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The Effect of Manipulating the IL-6 Response to Exercise on Biomarkers and Exercise Performance

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Abstract

Interleukin-6 (IL-6) is a pluripotent cytokine which has inflammatory properties. It is suggested to mediate a variety of processes including increased perception of fatigue during inflammatory states. In addition, prolonged exercise can cause a marked increase in circulating IL-6, and although there is a widely reported association between plasma IL-6 and fatigue in disease and inflammatory conditions, this relationship has remained relatively unexplored in healthy individuals during exercise. Therefore, the aims of this thesis were firstly, to develop a repeatable pre-load time trial in order to examine the variability of circulating IL-6 and other signalling molecules in response to an exercise challenge; secondly, to investigate the effect of plasma IL-6 and signalling molecules on fatigue and performance during a pre-loaded time trial; and thirdly, to evaluate the impact of nutritional interventions (glutamine intake during exercise, manipulation of pre-exercise diet and carbohydrate intake during exercise) on the response of circulating IL-6, IL-6 signalling molecules and biomarkers associated with IL-6 during exercise and their effect on pre-load time trial performance.

Results from the studies determined that there was large variability in the plasma IL-6 and signalling receptors response to the pre-loaded time trial (8-20%) but that the exercise protocol was repeatable. Nutritional interventions did not alter the signalling receptor response, nor biomarkers associated with IL-6, including hepcidin. However, carbohydrate intake during exercise attenuated the circulating IL-6 response to exercise by

I

49% which correlated well with an improved time trial performance. Regardless of the intervention, a consistent finding in all studies indicated that a greater plasma IL-6 response to the preload exercise bout correlated well with a reduced relative exercise performance as a percentage of velocity at $\dot{V}O_{2max}$ during the subsequent time trial.

To summarise, the findings from this thesis indicate that elevated levels of plasma IL-6 are associated with a decrement in exercise performance. Associated IL-6 signalling molecules are elevated in response to exercise but are not associated with performance and are unaltered by nutritional interventions.

Contents

Chapter 5

The Effect of Pre-exercise Feeding Protocols on the IL-6 and Signalling Receptor Response to Exercise 84

List of Tables

List of Figures

List of Abbreviations

1hPost	1 h post time trial
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
24hPost	24 h post baseline
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
BBB	blood brain barrier
BSF-2	B-cell stimulatory factor-2
C/EBP	CCAAT enhancer binding protein
CRH	corticotropin-releasing hormone
CRP	c-reactive protein
CVg	inter-individual coefficient of variation
CVi	intra-individual coefficient of variation
CVO	circumventricular organs
DepHi	depletion of glycogen and high carbohydrate diet
DS-sIL-6R	differential mRNA spliced soluble interleukin-6 receptor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinases
GLUT-4	glucose transporter type 4
Hi	high carbohydrate diet
HPA axis	hypothalamic-pituitary-adrenal axis

ICC	intraclass correlation
IFN- β2	interferon-β2
IFNγ	interferon-γ
IL-1β	interleukin-1β
IL-1ra	interleukin-1 receptor agonist
IL-6	interleukin-6
IL-10	interleukin-10
IRS-1	insulin receptor substrate-1
JAK	janus kinase
JNK	c-Jun NH_2-terminal kinases
kDa	kilo-dalton
LEAP-1	liver-expressed antimicrobial peptide
Lo	low carbohydrate diet
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinases
mRNA	messenger ribonucleic acid
NF-κβ	nuclear factor-κβ
PC-sIL-6R	proteoyltic cleaved soluble interleukin-6 receptor
PEPCK	phosphoenolpyruvate-carboxykinase
PGE_2	prostaglandin E_2
PL	preload
PostTT	post time trial

PPAR	peroxisome proliferator activator receptors
PVN	paraventricular nucleus
rhIL-6	recombinant human interleukin-6
ROS	reactive oxygen species
RPE	ratings of perceived exertion
sgp130	soluble glycoprotein 130
sIL-6R	soluble IL-6 receptor
STAT	signal transducers and activators of transcription
sTNFR	soluble tumor necrosis factor receptor
TNF-α	tumor necrosis factor- α
$\dot{V}O_{2max}$	maximal oxygen uptake
v$\dot{V}O_{2max}$	velocity at maximal oxygen uptake

Publications

Data within this thesis has formed the basis of the following

peer reviewed publications and conference proceedings:

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K.G. and Ansley, L. (2010). The reliability of the IL-6, sIL-6R and sgp130

response to a preloaded time trial. *Eur J Appl Physiol*; 110. pp 619–25.

Robson-Ansley, P; Walshe, I. and Ward, D. (2011) The effect of

carbohydrate ingestion on plasma interleukin-6, hepcidin and iron

concentrations following prolonged exercise. *Cytokine*, 53. pp196-200

Conference Proceedings

Walshe, I; Ansley, L; Robson-Ansley, P. (2009) Carbohydrate

supplementation does not alter the plasma soluble gp130 concentration in

response to prolonged running. In: Ninth symposium of the international

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Authors Declaration

I declare that the work contained in this thesis is all my own work with the exception where due acknowledgement has been made. This work has not been submitted for any other award.

Name:

Signature:

Date:

Chapter 1

Introduction and Literature Review

1.1 Introduction

Endurance exercise can place a high demand on the body which requires an increase in oxidative metabolism that can be met by utilisation of a selection of fuel substrates, including a proportion of lipids and carbohydrates (Maughan and Gleeson, 2004). During a prolonged period of moderate intensity exercise, liver and muscle glycogen stores can become depleted (Bergstrom and Hultman, 1967; Baldwin et al., 1975) which can not only lead to fatigue and a decrement in performance, but can also cause a disruption in homeostasis to biological systems relating to circulating glucose regulation.

In order to maintain homeostasis from such a biological stressor, a number of hormones and cytokines are released into the circulation which counteracts the actions of metabolic stress (Galbo et al., 1975; Lundberg et al., 1985; Ostrowski et al., 1998; Febbraio et al., 2004). During prolonged exercise, an increase in different subtypes of cytokines can be observed in the circulation, including interleukin-1 receptor antagonist (IL-1ra), interleukin-10 (IL-10) and soluble tumor necrosis factor receptor (sTNFR). However, interleukin-6 (IL-6) demonstrates the largest circulating increase during prolonged exercise which can increase up to 100 fold (Fischer, 2006).

Interleukin-6 is a cytokine that was originally thought to be a protein released by immune cells for the sole purpose to initiate the response of the immune system. However, it has been reported more recently that IL-6 can act in a hormone like manner to mediate the actions of non-immune

2

cells and organs (Lancaster, 2006). IL-6 is involved in cellular communication that initiates the function of a range of organs throughout the body, including the brain (Tsigos et al., 1997; Sparkman et al., 2006).

Circulating IL-6 can communicate with the brain through both direct and indirect mechanisms. IL-6 has been shown to cross the blood brain barrier (Banks et al., 1994) where it can interact with the brain. IL-6 may also interact with neurons extending from the brain where concentrations of blood borne proteins and peptides can be detected. Greater circulating levels of IL-6 have often been associated with fatigue and poor functional performance in healthy populations (Cappola et al., 2003; Cesari et al., 2004). However, despite a widely reported association between circulating IL-6 and fatigue in inflammatory states, the association between circulating IL-6 and fatigue in exercise performance in healthy trained runners during prolonged exercise is yet to be explored.

1.1.1 Aims and Objectives

Accordingly, the aims of this thesis were explore the association between increased circulating IL-6 from prolonged exercise and subsequent exercise performance.

- The primary purpose of this thesis was to examine the influence of increased circulating IL-6 from an exercise challenge on subsequent time trial performance.

- In addition, a second aim of this thesis was to investigate the role of the circulating soluble receptors of IL-6 (sIL-6R and sgp130) and their association with fatigue in response to the exercise challenge.

- Nutirional interventions employed to manipulate the exercise induced circulating IL-6 and signaling receptor response will determine the role of IL-6 and sIL-6R in exercise performance.

- Additionally, a further objective was to assess the influence of manipulated circulating IL-6 and sIL-6R response to exercise on circulating biomarkers that are associated with an increase in plasma IL-6.

1.2 Interleukin-6

1.2.1 Background of IL-6

IL-6 is a polypeptide molecule that is relatively small in mass, ranging from 21-30 kDa in size and is initially generated from 212 amino acids which are cleaved during the post-translational process to become a 184 amino acid peptide (Hirano et al., 1985; Van Snick et al., 1986). Although initially named interferon-β2 (IFN- β2) (Gauldie et al., 1987), the cytokine has also been termed B-cell stimulatory factor-2 (BSF-2) (Hirano et al., 1985), 26 kDa protein (Haegeman et al., 1986) and hepatocyte-stimulating factor (Gauldie et al., 1987) amongst others before its current identity as IL-6 with a diverse role in biology (Haegemen et al., 1986).

IL-6 can be released from various cell types and this release is dependent on the circumstance of cell stimulation. Monocytes (Maloponte et al., 2002; Neuner et al., 1991), smooth muscle cells (Wang et al., 2001), adipose tissue (Mohamed-Ali et al., 1997; Fain et al., 2004) and skeletal muscle cells (Steensberg et al., 2000; Keller et al., 2001) have all been shown to stimulate IL-6 release along with other cells which can contribute to a pro-inflammatory or anti-inflammatory environment (Opal and DePalo, 2000). The pro-inflammatory properties of IL-6 have been previously reported with functions such as a pyrogenic action which is associated with fever (Weber et al., 1993; Tsigos et al., 1997). Furthermore, circulating IL-6 has also been associated with many inflammatory states such as rheumatoid arthritis (Knudsen et al., 2008), cancer (Coussens and Werb, 2002) as well as type 2 diabetes mellitus and cardiovascular disease (Abeywardena et al., 2009). Adipose tissue can also secrete IL-6

5

(Mohamed-Ali et al., 1997; Fain et al., 2004) which has led to the proposal that this can lead to chronic inflammation in obese individuals (Abeywardena et al., 2009), thus, increasing the risk of type 2 diabetes mellitus and cardiovascular disease (McKeigue et al., 1991; Goran et al., 2003). Moreover, greater basal levels of systemic IL-6 have been associated with an increased prevalence of type 2 diabetes mellitus which is independent of body mass index (Pradhan et al., 2001). In cell culture, IL-6 has been shown to reduce insulin receptor substrate-1 (IRS-1) in human adipose cells (Rotter et al., 2003) and in murine hepatocytes (Senn et al., 2002) which may lead to insulin resistance. Although insulin resistance can be a risk factor for cardiovascular disease, the association between high levels of IL-6 and prevalence of cardiovascular disease could also be due to direct mechanisms of atherosclerosis. Increased expression of IL-6 is found in a number of atherosclerotic plaques (Schieffer et al., 2000) and also in vascular smooth muscle cells (Wang et al., 2001). Pro-inflammatory cytokines such as IL-1β and TNF-α have also been shown to initiate an increase in systemic IL-6 (Akira et al., 1993) which can disrupt the permeability of the vascular wall and increase fatty deposits (Abeywardena et al., 2009), suggesting that IL-6 may exert negative actions in chronic low level inflammation.

In contrast, IL-6 has also been reported to have anti-inflammatory properties with positive health effects (Pedersen and Fischer, 2007; Mathur and Pedersen, 2008). Infusion of recombinant human (rh) IL-6 into humans improves insulin-stimulated glucose uptake (Carey et al., 2006) and furthermore can increase fat oxidation (Petersen et al., 2005).

Studies have also shown that IL-6 down-regulates expression of TNF-α and also initiates the release of the anti-inflammatory cytokines IL-1 receptor antagonist (IL-1ra) and IL-10 (Schindler et al., 1990; Jones et al 2001; Ostrowski et al., 1998). Lipopolysaccharide (LPS) induced TNF-α production can be suppressed by IL-6 (Schindler et al., 1990) which may indicate that the purpose of the induction of IL-6 could be to prevent excessive pro-inflammatory actions. During prolonged exercise, a similar anti-inflammatory pattern is also seen as a large increase in systemic IL-6 is followed by increases in IL-1ra and IL-10 (Ostrowski et al., 1998). Furthermore, only slight increases in pro-inflammatory cytokines are seen following prolonged exercise, such as TNF-α (Ostrowski et al., 1998). Therefore, when taken together, these apparently contradicting findings may indicate that although IL-6 may contribute to chronic low level inflammation; exercise induced IL-6 release may initiate anti-inflammatory effects that may be beneficial to health.

During prolonged exercise, skeletal myocytes are a predominant source of circulating IL-6 (Steensberg et al., 2000); however other tissues can contribute to the circulating pool of IL-6 (Langberg et al., 2002; Fischer et al., 2004). Systemic IL-6 appears to increase in accordance with the intensity and duration of exercise (Fischer, 2006), and may also be affected by the mode of exercise (Nieman et al., 1998) as well as individual endurance capacity (Fischer et al., 2004). As the duration of exercise increases, so does the response of circulating IL-6 levels. Fischer (2006) illustrated that a linear regression of results from 67 trials showed that 51% of the increase seen in IL-6 during the trials can be

explained by the duration of exercise. Furthermore, the author reported

that the relationship could be even more pronounced if the data were

adjusted for relative exercise intensity. Exercise intensity has also been

shown to be related to increases in circulating IL-6 among marathon

participants when expressed in relative terms (Ostrowski et al., 2000).

However, when running is compared to cycling at the same intensity

running appears to increase plasma IL-6 to a greater extent than cycling

(Nieman et al., 1998) suggesting that mode of exercise can impact on the

IL-6 response which can be attributed to larger muscle groups being

activated during exercise. Table 1.1 shows typical circulating IL-6

increases following prolonged running.

Table 1.1 Circulating IL-6 Increases Following Prolonged Running

Duration	Intensity	Fold increase	(n)	Reference
2.5 h	75-80% VO_{2max}	8	30	Nehlsen Cannarella et al., 1997
2.5 h	75% VO_{2max}	25	10	Ostrowski et al., 1998
2.5 h	75% VO_{2max}	29	7	Steensberg et al., 2001
2.5 h	75% VO_{2max}	30	9	Steensberg et al., 2001
2.5 h	75% VO_{2max}	52	10	Nieman et al., 1998
2.5 h	16.4 $km \cdot h^{-1}$	109	16	Suzuki et al., 2000
2.6 h	16.1 $km \cdot h^{-1}$	80	10	Suzuki et al., 2003
3 h	70% VO_{2max}	10	16	Nieman et al., 2002
3 h	12 $km \cdot h^{-1}$	50	6	Langberg et al., 2002
3.75 h	11.2 $km \cdot h^{-1}$	44	12	Castell et al., 1996

1.2.2 Production of IL-6

The relationships between the duration, intensity and mode of exercise and circulating IL-6 levels may be accounted for by the extent of metabolic stress produced by exercise, since greater demands of exercise require greater CHO oxidation rates. During prolonged exercise, it is often observed that circulating glucose concentrations are maintained with a simultaneous increase in circulating IL-6 (Nhelsen-Cannarella et al., 1997; Steensberg et al., 2001; Ronsen et al., 2002; Pedersen et al., 2003), suggesting that muscle glycogen is the stimulus for increased circulating IL-6 production, rather than blood glucose concentration (Ronsen et al 2002). Therefore, it has been suggested that there is a relationship between systemic IL-6 and levels of muscle glycogen. Work by Keller et al. (2001) and Steensberg et al. (2001) have confirmed this hypothesis by demonstrating that contracting muscle releases IL-6 in relation to muscle glycogen levels. Keller et al. (2001) demonstrated that a ~40% reduction in muscle glycogen led to a 40% increase in circulating IL-6 during the exercise bout when compared to a control condition. Furthermore, transcription of the IL-6 gene and increased IL-6mRNA were enhanced during low glycogen conditions. Although this indicated that IL-6 production was receptive to muscle glycogen levels, it could not discount that exposure to an increase in hormonal activity or substrate was accountable for the increase in circulating IL-6 and IL-6mRNA. Steensberg et al. (2001) overcame this by depleting muscle glycogen levels in one leg and maintaining glycogen levels in the other leg. The results showed that an increased release of IL-6 from the glycogen

depleted leg was in accordance with a marked increase in IL-6mRNA compared to the control leg.

Increases in IL-6mRNA in glycogen depleted muscle following prolonged exercise has been demonstrated in numerous studies (Keller et al., 2001; Steensberg et al., 2001; Chan et al., 2004; Hiscock et al., 2004) and could be due to a number of signalling events. Mitogen-activated protein kinases (MAPK) and nuclear transcription factors are involved in signalling pathways in a variety of circumstances including cellular stress as a result of prolonged exercise (Kramer and Goodyear, 2007). Subclasses of MAPK such as extracellular signal-regulated kinases (ERK), p38 MAPK and c-Jun NH_2-terminal kinases (JNK) can increase in response to exercise according to the duration (Sakamoto and Goodyear, 2002), intensity (Widegreen et al., 2000) and mode of exercise (Nader et al., 2001). p38 MAPK is expressed in skeletal muscle (Baeza-Raja et al., 2004; Chan et al., 2004; Akimoto et al., 2005) and phosphorylation of p38 MAPK can increase in response to cycling after 30 min (Widegren et al., 1998; Widegren et al., 2000) which is in accordance with increases observed in IL-6 (Keller et al., 2001). Therefore, this could suggest that exercised induced IL-6 could be related to p38 MAPK phosphorylation. Chan et al. (2004) investigated this relationship and showed that exercise with low muscle glycogen resulted in an increase in phosphorylated p38 MAPK within myocytes; moreover, a significant correlation between pre-exercise p38 MAPK and the IL-6 mRNA response to exercise was

observed, providing support for the proposal that p38 MAPK may, in part, regulate IL-6mRNA expression.

Marked increases in p38 MAPK and JNK phosphorylation are also seen during high intensity exercise (Widegreen et al., 2000; Martineau and Gardiner, 2001; Yu et al., 2003) which could be a result of Ca^{2+} release during exercise, acting as a potent stimulus for a variety of biological processes (Berridge et al., 2001). In vitro studies have shown that Ca^{2+} ionophore ionomycin increases p38 MAPK phosphorylation in L6 myotubes (Elzi et al., 2001; Chan et al. 2004), and furthermore, work by Chan et al. (2004) also showed that 60 min of cycling increased phosphorylation of JNK in skeletal muscle in vivo. Therefore, collectively these findings may indicate that an increase in Ca^{2+} levels from skeletal muscle contraction can increase p38 MAPK phosphorylation, which in turn may lead to an increase in IL-6mRNA.

Prolonged exercise can lead to an increase in oxidative stress (Ji, 1999), particularly if the individual performing the exercise bout is unaccustomed to the exercise (Kramer and Goodyear, 2007). As contracting skeletal muscle can increase reactive oxygen species (ROS), this can stimulate activation of the transcription factor NF-κβ (Schmidt et al., 1995; Zhang et al., 2001). It has been previously been shown that NF-κβ can stimulate IL-6 expression. Kosmidou et al. (2002) demonstrated that in skeletal myotubes, IL-6 production can be triggered by ROS, and furthermore, a pharmacological inhibitor of NF-κβ inhibited ROS stimulated IL-6. Whether this phenomenon occurs *in vivo* in response to

exercise or not is unclear, but work from Fischer et al. (2004) demonstrated that antioxidant supplementation blunted the exercise induced circulating IL-6 response to exercise, although it did not significantly blunt IL-6mRNA within skeletal muscle.

Taken together, it is clear that IL-6mRNA transcription in skeletal muscle can be stimulated by a variety of factors including low skeletal muscle glycogen, a rise in Ca^{2+} levels from skeletal muscle contraction and increased ROS activity associated with prolonged exercise. Although a combination of these factors could be responsible for IL-6 transcription, it is possible that p38 MAPK, NF-κβ and JNK could all play a role in transcription of IL-6, perhaps through a cross-talk pathway (Febbraio and Pedersen, 2002).

1.3 Interleukin-6 Receptors

For IL-6 to instigate any biological actions, it must interact with the receptor which is situated on the surface of the target cell. Interaction must occur via two different membrane bound glycoproteins, firstly, IL-6 must bind with its receptor (IL-6R), then to the non ligand binding receptor, glycoprotein 130 (gp130). As a complex, signaling can then occur on cells expressing gp130 (figure 1.1) (Heirich et al., 1998; Jones et al., 2001). Many cells can express IL-6R including different regions of the brain such as the hippocampus and hypothalamus (Schobitz et al., 1993; Gadient and Otten, 1993), skeletal muscle cells (Keller et al. 2005), hepatocytes and leukocytes (Jones et al., 2001); whereas the gp130 receptor is ubiquitously expressed throughout the body (Hibi et al., 1990). This can

render the actions of IL-6 dependent and limited to the amount of IL-6R expression on target cells. However, a cognate receptor, soluble IL-6 receptor (sIL-6R) can shed from the cell surface and act in the same manner as IL-6R which can 'transsignal' through membrane bound gp130 (Muller-Newen et al., 1998). Therefore this can potentially increase the range of cells that can be stimulated from IL-6.

1.3.1 sIL-6R

sIL-6R is a 50-55 kDa protein which binds to IL-6 with a similar affinity as the membrane bound IL-6 receptor (Mitsuyama et al., 1995; Muller-Newen et al., 1998) to form an IL-6/sIL-6R complex which increases the duration of biologically active IL-6 by preventing its degradation (Gerhartz et al., 1994). Several studies have shown that sIL-6R can augment the actions of IL-6 in various biological processes. The presence of sIL-6R can increase the sensitisation of many cells to IL-6 including neuronal cells (Marz et al., 1999), hepatic cells (Mackiewicz et al., 1992) and muscle cells (Gray et al., 2009) when associated with sIL-6R. Schobitz et al. (1995) demonstrated that administration of IL-6 intracerebroventricularly into rats increased core temperature which was accompanied by a decrease in activity and food intake. When sIL-6R was also administered, symptoms were enhanced and prolonged. This phenomenon was also observed with skeletal muscle glucose uptake (Gray et al., 2009). Mouse skeletal muscle was incubated with IL-6 which showed no effect on glucose uptake. However when skeletal muscle was incubated with both IL-6 and sIL-6R, glucose uptake was increased.

When greater concentrations of IL-6 and sIL-6R were incubated, this augmented glucose uptake further. Mackiewicz et al. (1992) also illustrated this phenomenon by demonstrating that HepG2 cells that were chronically exposed to IL-6 were desensitised to the actions of IL-6; however, the addition of sIL-6R to the incubation increased the responsiveness, potentiating the effects of IL-6. Collectively, this indicates that sIL-6R increases the biological potency of IL-6 and can also increase the duration of its activity, which illustrates the importance of the interaction between sIL-6R and IL-6 and must be considered when examining the effects of IL-6.

sIL-6R can be produced via two different pathways comprising of alternative or differential mRNA splicing (DS-sIL-6R) or proteolytic cleavage of a membrane bound receptor (PC-sIL-6R). Membrane bound receptors that encode mRNA transcripts differentially splice to yield DS-sIL-6R production (Horiuchi et al., 1994) which can be detected 8-24 h after stimulation using specific antibodies against the peptide (Cichy et al., 1997). In contrast to DS-sIL-6R, PC-sIL-6R production is rapid and can be detected within 30-120 min of stimulation and is produced as a result of shedding from membrane bound receptors at a site close to the cell surface (Jones et al., 1999). PC-sIL-6R has been shown to increase with age (Muller-Newen et al., 1996), during certain conditions such as an inflammatory state (Hurst et al., 2001) and also during exercise (Leggate et al., 2010). The precise mechanism for proteolytic cleavage of IL-6R is unclear with a number of mechanisms proposed to be responsible for IL-6R shedding. IL-6R may be cleaved via a number of enzymes including

protein kinase C (Mullberg et al. 1992), mellatoproteases (Gallea-Robache et al., 1997), or could be as a result of Ca^{2+} mobilisation (Jones et al., 1998). Neutrophil apoptosis can increase shedding which is regulated by metalloproteases (Gaellea-Robache et al., 1997; Chalaris et al., 2007). However, neutrophils exposed to CRP demonstrated an increase in shedding which was not regulated by a metalloprotease inhibitor (Jones et al., 1999).

Numerous studies have previously shown that during prolonged exercise plasma sIL-6R increases (Gray et al., 2008; Gray et al., 2009; Leggate et al., 2010), but this is not consistently shown (Keller et al., 2005) which may be a consequence of low subject numbers and large inter-individual variability. The cause of the release of plasma sIL-6R in response to prolonged exercise is unknown, however, Robson-Ansley et al. (2009) observed that very prolonged exercise (>6 h) each day, over 6 days resulted a large increase in plasma CRP which was maintained and correlated with sIL-6R. This may suggest that CRP increased the circulating sIL-6R increase; however it is uncertain which isoform demonstrated the predominant increase. During and immediately following prolonged exercise, plasma PC-sIL-6R significantly increases whereas increases in DS-sIL-6R are observed during the recovery period at 6 h post exercise (Leggate et al., 2010) which may be reflected by the time course of stimulation of each of the isoforms.

1.3.2 sgp130

For transsignaling to occur as a complex, IL-6, (s)IL-6R and gp130 are required to construct a hexamer of two IL-6, two IL-6 receptor and two gp130 proteins (Boulanger et al., 2003). Furthermore, like IL-6R, a soluble form of gp130 (sgp130) has been detected in human plasma, of which different isoforms of sgp130 exist, including differential mRNA splicing and as well as proteolytic cleaved sgp130. Both isoforms can act as an antagonist to the actions of the IL-6/sIL-6R complex (Montero-Julian et al., 1997; Muller-Newen et al., 1998). Since transsignaling occurs through gp130, the IL-6/sIL-6R complex binds to sgp130 with the same affinity to form an IL-6/sIL-6R/sgp130 complex. The molar excess of this complex leads to biological inactivity (Jostock et al., 2001) thus, regulating excessive signalling (figure 1.1). The source of sgp130 is unclear with only a small number of sites known to release sgp130 (Montero-Julian et al. 1997) however, an increase in plasma sgp130 has been shown in response to exercise (Gray et al., 2008). The relevance of the increase in sgp130 is unknown, however it could be postulated that sgp130 may regulate the actions initiated by the IL-6/sIL-6R complex by inhibiting transsignalling via membrane bound gp130. Evidence to support this from Rebouissou et al. (1998) demonstrated that an increase of sIL-6R preceded an increase in sgp130. Furthermore, the actions of sgp130 have been shown to inhibit the response of IL-6 mediated actions through sIL-6R without impeding the responses through membrane-bound IL-6R (Hurst et al., 2001; Jostock et al., 2001). Together, this can suggest that the response of sgp130 reacts to the actions of sIL-6R rather than IL-6.

Figure 1.1a.

gp130

IL-6

IL-6R gp130 IL-6R

Figure 1.1b.

gp130

sIL-6R

sIL-6R

IL-6
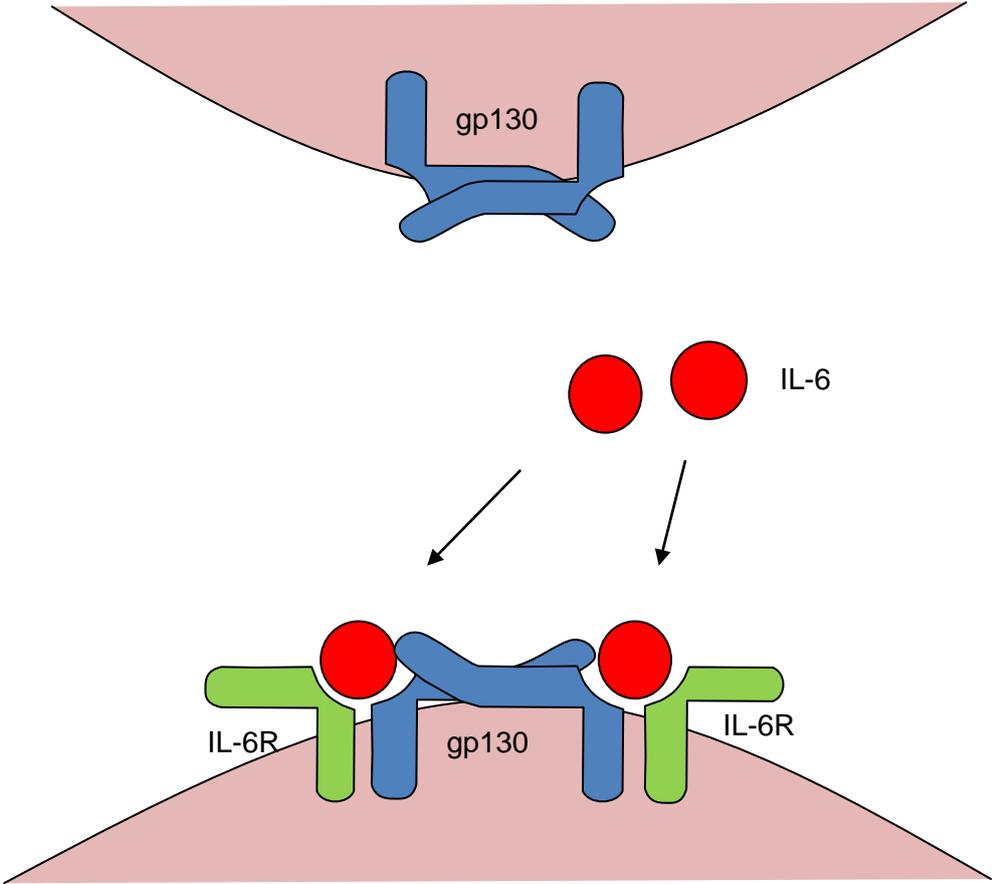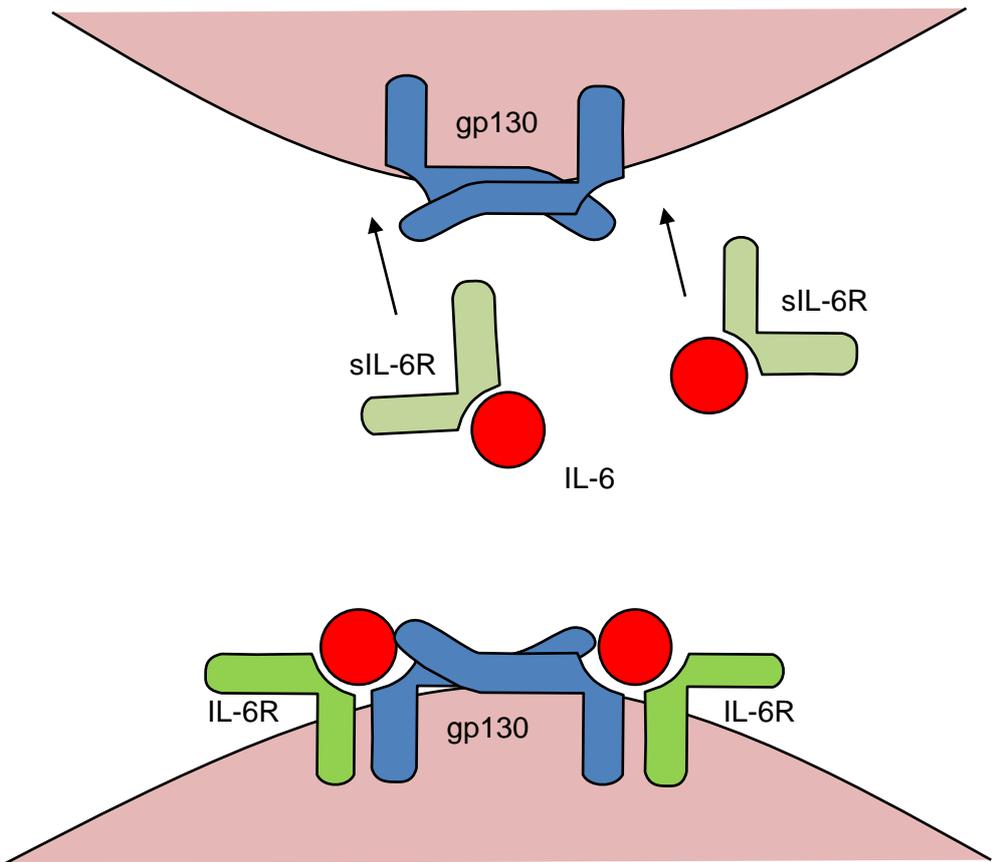
IL-6R gp130 IL-6R
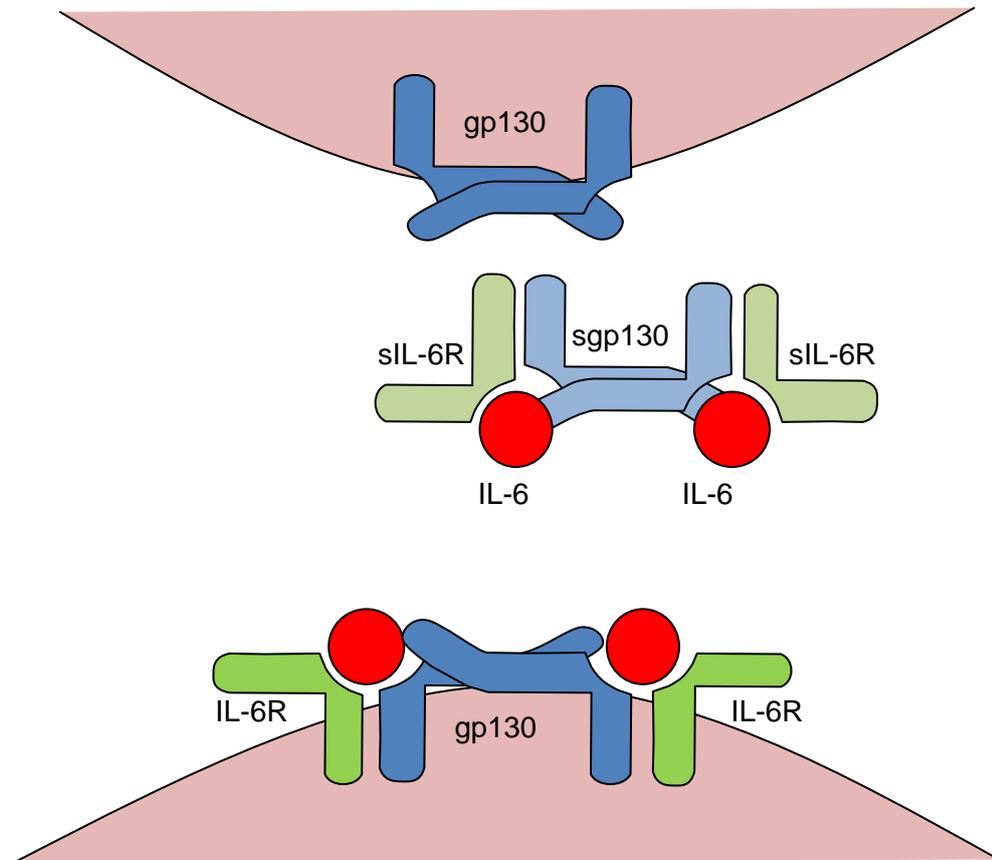
Figure 1.1c.

Figure 1.1 The signalling process of IL-6, sIL-6R and gp130. IL-6 in circulation readily binds onto IL-6R and gp130 on the cell surface to initiate transsignaling, however IL-6 is unresponsive to gp130 alone (figure 1.1a). In circulation, sIL-6R binds to IL-6 to form an IL-6/sIL-6R complex which can associate with membrane bound gp130 increasing IL-6 signalling (figure 1.1b). sgp130 binds to the IL-6/sIL-6R complex disrupting transsignaling of membrane bound gp130, therefore acting as an antagonist to the IL-6/sIL-6R complex (figure 1.1c).

1.3.3 Signalling

The signal transduction process allows for target cells to be activated from IL-6 signalling. Following IL-6 binding to the receptor IL-6R to form a complex, the IL-6/IL-6R complex then binds to the signalling receptor gp130; and through homodimerization of two membrane bound gp130 molecules, signaling occurs on the target cell (Murakami et al., 1993; Davis et al., 1993). Homodimerization activates the tyrosine kinases of the janus kinase family (JAK) JAK1, JAK2 and TYK2 (Vila-Coro et al., 1999) which associate with the receptor at multiple sites. Activation of JAKs then phosphorylate signal transducers and activators of transcription (STAT) in the cytoplasm, in particular, STAT1 and STAT3 (Gerhartz et al., 1996). STAT1 and STAT3 then translocate to the nucleus possibly with a shuttle protein carrying a nuclear localisation sequence for gene transcription (Strehlow and Schindler, 1998) allowing for tissue specific actions to take place.

1.4 Biological Roles of IL-6

IL-6 can exert both a local and global effect in the body. When in the circulation, IL-6 can exert a multitude of biological roles; therefore, increasing the potential to render IL-6 a stimulus to almost any cell in the body when in the presence of sIL-6R and membrane bound gp130. This reflects the pluripotent nature of the cytokine.

1.4.1 Metabolism

IL-6 plays a role in energy balance (Hoene and Weigert, 2007) which is enhanced during an energy crisis when energy substrates are low (Gleeson, 2000). IL-6 mobilizes extracellular substrates and also aids the delivery of substrates to target organs (Pedersen et al., 2003) which suggests an important role for IL-6 in metabolic control. Adipose tissue releases IL-6 into the circulation in healthy humans without the presence of inflammation; of which a greater proportion is secreted from visceral adipose tissue than subcutaneous adipose tissue (Bastard et al., 2006). At rest, 10-35% of the body's basal plasma IL-6 is derived from adipose tissue (Febbraio and Pedersen, 2002) and furthermore, it has been reported that infusion of IL-6 increases triglyceride and free fatty acid concentration in the circulation (Petersen et al., 1995; Southard et al., 1995; van Hall et al., 2003) and also affects liver metabolism, with increased VLDL secretion and hypertriglyceridemia (Bastard et al., 2006). Southard et al. (1995) demonstrated that infusion of rhIL-6 increased fatty acid oxidation, although it could not be determined if this increase was due to IL-6 *per se* or due to the IL-6 mediated increases in adrenaline and nor-adrenaline. A more recent study has shown an increase in fat oxidation without an increase in catecholamines, cortisol and glucagon when humans are infused with a low dose of rhIL-6. However, the fat oxidation was not enhanced when infused a high dose of rhIL-6 (van Hall et al., 2003). This is in accordance with a study from Hiscock et al. (2005) who demonstrated that during low intensity exercise, infusion of IL-6 did not further increase lipolysis when compared to exercise alone. Collectively,

these studies suggest that IL-6 can play a role in lipolysis at rest but the role of IL-6 in lipolysis is not effective during exercise. Although the effects of IL-6 on lipolysis are clear, the effects of IL-6 on insulin actions appear to be more controversial (Pedersen and Febbraio, 2007; Mooney, 2007).

IL-6 is elevated in the circulation during conditions that are associated with low grade inflammation, such as obesity, type 2 diabetes mellitus and is often associated with insulin resistance (DeFronzo and Ferrrannini, 1991; Kern et al., 2001). It has been demonstrated that IL-6 can inhibit the phosphorylation and the transcription of the insulin receptor IRS-1 and the glucose transporter GLUT-4 in cell cultured adipocytes and hepatocytes (Senn et al., 2002; Rotter et al., 2003). However in contrast, IL-6 can have mobilising properties by promoting glucose uptake and increasing insulin sensitivity in myocytes (Carey et al., 2006). Furthermore, chronic exposure to IL-6 in cell culture appears to increase the mRNA expression of GLUT-4 and the transcription regulators peroxisome proliferator activator receptors (PPARα, PPARγ and PPARδ) in skeletal muscle (Al-Khalili et al., 2006). Incubation of IL-6 also increases skeletal muscle glucose incorporation into glycogen (Glund et al., 2007). In addition, IL-6 and sIL-6R combined can increase glucose uptake in mouse skeletal muscle which could not be stimulated by IL-6 alone (Gray et al., 2009). The apparent discrepancy may be accounted for by differences in the expression of IL-6R as previously discussed; or by tissue specific insulin sensitivity. Skeletal muscle is responsible for over 75% of insulin actions *in vivo* (Stump et al., 2006); therefore it could be

possible that myocytes are more sensitive to insulin compared to other organs. This is in accordance with the work by Weigert and colleagues (2005) who reported that the effects of IL-6 on insulin are tissue specific.

As well as the role of a glucose transporter, previous studies have also demonstrated the actions of IL-6 to be associated with endogenous glucose production (Southard et al.,1995; Tsigos et al., 1997; Kanemaki et al., 1998), although the mechanisms for this action is unclear and is not consistently demonstrated (Steensberg et al., 2003; Carey et al., 2006). Infusion of rhIL-6 has been shown to increase endogenous glucose production during exercise when compared to exercise at the same intensity when insulin, glucagon and catecholamines remained constant (Febbraio et al., 2004). Furthermore, Banzet et al. (2009) have also demonstrated that murine muscle derived IL-6 contributes to hepatic glucose production in a dose dependant manner, with observed increases in phosphoenolpyruvate-carboxykinase (PEPCK), a key enzyme for gluconeogenesis. However, IL-6 does not increase glucose-6-phosphatase concentrations which allow glucose to leave the hepatocyte (Mithieux, 1997). Together, this may suggest that the actions of IL-6 in glucose metabolism may differ between resting and exercising conditions or possibly that muscle contraction may induce a cofactor concurrently with IL-6 for hepatic glucose release (Febbraio et al., 2004). Collectively, these results indicate that the liver can be a target organ for IL-6 (figure 1.2). Work from Castell et al. (1988) support this concept by demonstrating that rats infused with ^{125}I-labelled rhIL-6 had a high clearance rate from the circulation, and that within 20 min, the majority of

IL-6 had disappeared from circulation with 80% subsequently being present in the liver. Although this suggests that the liver is a target organ for glucose production, this does not fully elucidate the function of IL-6 in the liver due to the complex roles that the liver is responsible for such as clearance of cytokines, glycogenolysis and the acute phase response.

1.4.2 Acute Phase Response

An inflammatory stimulus such as injury or disruption of homeostasis leads to an increase in acute phase proteins which are synthesised predominantly from hepatocytes that are released into the circulation (Baumann and Gauldie, 1994). A 25% change from baseline values of acute phase proteins has been defined as an acute phase response (Gabey and Krushner, 1999) with Interleukin-1β (IL-1β), Tumor Necrosis Factor α (TNFα), Interferon-γ (IFNγ) and IL-6 all shown to be activators (Heinrich et al., 1990). IL-6 has a major role in the acute phase response, stimulating a range of acute phase proteins in humans via the JAK/STAT3 signalling pathway (Gao, 2005) which can be amplified by sIL-6R when cells are desensitised to IL-6 (Mackiewicz et al., 1992). During an acute phase response, IL-6 is responsible for deceases in acute phase proteins such as albumin and transferrin whilst also being responsible for increases in several proteins including serum amyloid A and C-reactive protein (CRP) which can increase up to 1000 fold during inflammatory conditions (Heinrich et al., 1990).

CRP is an acute-phase protein that can be produced from multiple sites in the body (Kuta and Baum 1986; Calabro et al., 2003) but is largely

produced in the liver (Hurlimann et al., 1966). CRP consists of five 23-kDa subunits (Agrawal et al., 2002) and has multiple biological roles including pro and anti-inflammatory functions depending on the context of it being released. These include the clearance of damaged and apoptic cells (Gershov et al., 2000), as well as opsonisation and initiation of the complement cascade to play a role in host defence (Kilpatrick et al., 1985; Mold et al., 2002). Both IL-1β and IL-6 in combination stimulates maximal levels of CRP (Calabro et al., 2003), however, when measured independently, only IL-6 alone stimulates CRP (Castell et al., 1990) which can be regulated not only by the STAT3 pathway, but also by a CCAAT enhancer binding protein (C/EBP) (Agrawal et al., 2001). Disrupting phosphorylation of STAT3 attenuates the signalling of IL-6 induced CRP synthesis in hepatocytes (Arnaund et al., 2005). Together, this is in accordance with *in vivo* work since infusion of rhIL-6 into humans increased plasma levels of CRP 3 h post infusion with peak levels occurring 24 h post infusion (Steensberg et al., 2003); a similar pattern to those observed following prolonged exercise.

Exercise induced plasma CRP can increase 4 h post exercise (Seigal et al., 2001) with peak levels occurring 24 h post exercise remaining elevated at 72 h post exercise (Strachen et al., 1984). Strachan et al. (1984) reviewed the effect of long distance running on circulating CRP levels and found that greater levels of circulating CRP post exercise were present in those running longer distances. Since IL-6 is the predominant cytokine released during exercise it can be assumed that IL-

6 is responsible for the plasma response of CRP (figure 1.2). Evidence to support this is found in the study from Miles et al. (2006) who demonstrated that participants who took part in a 32 km mountain trail race had increased plasma IL-6 levels immediately following the race with significant increases seen with plasma CRP the following day. In contrast, Scharhag et al. (2006) found that following 4 h of cycling an increase in plasma IL-6 did not result in a significant elevation in plasma CRP 19 h post exercise. However, it could be speculated that the lack of increase is possibly due to the relatively low levels of IL-6 observed following exercise (~4 pg·ml^{-1}).

A type II acute phase protein, hepcidin has also been shown to increase in the circulation as part of the acute phase response (Nemeth et al., 2003; Nemeth et al., 2004; Lee et al., 2005). Like CRP, hepcidin has been suggested to have a role in the defence against invading pathogens thus leading to its original name liver-expressed antimicrobial peptide (LEAP-1) before later being termed hepcidin (Park et al., 2001). Hepcidin protects against pathogens by depriving pathogenic bacterium of iron by inhibiting iron release from macrophages (Lee et al., 2005) which can in turn, reduce circulating plasma iron levels. Over expression of hepcidin in mice leads to anaemia (Nicolas et al., 2002) and conversely, iron overload has been shown to increase hepcidin release. Whether this is a direct action of hepcidin or not is unclear (Nemeth et al., 2003).

