chapter 7, Total DCs were then gated for TLR vs CD123 quadrants for identification of TLR7 pDCs ($CD123^+$ $TLR7^+$), TLR8 mDCs ($CD123^-$ $TLR8^+$) and TLR9 pDCs ($CD123^+$ $TLR9^+$). The same gating strategy was used for DC cytokines such that each cytokine was plotted against $CD123^+$ for identification of IFN-α^+ pDCs ($CD123^+$ IFN-α^+), TNF-α^+ pDCs ($CD123^+$ TNF-α^+) and TNF-α^+ mDCs (TNF-α^+ $CD123^-$). $CD4^+$ T cells were further gated for IL-10 to identify IL-10^+ $CD4^+$ T cells (Figure 3.3).

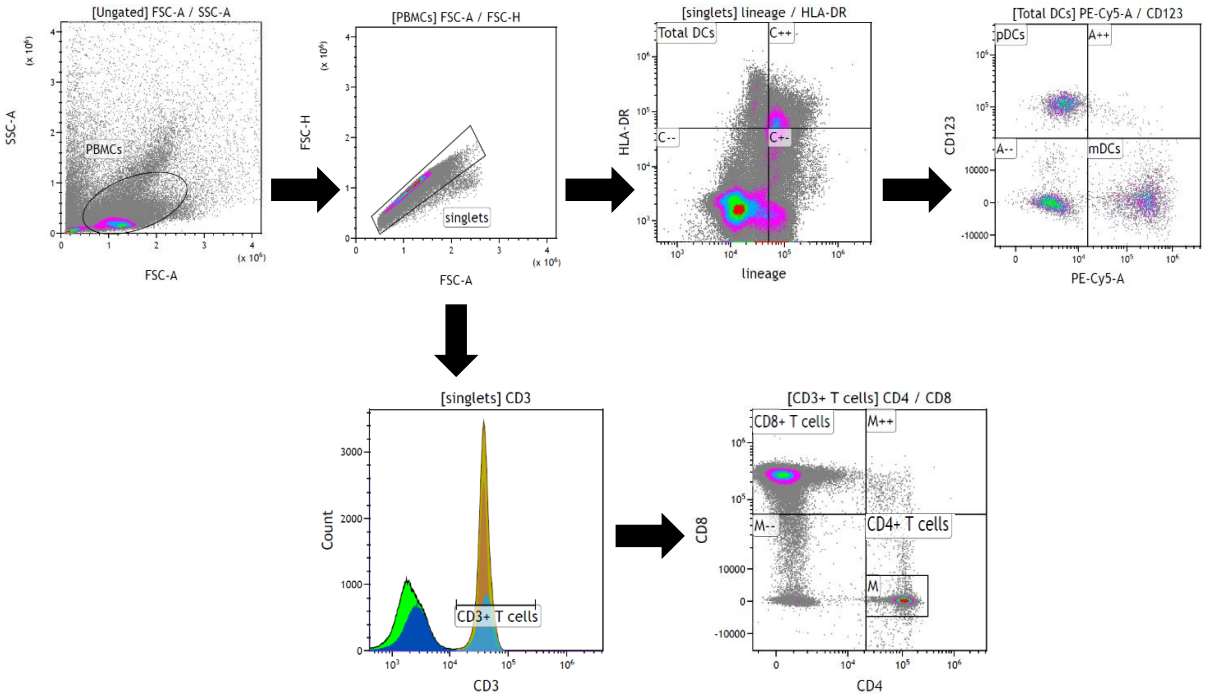


Figure 3.2. Gating strategy used in Chapter 6 and 7 to identify total dendritic cells, plasmacytoid Dendritic cells, myeloid Dendritic cells, T cells, T helper cells and T cytotoxic cells.

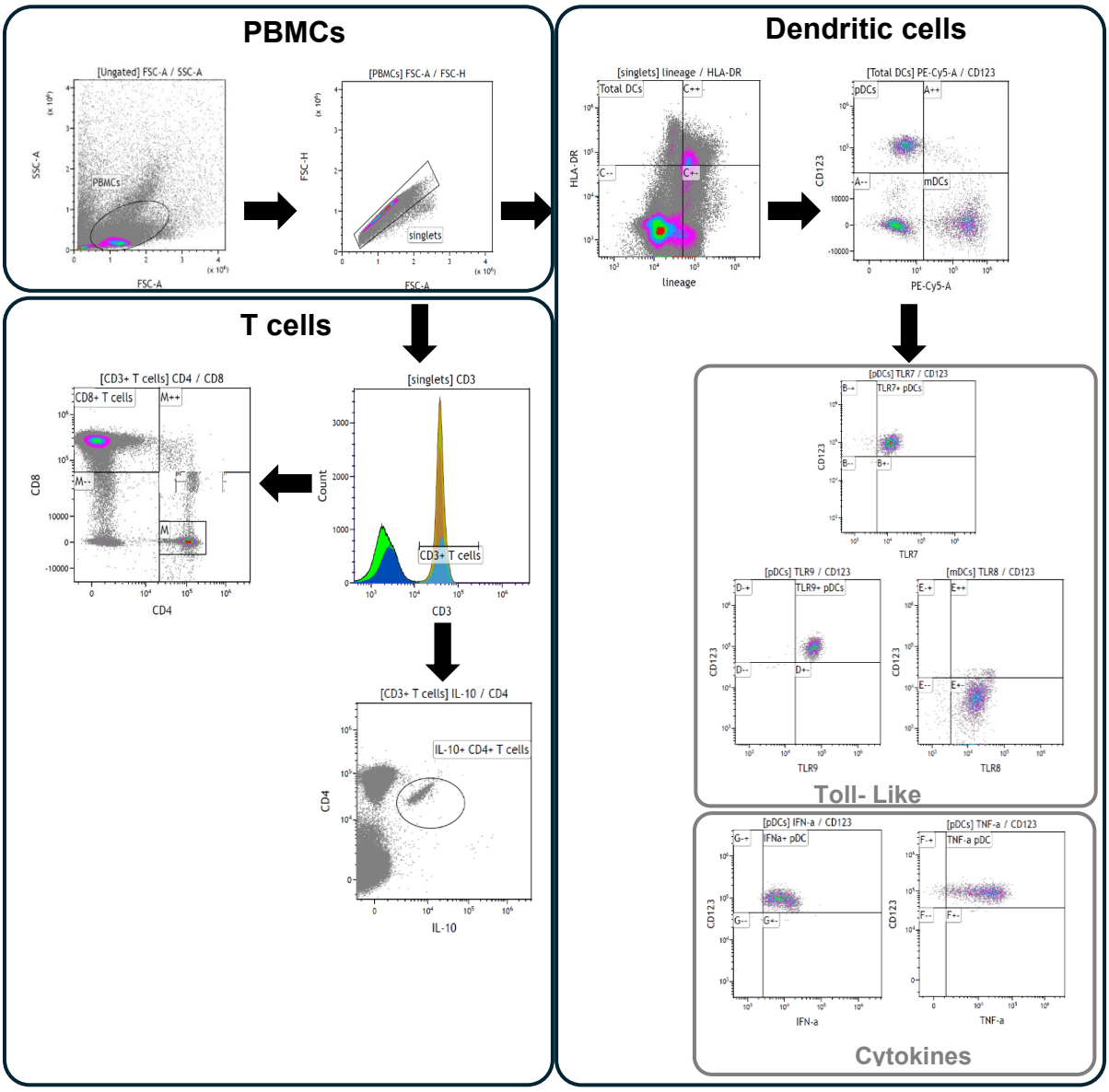


Figure 3.3. Gating strategy used in Chapter 7 to identify dendritic cells and their toll-like receptors and cytokines, and T Cells and their $CD4^+$ T cell IL-10 expression. Cells were stimulated with Resiquimod (R848) and ODN CPG 2395 for 5 hours.

Unstained controls and single stained reference controls were used to identify positive and negative cell populations and set the gates (Figure 3.5). Unmixing and compensation was automatically applied for Chapter 7 on the Cytek Aroura based on single stained controls and unstained controls and checked manually by assessing the positive and negative gates on the unmixing panel (Figure 3.4). For Chapter 6, compensation was performed manually. Spectral overlap values were measured for all fluorophores and in all detectors via single stained controls, and the spectral overlap values inverted to the built-in matrix function on Kaluza software (Figure 3.6).

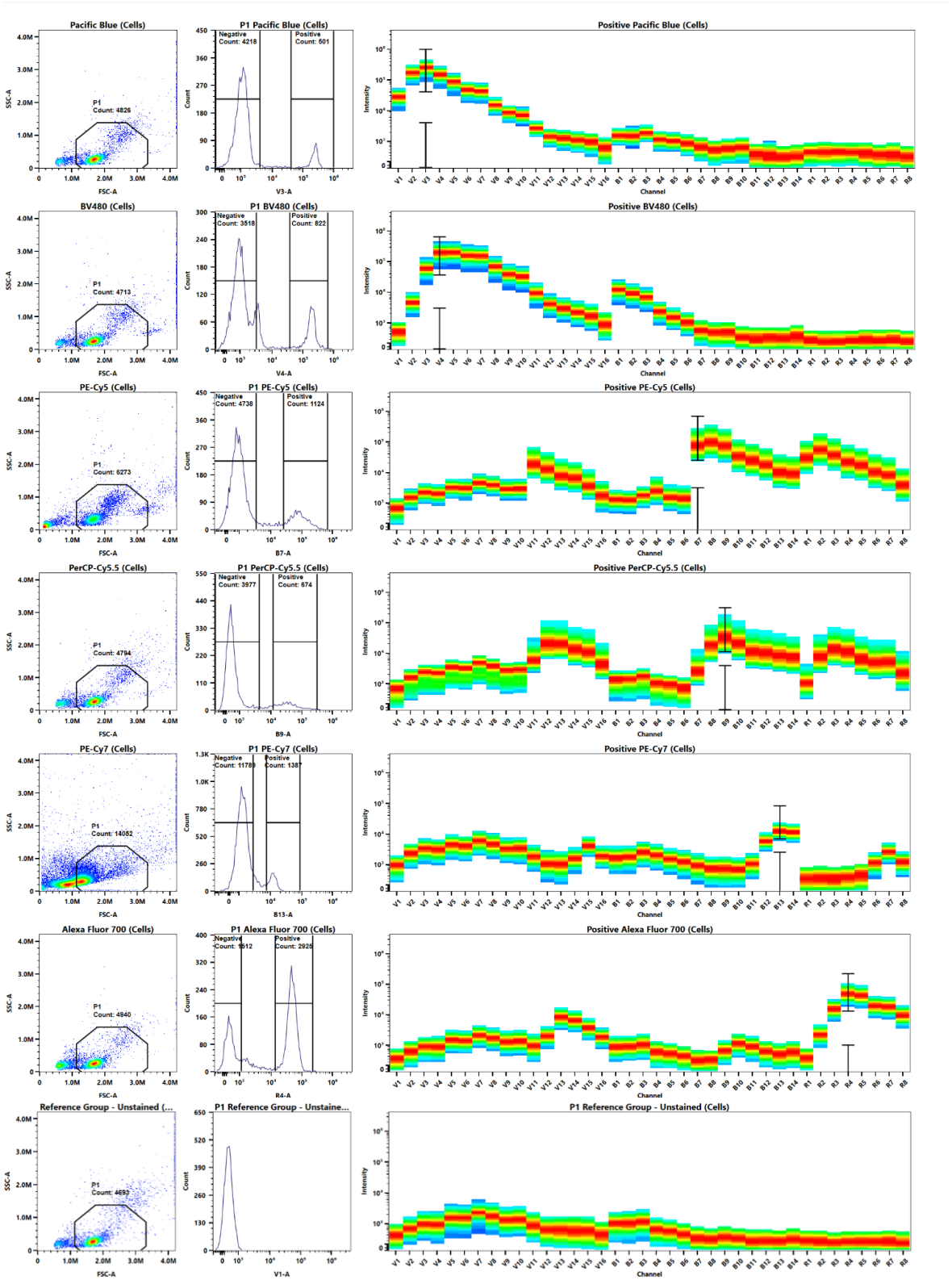


Figure 3.4. An example of the un-mixing procedure for antibodies used in Chapter 7. The unstained control (bottom tile) was used to set the negative gates on all histograms.

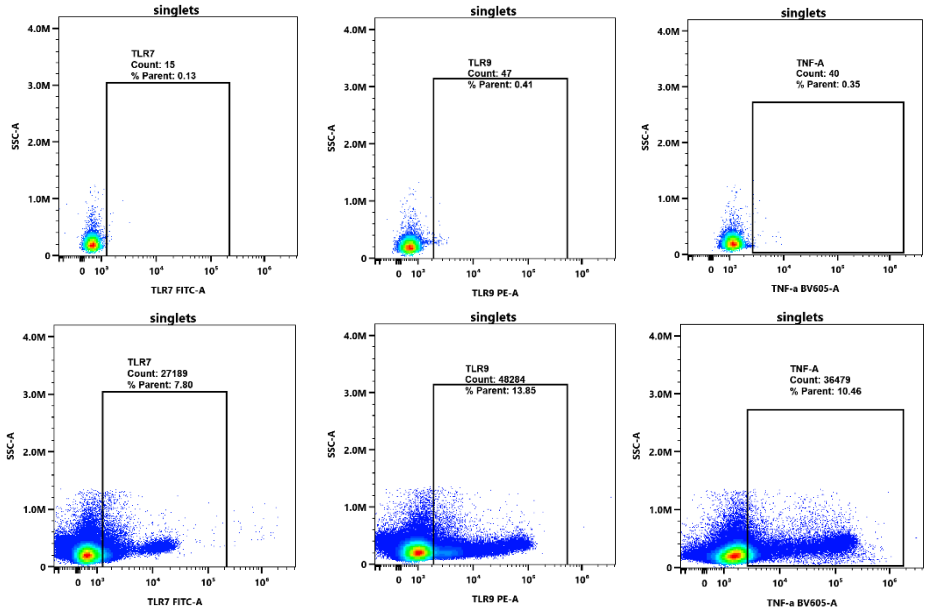


Figure 3.5. An example of the single stained (bottom row) and unstained controls (top row) used to set the positive and negative gates for sample gating and for the un-mixing process.

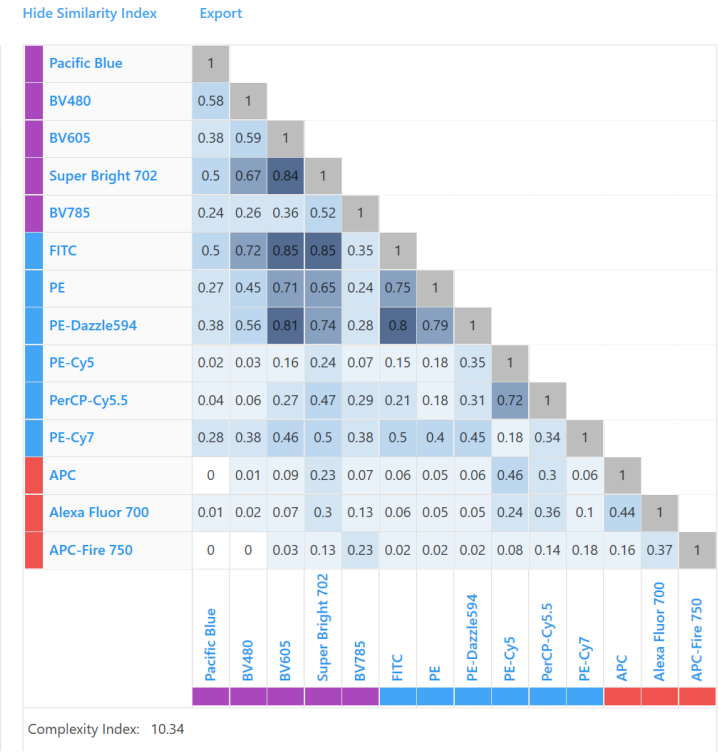


Figure 3.6. The spectral overlap matrix showing the level of spectral overlap of the fluorochromes in Chapter 7.

3.8.4 Optimisation of antibodies

All antibodies used in Chapters 6 and 7 were titrated to ensure optimal concentrations were used for staining. The aim of antibody titration is to minimise the loss of resolution caused by non-specific binding and ensure

sufficient identification of positive populations. If the antibody dilution is too low, the signal may be too weak to be accurately determined, resulting in poor data resolution, increased variability across measurements and an underestimation of the frequency of cells expressing a particular marker (Bonilla et al., 2024). Conversely, using excessive concentrations of antibodies can lead to non-specific binding, inefficient reagent use and high spillover (Bonilla et al., 2024).

After following the same PBMC isolation and counting procedures described above, individual tubes of 1 x 10^6 cells were stained with varying quantities of each antibody utilising the same extracellular and intracellular staining processes as previously described. The titration concentrations and stain indexes for Chapter 7 are displayed in Tables 3.3 and 3.4, and for Chapter 6 in Table 3.5 and 3.6, respectively.

Chapter 7 titrations

Table 3.3. Fluorescently conjugated monoclonal antibody concentrations for panel optimisation used in Chapter 7.

Extracellular/ Intracellular	Antibody	Protocol Volume (µL)	Titration (µL)	Optimised Volume (µL)
Extracellular	PerCP-Cy5.5 HLA-DR	5	2.5	2.5
	BV785 CD123	5	2.5	2.5
	PE CY-5 CD11c	20	10	20
	APC-Fire 750 CD4	5	2.5	2.5
	BV480 CD8	5	2.5	2.5
	Alexa Fluor 700 CD3	5	2.5	2.5
	Pacific Blue CD14 (HCD14)	4	2	2
	Pacific Blue CD16 (3G8)	5	2.5	5
	Pacific Blue CD19 (HIB19)	5	2.5	5
	Pacific Blue CD20 (2H7)	5	2.5	2.5
	Pacific Blue CD56 (HCD56)	4	2	4
Intracellular	APC IFN- α	10	5	10
	BV605 TNF-α	5	2.5	5
	Pe-Dazzle594 IL-10	5	2.5	5
	FITC TLR7	5	2.5	5
	PE-Cy7 TLR8	5	2.5	2.5
	PE TLR9	5	2.5	2.5

Table 3.4. Stain index values calculated for the antibody titrations used in Chapter 7. Stain index is calculated as the median fluorescent intensity (MFI) of the positive peak minus the MFI of the negative peak, divided by 2*SD of the negative peak. Red font represents the highest stain index, and therefore the optimised quantity used in the experiment.

Extracellular/ Intracellular	Antibody	Titration (µL)	Stain Index
Extracellular	BV480 CD8	5	184.037
		2.5	210.30
	BV785 CD123	5	4.94
		2.5	7.90
	PE CY-5 CD11c	10	4.37
		20	4.49
	APC-Fire 750 CD4	5	323.77
		2.5	329.59
	PerCP-Cy5.5 HLA-DR	5	20.37
		2.5	34.56
	Alexa Fluor 700 CD3	5	71.94
		2.5	74.91
	Pacific Blue CD14 (HCD14)	2	7.12
		4	6.19
	Pacific Blue CD16 (3G8)	5	121.17
		2.5	84.80
	Pacific Blue CD19 (HIB19)	5	39.58
		2.5	38.93
	Pacific Blue CD20 (2H7)	5	193.60
		2.5	220.72
	Pacific Blue CD56 (HCD56)	4	8.63
		2	7.57
Intracellular	APC IFN- α	10	2.99
		5	2.90
	BV605 TNF- α	5	4.64
		2.5	4.38
	Pe-Dazzle594 IL-10	5	5.57
		2.5	3.89
	FITC TLR7	5	3.17
		2.5	3.09
	PE-Cy7 TLR8	5	2.97
		2.5	3.19
	PE TLR9	5	4.83
		2.5	4.97

Table 3.5. Fluorescently conjugated monoclonal antibody concentrations for panel optimisation used in Chapter 6.

Pannel	Antibody	Protocol Volume (µL)	Titration 1 (µL)	Titration 2 (µL)	Optimised Volume (µL)
T cells	FITC CD3	20	10	5	5
	PE CD4	20	10	5	5
	APC CD8	5	2.5	1	1
Dendritic Cells	FITC Lineage Cocktail 2 (CD3; CD14; CD19; CD20; CD56)	20	10	5	20
	PE CD123	5	2.5	1	2.5
	PerCP-Cyanine 5.5 HLA-DR	20	10	5	5
	APC CD11c	5	2.5	1	5

Table 3.6. Stain index values calculated for the antibody titrations used in Chapter 6. Stain index is calculated as the median fluorescent intensity (MFI) of the positive peak minus the MFI of the negative peak, divided by 2*SD of the negative peak. Red font represents the highest stain index, and therefore the optimised quantity used in the experiment.

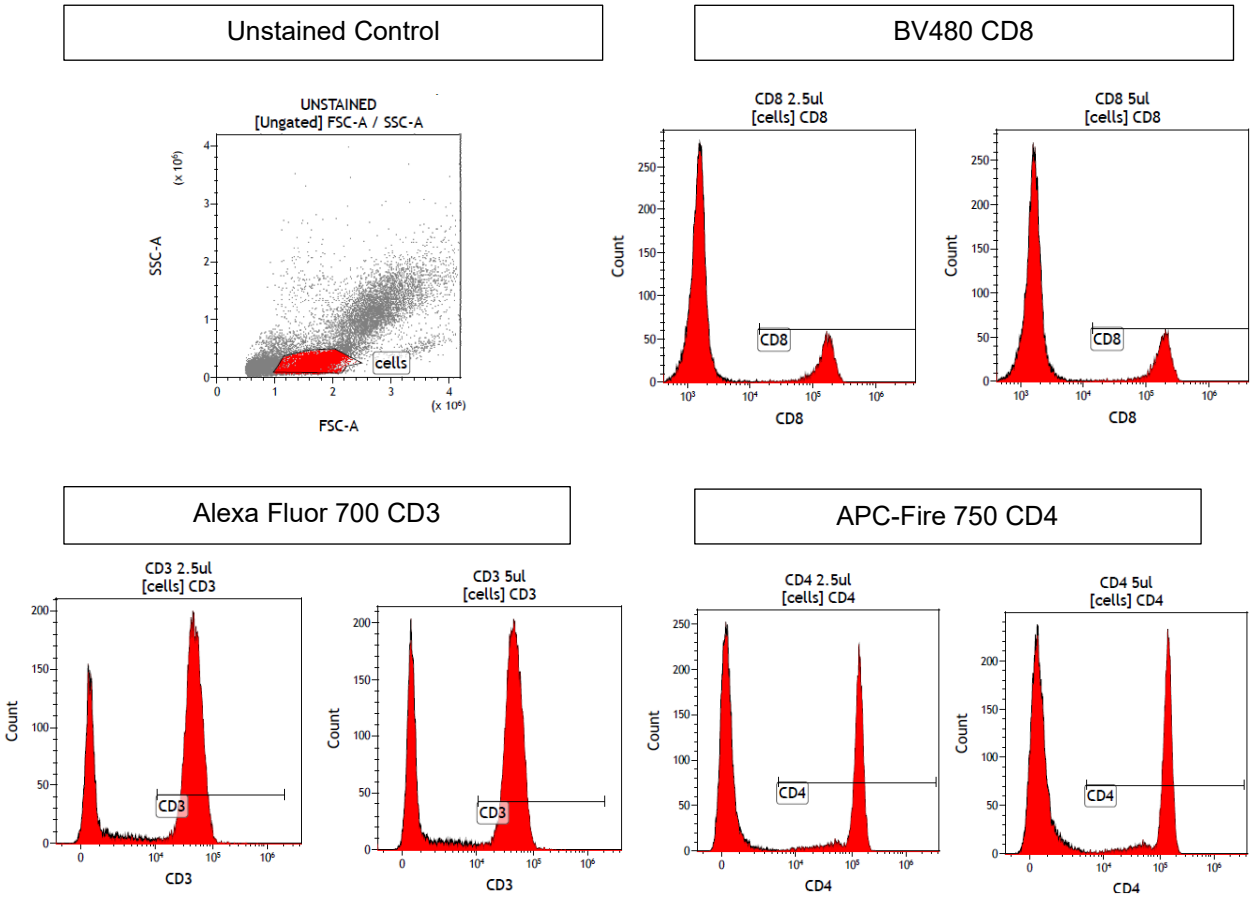
Pannel	Antibody	Titration (µL)	Stain Index
T cells	FITC CD3	20	45.90
		10	81.06
		5	102.57
	PE CD4	20	444.03
		10	458.91
		5	504.22
	APC CD8	5	306.41
		2.5	309.03
		1	337.28
Dendritic Cells	FITC Lineage Cocktail 2 (CD3; CD14; CD19; CD20; CD56)	5	257.61
		10	287.66
		20	343.58
	PE CD123	1	16.03
		2.5	19.09
		5	23.37
	PerCP-Cyanine 5.5 HLA-DR	5	5.40
		10	3.56
		20	4.08
	APC CD11c	1	18.93
		2.5	33.48
		5	45.70

The identification of optimal separation between positive and negative populations was completed via calculating a stain index using the calculation (Maecker et al., 2004; Bonilla et al., 2024):

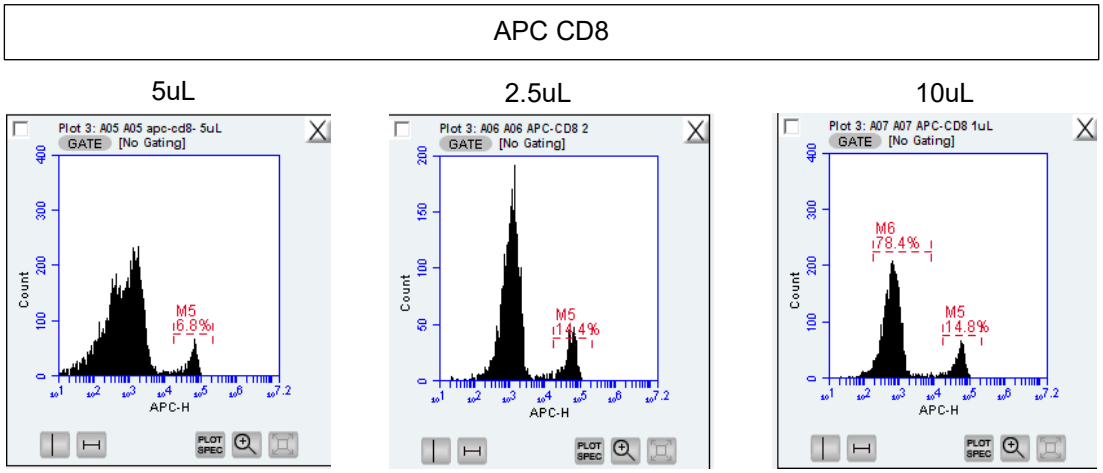
$$\frac{\text{Median Fluorescent Intensity (MFI) positive} - \text{MFI negative}}{2^* \text{ SD negative}}$$

The optimised titration is identified as the one resulting in the highest stain index as it is the value that provides the greatest discrimination between positive and negative signals (Bonilla et al., 2024; Maecker, 2004. Examples of the titration histograms used to calculate the stain index for the extracellular T cell antibodies used in Chapter 7 and 6 are shown below (Figure 3.7).

Chapter 7 (Cytek Aurora)



Chapter 6 (Accuri C6)



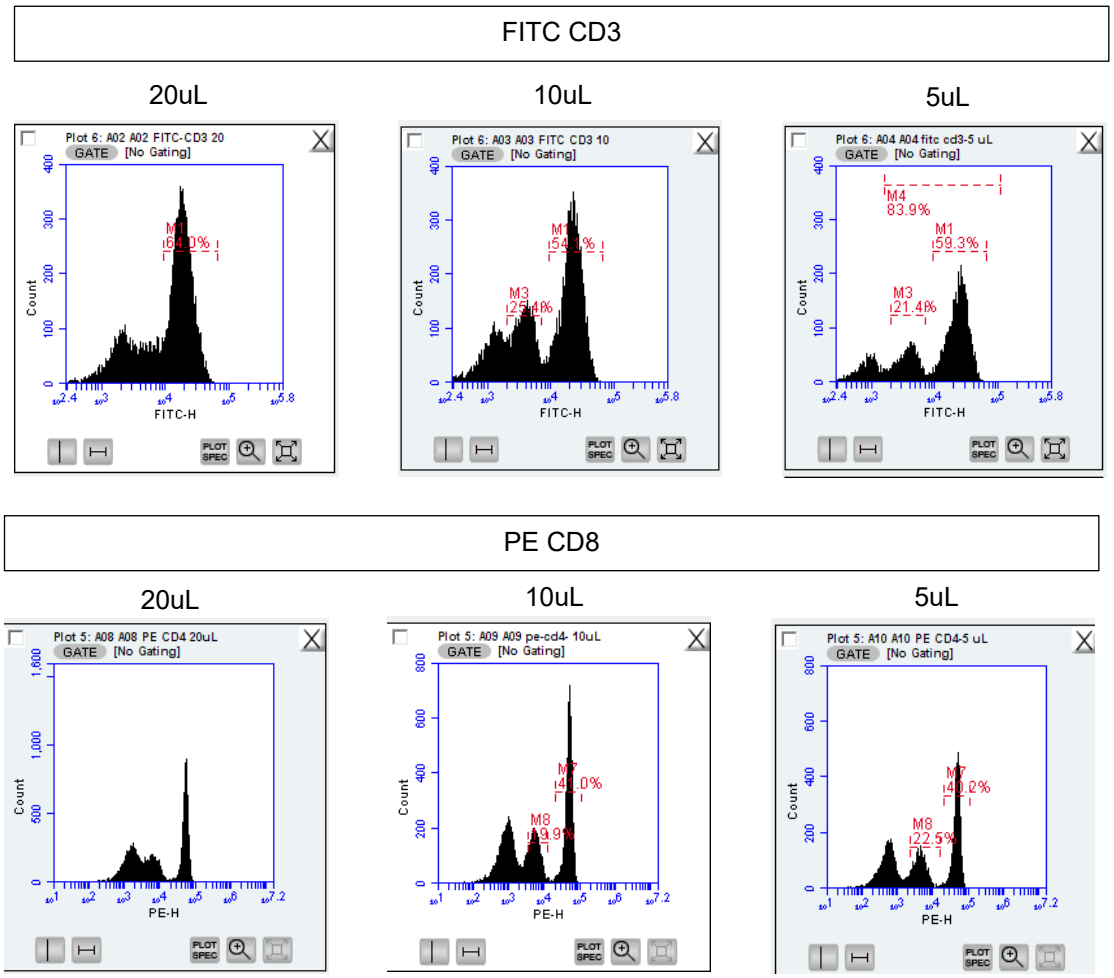


Figure 3.7. Titration histograms used to calculate the stain index for the extracellular T cell fluorescently conjugated monoclonal antibodies used in Chapter 7 and 6.

3.9 Menstrual cycle identification and control

In Chapter 4, both main trials took place during the early- mid luteal phase of the menstrual cycle. This was identified as being between 6 ± 1 days post-ovulation (resting control trial) and 9 ± 1 days post-ovulation (55/80 trial) for natural menstruators. Participants were given daily ovulation status urinary kits to use following the final day of bleed to confirm ovulation (Clearblue®, Bedford, UK). Oral contraceptive users completed their first main experimental trial in the middle of the pill taking phase (day 17-20 following beginning of withdrawal bleed), and their second 2 days later, as this is when endogenous sex hormones are most stable. Phase verification was confirmed via blood analysis of progesterone and oestrogen.

In Chapter 7, female participants tracked their menstrual cycle in a diary (Appendix 10.6) to identify the lengths of the luteal phase, ovulation, follicular phase, and menstrual bleed over one full cycle. Ovulation was confirmed using daily ovulation status urinary kits (Clearblue®, Bedford, UK). Female participants completed

both of their 20/50 main trials during menses, separated by training sessions occurring during the luteal phase (Figure 3.8).

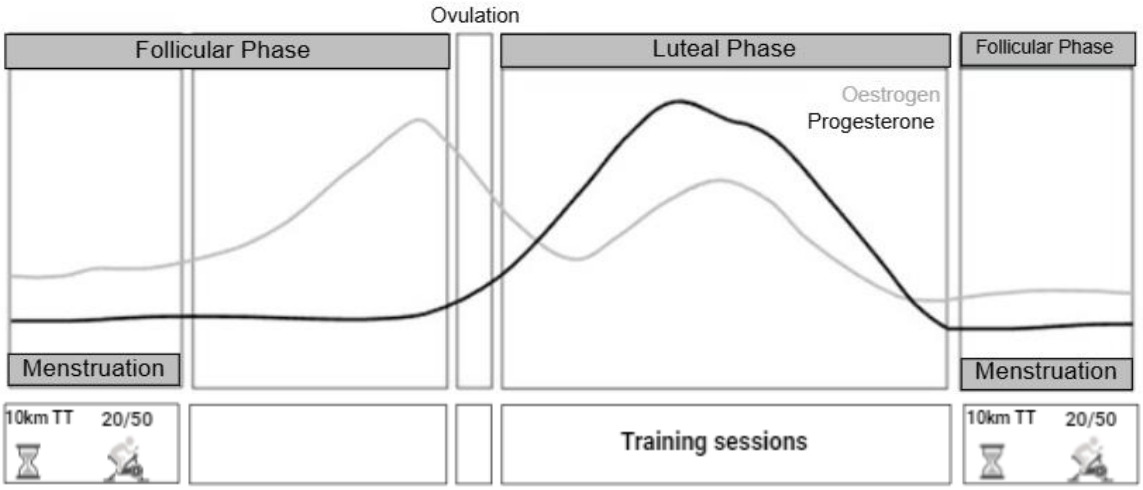


Figure 3.8. Schematic showing the study layout for naturally menstruating females in relation to oestrogen and progesterone levels across the menstrual cycle.

3.10 Questionnaires

3.10.1 Recovery stress questionnaire for athletes (REST-Q)

The REST-Q assesses perceived stress and recovery over the last three days and nights, further distinguishing differences between sport-specific and general stress and recovery (Kellmann & Kallus, 2001). The questionnaire comprises of 19 scales in total; 7 pertaining to general stress, 3 sport-specific stress, 5 general recovery and 4 sport-specific recovery. The version used in all experimental studies of this thesis was the 76 item REST-Q: thus containing 76 questions (Appendix 10.3). REST-Q scores were summed to give a general stress score, a sport stress score and an overall stress score. This was the same for recovery.

4 Chapter 4: Plasma and salivary hormone responses to a 30-minute exercise stress test in young, healthy, physically active females.

This Chapter is a modified version of the peer-reviewed study published in Physiological Reports: *Baker, C., Piasecki, J., Hunt, J. A., Foulds, G., & Hough, J. (2024). Plasma and salivary hormone responses to a 30-min exercise stress test in young, healthy, physically active females. Physiological reports, 12(24), e70168. https://doi.org/10.14814/phy2.70168*

4.1 Introduction

Overreaching is often used by athletes during a typical training cycle to enhance performance (Meeusen et al., 2013). Whilst initially the intensified training can result in a decline in performance, when appropriate periods of recovery are implemented, a super-compensatory effect resulting in enhanced performance above baseline levels can occur (Meeusen et al., 2013). However, if the stress/recovery balance is not carefully monitored, then NFOR, or more severely, the OTS, can occur (Meeusen et al., 2013). The OTS is surprisingly not infrequent amongst the athletic population and can take months- to- years for full recovery to occur (Meeusen et al., 2013). Previously reported symptoms of NFOR/OTS include poor sleep quality, increased illness incidence, a decline in performance and low moods or depression to name a few (Weakley et al., 2022; Witard et al., 2014). Despite the high prevalence of the OTS amongst those with highly physical occupations i.e. athletes and military personnel, there are currently no clear biomarkers to identify its occurrence, and it is often retrospectively diagnosed, prolonging recovery time (Weakley et al., 2022; Tanskanen et al., 2011).

Previous groups have identified hormones associated with the hypothalamus and pituitary glands, such as cortisol and testosterone as possible biomarkers of the OTS (Meeusen et al., 2013; Hough et al., 2021). Cortisol is a glucocorticoid hormone secreted by the adrenal cortex in response to stresses, such as heavy exercise (McMurray & Hackney, 2000). As described in Chapter 2, stress causes the hypothalamus to produce CRH, which stimulates the release of ACTH from the anterior pituitary gland, and in turn, the release of cortisol from the adrenal cortex; coined the HPA axis (Viru & Viru, 2004). The HPG axis is responsible for the production of testosterone and progesterone, thus both hormones follow a similar pathway of synthesis. The hypothalamus releases GnRH which stimulates the secretion of the gonadotropins LH and FSH from the pituitary gland, which in turn stimulates the production of testosterone from the testes in males and ovaries in females (Sharma et al., 2022). In females, the majority of testosterone is produced by the ovaries (25-50%) and adrenal glands (25%), with the remaining testosterone synthesised by the conversion of androstenedione to testosterone (Parish et al., 2021). Although limited differences between 'healthy' and overtrained athletes occur at rest, studies have shown that in response to a stress stimulus, cortisol and testosterone are blunted (Meeusen et al., 2010; Hough et al., 2013 & 2015). It is therefore suggested that hormone levels behave more homogenously upon stimulation compared to an "at rest" measure between 'healthy' and overtrained cohorts (Carrard et al., 2022).

Regarding the development of a laboratory protocol required for the study of the OTS, Meeusen et al. (2004) developed an exercise stress protocol consisting of two maximal cycling exercise bouts separated by a 4-hour resting recovery. They reported a ~118% reduction in exercise-induced plasma cortisol concentrations to the second maximal exercise bout after a 10-day period where training volume was increased by ~58%. However, the two-exercise bout protocol and lengthy recovery time used may make this an impractical tool for athletes. Hough et al. (2011) therefore developed a 30-minute high intensity cycling protocol, the 55/80, consisting of 1 minute at 55% $\dot{V}O_{2max}$ and 4 minutes at 80% $\dot{V}O_{2max}$. This stress test induced robust elevations in both salivary and plasma cortisol (~210% and ~91% from pre- to- post- 55/80, respectively) and salivary testosterone (~58% pre- to- post- 55/80) in healthy males (Hough et al., 2011). When implemented before and after an 11-day intensified training period, the 55/80 highlighted a ~166% (cortisol) and ~21% (testosterone) reduction in the peak salivary hormonal responses (Hough et al., 2013). These maladaptive hormone findings were coupled with increased fatigue and burnout scores reported via the REST-Q, indicating a possible state of NFOR/OTS (Hough et al., 2013 & 2015).

To date, research in the field of overreaching/overtraining took the path of least confounding variables, placing an emphasis on male physiology. All aforementioned studies investigating the usefulness of exercise stress tests as a tool to highlight the OTS utilised either physically active males, or elite male athletes (Meeusen et al., 2004; Hough et al., 2013 & 2015). A recent review has established that female athletes are underrepresented in overtraining studies, likely due to complications associated with the biological complexity of the menstrual cycle (Carrard et al., 2022). Albeit female inclusion makes the science more complicated, that leaves ~50% of the population for which the necessary is still missing. We know that both the natural menstrual cycle phases, and oral contraceptive use impacts circulating cortisol concentrations (Hertel et al., 2017). Specifically, in oral contraceptive users, the oral contraceptives have been shown to elevate circulating cortisol levels (Hertel et al., 2017). Additionally, in naturally menstruating (eumenorrheic) females, cortisol increases in response to mental stress activation of the HPA axis, are greater in the luteal phase (where progesterone is high) compared to other phases (Montero-Lopez et al., 2018). It is therefore not appropriate to assume that the findings shown in previous studies utilising male participants can be directly applied to female athletes. Matos et al. (2011) identified that of 376 screened young English athletes, the incidence of self-reported NFOR or the OTS was significantly higher in females than males, highlighting the importance of including females into future investigations, even more so given the increased female participation in sport in general. As such, to develop female inclusive tools in the identification of the OTS, it is first necessary to assess whether the 55/80 can also induce robust elevations in cortisol and testosterone levels in females.

Therefore, the current study aimed to establish the salivary and plasma cortisol and testosterone responses in young, healthy, physically active females to the 55/80 to assess its usefulness as a tool in the diagnosis of the OTS in females. Additionally, as the main female sex hormone and precursor to testosterone, we will also investigate the 55/80 induced progesterone changes to equally establish its usefulness.

4.2 Methods

4.2.1 Participants

Thirteen healthy, physically active, non-smoking females volunteered to take part in this study. Six participants were oral contraceptive users taking a combined oral contraceptive pill (for at least 6 months prior to the study). The type of oral contraceptive pill was regulated (150mg/30mg, Levonorgestrel/Ethinylestradiol). Seven participants were eumenorrheic (natural menstruators) and had not used oral contraceptives for a minimum of 6 months prior to the study and must have had at least 9 menstrual cycles in the past 12 months. The participant characteristics are outlined in Table 4.1. The study was approved by the Nottingham Trent University Invasive ethics committee (Ethics approval #573). After providing a detailed verbal and written explanation of the study, written informed consent (Appendix 10.1) and a health screen (Appendix 10.2) was obtained from each participant prior to testing.

Table 4.1. Descriptive characteristics of participants.

	Group	Oral Contraceptive Users	Natural Menstruators	P- Value
Age (y)	26 ± 4	24 ± 5	28 ± 2	0.114
Height (cm)	163.1 ± 11.8	157.3 ± 12.2	168.0 ± 8.9	0.088
Weight (kg)	62.0 ± 8.5	57.6 ± 3.3	65.8 ± 9.6	0.164
BMI (kg/m^2)	23.6 ± 3.8	23.8 ± 4.8	23.4 ± 2.6	0.857
$\dot{V}O_{2peak}$ (ml/kg/min)	40.95 ± 5.68	39.97 ± 4.33	41.93 ± 6.62	0.592

Data are presented as mean ± standard deviation, n = 13. Independent samples t-tests were used to identify significant differences in participant characteristics between oral contraceptive users and natural menstruators ($p < 0.05$).

4.2.2 Experimental design

This study was a non-randomised repeated measures-controlled trial. Each participant completed three separate visits to the laboratory: one session for preliminary measures, and two main experimental trials (Figure 4.1). To avoid any effect of circadian rhythm, the two main experimental trials (visits 2 and 3) occurred at the same time of day. Participants were instructed to abstain from exercise, caffeine, and alcohol 24 hours prior to testing. Both main experimental trials were completed within 3 weeks of visit 1, and two days apart from each other. On the main experimental trial days, participants were instructed to consume a standardised breakfast at the same time of day and drink at least 500 mL of water on the morning before each visit to ensure hydration, as hypohydration can lead to artificially elevated hormone levels. Once in the laboratory, as described in Chapter 3, urine osmolality was assessed.

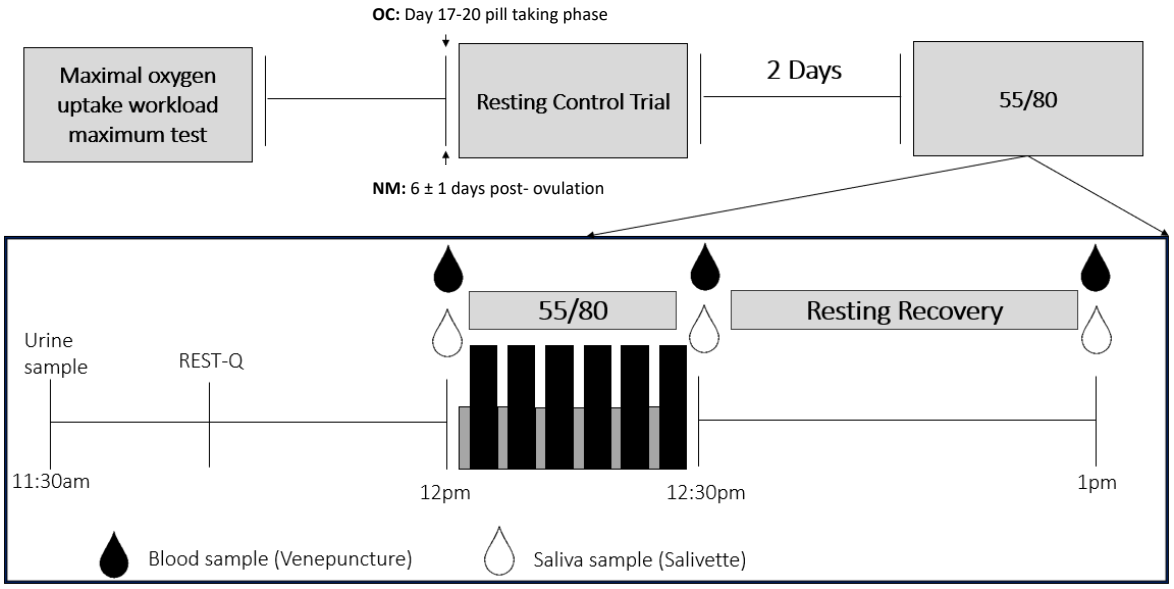


Figure 4.1. Schematic of the experimental design and specifics of the 55/80 trial. The resting control trial was the same as the 55/80 trial, but participants completed seated rest instead of the 55/80 cycle bout.

4.2.3 Menstrual cycle control

Both main trial days occurred within the early- mid luteal phase of the menstrual cycle. Chapter 3 details the menstrual cycle tracking procedure for identification of the luteal phase in natural menstruators, and the timeline of study visits for oral contraceptive users.

4.2.4 Preliminary measures

Height (Seca 217 stadiometer, Seca, Hamburg, Germany) and body mass (Seca 761 scales, Seca, Hamburg, Germany) were collected using standard methods. $\dot{V}O_{2peak}$ was assessed on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands), using a continuous step protocol as described in Chapter 3 for determination of 55/80 workloads.

4.2.5 Main trial 1: resting control

7-21 days after preliminary testing, participants reported to the laboratory for their first main experimental trial at 11:30am. Participants undertook seated-rest whilst completing the 76 item REST-Q (Appendix 10.3) (Kellmann & Kallus, 2001). Similar REST-Q scores between trials indicates participants were in a similar stressed/recovered state before completing each experimental trial. At 12pm, 4-6 mL blood was collected via venepuncture from the antecubital fossa into two EDTA vacutainers, and a saliva sample was collected into a Salivette (Sarstedt, Leicester, UK) as described in Chapter 3. This was repeated at 12:30pm and 1pm. Participants were allowed water *ad-libitum* but not during the 10 minutes prior to saliva sampling to avoid the possibility of saliva sample dilution.

4.2.6 Main trial 2: 55/80

2 days after the completion of the resting control, participants completed their second and final main experimental trial. All procedures were the same as during the resting control, but between 12:30pm and 1pm, participants completed the 55/80 as detailed in Chapter 3, Section 3.3.1.

4.2.7 Blood and saliva analysis

All blood and saliva samples were treated, stored and analysed as described in Chapter 3, Section 3. The mean intra-assay CV for all ELISA plates was <10.4%, and the inter-assay CV was <13.4%, apart from the plasma cortisol plate which was <13.2% and <17.5%, respectively.

4.2.8 Statistical analyses

Data were examined using SPSS statistical package version 28 (IBM Corporation, Armonk NY USA) for normal distribution using the Kolmogorov-Smirnov test. Non-normally distributed data were logarithmically transformed and re-examined for normality. A two-way repeated measures analyses of variance (ANOVA) with a Bonferroni correction was used to examine the effects of trial (resting control vs. 55/80), time and a trial*time interaction for the cortisol, testosterone, and progesterone concentrations. When the assumption of sphericity was violated, a Greenhouse-Geisser correction was applied. Paired samples t-tests were used for post hoc analysis, and to examine differences between plasma oestrogen and progesterone at rest, and REST-Q questionnaire scores between trials. Statistical significance was accepted at the $P< 0.05$ level. Data are presented as mean ± standard deviation (SD). One participant only provided saliva samples and therefore did not contribute to plasma oestrogen, progesterone, cortisol or testosterone. Therefore, plasma cortisol and testosterone have an $n = 12$. In addition to the above, as oestrogen was only measured at rest, 1 participant (natural menstruator) was excluded from oestrogen analysis due to a missed resting blood sample and 1 (oral contraceptive user) because all triplicates were out-of-range for the ELISA plate. Thus, for oestrogen analysis $n = 10$. For progesterone, 1 participant was excluded as their pre, post and 30-minute post samples were 17.5 fold higher than the group average, significantly skewing the distribution. Therefore, for progesterone analysis, $n =11$. N numbers in the figure legends reflect this.

4.2.9 Justification of sample size

Sample size estimations were based on our primary outcome variables, cortisol and testosterone. Completing an a priori power calculation, it was estimated that 12 participants would be required to see a meaningful difference in the main outcome variables in response to the exercise stress test (Cohen's $d = 0.90$) at 80% power and an α level of 0.05. To account for a 10% drop out rate, 13 participants were recruited.

4.3 Results

4.3.1 Confirmation of menstrual cycle phase

The plasma progesterone to oestrogen ratio at rest during the resting control and 55/80 trials were 1.94 ng/mL and 3.34 ng/ml, respectively, confirming that testing took place during the early to mid-luteal phase (higher progesterone vs. oestrogen levels). There were no significant differences in mean resting plasma oestrogen ($t(9)= 0.188$, $P= 0.855$) or progesterone concentrations between the 55/80 and resting control trials ($t(10)= -1.132$, $P= 0.284$) (Table 4.2).

Table 4.2. Resting plasma progesterone to oestrogen ratio (n=10), plasma progesterone (n=11) and plasma oestrogen (n=10) during the resting control and 55/80 trial visits. Significance is accepted as $P< 0.05$.

	Resting Control			**55/80**			
Plasma Progesterone (ng/mL): Oestrogen Ratio (ng/mL)	1.94 (0.81)			3.34 (1.87)			
	Group Mean	**Oral Contraceptive Users**	**Natural Menstruators**	**Group Mean**	**Oral Contraceptive Users**	**Natural Menstruators**	**P- Value**
Plasma Progesterone (ng/mL)	1.37 (1.39)	0.68 (0.29)	5.69 (7.89)	2.29 (3.63)	0.68 (0.34)	10.28 (12.59)	0.284
Plasma Oestrogen (pg/mL)	705.90 (179.21)	604.47 (76.49)	807.33 (194.44)	685.89 (109.92)	663.51 (133.28)	708.27 (73.50)	0.855

Data are presented as mean (standard deviation).

4.3.2 55/80 HR, RPE and %W_{max}

The mean HR during the 55/80 trial was 165 ± 9 bpm and the mean RPE was 15 ± 1. The average 55% W_{max} was 108 ± 18 W, and the mean 80% W_{max} was 156 ± 27 W.

4.3.3 REST-Q

There were no significant differences in the total recovery or total stress REST-Q scores between trials ($t(9)= -0.043$, $P= 0.967$). The average total recovery and total stress scores for the 55/80 trial were 1.75 ± 1.02 and the resting control trial was 1.74 ± 0.91.

4.3.4 Salivary cortisol

There was a significant main effect of trial (F(1,12)= 38.873, P< 0.001) whereby mean salivary cortisol levels were higher in the 55/80 trial compared to the resting control trial (19.09 ± 1.65 vs. 10.24 ± 1.71 nmol/L, P< 0.001). There was also a significant main effect of time wherein salivary cortisol levels were elevated in response to the 55/80 exercise bout (F(2,24)= 11.009, P= 0.004) (Figure 4.2). Post hoc analysis revealed that salivary cortisol was significantly elevated above baseline (11.18 ± 6.64 nmol/L) immediately post (19.20 ± 5.44 nmol/L) and 30 minutes post (26.89 ± 10.18 nmol/L) 55/80 (P< 0.001). Salivary cortisol was also significantly elevated from post 55/80 to 30 minutes post 55/80 (P= 0.002). The significant interaction between time and trial (F(2,24)= 36.335, P< 0.001) indicates that the change in salivary cortisol across the various timepoints were different in the two trials.

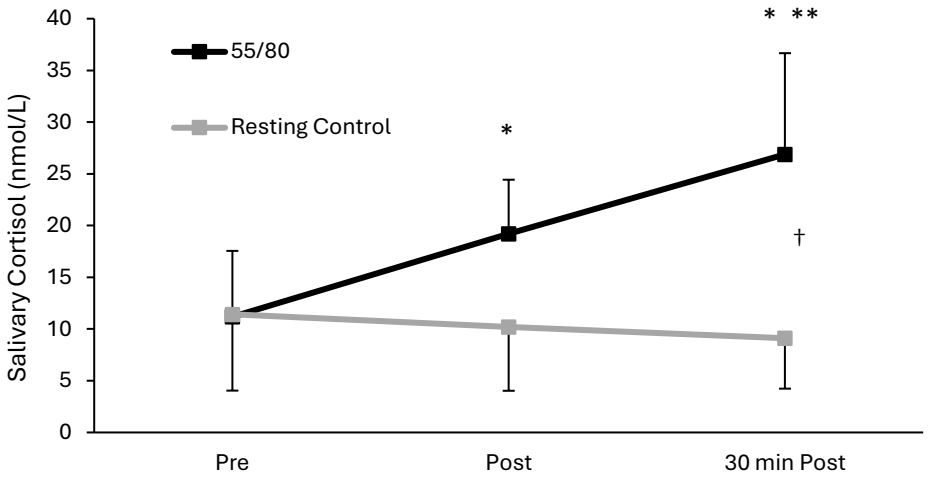


Figure 4.2. Salivary cortisol concentrations during the resting control trial and the 55/80 trial.
Data are presented as mean ± standard deviation.
* Significantly different from baseline. ** Significantly different from Post. † Significant effect of condition.
n=13.

