The IL-6 system and its interaction with chronic low-grade inflammation and high intensity intermittent exercise.

The IL-6 system and its interaction with chronic low-grade inflammation and high intensity intermittent exercise.

by

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Abstract

The IL-6 system is key in the development of chronic low-grade inflammation. It is known to be upregulated in response to acute exercise and lowered at rest after exercise training. IL-6 has both anti- and pro-inflammatory properties and moderation of this cytokine could alleviate chronic low-grade inflammation which is associated with obesity and Type 2 diabetes mellitus (T2DM). This thesis investigated the interplay between inflammation, glycaemic control and high intensity intermittent training (HIIT) - an exercise regimen that has been shown to yield many health benefits.

There was a greater increase in IL-6 after an acute bout of HIIT than continuous moderate intensity exercise, where external work was matched (Chapter 4). Although sIL-6R and the IL-6/sIL-6R complex were both significantly increased after acute exercise there were no differences between HIIT and moderate intensity exercise. In response to 2 weeks HIIT there was a significant reduction in IL-6 and increase in IL-6R in adipose tissue in overweight and obese males (Chapter 5). It was also determined that IL-6R present in adipose tissue is at least partly composed of the membrane-bound IL-6R isoform (Chapter 6). Reductions in circulating sIL-6R, the IL-6/sIL-6R complex, MCP-1 and adiponectin, as well as a decrease in waist circumference and increase in peak oxygen uptake during exercise were also induced after 2 weeks HIIT (Chapter 5). Young adults with T2DM (< 40 y) displayed elevated levels of inflammatory proteins in comparison to lean controls, however there were no significant differences in comparison to obese controls (Chapter 7).

In conclusion, the findings of this thesis demonstrate that acute and repeated bouts of HIIT have positive effects on the inflammatory profile in the circulation and adipose tissue, particularly in relation to the IL-6 system. It should be determined if HIIT is an achievable mode of exercise for patient populations, including T2DM patients, in order to downregulate the inflammatory profile.

Keywords: IL-6; inflammation; high intensity intermittent training; obesity; Type 2 diabetes mellitus

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List of abbreviations

AKP	alkaline phosphatase
ADAM	a disintegrin and metalloprotease
AMPK	adenosine monophosphate activated protein kinase
BME	black or minority ethnicity
BMI	body mass index
CD	cluster of differentiation
CI	confidence interval
CO_2	carbon dioxide
CRP	C-reactive protein
CV	coeffient of variance
CVD	cardiovascular disease
DEXA	dual-energy X-ray absorptiometry
dl	decilitre
DS-sIL-6R	differentially spliced soluble interleukin 6 receptor
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPOC	excess post-exercise oxygen consumption
gp	glycoprotein
GIR	glucose infusion rate
Hb	haemoglobin
HbA1c	glycated haemoglobin
HCl	hydrochloric acid
Hct	haematocrit
HDL	high density lipoprotein
HIIT	high intensity intermittent training
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
HRP	horse radish peroxidise
ICAM	intercellular adhesion molecule

IL-1ra	IL-1 receptor antagonist
IL	interleukin
IL-6R	IL-6 receptor
IP	immunoprecipitation
IRS	insulin receptor substrate
Jak	Janus kinase
kg	kilogram
kJ	kilojoule
LDL	low density lipoprotein
LDS	lithium dodecyl sulphate
M	molar
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MES	2-(N-morpholino)ethanesulfonic acid
MET	metabolic equivalent of task
mg	milligram
min	minute
MIP	macrophage inflammatory protein
ml	millilitre
MOD	continuous moderate intensity exercise
mol	mole
MRI	magnetic resonance imaging
ng	nanogram
NSAIDs	nonsteroidal anti-inflammatory drugs
O_2	oxygen
OGTT	oral glucose tolerance test
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with 0.05% Tween 20
PC-sIL-6R	proteolytically cleaved soluble interleukin 6 receptor
pg	picogram
PVDF	polyvinylidene fluoride
rev	revolution

rh	recombinant human
s	second
SD	standard deviation
SDS	sodium dodecyl sulfate
sgp	soluble glycoprotein
sIL-6R	soluble interleukin 6 receptor
SIT	sprint interval training
SOCS	suppressor of cytokines
STAT	signal transducer and activator of transcription
TBS	Tris buffered saline
TBS-T	Tris buffered saline with 0.05% Tween 20
TG	triacylglycerol
TNF	tumour necrosis factor
TNF-R	TNF-α receptor
T2DM	Type 2 diabetes mellitus
V	volt
VLDL	Very-low-density lipoprotein
$\dot{V}O_{2max}$	maximal oxygen uptake
$\dot{V}O_{2peak}$	peak oxygen uptake
v/v	volume to volume
W	watt
w/v	weight to volume
y	years
1D-PAGE	one-dimensional polyacrylamide gel electrophoresis
25(OH)D	25-hydroxyvitamin D
µg	microgram
µl	microlitre
µm	micrometre
°C	degree Celsius
%	percent

CHAPTER 1

INTRODUCTION

Physical activity is related to health, with a positive dose-response relationship existing between the amount of physical activity completed and the health benefits incurred (Blair et al. 2001), and physical inactivity is classed as the fourth leading risk factor for global mortality. Physical activity is a key determinant of energy expenditure and is therefore fundamental to energy balance and weight control. An imbalance in this energy equation has led to a prevalence of obesity which is increasing at an alarming rate, particularly in Western countries, with over 60% of the population in England overweight and approximately 25% obese in 2009 (Statistics on Obesity, Physical Activity and Diet: England, 2011). Obesity is a risk factor for many chronic diseases including cardiovascular disease (CVD), cancer and Type 2 diabetes mellitus (T2DM) which are the largest cause of death in the world, representing 63% of all deaths worldwide (World Health Organisation, Global status report on non-communicable diseases 2010). The prevalence of T2DM in particular is rapidly increasing and is expected to increase from 171 million in 2000 to 366 million people in 2030 worldwide (Wild et al. 2004), and physical inactivity is estimated to be the main cause of 27% of diabetes cases. In addition the age of diagnosis of T2DM is decreasing (Koopman et al. 2005), which is likely due to the increasing prevalence of obesity.

Exercise is a subcategory of physical activity that is planned, structured and repetitive and participation in exercise can decrease the risk of chronic diseases, including CVD and T2DM (Oguma and Shinoda-Tagawa 2004; Laaksonen et al. 2005; Sundquist et al. 2005). These diseases are also underpinned by chronic low-grade inflammation (Pradhan et al. 2001; Vasan et al. 2003; Berg and Scherer 2005) which has been defined as a 2-4 fold increase in circulating inflammatory and anti-inflammatory proteins, including cytokines, acute-phase proteins and chemokines (Bruunsgaard 2005).

The cytokine, interleukin-6 (IL-6) is central to the inflammatory process and is known to have anti- as well as pro-inflammatory properties, and has the ability to antagonise the effects of tumor necrosis factor-α (TNF-α) (Starkie et al. 2003), a known pro-inflammatory cytokine that can induce insulin resistance (Hotamisligil et al. 1993). Figure 1.1 illustrates the pro- and anti-inflammatory

cascade that occurs during sepsis. This differs from the cytokine cascade that occurs with exercise which is only anti-inflammatory (Petersen and Pedersen 2005). This anti-inflammatory response to exercise is at least partly due to the exponential release of IL-6 from skeletal muscle, which is released into the circulation. This elevation in IL-6 drives the anti-inflammatory cascade by inhibiting pro-inflammatory cytokines TNF-α and IL-1, due to an increase in the production of the antagonistic TNF-α receptors (Ostrowski et al. 1999) and IL-1 receptor antagonist (Steensberg et al. 2003) and stimulating production of the anti-inflammatory cytokine IL-10 (Steensberg et al. 2003). There has been much debate on the “good” and “bad” effects of IL-6 (Mooney 2007; Pedersen and Febbraio 2007). Whilst it is thought that IL-6 has the ability to induce insulin resistance in the liver (Klover et al. 2003), there is strong evidence that acute elevations of IL-6 can have positive effects, including increasing glucose transport and fatty acid oxidation in skeletal muscle (Carey et al. 2006). IL-6 is also thought to play a central role in the resolution of inflamed tissue during acute inflammation by inducing the expression of TNF-α receptor (TNF-R) and IL-1 receptor antagonist (IL-1ra) and therefore antagonising the functions of the pro-inflammatory cytokines IL-1 and TNF-α (Schindler et al. 1990; Tilg et al. 1994).

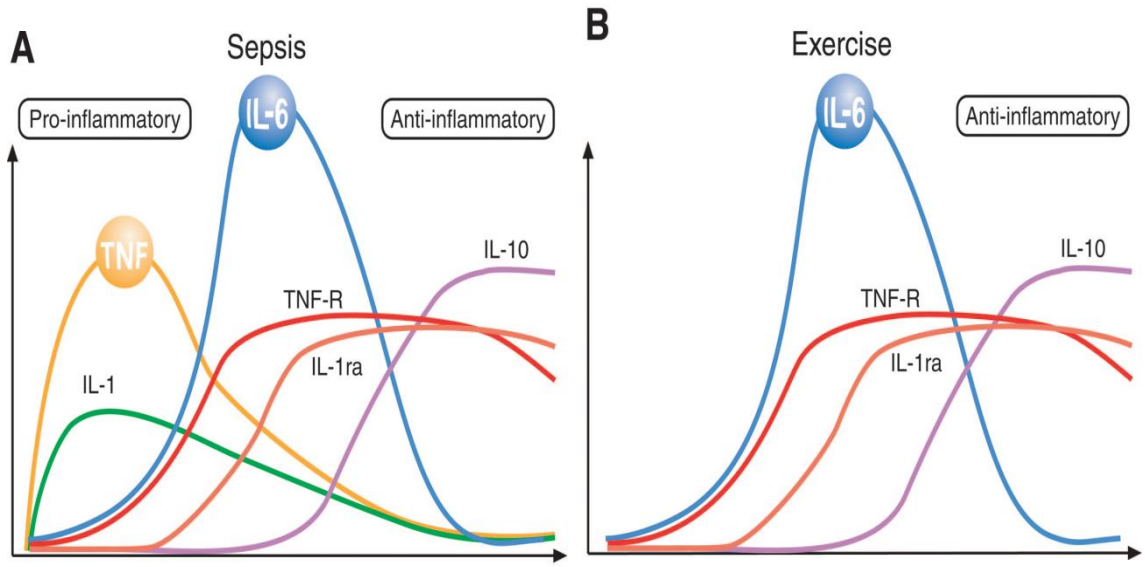

Figure 1.1 The cytokine cascade during sepsis and exercise. In sepsis (**A**), the cytokine cascade within the first few hours consists of TNF-α, IL-1, IL-6, IL-1ra, TNF-R and IL-10. The cytokine response to acute exercise (**B**) does not include TNF-α and IL-1 but does show a marked increase in IL-6, which is followed by IL-1ra, TNF-R and IL-10. Increased C-reactive protein (CRP) levels do not appear until 8–12 h later. Taken from Petersen and Pedersen (2005).

The acute increase in IL-6 found after a single bout of exercise (Keller et al. 2005a; Gray et al. 2009b; Robson-Ansley et al. 2009) is related to exercise intensity (Helge et al. 2003; Ostrowski et al. 2000), however, the biological activity of IL-6 is dependent on binding to its receptors, IL-6 receptor (IL-6R) and glycoprotein 130 (gp130). A soluble form of IL-6R exists, sIL-6R, which allows IL-6 signalling to occur in tissues lacking membrane-bound IL-6R, a process termed trans-signalling (Rose-John and Heinrich 1994). Recent studies have shown that sIL-6R is also elevated after exercise (Gray et al. 2009b; Robson-Ansley et al. 2009), although it is unclear whether sIL-6R release is related to exercise intensity.

In contrast to an acute anti-inflammatory response after a single bout of exercise, exercise training has been shown to aid the reduction of basal inflammatory proteins in a number of studies, however other studies have failed to see an effect of chronic exercise training on inflammatory proteins (Adamopoulos et al. 2002; Balducci et al. 2010b; Church et al. 2010; Gray et al. 2009a; Oberbach et al. 2008; Thompson et al. 2010; Zoppini et al. 2006).

Adipose tissue is one of the main sources of many of the proteins associated with inflammation and its resolution, with elevated circulating levels common in obesity due to excess adiposity. Despite this, there is no research investigating the effects of exercise training on inflammation in adipose tissue. Much of the literature has investigated the effects of low to moderate intensity exercise (less than 6 metabolic equivalent of task (METs)) on health, in line with the public health recommendations stating that adults should accumulate 30 min of moderate exercise 5 days per week (Chief Medical Officers, 2004). These recommendations were recently updated, with the new guidelines recommending 150 min of moderate, or 75 min vigorous exercise per week (or a combination), with an aim to do some physical activity every day, and in addition to include muscle strengthening exercises twice per week (Chief Medical Officers, 2011). In line with the new physical activity recommendations, there are numerous publications supporting a higher intensity of exercise (Tremblay et al. 1990; O'Donovan et al. 2005; Swain and Franklin 2006; Tjønna et al. 2008).

High intensity intermittent exercise enables individuals to exercise at a higher intensity than they could maintain during continuous exercise due to exercise intervals separated by rest periods or low intensity exercise. There are different modes of intermittent exercise, with one protocol, high intensity intermittent training (HIIT), finding a 36% increase in fat oxidation during exercise after a 2 week training period (Talanian et al. 2007). Additionally, high intensity intermittent exercise has induced improvements in some health factors after 3-4 months training in chronic disease states such as the metabolic syndrome and CVD, including improved endothelial function and insulin signalling, to a greater extent than continuous moderate intensity exercise with equal energy expenditure (Wisløff et al. 2007; Tjønna et al. 2008). As not all studies have reported a positive effect of exercise training on chronic low-grade inflammation it is possible that the differences reported between studies could be due to the exercise intensity and that high intensity exercise may be required to induce a reduction in inflammation. Since high intensity intermittent exercise has been shown to have some additional health benefits in comparison to traditional

moderate intensity exercise it could also be an appropriate mode of exercise to reduce basal inflammatory proteins in the circulation and in adipose tissue.

Aims of thesis:

- to establish whether a single bout of HIIT or continuous moderate intensity exercise elicits the greatest response of the IL-6 system;
- to determine if short-term exercise training can reduce inflammatory proteins in adipose tissue and the circulation in overweight and obese men;
- to develop a method to detect membrane-bound IL-6R in adipose tissue and determine if these isoforms are altered after a period of short-term exercise training;
- to phenotype the inflammatory, glycaemic and lipid profiles, and peak oxygen uptake during exercise in young adults with T2DM (< 40 y), in comparison to age-matched lean and obese individuals.

CHAPTER 2

LITERATURE REVIEW

2.1 Acute exercise and the IL-6 system

A large number of studies have looked at the acute effects of exercise on the pleiotropic cytokine IL-6 over the last 20 years (For review see Fischer 2006). IL-6 has been described as both a pro- and anti-inflammatory protein (Pedersen and Febbraio 2007), and has been identified in carcinoma cells, T and B cells, endothelial cells, adipocytes and skeletal muscle (Yasukawa et al. 1987; Hirano et al. 1987; Jirik et al. 1989; Mohamed-Ali et al. 1997; Hiscock et al. 2004; Keller et al. 2005b).

Research has shown that endurance and resistance exercise result in an acute increase in IL-6 (Fischer 2006; Izquierdo et al. 2009). The stimulated increase in IL-6 through exercise is transient and peaks at the end of exercise or shortly thereafter, before rapidly decreasing back to baseline levels (Keller et al. 2005a; Gray et al. 2009b; Robson-Ansley et al. 2009), with a similar response seen in both young and older adults (Sacheck et al. 2006).

As little as 25 min cycling at 60% peak oxygen uptake ($\dot{V}O_{2peak}$) has been shown to cause an elevation in systemic IL-6 (Febbraio et al. 2003), and it has been reported to increase over 100-fold with prolonged exercise (Ostrowski et al. 1999; Nieman et al. 2005), although more modest increases are generally reported. Elevated IL-6 after exercise is due to an increase in IL-6 production in skeletal muscle which is released into the circulation (Penkowa et al. 2003; McKay et al. 2009), as shown by increased IL-6 mRNA expression and protein expression after exercise, which is returned to baseline by 24 h after exercise. Increased IL-6 transcription and mRNA expression in contracting skeletal muscle has been shown to be increased further with low glycogen availability (Keller et al. 2001; Steensberg et al. 2001). IL-6 can also be elevated due to increased intracellular calcium content that results in the activation of p38 mitogen-activated protein kinase (MAPK) and subsequently activation of transcription factors that induce an increase in IL-6 production (Pedersen and Febbraio 2008).

IL-6 production is dependent on exercise intensity and duration, as well as the mass of muscle recruited (Fischer 2006). The original data identifying an

intensity-dependent release of IL-6 was based on an association between running speed during a marathon and IL-6 concentration (Ostrowski et al. 2000). This data has been supported by research showing that IL-6 increases in an intensity dependent manner during knee extensor exercises (Helge et al. 2003). Conversely, data gathered during treadmill running at two differing intensities, proposed that exercise intensity was not a major factor in IL-6 release (Tartibian et al. 2009), although IL-6 increased by 28% running at 60% maximal oxygen uptake ($\dot{V}O_{2max}$) and by 44% running at 75% $\dot{V}O_{2max}$, suggesting that intensity may still be relevant to IL-6 production, despite no significant difference between trials. This latter study would have benefited from using a within-sample study design as opposed to a between-sample design in order to compare the individual inflammatory response to exercise intensity, due to the large inter-individual variation in IL-6 levels (Walshe et al. 2010). Perhaps of more relevance to IL-6 production during exercise is duration, with over 50% of the variation in IL-6 being attributable to the duration of exercise (Fischer 2006). As previously discussed, IL-6 production during exercise occurs in skeletal muscle, therefore, it is also logical that some of the variation in IL-6 production will depend on the mass of muscle recruited during exercise. Hence, the type of exercise as well as the intensity and duration are important factors for IL-6 release from skeletal muscle.

Despite extensive research on the IL-6 response to exercise, less is known about the effects of exercise on IL-6R, which is fundamental to the biological activity of IL-6. More recently, some studies have quantified both IL-6R and IL-6 (Keller et al. 2005a; Gray et al. 2009b; Robson-Ansley et al. 2009; Keller et al. 2005b; Walshe et al. 2010; Gray et al. 2008; Patterson et al. 2008; Robinson et al. 2009; Robson-Ansley et al. 2010). In order for IL-6 to signal it must bind to the membrane-bound IL-6R, an 80 kDa glycoprotein, found mainly in leukocytes and hepatocytes (Hibi et al. 1990), or alternatively it can bind to a soluble version of the receptor, sIL-6R, found in plasma (Müllberg et al. 1993; Müller-Newen et al. 1998) (Figure 2.1). IL-6R availability is low or absent in many tissues including resting skeletal muscle (Keller et al. 2005b), therefore, IL-6 must bind to sIL-6R allowing signalling to occur in tissues deficient in IL-6R, a process termed trans-signalling (Rose-John and Heinrich 1994). Once

bound, the active IL-6/IL-6R complex must bind to a secondary receptor, gp130, which is ubiquitously expressed on almost all cell membranes (Hibi et al. 1990) and allows activation signals to be transmitted (Jones et al. 2001). A soluble version of gp130 also exists (sgp130), but has been shown to be a natural antagonist of IL-6/IL-6R signalling (Jostock et al. 2001). This antagonist can bind to sIL-6R in the absence of IL-6, rendering sIL-6R inactive and unable to bind to IL-6 to induce signalling, therefore reducing the amount of sIL-6R available for IL-6 signalling (Gaillard et al. 1999).

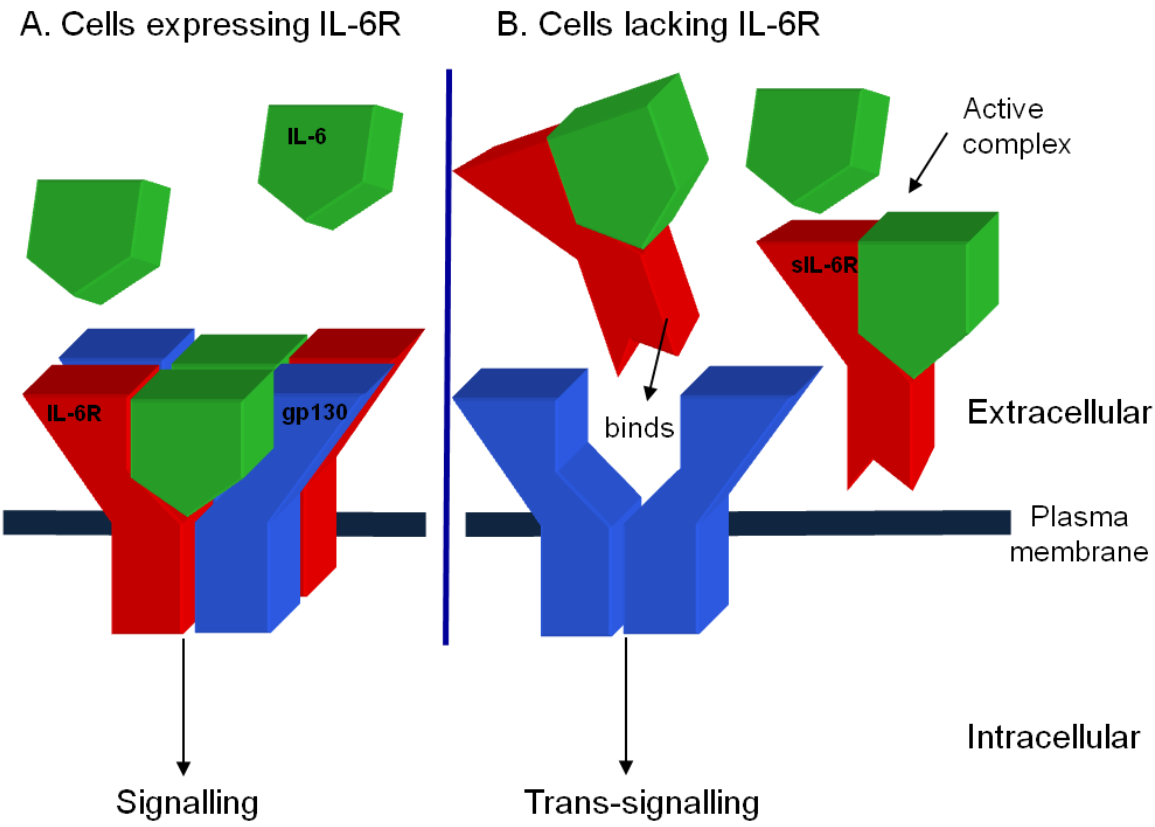

Figure 2.1. Schematic representation of: A. the membrane-bound IL-6/IL-R/gp130 complex and B. the IL-6/sIL-6R complex. Adapted from Paonessa et al. 1995.

Where there is some evidence to show that sIL-6R is increased after exercise (Sacheck et al. 2006; Gray et al. 2008; Gray et al. 2009b; Robson-Ansley et al. 2009), others have found no effect of exercise on sIL-6R in plasma (Keller et al. 2005a; Keller et al. 2005b; Patterson et al. 2008; Robinson et al. 2009). Unlike

IL-6, sIL-6R may not peak until several hours after the cessation of exercise (Keller et al. 2005a; Robson-Ansley et al. 2009) and sampling time could be an important issue.

The first study to investigate sIL-6R during exercise involved 3 h cycling at 60% of maximum power output and did not find any significant change in sIL-6R after exercise, however the sample size was very small (N = 6), and there was a mean increase of sIL-6R of ~9% post-exercise (Keller et al. 2005a). In the same study IL-6R mRNA and protein expression in skeletal muscle were significantly increased at 3 and 6 h post-exercise. This finding has been supported by another study where IL-6R mRNA expression in skeletal muscle was found to increase approximately 5-fold, 4 h after 300 knee extensions to maximal effort (McKay et al. 2009). The elevation was short lived and IL-6R mRNA expression had returned to baseline levels by 24 h post-exercise. Other researchers have found that acute exercise does induce an increase in sIL-6R (Gray et al. 2008; Gray et al. 2009b; Robson-Ansley et al. 2009). Gray et al. (2008; 2009b) found sIL-6R to increase immediately post-exercise after cycling to volitional exhaustion (96% lactate threshold or 68% $\dot{V}O_{2max}$) and after 1 h cycling at 90% lactate threshold (63% $\dot{V}O_{2peak}$). Findings by (Robson-Ansley et al. 2009) also reported an elevation in sIL-6R the morning after prolonged cycling during a mountain bike event. In addition to these studies, a single bout of muscle damaging eccentric exercise resulted in a significant reduction in sIL-6R at 48 and 72 h post-exercise (Robson-Ansley et al. 2010). The only other time point to be sampled in this study was at 24 h post-exercise, where sIL-6R was not significantly different to pre-exercise.

The differences reported in sIL-6R after exercise may be due to the timing of the blood sampling, and it is not clear when sIL-6R peaks in response to an acute bout of exercise which may vary depending on the nature of the exercise. As previously discussed, IL-6 production is dependent on exercise duration, intensity and mode or exercise, however, the effect of exercise intensity and duration on sIL-6R has not been determined.

Soluble IL-6R is produced by 2 different mechanisms; proteolytic cleavage, which is the shedding/cleavage of membrane-bound IL-6R (PC-sIL-6R)

(Müllberg et al. 1993), by enzymes of the ADAM family (a disintegrin and metalloprotease), including ADAM17 (Matthews et al. 2003), or secondly by differential mRNA splicing (DS-sIL-6R), which results from the deletion of 94 base pairs corresponding to the transmembrane domain of IL-6R (Horiuchi et al. 1994) (Figure 2.2). This deletion of the transmembrane domain results in a reading frameshift leading to a unique COOH-terminal sequence. Soluble IL-6R produced by shedding occurs rapidly, approximately 30-120 min after stimulation, whereas sIL-6R produced by differential mRNA splicing is much slower and has been found to occur between 6 and 24 h after activation (Jones et al. 2001; Dimitrov et al. 2006). The mechanism responsible for the increased production in sIL-6R after exercise is still unknown.

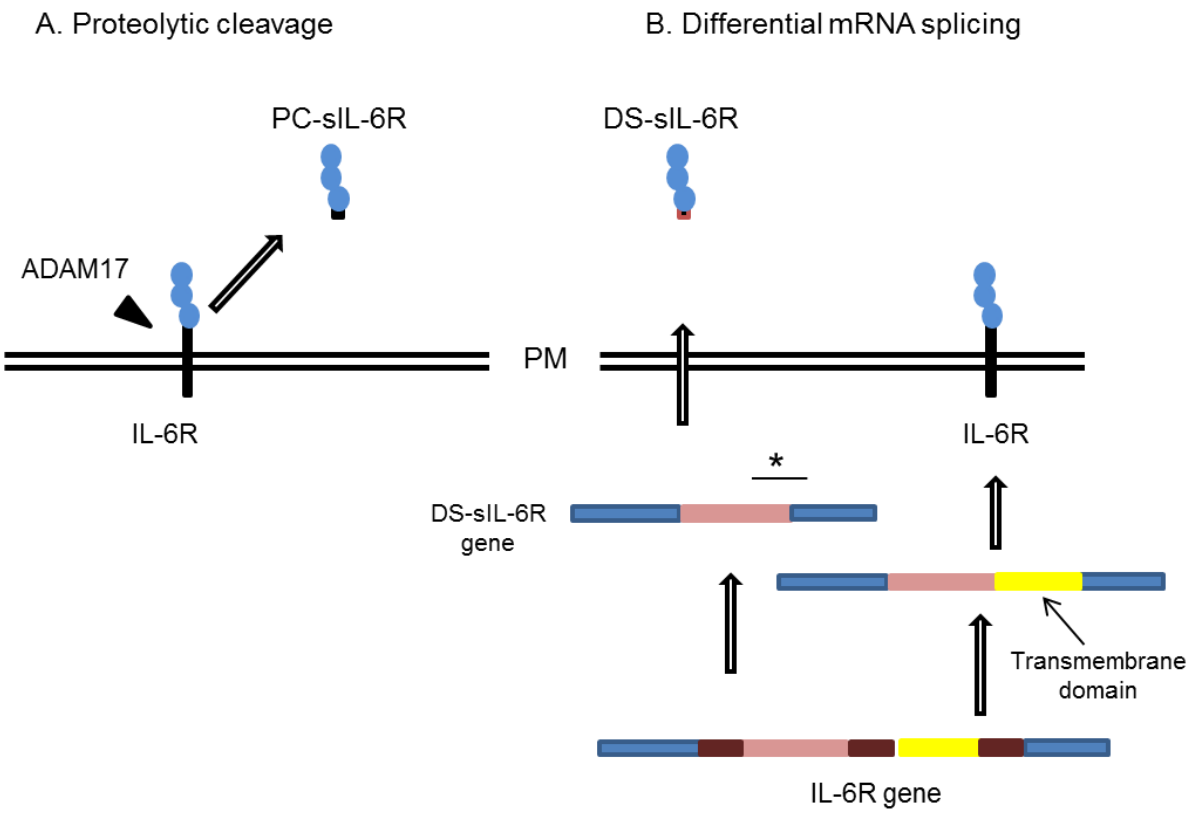

Figure 2.2 Schematic representation of the 2 mechanisms of sIL-6R production. A. membrane-bound IL-6R is cleaved into the circulation (PC-sIL-6R) by ADAM17 and **B.** deletion of the transmembrane region results in mRNA splicing and the release of DS-sIL-6R. * denotes the reading frameshift. PM; plasma membrane. Adapted from Jones et al. 2001.

Since IL-6 is biologically active when bound to IL-6R or sIL-6R, changes in the IL-6/sIL-6R complex after exercise will give an indication of how much IL-6 is biologically active. An acute 1 h bout of exercise at 90% lactate threshold resulted in over a 2-fold increase in the IL-6/sIL-6R complex after exercise, and was still significantly elevated at 1.5 h post-exercise (Gray et al. 2009b). This is the only study that has quantified the IL-6/sIL-6R complex in response to exercise. It remains unclear what happens to the concentration of the IL-6/sIL-6R complex after 1.5 h post-exercise, or if altering the intensity of exercise will have an effect on the IL-6/sIL-6R complex.

2.2 Obesity, adipose tissue and inflammation

Adipose tissue is a heterogenous organ and following the discovery of leptin secretion from the tissue in 1994 (Zhang et al. 1994), it is now known to form the largest endocrine organ in the body (Trayhurn 2005) and can produce and secrete over 75 inflammatory proteins (Wood et al. 2009). Approximately half of adipose tissue is made up of adipocytes, whilst the remainder consists of blood cells, endothelial cells, macrophages, pre-adipocytes and fibroblasts (Compher and Badellino 2008). Excess adipose tissue is related to an increased risk of chronic diseases such as CVD and T2DM (Sowers 2003). This superfluous adipose tissue results in a marked secretion of many inflammatory cytokines and cell adhesion molecules, including IL-6, TNF-α and intercellular adhesion molecule-1 (ICAM-1) in comparison to lean individuals (Hotamisligil et al. 1995; Kern et al. 1995; Kern et al. 2001; Bošanská et al. 2010). The increased production by adipose tissue contributes to a chronic state of inflammation and metabolic disorders (Maachi et al. 2004), which includes insulin resistance and progression to the metabolic syndrome and T2DM (Shoelson et al. 2006).

2.3 Mechanisms of increased inflammation in adipose tissue

Macrophages are mononuclear phagocytes and infiltration into the adipose tissue has been shown to be increased in obese compared with normal weight individuals (Weisberg et al. 2003; Cancello et al. 2005). These cells reside in the stromal vascular region of adipose tissue and produce many cytokines including IL-6 and TNF-α (Weisberg et al. 2003). It seems that the size of the adipocytes triggers macrophage infiltration rather than overall obesity (Cinti et al. 2005), and it has been speculated that recruitment of the macrophages may be stimulated by the chemokines, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) (Bruun et al. 2005; Xu et al. 2003). Adipocyte size has also emerged to be important in the release of inflammatory proteins, with the greatest secretion of IL-6, TNF-α, MCP-1, MIP-1α and IL-1ra found in the largest adipocytes, which is probably due to a greater mean surface area and cell volume (Skurk et al. 2007). It is difficult to

completely distinguish the contribution of inflammatory mediators from different cell types within adipose tissue, although of the ~30% of circulating IL-6 claimed to be secreted from adipose tissue at rest (Mohamed-Ali et al. 1997), only ~10% of this can be accountable to adipocyte production (Fried et al. 1998). Gene expression of IL-6 and its receptors IL-6R and gp130, as well as the acute phase protein CRP, have also been shown to be significantly elevated in adipose tissue of patients with chronic low-grade inflammation in comparison with healthy controls (Memoli et al. 2007), suggesting that adipose tissue may contribute to the elevated inflammatory proteins in the circulation. In addition, IL-6 is significantly elevated in obese individuals with T2DM in comparison to both a healthy control group and a group of non-obese T2DM individuals (Hansen et al. 2010), suggesting that adipose tissue is one of the main sources IL-6 production at rest. Although IL-6 levels are clearly related to the amount of adipose tissue, further regulation from other mediators are also likely, including dietary fat (García-Escobar et al. 2010) and hypoxia (Trayhurn and Wood 2004).

In vitro studies in rat adipocytes have shown that fatty acid composition in the diet is a strong mediator of IL-6 regulation despite no differences in body mass or fat mass (García-Escobar et al. 2010), therefore IL-6 regulation is likely to be moderated by more than one single factor. Another possible explanation for an increase in localised inflammation within adipose tissue of obese individuals is due to cellular hypoxia, proposed by Trayhurn and Wood (2004). Adipose tissue has poor vascularisation and obese individuals have lower tissue oxygenation in subcutaneous fat tissue than non-obese individuals (Fleischmann et al. 2005). Furthermore, adipocytes can reach up to 200 μm in diameter (Skurk et al. 2007), therefore exceeding the normal oxygen diffusion rate of ~100-200 μm (Brahimi-Horn and Pouysségur 2007). Elevated gene expression of many inflammatory proteins, including IL-6 and TNF-α have been found in hypoxic adipose tissue and macrophages in obese mice (Ye et al. 2007).

2.4 Subcutaneous and visceral adipose tissue

Adipose tissue is made up of white and brown tissue, where white adipose tissue cells contain a single large fat droplet and are used as a major energy store, whilst brown adipose tissue cells have numerous small lipid droplets and more mitochondria than white cells. There are two types of white adipose tissue, subcutaneous and visceral. Subcutaneous adipose tissue is stored just below the surface of the skin, whereas visceral adipose tissue is found deeper in the body, around the internal organs. There are differences in inflammatory protein release between subcutaneous and visceral adipose tissue. Evidence suggests that the dominant source of inflammation is visceral adipose tissue (Fried et al. 1998; Bruun et al. 2005; Bošanská et al. 2010), although one *ex-vivo* study found more IL-6 in subcutaneous than visceral fat (Gletsu et al. 2006), and similarly adiponectin abundance is greater in subcutaneous adipose tissue (Fain et al. 2004; Lihn et al. 2004). Despite the majority of evidence suggesting inflammatory proteins are present at greater concentrations in visceral than subcutaneous adipose tissue, visceral adipose tissue accounts for only 13% of total adipose tissue in obese men and 6% in obese women (Ross et al. 1994), therefore the contribution of subcutaneous adipose tissue to chronic low-grade inflammation could be substantial.

2.5 Chronic low-grade inflammation, insulin resistance and T2DM

Chronic low-grade inflammation has been shown to precede and predict the risk of T2DM (Schmidt et al. 1999). In particular elevated CRP and IL-6 are strong predictors of T2DM in those at risk of developing the disease (Pradhan et al. 2001; Dehghan et al. 2007). There is some evidence from animal studies to suggest that the pancreas produces inflammatory proteins, including IL-6, TNF-α and MCP-1 (Ehses et al. 2009), however, the pancreas is a relatively small organ, and it is likely that only a small proportion of chronic low-grade inflammation is a result of pancreatic inflammation. Adipose tissue is an important mediator of insulin sensitivity and many of the inflammatory proteins released from adipose tissue are involved in glycaemic control and the

development of insulin resistance. Both elevated subcutaneous and visceral adipose tissue are associated with insulin resistance, although there is a greater positive correlation between insulin resistance and visceral adipose tissue (Preis et al. 2010).

In chronic low-grade inflammatory states, such as in obesity and insulin resistance, the pro-inflammatory cytokine TNF-α is elevated in the circulation (Dandona et al. 1998; Dandona et al. 2004), which is a cytokine known to induce insulin resistance. A study published in 1993 was the first to show that inflammatory proteins played an important role in the development of insulin resistance (Hotamisligil et al. 1993). The study found that TNF-α mRNA and protein expression in epididymal adipose tissue of obese rodents was elevated. The authors derived that TNF-α was an important factor in insulin resistance, as neutralisation of TNF-α lead to an increase in insulin sensitivity. The same research group went on to demonstrate that TNF-α was significantly higher in subcutaneous adipose tissue of obese compared to lean females and that TNF-α was significantly reduced after weight loss (Hotamisligil et al. 1995). These findings suggested that the functional role of TNF-α in insulin resistance shown in their earlier animal studies is likely to be similar in humans. Evidence has shown that TNF-α may induce insulin resistance through the induction of serine phosphorylation of insulin receptor substrate 1 (IRS-1) (Hotamisligil et al. 1996), and it has been speculated that IL-6 can also induce insulin resistance via this mechanism (Bastard et al. 2002).

In contrast to an acute bout of exercise causing a short-term elevation in IL-6, elevated basal IL-6 found with chronic low-grade inflammation is associated with chronic conditions including obesity (Bastard et al. 2000) and T2DM (Duncan et al. 2003; Hu et al. 2004). There is evidence to suggest that sIL-6R is also elevated in T2DM in comparison to a control group (Müller et al. 2002), although another study found no difference between groups (Kado et al. 1999). However, in both studies, sIL-6R concentration was extremely high in all groups (~100 $ng \cdot ml^{-1}$), which is more than double the range reported in more recent years (~30-50 $ng \cdot ml^{-1}$), possibly due to the variation in antibodies and modern antibodies being more specific to the target protein. Polymorphisms of the IL-6 (Huth et al. 2009) and IL-6R genes (Hamid et al. 2004) may also contribute to

the genetic susceptibility of T2DM. Although Kado et al. (1999) found no difference in sIL-6R between T2DM and healthy controls, the study reported significantly higher levels of the IL-6/sIL-6R complex in T2DM. However, it should be noted that body mass was not reported in this study and the T2DM patients were significantly older than the controls.

The role of IL-6 in insulin sensitivity is complex, with contradictory findings reported in the literature (Mooney 2007; Pedersen and Febbraio 2007). It has been argued that although elevated IL-6 is found in insulin resistant states, most of the data comes from correlation or animal studies, or from tissue culture studies using supraphysiological IL-6 concentrations (Pedersen and Febbraio 2007). In adipocytes, oxidative stress occurs with insulin resistance and can induce the production of IL-6 (Lin et al. 2005). Elevated IL-6 in obese individuals has been shown to have a strong negative correlation with maximal insulin glucose transport rate, and has been speculated to be due to IL-6 inducing IRS-1 serine phosphorylation (Bastard et al. 2002), which is known to inhibit the insulin signalling pathway. However, this study found that TNF-α did not correlate with insulin resistance, contradicting the earlier findings by Hotamisligil et al. (1995). Another mechanism for cytokine-induced insulin resistance, could be that the expression of the suppressor of cytokines (SOCS)3 is activated by IL-6 in adipose tissue, which could induce insulin resistance through inhibition of IRS transduction (Rieusset et al. 2004). IL-6 is known to have different actions in adipose tissue and skeletal muscle. Although IL-6 in adipose tissue appears to have detrimental effects on insulin signalling, it is thought that IL-6 in skeletal muscle has a positive influence. It was previously shown that IL-6 increased glucose uptake in skeletal muscle at supraphysiological concentrations (Geiger et al. 2007) in rodent muscle, however this effect was not found in human tissue (Glund et al. 2007). More recently it has been shown sIL-6R plays a crucial role in glucose metabolism, since IL-6 in combination with sIL-6R, both at physiological concentrations, was shown to directly stimulate glucose transport, partially through adenosine monophosphate activated protein kinase (AMPK) signalling in mouse soleus muscle (Gray et al. 2009c). Activation of AMPK signalling also comes about via stimulation by adiponectin bound to its receptors, AdipoR1 and AdipoR2, and

results in an increase in fat oxidation and glucose utilisation in skeletal muscle (Yamauchi et al. 2002).

In a state of chronic low-grade inflammation adiponectin is reduced and it has been clearly demonstrated that BMI is inversely associated with circulating adiponectin concentration in both healthy and T2DM individuals (Arita et al. 1999; Weyer et al. 2001; Bruun et al. 2003; Kern et al. 2003; Ryan et al. 2003; Vilarrasa et al. 2005; Bluher et al. 2006). Adiponectin has been proposed to have insulin-sensitising effects via AMPK signalling (Berg et al. 2001; Fruebis et al. 2001; Yamauchi et al. 2001 Yamauchi et al. 2002), and a reduction in adiponectin production within adipose tissue results in both local and systemic insulin resistance. High adiponectin levels can also induce an anti-inflammatory environment by stimulating the production of IL-10 and IL-1ra in monocytes and macrophages (Kumada et al. 2004; Wolf et al. 2004) and can inhibit mRNA expression of ICAM-1 induced by TNF-α stimulation in human aortic endothelial cells (Ouchi et al. 1999).

A disruption in adiponectin signalling has been shown to induce an increase in the expression of the chemokine MCP-1 (Yamauchi et al. 2007; Yamauchi and Kadowaki 2008). MCP-1 and ICAM-1 production are upregulated in obesity and insulin resistance (Strączkowsk et al. 2002; Leinonen et al. 2003; Kim et al. 2006) and can induce macrophage recruitment into adipose tissue which leads to insulin resistance (Brake et al. 2006; Kanda et al. 2006). Mice overexpressing MCP-1 induced local insulin resistance in adipose tissue, due to an increase in macrophages infiltration which led to an increased expression of IL-6 and TNF-α (Kamei et al. 2006). Overexpression of MCP-1 can also lead to systemic effects on insulin resistance, as the authors in this study also showed that MCP-1 blunted insulin signalling in myotube cells and insulin-stimulated glucose uptake into skeletal muscle. The data suggests that adiponectin may be an important upstream regulator of chronic low-grade inflammation. In addition, inhibition of MCP-1 in obese mice reduced macrophage infiltration into adipose tissue and insulin resistance (Kanda et al. 2006), therefore emphasising the importance of this chemoattractant protein in the progression of T2DM.

Inflammatory proteins including IL-6 and TNF-α are associated with a number of downstream effects that can lead to the development of atherosclerosis (Libby et al. 2002) and it has been proposed that these inflammatory proteins can act on the liver to induce the production of certain lipoproteins (Sjöholm and Nyström 2006). Therefore, inflammation may cause dyslipidaemia, a major risk factor for atherosclerosis, and an association has been shown with increased very low density lipoprotein (VLDL), which is another risk factor for insulin resistance (Esteve et al. 2005). There is also some evidence that there could be a link between chronic low-grade inflammation and 25-hydroxyvitamin D (25(OH)D) deficiency. TNF-α has been shown to be negatively correlated with 25(OH)D in healthy women (Peterson and Heffernan 2008), and furthermore there was a tendency for an inverse correlation between IL-6 and 25(OH)D, although no correlation was found between CRP or IL-10 with 25(OH)D. It is thought that 25(OH)D plays a role in T2DM, and correlates with insulin sensitivity and BMI in obesity (Muscogiuri et al. 2010). Future research is therefore required to determine to relationship between raised inflammation and 25(OH)D deficiency. Moreover, T2DM patients are known to have poorer glycaemic control in the winter (Campbell et al. 1975), the time of year that hypovitaminosis D is most common. In addition to 25(OH)D correlating with some inflammatory mediators, it has been established that 25(OH)D has anti-inflammatory properties by suppressing TNF-α secretion in macrophages (Cohen et al. 2001). It does not appear clear whether the increased prevalence of 25(OH)D deficiency in T2DM is due to increased levels of adiposity, and moreover, it remains unclear whether there is a relationship between inflammation and 25(OH)D in T2DM.