Hepcidin is predominantly produced and released from hepatocytes (Park et al., 2001) which is triggered by IL-6 in cell culture (Nemeth et al.,

2003) and is blocked when IL-6 antibodies are added to the cell culture (Nemeth et al., 2004). However, in contrast, Lee and co-workers (2005) have reported that IL-1α and IL-1β stimulates hepcidin in IL-6 deficient cells, proposing that hepcidin is released in the absence of IL-6, thus providing equivocal findings *in vitro*. An increase in circulating IL-6 following administration of LPS in humans also results in an increase in plasma hepcidin. Circulating IL-6 levels peaked at 3 h with peak urinary hepcidin concentrations at 6 h post injection which resulted in a decrease in serum iron the following morning (Kemna et al., 2005). The findings from Kemna et al. (2005) support previous work by Nemeth et al. (2004) who have demonstrated that infusion of rhIL-6 resulted in a large increase in urinary hepcidin and a decrease in serum iron concentrations following the infusion.

Since IL-6 stimulates an increase in concentrations of hepcidin, it would reasonable to assume that exercise induced increases in IL-6 can initiate a similar hepcidin response. Roecker et al. (2005) examined female athletes after a marathon race and found an increase in urinary hepcidin the following day after the race. However, it is not clear if urinary hepcidin was related to IL-6 levels since plasma IL-6 and iron were not reported in the study by Roecker et al. (2005). A later study by Peeling et al. (2009) examined the effects of cumulative running sessions in a day and found an increase in IL-6, with urinary concentrations of hepcidin following a similar pattern 3 h post exercise.

Taken together, this can indicate that an increase of IL-6 levels can initiate an acute phase response leading to an increase in plasma CRP and hepcidin which is also seen when examining the effects of prolonged exercise (figure 1.2). Although muscle derived IL-6 interacts with the liver to initiate the acute phase response and mediate an increase in glucose production during prolonged exercise; IL-6 in the circulation pathways also interacts with brain to mediate central effects.

1.4.3 Central Effects of IL-6

IL-6 is well known to interact with the brain mediating many central effects such as initiating fever (Chai et al., 1996; Lenczowski et al., 1999), altering sleep architecture (Spath-Schwalbe et al., 1998; Hong et al., 2005) and inducing sickness behaviour (Bluthe et al., 2006; Konsman et al., 2002; Harden et al., 2006), all of which can possibly be attributed to activation of the hypothalamus (Mastorakos et al., 1993; Lenczowski et al., 1999; Spath-Schwalbe et al., 1994). To activate regions of the brain such as the hypothalamus, IL-6 may communicate with the brain through direct and indirect mechanisms including crossing the blood brain barrier (BBB), activation of neurons extending to the hypothalamus and also binding to the endothelial cells at the BBB to induce central mediators. Studies have demonstrated that IL-6 can directly cross the BBB in murine models (Banks et al., 1994; Blatteis and Sehic, 1997). Banks and colleagues demonstrated that infusion of radioactively labelled IL-6 at the periphery can be found in particular regions of the murine brain and can also be transported through a different system from those of TNF-α and IL-1α.

The authors also reported that IL-6 is able to cross the BBB in larger quantities when compared to TNF-α and IL-1α. Peripheral infusion of IL-6 resulted in ~0.2% entering the brain (Banks et al., 1994) whereas ~0.05% was observed with TNF-α and ~0.07% with IL-1α (Banks et al., 1991). In addition, IL-6R mRNA is found in the hypothalamus of rats as well as in neurons of the habenular nucleus, known to release dopamine and serotonin (Gadient and Otten, 1993; Schobitz et al., 1993). Low expression of IL-6R in the paraventricular nucleus (PVN) of the hypothalamus was also found to be rapidly upregulated during an endotoxemia challenge (Vallieres and Rivest, 1999). Therefore, collectively this provides evidence that IL-6 can cross the BBB where it interacts at particular sites of the brain that express IL-6 receptors.

Another possible mechanism for cytokines to communicate with the brain is via circumventricular organs (CVO) which are known to lack a BBB (Gross et al., 1987; Watkins et al., 1995). Previous research has shown that cytokines are transported to the brain passing through CVOs (Stitt, 1990; Katsuura et al., 1990) where concentrations of blood borne proteins and peptides can be detected, which subsequently, signal to the brain through neurons situated at the CVO site extending to the PVN in the hypothalamus (Xin and Blatteis., 1992; Marvel et al., 2003). In support of this notion, inhibition thermosensitive neurons and the dorsal vagal complex can block the actions of infused rhIL-6 and LPS. This consequently reduces c-fos expression in the PVN and also reduces fever and social withdrawal in rats (Marvel et al., 2003).

Cells that form the BBB are astrocytes, microglia and endothelial cells, all of which form tight junctions which create a barrier between the circulation and the central nervous system and maintain the functionality of the brain (Ballabh et al., 2004). At the BBB, IL-6 appears to interact with its corresponding receptors IL-6R and gp130 situated on endothelial cells (Vallieres and Rivest, 1997) which subsequently produces central mediators such as prostaglandins, which are well known to induce central effects such as fever (Xin and Blatteis, 1992; Blatteis and Sehic, 1997; Yamagata et al., 2001). It has been shown previously that IL-6 induces prostaglandin E_2 (PGE_2) both *in vivo* (Dinarello et al., 1991) and *in vitro* (Navarra et al., 1992) which is known to be a potent mediator of fever.

Although it is not known what the predominant pathway for IL-6 communication with the brain is, it is possible that IL-6 may use all the discussed pathways (figure 1.2). This underlines the role that IL-6 has in psychoneuroimmunology linking the endocrine system with the central nervous system. Thus, activation of the hypothalamic-pituitary-adrenal (HPA) axis by IL-6 can initiate many biological actions through secretion of hormones.

1.4.4 Cortisol Production

An increase in corticotropin-releasing hormone (CRH) and IL-6 secretion is observed in response to a stressor such as exercise; therefore, it could be assumed that IL-6 initiates CRH production as part of a cascade of events in response to stress. Activation of the hypothalamus can produce CRH in response to stress (Bethin et al., 2000; Inder et al.,

1998) which regulates the activation of the HPA axis stimulating the production and the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland (Farell et al., 1983; Swaine, 2000). ACTH in turn, can produce glucocorticoids such as cortisol (Farraell et al., 1983; Tsigos et al., 1997) to initiate a range of biological actions throughout the body.

IL-6 can stimulate cortisol production at various levels of the central pathway and although IL-6 alone does not initiate PVN stimulated CRH production (Vallieres et al., 1997); upregulation of IL-6R increases the sensitivity of the PVN to IL-6, consequently elevating CRH release (Vallieres and Rivest, 1999). Therefore an increase in CRH is dependent not only on IL-6 but also IL-6R or possibly sIL-6R to initiate an increase in ACTH. IL-6 also stimulates the release of ACTH independently of CRH. CRH deficient mice produces ACTH in response to IL-6 stimulation which can be attributed to IL-6 receptor expression on the pituitary (Bethin et al., 2000) indicating that although ACTH is stimulated through the PVN-CRH pathway, direct stimulation of ACTH is also possible depending on the dose of IL-6 present. Tsigos et al. (1997) demonstrated that ACTH increased in response to IL-6 in a dose dependent manner. Humans infused with IL-6 at doses ranging from 0.1 $\mu g \cdot kg^{-1}$ to 10 $\mu g \cdot kg^{-1}$ showed that doses of ≥3 $\mu g \cdot kg^{-1}$ resulted in stimulated ACTH release in a dose dependent manner. Moreover, plasma cortisol release followed a corresponding pattern to ACTH release in the same dose dependent manner. Recombinant human IL-6 infusion into humans subsequently increases plasma cortisol (Tsigos et al., 1997; Steensberg et al., 2003),

however, unlike CRH, IL-6 must depend on the actions of ACTH to produce cortisol (Silverman et al., 2004); therefore the cortisol response to plasma IL-6 appears to be an indirect action through ACTH release (Zarcovic et al., 2008).

During prolonged exercise, an increase in CRH (Tabata et al., 1991; Inder et al., 1998), ACTH (Fraioli et al., 1980; Inder et al., 1998) and cortisol is observed (Few, 1974; Kuoppasalmi et al., 1980; Kindermann et al., 1982; Utter et al., 1999) which could be attributed at least in part to exercise induced plasma IL-6 mediating the activation of the HPA axis. Typical roles for cortisol in exercise include a metabolic role (Del Corral et al., 1998; Khani andTayek, 2001) and also an anti-inflammatory role (Steensberg et al., 2003; Rhen and Cidlowski, 2005). Since exercise induced IL-6 has been shown to have similar roles as cortisol, it would be conceivable that IL-6 can stimulate the secretion of cortisol as part of a cascade to regulate metabolism and inflammation during exercise.

1.4.5 Fatigue

IL-6 is well known for its association with fatigue in both chronic (Wratten et al., 2004; Nishimoto et al., 2005; Genovese et al., 2008) and acute conditions (Weber et al., 1993; Mastorakos et al., 1993; Gordon et al., 1995). IL-6 mediated acute fatigue is associated with IL-6 infusion where fatigue usually begins within an hour of the infusion and is accompanied with fever and chills which subside within a few hours. In contrast, IL-6 mediated chronic fatigue is often accompanied by a medical

illness lasting longer than 6 months which can not be relieved by rest (Swaine, 2000).

In chronic inflammatory conditions, patients experience increased levels of fatigue which can be debilitating. Cancer patients experience fatigue during therapy which can often persists for months and even years following treatment (Schubert et al., 2007).   Rheumatoid arthritis is an autoimmune disorder which is characterised by joint pain, stiffness and fatigue (Davis et al., 2008) and furthermore, Castleman disease, a rare lymphoproliferative disorder is characterised by fever and fatigue (Nishimoto et al., 2005). Many of the inflammatory conditions show elevated plasma levels of cytokines such as TNF-α, IL-1and IL-6 which are either correlated with fatigue or sensations of fatigue which are reduced when an IL-6 antibody is administered.

A study by Wratten and colleagues (2004) demonstrated that plasma IL-6 was associated with levels of fatigue during treatment of breast cancer which was in accordance with a later study by Costanzo et al. (2005) and Meyers et al. (2005) which showed that plasma IL-6 correlated with levels of fatigue in ovarian cancer and leukemia patients respectively. Although these findings demonstrate an association between IL-6 and fatigue, this does not specify a causal effect and furthermore these findings are not consistently seen (Ahlberg et al., 2004). However, studies have shown that when IL-6 antibodies are administered to patients with chronic disorders, fatigue is alleviated. For example, rheumatoid arthritis patients treated with an IL-6 antibody have shown to improve the condition of the disease with diminished symptoms of fatigue

(Genovese et al., 2008); a finding that is also seen when an IL-6 antibody is administered to patients with Castleman disease (Nishimoto et al., 2005).

When IL-6 is infused into humans, it has been consistently shown to induce fever, chills as well as increase of sensations fatigue (Weber et al., 1993; Mastorakos et al., 1993; Gordon et al., 1995). Furthermore, the acute effects of IL-6 can mediate loss of appetite, reduce physical activity and lower mood states (Spath-Schwalbe et al., 1998); all of which can collectively be termed as sickness behaviour (Dantzer et al., 1998).

A range of doses of rhIL-6 infusion (1-30 $\mu g \cdot kg^{-1}$) into hospitalised patients during clinical trials have induced symptoms of anorexia, headaches, fatigue and fever in all patients which increases in a dose dependent manner (Mastorakos et al., 1993; Weber et al., 1993; Gordon et al., 1995). Spath-Schwalbe et al. (1998) demonstrated in a blinded crossover study that even a low dose of rhIL-6 (0.5 $\mu g \cdot kg^{-1}$) reduces mood state, increases fatigue and also reduces sleep quality compared to a placebo.

As well as directly causing fever, chills and fatigue, IL-6 can also potentiate the sickness behaviour response of LPS and IL-1. Bluthe et al. (2000) showed that IL-6 deficient mice reduced the response of sickness behaviour to LPS and IL-1 infusion when compared to wild type mice. Furthermore the work of Harden et al. (2006) demonstrated that LPS infusion reduced food intake and voluntary wheel running in mice. These symptoms of sickness behaviour were diminished when an antibody

against IL-6 was administered, suggesting that IL-6 could be necessary to mediate the activities of LPS and IL-1.

When taken together, these findings indicate that IL-6 can initiate fatigue directly in humans and can also potentiate the activities of LPS and IL-1 mediated sickness behaviour in rats. As well as the potential for IL-6 to directly and indirectly interact with the brain as discussed earlier; IL-6 may also cause fatigue by initiating monoamine neurotransmitters associated with reduced appetite or fatigue. Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter that is found throughout the body including the central nervous system which is associated with depressed mood, lethargy, sleep and reduced appetite (Davis et al., 2000; Young and Leyton, 2002). 5-HT is derived from tryptophan and although 5-HT cannot cross the BBB, tryptophan and another precursor of 5-HT, 5-hydroxytryptophan (5-HTP) can cross the BBB (Bell and Janzen, 1971). This subsequently leads to an increase in brain and neuronal 5-HT synthesis (Fernstrom and Wurtman, 1971; Davis et al., 2000; Young and Leyton, 2002; Fernstrom and Fernstrom, 2006) which has been shown to mediate central fatigue (Yamamoto and Newsholme, 2000).

Increased 5-HT in the brain is associated with negative mood, fatigue, altered sleep cycles and appetite regulation changes (Davis et al., 2000; Young and Leyton, 2002) and is also implicated as inducing central fatigue during exercise (Davis et al., 2000; Yamamoto and Newsholme, 2000). Bailey et al. (1993) demonstrated that a 5-HT agonist decreased exercise time to exhaustion in a dose dependent manner in rats;

conversely, in the same study, rats that were administered a 5-HT antagonist increased time to exhaustion also in a dose dependent manner.

IL-6 has also been shown to induce serotonergic pathways both at rest in rats (Yamaguchi et al., 1990; Wang and Dunn, 1998; Song et al., 1999; Zhang et al., 2001) and also during exercise in humans (Robson-Ansley et al., 2004). Yamaguchi et al. (1990) demonstrated that infusion of rhIL-6 infused into rats increased plasma levels of prolactin from the rat pituitary. In contrast with the findings from Yamaguchi and co-workers, Robson-Ansley et al. (2004) infused a low dose of IL-6 (0.5 µg·kg^{-1}) into humans and found no difference in prolactin levels at rest. However following exercise, prolactin levels were significantly elevated following rhIL-6 infusion compared to a placebo. Prolactin secretion is regulated by 5-HT neurons therefore it could be postulated that brain 5-HT activity can reflect the activity of prolactin in plasma (Meeusen et al., 2001). Infusion of IL-6 into rats alters neurotransmitter levels including elevating levels of 5-HT (Song et al., 1999; Wang and Dunn, 1998; Zhang et al., 2001) in the rat striatum. Moreover, infusion of IL-6 appears to not only increase 5-HT levels, but also concurrently reduce levels of dopamine (Song et al., 1999), which is linked to elevated motivation and arousal (Davis et al., 2000).

From the data it is clear that IL-6 induces sensations of fatigue which could be a result of direct interaction with the brain or possibly through indirect actions through central mediators or neurotransmitters such as serotonin and dopamine. An alteration in such neurotransmitters

appears to alter perception of effort and/or reduce motivational drive, ultimately which can lead to a decrement in performance of a given task.

1.4.6 Physical Performance

Given that IL-6 mediates increased sensations of fatigue among other biological roles, it would seem plausible that this in turn may have an affect on both functional and athletic performance. Chronic low grade inflammatory conditions that are characterised by elevated levels of plasma IL-6 are commonly associated with poor functional performance. Nicklas et al (2005) reported that in a cross sectional study of individuals with osteoarthritis, walking distance and stair climbing speed were negatively associated with plasma levels of IL-6. A finding that is in accordance with studies that have examined the functionality of aging populations (Taaffe et al., 2000; Cappola et al., 2003; Cesari et al., 2004). Markers of low grade inflammation including plasma levels of IL-6 tend to increase with age (Wei et al., 1992; Ferrucci et al., 2002). However, within a given elderly population, it has been shown that individuals with greater levels of IL-6 tend to have poorer physical functional performance such as slower walking speed, stair climbing speed and lower grip strength (Taaffe et al., 2000; Cesari et al., 2004; Pereira et al., 2008).

Hospitalised elderly patients have shown that fatigue resistance using hand grip strength negatively correlates with plasma IL-6 (Mets et al., 2004); a finding that was also shown in a later study by Bautmans et al. (2010) who demonstrated that greater surgery-induced IL-6 concentrations correlated with poorer muscular endurance using grip

strength. This finding is also consistent with those found from community

dwelling studies (Pereira et al., 2008; Cesari et al., 2004). A study

examining the performance of elderly persons demonstrated not only that

plasma IL-6 negatively correlated with grip strength but also with other

physical functional tasks such as walking speed (Cesari et al., 2004).

Cross sectional studies by Taaffe et al. (2000), Ferrucci et al. (2002) and

Cappola et al. (2003) have all shown that walking speed is negatively

correlated with plasma IL-6 concentrations in older persons. Furthermore,

this negative correlation can be attenuated by exercise training which has

been shown to reduce systemic IL-6 and improve functional performance

in individuals with the highest levels of IL-6 and lowest functional

performers (Nicklas et al., 2008).

Although there is a plethora of literature examining the effects of

plasma IL-6 in chronic low grade inflammation and the association with

functional performance, the literature concerning the acute effects of

plasma IL-6 in athletic performance is limited. Although Papanicolaou et

al. (1998) has reported that IL-6 correlates with exercise induced fatigue;

only one study to date has examined the effects of acute IL-6 on exercise

performance. Robson-Ansley et al. (2004) infused athletes with a small

dose of rhIL-6 ($0.5 \ \mu g \cdot kg^{-1}$) to produce levels in circulation to those

typically seen following prolonged running. The study found that athletes

ran a 10 km time trial slower following rhIL-6 infusion when compared to a

placebo trial. The authors also observed increases cortisol, ACTH and

prolactin, suggesting the infusion caused an activation of the HPA axis as

well as serotonergic pathways which may mediate the decrement in performance.

When taken together, it is clear that circulating IL-6 performs a myriad of biological roles at rest and during prolonged exercise which can be potentiated by the effects of sIL-6R. At present, it is not known what the effects of sIL-6R are with regard to exercise performance, and furthermore, it is not known if the sIL-6R can be manipulated with non pharmacological methods. If the IL-6 and signalling receptor response to prolonged exercise can be blunted through an intervention, this may in turn attenuate markers of the acute phase response, reduce stress markers associated with activation of the HPA axis, and attenuate the sensations of fatigue, potentially improving performance. Conversely, if the response of IL-6 and signalling receptor can be augmented, this can increase these effects and potentially cause a decrement in performance (figure 1.2).

Since nutritional interventions have been shown to manipulate the IL-6 response to exercise (Starkie et al., 2001; Keller et al., 2001; Hiscock et al., 2003) and also alter exercise performance (Coggan and Coyle, 1991; El-Sayed et al., 1997; Rauch et al., 2005), it could be hypothesised that plasma IL-6 and its signalling receptors act as an integrator between metabolism of skeletal muscle and the central nervous system. Therefore, the aims of this thesis were to investigate the response of circulating IL-6, sIL-6R and sgp130 to prolonged exercise and examine their relationship with exercise performance with nutritional manipulation.

Figure 1.2 A Schematic Representation of IL-6 Production and Possible Roles in Circulation

↑ **5-HT**

↑ **Fatigue**

↓ **Performance**

↑ **CRP**

↑ **Glucose**

↑ **Hepcidin**

↓ **Iron**

Low Muscle Glycogen

↑ **Reactive Oxygen Species**

↑ **IL-6**
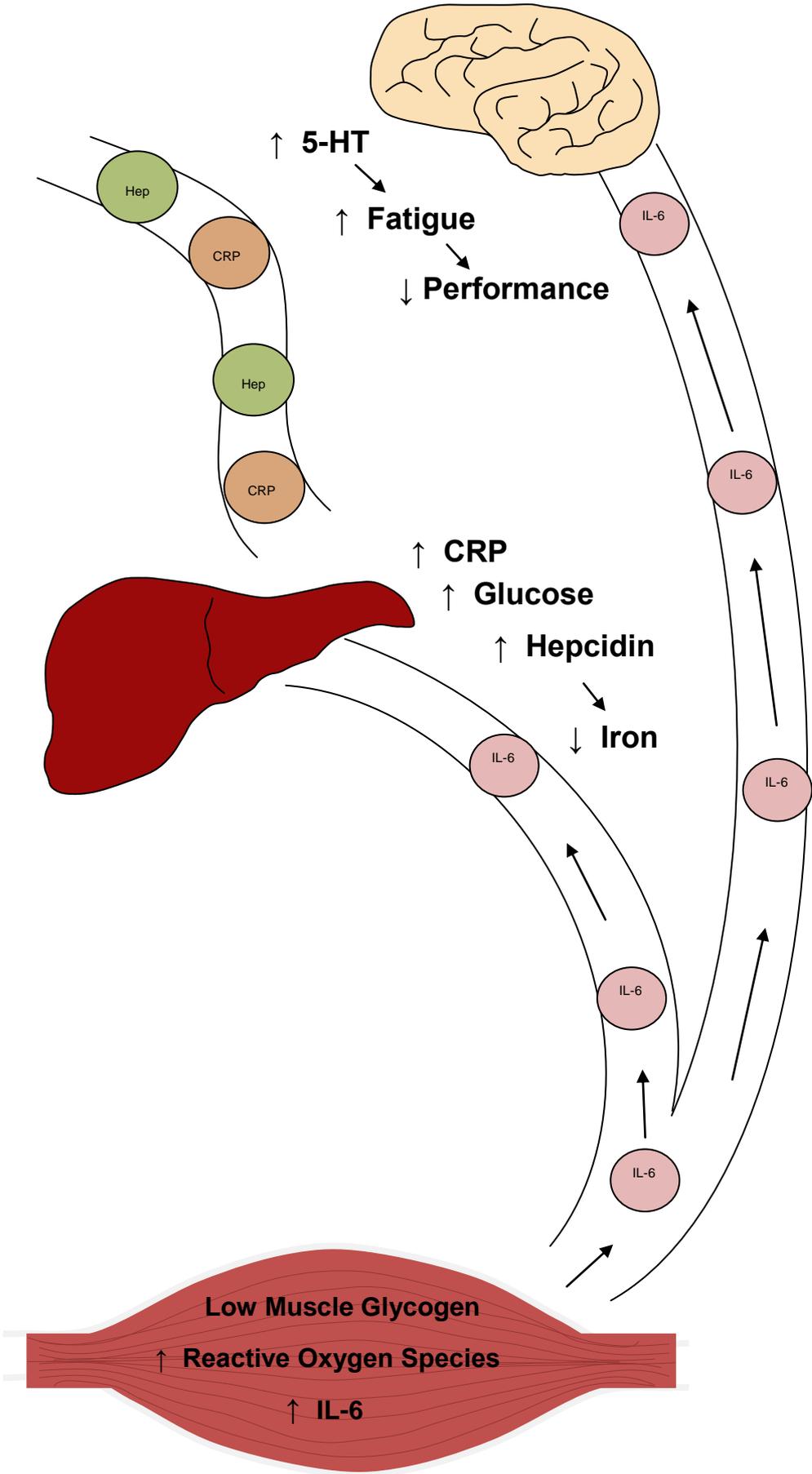
Chapter 2

General Methods

2.1 Exercise Protocol

2.1.1 Preliminary Testing

Before the preloaded time trials, preliminary testing was conducted to obtain participants' maximal oxygen uptake ($\dot{V}O_{2max}$) and running velocity at $\dot{V}O_{2max}$ (v$\dot{V}O_{2max}$) on a motorised treadmill (hp Cosmos Pulsar, Germany). Participants performed an incremental exercise test on a treadmill with the gradient set at 1%. Treadmill speed was initially set at 12 km·h^{-1} and was increased by 1 km·h^{-1} for 3 min stages until volitional exhaustion. It was considered a maximal effort if participants met any two of the following criteria: a change in $\dot{V}O_2$ < 2 ml·kg^{-1}·min^{-1} across the last two stages of the incremental test; a respiratory exchange ratio of 1.15 or greater, or ≥ 90% age predicted maximum heart rate (220-age). Expired air was measured using an online gas analyser (Cortex meta analyser 3B, Germany) and heart rate was measured using short range telemetry (Polar RS400, Finland).

2.1.2 Experimental Protocol

Participants performed the trials which were separated by one week. They were asked to ensure they were hydrated before the exercise protocol and to refrain from exercise, alcohol and caffeine 24 h prior to the study and during the 24 h post exercise.

Participants arrived at the laboratory in the morning after a 12 h fast. Following a void, body mass was recorded and subjects sat quietly for 10 min before a baseline venous blood sample was obtained.

41

2.1.3 Preload Exercise Bout

Before the exercise bout participants completed a 5-min standardised warm up. The preload component required participants to run at 60% v$\dot{V}O_{2max}$ for 2 h. To ensure a marked circulating IL-6 response from a mixture of muscle fiber types, the preload component was interspersed with a 30 s run at 90% v$\dot{V}O_{2max}$ every 10 min. Heart rate was measured immediately before each 30 s run at 90% v$\dot{V}O_{2max}$. Expired gas samples were obtained for 60 s along with rating of perceived exertion (RPE) (Borg, 1982) at 20 min intervals prior to every alternate sprint. Background music was allowed only during the preload component. On completion of the preload component, subjects were allowed to rest for 5 min whilst a venous blood sample (PL) was taken.

2.1.4 Performance Trial

Prior to the time trial component, participants were instructed to complete a 5 km time trial as fast as possible. During the time trial participants controlled the speed manually but were not provided any performance feedback or encouragement and were only informed of every kilometre completed at which point heart rate, RPE and time elapsed were recorded. On completion of the time trial a further blood sample was obtained (PostTT) and body mass was recorded. Further blood samples were taken one hour post time trial (1hPost) and a final blood sample was taken the following morning, 24 h from baseline (24hPost). Figure 2.1 shows a schematic time line for each trial.

2.1.5 Blood Sampling

At each blood sampling point, 18 ml of blood was taken from an antecubital vein and collected into monovette tubes (9 ml K$_3$EDTA and 9 ml serum). Blood samples were immediately centrifuged at 3000 revs·min^{-1} for 10 min at 4°C, aspirated, and the supernatant was stored at -80°C until the day of analysis.

Figure 2.1 A schematic representation for the exercise protocol

| Baseline | PL | PostTT | 1hPost | 24hPost |

| 2 h Preload Bout | | Time Trial | | |
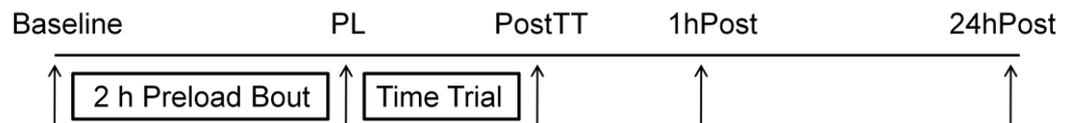
2.2 Blood Plasma Analysis

2.2.1 IL-6 Analysis

IL-6 was measured from K$_3$EDTA treated tubes, in duplicate, with a commercially available high sensitivity chemiluminescent Enzyme-Linked Immunosorbant Assay (ELISA) kit (R&D Systems, Abingdon, UK). 100 µl of a buffered protein base and 100 µl of plasma were added with to the plates which were pre-coated with a mouse monoclonal antibody against IL-6. The plates were incubated on a microplate shaker (Heidolph, Saffron Walden, UK) at 500 rev·min^{-1} for 2 h. The plates were washed in buffered surfactant solution (Bio Tek, Potton, UK), 200 µl of polyclonal antibody against IL-6 conjugated to horseradish peroxidase was added to

the wells and incubated for 3 h on the microplate shaker at 500 rev·min^{-1}.

The plates were washed and added with 100 µl of glo reagent (one part

stabilised enhanced luminal and two parts stabilised hydrogen peroxide)

which was incubated for 10 min. The relative luminescence units (RLU)

was then determined using a luminometer (Bio Tek, Potton, UK).

Manufacturer's instructions report minimum detection limit of 0.16 pg·ml^{-1}

and inter-assay and intra-assay variation of 7.7-9.6% and 3.0-5.8%

respectively for IL-6.

2.2.2 sIL-6R Analysis

sIL-6R was measured in duplicate from blood plasma using an

ELISA technique from a commercially available kit (R&D systems,

Abingdon, UK). Plasma was collected from K_3EDTA treated tubes and

diluted 1:100 in a buffered protein base and then added to plates pre-

coated with a mouse monoclonal antibody against sIL-6R. The plates

were then incubated for 2 h and washed (Bio Tek, Potton, UK) in a

buffered surfactant. 200 µl of polyclonal antibody against sIL-6R

conjugated to horseradish peroxidase was added to the plates and

incubated for a further 2 h, then washed. Stabilised hydrogen peroxide

and stabilised tetramethylbenzidine were mixed in equal parts and added

to the plates then incubated for 20 min. 50 µl of sulphuric acid was then

added and the absorbance was then read at 450 nm with a correction at

540 nm (Biochrom Ltd, Cambridge, UK). The minimum detection limits

were reported as 6.5 pg·ml^{-1} with an inter-assay variation of 4.7% and

intra-assay variation of 2.3%.

2.2.3 sgp130 Analysis

Plasma sgp130 was measured in duplicate from K_3EDTA treated tubes and measured in a commercially available ELISA kit (R&D systems, Abingdon, UK). Samples were diluted 1:100 in calibrator diluent and were added to plates coated with mouse monoclonal antibody against sgp130 with a buffered protein base. The plates were incubated for 3 h and then washed in buffered surfactant. A polyclonal antibody against sgp130 conjugated to horseradish peroxidase was added to the plates and then incubated for 1 h and washed. Stabilised hydrogen peroxide and stabilised tetramethylbenzidine were mixed together in equal parts and added to the plates which were incubated for 30 min. Sulphuric acid was then added to the plates and the absorbance was read at 450 nm with a correction of 540 nm. The minimum detection limit for sgp130 was reported as 0.08 ng·ml^{-1} with an inter-assay variation of 3.6% and intra-assay variation of 5.5%.

2.2.4 Glucose and Lactate Analysis

Plasma glucose and lactate was measured in duplicate from K_3EDTA treated tubes with a Biosen C_line sport glucose/lactate analyser (EKF diagnostic, Magdeburg, Germany). 20 µl of plasma was added to a pre filled hemolysis solution and measured using an electro-chemical measuring principle.

2.2.5 Cortisol Analysis

Plasma cortisol was measured in duplicate from serum tubes from a commercially available ELISA kit (BioVendor, Oxford, UK). Samples

and standards were added to plates that were pre-coated with rabbit polyclonal antibody against cortisol followed by 100 µl of cortisol horseradish peroxidase. The plates were incubated on a microplate shaker (Heidolph, Saffron Walden, UK) at 200 rev·min^{-1} for 45 min and then washed with a buffered surfactant (Bio Tek, Potton, UK). 150 µl of tetramethylbenzidine with hydrogen peroxide was added, then incubated on a microplate shaker for 15 min at 200 rev·min^{-1}. 50 µl of sulphuric acid was then added to the plates and the absorbance was read at 450 nm (Biochrom Ltd, Cambridge, UK). The manufacturer's instructions report a minimum detection limit of 0.4 µg·dl^{-1} with an inter-assay and intra-assay variation 5.0-3.8% and 2.9-5.0% respectively.

2.2.6 C Reactive Protein Analysis

Plasma C reactive protein was measured in duplicate from serum tubes using a high sensitivity C reactive protein (hsCRP) ELISA kit that is commercially available (Kalon Biological, Guildford, UK). Samples were diluted 1:100 in stabilised buffered saline and added to plates that were pre-coated with sheep antibody against CRP. The plates were incubated for 1 h then washed (Bio Tek, Potton, UK) with buffered surfactant. 100 µl of affinity purified sheep anti-CRP labelled with alkaline phosphatase was added and incubated for 1 h. The plates were washed and 100 µl of substrate solution was added (4-nitrophenylphosphate 20 mg tablet dissolvedin 12 ml substrate buffer) and then incubated for 30 min. 100 µl of stop solution was added (50 g·L^{-1} EDTA solution) and the absorbance was read at 405 nm (Biochrom Ltd, Cambridge, UK). Manufacturers

report inter-assay and intra-assay variation to be 13% and 5.1% respectively.

Chapter 3

The Reliability of the IL-6, sIL-6R and sgp130 Response to a Preloaded Time Trial

3.1 Abstract

Interleukin-6 (IL-6) is a cytokine that can mediate numerous biological actions including fatigue. Circulating IL-6 increases during prolonged exercise, and furthermore, the signalling receptors sIL-6R and sgp130 are also increased. The variability of the response of these markers to exercise is unknown; therefore, we examined the changes in these markers to a preloaded time trial bout of running. Nine males performed three identical trials where participants ran at 60% vVO2max for 2 h interspersed with 30 s at 90% vVO2max every 10 min, followed by a 5-km time trial. Blood samples were drawn at baseline, following the 2-h bout, post time trial, 1 h post time trial and the following morning. Results showed that between subject variability (CVg) was greater than within-subject variation (CVi) for the three markers. IL-6, sIL-6R and sgp130 demonstrated a CVi of 15.3–25.5%, 15.0–17.6% and 6.2–9.4% variation, respectively, across the time points. When the data from the second and third trials were analysed independently, CVi was reduced which is supported by the time trial results for which CVi improve (4.7–2.4%). In conclusion, the results indicate that a large variation in response to exercise can be reduced following a habituation trial.

3.2 Introduction

Interleukin-6 (IL-6) is a multifunctional cytokine that has been associated with fatigue in healthy humans at rest (Spath-Schwalbe et al., 1998) and during exercise (Robson et al., 2004). During prolonged exercise IL-6 is released from contracting muscle into the circulation (Steensberg et al., 2000); in addition the soluble form of the IL-6 receptor (sIL-6R) is increased in circulation during (Gray et al., 2008) and after (Robson-Ansley et al., 2009) exercise which can act to mediate the actions and prolong the half life of IL-6 when it is combined in a IL-6/sIL-6R complex (Levine et al., 2004). This complex can instigate trans-signalling through the ubiquitously expressed membrane bound receptor glycoprotein 130 (gp130) which would otherwise be unresponsive to IL-6 alone (Jones et al., 2001). The soluble form of gp130 (sgp130) may also increase in the circulation during exercise (Gray et al., 2008) and has the same affinity for IL-6/sIL-6R as the membrane bound receptor, therefore it has been suggested that sgp130 has a role as a regulator of the IL-6/sIL-6R complex (Jones and Rose-John, 2002).

Robson-Ansley et al. (2009) have proposed that if the circulating IL-6 response to exercise can be blunted, the sensations of fatigue will be reduced, therefore potentially improving performance. However in order to assess the effectiveness of attenuating plasma IL-6, it is important to first quantify the degree of biological variability of the response to exercise. A small number of studies have examined the variability of repeated measurements of circulating plasma cytokines at rest including

IL-6 and report a coefficient of variation of 26-48% (Knudsen et al., 2008 and Cava et al., 2000). However at rest, circulating levels of IL-6 in healthy humans are typically very low (~1 pg·ml^{-1}) and even a 50% increase is unlikely to be clinically significant. To date, there is no published data on the biological variability of the plasma IL-6, sIL-6R and sgp130 response to prolonged exercise in healthy humans.

Traditionally, time to exhaustion exercise trials have been used to quantify the response of biological systems to imposed stress and the effect of various interventions. However, the inherent variability of open loop trials, such as time to exhaustion trials, is so large that it can often mask meaningful performance changes. This has led to the utilisation of closed loop performance protocols where the distance or total amount of work is set (Jeukendrup et al., 1996). Certainly, time trials are more ecologically valid (Currell and Jeukendrup, 2008), but often the kinetics of a biological variable, such as plasma IL-6, would require a trial of such a long duration as to be unrealistic in a laboratory setting. To overcome this, researchers have adopted a preload time trial format in which an initial constant load exercise component stresses the biological systems in order to initiate a response, and the efficacy of any intervention is assessed in a subsequent time trial (Russell et al., 2004). Therefore, the purpose of this study was to investigate the variability of plasma IL-6 and soluble receptor response to a preloaded time trial protocol and the performance repeatability of the time trial.

51

3.3 Methods

3.3.1 Participants

Nine trained male club runners and triathletes took part in the study. Subject characteristics are presented in Table 3.1. Data are expressed as mean ± SD.

Table 3.1. Subject characteristics

Age	27 ± 5 yr
Height	181 ± 4 cm
Body mass	72.4 ± 8.2 kg
Maximal oxygen uptake	59 ± 5 ml·kg^{-1}·min^{-1}
Habitual training distance (running)	38 ± 22 km per week

Exclusion criteria included symptoms of infection and administration of anti-inflammatory drugs or vitamin supplementation in the four weeks prior to the study. Participants gave written informed consent to the study, which was approved by the school research ethics committee at Northumbria University.

3.3.2 Experimental Protocol

The participants performed three of the same trials which were separated by one week. The first, second and third trial are designated as trial A, B and C respectively. To ensure subjects began each trial with similar levels of muscle glycogen, participants were given a high carbohydrate (8 g·kg^{-1}) standardised diet (68% carbohydrate, 16% fat and 16% protein) 24 h prior to each of the experimental trials. They were asked to ensure they were well hydrated and to refrain from exercise, alcohol and caffeine 24 h prior to the study and during the 24 h post

exercise. Participants began the trial in the morning before 9.00 am which is outlined in detail in chapter 2. Water was consumed *ad libitum* during the first trial; the volume was recorded and replicated for the subsequent trials. Following each trial, body mass was recorded and a standardised cereal bar was consumed (Nutragrain, Kellogs). Laboratory environmental conditions were maintained throughout the study at 21.1 ± 2.4°C and 45 ± 9% relative humidity.

3.3.3 Blood Plasma Analysis

IL-6, sIL-6R, sgp130, cortisol, glucose and lactate were all measured from blood plasma which is outlined in detail in chapter 2. Plasma C reactive protein was analysed on a high sensitivity Siemens Medical solutions Advia 2400, UK with intra- and inter-assay coefficient of variations of less than 4% and 5% respectively.

3.3.4 Statistical Analysis

Sample size was calculated using a spreadsheet from Hopkins (2006). The data was analysed using the statistical package SPSS v. 16 (SPSS inc., Chicago, IL). To investigate if data were spherical, Mauchly's test of sphericity was applied, with a Greenhouse-Geisser correction for any violations. For trial and time analysis, a 3 x 5 (trial x time) repeated measures ANOVA was conducted with Bonferroni adjustments for multiple comparisons with a criterion for significance set at $p < 0.05$. Analysis of reliability was measured first with 3 trials, then the latter trials independently with trial A being stipulated as a habituation trial. The within-subject (CVi) and between-subject (CVg) coefficient of variation

were analysed at each time point. Within-subject intraclass correlation coefficient (ICC) was used to calculate reliability at each time point (Hopkins, 2000). All data are expressed as mean ± SD.

3.4 Results

3.4.1 Physiological and Performance Data

3.4.2 Preload

During the preload component mean exercise intensity was 70 ± 7%, 67 ± 5%, and 69 ± 6% of $\dot{V}O_{2max}$ for Trial A, B and C, respectively and was not significantly different between trials (p=0.10). Mean heart rate (Trial A =149 ± 19 b·min^{-1}, Trial B =148 ± 17 b·min^{-1}; Trial C = 148 ± 19 b·min^{-1} p=0.78), rating of perceived exertion (Trial A = 12 ± 1, Trial B = 12 ± 2, Trial C = 12 ± 2; p=0.19) and respiratory exchange ratio (Trial A = 0.89 ± 0.04, Trial B = 0.88 ± 0.03, Trial C = 0.86 ± 0.06; p=0.35) were all similar for the preload components between trials.

3.4.3 Time Trial

During the time trial, mean heart rate was similar between trials (Trial A =173 ± 14 b·min^{-1}, Trial B =172 ± 14 b·min^{-1}; Trial C = 172 ± 20 b·min^{-1} p=0.81) along with rating of perceived exertion (Trial A = 16 ± 2, Trial B = 16 ± 1, Trial C = 16 ± 1; p=0.37). The time trial was completed in mean times of 1406 ± 165 s (Trial A), 1381 ± 226 s (Trial B) and 1389 ± 224 s (Trial C), which were not significantly different (p=0.85). Between-subject (CVg) coefficient of variation for time to completion for all three trials was 14.6% and within-subject (CVi) variation was 4.7% which

showed an intra-class correlation coefficient (ICC) score of 0.90. When the data was excluded from trial A, between-subject (CVg) coefficient of variation increased to 16% and within-subject (CVi) variation was reduced to 2.4%. The intra-class correlation coefficient (ICC) score was 0.96.

3.4.4 *IL-6 and Signalling Receptors*

Plasma IL-6 demonstrated a significant increase from baseline (0.7 pg·ml^{-1}) to PL (8.6 pg·ml^{-1})(p<0.001) and demonstrated a further significant increase (33.8%) (13.0 pg·ml^{-1}) at PostTT (p<0.05). IL-6 levels decreased 1hPost (10.9 pg·ml^{-}1) and returned to baseline levels the following day. No significant differences were observed between trials for IL-6 (p=0.24) (Figure3.1).

There was a non significant rise in plasma sIL-6R levels (13.6%) from baseline (25.3 ng·ml^{-1}) to PL (28.7 ng·ml^{-}1) with a further significant increase at PostTT (31.0 ng·ml^{-1}) (8.1%), which decreased at 1hPost from PostTT(29.3 ng·ml^{-1}) (p<0.05). No other significant differences were observed between trials for sIL-6R (p=0.87) (Figure 3.2). Differentially spliced isoform of sIL-6R (DS-sIL-6R) showed no significant differences from baseline (p=0.14) with no differences between trials (p=0.80) (figure 3.3).

sgp130 response to exercise is shown in figure 3.4. sgp130 was elevated in response to exercise at PL (p<0.05), there was a trend for sgp130 to remain elevated at PostTT (p=0.06) which decreased by 1hPost. Furthermore, there were no significant differences observed between trials (p=0.42).

Circulating IL-6 levels prior to the time trial (PL) and percentage of velocity at $\dot{V}O_{2max}$ showed a significant negative correlation for trials A and B (trial A r -0.83, p<0.01. trial B r -0.72, p<0.05) while trial C showed a trend of a correlation (r -0.61, p=0.07) (figure 3.5).

Figure 3.1. The interleukin-6 response to the preload time trial protocol.
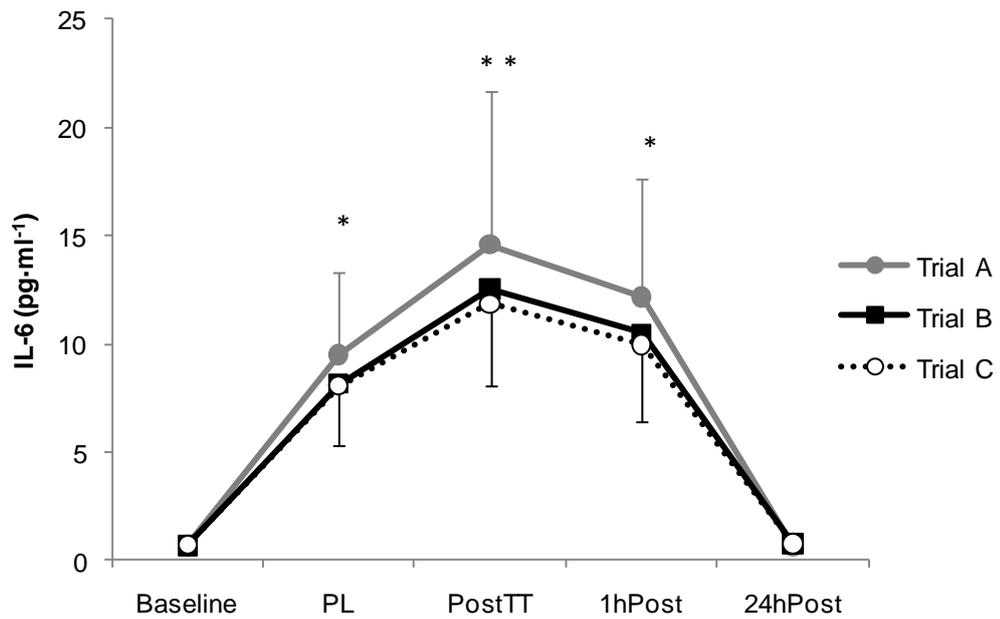
*Significantly higher than Baseline (effect of time) (p<0.001) **Significantly higher than Baseline and PL (effect of time)(p<0.05)

Figure 3.2. The soluble interleukin-6 receptor (sIL-6R) response to the preload time trial protocol.
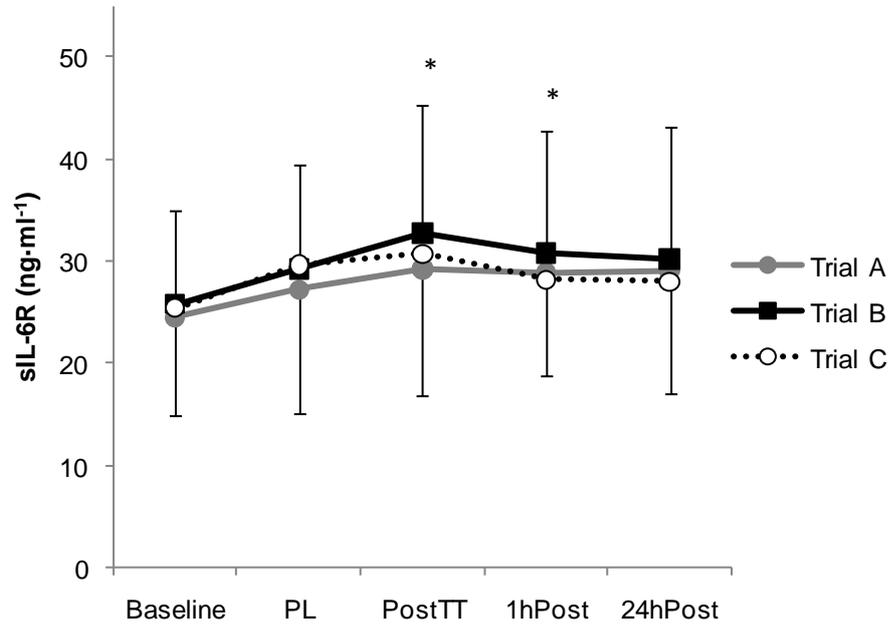
* Significantly higher than Baseline (effect of time) (P<0.05).