When oral contraceptive users (n=6) and natural menstruators (n=7) were compared, natural menstruators demonstrated a larger post exercise (~165%) and 30-minute post exercise (~265%) increase in salivary cortisol compared to oral contraceptive users (~30% and ~84%, respectively) from baseline (Figure 4.3a). For natural menstruators, there was a significant main effect of trial (F(1,6)= 30.188, P= 0.002) whereby mean salivary cortisol levels were higher in the 55/80 trial (15.82 ± 5.99 nmol/L) than the resting control trial (5.41 ± 1.83 nmol/L), time (F(1.053, 6.318)= 11.752, P= 0.012) and time*trial interaction (F(2, 12)= 31.494, P< 0.001) (Figure 4.3b). Post hoc analysis revealed that salivary cortisol was elevated above baseline (6.52 ± 5.90 nmol/L) immediately post (17.23 ± 5.27 nmol/L, P= 0.033) and 30 minutes post (23.72 ± 5.08 nmol/L, P=0.041) 55/80. Within the oral contraceptive users, there was a significant main effect of trial (F(1,5)= 11.710, P= 0.019), whereby mean salivary cortisol levels were higher in the 55/80 trial (22.90 ± 3.15 nmol/L) compared to the

resting control trial (15.87 ± 4.11 nmol/L) but no main effect of time (F(1.099,5.495)= 2.274, P= 0.188) was seen on salivary cortisol concentrations.

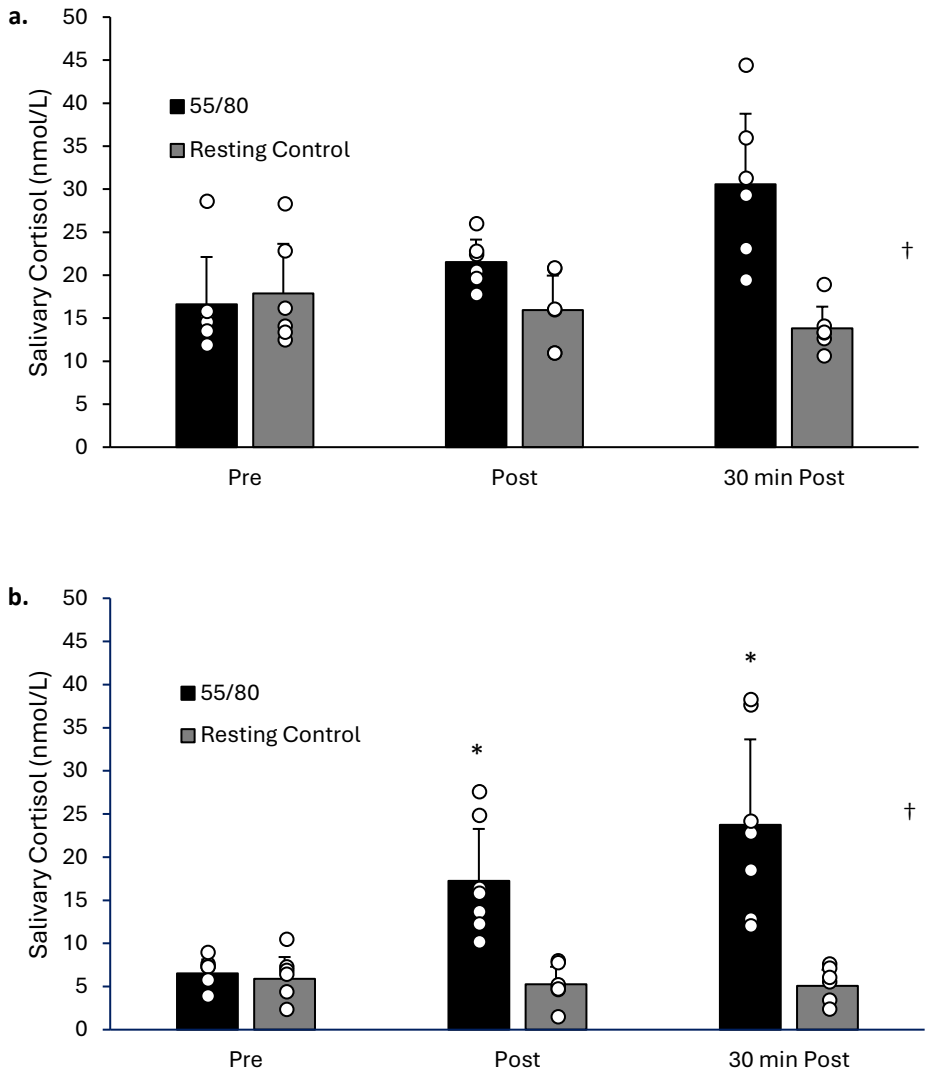


Figure 4.3. Salivary cortisol concentrations during the resting control trial and the 55/80 trial in (a) oral contraceptive users (n=6) and (b) natural menstruators (n=7).
Data are presented as mean ± standard deviation.
*Significantly different from Pre. † Significantly different between conditions.

4.3.5 Salivary testosterone

There was a significant main effect of trial (F(1,12)= 151.216, P< 0.001), whereby mean salivary testosterone levels were higher in the 55/80 trial compared to the resting control trial (316.59 ± 127.77 vs. 228.22 ± 88.96 pmol/L, P< 0.001). There was also a significant main effect of time whereby salivary testosterone levels were elevated in response to the 55/80 exercise bout (F(2,24)= 9.680, P< 0.001) (Figure 4.4). Post hoc analysis revealed that salivary testosterone was significantly elevated above baseline (216.55 ± 63.39 pmol/L)

immediately post (417.23 ± 140.03 pmol/L, P < 0.001) and 30 minutes post 55/80 (315.99 ± 87.25 pmol/L, P= 0.001). Salivary testosterone was significantly lower 30 minutes post compared to immediately post 55/80 (P= 0.005). The significant interaction between timepoint and trial (F(2,24)= 68.754, P< 0.001) indicates that the change in salivary testosterone across the various timepoints differed between the 55/80 trial and the resting control trial.

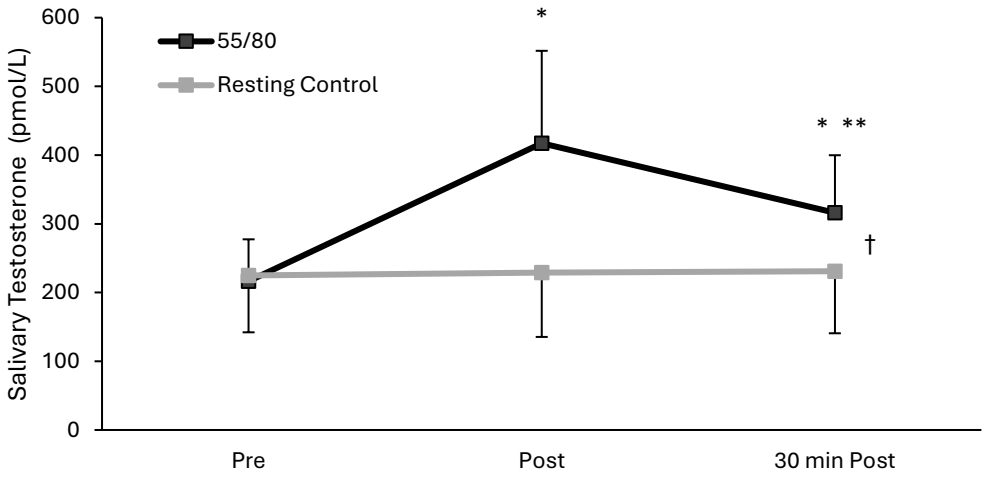


Figure 4.4. Salivary testosterone concentrations during the resting control trial and the 55/80 trial. Data are presented as mean ± standard deviation.
*Significantly different from Pre. ** Significantly different from Post † Significant effect of condition. (n=13) .

Within the oral contraceptive users, there was no significant main effect of trial (F(1,5)= 5.289, P= 0.070), but there was a significant main effect of time (F(2, 10)= 5.208, P= 0.028) and time*trial interaction (F(2, 10)= 25.522, P< 0.001). Post hoc analysis revealed that salivary testosterone was significantly elevated above baseline (218.33 ± 53.19 pmol/L) immediately post (332.14 ± 77.16 pmol/L, P= 0.007) and 30 minutes post 55/80 (261.33 ± 58.79 pmol/L, P= 0.005) (Figure 4.5a).

For natural menstruators, there was a significant main effect of trial (F(1,6)= 27.789, P= 0.002), whereby mean salivary testosterone levels were higher in the 55/80 trial (356.01 ± 85.78 pmol/L) compared to the control trial (241.97 ± 83.68 pmol/L), time (F(2,12)= 2.274, P< 0.001) and time*trial interaction (F(2,12)= 8.325, P= 0.005) (Figure 5b). Post-hoc analysis revealed that salivary testosterone was significantly elevated above baseline (215.03 ± 75.31 pmol/L) immediately post (490.17 ± 144.25 pmol/L) (both P< 0.001) and 30 minutes post 55/80 (362.85 ± 82.43 pmol/L, P= 0.006) (Figure 4.5b). When oral contraceptive users (n=6) and natural menstruators (n=7) were compared, natural menstruators demonstrated a larger post exercise (~128%) and 30 minutes post exercise (~68%) increase in salivary testosterone compared to oral contraceptive users (~52% and ~20%, respectively) from baseline.

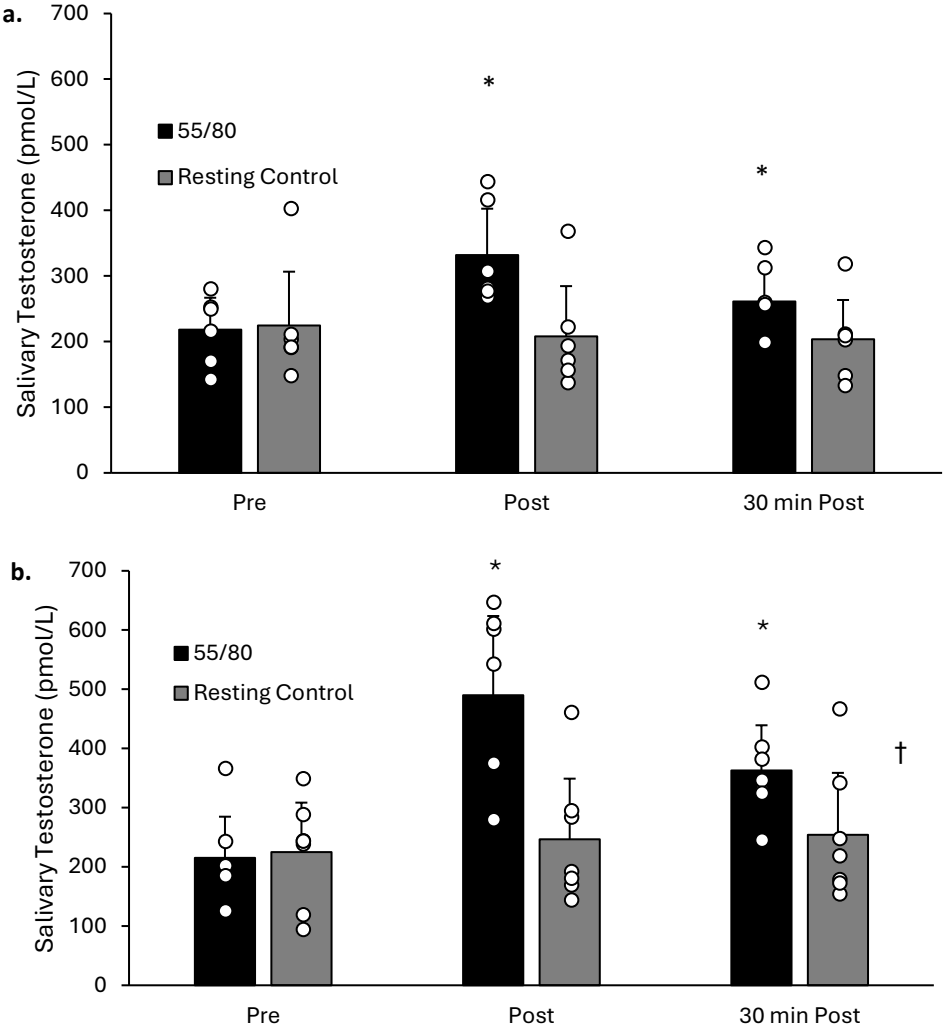


Figure 4.5. Salivary testosterone concentrations during the resting control trial and the 55/80 trial in (a) oral contraceptive users (n=6) and (b) natural menstruators (n=7).
Data are presented as mean ± standard deviation.
*Significantly different from Pre. † Significant effect of condition.

4.3.6 Plasma testosterone

There were no significant differences in plasma testosterone between trials ($F(1,11)= 0.646$, $P= 0.439$) or time ($F(2,22)= 0.1.586$, $P= 0.227$) (Figure 4.6).

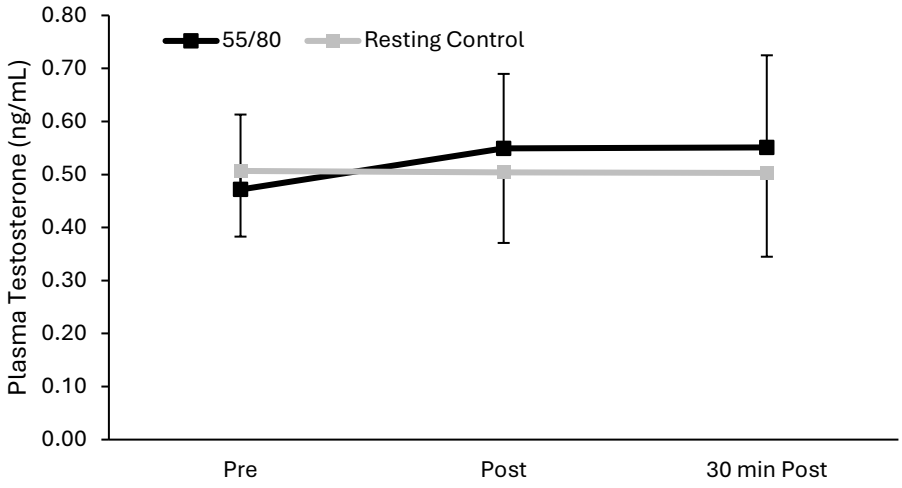


Figure 4.6. Plasma testosterone concentrations during the resting control trial and the 55/80 trial. Data are presented as mean ± standard deviation. n=13.

4.3.7 Plasma cortisol

There was a significant main effect of trial ($F(1,11)= 20.855$, $P< 0.001$), whereby mean plasma cortisol levels were higher in the 55/80 trial compared to the resting control trial (367.01 ± 146.37 vs. 210.07 ± 152.23 nmol/L, $P< 0.001$) (Figure 4.7). There was also a significant main effect of time whereby plasma cortisol levels elevated in response to the 55/80 exercise bout ($F(2,22)= 12.553$, $P< 0.001$). Post hoc analysis revealed that plasma cortisol levels were significantly elevated above baseline (246.61 ± 159.71 nmol/L) both immediately post (395.32 ± 82.86 nmol/L) (both $P= 0.002$) and 30 minutes post 55/80 (459.65 ± 107.45 nmol/L, $P< 0.001$). Plasma cortisol was also significantly elevated from post 55/80 to 30 minutes post 55/80 ($P= 0.009$). The significant interaction between time point and trial ($F(2,11.964)= 20.540$, $P< 0.001$) indicates that the change in plasma cortisol across the various timepoints differed between the 55/80 trial and the resting control trial.

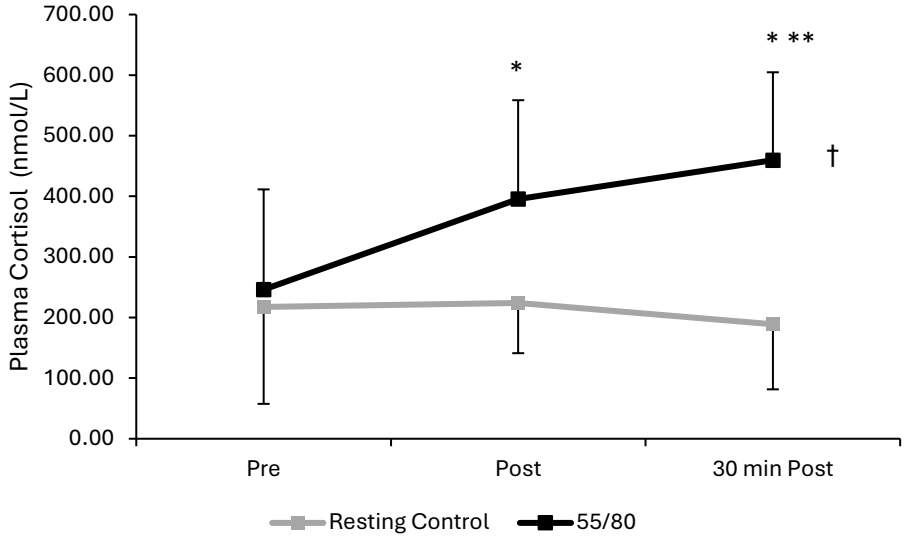


Figure 4.7. Plasma cortisol concentrations during the resting control trial and the 55/80 trial.
Data are presented as mean ± standard deviation.
* Significantly different from Pre. ** Significantly different than Post. † Significant effect of condition. n=12.

Within the oral contraceptive users, there was no significant effect of trial ($F(1,5)= 6.065$, $P= 0.057$) or time ($F(2,10)= 1.294$, $P= 0.316$) on plasma cortisol concentrations (Figure 4.8a). For natural menstruators, there was a significant main effect of trial ($F(1,5)= 57.652$, $P< 0.001$), whereby mean plasma cortisol levels were higher after the 55/80 trial (420.98 ± 131.28 nmol/L) compared to the control trial (314.86 ± 149.57 nmol/L), time ($F(2,10)= 24.965$, $P< 0.001$) and time*trial interaction ($F(2,10)= 31.866$, $P< 0.001$) (Figure 4.8b). Post hoc analysis revealed that plasma cortisol was significantly elevated above baseline (158.54 ± 92.74 nmol/L) immediately post (365.00 ± 74.42 nmol/L, $P= 0.002$) and 30 minutes post 55/80 (415.59 ± 45.83 nmol/L, $P< 0.001$).

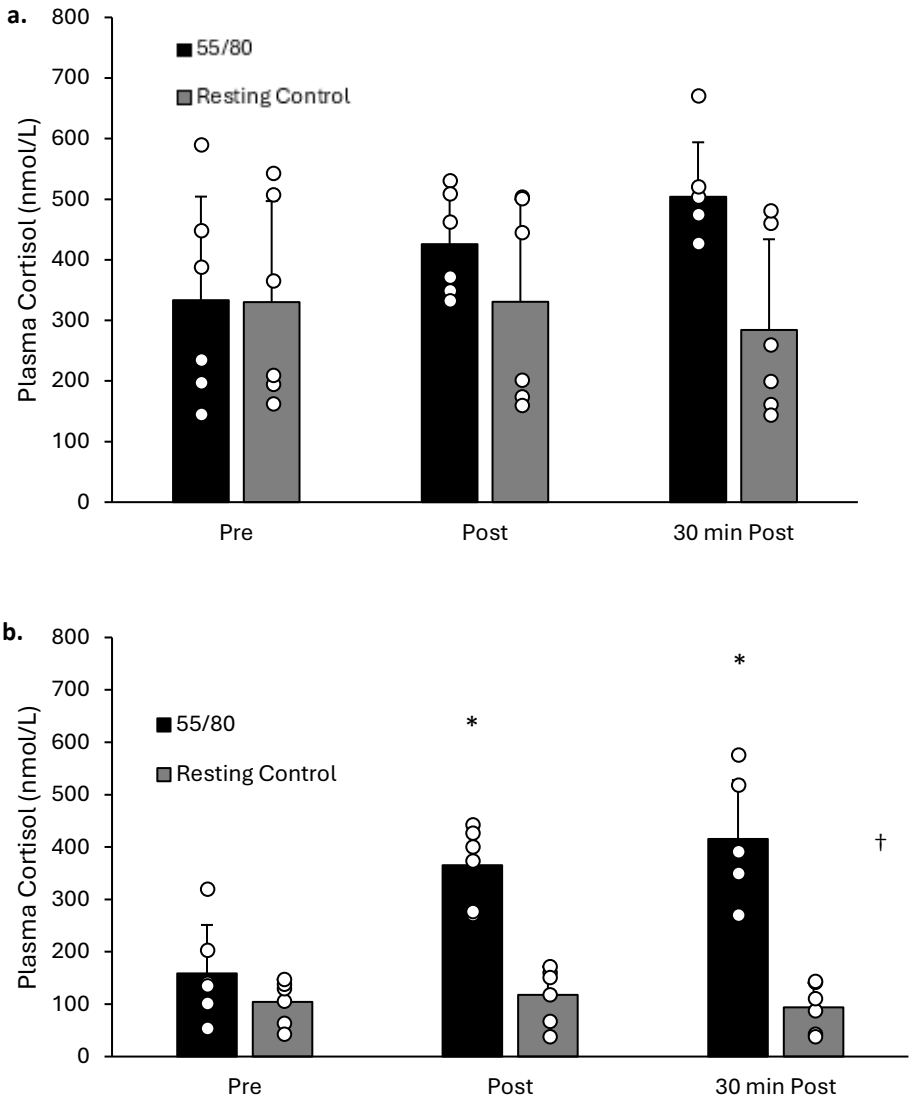


Figure 4.8. Plasma cortisol concentrations during the resting control trial and the 55/80 trial in (a.) oral contraceptive users (n=6) and (b.) natural menstruators (n=6).
Data are presented as mean ± standard deviation.
* Significantly different from baseline.† Significant effect of condition.

4.3.8 Plasma progesterone

There was a significant main effect of trial ($F(1,10)= 14.083$, $P= 0.004$), whereby mean plasma progesterone levels were higher in the 55/80 trial compared to the resting control trial (2.87 ± 0.56 vs. 1.22 ± 0.15 ng/mL). There was no significant main effect of time ($F(1.302,13.023)= 1.266$, $P= 0.296$), but a significant time*trial interaction ($F(1.146,11.458)= 4.910$, $P= 0.044$), indicating that the change in plasma progesterone across the various timepoints differed between the 55/80 trial and the resting control trial (Figure 4.9).

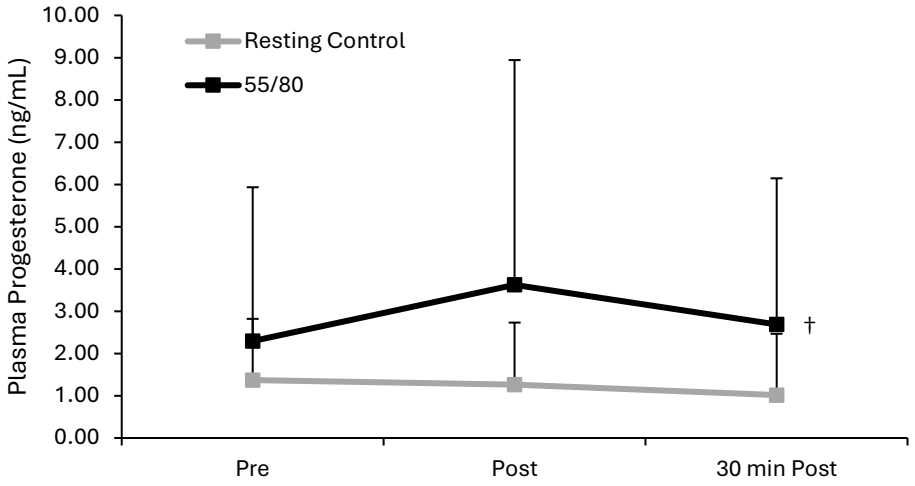


Figure 4.9. Plasma progesterone concentrations during the resting control trial and the 55/80 trial (n=11). Data are presented as mean ± standard deviation.
† Significant effect of condition.

When oral contraceptive users and natural menstruators were analysed separately, within the oral contraceptive users, there was no significant effect of trial ($F(2,5) = 14.554$, $P= 0.012$), or time ($F(2,10)= 0.256$, $P=0.779$) on plasma progesterone concentrations, but there was a significant interaction effect ($F(2,10)= 6.949$, $P= 0.013$).

For natural menstruators, however, there was a significant main effect of trial ($F(1,4) =13.412$, $P =0.022$), whereby mean plasma progesterone levels were higher after the 55/80 trial (5.38 ± 5.12 ng/mL) compared to the control trial (1.94 ± 1.80 ng/mL). There was no significant main effect of time ($F(2,8)=1.152$, $P=0.363$) or time*trial interaction ($F(2,8)=1.837$, $P=0.221$).

4.4 Discussion

The aim of this study was to examine whether the previously developed 55/80 stress test induced elevations in salivary and/or plasma cortisol and testosterone in females, as previously demonstrated in males, as well as in plasma progesterone. The goal was to determine and propose the 55/80 as a useful diagnostic tool to highlight the OTS in females. The current study experimentally evidenced that the 55/80 stress test induced significant elevations in salivary and plasma cortisol, salivary testosterone, and plasma progesterone in naturally menstruating females, but not in plasma testosterone. In oral contraceptive users, however, the 55/80 did not result in any significant hormonal responses.

Specifically, the 55/80 elevations in salivary cortisol (~141%) and testosterone (~93%) from baseline to peak-post exercise level were in line with those previously identified for the 55/80 in males, wherein both salivary cortisol and testosterone were elevated by ~210% and ~58%, respectively (Hough et al., 2013). Similarly, to

the results of Hough et al. (2011) in males, we found a robust elevation in plasma cortisol (~87%), but no change in plasma testosterone in females. Our results do not align with all previously published research comparatively to studies using similar exercise durations and intensities, however. Duclos et al. (1997) found that 20 minutes of running exercise at either 50% or 80% $\dot{V}O_{2max}$ in highly trained males did not alter plasma cortisol levels. Training background was proposed as a possible cause for differences in cortisol perturbations to exercise (Wittert et al., 1996). It was argued that the stress intensity threshold required to provoke cortisol increases was typically higher in more highly trained individuals when compared to those who are relatively sedentary (Hill et al., 2008; Hackney & Walz, 2013). The participants in the current study, and those used by Hough et al. (2011) were recreationally active with an average $\dot{V}O_{2max}$ classified as 'Good' according to the ACSM (ACSM, 2017). Duclos et al. (1997), although did not state the $\dot{V}O_{2max}$ of their participants, utilised highly trained long-distance runners who ran 60-80 km/week for > 4 years, and were able to complete a marathon in < 4 hours. Therefore, differences in the training status of participants could explain why our study found plasma and salivary cortisol level increases, whereas other studies utilising exercise of a similar intensity and duration may not have.

When the analysis of the hormone response to the 55/80 was conducted separately for oral contraceptive users and natural menstruators, there were no significant elevations in plasma cortisol or salivary testosterone in the oral contraceptive users, whereas salivary testosterone and cortisol, and plasma cortisol were significantly elevated in the natural menstruators. The natural menstruators had lower average resting salivary and plasma cortisol levels compared to the oral contraceptive users. It is known that oral contraceptives elevate circulating cortisol levels in females by causing stress-like alterations in the F056 binding protein FKBP5; a central regulator of the HPA axis (Hertel et al., 2017). This elevated baseline most likely explains why the oral contraceptive user group did not see significant elevations in their plasma cortisol levels to the 55/80. A ceiling effect of exercise induced plasma cortisol elevations has previously been shown in males (Behr et al., 2009). In this particular study, supra-maximal exercise was used at varying intensities in male participants to identify a ceiling in plasma cortisol occurring ~543-600 nmol/L (Behr et al., 2009). In the current study, the 55/80 elevated plasma cortisol to ~ 503 nmol/L in oral contraceptive users, nearing the ceiling level found in males. Therefore, starting with higher circulating plasma cortisol levels may have reduced the capacity for plasma cortisol elevation in the oral contraceptive users. Combined oral contraceptives have also been shown to reduce levels of androgen, especially testosterone, in females by inhibiting ovarian and adrenal androgen synthesis (Zimmerman et al., 2014). Therefore, the 55/80 may only induce significant plasma cortisol and salivary testosterone elevations in natural menstruators making it an unsuitable tool for highlighting the negative states of overtraining in oral contraceptive users. It must be emphasised however, that although the overall study is sufficiently powered, when classifying participants as oral contraceptive users or natural menstruators, the analyses conducted are underpowered. Whilst the comparisons between oral contraceptive users and natural menstruators remain interesting, strong conclusions cannot be drawn.

In the current study, plasma testosterone was not significantly elevated by the 55/80. Hough et al. (2011) also found that the 55/80 failed to elevate plasma testosterone in males. Research has demonstrated that strength training elicits more pronounced elevations in circulating testosterone levels compared to aerobic exercise, attributed to the robust influence of the anaerobic glycolytic pathway in precipitating acute hormonal surges following physical exertion (Tremblay et al., 2004; Kraemer & Ratamess, 2005). It has also been shown that testosterone is more responsive to higher intensity exercise of longer durations (Cadore & Kruel, 2012). Consequently, the 55/80 protocol might lack the required potency to elicit increases in plasma testosterone levels. Additionally, a significant negative relationship, although low in magnitude, between circulating cortisol and total testosterone in males occurred in response to 60-90 minutes of either running, cycling, or rowing at ~65-75% $\dot{V}O_{2max}$ (Brownlee et al., 2005). The authors proposed that a critical cortisol increase threshold of ~160% must be reached to substantially influence circulating testosterone levels; a threshold not reached in our research (Brownlee et al., 2005). Previous research in female runners also indicated that 30 minutes of running at a self-selected pace, elevated plasma testosterone levels and these were significantly greater when testing in the follicular phase compared to the luteal phase, despite baseline testosterone levels being similar in both phases (Shangold et al., 1981). The research presented from our study was taken from exercised female participants during the early-mid luteal phase, which could have therefore contributed to the limited plasma testosterone responsiveness to the 55/80.

Plasma progesterone levels were significantly higher in the 55/80 trial compared to the resting control trial, with a significant time*trial interaction indicating that plasma progesterone responded differently in the 55/80 trial compared to the rest control trial. As a secondary hypothesis, it was theorised that progesterone, as one of the two predominant female sex hormones and because it shares the same synthesis pathway as testosterone in females, may be altered in response to the 55/80 (Batth et al., 2020). Previous research has also found that 30 minutes of intense cycling is a strong enough stimulus to elevate serum progesterone levels in young healthy females (Bonen et al., 1975). Additionally, physical stress instilled via the cold pressor test, which requires participants to immerse their hand in ice cold water for 1-3 minutes, also led to elevations in salivary progesterone in young healthy females (Herrera et al., 2016). Similarly to testosterone, as previously described, it is suggested that the progesterone response to stress is higher during the follicular phase of the menstrual cycle, rather than the luteal phase, thus larger plasma progesterone elevations to the 55/80 may be seen if performed during the follicular phase (Herrera et al., 2016). Despite the plasma progesterone levels being significantly higher in the 55/80 trials compared to the resting control trial, there were large inter-individual differences in the progesterone response to the 55/80. This could be because OC users and natural menstruators were initially grouped together. Natural menstruators, as expected, have the highest concentrations and show the largest inter-individual variation (pre: 4.23 ± 4.41 to peak post: 6.96 ± 6.72) at rest and in response to the 55/80 compared to OC users (pre: 0.68 ± 0.37 to peak post: 0.84 ± 0.79). This is because OCs contain exogenous progestins that supress endogenous progesterone production in these females, creating a more uniform progesterone response in the OC users (Hirschberg, 2022). Therefore, although, the significant elevations in plasma progesterone seen in the current study suggests it may be a more useful biomarker of the OTS in females than plasma testosterone when testing is completed in the

luteal phase, caution must be taken if using this hormone as a biomarker due to the large inter-individual variation in response to exercise.

Importantly, hormonal measurements were analysed in euhydrated participants, meaning hydration status is unlikely to have influenced any hormonal changes. Additionally, the difference between total stress and total recovery measured by the REST-Q scores were not significantly different between trials meaning the stress induced hormonal changes seen in the current study are likely due to the 55/80 exercise stress and not due to external stressors.

In conclusion, the results of this study support the use of the 55/80 as a potentially valuable tool, capable of highlighting differences in hormonal biomarkers associated with the negative states of overtraining in females. Specifically, plasma and salivary cortisol, salivary testosterone and plasma progesterone were demonstrated to be indicative biomarkers of NFOR/OTS in females. However, whilst inducing robust hormonal elevations in natural menstruators, oral contraceptive users did not experience the same significant hormonal elevations in response to the 55/80. It must be noted, however, that analytes were not adjusted for changes in plasma volume to the 55/80. Although, it is predicted that the shift in plasma volume (~14%) will be lower than the percentage increases of hormones in response to the 55/80, thus not likely to change outcomes overall (Bjerre-Bastos et al., 2022). The utilisation of this tool is important considering the high prevalence NFOR/OTS amongst the under researched and thus under-represented female athlete population (Matos et al., 2011; Carrard et al., 2022). To improve the applicability of the 55/80 as a stress test, future research should investigate the hormonal perturbations in all phases of the menstrual cycle i.e., in the follicular phase, across the ovulatory period and during menses to ensure a robust hormonal stress response remains. It is also vital that the 55/80 is validated in an overreached female population, and further investigation into the effects of oral contraceptives is completed before its use in practice.

Given the known regulatory roles both cortisol and testosterone exert on the immune system, a dysfunctional endocrine system caused by intensified training may lead to impaired immune function. Before investigations into how intensified training affects the immune system are conducted, it is important to understand current knowledge, as well as areas of research that are currently lacking. Therefore Chapter 5 will systematically assess the existing evidence in relation to human and rodent immune alterations that occur with periods of heavy training and highlight areas requiring further investigation. The findings from this systematic review/meta-analysis will inform further investigations in this thesis.

5 Chapter 5: Lymphocyte and dendritic cell response to a period of intensified training in young, healthy humans and rodents: A systematic review and meta-analysis.

This Chapter is a modified version of the systematic review/meta- analysis published in Frontiers in Physiology:

Baker, C., Hunt, J., Piasecki, J., & Hough, J. (2022, November 11). Lymphocyte and dendritic cell response to a period of intensified training in young healthy humans and rodents: A systematic review and meta-analysis. Frontiers in Physiology, 13, 998925. https://doi.org/10.3389/fphys.2022.998925

5.1 Introduction

As described in Section 2.1, overloading the body whilst preventing inadequate recovery is a necessary process implemented within an athletes' training program to improve athletic performance (Whyte, 2006). If there is a prolonged imbalance of stress and recovery, negative states of overtraining may occur i.e. NFOR/OTS, which can take weeks- to- years for full recovery to occur (Meeusen et al., 2013).

Symptoms of NFOR/OTS occur in individual (37%) and team (17%) sport athletes (Matos et al., 2011), with the incidence in an athletes' career ranging from 30% to 60% (Birrer et al., 2013; Morgan et al., 1987). Despite the high incidence of these negative states of overtraining, little progress has been made on establishing objective and reliable biological markers for identifying when an athlete may be entering NFOR/OTS following periods of intensified training (Armstrong & VanHeest, 2002).

As previously explained, cortisol is a hormone that is synthesised and released in response to physical and mental stress via the HPA axis. During periods of intensified training, a ~72% blunting of the cortisol response to the 30-minute 55/80 stress test, when comparing before to after an 11-day intensified training period has been reported (Hough et al., 2013). This disrupted functioning of the HPA axis following an intensified training period has previously been highlighted by other groups. Meeusen et al. (2004) examined the hormonal responses to an exercise stress test composed of two maximal cycle tests separated by 4 hours of resting recovery in well-trained athletes before and after a 10-day intensified training period. They reported a ~118% and ~73% reduction in the response of cortisol and ACTH (a precursor hormone to cortisol) in the athletes in response to the second maximal cycling bout after the 10-day training period compared to before the training period (Meeusen et al., 2004). Meeusen et al. (2010) also reported that athletes in a state of OTS (classified according to the duration and severity of symptoms and underperformance experienced) show little or no exercise-induced increases in ACTH in response to the second maximal exercise bout in their exercise stress test. This suggests that the exercise-induced response of the HPA hormones, specifically cortisol and ACTH, may be lowered following periods of intensified training.

Cortisol plays an important role in the anti-inflammatory response of the immune system to exercise by increasing the phagocytic potential of neutrophils and monocytes (Blannin et al., 1996; Ortega et al., 1996), supressing pro-inflammatory mediators such as ROS (Franchimont, 2004), regulating the maturation and migration of DCs towards lymph nodes (Liberman et al., 2018) and inducing lymphocytopenia (Okutsu et al., 2005). As previously described in Chapter 2, lymphocytopenia refers to the lowering of lymphocytes in the blood, and most likely is a reflection of their increased migration into the tissues for increased immune-surveillance (Kruger et al., 2007). Therefore, a temporarily dysfunctional HPA axis caused by a period of intensified training may lead, in part, to an impaired immune response during intensified exercise.

The impact of heavy periods of training on the immune system remains unclear, with some evidence suggesting a decline in immunity after repeated arduous exercise bouts (Walsh, 2019). As debated in Simpson et al. (2020), it is suggested that the reduced post exercise immunosurveillance that occurs after prolonged (> 5 days) and intensive (> 60% $\dot{V}O_{2max}$) (Hoffman-Goetz et al., 1990) endurance training, in addition to the post exercise decline in Tc cells (Steensberg et al., 2001) introduces a 'window of opportunity' for infection. Repeated exposures to these acute declines in immunity may bare additive negative consequences to infection risk (Pedersen & Ullum, 1994). In line with this, it has been reported that elite athletes that undergo heavy training regimes experience significantly higher episodes of URIs than recreational athletes (Spence et al., 2007), with a small proportion of athletes experiencing recurrent episodes at higher rates than the general population (Fricker et al., 2000). These recurring URIs have been associated with persistent fatigue that can hinder an athletes training (Reid et al., 2004). Moreover, it has been shown that elite endurance athletes prone to recurrent URIs i.e. more than 4 episodes per year, have an altered cytokine response, suggestive of impaired inflammatory regulation compared to healthy athletes (Cox et al., 2007). Similarly, a reversible defect in $CD4^+$ T cell IFN-γ secretion, a cytokine known to affect illness severity and duration, has been associated with illness-prone athletes experiencing fatigue (Clancy et al., 2006). Furthermore, suppression of immune parameters can occur in elite athletes over years of training, which can result in reactivation of viruses (Gleeson et al., 2002; Reid et al., 2004). At a cellular level, studies have reported a reduced $CD4^+/CD8^+$ ratio in response to a 4-week strength training program involving progressive intensity increases each week from 75%-85% HR_{max} (Dongqing, 2013), a reduction in T cell proliferation immediately after a 30-minute treadmill run at 80% $\dot{V}O_{2max}$ following a 3-week intensified training period (25% above normal training load) when compared to before the training (Verde et al., 1992), and reduced NK cell cytotoxicity after 1 month of intense volleyball pre-season compared to before pre-season (Suzui et al., 2004).

However, the 'Open Window' theory is not accepted by all, with suggestions that reductions in immune cell function post exercise could reflect the lowered number of immune cells in the circulation after exercise, which are redistributed into tissues for enhanced immunosurveillance at sites of infection risk (Campbell et al., 2009; Kruger et al., 2007). For example, Green et al. (2002) showed a significant decrease in lymphocyte proliferation – an important first step to create effector lymphocytes - after a 60-minute, high-intensity run, but found no significant differences between the exercise and control groups when assessing lymphocyte proliferation in an NK cell depleted culture, or when adjusted per T cell. This suggests that the decreased

proliferation found initially was likely due to an exercise induced increase in NK cells within the sample, thus a reduction in the proportion of T cells that can be stimulated, rather than the exercise bout causing an actual reduction in T cell proliferation. Therefore, it is argued that studies reporting changes in immune cell function that coincide with changes in immune cell count cannot use lymphocytopenia as evidence for a decline in immunity. This is because the fall in cell number does not reflect mass apoptosis but a redistribution of highly functional T cells and NK cells from the bloodstream into the tissues and organs (Kruger et al., 2007; Campbell et al., 2009). This redistribution enhances the identification and eradication of tissue tumour cells; a clear benefit to the host. It has been shown that cancer cells incubated with exercised serum form less tumours when inoculated into mice (Hojman et al., 2018), and 4 weeks of voluntary wheel running prior to tumour cell inoculation reduced tumour growth by ~61%, attributed to the redistribution of NK cells after exercise causing an increased infiltration of NK cells to tumour sites (Pedersen et al., 2016). Another commonly used measure of URI susceptibility in athletes is salivary immunoglobulin A (sIgA). Although there are reports that the lowered sIgA seen with intense periods of training is associated with increased URIs in athletes (Fahlman & Engels, 2005), this hasn't been shown consistently (Antualpa et al., 2018; Gill et al., 2014; Pacque et al., 2007). Moreover, studies that do relate URI with decreased sIgA levels rarely consider confounding factors that may impact sIgA secretion and concentration, such as the profound intra- and inter-individual variation, likely due to oral health, psychological stress or sleep, and diurnal or seasonal changes (Brandtzaeg, 2013). Finally, immune competency is also influenced by non-exercising factors, and without clinical confirmation that an infection is present during URIs, symptoms could be due to allergy (Kennedy et al., 2016) or caused by variables such as psychological stress (Cohen et al., 1991), low energy availability (Bromley et al., 2018), or low sleep efficiency (Prather et al., 2015). Evidently the arguments for both an increased and reduced immune response post exercise are well supported, and more definitive research is required to provide a firm conclusion.

The HPA axis is known to be involved in the regulation of the previously introduced antigen presenting cells involved in linking the innate and adaptive immune responses; DCs (Liberman et al., 2018). Glucocorticoids, such as cortisol can regulate the maturation, survival, and migration toward the lymph nodes of DCs, but can also inhibit their immunogenic functions such as their TLR expression (Liberman et al., 2018). Cortisol itself has been shown to downregulate DC costimulatory molecules and dampen pro-inflammatory cytokine production, such as IL-6, IL-12 and TNF-α, which subsequently reduces the ability of the DCs to prime naïve $CD8^+$ T cells (Elftman et al., 2007). Given the importance of these cytokines in orchestrating the immune response, the measure of cytokines, such as TNF-α, IFN-γ and IL-1β, as pro-inflammatory orchestrators of a type 1 immune response, and IL-10 and IL-2, as key anti-inflammatory immune-regulators, can act as a measure of immune function. Specifically, these cytokines are released from and also activate T cells and DCs (Blanco et al., 2008; Shaw et al., 2018).

Not only do DCs prime naive T cells as detailed in Section 2.16, they also operate a bi-directional link with NK cells; a lymphocyte functioning within the innate immune system (Thomas & Yang, 2016). DCs can induce NK cell proliferation and cytotoxicity via the release of cytokines such as IL-12, IL-15 and IL-18 (Ferlazzo &

Morandi, 2014). Conversely, NK cells can induce DC maturation via the secretion of IFN-γ and TNF-α (Moretta et al., 2006), and eliminate DCs that do not mature properly, a process known as 'DC editing', through engagement with the activating receptor NKp30 (Moretta et al., 2006). To our knowledge, there are currently four reports investigating the DC response to exercise training, indicating that after chronic exercise training in rats, DC function; as a measure of the expression of co-stimulatory molecules and MHC II receptors, and IL-12 production, remains unchanged (CD80) (Liao et al., 2006; Chiang et al., 2007; Mackenzie et al., 2016; Fernandes et al., 2019), increased (CD86) (Mackenzie et al., 2016; Chiang et al., 2007) or conflicting (MHC II) (Chiang et al., 2007; Mackenzie et al., 2016). Despite the known DC changes with HPA axis alterations, the lack of evidence surrounding DCs leaves the question of how DCs respond to periods of intensified training unanswered. With evidence that DCs are in part regulated by the HPA axis, and the knowledge that the HPA axis response to exercise stress may be blunted following a period of intensified exercise, it is important to examine the impact that intensified exercise has on DCs. As these cells orchestrate the immune response, specifically, the direct nature of the relationship DCs have with both T lymphocytes and NK cells, it is logical to review evidence surrounding all three immune cells, providing further direction towards a conclusion of how the immune system responds to periods of intensified training.

Therefore, the aim of this systematic review/meta-analysis was to assess the current literature examining the effects of a period of intensified training on lymphocyte (T cells and NK cells) and DC number and function, in both humans and rodents. This review focuses on the normal impact of high intensity training due to the difficulties surrounding confirmation of NFOR/OTS diagnosis. However, heavy training is a factor involved in the establishment of NFR/OTS, and as such, any highlighted immune biomarkers could potentially indicate NFOR/OTS has occurred. The main purpose being to highlight areas already studied, indicate potential gaps requiring further investigation, and assess if there is scope for the future use of immune biomarkers in the diagnosis of overtraining.

5.2 Methods

This review conforms to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Figure 5.1) (Moher et al., 2009) and was registered with PROSPERO international prospective register for systematic reviews (CRD42021248776; 21 May 2020).

5.2.1 Inclusion and exclusion criteria

To develop the inclusion and exclusion criteria for this review a consideration of Population, Intervention, Comparison and Outcome (PICO) was used (Richardson et al., 1995).

5.2.1.1 Eligibility criteria

Population

Humans aged 18-50 years with a $\dot{V}O_{2max}$ of fair or higher (> 38.5 ml/kg/min) according to ACSM guidelines for cardiorespiratory fitness (ACSM, 2017) or Rodents aged 6 weeks - 5 months were included in this review.

Human studies using females must have controlled for menstrual cycle to be included in the review. The menstrual cycle is known to impact certain elements of the immune system e.g. lowered $CD4^+$ T cell numbers and increased type 1 cytokine production during the luteal phase compared to the follicular phase (Oertelt-Prigione et al., 2012; Timmons et al., 2005).

Intervention

Studies must include an increased training load compared to their regular training load, completed over multiple days.

Comparison

Studies included were required to have a comparative control. In human studies, participants were used as their own controls, comparing their pre and post training biomarker values. Where no pre training values were given in rodent studies, the control group was used as a comparison.

Outcome

Studies must have measured at least one immunological biomarker relating to lymphocytes, DCs, or cytokines before and after a period of training. The immune biomarkers could be measured at rest, or in response to an acute bout of exercise; this will be referred to as 'exercise-induced' and indicates that the biomarker was measured immediately after an acute exercise bout both before and after a period of intensified training. Data must have been presented as mean and standard deviation to allow the calculation of the standardised mean difference (SMD) of the change in biomarker from pre to post training. A minimum of two studies measuring the same biomarker, using the same measurement units, were required to include that biomarker in the meta-analysis component. Where possible, differing units of measurements were converted into the same 'gold standard' units for comparison. If this was not possible, it was excluded from the meta-analysis.

5.2.2 Search strategy for identification of studies

A literature search was conducted in the following databases on 26 May 2021: SPORTDiscus, PUBMED, Academic Search Complete, Scopus and Web of Science. Databases were searched from inception up until May 2021 for articles published in English. In addition to database searches, reference lists of relevant studies were screened for eligible studies. The search was re-run in June 2022 to identify any additional articles meeting the inclusion criteria.

Titles, abstract and keywords were searched using the following search terms:

1. "chronic exercise*" OR "training volume" OR "intensified training" OR "exercise training" OR "overtrain*" OR "endurance training*" OR "physical education and training" OR "high intensity training" OR "chronic exercise training" OR "physical conditioning, animal*" OR "Physical exertion"
2. "lymphocyte function" OR "immune response" OR "dendritic cell function" OR "immune function" OR "dendritic cell" OR "myeloid" OR "plasmacytoid" OR "t cell*" OR "cd4*" OR "cd8*" OR "T helper" OR "T cytotoxic" OR "lymphocyte*" OR "NK cell" OR "natural killer cell" OR "cd56*" OR "T regulatory" OR

"cd25*" OR "lymphocyte proliferation" OR "T cell proliferation" OR "CD80*" OR "CD86*" OR "cd80*/86*" OR "NK-cell" OR "NKCA" OR "Natural Killer cell cytotoxic activity" OR "killer cells, natural" OR "cytotoxicity, immunologic" OR "lymphocyte activation" OR "antigen presenting cell*" OR "dendritic cells" OR "genes, mhc class i" OR "genes, mhc class ii" OR "interleukin"

3. "athlete*" OR "Mice" OR "animals"
4. "elderly" OR "Cancer" OR "Elder" OR "older" OR "geriatric" OR "aged"

1 AND 2 AND 3 NOT 4

5.2.3 Study selection

Articles retrieved through the systematic search were exported to ProQuest RefWorks, a reference management software (RefWorks 3.0, Pro-Quest LLC, Michigan U.S.), and further exported to Excel (Microsoft 365, Microsoft, Washington, USA), whereby duplicates were removed and assessment for eligibility began. Two investigators (CB and JH) independently screened articles by title and abstract, and full text, when necessary, against the inclusion criteria. Full texts from the eligible studies were then independently screened (CB and JH) for inclusion into the review.

5.2.4 Data extraction and management

Data extraction was conducted by one reviewer (CB) whereby the following data from all eligible articles were extracted into an Excel document: Title, publication details (year and author), participant characteristics (sex, age, number, $\dot{V}O_{2max}$), intensified training period details (mode and duration) and assessed biomarker information (biomarker assessed, method of assessment and units of measurement). Pre and post training values were extracted for each relevant biomarker in the form of mean and SD. Where appropriate data was not presented, the authors were emailed and were allocated 4 weeks to reply. If no reply was received after 4 weeks, the study was excluded. Any variables included in the search string that didn't have sufficient studies to perform a meta-analysis were not included in the results. Where figures were used displaying the mean and SD, data was extracted by eye.

5.2.5 Risk of bias

Risk of bias was assessed by one reviewer (CB) and independently verified by one member of the review team (JH). Three Cochrane Collaboration tools were used for assessing risk of bias; ROBINS-1 for non-randomised controlled trials (Appendix 10.7), ROB-2 for randomised controlled trials (Appendix 10.9) and ROB-2 (Crossover) for randomised crossover trials (Appendix 10.8) (Cochrane Collaboration 2021, Oxford, UK). Specific study components assessed for risk of bias using the ROBINS-1 tool included confounding, selection of participants, classification of intervention, deviations from intended interventions, missing data, measurement of outcomes and reporting of results. Study components assessed using the ROB-2 tool included the randomisation process, deviations from intended interventions, missing outcome data, measurement of outcomes and reporting of results. The ROB-2 crossover tool assessed the same components as the ROB-2 tool with the addition of carryover effects.

5.2.6 Statistical analysis

Inverse variance, random effects meta-analysis was then conducted on immune biomarker data in Review Manager Software (RevMan, Version 5.3, Cochrane Collaboration, Oxford, UK). Hedge's *g* SMD was calculated via the RevMan software (RevMan, Version 5.3, Cochrane Collaboration, Oxford, UK).

A separate meta-analysis was conducted for each biomarker where > 2 studies measured the same biomarker using the same method and units of measurement. Human and rodent studies were analysed together for all biomarkers, apart from 'lymphocyte proliferation' due to human studies measuring peripheral blood lymphocytes, and rodent studies measuring spleenocytes. Effect sizes were classified based on the magnitude of change from pre to post intervention. Classifications included very small (0.01-0.19), small (0.20-0.49), moderate (0.50-0.79), large (0.80-1.19), very large (1.20-1.99) and huge (> 2.0) (Cohen, 1988; Turner & Bernard, 2006; Sawilowsky, 2009). Statistical heterogeneity was determined using the I^2 statistic; 0-40% indicated non-important (low) heterogeneity, 40-60% indicted moderate heterogeneity, 50-75% indicated substantial heterogeneity and 75-100% indicated considerable heterogeneity (Cochrane, 2021). All results were reported as Hedge's *g* with 95% confidence intervals (CI). Additional sub-group analysis was conducted on resting immune cell count biomarkers based on the duration of intensified training periods i.e. $\leqslant$ 7 days, 8 days-2 weeks, 15 days- 4 weeks or > 4 weeks.

Table 5.1. Risk of bias assessment of included studies using Cochranes ROB-2, ROBIN-2 Cross-Over and ROBINS-1 tools. ROB-2 and ROB-2 Cross-Over: Low (✓), Some concern (~), High (X), Not enough Information (?). ROBINS-1: Low(✓), Moderate (~), Serious (S), Critical (X), Not enough information (?).