2.6 Chronic low-grade inflammation and exercise training

A number of studies have investigated the effects of exercise training on inflammatory proteins in the circulation in both healthy and disease states (Adamopoulos et al. 2002; Zoppini et al. 2006; Oberbach et al. 2008; Gray et al. 2009a; Balducci et al. 2010b; Church et al. 2010; Thompson et al. 2010), with both positive and no effect of exercise training on inflammation reported.

However, most studies that have investigated the effects of exercise training on inflammation have used low to moderate intensity exercise, generally less than 60% $\dot{V}O_{2max}$, which could be below the lactate threshold even for some untrained individuals (Demello et al. 1987; Hagberg et al. 1988).

Non-disease cohorts have been used in a number of studies to investigate the effects of exercise training on systemic inflammation. A 12 week community based walking intervention did not induce changes in plasma CRP, IL-6, sIL-6R, TNF-α or its receptors in individuals who initially did less than 30 min of moderate intensity exercise, 5 days per week (Gray et al. 2009a). Participants were encouraged to increase walking by 3000 steps on 5 days per week during the 12 week period, in order to meet the physical activity guidelines. Two factors that could explain why no reduction in any of the inflammatory parameters were found were that adherence to the programme was not monitored and also the exercise intensity or volume may not have been sufficient to induce a reduction in the inflammatory proteins. In support of these findings Keller et al. (2005b) carried out a 10 week training study in untrained males, who undertook 1 h knee extensor training sessions at 75% maximal power output 5 days per week, and although this resulted in a 12% decrease in sIL-6R after training, this was not statistically significant, however this was possibly due to a small sample size (N = 7).

Another study that utilised a higher exercise intensity than that of Gray et al. (2009a) and was conducted in sedentary and overweight males, who were otherwise healthy, found that IL-6 was reduced after 12 and 24 weeks participation in a progressive moderate intensity exercise training programme (50-70% $\dot{V}O_{2max}$), although no change in CRP or ICAM-1 was detected (Thompson et al. 2010). This study also demonstrated that the effects of exercise training on inflammatory proteins could be relatively short-lived, since IL-6 had increased again after 2 weeks detraining. Since (Gray et al. 2009a) and (Thompson et al. 2010) both measured IL-6 after 12 weeks exercise training in similar populations and with a similar total exercise duration, it could be hypothesised that the induced decrease in IL-6 in (Thompson et al. 2010) was due to the higher exercise intensity.

Similarly to Gray et al. (2009a) and (Thompson et al. 2010), another study looked at the effects of 4 weeks moderate intensity exercise in healthy individuals and found no change in IL-6, CRP, IL-10 or adiponectin after the intervention, despite a reduction in inflammation in those with metabolic abnormalities (Oberbach et al. 2006). However, in this study there is no information on exercise intensity, and again it could be that it was not high enough to elicit any changes in inflammatory proteins.

Although most research has looked at the effects of aerobic exercise on inflammatory proteins, there is also evidence that resistance training can have a beneficial effect on some inflammatory mediators. A one year moderate intensity resistance training intervention was shown to cause a reduction in CRP and increase adiponectin, despite there being no effect on IL-6, ICAM-1, body mass or fat mass in overweight women who trained at least twice a week (Olson et al. 2007). Similarly, resistance training but not aerobic training elicited a reduction in circulating CRP after a 10 week period in sedentary, overweight individuals, however IL-6 was unaltered in both groups (Donges et al. 2010). Therefore, there does appear to be some benefit of resistance training on inflammatory proteins, particularly regarding CRP, which has been shown to have an inverse relationship with physical activity (Abramson and Vaccarino 2002), however, it may not be suitable to induce an improvement in other inflammatory proteins, including IL-6, in non-disease populations.

In conclusion, a number of studies have looked at the effects of exercise training in healthy individuals with regards to basal inflammatory proteins. There is some evidence suggesting that exercise can be a suitable treatment or prevention strategy for inflammation in the circulation, however as this finding is not consistent, it is possible that individuals may benefit from incorporating higher intensity exercise into an exercise regimen, as the studies described utilised mainly low to moderate intensity exercise. The lack of effect of exercise on systemic inflammation could also be due to these studies being in non-disease populations that already have relatively normal levels of inflammatory proteins prior to an intervention.

In addition to studies investigating the effects of exercise training in non-disease groups, there are a number of studies that have investigated the effects of exercise interventions on inflammatory proteins in patient populations, including those with metabolic diseases such as T2DM and the metabolic syndrome who have chronic low-grade inflammation.

IL-6, sIL-6R and TNF-α were successfully reduced after a 12 week exercise intervention in chronic heart failure patients who exercised for 30 min at 60-80% of maximal heart rate, 5 days per week (Adamopoulos et al. 2002). Although a decrease was found in chronic heart failure patients, another study that investigated the effects of 4 weeks moderate intensity exercise, 4 days per week, in T2DM and impaired glucose tolerance patients found no change in IL-6 or IL-10 post-training, however the intervention did induce a significant reduction in CRP and an increase in adiponectin, coinciding with a small but significant weight loss in both groups (Oberbach et al. 2006).

A longer term exercise training programme (6 months) has been shown to lead to an increase in IL-10 in T2DM patients, thereby exerting anti-inflammatory protection (Kadoglou et al. 2007). The authors also reported a reduction in CRP and a slight but not statistically significant decrease in TNF-α and increase in adiponectin. Increased IL-10 with exercise training could induce a decrease in ICAM-1, as shown to occur in other studies, since IL-10 has been shown to downregulate ICAM-1 (Willems et al. 1994), although this study did not measure ICAM-1.

Low to moderate intensity exercise training can be a beneficial aid for decreasing ICAM-1 in obesity, as well as in those with impaired glucose tolerance. In T2DM patients, ICAM-1 was reduced after exercise training interventions lasting 3/4 weeks or 6 months, with sessions ranging from twice per week to daily (Roberts et al. 2006a; Roberts et al. 2006b; Zoppini et al. 2006; Tönjes et al. 2007), however, the training had no effect in healthy individuals (Zoppini et al. 2006). Moderate intensity exercise interventions involving aerobic and resistance training have also significantly reduced MCP-1 in men with the metabolic syndrome (Troseid et al. 2004), which could be due to an inhibitory effect via an increase in adiponectin. Therefore exercise training

in these populations has a positive effect on chronic low-grade inflammation. In all but one of these studies, the reduction in MCP-1 or ICAM-1 was accompanied by a significant weight loss, therefore it is not possible to conclude whether the reduced inflammation is entirely due to a direct effect of exercise training or to a reduction in adiposity. Despite not all studies showing that all inflammatory proteins are reduced after every exercise intervention in clinical populations, overall, exercise seems to be a suitable form of treatment to reduce chronic low-grade inflammation.

Although some of the previously discussed exercise interventions induced a reduction in body mass, diet was not controlled in these studies. Calorie restriction is an additional component which may influence the outcome of exercise training on inflammation, and some studies have looked at the effects of exercise training and energy restriction on chronic low-grade inflammation. Bruun et al. (2006) carried out a study that involved a 15 week lifestyle intervention, and incorporated a large volume of moderate intensity exercise (2-3 h, 5 days/week) and a hypocalorific diet in a morbidly obese population (Bruun et al. 2006). This lifestyle intervention resulted in reductions in IL-6, CRP and MCP-1 in the circulation, as well as an increase in adiponectin, although no change in TNF-α was found. In this study participants significantly reduced their body mass, with a mean loss of ~18 kg (~13% of body mass). Therefore, it is not possible to conclude whether the reduction in inflammatory proteins and increase in adiponectin was in response to the exercise training or due to the weight loss as the mean BMI in this study was also extremely high (~45 $kg \cdot m^{-2}$).

A similar lifestyle intervention induced a weight loss of ~12 kg (12% of body mass) after a 3 month period by combining exercise and energy restriction (Christiansen et al. 2010a), in obese individuals, although their baseline BMI was much lower (~34 $kg \cdot m^{-2}$) than Bruun et al. (2006). The findings by Christiansen et al. (2010a) supported those of Bruun et al. (2006) as they found that IL-6 and MCP-1 were significantly reduced and that adiponectin was increased after training. In addition, Christiansen et al (2010a) included an exercise only group that resulted in a far smaller weight loss of ~3.5 kg (3% of body mass) and did not alter any of the inflammatory proteins or adiponectin after training. The findings of both Bruun et al. (2006) and Christiansen et al.

(2010a) suggest that with moderate intensity exercise, weight loss may also be required to induce positive effects on chronic low-grade inflammation in obese populations.

Weight loss appears to be particularly important with regards to adiponectin. One study found that a weight loss programme via dieting caused a significant increase in adiponectin, however exercise without weight loss was insufficient to increase adiponectin (Hulver et al. 2002). Although these findings are important, it should be noted that the exercise group consisted of healthy individuals, whereas individuals in the diet group were morbidly obese, and therefore it is difficult to directly compare the effects of diet and exercise on adiponectin, albeit the post-intervention adiponectin concentration was far greater in the diet than exercise group.

In addition to the above lifestyle interventions, another study in obese and overweight post-menopausal women found that a 6 week diet and exercise programme had no effect on CRP and TNF-α, although there was a significant reduction in IL-6 (Ryan and Nicklas 2004). Despite a significant reduction in IL-6 there was no change in sIL-6R after training, however the mean pre-training sIL-6R of ~23 $ng \cdot ml^{-1}$ was very low. Exercise sessions were completed 3 times per week and involved 45 min sessions >60% $\dot{V}O_{2max}$ on a cycle ergometer or treadmill, however, it should be highlighted that only 25 of the 37 participants took part in the exercise training programme in addition to the weight loss programme. In addition, the weight loss in this study was less than in the previously discussed studies, where the mean weight loss was ~6 kg (~7% body mass) and initial body mass was ~50 and ~20 kg less than Bruun et al. (2006) and Christiansen et al. (2010a) respectively. The same research group carried out a longer intervention lasting 6 months and demonstrated that IL-6, sIL-6R and CRP were significantly reduced, although there was still no change in TNF-α (You et al. 2004), where body mass was reduced by 7.3 kg (~9%).

Whether exercise training is sufficient to reduce circulating TNF-α is not clear. Even when exercise training was accompanied by a large weight loss, studies have found no change in TNF-α in obese populations (Ryan and Nicklas 2004; Bruun et al. 2006). One study did find a reduction in TNF-α after a 12 week

supervised training programme in obese women, with and without impaired glucose tolerance (Strączkowsk et al. 2001), although pre-training TNF-α concentration was far greater than reported by Ryan and Nicklas (2004). In addition to the five 30 min supervised training sessions per week at 70% maximum heart rate, the participants were also encouraged to increase their leisure time physical activity throughout the duration of the study (Strączkowsk et al. 2001), which could account for the differences in the findings reported in this study and others that have found no change in TNF-α with exercise training. Further research is required to determine whether exercise is capable of inducing a decrease in TNF-α in chronic low-grade inflammation in conditions such as obesity.

In all of the previously discussed studies the exercise intensity has been of a low to moderate intensity, generally below 60% $\dot{V}O_{2max}$. Since some, but not all studies have found exercise to be a useful aid to maintain low levels of inflammatory proteins in the circulation in healthy populations and to reduce the inflammatory proteins in populations with chronic low-grade inflammation, it is important to consider the exercise intensity and whether the chosen intensity provides the optimum health benefits. One study that utilised a higher exercise intensity (70-80% $\dot{V}O_{2max}$) training programme in T2DM and those with the metabolic syndrome, twice per week for 12 months, found many inflammatory proteins to be reduced (Balducci et al. 2010b). Reductions in IL-6, TNF-α and CRP were reported as well as an increase in the anti-inflammatory cytokine IL-10. The increase in IL-10 was accompanied by an increase in adiponectin after exercise training, which has been shown to induce production of IL-10 in macrophages and monocytes (Wolf et al. 2004). Many of the studies discussed have shown that exercise training has not reduced TNF-α and therefore it is possible that the higher exercise intensity elicited in this study could have induced a reduction in TNF-α. This study suggests that a higher exercise intensity can induce greater reductions in inflammatory status than low or moderate intensity training programmes. It is likely that the changes in inflammatory proteins in this study were at least in part down to a direct effect of exercise since there was no change in fat mass, as measured via a bio-impedance monitor, although there was a significant reduction in waist

circumference. In the earlier studies discussing adiponectin, it appeared an increase in most studies was only found with weight loss, however, from the findings by Balducci et al. (2010b) it could be hypothesised that in order to induce a change in adiponectin, without a reduction in body mass, higher intensity exercise is required.

For many of these inflammatory proteins and adiponectin, there are inconsistencies with regards to the effects of exercise training between studies. Most of the studies that did induce a reduction in inflammation were in clinical populations that had higher levels of inflammatory proteins than those in non-clinical populations. There are a number of other factors that could explain the inconsistencies between studies, such as exercise duration and frequency, supervision of exercise training and dietary control. One of the key elements in all of the studies is the exercise intensity. Nearly all studies utilised a low to moderate intensity training programme (< 60% $\dot{V}O_{2max}$), often unsupervised, that may not be sufficient to induce reductions in inflammatory proteins, and therefore it is possible that higher intensity exercise is often required to decrease the inflammatory state in those with chronic low-grade inflammation. In addition, a number of the existing training protocols induced a significant weight loss in obese cohorts and therefore it is not clear whether any changes found in inflammatory proteins are directly due to the exercise or due to a weight loss induced by the exercise training.

2.7 Inflammatory proteins in adipose tissue and exercise training

As previously discussed adipose tissue contributes to inflammatory proteins in the circulation and this is particularly important in disease states where obesity is a risk factor, since there will be greater potential for production of inflammatory proteins. Inflammatory proteins produced within adipose tissue can have autocrine, paracrine and endocrine functions (Trujillo and Scherer 2006). Despite adipose tissue being a major source of many inflammatory mediators, and evidence that exercise has been shown to reduce levels of circulating inflammatory proteins in many studies, there is limited research

investigating the effects of repeated exercise on local inflammation within adipose tissue. Some studies have looked at the effects of exercise and lifestyle interventions on mRNA expression of inflammatory mediators in subcutaneous adipose tissue, however mRNA is not always reflective of the functional protein expression, with only moderate correlations shown (Guo et al. 2008).

Of these studies investigating mRNA expression, no change in IL-6, TNF-α or adiponectin mRNA expression in subcutaneous adipose tissue was found after 12 weeks of aerobic training at ~50% $\dot{V}O_{2max}$ (Polak et al. 2006) or resistance training (Klimcakova et al. 2006) in obese individuals, despite the aerobic training inducing a significant reduction in body mass. Christiansen and colleagues (2010a) however, found an increase in adiponectin mRNA expression in adipose tissue, after 12 weeks aerobic exercise training at an estimated 60% $\dot{V}O_{2max}$, but no change in IL-6, TNF-α, MCP-1 or macrophage specific markers, CD-14 and CD-68. In contrast, lifestyle interventions over 12 (Christiansen et al. 2010a; Christiansen et al. 2010b) and 15 (Bruun et al. 2006) weeks, incorporating moderate intensity exercise and a hypocaloric diet found a decreased expression of IL-6, TNF-α and MCP-1 mRNA in subcutaneous adipose tissue and an increase in adiponectin mRNA, alongside a weight loss of 5-14% in obese individuals. Despite these studies investigating the effects of exercise training on mRNA expression of these inflammatory mediators in adipose tissue, only one study has investigated the effect of weight loss induced by a hypocaloric diet in obese women who found that IL-6 protein in subcutaneous adipose tissue was reduced (Bastard et al. 2002), however this study did not involve an exercise intervention.

The existing literature on mRNA expression of inflammatory mediators in adipose tissue after periods of exercise training, alongside evidence of a decrease in IL-6 in adipose tissue induced by weight loss, suggests that exercise alone may not be sufficient to reduce inflammation in adipose tissue. However, further studies are needed to determine whether exercise can reduce inflammation at the protein level. In addition, the exercise training was of a relatively low to moderate intensity (~50-60% $\dot{V}O_{2max}$) in the above studies. As

many of the changes in the inflammatory proteins appear to be due to weight loss, future studies are required to see if a higher intensity exercise could reduce inflammation within adipose tissue, without weight loss. There is some evidence from clinical populations to support a higher exercise intensity, with reductions in many inflammatory proteins in the circulation found after a 12 month exercise regimen at 70-80% $\dot{V}O_{2max}$ in those with impaired glucose tolerance and T2DM (Balducci et al. 2010b), therefore it is plausible that a higher intensity exercise may reduce inflammation in adipose tissue.

2.8 High intensity intermittent training or traditional endurance training?

In a number of the studies discussed in the previous section, the effects of exercise on chronic low-grade inflammation and mRNA expression of inflammatory proteins in adipose tissue were discussed. Although many studies have found that exercise training can reduce inflammation, not all studies have reported a positive effect. Exercise intensity, duration and frequency are key components of training protocols that could determine whether inflammation is reduced. There is currently no research on the effects of high intensity intermittent exercise on inflammatory proteins in the circulation or adipose tissue.

The additional health benefits of high intensity intermittent exercise above traditional aerobic training are increasingly emerging in the health literature, with reports of increased $\dot{V}O_{2peak}$, insulin sensitivity, fat oxidation and fat loss. High intensity intermittent exercise has been shown to have greater cardioprotective effects than moderate exercise, including greater improvements in lipid profile, risk of developing T2DM and all-cause mortality (Swain and Franklin 2006).

The Wingate test is currently the most common form of intermittent training used in exercise physiology research and is a time efficient strategy in comparison to prolonged continuous aerobic training (90-120 min per session), to elicit similar changes in exercise performance, skeletal muscle oxidative

capacity and buffering capacity of skeletal muscle (Gibala 2006; Burgomaster 2008). This training stimulus has also been shown to reduce risk factors associated with CVD, through an improvement in peripheral artery distensibility and endothelial function to a similar degree as continuous endurance training (Rakobowchuk et al. 2008). However, in addition to these benefits, high intensity intermittent exercise includes anaerobic aspects which can result in greater improvements in cellular adaptations involved in energy metabolism (Earnest 2008). Each Wingate training session consists of 4-6 thirty second 'all out sprints', separated by 4 min cycling at a low cadence against a light resistance (~30 W) on a stationary bike, and will be referred to as sprint interval training (SIT) throughout this thesis.

In addition to the adaptations mentioned above, there is some evidence to suggest that intermittent exercise training can induce improvements in insulin sensitivity. Three studies have investigated the effects of 2 weeks SIT on insulin sensitivity with variations in the results reported (Babraj et al. 2009; Richards et al. 2010; Whyte et al. 2010). One group found an improvement in the insulin sensitivity index, and the response of insulin and glucose to an oral glucose tolerance test (OGTT) in healthy sedentary young males (Babraj et al. 2009). Participants completed a post-training OGTT either 2 or 3 days after the last training session to ensure any improvements were not attributable to the last training session (Hawley and Lessard 2008). Richards et al. (2010) also reported an increase in insulin sensitivity 72 h after training, as measured by an increase in the glucose infusion rate (GIR) after training, using the gold standard methodology – the hyperinsulinaemic euglycaemic clamp. This study benefited from a control group, and a third group that completed an acute bout of SIT to ensure any change in the GIR was not due to an acute effect from the last bout of exercise. However, the pre-training GIR appeared low in the SIT group in comparison to the pre GIR in the control group and the acute exercise group, and although post-training GIR was significantly increased from pre-training in the SIT group, the post GIR was comparable to the control groups. In contrast to the above findings a similar study has reported that the augmented insulin sensitivity at 24 h after the last training session was abolished at 72 h post-training in overweight and obese males (Whyte et al. 2010). These studies

indicate that it has not been fully established if intermittent training, and in particular if SIT can improve insulin sensitivity.

Although SIT improves many physiological factors, it is a very intense type of exercise that may not be achievable for the general population (Hawley and Gibala 2009), with feelings of nausea and light headedness reported (Richards et al. 2010). Other modes of intermittent exercise involve longer intervals (3-4 min) at a relatively high intensity (~80-90% $\dot{V}O_{2max}$) and will be referred to as high intensity intermittent training (HIIT). A pilot study in individuals with metabolic syndrome found that HIIT, involving 4 min running intervals at 90% heart rate max interspersed with 3 min active recovery periods at 70% heart rate max, resulted in greater improvements in $\dot{V}O_{2peak}$, endothelial function, insulin signalling in adipose tissue and skeletal muscle, as well as larger reductions in blood glucose and lipogenesis in comparison to moderate intensity exercise of equal volume (Tjønna et al. 2008). After 16 weeks training, 45% of the HIIT group were no longer classed as having metabolic syndrome, therefore, HIIT could be an important prevention strategy for T2DM. Similar findings have also been reported in older patients with postinfarction heart failure, where HIIT improved endothelial function, $\dot{V}O_{2max}$ and left ventricular ejection fraction to a greater extent than moderate continuous exercise after 12 weeks training (Wisløff et al. 2007).

A reduction in adipose tissue has also been reported with different high intensity intermittent exercise protocols to a greater extent than training involving continuous moderate intensity exercise (Tremblay et al. 1994; Trapp et al. 2008). The authors of one study found that 15 weeks interval training (8 s sprints and 12 s slow pedalling for a maximum of 20 min) resulted in a significantly greater fat loss than steady state exercise, measured by dual-energy X-ray absorptiometry (DEXA), and that abdominal fat was significantly reduced as well as an increase in abdominal lean mass (Trapp et al. 2008). Energy expenditure was not significantly different between groups, however the reason for the differences in fat loss are unclear. An increase in excess post-exercise oxygen consumption (EPOC), post-exercise fat oxidation and appetite suppression have all been suggested to be mechanisms by which fat loss may

be increased to a greater extent after HIIT than after moderate intensity exercise training, although these have not been investigated.

The studies utilising protocols with longer intervals (~3-4 min) could be classed as a middle ground between SIT and prolonged continuous endurance exercise. Talanian et al. (2007) carried out a 2 week training study in recreationally active women, where one session of HIIT consisted of ten 4 min intervals cycling at ~90% $\dot{V}O_{2peak}$, interspersed by 2 min rest. This type of training elicited similar improvements in muscle oxidative potential and $\dot{V}O_{2peak}$ as SIT (Burgomaster 2005; Whyte et al. 2010). The authors concluded that 7 sessions of HIIT over a 2 week period increased whole body fat oxidation during exercise and described the training as suitable for untrained individuals. Longer intervals enable the desired percentage of $\dot{V}O_{2peak}$ to be reached during the interval period and have been suggested to be one of the best forms of interval training due to all cardiorespiratory parameters being maximal (Astrand et al. 1960), with optimal improvements in $\dot{V}O_{2max}$ found with intervals at 90-100% $\dot{V}O_{2max}$ in runners (Robinson et al. 1991). In addition to the performance and health benefits associated with high intensity intermittent exercise it has also been reported as 'more enjoyable' than continuous moderate intensity exercise in both healthy young males (Bartlett et al. 2011) and in coronary heart disease patients (Guiraud et al. 2011).

2.9 Summary

In summary this literature review has demonstrated the importance of the IL-6 system in relation to both acute and chronic exercise and the potential effects that regular exercise may have on chronic low-grade inflammation in disease states such as obesity and T2DM. It has also raised important questions on the potential health benefits of HIIT, however the effects of HIIT in relation to the IL-6 system and inflammation are currently unknown. This thesis aims to answer some of the important questions regarding HIIT in relation to the IL-6 system, chronic low-grade inflammation and glycaemic control.

Chapter 3

GENERAL MATERIALS AND METHODS

3.1 Participants and ethical approval

All of the studies presented in this thesis were approved by the Loughborough University Ethical Advisory Committee and were conducted in accordance with the Declaration of Helsinki (2008). The volunteers gave informed written and verbal consent (Appendix A) after being advised of all possible risks and discomforts associated with the procedures used in the study designs.

All participants were asked to complete a health-screening questionnaire at the time of consent (Appendix B) to ascertain their suitability for the study. Any participants who reported any haematological, inflammatory disorder or were taking any nonsteroidal anti-inflammatory drugs (NSAIDs) were excluded from taking part.

3.2 Anthropometric measurements

Participant's weight and height were measured prior to all experimental tests. In Chapters 5 and 7 participants had their waist and hip circumference measured with a measuring tape. Waist circumference was measured half way between the iliac crest and the lowest rib and hip circumference was measured at the widest part of the hips. These measurements were then used to calculate the waist-hip ratio.

3.3 Exercise tests

3.3.1 Maximal oxygen uptake

In Chapters 4 and 5 participants had their peak oxygen uptake ($\dot{V}O_{2peak}$) determined using a continuous incremental exercise test on an electromagnetically-braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands), performed to volitional exhaustion. Expired air was measured continuously using an on-line breath-by-breath gas analysis system (Ultima CPX, MedGraphics, MN, USA), as well as continuous monitoring of heart rate

throughout the test (RS200, Polar Electro, Kempele, Finland). The Ultima CPX was calibrated using a 3 litre syringe, a calibration gas of known composition (5% CO_2 and 12% O_2) and a reference gas (21% O_2). The power output for the incremental exercise test started at 100 watts (W) and increased by 35 W every 3 min, with participants cycling at a pedal cadence of 70 revs·min^{-1}. $\dot{V}O_{2peak}$ was identified as the $\dot{V}O_2$ averaged over the highest 30 s period during the test.

3.3.2 High intensity intermittent training (HIIT)

The HIIT protocol utilised in Chapters 4 and 5 consisted of 10 intervals at ~85% $\dot{V}O_{2peak}$ lasting 4 min each and separated by 2 min recovery on the cycle ergometer. Participants completed a familiarisation trial for the HIIT protocol before all main trials. During this visit participants completed five 4 min intervals separated by 2 min rest at a load corresponding to that which would elicit 85-90% $\dot{V}O_{2peak}$. This session allowed the external work to be calculated for the HIIT trial.

3.4 Food intake and physical activity control

Participants abstained from caffeine, alcohol and strenuous exercise during the 24 h period prior to all experimental trials. Where participants had to take part in more than one experimental trial they filled in food and physical activity diaries for the 24 h period beforehand and then were asked to replicate the diaries during any subsequent trials.

3.5 Blood pressure

Arterial blood pressure was measured using a digital automatic blood pressure monitor (Omron M7, Omron Healthcare UK Ltd, Milton Keynes, UK). Participants remained in a supine position for 5 min before the 1st

measurement. A cuff was placed around the upper right arm and the participant rested their arm on a firm surface during all measurements. Blood pressure was measured 3 times and the reported results are an average of the 2nd and 3rd readings.

3.6 Blood sampling and handling

Blood samples were collected from an antecubital vein via venepuncture using a 21 G butterfly winged infusion set (Hospira UK Ltd, Warwickshire, UK). Where repeated sampling during a short period of time was required an 18 G BD venflon cannula (Becton Dickinson Infusion Therapy, Helsingborg, Sweden) was used instead. The cannula was kept patent via regular flushing with 0.9% (*w/v*) saline solution. The first 2 ml of blood extracted from the cannula via a syringe was discarded. Blood samples were collected into BD vacutainers (BD, Plymouth, UK) containing 1.8 mg EDTA per ml of blood. Blood samples were gently inverted 8 times and then placed on an SRT6 Stuart roller mixer (Bibby Scientific Ltd, Stone, UK) to ensure mixing. Whole blood was used to determine haemoglobin (Hb) concentration and haematocrit (Hct) content. For plasma, whole blood was centrifuged at 4,000 x g for 10 min at 4°C (Heraeus Labofuge 400 R, Kendro Laboratory Products, Langenselbold, Germany), and the resulting plasma aliquoted into eppendorfs and stored at -80°C for subsequent analysis. Participants were in a seated position for all blood sampling. Where blood samples were taken during acute exercise trials the participant's forearm was heated prior to sampling in order to collect an arterialised venous sample which was comparable to post-exercise samples. The forearm was inserted into warm water (~42ºC) for 10 min prior to sampling.

3.7 Haematological analysis

3.7.1 Haemoglobin

Hb concentration was measured in whole blood by a colorimetric method using a commercially available kit (Randox, Co Antrim, UK). This method is linear up to 21 $g \cdot dl^{-1}$. The reagent provided in the kit contained:

- Potassium Ferricyanide 0.61 $mmol \cdot l^{-1}$
- Potassium Cyanide 0.77 $mmol \cdot l^{-1}$
- Potassium Phosphate 1.03 $mmol \cdot l^{-1}$
- Surfactant 0.1 % v/v

Samples were prepared in duplicate by adding 10 µl of whole blood to 2.5 ml of reagent and mixing. Samples were left to incubate for 10 min and the sample absorbance (A_{sample}) of cyanmethaemoglobin was read at 540 nm on a UV mini 1240 spectrophotometer (Shimadzu UK Ltd, Milton Keynes, UK). The Hb concentration was calculated using the following equation:

$$\text{Hb concentration } (g \cdot dl^{-1}) = A_{sample} \times 36.77$$

The intra-assay coeffients of variance (CV) are reported in Table 3.1.

3.7.2 Haematocrit

Hct content was measured in triplicate using the microcapillary method and the mean was calculated. Whole blood was drawn into non-heparinised microhaematocrit tubes and the tubes were sealed with Cristaseal capillary tube sealant. Samples were centrifuged for 4 min in a HaematoSpin1300 centrifuge and Hct content was measured using a microhaemoatocrit tube reader (Hawksley, Sussex, UK). The intra-assay CVs for Hct are reported in Table 3.1.

Table 3.1 Intra-assay coefficients of variance (CV) for haemoglobin and haematocrit analysis.

	CV (%)
Haemoglobin	2.7
Haematocrit	0.6

3.7.3 Plasma volume changes

Protein concentrations in plasma were adjusted to account for any changes in plasma volume from baseline according to the methods outlined previously by Dill and Costill (1974).

3.8 Enzyme-linked immunosorbent assays

Adiponectin, MCP-1 and ICAM-1 were quantified using commercial sandwich enzyme-linked immunosorbent assays (ELISAs), and TNF-α and IL-10 were measured via high-sensitivity ELISAs (R & D Systems, Minneapolis, MN, USA).

Plasma IL-6, sIL-6R, the IL-6/sIL-6R complex and CRP were analysed via ELISAs. All materials and chemical reagents were obtained from Sigma-Aldrich Ltd (Poole, UK) unless otherwise specified. All incubation periods were at room temperature and during each incubation stage the plate was placed on a Stuart Mini Orbital Shaker (Bibby Scientific Ltd, Stone, UK) at 60 revs·min^{-1} unless otherwise stated. Wash steps for ELISAs were carried out using an automated Wellwash AC microplate washer (Thermo Scientific, Vantaa, Finland). The absorbance of wells was read using a Varioskan Flash Multimode Reader (Thermo Scientific, Vantaa, Finland). Protein concentration of samples was determined in relation to a 4-parameter logistic standard curve (GraphPad Prism version 4.00, San Diego California, USA). The equation of the curve is given below:

$$X = \log EC50 - LOG10((Top - y) / (y - Bottom))*(1/Hillslope)$$

Where X is the sample concentration; y is the absorbance; EC50 is the middle of the curve. All samples were analysed in duplicate and were repeated if the CV between duplicates was more than 10%. The intra-assay CVs for the inflammatory proteins throughout this thesis are reported in Table 3.2.

Table 3.2 Intra-assay coefficients of variance (CV) for inflammatory protein analysis in plasma.

Inflammatory protein	CV (%)
IL-6	6.1
sIL-6R	2.4
IL-6/sIL-6R complex	3.8
Adiponectin	2.7
TNF-α	7.5
IL-10	8.0
CRP	3.8
MCP-1	2.4
ICAM-1	3.2

3.8.1 Interleukin-6 assay

An ELISA for the detection of plasma IL-6 was optimised using a human IL-6 antibody set (OptEIA, BD Biosciences, Oxford, UK) containing a primary and secondary antibody and recombinant human IL-6 (rhIL-6) for standards. Immulon 4HBX Flat 96-well microtiter plates (Nunc, Thermo Scientific, Roskilde, Denmark) were coated with 100 µl anti-human IL-6 monoclonal capture antibody diluted 1:250 in a 0.1 M sodium carbonate buffer (see Table 3.3). The next day the plates were washed with Tris Buffered Saline (TBS), pH 7.5 with 0.05% Tween 20 (TBS-T) 3 times, and then blocked with 5% bovine albumin serum (BSA; Probumin, Millipore, Illinois, USA) in TBS. The plates were incubated for 1 h at room temperature. Afterwards, plates were washed

and 100 µl of samples or standards were added to the wells in duplicate. Plasma samples were diluted 1:5 in TBS with 10% fetal calf serum (FCS). The rhIL-6 was serially diluted in TBS with 10% FCS from 20 to 0.156 $pg \cdot ml^{-1}$. TBS with 10% FCS served as the zero standard. After 2 h plates were washed 6 times and the 100 µl of biotinylated anti-human IL-6 monoclonal antibody detection antibody diluted 1:250 in TBS-T with 1% BSA was added per well. Plates were incubated for a further 1 h before being washed 7 times with 30 s soak periods. The enzyme Streptavidin Alkaline Phosphatase (AKP) was diluted 1:2000 in TBS with 1% BSA and 100 µl was added per well. Plates were then incubated for 45 min. Washes were repeated as in the previous step before the addition of an ELISA amplification system (Invitrogen, Paisley, UK). In the first step 50 µl of substrate solution was added to all wells and plates were incubated on the bench top before adding 50 µl of the amplification solution. The reaction was stopped within 15 min of the amplification solution being added by the addition of 50 µl of 10% sulphuric acid (stop solution) and the absorbance of the wells was read at 490 nm with a correction wavelength of 690 nm. This assay measures total IL-6 content.

3.8.2 Soluble interleukin-6 receptor assay

Plasma sIL-6R concentration was determined by ELISA via a method adapted from Gray et al. (2008). Antibody pairs M5 (capture) and M182 (detection) were used to detect sIL-6R (BD Biosciences, San Diego, USA). This ELISA detects both free and bound sIL-6R. Immulon 4HBX Flat 96-well microtiter plates were coated with 100 µl Purified Mouse Anti-Human CD126 capture antibody (Clone M5, BD Biosciences, San Diego, USA) diluted in 0.1 M sodium carbonate buffer (see Table 3.3) to give a concentration of 2 $\mu g \cdot ml^{-1}$ and were stored at 4ºC overnight. The next day plates were washed 3 times with phosphate buffered saline (PBS) with 0.05% Tween 20 (PBS-T) and wells were subsequently blocked with PBS with 10% FCS (assay diluent). Plates were incubated for 1 h before being washed again and adding 100 µl standards or samples to wells. Samples were diluted 1:200 in assay diluent. The rhIL6-R (R&D systems, Minneapolis, MN, USA) was serially diluted in assay diluent to give standards

ranging from 500 to 0.5 pg·ml^{-1}. After 2 h the plates were washed 5 times with PBS-T and 100 µl of the detection antibody, Biotin Mouse Anti-Human CD126 (Clone M182, BD Biosciences, San Diego, USA) diluted in assay diluent to give a final concentration of 0.5 µg·ml^{-1}, was added to each well. Plates were incubated for a further hour before washing 5 times. The enzyme Streptavidin Horse Radish Peroxidise (HRP) (BD Biosciences) was diluted 1:4000 in assay diluent and 100 µl was added per well. Meanwhile a substrate solution was prepared (see Table 3.6). After 45 min the plate was washed a further 7 times with 30 s soaks in between and 100 µl of the working substrate solution was added for 30 min before the addition of 50 µl of stop solution. The final absorbance was then read at 450 nm with a correction wavelength of 570 nm.

3.8.3 IL-6/sIL-6R complex assay

An ELISA for the determination of the IL-6/sIL-6R complex has previously been described by Gray et al. (2009b). This ELISA measures the biologically active binary form of the IL-6/sIL-6R complex and does not measure the biologically inactive tertiary IL-6/sIL-6R/sgp130 complex. The protocol for this method is similar to the sIL-6R assay, however, the wells were coated with 100 µl anti-human IL-6 monoclonal capture antibody diluted 1:250 in coating buffer and plasma samples were diluted 1:2 in assay diluent. There are no standards available for this assay, therefore the results are presented either as a fold-change or as arbitrary units.

3.8.4 C-reactive protein assay

CRP was quantified via an ELISA method adapted from (Pawluczyk et al. 2011). Immulon 4HBX Flat 96-well microtiter plates were coated with 70 µl of primary anti-CRP rabbit polyclonal antibody (Calbiochem, EMD Biosciences, Inc., La Jolla, CA, USA), diluted to a working concentration of 3.5 µg·ml^{-1} in 0.05 M sodium carbonate, pH 9.6 (Table 3.7). The plates were incubated at 4˚C overnight. The following morning the plates were washed 4 times with PBS-T

and the wells were blocked with 100 µl PBS with 1% BSA (Probumin, Millipore, Illinois, USA). The plates were then incubated for 1 h at room temperature and then washed as before. Afterwards, 50 µl of plasma samples (diluted 1:100 in PBS) or standards were added to the wells in duplicate. Human CRP (NIBSC, Potters Bar, UK) was used as a calibrant for this ELISA method. A CRP stock concentration of 50 $\mu g \cdot ml^{-1}$ was serially diluted in PBS to give a standard curve ranging from 1000 to 1 $ng \cdot ml^{-1}$. The plate was incubated at 4˚C overnight. On the 3rd day the plates were washed as before and 50 µl of a secondary mouse monoclonal antibody to CRP (Abcam, Cambridge, UK) diluted to a working concentration of 2.7 $\mu g \cdot ml^{-1}$ in PBS was added to each well. Plates were then incubated for 2 h and the wash step was repeated. For the next step polyclonal rabbit anti-mouse IgG conjugated to HRP (Dako, Ely, UK) was diluted 1:1000 in PBS and 50 µl of the diluted solution was added to the wells and plates were incubated for 1 h. A soluble substrate solution for the detection of peroxidase activity was prepared using SIGMAFAST OPD tablets shortly before the next wash step. One *o*-Phenylenediamine dihydrochloride (OPD) tablet and one urea hydrogen peroxide tablet were dissolved in 20 ml deionised water to give a final concentration of 0.4 $mg \cdot ml^{-1}$ OPD, 0.4 $mg \cdot ml^{-1}$ urea hydrogen peroxide, and 0.05 M phosphate-citrate, pH 5.0. The plates were washed a further 4 times and 50 µl of the substrate solution was immediately added to all wells and the colour was left to develop for 5 min before stopping the reaction with 70 µl of 10% sulphuric acid. The absorbance of the wells was read at 490 nm with a correction wavelength at 650 nm.

3.8.5 ELISA buffers and solutions

Table 3.3 0.1 M Sodium carbonate (coating buffer).

Component	Mass/Volume
$NaHCO_3$ Sodium bicarbonate	0.42 g
Na_2CO_3 Sodium carbonate	0.178 g
Deionised water to	50 ml
pH 9.5 with HCl	

Table 3.4 Tris buffered saline.

Component	Mass/Volume
Tris base (50 mM)	6.05 g
NaCl Sodium chloride (150 mM)	8.76 g
Deionised water to:	1000 ml
pH 7.5 with HCl	

Table 3.5 Phosphate buffered saline.

Component	Mass/Volume
NaCl Sodium chloride	8 g
Na_2HPO_4 Sodium phosphate, dibasic	1.16 g
KH_2PO_4 Potassium phosphate, monobasic	0.2 g
KCl Potassium chloride	0.2 g
Deionised water to	1000 ml
pH 7.4 with HCl	

Table 3.6 Working substrate solution for the sIL-6R and IL-6/sIL-6R complex ELISA.

Component	Mass/Volume
TMB in DMSO (6 mg·ml^{-1})	100 µl
Sodium acetate (0.1 M)	10 ml
H_2O_2 Hydrogen peroxide (30 w/w)	5 µl
pH 5 with Solid citric acid crystals	

TMB, 3,3',5,5'-Tetramethylbenzidine; DMSO, Dimethyl sulfoxide.
Hydrogen peroxide was added immediately before use.

Table 3.7 0.05 M Sodium carbonate (coating buffer) for the C-reactive protein ELISA.

Component	Mass/Volume
$NaHCO_3$ Sodium bicarbonate	0.189 g
Na_2CO_3 Sodium carbonate	0.027 g
Deionised water to	50 ml
pH 9.6 with HCl	

3.9 Subcutaneous abdominal adipose tissue sampling and analysis

3.9.1 Adipose tissue biopsy procedure

Subcutaneous abdominal adipose tissue biopsy samples were collected for Chapters 5 and 6, before and after 2 weeks of HIIT. After a 12 h overnight fast participants reported to the lab. Participants lay in a semi-supine position and povidone-iodine was used to clean the inferior border of the costal margin to the anterior superior iliac spine. 10 ml of 1% (*w/v*) lidocaine was administered under sterile conditions to the area before the adipose tissue was extracted ~10-15 cm laterally from the umbilicus, using a percutaneous needle biopsy technique (Christiansen et al. 2010a) with a 14 G needle and 20 ml syringe. A vacuum was applied to the syringe resulting in the collection of adipose tissue. The excised adipose tissue was immediately washed with 0.9% (*w/v*) saline

solution to limit blood levels within the biopsy sample before it was aliquoted into eppendorfs using sterile forceps. The tissue was snap-frozen in liquid nitrogen before being transferred to -80 ºC freezer until analysis. The post 2 weeks HIIT sample was taken 46-48 hours after the last training session in order to abolish any effects of acute exercise (Hawley and Lessard 2008).

3.9.2 Homogenisation of subcutaneous adipose tissue

Adipose tissue samples (typically ~200 mg) were homogenised in 500 µl buffer of 5 mM Tris/HCl, pH 7.5 (Table 3.8), containing protease inhibitor cocktail (Roche Diagnostics Ltd, Mannheim, Germany), for 30 s using a handheld TissueRuptor (Qiagen Ltd, Crawley, UK). The probe on the TissueRuptor was changed between samples. After homogenisation the samples were clarified by centrifugation at 10,000 x g for 10 min at 4ºC and the internatant was transferred to a fresh eppendorf and then stored and frozen at -80 ºC prior to protein analysis.

Table 3.8 Tris/HCl homogenisation buffer for adipose tissue.

Component	Final concentration	Mass/Volume
Tris/HCl (500 mM)	5 mM	100 µl
EDTA (50 MM)	1 mM	100 µl
Sucrose (10 % w/v)		1 g
DTT (100 mM)	1 mM	100 µl
Deionised water to		10 ml
Protease inhibitor cocktail tablet		1 tablet
pH 7.4 with HCl		

Components were added in this order. Samples were homogenised immediately after protease inhibitor cocktail tablet had dissolved in the buffer.

3.9.3 Determination of adipose tissue protein concentration

Internatant protein concentration was determined using the DC Protein Assay Kit (Bio-Rad Laboratories, Hemel Hempstead, UK) with a bovine serum albumin standard set (Bio-Rad Laboratories) used as protein standards. Samples were diluted 1:50 in 5 mM Tris/HCl, pH 7.5. To 96-well microtest plates (Sarstedt, Nümbrecht, Germany) 40 µl of standards, ranging from 1.25-10 $\mu g \cdot ml^{-1}$ or samples were added to wells, followed by 20 µl of Reagent A and 160 µl of Reagent B. Tris/HCl buffer served as the blank. Wells were incubated at room temperature for 15 min and the absorbance measured at 750 nm (Varioskan Flash, Thermo Scientific, Vantaa, Finland). The absorbance of standards was plotted and the linear regression relationship was determined which was used to calculate the protein concentration of adipose tissue samples. R^2 was >0.98 for all linear regression relationships.

3.10 Statistical analysis

All statistical analysis was performed using SPSS 16.0 software (Statistical Package for the Social Sciences Inc., Chicago, Illinois, USA) and data is presented as mean and standard deviation (± SD) with 95% confidence intervals (CI). All data was checked for normality using the Shapiro-Wilk test before the main analysis. If data was not normally distributed the data was transformed and checked for normality again. The transformed, normally distributed data was then used for any subsequent statistical analysis, to comply with the assumptions of parametric models of analysis. Where there were more than 3 comparisons Mauchly's test of sphericity was also checked. Sphericity was assumed where $p > 0.05$. Where sphericity was not met the Greenhouse-Geisser correction was used.

Data was analysed using a within group, repeated measures analysis of variance (ANOVA) model where there were more than two comparisons. Post-hoc pair-wise comparisons were performed where appropriate according to the Bonferroni adjustment method. Paired sample t-tests were used to analyse

within group differences when there were only two comparisons. Statistical significance was accepted at $p \leq 0.05$.