Figure 3.3 The differentially spliced soluble IL-6 receptor response to the preload protocol.
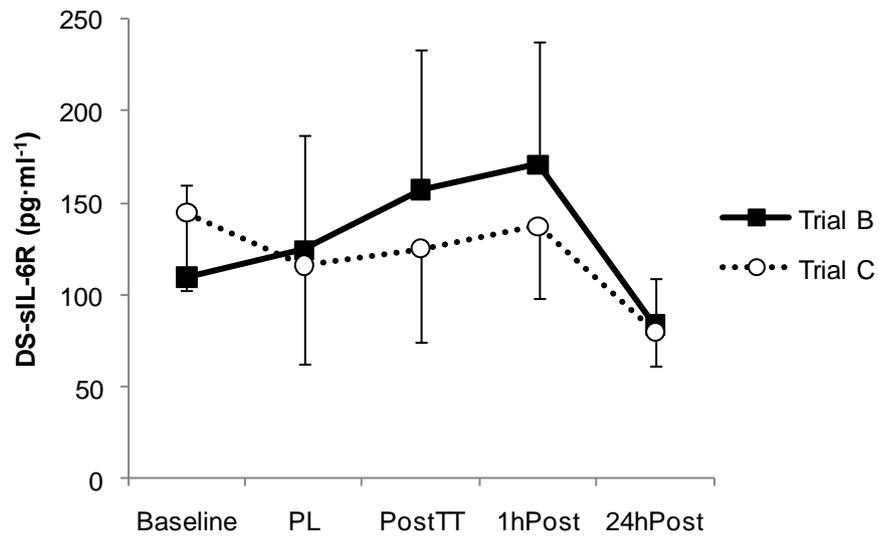
Data are expressed as mean±SEM

Figure 3.4 The sgp130 receptor response to the preload time trial protocol.
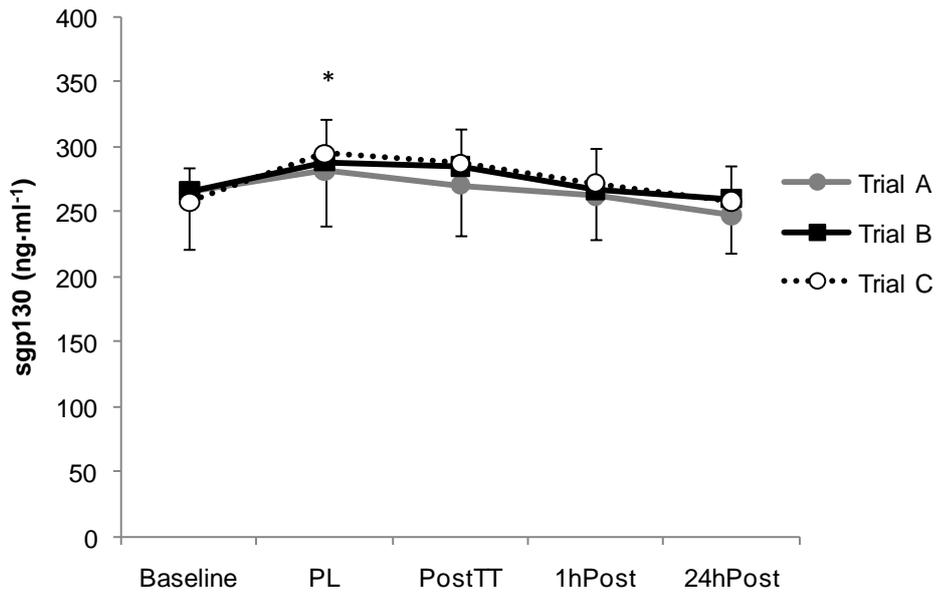
* Significantly higher than Baseline (effect of time) (P<0.05).

Figure 3.5 Correlations between IL-6 at PL and running performance during the time trials.
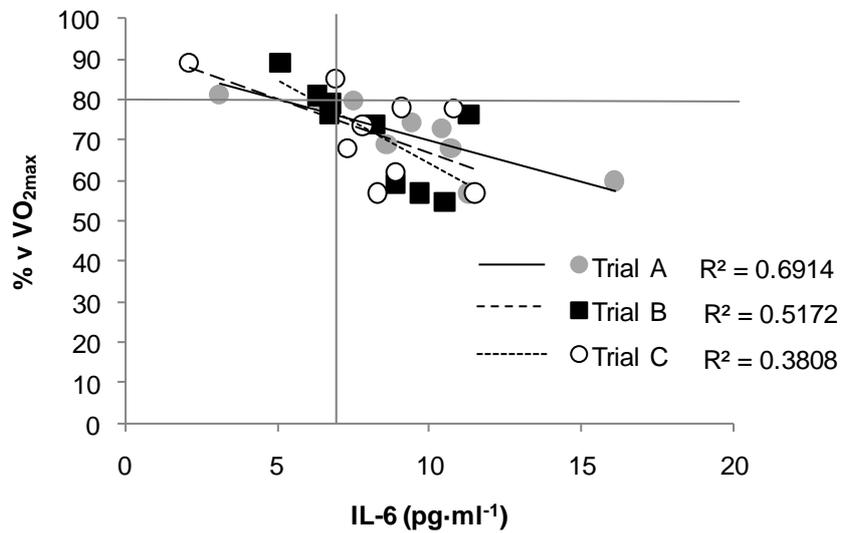
Trial A r -0.83, p<0.01; Trial B r -0.72, p<0.05; Trial C r -0.61, p=0.07

3.4.5 Variability of IL-6 and Signalling Receptors

Between-subject variability was greater than within-subject variation for IL-6 and both sIL-6R and sgp130 (Table 3.2). IL-6 CVg ranged from 28.3-39.3% across the time points whereas CVi ranged from 15.3-25.5%. ICC showed a variable reliability across all time points with a range from 0.18-0.80. Results for sIL-6R demonstrated greater CVg than IL-6 (range 36.1-39.6%) but a lower CVi than IL-6 (range 15.0-17.6%). ICC was also higher for sIL-6R compared to IL-6 (range 0.65-0.80). sgp130 had the lowest CVg (range 10.5-14.1%) and a CVi (range 6.2-9.4%) whilst the ICC range was between 0.47-0.75 across the time points.

When the data from Trials B and Trial C were analysed independently from Trial A, IL-6 CVg ranged from 27.1-36.6% across the time points whereas CVi ranged from 13.9-19.7%. ICC showed a varied reliability across all time points with a range from 0.47-0.8. Results for sIL-6R demonstrated greater CVg than IL-6 (range 34.5-38.5%) but showed less CVi than IL-6 (range 7.6-13.7%). A higher ICC was also displayed by sIL-6R compared to IL-6 (range 0.67-0.88). The lowest coefficient of variation was demonstrated in sgp130 with a CVg range of 9.7-12.8% and a CVi range of 4.5-7.9%. However, sgp130 also demonstrated the lowest ICC range of 0.31-0.67 (Table 3.3).

Table 3.2. Variability of IL-6, sIL-6R and sgp130 of all three trials.

	Baseline	PL	PostTT	1hPost	24hPost
IL-6					
CVi%	15.3	19.1	25.5	21.5	22.9
CVg%	31.4	33.1	34.2	39.3	28.3
ICC	0.80	0.76	0.55	0.67	0.18
sIL-6R					
CVi%	15.3	15.9	15.0	15.7	17.6
CVg%	36.1	38.6	39.6	37.4	38.1
ICC	0.80	0.79	0.75	0.76	0.65
sgp130					
CVi%	8.4	9.4	7.1	6.8	6.2
CVg%	14.1	13.6	11.2	11.1	10.5
ICC	0.72	0.47	0.75	0.73	0.70

Preload component (PL) Post time trial (PostTT) 1 h post time trial (1hPost) 24 h post baseline (24hPost)

CVi within subject variation. CVg between subject variation. ICC Intraclass correlation coefficient.

Table 3.3. Variability of IL-6, sIL-6R and sgp130 of trial B and trial C.

	Baseline	PL	PostTT	1hPost	24hPost
IL-6					
CVi%	15.2	13.9	19.7	16.5	17.5
CVg%	31.4	29.6	27.1	36.6	29.3
ICC	0.70	0.81	0.48	0.80	0.47
sIL-6R					
CVi%	10.7	11.1	11.3	7.6	13.7
CVg%	34.5	35.8	38.5	36.5	36.4
ICC	0.88	0.88	0.82	0.88	0.67
sgp130					
CVi%	7.5	7.9	4.5	5.5	6.1
CVg%	12.7	12.8	9.7	10.3	9.9
ICC	0.60	0.31	0.67	0.49	0.48

Preload component (PL) Post time trial (PostTT) 1 h post time trial (Post1h) 24 h post baseline (Post24h)

CVi% within subject variation. CVg% between subject variation. ICC Intraclass correlation coefficient.

3.4.6 Other Plasma Variables

Blood metabolites, cortisol and C reactive protein (CRP) levels displayed a main effect of time, but no significant differences were observed between trials for glucose (p=0.15), lactate (p=0.29), cortisol (p=0.35) or CRP (p=0.53) at any time point. Plasma cortisol levels were elevated PostTT which remained elevated at 1hPost compared to baseline (p<0.05) (table 3.4). Circulating levels of CRP did not change PostTT but were elevated 24hPost (p<0.05). Plasma glucose did not change significantly during or following exercise. Lactate levels were higher at PL and PostTT compared to baseline (p<0.05) and had returned to baseline levels by 1hPost (table 3.4). The reproducibility of the other circulating plasma variables for all three trials are shown in table 3.5. The reproducibility of the other plasma variables were reduced when analysed for trials B and C alone.

Table 3.4. Plasma glucose, lactate, cortisol and C reactive protein.

	Baseline	PL	PostTT	1hPost	24hPost
Glucose (mmol·L^{-1})					
Trial A	4.74±0.75	5.00±0.81	5.17±0.32	5.68±1.00	5.31±0.88
Trial B	4.32±0.41	4.77±0.53	4.68±0.93	5.73±0.71*	4.53±0.88
Trial C	5.12±0.75	5.24±0.50	5.49±1.14	5.30±0.55	5.06±0.70
Lactate (mmol·L^{-1})					
Trial A	1.73±0.67	2.49±0.80	3.48±1.09*	2.12±0.40	1.78±0.34
Trial B	1.22±0.38	2.13±0.45*	3.55±1.15*	1.69±0.35	1.49±0.22
Trial C	1.41±0.59	2.09±0.43*	3.73±1.44*	1.53±0.50	1.61±0.35
Cortisol (nmol·L^{-1})					
Trial A	309±60	369±142	469±151	526±160**	290±95
Trial B	281±93	345±90	460±66*	426±92*	274±56
Trial C	301±74	285±90	463±88**	439±122**	267±75
CRP (mg·L^{-1})					
Trial A	0.2±0.1	-	0.2±0.1	-	2.7±1.6*
Trial B	0.4±0.5	-	0.4±0.5	-	2.4±1.1*
Trial C	0.3±0.2	-	0.3±0.1	-	2.2±0.7*

Preload component (PL) Post time trial (PostTT) 1 h post time trial (1hPost) 24 h post baseline (24hPost)

* Significantly higher than Baseline (effect of time) ($p<0.05$). ** Significantly higher than Baseline and PL (effect of time) ($p<0.05$).

3.5 Discussion

The aims of the present study were to examine the reproducibility of the IL-6, sIL-6R and sgp130 response to a preload exercise protocol and the repeatability of a subsequent time trial performance. Within-subject variation (CVi) illustrates the relative dispersion of individual variability between trials whereas between-subject variation (CVg) represents group discrepancy; these measurements of reliability have been used in conjunction with ICC which allows the assessment of absolute agreement between trials to further elucidate the reliability of the inflammatory response.

The IL-6 response showed a higher CVg than CVi, which may be indicative of individual endurance capacity since the participants were given a standardised diet for the 24 h prior to the exercise bout to control nutritional status and glycogen content.   CVi was highest PostTT which is possibly due to differences in the distribution of relative intensities during the time trial whereas a lower CVi was observed at PL where the intensity and duration had been externally imposed.

The response of sIL-6R displayed high variability, but the coefficient of variation and ICC remained relatively constant for all time points. The reliability of sIL-6R response to exercise is unreported, however, the baseline results from the present study are consistent with others (Dugue and Leppanen, 1998) although Ho et al. (2005) described a lower reliability (ICC 0.36) at rest than the present study.

A lower variation was observed for sgp130 than IL-6 and sIL-6R, furthermore, the ICC score remained constant throughout. This could possibly be due to concentrations of spg130 being much greater than those for IL-6 and sIL-6R, therefore sgp130 is less affected by small changes induced by exercise. To the best of our knowledge this is the first study to examine the variability of sgp130 at rest or during exercise.

Excluding the data from Trial A improved the reliability scores for IL-6 and sIL-6R illustrating a reduced variation to an external factor such as exercise and furthermore, this indicates the importance of a habituation trial. This interpretation is supported by the time trial results for which CVi and ICC improve (4.7% to 2.4% and 0.90 to 0.96 respectively) for which a habituation trial has been suggested previously (Currell and Jeukendrup, 2008).

The time trial data from the current study are consistent with other studies that found a 1-2.4% variation with preload treadmill protocols [Russell et al. 2004, Tyler and Sunderland, 2008) demonstrating that, following a habituation trial, the current protocol provides a reliable measure of performance. Furthermore, greater circulating levels of IL-6 negatively correlate with relative running performance. This not only demonstrates that greater IL-6 levels can impair performance which is in accordance with previous work (Robson et al., 2004) but also reveals this effect in a dose dependant manner. Furthermore, analysis of the findings demonstrated that a plasma IL-6 response $<7pg \cdot ml^{-1}$ was associated with a time trial performance $>80v\dot{V}O_{2max}$ which may be a result of exercise

training. Alternatively, these findings may also suggest that a low response of IL-6 may not impact on performance whereas a response $>7pg·ml^{-1}$ may begin to impair performance. Therefore, if IL-6 can be manipulated above the range of variation during a preload, this may alter the performance throughout the time trial.

The results from the study have demonstrated that within-subject variation is lower than between-subject variation for IL-6, sIL-6R and sgp130 in response to exercise. This underlines the need to use a crossover design rather than a parallel separate group design when assessing biological markers in studies with low subject numbers. Although the variation of the circulating IL-6, sIL-6R and sgp130 response to exercise is large, these results provide boundaries for the interpretation of exercise induced changes in IL-6, sIL-6R and sgp130 and can provide a useful point of reference for meaningful differences when examining intervention protocols.

DS-sIL-6R showed no effect from exercise in the present study. To date, only one other study has examined the effect of exercise on DS-sIL-6R who showed an increase 6 h following exercise (Leggate et al., 2010) indicating that the response of DS-sIL-6R is delayed. The cause of circulating DS-sIL-6R is unknown, however the results from the present study demonstrate that a small proportion of the total sIL-6R is derived from DS-sIL-6R (~0.5%), therefore the majority of the increase in plasma sIL-6R can be attributed to PC-sIL-6R.

In conclusion, this study has illustrated a large variation of IL-6, sIL-6R and sgp130 in response to exercise and has also shown that within-subject variability is lower than between-subject variation. A habituation trial is needed to improve reliability of these biomarkers, and to reduce the variation of time trial performance. Furthermore, the results of the study demonstrate that circulating levels of IL-6 negatively correlate with relative running performance in a dose dependant manner.

Chapter 4

Effect of Glutamine Supplementation on the Response of Plasma IL-6 and sIL-6R to Prolonged Exercise

4.1 Abstract

Glutamine supplementation during exercise has often been reported to maintain or even elevate plasma glutamine levels but the impact on immune function is less clear. It has been previously been shown that glutamine supplementation augments the exercise induced increase in circulating IL-6 by ~50%. In addition, increased circulating IL-6 levels have been previously shown to heighten sensations of fatigue and impair exercise performance. Therefore, we examined the effects of glutamine supplementation on circulating IL-6 and the subsequent effect on exercise performance. Ten trained male runners ran at 60% vVO2max for 2 h interspersed with 30 s at 90% vVO2max every 10 min, followed by a 5-km time trial, supplemented with either glutamine or a placebo in a double blind randomised crossover manner. Blood samples were drawn at baseline, following the 2-h bout, post time trial, 1 h post time trial and the following morning. No significant differences were observed between trials for circulating IL-6, sIL-6R or sgp130. In addition, there was no significant difference between conditions for time trial performance; however the plasma IL-6 response to the preload was inversely correlated with relative time trial performance regardless of trial. We conclude that glutamine supplementation during prolonged exercise does not alter the circulating IL-6, sIL-6R or sgp130 response. However, the plasma IL-6 response to the preload bout of exercise is negatively associated with time trial performance.

4.2 Introduction

A high proportion of athletes have been reported to consume dietary supplements during training periods with the view to improve performance or to reduce the risk of illness (Froiland et al., 2004). In particular, glutamine supplementation is used by athletes in order prevent muscle catabolism as well as improve immune function based on scientific literature. Studies have shown the versatility of glutamine, which holds a range of functions including a role in neurological activity (Newsholme et al., 2003), acid-base balance (Taylor and Curthoys, 2003), maintenance of gastrointestinal integrity (van der Hulst, 1993) and also has an important metabolic role for immune cells (Newsholme and Parry-Billings, 1990).

Glutamine is a conditionally essential amino acid found within the body where plasma concentrations have typically been reported to be 500-750 µmol·L^{-1} in fasted healthy humans (Walsh et al., 1998; Gleeson, 2008), predominantly supplied by skeletal muscle. Following prolonged exercise, plasma glutamine levels can be reduced (Robson et al., 1999; Parry-Billings et al., 1992) and one hypothesis for this reduction in plasma glutamine is due to an increased uptake by other organs, such as increased hepatic uptake to facilitate an increase in gluconeogenesis (Walsh et al., 1998). Indeed, glutamine supplementation during exercise can enhance hepatic uptake of glutamine (Iwashita et al., 2005), and furthermore, gluconeogenesis in the kidneys has been shown to use glutamine as a precursor (Stumvoll et al., 1998) illustrating that an increased demand for glutamine during prolonged exercise could be due a

greater reliance on glutamine stimulated gluconeogenesis when muscle and liver glycogen levels become depleted.

Glutamine supplementation during exercise has often been reported to maintain or even elevate plasma glutamine levels but the impact on immune function is less clear (Rhode et al., 1998; Walsh et al., 2000; Krzywkowski et al., 2001). However, Hiscock et al. (2003) have demonstrated that glutamine supplementation augments the exercise induced increase in circulating IL-6 by ~50%, speculating that an increased uptake of glutamine into skeletal muscle from supplementation results in an increased IL-6 release into the circulation. Increased circulating IL-6 levels have been previously shown to heighten sensations of fatigue (Spath-Schwalbe et al., 1998) and impair exercise performance (Robson et al., 2004). Therefore, it is possible that glutamine intake during exercise may adversely affect performance through IL-6 mediated fatigue.

IL-6 typically increases its effectiveness in a range of biological actions when in the presence of its soluble receptor, sIL-6R. Sickness behaviour symptoms that are commonly associated with IL-6 infusion are heightened and prolonged with the addition of sIL-6R (Schobitz et al., 1995). Therefore, the agonistic properties of sIL-6R may have a negative impact on exercise performance when combined in an IL-6/sIL-6R complex (Levine et al., 2004). In chapter 3 we have demonstrated that circulating IL-6 negatively correlates with exercise performance. However,

the effect of glutamine intake during prolonged exercise on IL-6 mediated performance is unknown.

Since the effect of glutamine supplementation on both the plasma IL-6 and sIL-6R have not been examined during prolonged exercise, we aimed to investigate the effect of glutamine intake on the response of these markers in relation to exercise performance. We hypothesised that glutamine ingestion during prolonged exercise will result in an increase in plasma IL-6 and sIL-6R response which will impair subsequent time trial performance.

4.3 Methods

4.3.1 Participants

Ten trained male runners took part in the study. Mean ± SD age was 27 ± 6 yr height and body mass were 181 ± 4 cm and 73.4 ± 8.8 kg respectively and maximal oxygen uptake was 60 ± 4 ml·kg·min^{-1} whilst mean habitual training distance for running was 38 ± 28 kilometres per week. Exclusion criteria included those taking anti-inflammatory drugs or vitamin supplementation. All participants were free from any symptoms of infection in the 4 weeks prior to the study.   Participants gave written informed consent to the study which was given approval by the Northumbria University school ethics committee.

4.3.2 Experimental Protocol

Before the main trials, participants performed a familiarisation trial with at least one week separating each trial. To ensure nutritional status

remained constant, a food diary was recorded 24 h prior to experimental trials with participants encouraged to consume foods that were rich in carbohydrates. For the following trial, participants were instructed to replicate their diet they had previously recorded prior to trial 1. Each participant was also asked to refrain from exercise, alcohol and caffeine 24 h prior to the study and during the 24 h post exercise period. Each participant arrived at the laboratory in the morning before 9:00 am after a 12h fast. Participants then began the trial which is outlined in detail in chapter 2. Immediately before and at 30 min intervals during the preload bout, participants consumed 3 ml·kg^{-1} body mass of L-Glutamine (0.05 g·kg^{-1}) (MyProtein, UK) dissolved in lemon flavoured cordial or a taste matched placebo in a randomised crossover manner. Laboratory environmental conditions throughout the study were 20.1 ± 1.2°C and 38 ± 3% relative humidity

4.3.3 Blood Plasma Analysis

Blood samples were immediately centrifuged at 3000 rpm for 10 min aspirated and the supernatant was stored at -80°C until the day of analysis.

Plasma glutamine was measured in duplicate using a commercially available assay kit (Bioassay systems, Hayward, CA, USA). Samples were added to plates with 80 µl of working reagent including 2.5 µl of NAD and 14 µl MTT. Samples were then incubated for 40 min before 100 µl stop reagent was added to the plates. Samples were then ascertained through colorimetric determination at 540 nm

IL-6, sIL-6R, sgp130, cortisol, CRP, glucose and lactate were all analysed from blood plasma which is outlined in detail in chapter 2.

4.3.4 Statistical Analysis

The data was analysed using the statistical package SPSS v. 16 (SPSS inc., Chicago, IL). To analyse if data were spherical, Mauchly's test of sphericity was used, with a Greenhouse-Geisser correction for any violations. For trial and time analysis, a 2x5 (trial x time) repeated measures ANOVA was conducted; post hoc analysis were used with Bonferroni corrections for multiple comparisons with a criterion for significance set at $p<0.05$. For analysis of other physiological variables and time trial data, a paired samples t test was used with Bonferroni corrections used when appropriate.   All data are expressed as mean ± SD.

4.4 Results

4.4.1 Physiological Variables

Table 4.1 illustrates the physiological variables during the preload bout. No significant differences were observed between PLA and GLN for exercise intensity, heart rate, rating of perceived exertion or respiratory exchange ratio.

Table 4.1 Physiological variables during the preload bout.

	Placebo	Glutamine	P value
% $\dot{V}O_{2max}$	69 ± 7	68 ± 5	0.33
Heart rate (b·min^{-1})	146 ± 19	146 ± 20	0.96
RPE	12 ± 1	12 ± 1	0.67
RER	0.81 ± 0.05	0.81 ± 0.03	0.66

4.4.2 Plasma Glutamine

Plasma glutamine levels did not show a significant effect of trial between the treatments (p=0.06). However, during PLA, plasma glutamine levels fell from Baseline to PL (p=0.01) and remained low at PostTT whereas during GLN, plasma glutamine levels were maintained from Baseline to PL (p=0.70) which was reduced by PostTT (table 4.2).

4.4.3 Plasma IL-6, sIL-6R and sgp130

Plasma IL-6 showed a significant increase from Baseline to PL (p<0.01) and increased further by PostTT (p<0.001). Plasma levels remained elevated at 1hPost (p<0.001) and returned to Baseline levels by 24hPost. No significant differences were observed between trials (p=0.33) (figure 4.1).

Circulating sIL-6R increased 5% from Baseline to PL however the results were not significant (p=0.49). Furthermore, there was no significant difference in sIL-6R levels at PostTT compared to Baseline (p=0.08) following a 8.3% increase. No differences were observed between the treatments for the sIL-6R response to exercise (p=0.85) (figure 4.2).

Plasma sgp130 demonstrated no significant differences between trials (p=0.18). A non significant increase of 4.3% from Baseline to PL (p=0.10) was observed and furthermore, the increase of 7.6% at PostTT and 1hPost compared to Baseline was not significant (p=0.06). Levels had returned to baseline the following morning (figure 4.3).

Figure 4.1 Effect of glutamine supplementation on IL-6 response to exercise.
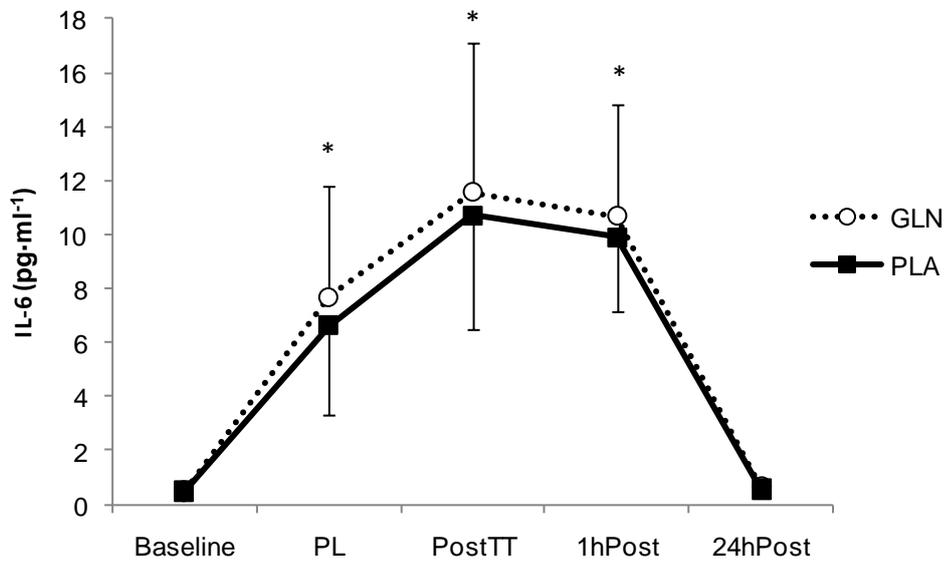
* Significantly higher than Baseline (effect of time) (p<0.01)

Figure 4.2 Effect of glutamine supplementation on the plasma sIL-6R response to exercise.
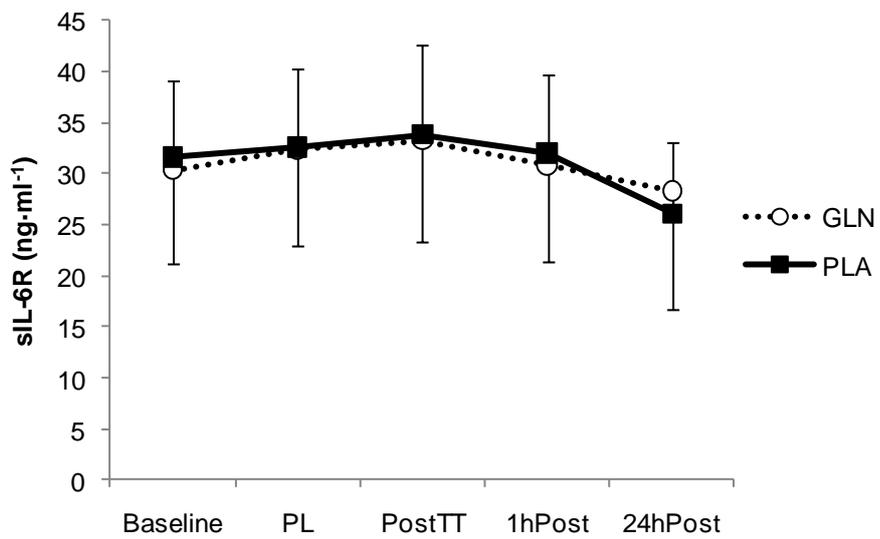
Figure 4.3 Effect of glutamine supplementation on the sgp130 response to exercise
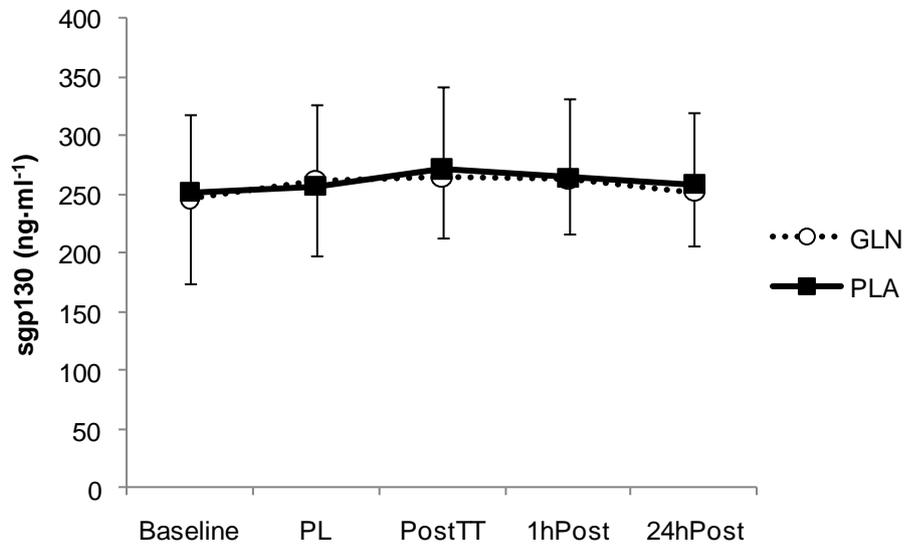
4.4.4 Time Trial

During the time trial there was no significant difference between trials for heart rate (PLA, 180 b·min^{-1}, GLN, 186 b·min^{-1}) (p=0.21) or for RPE (PLA, 16; GLN, 16) (p=0.75). Time to complete the time trials were 1456 s for PLA and 1375 s for GLN which were not significantly different (p=0.39) (figure 4.4). A significant negative correlation between IL-6 at PL and relative running speed throughout the time trial was observed for both PLA (r -0.75 p<0.05) and GLN (r -0.79 p<0.01) (figure 4.5).

Figure 4.4 Time completed for each time trial.
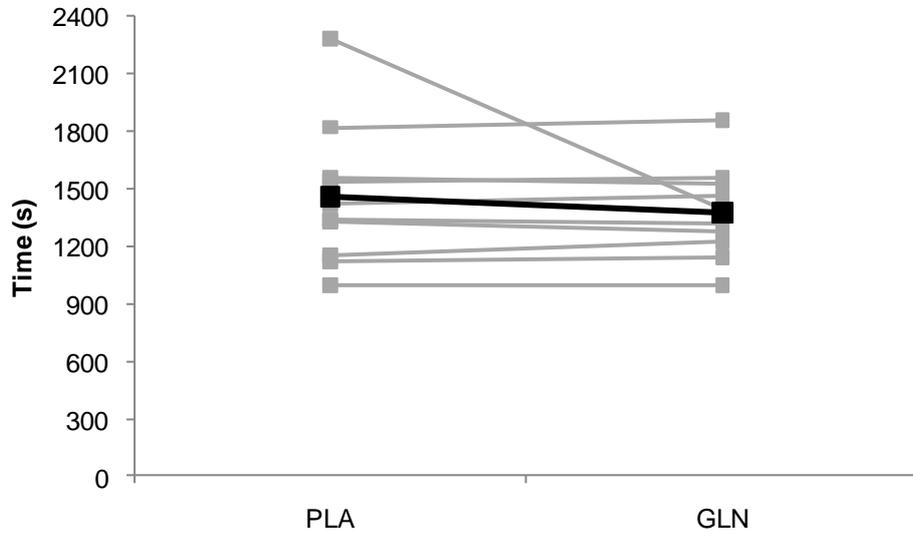
Individual performance times in grey. Group mean in black

Figure 4.5 Correlations between IL-6 at PL and running performance during the time trial.
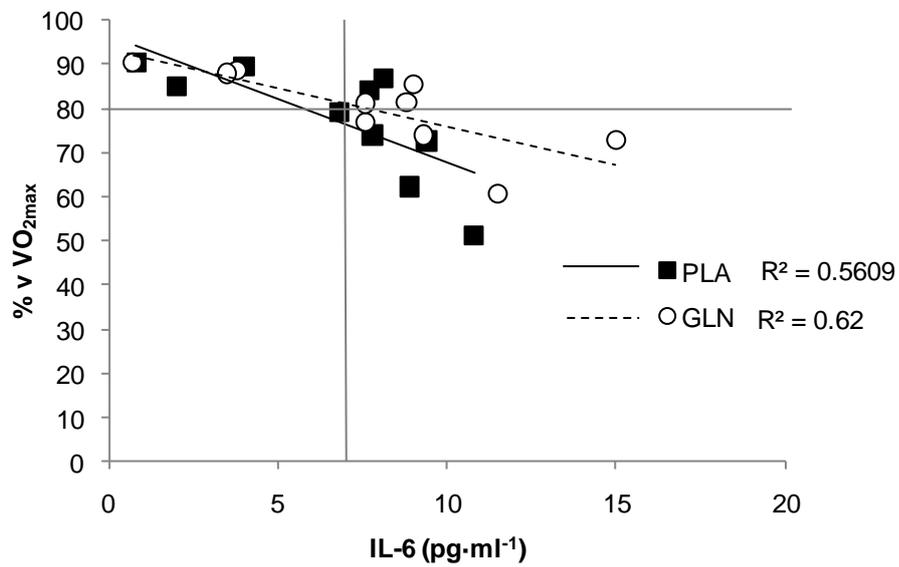
*Significant negative correlation (PLA $p < 0.05$; GLN $p < 0.01$)

4.4.5 Other Plasma Variables

The findings showed elevated plasma cortisol through an effect of trial (p=0.01). Cortisol increased from Baseline to PL which was not significant (p=0.44) but showed a further significant increase PostTT (p<0.001) which remained elevated 1hPost (p<0.05) then returned to Baseline values at 24hPost. CRP did not increase at PostTT (p=0.34) but did significantly increase 24hPost (p<0.01), however, no differences were shown between trials (p=0.55) (table 4.2).

Plasma lactate increased from Baseline to PL (p=0.05) which increased further at PostTT (p<0.01). Lactate levels were reduced at 1hPost and returned to Baseline levels the following morning with no significant differences between trials. Plasma glucose remained relatively unchanged throughout exercise then significantly decreased at 1hPost compared to PostTT (p<0.05). Statistical analysis demonstrated elevated plasma glucose through an effect of trial during GLN (p<0.05) (table 4.2).

Table 4.2. Plasma glucose, lactate, cortisol and C reactive protein.

	Baseline	PL	PostTT	1hPost	24hPost
Glutamine (µmol)					
PLA	816±105	729±109[a]	745±76	-	-
GLN	856±113	877±88	776±95	-	-
Glucose (mmol·L^{-1})					
PLA	4.79±0.45	4.67±0.81	5.18±0.82	4.57±0.59	4.92±0.45
GLN[†]	5.03±0.38	5.22±0.39	5.62±1.12	4.44±0.28	4.90±0.64
Lactate (mmol·L^{-1})					
PLA	2.18±0.53	3.67±1.33*	6.32±2.50**	3.81±1.89	1.89±0.80
GLN	1.85±0.91	3.61±1.68*	5.06±2.92**	3.24±1.70	2.10±1.13
Cortisol (nmol·L^{-1})					
PLA	330±71	372±85	704±231**	471±107*	265±68
GLN[†]	329±80	427±178	823±169**	663±262*	306±121
CRP (mg·L^{-1})					
PLA	0.8±0.9	-	0.8±1.0	-	2.8±1.5*
GLN	0.7±1.1	-	0.9±1.4	-	3.1±2.2*

Preload component (PL) Post time trial (PostTT) 1 h post time trial (1hPost) 24 h post baseline (24hPost)

*Significantly higher than Baseline (effect of time)(p<0.05) **Significantly higher than PL and Baseline (effect of time) (p<0.01) [a]Significant reduction from Baseline (effect of time) (p<0.05)
[†] GLN higher than PLA (effect of trial) (p<0.05)

4.5 Discussion

The findings in this study show that glutamine intake during exercise does not alter the response of circulating IL-6 or sIL-6R when compared to a placebo, despite maintaining plasma glutamine concentrations. Nor does glutamine intake alter exercise performance in a subsequent time trial.

Following prolonged exercise, plasma glutamine levels are typically reduced. We showed a 10.6% reduction in plasma glutamine following exercise which is consistent with Robson et al. (1999) who demonstrated that during prolonged exercise, plasma glutamine can fall by 11%. On the other hand, the present study showed that the glutamine treatment maintained circulating concentrations which is in accordance with previous work (Rhode et al., 1998; Walsh et al., 2000; Krzywkowski et al., 2001), indicating that supplementation of this nature at regular intervals are sufficient to maintain plasma glutamine levels during prolonged exercise. However, no effect on the circulating IL-6 response to exercise is observed.

In contrast to our findings, work by Hiscock et al. (2003) demonstrated an augmented plasma IL-6 response following glutamine supplementation and reported that plasma glutamine levels could influence circulating IL-6 by means of either decreasing IL-6 clearance or by increasing IL-6 production due to increased uptake of glutamine to skeletal muscle. It is unclear why the results differ between the present study and the study of Hiscock and colleagues (2003); differences in the mode of exercise employed would seem an unlikely explanation, however, differences in plasma IL-6 between studies may have occurred due to the time point at which the supplement was given. In the present study, participants were given glutamine at baseline and then at 30 min intervals during the 2 h preload bout; whereas participants were supplemented at 60 min and 105 min during the study by Hiscock and colleagues. It could

be speculated that the augmented plasma IL-6 response from glutamine intake is transient which was captured by Hiscock and co workers and missed in the present study.

Although our findings showed no differences in circulating IL-6 between treatments, the plasma IL-6 response to the preload was inversely correlated with relative time trial performance regardless of trial. These findings indicate that the magnitude of the plasma IL-6 response to an exercise preload bout can impact on subsequent exercise performance which is consistent with findings from the previous chapter. The release of IL-6 into the circulation may act as a distress signal as a result of cellular or metabolic stress within skeletal muscle (Jurimae et al., 2011). IL-6 has been shown to cross the blood brain barrier (Banks et al., 1994) which has been hypothesised to act as a fatigue signal (Gleeson, 2000). The increased circulating IL-6 may interact with the brain and alter perception of effort during exercise in order to preserve energy.

Further analysis of the findings demonstrate that a circulating IL-6 response below a threshold of $\sim$7 pg·ml^{-1} is associated with a time trial performance above $\sim$80% v$\dot{V}O_{2max.}$ This suggests that there is a level of cellular or metabolic stress below which any IL-6 interaction with the brain could be tolerable, allowing participants to increase their performance throughout the time trial. However, it appears that when an IL-6 response to the preload is above the threshold of $\sim$7 pg·ml^{-1}, it influences perception of effort at the relative intensity during the time trial.

No change in either sIL-6R or sgp130 was observed in either condition. Although increases in the signalling receptors have been previously reported (Gray et al., 2008; Leggate et al., 2010), The small change in the circulating receptors are in accordance with the findings in chapter 3. It is likely that a significant difference was not seen due to the large inter-variability of sIL-6R and sgp130 and relatively low participant number. Notably, neither sIL-6R nor sgp130 increased with exercise which may indicate that sgp130 reflects the activity of sIL-6R. Rebouissou et al. (1998) have previously shown that an increase in sIL-6R activity is followed by an increase in sgp130. The delayed actions of sgp130 could be to regulate the actions of the IL-6 complex to prevent excessive signalling.

The effects of nutritional interventions on the response of signalling receptors to exercise are unknown. Since there was no increase in the signalling receptors, and there was no significant difference in plasma IL-6 following glutamine supplementation, it is difficult to interpret whether nutritional interventions alter the signalling receptor response to exercise. Therefore, this needs further investigation.

Our findings showed increased cortisol concentrations following glutamine intake. Since cortisol plays a role in glutamine transport (Jones et al., 2006), it could be speculated that excess glutamine following supplementation stimulates an increase in plasma cortisol levels in order to transport the excess glutamine to the liver. In addition, cortisol plays a role in gluconeogenesis (Tayek and Katz, 1997); therefore it is possible

that cortisol can concurrently stimulate hepatic glucose production, thereby increasing plasma glucose concentrations, which was observed in our study.

Circulating CRP was not significantly different between glutamine ingestion and a placebo in response to exercise. Our findings are in accordance with Castell et al. (1996) who found that the increase in circulating IL-6 following a marathon corresponded with circulating CRP 16 h post marathon with no differences observed between glutamine supplementation and a placebo.

Our results indicate that glutamine supplementation during prolonged exercise does not alter circulating IL-6, its associated signalling receptors, nor does it affect exercise performance. However, in accordance with previous work, the circulating IL-6 response to exercise was negatively correlated with time trial performance regardless of glutamine ingestion.

Chapter 5

The Effect of Pre-exercise Feeding
Protocols on the IL-6 and Signalling
Receptor Response to Exercise

5.1 Abstract

Carbohydrate loading protocols can maximise muscle glycogen levels and delay fatigue. In addition, low muscle glycogen levels can increase plasma IL-6 levels which are associated with a reduction in time trial performance. It is not known if a CHO loading protocol can reduce the plasma IL-6 response to exercise, subsequently improving time trial performance. Therefore, we investigated the effects of two high CHO loading protocols (with and without a prior bout of glycogen depleting exercise), compared to a low CHO dietary protocol on the plasma response of IL-6 to exercise and the subsequent impact on exercise performance. Eleven trained males performed a preloaded time-trial, running at 60% vVO2max for 2 h interspersed with 30 s at 90% vVO2max every 10 min, followed by a 5-km time trial. Prior to each trial, participants consumed either a low carbohydrate diet (3 g·kg^{-1} body mass,)(Lo); a high carbohydrate diet (10 g·kg^{-1} body mass) (Hi) or performed a glycogen depleting exercise bout followed by a high carbohydrate diet (DepHi) prior to the exercise trials. Results found improved time trial performance for Hi and DepHi compared to Lo, however, there were no significant differences between trials for the circulating IL-6 response to exercise. However, plasma IL-6 levels prior to the time trial and percentage of velocity at $\dot{V}O_{2max}$ achieved during the time trial showed a negative correlation in all three trials. Therefore, we conclude that a negative association between the plasma IL-6 response to a preload bout and subsequent time trial performance is not affected by altered dietary CHO intake.

5.2 Introduction

It is widely accepted that an athletes diet is an important aspect to the preparation regimen in the days prior to competition to increase muscle glycogen content and improve exercise performance (Rauch et al., 1995) since CHO availability as a substrate can be a limiting factor to performance in prolonged sessions >90 min (Burke et al., 2004). Therefore, to optimise endurance exercise performance, athletes will consume a carbohydrate rich diet in order to maximise muscle glycogen levels and delay the onset of fatigue (Karson and Saltin, 1971; Hargreaves et al., 1984).

A variety of carbohydrate loading protocols have been developed in order to supercompensate muscle glycogen content (Bergstrom et al. 1967; Sherman et al., 1981; Bishop et al., 2001).   CHO loading protocols may differ according to the timing, amount, type and form of ingested CHO, while other strategies include depletion of muscle glycogen followed by restoration of muscle glycogen with a high CHO diet (Goforth et al., 1997). A glycogen depleting exercise bout can increase muscle glycogen synthesis to a greater extent compared to a high CHO only diet (without glycogen depletion) (Roedde et al,. 1986; Goforth et al., 2003). Therefore, it appears that a bout of glycogen depleting exercise prior to CHO loading is key to optimising muscle glycogen synthesis.

Skeletal muscle glycogen possesses several signalling factors that have a glycogen binding domain, and when activated, particularly during metabolic stress, are linked with an increase in IL-6 production

(MacDonald et al., 2003; Chan et al., 2004). Steensberg et al., (2000) demonstrated that contracting skeletal muscle accounts for the increase in plasma IL-6 which is observed when muscle glycogen levels are low. In the view of this, it would be reasonable to assume that a bout of glycogen depleting exercise followed by CHO loading would result in an increase of muscle glycogen levels and subsequently reduce the response of plasma IL-6 to exercise. Although the impact of various glycogen depletion and restoration diet protocols on muscle glycogen levels have been investigated with the view to improve subsequent exercise performance, the effect of this type of protocol on the plasma IL-6 response to exercise has not been examined.

Previously we have shown that the magnitude of plasma IL-6 response during an exercise preload bout was negatively associated with exercise performance during a subsequent time trial. Consequently, if the plasma IL-6 response can be altered by manipulation of dietary CHO intake, then this may further clarify the link between plasma IL-6 and exercise performance. In addition, the plasma concentration of the signalling receptor of IL-6 (sIL-6R) increases in response to prolonged exercise (Gray et al., 2008; Leggate et al., 2010) which increases the effectiveness of IL-6 as a CHO metabolism regulator and prolongs the half life of circulating IL-6 (Jones et al., 2001). However, it is not known whether this occurs in humans during prolonged exercise.

Therefore, the aims of this study were to examine the effects of high CHO loading protocols, with and without a prior bout of muscle

glycogen depleting exercise, compared to a low CHO dietary protocol on the plasma response of IL-6 and sIL-6R to exercise and the subsequent impact on exercise performance. It is hypothesised that the high CHO loading protocols will induce a lower plasma IL-6 and sIL-6R response to exercise compared to a low CHO diet with the glycogen depletion and restoration protocol being most effective.

5.3 Methods

5.3.1 Participants

Eleven trained males volunteered for the study for which they gave written informed consent and was approved by the school research ethics committee at Northumbria University. Exclusion criteria included symptoms of infection and administration of anti-inflammatory drugs including medication or vitamin supplementation in the four weeks prior to the study. Subject characteristics are presented in Table 5.1. Data are expressed as mean ± SD.