	ROB-2					
Study	**Domain 1** **Risk of Bias arising from the randomisation process**	**Domain 2** **Risk of Bias due to deviations from the intended interventions**	**Domain 3** **Risk of Bias due to missing outcome data**	**Domain 4** **Risk of Bias in measurement of the outcome**	**Domain 5** **Risk of Bias in selection of the reported result**	**Overall ROB**
Croft et al., 2009	✓	~	✓	✓	✓	~
Gholamnezhad et al., 2014	✓	✓	✓	✓	✓	✓
Hack et al., 1997	✓	✓	?	✓	✓	✓
Hasanli et al., 2021	✓	✓	?	✓	✓	✓
Hoffman-Goetz, 1986	✓	✓	?	✓	✓	~
Hoffman-Goetz et al., 1988	✓	✓	?	✓	✓	✓
Hwang et al., 2007	✓	✓	?	✓	✓	✓
Kaufaman et al., 1994	✓	✓	✓	?	✓	✓

Koyama et al., 1997	✓	✓	?	✓	✓	✓
Kwak, 2006	✓	✓	?	✓	✓	✓
Louis et al., 2016	✓	✓	?	✓	✓	✓
Mackenzie et al., 2016	✓	✓	?	✓	✓	✓
Mitchell et al., 1996	✓	✓	✓	✓	✓	✓
Peijie et al., 2003	✓	✓	✓	✓	✓	✓
Poffe, 2019	✓	?	✓	✓	✓	✓
Sheyklouvand et al., 2018	✓	✓	?	✓	✓	✓
Wang et al., 2011	✓	~	?	✓	✓	~
Wang and Weng, 2011	✓	~	?	✓	✓	~
Watson et al., 1986	~	✓	X	✓	✓	x
Weng, 2013	✓	✓	✓	✓	✓	✓
Zhang et al., 2019	✓	?	✓	✓	✓	✓

ROB-2 Cross-Over

Study	Domain 1 Risk of Bias arising from the randomisation process	Domain S Risk of Bias arising from period and carryover effects	Domain 2 Risk of Bias due to deviations from the intended interventions	Domain 3 Risk of Bias due to missing outcome data	Domain 4 Risk of Bias in measurement of the outcome	Domain 5 Risk of Bias in selection of the reported result	Overall ROB
Li et al., 2013	✓	✓	✓	✓	~	✓	~
Meyer et al., 2004	~	~	✓	✓	✓	✓	~
Pizza et al., 1995	?	✓	~	✓	✓	✓	~

ROBINS-1

Study	Domain 1 Bias due to confounding	Domain 2 Bias in selection of participants into the study	Domain 3 Bias in classification of interventions	Domain 4 Bias due to deviations from intended interventions	Domain 5 Bias due to missing data	Domain 6 Bias in measurement of outcomes	Domain 7 Bias in selection of the reported result	Overall ROB
Baj et al., 1994	✓	✓	✓	✓	✓	✓	✓	✓
Baum et al., 1994	~	✓	✓	?	?	~	✓	~
Blank et al., 1994	✓	✓	✓	✓	✓	✓	✓	✓

Borges et al., 2012	~	✓	✓	?	✓	~	✓	~
Borges et al., 2018	✓	✓	✓	?	✓	✓	✓	✓
Bresciani et al., 2011	✓	?	✓	?	?	~	✓	?
Bury et al., 1998	~	?	✓	✓	✓	✓	✓	~
Chiang et al., 2007	✓	✓	✓	✓	✓	✓	✓	✓
Chung et al., 2021	✓	✓	✓	✓	?	✓	✓	✓
Córdova et al., 2015	✓	✓	✓	✓	~	~	✓	~
Dongqing, 2013	✓	✓	✓	✓	?	✓	✓	~
Dressendorfer, 2002	✓	✓	✓	✓	✓	~	✓	~
Ferry et al., 1990	✓	✓	?	?	?	✓	✓	?
Fry et al., 1991	✓	?	?	?	?	~	✓	?
Halson et al, 2003	?	✓	✓	?	?	✓	✓	?
Heisterberg et al., 2013	✓	✓	✓	?	✓	✓	✓	✓

Jurimae and Purge, 2021	✓	✓	?	?	?	✓	✓	?
Kajiura et al., 1995	✓	✓	✓	?	?	?	✓	?
Lancaster et al., 2004	✓	✓	✓	?	✓	✓	?	✓
Leal et al., 2021	?	✓	✓	✓	?	✓	✓	✓
Main et al., 2010	✓	✓	✓	?	?	~	?	?
Mueller et al., 2001	✓	✓	✓	?	?	?	✓	?
Mujika et al., 1996	✓	✓	✓	?	✓	✓	✓	✓
Ndon et al., 1992	✓	✓	✓	?	~	✓	✓	~
Nickel et al., 2011	?	✓	✓	✓	✓	✓	✓	✓
Peake et al., 2003	✓	✓	✓	?	?	✓	✓	✓
Rebello et al., 1998	✓	✓	✓	?	?	✓	✓	✓
Ronsen et al., 2001	✓	✓	✓	✓	~	✓	~	~

Shing et al., 2007	✓	✓	✓	?	?	✓	✓	✓
Smith and Myburgh, 2006	✓	✓	✓	?	?	✓	✓	✓
Tanimura et al., 2009	✓	✓	✓	?	?	✓	✓	✓
Verde, 1992	✓	✓	✓	?	?	✓	✓	✓
Witard, 2012	✓	✓	✓	**?**	**?**	✓	✓	✓

5.3 Results

5.3.1 Risk of bias

A complete analysis of ROB is displayed in Table 5.1. For studies assessed with the ROBINS-1 tool, bias in 'selection of participants to the study' was deemed as 'not applicable' (n= 3) or 'Low' (n= 30) because most studies followed a group of athletes over time or assessed the same group of participants before and after a period of intensified training. The bias arising from participant awareness of intervention encapsulated in ROBINS-1 domain 6; bias in measurement of outcome, was judged as being negligible in most studies (n= 24). It is difficult to blind participants from intervention when intensified training is the independent variable and training loads were often monitored or implemented by the investigators themselves, so knowledge of intervention was necessary. It could be argued that as objective immune biomarkers were measured, results are unlikely to be affected by knowledge of intervention, especially in the rodent studies.

Despite this, it has been suggested that anticipatory stress may cause alterations to the immune system, such as decreased lymphocyte counts and reduced lymphocyte proliferation (Lekander, 2001; Ironson et al., 1990; Ader & Cohen, 1991). However, studies investigating this phenomenon tend to use the anticipatory stress surrounding major life events such as cancer patients waiting for chemotherapy treatment (Lekander, 2001), and homosexual men waiting for HIV test results (Ironson et al., 1990). The evoked stress response caused by such serious events could be deemed as incomparable to the anticipation of undertaking exercise, especially when undertaken by trained athletes. Therefore, whilst we acknowledge that the anticipation of undertaking exercise may elicit a stress response to some extent, perhaps more so in untrained personnel, it is an unavoidable, and potentially non-significant bias. It is impossible to know the true effect anticipatory stress may have on the measured immunological outcomes without studies undertaking measures of stress scores.

Bias due to missing outcome data and attrition rate was mainly low (n= 22) or unclear (n= 31) in most studies, mainly because no information regarding excluded participants or reasons for missing data were highlighted. Only one study (Watson et al., 1986) was rated as 'high' for bias due to missing data as Table 4 in their publication only included n= 5 results for the placebo group's % T lymphocytes, when the placebo group consisted of 15 participants. A 'moderate' rating for bias due to missing outcome data was given for Córdova et al. (2015), as although they stated blood samples were collected before and after each stage of the cycling competition, unlike the other blood markers, only pre and post competition values were reported for cytokines. Ronsen et al. (2001) and Ndon et al. (1992) were also rated as 'moderate' for bias due to missing outcome data as participants with incomplete data sets were still included in the final analysis (Ronsen et al., 2001) and participants were excluded from analysis by the investigators after final outcome measures were taken as they were perceived to be overtrained (Ndon et al., 1992).

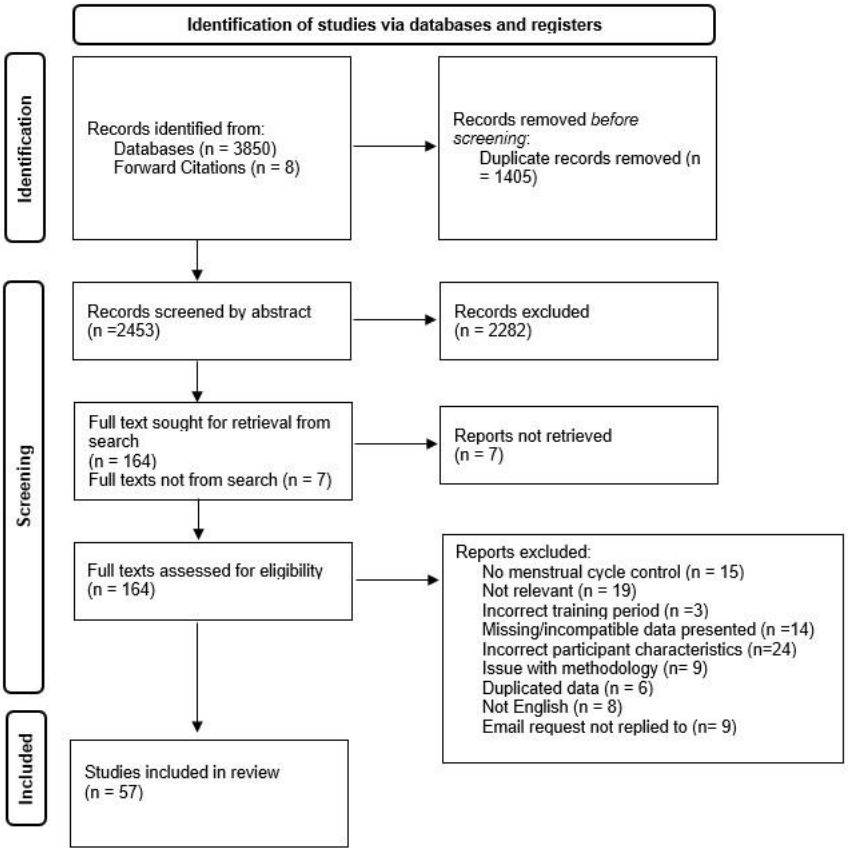


Figure 5.1. Systematic literature search PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flow chart.

5.3.2 Study outcomes

Across the 57 included studies (Table 5.2), the variables used to assess immune cell changes included immune cell counts (Baj et al., 1994; Baum et al 1994; Borges et al., 2012; Bresciani, 2011; Bury et al., 1998; Chung et al., 2021; Dressendorfer, 2002; Ferry et al., 1990; Fry et al., 1992; Hack et al., 1997; Halson et al., 2003; Heisterberg et al., 2013; Lancaster et al., 2004; Leal et al., 2021; Li et al., 2013; Louis et al., 2016; Meyer et al., 2004; Mitchell et al., 1996; Mueller et al., 2001; Mujika et al., 1996; Ndon et al., 1992; Peake et al., 2003; Pizza et al., 1995; Poffe, 2019; Rebelo et al., 1998; Ronsen et al., 2001; Sheyklouvand et al., 2018; Shing et al., 2007; Smith & Myburgh, 2006; Tanimura et al., 2009; Wang et al., 2011; Wang and Weng, 2011; Weng, 2013; Witard, 2012), lymphocyte proliferation (Bury et al., 1998; Hoffman-Goetz, 1986; Hoffman-Goetz et al., 1988; Hwang et al., 2007; Koyama et al., 1998; Kwak, 2006; Mitchell et al., 1996; Peake et al., 2003; Peijie et al., 2003; Verde, 1992; Watson et al., 1986), CD4/CD8 ratio (Blank et al., 1994; Dongqing, 2013; Dressendorfer, 2002 ; Ferry et al., 1990; Fry et al., 1992; Hack et al., 1997 ; Kajiura et al., 1995; Kaufman et al., 1994 ; Leal et al., 2021; Li et al., 2013 ; Mueller et al., 2001 ; Pizza et al., 1995 ; Poffe, 2019; Shing et al., 2007 ; Smith and Myburgh, 2006; Verde, 1992; Wang et al., 2011; Weng, 2013; Zhang et al., 2019), cytokine secretion (Borges, 2018; Bresciani, 2011; Córdova et al., 2015; Croft et al., 2009; Dressendorfer, 2002; Gholamnezhad et al.,

2014 ; Halson et al., 2003; Hasanli et al., 2021; Juirmae & Purge, 2021; Main et al., 2010 ; Nickel et al., 2011 ; Shing et al., 2007), DC co-stimulatory molecule and MHC II expression (Chiang et al., 2007; Mackenzie et al., 2016) and NK Cytolytic activity (Bury et al., 1998 ; Shing et al., 2007 ; Watson et al., 1986).

Table 5.2. Return of relevant studies from the systematic search. Studies in ***Bold Italics*** were eligible for inclusion from the systematic search but were not suitable for inclusion into the meta-analysis.

Study	Participants	Age	$\dot{V}O_{2max}$	Training status	Intensified Training	Duration	Measurement method	Biomarker
Anomasiri et al. 2002	***Humans (Males)***	***21.1 ± 0.4***	***40.2 ± 8.7***	***Trained***	***Military Training, 5d/wk***	***8 weeks***	***Flow Cytometry***	***CD3+, CD4+, CD8+, NK cell, lymphocyte counts***
Baj *et al.* 1994	Humans (males)	21.0 ± 1.5	74.0 ± 1.4	Trained	Cycling training and competition; 500km/wk training & 12,000km during competition	24 weeks	Flow cytometry	Lymphocyte, CD3+, CD4+, CD8+ and NK cell counts, CD3/CD4 Ratio, lymphocyte proliferation, IL-2
Baum *et al.* 1994	Humans (males)	20.8 ± 3.0	No Information	Trained	Training 3 phases: endurance runs (60-160 km/wk), 8 weeks anaerobic phase (intensive training- submax and max runs, uphill runs) & competition phase.	8 weeks	Flow cytometry	Lymphocyte, CD3+, CD4+, CD8+ Counts

					(Pre and post phase 2 used)			
Blank *et al.* 1994	Rodents (females)	8-10 weeks	Not applicable	Not applicable	Treadmill running 60min.d, 5d/wk at 12 m/min, 8 degree gradient	10 weeks	Automated cell counter	CD4/CD8 Ratio, NK cell, CD4+ and CD8+ counts
Blank et al. 1997	***Rodents (females)***	***9-10 weeks***	***Not applicable***	***Not applicable***	***Treadmill running 60min.d, 5d.wk at 12 m/min, 8 degree gradient***	***10 weeks***	***51Cr-release assay in lytic units and Flow cytometry.***	***Spleen NK cell, CD4+, CD8+ counts and NK cytolytic activity.***
Borges, 2012	Humans (males)	22.0 ± 4.2	61.2 ± 5.5	Trained	Kayak season (t0-t2 timepoint used)	26 Weeks	Automatic cell counter.	Lymphocyte count.
Borges *et al.* 2018	Humans (males)	22.0 ± 4.3	61.2 ± 5.5	Trained	11 weeks high volume, 15 weeks high intensity; (kayaking, running, swimming & strength).	26 Weeks	ELISA (unstimulated)	TNF-α, IFN-γ, IL-1β.

Bresciani, 2011	Humans (males)	22.3 ± 1.4	45.2 ± 2.3	Recreationally Active	running 3d.wk starting at 40, 30 ,30 min per session and increasing weekly volume by 5 mins each session every week. (T1-13 used). Intensity started at 42.5% and increased to 80% TRIMP.	9 weeks	ELISA (unstimulated) and Automatic cell counter	Resting Lymphocyte count, TNF-α
Bury *et al.* 1998	Humans (males)	24.2±2.6	62.8±4	Trained	Football season	40 weeks	Resting PHA stimulated labelled thymidine incorporation via liquid scintillation (proliferation). Immunofluorescent staining and microscope (count)	Lymphocyte, CD4+, CD8+, CD56+ Counts, lymphocyte proliferation.
Cardoso et al. 2018	***Rodents (males)***	***6-8 weeks***	***Not applicable***	***Not applicable***	***30 mins swimming/d***	***15 days***	***ELISA (unstimulated)***	***IL-10, IL-1β, TNF-α***
Cardova *et al.* 2015	Humans (males)	20.0 ± 0.9	73.2 ± 6.7	untrained	460km cycling: 4 stages (Basal and post 3rd stage used)	3 days	ELISA (unstimulated)	TNF-α, IFN-γ, 1L-1β

Chiang *et al.* 2007	Rodents (males)	9 weeks	Not applicable	Not applicable	Treadmill endurance training 6d.wk. (progressively increased from 10m/min to 25 m/min and 5 min to 30 min sessions)	5 weeks	LPS stimulated, measured via flow cytometry	BMD MHC II, Myeloid DC CD80 and CD86, DC IL-12
Croft *et al.* 2009	Humans (males)	20 ± 1	55.9 ± 6.8	untrained	High intensity interval running 4xwk: 10 mins at 70% $\dot{V}O_{2max}$, 3x5min at 90%, 1.5 min at 50%, then 10 min cooldown at 70%	6 weeks	Unstimulated Bead assay (flow cytometry)	TNF-α
Dos Santos Cunha et al. 2004	***Rodents (males)***	***6-8 weeks***	***Not applicable***	***Not applicable***	***Treadmill running, 5 d/wk at 60-65% $\dot{V}O_{2max}$***	***5 weeks***	***CON A stimulated proliferation, Thymidine incorporation measured by liquid scintillation (dpm) and ELISA (PHA stimulated).***	***Resting and exercise induced spleen Lymphocyte proliferation, IL-2, IL-10 and TNF-α***

Dressendorfer, 2002	Humans (males)	24.4 ± 2.1	59.3 ± 5.0	trained	HIIT at 100%HRmax 4d/wk on a bike, plus one wind tunnel cycle and one weight session per week. (Baseline to end of I phase used)	6.5 weeks	Flow cytometry.	Lymphocyte count Resting: CD3+, CD8+, CD4+ Counts Exercising: CD3+, CD8+, CD4+ Counts
Fernandes et al. 2019	***Rodents (males)***	***6-8 weeks***	***Not applicable***	***Not applicable***	***Treadmill running 1 hr/d at 50% average max speed.***	***5 weeks***	***Flow cytometry.***	***pDC and mDC count, CD80 and CD86 expression of lung and lymph***
Ferry *et al.* 1990	Humans (males)	20.1 ± 2.9	63.2 ± 4.3	trained	Across a cycling training cycle	20 weeks	Flow cytometry	Resting: Lymphocyte, CD8+, CD4+, CD56+ Counts Exercising: Lymphocyte, CD8+, CD4+, CD56+ Counts. CD4/CD8 Ratio
Ferry et al. 1992	***Rodents (males)***	***12 weeks***	***Not applicable***	***Not applicable***	***Treadmill running 6 d.wk, duration***	***4 weeks***	***Flow cytometry***	***Spleen CD4+ (resting and exercise) and***

					increased from 30-60 min and speed from 20-30 m.min			***CD8+ (resting) counts***
Fry *et al.* 1991	Humans (males)	31.6 ± 3.5	3.71 ± 0.14 (L.min) (No body mass (kg) provided to convert to ml/kg/min)	trained	Army training: 10 d treadmill intervals 2x day (15 x 1 min exercise period 2 mins rest in AM, PM= 10 x 1 min intervals 1 min rest), 5 d active recovery. (Day 1-10 used).	10 days	Flow cytometry	Lymphocyte, CD3+, CD8+, CD4+ CD56+ Counts, CD4/CD8 ratio, lymphocyte proliferation.
Gao, 2013	Humans (males)	20.2 ± 1.3	No Information	Trained	Weightlifting; progressive increase each week@ 75%, 80%, 80%, 85% HRmax, 5.5 d.wk.	4 weeks	Fluorescent double labelling method using Immunoassay	Resting CD4+, CD8+ counts, CD4/CD8 Ratio
Gholamnezhad *et al.* 2014	Rodents (males)	6-8 weeks	Not applicable	Not applicable	Treadmill running at 25 m.min, 60 min.d, 6d.wk	11 weeks	ELISA (unstimulated)	IL-10, IFN-γ, TNF-α
Hack *et al.* 1997	Humans (males)	23.4 ± 0.8	No Information	untrained	Anaerobic training, 60 min sessions 3d.wk. 2x sprint sessions	8 weeks	Automated cell counter and flow cytometry	Lymphocyte, CD3+, CD4+, CD8+ and CD4+CD45RA+

					90-110% $\dot{V}O_{2max}$ with 5-8 min recovery; 5 x 80-300m), 1x 60min weights session			counts, CD4/CD8 Ratio
Halson *et al.* 2003	Humans (males)	21.1 ± 3.0	58.0 ± 1.7	trained	2 week normal cycling training, 2 weeks intensified cycling period (7d.wk 150% normal load)	4 weeks	ELISA (unstimulated) and flow cytometry	Lymphocyte count, TNF-a
Heisterberg *et al.* 2013	Humans (males)	26.3 ± 1.1	62.5	trained	Professional soccer season (5-8 x wk, 1.5- 2 hr. session)	24 weeks	Automated cell counter	Lymphocyte count
Hoffman-Goetz, 1986	Rodents (males)	12 weeks	Not applicable	Not applicable	Treadmill running at 28 m/min, 6d. wk at gradient 8 degree	6 weeks	CON A stimulated thymidine incorporation via liquid scintillation (CPM)	Spleen lymphocyte proliferation
Hoffman-Goetz *et al.* 1988	Rodents (males)	8 weeks	Not applicable	Not applicable	Treadmill running (2 wks; 12-30 m-min, 0-8 degree gradient, 30 min. d, 5d/wk and 6 wks; 30m.min, 8 degree gradient, 20min.d, 5d.wk)	8 weeks	LPS and PWM stimulated thymidine incorporation via liquid scintillation (cpm)	Lymphocyte proliferation

Hoffman-Goetz et al. 1990	***Humans (males)***	***24.5 ± 0.9***	***46.4 ± 6.4***	***untrained***	***Cycling 65% $\dot{V}O_{2max}$, 1 hr.d***	***5 days***	***Flow cytometry***	***Resting and exercising CD3+, CD4+, CD8+ and NK cell counts***
Hwang *et al.* 2007	Rodents (males)	6 weeks	Not applicable	Not applicable	Swimming 30 min/d, 5d/wk in week 1, then extended by 10 min.d, up to 60 min.d, 5d.wk	10 weeks	CON A and LPS induced thymidine incorporation via liquid scintillation (cpm)	Spleen lymphocyte proliferation
Jurimae *et al.* 2021	Humans (males)	25.0 ± 6.5	64.0 ± 3.5	trained	Rowing training, starting at 11.6±1.4 hr/wk and increasing to 18.4±1 hr.wk	24 weeks	ELISA (unstimulated)	IFN-γ, TNF-α, IL-1β, IL-2
Kajiura *et al.* 1995	Humans (males)	20.2 ± 1.8	60.13 ± 5.23	trained	High volume, high intensity running phase (100% increase in normal running load, with 1000m intervals at 95-100% $\dot{V}O_{2max}$ every other day)	10 days	Flow cytometry	Resting and exercising CD4+ count, CD4/CD8 ratio
Kaufman *et al.* 1998	Rodents (males)	6 weeks	Not applicable	Not applicable	Swimming 15 min intervals, increased over 2.5 wks to 2hr/d	4 weeks	Flow cytometry	Spleen CD4/CD8 ratio

					5d/wk, with an additional 1.5 wk at 2hr/d 5d/wk			
Kilgore et al. 2002	***Humans (males)***	***28.3 ± 6.3***	***No Information***	***trained***	***Weightlifting; 57-90% 1RM ranging from 3-5 d/wk***	***6 weeks***	***Automated cell counter***	***Lymphocyte count***
Koyama *et al.* 1998	Rodents (males)	7 weeks	Not applicable	Not applicable	Progressive wheel running; 1 wk (60-120 min/d), 3 wks (120 min/d), 6d/wk), average distance increased from 1500-2500m/d	4 weeks	CON A induced thymidine incorporation via liquid scintillation (cpm)	Peripheral lymphocyte proliferation
Kwak, 2006	Rodents (males)	6 weeks	Not applicable	Not applicable	Swimming 1 week; 30 min/d, increased by 10 min/wk up to 60 min/d	10 weeks	CON A and LPS induce thymidine incorporation via liquid scintillation (cpm)	Spleen lymphocyte proliferation
Lancaster *et al.* 2004	Humans (males)	30 ± 2	60.6 ± 1.5	trained	Cycling training every day at 150% normal volume with $\dot{V}O_{2max}$ tests before and after.	6 days	Flow cytometry and geometric mean fluorescence intensity	CD3+, CD4+, CD8+, CD4+CD45RO+, CD8+CD45RO+ and lymphocyte counts, IFN-γ

Leal *et al.* 2021	Humans (males)	21 ± 5	59 ± 6	untrained	Running session repeated in the order of; 90 min continuous treadmill; 70 min @55% v $\dot{V}O_{2max}$ & 20 min @75% v $\dot{V}O_{2max}$; 5km TT; 70 min treadmill at 12 RPE (borg) for 30 mins, 13 RPE for 30 mins &15 RPE for 10 mins.	12 days	Flow cytometry and Automated cell counter	CD4+, CD3+, CD8+, NK cell counts, CD4/CD8 ratio, DC CD11c Expression
Leandro et al. 2006	***Rodents (male)***	***No Information***	Not applicable	Not applicable	***Treadmill running 5d/wk, 60 min/d at 70% $\dot{V}O_{2max}$***	***8 weeks***	***CON A induced thymidine incorporation via liquid scintillation***	***Lymphocyte proliferation***
Li *et al.* 2013	Humans (male)	19.2 ± 1.6	No Information	trained	Military training	1 week	Flow cytometry	CD4/CD8 ratio, Resting NK cell, CD3+, CD4+ count
Liao et al. 2006	***Rodents (males)***	***6-8 weeks***	Not applicable	Not applicable	***Treadmill running 6 d/wk, timing increased from 15-35 min/d, speed increased from 10-25 m/min. 2% incline increase***	***5 weeks***	***Flow cytometry***	***mDC CD80 and CD86 expression, mDC count***

					in last week. 30% intensity every 3rd day of each week.			
Louis *et al.* 2016	Humans (males)	31.0 ±4.7	58.7 ± 5.6	trained	6 sessions over 4 consecutive days, 10-15h/wk; High intensity afternoon session (8x5 min cycling @85% MAP and 6x5 min running at 10km intensity); low intensity the next morning (60 min cycle at 65% MAP). Light sessions 3 d/wk.	3 weeks	Automated cell counter	Lymphocyte count
Mackenzie *et al.* 2016	Rodents (males)	6-8 weeks	Not applicable	Not applicable	Treadmill running at 60% max velocity, 1hr.d, 5d.wk	4 weeks	Flow cytometry	Bone marrow dendritic cell count
Main *et al.* 2010	Humans (males)	26.6 ±4.1	65.0 ±3.4	trained	14 sessions. Wk; 7d/wk (10 x rowing, the rest weights, running and ergometer). 24hrs/wk,80% endurance	8 weeks	Flow cytometry (unstimulated)	IL-1β, TNF-α, IL-12p70, IL-10

					based, 20% at LT and max sprint efforts			
Meyer *et al.* 2004	Humans (males)	24.8 ± 3.8	68.4 ± 10.0	trained	Cycling training 20h/wk (40% increase from normal training volume)	13 days	Flow cytometry	Lymphocyte and NK cell counts
Mitchell *et al.* 1996	Humans (males)	23.4 ± 7.0	40.4 ± 1.0	untrained	Cycle ergometer; 30 min/d, 2d/wk at 75% $\dot{V}O_{2max}$	12 weeks	PHA and PWM induced thymidine incorporation via liquid scintillation	Lymphocyte count, lymphocyte proliferation
Mueller *et al.* 2001	Humans (males)	25.8 -37.9	73.7 ± 4.7 (Ex) 44.8 ± 5.2 (con)	trained	Season of cross country endurance skiing	8 weeks	ELISA, flow cytometry	Lymphocyte, CD3+, CD4+, CD8+ counts, CD4/CD8 ratio, IFN-γ, IL-10, IL-12
Mujika *et al.* 1996	Humans (males)	21.1 ± 3.4	No Information	trained	Swimming season; 12 weeks training, 4 weeks taper	16 weeks	Automated cell counter	Lymphocyte count
Ndon *et al.* 1992	Humans (males)	25.6 ± 2.6	67.9 ± 2.3	trained	Cycling, swimming, running and weights training; 150% normal training duration	4 weeks	Automated cell counter	Lymphocyte count

Nickel *et al.* 2011	Humans (males)	40 ± 7	No Information	trained	Continuous aerobic running and interval training, gradual increase in training and intensity (week 1: 38 ± 1km/wk – week 10: 54±2 km/wk)	10 weeks	ELISA (unstimulated)	TNF-α
Peake *et al.* 2003	Humans (males)	28 ± 7	No Information	trained	16% increase normal running training volume; 104 ± 48 km average distance	4 weeks	Flow cytometer, CON A and PWM induced thymidine incorporation via liquid scintillation	Lymphocyte, CD3+, CD4+, CD8+, NK cell counts, lymphocyte proliferation
Peijie *et al.* 2003	Rodents (males)	6 weeks	Not applicable	Not applicable	Swimming; Week 1 (1.4m/s for 30 min) increased by 5 min/d until 120 min/d. intensity increased to 1.6m/s at week 2 and 1.8 m/s at week 5.	6 weeks	CON A induced thymidine incorporation via liquid scintillation	Spleen lymphocyte proliferation
Pizza *et al.* 1995	Humans (males)	34.8 ± 7.6	65.1 ± 4.9	trained	200% increased running training load than normal	10 days	Flow cytometry	Lymphocyte, CD4+, CD3+, CD8+ and NK cell

								counts, CD4/CD8 Ratio
Poffe, 2019	Humans (males)	21.2 ± 2.9	55.3 ± 6.1	trained	Cycling 6d/wk; HIIT (30 s max sprint 100 rpm, 4.5 min active recovery at 50W; sprints increased from 4-6 over 3 weeks); intermittent endurance (5x6 min 100-110% av. PO, 8 min 55-85% recovery periods); constant load endurance (70-95% av. PO 30 min TT for 60-150 min)	3 weeks	Flow cytometry	CD3+, CD4+, CD8+ counts, CD4/CD8 ratio
Rebello *et al.* 1998	Humans (males)	26.3 ± 3.7	No Information	trained	Across an entire Portuguese football season	44 weeks	Flow cytometry	Lymphocytes, CD3+, CD4+ counts
Ronsen *et al.* 2001	Humans (males)	21-29	70-82	trained	Nordic skiing season	8 weeks	Automated cell counter	Lymphocyte count
Sheyklouvand *et al.* 2018	Humans (males)	24 ± 3	No Information	trained	Canoe paddling based HIIT 3d/wk, variable	3 weeks	Automated cell counter	Lymphocyte count

					intensity (6 x 60s at 100%, 110%, 120%, 130%, 130%, 130%, 120%, 130%, 100% v$\dot{V}O_{2peak}$) across 9 sessions			
Shing *et al.* 2006	Humans (males)	27 ± 2	69.3 ± 1.3	trained	High intensity cycle training >VT. Day 1: 20x1min at PPO, 2 min recovery at 50 W; Day 2: 60 min at 100%VT; Day 3: 12x30s sprints at 175% PPO, 4.5min at 50W recovery; Day 4: 30 mins at 80%VT, 45 min at 100%VT; Day 5: 40 min TT	5 days	Flow cytometry	Lymphocyte, CD3+, CD4+, CD8+, NK cell counts, CD4/CD8 ratio, TNF-α, IFN-γ, IL-12p70
Shing et al. 2007	***Humans (males)***	***27 ± 2***	***69.3 ± 1.3***	***trained***	***High intensity cycle training >VT. Day 1: 20x1min at PPO, 2 min recovery at 50W; Day 2: 60 min at***	***5 days***	***Flow cytometry***	***NKCC %lysis***

					100%VT; Day 3: 12x30s sprints at 175% PPO, 4.5min at 50W recovery; Day 4: 30 mins at 80%VT, 45 min at 100%VT; Day 5: 40 min TT			
Smith & Myburgh, 2006	Humans (males)	22.6 ± 4.7	56.1 ± 4.7	trained	Cycling; 2d/wk alternating sessions of (1) 80% PPO for 5 min, 1 min rest x 5-8 reps (increased by 1 bout.wk), and (2) 90% 5kmTT speed for 5km, 50% 5km speed for 20min, 90% 5kmTT speed 5km.	4 weeks	Flow cytometry	CD3+, CD4+, CD8+, NK cell counts, CD4/CD8 ratio
Sugiura et al. 2002	***Rodents (males)***	***6 weeks***	***Not Applicable***	***Not Applicable***	***Wheel running 3d/wk, 12h/d. Distance increased from 7km/d to 8km/d, peaking at 10km/d at 5 wk***	***8 weeks***	***CON A and PHA induced thymidine incorporation via liquid scintillation***	***Lymphocyte proliferation***

Tanimura *et al.* 2009	Humans (males)	19.6 ± 0.9	46.8 ± 3.4	trained	Kendo training; 310 min/d	6 days	Flow cytometry	Lymphocyte, CD4+, CD8+ counts
Verde, 1992 (immune resp..)	Humans (males)	28.8 ± 1.7	>60	trained	Increased running training load by 38%	3 weeks	Flow cytometer, PHA and CON A induced thymidine incorporation via liquid scintillation	CD4/CD8 ratio, lymphocyte proliferation, resting CD3+ count
Verde et al. 1992a	***Humans (males)***	***28.8±1.7***	***65.3±4.9***	***trained***	***Increased running training load by 38%***	***3 weeks***	***Flow cytometry***	***CD4+, CD8+ counts***
Wang & Weng, 2011	Humans (males)	23.1 ± 0.8	43.9 ± 2.3	untrained	Cycling at 50% Wmax, 30min.d, 5d/wk	4 weeks	Flow cytometry	NK cell count, NK CD45RO/RA+ count
Wang *et al.* 2011	Humans (males)	21.5 ± 0.7	44.1 ± 2.5	untrained	Cycling at 50% W_{max}, 30min/d, 5d/wk	4 weeks	Flow cytometry	Lymphocyte, CD4+, CD3+, CD8+ counts, CD4/CD8 ratio
Watson *et al.* 1986	Humans (males)	22.8 ± 4.7	54.0 ± 3.1	untrained	Running 40-50 min.d, 5d/wk at 70-85% $\dot{V}O_{2max}$	15 weeks	Haemocytometer, Flow cytometry, NK cell ^{51}Cr release	Lymphocyte and CD3+ counts, NKCC, T cell proliferation
Weng, 2013	Humans (males)	22.3 ± 0.2	46.5 ± 1.7	untrained	Cycling 5d/wk; 3 min intervals at 40 and 80% $\dot{V}O_{2max}$, 30min/d	5 weeks	Flow cytometry	Lymphocyte, CD4+, CD8+ counts, CD4.CD8 ratio

Witard, 2012	Humans (males)	27 ± 8	64.2 ± 6.5	trained	1 wk normal, 1 wk high intensity cycling (increase volume and intensity by 70% vs normal), 1-2 sessions/d, 7d/wk. End of each week; 120min at 60% $\dot{V}O_{2max}$ and 45 min TT at 85-100% $\dot{V}O_{2max}$	2 weeks	Flow cytometry	Lymphocyte and CD8+ counts
Zhang *et al.* 2019	Humans (males)	20.1 ± 2.4	No Information	trained	High intensity training, 8h.d at grade 5-6 intensity	4 weeks	Flow cytometry	Lymphocyte, CD4+, CD8+ and CD3+ counts, and CD4/CD8 ratio.

5.4 Meta-analysis

5.4.1 Immune cell counts

Total lymphocytes

Of the 57 included studies, 29 studies assessed lymphocyte count at rest. Overall, a period of intensified training significantly (Z= 4.06 (P < 0.0001)) reduced resting lymphocyte number with a moderate effect (*d*= -0.56, 95% CI [-0.84, -0.29]; Figure 5.2). However, there is substantial heterogeneity amongst the studies (Chi^2= 79.90, df= 28 (P < 0.00001), I^2= 65%). Subgroup analysis indicated significant decreases in resting lymphocyte count in all exercise durations of >7 days (8 days- 2 weeks (n= 4): Z= 2.13 (P= 0.03), *d*= -1.23, 95% CI [-2.35, -0.10]; 15 days- 4 weeks (n= 7): Z= 3.21 (P= 0.001), *d*= -0.65, 95% CI [-1.05, -0.26]; > 4 weeks (n= 15): Z= 2.08 (P= 0.04), *d*= -0.38, 95% CI [-0.73, -0.02]). Exercise durations of ⩽ 7 days (n= 3) did not alter lymphocyte counts at rest (Z= 1.04 (P= 0.30), *d*= -0.71, 95% CI [-2.04, -0.63]).

Of the 57 included studies, 8 studies assessed lymphocyte count in response to exercise. Overall, a period of intensified training did not change the total lymphocyte count immediately post exercise (Z= 1.47, (P = 0.14), *d*= -0.64, 95% CI [-1.50, 0.21]). There is substantial heterogeneity amongst the studies (Chi^2= 34.43, df= 7 (P < 0.0001), I^2= 80%).

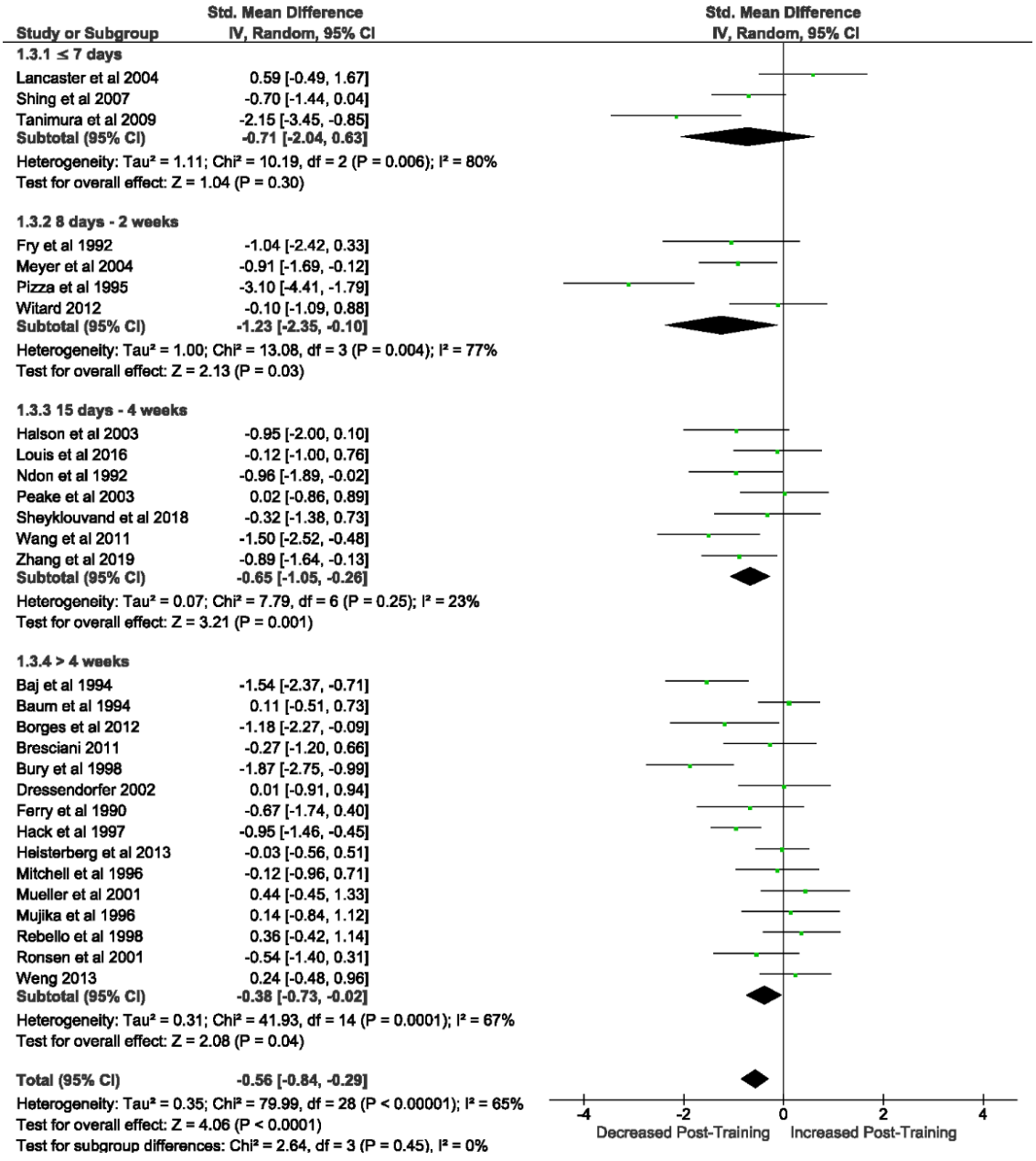


Figure 5.2. Effect of intensified training on resting lymphocyte counts, measured by FACS or Automated cell counter. Subgroup analysis is based on the duration of intensified training period; ⩽7 days, 8 days—2 weeks, 15 days—4 weeks and > 4 weeks.
All studies used human participants. Confidence interval (CI).

5.4.2 T cells

$CD3^+$ T cells

Of the 57 included studies, 14 studies assessed $CD3^+$ count at rest. Overall, a period of intensified training did not change $CD3^+$ count at rest (Z= 1.67, (P= 0.10), *d*= -0.50, 95% CI [-1.08, 0.09]; Figure 5.3). However, there is considerable heterogeneity amongst the studies (Chi^2= 77.41, df= 13 (P< 0.00001), I^2= 83%). Subgroup

analysis revealed that intensified training periods of 8 days- 2 weeks (n= 2) significantly decreased $CD3^+$ T cell count at rest (Z= 2.14 (P= 0.03), *d*= -0.80, 95% CI [-1.53, -0.07]).

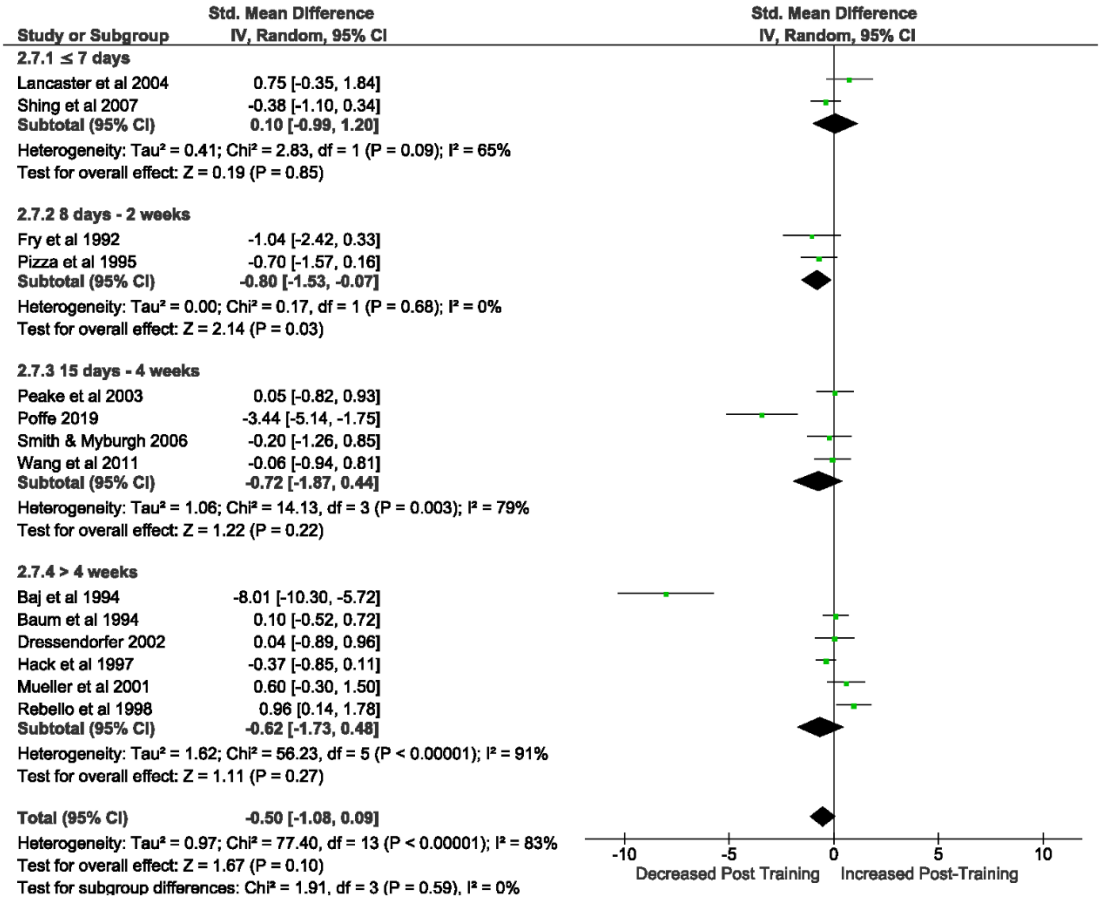


Figure 5.3. Effect of intensified training on resting $CD3^+$ T cell counts, measured by FACS or Automated cell counter. Subgroup analysis is based on the duration of intensified training period; ⩽ 7 days, 8 days—2 weeks, 1 days—4 weeks and > 4 weeks.
All studies used human participants. Confidence interval (CI).

Of the 57 included studies, 3 studies assessed $CD3^+$ count immediately post exercise. Overall, a period of intensified training did not change $CD3^+$ count immediately post exercise (Z= 0.82, (P = 0.41), *d*= -1.16, 95% CI [-3.93, 1.16]). However, there is considerable heterogeneity amongst the studies (Chi^2= 35.40, df= 2 (P< 0.00001), I^2= 94%).

CD4$^+$ T Cells

Of the 57 included studies, 19 studies assessed CD4$^+$ count at rest. Overall, a period of intensified training did not change CD4$^+$ count at rest (Z= 1.41, (P= 0.16), *d*= -0.30, 95% CI [-0.71, 0.12]; Figure 5.4). However, there is considerable heterogeneity amongst the studies (Chi^2= 74.06, df= 18 (P< 0.00001), I^2= 76%). Subgroup analysis revealed that intensified training periods of 8 days- 2 weeks (n= 3) significantly decreased CD4$^+$ T cell count at rest (Z= 2.53 (P= 0.01), *d*= -1.17, 95% CI [-2.08, -0.26]).

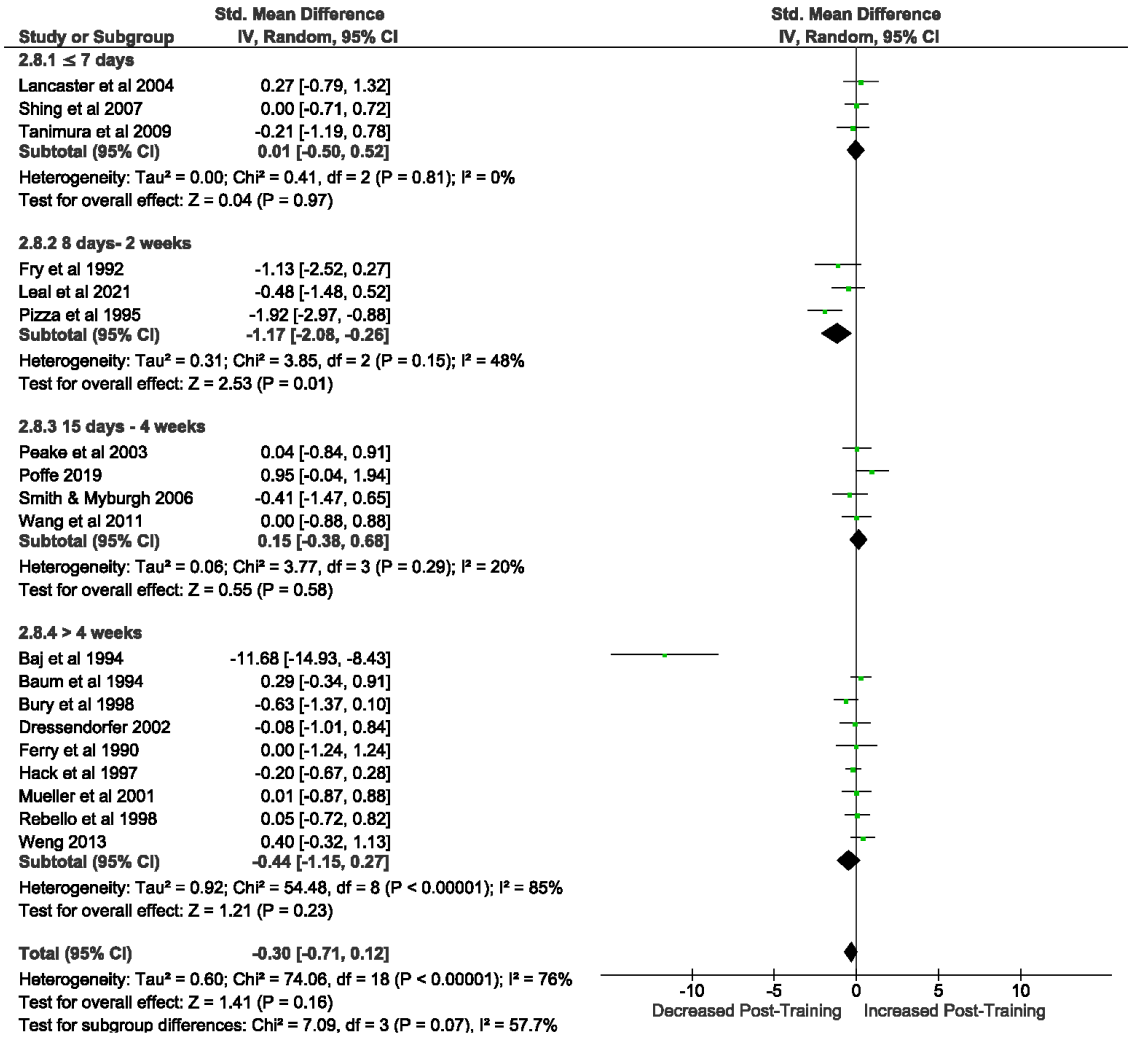


Figure 5.4. Effect of intensified training on resting CD4$^+$ T cell counts, measured by FACS or Automated cell counter. Subgroup analysis is based on the duration of intensified training period; ⩽ 7 days, 8 days—2 weeks, 15 day—4 weeks and > 4 weeks.
All studies used human participants. Confidence intervals (CI).

Of the 57 included studies, 5 studies assessed CD4$^+$ count immediately post exercise. Overall, a period of intensified training did not change CD4$^+$ count immediately post exercise (Z= 0.80, (P= 0.42), *d*= 0.35, 95% CI [-0.50, 1.19]). However, there is substantial heterogeneity amongst the studies (Chi^2= 12.92, df= 4 (P= 0.01), I^2= 69%).

CD8$^+$ T cells

Of the 57 included studies, 18 studies assessed CD8$^+$ count at rest. Overall, a period of intensified training significantly (Z= 2.18, (P = 0.03)) reduced CD8$^+$ count at rest with a small effect (*d*= -0.37, 95% CI [-0.7, -0.04]; Figure 5.5). However, there is substantial heterogeneity amongst the studies (Chi^2= 44.13, df= 17 (P= 0.0003), I^2= 61%). Subgroup analysis revealed that intensified training periods of 8 days- 2 weeks (n= 4) were the only duration to significantly alter resting CD8$^+$ T cell count (Z= 2.98 (P= 0.003), *d*= -0.79, 95% CI [-1.30, -0.27]).