Chapter 4

IL-6 AND sIL-6R RESPONSES TO INTERMITTENT HIGH INTENSITY AND CONTINUOUS MODERATE INTENSITY EXERCISE

4.1 Abstract

As IL-6, sIL-6R and the IL-6/sIL-6R complex are transiently elevated in response to continuous moderate intensity exercise, this study investigated how these biological parameters would be modulated by an acute bout of HIIT in comparison to continuous moderate exercise (MOD). The study also investigated the response of differentially spliced sIL-6R (DS-sIL-6R) to exercise. Eleven healthy males completed 2 exercise trials matched for external work done [582 (82) kJ]. During MOD participants cycled at 62 (3) % $\dot{V}O_{2peak}$ for 59 (2) min, whilst HIIT consisted of ten 4 min intervals cycling at 88 (3) % $\dot{V}O_{2peak}$ separated by 2 min rest. Blood samples were collected pre-exercise, post-exercise and 1.5, 6 and 23 h post-exercise. Plasma IL-6, sIL-6R, the IL-6/sIL-6R complex and DS-sIL-6R levels were measured by ELISA. HIIT caused a significantly greater increase in IL-6 than MOD (p = 0.018). Both MOD and HIIT resulted in an increase in sIL-6R and IL-6/sIL-6R complex (p < 0.001), however, this was not significantly different between trials. The sIL-6R was significantly increased at 6 h post-exercise in both trials (p < 0.05). DS-sIL-6R was also significantly increased after exercise (p = 0.020), representing 0.5% of the total sIL-6R increase. This investigation has demonstrated that the IL-6 response is greater after intermittent high intensity exercise than comparable moderate exercise, however, increased IL-6/sIL-6R complex and sIL-6R did not differ between HIIT and MOD. The current study has also shown for the first time that elevated sIL-6R after exercise is derived from both proteolytic cleavage and differential splicing of membrane-bound IL-6R.

4.2 Introduction

A comprehensive review of the cytokine, IL-6, has provided evidence to support the concept that acute elevations of IL-6 can have beneficial consequences on health through metabolic and anti-inflammatory mechanisms (Pedersen 2009). IL-6 has been classified as having both pro- and anti-inflammatory properties (Scheller et al. 2011). Unlike pro-inflammatory cytokines, IL-6 appears to be the primary inducer of acute-phase proteins, many of which have anti-inflammatory properties, as well as inhibiting TNF-α and IL-1 expression (reviewed in Pedersen et al. 2001). Prolonged moderate intensity exercise is capable of elevating levels of circulating IL-6 (Keller et al. 2001), which binds to IL-6R to form a binary complex (IL-6/IL-6R) (Taga et al. 1989). Soluble IL-6R allows IL-6 signalling to occur in tissues lacking membrane-bound IL-6R (Rose-John and Heinrich 1994) and can be generated by differential mRNA splicing (DS-sIL-6R) (Horiuchi et al. 1994) or proteolytic cleavage (PC-sIL-6R) of the membrane bound receptor (Müllberg et al. 1994). Although DS-sIL-6R represents less than 1% of sIL-6R at rest (Dimitrov et al. 2006), the source of the elevated sIL-6R in response to exercise stress is unknown.

While there is considerable knowledge regarding moderate intensity exercise and the response of the IL-6 system, there is increasing evidence that high intensity exercise may have a greater cardioprotective effect (Swain and Franklin 2006). This is in parallel to evidence indicating that IL-6 release is intensity dependent (Ostrowski et al. 2000; Helge et al. 2003), and that over 50% of the variation can be contributed to the duration of exercise (Fischer 2006). Intermittent exercise offers a solution to both these criteria in that it combines periods of high intensity exercise intervened with rest periods allowing the duration to be extended beyond that of continuous high intensity exercise. Although intermittent exercise is normally the domain of athletes, there is evidence that this form of exercise can be accomplished by recreationally active women (Talanian et al. 2007) and in patient populations including those with the metabolic syndrome and postinfarction heart failure patients (Wisløff et al. 2007; Tjønna et al. 2008). If IL-6 is considered a

mediating factor for health then it is important to investigate the response of the IL-6 system to this type of exercise.

This study aimed to test the hypothesis that an acute bout of HIIT will elevate all components of the IL-6 system above that of continuous moderate intensity exercise. In addition, a secondary hypothesis, that acute exercise will increase DS-sIL-6R was investigated.

4.3 Materials and methods

4.3.1 Participant characteristics

Eleven healthy male volunteers participated in this study [age 22.3 (4.0) y, body mass 73.5 (5.4) kg, height 1.79 (0.1) m, BMI 23.0 (1.8) $kg \cdot m^{-2}$]. Participants were not specifically trained to a particular sport, however, all were physically active and reported participating in exercise equating to 3 or 4 thirty minute exercise sessions per week.

4.3.2 Preliminary measurements

Participants completed a $\dot{V}O_{2peak}$ test 2 weeks before the first main trial and a familiarisation trial for the HIIT protocol 1 week prior to the first main trial (Section 3.3). The familiarisation trial allowed the external work to be calculated for the HIIT trial. This was used to determine the duration required during the MOD trial (undertaken at ~60% $\dot{V}O_{2peak}$), in order for the same external work to be completed in both trials.

4.3.3 Experimental protocol

Participants attended the laboratory on 2 other occasions separated by 1 week, to complete a HIIT and a MOD trial in a randomised order. On both occasions, participants arrived at the laboratory at 8 am following a 10 h overnight fast. A

cannula provided blood samples at pre-, post- and 1.5 h post-exercise. After pre-exercise blood sampling, the participant moved to the cycle ergometer and completed ten 4 min intervals at a power output to elicit ~85-90% of $\dot{V}O_{2peak}$ or cycled continuously at ~60% $\dot{V}O_{2peak}$. Participants completed the same external work in both experimental trials. Immediately upon the cessation of exercise a post-exercise blood sample was taken. Participants then rested in a seated position for 1.5 h at which point another blood sample was taken. Following this sample, the cannula was removed and the participant was permitted to leave the laboratory, and was requested to reduce physical activity to a minimum for the remainder of the trial. The participant returned to the laboratory at 6 h post-exercise and 24 h from the start of the exercise for blood sampling. The average temperature and relative humidity throughout the study were 20.4 (0.4) ºC and 33.8 (6.3) % respectively. Figure 4.1 illustrates the HIIT and MOD trials.

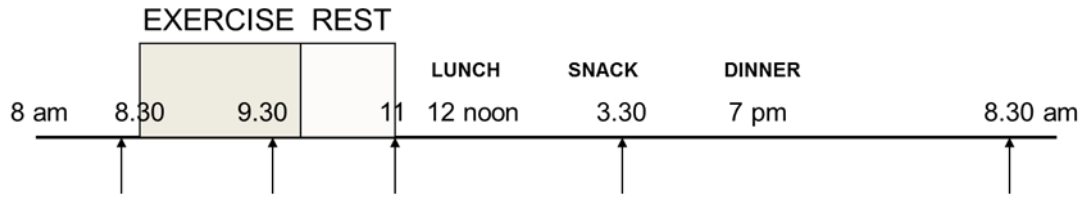

Figure 4.1 Schematic representation of the high intensity intermittent and the moderate continuous intensity exercise trials. ↑ represents blood sampling.

4.3.4 Blood sampling

Blood samples were collected and handled as previously described (Section 3.6). Haematological analysis was carried out and results were corrected for changes in plasma volume from pre-exercise (Section 3.7).

4.3.5 Meals for experimental trials

Participants were provided with standardised meals for both experimental trials, consisting of lunch, a snack, dinner and drinks. Energy intake was the same for every participant and totalled 10,962 kJ per trial day. Nutrient content of the meals can be found in Appedix C.

4.3.6 IL-6, sIL-6R and the IL-6/sIL-6R complex analysis

Plasma IL-6, sIL-6R and the IL-6/sIL-6R complex were analysed as described in Chapter 3.

4.3.7 Differentially spliced sIL-6R analysis

An ELISA for the detection of DS-sIL-6R was adapted from Horiuchi et al. (1998). As sIL-6R peaked at 6 h post-exercise, blood samples at pre-exercise and 6 h post-exercise were analysed from the HIIT trial for DS-sIL-6R concentration, in order to determine the mechanism of sIL-6R production at rest and after exercise. Briefly 96-well plates were coated overnight with 100 µl of 0.5 $\mu g \cdot ml^{-1}$ anti-DS-sIL-6R monoclonal antibody (mAb 2F3, which was raised against the unique COOH-terminal sequence of DS-sIL-6R, GSRRRGSCGL) in PBS. The following morning the plate was washed 3 times with PBS-T and the wells were blocked with 200 µl PBS with 5% BSA for 1 h. After washing the plate 3 times, 100 µl of samples or standards were added to wells in triplicate and incubated for 2 h at room temperature. Recombinant DS-sIL-6R was serially diluted in PBS with 1% BSA to provide a standard curve ranging from 2000 to 31.25 $pg \cdot ml^{-1}$. Afterwards, the plate was washed a further 3 times and wells were incubated with the secondary antibody, human IL-6R biotinylated antibody, clone *Sf* 21 (R & D Systems, Minneapolis, MN, USA) in PBS with 1% BSA. After 1 h the plate was washed 3 times and 100 µl of ready-to-use HRP (Vector Laboratories Ltd, Peterborough, UK) was added to each well for 20 min before washing the plate a further 3 times. The color was developed by the

addition of 100 µl SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL, Gaithersburg, MD, USA). The reaction was stopped with the addition of 50 µl 1 M sulphuric acid and the optical density was read at 450 nm. There was an intra assay CV of 7.6%.

4.3.8 Substudy: IL-6 response to an indwelling cannula

During this study Dixon et al. (2009) published a paper identifying that IL-6 was significantly greater when an indwelling cannula was used in comparison to a single-use needle. In the current study this would apply to our post-exercise and 1.5 h post-exercise samples and would suggest that our values may be slightly high at these time points. However, as a cannula was used in both trials this should not affect trial differences. To investigate this further, in 4 participants an additional blood sample was collected from the contralateral forearm via venepuncture at 1.5 h post-exercise.

4.3.9 Statistical analysis

Full statistical analysis is outlined in Section 3.10.

4.4 Results

4.4.1 Exercise intensity and heart rate

The mean $\dot{V}O_{2peak}$ for the participants was 51 (6) $ml \cdot kg^{-1} \cdot min^{-1}$ and occurred at a mean power output of 294 (33) W. Table 4.1 summarises the exercise outcomes for both HIIT and MOD trials.

Table 4.1 Descriptive data for HIIT and MOD exercise trials.

	$\dot{V}O_2$ peak (%)	Heart rate (beats·min^{-1})	Power output (W)	Work done (kJ)	Exercise duration (min)
HIIT	88 (3)	172 (9)	242 (34)	582 (82)	40 (0)
MOD	62 (3)	146 (12)	165 (19)	582 (82)	59 (2)

Values are mean (SD), N = 11.

In addition, Figure 4.2 illustrates the percentage of peak heart rate and $\dot{V}O_2$ that participants were cycling at for each interval during the HIIT trial.

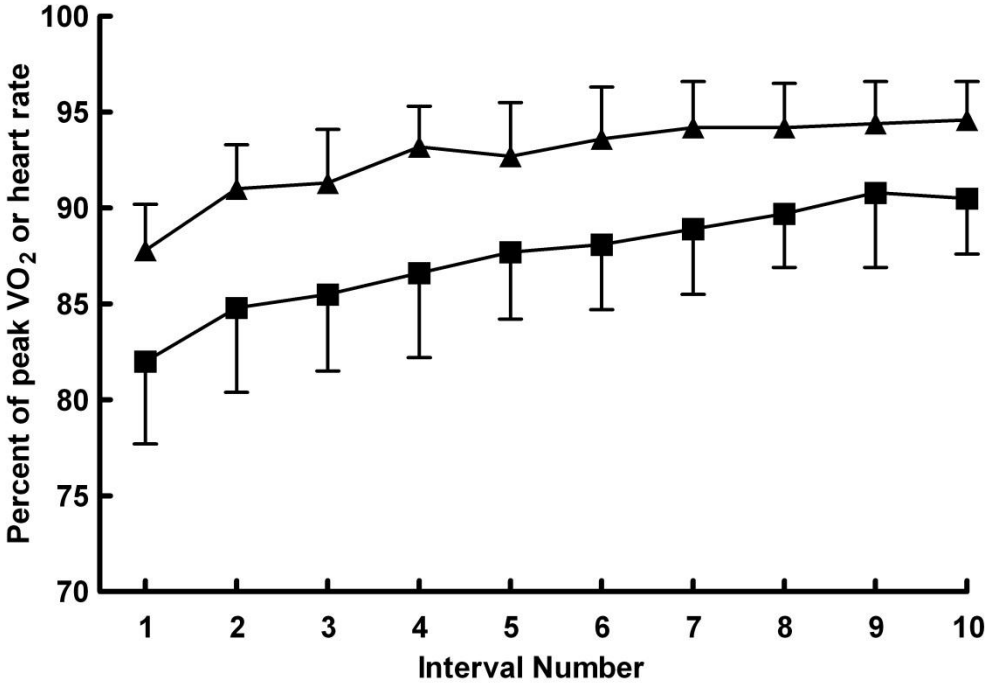

Figure 4.2 The percentage of peak heart rate (▲) and $\dot{V}O_2$ (■) that participants were cycling at during individual intervals in the HIIT trial.

4.4.2 IL-6

A main trial effect was found for IL-6 (p = 0.018), with significantly higher IL-6 immediately post-exercise during HIIT compared to MOD (p = 0.004; Figure 4.3). In addition, there was a main effect of time (p < 0.001), with IL-6 peaking immediately post-exercise in both trials (10.2 (6.8) [5.6-14.7 95% CI] pg·ml^{-1} and 7.2 (3.6) [4.8-9.6 95% CI] pg·ml^{-1} after HIIT and MOD respectively). IL-6 was significantly elevated post- and 1.5 h post-exercise during both trials (p < 0.05).

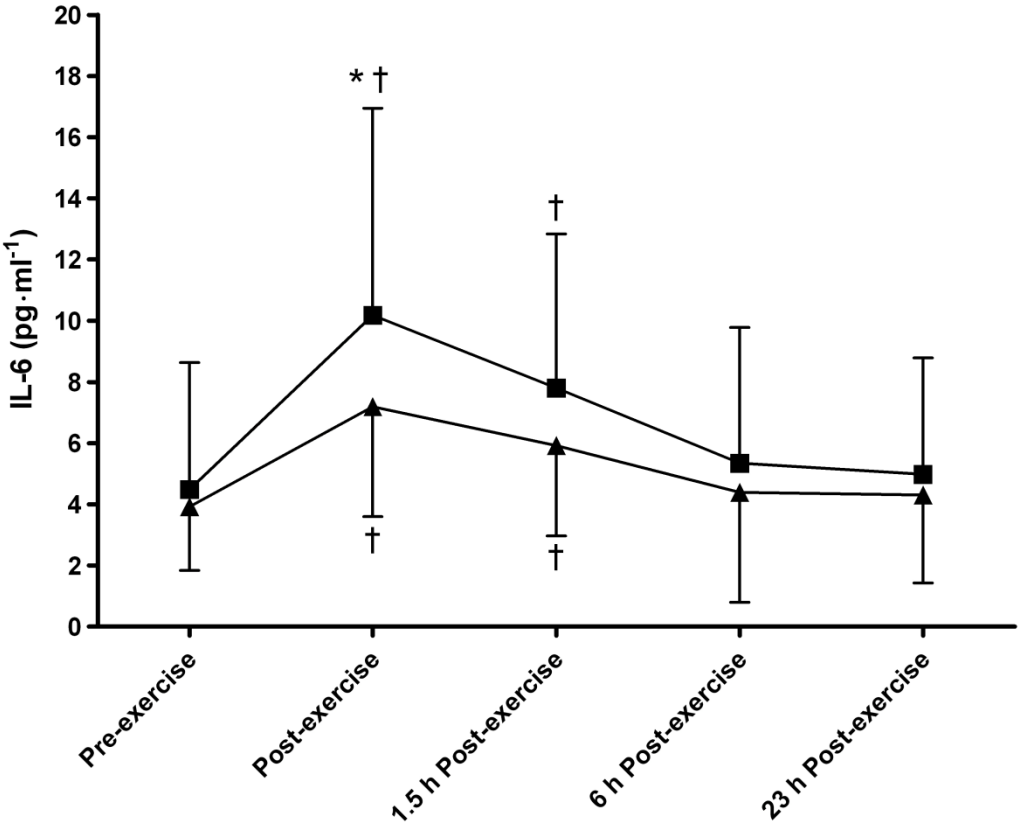

Figure 4.3 The IL-6 response to MOD (▲) and HIIT (■) exercise trials. Mean (SD). * significantly higher than the MOD (p = 0.004). † significantly different to pre-exercise (p < 0.001).

4.4.3 Soluble IL-6R

No differences were found for sIL-6R between trials (p = 0.214; Figure 4.4), however, a main effect of time was found (p < 0.001), where sIL-6R was significantly higher than pre-exercise at 6 h post-exercise during both trials (sIL-6R was 49.1 (16.5) [38.1-60.2 95% CI] ng·ml^{-1} and 47.5 (15.0) [37.4-57.6 95% CI] ng·ml^{-1} during HIIT and MOD respectively).

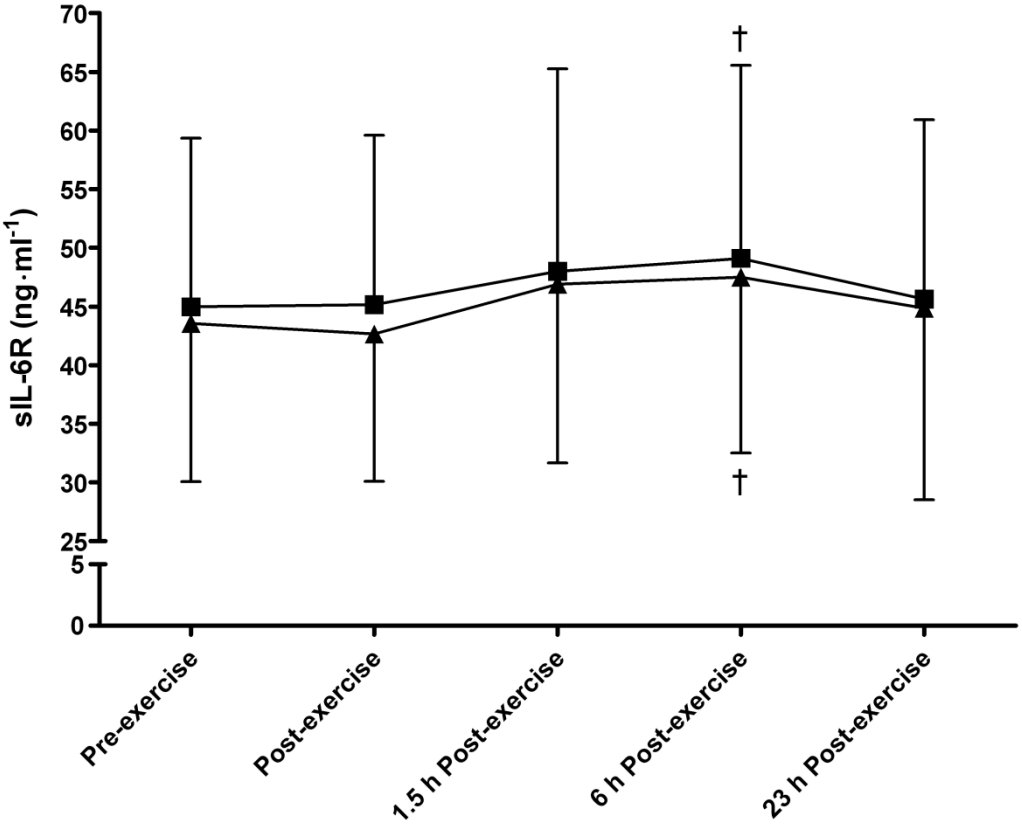

Figure 4.4 The sIL-6R response to MOD (▲) and HIIT (■) exercise trials. Mean (SD). † significantly different to pre-exercise (p < 0.05).

4.4.4 IL-6/sIL-6R complex

No significant differences were found for the IL-6/sIL-6R complex concentration between HIIT and MOD trials (p = 0.215), although a main effect of time was found (p < 0.001; Figure 4.5). Peak IL-6/sIL-6R complex concentration occurred immediately post-exercise during both trials (59 (62) [17-100 95% CI] % and 26 (41) [-2.2-53 95% CI] % increase from pre-exercise for HIIT and MOD respectively).

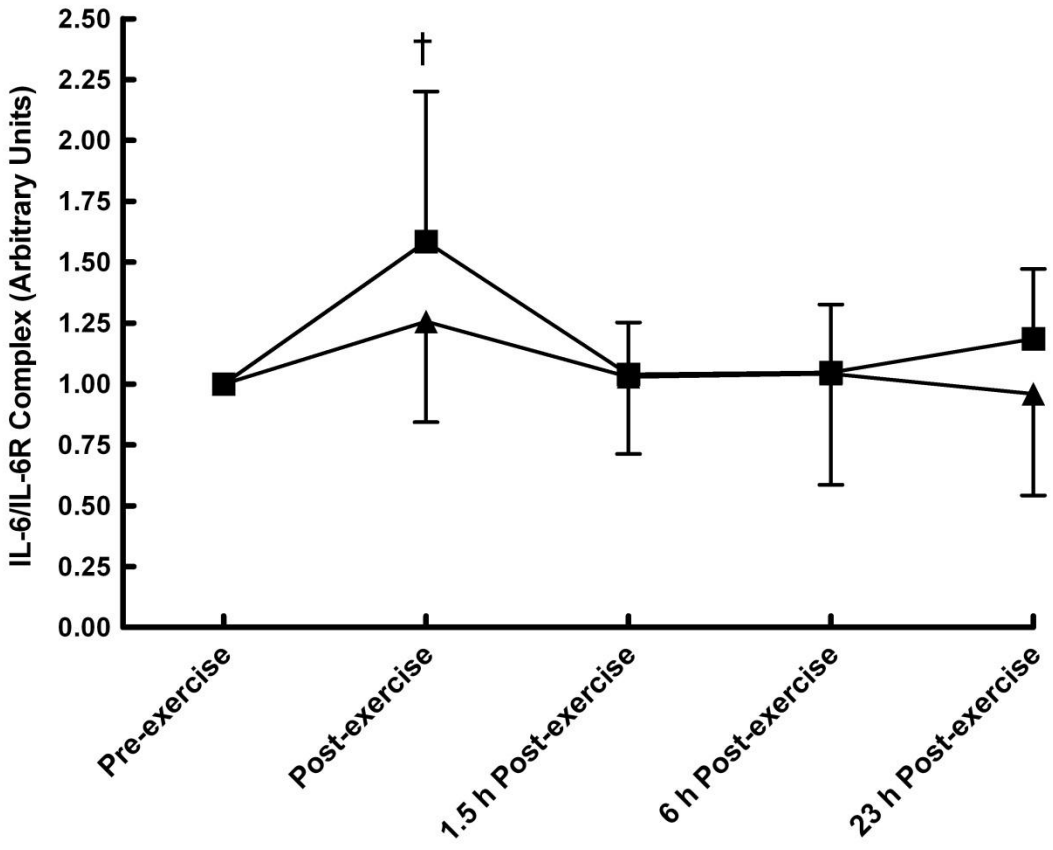

Figure 4.5 Fold-change of the IL-6/sIL-6R complex from pre-exercise in response to MOD (▲) and HIIT (■) exercise trials. Mean (SD). † Significantly different from pre-exercise during HIIT (p < 0.05).

4.4.5 Differentially spliced sIL-6R

DS-sIL-6R was significantly elevated 6 h post-exercise (155 (80) [79-190 95% CI] pg·ml^{-1} in comparison to pre-exercise (135 (83) [101-209 95% CI] pg·ml^{-1}; p = 0.020), however, this contributed to less than 1% of sIL-6R at both time points. There was also a large inter subject variation in DS-sIL-6R concentration, ranging from 25 - 290 pg·ml^{-1} (Figure 4.6).

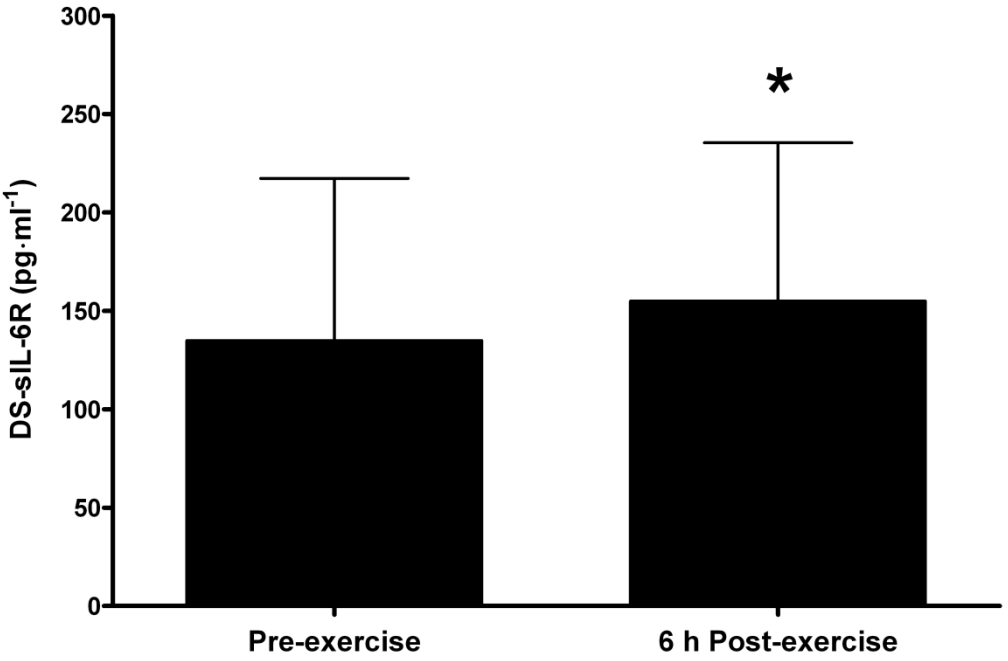

Figure 4.6 DS-sIL-6R in response to the HIIT exercise trial. Mean (SD). * significantly different to pre-exercise (p < 0.05).

4.4.6 Sub-study: The IL-6 response to an indwelling cannula

IL-6 was 0.88 pg·ml^{-1} higher when sampled via a cannula than by venepuncture at 1.5 h post-exercise, however this was not statistically significant (p = 0.088; Figure 4.7). If the 1.5 h post-exercise samples from the remaining 7 subjects are corrected to reflect a theoretical venepuncture value, a significant main trial effect for IL-6 is still detected (p = 0.029).

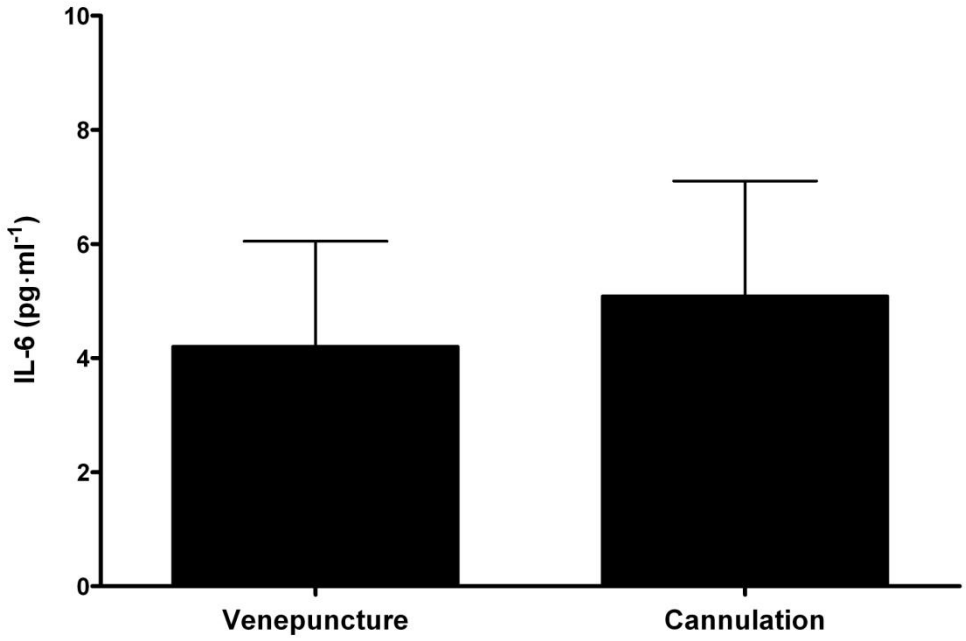

Figure 4.7 IL-6 concentration at 1.5 h post-exercise in samples collected via venepuncture or a cannula. Mean (SD), N = 4 (p = 0.088).

4.5 Discussion

The main findings of this Chapter were that IL-6 was increased to a greater extent after HIIT than MOD when the same external amount of work was undertaken, although sIL-6R and the IL-6/sIL-6R complex were not significantly different between trials. In addition, the findings of the study provide a novel indication that the increase in sIL-6R after exercise is derived from both proteolytic cleavage and differential IL-6R mRNA splicing, though the latter contributes less than 1% of total sIL-6R.

To our knowledge the present study is the first to have shown a higher IL-6 response after an acute bout of high intensity intermittent exercise than continuous moderate exercise. Previous intermittent exercise protocols have shown that IL-6 increased in distance runners completing ten 1000 m sprints with 2 min recovery (Niess et al. 2003), and again in handball players after four 250 m sprints (Meckel et al. 2009). In addition to these protocols not being applicable to the general population there was no indication to whether elevated IL-6 was comparable to moderate intensity exercise. Elevated IL-6 after HIIT could be due to increased glycogen usage compared to MOD, as circulating IL-6 is increased to a greater extent during exercise when muscle glycogen levels are low compared to normal levels (Keller et al. 2001; Steensberg et al. 2001). However, in order to confirm this muscle glycogen levels after HIIT and MOD would need to be quantified.

The literature is variable regarding how IL-6 concentration is reported following exercise. All data in this study has been corrected for changes in plasma volume in order to accurately reflect alterations in the amount of protein present. However, a substantial amount of the literature on IL-6 has not been corrected for PVC, therefore the data was also analysed before correction for PVC and the main outcome is not altered (main trial effect, p = 0.013). Interestingly, correcting for PVC alters the timing of peak levels of the sIL-6R due to haemoconcentration associated with exercise and the subsequent haemodilution (Kargotich et al. 1998). Before correction, sIL-6R peaks immediately post-exercise in both trials, compared to 6 h post-exercise when corrected for PVC (graphs and statistics for the data before correcting for PVC can be found in Appendix D).

In a previous study, plasma volume corrected peak sIL-6R occurred immediately after exercise (Gray et al. 2009b), although there was no significant difference between this point and that taken at 1.5 h post-exercise - the only other time point to be sampled. In the current study although sIL-6R is significantly increased at 6 h post-exercise, there is no significant difference between this point and the other time points. Keller et al. (2005a) reported peak IL-6R mRNA levels in skeletal muscle at 6 h after the cessation of exercise, however, did not identify any significant differences in circulating sIL-6R,

possibly due to a small sample size (N = 6). In addition, Robson-Ansley et al. (2009) identified significantly elevated sIL-6R the morning following strenuous exercise, yet the current study was unable to identify an elevation in sIL-6R 24 h from the start of exercise. Discrepancies between this study and Robson-Ansley et al. (2009) could be explained by the extremely large volume of exercise (468 km cycled over 6 days) completed in comparison to the current study and repeated exercise may extend the elevation of the receptor. In contrast to these findings, a decrease in sIL-6R has been reported 48 and 72 h after eccentric exercise (Robson-Ansley et al. 2010). If our data is not corrected for plasma volume changes and is analysed similarly to that of Robson-Ansley et al. (2010) then our data show that during HIIT sIL-6R was significantly decreased at 23 h post-exercise when compared to pre-exercise levels (p = 0.034).

Although there are no differences between trials for the IL-6/sIL-6R complex, both experimental trials in the current study are consistent with a previous report of elevations in the IL-6/sIL-6R complex after exercise (Gray et al. 2009b), which will prolong the half-life of IL-6 (Peters et al. 1996).

To determine the contribution of sIL-6R isoforms of the exercise-dependent elevation in sIL-6R, DS-sIL-6R was quantified. At rest, DS-sIL-6R represents less than 1% of total sIL-6R which is consistent with previous studies (Dimitrov et al. 2006). The novel finding of this study is that DS-sIL-6R increases significantly with exercise, although it remains less than 1% of total sIL-6R. As the increase in DS-sIL-6R does not account for the total increase in sIL-6R then PC-sIL-6R must also increase. There remains the possibility that proportionally the contributions of the two sIL-6R isoforms varied at different time points, as previously shown with sleeping patterns (Dimitrov et al. 2006), however, a more detailed study would be needed to comprehensively answer this question. Functionally, the two sIL-6R isoforms are alike in that they appear to mediate IL-6 signalling in a similar fashion (Nowell et al. 2003; McLoughlin et al. 2004), however they are produced by different cell types. DS-sIL-6R has been shown to be produced by a defined subset of monocytic cells (McLoughlin et al. 2004), as well as T cells (Horiuchi et al. 1994), whereas PC-sIL-6R appears to be shed from all cells expressing membrane-bound IL-6R. PC-sIL-6R activation is rapid

and is shed by a number of activators, including CRP (Jones et al. 1999), whereas few activators have been found to regulate DS-sIL-6R (reviewed in Jones et al. 2008).

In conclusion, the present study is the first to compare the acute effects of high intensity intermittent exercise and continuous moderate intensity exercise on the IL-6 system and found that there was a greater IL-6 production in response to HIIT compared to MOD. This is the first study to show that sIL-6R produced by differentially splicing increases in response to an acute bout of exercise. The study lends support to advocating HIIT as an appropriate and achievable exercise intervention for recreationally active individuals that induces a greater IL-6 response than moderate intensity continuous exercise.

Chapter 5

INFLAMMATION IN THE CIRCULATION AND ADIPOSE TISSUE IN RESPONSE TO HIGH INTENSITY INTERMITTENT TRAINING IN OVERWEIGHT AND OBESE MALES

5.1 Abstract

This study aimed to determine whether two weeks of HIIT altered the inflammatory profile in plasma and adipose tissue in overweight and obese males. Twelve participants (mean (SD); age 23.7 (5.2) y, body mass 91.0 (8.0) kg, BMI 29.1 (3.1) $kg \cdot m^{-2}$) undertook 6 HIIT sessions over 2 weeks. Resting blood and subcutaneous abdominal adipose tissue samples were collected pre- and post-training. Inflammatory proteins were quantified in plasma and adipose tissue via ELISAs and insulin sensitivity was determined. There was a significant decrease in sIL-6R (p = 0.050), the IL-6/sIL-6R complex (p = 0.047), MCP-1 (p = 0.047) and adiponectin (p = 0.041) in plasma post-training. Plasma IL-6, ICAM-1, TNF-α, lL-10, CRP and insulin sensitivity did not change. In adipose tissue, IL-6 significantly decreased (p = 0.036) and IL-6R increased (p = 0.037), whilst adiponectin tended to decrease (p = 0.056), with no change in ICAM-1 post-training. TNF-α, MCP-1 and IL-10 were not detectable in adipose tissue. The current investigation suggests two weeks of HIIT is sufficient to induce some beneficial alterations in the resting inflammatory profile of an overweight and obese male cohort.

5.2 Introduction

Adipose tissue produces a number of inflammatory cytokines and cell adhesion molecules that contribute to chronic low-grade inflammation. Increased adiposity will drive localised inflammation deriving from cellular hypoxia (Trayhurn and Wood 2004) and macrophage infiltration into adipose tissue (Weisberg et al. 2003; Cancello et al. 2005), which increases the capacity for production of inflammatory proteins. As a consequence, adipose tissue in obese individuals produces greater amounts of inflammatory proteins including TNF-α, IL-6 and ICAM-1 than lean individuals (Hotamisligil et al. 1995; Kern et al. 1995; Kern et al. 2001; Bošanská et al. 2010). These inflammatory proteins can be released into the circulation resulting in chronic low-grade inflammation which is associated with insulin resistance (Kern et al. 2001).

Exercise has many health benefits, including maintaining a healthy weight, reducing the risk of developing chronic diseases such as T2DM and subsequent CVD (Warburton et al. 2006), and reducing chronic low-grade inflammation in both healthy and disease states (Adamopoulos et al. 2002; Zoppini et al. 2006; Balducci et al. 2010b; Thompson et al. 2010). In addition, a lifestyle intervention involving exercise has been shown to improve insulin sensitivity in individuals with elevated fasting glucose levels, above that of application of the anti-hyperglycaemic drug metformin (Knowler et al. 2002). Therefore, exercise plays an important role in the prevention of T2DM and other associated co-morbidities.

Despite adipose tissue being a major source of inflammatory mediators, there is limited research investigating the effects of repeated exercise on inflammatory proteins. No changes in IL-6, TNF-α or adiponectin mRNA expression in subcutaneous adipose tissue were found after 12 weeks of aerobic training (Polak et al. 2006) or strength training (Klimcakova et al. 2006) in obese groups, despite aerobic training causing a significant reduction in body mass. Christiansen and colleagues (2010a) however, did find an increase in adiponectin mRNA expression in adipose tissue, in an exercise only group, but no change in any cytokine mRNA expression. In contrast, lifestyle interventions over 12 (Christiansen et al. 2010a; Christiansen et al. 2010b) and 15 (Bruun et

al. 2006) weeks, incorporating exercise and a hypocaloric diet found a decreased expression of IL-6, TNF-α and MCP-1 mRNA in adipose tissue and an increase in adiponectin mRNA alongside a weight loss of 5-14% in obese individuals. Studies investigating protein levels have shown IL-6 to be reduced in adipose tissue of obese women after weight loss induced by a hypocaloric diet (Bastard et al. 2000). The consensus in the literature appears to indicate therefore that exercise alone is not sufficient to reduce inflammation.

However, in all of these studies the exercise protocol is of a relatively low to moderate intensity and the influence of HIIT has not been tested. Positive training outcomes, including improved insulin sensitivity (Richards et al. 2010), can be achieved with just 2 weeks training of 6 or 7 exercise sessions. However, this protocol involved 4-6 thirty second maximal Wingate sprints per session and participants reported feelings of nausea and light-headedness. In Chapter 4 an alternate intermittent protocol found that there was a greater response of the IL-6 system to a single bout of HIIT compared to continuous moderate intensity exercise and a marked increase in whole body and skeletal muscle capacity for fatty acid oxidation has been shown after 2 weeks HIIT training (Talanian et al. 2007), without any report of the negative outcomes associated with Wingate sprints.

To investigate whether increasing the intensity of exercise is sufficient to drive decreases in inflammatory proteins this study examined the effects of two weeks of HIIT on metabolic and inflammatory changes in the circulation and subcutaneous adipose tissue in a cohort of overweight and obese males.

5.3 Materials and methods

Twelve overweight and obese males (age 23.7 (5.2) y; body mass 91.0 (8.0) kg; BMI 29.1 (3.1) $kg \cdot m^{-2}$) participated in this study. All participants had a BMI greater than 25 $kg \cdot m^{-2}$ but were otherwise healthy and reported taking part in no more than 2 bouts of light to moderate intensity exercise per week.

5.3.1 Preliminary measurements

Participants performed a $\dot{V}O_{2peak}$ test and a familiarisation of the HIIT protocol, separated by 5-7 days, as outlined in Section 3.3. Waist-hip ratio and blood pressure measurements were also taken prior to training (Sections 3.2 and 3.5 respectively).

5.3.2 Subcutaneous abdominal adipose tissue biopsy and oral glucose tolerance test (OGTT)

One week after the familiarisation, participants underwent a subcutaneous abdominal adipose tissue biopsy as outlined in Section 3.9.1. After, a cannula was inserted into a participant's antecubital vein and a resting blood sample was collected. Participants then consumed a 75 g glucose load (82.5 g dextrose monohydrate) in 300 ml liquid (290 ml water and 10 ml lemon juice for flavouring) within a 5 min period. Further venous blood samples were collected every 30 min over a 2 h period. Blood samples were collected and handled as previously described (Section 3.6) and haematological analysis was carried out (Section 3.7).

5.3.3 High intensity intermittent training (HIIT)

Participants completed 3 sessions of HIIT per week for 2 weeks, with 1 or 2 days rest between sessions, as outlined in Section 3.3. Expired gas samples were collected during each 4 min interval during the first training session to determine exercise intensity. The mean $\dot{V}O_2$ during the intervals of the first HIIT session was 85.0 (4.6) % $\dot{V}O_{2peak}$, which equated to 89.5 (2.4) % of maximal heart rate. Subsequently, during the remaining HIIT sessions the power output was kept the same as the first session and heart rate was recorded throughout. The power output was adjusted if heart rate dropped below 80% of maximal levels in the subsequent sessions. Forty-six to forty-eight hours after the last training session a subcutaneous adipose tissue biopsy was taken, and fasting

resting blood collected and an OGTT undertaken. The post-training adipose tissue biopsy was taken from the contralateral side of the abdomen in order to reduce any effect of the pre-training biopsy on localised inflammation. This time delay from the last training session was employed to minimise any influence of acute exercise (Hawley and Lessard 2008). Fluid and dietary intake were standardised 24 h before both visits as outlined previously (Section 3.4). Seventy-two hours after the last training session, blood pressure, waist and hip circumference, and $\dot{V}O_{2peak}$ measurements were repeated. All participants were asked to maintain their normal diet and physical activity routine throughout the training period. The average temperature and relative humidity throughout the study were 20.6 (1.0) ºC and 26.6 (7.6) % respectively.

5.3.4 Adipose tissue homogenisation

Adipose tissue samples were homogenised and internatant protein concentration was determined as previously described (Sections 3.9.2 and 3.9.3 respectively).

5.3.5 ELISAs and biochemical analysis

Plasma IL-6, sIL-6R, the IL-6/sIL-6R complex and CRP were analysed via ELISA at rest before and after 2 weeks HIIT (Section 3.8). IL-6 and IL-6R concentration in 40 µg protein in adipose tissue internatant was also quantified using the same ELISA protocols. Adiponectin, TNF-α, MCP-1, ICAM-1 and IL-10 levels were determined in plasma and to quantify the amount of each protein present in subcutaneous adipose tissue using commercially available kits (Section 3.8). Plasma insulin concentration was also determined using a commercially available kit, with human low and high controls (Mercodia, Uppsala, Sweden) and plasma glucose concentration was determined by an enzymatic, colorimetric method using a bench top analyser (Pentra 400, HORIBA ABX Diagnostics, Montpellier, France). Intra-assay CVs for inflammatory proteins in adipose tissue are shown in Table 5.1. IL-6/sIL-6R and

CRP were not quantified in adipose tissue and MCP-1, TNF-α and IL-10 were not detectable in adipose tissue.

Table 5.1 Intra-assay coefficients of variance (CV) for inflammatory protein analysis in adipose tissue.

Inflammatory protein	CV (%)
IL-6	5.8
sIL-6R	2.7
Adiponectin	2.6
ICAM-1	3.4

5.3.6 Insulin sensitivity index

Insulin sensitivity was assessed as the insulin sensitivity index (ISI) calculated using the OGTT results and the formula proposed by Matsuda and DeFronzo (1999).

$$\text{Insulin Sensitivity Index} = \frac{10{,}000}{\sqrt{(FPG*FPI)*(G*I)}}$$

Where:
FPG is the fasting plasma glucose
FPI is the fasting plasma insulin
G is the mean plasma glucose during the OGTT
I is the mean plasma insulin

5.3.7 Statistical analysis

Full statistical analysis is outlined in Section 3.10.

5.4 Results

5.4.1 Exercise intensity and heart rate

Figure 5.1 illustrates the percentage of peak heart rate and $\dot{V}O_2$ that participants were cycling at for each interval during the first HIIT session.