Table 5.1 Subject characteristics

Age	27	± 7 yr
Height	182	± 6 cm
Body mass	80.2	± 8.4 kg
Maximal oxygen uptake	57	± 6 $ml \cdot kg^{-1} \cdot min^{-1}$
Habitual training distance (running)	22	± 8 $km \cdot week^{-1}$

5.3.2 Experimental Protocol

All participants performed a familiarisation trial before completing the three experimental trials with at least one week separating each trial. Trials were conducted in a randomised crossover design that was counterbalanced. Prior to each trial, participants consumed isocaloric

diets that comprised a different proportion of carbohydrates. Participants consumed either a low carbohydrate diet (Lo) (3 g·kg^{-1} body mass, 20% carbohydrate, 68% fat and 12% protein); a high carbohydrate diet (Hi) (10 g·kg^{-1} body mass, 68% carbohydrate, 16% fat and 16% protein) or performed a glycogen depleting exercise bout followed by a high carbohydrate diet (DepHi) 48 h prior to the exercise trials outlined in chapter 2 (General Methods). For the DepHi trial, participants arrived at the laboratory approximately 60 h prior to the main trial to complete an exercise protocol which aimed to deplete muscle glycogen stores. Participants ran at 70% $\dot{V}O_{2max}$ for 90 min which has previously been shown to deplete muscle glycogen (Tsintzas et al., 1996) then consumed the high carbohydrate diet the following morning and for the next 48 h. All diets were calculated according to dietary analysis software (Microdiet, Downlee Systems Ltd, UK).

48 h prior to the study and during the 24 h post exercise, participants were asked to ensure they were well hydrated and to refrain from exercise, alcohol and caffeine. Participants began the trial in the morning before 9:00 am (outlined in detail in Chapter 2). Water was consumed *ad libitum* during the trials and recorded. Water consumed was similar for all three trials. Following each trial, body mass was recorded. Laboratory environmental conditions throughout the study were 21.4 ± 2.2°C and 42 ± 9% relative humidity.

5.3.3 Blood Plasma Analysis

IL-6, sIL-6R, sgp130, cortisol, CRP, glucose and lactate were all measured from blood plasma (outlined in detail in Chapter 2).

5.3.4 Statistical Analysis

The data was analysed using the statistical package PASW v.18 (SPSS inc., Chicago, IL). To investigate if data were spherical, Mauchly's test of sphericity was applied, with a Greenhouse-Geisser correction for any violations. For trial and time analysis, a 3 x 5 (trial x time) repeated measures ANOVA was conducted with Bonferroni adjustments for multiple comparisons with a criterion for significance set at $p<0.05$, while a one-way ANOVA was used to assess time trial times. General estimating equation was applied to assess within-subject factors for correlation analysis. All data are expressed as mean ± SD.

5.4 Results

5.4.1 Physiological and Performance Data

5.4.2 Preload

Exercise intensity during the preload was 66 ± 5%, 67 ± 6%, and 64 ± 7% of $\dot{V}O_{2max}$ for Lo, Hi and DepHi trials respectively and was not significantly different between trials (p=0.73). Respiratory exchange ratio was also not significantly different (Lo = 0.89 ± 0.05, Hi = 0.91 ± 0.05, DepHi = 0.93 ± 0.08; p=0.35). Mean heart rate (Lo =149 ± 16 b·min^{-1}, Hi =149 ± 16 b·min^{-1}; DepHi = 146 ± 18 b·min^{-1} p=0.14) were similar for the preload components between trials. For ratings of perceived exertion, no

significant differences were observed between the trials (Lo = 13 ± 2; Hi =
12 ± 1; DepHi = 12 ± 2, p=0.14).

5.4.3 Time Trial

Repeated measures ANOVA showed that running speed and time
to complete the Lo time trial was significantly impaired compared to Hi
(p=0.01) and DepHi (p<0.01) however, there were no differences
observed between Hi and DepHi (p=1.00) (figure 5.1and figure 5.2). A
lower mean heart rate was recorded during the Lo time trial compared to
Hi (p<0.05) (Lo =168 ± 9 b·min^{-1}; Hi =180 ± 17 b·min^{-1}; DepHi = 177 ± 17
b·min^{-1}). There were no differences between trials for RPE were observed
during the time trial for RPE (Lo = 17 ± 1, Hi = 17 ± 1, DepHi = 17 ± 2,
p=0.12).

Figure 5.1 Speed during the time trials
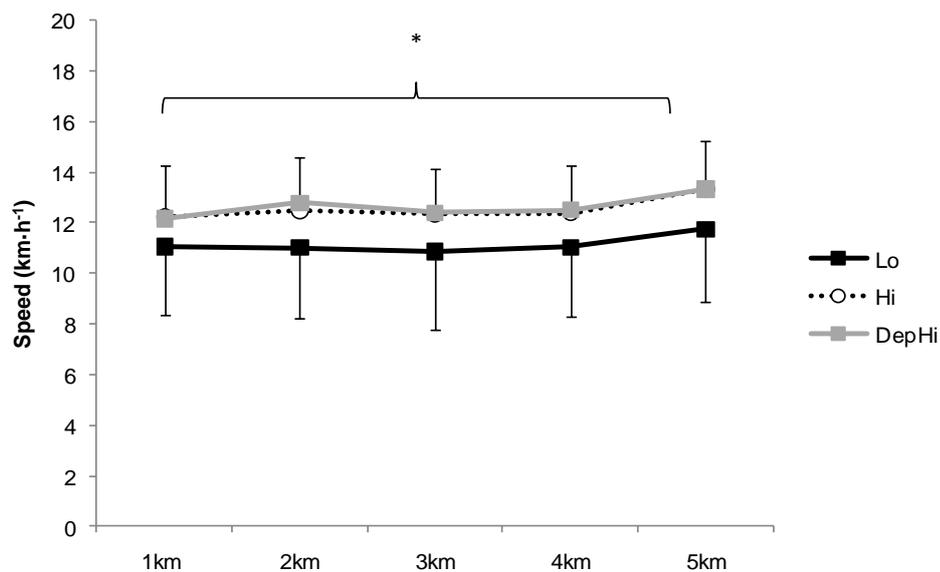
*Lo significantly lower speed than Hi and DepHi (effect of trial) (p≤0.01)

91

Figure 5.2 Individual times for the time trials
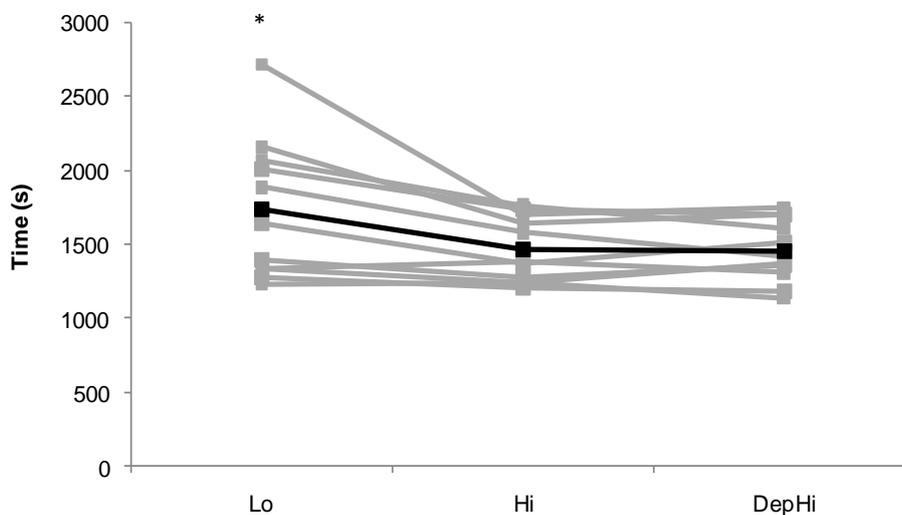
*Significantly slower than Hi and DepHi (effect of trial). Individual times (grey), geometric mean of group (black)

5.4.4 IL-6 and Signalling Receptors

Analysis of plasma IL-6 revealed an outlier that influenced the statistical analysis when examining for differences. Therefore the outlier was excluded from the statistical analysis and was presented on a separate figure.

Plasma IL-6 demonstrated a 15 fold increase from baseline (0.5 pg·ml^{-1}) at PL (7.6 pg·ml^{-1})(p<0.01) and a 30 fold increase (14.8 pg·ml^{-1}) at PostTT (p<0.01). IL-6 levels decreased 1hPost to 11.0 pg·ml^{-1} and returned to baseline levels by the following morning. No significant differences were observed between trials for IL-6 (p=0.11) (Figure 5.3a and Figure 5.3b).

An increase in plasma sIL-6R levels from baseline (36.4 ng·ml^{-1}) to
PL (39.8 ng·ml^{-1}) (p<0.01) was observed with a further significant increase
at PostTT (40.7 ng·ml^{-1}) (p<0.01). These levels remained elevated at
1hPost (39.3 ng·ml^{-1}) and returned to baseline levels by 24hPost. No trial
effect was observed for plasma sIL-6R (p=0.08) (figure 5.4).

The plasma response of sgp130 showed no differences between
trials (p=0.46) and although no significant difference in plasma sgp130
was observed from Baseline to PL (p=0.06), sgp130 was elevated in
response to exercise PostTT compared to Baseline (p<0.01). No other
differences were observed for plasma sgp130 (figure 5.5).

Figure 5.3a. Group data for the interleukin-6 response to the preloaded time trial protocol
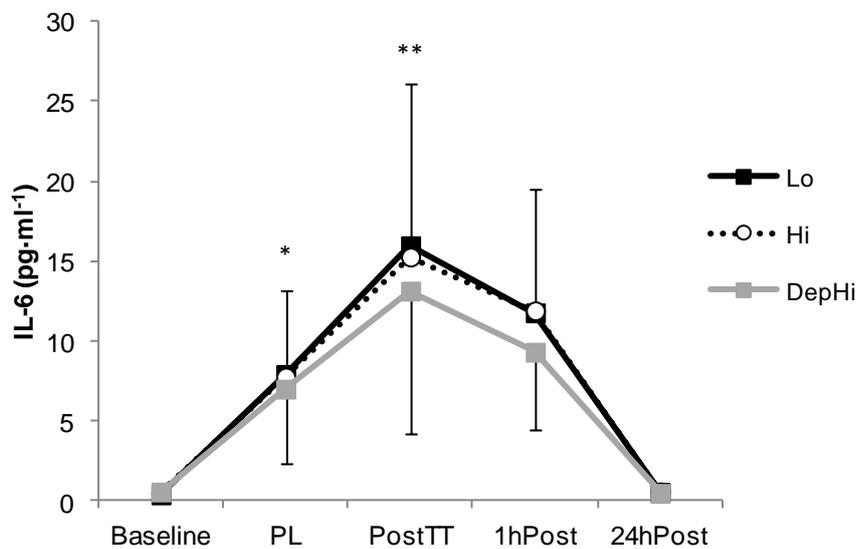
*Significantly higher than Baseline (effect of time) (p<0.01) **Significantly higher than
Baseline and PL (effect of time) (p<0.05)

Figure 5.3b. Outlier data for the interleukin-6 response to the preloaded time trial protocol
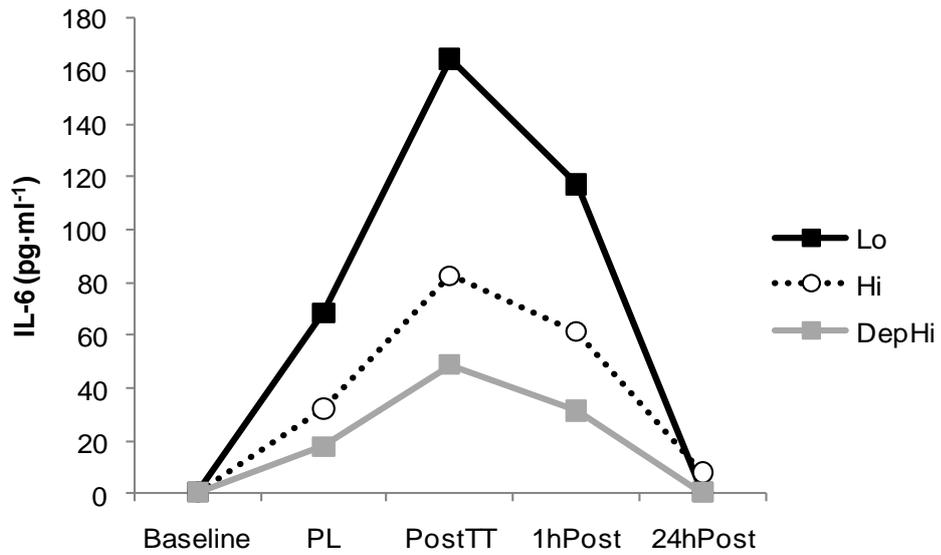
Figure 5.4. The soluble interleukin-6 receptor (sIL-6R) response to the preload time trial protocol.
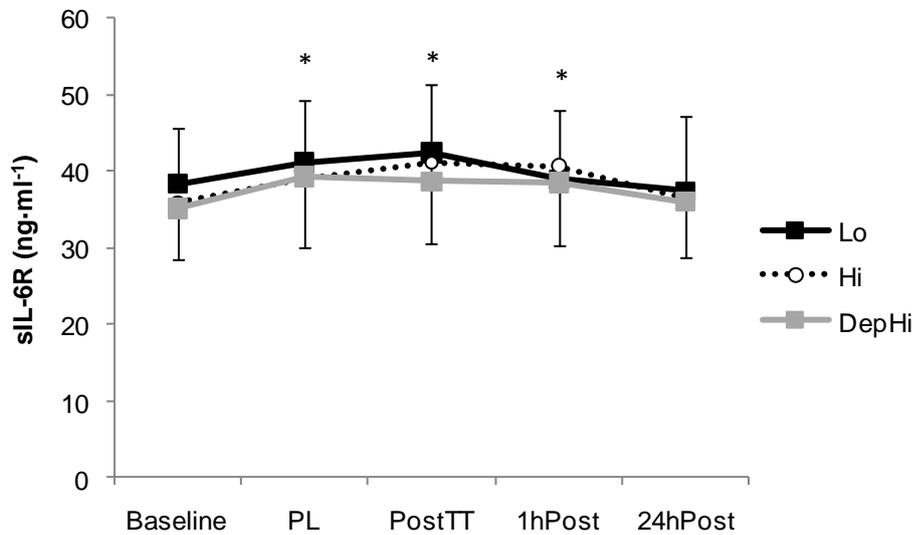
* Significantly higher than Baseline (effect of time) (P<0.05).

Figure 5.5. The sgp130 receptor response to the preload time trial protocol.
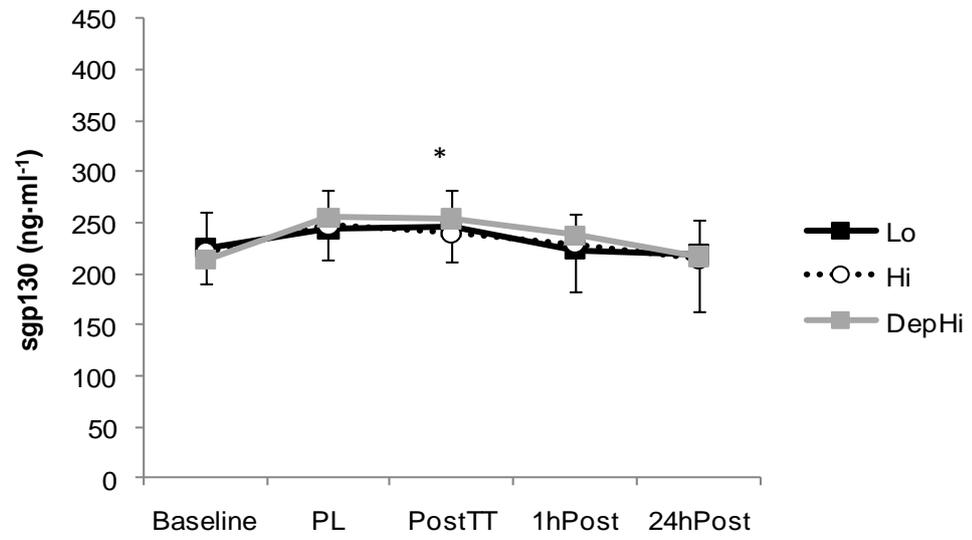
* Significantly higher than Baseline (effect of time) (P<0.01).

Plasma IL-6 levels prior to the time trial (PL) and percentage of velocity at $\dot{V}O_{2max}$ achieved during the time trial showed a significant negative correlation in all three trials (Lo r -0.68, p<0.05; Hi r -0.63, p=0.05; DepHi r-0.67, p<0.05). Quadrant analysis illustrated that plasma IL-6 at a threshold of ≥7 pg·ml^{-1} was associated with achieving a relative performance of <80% v$\dot{V}O_{2max}$ during the time trial (figure 5.6a and 5.6b). Furthermore, the mean circulating IL-6 response was not associated with habitual running distance and $\dot{V}O_{2max}$ (figures 5.7 and 5.8)

Figure 5.6a. Group data correlations and quadrant analysis between IL-6 at PL and running performance during the time trials.
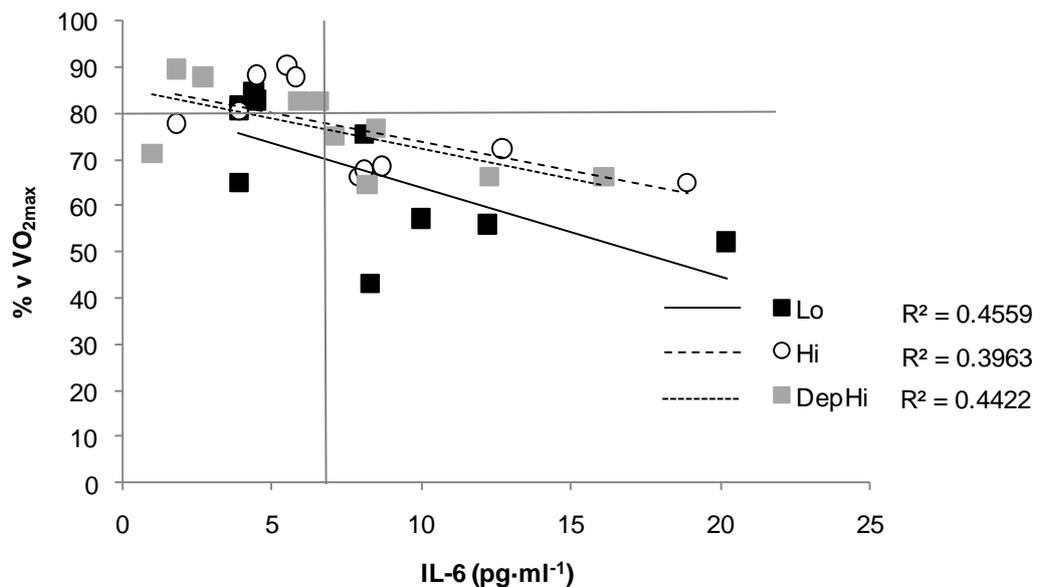
Lo p<0.05; Hi p=0.05; DepHi p<0.05

Figure 5.6b. Outlier data correlations and quadrant analysis between IL-6 at PL and running performance during the time trials.
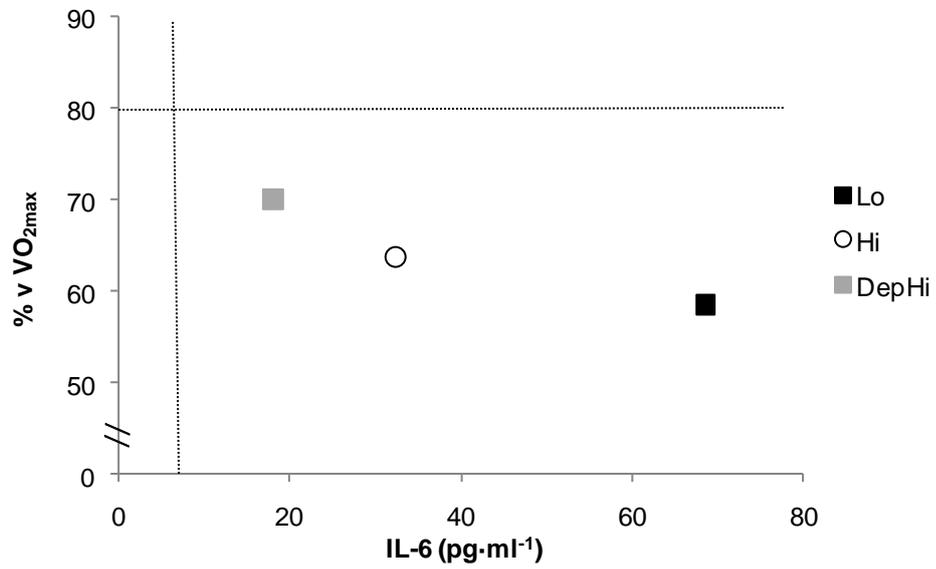
Figure 5.7 Correlation between mean circulating IL-6 response to the preload and habitual running distance
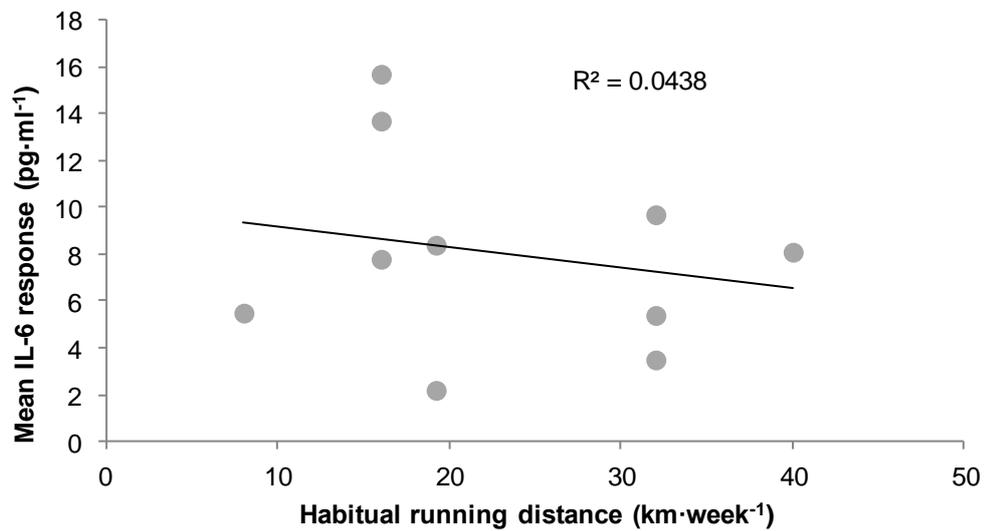
Figure 5.8 Correlation between mean circulating IL-6 response to the preload and and maximal oxygen uptake
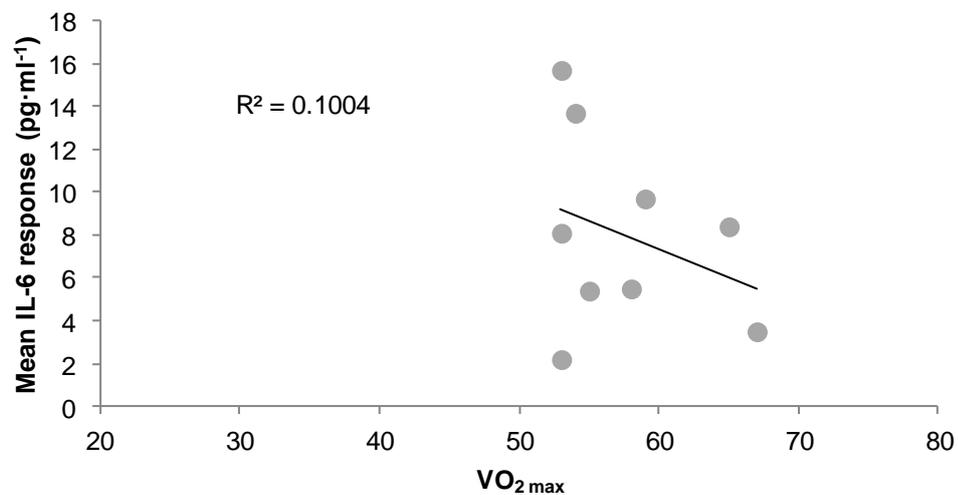
5.4.5 Other plasma variables

No significant differences were observed between trials for plasma glucose and furthermore plasma glucose did not change significantly during or following exercise (p=0.30). Circulating lactate significantly increased at PL compared to Baseline (p<0.05) and showed a further significant increase PostTT (P<0.01), which began to fall at 1hPost. Plasma lactate was lower at PostTT during Lo compared to Hi (p<0.05). Plasma cortisol levels showed a non significant rise at PL (p=0.39) which was significantly elevated at PostTT and remained elevated at 1hPost compared to Baseline (p<0.05). Circulating levels of CRP were not significantly different between trials and did not significantly change at PostTT (p=0.22) or 24hPost (p=0.09) (table 5.2).

Table 5.2. Plasma glucose, lactate, cortisol and CRP responses to the pre-exercise feeding protocols

	Baseline	PL	PostTT	1hPost	24hPost
Glucose (mmol·L^{-1})					
Lo	6.25±0.74	6.01±0.92	5.56±1.53	5.73±0.88	6.10±0.86
Hi	6.84±1.42	6.54±1.09	6.71±1.15	5.95±0.97	6.39±0.89
DepHi	5.79±0.88	6.61±0.69	6.59±1.31	5.83±0.67	6.23±0.49
Lactate (mmol·L^{-1})					
Lo	1.07±0.28	2.10±0.81*	4.64±2.50**[†]	1.78±0.46	1.25±0.35
Hi	1.60±0.57	2.59±1.59*	7.22±2.61**[†]	2.15±0.70	1.65±0.60
DepHi	1.35±0.41	2.52±1.20*	6.24±2.21**	1.84±0.65	1.32±0.31
Cortisol (nmol·L^{-1})					
Lo	624±243	852±349	1280±290**	1089±291**	419±205
Hi	565±173	707±311	1084±353**	958±347**	410±190
DepHi	579±234	765±371	1079±339**	981±356**	460±260
CRP (mg·L^{-1})					
Lo	0.7±0.6	-	0.8±0.6	-	4.3±3.4
Hi	0.5±0.4	-	0.5±0.5	-	4.8±4.3
DepHi	0.5±0.4	-	0.5±0.2	-	4.0±3.0

* Significantly higher than Baseline (effect of time) ($p<0.05$) ** Significantly higher than Baseline and PL (effect of time) ($p<0.05$) [†] Significantly lower during Lo compared to Hi ($p<0.01$)

5.5 Discussion

The present study sought to examine the effects of different pre-exercise dietary protocols on the plasma IL-6 response to exercise and what impact this response had on subsequent time trial performance. Although plasma IL-6 and sIL-6R increased in response to exercise, no significant differences were observed between the trials. An improved performance during the time trial was observed for the high carbohydrate

loading protocols, with and without prior glycogen depletion, compared to the low carbohydrate diet.

An improved exercise performance following high CHO diets compared to a low CHO diet has been reported extensively (Walker et al., 2000; Lambert et al., 2001; Rauch et al., 2005) with muscle glycogen levels being the predominant factor related to the differences in exercise performance. The increased CHO availability from muscle glycogen allows for spared CHO oxidation rate from circulating blood glucose throughout prolonged exercise (Coggan and Coyle, 1991). This also allows the participant to maintain a higher intensity during the time trial when compared to a low glycogen trial. Our findings also demonstrate that no significant difference in performance was seen between the two high carbohydrate trials, indicating that a glycogen depletion and restoration protocol is no more beneficial than a high CHO diet alone in terms of exercise performance of this nature. Whether this is due to a lack of difference in muscle glycogen, or indeed, a difference in muscle glycogen, which did not translate to an increase in performance is unknown since we did not measure muscle glycogen in the current study.

Although there were no significant differences observed in the plasma IL-6 response to exercise between the trials, the plasma IL-6 response showed a strong negative correlation with relative exercise performance during the time trial in all three trials. Indeed, individuals who exhibited a greater plasma IL-6 response to the preload tended to perform

a slower time trial performance when expressed as a percentage of maximal aerobic velocity, regardless of dietary CHO intake.

Our data suggests that a higher circulating IL-6 concentration following a bout of prolonged exercise is associated with lower subsequent relative exercise performance. Since elevations in IL-6 have been shown to induce fatigue at rest (Spath-Schwalbe et al., 1998), during daily activities (Ferrucci et al., 2002; Cappola et al., 2003) and during exercise (Robson et al., 2004), it is plausible that IL-6 acts as a fatigue signal, interacting with the brain, subsequently altering perception of effort during the time trial. The relationship between IL-6 and fatigue during exercise has been previously demonstrated (Robson et al., 2004) and it has since been suggested that IL-6 acts as a mediator between muscle and brain (Pedersen and Febbraio, 2005). Therefore, the concentration of circulating IL-6 may indicate to the brain the magnitude of stress and result in an altered pacing strategy during the time trial.

Analysis of the findings indicates that a circulating IL-6 response below a threshold of ~7 pg·ml^{-1} is associated with a time trial performance above ~80% v$\dot{V}O_{2max.}$ This may be interpreted that a low IL-6 response to exercise, below 7 pg·ml^{-1}, indicates a low stress response and little disruption to homeostasis, therefore allowing participants to maintain their relative performance throughout the time trial. When the circulating IL-6 response is >7 pg·ml^{-1}, it increases the perception of effort for a given intensity and subsequently lead reduce relative exercise intensity during the time trial.

101

Endurance training is associated with a multitude of adaptations including changes to metabolic enzymes (Gollnick et al., 1982), increases in muscle glycogen storage (Hickner et al., 1997), increases in fatty acid transport and an increased reliance on lipids as a substrate during sub maximal exercise (Kiens et al., 1993); all potentially resulting in a lower plasma IL-6 response to a bout of prolonged exercise (Fischer et al., 2004; Gokhale et al., 2007). Therefore, a reduced plasma IL-6 response to prolonged exercise could be due to differences in habitual training volume. However, we found no association between habitual weekly running distance and the plasma IL-6 response to exercise. Furthermore, the plasma IL-6 response was not associated with maximal oxygen uptake, suggesting that neither training volume nor aerobic fitness attributed to the differences in the IL-6 association with time trial performance.

Circulating sIL-6R levels increased following the preload exercise bout, peaking at PostTT which is consistent with previous work (Gray et al., 2008; Leggate et al., 2010). The role of upregulated sIL-6R levels in prolonged exercise is unknown. One hypothesis is that an upregulation of sIL-6R can improve the binding capacity to IL-6 and therefore improve IL-6 signalling as a complex (Booth and Company, 2009). As sIL-6R is an agonist of IL-6, it may influence time trial performance. However, no differences were observed in plasma sIL-6R between the trials and furthermore, no correlations were observed between sIL-6R and exercise

performance, indicating that sIL-6R is not influenced by CHO ingestion during prolonged exercise.

Plasma sgp130 increased significantly at PostTT which is consistent with our previous work (Chapter 3) and research elsewhere (Gray et al., 2008). The effect of sgp130 is in contrast to the cognate membrane bound gp130 by acting antagonistically, preventing IL-6/sIL-6R signalling. In the circulation, sgp130 can bind to the IL-6/sIL-6R complex, presenting a molar excess which is biologically inactive (Jostock et al., 2001). Therefore, the similar response between the trials is probably explained by the plasma sIL-6R response to exercise. Increases in sgp130 follow increases in sIL-6R in order to modulate the actions of sIL-6R, possibly regulating excessive signalling (Rebouissou et al., 1998).

As has been previously demonstrated, circulating cortisol increased in response to prolonged exercise (Farrell et al., 1983; Buono et al., 1986; Coiro et al., 1988; Tabata et al., 1991). Plasma cortisol increases in response to prolonged exercise have been associated with metabolic stress, resulting in activation of the HPA axis. However, since no significant differences were observed between the trials and blood glucose was similar, it would suggest that muscle and liver glycogen levels were sufficient to maintain circulating blood glucose levels regardless of pre CHO loading regimen. This is also reflected by similar responses observed in circulating IL-6 which can also act as an independent stimulator of cortisol (Tsigos et al.,1997; Bethin et al., 2000)

Circulating C reactive protein was not different between trials, which is most likely due to the similar response observed in plasma IL-6. Although there was an approximate 7-fold increase in CRP the following morning, the concentrations were not significantly different from baseline ($p=0.09$) which is in contrast to previous work (Strachen et al., 1984; Castell et al., 1996; Seigal et al., 2001; Miles et al., 2006). It is likely that the lack of significance is due to the large variability between participants and relatively low number of participants.

In conclusion, our findings demonstrate a strong inverse relationship between the plasma IL-6 response to a preload bout and subsequent relative time trial performance, which was not affected by altered dietary CHO intake. Furthermore, we have demonstrated that a prior bout of glycogen depleting exercise before a high CHO diet is no more beneficial than a high CHO alone for exercise performance following a preload bout of exercise.

Chapter 6

The Effect of Carbohydrate Supplementation on Time Trial Performance and its Association with   IL-6 and sIL-6R

6.1 Abstract

Carbohydrate supplementation can delay the onset of fatigue and improve exercise performance through different pathways including suppressing fatigue signals. Furthermore, increased levels of plasma IL-6 have fatigue inducing properties which are associated with reduced time trial performance. CHO supplementation during prolonged exercise can markedly reduce the appearance of IL-6 in circulation in response to prolonged exercise. However, it is unknown if attenuating circulating IL-6 will subsequently improve exercise performance. Therefore, we investigated the effects of CHO supplementation on the circulating IL-6 response to exercise and the subsequent impact on performance. Nine trained male runners performed a preloaded time-trial, consisting of a 2 h run at 60 vVO2max interspersed with 30 s runs at 90% vVO2max at 10 min intervals, followed by a 5 km time trial. Participants were supplemented with 2 ml·kg^{-1} body mass of an 8% carbohydrate solution or a taste matched placebo in a randomised crossover manner at 20 min intervals. Results showed that CHO supplementation attenuated the plasma IL-6 response post preload by 49% which correlated well with an 8% improvement in time-trial performance. In conclusion, we have shown that the CHO-related improvement in performance is associated with the atteunuation of circulating IL-6 during exercise.

6.2 Introduction

During prolonged exercise, fatigue is inevitable and results in the inability to maintain a given work output. CHO supplementation can reverse or delay the onset of fatigue and improve exercise performance which is achieved through central and peripheral pathways. Mouth rinse of a CHO solution has been shown to improve 1 h running performance (Rollo et al., 2010), acting possibly through activation of reward centres of the brain (Chambers et al., 2009) or by suppressing fatigue signals (Pottier et al., 2010). Furthermore, CHO ingestion during exercise may also reduce fatigue by maintaining blood glucose concentration, allowing CHO oxidation rates from circulating glucose to be maintained (Coyle et al., 1986) while sparing both liver glycogen (Jeukendrup et al., 1999) and muscle glycogen stores (Erickson et al., 1987; Yaspelkis et al., 1993; Stellingwerff et al., 2007).

When intramuscular glycogen levels become depleted from prolonged exercise contracting skeletal muscle releases IL-6 into the circulation (Nieman et al., 1998; Steensberg et al., 2000; Keller et al., 2001; Cox et al., 2007), which increases in a dose dependant manner relative to both the intensity (Ostrowski et al., 2000) and the duration of exercise (Fischer, 2006) as the body relies more on blood glucose as a source of energy (Coggan and Coyle, 1991; Stellingwerf et al., 2007). When released into the circulation, IL-6 can act as a glucose regulator to stimulate hepatic glucose production (Febbraio et al., 2004)

As well as a glucose regulator, increased plasma IL-6 has fatigue inducing properties and furthermore, worsens mood state and reduces the ability to concentrate when in the circulation (Spaith-Schwalbe et al., 1998). Resting plasma IL-6 levels have been associated with impaired functional performance such as lower walking speed (Taafe et al., 2000), lower muscle strength of the lower extremities (Visser et al., 2002) and lower grip strength (Bautmans et al., 2007). Furthermore, an increase in circulating IL-6 can also impair athletic performance in athletes (Robson et al., 2004).

Carbohydrate supplementation during prolonged exercise can markedly reduce the appearance of IL-6 in circulation in response to prolonged exercise (Nehlsen-Cannarella et al. 1997; Utter et al., 1999; Starkie et al., 2001; Febbraio et al., 2003). Since IL-6 mRNA is unaffected by CHO ingestion during prolonged exercise within skeletal muscle (Starkie et al., 2001; Febbraio et al., 2003), it has been speculated that CHO supplementation attenuates the release of IL-6 into the circulation and not the production of IL-6. In addition, CHO ingestion during exercise reduces liver glycogenolysis and gluconeogenesis (Jeukendrup et al., 1999) therefore reducing the need for IL-6 to stimulate glucose production.

Therefore, it is possible that CHO supplementation during prolonged exercise will result in a lower circulating IL-6 response, attenuating a stress signal which will subsequently improve exercise performance. Accordingly, the present study intended to determine the

impact of CHO ingestion during prolonged exercise on the responses of plasma IL-6 and the subsequent time trial performance.

6.3 Methods

6.3.1 Participants

Nine trained male runners took part in the study. Participant characteristics are displayed in table 6.1. Exclusion criteria included those taking medication including anti-inflammatory drugs or vitamin supplementation. All participants were free from any symptoms of infection in the four weeks prior to the study. Participants gave written informed consent to the study which was given approval by the Northumbria University school ethics committee.

Table 6.1 Subject characteristics

Age (yr)	26	±	6
Height (cm)	181	±	6
Body mass (kg)	73.5	±	7.4
Maximal oxygen uptake (ml·kg·min^{-1})	58	±	4
Weekly habitual training (km)	40	±	18

Data are expressed as mean ± SD

6.3.2 Experimental Protocol

Participants performed a familiarisation trial prior to the experimental trial. To ensure nutritional status was similar, participants were given a food diary to record dietary intake 24 h prior to experimental trials. For the following trial, participants were instructed to replicate their diet as previously recorded. Participants were also asked to refrain from

exercise, alcohol and caffeine 24 h prior to each trial and during the 24 h following the exercise period. Each participant arrived at the laboratory in the morning before 09:00 after a 12 h fast and began the trial which is outlined in detail in Chapter 2. Immediately before and at 20 min intervals during the preload bout, participants consumed 2 ml·kg^{-1} body mass of an 8% carbohydrate solution or a taste matched placebo in a randomised crossover manner. Substrate oxidation rates were calculated according to Frayn (1983). Following the time trial, blood samples were taken, body mass was recorded and a further 2 ml·kg^{-1} of carbohydrate or placebo was consumed. Laboratory environmental conditions throughout the study were 20.0 ± 1.4°C and 38 ± 6% relative humidity

6.3.3 Blood Plasma Analysis

Blood samples were immediately centrifuged at 3000 rpm for 10 min aspirated and the supernatant was stored at -80°C until the day of analysis. IL-6, sIL-6R, sgp130, cortisol, CRP, glucose and lactate were all analysed from blood plasma - see general methods (Chapter 2).

6.3.4 Statistical Analysis

The data were analysed using the statistical package SPSS v. 16 (SPSS inc., Chicago, IL). To analyse if data were spherical, Mauchly's test of sphericity was used, with a Greenhouse-Geisser correction for any violations. For trial and time analysis, a 2 x 5 (trial x time) repeated measures ANOVA was conducted; post hoc analysis were used with Holme Bonferroni corrections for multiple comparisons with a criterion for significance set at $p < 0.05$. For analysis of other physiological variables

and time trial data, a paired samples t test was used with Holme

Bonferroni corrections applied when appropriate.   All data are expressed

as mean ± SD.

6.4 Results

6.4.1 Physiological Data

During the preload there were no significant differences in mean

exercise intensity, heart rate, respiratory exchange ratio or substrate

oxidation rate between trials. Mean RPE during the preload was lower for

CHO compared to PLA (table 6.2).

Table 6.2 Physiological variables and substrate oxidation during the preload.

	Placebo	Carbohydrate	P value
% $\dot{V}O_{2max}$	73 ± 4	75 ± 3	0.52
Heart rate (b·min^{-1})	151 ± 17	149 ± 15	0.37
RPE	13 ± 1	12 ± 1	0.05 [†]
RER	0.84 ± 0.04	0.83 ± 0.04	0.65
CHO oxidation rate (g·min^{-1})	1.83 ± 0.63	1.85 ± 0.47	0.47
Fat oxidation rate (g·min^{-1})	0.79 ± 0.25	0.84 ± 0.20	0.84

[†] Significantly lower in CHO than PLA (p≤0.05)

6.4.2 Plasma IL-6 and Receptor Responses

Figure 6.1 shows that during PLA, circulating IL-6 increased 9.2

fold from baseline to PL (p<0.01) with a further increase observed PostTT

(p<0.001). IL-6 levels were lower by 1hPost but remained elevated

(p<0.001), and had returned to baseline levels by the following morning.

CHO attenuated the IL-6 response post preload by 49% (p<0.05) which

remained 41% lower at PostTT (p<0.01) and 39% (p<0.01) lower at
1hPost.

Plasma sIL-6R levels were significantly increased by 12.8% in both
trials from baseline to PostTT (p<0.05) and 1hPost (p<0.05) but returned
to baseline values by the following morning. No differences were
observed between trials (p=0.88) (figure 6.2).

The plasma sgp130 response to exercise is shown in figure 6.3. Plasma
sgp130 showed a significant increase at PostTT (9.7%) (p<0.05) which
were maintained at 1hPost (p<0.05) but returned to baseline by 24hPost.
Although sgp130 increased with exercise, no differences were observed
between trials (p=0.35).

Figure 6.1. Effect of CHO supplementation on IL-6 responses.
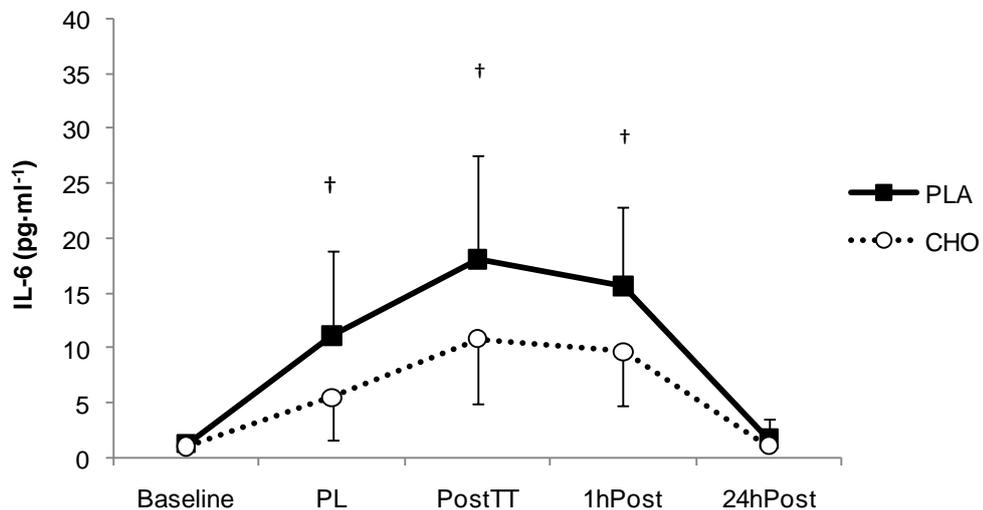
†CHO signifcantly lower than PLA at the corresponding time points
(effect of trial)(p<0.05).

Figure 6.2 Effect of CHO supplementation on sIL-6R responses
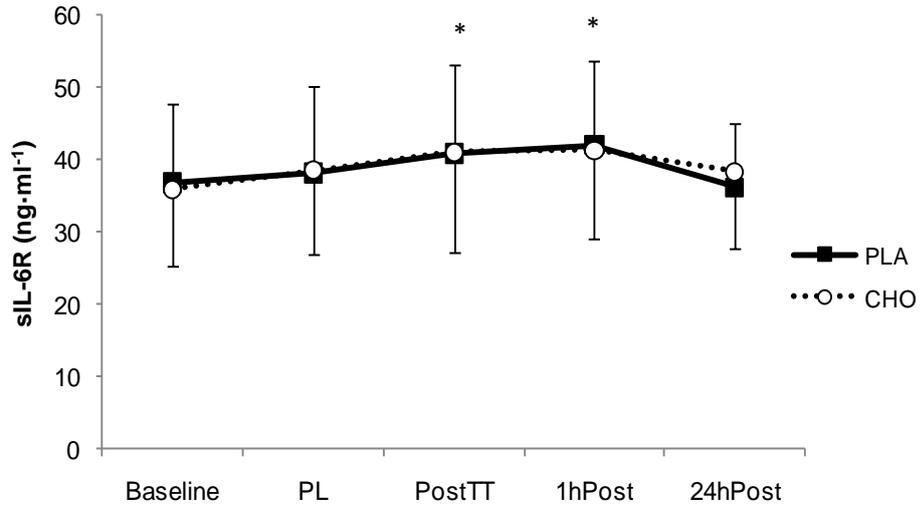
* Significantly higher than baseline (effect of time) (p<0.05)

Figure 6.3 The effect of CHO supplementation on plasma sgp130 response
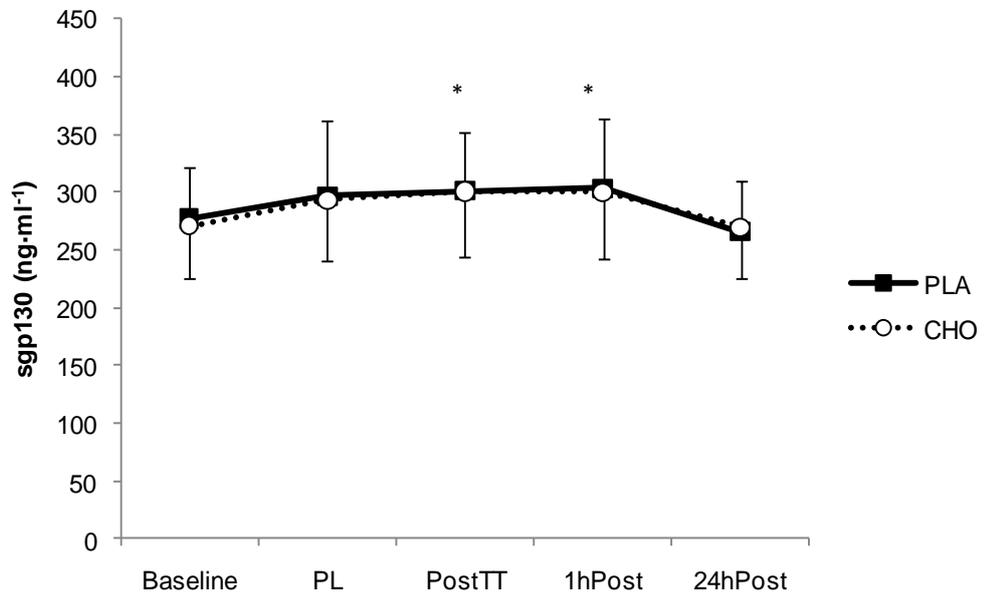
* Significantly higher than baseline (effect of time) (p<0.05)

6.4.3 Time Trial Data

The mean running speed during the 5 km time trial was higher during CHO (13.2 ± 1.9 km·h^{-1}) compared to PLA (12.3 ± 2.2 km·h^{-1}) (p<0.01) (figure 6.4) resulting in a faster completion time (p<0.05) (figure 6.5). During the time trial heart rate was significantly higher in CHO (184 ± 13 b·min^{-1} vs. 174 ± 12 b·min^{-1}) (p<0.05) however there were no differences in RPE (p=1.00).