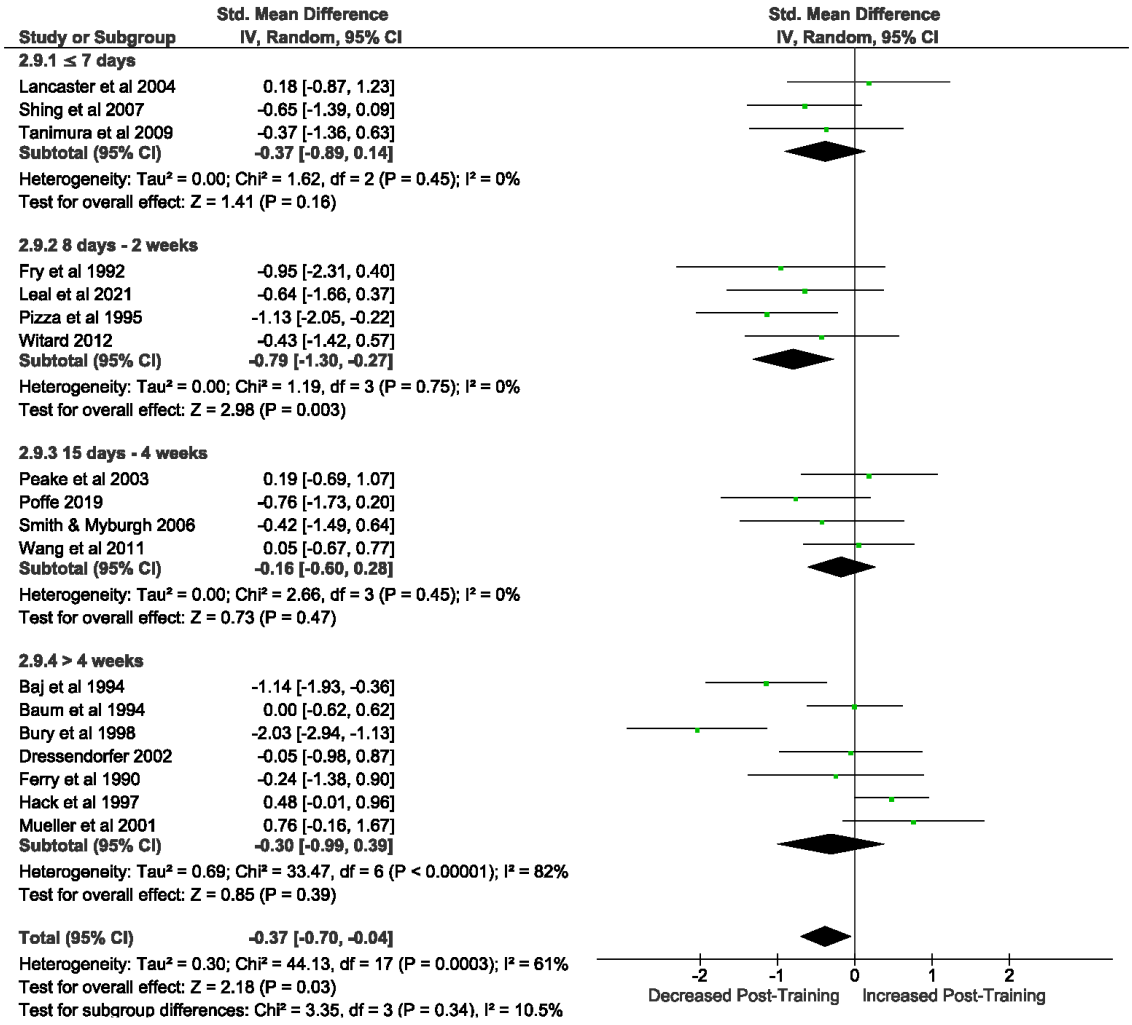


Figure 5.5. Effect of intensified training on resting CD8$^+$ T cell counts, measured by FACS or Automated cell counter. Subgroup analysis is based on the duration of intensified training period; ⩽ 7 days, 8 days—2 weeks, 15 day—4 weeks and > 4 weeks.
All studies used human participants. Confidence Interval (CI).

Of the 57 included studies, 6 studies assessed CD8$^+$ count immediately post exercise, before and after a period of intensified training. Overall, a period of intensified training did not change CD8$^+$ count immediately post exercise (Z= 0.54, (P= 0.59), *d*= 0.37, 95% CI [-0.97, 1.72]). However, there is considerable heterogeneity amongst the studies (Chi^2= 45.96, df= 6 (P< 0.00001), I^2= 89%).

CD4/CD8 ratio

Of the 57 included studies, 19 studies assessed CD4/CD8 Ratio at rest. Overall, a period of intensified training did not change the resting CD4/CD8 ratio (Z= 0.91, (P= 0.36), *d*= -0.15, 95% CI [-0.49, 0.18]; Figure 5.6). However, there is substantial heterogeneity amongst the studies (Chi^2= 59.81, df= 18 (P< 0.00001), I^2= 70%).

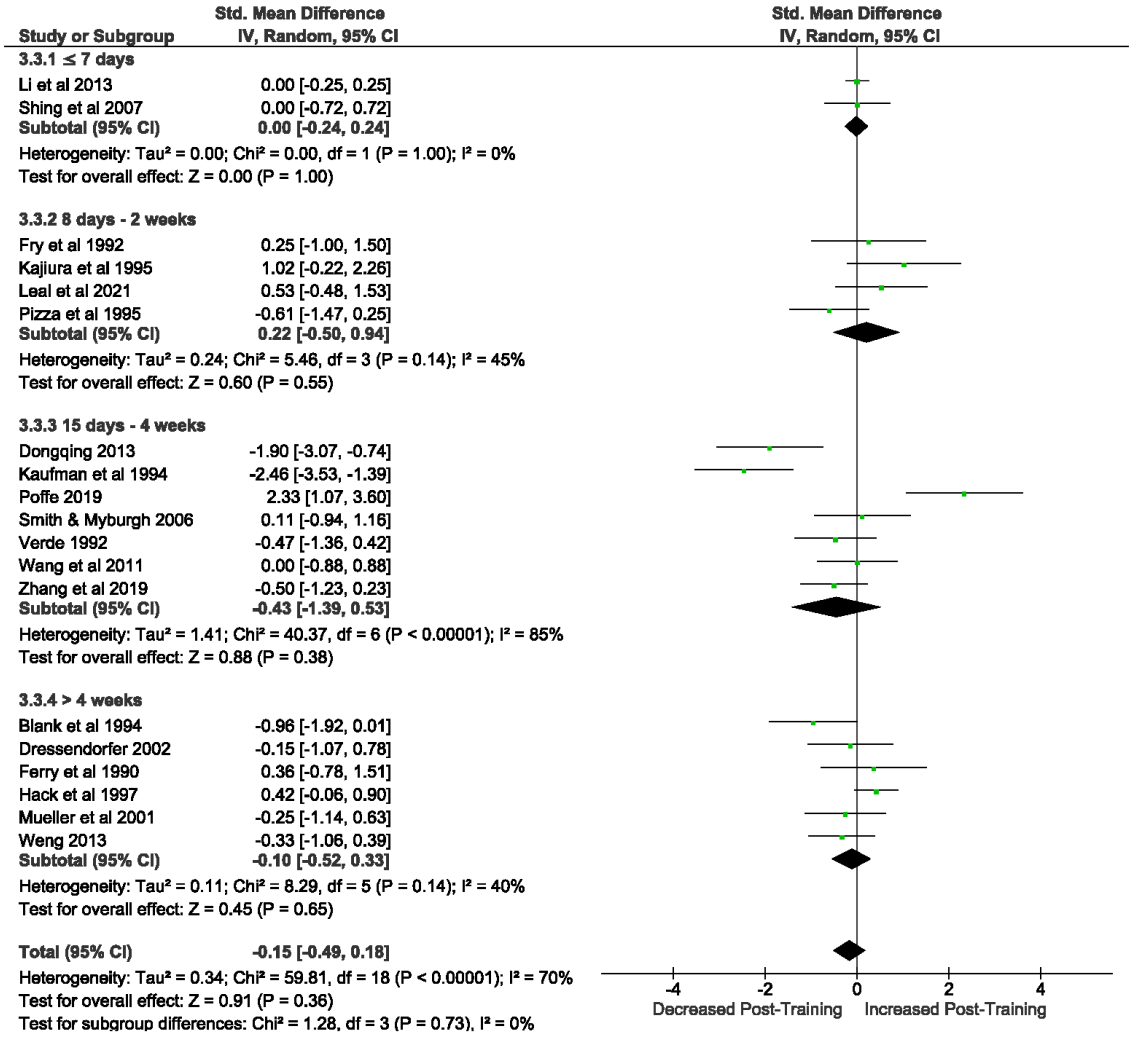


Figure 5.6. Effect of intensified training on resting CD4/CD8 ratio. Subgroup analysis is based on the duration of intensified training period; ⩽ 7 days, 8 days—2 weeks, 15 days—4 weeks and > 4 weeks. Studies used a mixture of human and rodent (Blank et al., 1994; Kaufman et al., 1994) participants. Confidence Intervals (CI).

Of the 57 included studies, 7 studies assessed the CD4/CD8 ratio immediately post exercise. Overall, a period of intensified training did not change the CD4/CD8 ratio immediately post exercise (Z= 0.19, (P= 0.85), *d*= -0.04, 95% CI [-0.43, 0.35]). There is low heterogeneity amongst the studies (Chi^2= 7.15, df= 6 (P= 0.31), I^2= 16%).

5.4.3 Natural killer cells

Of the 57 included studies, 10 studies assessed NK cell count at rest based on $CD56^+$ expression. Overall, a period of intensified training did not change NK cell count at rest (Z= 1.18, (P= 0.24), *d*= -0.25, 95% CI [-0.67, 0.16]; Figure 5.7). However, there is substantial heterogeneity amongst the studies (Chi^2= 24.78, df= 10 (P= 0.003), I^2= 64%). Subgroup analysis revealed that resting NK cell count did not alter after a period of intensified training of any duration (i.e. ⩽7 days (P= 0.36), 8 days-2 weeks (P= 0.18), 15 days-4 weeks (P= 0.62) or > 4 weeks (P= 0.17)).

Of the 57 included studies, 3 studies assessed NK cell count based on $CD56^+$ expression immediately post exercise. Overall, a period of intensified training did not change NK cell count immediately post exercise (Z= 0.10, (P= 0.92), *d= 0.04*, 95% CI [-0.74, 0.82]). However, there is substantial heterogeneity amongst the studies (Chi^2= 4.35, df= 3 (P = 0.11), I^2= 54%).

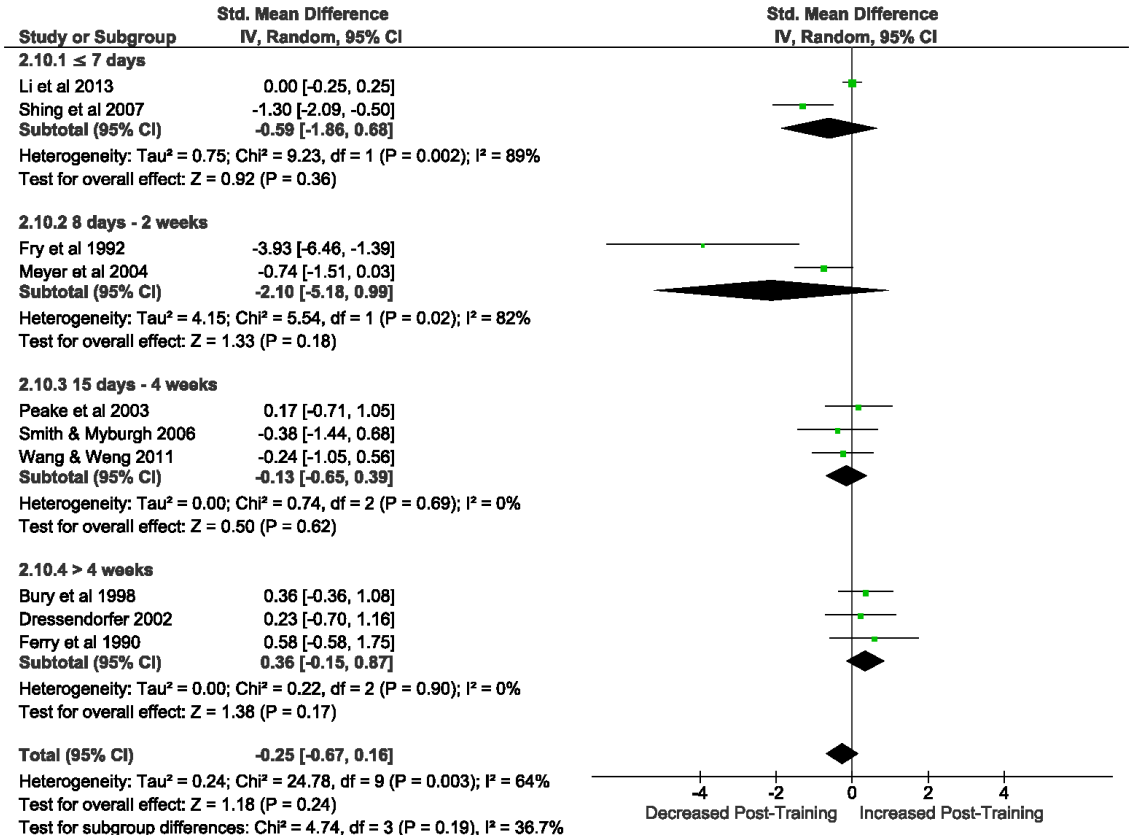


Figure 5.7. Effect of intensified training on resting Natural Killer cells, measured by FACS or Automated cell counter. Subgroup analysis is based on the duration of intensified training period;⩽ 7 days, 8 days—2 weeks, 15 days—4 weeks and > 4 weeks.
All studies used human participants. Confidence Intervals (CI).

5.4.4 Immune cell function

Lymphocyte proliferation

Resting stimulated human peripheral blood (counts per minute)

Of the 57 included studies, 5 different studies assessed human peripheral blood lymphocyte proliferation at rest, with 3 studies assessing lymphocyte proliferation to more than one stimulant, thus 8 results were entered into the meta-analysis. Overall, a period of intensified training did not change resting lymphocyte proliferation (Z= 0.04, (P= 0.97), *d*= -0.02, 95% CI [-1.10, 1.05]). However, there is considerable heterogeneity amongst the studies (Chi^2= 75.72, df= 7 (P< 0.00001), I^2= 91%).

Resting stimulated rodent spleen lymphocytes (counts per minute)

Of the 57 included studies, 6 studies assessed rodent spleen lymphocyte proliferation at rest with 2 studies assessing lymphocyte proliferation to more than one stimulant, thus 8 results were entered into the meta-analysis. Overall, a period of intensified training did not change resting spleen lymphocyte proliferation (Z= 0.10, (P= 0.92), *d*= 0.09, 95% CI [-1.79, 1.97]). However, there is considerable heterogeneity amongst the studies (Chi^2= 94.88, df= 7 (P< 0.00001), I^2= 93%).

Exercise Induced CON A stimulated rodent spleen lymphocytes (counts per minute)

Of the 57 included studies, 3 studies assessed stimulated spleen lymphocyte proliferation immediately post exercise. Overall, a period of intensified training did not change spleen lymphocyte proliferation immediately post exercise (Z= 0.77, (P = 0.44), *d*= -2.38, 95% CI [-8.44, 3.68]). However, there is considerable heterogeneity amongst the studies (Chi^2= 44.46, df= 2 (P< 0.00001), I^2= 96%).

NK cell cytolytic activity

Of the 57 included studies, 3 studies assessed NK cytolytic activity as %lysis of K-562 tumour cells. Overall, a period of intensified training did not change NK cell cytolytic activity (Z= 1.63, (P= 0.10), *d*= 1.13, 95% CI [-0.23, 2.49]). However, there is considerable heterogeneity amongst the studies (Chi^2= 16.75, df= 2 (P< 0.0002), I^2= 88%).

Cytokines

Unstimulated TNF-α

Of the 57 included studies, 10 studies assessed unstimulated TNF-α secretion at rest. Overall, a period of intensified training did not change resting TNF-α secretion (Z= 0.39, (P= 0.70), *d*= -0.13, 95% CI [-0.76, 0.51];

Figure 5.8). However, there is substantial heterogeneity amongst the studies (Chi^2= 35.96, df= 9 (P< 0.00001), I^2= 75%).

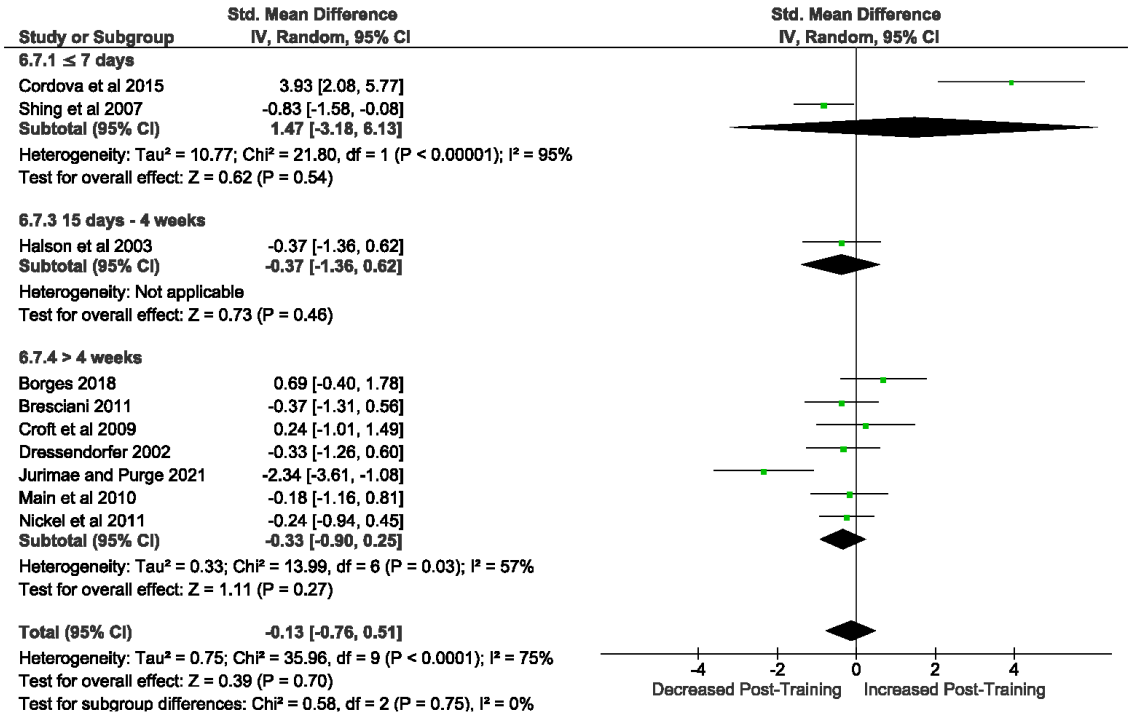


Figure 5.8. Effect of intensified training on resting unstimulated plasma TNF-α. Subgroup analysis is based on the duration of intensified training period; ≤ 7 day, 8 days—2 weeks, 15 days—4 weeks and > 4 weeks. All studies used human participants. Confidence Intervals (CI).

Unstimulated IFN-γ

Of the 57 included studies, 5 studies assessed unstimulated IFN-γ secretion at rest. Overall, a period of intensified training did not change resting IFN-γ secretion (Z= 1.33, (P= 0.18), *d*= 0.70, 95% CI [-0.33, 1.74]). However, there is considerable heterogeneity amongst the studies (Chi^2= 19.42, df= 4 (P = 0.0006), I^2= 79%).

Unstimulated IL-1β

Of the 57 included studies, 5 studies assessed unstimulated IL-1β secretion at rest. Overall, a period of intensified training significantly (Z= 2.69, (P = 0.007)) decreased resting IL-1 β secretion with a moderate effect (*d*= -0.63, 95% CI [-1.09, -0.17]; Figure 5.9). There is very low heterogeneity amongst the studies (Chi^2= 4.05, df= 4 (P= 0.40), I^2= 1%).

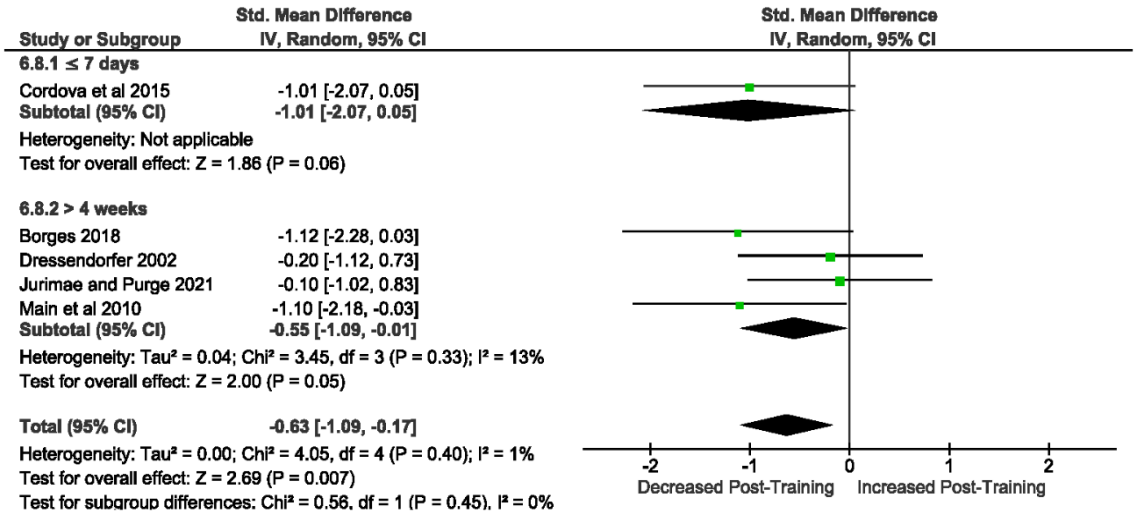


Figure 5.9. Effect of intensified training on resting unstimulated plasma IL-1β. Subgroup analysis is based on the duration of intensified training period; ⩽ 7 days, 8 days—2 weeks, 15 days—4 weeks and > 4 weeks. All studies used human participants. Confidence Intervals (CI).

Unstimulated IL-10

Of the 57 included studies, 3 studies assessed unstimulated IL-10 secretion at rest. Overall, a period of intensified training did not change resting IL-10 (Z= 1.62, (P= 0.11), *d= 1.52*, 95% CI [-0.32, 3.37]). However, there is considerable heterogeneity amongst the studies (Chi^2= 12.59, df= 2 (P= 0.002), I^2= 84%).

Unstimulated IL-2

Of the 57 included studies, 2 studies assessed unstimulated IL-2 secretion at rest. Overall, a period of intensified training did not change resting IL-2 secretion (Z= 1.62, (P= 0.11), *d= 1.41*, 95% CI [-0.30, 3.12]). However, there is considerable heterogeneity amongst the studies (Chi^2= 4.26, df= 1 (P= 0.04), I^2= 77%).

5.4.5 Dendritic cells (all rodent studies)

DC CD80 expression

Of the 57 included studies, 2 studies assessed DC CD80 expression as a percentage of fluorescent intensity. Overall, a period of intensified training did not change rodent bone marrow derived DC CD80 expression (Z= 0.82, (P= 0.41), *d*= 0.33, 95% CI [-0.46, 1.12]; Figure 5.10). There is low heterogeneity amongst the studies (Chi^2= 1.64, df= 1 (P= 0.20), I^2= 39%).

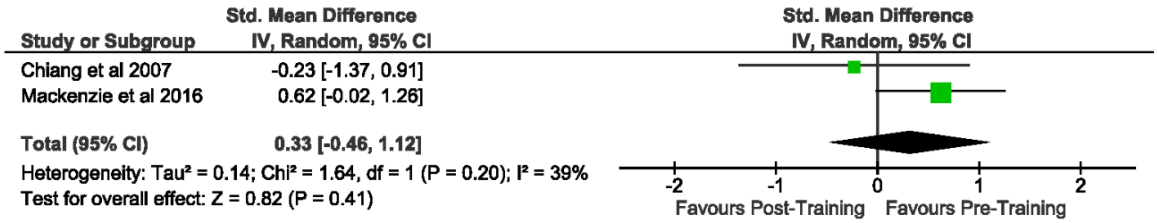


Figure 5.10. Effect of intensified training on resting dendritic cell CD80 expression.
All studies used rodent participants. Confidence Intervals (CI).

DC MHC II expression

Of the 57 included studies, 2 studies assessed DC MHC II expression. Overall, a period of intensified training did not change rodent DC MHC II expression (Z= 0.31, (P= 0.76), *d*= 2.30, 95% CI [-12.43, 17.04]; Figure 5.11). However, there is considerable heterogeneity amongst the studies (Chi^2= 65.24, df= 1 (P< 0.00001), I^2= 98%).

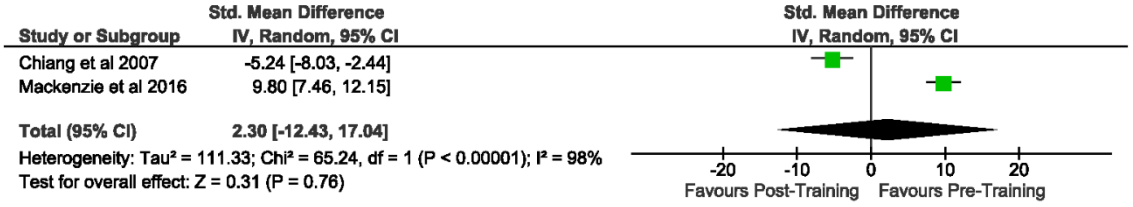


Figure 5.11. Effect of intensified training on resting dendritic cell MHC II expression.
All studies used rodent participants. Confidence Intervals (CI).

DC CD86 expression

Of the 57 included studies, 2 studies assessed rodent DC CD86 expression as a percentage of fluorescent intensity. Overall, a period of intensified training significantly (Z= 2.27, (P= 0.020)) increased DC CD86 expression with a large effect (*d*= 2.18, 95% CI [0.29, 4.07]; Figure 5.12). However, there is substantial heterogeneity amongst the studies (Chi^2= 5.71, df= 1 (P= 0.02), I^2= 83%).

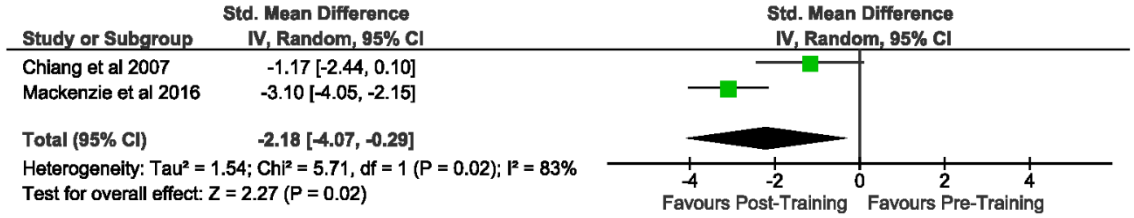


Figure 5.12. Effect of intensified training on resting dendritic cell CD86 expression.
All studies used rodent participants. Confidence Intervals (CI).

5.5 Discussion

5.5.1 Overview

The purpose of this study was to bring together a body of research to characterise the T lymphocyte, NK cell and DC activity at rest and in response to exercise stress following periods of intensified training. To assess whether these immune biomarkers could provide insight into their use as diagnostic indicators of the negative states of overtraining, it was necessary to focus on research with appropriate intensity of training. Training protocols of >7 days are more likely to induce NFOR than shorter training periods of the same intensity (Halson et al., 2002). With 93% of included studies utilising a training protocol of >7 days in duration, we can be confident that NFOR was a possibility. In agreement with this, subgroup analysis conducted on resting immune cell counts within this review indicated that training durations of <7 days did not induce alterations in immune cell counts, whereas durations above this did. Understanding the relationship between resting and exercise induced immune cell markers and intensified training periods may be useful in the diagnosis of the negative states of overtraining e.g. NFOR or OTS. Currently no clear biomarker has been uncovered to support diagnosis of these states (Meeusen et al., 2013). Additionally, this review highlights gaps within the literature where further experimental research is required.

In total this meta-analysis examined 16 immune biomarkers; 7 were assessed at rest and in response to a bout of exercise, with the remaining 9 assessed at rest only. When comparing each variable from before to after an intensified training period, significant decreases in resting total lymphocyte and $CD8^+$ T cell counts, and unstimulated plasma IL-1β levels were found. In addition, resting DC CD86 expression was significantly increased in rodents only. The time periods of training used to stratify the data were <7 days, 8 day – 2 weeks, 15 days – 4 weeks and > 4 weeks. The <7-day duration was chosen as the lowest training intervention period because it has been shown that overtraining can occur in as little as 7 days (Halson et al., 2002). Additionally, it was projected that Chapter 7 would likely use a training duration of 8 days – 2 weeks, thus it was important that immune alterations occurring in this time frame was captured. Finally, based on ensuring enough studies could be grouped for meta-analysis, the time points 15 days – 4 weeks and > 4 weeks were chosen.

5.5.2 Total lymphocyte and $CD8^+$ T cell count at rest

The meta-analysis found a significant decrease in resting total lymphocyte and $CD8^+$ T cell counts after a period of intensified training. The magnitude, direction, and length of immune recovery after a period of intensified training is dependent upon the intensity, duration, and load of training (Simpson et al., 2020). Therefore, differences in exercise protocols used by studies utilised in this review may account for the inter-study variability in findings. To examine this further, we note that of the studies reporting intensity increases, the study displaying the largest effect size for a significant decrease in resting lymphocyte count elevated running training load by 200% across a 10-day period in trained participants (Pizza et al., 1995). Whereas the study with the lowest effect size increased cycling training load by 40% across a 13-day period in trained

participants (Meyer et al., 2004). The use of sudden increases in workloads as large as 200% may not be reflective of normal practice within elite sport which could be suggested to limit the relevance of the findings to the wider athletic population. However, inducing high levels of stress on the immune system does provide insight into its response to training stress and helps highlight whether immune biomarkers could act as diagnostic indicators of NFOR or OTS. Although this may not be reflective of best practice in an athletes every-day regime, some individuals may experience this overload. Additionally, the HR response and number of active muscles during running is known to be considerably higher than that of cycling exercise. In triathletes, it has been observed that the HR_{max} achieved during cycling is often 6-10 bpm lower than obtained during running (Millet et al., 2009). Therefore, aside from the obvious intensity differences between Pizza et al. (1995) and Meyer et al. (2004), the use of running exercise in Pizza et al. (1995) induces larger internal stress and is thus more likely to push an athlete towards a state of NFOR than cycling protocols for the same given intensity and/or duration.

During exercise, a transient period of lymphocytosis occurs followed by a period of lymphocytopenia after cessation of exercise (Shek et al., 1995). Traditionally, as explained in Chapter 2, it is thought that this lymphocytopenia creates a 3 -72-hour window of opportunity for infection (Pedersen & Ullum, 1994) which is prolonged and more severe if a second bout of exercise is performed within this time frame (Simpson et al., 2015). Exercise-induced lymphocyte apoptosis (Phaneuf & Leeuwenburgh, 2001) that can still be evident 24 hours after a single bout of treadmill exercise to exhaustion (Mars et al., 1998) has been suggested as a possible cause for this. This mechanism is thought to be mediated by cortisol (Riccardi et al., 1999) by binding to glucocorticoid receptors within immune cells, leading to increased cell apoptosis (Cain & Cidlowski, 2017). A single bout of exercise (< 1.5 hours) has been shown to increase ROS which is further increased with higher intensities (Thirupathi & Pinho, 2021), often peaking 2-3 days after exercise (Theodorou et al., 2011). This increase in ROS has also been attributed to initiating lymphocyte apoptosis via damaging the DNA of the immune cell (Mooren et al., 2004). ROS are oxygen containing molecules that are capable of independent existence, containing at least one or more unpaired electrons (Jakubczyk et al., 2020). Although at low levels ROS may function in cell signalling processes i.e. regulating cell growth and differentiation, inflammation, immune responses and immune survival (Romero & Agostinis, 2014), at higher levels, ROS may damage cellular DNA of immune cells and thus play a role in apoptosis (Mooren et al., 2004).

As the studies used in this review examined repeated exposures to exercise stress, exceeding the aforementioned duration and/or intensities, apoptosis could be a potential reason for the decrease in resting lymphocyte numbers found after a period of intensified training. Despite earlier investigations suggesting that post exercise lymphocytopenia is a result of apoptosis, Simpson et al. (2007) reported limited lymphocyte markers of apoptosis (Annexin-V(+) or HSPA60) 1 hour after treadmill exercise to fatigue completed at 80% $\dot{V}O_{2max.}$, yet lymphocytopenia was evident. Moreover, the levels of apoptosis reported in studies indicating an increase in cell death are usually very small i.e. < 5% (Mooren et al., 2002; Simpson et al., 2007), and as such is unlikely to account for the 30-40% reductions in blood lymphocyte count witnessed after exercise (Peake et al., 2016). Therefore, the reduced resting lymphocyte and $CD8^+$ T cell counts after a period of

intensified training could instead be due to an exercise-driven redistribution of highly functional effector cells, such as $CD8^+$ T cells from the blood stream into the tissues and organs for heightened identification and eradication of tissue tumour cells (Simpson et al., 2020).

This redistribution has been demonstrated in rodents via fluorescent cell tracking following both running and swimming exercise (Kruger et al., 2007). In humans, cycling at 85% W_{max} for 20 minutes prompted the preferential mobilisation of highly cytotoxic $CD8^+$ T cells possessing a high propensity to migrate into the peripheral tissues during exercise recovery (Campbell et al., 2009). The redistribution of highly functional effector cells is driven by increased haemodynamics and the release of catecholamines and glucocorticoids following the activation of the SNS and HPA axis (Simpson et al., 2015).

Catecholamines, for example adrenaline and noradrenaline, influence the mobilisation of $CD8^+$ T cells both directly, via the action of adrenaline on lymphocyte β_2-adrenergic receptors (Graff et al., 2018) and expression of adhesion molecules (Shephard, 2003), and indirectly, via increased cardiac output and shear stress mobilising lymphocytes from endothelial walls (Shephard, 2003). Both mechanisms result in the demargination of highly cytotoxic effector cells into the circulation (Dimitrov et al., 2010). $CD8^+$ cells are the T cell subset expressing the most β_2-adrenergic receptors and are therefore more susceptible to change with increased exposure to catecholamines across the training period (Shephard, 2003). Whilst catecholamines drive the lymphocytosis of $CD8^+$ T cells during exercise, glucocorticoids such as cortisol are thought to influence the egress of $CD8^+$ T cells out of the peripheral blood and into the peripheral tissues and organs during exercise recovery. This is believed to be via heightened expressions of certain cell activation and adhesion molecules that facilitate migration, enabling them to pass through endothelial cells and into tissues (Simpson et al., 2006). During prolonged recovery from intensified exercise, substantial infiltration of certain subsets of T lymphocytes into damaged skeletal muscles also occur in order to enhance muscle repair (Jones et al., 1986). It is therefore possible that the reduction in resting lymphocytes, and more specifically $CD8^+$ T cells, is the result of a redistribution into damaged muscles, caused by repeated bouts of exercise over the training period (Pizza et al., 1995).

Subgroup analysis consistently showed that training periods of 8 days – 2 weeks in duration led to lowered resting lymphocyte, $CD3^+$, $CD4^+$ and $CD8^+$ T cell counts (Figure 5.2, 5.3, 5.4 and 5.5, respectively). As such, this duration of training will form the basis of the training intervention used in Chapter 7.

5.5.3 Exercise induced lymphocyte counts

Despite significant decreases in resting lymphocyte counts after a period of intensified training, there was no overall significant change in lymphocyte counts in response to an acute bout of exercise. This suggests that the lymphocyte response to exercise stress remains unchanged both before and after a period of intensified training. 8 studies investigated the exercise induced changes in lymphocyte count after a period of intensified training. Of those 8 studies, only 1 found a significant increase in exercise induced lymphocyte count (Wang

et al., 2011). All other studies included either found no change (Ferry et al., 1990; Ndon et al., 1992; Ronsen et al., 2001; Witard et al., 2012) or a significant decrease (Hasanli et al., 2021; Lancaster et al., 2004; Shing et al., 2007).

On examination of the acute exercise bouts used before and after the intensified training period to assess the exercise induced lymphocyte changes in each study; all were all-out tests until volitional exhaustion. Differences in exercise intensities of these acute exercise bouts are therefore not the cause of the differences in lymphocyte response between Wang et al. (2011) and the rest of the studies. However, Wang et al. (2011) was the only study that used untrained, sedentary participants, with an average $\dot{V}O_{2max}$ of 44.1 ml/kg/min (classed as 'Fair') (ACSM, 2017). All of the other studies utilised participants with a $\dot{V}O_{2max}$ > 60 ml/kg/min; classed as 'Superior' (ACSM, 2017). It is known that trained and untrained individuals undergoing a period of intensified training show different cellular responses to exercise, thought to be related to differences in the elevated cortisol levels and alterations in the pro/anti-inflammatory balance in response to exercise (Walsh et al., 2011). Specifically, T cell counts appear to be sensitive to exercise load in well trained individuals undertaking a period of intensified training, but this sensitivity is reduced in sedentary individuals undertaking the same training (Walsh et al., 2011). If Wang et al. (2011) was to be removed, and the meta-analysis re-run using only trained participants, the total lymphocyte count response to an acute bout of exercise would be significantly attenuated.

Additionally, the intensity of the training intervention used by Wang et al. (2011) was lower than those used by the other studies assessing exercise induced lymphocyte counts. Wang et al. (2011) used a training intensity of 50% W_{max} which has previously been likened to ~55% $\dot{V}O_{2max}$ (Van Loon et al., 1999). According to Gore et al. (2013), any exercise < 60% $\dot{V}O_{2max}$ is classed as light aerobic and represents the lowest training zone. The two studies displaying significant increases in exercise induced lymphocyte counts utilised intensities above the VT_1 level (Shing et al., 2007) and 70-95% HR_{max} for most of the training duration (Lancaster et al., 2004). It is suggested that anaerobic exercise during maximal effort is the most powerful catecholamine and cortisol stimulator (Baj et al., 1994). Therefore, the differences in responses between these studies could be because the training protocol adopted by Wang et al. (2011) was not intense enough to elicit such hormonal changes that may blunt the exercise induced lymphocytosis post training that was seen in Lancaster et al. (2004) and Shing et al. (2007).

5.5.4 Resting unstimulated IL-1β

A significant decrease in resting unstimulated plasma IL-1β levels after a period of intensified training was found. High serum levels of IL-1β are thought to exacerbate damage during chronic disease and acute tissue injuries (Lopez-Castejon & Brough, 2011); commonly implicated in the pathogenesis of chronic diseases such as rheumatoid arthritis (Almonte et al., 1992), atherosclerosis (Kirii et al., 2003) and chronic obstructive pulmonary disease (Hammad et al., 2015). As such, a reduction in resting serum IL-1β has been implicated in reducing low grade inflammation and is a target for many anti-inflammatory treatments (Dinarello et al.,

2012). This indicates that reduced resting IL-1β levels after a period of training may be seen as a positive anti-inflammatory effect of exercise training. Conversely, IL-1β is essential for resistance to infections, and lower resting levels of IL-1β may reduce the ability to initiate a type 1 immune response (Murray, 2013). However, in order to understand the true effects this reduction may have on immunity, stimulated cytokine release needs to be assessed.

On examination of the individual studies there needs to be a consideration of how resting IL-1β was defined. Córdova et al. (2015) took their post training, resting sample 3 hours after a cycling race and Main et al. (2010) collected what they referred to as a resting sample 30 minutes after a water-based rowing session. A study investigating plasma and mononuclear mRNA IL-1β levels in response to a 3-hour mixed cycling and running bout at 60-65% $\dot{V}O_{2max}$ reported that plasma IL-1β levels were still elevated compared to resting pre-exercise levels at both 300 minutes and 24 hours after cessation of exercise, but no change in mRNA was detected (Moldoveanu et al., 2000). Similarly, an acute bout of plyometric exercises consisting of 50 jumps and 50 drop jumps (Chatzinikolaou et al., 2010), and a marathon race (Ostrowski et al., 1999) have been shown to elevate plasma IL-1β levels immediately after exercise. It could therefore be argued that the true resting plasma IL-1β responses have not been shown and may account for the large differences in effect sizes between these studies and the two studies showing the smallest effect sizes. The two studies showing the smallest effect sizes, indicating little to no change in resting IL-1β levels, collected blood samples after at least 24 hours of rest (Dressendorfer, 2002; Jurimae & Purge, 2021).

In both of the studies displaying little to no change in IL-1β, markers of performance were shown to increase. For example, in a group of competitive endurance cyclists, after 6.5 weeks of intensified training, HR decreased at submaximal levels, cycling economy improved and no change in the testosterone:cortisol ratio was observed that would indicate any physiological stress that may evoke immune changes (Dressendorfer, 2002). Likewise, a group of elite rowers undergoing 6 months of volume extended training saw an improvement in performance in the form of increased aerobic power, indicating normal training adaptation (Jurimae & Purge, 2021). Therefore, regardless of sample timing, the results of these two studies may not be representative of the overtrained athlete, and insight into their use as a biomarker of overtraining may be limited.

Although acute bouts of exercise have been shown to elevate unstimulated plasma IL-1β, this review found that a period of intensified training led to significantly reduced levels. Pro-inflammatory cytokines are mediated by both anti-inflammatory cytokines, such as IL-1ra and IL-6, and cytokine inhibitors, such as cortisol and adrenaline, which are known to increase markedly in the circulation following endurance exercise (Suzuki et al., 2002). Cortisol is known to possess anti-inflammatory effects (Blannin et al., 1996; Ortega et al., 1996) and adrenaline has been shown to downregulate the stimulated production of IL- 1β (Bergmann et al 1999). Additionally, IL-6, the most notable cytokine secreted from contracting muscles, increases up to 100-fold during exercise, resulting in increased anti-inflammatory cytokine production, and decreased IL-1β

production (Beavers et al., 2010). As such, the triggered anti-inflammatory effects of exercise could explain the significant decrease in resting IL-1β levels found after a period of intensified training in this review.

Whilst this review focused on unstimulated IL-1β concentrations, it is apparent that stimulated cytokine production from immune cells may be more informative of the overall immune state (Gleeson et al., 2013). This is because IL-1β does not increase exponentially during exercise, which is different when compared to infections (Pedersen & Hoffman-Goetz, 2000). Therefore, stimulating the cytokine response from immune cells after exercise with stimulants such as LPS, mimics the initial innate immune response to bacterial infection. In line with this, Nielsen et al. (2016) found no significant changes in unstimulated IL-1β immediately after a half-marathon, but when measuring LPS-stimulated cytokines after the same bout of exercise, a significant decrease in plasma IL-1β was found. Despite this, only unstimulated cytokine responses were included in the meta-analysis because limited papers using the same stimulants were available for grouping.

5.5.5 Dendritic cell markers

The meta-analysis revealed a significant upregulation of stimulated CD86 expression after a period of intensified training in rodents, yet no significant changes were found for CD80 or MHC II expression. Only two papers satisfied the search criteria for this analysis, and both were in rodents. As such, it is reasonable to suggest that strong conclusions cannot be drawn.

When looking at the trends across all papers that assessed DC markers, including those not suitable for meta-analyses, it is apparent that overall, there is a trend for increased CD86 (Mackenzie et al., 2016; Fernandes et al., 2019; Chiang et al., 2007) expression after a period of training, with conflicting results for MHC II (Chiang et al., 2007; Mackenzie et al., 2016) and CD80 (Chiang et al., 2007; Fernandes et al., 2019; Liao et al., 2006; Mackenzie et al., 2016). Chiang et al. (2007) found a significant increase in DC MHC II expression and IL-12 secretion in male sprawly rats in response to 5 weeks progressive endurance treadmill running, but no significant increase in CD80/86 expression. They suggested the upregulation in MHC II and IL-12 indicates enhanced DC differentiation and maturation, potentially implicating greater antigen presentation ability, and a greater Th1 response to elicit antitumor immunity (Chiang et al., 2007). Chiang et al. (2007) utilised a periodised endurance protocol; a well-designed training programme consisting of progressive intensity increases and sufficient active recovery periods. Periodised endurance training has been shown to modulate immunity in human models (Liao et al., 2006), allowing for sufficient recovery before the next training session. It could therefore be argued that the favourable outcomes seen in Chiang et al. (2007) may not represent the responses that would be seen in an overtrained athlete. Additionally, differences between these studies' findings could also be due to the use of different DC stimulants i.e. Chiang et al. (2007) used LPS, whereas Mackenzie et al. (2016) used OVA stimulation. It has been shown that the OVA-stimulated and LPS-stimulated DC cytokine responses are different in rodents, with LPS inducing a larger response (Huang et al., 2013). As

such, these stimulants may also differently affect the expression of DC cell co-stimulatory molecules and MHC II on DCs upon stimulation.

Mackenzie et al. (2016) found a significant increase in DC CD86 expression, a significant decrease in MHC II expression and no significant differences in CD80 expression in mice who underwent 4 weeks of treadmill running at 6% maximum velocity for 1 hour/day, 5 days/week. DCs transmigrate between peripheral blood and the lymphatic system acting as immune sentinels (Brown et al., 2018). When infection occurs, DCs undergo maturation which involves the upregulation of co-stimulatory molecules CD80 and CD86, the MHC complex and IL-12 cytokine secretion (Wehr et al., 2019). All three of these signals are required for T cell activation, therefore, it is unlikely that an upregulation of one of these signals alone will significantly alter DC function, and ability to induce a T cell response. The discrepancies between findings of these studies (Chiang et al., 2007; Mackenzie et al., 2016), in addition to the lack of studies investigating these DC markers is a cause for expansion upon this preliminary work in rodents towards human models, which becomes increasingly important as exercise training may hold the potential to increase DC maturation, and thus antitumor immunity (Chiang et al., 2007). It is acknowledged however, that much is left to speculation, due to the limited numbers of studies assessing the DC response to intensified training, and those studies that have assessed the DC response, only examined rodent models. Therefore, we cannot make any conclusions in humans, but it does suggest some research in human models is required.

The immune system is a complex system. We cannot assume that periods of intensified training, or the cortisol alterations it may induce, affects all immune markers in the same way. Therefore, it would not be unreasonable to see variation in the changes of different immune activation markers with intensified training. Furthermore, resilience is the ability of the body to resist, adapt to, recover or grow in response to stressors (Chow et al., 2022). For example, high levels of immune resilience can reduce illness episodes/hospitalisations and accelerate immune recovery, as shown in COVID-19 patients (Justice et al., 2021). Differences in levels of immune resilience within the participants used in these studies could therefore lead to variations in immune activation and recovery to the same stress, and as such, we cannot assume that all immune systems will respond in the same way to the same level of stressor.

5.6 Conclusion

This review identified numerous immune biomarkers that have been investigated before and after a period of intensified training. Although this review focuses on the normal impact of high intensity training due to the difficulties surrounding confirmation of NFOR/OTS diagnosis, heavy training is a factor involved in the establishment of NFR/OTS, therefore, the results presented could provide evidence that these immune biomarkers are potentially indicative of NFOR/OTS. Results suggest that although some biomarkers indicated significant alterations after a period of intensified training (resting $CD8^+$ T Cell and total lymphocyte number, unstimulated IL-1β secretion, and DC CD86 expression), definitive immune biomarkers indicative of the negative states of overtraining are limited. Incompatibilities in methodologies and units of measurements

between studies, as well as low study numbers contributed to the inability to identify more definitive immune biomarkers within the literature. It must also be acknowledged that whilst the majority of studies included controlled for recent infections and diet (i.e. no alcohol or caffeine), various other lifestyle factors could influence immune outcomes and cause large amounts of heterogeneity between studies. Previous studies have shown that inadequate sleep can impair immune function and increase susceptibility to infection (Prather et al., 2015), psychological stress can alter lymphocyte trafficking and cytokine production (Ishikawa & Furuyashiki, 2022) and nutritional status, particularly low energy availability, is associated with increased immune cell ROS production, reduced lymphocyte proliferation, lower exercise induced leukocyte mobilisation and altered T cell metabolism (Jeppesen et al., 2024; Sarin et al., 2019). Additionally, variation in methodological approaches, including the use of supervised versus remote or self-directed exercise protocols, also introduces compliance-based variability that may influence physiological responses.

This review highlights the need for further research into biomarkers specifically relating to DCs, especially in human models. Additionally, although this review aimed to include females, no study returned from the systematic search controlled for menstrual cycle meaning only male data could be included. Therefore, future research should aim to conduct a controlled study of immune biomarkers in female subjects in response to a period of intensified training in order to widen the applicability of findings. The inclusion of humoral immunity, such as the measure of sIgA was not considered for this review, however, future reviews should include this as a possible immune biomarker to provide a more holistic overview of the immune state after a period of intensified training. Overall, a period of intensified training has been shown to significantly decrease resting total lymphocyte counts, resting $CD8^+$ T cell counts and unstimulated IL-1β levels, and significantly increase DC CD86 expression.

This systematic search of the literature confirmed that definitive immune markers, indicative of the negative states of overtraining are lacking (Baker et al., 2021). The review highlighted the need for further research into biomarkers specifically relating to DCs, especially in human models including female participants. As discussed in Chapter 4, hormone alterations that occur with periods of heavy training are not seen at rest, only in response to a short exercise stress (Hough et al., 2013; Hough et al., 2015). These hormones are known to regulate immune cells, and as such, any immune alterations that occur with intensified training may also not be evident at rest. To identify the exercise-induced immune changes associated with intensified periods of training, the reproducibility of immune cell responses to the exercise stress test used firstly needs to be established and thus will be explored in Chapter 6. Once confirmed, any exercise-induced immune alterations that occur after periods of intensified training are likely due to the training stress itself, and not due to random variation associated with the exercise stress test.

6 Chapter 6: The reproducibility of dendritic cell and T cell counts to a 30-minute high-intensity cycling protocol as a tool to highlight overtraining

This Chapter is a modified version of the peer-reviewed research article published in Experimental Physiology: *Baker, C., Piasecki, J., Hunt, J. A., & Hough, J. (2024). The reproducibility of dendritic cell and T cell counts to a 30-min high-intensity cycling protocol as a tool to highlight overtraining. Experimental Physiology, 109(3), 380–392. https://doi.org/10.1113/EP091326*

6.1 Introduction

As sport continues to push the physiological boundaries of performance, there is a growing expectation for athletes to undertake higher training loads. It is established that successful training regimes involve the fine-tuned balancing of progressively overloading the body's physiological systems whilst also ensuring adequate recovery (Meeusen et al., 2010). If sufficient recovery is not achieved, short-term decrements in performance may arise in as little as 7 days (Halson et al., 2002). As explained in Section 2.1, if appropriate recovery is implemented at this point, a 'super-compensatory' response in performance can occur (FOR) (Birrer et al., 2013), however if adequate recovery is not implemented alongside the high training loads, the athlete may experience NFOR/OTS (Schwellnus et al., 2016). Despite the previously described high incidence of NFOR/OTS across an athlete's career (Birrer et al., 2013; Morgan et al., 1987), little progress has been made on uncovering objective and reliable biomarkers that focus on identifying the occurrence of NFOR/OTS and the underlying mechanisms leading to the associated illness symptoms (Armstrong & Vanheest, 2002; Schwellnus et al., 2016).

It seems that periods of heavy training, most notably those that induce NFOR/OTS, may alter aspects of immune function that could possibly lead to increased episodes of URIs (Spence et al., 2007; Walsh, 2019). The highly debated 'Open Window' theory described in Chapters 2 and 5 (Simpson et al., 2020), caused by a cortisol-driven reduction in circulating Tc cells (Steensberg et al., 2001) has been alluded to as the main culprit for this heightened infection risk. Two major subsets of the previously described DCs, are found in peripheral blood: mDCs and pDCs. mDCs mainly stimulate antigen-specific T cells, whereas pDCs produce high levels of type 1 interferons and play an important role in anti-viral defence (Suchanek et al., 2010). Despite their important role in orchestrating the innate and adaptive responses, and their potential as a biomarker in the identification of NFOR/OTS, they have not been readily researched.

As highlighted in previous Chapters, cortisol is a hormone secreted from the HPA axis in response to mental and physical stress (Hill et al., 2008) and possess anti-inflammatory properties in the immune system. For example, cortisol is a major regulator of the maturation and migration of DCs towards lymph nodes (Liberman et al., 2018). Chapter 4 describes the reported HPA axis maladaptation after periods of intensified training in more detail, however in brief, periods of intensified exercise have been shown to induce a blunted cortisol response to exercise (Meeusen et al., 2004). Due to its key regulatory role in the immune system, this blunted cortisol response to exercise may in part lead to impaired immune function during intensified training.

The maladaptive functioning of the HPA axis after periods of intensified training described briefly above, and in more detail in Section 4.1, is not apparent at rest but is highlighted when examining the HPA axis response to exercise stress. Hough et al. (2011 & 2021) identified robust and reproducible cortisol increases the 30-minute 55/80 in healthy male athletes. The same group found a ~72% blunting in the cortisol response to the 55/80 test after an 11-day intensified training period when compared with a test before the intensified training period (Hough et al., 2013). Similarly, Meeusen et al. (2004) used two maximal cycle tests separated by 4 hours in well-trained athletes to show that the exercise-induced responses of cortisol and ACTH to the second maximal cycle were reduced by ∼118% and ∼73%, respectively, after a 10-day training period compared to before.

However, the 55/80 stress test is based solely on the percentage of W_{max}, identified from work rate achieved at $\dot{V}O_{2max}$. It has been argued that using a percentage of maximum to prescribe exercise intensity assumes that all participants will experience the same homeostatic perturbations to the same relative intensity, not taking into account submaximal physiological thresholds (Jaminick et al., 2020). However, large differences in homeostatic perturbations, that is, oxygen uptake kinetics and blood lactate responses, have been reported across multiple studies using exercise within the 'moderate intensity' zone (60–80% $\dot{V}O_{2max}$) (Jamnick et al., 2020). As such, the use of submaximal anchors, such as the VT_1, is recommended to prescribe exercise intensity (Mann et al., 2013). Therefore, to ensure the stress test is stressful enough to highlight any immune alterations that may occur due to excessive exercise in all participants, an adjusted version of this 55/80 stress test that prescribes intensity based on the VT_1 has been developed for use in the current study.

