



PHD

The effect of diet on the acute and chronic responses to exercise with a particular focus on adipose tissue

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Award date:
2017

Awarding institution:
University of Bath

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**THE EFFECT OF DIET ON THE ACUTE AND
CHRONIC RESPONSES TO EXERCISE WITH A
PARTICULAR FOCUS ON ADIPOSE TISSUE**

YUNG-CHIH, CHEN

A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department for Health

October 2016

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.....

Abstract

Long-term excessive positive energy balance results in overweight and obesity, which is caused by adipose tissue deposition. This increases the occurrence of cardiovascular diseases and type 2 diabetes. Adipose tissue plays an active role in the development of these diseases and so it is important to understand how this tissue responds to relevant stimuli such as feeding, fasting and physical activity.

The study in **Chapter 4** examined the impact of fasting and feeding, on adipose tissue responsiveness to prolonged moderate intensity exercise. Ten healthy overweight men aged 26 ± 5 years (mean \pm SD) with a waist circumference of 105 ± 10 cm walked at 60% of maximum oxygen uptake under either fasted (12 h overnight fasting) or fed (70% carbohydrate breakfast) conditions in a randomised, counterbalanced design. Feeding comprised 648 ± 115 kcal 2 h before exercise. The expression of several metabolism-related adipose tissue genes was acutely regulated whilst participants undertook fasted exercise, including up-regulation of lipolytic lipase and transporter (adipose triglyceride lipase, hormone sensitive lipase & fatty acid translocase/CD36), glycolytic inhibitor (pyruvate dehydrogenase 4), insulin signalling molecules (glucose transporter type 4 & insulin receptor substrate 2) as well as adipose insulin receptor substrate 2 protein contents (all $p \leq 0.05$), compared to exercise in the fed state. The results indicate that adipose tissue responsiveness to prolonged exercise is affected by the dietary conditions. The study in **Chapter 5** examined whether adipose tissue would be influenced by more modest changes in accumulated physical activity. Eleven overweight participants (7 men and 4 post-menopausal women) aged 50 ± 5 years (means \pm SD) completed two identical mixed meal ($\sim 1,700 \pm 360$ kcal in total) feeding trials (prolonged sitting *versus* breaking sitting) in a randomised, counterbalanced

Abstract

design. The breaking sitting intervention comprised walking for 2 min every 20 min over 5 h. The results demonstrated that postprandial insulin and glucose concentrations were attenuated (all $p \leq 0.05$) while participating in regular small bouts of walking but this did not affect adipose tissue metabolic- and insulin-associated pathways in adipose tissue. The study in **Chapter 6** examined the responsiveness to aforementioned different forms of physical activity (a single bout of prolonged exercise *versus* accumulation of small bout of physical activity) on a challenge imposed by 50% overfeeding. Twenty-four lean, active and healthy men aged 21 ± 3 years were recruited. Participants were randomised to either an overfeeding with restricted physical activity ($\leq 4,000$ steps per day) group (OVER, $n = 8$), overfeeding with restricted physical activity ($\leq 4,000$ steps per day) plus daily 45 min endurance moderate intensity walking group ($50\% \dot{V}O_{2max}$) (OVER + EN, $n = 8$) or overfeeding with restricted physical activity ($\leq 4,000$ steps per day) plus intermittent breaking sitting group (OVER + BREAKS, $n = 8$). All groups achieved the same overfeeding (50% of overfeeding based on their habitual diet). Notably, despite the impairment of insulin sensitivity as a result of the energy surplus, the accumulation of small bouts of physical activity blunted overfeeding induced up-regulation of adipose lipogenic activity (i.e. the down-regulation of sterol regulatory element binding protein 1c and fatty acid synthase) and circulating inflammation (i.e. no change of white blood cell count) compared to energy surplus with sedentary lifestyle and/or overfeeding plus a single bout of moderate intensity exercise. This could mean that the form of physical activity undertaken could play a key role in lipogenesis activation.

Based on the results from this thesis, it appears that energy consumption and physical activity are both capable of acutely and chronically influencing adipose tissue metabolic signalling and regulation.

PUBLICATIONS

Publication

1. **Yung-Chih Chen**, Rebecca Travers, Jean-Philippe Walhin, Javier T Gonzalez, Françoise Koumanov, James Betts, and Dylan Thompson. Feeding influences adipose tissue responses to exercise in overweight men. (2017). (Accepted by American Journal of Physiology-endocrinology and metabolism).