A significant negative correlation was observed between IL-6 and relative running performance during PLA (r=-0.76 p<0.05) however, the relationship was not maintained during CHO (r= -0.52 p=0.15) (figure 6.6). No correlation was found between the difference in running speed and the difference in IL-6 (r=0.06 p=0.88) when all participants were examined; however, when outliers more than three standard deviation away from the mean are excluded (Healy, 1979; Weiss and Hilgenfeld, 1997), the correlation reaches significance (r=0.92 p<0.01) (figure 6.7).

Figure 6.4 Running speed during the time trial.
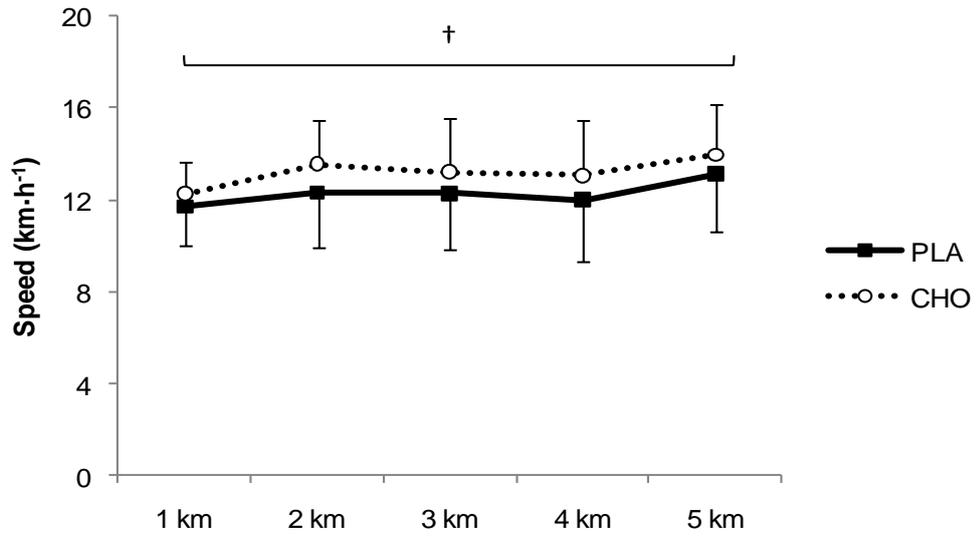
† CHO significantly higher than PLA (effect of trial) (p<0.01)

Figure 6.5 Individual times completed for the time trial
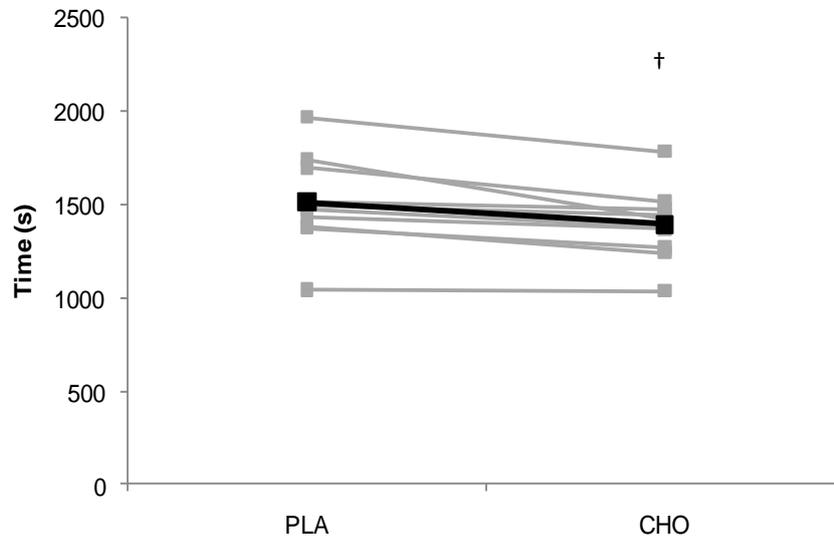
†CHO significantly faster than PLA (effect of trial) (p<0.05)

Figure 6.6 Correlation between circulating IL-6 at PL and running performance during the time trial.
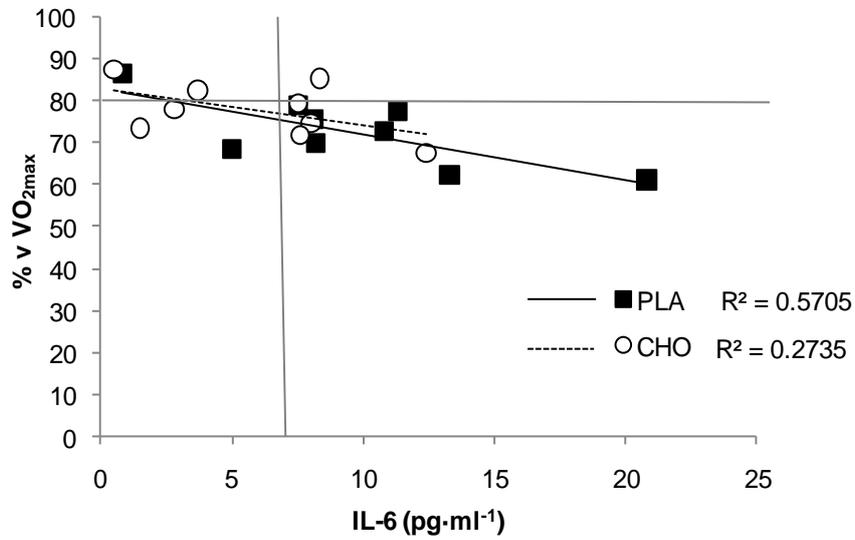
PLA p<0.05; CHO p=0.15

Figure 6.7 Correlation between difference in IL-6 at PL and difference in speed during the time trial
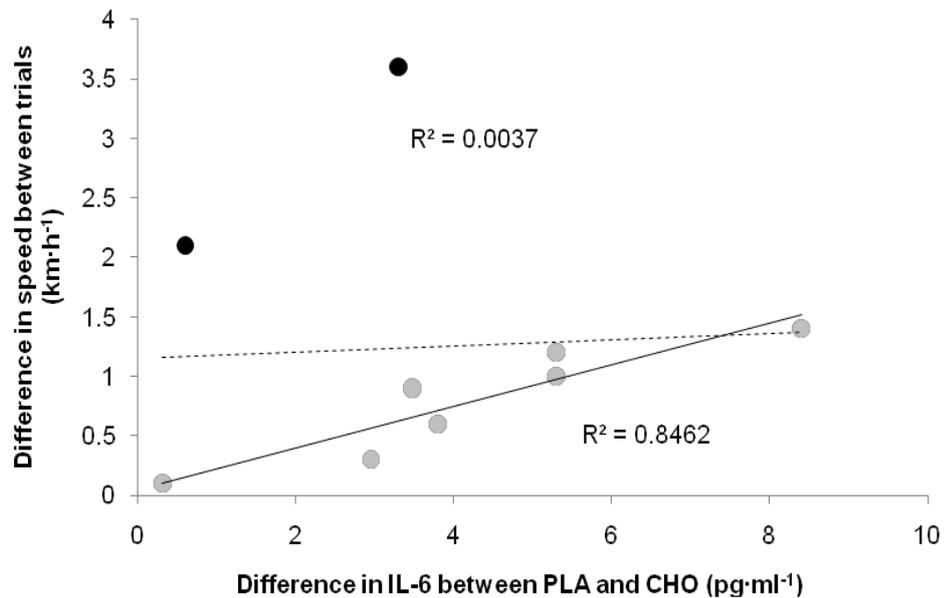
Correlations for all participants (broken line) (p=0.88) and with the excluded outliers who are more than three standard deviation away from the mean (bold line) (p<0.01)

6.4.4 Other Plasma Variables

Table 6.3 demonstrates that plasma glucose remained relatively unchanged throughout the exercise and time trial period during PLA. Plasma glucose increased during CHO and showed a significant difference compared to PLA at PL ($p < 0.01$). Plasma cortisol was not different between trials ($p = 0.14$) but did increase for both trials at PL ($p \leq 0.05$) and showed a further increase at PostTT ($p < 0.01$) which remained elevated at 1hPost ($p < 0.01$). Cortisol levels had returned to baseline levels by the following morning (Table 6.3). C reactive protein (CRP) remained unchanged post time trial compared to baseline ($p = 0.82$) however, a significant increase was observed 24hPost ($p < 0.01$). No significant differences were observed between trials for CRP ($p = 0.17$) (Table 6.3).

Table 6.3 Plasma glucose, cortisol and CRP responses to carbohydrate supplementation.

	Baseline	PL	PostTT	1hPost	24hPost
Glucose (mmol·L^{-1})					
PLA	5.01±1.17	5.14±1.39†	5.29±1.26	4.68±1.02	5.60±1.16
CHO	5.13±0.54	7.02±1.27†	6.20±1.18	5.80±1.93	5.69±0.98
Cortisol (nmol·L^{-1})					
PLA	456±152	801±373*	1212±412*	1047±407*	385±115
CHO	415±251	503±398*	849±519*	783±327*	371±144
CRP (mg·L^{-1})					
PLA	0.5±0.6	-	0.6±0.6	-	3.3±2.1*
CHO	0.3±0.2	-	0.3±0.2	-	2.6±1.8*

* Significantly higher than baseline ($p \leq 0.05$) † Significantly higher in CHO than PLA ($p < 0.01$)

6.5 Discussion

The aims of the present study were to investigate the effect of carbohydrate supplementation on the response of plasma IL-6 and the signalling receptor to exercise and the association with time trial performance. Our findings demonstrate that CHO ingestion reduced the plasma IL-6 response to exercise, which was associated with an improvement in exercise performance during the time trial. CHO supplementation during exercise did not alter the response of the circulating receptors sIL-6R or sgp130.

The finding that CHO intake during prolonged exercise reduces circulating IL-6 is in accordance with previous work (Nehlsen-Cannarella et al., 1997; Starkie et al., 2001; Bishop et al., 2002) suggesting that IL-6 release is related to CHO metabolism during exercise. CHO supplementation has been shown to spare muscle glycogen content during prolonged exercise (Yaspelkis et al., 1993; Tsintzas et al., 1996; Stellingwerf et al., 2007). However, this has not consistently been observed (Coyle et al., 1986; Flynn et al., 1987; Starkie et al., 2001; Febbraio et al., 2003). Furthermore, work from Starkie et al. (2001) and Febbraio et al. (2003) showed that the IL-6 mRNA within contracting skeletal muscle is unaffected by CHO intake during exercise whilst a reduction in IL-6 was seen in the circulation. This indicates that CHO intake suppresses the release of IL-6 into the circulation from skeletal muscle, rather than IL-6 synthesis. It is presently unclear how CHO supplementation reduces circulating IL-6 levels; although, it could be

speculated that CHO ingestion reduces the number of transporters allowing the IL-6 protein to transport from skeletal muscle into the circulation. Nonetheless, inhibiting the release of IL-6 from skeletal muscle attenuates the global actions of IL-6.

6.5.1 *IL-6 as a Mediator between Muscle and Central Regions*

IL-6 can interact between muscle cells and other organs, such as the brain (Banks et al., 1994; Watkins et al., 1995). Since IL-6 in circulation can induce sensations of fatigue (Spaith-Schwalbe et al., 1998) and also impair exercise performance (Robson et al., 2004). We propose that the release of IL-6 into the circulation from skeletal muscle during prolonged exercise acts as a signalling protein to provide feedback on metabolic stress when CHO availability stores are compromised. This may in turn protect the body from energy depletion during exercise. Work from Rauch et al. (2005) demonstrated that reduced CHO availability alters pacing strategy and they speculated that exercising muscle releases a metabolic signal or 'glycostat'. In the present study, seven of the nine participants demonstrated a reduction in circulating IL-6 with CHO ingestion which correlated with an increase in speed during the time trial run. This supports the proposal that an attenuated IL-6 response following CHO intake reduces the central effect whereby attenuating the sensation of fatigue or effort results in an increase in performance.

CHO supplementation during prolonged exercise reduces ratings of perceived exertion (RPE), particularly towards the end of exercise (Burgess et al., 1991; Utter et al., 1999; Utter et al., 2004). In the present

study, ratings of perceived exertion were lower during CHO following the preload bout of exercise which was accompanied with a 49% reduction of circulating IL-6. No differences were observed in RPE during the time trial; however, although perception of effort was the same, the mean speed was higher during the CHO trial. Therefore the circulating IL-6 response may influence perception of effort which impacts on subsequent exercise performance.

The mechanisms regulating IL-6, perception of effort and performance could be the physiological link of IL-6 with serotonergic pathways. Increased serotonin or 5-hydroxytryptophan (5-HT) activity in the brain is known to be associated with depressed mood, lethargy, sleep and appetite regulation (Cooper et al., 2003) and centrally mediated fatigue. Increased 5-HT activity during prolonged exercise can alter sensation of effort leading to a reduction in motor unit recruitment (Davis and Bailey, 1997) and alter exercise performance (Bailey et al., 1993). Intravenous infusion of IL-6 into rats increases concentrations of tryptophan, a precursor of 5-HT synthesis (Wang and Dunn et al., 2001), which increases 5-HT release from particular regions of the rat brain (Zhang et al., 2001). Furthermore, an increase in 5-HT following IL-6 infusion is concurrent with a reduction in dopamine (Song et al., 1999), a neurotransmitter linked to increased motivation and arousal (Davis et al., 2000). Robson et al. (2004) demonstrated that infusion of IL-6 into well trained athletes potentiated the release of prolactin, a marker of serotonergic activity (Chandler and Blair, 1980), and impaired exercise

performance. Therefore, although we did not measure any markers of central activity, it could be possible in the present study that attenuating peripherally produced IL-6 inhibits centrally mediated effects by reducing serotonergic activity and the associated sensations of fatigue and consequently, preserves performance.

6.5.2 Signalling Receptors

An increase in circulating sIL-6R levels post time trial and 1 h following the time trial was unaltered by CHO intake. This indicates that sIL-6R lacks a direct role in CHO metabolism during exercise. These findings are in accordance with Leggate and colleagues (2010) who found that an increase in plasma sIL-6R in response to exercise is not intensity related which lends support to the notion that sIL-6R does not play a direct role in CHO metabolism during exercise.

Circulating sgp130 increased post time trial however there were no differences between trials indicating that circulating sgp130 is not related to CHO metabolism during exercise, therefore, the mechanisms for sgp130 production and release into the circulation in response to exercise remain unclear. sgp130 has been previously reported as an antagonist to IL-6/sIL-6R signalling since sgp130 can bind to the IL-6/sIL-6R complex, presenting a molar excess which is biologically inactive (Jostock et al., 2001). It is possible that the increase in sgp130 regulates the actions of sIL-6R signalling since an increase in sIL-6R is accompanied by an increase in sgp130 (Rebouissou et al., 1998).

6.5.3 Other Systemic Markers

Plasma cortisol significantly increased during exercise but was not significantly different between trials, which supports previous work (Tsintzas et al., 1996; Bishop et al., 2001). Prolonged exercise has been shown to consistently increase CRH (Tabata et al., 1991; Inder et al., 1998) leading to an increase in ACTH (Fraioli et al., 1980; Inder et al., 1998) subsequently raising glucocorticoid concentrations such as cortisol (Few, 1974; Kuoppasalmi et al., 1980; Kindermann et al., 1982; Utter et al., 1999) when exercise is at an intensity >60% $\dot{V}O_{2max}$ (McCarthy and Dale, 1988). Although the majority of research has shown a reduced response of plasma cortisol following CHO supplementation during exercise (Murray et al., 1991; Utter et al., 1999; Bishop et al., 2002; Chen et al., 2009), our data did not show any significant effect (p=0.14). Therefore, it is likely that the large inter-individual variation and low subject number are the basis of the lack of statistical significance required.

Circulating CRP significantly increased the following morning but was not significantly different between trials. An increase in circulating CRP 16 h post exercise has been previously shown (Castell et al., 1996; Robson-Ansley et al., 2009) with peak concentrations occurring at 24 h post exercise (Strachan et al., 1984). Our observation that plasma CRP is not altered by CHO intake is in accordance with previous work (Scharhag et al., 2006); indicating that attenuation of IL-6 does not translate to a reduced CRP response. Since plasma IL-6 was blunted by 43% across all

the time points, this suggests that during exercise, plasma IL-6 is not the sole stimulator of CRP.

In conclusion, the results from the study show that CHO supplementation during prolonged exercise can improve time trial performance during prolonged exercise. The findings suggest that the CHO-related atteunuation of circulating IL-6 during exercise may be another mechanism to explain the previously reported ergogenic effect of CHO ingestion during exercise. The improvement in performance does not appear to be related to the IL-6 signalling receptors.

Chapter 7

Part II. The Effect of Carbohydrate Supplementation on the Plasma Hepcidin Response

7.1 Abstract

Hepcidin is an antimicrobial peptide which regulates circulating iron to control iron homeostasis. Previous research has shown that cytokines play a role in the release of hepcidin. In particular, IL-6 is a key mediator, which can induce hepcidin during inflammatory states and during exercise. Previously, we have shown that CHO ingestion during prolonged exercise attenuates the circulating IL-6 response. However it is unknown what impact this has on circulating hepcidin and iron levels. Therefore, the purpose of this study was to investigate the impact of attenuated plasma IL-6 on circulating hepcidin and iron responses. The samples taken for chapter 6 were analysed for plasma hepcidin, iron and ferritin. Results found that hepcidin increased in response to exercise. However, although CHO supplementation attenuated plasma IL-6, no differences were observed for plasma hepcidin, iron or ferritin. Therefore, in conclusion, we have shown that plasma hepcidin increases in a similar fashion to IL-6 during prolonged exercise; however, the hepcidin response was not blunted by CHO ingestion.

7.2 Introduction

Iron deficiency is a common disorder in both trained and untrained populations and is particularly prevalent among athletes who participate in endurance type events (Looker et al., 1997; Schumacker et al., 2002). Iron is essential for oxygen delivery and neurotransmitter synthesis, therefore a deficiency in this mineral may impair athletic performance. Individuals with iron deficiency or anaemia have a lower $\dot{V}O_{2max,}$ and lower endurance capacity (Haas and Brownlie, 2001; Hinton et al., 2000) which can result in the early onset of fatigue and reduce exercise performance. Causes of exercise associated iron deficiency are multifaceted and include damage to red blood cells as a result of vigorous muscle contraction and foot strike during running (Telford et al., 2003), however, the peptide, hepcidin may play a key role in the regulation of iron metabolism during exercise (Nemeth et al., 2003).

Hepcidin is an antimicrobial peptide which plays a role in host defence against invading pathogens (Krause et al., 2000; Park et al., 2001) which is predominantly produced and released from hepatocytes (Park et al., 2001) regulating circulating iron to control iron homeostasis (Nicolas et al., 2002, Nemeth et al., 2003; Nemeth et al., 2004; Rivera et al., 2005). In the murine model, over expression of hepcidin leads to anaemia (Nicolas et al., 2002) and mice that are infused with hepcidin can display an 80% reduction of serum iron (Rivera et al., 2005). In humans, increased levels of hepcidin are subsequently followed by a delayed decrease in serum iron levels (Kemna et al., 2005).

Research indicates that cytokines play a role in the hepatic release of hepcidin both *in vitro* and *in vivo* (Nemeth et al., 2003; Nemeth et al., 2004; Kemna et al., 2005). When IL-6 antibodies are added to cell culture, LPS induced hepcidin is blocked (Nemeth et al., 2004). Infusion of LPS into humans also increases urinary hepcidin concentrations which are mediated by an increase in circulating IL-6 (Kemna et al., 2005). Furthermore, this effect is also observed when humans are infused with rhIL-6 (Nemeth et al., 2004), indicating that IL-6 is both a key mediator in the inflammatory induced hepcidin response and can also induce hepcidin *per se.*

Prolonged exercise can stimulate an increase in circulating IL-6 from skeletal muscle, particularly when muscle glycogen levels are low (Steensberg et al., 2000, Keller et al., 2001). Since IL-6 can play a key role in the release of hepcidin from hepatocytes, it can be assumed that the exercise induced IL-6 can initiate a hepcidin response. Research from Roecker et al. (2005) established from female athletes that after a marathon, increases in urinary hepcidin occur immediately post race with peak hepcidin concentrations occurring the following day. Later work by Peeling et al. (2009) demonstrated that an increase in circulating IL-6 following exercise preceded an increase in urinary hepcidin.

Taken together, the literature suggests that an association between plasma IL-6 and hepcidin concentrations exist which can subsequently reduce plasma iron concentrations. Therefore we hypothesised that a reduced plasma IL-6 response to exercise following CHO intake will

reduce the hepcidin response and subsequently maintain plasma iron levels the following morning. Accordingly, the aim of this study was to examine the effect of CHO supplementation during prolonged exercise on the plasma response of hepcidin and its subsequent effect on plasma iron and ferritin concentrations.

7.3 Methods

7.3.1 Participants

Nine trained male runners took part in the study, of which the participant characteristics and methods are outlined in chapter 6. All participants were free from any symptoms of infection in the 4 weeks prior to the study and gave written informed consent to the study which was given approval by the Northumbria University school ethics committee.

7.3.2 Blood Analysis

Blood samples were immediately centrifuged at 3000 rpm for 10 min aspirated and the supernatant was stored at -80°C until the day of analysis. IL-6 was analysed from blood plasma which is outlined in detail in chapter 2.

7.3.3 Hepcidin Analysis

Plasma hepcidin was analysed from K_3EDTA treated tubes and was measured by mass spectrometry incorporating stable isotope labelled synthetic hepcidin as an internal standard as described previously (Ward et al., 2008). Briefly, 100 ng labelled hepcidin was added per ml of plasma. The spiked plasma was then diluted 5-fold in 8 M urea, 1%

CHAPS, 20 mM ammonium bicarbonate and incubated at room temperature for 30 min. Following a 10-fold dilution in 20 mM ammonium bicarbonate, the samples were applied to Cu^{2+} loaded ProteinChip Arrays. After a 60 minute incubation the arrays were washed with 20 mM ammonium bicarbonate, rinsed with distilled water, dried and the samples overlayed with 1 µl saturated sinapinic acid in 50% acetonitrile, 0.5% trifluoroacetic acid. Mass spectra were acquired on a PBS IIc time-of-flight analyser (Ciphergen) and the hepcidin concentration in the plasma calculated from the intensity ratio of the endogenous and spiked hepcidin peaks (m/z 2790 and 2800 respectively). The hepcidin in each plasma sample was measured in duplicate using independent technical replicates.

7.3.4 Ferritin Analysis

Serum ferritin was measured in duplicate on the Adiva Centaur which is comprised of a two site sandwich immunoassay using two anti-ferritin antibodies. The first antibody is a polyclonal goat antibody against ferritin, labelled with acridinium ester. The second antibody is a monoclonal mouse antibody against ferritin that is covalently coupled to paramagnetic particles. The samples were incubated, exposed to a magnetic field and then washed. Hydrogen peroxide was then added and the RLU was then measured in the luminometer.

7.3.5 Iron Analysis

Serum iron was measured using the Olympus method. TPTZ (2,4,6-Tri(2-pyridyl)-5-triazine) was used as the chromogen in an acidic medium.

Hydrochloric acid and sodium ascorbate was added and was
bichromatically measured at 590 nm.

7.3.6 Statistical Analysis

The data were analysed using the statistical package SPSS v. 16
(SPSS inc., Chicago, IL). To analyse if data were spherical, Mauchly's
test of sphericity was used, with a Greenhouse-Geisser correction for any
violations. For trial and time analysis, a 2x5 (trial x time) repeated
measures ANOVA was conducted; post hoc analysis were used with
Holme Bonferroni corrections for multiple comparisons with a criterion for
significance set at $p<0.05$. For analysis of other physiological variables
and time trial data, a paired samples t test was used with Holme
Bonferroni corrections used when appropriate.   All data are expressed as
mean ± SD.

7.4 Results

7.4.1 Physiological and Time Trial Data

During the preload there were no significant differences for mean
exercise intensity, heart rate or respiratory exchange ratio between trials.

Table 7.1 Physiological variables and substrate oxidation during the preload

	Placebo	Carbohydrate	P value
% $\dot{V}O_{2max}$	73 ± 4	75 ± 3	0.52
Heart rate (b·min^{-1})	151 ± 17	149 ± 15	0.37
RER	0.84 ± 0.04	0.83 ± 0.04	0.65

7.4.2 Plasma Variables

Circulating IL-6 increased 9.2 fold from pre exercise to PL (p<0.01) during PLA with a further increase observed PostTT (p<0.001). IL-6 levels were reduced 1hPost but remained elevated (p<0.001) and had returned to baseline levels the following morning. CHO attenuated the IL-6 response post exercise by 49% (p<0.05) which was also 41% lower PostTT (p<0.01) and 39% (p<0.01) lower 1hPost (Chapter 6).

Plasma hepcidin showed a significant increase from Baseline to PL (p<0.01) which remained elevated until 1hPost (p<0.05) then returned to baseline values 24hPost. Although a significant increase in plasma hepcidin was observed, no significant differences were seen between PLA and CHO (p=0.45) (figure 7.1)

Figure 7.1. Effect of CHO supplementation on plasma hepcidin
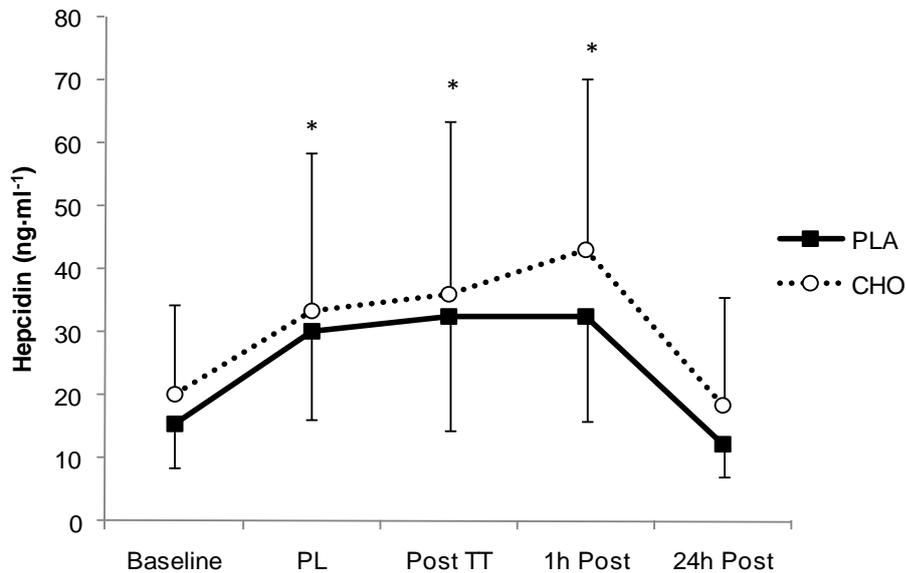
*Significantly higher than Baseline (effect of time) (p<0.05)

Plasma iron increased from Baseline to PL (p<0.05), however, plasma levels remained reletively constant until 1hPost and was significantly reduced at 24hPost (p<0.05) compared to other time points (figure 7.2). No significant differences were observed between trials for plasma iron.

Figure 7.2. Effect of CHO supplementation on plasma iron
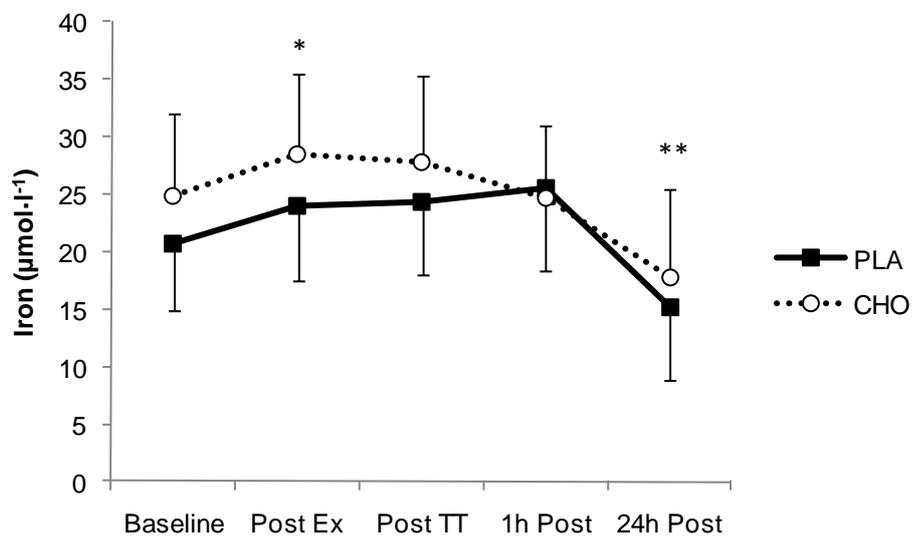
*Significantly higher than baseline (p<0.05) **Significantly lower than any other time point (effect of time) (p<0.05)

Plasma ferritin remained relatively constant throughout the trial. There was no effect of time (p=0.61) and furthermore, there were no differences between PLA and CHO (p=0.91) (figure 7.3).

Figure 7.3. Effect of CHO supplementation on plasma ferritin
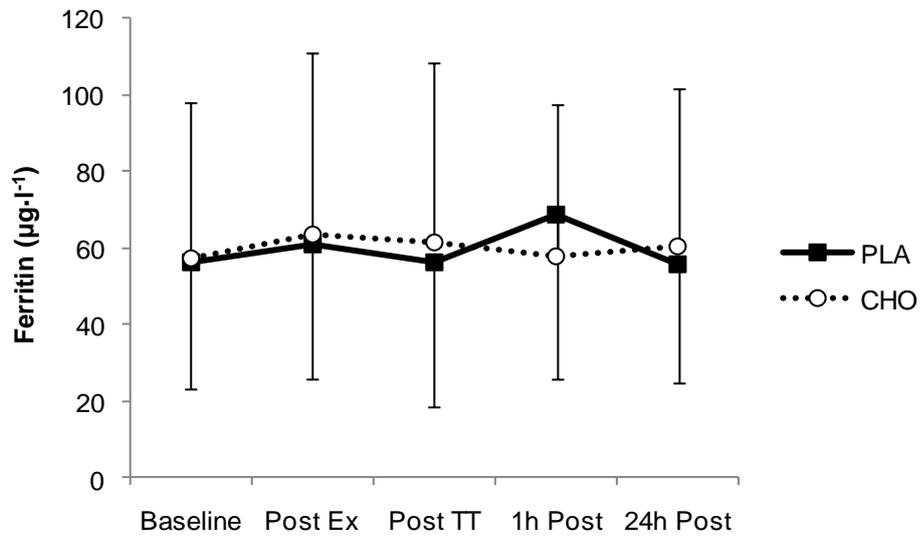
During PLA plasma hepcidin showed a good correlation with plasma IL-6 (r=0.76, p<0.001) however, during CHO a lower correlation was observed (r=0.33, p<0.02) (figure 7.4 and 7.5).

Figure 7.4. Correlation betweeen hepcidin and IL-6 during PLA
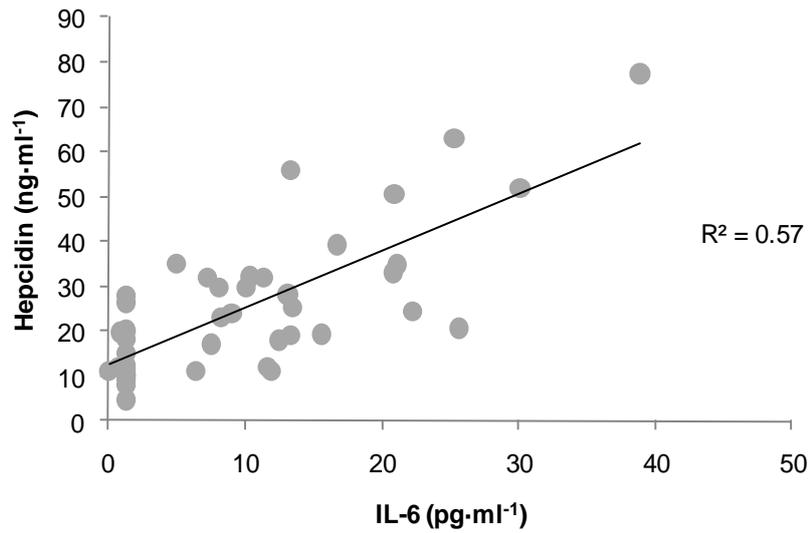
Significant correlation between plasma hepcidin and plasma IL-6 during PLA (p<0.001)

Figure 7.5. Correlation between hepcidin and IL-6 during CHO
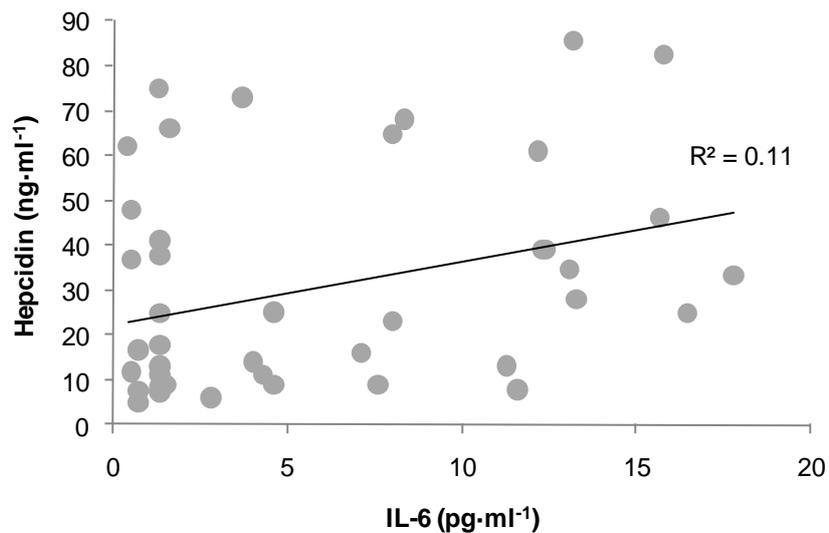
Correlation between plasma hepcidin and plasma IL-6 during CHO (p<0.02)

7.5 Discussion

The aim of the present study was to examine the effect of the attenuated IL-6 response to exercise from CHO supplementation on the subsequent response of plasma hepcidin, iron and ferritin. An increase in plasma IL-6 correlated well with an increase in plasma hepcidin which led to a subsequent decrease in plasma iron the following morning during the placebo; however, the reduced plasma IL-6 response from CHO intake did not result in an attenuated response of plasma hepcidin which subsequently, did not alter the plasma iron or ferritin response to exercise.

In agreement with other studies, we found that plasma IL-6 and hepcidin increased in response to exercise during PLA. Furthermore, a strong correlation was observed between the two circulating peptides. Previous work has demonstrated increases in plasma IL-6 and urinary hepcidin (Peeling et al., 2009a; Peeling et al., 2009b) which supports the concept that exercise induced hepcidin is linked to the plasma IL-6 response to prolonged exercise.

Although we have illustrated a strong correlation between plasma IL-6 and plasma hepcidin, this relationship is weakened when CHO is consumed during exercise. On this basis we speculate that circulating IL-6 is not solely responsible for the plasma hepcidin response to prolonged exercise, and other mechanisms are involved in the increase in circulating hepcidin. Work by Wrighting and Andrews (2006) demonstrated that IL-6 induces hepcidin through the STAT3 signalling pathway and reported that STAT3 is necessary for increased hepcidin expression. This was later

confirmed by Pietrangelo et al. (2007) who illustrated that IL-6 did not increase hepcidin expression in hepatocytes in STAT3 deficient mice. Since IL-6 activates the JAK/STAT signalling pathway (Heinrich et al., 2003), this provides a rationale for the association between IL-6 and hepcidin levels; however other peptides can stimulate the JAK/STAT signalling pathway which results in an increase in hepcidin concentrations.

Hepcidin transcription in macrophages and hepatocytes occurs in IL-6 deficient mice, indicating that hepcidin expression occurs through both IL-6 dependent and independent pathways (Lee et al., 2005). It can therefore be considered that the increase in exercise induced hepcidin is due to the activation of STAT3. STAT3 has been shown to have multiple stimulators including cytokines, growth factors amongst other stimuli (Campbell et al., 1995; Heinrich et al., 1998; Zong et al., 2000). Furthermore, prolonged exercise is associated with a multitude of hormonal, immune and biochemical changes which can potentially activate STAT3 signalling. For instance, growth hormone (GH) and insulin growth factor I (IGF-I) have been shown to increase with exercise (Schwarz et al., 1996; Kraemer et al., 2004) both of which can activate STAT3 (Campbell et al., 1995; Zong et al., 2000). Therefore, it is possible that another exercise induced peptide is involved with hepcidin release through activation of STAT3.

The finding that plasma iron levels were reduced 24 h following exercise is in accordance with previous work (Pattini et al., 1990) but is in contrast with others (Peeling et al., 2009a; Peeling et al., 2009b). The

studies by Peeling and colleagues involved an exercise bout of shorter duration than that of the present study resulting in a lower hepcidin response which may have had no impact on circulating iron concentrations. A number of studies have reported that hepcidin is responsible for a reduction in serum iron (Nemeth et al., 2004; Ramey et al., 2010; Kemna et al., 2005); caused by the actions of ferroportin (Ramey et al., 2010). Ferroportin is responsible for the export of iron and acts to regulate the release of iron into the circulation, and is situated on the surface of many cells such as intestinal enterocytes, macrophages and hepatocytes. Hepcidin binds to ferroportin causing it to internalise and degrade (Nemeth et al., 2004). This results in iron being retained within the cell and subsequently leads to a decrease in circulating free iron levels. These events counter regulate iron overload but also play a role in host defence against invading pathogens. Depriving pathogenic bacterium of iron can impair growth of the bacterium since an increase in iron is required with increased production of bacteria (Abdul-Tehrani et al., 1999). However, the stored form of iron, ferritin was not altered by exercise or by CHO intake which provides further support of this, indicating that plasma hepcidin alters plasma free iron concentrations, whereas the stored form of iron is not altered.

Since the attenuated response of IL-6 from CHO intake did not alter the plasma iron response compared to a placebo, this highlights the strong relationship between serum iron and hepcidin. Although CHO intake during prolonged exercise did not reduce the plasma hepcidin

response, further research is warranted to determine if other interventions could influence hepcidin concentrations to help maintain iron levels during the post exercise recovery period.

In conclusion, we have shown that plasma hepcidin increases in a similar fashion to IL-6 during prolonged exercise; however, unlike IL-6, the hepcidin response was not blunted by CHO ingestion. This indicates that exercise induced IL-6 is not the predominant cause of the increase in plasma hepcidin and is due to other mediators such as STAT3. Attenuation of hepcidin by other supplements or interventions is warranted in order to prevent iron deficiency occurring which is commonly reported in endurance athletes.

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Chapter 8

General Discussion

The primary aim of the thesis was to examine the influence of the systemic response of IL-6 to exercise on subsequent time trial performance using a preloaded time trial protocol. Findings from chapter 3 initially determined the magnitude of the plasma IL-6 response to the preloaded time trial protocol, of which, we have quantified the variation. The findings also show that a negative correlation occurs between the response of plasma IL-6 to the preload bout and the subsequent performance during the time trial. Consequently, it was hypothesised that manipulation of the IL-6 response to the preload using nutritional interventions would clarify the influence of circulating IL-6 on subsequent time trial performance.

Glutamine supplementation during prolonged exercise does not impact on the circulating IL-6 response to the preload and furthermore, time to complete the time trial is not altered by glutamine supplementation (Chapter 4). In addition, altered dietary CHO intake prior to the protocol does not alter the mean plasma IL-6 response (Chapter 5). Conversely, CHO intake during a preload bout of exercise attenuates the plasma IL-6 response by 49% which is also accompanied by an 8% improvement in performance in the subsequent time trial (Chapter 6). However, the reduction in plasma IL-6 did not translate to a reduction in plasma hepcidin (Chapter 7).

Regardless of the nutritional intervention employed, a consistent finding in the series of studies is that the systemic IL-6 response to the preload bout negatively correlates with relative exercise performance

during the time trial. This indicates that the magnitude of the IL-6 response to the preload bout is associated with performance when expressed in relative terms. Furthermore, the correlations demonstrate that a low IL-6 response to the preload bout of exercise (<7 pg·ml^{-1}) is often associated with a higher fractional aerobic utilisation during the time trial (>80% $v\dot{V}O_{2max}$) indicating a relationship between circulating IL-6 levels and exercise performance. Although these findings show an association between plasma IL-6 responses and exercise performance, the results do not specify causality. Therefore, assessing the manipulated plasma IL-6 response to exercise, and the subsequent influence on time-trial performance can give more indication of the role of circulating IL-6 in exercise.

8.1 *Nutritional Manipulation of the IL-6 Response*

It has been previously established that CHO supplementation during exercise improves performance; however mechanisms responsible for this improvement are unclear (Coyle and Coggan, 1991; Stellingwerff et al., 2007; Rollo et al., 2010). Our findings from chapter 6 show that CHO intake during exercise reduces the circulating IL-6 response. We speculate that this may be one of several potential mechanisms by which CHO supplementation can improve performance.

The level of circulating IL-6 has been suggested to be a marker of altered homeostasis during prolonged exercise and intensive training periods (Ostrowski et al., 1998; Robson-Ansley et al., 2007; Jurimae et al., 2011). Since IL-6 can cross the blood brain barrier (Banks et al., 1994), it

is speculated that IL-6 acts as a 'distress' signal, indicating peripheral cellular stress relating to increased ROS activity and also metabolic stress relating to substrate availability. Increases in circulating IL-6 induced by the preload bout of exercise may therefore increase signalling to regions of the brain, influencing pacing in the subsequent time trial in order to conserve energy stores. In contrast, the attenuation of the plasma IL-6 response with CHO intake could lower the distress signal and reduce the sensations of fatigue, which could translate to a potential improvement in speed during the time trial. The findings of this thesis may lend support to the previous hypothesis that feedback from the peripheries can influence efferent command, taking into account metabolic status, in order to complete a physical task (Ulmer, 1996; Lambert et al., 2005).

Findings from chapter 4 demonstrate that glutamine supplementation does not alter the circulating IL-6 response to exercise. However, there was a significant increase in circulating glucose levels. Since circulating IL-6 was not influenced by an increase in circulating glucose levels *per se*, this highlights the fact that the synthesis and release of IL-6 is influenced by other forms of CHO availability such as muscle glycogen (Steensberg et al., 2000; Keller et al., 2001). As a result of prolonged exercise, critically low muscle glycogen levels can reduce exercise performance; and it has been reported that cyclists pace themselves according to some form of metabolic signal or 'glycostat' related to muscle glycogen concentrations (Rauch et al., 2005). Therefore, in accordance with this previous work, we propose that low

muscle glycogen can instigate a release of IL-6 into the circulation which can alter the sensation of fatigue and influence pacing strategy. It is possible that IL-6 exerts its effects through a number of different pathways including neurotransmitter activity, the neural circuitry level or exerting its effects at the subconscious level.

Chapter 5 demonstrates that altering dietary CHO intake prior to exercise in order to manipulate muscle glycogen levels did not alter the mean IL-6 response to exercise. This may indicate that trained runners have sufficient CHO availability to complete a protocol of this nature, therefore, the requirement of IL-6 as a mediator of glucose production is not altered. Indeed, it is possible that CHO supplementation during rather than prior to exercise has a more pronounced effect on IL-6 concentrations. Nonetheless, in accordance with results from previous chapters, the plasma IL-6 response showed a strong negative correlation with relative exercise performance during the time trial, indicating that a large circulating IL-6 response is associated with a noticeable decrement in performance.

8.2 *Threshold Analysis of IL-6 Mediated Performance*

A consistent finding in this series of studies is that regardless of the intervention employed, the systemic IL-6 response to the preload bout negatively correlates with relative exercise performance during the time trial. However, it is noticeable that performance does not decline in a strict dose dependant manner with increasing IL-6 concentration, since the error from the correlation trendline increases with the increases in the IL-6

response (figures 3.5, 4.5, 5.6a and 6.6). In light of this, we propose that

a threshold may exist where a plasma IL-6 response above this threshold

may indicate substantial cellular stress and/or limited CHO availability,

subsequently impacting on performance, although the performance

decrement varies between individuals. In contrast, a response below this

threshold has minimal, if any, impact on exercise performance.

The reasons for the individual differences in performance to

elevated IL-6 concentrations are unclear. An obvious explanation is

differences in training status. An interaction between the circulating IL-6

response to exercise and endurance training has previously been

established (Fischer et al., 2004; Gokhale et al., 2007). Endurance

training can lead to improvements in exercise metabolism, including

spared muscle glycogen during exercise and increased muscle glycogen

storage capacity (Gollnick et al., 1973; Henriksson and Reitman, 1976;

Hurley et al., 1986), which can reduce and/or delay the plasma IL-6

response to exercise. In accordance with this, we have found that there

are negative correlations between markers of training status (habitual

weekly running distance and $\dot{V}O_{2max}$), the IL-6 response to exercise and

time to complete the 5 km time trial.