It has been shown that the total number of DCs can be elevated and phenotypic changes induced by 20–30 minutes of exercise (Brown et al., 2018; Deckx et al., 2015). Additionally, the 55/80 stress test used by Hough et al. (2011 & 2021) to show reproducible cortisol increases induced a mean HR of ∼162 bpm, and an RPE of ∼13, which is similar to the mean HR of ∼152 bpm and mean RPE of ∼13 elicited by the 20/50 in the current study. These studies suggest that the 30-minute adapted version of the 55/80 stress test; the 20/50, may also be stressful enough to elicit robust and reproducible changes in immune cell number. If reproducible DC and T cell count responses also occur in response to the 20/50 exercise stress test, when implemented before and after a period of intensified training, changes in immune cell count and/or function could be identified as possible biomarkers of NFOR/OTS.

The aim of the current study was to establish the reproducibility of immune cell count responses to an adapted version of the 55/80 stress test that was previously developed by Hough et al. (2011) that has

previously been shown to elicit reproducible cortisol changes. Specifically, the reproducibility of peripheral blood total DCs, mDCs, pDCs, total $CD3^+$ T cells, $CD8^+$ T cells and $CD4^+$ T cells to the 20/50 was examined. It was hypothesised that the 30-minute 20/50, would induce robust and reproducible immune cell count changes, increasing post exercise from baseline and declining back towards baseline 30 minutes post exercise.

6.2 Methods

In this experimental trial twelve healthy males volunteered to complete the study. As detailed in the general methods, all participants gave their written informed consent (Appendix 10.1) and completed a health questionnaire (Appendix 10.2) before taking part in all experimental trials. The participant characteristics at baseline are outlined in Table 6.1. All were free from URIs for at least 2 weeks prior to testing and none were taking any medications. All participants were required to undertake structured exercise at least three times per week to be included.

Table 6.1. Descriptive characteristics of participants.

	Mean ± SD
Age (y)	26.4 ± 5.8
Height (cm)	182.5 ± 5.3
Weight (kg)	81.6 ± 8.3
BMI (kg/m^2)	24.48 ± 1.98
$\dot{V}O_{2peak}$ (ml/kg/min)	48.58 ± 7.14

n = 12

6.2.1 Pre-experimental procedures and determination of exercise intensity

Height (Seca 217 stadiometer, Seca, Hamburg, Germany) and body mass (Seca 761 scales, Seca, Hamburg, Germany) were collected using standard methods, and $\dot{V}O_{2peak}$ was assessed on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands), using the protocol described in Section 3.2.2 of the general methods (Chapter 3), for the identification of W_{max} and VT_1.

6.2.2 Exercise trial and blood sampling

At least 7 days after participants completed their $\dot{V}O_{2peak}$ test, participants reported to the laboratory for their first exercise trial (Figure 6.1). Participants completed both exercise trials at the same time of day to ensure limited influence of circadian rhythms (commencement ranging from 09.20 to 13.30 hours), 2–7 days apart and were instructed to consume the same foods and to drink at least 500 mL of water on the morning before each visit to ensure hydration. Once in the laboratory, urine osmolality was assessed. Participants then undertook seated-rest whilst completing the 76 item REST-Q (Kellmann & Kallus, 2001) (Appendix 10.3). After ~15-minute seated-rest, 8–12 mL blood was collected via venepuncture from the antecubital fossa into two

4– 6 mL EDTA vacutainers following procedures outlined in Chapter 3. After a 3-minute warm up at 50 W, the 30-minute 20/50 began, with HR and RPE measured 30 seconds before the end of each interval. Immediately after the 20/50 (within ~2 minutes), a venepuncture blood sample was collected. Participants rested, then 30 minutes later the final blood sample was collected. The same protocol was followed for the second exercise trial.

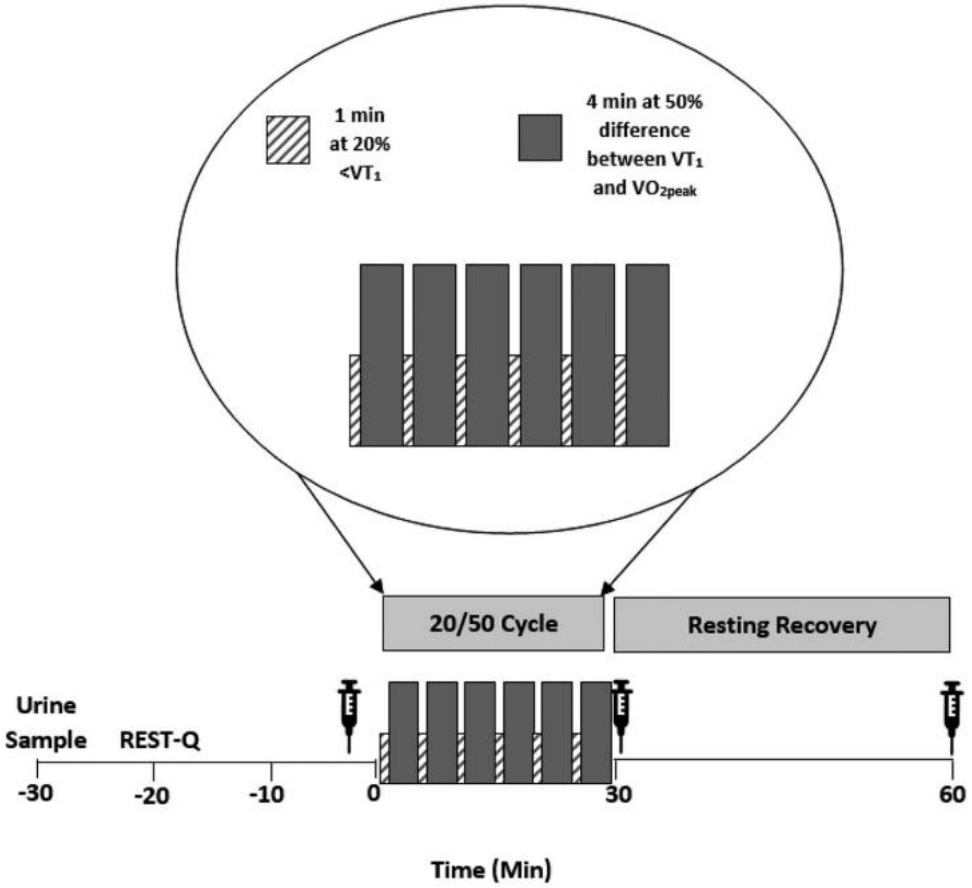


Figure 6.1. Schematic of the 20/50 exercise trials. The syringe symbol represents a venepuncture blood sample.

6.2.3 Peripheral blood mononuclear cell isolation and flow cytometry

PBMCs were isolated from whole blood, counted and stained as described in Section 3.8. The PBMCs were stained to identify total $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, total DCs ($HLA\text{-}DR^+$ $Lineage^-$), pDCs ($CD123^+$ $CD11c^-$) and mDCs ($CD123^-$ $CD11c^+$). The exact antibodies and quantity of antibodies used, alongside the gating strategy is described in Section 3.8.

The Accuri C6 flow cytometer calculates cells/ul of sample run automatically. Therefore, making it a single platform flow cytometer. This uses a peristaltic pump system to measure the volume of sample used during a run. It then uses the volume of measurement with the number of events to calculate cells/uL. Cell counts are therefore presented as cells/mL cell suspension.

6.2.4 Statistical analysis

Data were examined using SPSS Statistics version 28 (IBM Corp., Armonk, NY, USA) for normal distribution using the Kolmogorov–Smirnov test. Non-normally distributed data were logarithmically transformed and re-examined for normality. Data that were still non-normally distributed were analysed using non-parametric statistical tests, such that a Wilcoxon's signed-rank test was used to assess main effect of trial and post hoc time effects, and a Friedman's test was used to assess main effects of time. This was performed for pDCs only. Normally distributed immune cell count responses to the 20/50 exercise test were assessed using a two-way repeated ANOVA to examine the effects of trial (20/50 trials 1 and 2) and time on the immune cell counts. When the assumption of sphericity was violated, a Greenhouse–Geisser correction was applied. Student's paired samples t-test was used to examine differences between HR, RPE and REST-Q questionnaire scores between trials. Statistical significance was accepted at the $P< 0.05$ level. Data are presented as mean ± SD.

Relative reliability between the two 20/50 trials was assessed by calculating the ICCs and the intra-individual CVs. ICC was calculated via the $ICC_{2,1}$ model for the post-exercise cell counts using IBM SPSS Statistics version 28. ICC values < 0.40 indicate poor reliability, 0.40-0.59 indicate fair reliability, 0.60-0.74 good reliability and > 0.75 indicates excellent reliability (Fleiss, 1981). CV (%) was calculated from intra-individual delta pre to peak post cell counts and is presented alongside 95% confidence intervals (Table 6.3). Absolute reliability was assessed by Bland–Altman plot analysis and limits of agreements, and calculation of the closely related SRD was performed as described in Madsen et al. (2023) (Table 6.3).

6.3 Results

6.3.1 Physiological and recovery-stress data: HR, RPE and REST-Q questionnaire

Heart rate

Paired samples t-tests showed no significant difference in mean HR between the two 20/50 exercise trials ($t(11= 0.610$, $P = 0.554$) (Table 6.2).

RPE

Paired samples t-tests showed no significant difference in RPE between the trial 20/50 exercise trials ($t(11)= 1.887$, $P= 0.086$) (Table 6.2).

REST-Q

Paired samples t-tests showed no significant differences in the mean of total stress ($t(10)= 0.800$, $P= 0.442$) or the mean of total recovery ($t(10)= 0.740$, $P= 0.476$) (Table 6.2).

Table 6.2. Heart Rate (bpm) (n=12), Rating of Perceived Exertion (n=12) and REST-Q (n=11) mean total stress and recovery scores for the 20/50 trial 1 and trial 2.

	20/50 Trial 1	**20/50 Trial 2**	**Significance (P-Value)**
Heart Rate (bpm)	153 ± 14	152 ± 15	0.554
Rating of Perceived Exertion	14 ± 1	13 ± 1	0.086
REST-Q			
Total Stress	1.7 ± 0.5	1.6 ± 0.5	0.442
Total Recovery	3.0 ± 1.1	2.9 ± 1.1	0.476

Values are means ± standard deviation.

6.3.2 T cells

Total T cells ($CD3^+$)

The $CD3^+$ T cell response to both 20/50 exercise trials was similar ($F(1,11)= 1.191$, $P= 0.299$). There was a significant main effect of time ($F(2,22)= 39.237$, $P< 0.001$) with acute increases in peripheral blood $CD3^+$ from pre (1.49×10^6 cells/mL cell suspension) to post exercise (2.53×10^6 cells/mL cell suspension; $P< 0.001$), followed by a decrease towards baseline at 30 minutes post exercise (1.51×10^6 cells/mL cell suspension; $P< 0.001$). There was no significant interaction effect between trial and time point ($F(2,22)= 0.524$, $P= 0.600$) (Figure 6.2a).

An excellent reliability in the responses of $CD3^+$ T cell counts to the 20/50 exercise trials was found with an ICC of 0.970 (95% CI: 0.891, 0.992). Bland–Altman analysis indicates that the differences in $CD3^+$ T cell counts between trials ranged from 1.02 to -0.69×10^6 cells/mL blood. 100% of $CD3^+$ T cell measurements lay within the upper (1.19×10^6 cells/mL cell suspension) and lower (-0.88×10^6 cells/mL cell suspension) limits of agreement (Figure 6.2b).

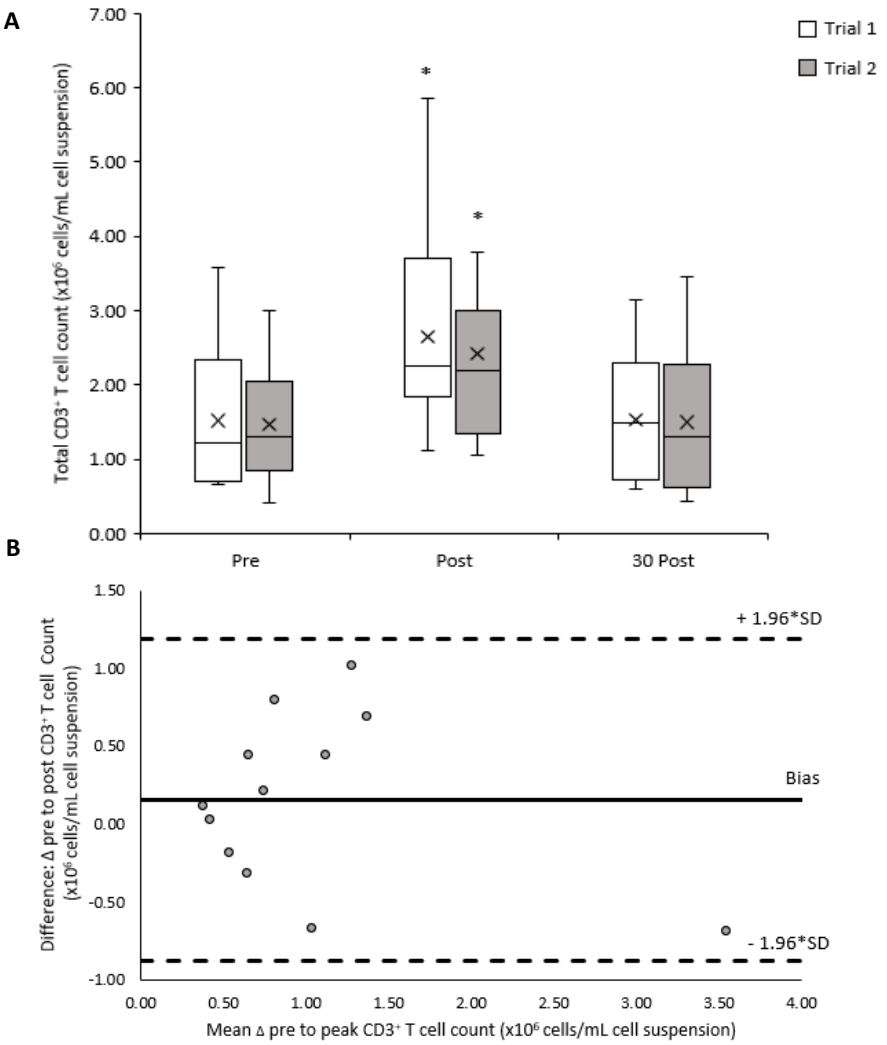


Figure 6.2a. Box plots of $CD3^+$ T cell counts pre, post and 30 minutes post 20/50 exercise bouts in both repeated trials. X denotes the mean.
* significantly different to baseline (pre exercise), n=12.

6.2b. Bland -Altman plot: Differences between delta (Δ) pre to post $CD3^+$ T Cell counts between trial 1 and 2 by the average of the two.
Dotted lines indicate the upper and lower limits of the 95% confidence interval for the average differences, n=12.

When $CD3^+$ T cells were measured as a percentage of PBMCs, There was a significant main effect of time ($F(2, 22)= 37.365$, $P< 0.001$), but not trial ($F(1,11)= 0.379$, $P= 0.551$) or time*trial interaction ($F(2, 22)= 0.986$, $P= 0.389$). Specifically, the percentage of $CD3^+$ T cells significantly decreased ($P< 0.001$) in response to the 20/50, returning to baseline in both trials ($P= 1.00$) (Figure 6.3).

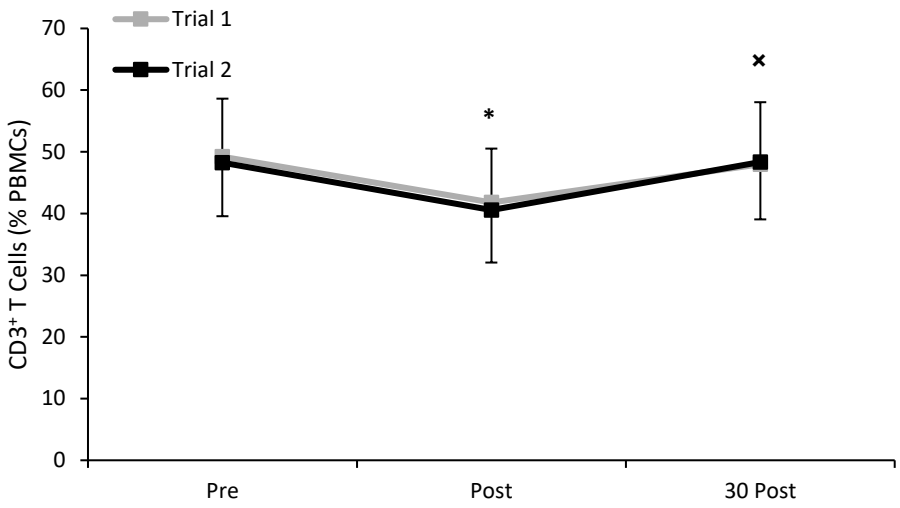


Figure 6.3. $CD3^+$ T cells as a percentage of PBMCs pre, post and 30 minutes post 20/50 exercise bouts in both repeated trials.
Data presented as mean ± standard deviation (n=12).
* significantly different to baseline (pre exercise)
X significantly different to post exercise

$CD4^+$ T cells

The $CD4^+$ T cell response to both 20/50 exercise trials was similar ($F(1,11)= 0.995$, $P= 0.340$). There was a significant main effect of time ($F(2, 22)= 25.544$, $P< 0.001$) with acute increases in peripheral blood $CD4^+$ from pre (0.86×10^6 cells/mL) to post exercise (1.23×10^6 cells/mL cell suspension) ($P< 0.001$), followed by a decrease towards baseline at 30 minutes post exercise (0.86×10^6 cells/mL cell suspension) ($P= 0.001$). There was no significant interaction effect between trial and time point ($F(2, 22)= 1.539$, $P= 0.237$) (Figure 6.4a)

An excellent reliability in the response of $CD4^+$ T cell counts to the 20/50 exercise trials was found with an ICC of 0.951 (95% CI: 0.814, 0.986). Bland–Altman analysis indicates that the differences in $CD4^+$ T cell counts between trials ranged from 0.45 to -0.33×10^6 cells/mL cell suspension. 100% of $CD4^+$ T cell measurements lay within the upper (0.53×10^6 cells/mL cell suspension) and lower (-0.38×10^6 cells/mL cell suspension) limits of agreement (Figure 6.4b).

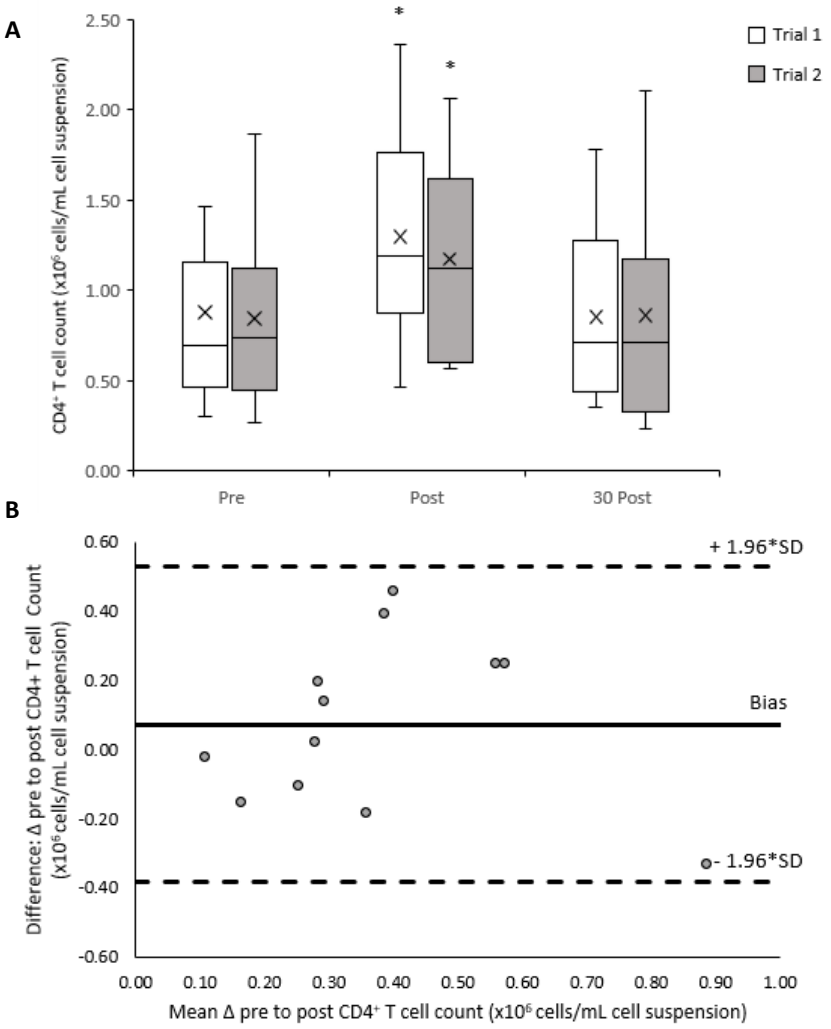


Figure 6.4a. Box plots of $CD4^+$ T cell counts pre, post and 30 minutes post 20/50 exercise bouts in both repeated trials. X denotes the mean.
* means significantly different to baseline (pre exercise), n=12.

6.4b. Bland -Altman plot: Differences between delta (Δ) pre to post CD4+ T Cell counts between trial 1 and 2 by the average of the two.
Dotted lines indicate the upper and lower limits of the 95% confidence interval for the average differences, n=12.

When $CD4^+$ T cells were measured as a percentage of lymphocytes, There was a significant main effect of time ($F(2,22)= 44.440$, $P< 0.001$), but not trial ($F(1,11)= 0.190$ $P= 0.671$) or time*trial interaction ($F(2, 22)= 0.479$, $P= 0.625$). Specifically, the percentage of $CD4^+$ T cells significantly decreased ($P< 0.001$) in response to the 20/50, returning to baseline in both trials ($P= 1.00$) (Figure 6.5).

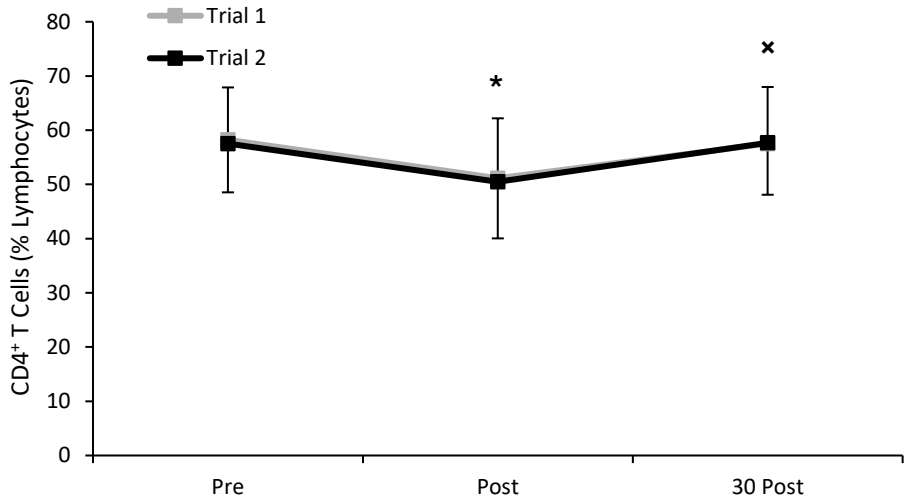


Figure 6.5. $CD4^+$ T cells as a percentage of lymphocytes pre, post and 30 minutes post 20/50 exercise bouts in both repeated trials.
Data presented as mean ± standard deviation (n=12).
* significantly different to baseline (pre exercise)
X significantly different to post exercise

$CD8^+$ T cells

The $CD8^+$ T cell response to both 20/50 exercise trials was similar ($F(1,11)= 0.177$, $P= 0.682$). There was a significant main effect of time ($F(2,22)= 45.123$, $P< 0.001$) with acute increases in peripheral blood $CD8^+$ from pre (0.45×10^6 cells/mL cell suspension) to post exercise (0.86×10^6 cells/mL cell suspension) ($P< 0.001$), followed by a decrease towards baseline at 30 minutes post-exercise (0.46×10^6 cells/ml cell suspension) ($P< 0.001$). There was no significant interaction effect between trial and time point ($F(2, 22)= 0.496$, $P= 0.615$) (Figure 6.6a).

An excellent reliability in the response of $C8^+$ T cell counts to the 20/50 exercise trials was found with an ICC of 0.971 (95% CI: 0. 901, 0.991). Bland–Altman analysis indicates that the differences in $CD8^+$ T cell counts between trials ranged from 0.42 to -0.42×10^6 cells/mL cell suspension. 100% of $CD8^+$ T cell measurements lay within the upper (0.49×10^6 cells/mL cell suspension) and lower (-0.42×10^6 cells/mL cell suspension) limits of agreement (Figure 6.6b).

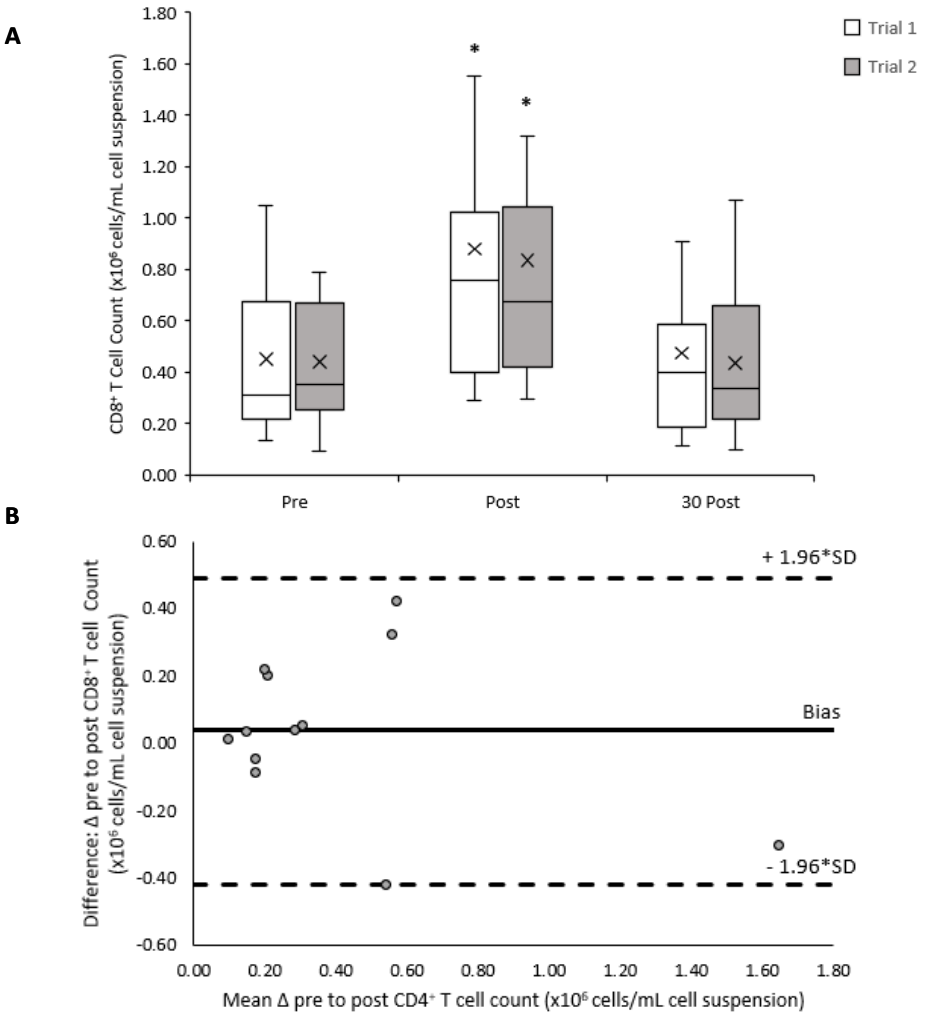


Figure 6.6a. Box plots of $CD8^+$ T cell counts pre, post and 30 minutes post 20/50 exercise bouts in both repeated trials. X denotes the mean.
* means significantly different to baseline (pre exercise), n=12.

6.6b. Bland -Altman plot: Differences between delta (Δ) pre to post CD8+ T Cell counts between trial 1 and 2 by the average of the two. Dotted lines indicate the upper and lower limits of the 95% confidence interval for the average differences, n=12.

When $CD8^+$ T cells were measured as a percentage of lymphocytes, There was a significant main effect of time ($F(2,22)= 9.354$, $P= 0.001$), but not trial ($F(1,11)= 1.718$ $P= 0.217$) or time*trial interaction ($F(2,22)= 0.636$, $P= 0.539$). Specifically, the percentage of $CD8^+$ T cells significantly increased ($P= 0.012$) in response to the 20/50, returning to baseline in both trials ($P= 1.00$) (Figure 6.7).

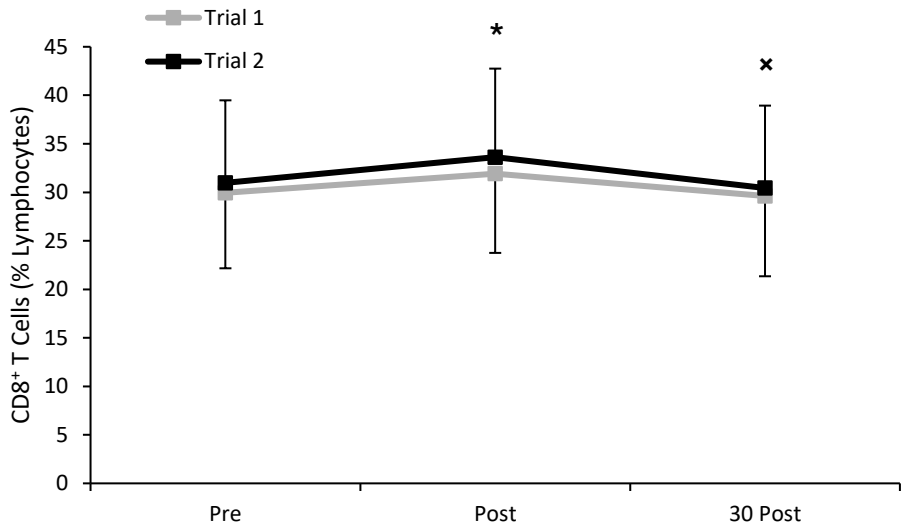


Figure 6.7. $CD8^+$ T cells as a percentage of lymphocytes pre, post and 30 minutes post 20/50 exercise bouts in both repeated trials.
Data presented as mean ± standard deviation (n=12).
* significantly different to baseline (pre exercise)
X significantly different to post exercise

6.3.3 Dendritic cells

Total DCs (lineage$^-$ HLA-DR$^+$)

The total DC response to both 20/50 exercise trials was similar ($F(1,11)= 1.254$, $P= 0.287$). There was a significant main effect of time ($F(2,22)= 51.000$, $P< 0.001$) with acute increases in peripheral blood total DCs from pre (1.03×10^5 cells/mL cell suspension) to post exercise (2.21×10^5 cells/mL cell suspension) ($P< 0.001$), followed by a decrease towards baseline at 30 minutes post exercise (1.05×10^5 cells/mL cell suspension) ($P< 0.001$). There was no significant interaction effect between trial and time point ($F(2,22)= 3.596$, $P= 0.058$) (Figure 6.8a).

A good reliability in the response of total DC counts to the 20/50 exercise trials was found with an ICC of 0.745 (95% CI: 0.189, 0.925). Bland–Altman analysis indicates that the differences in total DC counts between trials ranged from 0.85 to -0.83×10^5 cells/mL cell suspension. 92% (11 out of 12) of total DC measurements lay within the upper (1.34×10^5 cells/mL cell suspension) and lower (-0.67×10^5 cells/mL cell suspension) limits of agreement (Figure 6.8b).

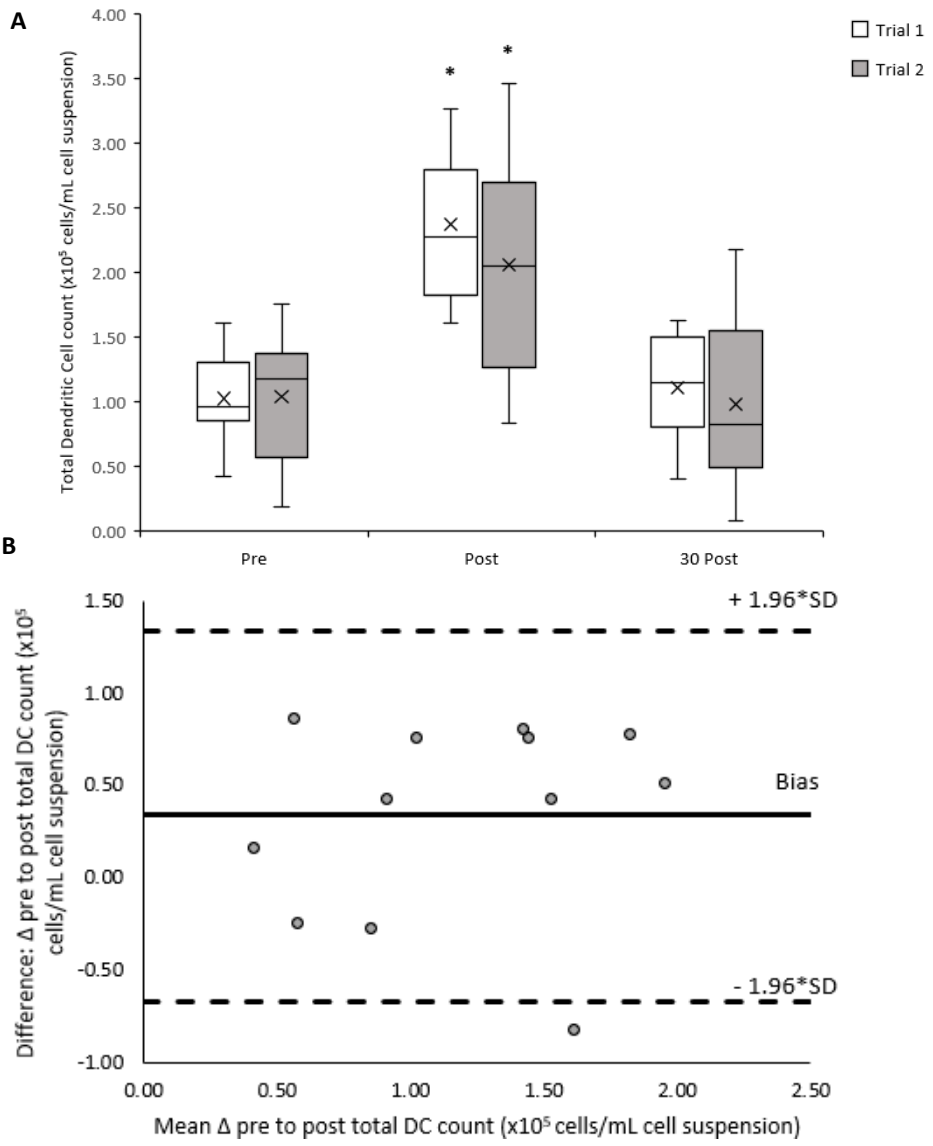


Figure 6.8a. Box plot of total dendritic cell counts pre, post and 30 minutes post 20/50 exercise bouts in both repeated trials.
X denotes the mean.
* means significantly different to baseline (pre exercise), n=12.

6.8b. Bland -Altman plot: Differences between delta (Δ) pre to post Total DC counts between trial 1 and 2 by the average of the two.
Dotted lines indicate the upper and lower limits of the 95% confidence interval for the average differences, n=12.

When total DCs were measured as a percentage of PBMCs, there was no significant main effect of time ($F(2, 22)= 1.902$, $P=0.173$), trial ($F(1,11)= 0.402$ $P= 0.539$) or time*trial interaction ($F(2,22)= 0.289$, $P=0.752$). (Figure 6.9).

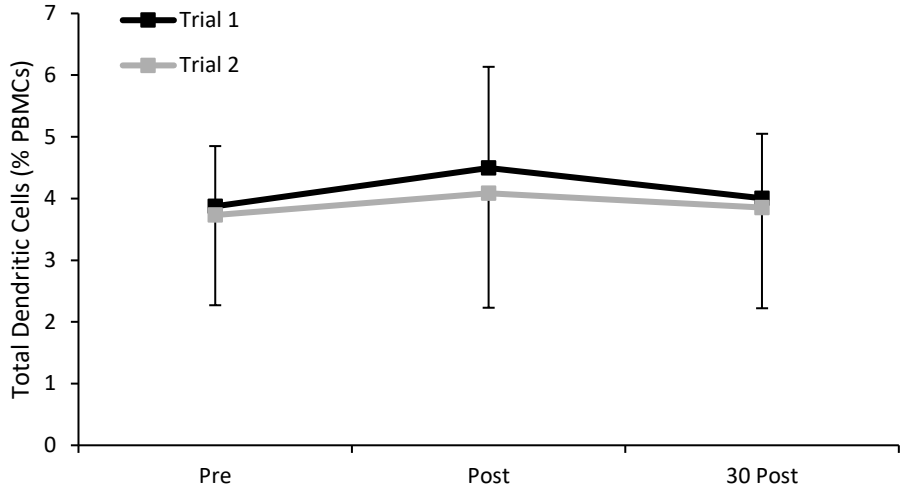


Figure 6.9. Total DCs as a percentage of PBMCs pre, post and 30 minutes post 20/50 exercise bouts in both repeated trials.
Data presented as mean ± standard deviation (n=12).

Myeloid DCs ($CD11c^+$ $CD123^-$)

The mDC response to both 20/50 exercise trials was similar ($F(1,11)= 0.423$, $P= 0.529$). There was a significant main effect of time ($F(2,22)= 41.233$, $P< 0.001$) with acute increases in peripheral blood mDCs from pre (0.74×10^5 cells/mL cell suspension) to post exercise (1.61×10^5 cells/mL cell suspension) ($P< 0.001$), followed by a decrease towards baseline from at 30 minutes post exercise (0.74×10^5 cells/mL cell suspension) ($P< 0.001$). There was no significant interaction effect between trial and time point ($F(2, 22)= 1.337$, $P= 0.282$) (Figure 6.10a).

A good reliability in the response of mDC counts to the 20/50 exercise trials was found with an ICC of 0.674 (95% CI: −0.111, 0.906). Bland–Altman analysis indicates that the differences in mDC counts between trials ranged from 0.78 to -0.70×10^5 cells/mL cell suspension. 100% of mDC measurements lay within the upper (1.14×10^5 cells/mL cell suspension) and lower (-0.74×10^5 cells/mL cell suspension) limits of agreement (Figure 6.10b).

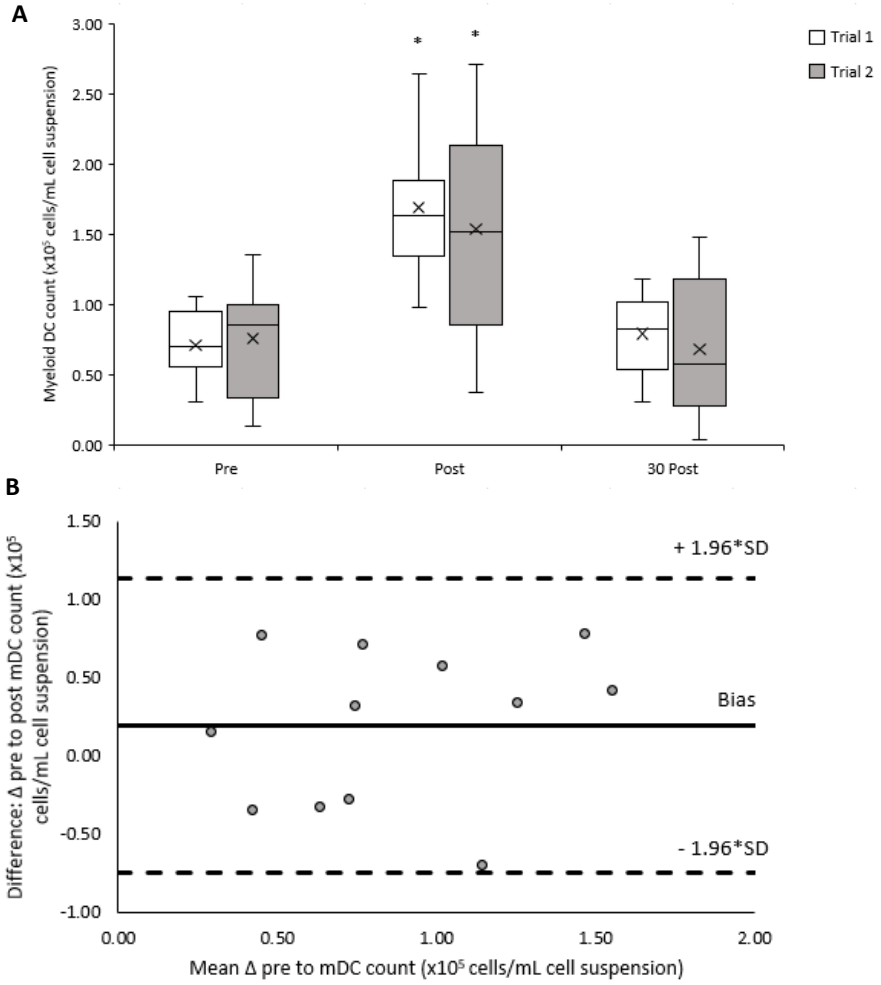


Figure 6.10a. Box plot of myeloid dendritic cell counts pre, post and 30 minutes post 20/50 exercise bouts in both repeated trials. X denotes the mean.
* means significantly different to baseline (pre exercise), n=12.

6.10b. Bland -Altman plot: Differences between delta (Δ) pre to post myeloid DC counts between trial 1 and 2 by the average of the two.
Dotted lines indicate the upper and lower limits of the 95% confidence interval for the average differences, n=12.

When mDCs were measured as a percentage of total DCs, There was a significant main effect of time $X^2(5)= 14.190$, P= 0.014, but not trial (Z= -0.228, P= 0.082). Specifically, the percentage of mDCs was significantly lower 30 minutes post 20/50 compared to pre (P= 0.028) and immediately post (P= 0.003) (Figure 6.11).

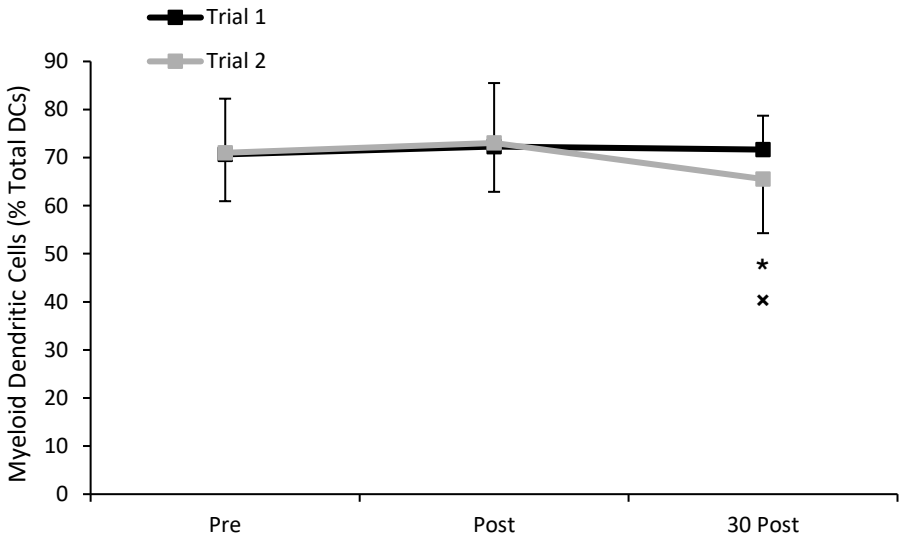


Figure 6.11. mDCs as a percentage of total DCs pre, post and 30 minutes post 20/50 exercise bouts in both repeated trials.
Data presented as mean ± standard deviation (n=12).
* significantly different to baseline (pre exercise)
X significantly different to post exercise

Plasmacytoid DCs ($CD11c^{-}$ $CD123^{+}$)

The pDC response to both 20/50 exercise trials was similar (Z= −1.225, P= 0.220). There was a significant main effect of time ($\chi^2(5)$= 39.511, P< 0.001), with acute increases in peripheral blood pDCs from pre (0.12×10^5 cells/mL cell suspension) to post exercise (0.25×10^5 cells/mL cell suspension) (Z= −3.059, P= 0.002) that remained elevated above baseline 30 minutes post exercise (0.13×10^5 cells/mL cell suspension) (Z= −2.510, P= 0.012) in trial 1. Acute increases in peripheral blood pDCs from pre- to- post exercise (Z= −3.059, P= 0.002) were also found in trial 2 but decreased to baseline 30 minutes post exercise (Z= −0.628, P= 0.530) (Figure 6.12a).

An excellent reliability in the response of pDC counts to the 20/50 exercise trials was found with an ICC of 0.821 (95% CI: 0.384, 0.949). Bland–Altman analysis indicates that the differences in pDC counts between trials ranged from 0.16 to -0.08×10^5 cells/mL cell suspension. 92% (11 out of 12) of pDC measurements lay within the upper (0.14×10^5 cells/mL cell suspension) and lower (-0.11×10^5 cells/mL cell suspension) limits of agreement (Figure 6.12b).

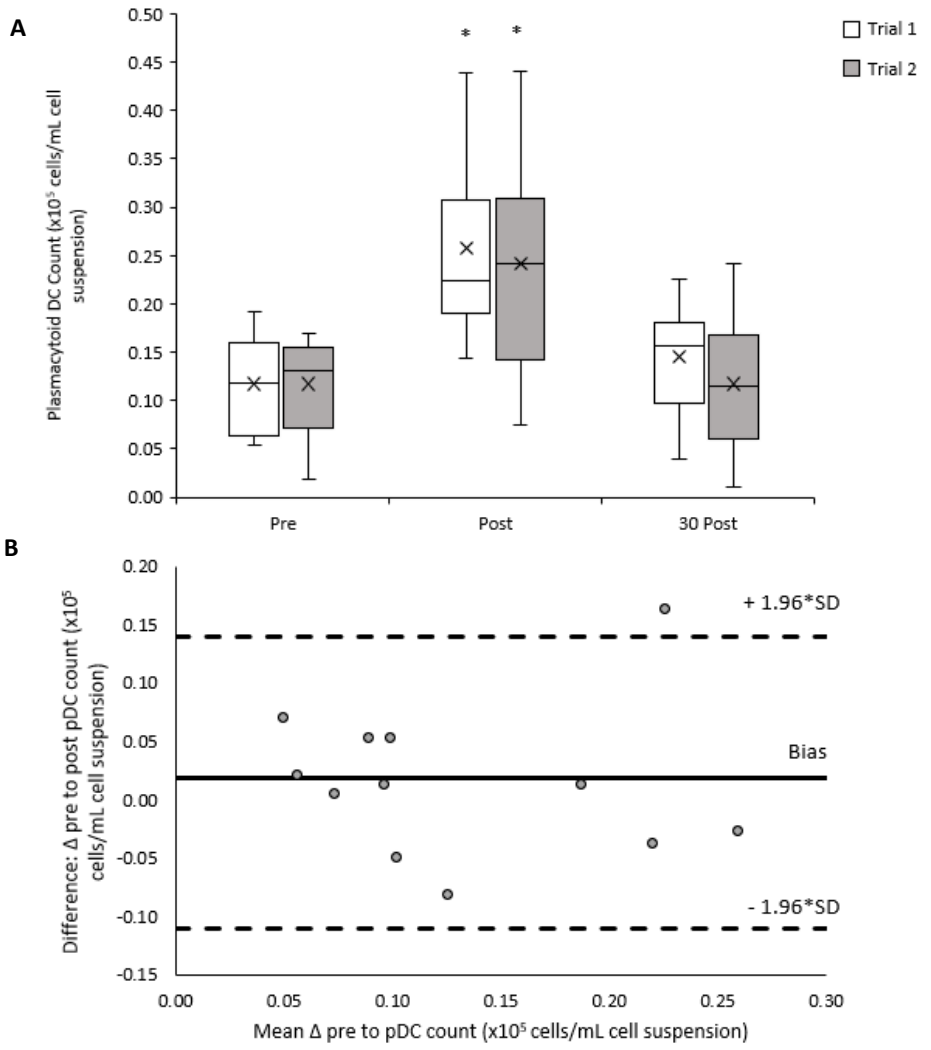


Figure 6.12. Box plot of Plasmacytoid dendritic cell counts pre, post and 30 minutes post 20/50 exercise bouts in both repeated trials. X denotes the mean.
Counts are presented as mean ± standard deviation x10^5 cells/mL cell suspension.
* means significantly different to baseline (pre exercise), n=12.

6.12b. Bland -Altman plot: Differences between delta (Δ) pre to post pDC counts between trial 1 and 2 by the average of the two.
Dotted lines indicate the upper and lower limits of the 95% confidence interval for the average differences, n=12.

When pDCs were measured as a percentage of total DCs, there was a significant main effect of time ($F(2,22)= 4.685$, $P= 0.020$), but not trial ($F(1,11)= 0.044$, $P= 0.838$) or time*trial interaction ($F(2,22)= 0.783$, $P= 0.469$). Post hoc analysis revels no significant pairwise comparisons for time ($P> 0.05$).

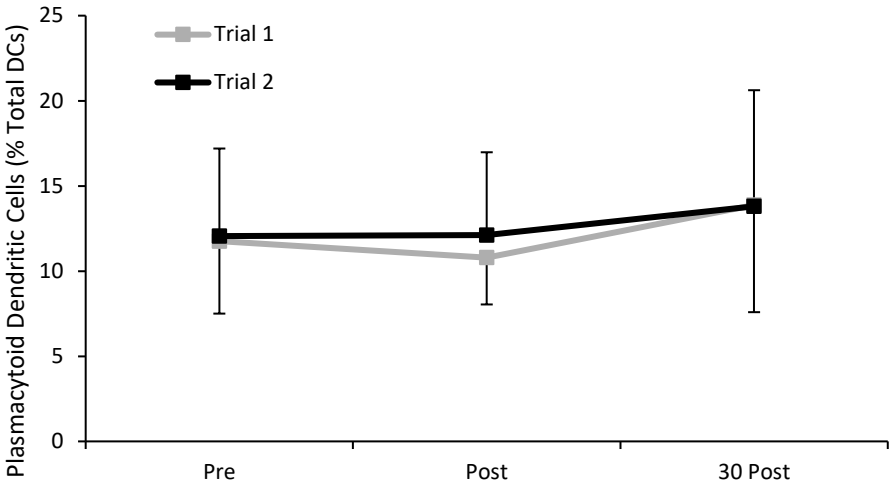


Figure 6.13. Plasmacytoid dendritic cells as a percentage of total DCs pre, post and 30 minutes post 20/50 exercise bouts in both repeated trials.
Data presented as mean ± standard deviation (n=12).

Table 6.3. Mean Intra-individual coefficients of variation (%) with 95% confidence intervals for pre, post and 30 minutes post 20/50, and the smallest real difference (SRD) for delta pre- to- peak post exercise cell counts. Variation (SD) around the mean fold change (pre to post 20/50) cell counts and cell ingress (pre to post 20/50), presented as mean ± SD

	$CD3^+$ T cells	**$CD4^+$ T cells**	**$CD8^+$ T cells**	**Total DCs**	**mDCs**	**pDCs**
			Coefficient of Variation (%) (CI Upper – CI Lower)			
Pre 20/50	12.9 (9.8 -16.0)	13.6 (10.0- 17.2)	12.8 (9.9-15.7)	19.1 (10.0-28.2)	21.2 (11.02-31.3)	14.0 (5.7-22.2)
Post 20/50	7.7 (4.0-11.4)	8.9 (5.5-12.3)	8.9 (4.7-13.1)	15.1 (7.4-22.8)	18.1 (8.0-28.3)	15.3 (8.3-22.2)
30 min Post 20/50	14.1 (8.3-19.8)	13.9 (8.3-19.4)	14.5 (8.8-20.2)	25.2 (14.3-36.2)	31.5 (19.0-44.1)	23.43 (15.5-31.4)
			SRD (units)			
	2.7 (2.2-3.2)	0.7 (0.6-0.9)	1.3 (1.1-1.6)	1.8 (1.5-2.1)	1.5 (1.2-1.7)	0.2 (0.2-0.3)
			Mean Fold Change (Pre to Post 20/50)			
Mean ± SD	1.9 ± 0.6	1.7 ± 0.5	2.1 ± 0.7	2.5 ± 1.0	2.6 ± 1.0	2.4 ± 0.8
			Cell Ingress (Pre to Post 20/50)			
Mean ± SD $X10^6$ Cells/uL cell suspension	1.5 ± 0.8	0.9 ± 0.5	0.4 ± 0.3	0.3 ± 0.09	0.1 ± 0.05	0.01 ± 0.007

n=12

6.4 Discussion

This study aimed to establish the reproducibility of (1) immune cell counts, specifically T cells and DCs, and (2) physiological and perceived exertion responses to a 30-minute, high intensity cycle bout (20/50) to determine its usefulness as an exercise stress test to highlight immune alterations that may occur after periods of intensified training, or during NFOR/OTS. All T cell and DC numbers were elevated post exercise compared to pre exercise, and these elevations did not differ between the repeated 20/50 trials. Therefore, the hypothesis that the 20/50 would induce robust and reproducible immune cell count changes can be accepted. It was also hypothesised that immune cell counts would increase during exercise from baseline and decline back to baseline 30 minutes post exercise. This can be accepted for total T cells ($CD3^+$), $CD4^+$ T cells, $CD8^+$ T cells, total DCs and mDCs, but not for pDCs. pDCs remained elevated above baseline 30 minutes after the 20/50 bout in both trials. When proportions of gated cells were measured, $CD3^+$ and $CD4^+$ T cell percentage decreased in response to the 20/50, returning to baseline 30 minutes post, whereas the percentage of $CD8^+$ T cells elevated in response to the 20/50 before returning to baseline 30 minutes post. Dendritic cell proportions were more variable however, with no real alterations in the proportions of total DCs, mDCs or pDCs to the 20/50. Therefore, whilst cell counts may be useful biomarkers to highlight overtraining, cell proportions, at least in dendritic cells, may not be. A secondary aim was to measure the reproducibility of the physiological (HR) and perceptual (RPE) strain of the 20/50. Similar strains across both 20/50 exercise trials were found, which confirms that the physiological strain induced by the 20/50 may not be altered by repeated exposures. This is important if the 20/50 is to be used as a test to examine alterations of immune function during periods of heavy training stress.