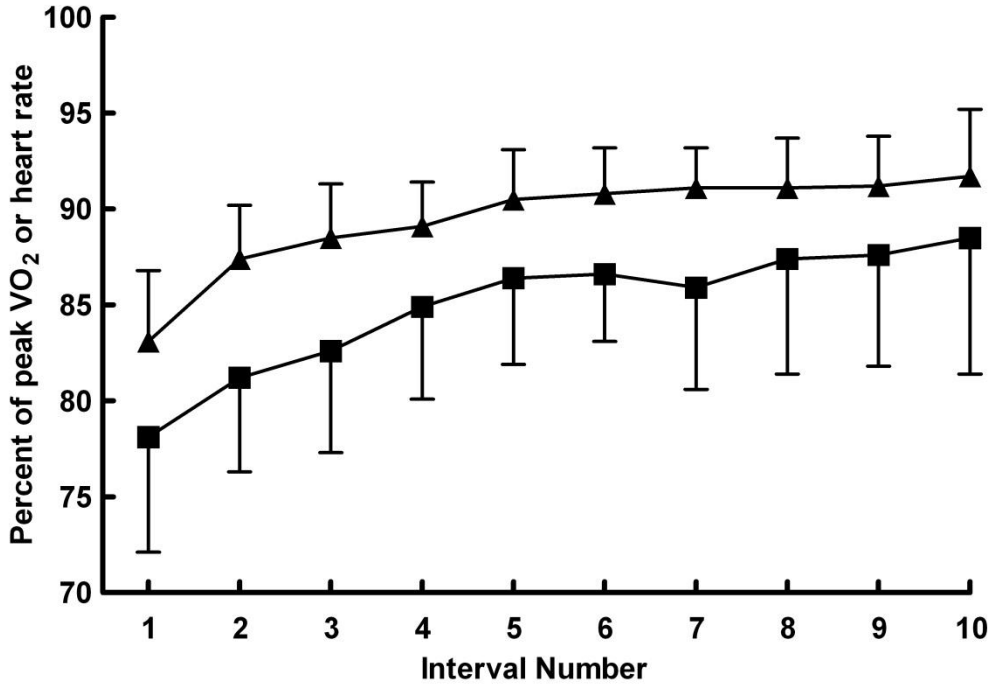

Figure 5.1 The percentage of peak heart rate (▲) and $\dot{V}O_2$ (■) that participants were cycling at during individual intervals of the first HIIT session.

5.4.2 Anthropometry, blood pressure and peak oxygen uptake during exercise

As a result of the 2 weeks of HIIT training there was a significant reduction (p = 0.029) in waist circumference as well as a tendency for a decrease in hip circumference (p = 0.052), despite no change in body mass or BMI (p > 0.05). There was also a significant increase in $\dot{V}O_{2peak}$ expressed in absolute or relative terms (Table 5.2).

Table 5.2 Anthropometry, blood pressure and peak oxygen uptake during exercise at pre and post 2 weeks HIIT.

	Pre-training			Post-training			
	Mean (SD)	**95% Confidence interval for mean**		**Mean (SD)**	**95% Confidence interval for mean**		***p***
		Lower	Upper		Lower	Upper	
Body mass (kg)	91.0 (8.0)	85.9	96.1	90.7 (7.8)	85.8	95.7	0.518
BMI ($kg \cdot m^{-2}$)	29.1 (3.1)	27.1	31.1	29.0 (3.2)	27.0	31.0	0.501
Waist circumference (cm)	96.3 (8.0)	91.2	101.4	94.9 (8.4)	89.6	100.3	0.029*
Hip circumference (cm)	109.8 (5.2)	106.5	113.1	108.6 (5.7)	105.0	112.2	0.052
Waist-to-hip ratio	0.88 (0.05)	0.85	0.91	0.87 (0.05)	0.84	0.90	0.269
Systolic BP (mmHg)	126 (8)	121	131	126 (9)	120	132	0.870
Diastolic BP (mmHg)	77 (12)	69	84	79 (11)	71	85	0.651
$\dot{V}O_{2peak}$ ($l \cdot min^{-1}$)	3.4 (0.6)	3.1	3.8	3.7 (0.5)	3.4	4.1	0.022*
$\dot{V}O_{2peak}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	38 (6)	34	43	42 (5)	38	45	0.037*

N = 12. * Significantly different compared to pre-training ($p \leq 0.05$).
BMI, body mass index; BP, blood pressure

5.4.3 Inflammatory proteins in the circulation and adipose tissue at rest

After training, there were decreases in plasma sIL-6R, the IL-6/sIL-6R complex, adiponectin and MCP-1 of approximately 10%, 13%, 11% and 12% respectively, yet no significant changes in IL-6, TNF-α, ICAM-1, IL-10 or CRP (Table 5.3).

Table 5.3 Inflammatory proteins in plasma at pre and post 2 weeks HIIT.

	Pre-training			Post-training			*p*
	Mean (SD)	**95% Confidence interval for mean**		**Mean (SD)**	**95% Confidence interval for mean**		
		Lower	Upper		Lower	Upper	
IL-6 (pg·ml^{-1})	3.1 (3.0)	1.2	5.0	2.6 (2.2)	1.2	3.9	0.373
sIL-6R (ng·ml^{-1})	42.0 (12.3)	34.2	49.8	37.6 (9.6)	31.6	43.7	0.050*
IL-6/sIL-6R complex (arbitrary units)	7.6 (3.5)	5.4	9.7	6.6 (3.2)	4.6	8.6	0.047*
MCP-1 (pg·ml^{-1})	145 (50)	114	177	128 (38)	104	152	0.047*
Adiponectin (µg·ml^{-1})	7.5 (3.5)	5.3	9.7	6.7 (3.4)	4.5	8.9	0.041*
TNF-α (pg·ml^{-1})	1.3 (0.4)	1.0	1.6	1.3 (0.5)	1.0	1.6	0.918
ICAM-1 (pg·ml^{-1})	161 (25)	145	177	154 (19)	143	166	0.373
IL-10 (pg·ml^{-1})	2.1 (0.6)	1.7	2.4	1.9 (0.6)	1.5	2.3	0.455
CRP (µg·ml^{-1})	1.8 (1.9)	0.5	3.0	1.2 (1.3)	0.4	2.0	0.340

N = 12. * Significantly different compared to pre-training ($p \leq 0.05$).

Within adipose tissue, IL-6 was reduced by 33% (p = 0.036; Figure 5.2) and IL-6R increased by 31% (p = 0.037; Figure 5.3). In addition there was a tendency for a reduction in adiponectin of 23% (p = 0.056; Figure 5.4). There was no change in ICAM-1 after training (p = 0.480; Figure 5.5). TNF-α, MCP-1 and IL-10 protein levels were below the limit of detection of the assay.

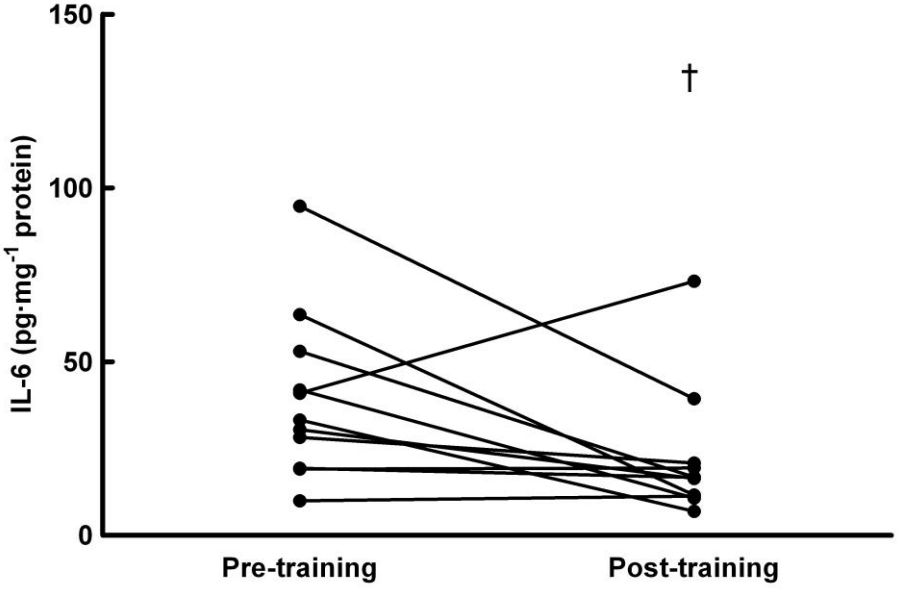

Figure 5.2 IL-6 in subcutaneous adipose tissue at pre and post 2 weeks HIIT. N = 12; † significantly different to pre-training ($p \leq 0.05$).

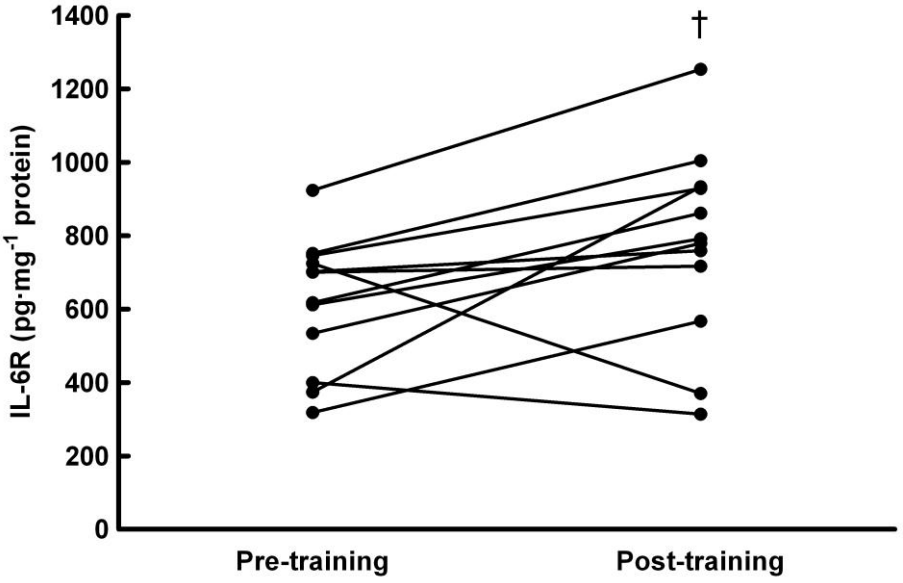

Figure 5.3 IL-6R in subcutaneous adipose tissue at pre and post 2 weeks HIIT. N = 12; † significantly different to pre-training ($p \leq 0.05$).

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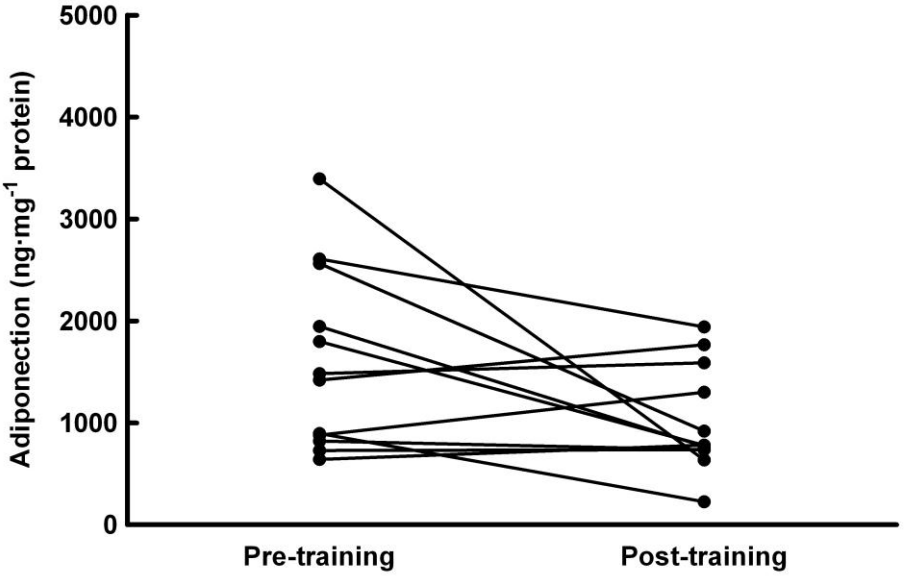


Figure 5.4 Adiponectin in subcutaneous adipose tissue at pre and post 2 weeks HIIT. N = 12; *p* = 0.056).

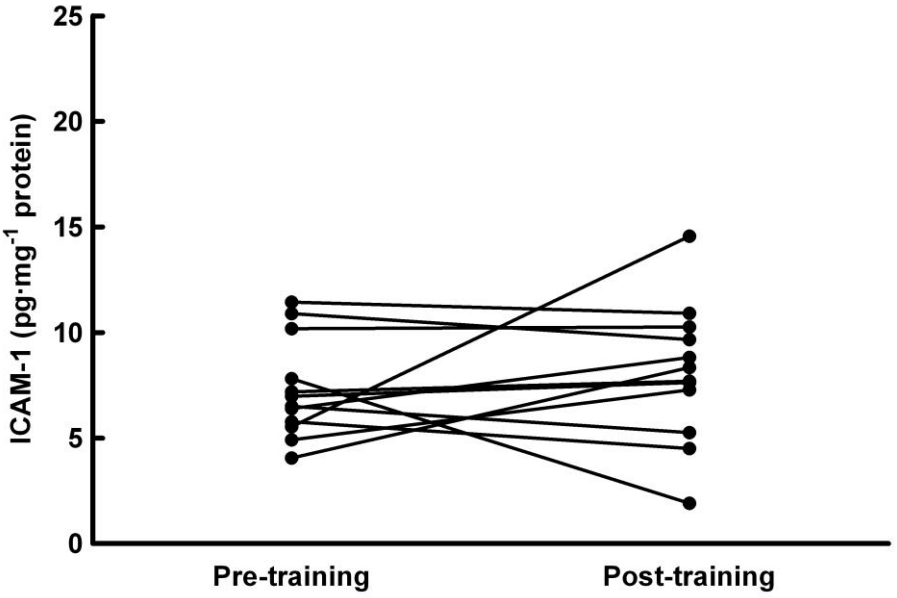


Figure 5.5 ICAM-1 in subcutaneous adipose tissue at pre and post 2 weeks HIIT. N = 12; *p* = 0.480).

5.4.4 Insulin sensitivity

There were no significant changes in fasting glucose, insulin or the insulin sensitivity index, nor were there any differences found for the area under the curve (AUC) in response to a 75 g OGTT (Table 5.4). The glucose and insulin response to the 2 h OGTT before and after training is shown in Figure 5.6. Although, there were significant time effects for both glucose and insulin (p < 0.001) during the OGTT, there were no differences between pre- and post-training for glucose or insulin (p = 0.842 and 0.831 respectively).

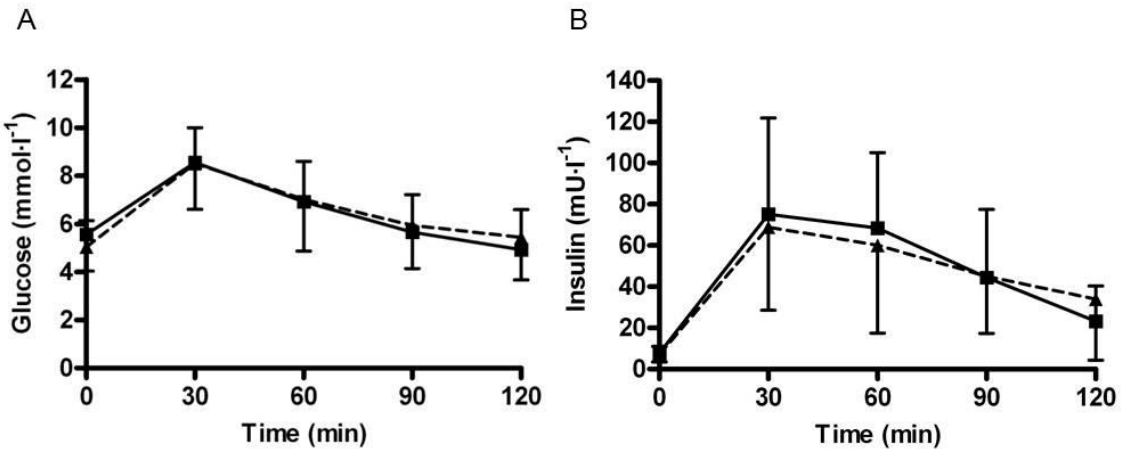


Figure 5.6 Plasma glucose (A) and insulin (B) response to a 75 g OGTT pre and post 2 weeks HIIT. ■ (solid line) represents pre-training. ▲(dashed line) represents post-training.

Table 5.4 Glycaemic control at pre and post 2 weeks HIIT.

	Pre-training			Post-training			
	Mean (SD)	**95% Confidence interval for mean**		**Mean (SD)**	**95% Confidence interval for mean**		***p***
		Lower	Upper		Lower	Upper	
Fasting glucose (mmol·l^{-1})	5.6 (0.6)	5.2	5.9	5.0 (1.0)	4.4	5.7	0.151
Fasting insulin (mU·l^{-1})	7.8 (3.2)	5.7	9.8	6.8 (3.5)	4.6	9.0	0.268
Glucose AUC (mmol·l^{-1}·120 min^{-1})	791 (110)	721	861	801 (165)	696	905	0.815
Insulin AUC (mU·l^{-1}·120min^{-1})	6105 (3303)	4007	8203	5730 (3459)	3533	7926	0.448
Insulin sensitivity index	6.7 (4.6)	3.8	9.7	7.7 (3.8)	5.2	10.1	0.374

N = 12
AUC, area under the curve.

5.5 Discussion

Exercise regimens of varying intensities may improve well-being and combat some of the basal increase in inflammation associated with excessive fat deposition and other adverse health conditions including T2DM and CVD (Adamopoulos et al. 2002; Balducci et al. 2010b; Thompson et al. 2010; Zoppini et al. 2006). The results of this study indicate that in overweight and obese males, a two week HIIT regimen can induce reductions in waist circumference and increase $\dot{V}O_{2peak}$, and that it is an achievable mode of exercise to reduce inflammation, with participants completing 100% of HIIT sessions during the study.

IL-6 is a pleiotropic cytokine and exerts both pro- and anti-inflammatory actions (Scheller et al. 2011). This is the first study to report a significant reduction in IL-6 in subcutaneous adipose tissue with exercise training. However, it is unclear whether this is due directly to the exercise or to a loss in fat, as reduced IL-6 in subcutaneous adipose tissue has been shown after weight loss in obese women through a hypocaloric diet but no exercise intervention (Bastard et al. 2000). The same research group has also shown that IL-6 in subcutaneous adipose tissue is negatively correlated with insulin sensitivity (Bastard et al. 2002). Previous studies have found no change in IL-6 mRNA expression in adipose tissue after 12 weeks aerobic training in obese females (Polak et al. 2006) or strength training in obese males (Klimcakova et al. 2006), although these studies did not examine IL-6 protein changes in adipose tissue, and the exercise intensity was substantially lower. The finding of a decrease in IL-6 protein in adipose tissue in the current study could therefore be due to the higher exercise intensity elicited in this study. In the present study there was no significant change in circulating IL-6 post-training which could explain why insulin sensitivity was unaltered, as systemic IL-6 is strongly correlated with insulin resistance (Bastard et al. 2000; Fernandez-Real et al. 2001; Kern et al. 2001; Bastard et al. 2002), however there was a significant reduction in the IL-6/sIL-6R complex suggesting that the biological activity of IL-6 has been reduced. It is important to note that the current study focussed on total changes in the protein concentration of inflammatory proteins within the adipose tissue

as opposed to changes in protein secretion. Therefore, although there was a significant reduction of IL-6 in adipose tissue the rate of IL-6 secretion from adipose tissue into the circulation may have been unaltered and could explain why there was no change in circulating IL-6. Other tissues and cells also contribute to circulating IL-6, with only ~15-35 % of circulating IL-6 at rest deriving from subcutaneous adipose tissue (Mohamed-Ali et al. 1997). The lack of correlation between the changes in adipose tissue and circulating levels is therefore understandable.

Within adipose tissue, only around 4-10% of IL-6 comes from adipocytes (Fried et al. 1998; Fain et al. 2004), therefore it is likely that other immune cells such as macrophages are the main source of IL-6 production. Macrophage recruitment into adipose tissue is greater in obese compared with lean individuals (Weisberg et al. 2003; Cancello et al. 2005), although, it seems that the size of the adipocytes triggers macrophage infiltration rather than overall obesity (Cinti et al. 2005). MCP-1 is a chemoattractant known specifically to stimulate macrophage and monocyte recruitment into adipose tissue. MCP-1 levels are increased in obesity resulting in an influx of macrophages and monocytes into the adipose tissue (Bruun et al. 2005). MCP-1 was not detectable in subcutaneous adipose tissue in the current study and furthermore, has been shown to be higher in visceral adipose tissue (Bruun et al. 2005), therefore it is likely that the decrease of this chemokine in the circulation is due to a reduced MCP-1 production in other tissues such as visceral adipose tissue.

IL-6R in subcutaneous adipose tissue was significantly increased post-training which is consistent with findings in skeletal muscle after 10 weeks of knee extensor exercise training (Keller et al. 2005b; Akerstrom et al. 2009). IL-6R has previously been shown to be expressed on the plasma membrane of approximately 60% of adipocytes (Bastard et al. 2002). The counter finding of a reduction in sIL-6R in plasma is also in support of previous studies showing similar changes in obese women after 6 months of training (You et al. 2004; Silverman et al. 2009) and in chronic heart failure patients after a 12 week exercise intervention (Adamopoulos et al. 2002). This finding is consistent with the current understanding that sIL-6R is independent of cell production of the

protein and is predominantly derived from proteolytic cleavage of the membrane-bound IL-6R (Chapter 4).

ICAM-1 is a vascular cell adhesion molecule that is used as a biomarker of endothelial dysfunction and can independently predict CVD (Ridker et al. 1998). In the current study, no alterations were found in ICAM-1 in plasma or adipose tissue. Some research has shown improvements in circulating ICAM-1 with exercise training in individuals with metabolic disorders and T2DM (Roberts et al. 2006a; Roberts et al. 2006b; Zoppini et al. 2006), however there were no changes in a group with normal glucose tolerance after a 4-week exercise intervention (Tönjes et al. 2007). In all of these studies pre-intervention ICAM-1 concentrations were 2-3 times greater than in the current study. As with MCP-1, it has been demonstrated that ICAM-1 is greater in visceral fat in obese compared with lean individuals, but there is no difference in subcutaneous adipose tissue (Bošanská et al. 2010). Therefore, the reason for the absence of a reduction in circulating ICAM-1 in the present study could be that decreases in circulating ICAM-1 found in other studies are caused by a reduction in ICAM-1 production in visceral as opposed to subcutaneous fat, as well as lower pretraining circulating ICAM-1 in the present study than in the aforementioned studies.

The literature shows clear evidence that adiponectin levels are inversely correlated with BMI (Arita et al. 1999; Weyer et al. 2001; Bruun et al. 2003; Kern et al. 2003; Ryan et al. 2003; Vilarrasa et al. 2005; Bluher et al. 2006) and is increased after a year of high intensity exercise in T2DM patients (Balducci et al. 2010b). The present finding of a reduction in adiponectin in plasma and a tendency for this decrease to be replicated in adipose tissue (p = 0.056) is inconsistent with some existing literature. However, in a recent review (Simpson and Singh 2008), only 3 out of 8 randomised control trials involving exercise training resulted in an increase in plasma adiponectin, and there is some evidence, supported by the current study, that in order to increase adiponectin levels, at least in plasma, dietary restriction with a 10% weight loss is required (Madsen et al. 2008). Christiansen and colleagues (2010b) support this concept presenting a small non-significant decrease in plasma adiponectin after a 3 month exercise regimen, yet adiponectin was increased in a diet only group and

a combined diet plus exercise group. This research group demonstrated adiponectin mRNA in subcutaneous adipose tissue was increased in all groups, whereas other exercise only interventions have found no changes in adiponectin mRNA in adipose tissue (Klimcakova et al. 2006; Polak et al. 2006), although none of these studies measured protein changes. An alternative explanation may relate to the fact that there are different isoforms of adiponectin expressing both anti- and pro-inflammatory actions (Ouchi et al. 1999; Ouchi et al. 2000; Haugen and Drevon 2007). Haugen and Drevon (2007) demonstrated that globular proteolyticaly cleaved adiponectin induced TNF-α secretion demonstrating pro-inflammatory properties whereas in studies measuring total adiponectin, it is thought to exhibit overall anti-inflammatory properties including enhancing insulin sensitivity (Berg et al. 2001;Yamauchi et al. 2001). Further work is required to determine whether functionality is related to changes in the various isoforms of adiponectin and to test the differences between dietary restriction and exercise interventions on the adiponectin response.

IL-10, MCP-1 and TNF-α were not detectable within subcutaneous adipose tissue, suggesting the dominant source of inflammation is visceral adipose tissue which is consistent with other studies (Fried et al. 1998; Bruun et al. 2005; Bošanská et al. 2010). Whereas one *ex-vivo* study found IL-6 to be greater in subcutaneous than visceral fat (Gletsu et al. 2006) and similarly adiponectin is more abundant in subcutaneous adipose tissue (Fain et al. 2004; Lihn et al. 2004). Despite the majority of evidence suggesting inflammatory proteins are present at greater concentrations in visceral than subcutaneous adipose tissue, visceral adipose tissue accounts for only 13% of total adipose tissue in obese men and 6% in obese women (Ross et al. 1994), therefore the contribution of subcutaneous adipose tissue to systemic low-grade inflammation could be substantial. Clarification of the protein concentration of inflammatory cytokines is therefore required to substantiate the existing literature.

A similar decrease in waist circumference was found after 2 weeks sprint interval training (Whyte et al. 2010), although it seems unlikely that the decrease in waist circumference is simply due to the increased energy

expenditure introduced due to the training protocol. Total energy expenditure in the current study was estimated to be ~14,500 kJ for the 6 HIIT sessions, with an estimated additional ~5000 kJ due to excess post-exercise oxygen consumption (Knab et al. 2011). This would theoretically cause a total body fat loss of ~600 g of adipose tissue, which is unlikely to induce a mean reduction in waist circumference of ~1.4 cm. Further studies are therefore required to determine the cause of the reduced waist circumference since abdominal adiposity was not measured in the current study.

Despite significant improvements in inflammatory proteins, waist circumference and $\dot{V}O_{2peak}$, the current investigation found no improvement of insulin sensitivity after HIIT. A possible explanation for the discrepancy in findings between this study and others that did detail an increase in insulin sensitivity after 2 weeks SIT (Richards et al. 2010; Whyte et al. 2010) could be due to the timing of the post-training OGTT. In the present study the OGTT took place 46-48 h after training to eradicate any acute effects on insulin sensitivity from the last training session (Hawley and Lessard 2008). A previous investigation in overweight and obese males has shown that insulin sensitivity although augmented 24 h after the last bout of exercise was lost at 72 h post-training suggesting the augmentation may be due to the effect of the last acute exercise bout (Whyte et al. 2010). In contrast to these findings, utilising the gold standard methodology of the hyperinsulinaemic euglycaemic clamp, before and after a 2 week sprint training protocol and sampling 72 h post-training, found insulin sensitivity to be increased (Richards et al. 2010). In this study, however, the pre-exercise sample of the training group appeared low and the post-training sample although significantly different from pre-training, was comparable to the sedentary control group and much lower than an acute exercise group suggesting the pre-training value was unusually low. It is clear that the timing of the post-training samples after the last exercise bout is critical when interpreting insulin sensitivity results.

In conclusion the present study provides novel evidence to support that HIIT, a high intensity intermittent training protocol, is an appropriate form of exercise to induce both metabolic and inflammatory changes after only 2 weeks. The

protocol was suitable for an overweight and obese cohort, with all HIIT sessions completed by the participants. Future research should determine whether this mode of training is suitable for different patient groups and if greater health benefits can be achieved over a longer training period.

Chapter 6

DEVELOPMENT OF A METHOD TO QUANTIFY MEMBRANE-BOUND IL-6R IN ADIPOSE TISSUE

6.1 Abstract

In Chapter 5, IL-6R in adipose tissue was found to be significantly increased after 2 weeks HIIT in overweight and obese males (p = 0.037). Since IL-6R is commonly identified as a membrane-bound glycoprotein as well as in a soluble form in the circulation (sIL-6R), this study aimed to investigate whether the IL-6R detected in adipose tissue was membrane-bound IL-6R, present on the surface of cells within adipose tissue or purely due to sIL-6R present in the adipose tissue matrix. Western blot analysis initially revealed many protein bands when probed with an antibody to detect membrane-bound IL-6R, with a range of molecular weights between ~45-220 kDa, questioning the specificity of the antibody. Control experiments were therefore carried out, including immunoprecipitation and blocking the antibody with the peptide it was raised against, which provided some evidence that the antibody was binding to IL-6R. The Western blot protocol was then optimised. After optimisation, some non-specific binding was reduced and a single band was visible at a molecular weight of ~90 kDa and several bands between ~45-55 kDa. Fold-change from pre-training found a similar increase in IL-6R between Western blotting analysis and that found via ELISA in Chapter 5 (1.22 and 1.31 fold-change respectively), however no significant differences were found in any of the IL-6R isoforms detected during Western blotting. This suggests that the increase in total IL-6R found in Chapter 5, is at least in part due to an increase in membrane-bound IL-6R. Full-length IL-6R has 6 potential *N*-linked glycosylation sites and the different bands detected during Western blotting could be due to a range of glycosylated forms of IL-6R. In conclusion this study has developed a robust measurement of membrane-bound IL-6R in adipose tissue and demonstrated that many forms of IL-6R exist in adipose tissue.

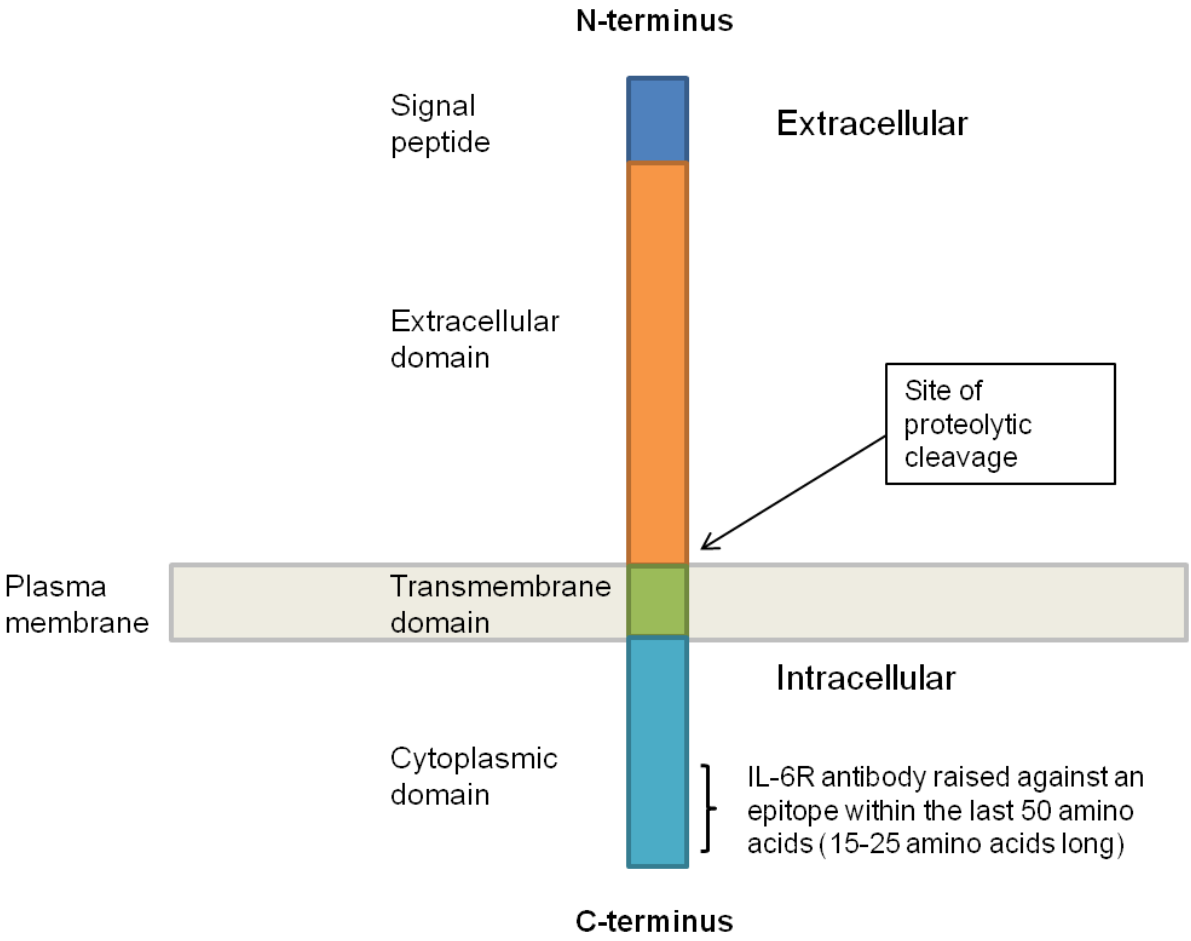
6.2 Introduction

In order for IL-6 to signal it must bind to the type I transmembrane receptor, IL-6R (CD126). This complex then associates with gp130 (CD130), a common signal-transducing membrane protein for many cytokines to form either a tetrameric [IL-6-IL-6R-$(gp130)_2$] or hexameric [$(IL\text{-}6)_2$-$(IL\text{-}6R)_2$-$(gp130)_2$] structure (Schroers et al. 2005). Once bound, gp130 can activate the Janus kinase (Jak) family (Lütticken et al. 1994; Stahl et al. 1994), followed by activation of signalling pathways, such as signal transducer and activator of transcription (STAT) 3 and 1, as well as activation of the MAPK pathway (Heinrich et al. 2003). This is known as classic IL-6 signalling. Membrane-bound IL-6R has been found to be expressed mainly on the plasma membrane of macrophages, monocytes, hepatocytes, neutrophils and some lymphocytes (Bauer et al. 1989; Oberg et al. 2006; Chalaris et al. 2007), as well as adipocytes (Bastard et al. 2002). The cytoplasmic and transmembrane domains of IL-6R are not essential for initiating IL-6 signalling, therefore the soluble form of the receptor (sIL-6R), that was quantified in Chapters 4 and 5, is an active isoform of this receptor, as it is composed of the signalling extracellular domain of full-length IL-6R. Soluble IL-6R can be produced by proteolytic cleavage or differential splicing, as quantified in Chapter 4, and allows IL-6 to bind and signal in tissues that are deficient in the membrane-bound IL-6R, as well as in tissues that express IL-6R on the plasma membrane, and is termed trans-signalling, as previously shown in Figure 2.1.

Results from Chapter 5 showed that IL-6R was present in adipose tissue homogenate and that it was increased after 2 weeks HIIT in overweight and obese males. This was quantified via ELISA, with the antibodies raised against an epitope in the extracellular domain, hence detecting membrane-bound and soluble IL-6R. Therefore, it was not clear whether the IL-6R detected was membrane-bound IL-6R present in cells such as adipocytes and macrophages or sIL-6R present in the adipose tissue matrix (blood and connective tissue), which can comprise up to 70% of adipose tissue (Fain et al. 2004), though this should be limited by washing the tissue with saline and removing connective tissue.

The 468 long amino acid sequence for the IL-6R is a precursor for the mature IL-6R, which is 449 amino acids in length (Yamasaki et al. 1988). IL-6R consists of a 19 amino acid signal peptide, a 339 amino acid extracellular region, a 28 amino acid transmembrane region and an 82 amino acid cytoplasmic region (Figure 6.1). Mature full-length IL-6R has a predicted molecular weight of 49.9 kDa, however, the observed molecular weight is consistently reported as ~80 kDa (Hirata et al. 1989). The observed molecular weight of proteins can be different from the predicted molecular weight for various reasons; post-translational modifications, post-translational cleavage, splice variants of the full-length protein, the composition of the amino acids and multimers. In the case of IL-6R the mature molecular weight is greater than predicted due to 6 potential *N*-linked glycoslation sites (Yamasaki et al. 1988), 5 in the extracellular domain and 1 in the cytoplasmic domain of the protein. Glycosylation is an enzymatic reaction where glycans attach to proteins at specific sites and is essential for protein folding to create its tertiary structure. Without this folding IL-6 would be unable to bind to IL-6R and signalling would not occur. During denaturing and preparation of the gels for Western blotting it is likely that some or all of these sites are cleaved, thus presenting with various molecular weights, however, *in vivo* these forms of the protein could be functional.

A.



B.

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1   mlavgcalla allaapgaal aprrcpaqev argvltslpg dsvtltcpgv epednatvhw
61  vlrkpaagsh psrwagmgrr lllrsvqlhd sgnyscyrag rpagtvhllv dvppeepqls
121 cfrksplsnv vcewgprstp slttkavllv rkfqnspaed fqepcqysqe sqkfscqlav
181 pegdssfyiv smcvassvgs kfsktqtfqg cgilqpdppa nitvtavarn prwlsvtwqd
241 phswnssfyr lrfelryrae rsktfttwmv kdlqhhcvih dawsglrhvv qlraqeefgq
301 gewsewspea mgtpwtesrs ppaenevstp mqalttnkdd dnilfrdsan atslpvqdss
361 svplptflva ggslafgtll ciaivlrfkk twklralkeg ktsmhppysl gqlvperprp
421 tpvlvplisp pvspsslgsd ntsshnrpda rdprspydis ntdyffpr
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Figure 6.1 A. Schematic structure of IL-6R and B. amino acid sequence for full-length IL-6R. The signal peptide is underlined in bold.

It is hypothesised that membrane-bound IL-6R constitutes the IL-6R detected in our adipose samples. This study aimed to develop a technique to quantify membrane-bound IL-6R in adipose tissue via Western blot techniques. The

antibody used in this study spans the –COOH cytoplasmic terminal and therefore should not detect sIL-6R. This antibody was selected as it has previously been shown to detect IL-6R in skeletal muscle (Akerstrom et al. 2009) and osteoblasts (Vermes et al. 2002). The second aim was to determine if protein expression of membrane-bound IL-6R in adipose tissue changed after 2 weeks HIIT, as found for total IL-6R (Chapter 5).

6.3 Overview of general methods

One-dimensional polyacrylamide gel electrophoresis (1D-PAGE) was carried out in order to separate the proteins in adipose tissue homogenate according to molecular weight. Proteins were transferred by electroblotting to polyvinylidene fluoride (PVDF) membranes and probed with an antibody to detect IL-6R. Materials were purchased from Invitrogen unless stated otherwise. Initially samples were prepared using existing protocols which are detailed below. A secondary aim of this study was to determine whether membrane-bound IL-6R changed in subcutaneous adipose tissue after 2 weeks HIIT in overweight and obese males (samples collected during Chapter 5).

Subcutaneous adipose tissue sampling

Methodology for adipose tissue sampling, tissue homogenisation and protein concentration determination are outlined in Chapter 3.8.

Sample preparation

Protein homogenate from adipose tissue samples was reduced by the addition of a quarter of a volume of NuPAGE Lithium dodecyl sulfate (LDS) Sample Buffer (4X) and a tenth of a volume of NuPAGE Sample Reducing Agent (10X, contains 500 mM dithiothreitol (DTT)), then heat-denatured by incubation for 10 min at 70ºC on a Techne Dri-Block DB-3 (Bibby Scientific Limited, Stone, UK)

to reduce the protein disulfide bonds (see Table 6.1 for sample preparation). Samples were left to cool before 1D-PAGE or frozen at -80ºC until required.

Table 6.1 Reagents and sample volume for adipose tissue preparation.

Component	Mass/Volume
NuPAGE Sample Reducing Agent (X10)	3 µl
NuPAGE Sample Buffer (X4)	7.5 µl
Homogenised adipose tissue protein	40 µg
Deionised water to	30 µl

One-dimensional polyacrylamide gel electrophoresis (1D-PAGE)

NuPAGE Novex 4-12% Bis-Tris gels (1.0 mm thick, 10 well) were inserted into an XCell SureLock Mini-Cell tank and the inner chamber was filled with 2-(N-morpholino)ethanesulfonic acid (MES) running buffer and 500 µl of NuPAGE antioxidant, which maintains proteins in a reduced state during protein gel electrophoresis. MES buffer is recommended for resolving small to medium proteins and is composed of 50 mM MES, 50 mM Tris Base, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, pH 7.3. The outer chamber was half-filled with MES buffer, and sample protein (40 µg/gel lane) was resolved on the gels for 1.5 h at 150 V.

Colloidal Blue staining

After electrophoresis, the gel cassette was removed from the tank and rinsed with deionised water and the gel was removed from the cassette using a gel knife. Proteins were fixed by washing for 10 min in 50 % methanol, 10 % acetic acid (*v/v*), and then stained with Colloidal Blue stain. The Colloidal Blue staining kit contains Stainer A and Stainer B. For each gel to be stained 5 ml of methanol, 13.75 ml of deionised water and 5 ml of Stainer A were mixed and

poured onto the gel and it was left to incubate on a Mini Orbital Shaker at room temperature at 20 rev·min^{-1}. After 10 min, 1.25 ml of Stainer B was added to the solution and the gels were left in the solution overnight on the shaker. The following morning the staining solution was disposed and the gels were washed with deionised water. The gels were left on the shaker and the water was replaced every hour for 3 h. Images of the stained gels were obtained using a scanner (Canon Ltd., Surrey, UK).

Protein Transfer

In order to carry out Western blot analysis 1D-PAGE was repeated for all samples and proteins were transferred to PVDF membranes for Western blotting. The PVDF membrane (Millipore Ltd, Billerica, MA, USA) was cut to the same size as the gel (9 X 8 cm) and soaked in methanol for 5 min. Methanol was gradually replaced with NuPAGE transfer buffer until the membrane was soaked in 100% transfer buffer. After 1D-PAGE, the gel was removed from the cassette and was left to equilibrate in transfer buffer for 15 min. After, the gel was assembled to prepare protein transfer from the gel to the PVDF membrane (Figure 6.2). When assembling the transfer unit, care was taken to ensure there were no air bubbles between the gel and the PVDF membrane, to allow equal protein transfer across the membrane. The unit containing the gel and membrane was inserted into a Mini-PROTEAN Tetra System tank (Bio-Rad, Hercules, CA, USA). The tank was placed in an ice box and a cooling unit was inserted into the tank. The tank was then filled with transfer buffer. Proteins were electrophorectically transferred onto the PVDF membrane for 2 h at 80 V.

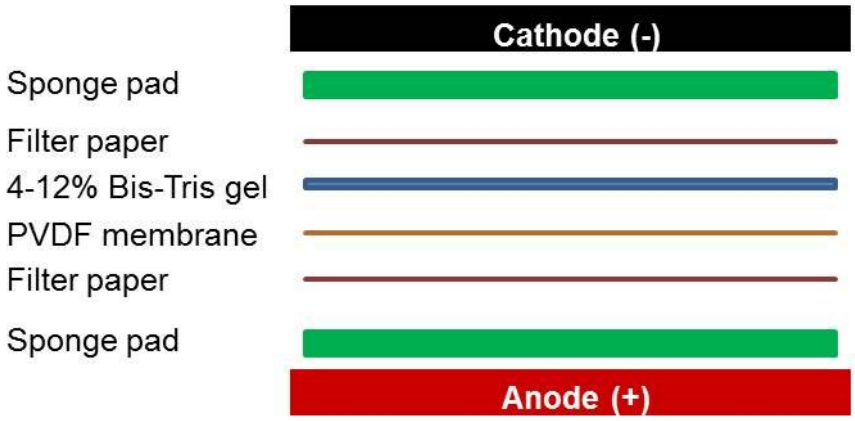

Figure 6.2 Assembly of unit for electrophoretic protein transfer. The PVDF membrane and gel are inserted between 2 sheets of filter paper and sponge pads before being tightly secured in the cassette for protein transfer. The gel proteins have been coated with LDS, rendering them negatively charged. Therefore the proteins will migrate out of the gel during protein transfer towards the positive electrode, where they will be captured onto the PVDF membrane.