On this basis, it is difficult to determine whether circulating IL-6

directly impacts on performance or if endurance training improves

performance and that a reduced IL-6 response is a consequence of

training. However, when time trial performance is expressed relative to

velocity at $\dot{V}O_{2max}$, there is still an inverse relationship between IL-6 and

performance but training status does not consistently correlate with performance. Not only does this provide further support that the IL-6 response to the preload influences subsequent performance; but this also indicates that this occurrence is independent of habitual running distance and $\dot{V}O_{2max.}$ Consequently, since training status is not solely responsible for the IL-6 mediated decrement in performance, the mechanisms for individual differences of the IL-6 response remain unclear and other factors must play a role. Indeed, fiber type composition will play a role in exercise metabolism which may influence the IL-6 response to exercise. Furthermore, it has been previously shown that genetic predisposition influences the IL-6 response to an endotoxemia challenge (Fishman et al., 1998) and that some individuals are predisposed to produce greater levels of IL-6 (Bonafe et al., 2001). Therefore, to decipher the differential response of plasma IL-6 to prolonged exercise, further work must be directed not only towards phenotype characteristics, but also genotypes of the IL-6 gene polymorphisms which may impact on the IL-6 response.

8.3 *Signalling Receptors*

The soluble signalling receptor of IL-6, sIL-6R, can bind to IL-6 to form an IL-6/sIL-6R complex which increases the half life of IL-6 (Gerhartz et al., 1994) and also increases its potency (Marz et al., 1999). However, to regulate excessive signalling, the soluble trans-signalling receptor of the complex, sgp130 can also bind to the complex leading to inactivity due to a molar excess (Jostock et al., 2001). During exercise, increases in the circulating receptors have been observed; however, a consistent finding of

this thesis was that nutritional interventions did not alter the systemic sIL-6R or sgp130 response to exercise. Although glutamine supplementation and CHO loading protocols did not alter the circulating IL-6 response, CHO supplementation blunted the IL-6 response by 49% whereas the signalling receptors were unaffected. Collectively this indicates that the responses of IL-6 signalling receptors are not responsive to metabolic changes associated with CHO oxidation during prolonged exercise. In addition, Leggate et al. (2010) have demonstrated that the plasma response of sIL-6R is not related to differences in exercise intensity when matched for total exercise load.

sIL-6R and sgp130 are also not related to relative or absolute exercise performance. Additionally, markers of training status such as habitual weekly running distance and $\dot{V}O_{2max}$ are not associated with resting levels or the exercise induced response of sIL-6R or sgp130. These findings are in contrast to those reported for skeletal muscle where the density of IL-6R locally is increased in response to exercise training (Keller et al., 2005; Akerstrom et al., 2009). It is possible that within skeletal muscle, the increase in IL-6R may have a sensitising effect on IL-6 following endurance training which could reduce the circulating response of IL-6 to exercise.

IL-6R is predominantly located on the cell surface within skeletal muscle (Keller et al., 2005), liver cells (Jones et al., 1999) and leukocytes (Jones et al., 2005). However during prolonged exercise, it is unknown which cells are the predominant source of circulating sIL-6R, making it

difficult to fully ascertain the role of sIL-6R during exercise. In addition, it is unknown what impact circulating sIL-6R has on tissue specific functions. However, the observation that circulating sIL-6R is unresponsive to manipulation of CHO metabolism, and is not influenced by exercise intensity (Leggate et al., 2010) may suggest that the increased circulating sIL-6R is not due to metabolic stress. A physical task such as exercise can induce many biochemical changes, and although circulating sIL-6R does not increase in response to infusion of rhIL-6 (Robson-Ansley et al., 2006) it is possible that circulating sIL-6R is upregulated by some other signalling factor to increase the potency of IL-6 which can respond to a diverse range of stress signals.

Leggate et al. (2010) have shown that the IL-6/sIL-6R complex is upregulated by two fold in circulation during exercise. However, the assay procedure used in the present series of studies was unable to differentiate between complex bound and free forms of IL-6 and sIL-6R. Therefore is it difficult to determine whether an upregulation of the complex occurred during exercise. From this, we do not know whether the attenuation of circulating IL-6 following CHO supplementation downregulates IL-6 and sIL-6R binding to form a complex, or indeed if the IL-6/sIL-6R is upregulated as a compensatory mechanism.

8.4 *Other Systemic Markers*

We have demonstrated that circulating CRP is consistently elevated the morning following the preloaded time trial (Chapter 3). However, no significant differences were observed in plasma CRP

between trials following either glutamine supplementation (Chapter 4) or manipulating dietary CHO prior to exercise (Chapter 5) or manipulating CHO intake during exercise (Chapter 6). This could be attributed to the similar responses that were observed in systemic IL-6 since it has been previously been shown that IL-6 can stimulate CRP production following exercise (Strachen et al., 1984; Scharhag et al., 2006). However, the observation that a reduced IL-6 response following CHO intake did not attenuate CRP production (Chapter 6) suggests that the exercise-induced CRP is not totally dependent on circulating IL-6; and that some other mechanism stimulates CRP production and release.

IL-1β, TNFα and IFNγ have all shown to be stimulators of CRP (Heinrich et al., 1990); however, it is unlikely that these pro-inflammatory cytokines are responsible for the increase in CRP in this series of studies since prolonged exercise produces a minimal response from pro-inflammatory cytokines (Ostrowski et al., 1999). It is also possible that that the exercise induced elevations in IL-6 may initiate CRP release, however, this may not be a dose dependent relationship and may be regulated on an individual basis which explains large inter-individual variances seen in the present series of studies.

The acute phase protein, hepcidin, has recently been proposed as a regulator of iron stores that may be involved in exercise-induced iron deficiency or anaemia (Peeling et al., 2008, Peeling et al., 2009). Since IL-6 has been shown to induce circulating hepcidin levels (Nemeth et al., 2004; Kemna et al., 2005), it seems plausible that a reduced IL-6

response to exercise would dampen the response of systemic hepcidin. However, in chapter 7 we demonstrated that the attenuated IL-6 response from CHO supplementation did not alter the hepcidin response. Since the plasma hepcidin response did not alter with CHO supplementation, it could be hypothesised that the exercise-induced plasma hepcidin response is related to some other signalling factor. For IL-6 signalling to occur, the JAK/STAT signalling pathway is employed through STAT1 and STAT3 (Gerhartz et al., 1996). It has been previously been shown that STAT3 is necessary for increased hepcidin expression (Wrighting and Andrews, 2006; Pietrangelo et al., 2007). Therefore the association between increased systemic IL-6 and hepcidin concentrations seen during the placebo trial could be attributed to activation of STAT3 signalling. However, STAT3 has been shown to have other stimulators such as growth factors (Campbell et al., 1995; Heinrich et al., 1998; Zong et al., 2000), which may increase in response to prolonged exercise and that are unresponsive to CHO intake. It is therefore possible that exercise-induced STAT3 expression increased hepcidin concentrations and not the IL-6 *per se.*

Collectively, these findings show that although the exercise-induced increases in circulating IL-6, CRP and hepcidin are observed; the circulating CRP and hepcidin concentrations are unaltered by manipulating circulating IL-6 levels. Therefore their release is not due to IL-6 *per se* but possibly other signalling factors.

8.5 *Conclusions*

In the present thesis we have demonstrated that the plasma IL-6 and signalling receptors display a repeatable response to a preloaded time trial protocol, of which, the increase in IL-6 concentration was attenuated by CHO intake during exercise. In addition, the magnitude of the reduced plasma IL-6 response following CHO intake is related to an improvement in running speed during the time trial. However, the attenuation of the plasma IL-6 response does not translate to a reduction of the circulating hepcidin response to exercise. Our findings also show that the IL-6 response to exercise is not altered by dietary CHO intake prior to exercise or by glutamine supplementation during exercise; but despite the nutritional intervention employed, the findings consistently show that a lower circulating IL-6 response to the preload is associated with improved relative exercise performance during the time trial, which is independent of training status.

The findings from the thesis highlight the complexity of IL-6 in the circulation and add further awareness of IL-6 as a stress marker. The magnitude of the IL-6 response to the preload bout can act as a marker to indicate an athlete's stress response to exercise and can also explain the differences observed in subsequent exercise performance. The improvement in performance following attenuation of the IL-6 response with CHO supplementation is in accordance with the literature of IL-6 as a fatigue inducing protein. Therefore, during competition of long duration, athletes should consume CHO products in order to minimise the IL-6

stress response to optimise performance. However, IL-6 has been previously shown in the literature to be a key mediator in the adaptation responses to exercise training and metabolism. Therefore, in light of the present findings, our results support the model of 'train low, compete high' where athletes should incorporate consuming low carbohydrates during particular training sessions to maximise training adaptations. However during competition, high carbohydrates should be consumed to minimise stress and fatigue signals such as IL-6.

Future research should be directed towards the differential response of circulating IL-6 to exercise, including phenotype and genotype characteristics. In addition, research should examine the threshold further at which circulating IL-6 impacts on performance.

In conclusion, effective manipulation of the circulating IL-6 response to exercise can improve performance. However, this does not alter the response of the IL-6 signalling receptors or biomarkers associated with IL-6.

References

Abbiss, C. & Laursen, P. (2005) 'Models to explain fatigue during prolonged endurance cycling', *Sports Medicine,* 35, pp. 865-898.

Abdul-Tehrani, H., Hudson, A. J., Chang, Y.-S., Timms, A. R., Hawkins, C., Williams, J. M., Harrison, P. M., Guest, J. R. & Andrews, S. C. (1999) 'Ferritin mutants of Escherichia coli are iron deficient and growth impaired, and fur mutants are iron deficient', *The Journal of Bacteriology,* 181 (5), pp. 1415-1428.

Abeywardena, M., Leifert, W., Warnes, K., Varghese, J. & Head, R. (2009) 'Cardiovascular biology of interleukin-6', *Current Pharmaceutical Design,* 15, pp. 1809-1821.

Agrawal, A., Cha-Molstad, H., Samols, D. & Kushner, I. (2001) 'Transactivation of C-reactive protein by IL-6 requires synergistic interaction of CCAAT/enhancer binding protein (C/EBP) and rel p50', *The Journal of Immunology,* 166 (4), pp. 2378-2384.

Agrawal, A., Simpson, M. J., Black, S., Carey, M. P. & Samols, D. (2002) 'A C-reactive protein mutant that does not bind to phosphocholine and pneumococcal C-polysaccharide', *The Journal of Immunology,* 169 (6), pp. 3217-3222.

Ahlberg, K., Ekman, T. & Gaston-Johansson, F. (2004) 'Levels of fatigue compared to levels of cytokines and haemoglobin during pelvic radiotherapy: a pilot study', *Biological Research for Nursing,* 5 (3), pp. 203-210.

Ainsworth, B., Serfass, R. & Leon, A. (1993) 'Effects of recovery duration and blood lactate level on power output during cycling', *Applied Physiology, Nutrition and Metabolism,* 18 (1), pp. 19-30.

Akerstrom, T. C. A., Krogh-Madsen, R., Petersen, A. M. W. & Pedersen, B. K. (2009) 'Glucose ingestion during endurance training in men attenuates expression of myokine receptor', *Experimental Physiology,* 94 (11), pp. 1124-1131.

Akimoto, T., Pohnert, S. C., Li, P., Zhang, M., Gumbs, C., Rosenberg, P. B., Williams, R. S. & Yan, Z. (2005) 'Exercise stimulates PGC-1 alpha transcription in skeletal muscle through activation of the p38 MAPK pathway', *Journal of Biological Chemistry,* 280 (20), pp. 19587-19593.

Akira, S., Taga, T., Kishimoto, T. & Frank, J. D. (1993) 'Interleukin-6 in biology and medicine', in *Advances in Immunology.* Academic Press, pp. 1-78.

Al-Khalil, L., Bouzakri, K., Glund, S., Lonnqvist, F., Koistinen, H. & Krook, A. (2006) 'Signalling specificity of interleukin-6 action on glucose and lipid metabolism in skeletal muscle', *Molecular Endocriniology,* 20, pp. 3364-3375.

Arnall, D., Nelson, A., Quigley, J., Lex, S., DeHart, T. & Fortune, P. (2007) 'Supercompensated glycogen loads persist 5 days in resting trained cyclists', *European Journal of Applied Physiology,* 99 (3), pp. 251-256.

Arnaud, C., Burger, F., Steffens, S., Veillard, N. R., Nguyen, T. H., Trono, D. & Mach, F. (2005) 'Statins reduce interleukin-6-induced C-reactive protein in human hepatocytes: New evidence for direct anti-inflammatory effects of statins', *Arteriosclerosis, Thrombosis, and Vascular Biology,* 25 (6), pp. 1231-1236.

Atkinson, G. & Nevill, A. (1998) 'Statistical methods for assessing measurement error (reliability) in variables relevant to sports medicine ', *Sports Medicine,* 26 (4), pp. 217-238.

Baeza-Raja, B. & Munoz-Canoves, P. (2004) 'p38 MAPK-induced nuclear factor-kappaB activity is required for skeletal muscle differentiation: Role of interleukin-6', *Molecular Biology of the Cell,* 15 (4), pp. 2013-2026.

Bailey, S. P., Davis, J. M. & Ahlborn, E. N. (1993) 'Serotonergic agonists and antagonists affect endurance performance in the rat', *International Journal of Sports Medicine,* 14 (06), pp. 330,333.

Baldwin, K. M., Fitts, R. H., Booth, F. W., Winder, W. W. & Holloszy, J. O. (1975) 'Depletion of muscle and liver glycogen during exercise', *Pflügers Archive of European Journal of Physiology,* 354 (3), pp. 203-212.

Ballabh, P., Braun, A. & Nedergaard, M. (2004) 'The blood-brain barrier: an overview: Structure, regulation, and clinical implications', *Neurobiology of Disease,* 16 (1), pp. 1-13.

Banks, W. (2005) 'Blood-brain barrier transport of cytokines: A mechanism for neuropathology', *Current Pharmaceutical Design,* 11, pp. 973-984.

Banks, W., Ortiz, L., Plotkin, S. & Kastin, A. (1991) 'Human interleukin (IL) 1 alpha, murine IL-1 alpha and murine IL-1 beta are transported from blood to brain in the mouse by a shared saturable mechanism', *The Journal of Pharmocology and Experimental Theraputics,* 259 (3), pp. 988-996.

Banks, W. A., Kastin, A. J. & Gutierrez, E. G. (1994) 'Penetration of interleukin-6 across the murine blood-brain barrier', *Neuroscience Letters,* 179 (1-2), pp. 53-56.

Banzet, S., Koulmann, N., Simler, N., Sanchez, H., Chapot, R., Serrurier, B., Peinnequin, A. & Bigard, X. (2009) 'Control of gluconeogenic genes during intense/prolonged exercise: hormone-independent effect of muscle-derived IL-6 on hepatic tissue and PEPCK mRNA', *Journal of Applied Physiology,* 107 (6), pp. 1830-1839.

Barkhudaryan. N & Dunn, A. (1999) 'Molecular mechanisms of actions of interleukin-6 on the brain, with special reference to serotonin and the hypothalamo-pituitary-adrenocortical axis.', *Neurochemical Research,* 14 (9), pp. 1169-1180.

Baskin, C. R., Hinchcliff, K. W., DiSilvestro, R. A., Reinhart, G. A., Hayek, M. G., Chew, B. P., Burr, J. R. & Swenson, R. A. (2000) 'Effects of dietary antioxidant supplementation on oxidative damage and resistance to oxidative damage during prolonged exercise in sled dogs', *American Journal of Veterinary Research,* 61 (8), pp. 886-891.

Bastard, J., Maachi, M., Lagathu, C., Kim, M., Caron, M., Vidal, H., Capeau, J. & Feve, B. (2006) 'Recent advances in the relationship between obesity, inflammation and insulin resistance', *European Cytokine Network,* 17 (1), pp. 4-12.

Baumann, H. & Gauldie, J. (1994) 'The acute phase response', *Immunology Today,* 15 (2), pp. 74-80.

Bautmans, I., Gorus, E., Njemini, R. & Mets, T. (2007) 'Handgrip performance in relation to self-percieved fatigue, physical functioning anc circulating IL-6 in elderly persons without inflammation', *BMC Geriatrics,* 7 (5).

Bautmans, I., Njemini, R., De Backer, J., De Waele, E. & Mets, T. (2010) 'Surgery-induced inflammation in relation to age, muscle endurance, and self-perceived fatigue', *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 65A (3), pp. 266-273.

Bell, E. A. & Janzen, D. H. (1971) 'Medical and ecological considerations of L-dopa and 5-HTP in seeds', *Nature,* 229 (5280), pp. 136-137.

Bergstrom, J., Hermansen, L., Hultman, E. & Saltin, B. (1967) 'Diet, muscle glycogen and physical performance', *Acta Physiologica Scandinavica,* 71 (2-3), pp. 140-150.

Bergstrom, J. & Hultman, E. (1967) 'A study of the glycogen metabolism during exercise in man', *Scandinavian Journal of Clinical & Laboratory Investigation,* 19 (3), pp. 218-228.

Bethin, K. E., Vogt, S. K. & Muglia, L. J. (2000) 'Interleukin-6 is an essential, corticotropin-releasing hormone-independent stimulator of the adrenal axis during immune system activation', *Proceedings of the National Academy of Sciences of the United States of America,* 97 (16), pp. 9317-9322.

Bishop, N., Gleeson, M., Nicholas, C. & Ali, A. (2002) 'Influence of carbohydrate supplementation on plasma cytokine and neutrophil degranulation responses to high intensity intermittent exercise.', *International Journal of Sport Nutrition and Exercise Metabolism,* 12 (2), pp. 145-156.

Bishop, N., Walsh, N., Haines, D., Richards, E. & Gleeson, M. (2001) 'Pre-exercise carbohydrate status and immune responses to prolonged cycling: II. Effect on plasma cytokine concentration ', *International Journal of Sport Nutrition and Exercise Metabolism,* 11 (4).

Bishop, N. C., Blannin, A. K., Walsh, N. P. & Gleeson, M. (2001) 'Carbohydrate beverage ingestion and neutrophil degranulation responses following cycling to fatigue at 75 % VO2 max', *International Journal of Sports Medicine,* 22 (03), pp. 226,231.

Blatteis, C. M. & Sehic, E. (1997) 'Fever: How may circulating pyrogens signal the brain?', *News in Physiological Sciences,* 12 (1), pp. 1-9.

Bluthe, R., Michaud, B., Poli, V. & Dantzer, R. (2000) 'Role of IL-6 in cytokine induced sickness behaviour: A study with IL-6 deficient mice', *Physiology and Behavior,* 70, pp. 367-373.

Bluthe, R.-M., Kelley, K. W. & Dantzer, R. (2006) 'Effects of insulin-like growth factor-I on cytokine-induced sickness behaviour in mice', *Brain, Behavior, and Immunity,* 20 (1), pp. 57-63.

Bonafè, M., Olivieri, F., Cavallone, L., Giovagnetti, S., Marchegiani, F., Cardelli, M., Pieri, C., Marra, M., Antonicelli, R., Lisa, R., Rizzo, M. R., Paolisso, G., Monti, D. & Franceschi, C. (2001) 'A gender–dependent genetic predisposition to produce high levels of IL-6 is detrimental for longevity', *European Journal of Immunology,* 31 (8), pp. 2357-2361.

Booth, F. W. & Company, J. M. (2009) 'Local adaptation in human trained skeletal muscle could preferentially bind blood interleukin-6', *Experimental Physiology,* 94 (11), pp. 1099-1100.

Borg, G. (1982) 'Ratings of perceived exertion and heart rates during short-term cycle exercise and their use in a new cycling strength test.', *International Journal of Sports Medicine,* 3 (3), pp. 153-158.

Boulanger, M. J., Chow, D.-c., Brevnova, E. E. & Garcia, K. C. (2003) 'Hexameric structure and assembly of the interleukin-6/IL-6 receptor/gp130 complex', *Science,* 300 (5628), pp. 2101-2104.

Buono, M. J., Yeager, J. E. & Hodgdon, J. A. (1986) 'Plasma adrenocorticotropin and cortisol responses to brief high-intensity exercise in humans', *Journal of Applied Physiology,* 61 (4), pp. 1337-1339.

Burgess, M., Robertson, R., Davis, M. & Norris, J. (1991) 'RPE, blood glucose, and carbohydrate oxidation during exercise: effects of glucose feedings', *Medicine & Science in Sports & Exercise,* 23 (3), pp. 353-359.

Burke, L. M., Kiens, B. & Ivy, J. L. (2004) 'Carbohydrates and fat for training and recovery', *Journal of Sports Sciences,* 22 (1), pp. 15 - 30.

Calabro, P., Willerson, J. T. & Yeh, E. T. H. (2003) 'Inflammatory cytokines stimulated C-reactive protein production by human coronary artery smooth muscle cells', *Circulation,* 108 (16), pp. 1930-1932.

Campbell, G. S., Meyer, D. J., Raz, R., Levy, D. E., Schwartz, J. & Carter-Su, C. (1995) 'Activation of acute phase response factor (APRF)/Stat3 transcription factor by growth hormone', *Journal of Biological Chemistry,* 270 (8), pp. 3974-3979.

Cappola, A. R., Xue, Q.-L., Ferrucci, L., Guralnik, J. M., Volpato, S. & Fried, L. P. (2003) 'Insulin-like growth factor I and interleukin-6 contribute synergistically to disability and mortality in older women', *Journal of Clinical Endocrinology Metabolism,* 88 (5), pp. 2019-2025.

Carey, A., Steinberg, G., Macaulay, S., Thomas, W., Holmes, A., Ramm, G., Prelovsek, O., Hohnen-Behrens, C., Watt, M., James, D., Kemp, B., Pedersen, B. & Febbraio, M. (2006) 'Interleukin-6 increases insulin stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase', *Diabetes,* 55 (10), pp. 2688-2697.

Carter, J., Jeukendrup, A. & Jones, D. (2004) 'The effect of carbohydrate mouth-rinse on 1 h cycle time-trial performance', *Medicine and Science in Sports and Exercise,* 36 (12), pp. 2107-2111.

Castell, J. V., Geiger, T., Gross, V., Andus, T., Walter, E., Hirano, T., Kishimoto, T. & Heinrich, P. C. (1988) 'Plasma clearance, organ distribution and target cells of interleukin-6/hepatocyte-stimulating factor in the rat', *European Journal of Biochemistry,* 177 (2), pp. 357-361.

Castell, J. V., Gómez-lechón, M. J., David, M., Fabra, R., Trullenque, R. & Heinrich, P. C. (1990) 'Acute-phase response of human hepatocytes: Regulation of acute-phase protein synthesis by interleukin-6', *Hepatology,* 12 (5), pp. 1179-1186.

Castell, L. M. (2003) 'Glutamine supplementation in vitro and in vivo, in exercise and in immunodepression', *Sports Medicine,* 33 (5), pp. 323-345.

Castell, L. M., Poortmans, J. R., Leclercq, R., Brasseur, M., Duchateau, J. & Newsholme, E. A. (1996) 'Some aspects of the acute phase response after a marathon race, and the effects of glutamine supplementation', *European Journal of Applied Physiology,* 75 (1), pp. 47-54.

Cava, F., Gonzalez, C., Pascual, M., Navajo, J. & Gonzalez-Buitrago, J. (2000) 'Biological variation of the interleukin-6 and soluble interleukin-2 receptor in serum of healthy individuals ', *Cytokine,* 12 (9), pp. 1423-1425.

Cesari, M., Penninx, B. W. J. H., Pahor, M., Lauretani, F., Corsi, A. M., Williams, G. R., Guralnik, J. M. & Ferrucci, L. (2004) 'Inflammatory markers and physical performance in older persons: The InCHIANTI study', *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 59 (3), pp. M242-M248.

Chai, Z., Gatti, S., Toniatti, C., Poli, V. & Bartfai, T. (1996) 'Interleukin (IL)-6 gene expression in the central nervous system is necessary for fever response to lipopolysaccharide or IL-1 beta: a study on IL-6-deficient mice', *The Journal of Experimental Medicine,* 183 (1), pp. 311-316.

Chalaris, A., Rabe, B., Paliga, K., Lange, H., Laskay, T., Fielding, C. A., Jones, S. A., Rose-John, S. & Scheller, J. (2007) 'Apoptosis is a natural stimulus of IL6R shedding and contributes to the proinflammatory trans-signaling function of neutrophils', *Blood,* 110 (6), pp. 1748-1755.

Chambers, E. S., Bridge, M. W. & Jones, D. A. (2009) 'Carbohydrate sensing in the human mouth: effects on exercise performance and brain activity', *The Journal of Physiology,* 587 (8), pp. 1779-1794.

Chan, M., McGee, S., Watt, M., Hargreaves, M. & Febbraio, M. (2004) 'Altering dietary nutrient intake that reduces glycogen content leads to phosphorylation of nuclear p38 MAP kinase in human skeletal muscle: association with IL-6 gene transcription during contraction', *Federation of American Societies for Experimental Biology,* 18 (14), pp. 1785-1787.

Chandler, J. & Blair, S. (1980) 'The effect of amphetamines on selected physiological components related to athletic success', *Medicine & Science in Sports & Exercise,* 12 (1), pp. 65-69.

Chen, Y. J., Wong, S. H. S., Chan, C. O. W., Wong, C. K., Lam, C. W. & Siu, P. M. F. (2009) 'Effects of glycemic index meal and CHO-electrolyte drink on cytokine response and run performance in endurance athletes', *Journal of Science and Medicine in Sport,* 12 (6), pp. 697-703.

Cichy, J., Rose-John, S., Potempa, J., Pryjma, J. & Travis, J. (1997) 'Oncostatin M stimulates the expression and release of the IL-6 receptor in human hepatoma HepG2 cells', *The Journal of Immunology,* 159 (11), pp. 5648-5653.

Coggan, A. & Coyle, E. (1991) 'Carbohydrate ingestion during prolonged exercise: Effects on metabolism and performance', *Exercise and Sport Sciences Reviews,* 19 (1), pp. 1-40.

Coiro, V., Passeri, M., Davoli, C., Bacchi-Modena, A., Bianconi, L., Volpi, R. & Chiodera, P. (1988) 'Oxytocin reduces exercise-induced ACTH and cortisol rise in man', *Acta Endocrinologica,* 119 (3), pp. 405-412.

Cooper, J., Bloom, F. & Roth, R. (2003) *The biochemical basis of neuropharmacology* 8th edn. New York: Oxford University Press.

Costanzo, E. S., Lutgendorf, S. K., Sood, A. K., Anderson, B., Sorosky, J. & Lubaroff, D. M. (2005) 'Psychosocial factors and interleukin-6 among women with advanced ovarian cancer', *Cancer,* 104 (2), pp. 305-313.

Cox, A., Pyne, D., Saunders, P., Callister, R. & Gleeson, M. (2007) 'Cytokine responses to treadmill running in healthy and illness-prone athletes', *Medicine and Science in Sports and Exercise,* 39 (11), pp. 1918-1926.

Coyle, E., Coggan, A., Hemmert, M. & Ivy, J. (1986) 'Muscle glycogen utilization during prolonged strenuous exercise when fed carbohydrate', *Journal of Applied Physiology,* 61, p. 165.

Currell, K. & Jeukendrup, A. (2008) 'Validity, reliability and sensitivity of measures of sporting performance', *Sports Medicine,* 38 (4), pp. 297-316.

Dantzer, R., Bluthe, R.-M., Gheusi, G., Cremona, S., Laye, S., Parnet, P. & Kelley, K. W. (1998) 'Molecular basis of sickness behaviour', *Annals of the New York Academy of Sciences,* 856 (1), pp. 132-138.

Davis, J., Bailey, S., Woods, J., Galiano, F., Hamilton, M. & Bartoli, W. (1992) 'Effects of carbohydrate feedings on plasma free tryptophan and branched-chain amino acids during prolonged cycling', *European Journal of Applied Physiology and Occupational Physiology,* 65 (6), pp. 513-519.

Davis, J. M., Alderson, N. L. & Welsh, R. S. (2000) 'Serotonin and central nervous system fatigue: nutritional considerations', *American Journal of Clinical Nutrition,* 72 (2), pp. 573S-578.

Davis, J. N., Kucuk, O., Djuric, Z. & Sarkar, F. H. (2001) 'Soy isoflavone supplementation in healthy men prevents NF-[kappa]B activation by TNF-[alpha] in blood lymphocytes', *Free Radical Biology and Medicine,* 30 (11), pp. 1293-1302.

Davis, M. & Bailey, S. (1997) 'Possible mechanisms of central nervous system fatigue during exercise', *Medicine & Science in Sports & Exercise,* 29 (1), pp. 45-57.

Davis, M. C., Zautra, A. J., Younger, J., Motivala, S. J., Attrep, J. & Irwin, M. R. (2008) 'Chronic stress and regulation of cellular markers of inflammation in rheumatoid arthritis: Implications for fatigue', *Brain, Behavior, and Immunity,* 22 (1), pp. 24-32.

Davis, S., Aldrich, T. H., Stahl, N., Pan, L., Taga, T., Kishimoto, T., Ip, N. Y. & Yancopoulos, G. D. (1993) 'LIFR beta and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor', *Science,* 260 (5115), pp. 1805-1808.

de Milander, L., Stein, D. & Collins, M. (2009) 'The interleukin-6, serotonin transporter, and monoamine oxidase A genes and endurance performance during the South African Ironman Triathlon', *Applied Physiology, Nutrition, and Metabolism,* 34, pp. 858-865.

deFronzo, R. & Ferrrannini, E. (1991) 'Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease', *Diabetes Care,* 14, pp. 173-194.

Del Corral, P., Howley, E. T., Hartsell, M., Ashraf, M. & Younger, M. S. (1998) 'Metabolic effects of low cortisol during exercise in humans', *Journal of Applied Physiology,* 84 (3), pp. 939-947.

Denadai, B., Ortiz, M., Greco, C. & de Mello, M. (2006) 'Interval training at 95% and 100% of the velocity at VO2 max: effects on aerobic physiological indexes and running performance.', *Applied Physiology, Nutrition and Metabolism,* 31 (6), pp. 737-743.

Dinarello, C. A., Cannon, J. G., Mancilla, J., Bishai, I., Lees, J. & Coceani, F. (1991) 'Interleukin-6 as an endogenous pyrogen: induction of prostaglandin E2 in brain but not in peripheral blood mononuclear cells', *Brain Research,* 562 (2), pp. 199-206.

Dolmetsch, R., Xu, K. & Lewis, R. (1998) 'Calcium oscillations increase the efficiency and specificity of gene expression', *Nature,* 392, pp. 933-936.

Dugue, B. & Leppanen, E. (1998) 'Short-term variability in the concentration of serum interleukin-6 and its soluble receptor in subjectively healthy persons', *Clinical Chemistry and Laboratory Medicine,* 36 (5), pp. 323-325.

Duthie, S. J., Ma, A., Ross, M. A. & Collins, A. R. (1996) 'Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes', *Cancer Research,* 56 (6), pp. 1291-1295.

El-Sayed, M., Balmer, J. & Rattu, A. (1997) 'Carbohydrate ingestion improves endurance performance during a 1 h simulated time trial', *Journal of Sport Sciences,* 15, pp. 223-230.

Elzi, D. J., Bjornsen, A. J., MacKenzie, T., Wyman, T. H. & Silliman, C. C. (2001) 'Ionomycin causes activation of p38 and p42/44 mitogen-activated protein kinases in human neutrophils', *American Journal of Physiology - Cell Physiology,* 281 (1), pp. C350-360.

Erickson, M., Schwarzkopf, R. & McKenzie, R. (1987) 'Effects of caffeine, fructose and glucose ingestion on muscle glycogen utilization during exercise', *Medicine and Science in Sports and Exercise,* 19, p. 579.

Fain, J. N., Madan, A. K., Hiler, M. L., Cheema, P. & Bahouth, S. W. (2004) 'Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans', *Endocrinology,* 145 (5), pp. 2273-2282.

Farrell, P. A., Garthwaite, T. L. & Gustafson, A. B. (1983) 'Plasma adrenocorticotropin and cortisol responses to submaximal and

exhaustive exercise', *Journal of Applied Physiology,* 55 (5), pp. 1441-1444.

Febbraio, M. & Pedersen, B. (2002) 'Muscle-derrived interleukin-6: Mechanisms for activation and possible biological roles.', *Federation of American Societies for Experimental Biology,* 16 (11), pp. 1335-1347.

Febbraio, M. & Pedersen, B. (2005) 'Contraction-induced myokine production and release: Is skeletal muscle an endocrine organ?', *Exercise and Sport Sciences Reviews,* 33 (3), pp. 114-119.

Febbraio, M. A., Hiscock, N., Sacchetti, M., Fischer, C. P. & Pedersen, B. K. (2004) 'Interleukin-6 is a novel factor mediating glucose homeostasis during skeletal muscle contraction', *Diabetes,* 53 (7), pp. 1643-1648.

Febbraio, M. A. & Koukoulas, I. (2000) 'HSP72 gene expression progressively increases in human skeletal muscle during prolonged, exhaustive exercise', *Journal of Applied Physiology,* 89 (3), pp. 1055-1060.

Febbraio, M. A., Steensberg, A., Keller, C., Starkie, R. L., Nielsen, H. B., Krustrup, P., Ott, P., Secher, N. H. & Pedersen, B. K. (2003) 'Glucose ingestion attenuates interleukin-6 release from contracting skeletal muscle in humans', *The Journal of Physiology,* 549 (2), pp. 607-612.

Fernstrom, J. D. & Fernstrom, M. H. (2006) 'Exercise, serum free tryptophan, and central fatigue', *The Journal of Nutrition,* 136 (2), pp. 553S-559S.

Fernstrom, J. D. & Wurtman, R. J. (1971) 'Brain serotonin content: Physiological dependence on plasma tryptophan levels', *Science,* 173 (3992), pp. 149-152.

Ferrucci, L., Penninx, B. W. J. H., Volpato, S., Harris, T. B., Bandeen-Roche, K., Balfour, J., Leveille, S. G., Fried, L. P. & Md, J. M. G. (2002) 'Change in muscle strength explains accelerated decline of physical function in older women with high interleukin-6 serum levels', *Journal of the American Geriatrics Society,* 50 (12), pp. 1947-1954.

Few, J. D. (1974) 'Effect of exercise on the secretion and metabolism of cortisol in man', *Journal of Endocrinology,* 62 (2), pp. 341-353.

Fischer, C. (2006) 'Interleukin-6 in acute exercise and training: what is the biological relevance?', *Exercise Immunology Review,* 12, pp. 6-33.

Fischer, C. P., Hiscock, N. J., Penkowa, M., Basu, S., Vessby, B., Kallner, A., Sjöberg, L.-B. & Pedersen, B. K. (2004) 'Supplementation with vitamins C and E inhibits the release of interleukin-6 from contracting human skeletal muscle', *The Journal of Physiology,* 558 (2), pp. 633-645.

Fischer, C. P., Plomgaard, P., Hansen, A. K., Pilegaard, H., Saltin, B. & Pedersen, B. K. (2004) 'Endurance training reduces the contraction-induced interleukin-6 mRNA expression in human skeletal muscle', *American Journal of Physiolology Endocrinology and Metabolism,* 287 (6), pp. E1189-1194.

Fishman, D., Faulds, G., Jeffery, R., Mohamed-Ali, V., Yudkin, J. S., Humphries, S. & Woo, P. (1998) 'The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis', *Journal of Clinical Investigation,* 102 (7), pp. 1369-1376.

Flynn, M. G., Costill, D. L., Hawley, J. A., Fink, W. J., Neufer, P. D., Fielding, R. A. & Sleeper, M. D. (1987) 'Influence of selected carbohydrate drinks on cycling performance and glycogen use', *Medicine & Science in Sports & Exercise,* 19 (1), pp. 37-40.

Fraioli, F., Moretti, C., Paolucci, D., Alicicco, E., Crescenzi, F. & Fortunio, G. (1980) 'Physical exercise stimulates marked concomitant release of β-endorphin and adrenocorticotropic hormone (ACTH) in peripheral blood in man', *Cellular and Molecular Life Sciences,* 36 (8), pp. 987-989.

Franch, J., Madsen, K., Djurhuus, M. & Pedersen, P. K. (1998) 'Improved running economy following intensified training correlates with reduced ventilatory demands', *Medicine & Science in Sports & Exercise,* 30 (8), pp. 1250-1256.

Frayn, K. N. (1983) 'Calculation of substrate oxidation rates in vivo from gaseous exchange', *Journal of Applied Physiology,* 55 (2), pp. 628-634.

Froiland, K., Koszewski, W., Hingst, J. & Kopecky, L. (2004) 'Nutritional supplement use among college athletes and their sources of information', *International Journal of Sport Nutrition and Exercise Metabolism,* 14 (1), pp. 104-120.

Gabey, C. & Krushner, I. (1999) 'Acute-phase proteins and other systemic responses to inflammation ', *The New England Journal of Medicine,* 340 (6), pp. 448-450.

Gadient, R. A. & Otten, U. (1993) 'Differential expression of interleukin-6 (IL-6) and interleukin-6 receptor (IL-6R) mRNAs in rat hypothalamus', *Neuroscience Letters,* 153 (1), pp. 13-16.

Galbo, H., Holst, J. J. & Christensen, N. J. (1975) 'Glucagon and plasma catecholamine responses to graded and prolonged exercise in man', *Journal of Applied Physiology,* 38 (1), pp. 70-76.

Gallea-Robache, S., Morand, V., Millet, S., Bruneau, J.-M., Bhatnagar, N., Chouaib, S. & Roman-Roman, S. (1997) 'A metalloproteinase inhibitor blocks the shedding of soluble cytokine receptors and processing of transmembrane cytokine precursors in human monocytic cells ', *Cytokine,* 9 (5), pp. 340-346.

Gao, B. (2005) 'Cytokines, STATs and liver disease', *Cellular and Molecular Immunology,* 2 (2), pp. 92-100.

Gauldie, J., Richards, C., Harnish, D., Lansdorp, P. & Baumann, H. (1987) 'Interferon beta 2/B-cell stimulatory factor type 2 shares identity with monocyte derived hepatocyte stimulating factor and regulates the major acute phase response in protein response in liver cells', *Proceedings of the National Acamedy of Sciences,* 84 (7251-7255).

Genovese, M. C., McKay, J. D., Nasonov, E. L., Mysler, E. F., da Silva, N. A., Alecock, E., Woodworth, T. & Gomez-Reino, J. J. (2008) 'Interleukin-6 receptor inhibition with tocilizumab reduces disease activity in rheumatoid arthritis with inadequate response to disease-modifying antirheumatic drugs: The tocilizumab in combination with traditional disease-modifying antirheumatic drug therapy study', *Arthritis & Rheumatism,* 58 (10), pp. 2968-2980.

Gerhartz, C., Dittrich, E., Stoyan, T., Rose-John, S., Yasukawa, K., Heinrich, P. C. & Graeve, L. (1994) 'Biosynthesis and half-life of the interleukin-6 receptor and its signal transducer gp130', *European Journal of Biochemistry,* 223 (1), pp. 265-274.

Gerhartz, C., Heesel, B., Sasse, J., Hemmann, U., Landgraf, C., Schneider-Mergener, J., Horn, F., Heinrich, P. & Graeve, L. (1996) 'Differential activation of acute phase response factor/STAT3 and STAT1 via the cytoplasmic domain of the interleukin 6 signal transducer gp130. I. Definition of a novel phosphotyrosine motif mediating STAT1 activation.', *The Journal of Biological Chemistry,* 271 (22), pp. 12991-12998.

Gershov, D., Kim, S., Brot, N. & Elkon, K. B. (2000) 'C-reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an anti-inflammatory innate immune response', *The Journal of Experimental Medicine,* 192 (9), pp. 1353-1364.

Gleeson, M. (2000) 'Interleukins and exercise', *The Journal of Physiology,* 529 (1).

Glund, S., Deshmukh, A., Long, Y. C., Moller, T., Koistinen, H. A., Caidahl, K., Zierath, J. R. & Krook, A. (2007) 'Interleukin-6 directly increases glucose metabolism in resting human skeletal muscle', *Diabetes,* 56 (6), pp. 1630-1637.

Goforth, H. W., Arnall, J., David A., Bennett, B. L. & Law, P. G. (1997) 'Persistence of supercompensated muscle glycogen in trained subjects after carbohydrate loading', *Journal of Applied Physiology,* 82 (1), pp. 342-347.

Goforth, H. W., Jr., Laurent, D., Prusaczyk, W. K., Schneider, K. E., Petersen, K. F. & Shulman, G. I. (2003) 'Effects of depletion exercise and light training on muscle glycogen supercompensation in men', *American Journal of Physiolology Endocrinology and Metabolism,* 285 (6), pp. E1304-1311.

Gokhale, R., Chandrashekara, S. & Vasanthakumar, K. C. (2007) 'Cytokine response to strenuous exercise in athletes and non-athletes--an adaptive response', *Cytokine,* 40 (2), pp. 123-127.

Gollnick, P. D., Armstrong, B., Saltin, B., Saubert, C., Sembrowich, W. & Shephard, R. (1973) 'Effect of training on enzyme activity and fiber composition of human skeletal muscle', *Journal of Applied Physiology,* 34 (1), pp. 107-111.

Gollnick, P. D. & Saltin, B. (1982) 'Significance of skeletal muscle oxidative enzyme enhancement with endurance training', *Clinical Physiology,* 2 (1), pp. 1-12.

Goran, M. I., Ball, G. D. C. & Cruz, M. L. (2003) 'Obesity and risk of type 2 diabetes and cardiovascular disease in children and adolescents', *Journal of Clinical Endocrinology and Metabolism,* 88 (4), pp. 1417-1427.

Gordon, M. S., Nemunaitis, J., Hoffman, R., Paquette, R. L., Rosenfeld, C., Manfreda, S., Isaacs, R. & Nimer, S. D. (1995) 'A phase I trial of recombinant human interleukin-6 in patients with myelodysplastic syndromes and thrombocytopenia', *Blood,* 85 (11), pp. 3066-3076.

Gray, S., Robinson, M. & Nimmo, M. (2008) 'Response of plasma IL-6 and its soluble receptors during submaximal exercise to fatigue in sedentary middle-aged men', *Cell Stress and Chaperones,* 13 (2), pp. 247-251.

Gray, S. R., Ratkevicius, A., Wackerhage, H., Coats, P. & Nimmo, M. A. (2009) 'The effect of interleukin-6 and the interleukin-6 receptor on glucose transport in mouse skeletal muscle', *Experimental Physiology,* 94 (8), pp. 899-905.

Gross, P. M., Blasberg, R. G., Fenstermacher, J. D. & Patlak, C. S. (1987) 'The microcirculation of rat circumventricular organs and pituitary gland', *Brain Research Bulletin,* 18 (1), pp. 73-85.

Grotzinger, J., Kurapkat, G., Wollmer, A., Kalai, M. & Rose-John, S. (1997) 'The family of the IL-6 type cytokines: Specificity and promiscuity of the receptor complexes', *Proteins: Structure, Function and Genetics,* 27, pp. 96-109.

Haas, J. D. & Brownlie, T. (2001) 'Iron deficiency and reduced work capacity: A critical review of the research to determine a causal relationship', *The Journal of Nutrition,* 131 (2), pp. 676S-690S.

Haegeman, G., Content, J., Volckaert, G., Derynck, R., Tavernier, J. & Fiers, W. (1986) 'Structural analysis of the sequence coding for an inducible 26-kDa protein in human fibroblasts', *European Journal of Biochemistry,* 159 (3), pp. 625-632.

Harden, L. M., du Plessis, I., Poole, S. & Laburn, H. P. (2006) 'Interleukin-6 and leptin mediate lipopolysaccharide-induced fever and sickness behaviour', *Physiology & Behavior,* 89 (2), pp. 146-155.

Hargreaves, M., Costill, D., Coggan, A., Fink, W. & Nishibata, I. (1984) 'Effect of carbohydrate feedings on muscle glycogen utilization and exercise performance', *Medicine & Science in Sports & Exercise,* 16 (3), pp. 219-222.

Hashizume, M., Uchiyama, Y., Horai, N., Tomosugi, N. & Mihara, M. (2010) 'Tocilizumab, a humanized anti-interleukin-6 receptor antibody, improved anaemia in monkey arthritis by suppressing IL-6-induced hepcidin production', *Rheumatology International,* 30 (7), pp. 917-923.

Haverty, M., Kenney, W. L. & Hodgson, J. L. (1988) 'Lactate and gas exchange responses to incremental and steady state running', *British Journal of Sports Medicine,* 22 (2), pp. 51-54.

Healy, M. J. (1979) 'Outliers in clinical chemistry quality-control schemes', *Clinical Chemistry,* 25 (5), pp. 675-677.

Heinrich, P., Castell, J. & Andos, T. (1990) 'Interleukin-6 and the acute phase response', *Journal of Biochemistry,* 265, pp. 621-636.

Henriksson, J. & Reitman, J. S. (1976) 'Quantitative measures of enzyme activities in type I and type II muscle fibres of man after training', *Acta Physiologica Scandinavica,* 97 (3), pp. 392-397.

Henritze, J., Weltman, A., Schurrer, R. & Barlow, K. (1985) 'Effects of training at and above the lactate threshold on the lactate threshold and maximal oxygen uptake', *European Journal of Applied Physiology and Occupational Physiology,* 54 (1), pp. 84-88.

Hibi, M., Murakami, M., Saito, M., Hirano, T., Taga, T. & Kishimoto, T. (1990) 'Molecular cloning and expression of an IL-6 signal transducer, gp130', *Cell,* 63 (6), pp. 1149-1157.

Hickner, R. C., Fisher, J. S., Hansen, P. A., Racette, S. B., Mier, C. M., Turner, M. J. & Holloszy, J. O. (1997) 'Muscle glycogen accumulation after endurance exercise in trained and untrained individuals', *Journal of Applied Physiology,* 83 (3), pp. 897-903.