Our findings showed robust increases in all T cell and DC counts from pre to post 20/50, followed by a reduction to baseline 30 minutes post 20/50 in all T cells and DCs apart from pDCs. In line with our findings, previous research has also shown a biphasic response in T cells such that during exercise, a transient period of lymphocytosis occurs, followed by a period of lymphocytopenia after cessation of exercise (Shek et al., 1995), with a similar pattern of mobilisation also shown in DCs (Brown et al., 2018; Ho et al., 2001). The increase in immune cells seen in our study in the peripheral blood was likely to be driven by increased haemodynamics and the release of catecholamines following the activation of the SNS, and the decline driven largely by the release of glucocorticoids, such as cortisol via the HPA axis (Dimitrov et al., 2010; Hill et al., 2008; Krueger & Mooren, 2007). In agreement with this, the pattern of immune cell response to the 20/50 mirrors that of the cortisol response to the 55/80 stress test, whereby cortisol increased from pre to post 55/80, and decreased back to baseline 30 minutes post (Hough et al., 2021). As discussed in Chapter 5, this post exercise decline in immune cell numbers could represent a redistribution of effector cells for enhanced immunosurveillance, and not exercise induced apoptosis (Peake et al., 2017; Simpson et al., 2020). The redistribution of immune cells with high effector functions is in line with the findings of the current study in which a preferential mobilisation of $CD8^+$ Tc cells occurred, increasing by ~92% from pre to post exercise compared to the ~43% increase in $CD4^+$ T cells.

The results of the current study indicate a ~114% increase in total DCs to the 20/50, with a ~113% and ~119% increase in pDC and mDCs, respectively. All returned to baseline 30 minutes after cessation of the 20/50 apart from pDCs, which although were on their way back down to baseline, were still significantly different to pre exercise pDC counts. Brown et al. (2018) showed that 20 minutes of cycling at 80% $\dot{V}O_{2max}$ increased total DC, pDC and mDC counts by ~150%, ~195% and ~131%, respectively, which all returned to baseline 30 minutes after cessation of exercise. The slight differences in magnitude of DC count changes may be due to the intensity of exercise used. The 20-minute steady state cycle at 80% $\dot{V}O_{2max}$ used by Brown et al. (2018) elicited a mean HR of 176 ± 7 bpm and an RPE of 16 ± 1. These physiological and perceptual strains were higher than the mean HR and RPE across the two trials induced by our 20/50 (153 ± 15 bpm and 14 ± 1, respectively). It has been experimentally evidenced that the degree of DC mobilisation is positively correlated with the concentration of catecholamines release into the blood during exercise, and that catecholamine release is increased with increasing exercise intensities (Suchanek et al., 2010; Zouhal et al., 2008). Therefore, differences in the magnitude of mobilisation of DCs into the circulation could be due to differences in exercise intensity.

Additionally, previous groups have indicated a preferential mobilisation of pDCs post exercise, compared to baseline (Brown et al., 2018; Suchanek et al., 2010). A vigorous ice-hockey training session increased pDCs two-fold compared to mDCs (~200% vs. ~100%, respectively) (Suchanek et al., 2010), and the 20-minute cycle at 80% $\dot{V}O_{2max}$ used by Brown et al. (2018) also induced a larger pDC mobilisation than mDC (~195% vs. ~131%, respectively). This preferential mobilisation of pDCs has been described as an adaptive process in which cells with potent anti-viral properties are redistributed (Brown et al., 2018). pDCs have been shown to possess greater inflammatory and migratory potential compared to mDCs (Liu et al., 2021), so it may be unsurprising that previous studies have found a preferential mobilisation of pDCs during exercise. As previously discussed, similarly to lymphocytes, the degree of DC mobilisation during intense exercise is positively correlated with the concentration of catecholamines released into the blood (Suchanek et al., 2010), operating via a dose dependent increase in exercise intensity, and relying upon density of β2-adrenorecptors on DCs (Nijhuis et al., 2014). However, our findings did not show a preferential mobilisation of pDCs. In fact, a slightly higher mDC mobilisation than pDC (~119% vs. ~113%) was highlighted.

Our findings do, however, coincide with Deckx et al. (2015) whereby patients with multiple sclerosis (MS) and healthy controls undertook a moderate to high intensity mixed endurance (15 minute cycle, 15 minute walk) and resistance (3 × 10 repetitions; 6 upper and lower body exercise) exercise bout. They found a lower increase in pDCs (50%) compared to mDCs (75%), with no differences between patients and healthy controls. Of particular interest, in line with our findings, this study also found that pDCs took longer than 30 minutes to return to baseline, specifically they returned to baseline after 2 hours of resting recovery. Differences in preferential mobilisation could be due to the differences in exercise format, that is, the 20/50 was interval based and may not elicit the same DC response as a continuous exercise bout, or the exercise intensity elicited by the 20/50 may have induced a lower catecholamine response than Brown et al. (2018) and Suchanek et al.

(2010). However, it is accepted that these hormones were not measured in the current study to confirm this argument.

Our analysis revealed that the reliability of T cell and DC count responses to the 20/50 can be interpreted as good (total DCs and mDCs) to excellent (total T cells, $CD4^+$ T cells, $CD8^+$ T cells and pDCs) according to ICC values, with no significant differences in cell count responses to exercise between the trials. All data points in the Bland–Altman analysis lay within the limits of agreement in 4 out of 6 cell types, with only one data point falling just outside for total DCs and pDCs, reinforcing conclusions drawn from the ICC values. The differences in the mean delta pre to peak post exercise cell counts between trial 1 and 2 for all cell types are lower than their respective SRD measurements, meaning we can conclude that these differences are likely caused by measurement error, and not systematic variability between trials (Vaz et al., 2013). These reliability results indicate that if implemented before and after a period of intensified training, any changes seen in the responses to the 20/50 are likely to represent actual immune alterations associated with negative states of overtraining and are not due to regular variation. Additionally, the mean fold change and mean ingress values for all cell types (signal) is larger than the standard deviations (noise) indicating that an effect (e.eg., in response to overtraining) would be observable again background variation (Table 6.3). As previously stated, there are currently no clear reliable biomarkers of overtraining capable of identifying the occurrence of NFOR/OTS. The results of the current study suggest that the 20/50 is a test capable of identifying whether immune alterations could be a useful biomarker in highlighting the occurrence of NFOR/OTS.

The ICC is commonly used as a measure of relative reliability, as it can conveniently categorise the reliability of a measure as poor, moderate, good or excellent (Hartmann et al., 2023; Koo & Li, 2016). However, caution must be taken when utilising ICC as a sole measure of reliability, as the high within-group SD associated with a very heterogeneous population may lead to a high ICC value regardless of how unreliable the method is (Hartmann et al., 2023). As such, it is argued that the ICC does not provide a comprehensive assessment of reliability when taken alone. Calculating the CV as another measure of relative reliability is therefore seen as beneficial for interpretation (Gomez & Gomez, 1984). Nevertheless, the CV is also not without its flaws, as when the mean is close to zero, inaccurate results can occur, which is likely the cause of the inflated CV values in the current study (Hartmann et al., 2023). As such, the addition of absolute reliability measurements via the Bland– Altman plot and calculation of the SRD is required to provide a more comprehensive review of overall reliability. Bland– Altman analysis provides a visual interpretation of measurement agreements whereby a reference range within which 95% of all differences between measurements are likely to lie (Mansournia et al., 2021). The SRD is easily interpretable because it provides an estimate of the maximal difference there will be between two measurements on 95% of occasions and is particularly important for clinical translation as it is reported in the same units as the measurement itself (Vaz et al., 2013). Vitally, our calculation of SRD took the n into account, rather than the more widely used $1.96 \times SEM \times \sqrt{2}$.

6.5 Conclusion

To conclude, the 20/50 exercise test elicited robust and reproducible DC and T cell responses. Consequently, when implemented before and after a period of intensified training, the 20/50 exercise test can identify whether exercise induced immune alterations are a useful biomarker in highlighting the occurrence of NFOR/OTS. The development of this test is important when considering the high incidence of NFOR/OTS in the athletic population. However, a limitation of the current work is that all participants were male. Females could potentially display a different immune response to the same stimulus, and therefore to make a viable test, both biological sexes should be assessed. Additionally, it must be noted that immune measures were not adjusted for haemoconcentration shifts that occur with exercise (Matomäki et al., 2018). Chapter 7 will investigate exercise induced immunological changes relating to DCs and T cells associated with intensified training by implementing the 20/50 before and after a period of intensive training, including both males and females. This will provide an overview of how the relationship between the innate and adaptive systems may be altered with intensified training, and/or when in a negative state of overtraining.

7 Chapter 7: The effects of a 9-day intensified training period on immune and endocrine biomarkers in healthy males and females.

7.1 Introduction

As explained in previous Chapters, the implementation of intensified training periods into an athlete's training cycle is common. During these periods, training volume/intensity is increased. This form of progressive overload is aimed at inducing positive training adaptations when coupled with sufficient recovery periods (FOR) (Meeusen et al., 2013). If mis-managed, athletes could encounter negative symptoms associated with NFOR or the OTS (Meeusen et al., 2013). These negative states of overtraining are described in more detail in Chapter 2, but symptoms may include increased risk of URI, poor sleep quality, low moods and importantly, a decline in performance (Cadegiani et al., 2018; Carrard et al. 2022; Lehmann et al., 1999). According to the ECSS and ACSM, the OTS is equally as debilitating as an orthopaedic injury, taking substantial time for recovery, thus highlighting the importance in establishing key biomarkers to detect when an athlete may be entering these negative states (Meeusen et al., 2013).

Both salivary and plasma cortisol and testosterone have previously been identified as potential biomarkers of overreaching. Whilst no differences in these hormones are seen at rest after intensified training periods, their response to acute exercise bouts are blunted after the training period (Hough et al., 2013; Uusitalo et al., 1998; Meeusen et al., 2013). Specifically, Hough et al. (2013) identified a ~166% and ~21% blunting in salivary cortisol and testosterone, respectively, in response to a 30-minute exercise stress test when comparing before to after an 11-day period cycling at 75% $\dot{V}O_{2max}$ for 1.5 hours daily (~143% intensification of training load). A similar blunting in salivary testosterone (~44%) has also been observed in elite triathletes after a 10-day intensified training period (Hough et al., 2015). Blunted plasma cortisol and testosterone responses to a $\dot{V}O_{2max}$ test after 4 weeks of intensified training (~130% intensification of training load) have also been observed in the order of ~20% and ~22%, respectively (Uusitalo et al., 1998). Interestingly, Uusitalo et al. (1998) found an inverse relationship between plasma testosterone concentrations and training volume. It has been reported that even smaller elevations in training intensity can lead to maladaptive endocrine responses to exercise, such that a ~38% increase in training load across 3 weeks in highly trained distance runners resulted in a ~122% blunted plasma cortisol response to a 30-minute treadmill run at 80% $\dot{V}O_{2max}$ (Verde et al., 1992). Importantly, both cortisol and testosterone are key regulators of the immune system, thus maladaptive endocrine responses to intensified training periods may lead to a dysfunctional immune system.

As described in previous Chapters, the process of DC antigen presentation relies on their ability to recognise, uptake, process and load antigen onto MHCs. Primarily, DCs use their intracellular and extracellular TLRs to

recognise invading microbes by their PAMPs (Dempsey et al., 2003; Palm & Medhitov, 2009; Yamamoto & Takeda, 2010). Once activated, TLRs control the generation of adaptive immunity through the induction of MHC and co-stimulatory (CD80/86) molecules, and pro-inflammatory cytokines (Lancaster et al., 2005).

TLR7 and 9 are located intracellularly in endosomal compartments of pDCs and TLR8 in mDCs. Specifically, TLR7/8 and 9 recognise viral RNA and DNA, respectively, and together form an evolutionarily related subgroup within the TLR super family (Kadowaki et al., 2001; Larangé et al., 2009; Kawai & Akira, 2006). Upon ligand engagement, a downstream of signalling events occur resulting in the activation of NF-κB and the Interferon regulatory factor 7; the master regulator of type 1 interferon production, resulting in the production of pro-inflammatory cytokines, such as TNF-α and IFN-α (Bao et al., 2012). The specific mechanisms in which DCs take up and process antigen are described in detail in Chapter 2. Despite their important role in orchestrating the immune response, very little is currently known about how they respond to periods of heavy training.

Glucocorticoids have been shown to regulate TLR expression by directly modulating NF-κB transcriptional activity which is required for an increase in the expression of several TLR controlled genes (Lancaster et al., 2005). In monocytes; another type of antigen presenting cell, a synthetic glucocorticoid, dexamethasone, was shown to induce TLR2 and 4 gene expression in PBMCs (Galon et al., 2002). Additionally, the upregulation of costimulatory (CD80/86) molecules and MHC class II on monocytes after TLR stimulation was greater in samples obtained in the evening vs. in the morning, coinciding with circulating cortisol circadian rhythm (Lancaster et al., 2005). Furthermore, 1.5 hours of cycling at 55% W_{max} in the heat also decreased TLR expression on monocytes, coinciding with elevated stress hormones such as cortisol (Lancaster et al., 2005). Cortisol has also been shown to modulate the TLR transcriptional pathways and pro-inflammatory cytokine release of DCs. Specifically, DCs treated with cortisol possessed increased expression of TLR2, 3 and 4, but their subsequent ability to stimulate T cells from their TLR-derived signals was lessened (Rozkova et al., 2006). The same group also evidenced that patients treated with high dose corticosteroids displayed significantly reduced DC IL-12 and TNF-α secretion, impaired T cell stimulatory function and skewed DC differentiation, favouring a distinct population incapable of inducing an efficient immune response (Rozkova et al., 2006). Whilst somewhat unsurprising given the known anti-inflammatory properties of cortisol, it highlights the importance of understanding how endocrine alterations associated with intense training periods may impact important immunological mechanisms.

Therefore, the aim of the current study was to utilise the established 20/50 stress test discussed in Chapter 6 to highlight endocrine and immune alterations that may occur with a 9-day intensified training period in recreationally active males and females. Specifically, plasma and salivary cortisol and testosterone, T cell and DC counts, DC TLR7/8/9 expression and stimulated TNF-α, IFN- α and IL-10 production will be investigated. Highlighting endocrine and immune alterations that occur with the intensified training period may make them suitable objective biomarkers for highlighting when athletes are falling into the negative states of overtraining. Importantly, this study will further the understanding within the literature of how short duration heavy

training periods can impact DC function. It was hypothesised that the 9-day intensified training period would lead to blunted salivary and plasma cortisol and testosterone responses to the 20/50, lowered resting T cell and DC counts, and reduced elevations in T cells and DCs to the 20/50.

7.2 Methods

7.2.1 Ethical Approval

Prior to study participation, written informed consent (Appendix 10.1) was obtained from all participants, and health questionnaires were completed (Appendix 10.2). The study conformed to the standards set by the Declaration of Helsinki, except for registration in a database, and procedures were approved by the NTU ethics committee (Ethical approval number 1748383; Nottingham Trent University, UK).

7.2.2 Participants

Thirteen healthy males and one healthy female participated in the study (Table 7.2). All were free from URIs for at least 2 weeks prior to testing and none were taking any medications. All participants completed a training diary and HR monitoring for 7 days prior to commencing the study to quantify normal training load as described in Chapter 3. The participants abstained from exercise, caffeine, and alcohol intake 24 hours before each main trial.

7.2.3 Female menstrual cycle tracking

To be eligible for the study, female participants had to be naturally menstruating and have had at least 9 cycles in the past 12 months. Female participants tracked their menstrual cycle in a diary (Appendix 10.6) over one full cycle as described in Chapter 3. Both 20/50 main trials were performed during menses and training sessions occurring during the luteal phase.

7.2.4 Preliminary measures

On laboratory visit 1, height (Seca 217 stadiometer, Seca, Hamburg, Germany) and body mass (Seca 761 scales, Seca, Hamburg, Germany) were collected using standard methods. $\dot{V}O_{2peak}$ was assessed on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands) using a continuous ramp protocol as described in Chapter 3.

7.2.5 10 km time trial (before and after the intensified training period)

On visit 2 (before the intensified training period) and visit 14 (after the intensified training period), participants arrived at the laboratory at 7:30am and were instructed to cycle 10 km as quickly as possible on an electronically braked cycle ergometer using a self-selected intensity (Lode Excalibur Sport, Groningen, The

Netherlands) under controlled conditions. Participants were blinded from the time. HR, RPE, time (min:sec) and power (W) were recorded every 1 km.

7.2.6 Main trials (Before and after the intensified training period)

On visits 3 (before the intensified training period) and 13 (after the intensified training period) participants arrived at the laboratory between 07:00-07:30am to ensure limited influence of circadian rhythms. Participants were instructed to consume the same breakfast and drink at least 500 mL of water on the morning before each visit to ensure hydration. Urine osmolality was then assessed as described in Chapter 3.

Participants undertook seated-rest whilst completing the REST-Q (Kellmann & Kallus, 2001). Following ~15 minutes of seated-rest, a venepuncture blood sample and saliva sample were collected as described in Chapter 3. Participants then completed the 30-minute 20/50 stress test as described in Chapter 3 and 6. Immediately after the 20/50, venepuncture blood and saliva samples were collected again. Participants drank water ad libitum during the main trials but were not permitted to drink in the 10 minutes before saliva sampling to avoid possible dilution of the saliva sample.

7.2.7 Training Days (visits 4-12)

Participants attended the laboratory at any time of day for 9 consecutive weekdays to undertake the intensified training period of 1.5 hour daily cycling at 75% $\dot{V}O_{2max}$ on top of their normal training load, as described in Chapter 3. As no supervised training was conducted over the weekend, participants were encouraged to continue their usual exercise routines away from the laboratory. Specifically, 70% of participants performed exercise over the weekend, with 30% of the reported TRIMP/day resulting from weekend training. In particular, most of the extra training sessions completed involved endurance running (> 5km) A schematic of study visits is shown in Figure 7.1.

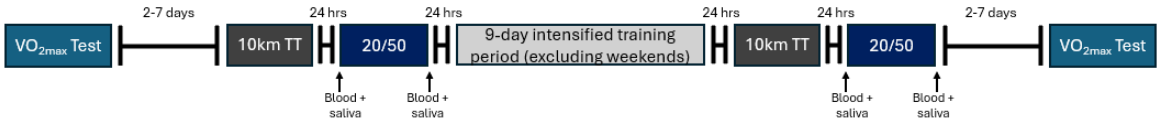


Figure 7.1. Schematic showing the layout of each laboratory visit.

7.2.8 Quantification of normal training load and intensified training load

Participants completed a training diary 7 days prior to commencing the study for the calculation of a TRIMP score to quantify normal training load, as described in Chapter 3 (Appendix 10.5). During the 9-day training period, participants were encouraged to continue normal exercise outside the laboratory in addition to the 1.5 hour supervised training sessions, which they recorded in a training diary. Participants were required to record the mode, duration and RPE of each session completed, alongside HR monitoring (average, minimum and maximum HR for the session) (Polar Beats, Polar Electro Oy, Kempele, Finland). TRIMP scores calculated

across the 9-day training period, including the 1.5 hour supervised cycles were compared to participants' TRIMP scores calculated from the pre training 7-day training diary to ensure an increase in normal training load was achieved.

7.2.9 Salivary and plasma analysis

Saliva and plasma samples were stored at -80˚C and analysed within two months of collection using commercially available ELISA kits as described in Chapter 3. The mean inter- assay CV for all ELISA plates was < 8.3%, apart from salivary testosterone which was 14.3%. All intra- assay CVs were < 8%.

7.2.10 Isolation and stimulation of PBMCs

PBMCs were then isolated, stimulated and stained in line with the protocol outlined in Chapter 3, Section 3.8.2, option B.

7.2.11 Flowcytometry analysis

As detailed in Chapter 3, Section 3.8.3, PBMCs were analysed via spectral flow cytometry (Cytek Auroura, Cytek Biosciences, Chester, UK) for total DCs, pDCs, mDCs, total T cells, $CD4^+$ T cells and $CD8^+$ T cells. DCs were further gated for TLR7, 8 and 9, IFN-α and TNF-α expression, and $CD4^+$ T cells were further gated for IL-10 expression.

Cell counts were calculated in cells/mL input blood for all cell types vie the following calculation:

$$\frac{\textbf{cell concentration (cells/mL) * volume of resuspension (mL)}}{\textbf{volume of whole blood isolated (mL)}}$$

Specifically, cells were counted via manual haemocytometer as described in Section 3.8.2 to provide a cell concentration of PBMCSs in cells/mL. As cells were suspended in 0.5 mL for counting, the cell concentration was multiplied by 0.5 mL. This provides the concentration of cells in the PBMC suspension (cells/mL). As 4 mL whole blood was isolated initially, this cell concentration was divided by 4 mL, resulting in the concentration of PBMCs in cells/mL of input blood.

Percentage gates were then used to calculate cells/mL input blood for each subtype i.e. if 60% of gated PBMCs were $CD3^+$ T cells, then 60% of the total PBMC concentration calculated above (cells/mL) would provide the cell concentration of $CD3^+$ T cells in cells/mL input blood.

7.2.12 Statistical Analysis

Data were examined using SPSS statistics version 28 (IBM CORP., Armonk, NY, USA). Normal distribution was assessed using the Kolmogorov-Smirnov test, with any non-normally distributed data logarithmically transformed and re-examined for normality. Data that remained non-normally distributed were analysed using non-parametric statistical tests. Wilcoxon's signed-rank test was used to assess main effect of trial and post hoc time effects, and a Friedman's test was used to assess main effects of time. Non-parametric tests

were performed for mDC count, TLR9 MFI, TLR8 MFI, pDC TNF-α and mDC TNF-α expression. All other data was normally distributed and assessed using a two-way repeated measures ANOVA to examine main effect of trial and time. When the assumption of sphericity was violated, a Greenhouse-Geisser correction was applied. All TLR and cytokine data are presented as MFI fold change which was calculated with respect to the unstimulated controls.

Student's paired samples t-test was used to examine differences between $\dot{V}O_{2peak}$, 10 km time trial, TRIMP and REST-Q scores before and after the intensified training period. One participant was not included in the TRIMP analysis due to an incomplete training diary, and one participant did not have a post training 10 km time trial result. Differences in mean HR and RPE scores during both 20/50 trials were also examined using student's paired samples t-tests. Statistical significance was accepted at the $P \leq 0.05$ level. All data are presented as mean ± SD.

Sample size estimations were based on our primary outcome variables, cortisol and testosterone, and T cell and DC counts. Completing an a priori power calculation, it was estimated that 10 participants would be required to see a meaningful difference in the main outcome variables in response to the exercise stress test (Cohen's *d* = 0.90) at 80% power and an α level of 0.05. To account for a 10% drop out rate, 14 participants were recruited.

7.3 Results

7.3.1 Participant characteristics

Table 7.1. Descriptive characteristics of participants.

	Pre Training	Post Training	P- Value
Age (y)	28 ± 6		
Height (cm)	166.9 ± 48.1		
Weight (kg)	81.5 ± 8.6	81.1 ± 8.7	0.136
BMI (kg/m^2)	25.5 ± 2.4	25.4 ± 2.4	0.130
$\dot{V}O_{2peak}$ (ml/kg/min)	49.37 ± 5.38	50.38 ± 6.59	0.446

Data are presented as mean ± standard deviation. All (n= 14), apart from post training $\dot{V}O_{2peak}$ (n= 8).

7.3.2 HR and RPE during the 20/50

Mean HR during the 20/50 was significantly lower post training (144 ± 19 bpm) compared to pre training (153 ± 24 bpm) ($t(13)= 4.613$, $P< 0.001$). Mean RPE during the 20/50 did not differ pre (14 ± 3) or post training (14 ± 3) ($t(13)= 0.552$, $P= 0.295$).

7.3.3 REST-Q

Sport related stress was significantly higher after the intensified training period (2.29 ± 0.97) compared to before (1.33 ± 0.80) (t(13)= -3.567, P= 0.002) (Figure 7.2). However, there were no significant differences in general stress (t(13)= -0.295, P= 0.773), general recovery (t(13)= -0.234, P=0.819) or sport related recovery (t(13)= 0.739, P= 0.237) after the training period.

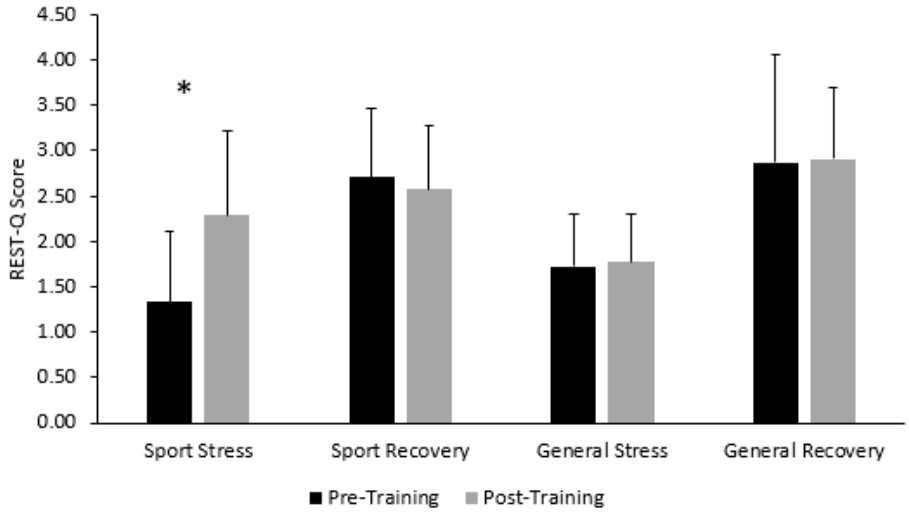


Figure 7.2.REST-Q scores for Sport related and general stress and recovery pre and post training. Data are presented as mean ± standard deviation, n=14.

7.3.4 10 km time trial performance

10 km time trial performance was significantly improved (t(12)= 2.489, P= 0.014) after the intensified training period (694.77 ± 141.50 seconds) compared to before (753.62 ± 178.51 seconds) (Figure 7.3).

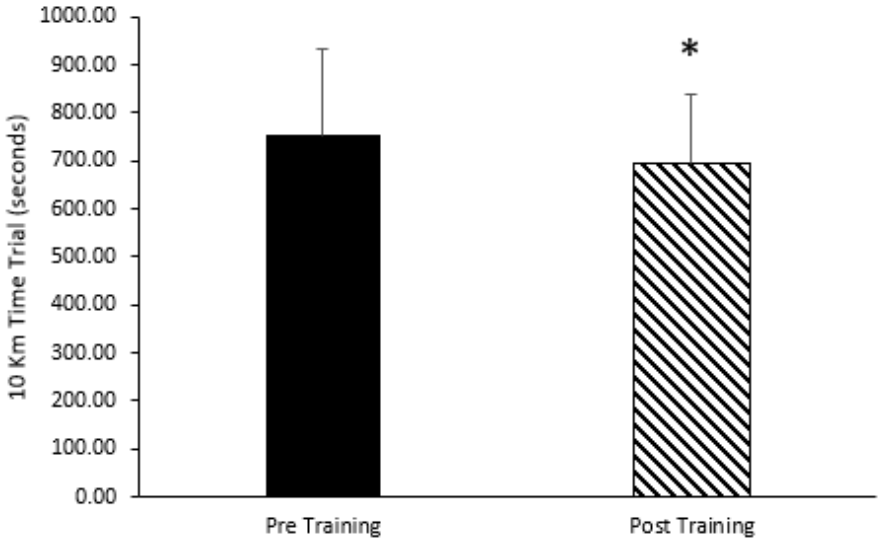


Figure 7.3. 10km time trial performance pre- and post- training.
Data are presented as mean ± standard deviation, n=13.
* significance (p<0.05).

7.3.5 $\dot{V}O_{2peak}$ (mL/kg/min)

For the 8 participants who also performed a post training $\dot{V}O_{2max}$ test, there were no significant differences in relative $\dot{V}O_{2peak}$ (mL/kg/min) after the intensified training period (49.4 ± 5.8 mL/kg/min) compared to before training (50.4 ± 7.0 mL/kg/min) (t(7)= -0.807, P= 0.223).

7.3.6 Training Impulse (TRIMP)

TRIMP/day scores were significantly elevated (t(12)= 25.587, P< 0.001) by ~84% during the intensified training period compared to 7 days of normal training (Figure 7.4).

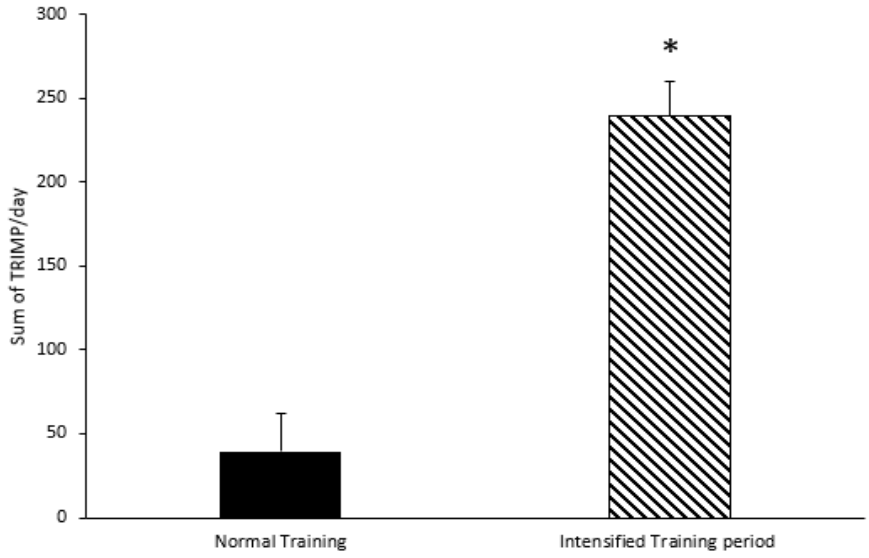


Figure 7.4. Average daily training impulse (TRIMP) scores over 7-days of normal training before the study and over the study period (TRIMP/day).
Data are presented as mean ± standard deviation, n=13.
* significance (p<0.05).

7.3.7 Immune measures

$CD3^+$ T cell counts

There was a significant main effect of time (F(1,13)= 28.090, P< 0.001), but not trial (F(1,13)= 3.681, P= 0.077) or time*trial interaction (F(1,13)= 0.030, P= 0.865). $CD3^+$ T cells were significantly elevated from pre to post 20/50 in both trials (1.43 to 1.99 $x10^6$ cells/mL input blood pre training and 1.24 to 1.77 $x10^6$ cells/mL input blood post training) (Figure 7.5).

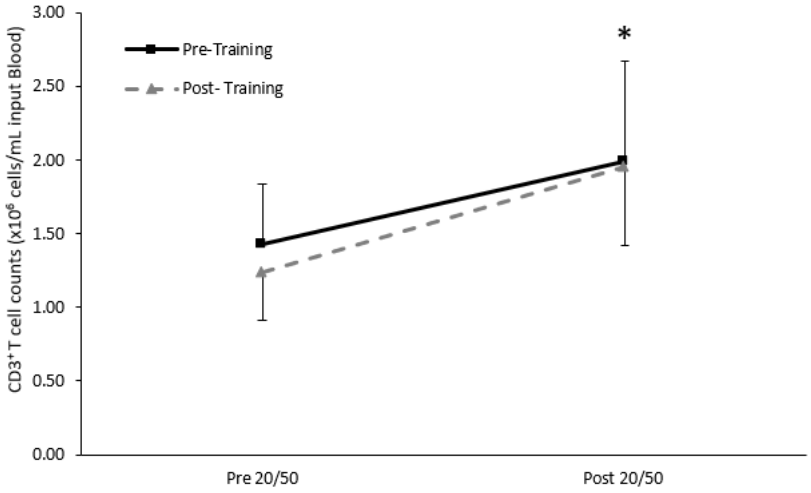


Figure 7.5. $CD3^+$ T cell counts ($x10^6$ cells/mL input blood) in response to the 20/50 pre and post training. Data are presented as mean ± standard deviation, n=14.
* significantly different to pre 20/50 ($P< 0.05$).

When assessing $CD3^+$ T cells as a percentage of the PBMC gate, there was a significant main effect of time ($F(1,13)= 13.674$, $P= 0.003$), whereby the percentage of $CD3^+$ T cells in the PBMC gate decreased in response to the 20/50 both pre (62.62 to 56.03%) and post training (65.26 to 60.29%). However, there was no significant main effect of trial ($F(1,13)= 0.093$, $P= 0.766$) or time*trial interaction ($F(1,13)= 0.428$, $P= 0.524$) (Figure 7.6).

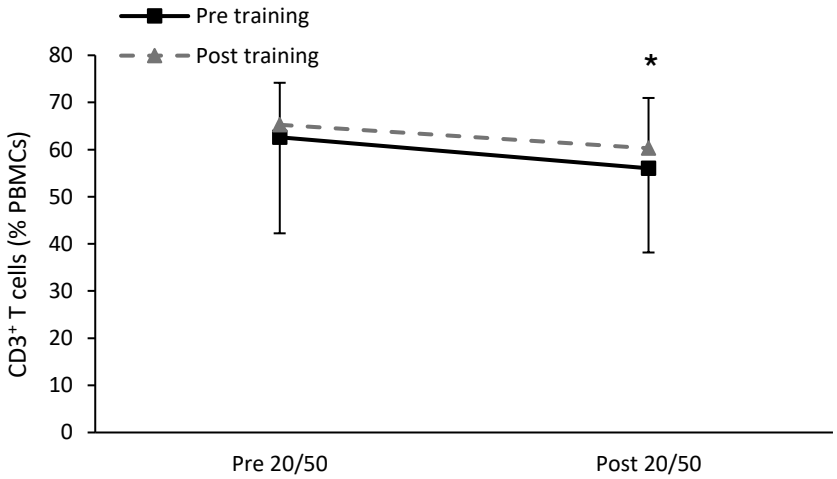


Figure 7.6. CD3+ T cells as a percentage of PBMCs in response to the 20/50 pre and post training. Data are presented as mean ± standard deviation, n=14.
* significantly different to pre 20/50 ($P< 0.05$).

$CD4^+$ T Cell counts

There was no significant main effect of time ($F(1,13)= 3.837$, $P= 0.072$), trial ($F(1,13)=3.230$, $P= 0.096$) or interaction ($F(1,13)= 0.033$, $P= 0.859$). $CD4^+$ T cells did not change from pre to post 20/50 in either trials (0.69 to 0.75 $x10^6$ cells/mL input blood pre training and 0.62 to 0.68 $x10^6$ cells/mL input blood post training) (Figure 7.6).

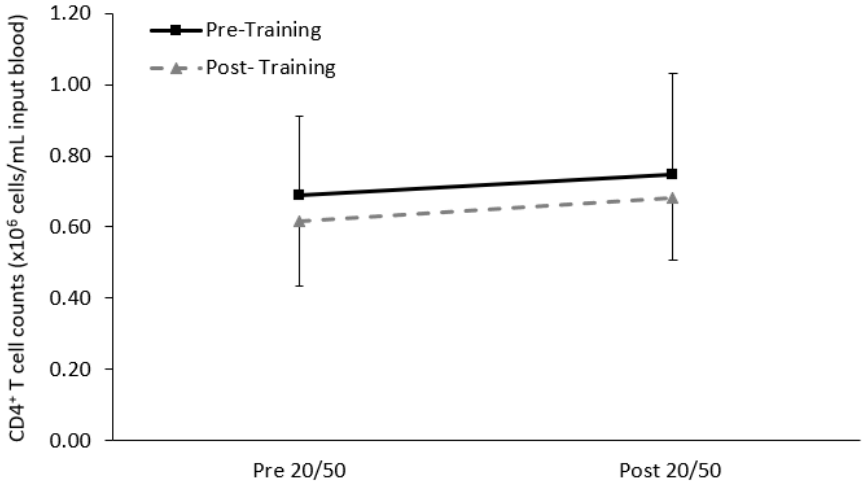


Figure 7.7. CD4$^+$ T cell counts (x10^6 cells/mL input blood) in response to the 20/50 pre and post training. Data are presented as mean ± standard deviation, n=14.

When assessing CD4$^+$ T cells as a percentage of the lymphocyte gate, there was a significant main effect of time ($F(1,13)= 76.139$, $P< 0.001$) whereby the percentage of CD4+ T cells in the lymphocyte gate decreased in response to the 20/50 both pre (45.45 to 35.97%) and post training (50.49 to 39.59%). However, there was no significant main effect of trial ($F(1,13)= 1.450$, $P= 0.250$) or time*trial interaction ($F(1,13)= 0.154$, $P= 0.701$) (Figure 7.8).

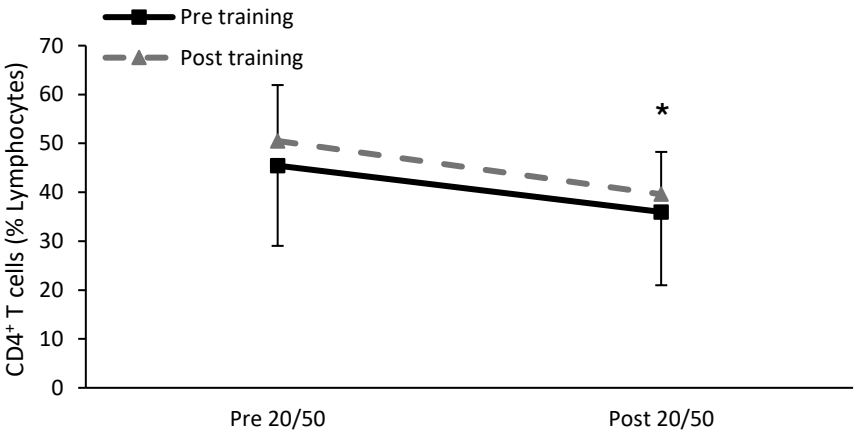


Figure 7.8. CD4+ T cell counts as a percentage of lymphocytes in response to the 20/50 pre and post training. Data are presented as mean ± standard deviation, n=14.
* significantly different to pre 20/50 ($P< 0.05$).

CD8$^+$ T cell counts

There was a significant main effect of time ($F(1,13)= 35.778$, $P< 0.001$), but not trial ($F(1,13)= 1.279$, $P= 0.279$) or time*trial interaction ($F(1,13)= 0.253$, $P= 0.624$). CD8$^+$ T cells were significantly elevated from pre to post 20/50 in both trials (0.47 to 0.77 x10^6 cells/mL input blood pre training, and 0.46 to 0.70 x10^6 cells/mL input blood post training) (Figure 7.9).

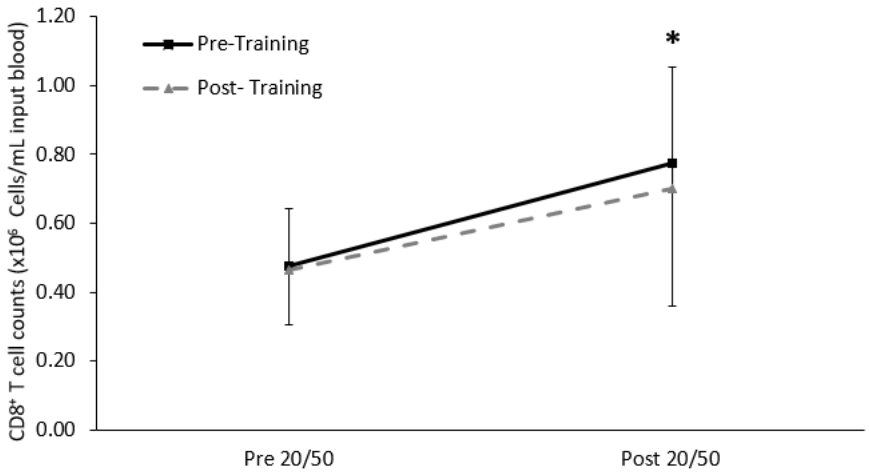


Figure 7.9. CD8$^+$ T cell counts (x10^6 cells/mL input blood) in response to the 20/50 pre and post training. Data are presented as mean ± standard deviation, n= 14.
* significantly different to pre 20/50 (P< 0.05).

When assessing CD8$^+$ T cells as a percentage of the lymphocyte gate, there was no significant main effect of time (F(1,13)= 3.620, P= 0.079), trial (F(1,13)= 0.118, P= 0.737) or time*trial interaction (F(1,13)= 4.135, P= 0.063) (Figure 7.10).

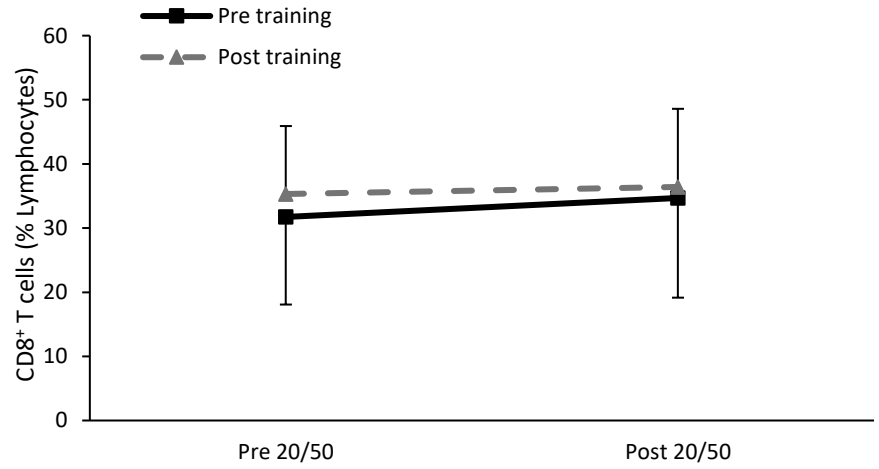


Figure 7.10. CD8+ T cell counts as a percentage of lymphocytes in response to the 20/50 pre and post training. Data are presented as mean ± standard deviation, n= 14.

Total DC counts

Wilcoxon's signed-rank test showed that there was a significant main effect of trial on total DC count (Z= -2.248, P= 0.025), whereby mean total DC count was higher pre training (0.70 ± 0.37 x10^5 cells/mL input blood) compared to post training (0.55 ± 0.19 x10^5 cells/mL input blood). Friedmans showed there was a significant effect of time (X^2(3)= 16.783, P< 0.001). Post hoc analysis revealed that the 55/80 significantly elevated total DC count post training (0.44 to 0.66 x10^5 cells/mL input blood, P< 0.001), but not pre training (0.62 to 0.78

x10^5 cells/mL input blood, P= 0.148) (Figure 7.8). Post hoc analysis also revealed that total DCs were significantly lower at rest post training compared to pre training (Z= -2.668, P= 0.008), but not significantly different post 55/80 between trials (Z= -0.565, P= 0.572).

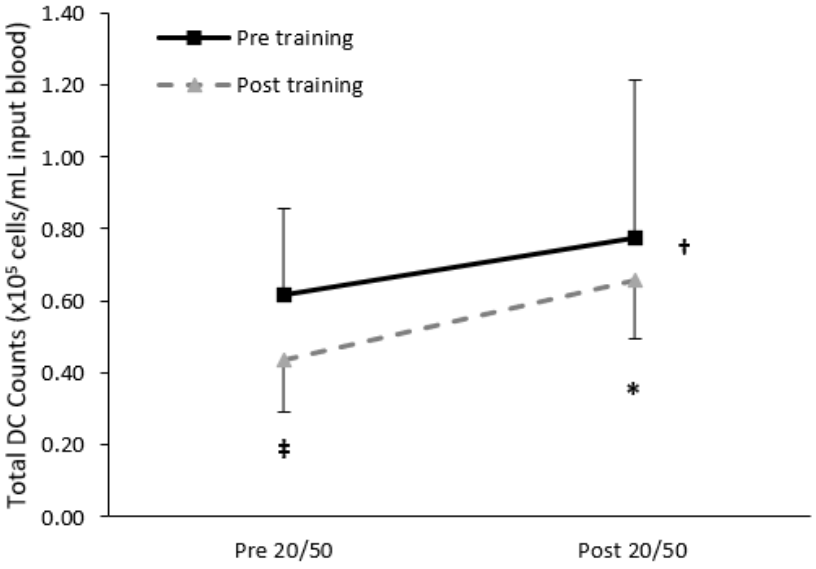


Figure 7.11. Total dendritic cell counts (x 10^5 cells/mL input blood) in response to the 20/50 pre and post training.
Data are presented as mean ± standard deviation, n=14.
* significantly different to pre 20/50 (P< 0.05).
† significant main effect of trial.
‡ significantly different to pre training.

When assessing total DCs as a percentage of PBMCs, there was no significant main effect of trial (F(1,13)= 1.615, P= 0.226) or time (F(1,13)= 3.733, P= 0.075), but a significant time*trial interaction (F(1,13) 13.335, P= 0.003) (Figure 7.12).

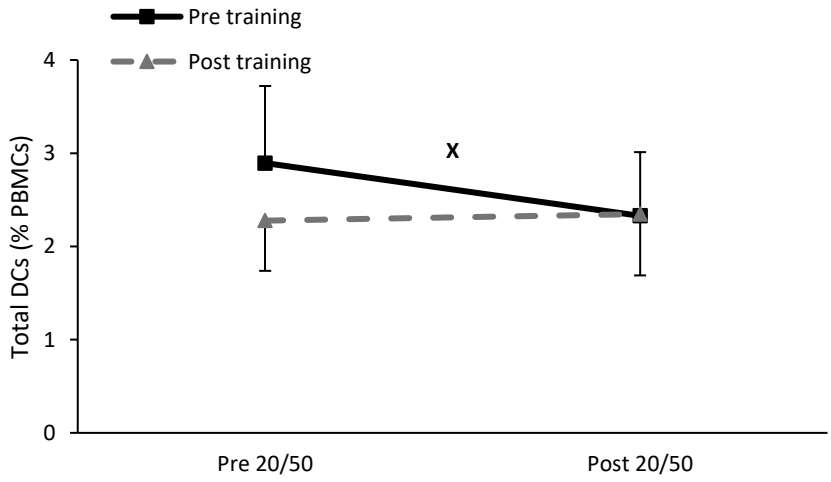


Figure 7.12. Total dendritic cells as a percentage of PBMCs in response to the 20/50 pre and post training.
Data are presented as mean ± standard deviation, n=14.
X significant interaction.

mDC counts

Wilcoxon's signed-rank test showed that there was no significant main effect of trial on mDC count (Z= -0.288, P= 0.773). Friedmans showed there was a significant effect of time ($X^2(3)$= 22.196, P< 0.001). mDCs were significantly elevated by the 20/50 in both trials (0.17 to 0.26 x10^5 cells/mL input blood pre training, and 0.18 to 0.27 x10^5 cells/mL input blood post training) (Figure 7.9).

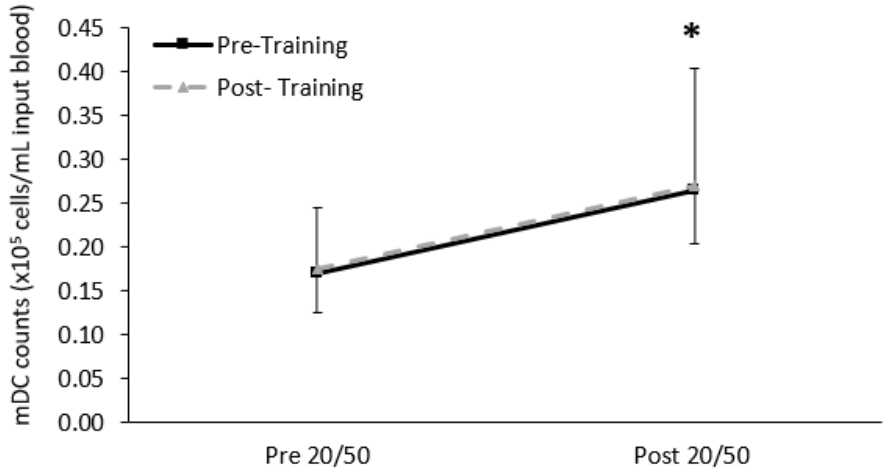


Figure 7.13. Myeloid dendritic cell counts (x 10^5 cells/mL input blood) in response to the 20/50 pre and post training.
Data are presented as mean ± standard deviation, n=14.
* significantly different to pre 20/50 (P< 0.05).

When assessing mDCs as a percentage of total DCs, there was no significant main effect of time (F(1,13)= 1.714, P= 0.213) or time*trial interaction (F(1,13)= 1.792, P= 0.204), but a significant main effect of trial (F(1,13)= 8.761, P= 0.011) whereby the percentage of mDCs in the lymphocyte gate was significantly higher post training (41.63 ± 2.43%) compared to pre training (33.19 ± 2.64%) (Figure 7.14).

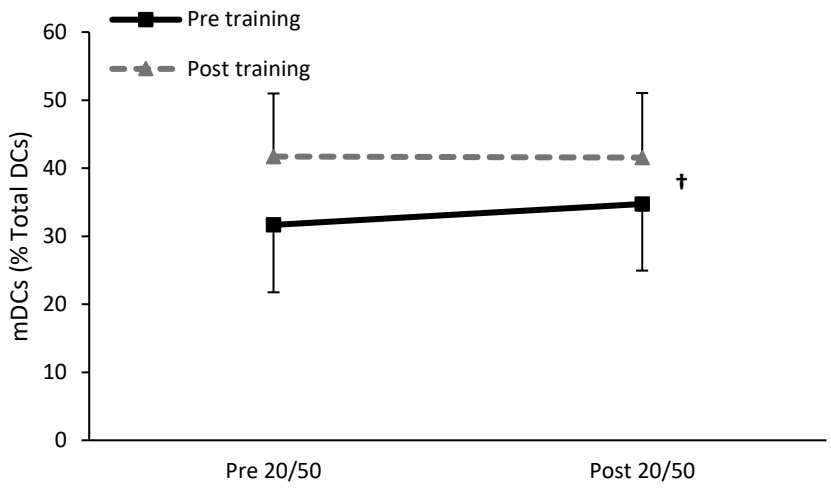


Figure 7.14. Myeloid dendritic cells as a percentage of total DCs in response to the 20/50 pre and post training. Data are presented as mean ± standard deviation, n=14.
† significant main effect of trial.

pDC counts

There was a significant main effect of time (F(1,13)= 140.082, P< 0.001), but not trial (F(1,13)= 0.062, P= 0.807) or time*trial interaction (F(1,13)= 1.064, P= 0.321). pDCs were significantly elevated from pre to post 20/50 in both trials (0.12 to 0.22 x10^5 cells/mL input blood pre training, and 0.11 to 0.23 x10^5 cells/mL input blood post training) (Figure 7.10).

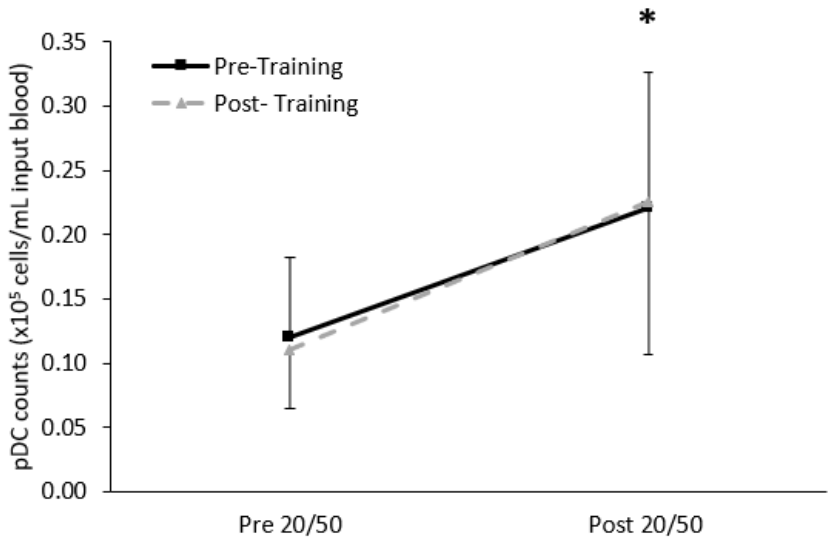


Figure 7.15. Plasmacytoid dendritic cell counts (x10^5 cells/mL input blood) in response to the 20/50 pre and post training. Data are presented as mean ± standard deviation, n=14.
* significantly different to pre 20/50 (P< 0.05).