After 2 h the cassette was removed from the tank and the membrane was removed. Protein transfer onto the membrane was confirmed using Simply Blue Safestain. Twenty five millilitres of Coomassie stain was poured onto the membrane and incubated at room temperature for 30 min on the orbital shaker at 20 rev·min^{-1}. The membrane was then removed from the solution and left to dry overnight. To confirm complete protein transfer, gels were also stained with the Colloidal Blue stain after electroblotting, as described earlier. If protein transfer was successful there should only be little protein visible on the gel after staining. Large proteins do not migrate as efficiently and therefore some are left on the gel after protein transfer, however proteins at these molecular weights (> 180 kDa) are not relevant to the current study. The following day the membranes were destained with 50% methanol, 10% acetic acid (*v/v*), to visualise protein bands and confirm that there was even protein transfer across the membrane. The blots were washed 3 X 10 min using PBS with 0.05% Tween 20 (PBS-T) as a wash buffer, on the orbital shaker at 20 rev·min^{-1}. All subsequent washes were the same as this.

Western blotting

After protein transfer, the PVDF membranes can be probed with antibodies for specific proteins to detect their presence in the adipose tissue homogenate. The primary antibody used in this study was an IL-6R rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., CA, USA) used at a concentration of 0.5 $\mu g \cdot ml^{-1}$ in PBS-T with 5% dried milk. The IL-6R antibody is approximately 15-25 amino acids long and was raised against a peptide mapping within the last 50 C-terminal amino acids of IL-6R of human origin (Figure 6.1). PVDF membranes were blocked with 25 ml of 5% dried milk in PBS-T for 1 h at room temperature. The membranes were then probed with 8 ml of the primary IL-6R antibody solution and the membranes were rotated at 40 $rev \cdot min^{-1}$, for 16 h at 4ºC. The following day the membranes were washed, and then incubated with a secondary antibody, a polyclonal goat anti-rabbit immunoglobulins-horseradish peroxidase conjugated antibody (Dako, Ely, UK) at 1:1000 dilution for 1 h at room temperature. Membranes were washed again and then antibody localisation was visualised by enhanced chemiluminescence using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific, Cramlington, UK). Equal amounts of the peroxide buffer and luminol/enhancer solution were mixed just prior to use. The membrane was placed on a sheet of overhead projector paper and 2 ml of the working substrate solution was poured onto the membrane. The membrane was incubated for 5 min and light was captured using a ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, USA). Relative levels of protein bands were quantified using Quantity One 1D analysis software (The Discovery Series, Bio-Rad, Hercules, CA, USA). The freehand tool was used to draw around the protein band, as well as an area of equal size at an unexposed region of the membrane to represent the background. The software gives the $intensity \cdot mm^2$ for each band, which were corrected for background $intensity \cdot mm^2$.

Protein Normalisation

To account for any differences in protein loaded between pre- and post-training samples for participants, the same membranes were also probed for actin which is expected to be uniformly expressed across these cell types. This allowed the post-training IL-6R results to be normalised to actin expression. After membranes were probed for IL-6R they were stripped with 25 ml of Restore PLUS Western Blot stripping buffer (Thermo Scientific, Cramlington, UK) and incubated at 50ºC for 30 min at 60 rev·min^{-1} in a benchtop incubating shaker (MaxQ 4000, Thermo Scientific, Cramlington, UK). The membranes were then washed and blocked again before re-probing for actin using the previous Western blot method. The primary antibody used in this application (I-19; Santa Cruz Biotechnology, Inc., CA, USA) detects a broad range of actin isoforms of human origin and is raised against a peptide mapping at the C-terminus. The rabbit polyclonal antibody was diluted in PBS-T with 5% dried milk to a working concentration of 0.8 µg·ml^{-1}. A single band was detected at 43 kDa. All changes in IL-6R between pre- and post-training are presented relative to actin expression. The intensity·mm^2 of each band was quantified and the fold-change from pre-training determined in order to normalise any changes in IL-6R. An example of a membrane probed for actin expression pre- and post-training is shown in Figure 6.3.

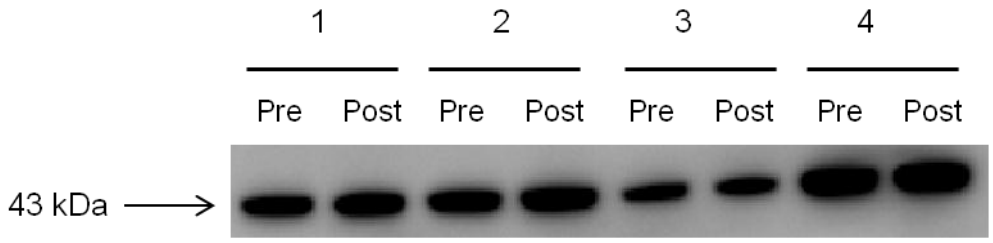

Figure 6.3 Actin expression in subcutaneous adipose tissue homogenate pre and post 2 weeks HIIT.

6.4 Experimental procedures

Experiment 1: 1D-PAGE running conditions

Initially, 1D-PAGE samples were resolved on the gels for 1.5 h at 150 V. This tended to give a ‘smile’ effect on the gel, with samples in the middle running quicker than samples at either end of the gel. Therefore, samples were run at a lower voltage of 125 V for a longer period of time of 2 h. This resulted in a more uniform migration of proteins through the gel and proteins had successfully run the full length of the gel after this time.

Experiment 2: Denaturation of protein samples

The aim of this experiment was to ensure that the protein samples were fully denatured and intact, and to assess relative protein levels, before proceeding with Western blots. Protein samples were loaded onto gels for electroblotting and then stained as described earlier. An image of a stained gel is shown in Figure 6.4.

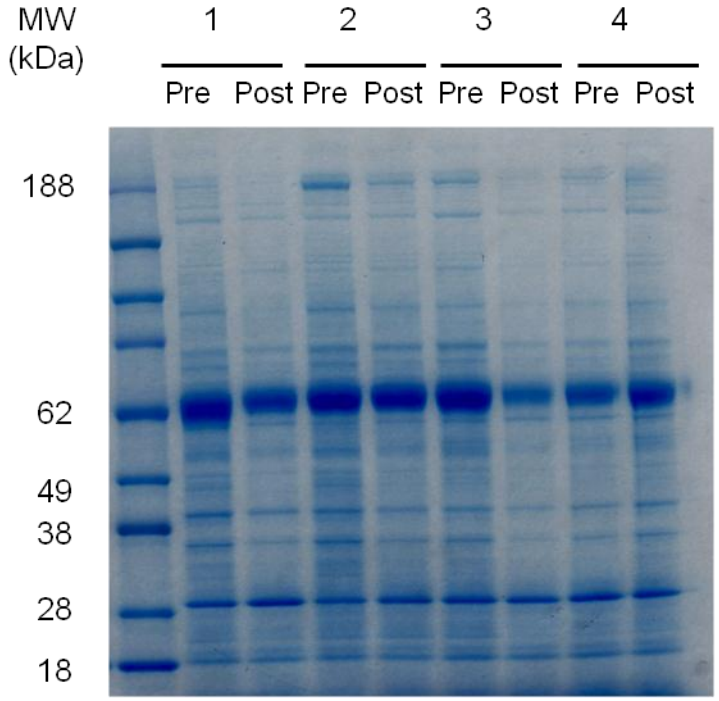

Figure 6.4 Colloidal Blue staining of adipose tissue homogenate. Samples resolved on NuPAGE 4-12% Bis-Tris gels, pre and post 2 weeks HIIT.

Figure 6.4 demonstrates that there are many bands visible between ~18-188 kDa representing individual proteins. This image provides some evidence that the protein samples are intact and appear to have been successfully reduced, and sufficiently resolved. To provide evidence that IL-6R specifically had been successfully reduced, samples were denatured at both 70ºC and 90ºC, prior to 1D-PAGE and Western blot analysis, as described previously. If samples are not fully denatured this could lead to proteins being detected at different molecular weights than those expected. Figure 6.5 shows that all of the same protein bands are detectable at both 70ºC and 90ºC, therefore the higher temperature did not cause additional denaturing of IL-6R.

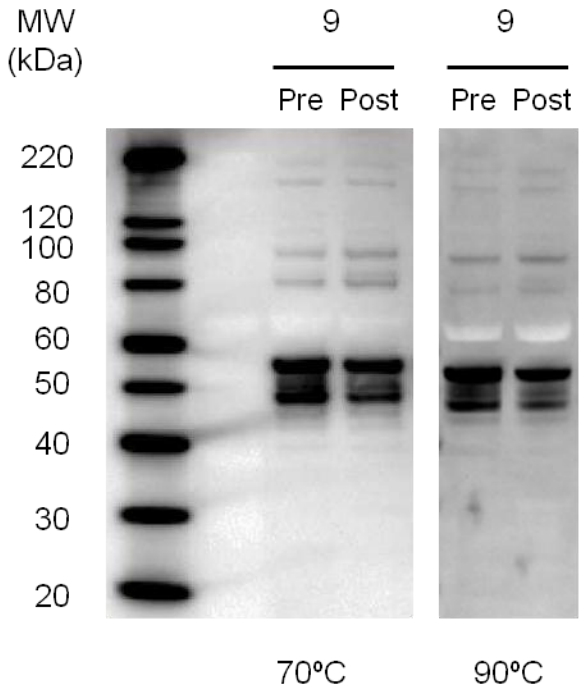

Figure 6.5 Western blots probed for IL-6R where adipose tissue homogenates were reduced at either 70ºC or 90ºC.

Experiment 3: IL-6R isoforms

The aim of this experiment was to determine whether membrane-bound IL-6R was detectable in the subcutaneous adipose tissue samples of all 12

participants before and after 2 weeks HIIT. 1D-PAGE was carried out for all samples, which were transferred to PVDF membranes and probed for IL-6R using a rabbit polyclonal antibody (see Western blot section). Figure 6.6 shows the protein bands identified as IL-6R by chemiluminescence.

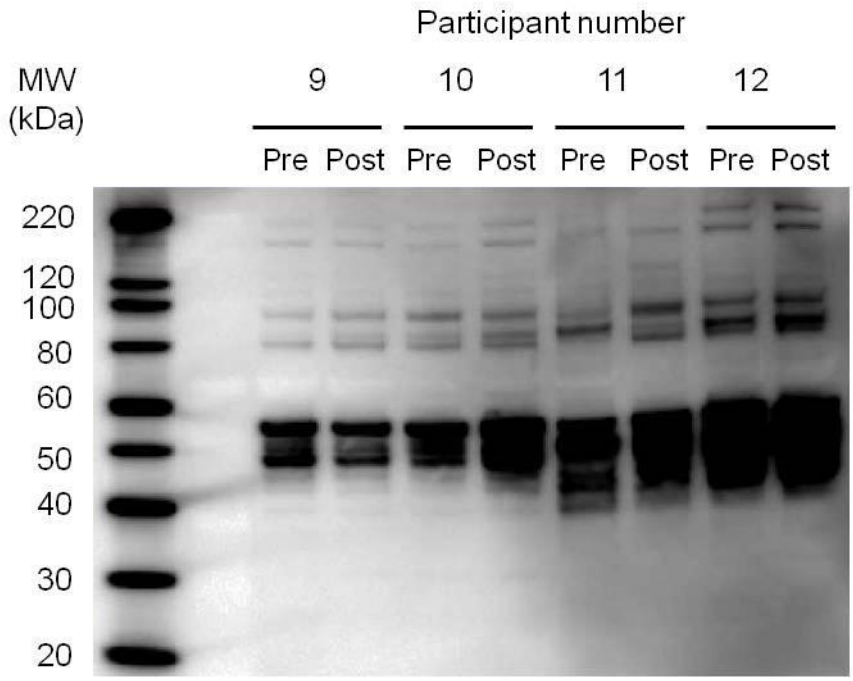

Figure 6.6 A Western blot showing IL-6R expression in subcutaneous adipose tissue homogenate, pre and post 2 weeks HIIT.

Figure 6.6 shows that there are a number of protein bands detected over a wide range of molecular weights (~40-220 kDA). The most prominent band identified spanned a molecular weight of approximately 45-55 kDa. Since 55 kDa is the molecular weight of heavy chain immunoglobulins (IgG) further experiments were carried out to ensure the antibody was not binding to non-specific antigens.

Experiment 4: Negative Controls

In order to determine the specificity of the IL-6R antibody two negative control experiments were carried out:

Peptide neutralisation

The IL-6R antibody was incubated with its blocking (neutralising) peptide. Affinity purified rabbit polyclonal antibodies can be raised against peptide antigens. Incubating the IL-6R antibody with the peptide (immunogen) prior to incubating the IL-6R antibody with the adipose tissue homogenate should block any IL-6R immunoreactivity present within the sample from binding to the polyclonal antibody and therefore provides a negative control. Four micrograms of IL-6R rabbit polyclonal antibody was added to 500 µl of PBS in an eppendorf. The neutralising peptide for IL-6R antibody was added to this solution at 5-fold the weight of the antibody, therefore 20 µg. The antibody and peptide mixture was left to incubate on a rotator overnight at 4°C at 40 rev·min^{-1}, to enable the immunogen to bind to the IL-6R antibody.

The following morning, a membrane with adipose tissue samples from 2 participants, pre- and post-training was blocked with PBS (5% dried milk) for 1 h. The peptide mixture was diluted in PBS (5% dried milk) to a total volume of 8 ml. This was then left to incubate on the membrane overnight on the rotator at 4°C. The membrane was then treated as previously described, where it was washed before incubation with a goat anti-rabbit HRP linked secondary antibody. An image of the blot incubated with IL-6R plus the neutralising peptide is shown in Figure 6.7B.

Control IgG

As a second negative control, another membrane with the same samples as used for the neutralising peptide experiment, was probed with normal rabbit IgG (sc-2027) (Santa Cruz Biotechnology, Inc., CA, USA). This control serum is not

raised against any specific antigens and therefore should have no or very low binding to proteins in the samples (Figure 6.7C).

As well as the membranes for the 2 negative control experiments, a third membrane with the same samples was treated as normal (A), as described in the Western blot section for comparison. In addition, as a further control a single adipose tissue sample was probed with only the goat anti-rabbit HRP linked secondary antibody at 1:1000 dilution, to ensure the secondary antibody was not binding non-specifically (D). Images for all 4 Western blots are shown in Figure 6.7.

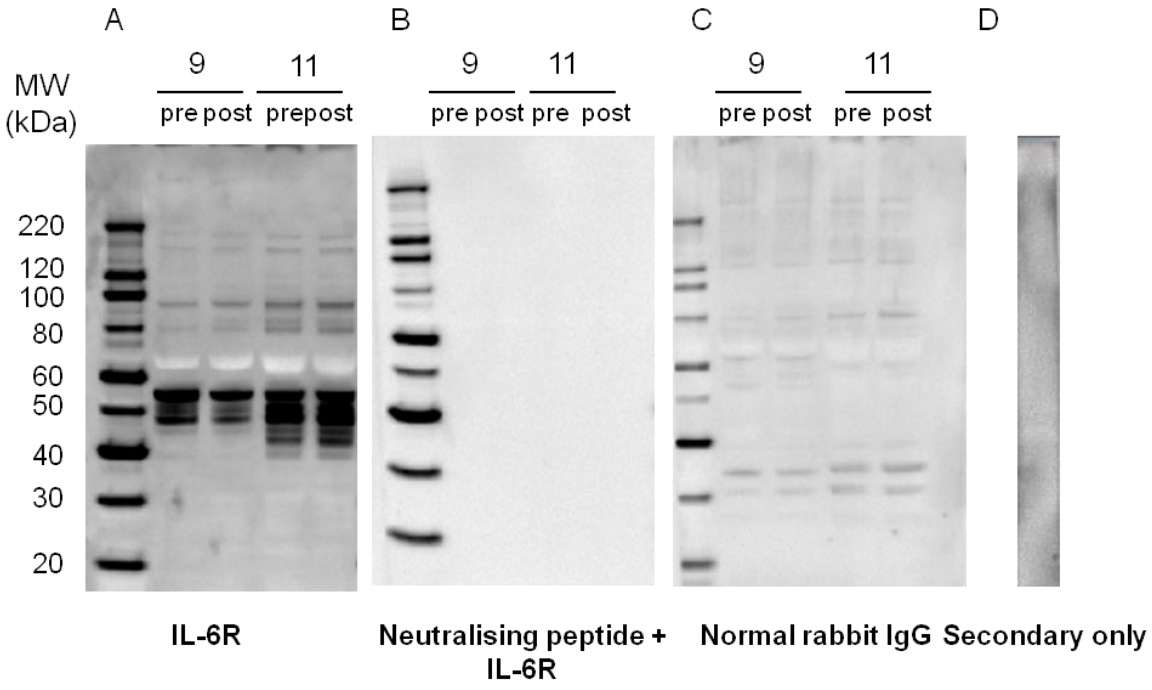

Figure 6.7 Negative control Western blots. Western blot were probed with: **A** IL-6R; **B** IL-6R + neutralising peptide **C** control serum **D** secondary antibody only. Adipose tissue samples were from 2 participants, pre and post 2 weeks HIIT.

Figure 6.7 shows there are no visible bands on the membrane incubated with the IL-6R antibody plus neutralising peptide (B). Therefore, the peptide has successfully bound to the IL-6R antibody, hence blocking it from binding to proteins on the membrane and supports the claim that the antibody used is

specific to IL-6R. The control serum (C), which should contain no or little IL-6R, shows some non-specific binding, however, this is not at the same molecular weights that IL-6R has been identified (A). Since the control serum and neutralising peptide showed no/little non-specific binding it seems likely that the IL-6R antibody used in these experiments is specific to IL-6R. In addition, there is no non-specific binding of the secondary antibody as no protein bands were identified during this experiment (D). In conclusion, these control experiments provide evidence that the antibody is specifically binding to IL-6R.

Experiment 5: Immunoprecipitation

Figure 6.6 showed several bands were detected when probed for IL-6R. Western blot procedures are constrained by the concentration of the specific protein of interest, and the specificity and sensitivity of the antibody used. Non-specific signals can occur if other proteins in the sample contain cross-reacting epitopes, due to the denaturation of proteins during sample preparation which can expose cross-reacting epitopes. By combining immunoprecipitation (IP) with Western blotting procedures this cross-reactivity can be reduced. The protein of interest is partially purified and concentrated during IP. IP allows the antibody to bind to native IL-6R in the adipose homogenate where cross-reacting epitopes are unlikely to be present. IP is then followed by Western blotting where the protein of interest (i.e. IL-6R) will become the predominant signal detected. This procedure was carried out in adipose tissue homogenate from 2 participants in this study. An outline of the procedure is below and is illustrated in Figure 6.8.

Immunoprecipitation Procedure:

- 200 µg of protein from adipose tissue homogenate was diluted in PBS to give a final volume of 500 µl.
- 1 µl of normal rabbit IgG-AC (25% agarose) (Santa Cruz Biotechnology, Inc., CA, USA) was added to each sample and rotated at 40 rev·min^{-1}, at

4°C for 30 min. This preclearing step prior to IP limits non-specific proteins in the homogenate. Samples were centrifuged at 1,000 g·min^{-1} for 30 s at 4°C, before transferring the supernatant to new eppendorfs.

- 2 µg of IL-6R antibody was added to the supernatant and rotated at 40 rev·min^{-1} overnight, at 4°C. The antibody will bind to IL-6R in the sample.
- The following morning 300 µl of protein A/G PLUS-agarose (50% suspension) (Santa Cruz Biotechnology, Inc., CA, USA) was centrifuged at 1,000 g·min^{-1} for 2 min at room temperature. The supernatant was discarded and 150 µl of PBS was added to the eppendorf to wash the agarose beads before centrifuging again. This step was repeated a further 3 times.
- After the final wash, the PBS was discarded and 50 µl of a 50% agarose bead solution was added to each eppendorf before rotating samples at 40 rev·min^{-1} for 1 h at 4°C. The IL-6R immune complex binds to the protein A/G agarose beads.
- Samples were centrifuged for at 1,000 g·min^{-1} for 3 min at 4°C. The supernatant was transferred to a fresh eppendorf, on ice. The pellet (containing the IL-6R immune complex) was washed 4 times with 150 µl of PBS and centrifuged in between at 1,000 g·min^{-1} for 3 min at 4°C. After the final wash all liquid was aspirated from the samples.
- Both the pellet and supernatant were prepared for 1D-PAGE.

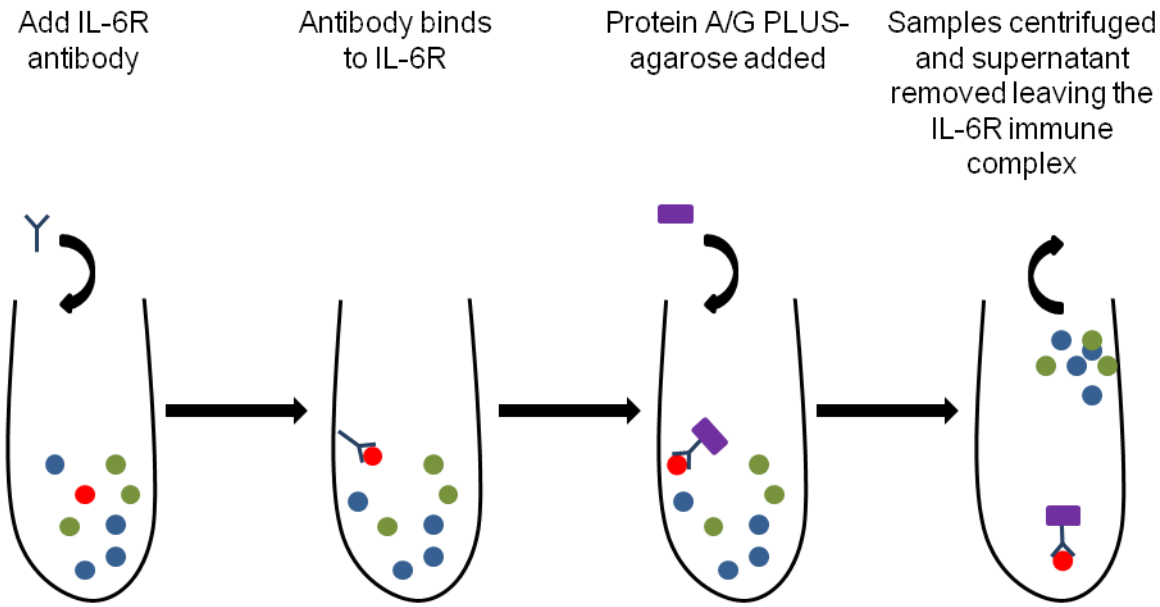

Figure 6.8 Immunoprecipitation process. Red circle represents IL-6R molecule (blue and green circles are non-specific proteins).

Figure 6.9 shows Western blots of 2 participants before and after IP for IL-6R, as well as the supernatant that was removed during the IP.

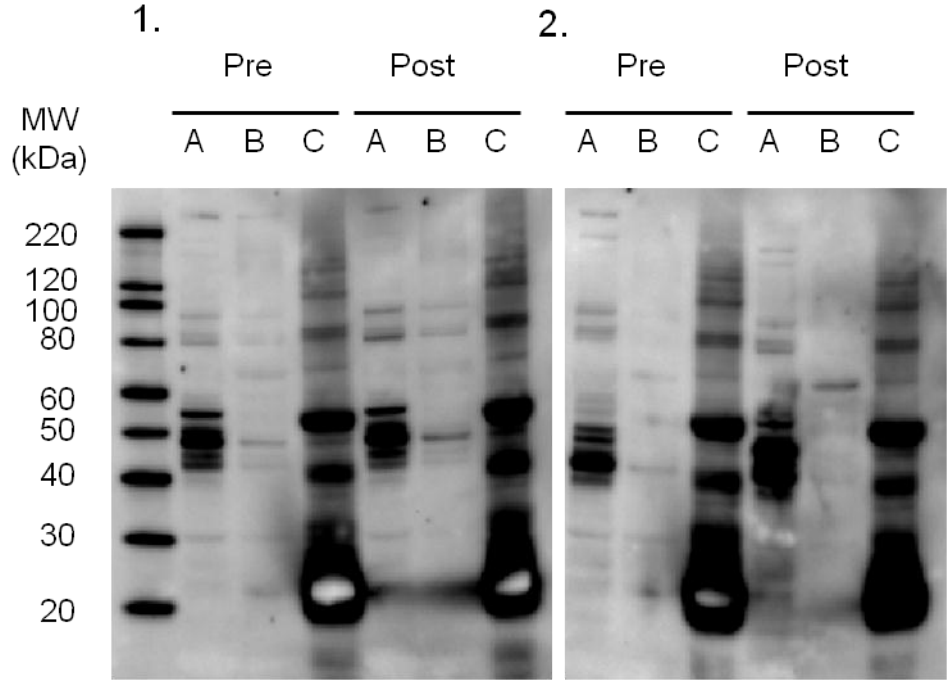

Figure 6.9 Western blots probed for IL-6R before and after immunoprecipitation, pre and post 2 weeks HIIT for 2 participants. Where **A** represents start material, **B** represents the supernatant from the immunoprecipitate and **C** represents the immunoprecipate material.

The supernatant should include any protein signals that the IL-6R antibody cross-reacts with, that were not precipitated during the IP. The supernatant (B) for all 4 samples confirms there is very little material identified, and therefore the material in A, i.e. the starting material, and the material used in the Western blots in Experiment 3, is likely to be IL-6R, rather than signals identified due to cross-reactivity with non-specific epitopes. In the lanes with the immunoprecipitate material (C) there are large bands at ~25 and ~55 kDa which are likely to be light and heavy chain IgG respectively, from the immunoprecipitating antibody. As heavy chain IgG is at the same molecular weight as the band we wish to detect (~45-55 kDa) it is not possible to quantify any changes in IL-6R from the immunoprecipitate material. This experiment is for illustrative processes only as different amounts of protein had to be used for the starting material (A) than for the IP. For the IP each sample started with

200 µg of protein to ensure when IL-6R was removed there was enough material immunoprecipitated that could be detectable. In conclusion, immunoprecipitation of IL-6R has provided evidence that the material detected in the previous Western blot experiment was IL-6R, as opposed to the IL-6R antibody cross-reacting with non-specific epitopes in the denatured adipose tissue samples. The immunoprecipitated material was unable to be quantified due to the IL-6R antibody reacting with light and heavy chain IgG.

Experiment 6: Immunoglobulin and albumin removal

As there is a large band detected at ~55 kDa in all of the adipose tissue samples (Figure 6.6), a ProteoExtract kit (Calbiochem, EMD Biosciences, Inc., La Jolla, CA, USA) was used to deplete IgG and albumin in adipose tissue homogenate from 2 participants. This was to ensure that the band detected at this molecular weight was IL-6R and not heavy chain IgG which also has a molecular weight of 55 kDa. To 540 µl of a binding buffer 60 µl of adipose tissue homogenate was added. To each column 850 µl of albumin/IgG binding buffer was added and allowed to pass through the resin bed by gravity-flow. The column was placed in a new collection tube and the 600 µl binding buffer and sample mixture was added to the column and left to pass through the resin bed into the collection tube. A further 600 µl of binding buffer was added to wash the column. The collected sample was depleted of IgG and albumin. As the sample has been diluted in a large volume of binding buffer and albumin and IgG have been depleted, the protein concentration was too low for 1D-PAGE. Therefore, the samples were concentrated using Amicon Ultra-0.5 centrifugal filter devices (Millipore, Billerica, MA, USA), which concentrates samples by filtering out proteins with a molecular weight less than 3 kDa (Figure 6.10).

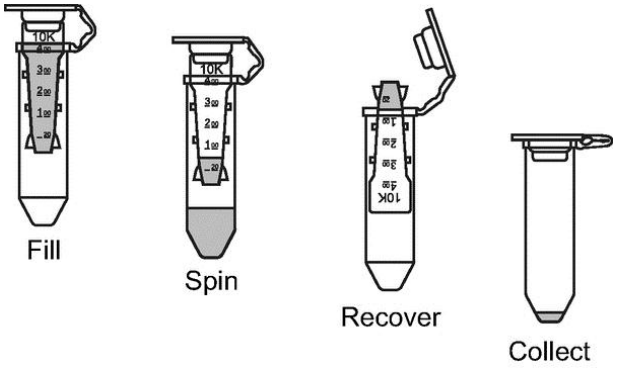

Figure 6.10 Procedure for concentrating the adipose tissue protein after IgG and albumin depletion. Up to 500 µl of the IgG and albumin depleted samples were added to the filter unit and centrifuged at 14,000 g·min^{-1} for 20 min at 4°C. Immediately afterwards, the filter unit was placed upside down in a new eppendorf and centrifuged at 1,000 g·min^{-1}, for 2 min at 4°C to recover the concentrated sample.

The protein concentration of the samples was determined again using the DC Protein Assay (Chapter 3.9.3) and samples were prepared for 1D-PAGE. Samples from before and after albumin and IgG removal were loaded onto 4-12% Bis-Tris gels as previously described and then transferred to PVDF membranes. One membrane was probed with the IL-6R antibody and another with normal rabbit IgG control serum to check for non-specific binding (Figure 6.11).

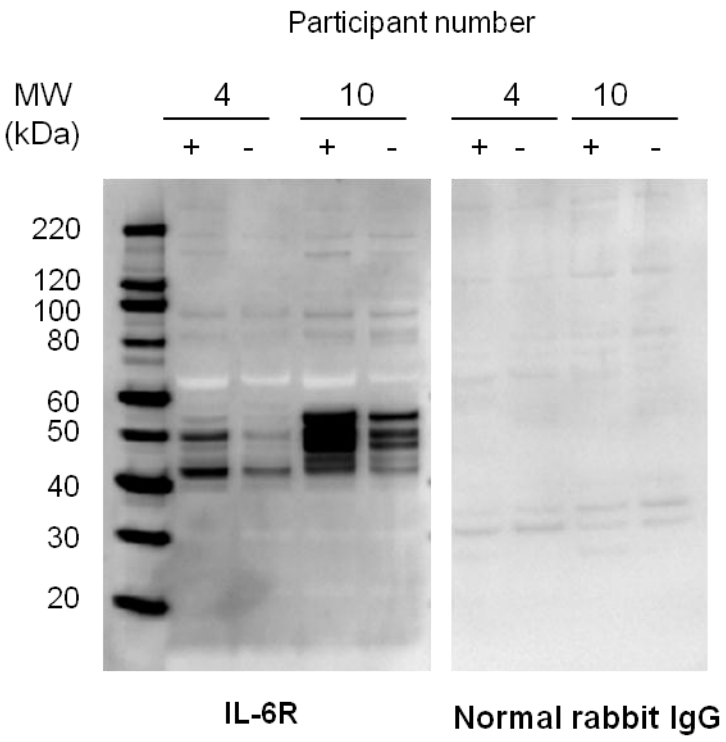

Figure 6.11 Western blot probed for IL-6R and normal rabbit IgG for adipose tissue homogenate before (+) and after (-) IgG and albumin depletion.

Figure 6.11 illustrates that after IgG and albumin have been removed from the samples there is less material present at 45-55 kDa. Therefore, the antibody used in these experiments could be binding non-specifically to heavy chain IgG. However, it is important to note that IgG removed samples have been treated differently to the other samples, although the amount of protein is the same across wells. The IgG depleted samples still have the same bands present, but at a lower concentration which could be due to the IgG and albumin depletion process or the concentration procedure. Since the same bands are still visible and in addition to the previous experiments it seems likely that the bands identified are IL-6R. In conclusion, all of the control experiments in this Chapter have supported that it is IL-6R detected in the adipose tissue homogenates. Since there is confidence that the protein detected is IL-6R, the Western Blot procedure was optimised in the following section.

Experiment 7: Optimisation of antibody concentration

Optimisation of this IL-6R Western blot protocol is required to ensure the best possible images are captured. The concentration of primary and secondary antibodies during Western blotting is important as too high a concentration will lead to non-specific binding and too low an antibody concentration will result in too weak a signal to allow detection of the protein of interest. In order to optimise the protocol, 2 adipose tissue samples were blotted with different concentrations of the primary IL-6R antibody. Membranes were incubated with IL-6R at 2 $\mu g \cdot ml^{-1}$, 0.5 $\mu g \cdot ml^{-1}$ (the concentration used in the previous experiments), 0.25 $\mu g \cdot ml^{-1}$ and 0.17 $\mu g \cdot ml^{-1}$ in PBS with 5% dried milk (Figure 6.12). All other variables remained constant.

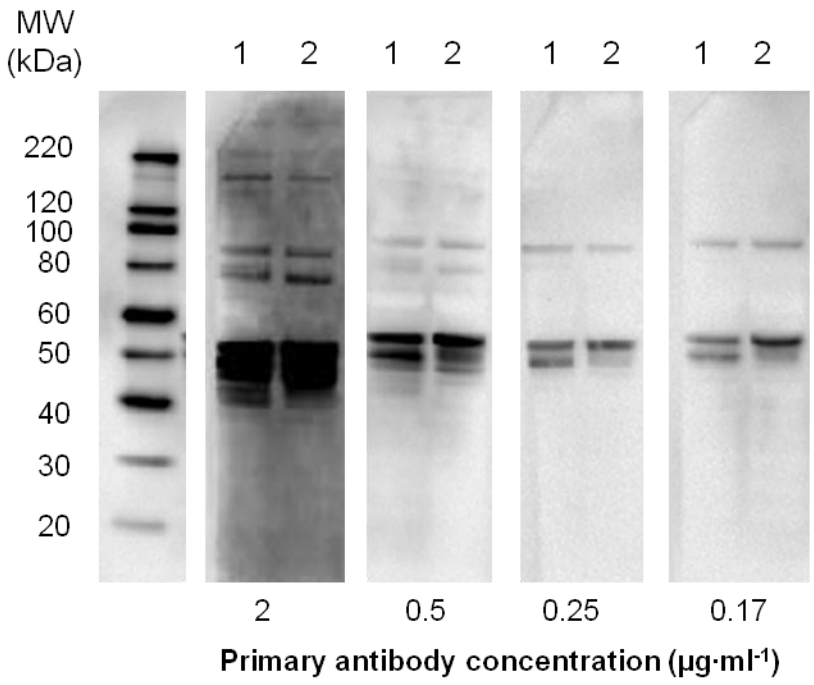

Figure 6.12 Optimisation of the primary IL-6R rabbit polyclonal antibody. Western blots for 2 adipose tissue samples probed with different concentrations of primary IL-6R antibody (2 $\mu g \cdot ml^{-1}$, 0.5 $\mu g \cdot ml^{-1}$, 0.25 $\mu g \cdot ml^{-1}$ and 0.17 $\mu g \cdot ml^{-1}$). The concentration of the goat anti-rabbit HRP linked secondary antibody was 1:1000.

IL-6R is detectable at all 4 concentrations. At the highest primary antibody concentration of 2 $\mu g \cdot ml^{-1}$ individual bands are not distinguishable and there appears to be some non-specific binding of protein bands at higher molecular weights which is reduced with dilution of the antibody. As Western blots with antibody concentrations of 0.25 $\mu g \cdot ml^{-1}$ and 0.17 $\mu g \cdot ml^{-1}$ give similar images it is more economical to use a working primary IL-6R antibody concentration of 0.17 $\mu g \cdot ml^{-1}$. There are now 2 distinct bands at ~45-55 kDa and a band at ~90 kDa.

To optimise the IL-6R Western blot protocol further, 4 membranes were incubated with the optimal primary IL-6R antibody concentration of 0.17 $\mu g \cdot ml^{-1}$, and a range of concentrations of the polyclonal goat anti-IgG-HRP conjugated secondary antibody. The concentration of the secondary antibody was 1:1000, 1:2000, 1:3000 or 1:4000 (Figure 6.13). Due to limited sample, the adipose tissue samples are different to the samples used to optimise the primary IL-6R antibody concentration and therefore there will be some differences in the images.

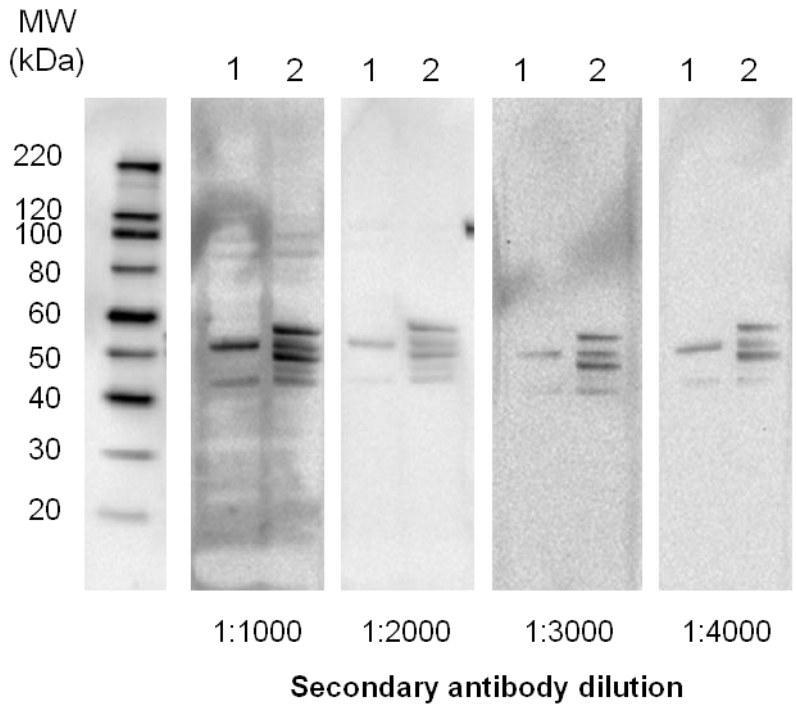

Figure 6.13 Optimisation of goat anti-rabbit HRP linked secondary antibody. Western blots for 2 adipose tissue homogenates probed with the following antibody dilutions: 1:1000, 1:2000, 1:3000 and 1:4000. The concentration of the primary IL-6R rabbit polyclonal antibody was 0.17 $\mu g \cdot ml^{-1}$.

Figure 6.13 shows that the same bands are visible for all of the blots, however, the clearest image is with the 1:1000 dilution. Therefore, the optimum concentrations for this IL-6R Western blot protocol is 0.17 $\mu g \cdot ml^{-1}$ for the primary IL-6R antibody and 1:1000 for the secondary antibody.

Experiment 8: Optimised Western blots for detection of IL-6R

1D-PAGE analysis and protein transfer to PVDF membranes were repeated for the pre- and post-training samples (N = 12), and membranes were probed for IL-6R using the new optimal antibody concentrations. After, membranes were stripped and probed for actin to normalise protein loading between pre- and post-training samples. Figure 6.14 is a Western blot for IL-6R for 4 participants pre- and post-training.

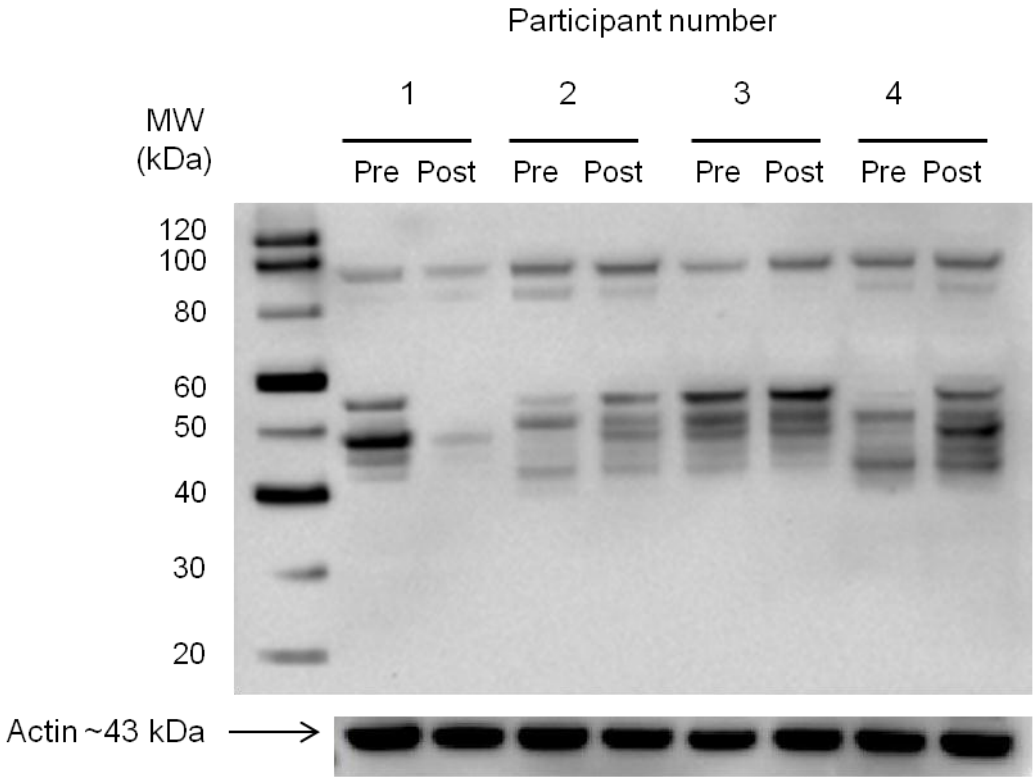

Figure 6.14 A Western blot showing IL-6R expression in subcutaneous adipose tissue homogenate, after optimising antibody concentrations, pre and post 2 weeks HIIT.

There is a clear band visible at ~90 kDa, similar to a band detected for membrane-bound IL-6R in osteoblasts at ~88 kDa (Vermes et al. 2002), and several isoforms identified between 45-55 kDa (Figure 6.14). Much of the cross-reactivity seen in earlier experiments has been reduced and the bands at 45-55 kDa are shown to be separate identifiable isoforms, whereas in previous

experiments were only visible as one large band. An example of the bands quantified for the participants is shown in Figure 6.15.

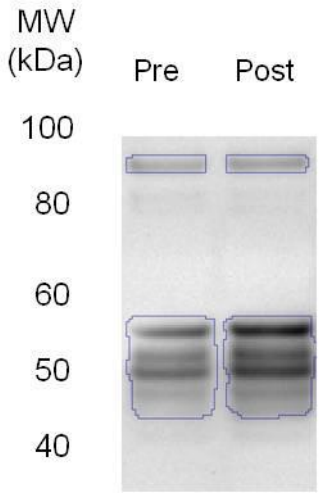

Figure 6.15 IL-6R isoforms quantified for one participant. IL-6R isoforms spanning ~45-55 kDa and ~90 kDa were quantified. The blue line represents the area that the intensity·mm^2 was determined. An area of equal size to the protein bands, from a similar unexposed region of the blot was subtracted, which represented the background intensity·mm^2.

Figure 6.16 shows the fold-change of the protein bands identified at ~45-55 kDa, ~90 kDa and the sum of these bands, pre- and post-training.

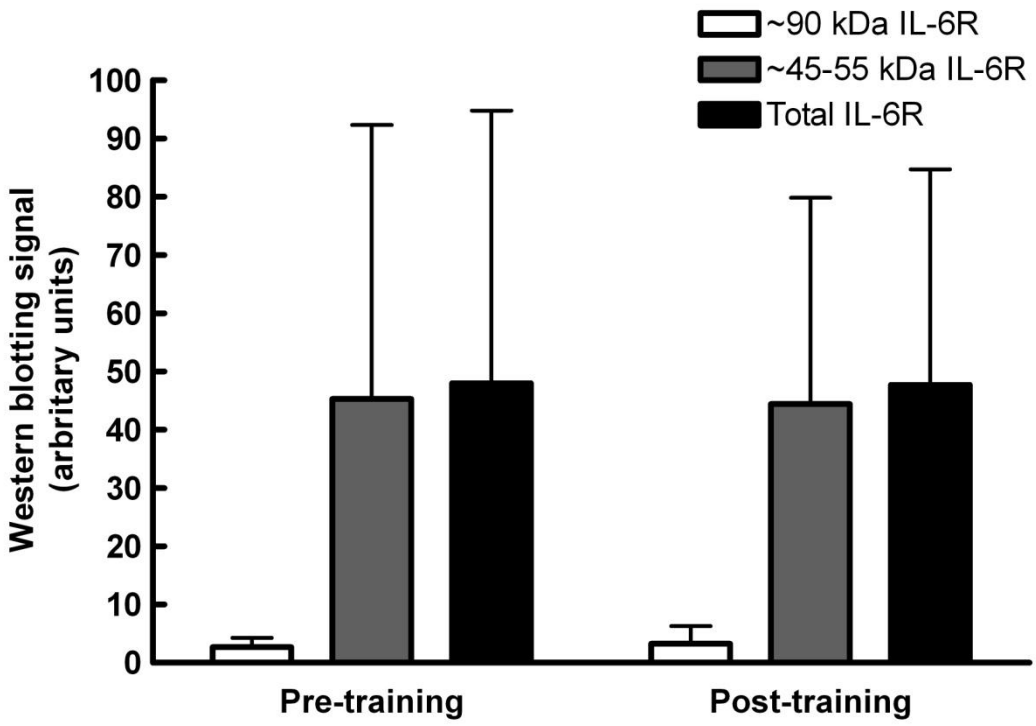

Figure 6.16 IL-6R isoforms at ~45-55 kDa, ~90 kDa and the sum of these isoforms in subcutaneous adipose tissue homogenate, pre and post 2 weeks HIIT (N = 12). Data normalised to actin expression.