Hickson, R. C., Hagberg, J. M., Ehsani, A. A. & Holloszy, J. O. (1981) 'Time course of the adaptive responses of aerobic power and heart rate to training', *Medicine & Science in Sports & Exercise,* 13 (1), pp. 17-20.

Hickson, R. C., Rosenkoetter, M. A. & Brown, M. M. (1980) 'Strength training effects on aerobic power and short-term endurance', *Medicine & Science in Sports & Exercise,* 12 (5), pp. 336-339.

Hinton, P. S., Giordano, C., Brownlie, T. & Haas, J. D. (2000) 'Iron supplementation improves endurance after training in iron-depleted, nonanemic women', *Journal of Applied Physiology,* 88 (3), pp. 1103-1111.

Hirano, T., Taga, T., Nakano, N., Yasukawa, K., Kashiwamura, S., Shimizu, K., Nakajima, K., Pyun, K. & Kishimoto, T. (1985) 'Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSFp-2)', *Proceedings of the National Acamedy of Sciences,* 82, pp. 5490-5494.

Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S., Nakajima, K., Koyama, K. & Iwamatsu, A. (1986) 'Complementary DNA for a novel human interleukin (BSF-2) that iduces B lymphocytes to produce immunoglobulin', *Nature,* 324, pp. 73-76.

Hiscock, N., Chan, M. H. S., Bisucci, T., Darby, I. A. & Febbraio, M. A. (2004) 'Skeletal myocytes are a source of interleukin-6 mRNA expression and protein release during contraction: evidence of fiber type specificity', *Federation of American Societies for Experimental Biology.*

Hiscock, N., Fischer, C., Sacchetti, M., Van Hall, G., Febbraio, M. & Pedersen, B. (2005) 'Recombinant human Interleukin-6 Infusion During Low-intensity Exercise Does Not Enhance Whole Body Lypolysis or Fat Oxidation in Humans', *American Journal of Physiology Endocrine and Metabolism,* 289, pp. E2-E7.

Hiscock, N., Petersen, E., Krzywkowski, K., Boza, J., Halkjaer-Kristensen, J. & Pedersen, B. (2003) 'Glutamine supplementation further enhances exercise-induced plasma IL-6', *Journal of Applied Physiology,* 95, pp. 145-148.

Ho, G., Xue, X., Burk, R., Kaplan, R., Cornell, E. & Cushman, M. (2005) 'Variability of serum levels of tumor necrosis factor alpha, interleukin-6, and soluble interleukin-6 receptor over 2 years in young women.', *Cytokine,* 30, pp. 1-6.

Hoene, M. & Weigert, C. (2008) 'The role of interleukin-6 in insulin resistance, body fat distribution and energy balance', *Obesity reviews,* 9 (1), pp. 20-29.

Hong, S., Mills, P. J., Loredo, J. S., Adler, K. A. & Dimsdale, J. E. (2005) 'The association between interleukin-6, sleep, and demographic characteristics', *Brain, Behavior, and Immunity,* 19 (2), pp. 165-172.

Hopkins, S. J. & Rothwell, N. J. (1995) 'Cytokines and the nervous system I: expression and recognition', *Trends in Neurosciences,* 18 (2), pp. 83-88.

Hopkins, W. (2000) 'Measures of reliability in sports medicine and science', *Sports Medicine,* 30 (4), pp. 1-25.

Hopkins, W. (2006) 'Estimating sample size for magnitude-based inferences', *Sportscience,* 10, pp. 63-70.

Hopkins, W., Schabort, E. & Hawley, J. (2001) 'Reliability of power in physical performance tests', *Sports Medicine,* 31 (3), pp. 211-234.

Horiuchi, S., Koyanagiu, Y., Zhouu, Y., Miyamotou, H., Tanakau, Y., Waki, M., Matsumoto, A., Yamamotou, M. & Yamamotof, N. (1994) 'Soluble interleukin-6 receptors released from T cell or granulocyte/macrophage cell lines and human peripheral blood mononuclear cells are generated through an alternative splicing mechanism', *European Journal of Immunology,* 24 (8), pp. 1945-1948.

Horowitz, J. F. & Klein, S. (2000) 'Lipid metabolism during endurance exercise', *The American Journal of Clinical Nutrition,* 72 (2), pp. 558S-563S.

Hurley, B. F., Nemeth, P. M., Martin, W. H., Hagberg, J. M., Dalsky, G. P. & Holloszy, J. O. (1986) 'Muscle triglyceride utilization during exercise: effect of training', *Journal of Applied Physiology,* 60 (2), pp. 562-567.

Hurlimann, J., Thorbecke, G. J. & Hochwald, G. M. (1966) 'The liver as the site of C-reactive protein formation', *The Journal of Experimental Medicine,* 123 (2), pp. 365-378.

Hurst, S. M., Wilkinson, T. S., McLoughlin, R. M., Jones, S., Horiuchi, S., Yamamoto, N., Rose-John, S., Fuller, G. M., Topley, N. & Jones, S. A. (2001) 'IL-6 and Its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation', *Immunity,* 14 (6), pp. 705-714.

Inder, W. J., Hellemans, J., Swanney, M. P., Prickett, T. C. R. & Donald, R. A. (1998) 'Prolonged exercise increases peripheral plasma ACTH, CRH, and AVP in male athletes', *Journal of Applied Physiology,* 85 (3), pp. 835-841.

Iwashita, S., Williams, P., Jabbour, K., Ueda, T., Kobayashi, H., Baier, S. & Flakoll, P. J. (2005) 'Impact of glutamine supplementation on glucose homeostasis during and after exercise', *Journal of Applied Physiology,* 99 (5), pp. 1858-1865.

Jeukendrup, A. (2004) 'Carbohydrate intake during exercise and performance.', *Nutrition,* 20 (7-8), pp. 669-677.

Jeukendrup, A., Raben, A. & Gijsen, A. (1999) 'Glucose kinetics during prolonged exercise in highly trained human subjects: effect of glucose ingestion', *Journal of Physiology,* 51 (part 2), p. 579.

Jeukendrup, A., Raben, A., Gijsen, A., Stegen, J., Brouns, F., Saris, W. & Wagenmakers, A. (1999) 'Glucose kinetics during prolonged exercise in highly trained human subjects: effect of glucose ingestion', *Journal of Physiology,* 515 (2), pp. 579-589.

Jeukendrup, A., Saris, W., Brouns, F. & Kester, A. (1996) 'A new validated endurance performance test', *Medicine and Science in Sports and Exercise,* 28 (2), pp. 266-270.

Jones, H. N., Ashworth, C. J., Page, K. R. & McArdle, H. J. (2006) 'Cortisol stimulates system A amino acid transport and SNAT2 expression in a human placental cell line (BeWo)', *American Journal of Physiolology Endocrinology and Metabolism,* 291 (3), pp. E596-603.

Jones, S., Horiuchi, S., Novick, D., Yamamoto, N. & Fuller, G. (1998) 'Shedding of the soluble IL-6 receptor is triggered by Ca2+ mobilization, while basal release is predominantly the product of differential mRNA splicing in THP-1 cells.', *European Journal of Immunology,* 28 (11), pp. 3514-3522.

Jones, S., Horiuchi, S., Topley, N., Yamamoto, N. & Fuller, G. (2001) 'The soluble interleukin 6 receptor: mechanisms of production and implications in disease', *Federation of American Societies for Experimental Biology,* 15, pp. 43-58.

Jones, S., Novick, D., Horiuchi, S., Yamamoto, N., Szalai, A. & Fuller, G. (1999) 'C-reactive protein: A physiological activator of interleukin-6 receptor shedding', *Journal of Experimental Medicine,* 189 (3), pp. 599-604.

Jones, S., Richards, P., Scheller, J. & Rose-John, S. (2005) 'IL-6 transsignalling: The in vivo consequences', *Journal of Interferon and Cytokine Research,* 25, pp. 241-253.

Jones, S. & Rose-John, S. (2002) 'The role of the soluble receptors in cytokine biology, the agonistic properties of the sIL-6R/IL-6 complex ', *Biochimica et Biophysica Acta,* 1592, pp. 251-263.

Jostock, T., Müllberg, J., Özbek, S., Atreya, R., Blinn, G., Voltz, N., Fischer, M., Neurath, M. F. & Rose-John, S. (2001) 'Soluble gp130 is the natural inhibitor of soluble interleukin-6 receptor transsignaling responses', *European Journal of Biochemistry,* 268 (1), pp. 160-167.

Joyner, M. J. & Coyle, E. F. (2008) 'Endurance exercise performance: the physiology of champions', *The Journal of Physiology,* 586 (1), pp. 35-44.

Jürimäe, J., Mäestu, J., Jürimäe, T., Mangus, B. & von Duvillard, S. P. (2011) 'Peripheral signals of energy homeostasis as possible markers of training stress in athletes: a review', *Metabolism,* 60 (3), pp. 335-350.

Kanemaki, T., Kitade, H., Kaibori, M., Sakitani, K., Hiramatsu, Y., Kamiyama, Y., Ito, S. & Okumura, T. (1998) 'Interleukin 1β and interleukin 6, but not tumor necrosis factor α, inhibit insulin-stimulated glycogen synthesis in rat hepatocytes', *Hepatology,* 27 (5), pp. 1296-1303.

Katsuura, G., Arimura, A., Koves, K. & Gottschall, P. E. (1990) 'Involvement of organum vasculosum of lamina terminalis and preoptic area in interleukin 1 beta-induced ACTH release',

American Journal of Physiolology Endocrinology and Metabolism, 258 (1), pp. E163-171.

Keller, C., Steensberg, A., Pilegaard, H., Osada, T., Saltin, B., Pedersen, B. & Neufer, P. (2001) 'Transcriptional activation of the IL-6 gene in human contracting skeletal muscle: influence of muscle glycogen content.', *Federation of American Societies for Experimental Biology,* 15 (14), pp. 2748-2750.

Keller, P., Penkowa, M., Keller, C., Steensberg, A., Fischer, C., Giralt, M., Hidalgo, J. & Pedersen, B. (2005) 'Interleukin-6 receptor expression in contracting human skeletal muscle: regulating role of IL-6', *Federation of American Societies for Experimental Biology,* 19, pp. 1181-1183.

Kemna, E., Pickkers, P., Nemeth, E., van der Hoeven, H. & Swinkels, D. (2005) 'Time-course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS', *Blood,* 106 (5), pp. 1864-1866.

Kemna, E. H. J. M., Kartikasari, A. E. R., van Tits, L. J. H., Pickkers, P., Tjalsma, H. & Swinkels, D. W. (2008) 'Regulation of hepcidin: Insights from biochemical analyses on human serum samples', *Blood Cells, Molecules, and Diseases,* 40 (3), pp. 339-346.

Kern, P. A., Ranganathan, S., Li, C., Wood, L. & Ranganathan, G. (2001) 'Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance', *American Journal of Physiolology Endocrinology and Metabolism,* 280 (5), pp. E745-751.

Khani, S. & Tayek, J. A. (2001) 'Cortisol increases gluconeogenesis in humans: its role in the metabolic syndrome', *Clinical Science,* 101 (6), pp. 739-747.

Kiens, B., Essen-Gustavsson, B., Christensen, N. J. & Saltin, B. (1993) 'Skeletal muscle substrate utilization during submaximal exercise in man: effect of endurance training', *The Journal of Physiology,* 469 (1), pp. 459-478.

Kilpatrick, J. M. & Volanakis, J. E. (1985) 'Opsonic properties of C-reactive protein. Stimulation by phorbol myristate acetate enables human neutrophils to phagocytize C-reactive protein-coated cells', *The Journal of Immunology,* 134 (5), pp. 3364-3370.

Kindermann, W., Schnabel, A., Schmitt, W., Biro, G., Cassens, J. & Weber, F. (1982) 'Catecholamines, growth hormone, cortisol, insulin, and sex hormones in anaerobic and aerobic exercise',

European Journal of Applied Physiology and Occupational Physiology, 49 (3), pp. 389-399.

Klein, S., Coyle, E. F. & Wolfe, R. R. (1994) 'Fat metabolism during low-intensity exercise in endurance-trained and untrained men', *American Journal of Physiolology Endocrinology and Metabolism,* 267 (6), pp. E934-940.

Knapik, J. J., Meredith, C. N., Jones, B. H., Suek, L., Young, V. R. & Evans, W. J. (1988) 'Influence of fasting on carbohydrate and fat metabolism during rest and exercise in men', *Journal of Applied Physiology,* 64 (5), pp. 1923-1929.

Knudsen, L., Christiensen, I., Lottenburger, T., Svendsen, M., Nielsen, L., Horslev-Petersen, K., Jensen, J., Kollerup, G. & Johansen, J. (2008) 'Pre-analytical and biological variability in circulating interleukin-6 in healthy subjects and patients with rheumatoid arthritis', *Biomarkers,* 13 (1).

Konsman, J. P., Parnet, P. & Dantzer, R. (2002) 'Cytokine-induced sickness behaviour: mechanisms and implications', *Trends in Neurosciences,* 25 (3), pp. 154-159.

Kosmidou, I., Vassilakopoulos, T., Xagorari, A., Zakynthinos, S., Papapetropoulos, A. & Roussos, C. (2002) 'Production of interleukin-6 by skeletal myotubes. Role of reactive oxygen species', *American Journal of Respiratory Cell and Molecular Biology,* 26 (5), pp. 587-593.

Kraemer, R. R., Durand, R. J., Acevedo, E. O., Johnson, L. G., Kraemer, G. R., Hebert, E. P. & Castracane, V. D. (2004) 'Rigorous Running Increases Growth Hormone and Insulin-Like Growth Factor-I Without Altering Ghrelin', *Experimental Biology and Medicine,* 229 (3), pp. 240-246.

Kramer, H. F. & Goodyear, L. J. (2007) 'Exercise, MAPK, and NF-kappaB signaling in skeletal muscle', *Journal of Applied Physiology,* 103 (1), pp. 388-395.

Krause, A., Neitz, S., Mägert, H.-J., Schulz, A., Forssmann, W.-G., Schulz-Knappe, P. & Adermann, K. (2000) 'LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity', *FEBS Letters,* 480 (2-3), pp. 147-150.

Krzywkowski, K., Petersen, E. W., Ostrowski, K., Kristensen, J. H. r., Boza, J. & Pedersen, B. K. (2001) 'Effect of glutamine supplementation on exercise-induced changes in lymphocyte function', *American Journal of Physiology - Cell Physiology,* 281 (4), pp. C1259-C1265.

Kuhad, A., Bishnoi, M., Tiwari, V. & Chopra, K. (2009) 'Suppression of NF-kappa beta signaling pathway by tocotrienol can prevent diabetes associated cognitive deficits', *Pharmacology Biochemistry and Behavior,* 92 (2), pp. 251-259.

Kulaksiz, H., Theilig, F., Bachmann, S., Gehrke, S. G., Rost, D., Janetzko, A., Cetin, Y. & Stremmel, W. (2005) 'The iron-regulatory peptide hormone hepcidin: expression and cellular localization in the mammalian kidney', *Journal of Endocrinology,* 184 (2), pp. 361-370.

Kuoppasalmi, K., Naveri, H., Harkanen, M. & Adlercreutz, H. (1980) 'Plasma cortisol, androstenedione, testosterone and luteinizing hormone in running exercise of different intensities', *Scandinavian Journal of Clinical & Laboratory Investigation,* 40 (5), pp. 403-409.

Kuta, A. E. & Baum, L. L. (1986) 'C-reactive protein is produced by a small number of normal human peripheral blood lymphocytes', *The Journal of Experimental Medicine,* 164 (1), pp. 321-326.

Lambert, E., Goedecke, J., Van Zyl, C., Murphym K, Hawley, J., Dennis, S. & Noakes, T. (2001) 'High-fat diet versus habitual diet prior to carbohydrate loading: Effects on exercise metabolism and cycling performance', *International Journal of Sport Nutrition and Exercise Metabolism,* 11 (2).

Lambert, E. V., St Clair Gibson, A. & Noakes, T. D. (2005) 'Complex systems model of fatigue: integrative homoeostatic control of peripheral physiological systems during exercise in humans', *British Journal of Sports Medicine,* 39 (1), pp. 52-62.

Lancaster, G. (2006) 'Exercise and cytokines', in Gleeson, M. (ed.) *Immune Function in Sport and Exercise.* London: Elselvier, pp. 205-218.

Langberg, H., Olesen, J. L., Gemmer, C. & Kjar, M. (2002) 'Substantial elevation of interleukin-6 concentration in peritendinous tissue, in contrast to muscle, following prolonged exercise in humans', *The Journal of Physiology,* 542 (3), pp. 985-990.

Lee, P., Peng, H., Gelbart, T., Wang, L. & Beutler, E. (2005) 'Regulation of hepcidin transcription by interleukin-1 and interleukin-6', *Proceedings of the National Academy of Sciences of the United States of America,* 102 (6), pp. 1906-1910.

Leggate, M., Nowell, M., Jones, S. & Nimmo, M. (2010) 'The response of interleukin-6 and soluble interleukin-6 receptor isoforms following

intermittent high intensity and continuous moderate intensity cycling', *Cell Stress and Chaperones*, pp. 1-7.

Lenczowski, M. J. P., Bluthe, R. M., Roth, J., Rees, G. S., Rushforth, D. A., van Dam, A. M., Tilders, F. J. H., Dantzer, R., Rothwell, N. J. & Luheshi, G. N. (1999) 'Central administration of rat IL-6 induces HPA activation and fever but not sickness behaviour in rats', *AJP - Regulatory, Integrative and Comparative Physiology,* 276 (3), pp. R652-658.

Levine, S. (2004) 'Mechanisms of soluble cytokine receptor generation', *Journal of Immunology,* 173, pp. 5343-5348.

Looker, A. C., Dallman, P. R., Carroll, M. D., Gunter, E. W. & Johnson, C. L. (1997) 'Prevalence of Iron Deficiency in the United States', *JAMA: The Journal of the American Medical Association,* 277 (12), pp. 973-976.

Lundberg, J. M., Martinsson, A., Hemsén, A., Theodorsson-Norheim, E., Svedenhag, J., Ekblom, B. & Hjemdahl, P. (1985) 'Co-release of neuropeptide Y and catecholamines during physical exercise in man', *Biochemical and Biophysical Research Communications,* 133 (1), pp. 30-36.

MacDonald, C., Wojtaszewski, J. F. P., Pedersen, B. K., Kiens, B. & Richter, E. A. (2003) 'Interleukin-6 release from human skeletal muscle during exercise: relation to AMPK activity', *Journal of Applied Physiology,* 95 (6), pp. 2273-2277.

Mackiewicz, A., Schooltink, H., Heinrich, P. & Rose-John, S. (1992) 'Complex of soluble human IL-6-receptor/IL-6 up-regulates expression of acute-phase proteins', *Journal of Immunolology,* 149 (6), pp. 2021-2027.

Madsen, K., Pedersen, P., Rose, P. & Richter, E. (1990) 'Carbohydrate supercompensation and muscle glycogen utilization during exhaustive running in highly trained athletes', *European Journal of Applied Physiology and Occupational Physiology,* 61 (5), pp. 467-472.

Martineau, L. C. & Gardiner, P. F. (2001) 'Insight into skeletal muscle mechanotransduction: MAPK activation is quantitatively related to tension', *Journal of Applied Physiology,* 91 (2), pp. 693-702.

Marvel, F. A., Chen, C.-C., Badr, N., Gaykema, R. P. A. & Goehler, L. E. (2004) 'Reversible inactivation of the dorsal vagal complex blocks lipopolysaccharide-induced social withdrawal and c-Fos expression in central autonomic nuclei', *Brain, Behavior, and Immunity,* 18 (2), pp. 123-134.

Marz, P., Otten, U. & Rose-John, S. (1999) 'Neural activities of IL-6-type cytokines often depend on soluble cytokine receptors', *European Journal of Neuroscience,* 11 (9), pp. 2995-3004.

Mastorakos, G., Chrousos, G. P. & Weber, J. S. (1993) 'Recombinant interleukin-6 activates the hypothalamic-pituitary-adrenal axis in humans', *Journal of Clinical Endocrinology Metabolism,* 77 (6), pp. 1690-1694.

Mathur, N. & Pedersen, B. (2008) 'Exercise as a mean to control low-grade systemic inflammation', *Mediators of Inflammation,* 2008.

Maughan, R. J. (1999) 'Nutritional ergogenic aids and exercise performance', *Nutrition Research Reviews,* 12 (02), pp. 255-280.

Maughan, R. J. & Gleeson, M. (2004) 'The endurance athlete', in *The Biochemical Basis of Sports Performance.* New York: Oxford University Press.

McCarthy, D. A. & Dale, M. M. (1988) 'The leucocytosis of exercise: A review and model', *Sports Medicine,* 6 (6), pp. 333-363.

McKeigue, P. M., Shah, B. & Marmot, M. G. (1991) 'Relation of central obesity and insulin resistance with high diabetes prevalence and cardiovascular risk in South Asians', *The Lancet,* 337 (8738), pp. 382-386.

Meeusen, R., Piacentini, M. & De Meirleir, K. (2001) 'Brain microdialysis in exercise research.', *Sports Medicine,* 31 (14), pp. 965-983.

Mets, T., Bautmans, P., Njemini, R., Lambert, M. & Demanet, C. (2004) 'The influence of celecoxib on muscle fatigue resistance and mobility in elderly patients with inflammation', *The American Journal of Geriatric Pharmacotherapy,* 2, pp. 230-238.

Meyers, C. A., Albitar, M. & Estey, E. (2005) 'Cognitive impairment, fatigue, and cytokine levels in patients with acute myelogenous leukemia or myelodysplastic syndrome', *Cancer,* 104 (4), pp. 788-793.

Miles, M., Walker, E., Conant, S., Hogan, S. & Kidd, J. (2006) 'Carbohydrate influences plasma interleukin-6 but not C-reactive protein or creatine kinase following a 32-km mountain trail race.', *International Journal of Sport Nutrition and Exercise Metabolism,* 16 (1), pp. 36-46.

Mithieux, G. (1997) 'New knowledge regarding glucose-6 phosphatase gene and protein and their roles in the regulation of glucose

metabolism', *European Journal of Endocrinology,* 136 (2), pp. 137-145.

Mitov, I., Kropec, A., Benzing, A., Just, H., Garotta, G., Galanos, C. & Freudenberg, M. (1997) 'Differential cytokine production in stimulated blood cultures from intensive care patients with bacterial infections', *Infection,* 25 (4).

Mitsuyama, K., Toyonaga, A., Sasaki, E., Ishida, O., Ikeda, H., Tsuruta, O., Harada, K., Tateishi, H., Nishiyama, T. & Tanikawa, K. (1995) 'Soluble interleukin-6 receptors in inflammatory bowel disease: relation to circulating interleukin-6', *Gut,* 36 (1), pp. 45-49.

Mittendorfer, B., Volpi, E. & Wolfe, R. R. (2001) 'Whole body and skeletal muscle glutamine metabolism in healthy subjects', *American Journal of Physiolology Endocrinology and Metabolism,* 280 (2), pp. E323-333.

Mohamed-Ali, V., Goodrick, S., Rawesh, A., Katz, D. R., Miles, J. M., Yudkin, J. S., Klein, S. & Coppack, S. W. (1997) 'Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo', *Journal of Clinical Endocrinology and Metabolism,* 82 (12), pp. 4196-4200.

Mold, C., Baca, R. & Du Clos, T. W. (2002) 'Serum amyloid P component and C-reactive protein opsonize apoptotic cells for phagocytosis through Fc gamma receptors', *Journal of Autoimmunity,* 19 (3), pp. 147-154.

Montero-Julian, F., Brailly, H., Sautes, C., Joyeux, I., Dorval, T., Mosseri, V., Yasukawa, K., Widjdenes, J., Adler, A., Gorin, I., Fridman, W. & Tartour, E. (1997) 'Characterization of a soluble gp130 released by melanoma cell lines: a polyvalent antagonist of cytokines from the interleukin-6 family.', *Clinical Cancer Research,* 3, pp. 1443-1451.

Mooney, R. (2007) 'Counterpoint: Interleukin-6 does not have a beneficial role in insulin sensitivity and glucose homeostasis', *Journal of Applied Physiology,* 102, pp. 816-818.

Mullberg, J., Schooltink, H., Stoyan, T., Heinrich, P. C. & Rose-John, S. (1992) 'Protein kinase C activity is rate limiting for shedding of the interleukin-6 receptor', *Biochemical and Biophysical Research Communications,* 189 (2), pp. 794-800.

Muller-newen, G., Kohne, C., Keul, R., Hemmann, U., Müller-esterl, W., Wijdenes, J., Brakenhoff, J. P. J., Hart, M. H. L. & Heinrich, P. C. (1996) 'Purification and characterization of the soluble interleukin-6 receptor from human plasma and identification of an isoform

generated through alternative splicing', *European Journal of Biochemistry,* 236 (3), pp. 837-842.

Muller-Newen, G., Kuster, A., Hemmann, U., Keul, R., Horsten, U., Martens, A., Graeve, L., Wijdenes, J. & Heinrich, P. C. (1998) 'Soluble IL-6 receptor potentiates the antagonistic activity of soluble gp130 on IL-6 responses', *The Journal of Immunology,* 161 (11), pp. 6347-6355.

Murakami, M., Hibi, M., Nakagawa, N., Nakagawa, T., Yasukawa, K., Yamanishi, K., Taga, T. & Kishimoto, T. (1993) 'IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase', *Science,* 260 (5115), pp. 1808-1810.

Murray, R., Paul, G., Seifert, J. & Eddy, D. (1991) 'Responses to varying rates of carbohydrate ingestion during exercise', *Medicine & Science in Sports & Exercise,* 23 (6), pp. 713-718.

Nader, G. A. & Esser, K. A. (2001) 'Intracellular signaling specificity in skeletal muscle in response to different modes of exercise', *Journal of Applied Physiology,* 90 (5), pp. 1936-1942.

Navarra, P., Pozzoli, G., Brunetti, L., Ragazzoni, E., Besser, M. & Grossman, A. (1992) 'Interleukin-1 beta and interleukin-6 specifically increase the release of prostaglandin E2 from rat hypothalamic explants in vitro', *Neuroendocrinology,* 56 (1), pp. 61-68.

Nehlsen-Cannarella, S., Fagoaga, O., Nieman, D., Hensen, D., Butterworth, D., Schmitt, R., Bailey, E., Warren, B., Utter, A. & Davis, J. (1997) 'Carbohydrate and the cytokine response to 2.5 h of running', *Journal of Applied Physiology,* 82 (5), pp. 1662-1667.

Nieman, D., Nehlsen-Cannarella, S., Fagoaga, O., Hensen, D., Utter, A., Davis, J., Williams, M. & Butterworth, D. (1998) 'Influence of mode and carbohydrate on the cytokine response to heavy exertion', *Medicine and Science in Sports and Exercise,* 30 (5), pp. 671-678.

Nemeth, E., Rivera, S., Gabayan, V., Keller, C., Taudorf, S., Pedersen, B. K. & Ganz, T. (2004) 'IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin', *The Journal of Clinical Investigation,* 113 (9), pp. 1271-1276.

Nemeth, E., Valore, E. V., Territo, M., Schiller, G., Lichtenstein, A. & Ganz, T. (2003) 'Hepcidin, a putative mediator of anaemia of inflammation, is a type II acute-phase protein', *Blood,* 101 (7), pp. 2461-2463.

Neuner, P., Urbanski, A., Trautinger, F., Moller, A., Kirnbauer, R., Kapp, A., Schopf, E., Schwarz, T. & Luger, T. A. (1991) 'Increased IL-6 production by monocyte and keratinocytes in patients with psoriasis', *Journal of Investigative Dermatology,* 97 (1), pp. 27-33.

Newsholme, E. A. & Parry-Billings, M. (1990) 'Properties of glutamine release from muscle and its importance for the immune system', *Journal of Parenteral and Enteral Nutrition,* 14 (4 suppl), pp. 63S-67S.

Newsholme, P., Procopio, J., Lima, M. M. R., Pithon-Curi, T. C. & Curi, R. (2003) 'Glutamine and glutamate—their central role in cell metabolism and function', *Cell Biochemistry and Function,* 21 (1), pp. 1-9.

Nicklas, B. J., Mychaleckyj, J., Kritchevsky, S., Palla, S., Lange, L. A., Lange, E. M., Messier, S. P., Bowden, D. & Pahor, M. (2005) 'Physical function and its response to exercise: Associations with cytokine gene variation in older adults with knee osteoarthritis', *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 60 (10), pp. 1292-1298.

Nicolas, G. I., Bennoun, M., Porteu, A., Mativet, S., Beaumont, C., Grandchamp, B., Sirito, M., Sawadogo, M. I., Kahn, A. & Vaulont, S. (2002) 'Severe iron deficiency anaemia in transgenic mice expressing liver hepcidin', *Proceedings of the National Academy of Sciences of the United States of America,* 99 (7), pp. 4596-4601.

Nishimoto, N., Kanakura, Y., Aozasa, K., Johkoh, T., Nakamura, M., Nakano, S., Nakano, N., Ikeda, Y., Sasaki, T., Nishioka, K., Hara, M., Taguchi, H., Kimura, Y., Kato, Y., Asaoku, H., Kumagai, S., Kodama, F., Nakahara, H., Hagihara, K., Yoshizaki, K. & Kishimoto, T. (2005) 'Humanized anti-interleukin-6 receptor antibody treatment of multicentric Castleman disease', *Blood,* 106 (8), pp. 2627-2632.

Noakes, T. D., St Clair Gibson, A. & Lambert, E. V. (2005) 'From catastrophe to complexity: a novel model of integrative central neural regulation of effort and fatigue during exercise in humans: summary and conclusions', *British Journal of Sports Medicine,* 39 (2), pp. 120-124.

Nurjhan, N., Bucci, A., Perriello, G., Stumvoll, M., Dailey, G., Bier, D. M., Toft, I., Jenssen, T. G. & Gerich, J. E. (1995) 'Glutamine: a major gluconeogenic precursor and vehicle for interorgan carbon transport in man', *The Journal of Clinical Investigation,* 95 (1), pp. 272-277.

Opal, S. M. & DePalo, V. A. (2000) 'Anti-inflammatory cytokines', *Chest,* 117 (4), pp. 1162-1172.

Ostrowski, K., Hermann, C., Bangash, A., Schjerling, P., Nielsen, J. & Pedersen, B. (1998) 'A trauma-like elevation of plasma cytokines in humans in response to treadmill running', *Journal of Physiology,* 513 (3), pp. 889-894.

Ostrowski, K., Rhode, T., Asp, S., Schjerling, P. & Pedersen, B. (1999) 'Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans', *Journal of Physiology,* 515, pp. 287-291.

Ostrowski, K., Schjerling, P. & Pedersen, B. (2000) 'Physical activity and plasma interleukin-6 in humans - effect of intensity of exercise', *European Journal of Applied Physiology,* 83, pp. 512-515.

Papanicolaou, D. A., Wilder, R. L., Manolagas, S. C. & Chrousos, G. P. (1998) 'The pathophysiologic roles of interleukin-6 in human disease', *Annals of Internal Medicine,* 128 (2), pp. 127-137.

Park, C. H., Valore, E. V., Waring, A. J. & Ganz, T. (2001) 'Hepcidin, a urinary antimicrobial peptide synthesized in the liver', *Journal of Biological Chemistry,* 276 (11), pp. 7806-7810.

Parry-Billings, M., Blomstrand, E., McAndrew, N. & Newsholme, E. A. (1990) 'A communicational link between skeletal muscle, brain, and cells of the immune system', *International Journal of Sports Medicine,* 11 (S 2), pp. S122,S128.

Parry-Billings, M., Budgett, R., Koutedakis, Y., Blomstrand, E., Brooks, S., Williams, C., Calder, P., Pilling, S., Baigrie, R. & Newsholme, E. (1992) 'Plasma amino acid concentrations in the overtraining syndrome: possible effects on the immune system', *Medicine & Science in Sports & Exercise,* 24 (12), pp. 1353-1358.

Pattini, A., Schena, F. & Guidi, G. (1990) 'Serum ferritin and serum iron changes after cross-country and roller ski endurance races', *European Journal of Applied Physiology and Occupational Physiology,* 61 (1), pp. 55-60.

Pedersen, B. & Febbraio, M. (2005) 'Muscle-derived interleukin-6. A possible link between skeletal muscle, adipose tissue, liver and brain', *Brain, Behavior and Immunity,* 19, pp. 371-376.

Pedersen, B. & Febbraio, M. (2007) 'Point: Interleukin-6 does have a benificial role in insulin sensitivity and glucose homeostasis', *Journal of Applied Physiology,* 102, pp. 814-816.

Pedersen, B. & Fischer, C. (2007) 'Physiological roles of muscle-derived interleukin-6 in response to exercise ', *Current Opinion in Clinical Nutrition and Metabolic Care,* 10 (3), pp. 265-271.

Pedersen, B., Steensberg, A., Keller, P., Keller, C., Fischer, C., Hiscock, N., Van Hall, G., Plomgaard, P. & Febbraio, M. (2003) 'Muscle-derived interleukin-6: lypolytic, anti-Inflammatory, and immune regulatory effects ', *European Journal of Physiology,* 446, pp. 9-16.

Pedersen, B. K., Steensberg, A., Fischer, C., Keller, C., Keller, P., Plomgaard, P., Febbraio, M. & Saltin, B. (2003) 'Searching for the exercise factor: is IL-6 a candidate?', *Journal of Muscle Research and Cell Motility,* 24 (2), pp. 113-119.

Peeling, P., Dawson, B., Goodman, C., Landers, G. & Trinder, D. (2008) 'Athletic induced iron deficiency: new insights into the role of inflammation, cytokines and hormones', *European Journal of Applied Physiology,* 103 (4), pp. 381-391.

Peeling, P., Dawson, B., Goodman, C., Landers, G., Wiegerinck, E., Swinkels, D. & Trinder, D. (2009) 'Cumulative effects of consecutive running sessions on hemolysis, inflammation and hepcidin activity', *European Journal of Applied Physiology,* 106 (1), pp. 51-59.

Peeling, P., Dawson, B., Goodman, C., Landers, G., Wiegerinck, E., Swinkels, D. W. & Trinder, D. (2009) 'Training surface and intensity: Inflammation, hemolysis, and hepcidin expression', *Medicine & Science in Sports & Exercise,* 41 (5), pp. 1138-1145

Pereira, L. S. M., Narciso, F. M. S., Oliveira, D. M. G., Coelho, F. M., Souza, D. d. G. d. & Dias, R. C. (2008) 'Correlation between manual muscle strength and interleukin-6 (IL-6) plasma levels in elderly community-dwelling women', *Archives of Gerontology and Geriatrics,* 48 (3), pp. 313-316.

Peters, M., Muller, A. & Rose-John, S. (1998) 'Interleukin-6 and soluble interleukin-6 receptor direct stimulation of gp130 and hematopoises', *Blood,* 92 (10), pp. 3495-3504.

Petersen, E., Carey, A., Sacchetti, M., Steinberg, G., Macaulay, S., Febbraio, M. & Pedersen, B. (2005) 'Acute IL-6 treatment increases fatty acid turnover in elderly humans in vivo and in tissue culture in vivo', *American Journal of Physiology Endocrine and Metabolism,* 288, pp. E155-E162.

Phillips, G. C. (2007) 'Glutamine: The nonessential amino acid for performance enhancement', *Current Sports Medicine Reports,* 6 (4), pp. 265-268

Pietrangelo, A., Dierssen, U., Valli, L., Garuti, C., Rump, A., Corradini, E., Ernst, M., Klein, C. & Trautwein, C. (2007) 'STAT3 is required for

IL-6-gp130-dependent activation of hepcidin in vivo', *Gastroenterology,* 132 (1), pp. 294-300.

Pinniger, G., Steele, J. & Groeller, H. (2000) 'Does fatigue induced by repeated dynamic efforts affect hamstring muscle function?', *Medicine & Science in Sports & Exercise,* 32 (3), pp. 647-653.

Pottier, A., Bouckaert, J., Gilis, W., Roels, T. & Derave, W. (2010) 'Mouth rinse but not ingestion of a carbohydrate solution improves 1-h cycle time trial performance', *Scandinavian Journal of Medicine & Science in Sports,* 20 (1), pp. 105-111.

Poupart, P., Vandenabeel, P., Cayphas, S., Van Snick, J., Haegerman, G., Kruys, V., Fiers, W. & Content, J. (1987) 'B cell growth modulating and differentiating activity of recombinant human 26-kd protein (BSF-2, HuIFN-beta 2, HPGF)', *The EMBO Journal,* 1219-1224.

Pradhan, A., Manson, J., Rifai, N., Buring, J. & Ridker, P. (2001) 'C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus ', *Journal of the American Medical Association,* 286 (3), pp. 327-334.

Ramey, G., Deschemin, J.-C., Durel, B., Canonne-Hergaux, F., Nicolas, G. & Vaulont, S. (2010) 'Hepcidin targets ferroportin for degradation in hepatocytes', *Haematologica,* 95 (3), pp. 501-504.

Raney, M. A. & Turcotte, L. P. (2006) 'Regulation of contraction-induced FA uptake and oxidation by AMPK and ERK1/2 is intensity dependent in rodent muscle', *American Journal of Physiolology Endocrinology and Metabolism,* 291 (6), pp. E1220-1227.

Rauch, H., Rodger, I., Wilson, G., Belonje, D., Dennis, S., Noakes, T. & Hawley, J. (1995) 'The effects of carbohydrate loading on muscle glycogen content and cycling performance', *International Journal of Sport Nutrition,* 5 (1), pp. 25-36.

Rauch, H., St Clair Gibson, A., Lambert, E. & Noakes, T. (2005) 'A signalling role for muscle glycogen in the regulation of pace during prolonged exercise', *British Journal of Sports Medicine,* 39, pp. 34-38.

Rebouissou, C., Wijdenes, J., Autissier, P., Tarte, K., Costes, V., Liautard, J., Rossi, J.-F., Brochier, J. & Klein, B. (1998) 'A gp130 interleukin-6 transducer-dependent SCID model of human multiple myeloma', *Blood,* 91 (12), pp. 4727-4737.

Rhen, T. & Cidlowski, J. A. (2005) 'Antiinflammatory action of glucocorticoids. New mechanisms for old drugs', *New England Journal of Medicine,* 353 (16), pp. 1711-1723.

Rhode, T., Maclean, D. & Pedersen, B. (1998) 'Effect of glutamine supplementation on changes in the immune system induced by repeated exercise', *Medicine and Science in Sports and Exercise,* 30, pp. 856-862.

Rivera, S., Nemeth, E., Gabayan, V., Lopez, M. A., Farshidi, D. & Ganz, T. (2005) 'Synthetic hepcidin causes rapid dose-dependent hypoferremia and is concentrated in ferroportin-containing organs', *Blood,* 106 (6), pp. 2196-2199.

Robson, P. J., Blannin, A. K., Walsh, N. P., Castell, L. M. & Cleeson, M. (1999) 'Effects of exercise intensity, duration and recovery on in vitro neutrophill function in male athletes', *International Journal of Sports Medicine,* 20 (02), pp. 128,130.

Robson-Ansley, P., Barwood, M., Canavan, J., Hack, S., Eglin, C., Davey, S., Hewitt, J., Hull, J. & Ansley, L. (2009) 'The effect of repeated endurance exercise on IL-6 and sIL-6R and their relationship with sensations of fatigue at rest.', *Cytokine,* 45 (2), pp. 111-116.

Robson-Ansley, P., Barwood, M., Eglin, C. & Ansley, L. (2009) 'The effect of carbohydrate ingestion on the interleukin-6 response to a 90-minute run time trial', *International Journal of Sports Physiology and Performance,* 4 (2), pp. 186-194.

Robson-Ansley, P., Blannin, A. & Gleeson, M. (2007) 'Elevated plasma interleukin-6 levels in trained male triathletes following an acute period of intense interval training', *European Journal of Applied Physiology,* 99 (4), pp. 353-360.

Robson-Ansley, P., de Milander, L., Collins, M. & Noakes, T. (2004) 'Acute interleukin-6 administration impairs athletic performance in healthy, trained male runners.', *Canadian Journal of Applied Physiology,* 29 (4), pp. 411-418.

Robson-Ansley, P., de Milander, L., Collins, M. & Noakes, T. D. (2006) 'Acute interleukin-6 administration does not alter plasma soluble interleukin-6 receptor concentration following an acute bout of exercise', *Proceedings of the 11th annual congress of the European college of sports sciences.*

Roecker, L., Meier-Buttermilch, R., Brechtel, L., Nemeth, E. & Ganz, T. (2005) 'Iron-regulatory protein hepcidin is increased in female athletes after a marathon', *European Journal of Applied Physiology,* 95 (5), pp. 569-571.

Roedde, S., MacDougall, J., Sutton, J. & Green, H. (1986) 'Supercompensation of muscle glycogen in trained and untrained subjects', *Canadian Journal of Applied Sport Sciences,* 11 (1), pp. 42-46.

Rohde, T., MacLean, D., Hartkopp, A. & Pedersen, B. (1996) 'The immune system and serum glutamine during a triathlon', *European Journal of Applied Physiology and Occupational Physiology,* 74 (5), pp. 428-434.

Rollo, I., Cole, M., Miller, R. & Williams, C. (2010) 'Influence of mouth rinsing a carbohydrate solution on 1-h running performance', *Medicine & Science in Sports & Exercise,* 42 (4), pp. 798-804

Ronsen, O., Lea, T., Bahr, R. & Pedersen, B. K. (2002) 'Enhanced plasma IL-6 and IL-1ra responses to repeated vs. single bouts of prolonged cycling in elite athletes', *Journal of Applied Physiology,* 92 (6), pp. 2547-2553.

Rotter, V., Nagaev, I. & Smith, U. (2003) 'Interleukin-6 (IL-6) induces insulin resistance in LT3-L1 adipocytes and is, like IL-8 and tumor necrosis factor alpha, overexpressed in human fat cells from insulin resistant subjects', *The Journal of Biological Chemistry,* 278 (46), pp. 45777-45784.

Russell, R., Redmann, S., Ravussin, E., Hunter, G. & Larson-Meyer, D. (2004) 'Reproducibility of endurance performance on a treadmill using a preloaded time trial', *Medicine and Science in Sports and Exercise,* 36 (4), pp. 717-724.

Sakamoto, K. & Goodyear, L. J. (2002) 'Exercise effects on muscle insulin signaling and action: Invited review: Intracellular signaling in contracting skeletal muscle', *Journal of Applied Physiology,* 93 (1), pp. 369-383.

Saltin, B., Hartley, L. H., Kilbom, A. & Astrand, I. (1969) 'Physical training in sedentary middle-aged and older men II. oxygen uptake, heart rate, and blood lactate concentration at submaximal and maximal exercise', *Scandinavian Journal of Clinical & Laboratory Investigation,* 24 (4), pp. 323-334.

Schabort, E., Hopkins, W. & Hawley, J. (1998) 'Reproducibility of self-paced treadmill performance of trained endurance runners', *International Journal of Sports Medicine,* 19, pp. 48-51.

Scharhag, J., Meyer, T., Auracher, M., Gabriel, H. & Kindermann, W. (2006) 'Effects of graded carbohydrate supplementation on the

immune response in cycling', *Medicine & Science in Sports & Exercise,* 38 (2), pp. 286-292

Schieffer, B., Schieffer, E., Hilfiker-Kleiner, D., Hilfiker, A., Kovanen, P. T., Kaartinen, M., Nussberger, J., Harringer, W. & Drexler, H. (2000) 'Expression of angiotensin II and interleukin 6 in human coronary atherosclerotic plaques : Potential implications for inflammation and plaque instability', *Circulation,* 101 (12), pp. 1372-1378.

Schindler, R., Mancilla, J., Endres, S., Ghorbani, R., Clark, S. & Dinarello, C. (1990) 'Correlations and interactions in the production of interleukin-6 (IL- 6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF', *Blood,* 75 (1), pp. 40-47.

Schmidt, K. N., Amstad, P., Cerutti, P. & Baeuerle, P. A. (1995) 'The roles of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF-kappaB', *Chemistry & Biology,* 2 (1), pp. 13-22.

Schobitz, B., de Kloet, E. R., Sutanto, W. & Holsboer, F. (1993) 'Cellular localization of interleukin 6 mRNA and interleukin 6 receptor mRNA in rat brain', *European Journal of Neuroscience,* 5 (11), pp. 1426-1435.

Schobitz, B., Pezeshki, G., Pohl, T., Hemmann, U., Heinrich, P. C., Holsboer, F. & Reul, J. M. (1995) 'Soluble interleukin-6 (IL-6) receptor augments central effects of IL-6 in vivo', *Federation of American Societies for Experimental Biology,* 9 (8), pp. 659-664.

Schubert, C., Hong, S., Natarajan, L., Mills, P. & Dimsdale, J. (2007) 'The association between fatigue and the inflammatory marker level in cancer patients: A quantitative review', *Brain, Behavior and Immunity,* 21, pp. 413-427.

Schumacker, Y., Schmid, A., Grathwohl, D., Bultermann, D. & Berg, A. (2002) 'Hematological indices and iron status in athletes of various sports and performances', *Medicine & Science in Sports & Exercise,* 34 (5), pp. 869-875.

Schwarz, A. J., Brasel, J. A., Hintz, R. L., Mohan, S. & Cooper, D. M. (1996) 'Acute effect of brief low- and high-intensity exercise on circulating insulin-like growth factor (IGF) I, II, and IGF-binding protein-3 and its proteolysis in young healthy men', *Journal of Clinical Endocrinology Metabolism,* 81 (10), pp. 3492-3497.

Senn, J., Klover, P., Nowak, I. & Mooney, R. (2002) 'Interleukin-6 induces cellular insulin resistance in hepatocytes', *Diabetes,* 51 (12), pp. 3391-3399.

Sewell, D., Gleeson, M. & Blannin, A. (1994) 'Hyperammonaemia in relation to high-intensity exercise duration in man', *European Journal of Applied Physiology and Occupational Physiology,* 69 (4), pp. 350-354.