When assessing pDCs as a percentage of total DCs, there was a significant main effect of time (F(1,13)= 29.822, P< 0.001), whereby the percentage of pDCs of total DCs increased to the 20/50 both pre (39.93 to 45.81%)

and post training (37.80 to 43.32%), but not trial (F(1,13)= 2.861, P =0.115) or time*trial interaction (F(1,13)= 1.573, P= 0.232) (Figure 7.16).

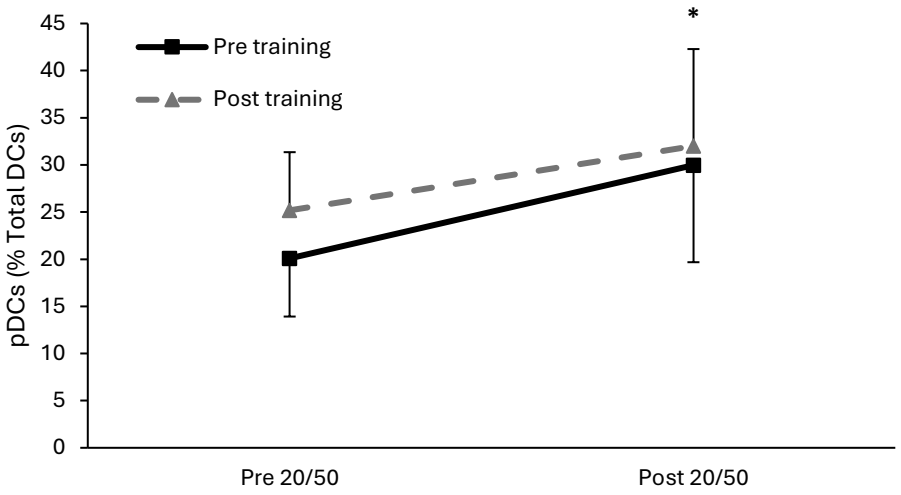


Figure 7.16. Plasmacytoid dendritic cells as a percentage of total DCs in response to the 20/50 pre and post training.
Data are presented as mean ± standard deviation, n=14.
* significantly different to pre 20/50 (P< 0.05).

TLR7 MFI mean fold change

There was a significant main effect of time (F(1,13)= 38.268, P< 0.001), but not trial (F(1,13)= 0.014, P= 0.907) or time*trial interaction (F(1,13)= 0.527, P= 0.481). TLR7 MFI mean fold change was significantly elevated from pre to post 20/50 in both trials (0.83 to 0.92 MFI mean fold change pre training, and 0.77 to 0.82 MFI mean fold change post training) (Figure 7.17).

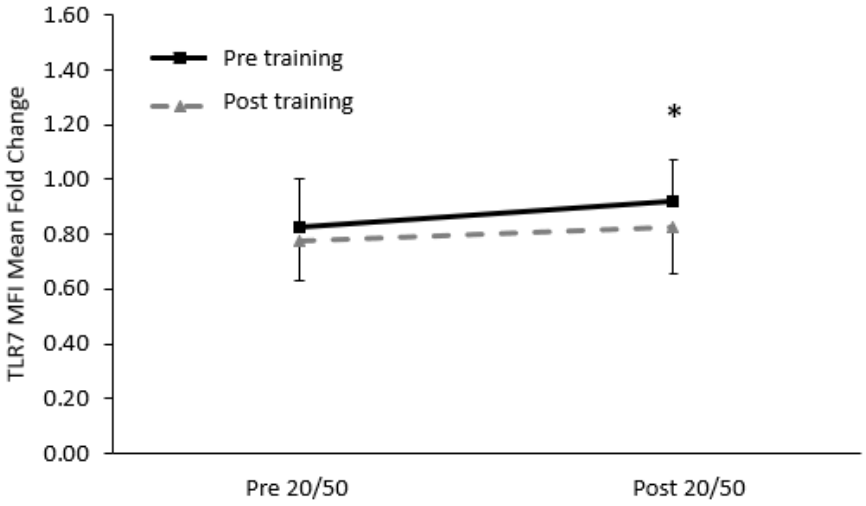


Figure 7.17. Dendritic cell toll-like receptor 7 median fluorescent intensity (mean fold change from unstimulated to stimulated) in response to the 20/50 pre and post training. Data are presented as mean ± standard deviation, n= 14.
* significantly different to pre 20/50 (P< 0.05).

TLR8 MFI mean fold change

Wilcoxon's signed-rank test showed that there was no significant main effect of trial on TLR8 MFI mean fold change (Z= - 0.672, P= 0.502). Friedmans showed there was also no significant main effect of time ($X^2(3)$= 0.285, P= 0.999) (0.96 to 1.10 MFI mean fold change pre training, and 0.92 to 1.00 MFI mean fold change post training) (Figure 7.18).

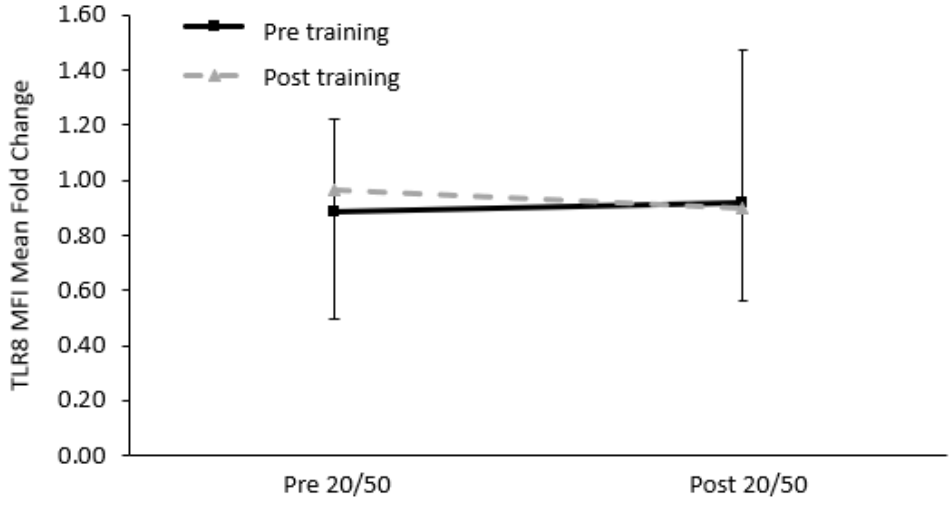


Figure 7.18. Dendritic cell toll-like receptor 8 median fluorescent intensity (mean fold change from unstimulated to stimulated) in response to the 20/50 pre and post training. Data are presented as mean ± standard deviation, n=14.

TLR9 MFI mean fold change

Wilcoxon's signed-rank test showed that there was no significant main effect of trial on TLR9 MFI mean Fold change (Z= -1.356, P= 0.175). Friedmans showed there was a significant main effect of time ($X^2(3)$= 7.921, P= 0.048). TLR9 MFI increased in response to the 20/50 in both trials (0.98 to 1.10 MFI mean fold change pre training, and 0.93 to 1.00 MFI mean fold change post training) (Figure 7.19).

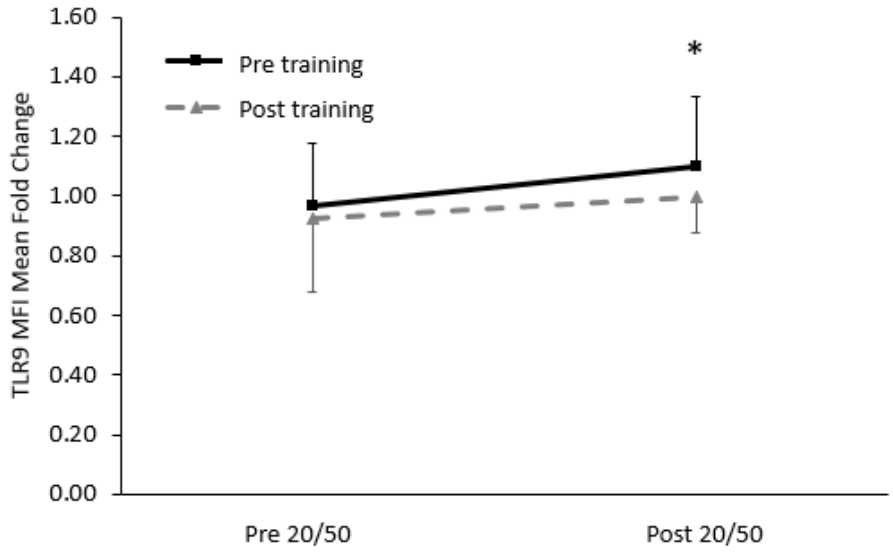


Figure 7.19. Dendritic cell toll-like receptor 9 median fluorescent intensity (mean fold change from unstimulated to stimulated) in response to the 20/50 pre and post training. Data are presented as mean ± standard deviation, n=14.
* significantly different to pre 20/50 (P< 0.05).

pDC TNF-α MFI mean fold change

Wilcoxon's signed-rank test showed that there was no significant main effect of trial on pDC TNF-α MFI mean fold change (Z= -0.558, P= 0.577). Friedmans showed there was no significant main effect of time ($X^2(3)$= 2.143, P= 0.543). (14.24 to 11.25 MFI mean fold change pre training, and 12.86 to 10.89 MFI mean fold change post training) (Figure 7.20).

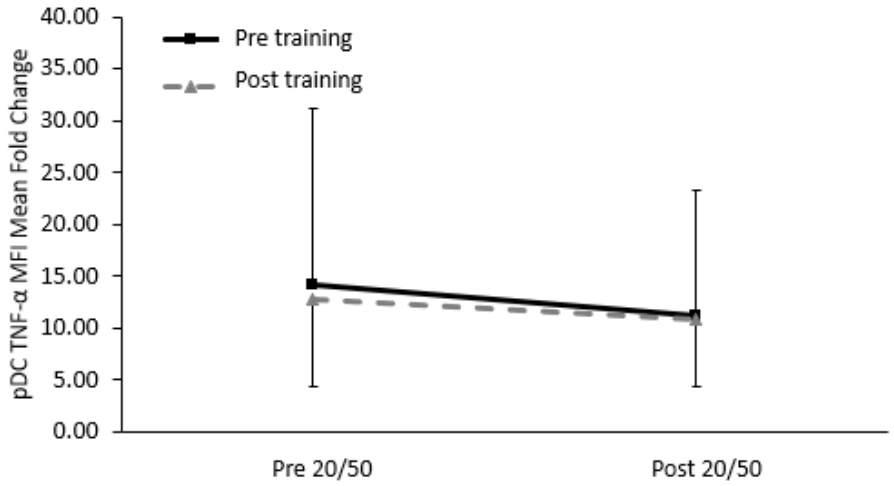


Figure 7.20. Plasmacytoid dendritic cell tumour necrosis factor- α (TNF- α) median fluorescent intensity (mean fold change from unstimulated to stimulated) in response to the 20/50 pre and post training. Data are presented as mean ± standard deviation, n=14.

mDC TNF-α MFI mean fold change

Wilcoxon's signed-rank test showed that there was no significant main effect of trial on mDC TNF-α MFI mean fold change (Z= -0.865, P= 0.387). Friedmans showed there was no significant main effect of time ($X^2(3)$= 3.173, P= 0.366); (4.93 to 3.61 MFI mean fold change pre training, and 5.88 to 3.17 MFI mean fold change post training) (Figure 7.21).

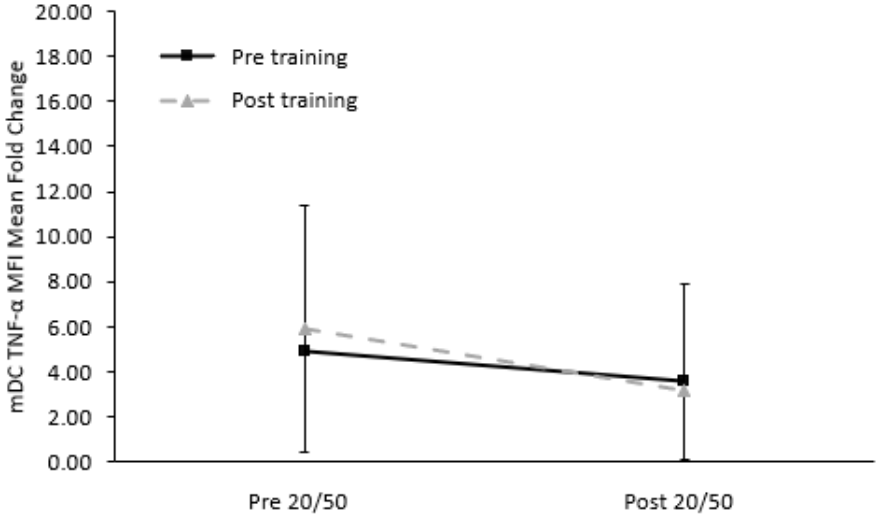


Figure 7.21. Myeloid dendritic cell tumour necrosis factor-α (TNF-α) median fluorescent intensity (mean fold change from unstimulated to stimulated) in response to the 20/50 pre and post training. Data are presented as mean ± standard deviation, n=14.

IFN-α pDC MFI mean fold change

There was a significant main effect of time (F(1,13)= 10.050, P= 0.007), but not trial (F(1,13)= 0.192, P= 0.669) or time*trial interaction (F(1,13)= 3.459, P= 0.086). IFN-α pDCs were significantly reduced from pre to post 20/50 in both trials (1.86 to 1.51 MFI mean fold change pre training, and 1.90 to 1.39 MFI mean fold change post training) (Figure 7.22).

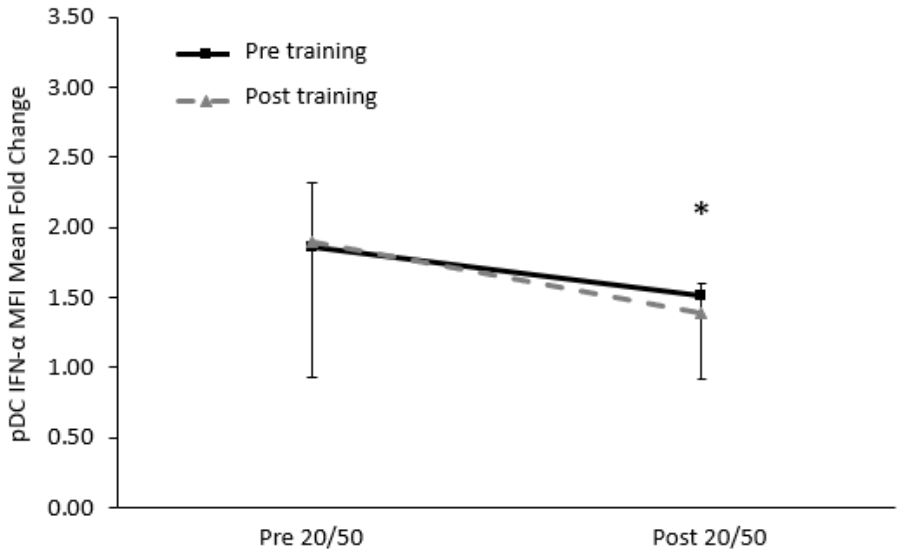


Figure 7.22. Plasmacytoid dendritic cell Interferon- α (IFN-α) median fluorescent intensity (mean fold change from unstimulated to stimulated) in response to the 20/50 pre and post training.
Data are presented as mean ± standard deviation, n=14.
* significantly different to pre 20/50 (P< 0.05).

IL-10 as a % of $CD4^+$ T cells

There was a significant main effect of time (F(1,13)= 5.060, P= 0.042), but not trial (F(1,13)= 0.242, P= 0.631) or time*trial interaction (F(1,13)= 0.192, P= 0.669). the percentage of $IL\text{-}10^+$ $CD4^+$ T cells were significantly increased from pre to post 20/50 in both trials (1.53 to 1.87% pre training, and 0.52 to 1.95% post training) (Figure 7.23).

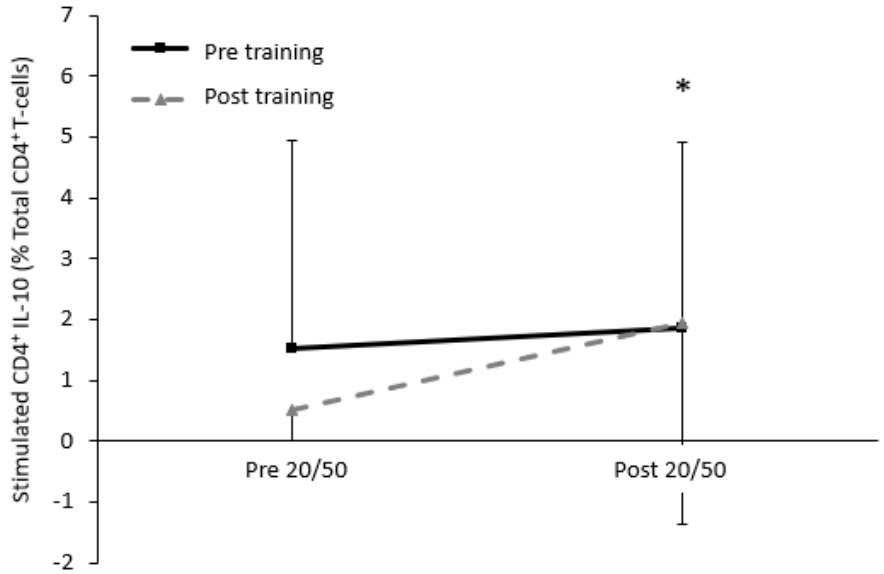


Figure 7.23. Stimulated $CD4^+$ T cell Interleukin- 10 (IL-10) expression as a percentage of total $CD4^+$ T cells (%) in response to the 20/50 pre and post training.
Data are presented as mean ± standard deviation, n=14.
* significantly different to pre 20/50 (P< 0.05).

7.3.8 Hormones

Plasma cortisol

There was a significant main effect of trial whereby mean plasma cortisol levels were higher pre training (417.64 ± 352.76 nmol/L) vs. post training (352.76 ± 135.08 nmol/L) ($F(13)= 5.524$, $P= 0.035$). There was no significant main effect of time ($F(13)= 0.161$, $P= 0.695$), but a significant interaction ($F(13)= 8.770$, $P= 0.011$), indicating that the plasma cortisol response to the 20/50 differed pre and post training (Figure 7.24).

There was a significant difference in delta pre to post 20/50 with training ($t(13)= 3.492$, $P= 0.014$). Plasma cortisol elevated in response to the 20/50 pre training (83.14 ± 165.09 nmol/L) compared to post training whereby plasma cortisol did not change (-0.07 ± 107.6 nmol/L), showing a significant blunting of the plasma cortisol response to the 20/50 post training.

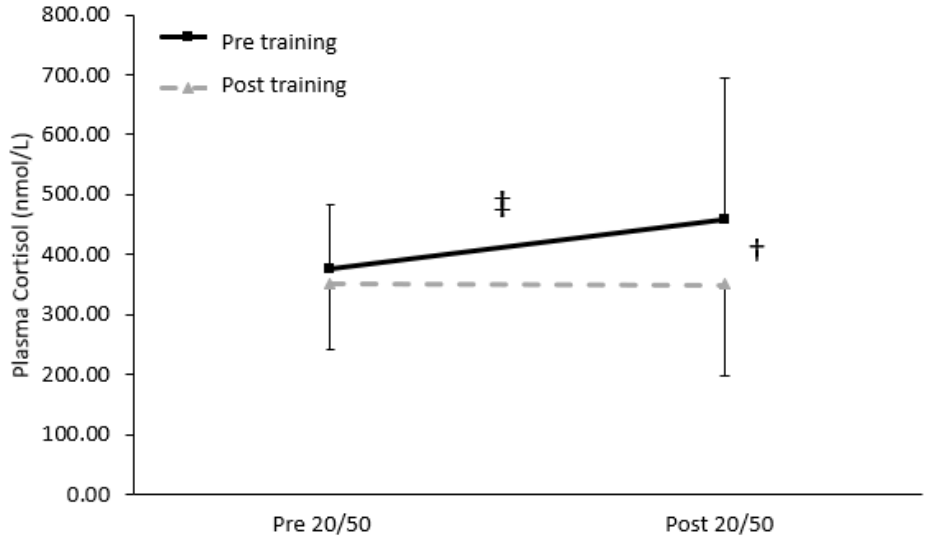


Figure 7.24. Plasma cortisol response to the 20/50 pre and post training (nmol/L).
Data are presented as mean ± standard deviation, n=14.
‡ significant interaction effect, † significantly different post training (P< 0.05).

Salivary cortisol

There was no significant main effect of trial (F(1,12)= 1.035, P= 0.329), whereby mean salivary cortisol levels were similar pre training (15.65 ± 0.78 nmol/L) and post training (14.36 ± 0.23 nmol/L). There was also no significant main effect of time (F(1,12)= 0.731, P= 0.409) (Figure 7.25).

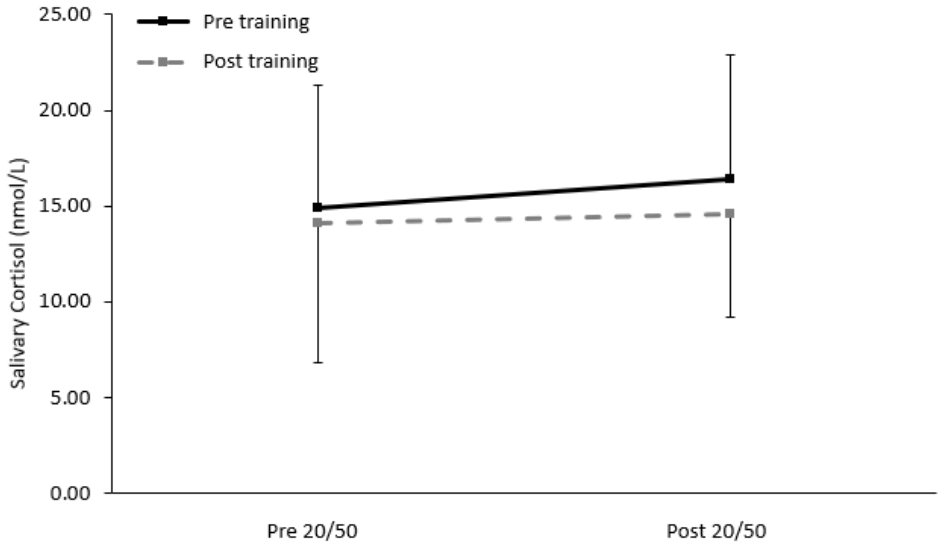


Figure 7.25. Salivary cortisol response to the 20/50 pre and post training (nmol/L).
Data are presented as mean ± standard deviation, n=14.

Plasma testosterone

There was a significant main effect of trial whereby mean plasma testosterone levels were higher pre training (15.81 ± 6.10 pmol/L) vs. post training (14.23 ± 6.45 pmol/L) ($F(13)= 9.534$, $P= 0.009$). There was a significant main effect of time ($F(13)= 20.183$, $P< 0.001$) whereby plasma testosterone levels were elevated by the 20/50, but no significant interaction ($F(13)= 3.237$, $P= 0.100$), indicating that the plasma testosterone response to the 20/50 did not differ pre and post training (Figure 7.26).

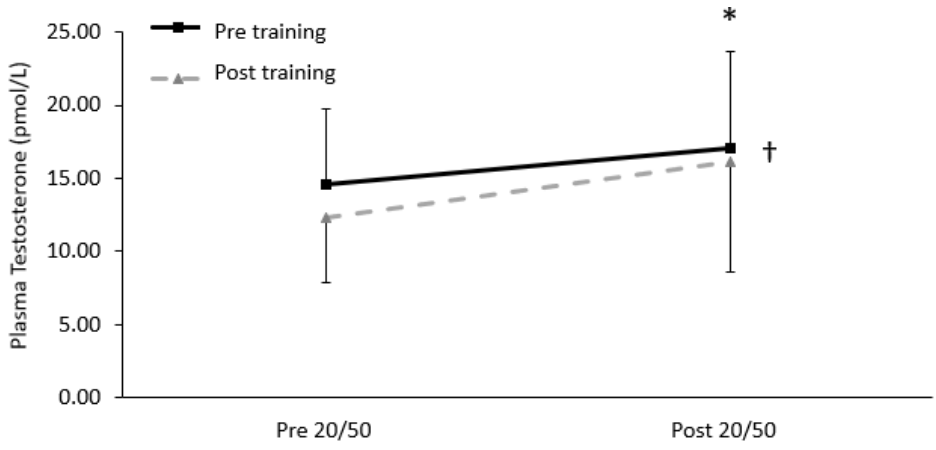


Figure 7.26. Plasma testosterone response to the 20/50 pre and post training (pmol/L).
Data are presented as mean ± standard deviation, n=14.
* significantly different to pre 20/50, † significantly different post training ($P< 0.05$).

Salivary Testosterone

There was a significant main effect of trial ($F(13)= 9.537$, $P= 0.009$), whereby mean salivary testosterone levels were higher pre training (959.49 ± 424.17 pmol/L) vs post training (830.77 ± 381.56 pmol/L). There was also a significant main effect of time ($F(13)= 22.990$, $P< 0.001$), whereby the 55/80 elevated salivary testosterone from 792.57 ± 328.29 to 1126.40 ± 443.03 pmol/L pre training, and from 736.23 ± 286.75 to 925.21 ± 437.12 pmol/L post training. There was no significant interaction effect ($F(13)= 3.665$, $P= 0.078$) (Figure 7.27).

There was a significant difference in delta pre to post 20/50 after the training period ($t(13)= 1.914$, $P= 0.039$). Specifically, salivary testosterone displayed a larger elevation in response to the 20/50 pre training (333.84 ± 260.76 pmol/L) compared to post training (189.08 ± 215.59 pmol/L).

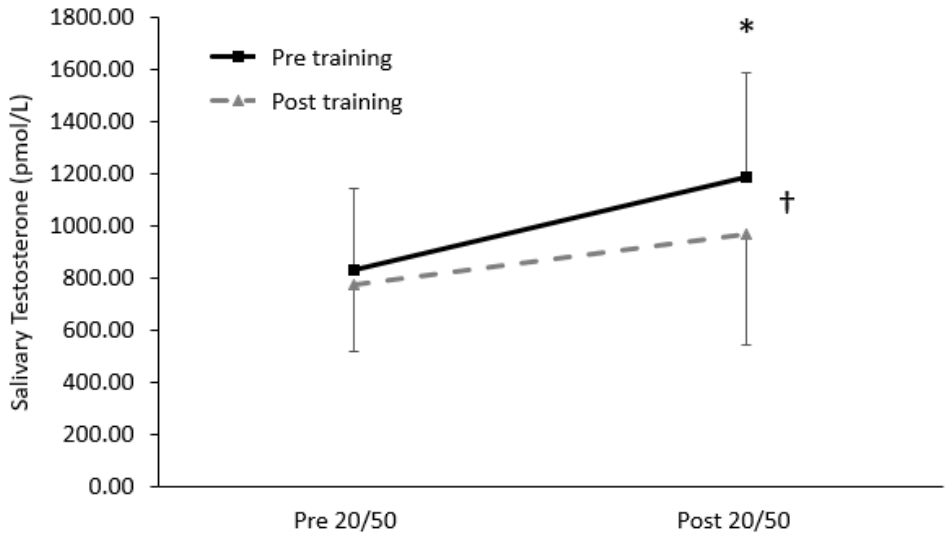


Figure 7.27. Salivary testosterone response to the 20/50 pre and post training (pmol/L). Data are presented as mean ± standard deviation, n=14.
* significantly different to pre 20/50, † significantly different post training (P< 0.05).

7.4 Discussion

This study aimed to investigate if immune and endocrine biomarkers alter following an intensified period of training. Specifically, stimulated DC TLR expression and the subsequent DC and T cell cytokine release was examined, alongside immune cell counts, and plasma and salivary cortisol and testosterone in response to the short duration exercise bout (20/50), before and after 9 days of intensified training. On average, the intensified training period resulted in an ~80% increase in training load compared to baseline and led to an improvement in 10 km time trial performance. Total T cell, $CD8^+$ T cell, total DC, pDC and mDC counts all increased in response to the 20/50, but only total DCs were altered by the intensified training period. Specifically, resting total DC count was significantly lower after the intensified training period. When looking at the proportion of cells in response to the 20/50, the percentage of $CD3^+$ and $CD4^+$ T cells declined, with no change in $CD8^+$. The percentage of total DCs and mDCs were not altered by the 20/50, but the percentage of pDCs increased. This highlights that measuring changes in the proportion of cells to the 20/50 is not a useful biomarker of overtraining. pDC TLR7 and 9 expression both elevated in response to the 20/50 but remined unchanged by the intensified training period. In response to the 20/50, pDC IFN-α decreased and the percentage of IL-10^+ $CD4^+$ T cells increased but were both also unchanged by the intensified training period. The exercise induced elevations in plasma cortisol and plasma and salivary testosterone were blunted by ~100%, ~10% and ~43%, respectively after the 9-day training period. Salivary cortisol was not elevated by the 20/50 or altered in response to training.

REST-Q and 10 km time trial

The purpose of the 9-day intensified training period was to induce a state of overreaching. According to the definitions in the joint ECSS and ACSM consensus statement, overreaching usually leads to a short-term performance decrement (Meeusen et al., 2013). Specifically, 4 weeks of a ~60% training intensification in elite swimmers resulted in a 6.5% reduction in 200m swimming time trial performance (Mackinnon et al., 1996), 2 weeks of a ~50% training intensification in elite cyclists resulted in a decline in 60-minute cycling time trial performance (Halson et al., 2003), and 3km running time trial performance was reduced in participants undergoing a 4-week, ~290% increase in training load, whereas those in the normal training group experienced improved performance (Coutts et al., 2007). Furthermore, it has been shown that overreaching can occur in as little as 7 days, whereby cycling time trial performance was significantly reduced after 7 days of a ~50% increase in the training volume above lactate threshold (Halson et al., 2002). However, in the current study, 10 km time trial performance was improved after the training period (Figure 7.3). It is therefore likely that the participants in the current study were in a state of FOR, resulting in acute fatigue and ultimately an improvement in performance (Meeusen et al., 2013). This theory is supported by the REST-Q scores in the current study, whereby an elevation in *sport related* stress occurred with no changes in *general stress*.

Endocrine System

Cortisol

The current study found that exercise induced plasma cortisol was blunted by ~100% after the 9-day intensified training period (Figure 7.24), whereas no alterations in the Salivary cortisol response was identified (Figure 7.25). Hough et al. (2013) utilised the same training procedure as the current study i.e. 9 days of supervised laboratory training of 1.5 hours cycling at 75% $\dot{V}O_{2max}$ daily, however, only measured salivary hormones. Specifically, they found a ~166% blunting in the salivary cortisol response to a similar exercise stress test (the 55/80) after the training period. Importantly, they took saliva samples pre, immediately post and 30 minutes post their exercise stress test, and saw that salivary cortisol continued to increase to peak levels 30 minutes post. It has been shown that salivary cortisol levels lag behind plasma cortisol levels by ~10 minutes in response to stress (Hough et al., 2011; Hernandez et al., 2014). For example, Hough et al. (2011) found that in response to a 30-minute cycling stress test, peak plasma cortisol levels were seen 20 minutes post exercise, whereas peak salivary cortisol levels were observed 30 minutes post exercise. The current study did not include a 30-minute post 20/50 saliva sample, so it is possible that we missed the peak, which contributed to not observing a significant salivary cortisol blunting after the training period. Additionally, although their intensified training period utilised the same protocol as the current study, our intensified training period only resulted in a ~80% intensification (Figure 7.4), whereas they reported a ~143% intensification, which could also explain why they observed salivary cortisol blunting, and we did not.

However, in line with the current study, Hough et al. (2015) found no significant blunting in salivary cortisol in response to a 10-day training camp in elite triathletes, despite measuring salivary hormones 30 minutes post 55/80 stress test. They suggested the lack of blunting was due to the elite triathlete's ability to cope with the elevated training load, but this is not the case for the current study, as our participants were not trained to an elite level. Additionally, whilst the literature highlights consistent positive relationships between plasma and salivary cortisol measurements both at rest and in response to exercise (Cadore et al., 2008), plasma cortisol represents total cortisol, not the free, biologically active cortisol that is measured in saliva, thus perhaps total cortisol is more sensitive to training load than free cortisol (Bozovic et al., 2013).

Interestingly, the 20/50 failed to elevate salivary cortisol, even before the intensified training period, whereas both Hough et al. (2011 & 2015) and Baker et al. (2024) observed a ~120%, ~200% and ~63% elevation in salivary cortisol to the 55/80 stress test from pre to immediately post 55/80. Whilst the 20/50 follows a similar layout to the 55/80 stress test used by Hough et al. (2011 & 2015) and Baker et al. (2024), i.e. 30 minutes in duration and alternating blocks of 1 minute 'easy' and 4 minutes 'hard' cycling; the anchoring of exercise intensities around submaximal thresholds in the 20/50 may have resulted in a lowered stress response compared to the 55/80 which utilised percentages of work rate at $\dot{V}O_{2max}$.

Despite no alterations in salivary cortisol, exercise induced plasma cortisol was blunted by ~100% after the training period in the current study (Figure 7.24). Uustalo et al. (1998) also observed a lowered plasma cortisol response (by ~20%) to a treadmill $\dot{V}O_{2max}$ test, after 4 weeks of a ~130% increase in intensive (above lactate threshold) endurance training in female athletes. In agreement, Verde et al. (1992) found that a ~38% increase in training load across 3 weeks in highly trained distance runners also resulted in a ~122% blunted plasma cortisol response to a 30-minute treadmill run at 80% $\dot{V}O_{2max}$. Two proposed mechanisms for this attenuated plasma cortisol response to exercise with intensified training are hypothalamic dysfunction, or changes in the sensitivity of the adrenal medulla to ACTH as a protective mechanism against consistently high cortisol levels that could be detrimental to the body, possibly causing high muscle degradation (Barron et al., 1985; Wittert et al., 1996). Unfortunately, the current study did not measure ACTH, thus cannot confirm if the 9-day intensified training period had an effect on hypothalamic-pituitary function. These findings suggest that plasma cortisol responses to exercise could be used as a biomarker to highlight the negative stages of overtraining.

Testosterone

Both salivary and plasma testosterone responses to the 20/50 were blunted after the intensified training period in the current study. Specifically, salivary testosterone was blunted by ~44% (Figure 7.27), and plasma testosterone by ~10% (Figure 7.26),. Uusitalo et al. (1998) also found a ~22% blunted plasma testosterone response to a $\dot{V}O_{2max}$ test after a 4-week intensified training period and highlighted an inverse relationship between plasma testosterone concentrations and training volume. In line with this, Hough et al. (2013) highlighted a ~21% blunted salivary testosterone response to their 30-minute 55/80 stress test after 11 days

of intensified training, with the same group also reporting a ~44% blunting after a 10-day intensive training block in elite triathletes (Hough et al., 2015). The consistently observed exercise induced blunting in plasma and salivary testosterone after intensified training periods within the literature highlights testosterone as a promising key biomarker of the negative states of overtraining.

This consistently observed blunting in exercise induced salivary and plasma testosterone may result from changes in testosterone synthesis and/or secretion in the testes. When gonadotropin releasing hormone was infused into a group of endurance trained runners, and a group of age matched non-active controls, it was found that those in the endurance trained group produced less testosterone than the non-active control group, thus highlighting reduced testosterone synthesis in the testes (Hackney et al., 2003). It is therefore possible that the increase in endurance-based cycling across the 9-day period in the current study could have caused a decrease in testicular testosterone production in the male participants. Dysfunction in testosterone production in males may also be associated with elevated levels of circulating cortisol. In a study where acute hypercortisolism was induced in participants through insulin or hydrocortisone administration, a rapid rise in cortisol levels coincided with a significant drop in circulating testosterone concentrations (Cumming et al., 1983). It was proposed that cortisol inhibits LH receptors on Leydig cells, thereby reducing testosterone production and secretion by the testes. It is possible that the repeated acute increases in cortisol that will have occurred across the training period in the current study exerted an inhibitory effect on the expression of LH receptors on the Leydig cells, which resulted in decreased LH induced testosterone production and secretion (Cumming et al., 1983; Hough et al., 2013).

The significant blunting of the exercise induced plasma cortisol and plasma and salivary testosterone after the 9-day intensified training period highlights the important potential that each of those hormones has as a biomarker of the negative states of overtraining. In addition, it provides evidence that the 20/50 is a useful stress test in practice to highlight these endocrine alterations. It must be noted, however, that analytes were not adjusted for changes in plasma volume to the 20/50. Although, it is predicted that the shift in plasma volume (~14%) will be lower than the percentage increases of hormones in response to the 55/80, thus not likely to change outcomes overall (Bjerre-Bastos et al., 2022).

Immune Function

Cytokines

The current study found that the 20/50 reduced pDC IFN-α expression (Figure 7.22) and elevated the percentage of IL-10^{+} CD4^{+} T cells (Figure 7.23) but were both unchanged after the 9-day training period. Exercise is known to exert anti-inflammatory effects on the immune system, thus the rising interest in exercise as a therapeutic route in managing autoimmune disorders, such as systemic lupus erythematosus (SLE) (Spinelli et al., 2023). SLE, characterised by pDC IFN-α over-signalling, is improved by moderate intensity (50-60% $\dot{V}O_{2max}$) aerobic exercise due to its ability to mitigate IFN-α over-expression, whilst simultaneously increasing anti-inflammatory cytokines such as IL-10 (Spinelli et al., 2023; Gleeson et al., 2012; Hashemi et al.,

2022). Conversely, Gleeson et al. (2012) provided evidence that across a 4-month endurance training period, illness-prone athletes (3 or more weeks of URI symptoms) had higher levels of IL-10 production in stimulated whole blood. This highlights the role that acute exercise plays in the delicate balancing of a reduction in chronic low-grade inflammation without increasing the risk of acute infection. However, the results of the current study suggest that short term intensified training periods (< 9-days in duration) may not lead to any chronic maladaptation to the stimulated pDC IFN-α or $CD4^+$ T cell IL-10 response. In contrast to the acute IL-10 increase observed in the current study to the 20/50, a ~50% decrease in CON A stimulated IL-10 production from circulating T cells was observed immediately after a marathon race, despite an elevation In plasma IL-10, in elite males completing the race in under 3 hours (Santos et al., 2013). Hoffman-Goetz et al. (2008), however found that continual treadmill running increased the expression of IL-10 in mouse intestinal T cells. It was therefore postulated that exercise-induced IL-10 is most likely produced by T lymphocytes from other tissues and lymphoid organs, rather than circulating T cells (Santos et al., 2013). However, our findings suggest the contrary and indicate that high intensity exercise of 30 minutes in duration elevates the percentage of circulating $IL\text{-}10^+$ $CD4^+$ T cells. It is also plausible that the observed increase in the proportion of $CD4^+$ $IL\text{-}10^+$ cells may reflect not only an upregulation of IL-10 expression but also potential shifts in the distribution of $CD4^+$ T cell subsets. Memory $CD4^+$ T cells are primed for rapid cytokine synthesis and can produce IL-10 more readily upon re-stimulation than naïve cells (Chang et al., 2007). Therefore, changes in the relative abundance of these subsets could contribute to the observed pattern.

Cabral-Santos et al. (2019) suggest a positive linear relationship exists between aerobic exercise duration and IL-10 production (r =1.00). A proposed mechanism for this is the increased β_2-adrenergic receptors signalling that occurs with exercise. It has been demonstrated that β_2-adrenergic receptor signalling supresses pro-inflammatory cytokine secretion from DCs in response to LPS stimulation by suppressing virtually all intracellular and extracellular TLR pathways (Sharma et al., 2020). Whilst most of these pathways are altered via direct blocking of NF-κB activation, IL-10, which is highly induced by adrenaline and noradrenaline, may also act in an autocrine fashion to block pro-inflammatory cytokine secretion (Sharma et al., 2020). Adrenaline or noradrenaline was not assessed in the current study, but our data suggests that the elevated percentage of $IL\text{-}10^+$ $CD4^+$ T cells in circulation were not due to dampened DC TLR pathways, as the 20/50 significantly increased TLR7 and 9 expression, concurrently.

Despite the percentage of $IL\text{-}10^+$ $CD4^+$ T cells increasing in response to the 20/50, no alterations were seen after the 9-day intensified training period. Gleeson et al. (2011) compared the antigen-stimulated cytokine response of groups of individuals who reported to take part in regular moderate amounts of exercise (3-6 hours per week) and those who engaged in substantially more hours of endurance-based training (7-10 hours per week and > 11 hours per week) across 4 months. They found that those engaging in > 11 hours of endurance training per week resulted in ~3 fold higher stimulated IL-10 production than those doing 3-6 hours per week, indicating that high volume exercise training increases stimulated IL-10 production. The duration of training intervention is a likely candidate for these disparities i.e. 9 days of increased training volume vs. 4 months but also differences in analytical techniques used such as the type of antigenic stimulant

and length of incubation period. The current study stimulated isolated PBMCs, whereas Gleeson et al. (2011) stimulated whole blood, which is suggested as being more representative of an in vivo immune challenge as it better maintains the native cellular and soluble environment i.e. the actual cellular makeup of the immune system, the plasma components, inter-cellular interactions and kinetics of cytokine responses (Shaw et al., 2018). Whilst we used ODN CPG 2395 and R848 as antigenic stimulants, incubated for 5 hours, Gleeson et al. (2011) used a multi-antigen vaccine which they incubated for 24 hours, likely influencing the potency and time course of observed cytokine concentration changes (Shaw et al., 2018).

No alterations in DC TLR stimulated TNF-α were observed in the current study, both in response to the 20/50 or after the intensified training period (Figure 7.20 & 7.21),. However, previous studies have shown that stimulated DC TLR induced TNF-α is reduced in response to 24 weeks of intensified training in swimmers (Morgado et al., 2012). Morgado et al. (2012) found a ~50% decrease in stimulated DC TNF-α MFI after 24 weeks of swimming training consisting of ~18 hours/week. The largest decline in stimulated DC TNF-α MFI was found between baseline (the end of the off-season), and week 6 which was considered a high-volume block, and the smallest decline was found after the taper week. A similar decrease in TLR7 induced TNF-α secretion was found in MS patients in response to 12 weeks of a combined endurance and resistance training programme (Deckx et al., 2016). Studies have demonstrated that stimulated PBMC TNF-α production is not significantly different between MS patients and healthy controls (Durán et al., 2001). It is therefore likely that 9 days of intensified training may not be long enough to induce the same stimulated TNF-α responses as seen in previous studies.

TLRs

The current study found that the stimulated DC TLR7 (Figure 7.17), and 9 (Figure 7.19), MFI mean fold change significantly increased in response to the 20/50. TLR7 and 9 are important intracellular pDC TLRs responsible for recognising viral and bacterial DNA, respectively, and initiating anti-viral T cell responses, indicating that pDCs may be more responsive to immunological challenge immediately post 20/50 (Iwasaki & Medzhitov, 2015). In contrast, Nickel et al. (2012) found a reduction in DC TLR7 mRNA expression immediately post marathon race, coupled with a reduction in TLR7 protein expression 24 hours later, however they did not stimulate the DC population. Deckx et al. (2015) observed a reduction in stimulated DC TLR responsiveness after a combined endurance/resistance exercise bout, evidenced by the less pronounced upregulation of activation markers; MHC II on pDCs and CD86 on mDCs, however, these variables were not assessed in the current study.

Despite elevating in response to the 20/50, stimulated DC TLR7 and 9 expression was not altered by the 9-day intensified training period. The lack of change in response to the intensified training period is not in line with the limited previous studies investigating human DC TLRs. 10 weeks of intensified endurance training in recreational marathon runners was shown to upregulate DC TLR7 mRNA and protein expression (Nickel et al., 2011), whereas a reduction in stimulated whole blood TLR7 induced TNF-α secretion was found after 12

weeks of a combined endurance and resistance training program in MS patients (Deckx et al., 2016). Both studies utilised running as a modality of training, whereas the current study utilised cycling. It is known that running induces larger internal stress for the same given intensity and/or duration than cycling, thus is more likely to push an athlete towards a state of NFOR than cycling training protocols and could be a contributing factor towards our differing findings (Millet et al., 2009). However, In line with our findings, the same group found no change in DC TLR stimulated IFN-α production after the 12-week training period in MS patients, although, it must be noted that IFN-α secretion by MS patients in response to DC TLR stimulation is usually lower in comparison to healthy individuals (Deckx et al., 2016; Stasiolek et al., 2006). It is therefore apparent that recreationally active males and females can tolerate at least an ~80% elevation in training load for 9 days without experiencing significant DC TLR dysregulation. It must however be noted that immune measures were not adjusted for haemoconcentration shifts that occur with exercise (Matomäki et al., 2018).

7.5 Conclusion

Recreationally active males and females can increase their habitual training plan by a substantial margin of around ~80% for a 9-day period without developing dysfunctional DC TLRs and their subsequent cytokine release. Whilst the intensified training period may not have been long enough or intense enough to induce decrements in exercise performance, participants reported increased *sport related* stress, possibly indicating a state of FOR. Most importantly, the endocrine results suggest that plasma cortisol and plasma and salivary testosterone are key biomarkers for the identification of overreaching, highlighted by their blunted exercise induced responses after the training period, yet the absence of a 30-minute post 20/50 saliva sample may be the reason for the lack of salivary cortisol blunting. The attenuated endocrine responses emphasise the suitability of the 20/50 in positively identifying endocrine dysregulation associated with intensified training. Overall, a 9-day intensified training period of 1.5-hour daily cycling at 75% $\dot{V}O_{2max}$ does not alter resting, or exercise induced, immune cell counts, stimulated DC TLR expression, cytokine expression or salivary cortisol concentration. However, it does lead to attenuated exercise-induced elevations in plasma cortisol, and salivary and plasma testosterone; highlighting their suitability as key biomarkers of overreaching.

8 General Discussion

The aim of this Chapter is to consolidate the findings and conclusions drawn from all experimental Chapters within this thesis to establish overall conclusions, highlight future directions and discuss practical implications. To aid reference to the main aims and findings for each study, Table 8.1 summarises the main aims and conclusions drawn from each Chapter.

Table 8.1. A summary of aims and conclusions drawn from each experimental Chapter in the thesis.

Chapter Number	Aim	Findings/Conclusions
Chapter 4	To examine the salivary and plasma cortisol and testosterone responses in females to the previously developed 30-minute cycling stress test (55/80), already validated in males, with the addition of plasma progesterone.	The 55/80 significantly elevated plasma and salivary cortisol, salivary testosterone and plasma progesterone in healthy females, thus indicating that the 55/80 can be used as a tool to examine if cortisol and testosterone alter following periods of overtraining in females. Oral contraceptive users failed to show elevated hormone responses to the 55/80, thus it may not be a useful tool for those prescribed oral contraceptives.
Chapter 5	To assess the current literature examining the effects of intensified training periods on lymphocyte and DC number and function.	The systematic review and meta-analysis indicated that resting $CD8^+$ T Cell and total lymphocyte number, unstimulated IL-1β secretion, and DC CD86 expression are significantly altered after intensified training periods. However, definitive immune biomarkers indicative of the negative states of overtraining are limited, mainly due to low study numbers. There is a requirement for human studies investigating dendritic cell alterations with intensified training periods. There is also a lack of studies including female participants whilst controlling for the menstrual cycle.
Chapter 6	To establish the reproducibility of T cell and DC count responses to a newly developed 30-minute stress test utilising submaximal	The 20/50 induced robust and reproducible DC and T cell count elevations, all with good- to-excellent ICCs, and with CVs, SRD and Bland-Altman analysis confirming high reliability

	physiological thresholds to prescribe intensity (20/50).	between 20/50 trials. When implemented before and after a period of intensified training, the 20/50 is capable of highlighting whether immune alterations are a useful biomarker of the negative states of overtraining. However, using changes in cell proportions to the 20/50 is not a useful measure to observe alterations that may occur with intensified training.
Chapter 7	To investigate the endocrine (salivary and plasma cortisol and testosterone) and immune (T cell and DC counts, TLRs and cytokines) alterations that may occur with a 9-day intensified training period in both males and females.	An ~80% intensification in training load over 9 days led to blunted plasma and salivary testosterone and plasma cortisol responses to the 20/50. No blunting was observed with salivary cortisol, likely due to the time lag between plasma and salivary cortisol responses to exercise. Whilst TLR7 and 9 and all DC and T cell (apart from $CD4^+$) counts elevated in response to the 20/50, they were not altered by the intensified training period, apart from total DC, which were significantly lower at rest after the training period. Similarly, pDC IFN-α MFI decreased and the percentage of $CD4^+$ $IL\text{-}10^+$ increased in response to the 20/50 but remained unchanged by the training period. This shows that the immune system may be more robust to elevated training loads than the endocrine system. Importantly, salivary and plasma testosterone and plasma cortisol are worthy biomarkers to highlight early-stage overreaching.

Meeusen et al. (2004) were the first to identify that exercise induced hormonal responses may be more indicative of overreaching than resting concentrations. They investigated ACTH, cortisol, growth hormone and prolactin responses to a double incremental cycle to fatigue, separated by 4 hours of rest, both before and after a period of intensified training. They reported blunted exercise induced ACTH and cortisol concentrations to the second cycle bout in overreached athletes (i.e. those showing larger performance declines) compared to when in a normally trained state. Whilst the findings of this study were novel and opened avenues for establishing objective biomarkers of overtraining, the lengthy protocol used is not practical for use in elite sport. The 55/80 was therefore developed as a short duration (30-minute) stress test

utilising percentage of W_{max}, also capable of acutely elevating plasma and salivary cortisol and testosterone in males when in a healthy state (Hough et al., 2011). The 55/80 also highlighted an exercise induced blunting of salivary cortisol and testosterone after 9 days of intensified training using a similar training protocol as Chapter 7 (Hough et al., 2013), and in salivary testosterone only, in response to 10 days of intensified training in elite male triathletes (Hough et al., 2015).

One of the main aims of this thesis was to establish whether the previously designed 55/80 stress test could elicit the same robust endocrine elevations in females as seen in males (Hough et al., 2011). As discussed in Chapter 4, despite the incidence of self-reported NFOR or OTS being significantly higher in females than males (Matos et al., 2011), a recent review identified that female athletes are underrepresented in overtraining studies due to biological complexity surrounding the menstrual cycle (Carrard et al., 2022). Both the natural menstrual cycle phases and the oral contraceptive pill can have a profound impact on circulating cortisol levels and its response to exercise (Hertel et al., 2017). Deeper understanding of the female response was therefore vital if the 55/80 is to be used as a tool to highlight endocrine alterations when overreached in female populations; thus, widening its useability. In accordance, the findings in Chapter 4 confirm that females are also able to use the 55/80 as a tool to highlight endocrine alterations that occur with overtraining. In addition, as one of the main female sex hormones, and sharing the same synthesis pathway as testosterone, plasma progesterone was also assessed in Chapter 4, alongside plasma and salivary cortisol and testosterone (Batth et al., 2020). Specifically, the 55/80 induced similar elevations in salivary and plasma cortisol and salivary testosterone in females as seen in males (Hough et al., 2011 & 2013). Plasma progesterone was also significantly elevated by the 55/80 in females and therefore has potential as an additional hormonal biomarker of overtraining in female athletes, possibly in the place of plasma testosterone. This is in line with Bonen et al. (1979) who also found that 30 minutes of intense cycling was a strong enough stimulus to elevate serum progesterone levels in young healthy females.

Importantly, in Chapter 4, oral contraceptive users displayed attenuated hormone responses to the 55/80. oral contraceptive pills are known to elevate resting levels of cortisol by causing stress-like alterations in the F056 binding protein FKBP5; a central regulator of the HPA axis, which potentially reduced the capacity for plasma cortisol to elevate before reaching the proposed ceiling (Hertel et al., 2017; Behr et al., 2009). In addition, combined oral contraceptives have also been shown to reduce levels of androgen, especially testosterone in females by inhibiting ovarian and adrenal androgen synthesis (Zimmerman et al., 2013). As such, utilising hormonal biomarkers to indicate the negative states of overtraining may therefore not be useful for female athletes utilising oral contraceptive methods.