Conference Presentation

1. **Chen Y.C.**, Travers R.L., Walhin J.P., Betts J.A., and Thompson D. (2015). The impact of feeding status on the adipose tissue responses to exercise in overweight men. *22nd Europe Congress on Obesity (ECO 2015), Prague, Czech Republic.*
2. **Chen Y.C.**, Betts J.A., and Thompson D. (2016). The effect of breaking prolonged sitting on metabolism and adipose tissue in middle-age overweight men and women. *Poster Presentation at 43rd Adipose Tissue Discussion Group Meeting (ATDG 2016), Oxford.*

ACKNOWLEDGEMENTS ☺

Firstly, I would like to thank my supervisor Professor Dylan Thompson for his great help throughout my PhD. The conversation, I will remember in my entire life between Dylan and I was, “I believe that you will succeed and I would like to see it; therefore, I would like to invest in your PhD”, he said, when my funding ran out and I did not know what to do. This trust is beyond the expression for me. I had many difficult periods during these 4 years PhD, without his understanding, assistance and encouragement; it would be hard for me to complete this degree. I would also like to thank my co-supervisor Dr. James Betts for his assistance for studies’ design and statistical help when I had problems.

Huge thanks to Becki Travers and Jean-Philippe Walhin for both your help and direction in various laboratory techniques. Thanks also to Francoise Koumanov for teaching me Western Blotting analysis. Thank you Aaron Hengist to contribute your time for my last project. I am also so grateful for all of participants who joined in my PhD projects. Great thanks to my viva examiners Professor Keith Stokes and Professor David Stensel, I really enjoyed our discussion, even though it was a bit intense for me.

Thanks to my type 2 diabetes group friends Harriet Smasher, Will Trim, Gareth Roberts (c), Iain Templeman and Pippa Heath for eating burgers with me, even though we are in the Department for Health. Also, my happy time with football teammates Richard Metcalfe, Tom Nightingale, Oly Perkin, Enhad Chowdhury and Max Western for playing football with me to promote my health. Thank you to Ben Lee, José Ruffino, Ching, Patacchini Francesco, Susy Echeverria and José Serra for your encouragement during my PhD. To all my friends in Taiwan, I miss you very much and your support inspired me to complete my study.

Lastly and very importantly, I really appreciate my family, my parents, sister and my two years old nephew. It means a lot to me to have your full support. Special thanks to the Ministry of Education Taiwan for providing financial support, I would not be in the UK for this PhD without this scholarship.

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Abbreviations

ACC1	Acetyl-coenzyme A carboxylase α
ACC- β	Acetyl-CoA carboxylase- β
ACSM	American College of Sports Medicine
AKT1	RAC-alpha serine/threonine-protein kinase
ALT	Alanine transaminase
AMPK	5' AMP-activated protein kinase
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
BMR	Basal metabolic rate
CPT-1	Carnitine palmitoyl transferase 1
Ct	Cycle threshold
CVD	Cardiovascular diseases
cAMP	Cyclic adenosine monophosphate
DEE	Daily energy expenditure
DEXA	Dual energy X-ray absorptiometry
DIT	Diet-induced thermogenesis
ECBM	Endothelial cell basal media
FAS	Fatty acid synthase
FAT/CD36	Fatty acid translocase/CD36
FOXO1	Forkhead box protein O1
G0S2	G0/G1 switch gene 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

Abbreviations

GI	Glycemic index
GLUT4	Glucose transporter type 4
GPAT	Glycerol-3-phosphate acyltransferase
HDL	High-density lipoprotein
HK II	Hexokinase II
HR _{max}	Maximum heart rate
HSL	Hormones-sensitive lipase
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-18	Interleukin 18
IRS1	Insulin receptor substrate 1
IRS2	Insulin receptor substrate 2
iAUC	Incremental area under the curve
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
MCP-1	Monocyte chemoattractant protein-1
METs	Metabolic equivalents
MGL	Monoglycerides lipase
mRNA	Messenger RNA
NEAT	Non-exercise activity thermogenesis
NEFA	Non-esterified fatty acids
PAEE	Physical activity-induced energy expenditure
PAI	Physical activity intensity
PAL	Physical activity level
PDH	Pyruvate dehydrogenase
PDK4	Pyruvate dehydrogenase 4