Sherman, W. M., Costill, D. L., Fink, W. J. & Miller, J. M. (1981) 'Effect of exercise-diet manipulation on muscle glycogen and its subsequent utilization during performance', *International Journal of Sports Medicine,* 02 (02), pp. 114,118.

Sierens, J., Hartley, J. A., Campbell, M. J., Leathem, A. J. C. & Woodside, J. V. (2001) 'Effect of phytoestrogen and antioxidant supplementation on oxidative DNA damage assessed using the comet assay', *Mutation Research/DNA Repair,* 485 (2), pp. 169-176.

Silverman, M. N., Miller, A. H., Biron, C. A. & Pearce, B. D. (2004) 'Characterization of an interleukin-6 and adrenocorticotropin-dependent, immune-to-adrenal pathway during viral infection', *Endocrinology,* 145 (8), pp. 3580-3589.

Song, C., Merali, Z. & Anisman, H. (1999) 'Variations of nucleus accumbens dopamine and serotonin following systemic interleukin-1, interleukin-2 or interleukin-6 treatment', *Neuroscience,* 88 (3), pp. 823-836.

Southard, J., Romijn, J., Van der Poll, T., Endert, E., Klein, S., Bakker, P., Veenhof, C. & Sauerwein, H. (1995) 'Endocrinilogic and metabolic effects of Interleukin-6 in humans', *American Journal of Physiology Endocrine and Metabolism,* 31, pp. E813-E819.

Sparkman, N., Buchanan, J., Heyen, J., Chen, J., Beverly, J. & Johnson, R. (2006) 'Interleukin-6 facilitates lipopolysaccharide-induced disruption in working memory and expression of other proinflammatory cytokines in hippocampal neuronal cell layers.', *The Journal of Neuroscience,* 18 (26), pp. 10709-10716.

Spath-Schwalbe, E., Born, J., Schrezenmeier, H., Bornstein, S. R., Stromeyer, P., Drechsler, S., Fehm, H. L. & Porzsolt, F. (1994) 'Interleukin-6 stimulates the hypothalamus-pituitary-adrenocortical axis in man', *Journal of Clinical Endocrinology Metabolism,* 79 (4), pp. 1212-1214.

Spath-Schwalbe, E., Hansen, K., Schmidt, F., Schrezenmeier, H., Marshall, L., Burger, K., Fehm, H. L. & Born, J. (1998) 'Acute effects of recombinant human interleukin-6 on endocrine and central nervous sleep functions in healthy men', *Journal of Clinical Endocrinology Metabolism,* 83 (5), pp. 1573-1579.

Starkie, R., Arkinstall, M., Koukoulas, I., Hawley, J. & Febbraio, M. (2001) 'Carbohydrate ingestion attenuates the increase in plasma interleukin-6, but not skeletal muscle interleukin-6 mRNA, during exercise in humans.', *The Journal of Physiology,* 1 (533), pp. 585-591.

Steensberg, A. (2003) 'The role of IL-6 in exercise-induced immune changes and metabolism.', *Exercise Immunology Review,* 9, pp. 40-47.

Steensberg, A., Fischer, C. P., Keller, C., Moller, K. & Pedersen, B. K. (2003) 'IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans', *American Journal of Physiolology Endocrinology and Metabolism,* 285 (2), pp. E433-437.

Steensberg, A., Van Hall, G., Osada, T., Sacchetti, M., Saltin, B. & Klarland, P. (2000) 'Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6', *Journal of Physiology,* 541, pp. 273-281.

Steinacker, J., Lormes, W., Reissnecker, S. & Liu, Y. (2004) 'New aspects of the hormone and cytokine response to training', *European Journal of Applied Physiology,* 91, pp. 382-391.

Stellingwerff, T., Boon, H., Gilsen, A., Stegen, J., Kuipers, H. & van Loon, L. (2007) 'Carbohydrate supplementation during prolonged cycling exercise spares musce glycogen but does not affect intramyocellular lipid use', *European Journal of Applied Physiology,* 454, pp. 635-647.

Stitt, J. (1990) 'Passage of immunomodulators across the blood-brain barrier', *The Yale Journal of Biology and Medicine,* 63 (2), pp. 121-131.

Stouthard, J. M. L., Oude Elferink, R. P. J. & Sauerwein, H. P. (1996) 'Interleukin-6 enhances glucose transport in 3T3-L1 adipocytes', *Biochemical and Biophysical Research Communications,* 220 (2), pp. 241-245.

Strachan, A. F., Noakes, T. D., Kotzenberg, G., Nel, A. E. & de Beer, F. C. (1984) 'C reactive protein concentrations during long distance running', *British Medical Journal (Clinical research ed.),* 289 (6454), pp. 1249-1251.

Strehlow, I. & Schindler, C. (1998) 'Amino-terminal signal transducer and activator of transcription (STAT) domains regulate nuclear

translocation and STAT deactivation', *Journal of Biological Chemistry,* 273 (43), pp. 28049-28056.

Stump, C. S., Henriksen, E. J., Wei, Y. & Sowers, J. R. (2006) 'The metabolic syndrome: Role of skeletal muscle metabolism', *Annals of Medicine,* 38 (6), pp. 389-402.

Stumvoll, M., Meyer, C., Perriello, G., Kreider, M., Welle, S. & Gerich, J. (1998) 'Human kidney and liver gluconeogenesis: evidence for organ substrate selectivity', *American Journal of Physiolology Endocrinology and Metabolism,* 274 (5), pp. E817-826.

Swain, M. G. (2000) 'Fatigue in chronic disease', *Clinical. Science.,* 99 (1), pp. 1-8.

Taaffe, D. R., Harris, T. B., Ferrucci, L., Rowe, J. & Seeman, T. E. (2000) 'Cross-sectional and prospective relationships of interleukin-6 and C-reactive protein with physical performance in elderly persons', *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 55 (12), pp. M709-M715.

Tabata, I., Ogita, F., Miyachi, M. & Shibayama, H. (1991) 'Effect of low blood glucose on plasma CRF, ACTH, and cortisol during prolonged physical exercise', *Journal of Applied Physiology,* 71 (5), pp. 1807-1812.

Tayek, J. A. & Katz, J. (1997) 'Glucose production, recycling, Cori cycle, and gluconeogenesis in humans: relationship to serum cortisol', *American Journal of Physiolology Endocrinology and Metabolism,* 272 (3), pp. E476-484.

Taylor, L. & Curthoys, N. P. (2004) 'Glutamine metabolism: Role in acid-base balance', *Biochemistry and Molecular Biology Education,* 32 (5), pp. 291-304.

Telford, R. D., Sly, G. J., Hahn, A. G., Cunningham, R. B., Bryant, C. & Smith, J. A. (2003) 'Footstrike is the major cause of hemolysis during running', *Journal of Applied Physiology,* 94 (1), pp. 38-42.

Tsigos, C. & Chrousos, G. P. (2002) 'Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress', *Journal of Psychosomatic Research,* 53 (4), pp. 865-871.

Tsigos, C., Papanicolaou, D. A., Defensor, R., Mitsiadis, C. S., Kyrou, I. & Chrousos, G. P. (1997) 'Dose effects of recombinant human interleukin-6 on pituitary hormone secretion and energy expenditure', *Neuroendocrinology,* 66 (1), pp. 54-62.

Tsintzas, O. K., Williams, C., Boobis, L. & Greenhaff, P. (1996) 'Carbohydrate ingestion and single muscle fiber glycogen metabolism during prolonged running in men', *Journal of Applied Physiology,* 81 (2), pp. 801-809.

Tyler, C. & Sunderland, C. (2008) 'The effect of ambient temperature on the reliability of a preloaded treadmill time-trial', *International Journal of Sports Medicine,* 29, pp. 812-816.

Ulmer, H. V. (1996) 'Concept of an extracellular regulation of muscular metabolic rate during heavy exercise in humans by psychophysiological feedback', *Cellular and Molecular Life Sciences,* 52 (5), pp. 416-420.

Utter, A., Kang, J., Nieman, D., Williams, F., Robertson, R., Henson, D., Davis, J. & Butterworth, D. (1999) 'Effect of carbohydrate ingestion and hormonal responses on ratings of percieved exertion during prolonged cycling and running', *European Journal of Applied Physiology,* 80, pp. 92-99.

Utter, A., Kang, J., Nieman, D., Dumke, C., Mcnaulty, S., Vinci, D. & Mcnaulty, L. (2004) 'Carbohydrate supplementation and perceived exertion during prolonged running', *Medicine & Science in Sports & Exercise,* 36 (6), pp. 1036-1041.

Vallieres, L., Lacroix, S. & Rivest, S. (1997) 'Influence of interleukin-6 on neural activity and transcription of the gene encoding corticotrophin-releasing factor in the rat brain: An effect depending upon the route of administration', *European Journal of Neuroscience,* 9 (7), pp. 1461-1472.

Vallieres, L. & Rivest, S. (1997) 'Regulation of the genes encoding interleukin-6, its receptor, and gp130 in the rat brain in response to the immune activator lipopolysaccharide and the proinflammatory cytokine interleukin-1β', *Journal of Neurochemistry,* 69 (4), pp. 1668-1683.

Vallieres, L. & Rivest, S. (1999) 'Interleukin-6 Is a needed proinflammatory cytokine in the prolonged neural activity and transcriptional activation of corticotropin-releasing factor during endotoxemia', *Endocrinology,* 140 (9), pp. 3890-3903.

Van der Hulst, R. R. W. J., Von Meyenfeldt, M. F., Deutz, N. E. P., Soeters, P. B., Brummer, R. J. M., Von Kreel, B. K. & Arends, J. W. (1993) 'Glutamine and the preservation of gut integrity', *The Lancet,* 341 (8857), pp. 1363-1365.

Van Hall, G., Steensberg, A., Sacchetti, M., Fischer, C., Keller, C., Schjerling, P., Hiscock, N., Moller, K., Saltin, B., Febbraio, M. &

Pedersen, B. (2003) 'Interleukin-6 stimulates lipolysis and fat oxidation in humans ', *Journal of Clinical Endocrinology and Metabolism,* 88, pp. 3005-3010.

Van Snick, J., Cayphas, S., Vink, A., Uyttenhove, C., Coulie, P., Rubira, M. & Simpson, R. (1986) 'Purification and NH2-terminal amino acid sequence of a T-cell-derived lymphokine with growth factor activity for B-cell hybridomas ', *Proceedings of the National Acamedy of Sciences,* 83, pp. 9679-9683.

Vila-Coro, A. J., Rodreguez-Frade, J. M., Marten De Ana, A. N. A., Moreno-Ortez, M. C., Martenez-A, C. & Mellado, M. (1999) 'The chemokine SDF-1 triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway', *Federation of American Societies for Experimental Biology,* 13 (13), pp. 1699-1710.

Visser, M., Pahor, M., Taaffe, D. R., Goodpaster, B. H., Simonsick, E. M., Newman, A. B., Nevitt, M. & Harris, T. B. (2002) 'Relationship of interleukin-6 and tumor necrosis factor-alpha with muscle mass and muscle strength in elderly men and women', *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 57 (5), pp. M326-M332.

Waguespack, P. J., Banks, W. A. & Kastin, A. J. (1994) 'Interleukin-2 does not cross the blood-brain barrier by a saturable transport system', *Brain Research Bulletin,* 34 (2), pp. 103-109.

Walker, J. L., Heigenhauser, G. J. F., Hultman, E. & Spriet, L. L. (2000) 'Dietary carbohydrate, muscle glycogen content, and endurance performance in well-trained women', *Journal of Applied Physiology,* 88 (6), pp. 2151-2158.

Waller, M. & Haymes, E. (1996) 'The effects of heat and exercise on sweat iron loss', *Medicine & Science in Sports & Exercise,* 28 (2), pp. 197-203.

Walsh, N. P., Blannin, A. K., Bishop, N. C., Robson, P. J. & Gleeson, M. (2000) 'Effect of oral glutamine supplementation on human neutrophil lipopolysaccharide-stimulated degranulation following prolonged exercise', *International Journal of Sport Nutrition,* 10 (1), pp. 39-50.

Walsh, N. P., Blannin, A. K., Robson, P. J. & Gleeson, M. (1998) 'Glutamine, exercise and immune function: Links and possible mechanisms', *Sports Medicine,* 26, pp. 177-191.

Walshe, I., Robson-Ansley, P., St Clair Gibson, A., Lawrence, C., Thompson, K. & Ansley, L. (2010) 'The reliability of the IL-6, sIL-6R

and sgp130 response to a preloaded time trial', *European Journal of Applied Physiology,* 110 (3), pp. 619-625.

Wang, J. & Dunn, A. J. (1998) 'Mouse interleukin-6 stimulates the HPA axis and increases brain tryptophan and serotonin metabolism', *Neurochemistry International,* 33 (2), pp. 143-154.

Wang, Z., Castresana, M. R. & Newman, W. H. (2001) 'Reactive oxygen and NF-kappaB in VEGF-induced migration of human vascular smooth muscle cells', *Biochemical and Biophysical Research Communications,* 285 (3), pp. 669-674.

Ward, D., Roberts, K., Stonelake, P., Goon, P., Zampronio, C., Martin, A., Johnson, P., Iqbal, T. & Tslepsis, C. (2008) 'SELDI-TOF-MS determination of hepcidin in clinical samples using stable isotope labelled hepcidin as an internal standard', *Proteome Science,* 6 (28).

Watkins, L., Maier, S. & Goehler, L. (1995) 'Cytokine-to-brain communication: A review & analysis of alternative mechanisms ', *Life Sciences,* 57 (11), pp. 1011-1026.

Weber, J., Yang, J. C., Topalian, S. L., Parkinson, D. R., Schwartzentruber, D. S., Ettinghausen, S. E., Gunn, H., Mixon, A., Kim, H. & Cole, D. (1993) 'Phase I trial of subcutaneous interleukin-6 in patients with advanced malignancies', *Journal of Clinical Oncology,* 11 (3), pp. 499-506.

Wei, J., Xu, H., Davies, J. L. & Hemmings, G. P. (1992) 'Increase of plasma IL-6 concentration with age in healthy subjects', *Life Sciences,* 51 (25), pp. 1953-1956.

Weiss, M. S. & Hilgenfeld, R. (1997) 'On the use of the merging R factor as a quality indicator for X-ray data', *Journal of Applied Crystallography,* 30 (2), pp. 203-205.

Welsh, R. S., Davis, M. J., Burke, J. R. & Williams, H. G. (2002) 'Carbohydrates and physical/mental performance during intermittent exercise to fatigue', *Medicine & Science in Sports & Exercise,* 34 (4), pp. 723-731.

Widegren, U., Jiang, X. J., Krook, A., Chibalin, A. V., Bjornholm, M., Tally, M., Roth, R. A., Henriksson, J., Wallberg-henriksson, H. & Zierath, J. R. (1998) 'Divergent effects of exercise on metabolic and mitogenic signaling pathways in human skeletal muscle', *Federation of American Societies for Experimental Biology,* 12 (13), pp. 1379-1389.

Widegren, U., Wretman, C., Lionikas, A., Hedin, G. & Henriksson, J. (2000) 'Influence of exercise intensity on ERK/MAP kinase signalling in human skeletal muscle', *Pflügers Archive European Journal of Physiology,* 441 (2), pp. 317-322.

Wratten, C., Kilmurray, J., Nash, S., Seldon, M., Hamilton, C. S., O'Brien, P. C. & Denham, J. W. (2004) 'Fatigue during breast radiotherapy and its relationship to biological factors', *International Journal of Radiation Oncology Biology Physics,* 59 (1), pp. 160-167.

Wrighting, D. M. & Andrews, N. C. (2006) 'Interleukin-6 induces hepcidin expression through STAT3', *Blood,* 108 (9), pp. 3204-3209.

Xin, L. & Blatteis, C. M. (1992) 'Hypothalamic neuronal responses to interleukin-6 in tissue slices: Effects of indomethacin and naloxone', *Brain Research Bulletin,* 29 (1), pp. 27-35.

Yamagata, K., Matsumura, K., Inoue, W., Shiraki, T., Suzuki, K., Yasuda, S., Sugiura, H., Cao, C., Watanabe, Y. & Kobayashi, S. (2001) 'Coexpression of microsomal-type prostaglandin E synthase with cyclooxygenase-2 in brain endothelial cells of rats during endotoxin-induced fever', *Journal of Neuroscience,* 21 (8), pp. 2669-2677.

Yamaguchi, M., Matsuzaki, N., Hirota, K., Miyake, A. & Tanizawa, O. (1990) 'Interleukin 6 possibly induced by interleukin 1beta in the pituitary gland stimulates the release of gonadotropins and prolactin', *Acta Endocrinolologica,* 122 (2), pp. 201-205.

Yaspelkis, B., Patterson, J., Anderla, P., Ding, Z. & Ivy, J. (1993) 'Carbohydrate supplementation spares muscle glycogen during variable-intensity exercise', *Journal of Applied Physiology,* 75 (4), pp. 1477-1485.

Young, S. N. & Leyton, M. (2002) 'The role of serotonin in human mood and social interaction: Insight from altered tryptophan levels', *Pharmacology Biochemistry and Behavior,* 71 (4), pp. 857-865.

Yu, M., Stepto, N. K., Chibalin, A. V., Fryer, L. G. D., Carling, D., Krook, A., Hawley, J. A. & Zierath, J. R. (2003) 'Metabolic and mitogenic signal transduction in human skeletal muscle after intense cycling exercise', *The Journal of Physiology,* 546 (2), pp. 327-335.

Zhang, J., Johnston, G., Stebler, B. & Keller, E. T. (2001) 'Hydrogen peroxide activates NFKB and the interleukin-6 promoter through NFKB-inducing kinase', *Antioxidants & Redox Signaling,* 3 (3), pp. 493-504.

Zhang, J.-j., Terreni, L., De Simoni, M. G. & Dunn, A. J. (2001) 'Peripheral interleukin-6 administration increases extracellular concentrations of serotonin and the evoked release of serotonin in the rat striatum', *Neurochemistry International,* 38 (4), pp. 303-308.

Zong, C. S., Chan, J., Levy, D. E., Horvath, C., Sadowski, H. B. & Wang, L.-H. (2000) 'Mechanism of STAT3 activation by insulin-like growth factor I receptor', *Journal of Biological Chemistry,* 275 (20), pp. 15099-15105.

Appendicies

Participant Information

PROJECT DETAILS

TITLE OF PROJECT: The repeatability of the cytokine response to prolonged exercise**.**

Principal Investigator: Ian Walshe

Investigator contact details: Phone: 07745520939
                               Email: ian.walshe@unn.ac.uk

1. What is the purpose of the project?
In this project we are investigating a model of an exercise bout that can induce a messenger molecule (cytokine) response and to ascertain the variation of the response from this model. Furthermore, we are investigating the performance effects of an induced cytokine response from this model.

2. Why have I been selected to take part?

You have asked to participate because you are male and aged between 18 and 35. You will also be involved in regular endurance running.

3. What will I have to do? For the experiment you will be asked to visit the laboratory on four occasions:

Visit One –

Suitability for the study:

Initially you will complete a health-history questionnaire concerning your past and present health status in private. It is important to be entirely honest and point out any known health problems, even if they seem trivial. We will also need to know if you have been on medication at any time during the four weeks prior to the study. At this time we will also ask you to fill out a questionnaire regarding your training habits. Any answers you give will be kept in strict confidence.

$\dot{V}$**O$_2$max Test:** Providing that you are suitable for the study and you feel that you want to be involved, you will then perform a treadmill exercise test to volitional exhaustion in order to determine your aerobic capacity ($\dot{V}$O$_2$max). You will start by running on a treadmill at 8.0 kph and the speed will be increased by 2.0 kph every three minutes until volitional fatigue. We will collect gas samples from you

(you will wear a nose clip and breathe through a mouthpiece and we will collect your expired air) and record your heart rate.

Visit one will last no more than 1 hour and can take place at any time of day.

Visits Two, Three and Four-

Pre- exercise: For the 24 hours before visits three and four we will ask you not to train or drink alcohol (it will affect your resting baseline immune values). We will also need you to make sure that you do not eat or drink any caffeine-containing foods and drinks for 24 hours before these visits. In addition we will provide you with standardised food to eat in the 24 hours before your trial, this helps to standardise your responses to the exercise test. We will also ask you to refrain from eating and drinking sports drinks12 hours before the trial but you can drink as much water as you like.

Before the test, you will be asked to go to the lavatory and then we will record your body mass. You will then be asked to provide a urine sample (in private) and a saliva sample. After sitting quietly for 10 minutes, we will then take a resting blood sample from the antecubital vein in the arm. You will then be asked to perform a test of memory (word recall).

Exercise Bouts: Visit two will give you a chance to familiarise yourself to the exercise test and to the methods of data collection that you may be unfamiliar with such as blood sampling and using the treadmill controls. On this visit, you will be asked to run at a speed that is equivalent to an exercise intensity that is 60% of your speed at $\dot{V}O_2$max for 2 hours. Every 10 minutes you will be required to run for 30 seconds at a speed that is 90% of your speed at $\dot{V}O_2$max. During the exercise we will analyse gas samples using a mouth piece and record your heart rate. Following this, you will be asked for another blood sample and allowed 5 minutes rest before performing a 5 kilometre time trial. You will be asked to run the time trial as fast as possible. During the trial, you will be informed of the distance you have run throughout the trial. Following the trial, you will be asked for al urine, saliva and blood sample. The test of memory will also be performed at this time. Final blood samples will then be taken one hour after exercise and approximately 24 hours after exercise.   Visits two, three and four must take place in the morning and will last no more than 3.5 hours each.

At all visits you will be asked to fill in a health questionnaire to confirm your fitness to participate in the study on that day.

4. What are the exclusion criteria (i.e. are there any reasons why I should not take part)?

Exclusion criteria will include being unaccustomed to vigorous exercise and experience of any symptoms of infection in the four weeks prior to the study or at any point during the study.

5. Will my participation involve any physical discomfort?

You may feel some discomfort towards the end of the exercise periods associated with long distance running due to the fatiguing nature of the tests

6. Will my participation involve any psychological discomfort or embarrassment?

No

7. Will I have to provide any bodily samples (i.e. blood, saliva)?

Pre exercise you will be asked to provide a saliva sample, blood sample from the antecubital vein in the arm and a urine sample in private. After the exercise bout you will be asked for a blood sample from the antecubital vein. After the time trial you will be asked for saliva, blood and urine samples as specified with pre exercise.

8. How will confidentiality be assured?

You will only be known by a number to protect anonymity. All data will be kept in a locked cabinet or computerised and accessed via a password which will be done in accordance with the Data Protection Act.

9. Who will have access to the information that I provide?

Only the principle investigator and supervisor will have access to information that you provide.

10. How will my information be stored / used in the future?

All data will be kept in a locked cabinet or computerised and accessed via a password which will be done in accordance with the Data Protection Act. Data may be used for publication in the form of a scientific paper, presented at a conference or both. Any data used can not be linked to you.

11. Has this investigation received appropriate ethical clearance?

Yes

12. Will I receive any financial rewards / travel expenses for taking part?

You will be paid £70 on completion of your participation. A further £30 will be given to the participant with the quickest average time trial.

13. Can I withdraw from the project?

You can withdraw from the project at any point without providing reasons for doing so and without prejudice.

14. If I require further information who should I contact and how?

For further information please contact myself by telephone on 07745520939 or by email at ian.walshe@unn.ac.uk

PARTICIPANT INFORMATION.

TITLE OF PROJECT: The effect of carbohydrate ingestion on the inflammatory response to exercise.

Principal Investigator: Ian Walshe

Investigator contact details:

Phone: 07745520939

Email: ian.walshe@unn.ac.uk

This project is funded by: Biotechnology and Biological Sciences Research Council and GlaxoSmithKline

Number of participant points / payment: £90

1. What is the purpose of the project? During prolonged exercise messenger molecules (cytokines) have been shown to increase in blood circulation and may cause fatigue. The purpose of this project is to examine the effect of carbohydrate ingestion on cytokines and subsequently the affect on fatigue.

2. Why have I been selected to take part?

You have asked to participate because you are male and aged between 18 and 40 years. You will also participate in regular endurance running and be able to complete a 2 hour bout of running

3. What will I have to do?

For the experiment you will be asked to visit the laboratory on four occasions:

Visit One –

Suitability for the study:

Initially you will complete a health-history questionnaire concerning your past and present health status in private. It is important to be entirely honest and point out any known health problems, even if they seem trivial. We will also need to know if you have been on medication at any time during the four weeks prior to the study. At this time we will also ask you to fill out a questionnaire regarding your training habits. Any answers you give will be kept in strict confidence.

$\dot{V}$**O₂max Test:** Providing that you are suitable for the study and you feel that you want to be involved, you will then perform a treadmill exercise test to volitional exhaustion in order to determine your aerobic capacity ($\dot{V}O_2$max). You will start by running on a treadmill at 8.0 kph and the speed will be increased by 2.0 kph every three minutes until volitional fatigue. We will collect gas samples from you (you will wear a mask and we will collect your expired air) and record your heart

rate. Visit one will last no more than 1 hour and can take place at any time of day.

Visit Two

Exercise Bout: Visit two will give you a chance to familiarise yourself to the exercise test and to the methods of data collection that you may be unfamiliar with such as using the treadmill controls. On this visit, you will be asked to run at a speed that is equivalent to an exercise intensity that is 60% of your speed at $\dot{V}O_2$max for 1 hour. Every 10 minutes you will be required to run for 30 seconds at a speed that is 90% of your speed at $\dot{v}O_2$max.

 You will also be asked to consume 2ml/kg of the carbohydrate drink every 20 minutes during the exercise bout. During the exercise bout we will analyse gas samples using a mouth piece and record your heart rate. Following this, you will be allowed 5 minutes rest before performing a 5 kilometre time trial. You will be asked to run the time trial as fast as possible. During the trial, you will be informed of the distance you have run throughout the trial. Following the trial you will be asked to perform another grip strength test. Visits two will last approximately 1.5 hours and can take place any time of day.

Visit 3 and 4

Pre- exercise: For the 24 hours before visit two we will ask you not to train or drink alcohol (it will affect your resting baseline immune values). We will also need you to make sure that you do not eat or drink any caffeine-containing foods and drinks for 24 hours before these visits (see the attached sheet). We will also ask you to refrain from eating 12 hours before the trial but you can drink water.

Before the test, you will be asked to go to the lavatory and then we will record your body mass. You will then be asked to consume 2ml/kg of a carbohydrate drink and will then be asked to perform a grip strength test using a hand grip dynamometer.

Visit 3 and 4 will consist of the same protocol as used in visit 2 however; the exercise trial will be 2 hours in duration followed by a 5km time trial. Before the exercise bout you will be asked to consume a carbohydrate drink or a taste matched placebo in a randomised order (2ml/kg) whilst during the exercise bout you will asked to consume the same drink (2ml/kg) every 20 minutes. Pre exercise you will be asked for a blood sample and a grip strength test as well as body mass. Following the exercise bout you will be asked for another blood sample and grip strength test before taking part in the time trial. Further blood samples and grip strength tests will be asked for immediately post time trial, 1 hour post and 24 hour post exercise.

At all visits you will be asked to fill in a health questionnaire to confirm your fitness to participate in the study on that day.

4. What are the exclusion criteria (i.e. are there any reasons why I should not take part)?

Exclusion criteria will include being unaccustomed to running for 2 hours and experience of any symptoms of infection in the four weeks prior to the study or at any point during the study.

5. Will my participation involve any physical discomfort?

You may feel some discomfort towards the end of the exercise periods associated with long distance running due to the fatiguing nature of the tests. You may experience some bruising from blood sampling, however this is normal and pain will be minimal.

6. Will my participation involve any psychological discomfort or embarrassment?

No

7. Will I have to provide any bodily samples (i.e. blood, saliva)?

You will be asked for blood samples at pre exercise, post exercise, post time trial, 1 hour post time trial and 24 hours post time trial.

8. How will confidentiality be assured?

You will only be known by a number to protect anonymity. All data will be kept in a locked cabinet or computerised and accessed via a password which will be done in accordance with the Data Protection Act.

9. Who will have access to the information that I provide?

Only the principle investigator and supervisor will have access to information that you provide

10. How will my information be stored / used in the future?

All data will be kept in a locked cabinet or computerised and accessed via a password which will be done in accordance with the Data Protection Act. Data may be used for publication in the form of a scientific paper, presented at a conference or both. Any data used cannot be linked to you.

11. Has this investigation received appropriate ethical clearance?

Yes

12. Will I receive any financial rewards / travel expenses for taking part?

You will be paid £90 on completion of your participation. A further £30 will be given to the participant with the quickest average time trial.

13. How can I withdraw from the project?

If you wish to withdraw from the project, you can inform me by email, telephone or in person. You can withdraw from the project at any point without providing reasons for doing so and without prejudice.

14. If I require further information who should I contact and how?

For further information please contact myself by telephone on 07745520939 or by email at ian.walshe@unn.ac.uk

PARTICIPANT INFORMATION.

TITLE OF PROJECT: The effect of glutamine ingestion on the inflammatory response to exercise.

Principal Investigator: Ian Walshe

Investigator contact details: 07745520939

Email: ian.walshe@unn.ac.uk

This project is funded by: Biotechnology and Biological Sciences Research Council and GlaxoSmithKline Nutritional Healthcare

 Payment: £90

1. What is the purpose of the project?
During prolonged exercise messenger molecules (cytokines) have been shown to increase in blood circulation and may cause fatigue. The purpose of this project is to examine the effect of glutamine (a form of protein) ingestion on cytokines and subsequently the affect on exercise performance.

2. Why have I been selected to take part?

You have asked to participate because you are male and aged between 18 and 40 years. You will also be endurance trained and participate in regular endurance running and be able to complete a 2 hour bout of running.

3. What will I have to do?

For the experiment you will be asked to visit the laboratory on four occasions:

Visit One –

Suitability for the study:

Initially you will complete a health-history questionnaire concerning your past and present health status in private. It is important to be entirely honest and point out any known health problems, even if they seem trivial. We will also need to know if you have been on medication at any time during the four weeks prior to the study. At this time we will also ask you to fill out a questionnaire regarding your training habits. Any answers you give will be kept in strict confidence.

$\dot{V}O_2$**max Test:** Providing that you are suitable for the study and you feel that you want to be involved, you will then perform a treadmill exercise test to volitional exhaustion in order to determine your aerobic capacity ($\dot{V}O_2$max). You will start by running on a treadmill at 8.0 kph and the speed will be increased by 2.0 kph every three minutes until volitional fatigue. We will collect gas samples from you (you will wear a mask and we will collect your expired air) and record your heart rate. Visit one will last no more than 1 hour and can take place at any time of day.

Visit Two

Pre- exercise: For the 24 hours before visit two we will ask you not to train or drink alcohol (it will affect your resting baseline immune values). We will also need you to make sure that you do not eat or drink any caffeine-containing foods and drinks for 24 hours before these visits (see the attached sheet). We will also ask you to refrain from eating and drinking 12 hours with the exception of water.

Before the test, you will be asked to go to the lavatory and then we will record your body mass. You will then be asked to consume 5ml/kg of body mass of flavoured water and will then be asked to perform a grip strength to fatigue test using a hand grip dynamometer.

Exercise Bout: Visit two will give you a chance to familiarise yourself to the exercise test and to the methods of data collection that you may be unfamiliar with such as using the treadmill controls. On this visit, you will be asked to run at a speed that is equivalent to an exercise intensity that is 60% of your speed at $\dot{V}O_2$max for 2 hours. Every 10 minutes you will be required to run for 30 seconds at a speed that is 90% of your maximum speed. You will be given lemon flavoured water at 30 minute intervals throughout the trial which will be the same for visits 3 and 4. During the exercise bout we will analyse gas samples using a mouth piece and record your heart rate. Following this, you will be allowed 5 minutes rest before performing a 5 kilometre time trial. You will be asked to run the time trial as fast as possible. During the trial, you will be informed of the distance you have run throughout the trial however, not made aware of the time elapsed. Following the trial you will be asked to perform another grip strength test. Visits two will last approximately 3.5 hours and can take place any time of day.

Visit 3 and 4: Visit 3 and 4 will consist of the same protocol as used in visit 2. Before the exercise bout you will be asked to consume 0.05g per kilogram of body mass of glutamine powder in flavoured water or a taste matched placebo in a randomised order before the exercise bout. Whilst during the exercise bout you will be given the same amount of flavoured water that was consumed in the familiarisation trial with added glutamine (0.05g per kilogram of body mass) in one of the trials. Pre exercise you will be asked for a venous blood sample from the arm and a grip strength test as well as body mass. Following the exercise bout you will be asked for another blood sample and grip strength test before taking part in the time trial. Further blood samples and grip strength tests will be asked for immediately post time trial, 1 hour post time trial and the following morning.

At all visits you will be asked to fill in a health questionnaire to confirm your fitness to participate in the study on that day.

4. What are the exclusion criteria (i.e. are there any reasons why I should not take part)?

Exclusion criteria will include being unaccustomed to running for 2 hours and experience of any symptoms of infection or injury in the four weeks prior to the study or at any point during the study.

5. Will my participation involve any physical discomfort?

You may feel some discomfort towards the end of the exercise periods associated with long distance running due to the fatiguing nature of the tests. You may experience some bruising from blood sampling, however this is normal and pain will be minimal.

6. Will my participation involve any psychological discomfort or embarrassment?

No

7. Will I have to provide any bodily samples (i.e. blood, saliva)?

You will be asked for blood samples at pre exercise, post exercise, post time trial, 1 hour post time trial and the following morning.

8. How will confidentiality be assured?

You will only be known by a number to protect anonymity. All data will be kept in a locked cabinet or computerised and accessed via a password which will be done in accordance with the Data Protection Act.

9. Who will have access to the information that I provide?

Only the principle investigator and supervisor will have access to information that you provide.

10. How will my information be stored / used in the future?

All data will be kept in a locked cabinet or computerised and accessed via a password which will be done in accordance with the Data Protection Act. Data may be used for publication in the form of a scientific paper, presented at a conference or both. Any data used cannot be linked to you. All records will be kept confidential except for review by representatives of GlaxoSmithKline and/or Northumbria University Ethics Committee representatives and regulatory authorities.

11. Has this investigation received appropriate ethical clearance?

Yes

12. Will I receive any financial rewards / travel expenses for taking part?

You will be paid £90 on completion of your participation. A further £30 will be given to the participant with the quickest average time trial compared to their maximum speed.

13. How can I withdraw from the project?

If you wish to withdraw from the project, you can inform me by email, telephone or in person. You can withdraw from the project at any point without providing reasons for doing so and without prejudice.

14. If I require further information who should I contact and how?

For further information please contact myself by telephone on 07745520939 or 0191 243 7018 or alternatively by email at ian.walshe@unn.ac.uk

PARTICIPANT INFORMATION

TITLE OF PROJECT: The effect of pre exercise feeding protocols on the inflammatory response to exercise.

Principal Investigator: Ian Walshe

Investigator contact details: 07745520939

Email: ian.walshe@unn.ac.uk

This project is funded by: Biotechnology and Biological Sciences Research Council and GlaxoSmithKline Nutritional Healthcare

 Payment: £100

1. What is the purpose of the project?

Many runners before competition may perform carbohydrate loading protocols to increase muscle glycogen and to increase performance. During prolonged exercise messenger molecules of the immune system (cytokines) have been shown to increase in blood circulation and may cause fatigue. The purpose of this project is to examine the effect of pre exercise feeding protocols on cytokine receptor and the affect on exercise performance.

2. Why have I been selected to take part?

You have been asked to participate because you are male and aged between 18 and 40 years. You will also be endurance trained and participate in regular endurance running and be able to complete a 2 hour bout of running.

3. **What will I have to do?**

For the experiment you will be asked to visit the laboratory on six occasions:

Visit One

Suitability for the study:

Initially you will complete a health-history questionnaire concerning your past and present health status in private. It is important to be entirely honest and point out any known health problems, even if they seem trivial. We will also need to know if you have been on medication at any time during the four weeks prior to the study. At this time we will also ask you to fill out a questionnaire regarding your training habits. Any answers you give will be kept in strict confidence.

$\dot{V}$**O$_2$max Test:** Providing that you are suitable for the study and you feel that you want to be involved, you will then perform a treadmill exercise test to volitional exhaustion in order to determine your aerobic capacity ($\dot{V}O_{2max}$). You will start by running on a treadmill at 8.0 kph and the speed will be increased by 2.0 kph every three minutes until volitional fatigue. We will collect gas samples from you

(you will wear a mask and we will collect your expired air) and record your heart rate. Visit one will last no more than 1 hour and can take place at any time of day.

Visit Two

Pre- exercise:

Before the test, you will be asked to go to the lavatory and then we will record your body mass. You will then be asked to perform a grip strength to fatigue test using a hand grip dynamometer using your dominant hand.

Exercise Bout: Visit two will give you a chance to familiarise yourself to the exercise test and to the methods of data collection that you may be unfamiliar with such as using the treadmill controls. On this visit, you will be asked to run at a speed that is equivalent to an exercise intensity that is 60% of your speed at $\dot{V}$ O_2max for 2 hours. Every 10 minutes you will be required to run for 30 seconds at a speed that is 90% of your maximum speed. You can consume water *ad libitum* which will be recorded and replicated for the main trials. During the exercise bout we will analyse gas samples using a mouth piece and record your heart rate. Following this, you will be allowed 5 minutes rest before performing a 5 kilometre time trial. You will be asked to run the time trial as fast as possible. During the trial, you will only be informed of the distance you have run throughout the trial however and not made aware of the time elapsed. Following the trial you will be asked to perform another grip strength test. Visit two will last approximately 3 hours and can take place any time of day.

Visits 3 to 6 (Main trials): During the remaining visits you will complete the same exercise task as in visit 2 under three separate conditions where we will manipulate your pre-exercise diet. The 3 conditions are as follows:

1. Low carbohydrate diet
2. High carbohydrate diet
3. High carbohydrate supercompensation diet (2 visits)

Pre-exercise diets: For the Low and High carbohydrate conditions you will be provided with food for the 48 hours prior to the trial. It is important that you only eat the foods given to you. For main trial 3 you will also be required to come in to the laboratory 3 days before the main trial to perform a glycogen depleting protocol which will consist of running at 70% of your maximal aerobic speed for 90 minutes to ensure that your muscles are depleted of glycogen. Following this you will be given the high carbohydrate diet to consume over the next 2 days, this will maximise the glycogen resynthesis and supercompensate your muscle glycogen levels. Please note the order of these conditions will be randomised so you might not complete them in the order shown here.

Exercise bout: The main trials will consist of the same exercise protocol as used in visit 2. We will also take some additional measures. Before the exercise bout you will be asked for a venous blood sample from the arm and a grip strength and fatigue index test as well as body mass. During the exercise bout you will be asked to consume the same amount of water that was consumed in visit 2. Following the exercise bout you will be asked for another blood sample and grip strength test before taking part in the 5 km time trial. Further blood samples and grip strength tests will be asked for immediately post time trial, 1 hour post time trial and the following morning, 24 hours from pre exercise. The main trials need to take place in the morning and will last approximately 3.5 hours.

Pre-test preparation: In addition to consuming the diets provided, for the 48 hours before the main trials we will also ask you not to train or drink alcohol (it will affect your resting baseline immune values). We will also need you to make sure that you do not eat or drink any caffeine-containing foods and drinks for 24 hours before these visits (see the attached sheet). We will also ask you to refrain from eating and drinking for the 12 hours before the start of the main trial with the exception of water.

At all visits you will be asked to fill in a health questionnaire to confirm your fitness to participate in the study on that day.

4. What are the exclusion criteria (i.e. are there any reasons why I should not take part)?

Exclusion criteria will include being unaccustomed to running for 2 hours and experience of any symptoms of infection or injury in the four weeks prior to the study or at any point during the study. Exclusion criteria also include if you have diabetes or metabolic disorder, allergies or suspected allergies to foods and any contraindications of exercise.

5. Will my participation involve any physical discomfort?

You may feel some discomfort towards the end of the exercise periods associated with long distance running due to the fatiguing nature of the tests. You may experience some bruising from blood sampling, however this is normal and pain will be minimal.

6. Will my participation involve any psychological discomfort or embarrassment?

No

7. Will I have to provide any bodily samples (i.e. blood, saliva)?

You will be asked for venous blood samples from the arm during the main trials at pre exercise, post exercise, post time trial, 1 hour post time trial and the following morning.

8. How will confidentiality be assured?

You will only be known by a number to protect anonymity. All data will be kept in a locked cabinet or computerised and accessed via a password which will be done in accordance with the Data Protection Act.

9. Who will have access to the information that I provide?

Only the principal investigator and supervisor will have access to information that you provide. All records will be kept confidential except for review by representatives of GlaxoSmithKline and/or Northumbria University Ethics Committee representatives and regulatory authorities.

10. How will my information be stored / used in the future?

All data will be kept in a locked cabinet or computerised and accessed via a password which will be done in accordance with the Data Protection Act. Data may be used for publication in the form of a scientific paper, presented at a conference or both. Any data used cannot be linked to you.

11. Has this investigation received appropriate ethical clearance?

Yes, the study and its protocol has received full ethical approval from the School of Psychology & Sport Sciences Ethics Committee. If you require confirmation of this please contact the Chair of this Committee, stating the title of the research project and the name of the principal investigator:

Chair of School of Psychology & Sport Science Ethics Committee, Northumberland Building, Northumbria University, Newcastle upon Tyne, NE1 8ST.

12. Will I receive any financial rewards / travel expenses for taking part?

You will be paid £100 on completion of your participation. A further £30 will be given to the participant with the quickest average time trial compared to their maximum speed.

13. How can I withdraw from the project?

If you wish to withdraw from the project, you can inform me by email, telephone or in person. You can withdraw from the project at any point without providing reasons for doing so and without prejudice.

14. If I require further information who should I contact and how?

If you would like further information on the study please contact the principal investigators on the contact emails listed above. If you would like to discuss the study, withdraw your data or register a complaint please contact the chair of the ethics committee on the address listed in section 11.

INFORMED CONSENT FORM

Principal Investigator: Ian Walshe

Participant Number: _____

I have read and understood the Participant Information Sheet. ☐

I have had an opportunity to ask questions and discuss this study and
I have received satisfactory answers. ☐

I understand I am free to withdraw from the study at any time, without having to
give a reason for withdrawing, and without prejudice. ☐

I agree to take part in this study. ☐

I would like to receive feedback on the overall results of the study at the email
address given below. I understand that I will not receive individual feedback on
my own performance. ☐

Email address………………………………………………

Signature of participant...
Date......………………..

(NAME IN BLOCK LETTERS)...

Signature of Parent / Guardian in the case of a minor

....

Signature of researcher...
Date......………………..

(NAME IN BLOCK LETTERS)...

FOR USE WHEN TISSUE IS BEING REMOVED <u>BUT NOT</u> STORED

Principal Investigator: Ian Walshe

Participant Number: _____

I agree that the following tissue or other bodily material may be taken and used for the study:

Tissue/Bodily material	Purpose	Removal Method
Blood plasma		Venous blood sample

I understand that if the material is required for use in any other way than that explained to me, then my consent to this will be specifically sought. I understand that I will not receive specific feedback from any assessment conducted on my samples, but should any kind of abnormality be discovered then the investigator will contact me.

Signature of participant..
Date.....………………..

Signature of Parent / Guardian in the case of a minor

....
Date.....………………..

Signature researcher..   Date.....………………..

Pre-exercise test questionnaire

Participant number ……………………………..

As you are participating in exercise within this laboratory, please can you complete the following questionnaire. Your co-operation is greatly appreciated.

All information within this questionnaire is considered confidential.

Where appropriate please circle your selected answer:

1. How would you describe your current level of activity?

   Sedentary   moderately active   highly active

2. How would you describe your current level of fitness?

   Very unfit   moderately fit   trained   highly trained

3. How would you consider your current body weight?

   Underweight   ideal   slightly overweight

   very overweight

4. Smoking habits:
   Currently non- smoker   **Yes / No**

   A previous non-smoker   **Yes / No**

   If previous smoker, how long is it since you stopped? ………… years

   A regular smoker   **Yes / No**   of ……… per day

   An occasional smoker   **Yes / No**   of ……… per day

5. Alcohol consumption:
   Do you drink alcohol   **Yes / No**

   If *yes* then do you:

   Have an occasional drink   **Yes / No**

   Have a drink every day   **Yes / No**

   Have more than one drink per day   **Yes / No**

6. Have you consulted your doctor within the last six months?   **Yes / No**
   If *yes* please give details to the test supervisor

7. Are you currently taking any medication   **Yes / No**
   If *yes* please give details to the test supervisor

   Are you currently taking any supplements   **Yes / No**

   If so, which one(s)

   _____
   _____
   _____

8. Do you suffer from any food intolerances or allergies?   **Yes / No**

9. Do you suffer from:

	Yes / No
Diabetes	
Asthma	
Bronchitis	
Epilepsy	

10. Do you suffer from, or have you suffered from, any heart complaint?

      Yes / No

11. Is there a history of heart disease in your family?   **Yes / No**

12. Do you currently have any form of muscle joint injury? **Yes / No**

13. Do you currently suffer with underlying infection?   **Yes / No**

14. Have you had any reason to suspend your training in the last two weeks?

      Yes / No

15. Is there anything to your knowledge that may prevent you from successfully completing the test(s) that have been outlined to you?

Yes / No

If yes please give details to the test supervisor:

Signature of test supervisor: …………………………..

Date: ………………

Physical Activity Questionnaire

The following questions are designed to give us an indication of your current level of physical activity and fluid intake during exercise.

Participant Number: _____   Date: _____

Are you currently ENDURANCE TRAINING?   YES ☐   NO ☐ .

If Yes, how many days each week do you usually train? _____

How many minutes does each session last? _____

What is your weekly mileage for running? _____

What is your weekly mileage for cycling? _____

Are you involved in any of the following training programmes?

Weight training ☐   Interval training ☐   Skills training ☐ .

If Yes, how many days each week do you usually train? _____

How many minutes does each session last? _____

Do you normally consume fluids during training? YES ☐   NO ☐ .

How much do you normally consume during your training session? _____