The 55/80, although able to induce robust hormonal elevations in both males (Hough et al., 2011, 2013 & 2015) and females (Baker et al., 2024; Chapter 4), and highlight exercise induced blunting after periods of intensified training (Hough et al., 2013 & 2015), does not ensure a standardised level of physiological load is placed on each individual. The 55/80 prescribes exercise intensity based solely on W_{max}, identified from the work rate achieved at $\dot{V}O_{2max}$, which fails to account for submaximal physiological thresholds such as the VT_1 (Jaminick et al., 2020). Large differences in homeostatic perturbations i.e. oxygen uptake kinetics and blood

lactate responses have been reported across multiple studies using exercise within the 'moderate intensity' zone (60-80% $\dot{V}O_{2max}$) (Jaminick et al., 2020). Utilising submaximal anchors such as VT_1 to prescribe exercise intensity is therefore recommended to ensure that each individual experiences the same level of physiological stress from the exercise bout (Mann et al., 2013). As such, Chapter 6 focused on developing a new exercise stress test to replace the 55/80 that shared the same structure i.e. 30 minutes of 1 minute 'easy' and 4 minutes 'hard' cycling, but utilises VT_1 as a submaximal physiological anchor to prescribe exercise intensities; the 20/50.

As explored in detail throughout this thesis, cortisol and testosterone both exert anti-inflammatory effects on the immune system and regulate key DC and T cell functions (Chrysohoou et al., 2013; Petrovsky et al., 1998). Despite their important role in orchestrating the immune response, Chapter 5 highlights that very little is currently known about how DCs are affected by periods of intensified training in humans. Therefore, in addition to establishing whether the 20/50 is a reliable exercise stress test, it was also used to examine whether alterations in immune cell counts could be a viable biomarker of overtraining in addition to endocrine markers. The findings from Chapter 6 showed that the 20/50 elicited robust and reproducible elevations in all DC and T cell counts, which all returned to baseline 30 minutes post 20/50 (apart from pDCs which remained elevated), with good to excellent ICCs, and CVs, SRD and Bland-Altman analysis verifying high reproducibility between both 20/50 trials. The physiological and perceived exertion responses were also similar between the 20/50 trials, confirming that the physiological strain induced by the 20/50 is not altered by repeated exposures. This biphasic response of immune cell counts to exercise has been previously well characterised in T cells (Shek et al., 1995), with similar patterns of mobilisation also shown in DCs (Brown et al., 2018; Ho et al., 2001). Increased haemodynamics and the release of catecholamines during exercise likely drive the increase in immune cells into the peripheral blood, whilst the release of glucocorticoids such as cortisol drive the redistribution of cells from the circulation into the tissues (Dimitrov et al., 2010; Hill et al., 2008). For example, the pattern of immune cell mobilisation in Chapter 6 mirrors that of the cortisol response to the 55/80 whereby cortisol increased from pre to post 55/80 and fell back to baseline 30 minutes post (Hough et al., 2021). The results of Chapter 6 indicate that if implemented before and after a period of intensified training, any observed differences in the responses to the 20/50 are likely to represent actual immune alterations associated with periods of heavy training.

In Chapter 7, the ~80% intensification of normal training load across 9 days led to a ~100% blunting of exercise-induced plasma cortisol and a ~10% and ~44% blunting of exercise-induced plasma and salivary testosterone, respectively, in response to the 20/50, but did not lead to any alterations in salivary cortisol. Hough et al. (2013) used the same intensified training protocol consisting of 9 days of supervised cycling at 75% $\dot{V}O_{2max}$ for 1.5 hours daily and observed blunted salivary cortisol (~166%) and testosterone (~21%) responses. Importantly, they collected saliva samples pre, immediately post and 30 minutes post their 55/80 stress test, and saw that salivary cortisol continued to increase to peak levels 30 minutes post. It has been shown that salivary cortisol levels lag behind plasma cortisol levels by ~10 minutes in response to stress (Hough et al., 2011; Hernandez et al., 2014). For example, Hough et al. (2011) found that in response to a 30-

minute cycling stress test, peak plasma cortisol levels were seen 20 minutes post exercise, whereas peak salivary cortisol levels were observed 30 minutes post exercise. The current study did not include a 30-minute post 20/50 saliva sample, so it is possible that we missed the peak, which contributed to not observing a significant salivary cortisol blunting after the training period. Additionally, although their intensified training period utilised the same protocol as the current study, our intensified training period only resulted in a ~80% intensification, whereas they reported a ~143% intensification, which could also explain why they observed salivary cortisol blunting, and we did not. Alternatively, the anchoring of exercise intensities around submaximal thresholds in the 20/50 may have resulted in a lowered stress response compared to the 55/80 which utilised percentages of work rate at $\dot{V}O_{2max}$. Whilst attempts were made to include females in the Chapter 7 study, only 1 female participant took part. Uustalo et al. (1998) however, observed a lowered plasma cortisol (~20%) and testosterone (~22%) response to a treadmill $\dot{V}O_{2max}$ test, after 4 weeks of a ~130% increase in intensive (above lactate threshold) endurance training in female athletes. The seemingly consistent exercise-induced blunting in plasma and salivary testosterone, and plasma cortisol observed in both males and females after intensified training periods within the literature highlights testosterone as a promising key biomarker of the negative states of overtraining.

Two potential mechanisms have been proposed to explain the blunted cortisol response to exercise following intensified training: hypothalamic dysfunction, and altered adrenal medulla sensitivity to ACTH (Barron et al., 1985; Wittert et al., 1996). These changes may serve as protective adaptations to prevent chronically elevated cortisol levels, which could otherwise lead to adverse outcomes such as excessive muscle catabolism. However, as ACTH levels were not assessed in Chapter 7, it is not possible to determine whether the 9-day period of intensified training impacted hypothalamic-pituitary function. The consistently observed suppression of exercise induced salivary and plasma testosterone levels may be attributed to alterations in testosterone synthesis and/or secretion within the testes. In a study where GnRH was administered to endurance-trained runners and age-matched sedentary controls, the endurance-trained individuals produced significantly less testosterone, suggesting impaired testicular testosterone synthesis in this population (Hackney et al., 2003). This finding supports the possibility that the increased training load of endurance cycling over the 9-day period in Chapter 7 may have contributed to a reduction in testosterone production by the testes. Additionally, impaired testosterone production in males has been linked to elevated circulating cortisol levels. In a study where acute hypercortisolism was induced via insulin or hydrocortisone administration, a rapid increase in cortisol corresponded with a significant decline in circulating testosterone levels (Cumming et al., 1983). This decline was hypothesised to result from cortisol-mediated inhibition of LH receptors on Leydig cells, thereby impairing testosterone synthesis and secretion. Consequently, it is plausible that the repeated acute elevations in cortisol throughout the training period in the current study suppressed the expression of LH receptors on Leydig cells, leading to diminished LH stimulated testosterone production (Cumming et al., 1983; Hough et al., 2013).

The aim of the 9-day intensified training period in the Chapter 7 was to induce a state of overreaching. As outlined in the joint consensus statement by the ECSS and the ACSM, overreaching typically results in a short-

term decline in performance (Meeusen et al., 2013). However, contrary to this expectation, 10 km time trial performance improved following the 9-day training period. Previous research has demonstrated that overreaching can occur within just 7 days; for example, Halson et al. (2002) reported a significant decrease in cycling time trial performance after a 7-day period during which training volume was increased by ~50% above the lactate threshold. Based on the ECSS and ACSM definitions, it could be argued that participants in Chapter 7 were experiencing a state of FOR, indicated by elevated acute fatigue, as evidenced by the increased REST-Q *sport related* stress scores, leading to enhanced performance (Meeusen et al., 2013).

Whilst acute elevations in DC and T cell counts, IL-10 secretion and TLR7 and 9 expression, and a decline in IFN-α were observed in response to the 20/50 in Chapter 7, no immune alterations were observed in response to the intensified training period, apart from lowered resting DC counts. Whilst these results are conflicting to observations made in previous studies (Nickel et al., 2011; Deckx et al., 2016), our results suggest that perhaps the immune system is more resistant to the effects of heavy training loads than the endocrine system. Whether heavy exercise training is immunosuppressive or not has been heavily debated in the literature (Simpson et al., 2020). There's no doubt that acute vigorous exercise has a profound impact on the phenotypic makeup and functional capacity of the immune system, such that the behaviour of almost all immune cell populations in the blood stream are altered in some way during and after exercise (Walsh et al., 2011), as shown to some extent in Chapter 6 and 7. Traditionally, it has been widely accepted that these immune changes lead to a temporary decline in immune competency during the hours post exercise (Campbell & Turner, 2018). This previously unchallenged view is based largely on early studies indicating that (i) infection risk is elevated after an acute bout of heavy prolonged exercise, (ii) a temporary reduction in sIgA occurs after vigorous exercise bouts leading to a higher risk of opportunistic infection and (iii) the transient decline in peripheral immune cell counts after excise represents supressed immunity (Campbell & Turner, 2018). As a collective, these observations led to the 'Open Window' theory, discussed in more detail in Chapter 2 and 5 (Pedersen & Ullum, 1994). Accordingly, one of the most consistently reproduced findings in exercise immunology is the transient time-dependant biphasic response of lymphocytes in the peripheral blood system in response to a single exercise bout; a phenomenon we also replicated in Chapter 6 (Walsh et al., 2011). Despite earlier investigations suggesting that this post exercise lymphocytopenia is a result of apoptosis, Simpson et al. (2007) reported limited lymphocyte markers of apoptosis (Annexin-V (+) or HSPA60) 1 hour after treadmill exercise to fatigue completed at 80% $\dot{V}O_{2max}$, yet lymphocytopenia was still evident. Moreover, the levels of apoptosis reported in studies indicating an increase in cell death are usually very small i.e. < 5% (Mooren et al., 2002; Simpson et al., 2007), and as such is unlikely to account for the 30%–40% reductions in blood lymphocyte count witnessed after exercise (Peake et al., 2016). As a result of the decline in lymphocyte frequency and the functional capacity of the lymphocyte pool post exercise, this phenomenon is now suggested to reflect a redistribution of the immune cells possessing the highest effector functions into the tissues for tumour eradication (Peake et al., 2017). The findings in Chapter 6 coincide with this view, such that a preferential mobilisation of $CD8^+$ T cells (~92%) compared to $CD4^+$ T cells (~43%) in response to the 20/50 was found. This debate is explored in more detail in Chapter 5, but in essence, the results in Chapter 7 indicate that intensified training may not lead to impaired DC immunity, and actually, the immune system is

able to tolerate large intensifications in habitual training loads without negative consequences on DC and T cell function.

The 20/50 has demonstrated its usefulness as a tool to highlight the exercise induced endocrine dysfunction that occurs with periods of intensified training (Chapter 7). However, despite its development ensuring a standardised approach to intensity prescription, the complex nature of assessing and identifying submaximal thresholds may limit the useability of it as a tool in practice. It would be interesting to investigate whether this tool could be redeveloped to base the 20/50 intensities on self-selected workloads based on RPE measurements. This approach has been used by Leal et al. (2019 & 2021) for development of a treadmill-based exercise test that does not require prior exercise intensity determination but only found reproducible salivary testosterone responses. The average RPE values for the 20/50's 1 minute 'easy' and 4 minutes 'hard' intervals were 12 ± 0 and 15 ± 0 (Chapter 7) and 12 ± 2 and 16 ± 2 (Chapter 6). The RPE scores for the 20/50 intervals were consistent across both Chapter 6 and 7, highlighting it could be a stable metric for exercise prescription; however, future studies are required to validate this against the 20/50.

8.1 Conclusion

This thesis provides evidence that salivary testosterone, and plasma cortisol and testosterone, with the potential addition of plasma progesterone in females can be used as diagnostic biomarkers to indicate when athletes are overreached and possibly experiencing the OTS (Chapter 4 and 7). Whilst immune biomarkers relating to DC and T cell counts and function may not be useful indicators of these states, this thesis provides evidence that the immune system is robust, with healthy, recreationally active males and females being able to withstand at least an ~80% intensification of normal training load over a 9-day period without experiencing significant declines in T cell and DC immunity. These findings add weight to the argument that heavy exercise may not be immunosuppressive (Chapter 7), with the preferential mobilisation and redistribution of effector cells possibly representing enhanced immunosurveillance at the tissue sites (Chapter 6). The results of Chapter 6 and 7 further add to the limited literature surrounding how DCs respond to intensified training as highlighted in Chapter 5; an important investigation given the important role they play in orchestrating the immune response. The 20/50, as a tool can highlight endocrine alterations that occur with intensified training and can be used in practice to monitor athlete training load during periods of heavy training, such as training camps.

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10 Appendices

10.1 Study consent form used in Chapters 4, 6 and 7

Nottingham Trent University

Study Title:	
Site Number / Ethics Number:	
Enrolment Identification Number for this trial:	

INFORMED CONSENT FORM FOR PARTICIPANTS

Study Title	T-cell And Dendritic Cell Response To High-intensity Cycling, Before And After A 12-day Intensified Training Period
Short Title	T-cell And Dendritic Cell Response To High-intensity Cycling, Before And After A 12-day Intensified Training Period
Name of Investigator	Carla Baker

Please Initial Each Box

1) I, [name of participant] agree to partake as a participant in the above study. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my rights being affected ☐

2) I understand from the participant information sheet (4th April 2023 Version 1), which I have read in full, and from my discussion(s) with Carla Baker or john Hough that this will involve me visiting the laboratory on 14 separate occasions (intervention group)/ 6 separate occasions (control group), with each visit lasting a maximum of 2 hours. ☐

3) It has also been explained to me by Carla Baker or John Hough that the risks and side effects that may result from my participation are as follows:
Fainting due to high-intensity exercise; Although extremely unlikely high intensity exercise has been known to reveal unsuspected heart or circulation problems and very rarely these have had serious or fatal consequences.
Discomfort due to blood sampling via venepuncture.
Discomfort from the mouthpiece required for gas sampling via a Douglas bag and metalyzer. ☐

4) I confirm that I have had the opportunity to ask questions about the study and, where I have asked questions, these have been answered to my satisfaction. ☐

5) I undertake to abide by University regulations and the advice of researchers regarding safety. ☐

1

Nottingham Trent University

6) I understand that any personal information regarding me, gained through my participation in this study, will be treated as confidential and only handled by individuals relevant to the performance of the study and the storing of information thereafter. Where information concerning myself appears within published material, my identity will be kept anonymous. ☐

7) I confirm that I have completed the health questionnaire and know of no reason, medical or otherwise that would prevent me from partaking in this research. ☐

The following is OPTIONAL and your choice will not affect your study participation in the current study

8) I agree and understand that the sample (s) I have given, and the information gathered about me can be stored by Carla Baker and John Hough under the HTA Research Licence at the Nottingham Trent University for possible use in future projects of a similar nature. I understand that some of these projects may be carried out by researchers other than Carla Baker and John Hough who ran the first project. I agree to my samples being stored for future ethically approved research. I understand that each additional consent below is entirely optional and does not affect my participation in the present study.

I consent to my samples being used for:

a) All future-ethically approved research projects ☐
OR,

b) Future ethically approved non-commercial (not for profit) research projects ☐

c) Future ethically approved commercial (for profit) research projects ☐

Participant name and signature		Date	
Independent witness name and signature		Date	
Primary Researcher name and signature		Date	

*When completed: 1 for participant; 1 for researcher site file; 1 to be kept in medical notes (if appropriate).

10.2 Health questionnaire used in Chapters 4, 6 and 7

Health screen

Name or Number .....

Please complete this brief questionnaire to confirm fitness to participate:

1. **At present,** do you have any health problem for which you are:

(a)	on medication, prescribed or otherwise	Yes ☐	No ☐
(b)	attending your general practitioner	Yes ☐	No ☐
(c)	on a hospital waiting list	Yes ☐	No ☐

2. **In the past two years,** have you had any illness which require you to:

(a)	consult your GP	Yes ☐	No ☐
(b)	attend a hospital outpatient department	Yes ☐	No ☐
(c)	be admitted to hospital	Yes ☐	No ☐

3. **Have you ever** had any of the following?

(a)	Convulsions/epilepsy	Yes ☐	No ☐
(b)	Asthma	Yes ☐	No ☐
(c)	Eczema	Yes ☐	No ☐
(d)	Diabetes	Yes ☐	No ☐
(e)	A blood disorder	Yes ☐	No ☐
(f)	Head injury	Yes ☐	No ☐
(g)	Digestive problems	Yes ☐	No ☐
(h)	Heart problems	Yes ☐	No ☐
(i)	Problems with bones or joints	Yes ☐	No ☐
(j)	Disturbance of balance / coordination	Yes ☐	No ☐
(k)	Numbness in hands or feet	Yes ☐	No ☐
(l)	Disturbance of vision	Yes ☐	No ☐
(m)	Ear / hearing problems	Yes ☐	No ☐
(n)	Thyroid problems	Yes ☐	No ☐
(o)	Kidney or liver problems	Yes ☐	No ☐
(p)	Allergy to nuts, alcohol etc.	Yes ☐	No ☐
(q)	Any problems affecting your nose e.g. recurrent nose bleeds	Yes ☐	No ☐
(r)	Any nasal fracture or deviated nasal septum	Yes ☐	No ☐

4. **Has any,** otherwise healthy, member of your family under the age of 50 died suddenly during or soon after exercise? Yes ☐ No ☐
5. Are there any reasons why blood sampling may be difficult? Yes ☐ No ☐
6. Have you had a blood sample taken previously? Yes ☐ No ☐
7. Have you had a cold, flu or any flu like symptoms in the last Month? Yes ☐ No ☐
8. Have you received the Covid-19 vaccination? Yes ☐ No ☐

If YES please state how long ago it was that you received the vaccine.

.....

9. Are you currently taking any form of contraception (i.e oral contraceptive pill?) Yes ☐ No ☐

If YES, please describe briefly which form of contraception you are on. If this is oral contraception, please detail which pill you are currently taking.

.....
.....
.....

10. Are you currently pregnant/ trying to fall pregnant or aim to fall pregnant at least 3 months after the study finishing? Yes ☐ No ☐

R E S T Q - 76 Sport

Single Code: _____ Group Code: _____

Name (Last): _____ **(First):** _____

Date: _____ Time: _____ Age: _____ Gender: _____

Sport/Event(s): _____

This questionnaire consists of a series of statements. These statements possibly describe your mental, emotional, or physical well-being or your activities during the past few days and nights.

Please select the answer that most accurately reflects your thoughts and activities. Indicate how often each statement was right in your case in the past days.

The statements related to performance should refer to performance during competition as well as during practice.

For each statement there are seven possible answers.

Please make your selection by marking the number corresponding to the appropriate answer.

Example:

In the past (3) days/nights

... I read a newspaper

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

In this example, the number 5 is marked. This means that you read a newspaper very often in the past three days.

Please do not leave any statements blank.

If you are unsure which answer to choose, select the one that most closely applies to you.

Please turn the page and respond to the statements in order without interruption.

From *Recovery-Stress Questionnaire for Athletes: User Manual* by Michael Kellmann and K. Wolfgang Kallus, 2001, Champaign, IL: Human Kinetics.

In the past (3) days/nights

1) ... *I watched TV*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

2) ... *I did not get enough sleep*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

3) ... *I finished important tasks*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

4) ... *I was unable to concentrate well*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

5) ... *everything bothered me*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

6) ... *I laughed*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

7) ... *I felt physically bad*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

8) ... *I was in a bad mood*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

9) ... *I felt physically relaxed*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

10) ... *I was in good spirits*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

11) ... *I had difficulties in concentrating*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

12) ... *I worried about unresolved problems*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

C.2 From *Recovery-Stress Questionnaire for Athletes: User Manual* by Michael Kellmann and K. Wolfgang Kallus, 2001, Champaign, IL: Human Kinetics.

In the past (3) days/nights

13) ... *I felt at ease*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

14) ... *I had a good time with friends*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

15) ... *I had a headache*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

16) ... *I was tired from work*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

17) ... *I was successful in what I did*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

18) ... *I couldn't switch my mind off*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

19) ... *I fell asleep satisfied and relaxed*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

20) ... *I felt uncomfortable*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

21) ... *I was annoyed by others*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

22) ... *I felt down*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

23) ... *I visited some close friends*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

24) ... *I felt depressed*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

From *Recovery-Stress Questionnaire for Athletes: User Manual* by Michael Kellmann and K. Wolfgang Kallus, 2001, Champaign, IL: Human Kinetics.

In the past (3) days/nights

25) ... *I was dead tired after work*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

26) ... *other people got on my nerves*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

27) ... *I had a satisfying sleep*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

28) ... *I felt anxious or inhibited*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

29) ... *I felt physically fit*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

30) ... *I was fed up with everything*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

31) ... *I was lethargic*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

32) ... *I felt I had to perform well in front of others*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

33) ... *I had fun*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

34) ... *I was in a good mood*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

35) ... *I was overtired*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

36) ... *I slept restlessly*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

C.4 From *Recovery-Stress Questionnaire for Athletes: User Manual* by Michael Kellmann and K. Wolfgang Kallus, 2001, Champaign, IL: Human Kinetics.

In the past (3) days/nights

37) ... *I was annoyed*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

38) ... *I felt as if I could get everything done*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

39) ... *I was upset*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

40) ... *I put off making decisions*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

41) ... *I made important decisions*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

42) ... *I felt physically exhausted*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

43) ... *I felt happy*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

44) ... *I felt under pressure*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

45) ... *everything was too much for me*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

46) ... *my sleep was interrupted easily*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	alwayss

47) ... *I felt content*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

48) ... *I was angry with someone*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

From *Recovery-Stress Questionnaire for Athletes: User Manual* by Michael Kellmann and K. Wolfgang Kallus, 2001, Champaign, IL: Human Kinetics. C.5

In the past (3) days/nights

49) ... *I had some good ideas*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

50) ... *parts of my body were aching*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

51) ... *I could not get rest during the breaks*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

52) ... *I was convinced I could achieve my set goals during performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

53) ... *I recovered well physically*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

54) ... *I felt burned out by my sport*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

55) ... *I accomplished many worthwhile things in my sport*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

56) ... *I prepared myself mentally for performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

57) ... *my muscles felt stiff or tense during performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

58) ... *I had the impression there were too few breaks*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

59) ... *I was convinced that I could achieve my performance at any time*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

60) ... *I dealt very effectively with my teammates' problems*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

C.6 From *Recovery-Stress Questionnaire for Athletes: User Manual* by Michael Kellmann and K. Wolfgang Kallus, 2001, Champaign, IL: Human Kinetics.

In the past (3) days/nights

61) ... *I was in a good condition physically*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

62) ... *I pushed myself during performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

63) ... *I felt emotionally drained from performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

64) ... *I had muscle pain after performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

65) ... *I was convinced that I performed well*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

66) ... *too much was demanded of me during the breaks*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

67) ... *I psyched myself up before performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

68) ... *I felt that I wanted to quit my sport*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

69) ... *I felt very energetic*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

70) ... *I easily understood how my teammates felt about things*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

71) ... *I was convinced that I had trained well*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

72) ... *the breaks were not at the right times*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

In the past (3) days/nights

73) ... *I felt vulnerable to injuries*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

74) ... *I set definite goals for myself during performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

75) ... *my body felt strong*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

76) ... *I felt frustrated by my sport*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

77) ... *I dealt with emotional problems in my sport very calmly*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

Thank you very much!

Scales and Items of the RESTQ-76 Sport

Scale 1: General Stress

22) ... I felt down
24) ... I felt depressed
30) ... I was fed up with everything
45) ... everything was too much for me

Scale 2: Emotional Stress

5) ... everything bothered me
8) ... I was in a bad mood
28) ... I felt anxious or inhibited
37) ... I was annoyed

Scale 3: Social Stress

21) ... I was annoyed by others
26) ... other people got on my nerves
39) ... I was upset
48) ... I was angry with someone

Scale 4: Conflicts/Pressure

12) ... I worried about unresolved problems
18) ... I couldn't switch my mind off
32) ... I felt I had to perform well in front of others
44) ... I felt under pressure

Scale 5: Fatigue

2) ... I did not get enough sleep
16) ... I was tired from work
25) ... I was dead tired after work
35) ... I was overtired

Scale 6: Lack of Energy

4) ... I was unable to concentrate well
11) ... I had difficulties in concentrating
31) ... I was lethargic
40) ... I put off making decisions

Scale 7: Somatic Complaints

7) ... I felt physically bad
15) ... I had a headache
20) ... I felt uncomfortable
42) ... I felt physically exhausted

Scale 8: Success

3) ... I finished important tasks
17) ... I was successful in what I did
41) ... I made important decisions
49) ... I had some good ideas

Scale 9: Social Relaxation

6) ... I laughed
14) ... I had a good time with my friends
23) ... I visited some close friends
33) ... I had fun

Scale 10: Somatic Relaxation

9) ... I felt physically relaxed
13) ... I felt at ease
29) ... I felt physically fit
38) ... I felt as if I could get everything done

Scale 11: General Well-being

10) ... I was in good spirits
34) ... I was in a good mood
43) ... I felt happy
47) ... I felt content

Scale 12: Sleep Quality

19) ... I fell asleep satisfied and relaxed
27) ... I had a satisfying sleep
36) ... I slept restlessly
46) ... my sleep was interrupted easily

Scale 13: Disturbed Breaks

51) ... I could not get rest during the breaks
58) ... I had the impression there were too few breaks
66) ... too much was demanded of me during the breaks
72) ... the breaks were not at the right times

Scale 14: Burnout/Emotional Exhaustion

54) ... I felt burned out by my sport
63) ... I felt emotionally drained from performance
68) ... I felt that I wanted to quit my sport
76) ... I felt frustrated by my sport

Scale 15: Fitness/Injury

50) ... parts of my body were aching
57) ... my muscles felt stiff or tense during performance
64) ... I had muscle pain after performance
73) ... I felt vulnerable to injuries

C.10 From *Recovery-Stress Questionnaire for Athletes: User Manual* by Michael Kellmann and K. Wolfgang Kallus, 2001, Champaign, IL: Human Kinetics.

Scale 16: Fitness/Being in Shape

53) ... I recovered well physically
61) ... I was in a good condition physically
69) ... I felt very energetic
75) ... my body felt strong

Scale 17: Burnout/Personal Accomplishment

55) ... I accomplished many worthwhile things in my sport
60) ... I dealt very effectively with my teammates' problems
70) ... I easily understood how my teammates felt about things
77) ... I dealt with emotional problems in my sport very calmly

Scale 18: Self-Efficacy

52) ... I was convinced I could achieve my set goals during performance
59) ... I was convinced that I could achieve my performance at any time
65) ... I was convinced that I performed well
71) ... I was convinced that I had trained well

Scale 19: Self-Regulation

56) ... I prepared myself mentally for performance
62) ... I pushed myself during performance
67) ... I psyched myself up before performance
74) ... I set definite goals for myself during performance

Note: The items 30 and 46 of the scale Sleep Quality have to be inverted for analysis.

From *Recovery-Stress Questionnaire for Athletes: User Manual* by Michael Kellmann and K. Wolfgang Kallus, 2001, Champaign, IL: Human Kinetics. C.11

10.5 Training diary used in Chapter 7

NOTTINGHAM TRENT UNIVERSITY

The effects of a 12 day training period on dendritic cell TLR function

Participant name:	

Training Diary

*Please record all of your training during the **7 days before your study visit, and everyday throughout your enrolment in the study.***

REMEMBER TO WEAR YOUR HR MONITOR FOR EACH SESSION AND RECORD YOUR RPE SCORE!

Date and day:	Time:	Exercise Type: *e.g Cycling, Weight Training, Running, Swimming.*	Duration (mins):	Intensity (low/moderate/high)	Cycling/running /walking specific: Distance covered (km)	RPE Score

10.6 Menstrual cycle diary used in Chapter 7

Menstrual Cycle Diary

Name:

	Date	Symptoms?
Start of Menstrual bleed		
End of menstrual bleed		
Ovulation Confirmation (Ovulation sticks)		
Ovulation no longer present (ovulation sticks)		
Start of Next Menstrual bleed		
End of Next menstrual bleed		

The Risk Of Bias In Non-randomized Studies – of Interventions (ROBINS-I) assessment tool

(version for cohort-type studies)

Version 19 September 2016

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ROBINS-I tool (Stage I): At protocol stage

Specify the review question

Participants	
Experimental intervention	
Comparator	
Outcomes	

List the confounding domains relevant to all or most studies

List co-interventions that could be different between intervention groups and that could impact on outcomes

Exposures an individual may receive after or during their intervention of interest that may affect the final outcome.

ROBINS-I tool (Stage II): For each study

Specify a target randomized trial specific to the study

Design	Individually randomized / Cluster randomized / Matched (e.g. cross-over)
Participants	
Experimental intervention	
Comparator	

Is your aim for this study…?

× to assess the effect of *assignment to* intervention

☐ to assess the effect of *starting and adhering to* intervention

Specify the outcome

Specify which outcome is being assessed for risk of bias (typically from among those earmarked for the Summary of Findings table). Specify whether this is a proposed benefit or harm of intervention.

Specify the numerical result being assessed

In case of multiple alternative analyses being presented, specify the numeric result (e.g. RR = 1.52 (95% CI 0.83 to 2.77) and/or a reference (e.g. to a table, figure or paragraph) that uniquely defines the result being assessed.

Preliminary consideration of confounders

Complete a row for each important confounding domain (i) listed in the review protocol; and (ii) relevant to the setting of this particular study, or which the study authors identified as potentially important.

"Important" confounding domains are those for which, in the context of this study, adjustment is expected to lead to a clinically important change in the estimated effect of the intervention. "Validity" refers to whether the confounding variable or variables fully measure the domain, while "reliability" refers to the precision of the measurement (more measurement error means less reliability).

the prognostic factors (predictors of the outcome) that also predict whether an individual receives one or the other intervention of interest

(i) Confounding domains listed in the review protocol				
Confounding domain	Measured variable(s)	Is there evidence that controlling for this variable was unnecessary?*	Is the confounding domain measured validly and reliably by this variable (or these variables)?	OPTIONAL: Is failure to adjust for this variable (alone) expected to favour the experimental intervention or the comparator?
			Yes / No / No information	Favour experimental / Favour comparator / No information

(ii) Additional confounding domains relevant to the setting of this particular study, or which the study authors identified as important				
Confounding domain	Measured variable(s)	Is there evidence that controlling for this variable was unnecessary?*	Is the confounding domain measured validly and reliably by this variable (or these variables)?	OPTIONAL: Is failure to adjust for this variable (alone) expected to favour the experimental intervention or the comparator?
			Yes / No / No information	Favour experimental / Favour comparator / No information

* In the context of a particular study, variables can be demonstrated not to be confounders and so not included in the analysis: (a) if they are not predictive of the outcome; (b) if they are not predictive of intervention; or (c) because adjustment makes no or minimal difference to the estimated effect of the primary parameter. Note that “no statistically significant association” is not the same as “not predictive”.

Preliminary consideration of co-interventions

Complete a row for each important co-intervention (i) listed in the review protocol; and (ii) relevant to the setting of this particular study, or which the study authors identified as important.

"Important" co-interventions are those for which, in the context of this study, adjustment is expected to lead to a clinically important change in the estimated effect of the intervention.

(i) Co-interventions listed in the review protocol		
Co-intervention	Is there evidence that controlling for this co-intervention was unnecessary (e.g. because it was not administered)?	Is presence of this co-intervention likely to favour outcomes in the experimental intervention or the comparator
		Favour experimental / Favour comparator / No information
		Favour experimental / Favour comparator / No information
		Favour experimental / Favour comparator / No information
		Favour experimental / Favour comparator / No information

(ii) Additional co-interventions relevant to the setting of this particular study, or which the study authors identified as important		
Co-intervention	Is there evidence that controlling for this co-intervention was unnecessary (e.g. because it was not administered)?	Is presence of this co-intervention likely to favour outcomes in the experimental intervention or the comparator
		Favour experimental / Favour comparator / No information
		Favour experimental / Favour comparator / No information

		Favour experimental / Favour comparator / No information
		Favour experimental / Favour comparator / No information

Risk of bias assessment

Responses underlined in green are potential markers for low risk of bias, and responses in red are potential markers for a risk of bias. Where questions relate only to sign posts to other questions, no formatting is used.

Signalling questions	Description	Response options
Bias due to confounding		
1.1 Is there potential for confounding of the effect of intervention in this study? **If N/PN to 1.1:** the study can be considered to be at low risk of bias due to confounding and no further signalling questions need be considered	All mice were housed in the same way and had the same lifestyles	Y / PY / PN / N
If Y/PY to 1.1: determine whether there is a need to assess time-varying confounding:		
1.2. Was the analysis based on splitting participants' follow up time according to intervention received? **If N/PN**, answer questions relating to baseline confounding (1.4 to 1.6) **If Y/PY**, go to question 1.3.		NA / Y / PY / PN / N / NI

1.3. Were intervention discontinuations or switches likely to be related to factors that are prognostic for the outcome? **If N/PN**, answer questions relating to baseline confounding (1.4 to 1.6) **If Y/PY**, answer questions relating to both baseline and time-varying confounding (1.7 and 1.8)		NA / Y / PY / PN / N / NI

Questions relating to baseline confounding only		
1.4. Did the authors use an appropriate analysis method that controlled for all the important confounding domains?		NA / Y / PY / PN / N / NI
1.5. **If Y/PY to 1.4**: Were confounding domains that were controlled for measured validly and reliably by the variables available in this study?		NA / Y / PY / PN / N / NI
1.6. Did the authors control for any post-intervention variables that could have been affected by the intervention?		NA / Y / PY / PN / N / NI
Questions relating to baseline and time-varying confounding		
1.7. Did the authors use an appropriate analysis method that controlled for all the important confounding domains and for time-varying confounding?		NA / Y / PY / PN / N / NI

1.8. **If Y/PY to 1.7**: Were confounding domains that were controlled for measured validly and reliably by the variables available in this study?		NA / Y / PY / PN / N / NI
Risk of bias judgement		Low / Moderate / Serious / Critical / NI
Optional: What is the predicted direction of bias due to confounding?		Favours experimental / Favours comparator / Unpredictable

Bias in selection of participants into the study		
2.1. Was selection of participants into the study (or into the analysis) based on participant characteristics observed after the start of intervention? **If N/PN to 2.1:** go to 2.4		Y / PY / PN / N / NI
2.2. **If Y/PY to 2.1**: Were the post-intervention variables that influenced selection likely to be associated with intervention?		NA / Y / PY / PN / N / NI
2.3 **If Y/PY to 2.2**: Were the post-intervention variables that influenced selection likely to be influenced by the outcome or a cause of the outcome?		NA / Y / PY / PN / N / NI
2.4. Do start of follow-up and start of intervention coincide for most participants?		Y / PY / PN / N / NI
2.5. **If Y/PY to 2.2 and 2.3, or N/PN to 2.4**: Were adjustment techniques used that are likely to correct for the presence of selection biases?		NA / Y / PY / PN / N / NI

Risk of bias judgement		Low / Moderate / Serious / Critical / NI
Optional: What is the predicted direction of bias due to selection of participants into the study?		Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Bias in classification of interventions		
3.1 Were intervention groups clearly defined?		Y / PY / PN / N / NI
3.2 Was the information used to define intervention groups recorded at the start of the intervention?		Y / PY / PN / N / NI
3.3 Could classification of intervention status have been affected by knowledge of the outcome or risk of the outcome?		Y / PY / PN / N / NI
Risk of bias judgement		Low / Moderate / Serious / Critical / NI
Optional: What is the predicted direction of bias due to classification of interventions?		Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Bias due to deviations from intended interventions		
If your aim for this study is to assess the effect of assignment to intervention, answer questions 4.1 and 4.2		
4.1. Were there deviations from the intended intervention beyond what would be expected in usual practice?		Y / PY / PN / N / NI

4.2. **If Y/PY to 4.1**: Were these deviations from intended intervention unbalanced between groups *and* likely to have affected the outcome?		NA / Y / PY / PN / N / NI
If your aim for this study is to assess the effect of starting and adhering to intervention, answer questions 4.3 to 4.6		
4.3. Were important co-interventions balanced across intervention groups?		Y / PY / PN / N / NI
4.4. Was the intervention implemented successfully for most participants?		Y / PY / PN / N / NI
4.5. Did study participants adhere to the assigned intervention regimen?		Y / PY / PN / N / NI
4.6. **If N/PN to 4.3, 4.4 or 4.5**: Was an appropriate analysis used to estimate the effect of starting and adhering to the intervention?		NA / Y / PY / PN / N / NI
Risk of bias judgement		Low / Moderate / Serious / Critical / NI
Optional: What is the predicted direction of bias due to deviations from the intended interventions?		Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Bias due to missing data		
5.1 Were outcome data available for all, or nearly all, participants?		Y / PY / PN / N / NI
5.2 Were participants excluded due to missing data on intervention status?		Y / PY / PN / N / NI

5.3 Were participants excluded due to missing data on other variables needed for the analysis?		Y / PY / PN / N / NI
5.4 **If PN/N to 5.1, or Y/PY to 5.2 or 5.3**: Are the proportion of participants and reasons for missing data similar across interventions?		NA / Y / PY / PN / N / NI
5.5 **If PN/N to 5.1, or Y/PY to 5.2 or 5.3**: Is there evidence that results were robust to the presence of missing data?		NA / Y / PY / PN / N / NI
Risk of bias judgement		Low / Moderate / Serious / Critical / NI
Optional: What is the predicted direction of bias due to missing data?		Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Bias in measurement of outcomes		
6.1 Could the outcome measure have been influenced by knowledge of the intervention received?		Y / PY / PN / N / NI
6.2 Were outcome assessors aware of the intervention received by study participants?		Y / PY / PN / N / NI
6.3 Were the methods of outcome assessment comparable across intervention groups?		Y / PY / PN / N / NI
6.4 Were any systematic errors in measurement of the outcome related to intervention received?		Y / PY / PN / N / NI

Risk of bias judgement		Low / Moderate / Serious / Critical / NI
Optional: What is the predicted direction of bias due to measurement of outcomes?		Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Bias in selection of the reported result		
Is the reported effect estimate likely to be selected, on the basis of the results, from... 7.1. ... multiple outcome *measurements* within the outcome domain?		Y / PY / PN / N / NI
7.2 ... multiple *analyses* of the intervention-outcome relationship?		Y / PY / PN / N / NI
7.3 ... different *subgroups*?		Y / PY / PN / N / NI
Risk of bias judgement		Low / Moderate / Serious / Critical / NI
Optional: What is the predicted direction of bias due to selection of the reported result?		Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Overall bias		
Risk of bias judgement		Low / Moderate / Serious / Critical / NI

	Optional: What is the overall predicted direction of bias for this outcome?		Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

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Revised Cochrane risk-of-bias tool for randomized crossover trials

Version of 18 March 2021

The development of the RoB 2 tool was supported by the MRC Network of Hubs for Trials Methodology Research (MR/L004933/2- N61), with the support of the host MRC ConDuCT-II Hub (Collaboration and innovation for Difficult and Complex randomised controlled Trials In Invasive procedures - MR/K025643/1), by MRC research grant MR/M025209/1, and by a grant from The Cochrane Collaboration.

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Study details	
Reference	
Study design	

☐ Individually-randomized parallel-group trial

☐ Cluster-randomized parallel-group trial

X Individually randomized cross-over (or other matched) trial

For the purposes of this assessment, the interventions being compared are defined as

Experimental: | Comparator:

Specify which outcome is being assessed for risk of bias

Specify the numerical result being assessed. In case of multiple alternative analyses being presented, specify the numeric result (e.g. RR = 1.52 (95% CI 0.83 to 2.77) and/or a reference (e.g. to a table, figure or paragraph) that uniquely defines the result being assessed.

Is the review team's aim for this result…?

☐ to assess the effect of *assignment to intervention* (the 'intention-to-treat' effect)

☐ to assess the effect of *adhering to intervention* (the 'per-protocol' effect)

If the aim is to assess the effect of *adhering to intervention*, select the deviations from intended intervention that should be addressed (at least one must be checked):

☐ occurrence of non-protocol interventions

☐ failures in implementing the intervention that could have affected the outcome

☐ non-adherence to their assigned intervention by trial participants

Which of the following sources were obtained to help inform the risk-of-bias assessment? (tick as many as apply)

☐ Journal article(s) with results of the trial

☐ Trial protocol

☐ Statistical analysis plan (SAP)

☐ Non-commercial trial registry record (e.g. ClinicalTrials.gov record)

☐ Company-owned trial registry record (e.g. GSK Clinical Study Register record)

☐ "Grey literature" (e.g. unpublished thesis)

☐ Conference abstract(s) about the trial

☐ Regulatory document (e.g. Clinical Study Report, Drug Approval Package)

☐ Research ethics application

☐ Grant database summary (e.g. NIH RePORTER or Research Councils UK Gateway to Research)

☐ Personal communication with trialist

☐ Personal communication with the sponsor

Risk of bias assessment

Responses underlined in green are potential markers for low risk of bias, and responses in red are potential markers for a risk of bias. Where questions relate only to sign posts to other questions, no formatting is used.

Domain 1a: Risk of bias arising from the randomization process

Signalling questions	Comments	Response options
1.1 Was the allocation sequence random?		Y / PY / PN / N / NI
1.2 Was the allocation sequence concealed until participants were enrolled and assigned to interventions?		Y / PY / PN / N / NI
1.3 Did baseline differences between intervention groups at the start of the first period suggest a problem with the randomization process?		Y / PY / PN / N / NI
Risk-of-bias judgement		Low / High / Some concerns
Optional: What is the predicted direction of bias arising from the randomization process?		NA / Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Domain S: Risk of bias arising from period and carryover effects

Signalling questions	Comments	Response options
S.1 Was the number of participants allocated to each of the two sequences equal or nearly equal?		Y/PY/PN/N/NI
S.2 If N/PN/NI to S.1: Were period effects accounted for in the analysis?		NA/Y/PY/PN/N/NI
S.3 Was there sufficient time for any carryover effects to have disappeared before outcome assessment in the second period?		Y/PY/PN/N/NI
Risk-of-bias judgement		Low / High / Some concerns
Optional: What is the predicted direction of bias arising from period and carryover effects?		NA / Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Domain 2: Risk of bias due to deviations from the intended interventions (effect of assignment to intervention)

Signalling questions	Comments	Response options
2.1. Were participants aware of their assigned intervention during each period of the trial?		Y / PY / PN / N / NI
2.2. Were carers and people delivering the interventions aware of participants' assigned intervention during each period of the trial?		Y / PY / PN / N / NI
2.3. If Y/PY/NI to 2.1 or 2.2: Were there deviations from the intended intervention that arose because of the trial context?		NA / Y / PY / PN / N / NI
2.4 If Y/PY to 2.3: Were these deviations likely to have affected the outcome?		NA / Y / PY / PN / N / NI
2.5. If Y/PY/NI to 2.4: Were these deviations from intended intervention balanced between groups?		NA / Y / PY / PN / N / NI
2.6 Was an appropriate analysis used to estimate the effect of assignment to intervention?		Y / PY / PN / N / NI
2.7 If N/PN/NI to 2.6: Was there potential for a substantial impact (on the result) of the failure to analyse participants in the group to which they were randomized?		NA / Y / PY / PN / N / NI
Risk-of-bias judgement		Low / High / Some concerns

Optional: What is the predicted direction of bias due to deviations from intended interventions?		NA / Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Domain 2: Risk of bias due to deviations from the intended interventions (effect of adhering to intervention)

Signalling questions	Comments	Response options
2.1. Were participants aware of their assigned intervention during each period of the trial?		Y / PY / PN / N / NI
2.2. Were carers and people delivering the interventions aware of participants' assigned intervention during each period of the trial?		Y / PY / PN / N / NI
2.3. [If applicable:] If Y/PY/NI to 2.1 or 2.2: Were important non-protocol interventions balanced between interventions?		NA / Y / PY / PN / N / NI
2.4. [If applicable:] Were there failures in implementing the intervention that could have affected the outcome?		NA / Y / PY / PN / N / NI
2.5. [If applicable:] Was there non-adherence to the assigned intervention regimen that could have affected participants' outcomes?		NA / Y / PY / PN / N / NI
2.6. If N/PN/NI to 2.3, or Y/PY/NI to 2.4 or 2.5: Was an appropriate analysis used to estimate the effect of adhering to the intervention?		NA / Y / PY / PN / N / NI
Risk-of-bias judgement		Low / High / Some concerns
Optional: What is the predicted direction of bias due to deviations from intended interventions?		NA / Favours experimental / Favours comparator /

		Towards null /Away from null / Unpredictable

Domain 3: Risk of bias due to missing outcome data

Signalling questions	Comments	Response options
3.1 Were data for this outcome available for all, or nearly all, participants randomized?		Y / PY / PN / N / NI
3.2 If N/PN/NI to 3.1: Is there evidence that the result was not biased by missing outcome data?		NA / Y / PY / PN / N
3.3 If N/PN to 3.2 Could missingness in the outcome depend on its true value?		NA / Y / PY / PN / N / NI
3.4 If Y/PY/NI to 3.3: Is it likely that missingness in the outcome depended on its true value?		NA / Y / PY / PN / N / NI
Risk-of-bias judgement		Low / High / Some concerns
Optional: What is the predicted direction of bias due to missing outcome data?		NA / Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Domain 4: Risk of bias in measurement of the outcome

Signalling questions	Comments	Response options
4.1 Was the method of measuring the outcome inappropriate?		Y / PY / PN / N / NI
4.2 Could measurement or ascertainment of the outcome have differed between interventions within each sequence?		Y / PY / PN / N / NI
4.3 If N/PN/NI to 4.1 and 4.2: Were outcome assessors aware of the intervention received by study participants?		NA / Y / PY / PN / N / NI
4.4 If Y/PY/NI to 4.3: Could assessment of the outcome have been influenced by knowledge of intervention received?		NA / Y / PY / PN / N / NI
4.5 If Y/PY/NI to 4.4: Is it likely that assessment of the outcome was influenced by knowledge of intervention received?		NA / Y / PY / PN / N / NI
Risk-of-bias judgement		Low / High / Some concerns
Optional: What is the predicted direction of bias in measurement of the outcome?		NA / Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Domain 5: Risk of bias in selection of the reported result

Signalling questions	Comments	Response options
5.1 Were the data that produced this result analysed in accordance with a pre-specified analysis plan that was finalized before unblinded outcome data were available for analysis?		Y / PY / PN / N / NI
Is the numerical result being assessed likely to have been selected, on the basis of the results, from...		
5.2. ... multiple eligible outcome measurements (e.g. scales, definitions, time points) within the outcome domain?		Y / PY / PN / N / NI
5.3 ... multiple eligible analyses of the data?		Y / PY / PN / N / NI
5.4 Is a result based on data from both periods sought, but unavailable on the basis of carryover having been identified?		Y / PY / PN / N / NI
Risk-of-bias judgement		Low / High / Some concerns
Optional: What is the predicted direction of bias due to selection of the reported result?		NA / Favours experimental / Favours comparator /

		Towards null /Away from null / Unpredictable

Overall risk of bias

Risk-of-bias judgement		Low / High / Some concerns
Optional: What is the overall predicted direction of bias for this outcome?		NA / Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

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Revised Cochrane risk-of-bias tool for randomized trials (RoB 2) TEMPLATE FOR COMPLETION

Edited by Julian PT Higgins, Jelena Savović, Matthew J Page, Jonathan AC Sterne
on behalf of the RoB2 Development Group

Version of 22 August 2019

The development of the RoB 2 tool was supported by the MRC Network of Hubs for Trials Methodology Research (MR/L004933/2- N61), with the support of the host MRC ConDuCT-II Hub (Collaboration and innovation for Difficult and Complex randomised controlled Trials In Invasive procedures - MR/K025643/1), by MRC research grant MR/M025209/1, and by a grant from The Cochrane Collaboration.

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Study design	

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Is the review team's aim for this result…?

☐ to assess the effect of *assignment to intervention* (the 'intention-to-treat' effect)

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If the aim is to assess the effect of *adhering to intervention*, select the deviations from intended intervention that should be addressed (at least one must be checked):

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☐ Conference abstract(s) about the trial

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☐ Research ethics application

☐ Grant database summary (e.g. NIH RePORTER or Research Councils UK Gateway to Research)

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Risk of Bias Assessment

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Domain 1: Risk of bias arising from the randomization process

Signalling questions	Comments	Response options

1.1 Was the allocation sequence random?		Y / PY / PN / N / NI
1.2 Was the allocation sequence concealed until participants were enrolled and assigned to interventions?		Y / PY / PN / N / NI
1.3 Did baseline differences between intervention groups suggest a problem with the randomization process?		Y / PY / PN / N / NI
Risk-of-bias judgement		Low / High / Some concerns
Optional: What is the predicted direction of bias arising from the randomization process?		NA / Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Domain 2: Risk of bias due to deviations from the intended interventions (*effect of assignment to intervention*)

Signalling questions	Comments	Response options
2.1. Were participants aware of their assigned intervention during the trial?		Y / PY / PN / N / NI
2.2. Were carers and people delivering the interventions aware of participants' assigned intervention during the trial?		Y / PY / PN / N / NI
2.3. If Y/PY/NI to 2.1 or 2.2: Were there deviations from the intended intervention that arose because of the trial context?		NA / Y / PY / PN / N / NI
2.4 If Y/PY to 2.3: Were these deviations likely to have affected the outcome?		NA / Y / PY / PN / N / NI
2.5. If Y/PY/NI to 2.4: Were these deviations from intended intervention balanced between groups?		NA / Y / PY / PN / N / NI
2.6 Was an appropriate analysis used to estimate the effect of assignment to intervention?		Y / PY / PN / N / NI
2.7 If N/PN/NI to 2.6: Was there potential for a substantial impact (on the result) of the failure to analyse participants in the group to which they were randomized?		NA / Y / PY / PN / N / NI
Risk-of-bias judgement		Low / High / Some concerns

Optional: What is the predicted direction of bias due to deviations from intended interventions?		NA / Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Domain 2: Risk of bias due to deviations from the intended interventions (*effect of adhering to intervention*)

Signalling questions	Comments	Response options
2.1. Were participants aware of their assigned intervention during the trial?		Y / PY / PN / N / NI
2.2. Were carers and people delivering the interventions aware of participants' assigned intervention during the trial?		Y / PY / PN / N / NI
2.3. [If applicable:] If Y/PY/NI to 2.1 or 2.2: Were important non-protocol interventions balanced across intervention groups?		NA / Y / PY / PN / N / NI
2.4. [If applicable:] Were there failures in implementing the intervention that could have affected the outcome?		NA / Y / PY / PN / N / NI
2.5. [If applicable:] Was there non-adherence to the assigned intervention regimen that could have affected participants' outcomes?		NA / Y / PY / PN / N / NI
2.6. If N/PN/NI to 2.3, or Y/PY/NI to 2.4 or 2.5: Was an appropriate analysis used to estimate the effect of adhering to the intervention?		NA / Y / PY / PN / N / NI
Risk-of-bias judgement		Low / High / Some concerns
Optional: What is the predicted direction of bias due to deviations from intended interventions?		NA / Favours experimental / Favours comparator /

		Towards null /Away from null / Unpredictable

Domain 3: Missing outcome data

Signalling questions	Comments	Response options
3.1 Were data for this outcome available for all, or nearly all, participants randomized?		Y / PY / PN / N / NI
3.2 If N/PN/NI to 3.1: Is there evidence that the result was not biased by missing outcome data?		NA / Y / PY / PN / N
3.3 If N/PN to 3.2: Could missingness in the outcome depend on its true value?		NA / Y / PY / PN / N / NI
3.4 If Y/PY/NI to 3.3: Is it likely that missingness in the outcome depended on its true value?		NA / Y / PY / PN / N / NI
Risk-of-bias judgement		Low / High / Some concerns
Optional: What is the predicted direction of bias due to missing outcome data?		NA / Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Domain 4: Risk of bias in measurement of the outcome

Signalling questions	Comments	Response options
4.1 Was the method of measuring the outcome inappropriate?		Y / PY / PN / N / NI
4.2 Could measurement or ascertainment of the outcome have differed between intervention groups?		Y / PY / PN / N / NI
4.3 If N/PN/NI to 4.1 and 4.2: Were outcome assessors aware of the intervention received by study participants?		NA / Y / PY / PN / N / NI
4.4 If Y/PY/NI to 4.3: Could assessment of the outcome have been influenced by knowledge of intervention received?		NA / Y / PY / PN / N / NI
4.5 If Y/PY/NI to 4.4: Is it likely that assessment of the outcome was influenced by knowledge of intervention received?		NA / Y / PY / PN / N / NI
Risk-of-bias judgement		Low / High / Some concerns
Optional: What is the predicted direction of bias in measurement of the outcome?		NA / Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Domain 5: Risk of bias in selection of the reported result

Signalling questions	Comments	Response options
5.1 Were the data that produced this result analysed in accordance with a pre-specified analysis plan that was finalized before unblinded outcome data were available for analysis?		Y / PY / PN / N / NI
Is the numerical result being assessed likely to have been selected, on the basis of the results, from...		
5.2. ... multiple eligible outcome measurements (e.g. scales, definitions, time points) within the outcome domain?		Y / PY / PN / N / NI
5.3 ... multiple eligible analyses of the data?		Y / PY / PN / N / NI
Risk-of-bias judgement		Low / High / Some concerns
Optional: What is the predicted direction of bias due to selection of the reported result?		NA / Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

Overall risk of bias

Risk-of-bias judgement		Low / High / Some concerns
Optional: What is the overall predicted direction of bias for this outcome?		NA / Favours experimental / Favours comparator / Towards null /Away from null / Unpredictable

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