Abbreviations

PGC-1 α	Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha
PIK3R1	PI3K-85 α
PKA	Protein kinase A
PPAR γ	Peroxisome proliferator-activated receptor gamma
PPIA	Peptidylpropyl isomerase A
RER	Respiratory exchange ratio
RMR	Resting metabolic rate
RPE	Rate of perceived exertion
RT	Reverse transcription
SCAT	Subcutaneous adipose tissues
SD	Standard deviation
SEM	Standard error of the mean
SPA	Spontaneous physical activity
SREBP1c	Sterol regulatory element binding protein 1c
T2DM	Type 2 diabetes mellitus
TEE	Total energy expenditure
TG	Triglycerides
TNF- α	Tumor necrosis factor alpha
UCP3	Uncoupling-protein 3
VAT	Visceral adipose tissues
VLDL	Very low-density lipoproteins
WAT	White adipose tissue
WHO	World Health Organization
$\dot{V}O_{2max}$	Maximal oxygen up take
$\dot{V}O_{2peak}$	Peak oxygen up take

Chapter 1: Introduction

1.1 Overview of physical activity

The balance of energy expenditure and intake dictates whether people will gain or lose weight. Daily energy expenditure (DEE) consists of basal metabolic rate (BMR), diet-induced thermogenesis (DIT) and physical activity-induced energy expenditure (PAEE). BMR accounts for approximately 60% of DEE and is influenced by body size, amount of skeletal muscle and health condition. DIT is typically approximately 10 % of DEE and is influenced by the kind of food ingested, as well as digestion and absorption processes. Relative to BMR and DIT, PAEE is the most unpredictable element of DEE that could range widely from inactive (i.e. a sedentary office worker) to very active (i.e. a professional athlete). Indeed, PAEE could vary by up to 2000 kcal·day⁻¹ (Levine, 2007).

Physical activity includes any types of body movement produced by skeletal muscle, leading to energy expenditure above resting level (Caspersen *et al.*, 1985), including structured (exercise) and unstructured physical activity (non-exercise activity thermogenesis, NEAT). Moreover, the intensity is an important component of physical activity and it consists of the concept of relative and absolute intensity. The percentage of maximal oxygen up take (% $\dot{V}O_{2max}$) is the concept of relative exercise intensity and, based on American College of Sports Medicine (ACSM) position, moderate intensity ranges from 46% to 63% $\dot{V}O_{2max}$ and vigorous intensity ranges from 64% to 90% $\dot{V}O_{2max}$ (Garber *et al.*, 2011). In terms of absolute physical activity intensity, metabolic equivalents (METs) are frequently used, representing fixed units of energy expenditure to define the intensity of physical activity where the value ranges from below 3 METs,

indicating sedentary behaviour (e.g. sitting or lying) to above 9 METs meaning high intensity exercise (e.g. running) (Norton *et al.*, 2010; Nimmo *et al.*, 2013) (Figure 1.1).

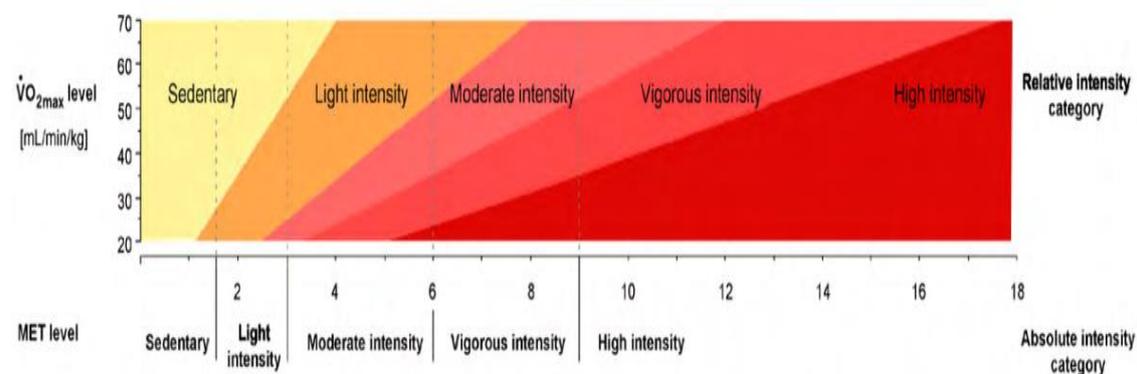


Figure 1.1 The definition of physical activity intensity
(Norton *et al.*, 2010)

1.1.1 Prolonged exercise

Physical activity induced thermogenesis can be further divided into two subcomponents including volitional exercise thermogenesis and non-exercise activity thermogenesis (NEAT). Exercise is a type of planned, structured physical activity resulting in skeletal muscle movement and aims to maintain or to promote physical fitness (Caspersen *et al.*, 1985). In the past few decades, participation in regular exercise has been confirmed as a useful strategy to maintain body weight and to prevent cardiovascular disease and type 2 diabetes. In general, the concept of ‘exercise’ is expressed in terms of frequency, intensity, type and time. The latest American College of Sports Medicine (ACSM) position stand published in 2011 stated that the aerobic exercise prescription for general individuals is to engage in moderate intensity activity (i.e. 46% to 63% $\dot{V}O_{2max}$) at least 30 min per day for 5 days a week or vigorous intensity (i.e. 64% to 90% $\dot{V}O_{2max}$) at least 20 min a day on 3 days a week, or to combine the variation of moderate- and vigorous- intensity exercise to accumulate ≥ 500 –1000 METs a week (Garber *et al.*, 2011).