The majority of IL-6R is present in the form of a cluster of protein bands detected at ~45-55 kDa. There were no changes found in IL-6R protein expression at ~45-55 kDa (p = 0.912), ~90 kDa (p = 0.710) or total (p = 0.975) IL-6R expression after 2 weeks HIIT. The fold-change from pre-training was compared to the fold-change in total IL-6R measured via ELISA in Chapter 5 (Figure 6.17).

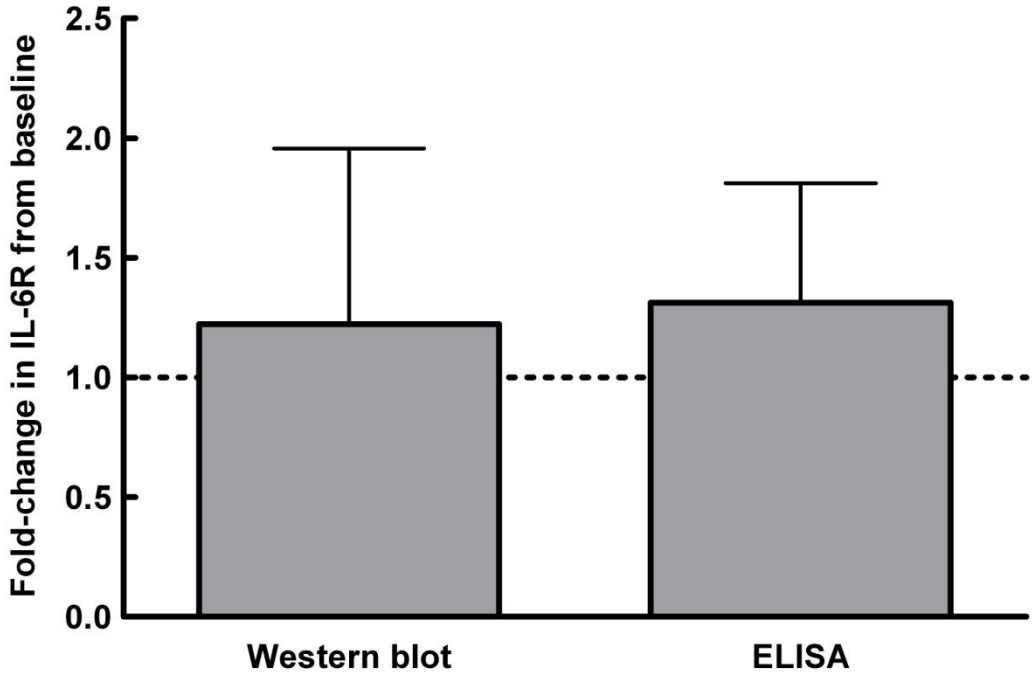

Figure 6.17 IL-6R fold-change in adipose tissue after 2 weeks HIIT using a polyclonal antibody during Western blot analysis and paired monoclonal antibodies during ELISA. Dotted line (1.0) represents baseline.

Figure 6.17 shows that both the ELISA and Western blot methods result in a similar fold-change in IL-6R of 1.31 and 1.22 respectively, although there were no significant differences between pre- and post-training for the different isoforms (Figure 6.16). It is likely that there was no significant difference in IL-6R post-training with Western blotting due to the larger variation found between individuals than was found with the ELISA. Therefore, the Western blot data has provided a similar mean fold-change result to the ELISA, suggesting that the assay is detecting changes in membrane-bound IL-6R and not the soluble form and represents a “true change” in the capability of IL-6 to signal.

6.5 Discussion

This Chapter has addressed many issues that arise when undertaking protein analysis. At the start of the study the aim was to investigate whether full-length membrane-bound IL-6R was present in adipose tissue. In Chapter 5, IL-6R was detected in adipose tissue, however it was unclear whether this was membrane-bound IL-6R or sIL-6R. The antibody in the current study has also been used to detect IL-6R in skeletal muscle (Akerstrom et al. 2009), where the authors reported detection of a single band at a molecular weight of ~80 kDa, although no images of the Western blots were provided in the publication. In a separate study this antibody was used to detect membrane-bound IL-6R in osteoblasts with a band detected at ~88 kDa (Vermes et al. 2002), approximately the same molecular weight as the band detected in the final Western blots in this Chapter.

Initial Western blots, following the published recipes, identified numerous bands at different molecular weights ranging from ~40-200 kDa. Control experiments, including blocking the IL-6R antibody with a neutralising peptide, were carried out to validate the specificity of the antibody. The control experiments provided supporting evidence that the bands identified during Western blotting were IL-6R, and the protocol was then optimised. After optimisation, fewer protein bands were detected, suggesting either the primary or secondary antibody or both were binding non-specifically. In the final set of Western blots, a band at ~88 kDa, similarly to that detected in osteoblasts (Vermes et al. 2002), and several bands detected at ~45-55 kDa were identified in adipose tissue. In most samples more than one band was detected between ~45-55 kDa.

Full-length IL-6R has 6 potential *N*-linked glycosylation sites, 5 in the extracellular domain and 1 in the cytoplasmic domain of the protein (Yamasaki et al. 1988). Glycosylation is an enzymatic reaction where glycans attach to proteins at specific sites and are essential for protein folding to create its tertiary structure. *N*-linked glycosylation is where glycans attach to a nitrogen of asparagine or arginine side-chains. Without this folding IL-6 would be unable to bind to IL-6R and IL-6 signalling would not occur. It is possible that when the adipose tissue homogenate was denatured there was less glycosylation, due to

heating and the addition of a denaturing detergent prior to 1D-PAGE. These conditions could break the *N*-glycosidic bonds between the peptide and carbohydrate, and therefore the molecular weight of the IL-6R would be reduced from the mature ~80 kDa to the predicted molecular weight of ~49.9 kDa if all bonds were broken. However, glycosylation may survive denaturing PAGE. The different forms of IL-6R detected in this Chapter could be the range of glycosylated forms of IL-6R. With specific antibodies for the carbohydrate side-chains this could be evaluated in future work. Native 1D-PAGE (i.e. without denaturing conditions) could also provide more information on the isoforms of IL-6R, as well as treating samples with deglycosylation enzymes to ensure all *N*-linked oligosaccharide chains have been removed. During denaturing conditions the proteins lose their tertiary and secondary structures, however, the primary amino acid structure remains intact. Therefore any bands detectable with a molecular weight lower than ~49.9 kDa cannot be accountable to the reducing conditions disrupting the amino acid sequence, however, they could be due to spliced variants of full-length IL-6R, if they consist of the cytoplasmic domain which the antibody has been raised against.

There is evidence that there are more than the 2 IL-6R isoforms (membrane-bound and soluble) commonly reported in the literature. In fact there is evidence of at least 5 spliced variants of full-length IL-6R, which is 468 amino acids. The spliced variants encode proteins of 365, 356, 293, 170 and 133 amino acids according to AceView (Thierry-Mieg and Thierry-Mieg 2006). However, based on the predicted molecular weights of these spliced variants along with their amino acid sequence it is unlikely these isoforms will be detected by the antibody used in these experiments. There is also the possibility that other spliced variants of the receptor exist that have not been identified yet.

After optimisation of the Western blot protocol a similar fold-change in IL-6R was found after 2 weeks HIIT, to that quantified via ELISA, although, this was not significantly different for the Western blot analysis. This could be due to the greater variation in fold-change for Western blot analysis in comparison to ELISA. There is the possibility that sIL-6R was present in the homogenate, which would have been detected via ELISA but not via Western blotting. This

could at least partially account for the discrepancy between methods, if the increase via ELISA was partly due to an increase in sIL-6R, however it is difficult to confirm this without an antibody specifically for sIL-6R.

This study has provided evidence that the increase in IL-6R after 2 weeks HIIT (as found in Chapter 5) is not purely due to an increase in sIL-6R in the adipose tissue matrix, but that cells within adipose tissue are expressing IL-6R on the plasma membrane. This supports previous findings that IL-6R is expressed on ~60% of adipocytes (Bastard et al. 2002) as well as expressed on macrophage cell surfaces (Bauer et al. 1989). Furthermore, a reliable Western blot method that has identified membrane-bound IL-6R in adipose tissue has been developed. This Chapter has revealed the complexity of the IL-6R protein and provided evidence that there are several isoforms of IL-6R present within subcutaneous adipose tissue that require further attention.

Chapter 7

THE INFLAMMATORY STATUS OF YOUNG ADULTS WITH TYPE 2 DIABETES MELLITUS – THE EXPEDITION STUDY

7.1 Abstract

The rising prevalence of obesity in Western populations has led to an epidemic of T2DM. The age of diagnosis of T2DM is decreasing and data describing this young adult T2DM population are currently lacking. The aim of this study was to investigate inflammatory status, $\dot{V}O_{2peak}$ and biochemical measurements associated with T2DM, including 25-hydroxyvitamin D, in young adults (< 40 y) diagnosed with T2DM. Twenty young adults with T2DM aged 18-40 years, 10 age-matched lean controls and 10 BMI and age-matched obese controls were recruited. Fasting, resting blood samples were collected and plasma IL-6, sIL-6R, CRP, IL-10, TNF-α and adiponectin concentration were measured via ELISA. Anthropometric measurements were taken, and lipid profiles, glycaemic control and $\dot{V}O_{2peak}$ were determined. T2DM had significantly higher plasma IL-6, TNF-α, IL-10 and CRP in comparison to the lean group ($p < 0.05$), but there were no significant differences compared with the OC group for any of the inflammatory proteins. Adiponectin in T2DM was significantly lower compared to the lean group ($p < 0.001$), however, there was no difference compared to obese controls ($p = 0.145$). T2DM had a significantly lower $\dot{V}O_{2peak}$ than lean controls relative to body mass ($p < 0.001$) but there was no difference compared to obese controls ($p = 0.255$). 85% of T2DM were 25-hydroxyvitamin D deficient, compared to 30% of lean controls and 80% of obese controls. This study has provided evidence that young adults with T2DM demonstrate chronic low-grade inflammation which is suspected to be due to increased adiposity.[1]

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7.2 Introduction

T2DM has traditionally been seen as a disease of middle to older age, however the age of diagnosis has fallen dramatically and the condition is now being seen in children, adolescents and young adults (Ehtisham et al. 2000; Ehtisham et al. 2004; Haines et al. 2007). The young adult with T2DM often has specific issues and represents an extreme phenotype. They are likely to be obese, many with morbid obesity, and are likely to have a strong family history of T2DM, lead a sedentary lifestyle, be of black or minority ethnic (BME) origin and come from less affluent socio-economic groups (Feltbower et al. 2003; Millett et al. 2008). In 2008 in a single specialist clinic at one site at University Hospitals Leicester there was over 30 cases of T2DM in those under 25 years, and 105 in those aged 30 or younger (Data from UHL Clinical Workstation July 2008).

As discussed in earlier chapters, chronic low-grade inflammation, including elevated CRP and IL-6, are known to be associated with T2DM (Hansen et al. 2010), and a number of studies have shown that there is a greater risk of developing T2DM in those with elevated inflammatory proteins (Pradhan et al. 2001; Hu et al. 2004; Pickup 2004). Until now, most of the literature has looked at inflammation in relation to insulin resistance and T2DM in older adults. However, one study investigated inflammatory proteins in young men with T2DM (aged 10-25 y), and CRP and TNF-α were elevated in obese T2DM but not lean T2DM, suggesting the increased CRP and TNF-α were associated with obesity (Su et al. 2010). This study included lean and obese groups with T2DM, as well as a lean control group, however they did not include an obese control group in the absence of T2DM to investigate the effects of obesity.

The aim of this study was to assess the inflammatory profile in young adults with T2DM, in comparison to lean and obese controls, and to determine if chronic low-grade inflammation in T2DM is attributable to obesity. Furthermore other risk factors associated with the development of T2DM including, biochemical, metabolic, anthropometric and $\dot{V}O_{2peak}$ were investigated to characterise which risk factors are associated with T2DM in young adults.

7.3 Materials and methods

7.3.1 Participants

Twenty young adults diagnosed with T2DM aged between 18-40 y, 10 age-matched lean controls, and 10 age and BMI matched obese controls were recruited from the Leicestershire region. T2DM patients were recruited from two specialist clinics at UHL based at the Leicester Royal Infirmary which specifically runs a young adult clinic and the Leicester General Hospital. Controls participants were recruited through advertisements in Loughborough and Leicester. Those with asthma or a body mass over 150 kg were not permitted to participate in the study. This study was granted ethical approval by the North Nottinghamshire Research Ethics Committee and Leicester NHS Research and Development. Participant characteristic are shown in Table 7.1.

Table 7.1 Participant characteristics

	T2DM (N = 20)	Lean (N = 10)	Obese (N= 10)
Sex, female/male	9/11	5/5	6/4
Age (y)	31.8 (6.6)	30.0 (6.7)	30.9 (5.6)
Body mass (kg)	100 (20.3)	63.3 (8.2)	93.9 (13)
BMI ($kg \cdot m^{-2}$)	33.9 (5.8)	21.9 (1.7)	33.4 (2.4)
Current smoker (%)	20	20	0
Family history of T2DM (%)	90	20	80
Black & minority ethnicity (%)	50	30	50

Mean (SD)
BMI, body mass index; T2DM, Type 2 diabetes mellitus.

Patients had been diagnosed with T2DM for a mean of 4.7 y (range 0.2 – 14 y) prior to participation in this study, with a mean age at diagnosis of 27.1 (6.1) y. All except one T2DM patient was on at least one antidiabetic drug. Current medications taken by the T2DM participants are listed in Table 7.2.

Table 7.2 Current medications taken by the Type 2 diabetes mellitus (T2DM) patients

	Medical use	Number of patients
Antihypertensive drugs	Lower blood pressure	6/20
Aspirin	Non-steroidal anti-inflammatory drug	1/20
Statins	Lower cholesterol	7/20
Fibrate	Lower cholesterol	4/20
Biguanide (Metformin)	Antidiabetic drug, decreases hyperglycaemia, lowers LDL cholesterol	16/20
Sulphonylurea	Antidiabetic drug, increases insulin production	3/20
GLP-1 analogue	Antidiabetic drug,	2/20
DPP – IV inhibitor	Antidiabetic drug, inhibits glucagon release, increase insulin production	2/20
Insulin	Administration will reduce blood glucose levels	5/20

T2DM, Type 2 diabetes mellitus; GLP-1, Glucagon-like peptide 1; DPP-IV, Dipeptidyl peptidase-IV

7.3.2 Study visits

All participants completed two study visits at least one week apart. The first visit took place in the exercise laboratory of the Clyde Williams building at Loughborough University. During this visit participants had their weight, height, blood pressure, waist and hip circumference measured, as described in Chapter 3. They went on to complete a $\dot{V}O_{2peak}$ test to volitional exhaustion. The equipment has previously been described in this thesis (Section 3.3). The starting power output and stage increments varied between participants depending on how active the individual reported they were during everyday life, with an aim to reach $\dot{V}O_{2peak}$ between ~8-12 min (Myers et al. 1991). The starting power output ranged from 25-100 W, and increased every 2 min by 20-

35 W. $\dot{V}O_{2peak}$ was identified as the $\dot{V}O_2$ averaged over the highest 30 s period. The average temperature and relative humidity throughout the study were 21.2 (1.2) ºC and 29.9 (7.3) % respectively.

For the second study visit, participants attended Glenfield Hospital in Leicester after an overnight fast, having abstained from alcohol, caffeine and exercise for 24 h prior to the visit. On arrival at the hospital a cannula was inserted into an antecubital vein and a resting blood sample was collected into vacutainers. Serum gel vacutainers (4.7 ml) were used to collect blood for the analysis of lipids and 25-Hydroxyvitamin D. A 2.5 ml EDTA vacutainer was used to sample blood for HbA1c. Fluoride oxalate vacutainers (2.5 ml) were collected for glucose measurement. A 4.9 ml vacutainer treated with lithium heparin was collected for the analysis of plasma insulin and c-peptide. Two 10 ml EDTA vacutainers were collected for the measurement of the inflammatory proteins and adiponectin. The vacutainers for the inflammatory protein analysis were immediately centrifuged at Glenfield Hospital before aliquoting plasma and storing at -80°C. Samples were later transported to Loughborough University where the analysis of inflammatory proteins was carried out. The analysis for all other biochemical parameters was carried out by the pathology department at Glenfield Hospital or Leicester Royal Infirmary.

7.3.3 Inflammatory protein analysis

IL-6, sIL-6R, the IL-6/sIL-6R complex, CRP, IL-10, TNF-α and adiponectin were measured by ELISAs as outlined earlier in this thesis (Chapter 3).

7.3.4 Biochemical analysis

Plasma glucose and serum cholesterol, HDL cholesterol and triacylglcerol were all measured using standard enzymatic endpoint methods on an ADVIA Chemistry System (Bayer Healthcare, NY, USA). The LDL cholesterol fraction was calculated by the Friedewald formula (1972). HbA1c was measured by ion exchange liquid chromatography (G7; Tosoh, Tokyo, Japan). Plasma insulin

and c-peptide concentration were determined using commercial ELISA kits (Mercodia, Uppsala, Sweden). Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was calculated using the recommended revised computer programme (Levy et al. 1998). 25(OH)D was quantified using liquid chromatography mass spectrometry (6410 Triple Quad, Agilent Technologies UK Ltd, Wokingham, UK).

7.3.5 Statistical analysis

Data was analysed using a between group analysis of covariance (ANCOVA) model, where sex and ethnicity were covariates in the model. Separate models were run for the comparison of T2DM and lean controls, and T2DM and obese controls. 25(OH)D results were categorised into deficient (<30 $nmol \cdot l^{-1}$) and non-deficient (>30 $nmol \cdot l^{-1}$) groups. Binary logistic regression models where sex and ethnicity were used as categorical covariates were used to estimate the odds ratio (95% CI) for 25(OH)D deficiency in T2DM. ANCOVA models were also used to determine whether inflammatory proteins differed in 25(OH)D deficient and non-deficient groups before and after adjusting for BMI.

7.4 Results

7.4.1 Anthropometry and blood pressure

T2DM had a significantly higher waist and hip circumference as well as waist-hip ratio than the lean controls ($p < 0.001$; Table 7.3). Matching BMI was achieved in the T2DM and obese control groups, with no significant difference in body mass, weight, waist and hip circumference or waist-hip ratio ($p > 0.05$; Table 7.4). No statistically significant differences in blood pressure were detected between groups.

Table 7.3 Anthropometric and blood pressure measurements for Type 2 diabetes mellitus (T2DM) patients and lean controls.

	T2DM (N=20)			Lean (N=10)			
	Mean (SD)	**95% Confidence interval for mean**		**Mean (SD)**	**95% Confidence interval for mean**		***p***
		Lower	Upper		Lower	Upper	
Waist (cm)	109.7 (12.6)	103.8	115.6	76.6 (7.2)	71.5	81.7	<0.001*
Hip (cm)	113.3 (11.1)	108.1	118.5	94.4 (4.1)	91.4	97.3	<0.001*
Waist: Hip ratio	0.97 (0.06)	0.94	1.00	0.81 (0.07)	0.76	0.86	<0.001*
Systolic BP (mmHg)	135 (14)	128	141	130 (11)	121	138	0.196
Diastolic BP (mmHg)	88 (10)	83	92	79 (12)	70	88	0.110

* Significantly different to T2DM group ($p \leq 0.05$)
BP, blood pressure.

Table 7.4 Anthropometric and blood pressure measurements for Type 2 diabetes mellitus (T2DM) patients and obese controls.

	T2DM (N=20)			Obese (N=10)			
	Mean (SD)	**95% Confidence interval for mean**		**Mean (SD)**	**95% Confidence interval for mean**		***p***
		Lower	Upper		Lower	Upper	
Waist (cm)	109.7 (12.6)	103.8	115.6	106.2 (8.1)	100.4	112.0	0.394
Hip (cm)	113.3 (11.1)	108.1	118.5	115.6 (8.0)	109.9	121.4	0.614
Waist: Hip ratio	0.97 (0.06)	0.94	1.00	0.92 (0.07)	0.87	0.97	0.082
Systolic BP (mmHg)	135 (14)	128	141	127 (14)	117	137	0.168
Diastolic BP (mmHg)	88 (10)	83	92	84 (9)	78	91	0.376

BP, blood pressure.

7.4.2 Peak oxygen uptake and power output

$\dot{V}O_{2peak}$ in T2DM was not significantly different compared to lean or obese control groups (p = 0.258 and 0.471 respectively; Figure 7.1). However, when expressed per kg of body mass, the T2DM group had a significantly lower $\dot{V}O_{2peak}$ than the lean individuals (p < 0.001), with no significant difference between T2DM and BMI matched, obese controls (p = 0.255). Peak power output at $\dot{V}O_{2peak}$ was significantly higher in lean controls, 221 (60) [178-264 95% CI] W, compared to T2DM, 162 (57) [135-190 95% CI] W (p = 0.043), although there was no difference between T2DM and obese controls, 178 (56) [138-218 95% CI] W (p = 0.404).

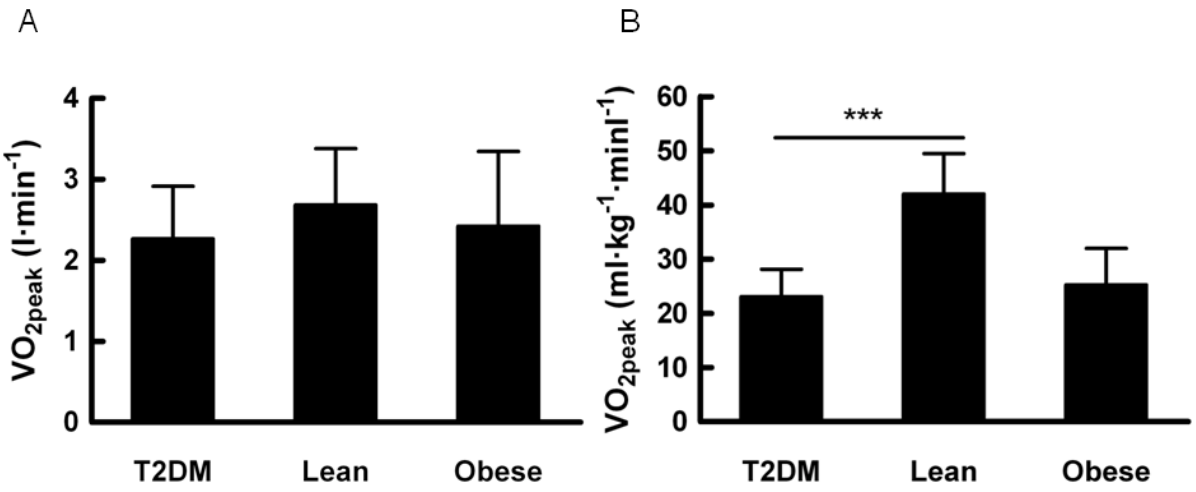


Figure 7.1 Absolute $\dot{V}O_{2peak}$ and $\dot{V}O_{2peak}$ relative to body mass. *** significant difference between groups, p <0.001.

7.4.3 Biochemical analysis

Fasting insulin, glucose, HbA1c and HOMA-IR were significantly higher in T2DM compared to lean and obese controls (Table 7.5 and 7.6). C-peptide was also significantly elevated in T2DM compared to lean controls, and tended to be higher than obese controls (p = 0.086). Lean controls had a significantly higher concentration of HDL cholesterol and lower triacylglycerols compared to T2DM. There were no difference in lipid profile between T2DM and obese controls.

Table 7.5 Glycaemic control and blood lipid profile for Type 2 diabetes mellitus (T2DM) patients and lean controls.

	T2DM (N=20)			Lean (N=10)			
	Mean (SD)	**95% Confidence interval for mean**		**Mean (SD)**	**95% Confidence interval for mean**		***p***
		Lower	Upper		Lower	Upper	
Glycaemic control							
Fasting glucose ($mmol \cdot l^{-1}$)[ab]	9.2 (4.0)	7.4	11.1	4.8 (0.5)	4.4	5.2	0.002*
Fasting insulin ($mU \cdot l^{-1}$) [ab]	28.3 (24.2)[c]	17.0	39.6	5.1 (1.1)[e]	4.4	5.9	<0.001*
Fasting C-peptide ($nmol \cdot l^{-1}$)[ab]	1.26 (0.76)	0.91	1.62	0.42 (0.05)[d]	0.38	0.46	<0.001*
HOMA-IR[ab]	3.7 (2.5)	2.5	4.8	0.9 (0.1)[d]	0.8	1.0	<0.001*
HbA1c (%)[ab]	8.2 (2.2)[c])	7.2	9.3	5.5 (0.3)	5.2	5.7	<0.001*
Blood lipid profile							
Total cholesterol ($mmol \cdot l^{-1}$)[a]	4.6 (1.3)	3.9	5.2	4.6 (1.1)	3.8	5.3	0.809
HDL cholesterol ($mmol \cdot l^{-1}$)[a]	1.1 (0.2)[c]	0.9	1.2	1.6 (0.4)	1.3	1.8	<0.001*
LDL cholesterol ($mmol \cdot l^{-1}$)[a]	2.7 (1.2)[d]	2.1	3.3	2.7 (1.0)	2.0	3.4	0.907
Triacylglycerols ($mmol \cdot l^{-1}$)[a]	2.0 (1.2)	1.4	2.5	1.1 (1.1)	0.3	1.8	0.014*

* significantly different to the T2DM group ($p \leq 0.05$)
[a] Adjusted for ethnicity and sex; [b] Based on transformed data; [c] 1 Missing value; [d] 2 missing values; [e] 3 missing values.
HOMA-IR, Homeostasis Model Assessment of Insulin Resistance HbA1c, Glycated haemoglobin; HDL, high density lipoprotein; LDL, low density lipoprotein.

Table 7.6 Glycaemic control and blood lipid profile for Type 2 diabetes mellitus (T2DM) patients and obese controls.

	T2DM (N = 20)			Obese (N = 10)			
	Mean (SD)	**95% Confidence interval for mean**		**Mean (SD)**	**95% Confidence interval for mean**		***p***
		Lower	Upper		Lower	Upper	
Glycaemic control							
Fasting glucose ($mmol \cdot l^{-1}$)[ab]	9.2 (4.0)	7.4	11.1	5.1 (0.4)	4.8	5.5	0.003*
Fasting insulin ($mU \cdot l^{-1}$) [ab]	28.3 (24.2)[c]	17.0	39.6	15.0 (8.1)	9.3	20.8	0.030*
Fasting C-peptide ($nmol \cdot l^{-1}$)[ab]	1.26 (0.76)	0.91	1.62	0.86 (0.30)	0.65	1.07	0.086
HOMA-IR[ab]	3.7 (2.5)	2.5	4.8	1.9 (0.7)	1.4	2.4	0.021*
HbA1c (%)[ab]	8.2 (2.2)[c])	7.2	9.3	5.6 (0.4)	5.3	5.9	<0.001*
Blood lipid profile							
Total cholesterol ($mmol \cdot l^{-1}$)[a]	4.6 (1.3)	3.9	5.2	4.3 (0.7)	3.8	4.9	0.830
HDL cholesterol ($mmol \cdot l^{-1}$)[a]	1.1 (0.2)[c]	0.9	1.2	1.2 (0.3)	1.0	1.3	0.336
LDL cholesterol ($mmol \cdot l^{-1}$)[a]	2.7 (1.2)[d]	2.1	3.3	2.6 (0.5)	2.2	3.0	0.851
Triacylglycerols ($mmol \cdot l^{-1}$)[a]	2.0 (1.2)	1.4	2.5	1.3 (0.8)	0.8	1.9	0.133

* significantly different to the T2DM group ($p \leq 0.05$)
[a] Adjusted for ethnicity and sex; [b] Based on transformed data; [c] 1 Missing value; [d] 2 missing values; [e] 3 missing values.
HOMA-IR, Homeostasis Model Assessment of Insulin Resistance HbA1c, Glycated haemoglobin; HDL, high density lipoprotein; LDL, low density lipoprotein.

Out of the T2DM patients, 85% were classified as being 25(OH)D (<30 nmol·l^{-1}) deficient in comparison to 30% of lean (p = 0.010) and 80% of obese controls (Figure 7.2). The odds ratio for the incidence of 25(OH)D deficiency in T2DM compared to lean controls was 13.9 [CI: 1.9, 101.5]. The number of individuals in the T2DM and obese groups with a 25(OH)D deficiency were not significantly different (p = 0.720), with an odds ratio of 1.6 [CI: 0.2, 11.4].

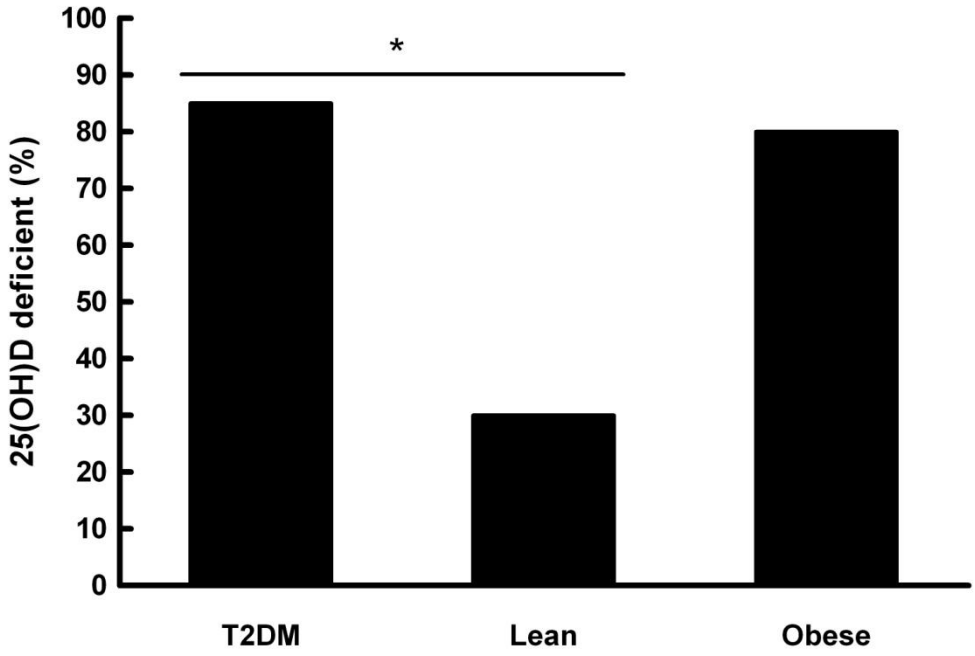


Figure 7.2 Percentage of individuals in T2DM, lean and obese groups who are deficient in 25-hydroxyvitamin D (25(OH)D) (* $p \leq 0.05$).

7.4.4 Inflammatory proteins

The T2DM group had significantly elevated levels of IL-6, TNF-α, IL-10 and CRP in comparison to the lean group (Table 7.7), although there were no differences in any of the inflammatory proteins between the T2DM and obese control groups (Table 7.8). There was also a tendency for the T2DM group to have higher sIL-6R in comparison to both lean and obese controls (p = 0.058 and 0.075 respectively), however there were no differences detected between T2DM and lean or obese controls for the IL-6/sIL-6R complex (p = 0.514 and

0.921 respectively). Adiponectin concentration was significantly lower in T2DM than the lean controls ($p < 0.001$), although there was no significant difference between T2DM and obese controls ($p = 0.145$).

Table 7.7 Fasted inflammatory proteins for Type 2 diabetes mellitus (T2DM) patients and lean controls.

	T2DM (N = 20)			Lean (N = 10)			
	Mean (SD)	**95% Confidence interval for mean**		**Mean (SD)**	**95% Confidence interval for mean**		***p***
		Lower	Upper		Lower	Upper	
IL-6 (pg·ml^{-1})	7.9 (9.1)	3.6	12.1	2.5 (2.8)[a]	0.4	4.7	0.004*
sIL-6R (ng·ml^{-1})	46.1 (12.1)	40.4	51.8	39.3 (8.8)	33.0	45.5	0.058
IL-6/sIL-6R complex (arbitrary units)	15.3 (8.4)	11.4	19.2	15.4 (8.8)	9.4	21.4	0.514
TNF-α (pg·ml^{-1})	1.7 (1.2)	1.1	2.3	1.1 (0.3)	0.9	1.3	0.016*
IL-10 (pg·ml^{-1})	2.3 (1.4)	1.7	3.0	0.9 (0.3)	0.7	1.1	<0.001*
CRP (µg·ml^{-1})	4.0 (3.1)	2.6	5.5	1.2 (0.7)	0.7	1.7	0.004*
Adiponectin (µg·ml^{-1})	2.9 (1.7)	2.1	3.7	7.3 (2.8)	5.2	9.3	<0.001*

* significantly different to the T2DM group ($p \leq 0.05$)
[a] 1 value missing; [b] 2 values missing.

Table 7.8 Fasted inflammatory proteins for Type 2 diabetes mellitus (T2DM) patients and obese controls.

	T2DM (N = 20)			Obese (N = 10)			
	Mean (SD)	95% Confidence Interval for Mean		**Mean (SD)**	95% Confidence Interval for Mean		***p***
		Lower	Upper		Lower	Upper	
IL-6 (pg·ml^{-1})	7.9 (9.1)	3.6	12.1	7.0 (8.5)	1.0	13.1	0.741
sIL-6R (ng·ml^{-1})	46.1 (12.1)	40.4	51.8	38.1 (8.5)	32.1	44.2	0.075
IL-6/sIL-6R complex (arbitrary units)	15.3 (8.4)	11.4	19.2	14.8 (8.9)	8.4	21.2	0.921
TNF-α (pg·ml^{-1})	1.7 (1.2)	1.1	2.3	1.4 (0.3)	1.2	1.6	0.003*
IL-10 (pg·ml^{-1})	2.3 (1.4)	1.7	3.0	1.5 (0.2)	1.3	1.7	0.087
CRP (µg·ml^{-1})	4.0 (3.1)	2.6	5.5	4.6 (2.9)	2.5	6.6	0.651
Adiponectin (µg·ml^{-1})	2.9 (1.7)	2.1	3.7	3.9 (1.7)	2.6	5.1	0.145

* significantly different to the T2DM group ($p \leq 0.05$)
[a] 1 value missing; [b] 2 values missing

7.4.5 25-hydroxyvitamin D deficiency and inflammation

There is some evidence to suggest that inflammation is associated with 25(OH)D deficiency (Peterson and Heffernan 2008), which has also been associated with T2DM (Muscogiuri et al. 2010), however, it is not clear whether this is due to excess adiposity. Therefore, all participants were split into 25(OH)D deficient or non-deficient groups and ANCOVAs were run to assess whether there were any differences in the inflammatory profiles between groups. The 25(OH)D deficient group had significantly lower adiponectin and significantly elevated IL-10 and CRP ($p < 0.05$), with a tendency for sIL-6R and the IL-6/sIL-6R complex to be elevated in 25(OH)D deficient individuals ($p = 0.080$ and 0.071 respectively), but there was no difference between groups for IL-6 or TNF-α concentration ($p > 0.05$; Table 7.9). Once adjusted for BMI, there were no differences between 25(OH)D deficient and non-deficient groups for any of the inflammatory proteins ($p > 0.05$).

Table 7.9 Inflammatory proteins in 25-hydroxyvitamin D deficient and non-deficient groups.

			p	
	Sufficient (N = 29)	**Deficient (N = 11)**	**Not BMI adjusted**	**BMI adjusted**
Adiponectin (μg·ml^{-1})	5.8 (3.0)	3.6 (2.4)	0.029*	0.488
IL-6 (pg·ml^{-1})	5.7 (5.6)	6.7 (8.8)	0.985	0.165
sIL-6R (ng·ml^{-1})	39.9 (9.8)	43.3 (11.4)	0.080	0.337
IL-6/sIL-6R complex (arbitrary units)	17.1 (7.5)	14.6 (8.6)	0.071	0.112
TNF-α (pg·ml^{-1})	1.4 (0.7)	1.5 (0.9)	0.649	0.797
IL-10 (pg·ml^{-1})	1.3 (0.7)	2.0 (1.3)	0.031*	0.488
CRP (μg·ml^{-1})	1.5 (1.1)	4.2 (3.1)	0.004*	0.438

Mean (SD); 25(OH)D results were categorised as deficient (<30 nmol·l^{-1}) and sufficient (>30 nmol·l^{-1}) amounts of 25(OH)D; N = 40.
* significantly different to the T2DM group ($p \leq 0.05$).
25(OH)D, 25-hydroxyvitamin D.

7.5 Discussion

The main finding of this study was that the development of chronic low-grade inflammation is at least in part mediated by excessive fat deposition. This study found that young adults with T2DM display signs of early onset chronic low-grade inflammation in comparison to healthy lean individuals, including elevated IL-6, IL-10, TNF-α and CRP and a reduction in adiponectin, although protein levels did not differ in comparison to obese individuals. In addition this is the first study to have shown that young obese adults with and without T2DM have an increased prevalence of 25(OH)D deficiency.

Elevated TNF-α may contribute to the induction of insulin resistance by inhibiting insulin-regulated glucose uptake and/or the phosphorylation of IRS-1. This is supported by previous studies that showed TNF-α was increased in both young lean and obese individuals with T2DM, aged between 10-25 y, compared to lean individuals without T2DM (Su et al. 2010). In the current study circulating TNF-α was raised in comparison to lean controls, however there was no difference compared to the obese control group. In contrast to the current study, Monroy and colleagues showed that adults (~42 y) with T2DM had increased circulatory TNF-α in compared to lean and obese controls (Monroy et al. 2009). This discrepancy could be due to the participants in this study being on average ~10 years older than in the current study.

There was a tendency for sIL-6R to be elevated in T2DM compared to lean and obese controls ($p = 0.058$ and 0.075 respectively). Significantly higher sIL-6R has previously been shown in T2DM (Monroy et al. 2009), although another study has found no difference between T2DM patients and controls (Kado et al. 1999). In the latter study sIL-6R was very high in both groups (~100 $ng \cdot ml^{-1}$) and therefore their findings may not be comparable to the current study. Despite only a tendency for sIL-6R to be elevated in T2DM, IL-6 was significantly higher in T2DM compared to lean individuals, however there was no difference between T2DM and obese controls ($p = 0.741$). This suggests that elevated IL-6 in T2DM is likely to be due to an increased adiposity and therefore greater potential for IL-6 production and secretion of IL-6 into the circulation, and supports the finding previously shown in older adults (Hansen

et al. 2010). Su et al. (2010) has also shown that IL-6 was only elevated in lean patients with T2DM and not obese patients with T2DM compared to lean controls, however IL-6 concentration was relatively low in all three groups (~1.3-2.2 pg·ml^{-1}), whereas in the current study IL-6 ranged between ~0.5-40 pg·ml^{-1}. The large range of IL-6 in the current study could be due to inclusion of BME participants and the large variation in BMI, however this factor was accounted for in the statistical analysis, therefore it will not affect the outcomes of the study.

IL-6 and TNF-α have been shown to induce production of lipoproteins in the liver (Sjöholm and Nyström 2006). Chronic low-grade inflammation is also associated with dyslipedmia, including an increase in VLDL, which is a risk factor for insulin resistance (Esteve et al. 2005). In the current study, all but two of the T2DM patients were on at least one cholesterol lowering medication which could explain why although IL-6 and TNF-α were both elevated in T2DM, there was not any difference in total or LDL cholesterol. Although there were no differences for total or LDL cholesterol between T2DM and lean controls, HDL cholesterol was significantly lower in T2DM and triacylglycerols were elevated. These differences are likely due to excess adipose tissue as no differences were found between T2DM in comparison to the obese group. In addition, all but one of the T2DM patients were currently being prescribed at least one antidiabetic drug and 6 patients were on antihypertensive medication. And it is therefore it possible that this could confound the findings of the study. However since inflammatory proteins were significantly elevated in T2DM group compared to the lean group, but there were no differences between T2DM and the obese control group which suggests that the medication may have had little effect on chronic low-grade inflammation.

There was a significant reduction in adiponectin in T2DM compared to lean controls, however adiponectin was not statistically different to obese controls (p = 0.145). This would suggest that the reduced adiponectin is due to an increase in adipose tissue, supporting the current literature that adiponectin is inversely correlated to BMI (Arita et al. 1999; Weyer et al. 2001; Bruun et al. 2003; Kern et al. 2003; Ryan et al. 2003; Vilarrasa et al. 2005; Bluher et al. 2006). Decreased adiponectin can cause insulin resistance due to a reduction in

AMPK activation and increase the concentration of triacylglycerols in the muscle (Yamauchi et al. 2001).

The finding of a significant increase in IL-10 in T2DM compared to lean controls is in contrast to another study where circulating IL-10 was lower in T2DM than individuals with impaired or normal glucose tolerance before an exercise intervention (Oberbach et al. 2006). The participants in this study were older than the current study (> 40 y) and it is possible that during obesity and the early stages of T2DM that the body tries to counteract the increase in pro-inflammatory cytokines and limit the overall inflammatory effect by increasing the production of the anti-inflammatory cytokine IL-10. Since chronic low-grade inflammation is defined as an increase in pro- as well as anti-inflammatory proteins, an elevation of IL-10 in chronic disease states seems plausible. Although speculative there is some evidence to support this theory since TNF-α has been shown to stimulate IL-10 release in subcutaneous adipose tissue which could act as a counter-regulatory measure as suggested by Juge-Aubry et al. (2005). Furthermore, a study has shown that IL-10 is elevated in obesity, however, is reduced in women with the metabolic syndrome, irrespective of body weight (Esposito et al. 2003). In addition it has been shown that exercise in those with T2DM induces an increase in IL-10 (Kadoglou et al. 2007; Balducci et al. 2010b), which is likely to be due to stimulation of IL-10 production through an increase in IL-6 after exercise.

The proportion of individuals with hypovitmanosis D was similar for both obese and T2DM participants (80% and 85% respectively), suggesting that 25(OH)D deficiency is associated with obesity. 25(OH)D deficiency has been suggested to be associated with inflammation and has been shown to correlate with TNF-α and a tendency to correlate with IL-6, but not CRP or IL-10 (Peterson and Heffernan 2008). In contrast to these findings, the current study found that IL-10 and CRP were significantly elevated and adiponectin significantly reduced in those that were 25(OH)D deficient. There was also a tendency for the 25(OH)D deficient group to have elevated sIL-6R and IL-6/sIL-6R ($p = 0.080$ and 0.071 respectively), but there were no differences for IL-6 or TNF-α. However, when the results were adjusted for BMI there were no differences for any of the

inflammatory proteins in the 25(OH)D deficient and non-deficient groups, therefore 25(OH)D deficiency is associated with obesity.

In conclusion, it seems that elevated IL-6, TNF-α, CRP, IL-10 and triacylglycerols as well as a decrease in adiponectin and HDL cholesterol in young adults with T2DM is likely to be caused by excessive fat tissue. This is the first study to investigate many of these inflammatory proteins in adults under 40 years with T2DM, as well as being the first study to show that 25(OH)D deficiency is common in this population and that it is likely due to an increase in adipose tissue.

Chapter 8

GENERAL DISCUSSION

8.1 Thesis outline

This thesis initially set out to investigate whether an acute bout of high intensity intermittent training (HIIT) was sufficient to induce a greater response of the IL-6 system in comparison to continuous moderate intensity exercise (MOD), where external work done was matched. Once it had been established that there was a greater IL-6 response after HIIT than MOD, subsequent studies were implemented to investigate:

- the effects of 2 weeks HIIT on inflammatory status in the circulation and adipose tissue, and glycaemic control in a cohort of overweight and obese males;
- whether IL-6R, detectable in subcutaneous adipose tissue, is membrane-bound or soluble IL-6R. If membrane-bound IL-6R is detected, to determine if it is altered after 2 weeks HIIT in overweight and obese males;
- the inflammatory, glycaemic and lipid profiles, and peak oxygen uptake during exercise in young adults with T2DM (< 40 y), in comparison to age-matched lean and obese individuals.