It is well known that participating in structured prolonged exercise training can change adipose tissue masses (Campbell *et al.*, 2013; Vissers *et al.*, 2013). It has been suggested that changes in adipose masses might due to the reduction of adipocyte size and/or number after exercise training (Campbell *et al.*, 2013). Adipose tissue is not just a storage tissue but plays an active role in health such as energy metabolism and inflammation-related adipokines regulation (Thompson *et al.*, 2012). And, it has been shown that adipose gene expression is modified after acute prolonged exercise irrespective of changes in adipose masses (Frydelund-Larsen *et al.*, 2007; Hojbjerre *et al.*, 2007; Christiansen *et al.*, 2013a). Most acute exercise studies are conducted after overnight fasting. Interestingly, diet impacts upon circulating substrate utilization (Wu *et al.*, 2003; Cluberton *et al.*, 2005; De Bock *et al.*, 2005; Backhouse *et al.*, 2007), and also alters skeletal muscle gene expression responses including energy metabolism, glucose and lipid transport as well as insulin signalling pathway (Civitarese *et al.*, 2005; Cluberton *et al.*, 2005; De Bock *et al.*, 2005; Nieman *et al.*, 2005; Treebak *et al.*, 2014). The effect of diet on acute skeletal muscle responses has been proposed to influence physiological adaptations after long-term exercise training (De Bock *et al.*, 2008; Stannard *et al.*, 2010; Van Proeyen *et al.*, 2010; Van Proeyen *et al.*, 2011). Thus, it is also possible that diet interacts with exercise to change the adipose tissue responses to exercise (especially given that adipose is very active in the postprandial period (Enevoldsen *et al.*, 2004) and also involved energy metabolism regulation during exercise (Horowitz, 2003)) But, surprisingly, adipose tissue responses to acute exercise under different dietary conditions has never been studied. *So, it is important to determine how adipose tissue responds to acute exercise under fasted or fed conditions and this will be the first step of this PhD.*

However, engaging in planned structured continuous exercise for at least 30 min is not feasible for everyone. This has led to the notion that exercise can be accumulated in shorter bouts 10 min. This has been proved as equally effective to reduce triglycerides (TG) concentrations as continuous exercise whether cycling at 60% maximum heart rate (HR_{max}) in obese males (Miyashita, 2008), jogging at 60% $\dot{V}O_{2max}$ in a sedentary population (Altena *et al.*, 2004), running at 70% $\dot{V}O_{2max}$ in healthy males (Miyashita *et al.*, 2006) or brisk walking at 40% $\dot{V}O_{2max}$ in self-selected pace physical activity (Miyashita *et al.*, 2008). Participating in regular exercise aims to maintain physical fitness and energy balance, and thus prevent the burden from obesity-related diseases. On the other hand, continuous or accumulated exercise takes up a small part of daily life – and there may be an alternative non-exercise way to enhance energy expenditure and/or improve health.

1.1.2 Non-exercise activity thermogenesis (NEAT)

NEAT is similar to spontaneous physical activity (SPA) and contains any type of physical activities, which increases energy expenditure, but excluding volitional sporting-like exercise. NEAT is the most variable component of physical activity energy expenditure ranging from 15% to 50% of total energy expenditure (TEE) (Levine, 2003) and can vary by approximately $2,000 \text{ kcal}\cdot\text{day}^{-1}$ between active and inactive people of the same body size (Levine, 2007). Indeed, for sedentary individuals, the role of NEAT is a key determinant of energy expenditure and the major source of physical activity (Hamilton *et al.*, 2007). As shown in Figure 1.2 (A), taking away exercise does not have a major impact on total energy expenditure. In contrast, for inactive people the manipulation of NEAT will have a major impact on their energy balance (Figure 1.2B). Therefore, enhancing NEAT could be another option for

maintaining body weight and preventing obesity-related disease, especially for those who are the most sedentary.

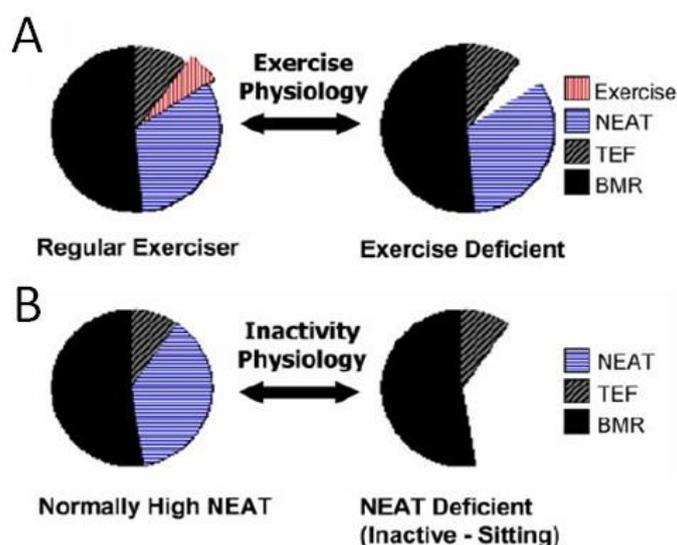


Figure 1.2 The component of total energy expenditure

(A) The proportion of energy expenditure in active people (B) The proportion of energy expenditure in inactive people (Hamilton et al., 2007)

In addition to the manipulation of energy balance, NEAT-type behaviours could have a profound metabolic and physiological effect, over-and-above the impact on energy balance. Prolonged sitting or sedentary behaviour shows a positive correlation with metabolic-related diseases (Ford *et al.*, 2005; Grontved & Hu, 2011; Dunstan *et al.*, 2012a) independent of physical activity (Biswas *et al.*, 2015). Recent research shows that breaking prolonged sitting and sedentary time (i.e., increasing NEAT-type behaviours) appears to offer various health benefits, including muscle health, fat oxidation and postprandial glycemia and insulinemia (Dunstan *et al.*, 2012b; Ando *et al.*, 2013; Latouche *et al.*, 2013; Peddie *et al.*, 2013; Bailey & Locke, 2015; Henson *et al.*, 2016). Even low intensity physical activity elicits powerful effects in adipose tissue (Thompson *et al.*, 2012). However, it is unclear whether one of the mechanisms through

which breaking prolonged sedentary behaviour and increasing NEAT improves health is through changes in adipose tissue physiology and function. Therefore, the second project of this PhD will explore this question.

1.1.3 Sedentary life style and overfeeding

Lack of physical activities and/or NEAT is part of cause resulting in energy imbalance which contributes overweight and obesity. Several studies have shown that increased sedentary behaviour is significantly associated with increased risk of abnormal glucose metabolism, metabolic syndrome, type 2 diabetes and cardiovascular disease (Ford *et al.*, 2005; Wilmot *et al.*, 2012). Apart from sedentary life style, overfeeding can also induce weight gain, glucose intolerance, insulin resistance, mitochondria dysfunction as well as adipose tissue remodelling after short-term overfeeding (Brons *et al.*, 2009; Tam *et al.*, 2010; Alligier *et al.*, 2012; Walhin *et al.*, 2013). However, high amount of NEAT (Levine *et al.*, 1999) or a single bout of 45 min high intensity exercise (70% $\dot{V}O_{2max}$) (Walhin *et al.*, 2013) can counteract such an overfeeding effect. But, studies have never compared the impact of these two different strategies (i.e. a single bout of exercise versus breaking sedentary behaviour) on the impact of overfeeding. Acutely participating in small bouts of walking throughout a day or single bout of continuous exercise impacts skeletal muscle responses at a cellular level (Civitarese *et al.*, 2005; Cluberton *et al.*, 2005; De Bock *et al.*, 2005; Latouche *et al.*, 2013). However, it is unclear how adipose tissue responds to such different physical activity strategies. Therefore, the final project of this PhD will examine how adipose tissue responds to two different types of physical activity intervention in the context of overfeeding and a positive energy balance.