8.2 Main findings

The key findings of this thesis were:

- that an acute bout of HIIT induced a greater IL-6 response than a bout of continuous moderate intensity exercise, where external work done was matched (Chapter 4);
- that sIL-6R and the IL-6/sIL-6R complex were both augmented after a single bout of exercise, however there were no significant differences between the HIIT and MOD trials (Chapter 4);
- plasma DS-sIL-6R was increased 6 h after an acute bout of HIIT. However, at both rest and after exercise, DS-sIL-6R contributes less

than 1% of total sIL-6R, therefore the majority of sIL-6R must be derived from proteolytic cleavage of the membrane-bound IL-6R (Chapter 4);

- after 2 weeks HIIT a reduction in waist circumference was induced as well as an increase in $\dot{V}O_{2peak}$ in overweight and obese males (Chapter 5);

- two weeks HIIT in an overweight and obese male cohort induced a reduction in sIL-6R, the IL-6/sIL-6R complex, MCP-1 and adiponectin in the circulation;

- that IL-6 was reduced and IL-6R was increased in subcutaneous adipose tissue after 2 weeks HIIT in overweight and obese males (Chapter 5);

- within subcutaneous adipose tissue, there are a number of different forms of IL-6R detected (Chapter 6), which are possibly due to varying degrees of glycosylation of the full-length IL-6R. This study demonstrated that membrane-bound IL-6R is present in subcutaneous adipose tissue, however was not significantly altered after 2 weeks HIIT. A method to quantify IL-6R within adipose tissue via Western blotting was optimised and the specificity of the antibody validated using control experiments;

- it was found that many inflammatory proteins were elevated in T2DM in comparison to a control group of lean participants (Chapter 7), yet when compared to a group of obese controls the concentration of inflammatory proteins was not significantly different. This suggests that the inflammatory proteins quantified in this study are at least in part dependent on adiposity, rather than a state of insulin resistance.

8.3 Main discussion

The research questions in this thesis recognised the increasing prevalence of obesity in the UK and in parallel the increasing evidence of the health benefits achieved from short exposures to high intensity exercise. The exercise regimen used in this thesis, HIIT, was selected since studies that have used sprint

interval training, i.e. a Wingate protocol, have reported feelings of nausea and light-headedness during the exercise, even in healthy individuals (Richards et al. 2010), therefore it may not be a feasible protocol to transfer to patient populations or even the general public. As the power output during HIIT is relative to individual aerobic capacity this should be a relatively safe and achievable mode of exercise for some patient groups, including those with T2DM. All of the participants in Chapters 4 and 5 successfully completed the HIIT protocol. In addition, other studies that have used a similar exercise intensity during intervals have induced a number of health benefits in patient populations, including heart failure patients and those with the metabolic syndrome (Wisløff et al. 2007; Tjønna et al. 2008). Intermittent exercise was also found to be more enjoyable than continuous exercise in coronary heart disease patients (Guiraud et al. 2011), therefore exercise adherence may be improved. More recently a new high intensity intermittent exercise protocol has been introduced, compromising of ten 60 s intervals on a cycle ergometer at 90% maximal heart rate, interspersed with 60 s rest (Little et al. 2011). This has been shown to improve glucose control after 2 weeks training using continuous glucose monitoring over a 24 h period in T2DM patients. Although energy expenditure is likely to be much lower than HIIT, and the potential for fat loss may be lower, further work could be carried out using this protocol to determine the effects on chronic low-grade inflammation, as it has been shown to be a time efficient strategy that is suitable for patient populations.

Chronic low-grade inflammation is characteristic of obesity and there is some evidence that exercise can reduce this inflammation in disease states. The major outcome throughout this thesis has been the inflammatory proteins and the IL-6 system in particular. The IL-6 system was selected for in depth investigation in this thesis due to its anti- and pro-inflammatory properties, and it represents a key link between acute and adaptive inflammation. IL-6 is also the most significant cytokine to increase after acute exercise and precedes the presence of others. It was originally hypothesised by the Copenhagen research group that IL-6 was the metabolic link between skeletal muscle and other tissues (Pedersen et al. 2001; Figure 8.1).

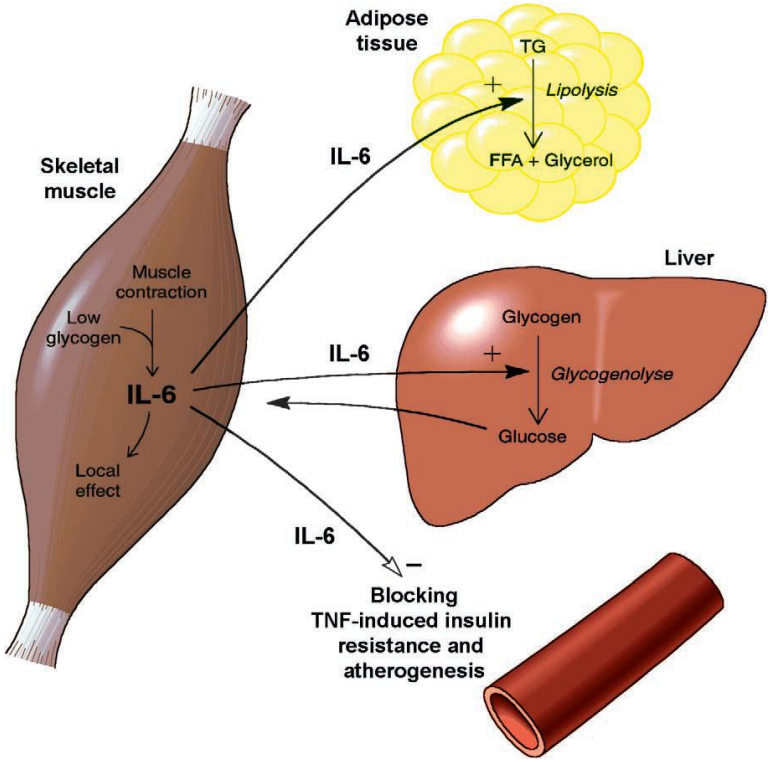


Figure 8.1 The proposed effects of skeletal muscle produced IL-6 on different tissues. The contracting muscle produces and releases IL-6, and this release is enhanced further if muscle glycogen is low. It was proposed that IL-6 induces lipolysis in the adipose tissue and through its effects on the liver, IL-6 also contributes to the maintenance of glucose homeostasis during exercise. IL-6 may also inhibit TNF-α induced insulin resistance. TG, triacylglycerol; FFA free fatty acid (Taken from Pedersen et al. 2001).

Steensberg et al. (2001) has suggested that the increase in IL-6 production in skeletal muscle during exercise, which is then released into the circulation, could have a direct or indirect effect on elevating hepatic glucose output. The increase in glucose release from the liver is required to maintain blood glucose levels during exercise to account for the increase in glucose uptake by the working muscles, and furthermore ingestion of glucose during exercise attenuates the increase in IL-6 (Nieman et al. 1998). Collectively this evidence suggests that IL-6 may have a role in glucose regulation during exercise.

It was shown that the increase in IL-6 was greater after HIIT than MOD at 1.5 h post-exercise and it was speculated that this was due to greater glycogen depletion induced by HIIT, since an increase in IL-6 transcription and mRNA expression in contracting skeletal muscle has been shown to be increased

further with low glycogen availability (Keller et al. 2001; Steensberg et al. 2001). Glycogen utilisation has previously been shown to be dependent on exercise intensity (Gollnick et al. 1974), although a counterargument available at that time (Essén et al. 1977), indicated that there was no difference in skeletal muscle glycogen content after 1 h high intensity intermittent or continuous exercise with similar external work outputs. However, in this latter study there was a small sample size (N = 5) and 6 months to 1 year between trials. A recently published study however, has compared an acute high intensity intermittent running protocol (six 3 min intervals at 90% $\dot{V}O_{2max}$ separated by 3 min recovery periods at 50% $\dot{V}O_{2max}$) with continuous exercise (50 min at 70% $\dot{V}O_{2max}$), matched for average intensity, duration and work done, and found no difference in glycogen depletion, or AMPK and p38 MAPK phosphorylation in skeletal muscle post-exercise (Bartlett et al. 2012). Some dubiety still exists however, as time spent at high intensity exercise is only 18 min as opposed to 40 min for the HIIT protocol used in this thesis, although the overall exercise duration is similar (50 and 58 min respectively). The authors of the study acknowledged that the difference in exercise intensity may not have been large enough to identify significant differences between the protocols (Bartlett et al. 2012), and therefore it is still plausible that the greater augmentation in IL-6 after HIIT is due to lower skeletal muscle glycogen content post-exercise than after MOD, but this requires further investigation.

IL-6 has been shown to increase glucose uptake into skeletal muscle in the presence of sIL-6R, partly through AMPK signalling (Gray et al. 2009c). Therefore if glycogen utilisation was greater during HIIT this could stimulate IL-6 production in order to increase the uptake of glucose into the exercising muscles. Reduced glycogen also leads to an increase in phosphorylation of p38 MAPK (Chan et al. 2004), and it is possible that p38 MAPK phosphorylation increased to a greater extent after HIIT, which could lead to subsequent activation of transcription factors which will induce an increase in IL-6 production (Pedersen and Febbraio 2008). MAPK signalling has been shown to be dependent on exercise intensity (Widegren et al. 2000) and has been significantly increased in skeletal muscle after a bout of intermittent exercise (Yu et al. 2003), similar to the protocol used in this thesis. Although Bartlett et

al. (2012) found no difference in p38 MAPK phosphorylation between trials, as previously discussed the time exercising at high intensity was less than half the time in the HIIT protocol used in this thesis, and in addition the exercise intensities of the two trials may not have differed sufficiently. Furthermore, another study has shown that although MAPK phosphorylation was not intensity dependent, activation of transcription factors and AMPK was (Egan et al. 2010), which could explain the greater increase in IL-6 after HIIT. Overall, indirect evidence suggests that glycogen depletion was greater after HIIT and therefore signalling pathways including AMPK and MAPK may have been enhanced which could have been linked to IL-6 production, however this warrants further investigation.

It was anticipated that the greater elevation in IL-6 after an acute bout of HIIT would initiate the anti-inflammatory cascade and lead to a greater activation of the anti-inflammatory system including an augmented production of IL-10, and inhibit the pro-inflammatory cytokines, TNF-α and IL-1. Although these cytokines were not measured it is anticipated that intermittent exercise may cause a greater anti-inflammatory drive than continuous exercise. The HIIT protocol was then used in an exercise training intervention, with 6 exposures to HIIT over a 2 week period. The main outcomes focused on were inflammation and glucose control. Because it is believed that a significant amount of many inflammatory proteins comes from adipose tissue this was studied to determine whether 2 weeks HIIT altered the inflammatory profile in adipose tissue. The population studied were overweight and obese who were not in regular training.

Although studies have found that some protocols of high intensity intermittent exercise are capable of improving glucose regulation (Babraj et al. 2009; Richards et al. 2010; Little et al. 2011), the results in Chapter 5 showed that 2 weeks HIIT in overweight and obese males did not induce such an improvement. On closer examination of the literature there are some inconsistencies as to whether 2 weeks intermittent exercise does improve glucose regulation (Babraj et al. 2009; Richards et al. 2010; Whyte et al. 2010). These discrepancies could be due to the differing populations studied, the timing of post-training measurements as well differences in the methodologies used. Two weeks SIT was shown have a positive response on insulin sensitivity

24 h post training, however this effect was eradicated at 72 h post-training, therefore suggesting the augmented insulin sensitivity was due to an acute exercise response (Whyte et al. 2010). Further research is required to determine if insulin sensitivity is improved after HIIT in those that have developed impaired glucose tolerance and T2DM. The lack of improvement in glucose regulation in this study coincided with a lack of change in circulatory IL-6 after 2 weeks HIIT. Despite no change in resting circulating IL-6 after the training period, there was a significant decrease in the IL-6/sIL-6R complex suggesting that IL-6 signalling may have been downregulated. However, it has been shown that IL-6R is increased in skeletal muscle post-training (Akerstrom et al. 2009), which could explain why although there was no difference in circulatory total IL-6, there is a reduction in sIL-6R and IL-6 bound to the soluble form of IL-6R in the circulation, as more IL-6R is present on the plasma membrane of specific tissues such as adipose tissue and skeletal muscle for signalling to occur.

IL-6R has previously been identified in adipocytes by Bastard et al. (2002) and this is the first study to have shown an increase in IL-6R in adipose tissue with exercise training. In Chapter 4 it was shown that sIL-6R increased with acute exercise, however there was no difference between HIIT and MOD. As the contribution of the two sIL-6R isoforms after exercise were unknown, DS-sIL-6R was quantified at rest and 6 h post HIIT, as this was the time point when total sIL-6R peaked. DS-sIL-6R was significantly increased at 6 h post-exercise, however, constituted less than 1% at rest and after exercise. Similarly Chapter 6 set out to evaluate the isoforms of IL-6R present in adipose tissue i.e. soluble and membrane-bound IL-6R. An antibody that binds to the intracellular domain of IL-6R in adipose tissue was used during Western blot analysis and concluded that membrane-bound IL-6R was present in subcutaneous adipose tissue. There was a multitude of IL-6R isoforms present, however it is unclear whether all of these forms would be functional *in vivo*.

IL-6R in adipose tissue increased after 2 weeks HIIT, yet there was a significant reduction in sIL-6R in plasma in overweight and obese males. Although speculative, the increase in IL-6R in adipose tissue could be due to a reduction in cleavage of the membrane-bound receptor, which could contribute to a

decrease in sIL-6R in plasma. Enzymes of the ADAM family have been shown to cleave IL-6R from the plasma membrane (Matthews et al. 2003). The membrane-bound IL-6R and the sIL-6R are therefore regulated by different processes and it is not surprising that they respond differently to an exercise stimulus. Future research should aim to establish if the activity of these enzymes are downregulated with exercise training. T2DM patients also tended to have elevated sIL-6R in comparison to both lean and obese controls (p = 0.058 and 0.075 respectively; Chapter 7) supporting the findings of Müller et al. (2002), who showed sIL-6R to be increased in older adults (mean age ~65 y) with T2DM in comparison to lean controls. Further work should be carried out to determine if the differences detected between T2DM and non-T2DM in sIL-6R extend to the adipose tissue.

In an obese state there is a greater infiltration of macrophages into adipose tissue (Cancello et al. 2005) which is regulated by MCP-1, although this was undetectable in subcutaneous adipose tissue pre- and post- 2 weeks HIIT. It is possible that macrophage infiltration was reduced after exercise training. The reduction in circulating MCP-1 could be due to a decrease in MCP-1 release from other tissues such as visceral adipose tissue, which has been shown to have a greater concentration of MCP-1 than subcutaneous adipose tissue (Bruun et al. 2005).

The reduction of adiponectin in the circulation and a tendency for a decrease in adipose tissue (p = 0.056) after 2 weeks HIIT was unexpected. Particularly as the classical outcome that adiponectin was highest in lean individuals was found in the EXPEDITION study Chapter 7, suggesting adiponectin is dependent on fat deposition. Lean controls had significantly higher adiponectin than T2DM patients, had but there was no significant difference with a group of BMI matched individuals. Exercise training has been reported to induce an increase in circulatory adiponectin, however, there are also studies that do not support this statement (Hulver et al. 2002; Boudou et al. 2003; Christiansen et al. 2010b). The study by Christiansen et al. (2010b) gives an insight that the differing responses may be due to fat loss as opposed to the exercise *per se*. They were able to show that a 3 month moderate intensity exercise intervention induced a non-significant decrease in circulating adiponectin, however, in a diet

group, and a diet and exercise combined group, adiponectin was elevated in obese individuals. Weight loss was approximately 4-fold higher in both the diet groups than the exercise only group. In Chapter 5 the effects of exercise on adiponectin in adipose tissue were shown for the first time. The tendency for a reduction in adiponectin in adipose tissue reflects the reduction in the circulation, as would be expected, since most adiponectin stems from adipose tissue. An additional complexity of adiponectin lies in the fact that there are different isoforms exerting both pro- and anti-inflammatory properties (Ouchi et al. 1999; Ouchi et al. 2000; Haugen and Drevon 2007). Further studies are required to decipher the roles of the different adiponectin isoforms and how exercise and diet affect these isoforms.

The final chapter phenotyped the inflammatory profile of young adults with T2DM as this is a growing population. There is little knowledge on whether they have similar metabolic and inflammatory traits to the traditional older population with T2DM. The T2DM patients were identified as having chronic low-grade inflammation in comparison to a group of age-matched lean controls, however, when compared to obese there were no differences detected, suggesting that many inflammatory proteins may be associated with adiposity. However, the finding of elevated TNF-α in comparsion to the lean control group is of importance as it is known to be a key player in the development of insulin resistance. The majority of both T2DM patients and obese controls were also identified as being 25(OH)D deficient, however the role that 25(OH)D plays in obesity, insulin resistance and chronic low-grade inflammation remains unclear and warrants further attention. An exercise intervention needs to be established that this population can successfully undertake. Future studies should be conducted to determine whether HIIT can reduce chronic low-grade inflammation in patient populations and what effect this has on 25(OH)D status. Other studies are required to determine whether this is an achievable mode of exercise in populations with chronic low-grade inflammation, such as in obesity and T2DM.

8.4 Remaining issues

The findings of this thesis have highlighted the beneficial effects of both acute and chronic HIIT on the IL-6 system as well as the effects on other inflammatory and metabolic parameters. In doing so the findings have brought about new issues and questions that future research should aim to address.

In Chapter 4, it was demonstrated that HIIT induced a greater IL-6 response than MOD, however the mechanism behind this augmented response is unclear. Further studies should be carried out to determine whether glycogen depletion in skeletal muscle differs between these protocols, as this could augment the IL-6 response. Furthermore, it should also be established if there is greater upregulation of the signalling pathways in skeletal muscle after HIIT, including AMPK and MAPK which have been linked to IL-6 production and glucose regulation.

Although HIIT was found to improve the inflammatory status in an obese and overweight cohort after 2 weeks, the optimum dosage should be established. Studies should be conducted to determine whether health benefits in relation to inflammatory status can be achieved with fewer intervals, and the number of training sessions per week required to achieve health benefits should also be optimised. A two week training period was chosen for this study as a number of other health benefits have previously been shown after the same length of time. However, it would also be useful to determine whether further health benefits can be achieved after longer training periods using this HIIT protocol. In addition, detraining studies should be carried out to establish how long these health benefits can be sustained.

A small, but significant reduction in waist circumference was induced after 2 weeks of HIIT in obese and overweight males. Waist circumference is correlated with abdominal visceral adiposity deposition and with CVD risk factors (Pouliot et al. 1994). However, further studies are warranted to ascertain if the decrease detected in waist circumference is due to a reduction in fat mass. This could be done via DEXA which gives localised lean and fat mass or by magnetic resonance imaging (MRI) scanning, which would allow the amount of subcutaneous and visceral adipose tissue to be quantified.

There is also some evidence that intermittent exercise will increase EPOC above continuous exercise, as research has shown that splitting aerobic sessions in two results in a significantly higher EPOC (Kaminsky et al. 1990; Almuzaini et al. 1998). However, it is thought that EPOC after aerobic exercise contributes 6-15% of total oxygen consumption (LaForgia et al. 2006), and therefore although it is likely to contribute to an increased fat loss after training it is unlikely to be the only factor to explain the differing fat loss. Future studies should aim to determine the effect of HIIT and MOD exercise on EPOC to determine the significance of this on energy expenditure.

A robust method was developed and validated for the detection of membrane-bound IL-6R in adipose tissue. Western blots revealed numerous IL-6R isoforms in adipose tissue. Continuing research in this area should evaluate the functionality of these isoforms and experiments should be carried out to determine whether the isoforms were the result of varying degrees of glycosylation. Similarly to the development of a method for IL-6R, it would be useful to do a comparable study for adiponectin to determine the contribution of the adiponectin isoforms in plasma and adipose tissue, particularly in response to an exercise intervention, to determine whether the adiponectin isoforms respond differently to an exercise stimulus.

8.5 Conclusions

In conclusion, this thesis has demonstrated that HIIT can significantly augment the IL-6 system after an acute bout of exercise, above that of moderate intensity exercise, matched for duration and external work done, however the mechanism behind this is unknown and requires further attention. For the first time it has been shown that exercise, and specifically HIIT, can induce changes in the inflammatory profile in subcutaneous adipose tissue in overweight and obese males after a 2 week training period, as well as reduce inflammatory mediators in the circulation. A protocol was optimised for the detection of membrane-bound IL-6R in adipose tissue. This protocol identified numerous forms of the IL-6 receptor and requires further attention to ascertain the

functionality of these isoforms. Future studies are needed to establish the functions of IL-6 in differing tissues and the role that IL-6R plays in mediating these functions. Young adults that have developed T2DM, as well as obese individuals that have not developed T2DM are in a state of chronic low-grade inflammation. These groups are at risk of chronic conditions and it is imperative that exercise protocols are developed which are achievable for such populations and can be adhered to. It should be established whether the HIIT protocol utilised in this thesis can be translated into patient populations, and if this type of training can induce changes in the inflammatory profile in those with T2DM, as it did with overweight and obese individuals.

REFERENCES

Abramson JL, Vaccarino V (2002) Relationship between physical activity and inflammation among apparently healthy middle-aged and older US adults. *Arch Intern Med* 162: 1286-1292

Adamopoulos S, Parissis J, Karatzas D, Kroupis C, Georgiadis M, Karavolias G, Paraskevaidis J, Koniavitou K, Coats AJ, Kremastinos DT (2002) Physical training modulates proinflammatory cytokines and the soluble Fas/soluble Fas ligand system in patients with chronic heart failure. *J Am Coll Cardiol* 39: 653-663

Akerstrom TC, Krogh-Madsen R, Petersen AM, Pedersen BK (2009) Glucose ingestion during endurance training attenuates expression of myokine receptor. *Exp physiol* 94: 1124-31

Almuzaini KS, Potteiger JA, Green SB (1998) Effects of split exercise sessions on excess postexercise oxygen consumption and resting metabolic rate. *Can J Appl Physiol* 23: 433-443

Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y (1999) Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 257: 79-83

Astrand I, Astrand PO, Christensen EH, Hedman R (1960) Intermittent muscular work. *Acta Physiol Scand* 48: 448-453

Babraj JA, Vollaard NB, Keast C, Guppy FM, Cottrell G, Timmons JA (2009) Extremely short duration high intensity interval training substantially improves insulin action in young healthy males. *BMC Endocr Disord* 9:3

Balducci S, Zanuso S, Nicolucci A, De Feo P, Cavallo S, Cardelli P, Fallucca S, Alessi E, Fallucca F, Pugliese G, Italian Diabetes Exercise Study (IDES) Investigators (2010a) Effect of an intensive exercise intervention strategy on modifiable cardiovascular risk factors in subjects with type 2 diabetes mellitus: a

randomized controlled trial: the Italian Diabetes and Exercise Study (IDES). *Arch Intern Med* 170: 1794-1803

Balducci S, Zanuso S, Nicolucci A, Fernando F, Cavallo S, Cardelli P, Fallucca S, Alessi E, Letizia C, Jimenez A, Fallucca F, Pugliese G (2010b) Anti-inflammatory effect of exercise training in subjects with type 2 diabetes and the metabolic syndrome is dependent on exercise modalities and independent of weight loss. *Nutr Metab Cardiovasc Dis* 20: 608-617

Bartlett JD, Close GL, MacLaren DP, Gregson W, Drust B, Morton JP (2011) High-intensity interval running is perceived to be more enjoyable than moderate-intensity continuous exercise: implications for exercise adherence. *J Sports Sci* 29: 547-553

Bartlett JD, Hwa-Joo C, Jeong TS, Louhelainen J, Cochran AJ, Gibala MJ, Gregson W, Close GL, Drust B, Morton JP (2012) Matched work high-intensity interval and continuous running induce similar increases in PGC-1alpha mRNA, AMPK, p38 and p53 phosphorylation in human skeletal muscle. *J Appl Physiol* doi:10.1152/japplphysiol.01040.2011

Bastard JP, Jardel C, Bruckert E, Blondy P, Capeau J, Laville M, Vidal H, Hainque B (2000) Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. *J Clin Endocrinol Metab* 85: 3338-3342

Bastard JP, Maachi M, Van Nhieu JT, Jardel C, Bruckert E, Grimaldi A, Robert JJ, Capeau J, Hainque B (2002) Adipose tissue IL-6 content correlates with resistance to insulin activation of glucose uptake both in vivo and in vitro. *J Clin Endocrinol Metab* 87: 2084-2089

Bauer J, Bauer TM, Kalb T, Taga T, Lengyel G, Hirano T, Kishimoto T, Acs G, Mayer L, Gerok W (1989) Regulation of interleukin 6 receptor expression in human monocytes and monocyte-derived macrophages. Comparison with the expression in human hepatocytes. *J Exp Med* 170: 1537-1549

Berg AH, Combs TP, Du X, Brownlee M, Scherer PE (2001) The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat Med* 7: 947-953

Berg AH, Scherer PE (2005) Adipose tissue, inflammation, and cardiovascular disease. *Circ Res* 96: 939-949

Blair SN, Cheng Y, Holder JS (2001) Is physical activity or physical fitness more important in defining health benefits? *Med Sci Sports Exerc* 33: S379-399; S419-420

Blüher M, Bullen JW, Jr, Lee JH, Kralisch S, Fasshauer M, Klöting N, Niebauer J, Schön MR, Williams CJ, Mantzoros CS (2006) Circulating adiponectin and expression of adiponectin receptors in human skeletal muscle: associations with metabolic parameters and insulin resistance and regulation by physical training. *J Clin Endocrinol Metab* 91: 2310-2316

Bošanská L, Michalský D, Lacinová Z, Dostálová I, Bártlová M, Haluzíková D, Matoulek M, Kasalický M, Haluzík M (2010) The influence of obesity and different fat depots on adipose tissue gene expression and protein levels of cell adhesion molecules. *Physiol Res* 59: 79-88

Boudou P, Sobngwi E, Mauvais-Jarvis F, Vexiau P, Gautier JF (2003) Absence of exercise-induced variations in adiponectin levels despite decreased abdominal adiposity and improved insulin sensitivity in type 2 diabetic men. *Eur J Endocrinol* 149: 421-424

Brahimi-Horn MC, Pouysségur J (2007) Oxygen, a source of life and stress. *FEBS Lett* 581: 3582-3591

Brake DK, Smith EO, Mersmann H, Smith CW, Robker RL (2006) ICAM-1 expression in adipose tissue: effects of diet-induced obesity in mice. *Am J Physiol Cell Physiol* 291: C1232-C1239

Bruun JM, Helge JW, Richelsen B, Stallknecht B (2006) Diet and exercise reduce low-grade inflammation and macrophage infiltration in adipose tissue

but not in skeletal muscle in severely obese subjects. *Am J Physiol Endocrinol Metab* 290: E961-E967

Bruun JM, Lihn AS, Pedersen SB, Richelsen B (2005) Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): implication of macrophages resident in the AT. *J Clin Endocrinol Metab* 90: 2282-2289

Bruun JM, Lihn AS, Verdich C, Pedersen SB, Toubro S, Astrup A, Richelsen B, (2003) Regulation of adiponectin by adipose tissue-derived cytokines: in vivo and in vitro investigations in humans. *Am J Physiol Endocrinol Metab* 285: E527-E533

Bruunsgaard H (2005) Physical activity and modulation of systemic low-level inflammation. *J Leukoc Biol* 78: 819-835

Burgomaster KA, Howarth KR, Phillips SM, Rakobowchuk M, MacDonald MJ, McGee SL, Gibala MJ (2008) Similar metabolic adaptations during exercise after low volume sprint interval and traditional endurance training in humans. *J Physiol* 586: 151-160

Burgomaster KA, Hughes SC, Heigenhauser GJF, Bradwell SN, Gibala MJ (2005) Six sessions of sprint interval training increases muscle oxidative potential and cycle endurance capacity in humans. *J Appl Physiol* 98: 1985-1990

Campbell IT, Jarrett RJ, Keen H (1975) Diurnal and seasonal variation in oral glucose tolerance: studies in the Antarctic. *Diabetologia* 11: 139-145

Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C, Coupaye M, Pelloux V, Hugol D, Bouillot JL, Bouloumié A, Barbatelli G, Cinti S, Svensson PA, Barsh GS, Zucker JD, Basdevant A, Langin D, Clément K (2005) Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes* 54: 2277-2286.

Carey AL, Steinberg GR, Macaulay SL, Thomas WG, Holmes AG, Ramm G, Prelovsek O, Hohnen-Behrens C, Watt MJ, James DE, Kemp BE, Pedersen BK, Febbraio MA (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: 2688-2697.

Chalaris A, Rabe B, Paliga K, Lange H, Laskay T, Fielding CA, Jones SA, 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: 1748-1755.

Chan MH, McGee SL, Watt MJ, Hargreaves M, Febbraio MA (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. F*ASEB J* 18: 1785-1787.

Christiansen T, Paulsen SK, Bruun JM, Pedersen SB, Richelsen B (2010a) Exercise-training versus diet-induced weight-loss on metabolic risk factors and inflammatory markers in obese subjects.A 12-week randomized intervention-study. *Am J Physiol Endocrinol Metab* 298: E824-E831.

Christiansen T, Paulsen SK, Bruun JM, Ploug T, Pedersen SB, Richelsen B (2010b) Diet-induced weight loss and exercise alone and in combination enhance the expression of adiponectin receptors in adipose tissue and skeletal muscle, but only diet-induced weight loss enhanced circulating adiponectin. *The J Clin Endocrinol Metab* 95: 911-919.

Church TS, Earnest CP, Thompson AM, Priest EL, Rodarte RQ, Saunders T, Ross R, Blair SN (2010) Exercise without weight loss does not reduce C-reactive protein: the INFLAME study. *Med Sci Sports Exerc* 42: 708-716.

Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin MS (2005) Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res* 46: 2347-2355.

Cohen ML, Douvdevani A, Chaimovitz C, Shany S (2001) Regulation of TNF-alpha by 1alpha,25-dihydroxyvitamin D3 in human macrophages from CAPD patients. *Kidney Int* 59: 69-75.

Compher C, Badellino KO (2008) Obesity and inflammation: lessons from bariatric surgery. *JPEN J Parenter Enteral Nutr* 32: 645-647.

Dandona P, Aljada A, Bandyopadhyay A (2004) Inflammation: the link between insulin resistance, obesity and diabetes. *Trends Immunol* 25: 4-7.

Dandona P, Weinstock R, Thusu K, Abdel-Rahman E, Aljada A, Wadden T, (1998) Tumor necrosis factor-alpha in sera of obese patients: fall with weight loss. *J Clin Endocrinol Metab* 83: 2907-2910.

Dehghan A, Kardys I, de Maat MP, Uitterlinden AG, Sijbrands EJ, Bootsma AH, Stijnen T, Hofman A, Schram MT, Witteman JC (2007) Genetic variation, C-reactive protein levels, and incidence of diabetes. *Diabetes* 56: 872-878.

Demello JJ, Cureton KJ, Boineau RE, Singh MM (1987) Ratings of perceived exertion at the lactate threshold in trained and untrained men and women. *Med Sci Sports Exerc* 19: 354-362.

Dill DB, Costill DL (1974) Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *J Appl Physiol* 37: 247-248.

Dimitrov S, Lange T, Benedict C, Nowell MA, Jones SA, Scheller J, Rose-John S, Born J (2006) Sleep enhances IL-6 trans-signaling in humans. *FASEB J* 20: 2174-2176.

Dixon NC, Hurst TL, Talbot DC, Tyrrell RM, Thompson D (2009) Active middle-aged men have lower fasting inflammatory markers but the postprandial inflammatory response is minimal and unaffected by physical activity status. *J Appl Physiol* 107: 63-68.

Donges CE, Duffield R, Drinkwater EJ (2010) Effects of resistance or aerobic exercise training on interleukin-6, C-reactive protein, and body composition. *Med Sci Sports Exerc* 42: 304-313.

Duncan BB, Schmidt MI, Pankow JS, Ballantyne CM, Couper D, Vigo A, Hoogeveen R, Folsom AR, Heiss G (2003) Low-grade systemic inflammation and the development of type 2 diabetes: the atherosclerosis risk in communities study. *Diabetes* 52: 1799-1805.

Earnest CP (2008) Exercise interval training: an improved stimulus for improving the physiology of pre-diabetes. *Med Hypotheses* 71: 752-761.

Egan B, Carson BP, Garcia-Roves PM, Chibalin AV, Sarsfield FM, Barron N, McCaffrey N, Moyna NM, Zierath JR, O'Gorman DJ (2010) Exercise intensity-dependent regulation of peroxisome proliferator-activated receptor coactivator-1 mRNA abundance is associated with differential activation of upstream signalling kinases in human skeletal muscle. *J Physiol* 588: 1779-1790.

Ehses JA, Lacraz G, Giroix MH, Schmidlin F, Coulaud J, Kassis N, Irminger JC, Kergoat M, Portha B, Homo-Delarche F, Donath MY (2009) IL-1 antagonism reduces hyperglycemia and tissue inflammation in the type 2 diabetic GK rat. *Proc Natl Acad Sci USA* 106: 13998-14003.

Ehtisham S, Barrett TG, Shaw NJ (2000) Type 2 diabetes mellitus in UK children--an emerging problem. *Diabetic Med* 17: 867-871.

Ehtisham S, Hattersley AT, Dunger DB, Barrett TG (2004) First UK survey of paediatric type 2 diabetes and MODY. *Arch Dis Child* 89: 526-529.

Esposito K, Pontillo A, Giugliano F, Giugliano G, Marfella R, Nicoletti G, Giugliano D (2003) Association of low interleukin-10 levels with the metabolic syndrome in obese women. *J Clin Endocrinol Metab* 88: 1055-1058.

Essén B, Hagenfeldt L, Kaijser L (1977) Utilization of blood-borne and intramuscular substrates during continuous and intermittent exercise in man. *J Physiol* 265: 489-506.

Esteve E, Ricart W, Fernández-Real JM (2005) Dyslipidemia and inflammation: an evolutionary conserved mechanism. *Clin Nutr* 24: 16-31.

Fain JN, Madan AK, Hiler ML, Cheema P, Bahouth SW (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: 2273-2282.

Febbraio MA, Steensberg A, Starkie RL, McConell GK, Kingwell BA (2003) Skeletal muscle interleukin-6 and tumor necrosis factor-alpha release in healthy subjects and patients with type 2 diabetes at rest and during exercise. *Metabolism* 52: 939-944.

Feltbower RG, McKinney PA, Campbell FM, Stephenson CR, Bodansky HJ (2003) Type 2 and other forms of diabetes in 0-30 year olds: a hospital based study in Leeds, UK. *Arch Dis Child* 88: 676-679.

Fernandez-Real JM, Vayreda M, Richart C, Gutierrez C, Broch M, Vendrell J, Ricart W (2001) Circulating interleukin 6 levels, blood pressure, and insulin sensitivity in apparently healthy men and women. *J Clin Endocrinol Metab* 86: 1154-1159.

Fischer CP (2006) Interleukin-6 in acute exercise and training: what is the biological relevance? *Exerc Immunol Rev* 12: 6-33.

Fleischmann E, Kurz A, Niedermayr M, Schebesta K, Kimberger O, Prager G, Sessler DI, Kabon B (2005) Tissue oxygenation in obese and non-obese patients during laparoscopy. *Obes Surg* 15: 813-819

Fried SK, Bunkin DA, Greenberg AS (1998) Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *J Clin Endocrinol Metab* 83: 847-850.

Friedewald WT, Levy RI, Fredrickson DS (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 18: 499-502.

Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE, Lodish HF (2001) Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc Natl Acad Sci USA* 98: 2005-2010.

Gaillard JP, Mani JC, Liautard J, Klein B, Brochier J (1999) Interleukin-6 receptor signaling. I. gp80 and gp130 receptor interaction in the absence of interleukin-6. *Eur Cytokine Netw* 10: 43-48.

García-Escobar E, Rodríguez-Pacheco F, García-Serrano S, Gómez-Zumaquero JM, Haro-Mora JJ, Soriguer F, Rojo-Martínez G (2010) Nutritional regulation of interleukin-6 release from adipocytes. *Int J Obes* 34: 1328-1332.

Geiger PC, Hancock C, Wright DC, Han DH, Holloszy JO (2007) IL-6 increases muscle insulin sensitivity only at superphysiological levels. *Am J Physiol Endocrinol Metab* 292: E1842-E1846.

Gibala MJ, Little JP, van Essen M, Wilkin GP, Burgomaster KA, Safdar A, Raha S, Tarnopolsky MA (2006) Short-term sprint interval versus traditional endurance training: similar initial adaptations in human skeletal muscle and exercise performance. *J Physiol* 575: 901-911.

Gletsu N, Lin E, Zhu JL, Khaitan L, Ramshaw BJ, Farmer PK, Ziegler TR, Papanicolaou DA, Smith CD (2006) Increased plasma interleukin 6 concentrations and exaggerated adipose tissue interleukin 6 content in severely obese patients after operative trauma. *Surgery* 140: 50-57.

Glund S, Deshmukh A, Long YC, Moller T, Koistinen HA, Caidahl K, Zierath JR, Krook A (2007) Interleukin-6 directly increases glucose metabolism in resting human skeletal muscle. *Diabetes* 56: 1630-1637.

Gollnick PD, Piehl K, Saltin B (1974) Selective glycogen depletion pattern in human muscle fibres after exercise of varying intensity and at varying pedalling rates. *J Physiol* 241: 45-57.

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

Gray SR, Baker G, Wright A, Fitzsimons CF, Mutrie N, Nimmo MA (2009a) The effect of a 12 week walking intervention on markers of insulin resistance and systemic inflammation. *Prev Med* 48: 39-44.

Gray SR, Clifford M, Lancaster R, Leggate M, Davies M, Nimmo MA (2009b) The response of circulating levels of the interleukin-6/interleukin-6 receptor complex to exercise in young men. *Cytokine* 47: 98-102.

Gray SR, Ratkevicius A, Wackerhage H, Coats P, Nimmo MA (2009c) The effect of interleukin-6 and the interleukin-6 receptor on glucose transport in mouse skeletal muscle. *Exp Physiol* 94: 899-905.

Guiraud T, Nigam A, Juneau M, Meyer P, Gayda M, Bosquet L (2011) Acute responses to high-intensity intermittent exercise in CHD patients. *Med Sci Sports Exerc* 43: 211-217.

Guo Y, Xiao P, Lei S, Deng F, Xiao GG, Liu Y, Chen X, Li L, Wu S, Chen Y, Jiang H, Tan L, Xie J, Zhu X, Liang S, Deng H (2008) How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes. *Acta Biochim Biophys Sin* 40: 426-436.

Hagberg JM, Seals DR, Yerg JE, Gavin J, Gingerich R, Premachandra B, Holloszy JO (1988) Metabolic responses to exercise in young and older athletes and sedentary men. *J Appl Physiol* 65: 900-908.

Haines L, Wan KC, Lynn R, Barrett TG, Shield JP (2007) Rising incidence of type 2 diabetes in children in the U.K. *Diabetes care* 30: 1097-1101.

Hamid YH, Urhammer SA, Jensen DP, Glumer C, Borch-Johnsen K, Jorgensen T, Hansen T, Pedersen O (2004) Variation in the interleukin-6 receptor gene associates with type 2 diabetes in Danish whites. *Diabetes* 53: 3342-3345.

Hansen D, Dendale P, Beelen M, Jonkers RA, Mullens A, Corluy L, Meeusen R, van Loon LJ (2010) Plasma adipokine and inflammatory marker concentrations are altered in obese, as opposed to non-obese, type 2 diabetes patients. *Eur J Appl Physiol* 109: 397-404.

Haugen F, Drevon CA (2007) Activation of nuclear factor-kappaB by high molecular weight and globular adiponectin. *Endocrinology* 148: 5478-5486.

Hawley JA, Lessard SJ (2008) Exercise training-induced improvements in insulin action. *Acta Physiol (Oxf)* 192: 127-135.

Hawley JA, Gibala MJ (2009) Exercise intensity and insulin sensitivity: how low can you go? *Diabetologia* 52: 1709-1713

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Müller-Newen G, Schaper F (2003) Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J* 374: 1-20.

Helge JW, Stallknecht B, Pedersen BK, Galbo H, Kiens B, Richter EA (2003) The effect of graded exercise on IL-6 release and glucose uptake in human skeletal muscle. *J Physiol* 546: 299-305.

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: 1149-1157.

Hirano T, Taga T, Yasukawa K, Nakajima K, Nakano N, Takatsuki F, Shimizu M, Murashima A, Tsunasawa S, Sakiyama F (1987) Human B-cell differentiation factor defined by an anti-peptide antibody and its possible role in autoantibody production. *Proc Natl Acad Sci USA* 84: 228-231.

Hirata Y, Taga T, Hibi M, Nakano N, Hirano T, Kishimoto T (1989) Characterization of IL-6 receptor expression by monoclonal and polyclonal antibodies. *J Immunol* 143: 2900-2906.

Hiscock N, Chan MH, Bisucci T, Darby IA, Febbraio MA (2004) Skeletal myocytes are a source of interleukin-6 mRNA expression and protein release during contraction: evidence of fiber type specificity. *FASEB J* 18: 992-994.

Horiuchi S, Ampofo W, Koyanagi Y, Yamashita A, Waki M, Matsumoto A, Yamamoto M, Yamamoto N (1998) High-level production of alternatively spliced soluble interleukin-6 receptor in serum of patients with adult T-cell leukaemia/HTLV-I-associated myelopathy. *Immunology* 95: 360-369.

Horiuchi S, Koyanagi Y, Zhou Y, Miyamoto H, Tanaka Y, Waki M, Matsumoto A, Yamamoto M, Yamamoto 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. *Eur J Immunol* 24: 1945-1948.

Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM (1995) Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* 95: 2409-2415.

Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM (1996) IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 271: 665-668.

Hotamisligil GS, Shargill NS, Spiegelman BM (1993) Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 259: 87-91.

Hu FB, Meigs JB, Li TY, Rifai N, Manson JE (2004) Inflammatory markers and risk of developing type 2 diabetes in women. *Diabetes* 53: 693-700.

Hulver MW, Zheng D, Tanner CJ, Houmard JA, Kraus WE, Slentz CA, Sinha MK, Pories WJ, MacDonald KG, Dohm GL (2002) Adiponectin is not altered with exercise training despite enhanced insulin action. *Am J Physiol Endocrinol Metab* 283: E861-E865.

Huth C, Illig T, Herder C, Gieger C, Grallert H, Vollmert C, Rathmann W, Hamid YH, Pedersen O, Hansen T, Thorand B, Meisinger C, Doring A, Klopp N, Gohlke H, Lieb W, Hengstenberg C, Lyssenko V, Groop L, Ireland H, Stephens JW, Wernstedt Asterholm I, Jansson JO, Boeing H, Mohlig M, Stringham HM, Boehnke M, Tuomilehto J, Fernandez-Real JM, Lopez-Bermejo A, Gallart L, Vendrell J, Humphries SE, Kronenberg F, Wichmann HE, Heid IM (2009) Joint analysis of individual participants' data from 17 studies on the association of the IL6 variant -174G>C with circulating glucose levels, interleukin-6 levels, and body mass index. *Ann Med* 41: 128-138.

Izquierdo M, Ibañez J, Calbet JA, Navarro-Amezqueta I, González-Izal M, Idoate F, Häkkinen K, Kraemer WJ, Palacios-Sarrasqueta M, Almar M, Gorostiaga EM (2009) Cytokine and hormone responses to resistance training. *Eur J Appl Physiol* 107: 397-409.

Jirik FR, Podor TJ, Hirano T, Kishimoto T, Loskutoff DJ, Carson DA, Lotz M (1989) Bacterial lipopolysaccharide and inflammatory mediators augment IL-6 secretion by human endothelial cells. *J Immunol* 142: 144-147.