Chapter 2: Literature review

2.1 Overweight/obesity and adipose tissue

2.1.1 The definitions of overweight and obesity

Obesity and being overweight are caused by energy consumption outweighing energy expenditure, so excess energy is stored as adipose tissue. The accumulation of excess adipose tissue is associated with insulin resistance, metabolic syndrome, cardiovascular disease and type 2 diabetes mellitus (T2DM). Generally, obesity can be estimated by body mass index (BMI) according to the calculation of height and weight of an individual. While a BMI ≥ 25 and ≥ 30 ($\text{kg}\cdot\text{m}^{-2}$) represents being overweight and obese respectively, this does not take into account the impact of variation in body composition. For example, high amounts of muscle mass can lead to an overweight classification using BMI but the person actually has a low fat proportion and a low risk of cardiovascular diseases (CVD)/T2DM. A 9-year follow-up study further demonstrated that abdominal obesity is a stronger predictor for coronary heart disease (CHD) compared to BMI (Canoy *et al.*, 2007). In addition, a great number of studies have shown that the accumulation of adipose tissue in the upper body (i.e. abdominal adipose tissue) has a strong positive correlation with insulin resistance (Carey *et al.*, 1996; Goodpaster *et al.*, 1997). Therefore, waist circumference could be a better way to examine metabolic risks. The World Health Organization (WHO) states that the risk of metabolic complications increases when waist circumference is greater than 94 cm for males and 80 cm for females (WHO, 2008).

2.1.2 Overview of adipose tissue

In general, there are two major types of adipose tissue in humans – namely white and brown adipose tissue (Giralt & Villarroya, 2013). Traditionally, the function of white adipose tissue (WAT) is responsible for energy storage and utilization whereas brown adipose tissue (BAT) operates thermogenesis and the generation of body heat.

Aside from different types, adipose tissue is stored in different depots and the distribution of adipose tissue is associated with metabolic function and health (Kissebah *et al.*, 1982). The accumulation of human adipose tissue in the upper body is significantly positively related to CVD risk factors (Williams *et al.*, 1997). On the other hand, depending on diverse depots, WAT can be further categorized into subcutaneous adipose tissues (SCAT) and visceral adipose tissues (VAT). SCAT is located underneath the skin and VAT surrounds the internal organs. Studies have shown that both VAT and abdominal SCAT have a substantial correlation with insulin resistance and that VAT is a stronger predictor when compared with abdominal SCAT (Preis *et al.*, 2010). The potential mechanism could be that when lipid is mobilized from visceral adipose tissue, it is released directly into the portal circulation, which has been suggested as a contributing factor in cardiovascular disease (Smith *et al.*, 2001).

It has been well documented that adipose tissue serves not only for energy storage and mobilization but also plays an active role as an endocrine organ. Adipose-secreted molecules have been termed “adipokines” and include adiponectin, leptin, visfatin, resistin, interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- α), etc. These undertake various roles including serving as pro- and anti-inflammatory mediators, signals of energy metabolism and appetite and so on (Calabro & Yeh, 2007; Galic *et al.*, 2010) by paracrine and autocrine regulations (Fain *et al.*, 2004b; Karastergiou &

Mohamed-Ali, 2010). In adipose tissue, both adipocytes and non-adipocytes are able to produce adipokines (Fain *et al.*, 2004b). In obese populations, the secretion of pro-inflammatory adipokines such as IL-6, TNF- α and interleukin 8 (IL-8) are increased and the secretion of anti-inflammatory adiponectin is decreased (Karastergiou & Mohamed-Ali, 2010).

2.2 Impact of fasting on adipose tissue metabolism

Lipid and glucose utilization and interaction are crucial in response to fasting and/or exercise. Energy metabolism and molecular responses in either adipose tissue (Palou *et al.*, 2008; Palou *et al.*, 2010; Nielsen *et al.*, 2011) or skeletal muscle (Tunstall *et al.*, 2002; Pilegaard *et al.*, 2003a; Spriet *et al.*, 2004; Wijngaarden *et al.*, 2014) is altered after overnight fasting prior to experimental manipulations and this further impacts circulating substrate utilization (Klein *et al.*, 1993; Samra *et al.*, 1996; Gjedsted *et al.*, 2007; Wijngaarden *et al.*, 2014). This could potentially influence how scientists interpret the results related to the mechanisms and/or pathways involved during and after exercise. Therefore, it is important to understand how adipose tissue (the main energy storage organ) and skeletal muscle (the biggest energy utilization organ) responds to overnight fasting.