Jones SA, Jones GW, Williams AS, Nowell MA (2008) Appreciating the balance between classical Interleukin (IL)-6 receptor signaling and IL-6 trans-signaling: Implications for arthritis progression. *Immunol Endocr Metabol Agents Med Chem* 8: 235-246.

Jones SA, Horiuchi S, Topley N, Yamamoto N, Fuller GM (2001) The soluble interleukin 6 receptor: mechanisms of production and implications in disease. *FASEB J* 15: 43-58.

Jones SA, Novick D, Horiuchi S, Yamamoto N, Szalai AJ, Fuller GM (1999) C-reactive protein: a physiological activator of interleukin 6 receptor shedding. *J Exp Med* 189: 599-604.

Jostock T, Müllberg J, Özbek S, Atreya R, Blinn G, Voltz N, Fischer M, Neurath MF, Rose-John S (2001) Soluble gp130 is the natural inhibitor of soluble interleukin-6 receptor transsignaling responses. *Eur J Biochem* 268: 160-167.

Juge-Aubry CE, Somm E, Pernin A, Alizadeh N, Giusti V, Dayer JM, Meier CA (2005) Adipose tissue is a regulated source of interleukin-10. *Cytokine* 29: 270-274.

Kado S, Nagase T, Nagata N (1999) Circulating levels of interleukin-6, its soluble receptor and interleukin-6/interleukin-6 receptor complexes in patients with type 2 diabetes mellitus. *Acta Diabetol* 36: 67-72.

Kadoglou NP, Iliadis F, Angelopoulou N, Perrea D, Ampatzidis G, Liapis CD, Alevizos M (2007) The anti-inflammatory effects of exercise training in patients with type 2 diabetes mellitus. *Eur J Cardiovasc Prev Rehabil* 14: 837-843.

Kamei N, Tobe K, Suzuki R, Ohsugi M, Watanabe T, Kubota N, Ohtsuka-Kowatari N, Kumagai K, Sakamoto K, Kobayashi M, Yamauchi T, Ueki K, Oishi Y, Nishimura S, Manabe I, Hashimoto H, Ohnishi Y, Ogata H, Tokuyama K, Tsunoda M, Ide T, Murakami K, Nagai R, Kadowaki T (2006) Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. *J Biol Chem* 281: 26602-26614.

Kaminsky LA, Padjen S, LaHam-Saeger J (1990) Effect of split exercise sessions on excess post-exercise oxygen consumption. *Br J Sports Med* 24: 95-98.

Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M (2006) MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest* 116: 1494-1505.

Kargotich S, Goodman C, Keast D, Morton AR (1998)bThe influence of exercise-induced plasma volume changes on the interpretation of biochemical parameters used for monitoring exercise, training and sport. *Sports Med* 26: 101-117.

Keller P, Penkowa M, Keller C, Steensberg A, Fischer CP, Giralt M, Hidalgo J, Pedersen BK (2005a) Interleukin-6 receptor expression in contracting human skeletal muscle: regulating role of IL-6. *FASEB J* 19: 1181-3.

Keller C, Steensberg A, Hansen AK, Fischer CP, Plomgaard P, Pedersen BK (2005b) Effect of exercise, training, and glycogen availability on IL-6 receptor expression in human skeletal muscle. *J Appl Physiol* 99: 2075-2079.

Keller C, Steensberg A, Pilegaard H, Osada T, Saltin B, Pedersen BK, Neufer PD (2001) Transcriptional activation of the IL-6 gene in human contracting skeletal muscle: influence of muscle glycogen content. *FASEB J* 15: 2748-2750.

Kern PA, Di Gregorio GB, Lu T, Rassouli N, Ranganathan G (2003) Adiponectin expression from human adipose tissue: relation to obesity, insulin resistance, and tumor necrosis factor-alpha expression. *Diabetes* 52: 1779-1785.

Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G (2001) Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am J Physiol Endocrinol Metabol* 280: E745-E751.

Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simsolo RB (1995) The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. *J Clin Invest* 95: 2111-2119.

Kim CS, Park HS, Kawada T, Kim JH, Lim D, Hubbard NE, Kwon BS, Erickson KL, Yu R (2006) Circulating levels of MCP-1 and IL-8 are elevated in human obese subjects and associated with obesity-related parameters. *Int J Obes* 30: 1347-1355.

Klimcakova E, Polak J, Moro C, Hejnova J, Majercik M, Viguerie N, Berlan M, Langin D, Stich V (2006) Dynamic strength training improves insulin sensitivity without altering plasma levels and gene expression of adipokines in subcutaneous adipose tissue in obese men. *J Clin Endocrinol Metab* 91: 5107-5112.

Klover PJ, Zimmers TA, Koniaris LG, Mooney RA (2003) Chronic exposure to interleukin-6 causes hepatic insulin resistance in mice. *Diabetes* 52: 2784-2789.

Knab AM, Shanely RA, Corbin K, Jin F, Sha W, Nieman DC (2011) A 45-minute vigorous exercise bout increases metabolic rate for 14 hours. *Med Sci Sports Exerc* 43: 1643-1648.

Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, Nathan DM (2002) Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med* 346: 393-403.

Koopman RJ, Mainous AG, Diaz VA, Geesey ME (2005) Changes in age at diagnosis of type 2 diabetes mellitus in the United States, 1988 to 2000. *Ann Fam Med* 3: 60-63.

Kumada M, Kihara S, Ouchi N, Kobayashi H, Okamoto Y, Ohashi K, Maeda K, Nagaretani H, Kishida K, Maeda N, Nagasawa A, Funahashi T, Matsuzawa Y (2004) Adiponectin specifically increased tissue inhibitor of metalloproteinase-1 through interleukin-10 expression in human macrophages. *Circulation* 109: 2046-2049.

Laaksonen DE, Lindström J, Lakka TA, Eriksson JG, Niskanen L, Wikström K, Aunola S, Keinänen-Kiukaanniemi S, Laakso M, Valle TT, Ilanne-Parikka P, Louheranta A, Hämäläinen H, Rastas M, Salminen V, Cepaitis Z, Hakumäki M, Kaikkonen H, Härkönen P, Sundvall J, Tuomilehto J, Uusitupa M (2005) Physical activity in the prevention of type 2 diabetes: the Finnish diabetes prevention study. *Diabetes* 54: 158-165.

LaForgia J, Withers RT, Gore CJ (2006) Effects of exercise intensity and duration on the excess post-exercise oxygen consumption. *J Sports Sci* 24: 1247-1264.

Leinonen E, Hurt-Camejo E, Wiklund O, Hultén LM, Hiukka A, Taskinen MR (2003) Insulin resistance and adiposity correlate with acute-phase reaction and soluble cell adhesion molecules in type 2 diabetes. *Atherosclerosis* 166: 387-394.

Levy JC, Matthews DR, Hermans MP (1998) Correct homeostasis model assessment (HOMA) evaluation uses the computer program. *Diabetes care* 21: 2191-2192.

Libby P, Ridker PM, Maseri A (2002) Inflammation and atherosclerosis. *Circulation* 105: 1135-1143.

Lihn AS, Bruun JM, He G, Pedersen SB, Jensen PF, Richelsen B (2004) Lower expression of adiponectin mRNA in visceral adipose tissue in lean and obese subjects. *Mol Cell Endocrinol* 219: 9-15.

Lin Y, Berg AH, Iyengar P, Lam TK, Giacca A, Combs TP, Rajala MW, Du X, Rollman B, Li W, Hawkins M, Barzilai N, Rhodes CJ, Fantus IG, Brownlee M, Scherer PE (2005) The hyperglycemia-induced inflammatory response in adipocytes: the role of reactive oxygen species. *J Biol Chem* 280: 4617-4626.

Little JP, Gillen JB, Percival M, Safdar A, Tarnopolsky MA, Punthakee Z, Jung ME, Gibala MJ (2011) Low-volume high-intensity interval training reduces hyperglycemia and increases muscle mitochondrial capacity in patients with type 2 diabetes. *J Appl Physiol* 111: 1554-1560.

Lütticken C, Wegenka UM, Yuan J, Buschmann J, Schindler C, Ziemiecki A, Harpur AG, Wilks AF, Yasukawa K, Taga T (1994) Association of transcription factor APRF and protein kinase Jak1 with the interleukin-6 signal transducer gp130. *Science* 263: 89-92.

Maachi M, Piéroni L, Bruckert E, Jardel C, Fellahi S, Hainque B, Capeau J, Bastard JP (2004) Systemic low-grade inflammation is related to both circulating and adipose tissue TNFalpha, leptin and IL-6 levels in obese women. *Int J Obes Relat Metab Disord* 28: 993-997.

Madsen EL, Rissanen A, Bruun JM, Skogstrand K, Tonstad S, Hougaard DM, Richelsen B (2008) Weight loss larger than 10% is needed for general improvement of levels of circulating adiponectin and markers of inflammation in obese subjects: a 3-year weight loss study. *Eur J Endocrinol* 158: 179-187.

Matsuda M, DeFronzo RA (1999) Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care* 22: 1462-1470.

Matthews V, Schuster B, Schutze S, Bussmeyer I, Ludwig A, Hundhausen C, Sadowski T, Saftig P, Hartmann D, Kallen KJ, Rose-John S (2003) Cellular cholesterol depletion triggers shedding of the human interleukin-6 receptor by ADAM10 and ADAM17 (TACE). *J Biol Chem* 278: 38829-38839.

McKay BR, De Lisio M, Johnston AP, O'Reilly CE, Phillips SM, Tarnopolsky MA, Parise G (2009) Association of interleukin-6 signalling with the muscle stem cell response following muscle-lengthening contractions in humans. *PLoS One* 4: E6027.

McLoughlin RM, Hurst SM, Nowell MA, Harris DA, Horiuchi S, Morgan LW, Wilkinson TS, Yamamoto N, Topley N, Jones SA (2004) Differential regulation of neutrophil-activating chemokines by IL-6 and its soluble receptor isoforms. *J Immunol* 172: 5676-5683.

Meckel Y, Eliakim A, Seraev M, Zaldivar F, Cooper DM, Sagiv M, Nemet D (2009) The effect of a brief sprint interval exercise on growth factors and inflammatory mediators. *J Strength Cond Res* 23: 225-230.

Memoli B, Procino A, Calabrò P, Esposito P, Grandaliano G, Pertosa G, Prete MD, Andreucci M, Lillo SD, Ferulano G, Cillo C, Savastano S, Colao A, Guida B (2007) Inflammation may modulate IL-6 and C-reactive protein gene expression in the adipose tissue: the role of IL-6 cell membrane receptor. *Am J Physiol Endocrinol Metab* 293: E1030-1035.

Millett C, Khunti K, Gray J, Saxena S, Netuveli G, Majeed A (2008) Obesity and intermediate clinical outcomes in diabetes: evidence of a differential relationship across ethnic groups. *Diabetic Med* 25: 685-691.

Mohamed-Ali V, Goodrick S, Bulmer K, Holly JM, Yudkin JS, Coppack SW (1999) Production of soluble tumor necrosis factor receptors by human subcutaneous adipose tissue in vivo. *Am J Physiol* 277: E971-E975.

Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S, Coppack SW (1997) Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J Clin Endocrinol Metab* 82: 4196-4200.

Monroy A, Kamath S, Chavez AO, Centonze VE, Veerasamy M, Barrentine A, Wewer JJ, Coletta DK, Jenkinson C, Jhingan RM, Smokler D, Reyna S, Musi N, Khokka R, Federici M, Tripathy D, DeFronzo RA, Folli F (2009) Impaired regulation of the TNF-alpha converting enzyme/tissue inhibitor of metalloproteinase 3 proteolytic system in skeletal muscle of obese type 2 diabetic patients: a new mechanism of insulin resistance in humans. *Diabetologia* 52: 2169-2181.

Mooney RA (2007) Counterpoint: Interleukin-6 does not have a beneficial role in insulin sensitivity and glucose homeostasis. *J Appl Physiol* 102: 816-819.

Müllberg J, Oberthür W, Lottspeich F, Mehl E, Dittrich E, Graeve L, Heinrich PC, Rose-John S (1994) The soluble human IL-6 receptor. Mutational characterization of the proteolytic cleavage site. *J Immunol* 152: 4958-4968.

Müllberg J, Schooltink H, Stoyan T, Günther M, Graeve L, Buse G, Mackiewicz A, Heinrich PC, Rose-John S (1993) The soluble interleukin-6 receptor is generated by shedding. *Eur J Immunol* 23: 473-480.

Müller S, Martin S, Koenig W, Hanifi-Moghaddam P, Rathmann W, Haastert B, Giani G, Illig T, Thorand B, Kolb H (2002) Impaired glucose tolerance is associated with increased serum concentrations of interleukin 6 and co-regulated acute-phase proteins but not TNF-alpha or its receptors. *Diabetologia* 45: 805-812.

Müller-Newen G, Kuster A, Hemmann U, Keul R, Horsten U, Martens A, Graeve L, Wijdenes J, Heinrich PC (1998) Soluble IL-6 receptor potentiates the antagonistic activity of soluble gp130 on IL-6 responses. *J Immunol* 161: 6347-6355.

Muscogiuri G, Sorice GP, Prioletta A, Policola C, Della Casa S, Pontecorvi A, Giaccari A (2010) 25-Hydroxyvitamin D concentration correlates with insulin-sensitivity and BMI in obesity. *Obesity* 18: 1906-1910.

Myers J, Buchanan N, Walsh D, Kraemer M, McAuley P, Hamilton-Wessler M, Froelicher VF (1991) Comparison of the ramp versus standard exercise protocols. *J Am Coll Cardiol* 17: 1334-1342.

Nieman DC, Dumke CL, Henson DA, McAnulty SR, Gross SJ, Lind RH (2005) Muscle damage is linked to cytokine changes following a 160-km race. *Brain Behav Immun* 19: 398-403.

NHS Information Centre, Lifestyle Statistics (2011) Statistics on obesity, physical activity and diet: England.

Nieman DC, Nehlsen-Cannarella SL, Fagoaga OR, Henson DA, Utter A, Davis JM, Williams F, Butterworth DE (1998) Influence of mode and carbohydrate on the cytokine response to heavy exertion. *Med Sci Sports Exerc* 30: 671-678.

Niess AM, Fehrenbach E, Strobel G, Roecker K, Schneider EM, Buergler J, Fuss S, Lehmann R, Northoff H, Dickhuth HH (2003) Evaluation of stress responses to interval training at low and moderate altitudes. *Med Sci Sports Exerc* 35: 263-269.

Nowell MA, Richards PJ, Horiuchi S, Yamamoto N, Rose-John S, Topley N, Williams AS, Jones SA (2003) Soluble IL-6 receptor governs IL-6 activity in experimental arthritis: blockade of arthritis severity by soluble glycoprotein 130. *J Immunol* 171: 3202-3209.

Oberbach A, Tönjes A, Klöting N, Fasshauer M, Kratzsch J, Busse MW, Paschke R, Stumvoll M, Blüher M (2006) Effect of a 4 week physical training program on plasma concentrations of inflammatory markers in patients with abnormal glucose tolerance. *Eur J Endocrinol* 154: 577-585.

Oberbach A, Lehmann S, Kirsch K, Krist J, Sonnabend M, Linke A, Tönjes A, Stumvoll M, Blüher M, Kovacs P (2008) Long-term exercise training decreases

interleukin-6 (IL-6) serum levels in subjects with impaired glucose tolerance: effect of the -174G/C variant in IL-6 gene. *Eur J Endocrinol* 159: 129-136.

Oberg HH, Wesch D, Grussel S, Rose-John S, Kabelitz D (2006) Differential expression of CD126 and CD130 mediates different STAT-3 phosphorylation in CD4+CD25- and CD25high regulatory T cells. *Int Immunol* 18: 555-563.

O'Donovan G, Owen A, Bird SR, Kearney EM, Nevill AM, Jones DW, Woolf-May K (2005) Changes in cardiorespiratory fitness and coronary heart disease risk factors following 24 wk of moderate- or high-intensity exercise of equal energy cost. *J Appl Physiol* 98: 1619-1625.

Oguma Y, Shinoda-Tagawa T (2004) Physical activity decreases cardiovascular disease risk in women: review and meta-analysis. *Am J Prev Med* 26: 407-418.

Olson TP, Dengel DR, Leon AS, Schmitz KH (2007) Changes in inflammatory biomarkers following one-year of moderate resistance training in overweight women. *Int J Obes* 31: 996-1003.

Ostrowski K, Rohde T, Asp S, Schjerling P, Pedersen BK (1999) Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. *J Physiol* 515: 287-291.

Ostrowski K, Schjerling P, Pedersen BK (2000) Physical activity and plasma interleukin-6 in humans--effect of intensity of exercise. *Eur J Appl Physiol* 83: 512-515.

Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y (1999) Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* 100: 2473-2476.

Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, Hotta K, Nishida M, Takahashi M, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y (2000) Adiponectin, an adipocyte-derived plasma

protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. *Circulation* 102: 1296-1301.

Paonessa G, Graziani R, De Serio A, Savino R, Ciapponi L, Lahm A, Salvati AL, Toniatti C, Ciliberto G (1995) Two distinct and independent sites on IL-6 trigger gp 130 dimer formation and signalling. *EMBO J* 14: 1942-1951.

Patterson S, Reid S, Gray S, Nimmo M (2008) The response of plasma interleukin-6 and its soluble receptors to exercise in the cold in humans. *J Sports Sci* 26: 927-933

Pawluczyk IZ, Yang B, Patel SR, Saleem MA, Topham PS (2011) Low-level C-reactive protein levels exert cytoprotective actions on human podocytes. *Nephrol Dial Transplant* 26: 2465-2475.

Pedersen BK, Steensberg A, Schjerling P (2001) Muscle-derived interleukin-6: possible biological effects. *J Physiol* 536: 329-337.

Pedersen BK, Febbraio MA (2007) Point: Interleukin-6 does have a beneficial role in insulin sensitivity and glucose homeostasis. *J Appl Physiol* 102: 814-816.

Pedersen BK, Febbraio MA (2008) Muscle as an endocrine organ: focus on muscle-derived interleukin-6. *Physiol Rev* 88: 1379-1406.

Pedersen BK (2009) Edward F. Adolph Distinguished Lecture: Muscle as an Endocrine Organ: IL-6 and other myokines. *J Appl Physiol* 107: 1006-1014

Penkowa M, Keller C, Keller P, Jauffred S, Pedersen BK (2003) Immunohistochemical detection of interleukin-6 in human skeletal muscle fibers following exercise. *FASEB J* 17: 2166-2168.

Peters M, Jacobs S, Ehlers M, Vollmer P, Müllberg J, Wolf E, Brem G, Meyer zum Buschenfelde KH, Rose-John S (1996) The function of the soluble interleukin 6 (IL-6) receptor in vivo: sensitization of human soluble IL-6 receptor transgenic mice towards IL-6 and prolongation of the plasma half-life of IL-6. *J Exp Med* 183: 1399-1406.

Petersen AM, Pedersen BK (2005) The anti-inflammatory effect of exercise. *J Appl Physiol* 98: 1154-1162.

Peterson CA, Heffernan ME (2008) Serum tumor necrosis factor-alpha concentrations are negatively correlated with serum 25(OH)D concentrations in healthy women. *J Inflamm* 5: 10.

Pickup JC (2004) Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. *Diabetes Care* 27: 813-823.

Polak J, Klimcakova E, Moro C, Viguerie N, Berlan M, Hejnova J, Richterova B, Kraus I, Langin D, Stich V (2006) Effect of aerobic training on plasma levels and subcutaneous abdominal adipose tissue gene expression of adiponectin, leptin, interleukin 6, and tumor necrosis factor alpha in obese women. *Metabolism* 55: 1375-1381.

Pouliot MC, Despres JP, Lemieux S, Moorjani S, Bouchard C, Tremblay A, Nadeau A, Lupien PJ (1994) Waist circumference and abdominal sagittal diameter: best simple anthropometric indexes of abdominal visceral adipose tissue accumulation and related cardiovascular risk in men and women. *Am J Cardiol* 73: 460-468.

Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM (2001) C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *JAMA J Am Med Assoc* 286: 327-334.

Preis SR, Massaro JM, Robins SJ, Hoffmann U, Vasan RS, Irlbeck T, Meigs JB, Sutherland P, D'Agostino Sr RB, O'Donnell CJ, Fox CS (2010) Abdominal subcutaneous and visceral adipose tissue and insulin resistance in the Framingham Heart Study. *Obesity* 18: 2191-2198.

Rakobowchuk M, Tanguay S, Burgomaster KA, Howarth KR, Gibala MJ, MacDonald MJ (2008) Sprint interval and traditional endurance training induce similar improvements in peripheral arterial stiffness and flow-mediated dilation in healthy humans. *Am J Physiol Regul Integr Comp Physiol* 295: R236-R242.

Richards JC, Johnson TK, Kuzma JN, Lonac MC, Schweder MM, Voyles WF, Bell C (2010) Short-term sprint interval training increases insulin sensitivity in healthy adults but does not affect the thermogenic response to beta-adrenergic stimulation. *J Physiol* 588: 2961-2972.

Ridker PM, Hennekens CH, Roitman-Johnson B, Stampfer MJ, Allen J, (1998) Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. *Lancet* 351: 88-92.

Rieusset J, Bouzakri K, Chevillotte E, Ricard N, Jacquet D, Bastard JP, Laville M, Vidal H (2004) Suppressor of cytokine signaling 3 expression and insulin resistance in skeletal muscle of obese and type 2 diabetic patients. *Diabetes,* 53: 2232-2241.

Roberts CK, Won D, Pruthi S, Kurtovic S, Sindhu RK, Vaziri ND, Barnard RJ (2006a) Effect of a short-term diet and exercise intervention on oxidative stress, inflammation, MMP-9, and monocyte chemotactic activity in men with metabolic syndrome factors. *J Appl Physiol* 100: 1657-1665.

Roberts CK, Won D, Pruthi S, Lin SS, Barnard RJ (2006b) Effect of a diet and exercise intervention on oxidative stress, inflammation and monocyte adhesion in diabetic men. *Diabetes Res Clin Pract* 73: 249-259.

Robinson DM, Robinson SM, Hume PA, Hopkins WG (1991) Training intensity of elite male distance runners. *Med Sci Sports Exerc* 23: 1078-1082.

Robinson M, Gray SR, Watson MS, Kennedy G, Hill A, Belch JJ, Nimmo MA (2009) Plasma IL-6, its soluble receptors and F-isoprostanes at rest and during exercise in chronic fatigue syndrome. *Scand J Med Sci Sports* 20: 282-290.

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: 111-116.

Robson-Ansley P, Cockburn E, Walshe I, Stevenson E, Nimmo M (2010) The effect of exercise on plasma soluble IL-6 receptor concentration: a dichotomous response. *Exerc Immunol Rev* 16: 56-76.

Rose-John S, Heinrich PC (1994) Soluble receptors for cytokines and growth factors: generation and biological function. *Biochem J* 300: 281-290.

Ross R, Shaw KD, Rissanen J, Martel Y, de Guise J, Avruch L (1994) Sex differences in lean and adipose tissue distribution by magnetic resonance imaging: anthropometric relationships. *Am J Clin Nutr* 59: 1277-1285.

Ryan Ad, Berman DM, Nicklas BJ, Sinha M, Gingerich RL, Meneilly GS, Egan JM, Elahi D (2003) Plasma adiponectin and leptin levels, body composition, and glucose utilization in adult women with wide ranges of age and obesity. *Diabetes Care* 26: 2383-2388.

Ryan AS, Nicklas BJ (2004) Reductions in plasma cytokine levels with weight loss improve insulin sensitivity in overweight and obese postmenopausal women. *Diabetes Care* 27: 1699-1705.

Sacheck JM, Cannon JG, Hamada K, Vannier E, Blumberg JB, Roubenoff R, (2006) Age-related loss of associations between acute exercise-induced IL-6 and oxidative stress. *Am J Physiol Endocrinol Metab* 291: E340-E349.

Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S (2011) The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta* 1813: 878-888.

Schindler R, Mancilla J, Endres S, Ghorbani R, Clark SC, Dinarello CA (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: 40-47.

Schmidt MI, Duncan BB, Sharrett AR, Lindberg G, Savage PJ, Offenbacher S, Azambuja MI, Tracy RP, Heiss G (1999) Markers of inflammation and

prediction of diabetes mellitus in adults (Atherosclerosis Risk in Communities study): a cohort study. *Lancet* 353: 1649-1652.

Schroers A, Hecht O, Kallen KJ, Pachta M, Rose-John S, Grötzinger J (2005) Dynamics of the gp130 cytokine complex: a model for assembly on the cellular membrane. *Protein Sci* 14: 783-790.

Shoelson SE, Lee J, Goldfine AB (2006) Inflammation and insulin resistance. *J Clin Invest* 116: 1793-1801.

Silverman NE, Nicklas BJ, Ryan AS (2009) Addition of aerobic exercise to a weight loss program increases BMD, with an associated reduction in inflammation in overweight postmenopausal women. *Calcif Tissue Int* 84: 257-265.

Simpson KA, Singh MA (2008) Effects of exercise on adiponectin: a systematic review. *Obesity* 16: 241-256.

Sjöholm A, Nyström T (2006) Inflammation and the etiology of type 2 diabetes. *Diabetes Metab Res Rev* 22: 4-10.

Skurk T, Alberti-Huber C, Herder C, Hauner H (2007) Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab* 92: 1023-1033.

Sowers JR (2003) Obesity as a cardiovascular risk factor. *Am J Med* 115: 37S-41S.

Stahl N, Boulton TG, Farruggella T, Ip NY, Davis S, Witthuhn BA, Quelle FW, Silvennoinen O, Barbieri G, Pellegrini S (1994) Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. *Science* 263: 92-95.

Starkie R, Ostrowski SR, Jauffred S, Febbraio M, Pedersen BK (2003) Exercise and IL-6 infusion inhibit endotoxin-induced TNF-alpha production in humans. *FASEB J* 17: 884-886.

Steensberg A, Febbraio MA, Osada T, Schjerling P, van Hall G, Saltin B, Pedersen BK (2001) Interleukin-6 production in contracting human skeletal muscle is influenced by pre-exercise muscle glycogen content. *J Physiol* 537: 633-639.

Steensberg A, Fischer CP, Keller C, Moller K, Pedersen BK (2003) IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. *Am J Physiol Endocrinol Metab* 285: E433-E437.

Strączkowsk, M, Kowalska I, Dzienis-Strączkowska S, Stępień A, Skibińska E, Szelachowska M, Kinalska I (2001) Changes in tumor necrosis factor-alpha system and insulin sensitivity during an exercise training program in obese women with normal and impaired glucose tolerance. *Eur J Endocrinol* 145: 273-280.

Strączkowsk M, Lewczuk P, Dzienis-Strączkowsk S, Kowalska I, Stępień A, Kinalska I (2002) Elevated soluble intercellular adhesion molecule-1 levels in obesity: relationship to insulin resistance and tumor necrosis factor-alpha system activity. *Metabolism* 51: 75-78.

Su SC, Pei D, Hsieh CH, Hsiao FC, Wu CZ, Hung YJ (2010) Circulating pro-inflammatory cytokines and adiponectin in young men with type 2 diabetes. *Acta Diabetol* 48: 113-119.

Sundquist K, Qvist J, Johansson SE, Sundquist J (2005) The long-term effect of physical activity on incidence of coronary heart disease: a 12-year follow-up study. *Prev Med* 41: 219-225.

Swain D, Franklin BA (2006) Comparison of cardioprotective benefits of vigorous versus moderate intensity aerobic exercise. *Am J Cardiol* 97: 141-147.

Taga T, Hibi M, Hirata Y, Yamasaki K, Yasukawa K, Matsuda T, Hirano T, Kishimoto T (1989) Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell* 58: 573-581.

Talanian JL, Galloway SD, Heigenhauser GJ, Bonen A, Spriet LL (2007) Two weeks of high-intensity aerobic interval training increases the capacity for fat oxidation during exercise in women. *J Appl Physiol* 102: 1439-47.

Tartibian B, Azadpoor N, Abbasi A (2009) Effects of two different type of treadmill running on human blood leukocyte populations and inflammatory indices in young untrained men. *J Sports Med Phys Fitness* 49: 214-223.

Thierry-Mieg D, Thierry-Mieg J (2006) AceView: a comprehensive cDNA-supported gene and transcripts annotation. *Genome Biol* 7: S12.1-14.

Thompson D, Markovitch D, Betts JA, Mazzatti DJ, Turner J, Tyrrell RM (2010) Time-course of changes in inflammatory markers during a 6-month exercise intervention in sedentary middle-aged men: A randomized-controlled trial. *J Appl Physiol* 108: 769-779.

Tilg H, Trehu E, Atkins MB, Dinarello CA, Mier JW (1994) Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. *Blood* 83: 113-118.

Tjønna AE, Lee SJ, Rognmo O, Stølen TO, Bye A, Haram PM, Loennechen JP, Al-Share QY, Skogvoll E, Slørdahl SA, Kemi OJ, Najjar SM, Wisløff U (2008) Aerobic interval training versus continuous moderate exercise as a treatment for the metabolic syndrome: a pilot study. *Circulation* 118: 346-354.

Tönjes A, Scholz M, Fasshauer M, Kratzsch J, Rassoul F, Stumvoll M, Blüher M (2007) Beneficial effects of a 4-week exercise program on plasma concentrations of adhesion molecules. *Diabetes Care* 30: e1.

Trapp EG, Chisholm DJ, Freund J, Boutcher SH (2008) The effects of high-intensity intermittent exercise training on fat loss and fasting insulin levels of young women. *Int J Obes* 32: 684-691.

Trayhurn P, Wood IS (2004) Adipokines: inflammation and the pleiotropic role of white adipose tissue. *Br J Nutr* 92: 347-355.

Trayhurn P (2005) White adipose tissue grafts--keeping in contact. *Am J Physiol Regul Integr Comp Physiol* 289: R297-R298.

Tremblay A, Despres JP, Leblanc C, Craig CL, Ferris B, Stephens T, Bouchard C (1990) Effect of intensity of physical activity on body fatness and fat distribution. *Am J Clin Nutr* 51: 153-157.

Tremblay A, Simoneau JA, Bouchard C (1994) Impact of exercise intensity on body fatness and skeletal muscle metabolism. *Metabolism* 43: 814-818.

Troseid M, Lappegard KT, Claudi T, Damas JK, Morkrid L, Brendberg R, Mollnes TE (2004) Exercise reduces plasma levels of the chemokines MCP-1 and IL-8 in subjects with the metabolic syndrome. *Eur Heart J* 25: 349-355.

Trujillo ME, Scherer PE (2006) Adipose tissue-derived factors: impact on health and disease. *Endocr Rev* 27: 762-778.

Vasan RS, Sullivan LM, Roubenoff R, Dinarello CA, Harris T, Benjamin EJ, Sawyer DB, Levy D, Wilson PW, D'Agostino RB (2003) Inflammatory markers and risk of heart failure in elderly subjects without prior myocardial infarction: the Framingham Heart Study. *Circulation* 107: 1486-1491.

Vermes C, Jacobs JJ, Zhang J, Firneisz G, Roebuck KA, Glant TT (2002) Shedding of the interleukin-6 (IL-6) receptor (gp80) determines the ability of IL-6 to induce gp130 phosphorylation in human osteoblasts. *J Biol Chem* 277: 16879-16887.

Vilarrasa N, Vendrell J, Maravall J, Broch M, Estepa A, Megia A, Soler J, Simón I, Richart C, Gómez JM (2005) Distribution and determinants of adiponectin, resistin and ghrelin in a randomly selected healthy population. *Clin Endocrinol* 63: 329-335.

Walshe I, Robson-Ansley P, St Clair Gibson A, Lawrence C, Thompson KG, Ansley L (2010) The reliability of the IL-6, sIL-6R and sgp130 response to a preloaded time trial. *Eur J Appl Physiol* 110: 619-625.

Warburton DE, Nicol CW, Bredin SS (2006) Health benefits of physical activity: the evidence. *Can Med Assoc J* 174: 801-809.

Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr (2003) Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112: 1796-1808.

Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, Tataranni PA (2001) Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab* 86: 1930-1935.

Whyte LJ, Gill JM, Cathcart AJ (2010) Effect of 2 weeks of sprint interval training on health-related outcomes in sedentary overweight/obese men. *Metabolism* 59: 1421-1428.

Widegren U, Wretman C, Lionikas A, Hedin G, Henriksson J (2000) Influence of exercise intensity on ERK/MAP kinase signalling in human skeletal muscle. *Eur J Physiol* 441: 317-322.

Wild S, Roglic G, Green A, Sicree R, King H (2004) Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care,* 27: 1047-1053.

Willems F, Marchant A, Delville JP, Gerard C, Delvaux A, Velu T, de Boer M, Goldman M (1994) Interleukin-10 inhibits B7 and intercellular adhesion molecule-1 expression on human monocytes. *Eur J Immunol* 24: 1007-1009.

Wisløff U, Støylen A, Loennechen JP, Bruvold M, Rognmo Ö, Haram PM, Tjønna AE, Helgerud J, Slørdahl SA, Lee SJ, Videm V, Bye A, Smith GL, Najjar SM, Ellingsen Ö, Skjærpe T (2007) Superior cardiovascular effect of aerobic interval training versus moderate continuous training in heart failure patients: a randomized study. *Circulation* 115: 3086-3094.

Wolf AM, Wolf D, Rumpold H, Enrich B, Tilg H (2004) Adiponectin induces the anti-inflammatory cytokines IL-10 and IL-1RA in human leukocytes. *Biochem Biophys Res Commun* 323: 630-635.

Wood IS, de Heredia FP, Wang B, Trayhurn P (2009) Cellular hypoxia and adipose tissue dysfunction in obesity. *Proc Nutr Soc* 68: 370-377.

World Health Organisation (2010) Global status report on non-communicable diseases.

Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 112: 1821-1830.

Yamasaki K, Taga T, Hirata Y, Yawata H, Kawanishi Y, Seed B, Taniguchi T, Hirano T, Kishimoto T (1988) Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor. *Science* 241: 825-828.

Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T (2001) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 7: 941-946.

Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, Kadowaki T (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 8: 1288-1295.

Yamauchi T, Nio Y, Maki T, Kobayashi M, Takazawa T, Iwabu M, Okada-Iwabu M, Kawamoto S, Kubota N, Kubota T, Ito Y, Kamon J, Tsuchida A, Kumagai K, Kozono H, Hada Y, Ogata H, Tokuyama K, Tsunoda M, Ide T, Murakami K, Awazawa M, Takamoto I, Froguel P, Hara K, Tobe K, Nagai R, Ueki K,

Kadowaki T (2007) Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. *Nat Med* 13: 332-339.

Yamauchi T, Kadowaki T (2008) Physiological and pathophysiological roles of adiponectin and adiponectin receptors in the integrated regulation of metabolic and cardiovascular diseases. *Int J Obes* 32: S13-S18.

Yasukawa K, Hirano T, Watanabe Y, Muratani K, Matsuda T, Nakai S, Kishimoto T (1987) Structure and expression of human B cell stimulatory factor-2 (BSF-2/IL-6) gene. *EMBO J* 6: 2939-2945.

Ye J, Gao Z, Yin J, He Q (2007) Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *Am J Physiol Endocrinol Metab* 293: E1118-E1128.

You T, Berman DM, Ryan AS, Nicklas BJ (2004) Effects of hypocaloric diet and exercise training on inflammation and adipocyte lipolysis in obese postmenopausal women. *J Clin Endocrinol Metab* 89: 1739-1746.

Yu M, Stepto NK, Chibalin AV, Fryer LG, Carling D, Krook A, Hawley JA, Zierath JR (2003) Metabolic and mitogenic signal transduction in human skeletal muscle after intense cycling exercise. *J Physiol* 546: 327-335.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM (1994) Positional cloning of the mouse obese gene and its human homologue. *Nature* 372: 425-432.

Zoppini G, Targher G, Zamboni C, Venturi C, Cacciatori V, Moghetti P, Muggeo M (2006) Effects of moderate-intensity exercise training on plasma biomarkers of inflammation and endothelial dysfunction in older patients with type 2 diabetes. *Nutr Metab Cardiovasc Dis* 16: 543-549.

APPENDICES

Appendix A

Example of the informed consent form to be completed by participants prior to taking part in any study.

INFORMED CONSENT FORM

(to be completed after Participant Information Sheet has been read)

The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the Loughborough University Ethical Advisory Committee.

I have read and understood the information sheet and this consent form.

I have had an opportunity to ask questions about my participation.

I understand that I am under no obligation to take part in the study.

I understand that I have the right to withdraw from this study at any stage for any reason, and that I will not be required to explain my reasons for withdrawing.

I understand that all the information I provide will be treated in strict confidence and will be kept anonymous and confidential to the researchers unless (under the statutory obligations of the agencies which the researchers are working with), it is judged that confidentiality will have to be breached for the safety of the participant or others.

I agree to participate in this study.

Your name _____

Your signature _____

Signature of investigator _____

Date _____

Loughborough University

Appendix B

Health Screen Questionnaire for Study Volunteers

As a volunteer participating in a research study, it is important that you are currently in good health and have had no significant medical problems in the past. This is (i) to ensure your own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.
If you have a blood-borne virus, or think that you may have one, please do not take part in this research.

Please complete this brief questionnaire to confirm your fitness to participate:

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

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

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

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

3. Have you ever had any of the following:

(a)	Convulsions/epilepsy	Yes		No	
(b)	Asthma	Yes		No	
(c)	Eczema	Yes		No	
(d)	Diabetes	Yes		No	
(e)	A blood disorder	Yes		No	
(f)	Head injury	Yes		No	
(g)	Digestive problems	Yes		No	
(h)	Heart problems	Yes		No	
(i)	Problems with bones or joints	Yes		No	
(j)	Disturbance of balance/coordination	Yes		No	
(k)	Numbness in hands or feet	Yes		No	
(l)	Disturbance of vision	Yes		No	
(m)	Ear / hearing problems	Yes		No	
(n)	Thyroid problems	Yes		No	
(o)	Kidney or liver problems	Yes		No	
(p)	Allergy to nuts	Yes		No	

4. Has any, otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise? ..... Yes ☐ No ☐

If YES to any question, please describe briefly if you wish (eg to confirm problem was/is short-lived, insignificant or well controlled.)

.....
.....

5. Are you,

exercising more than 3 x 30 min / week? ..... Yes ☐ No ☐

6. Allergy Information

(a) are you allergic to any food products? Yes ☐ No ☐
(b) are you allergic to any medicines? Yes ☐ No ☐
(c) are you allergic to plasters? Yes ☐ No ☐

If YES to any of the above, please provide additional information on the allergy

……

7. Additional questions for female participants

(a) are your periods normal/regular? ..... Yes ☐ No ☐
(b) are you on "the pill"? ..... Yes ☐ No ☐
(c) could you be pregnant? ..... Yes ☐ No ☐
(d) are you taking hormone replacement therapy (HRT)? Yes ☐ No ☐

Please provide contact details of a suitable person for us to contact in the event of any incident or emergency.

Name: ……

Telephone number:

……

Work ☐ Home ☐ Mobile ☐

Relationship to Participant:……

Are you currently involved in any other research studies at the University?

Yes ☐ No ☐

If yes, please provide details of the study

……

……

Appendix C

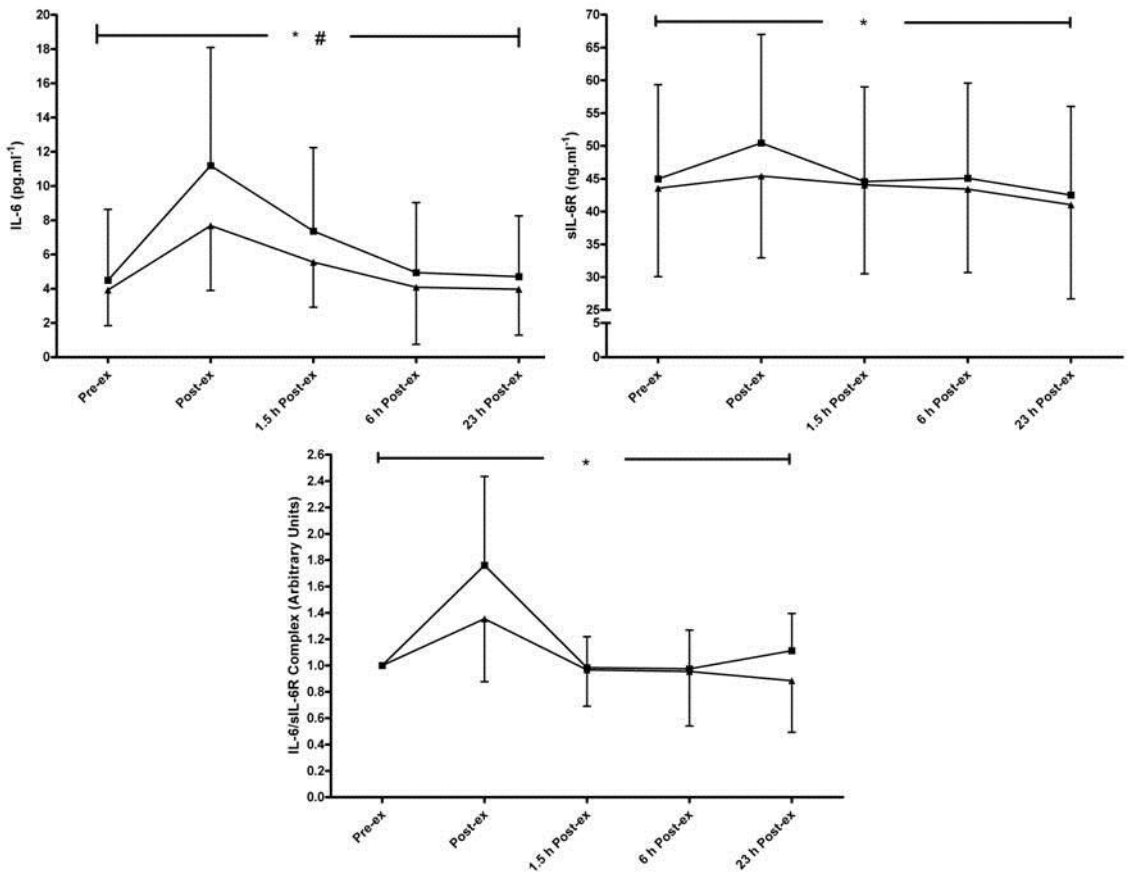
Dietary intake during HIIT and MOD trials in Chapter 4.

	Energy (kcal)	Protein (g)	Carbohydrate (g)	Fat (g)
LUNCH				
Sunflower spread (10 g)	222	trace	trace	5.9
Sliced chicken (65 g)	330	18.3	trace	0.7
Wholemeal bread (2 slice)	786	8.8	33	2.2
Gala apple (~160 g)	308	0.5	17.3	0.3
Orange squash (500 ml)	811	trace	47.6	trace
Kit Kat Chunky (50 g)	1101	2.6	31	14.3
SNACK				
Nutri-Grain Elevenses (45 g)	687	2.5	30	4
DINNER				
Thin & crispy margherita pizza (301g)	2888	34.2	82.4	24.8
Ready salted crisps (34.5 g)	758	2.2	16.9	11.7
Chocolate pudding (150 g)	2260	4.7	58.4	21
Orange squash (500 ml)	811	trace	47.6	trace
TOTAL	**10962**	**73.8**	**364.2**	**84.9**

Appendix D

Statistical analysis for Chapter 4 before correcting for changes in plasma volume.

There was a main trial effect for IL-6 (p = 0.013), with significantly higher IL-6 immediately post-exercise during HIIT compared to MOD (p = 0.002). In addition, there was a main effect of time (p < 0.001), with IL-6 peaking immediately post-exercise in both trials. IL-6 was significantly elevated post- and 1.5 h post-exercise during both trials (p < 0.05). No differences were found for sIL-6R between trials (p = 0.086), however, a main effect of time was found (p < 0.001). Soluble IL-6R was significantly higher than pre-exercise immediately post-exercise after the HIIT trial only. No significant differences were found for the IL-6/sIL-6R complex concentration between HIIT and MOD trials (p = 0.299), although a main effect of time was found (p < 0.001). Peak IL-6/sIL-6R complex concentration occurred immediately post-exercise during both trials.



The IL-6, sIL-6R and IL-6/sIL-6R complex response to MOD (▲) and HIIT (■) exercise trials without correcting for changes in plasma volume. Mean (SD). * main effect of time (p < 0.05). # main trial effect (p < 0.05).