2.2.1 The general responses after fasting

Fasting triggers physiological challenges that induce changes in substrate supply, mobilization and utilization (Klein *et al.*, 1993). In general, overnight fasting decreases circulating glucose and insulin levels, and increases non-esterified fatty acids (NEFA) levels (Tunstall *et al.*, 2002; Spriet *et al.*, 2004). This is shown when triglyceride is hydrolysed as an energy resource in order to save glucose for other vital organs such as brain. The overall processes of adipose tissue lipolysis is shown in Figure 2.1 and the details are described as follows. Catecholamine represents the primary hormone to stimulate lipolysis in a fasted state via the cyclic adenosine monophosphate (cAMP) pathway (Duncan *et al.*, 2007), following activation of protein kinase A (PKA) resulting in phosphorylation and activation of some pivotal lipases. Two receptors in the cell membrane, including β -adrenoceptors and α -adrenoceptors, can be activated by catecholamine via enhancing and inhibiting lipolytic processes respectively (Horowitz,

2003). Other hormones such as testosterone, cortisol, and growth hormone also affect the rate of lipolysis. In addition to these hormones, the reduction of circulating insulin level is another crucial factor that increases lipolysis (Klein *et al.*, 1993; Wijngaarden *et al.*, 2014).

Prior to lipid oxidation, stored triglyceride is hydrolysed into NEFA and glycerol. Adipose triglyceride lipase (ATGL) and hormones-sensitive lipase (HSL) are two crucial lipases to regulate lipolysis (Carmen & Victor, 2006; Duncan *et al.*, 2007), and are together responsible for up to 95% triglycerides hydrolysis in WAT (Schweiger *et al.*, 2006). The initial step of lipolysis is catalysed by ATGL for hydrolysis of triglycerides into diglycerides (Zimmermann *et al.*, 2004), followed by the next step from diglycerides to monoglycerides by functioning of HSL (Fredrikson *et al.*, 1981). The last step of lipolysis is completed by monoglycerides lipase (MGL) (Tornqvist & Belfrage, 1981), which releases the final fatty acid and glycerol. However, during long-term food deprivation of up to a few days, ATGL and HSL might not coordinate to regulate lipolysis (Bertile & Raclot, 2011). Of note, lipolysis is also much greater than fat oxidation after overnight fasting (Wolfe *et al.*, 1990; Horowitz *et al.*, 1997), which means that lipid resource is enough to supply fat utilization either during exercise, fasting or exercise under a fasted state.

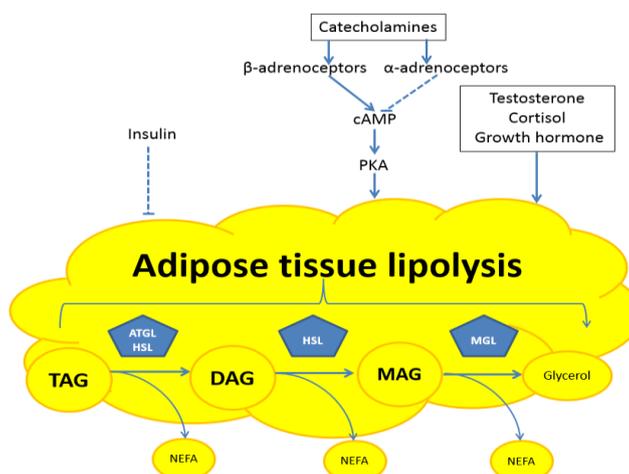


Figure 2.1 The process of adipose tissue lipolysis.

2.2.2 Short-term fasting – adipose tissue mRNA expression

Fat storage and mobilization are tightly controlled by a number of enzymatic steps involved with different molecular regulation (Frayn *et al.*, 1995). In human studies, it has been suggested that adipose tissue triglycerides mobilization is increased after 18 and 24 h of fasting (Klein *et al.*, 1993) and serum NEFA levels is increased after overnight fasting owing to increased lipolysis (Tunstall *et al.*, 2002; Wijngaarden *et al.*, 2014). When it comes to different populations, health status has been observed to influence adipose tissue gene expression (Costabile *et al.*, 2011; Weiss *et al.*, 2011). HSL mRNA regulation in adipose tissue was not different among lean normal weight, obese without T2DM and obese with T2DM groups after overnight fasting but lipoprotein lipase (LPL) messenger RNA (mRNA) in the lean normal weight group was higher than obese with T2DM patients (Costabile *et al.*, 2011). However, the evidence of short-term fasting in human adipose genes is relatively limited, so adipose gene expression in *rodents* in response to short-term fasting (shorter than 24 h) is discussed in the following section.

AMP-activated protein kinase (AMPK) is an energy homeostasis sensor and regulates lipid and glucose metabolism when the ratio of AMP/adenosine triphosphate (ATP) is low (Daval *et al.*, 2006). Fasting-induced energy imbalance in adipose tissue can be seen by showing increased AMPK and the AMP/ATP ratio after 15 h of fasting (Kajita *et al.*, 2008). The increase of adipose AMPK might potentially evoke the regulation of other adipose genes (Daval *et al.*, 2006; Anthony *et al.*, 2009). Unsurprisingly, 24 h of short-term fasting affects the expression of several key adipose tissue genes in rodents (Kim *et al.*, 1998; Li *et al.*, 2006; Palou *et al.*, 2008; Palou *et al.*, 2010), with depot-specific and age-related differences (Bertile *et al.*, 2003; Palou *et al.*, 2010; Wronska *et*

al., 2015). The abdominal subcutaneous adipose tissue is usually sampled in human studies, and this is an important consideration when interpreting the findings.

It has been found that the elevation NEFA levels was observed after 4 h of fasting and peaked at 8 h of fasting (Palou *et al.*, 2008; Palou *et al.*, 2010). In addition, the increases in carnitine palmitoyl transferase 1 (CPT-1) mRNA expression, which transports NEFA in to the mitochondria, have been seen after both 4 and 8 h of fasting and went up remarkably after 24 h of fasting (Palou *et al.*, 2008). The results of adipose lipolytic mRNA genes (ATGL & HSL) in animal models were inconsistent after short-term fasting (between 4-12 h of fasting) (Villena *et al.*, 2004; Palou *et al.*, 2008; Palou *et al.*, 2010), but one study found that ATGL mRNA was transiently increased after 12 h of fasting (Villena *et al.*, 2004). In addition, results have consistently shown that lipogenesis-related genes were decreased after 8 h and 24 h of fasting, including sterol regulatory element binding protein 1c (SREBP1c), fatty acid synthase (FAS), acetyl-coenzyme A carboxylase α (ACC1), glycerol-3-phosphate acyltransferase (GPAT) and peroxisome proliferator-activated receptor gamma (PPAR γ) (Palou *et al.*, 2008; Palou *et al.*, 2010). In particular, PPAR γ mRNA was shown to be more sensitive to fasting compared to other genes as it was reduced after just 4 h of fasting (Palou *et al.*, 2008). Based on the above observations, it appears that lipid-related energy metabolism is regulated via the elevation of lipolysis and the reduction of lipogenesis after short-term fasting. The subsequent rise in circulating NEFA is transported for oxidation. However, in rodent studies, the results of expression in lipolytic mRNA genes are not consistent after short-term fasting, and thus it is important to directly test these pathways in humans.

Apart from HSL and ATGL, LPL, another form of lipase, hydrolyses circulating triglyceride in capillary into NEFA, especially targeting chylomicrons and very low-density lipoproteins (VLDL). Fasting affects the expression of LPL in WAT. Short-term fasting induces the reduction of LPL activity in animal adipose tissue, without a change in gene expression and protein levels (Doolittle *et al.*, 1990; Bergo *et al.*, 2002). Its activity shows a different response to gene expression, and varies according to different adipose depots (Palou *et al.*, 2010). The down-regulation of adipose tissue LPL mRNA after fasting might be due to hydrolysis of circulating triglycerides into NEFA, and their reesterification for storage within adipocytes, so that the down-regulation of LPL mRNA might prevent further lipogenic processes.

Glucose-related pathways have also been shown to regulate energy metabolism in adipose tissue in response to fasting. The expression of glucose transporter type 4 (GLUT4) mRNA was reduced after 8 and 24 h of fasting (Palou *et al.*, 2008; Palou *et al.*, 2010). Hexokinase II (HK II) mRNA expression and protein levels were found to be down-regulated after fasting (Gosmain *et al.*, 2005). The expression of pyruvate dehydrogenase 4 (PDK4) mRNA, the enzyme which inhibits pyruvate dehydrogenase (PDH), was slightly increased after 24 h of fasting (Li *et al.*, 2006). This evidence indicates that not only lipid-related genes but also glucose transportation (GLUT4) and glycolysis regulation (HK II and PDK4) genes are regulated whilst under a fasted state. In addition, Markan *et al.* (2010) propose in their review paper, that adipose glycogen might also influence adipose tissue metabolism under a fasted state. Furthermore, transcription factors are regulated after short-term fasting including SREBP1c and PPARs (Vidal-Puig *et al.*, 1996; Kim *et al.*, 1998; Bertile *et al.*, 2003; Kajita *et al.*, 2008; Palou *et al.*, 2008; Palou *et al.*, 2010).

Taken together, as shown in Figure 2.2, under a fasted state in rodents, it is possible that the elevation of lipolytic adipose gene expression, combined with the reduction of lipogenic genes for releasing NEFA into circulation. In addition to these changes in lipid-related pathways, a reduction in glycolysis in adipose tissue could also influence adipose metabolism. Moreover, transcription factors such as SREBP1c and PPAR γ can also play an important role to regulate adipose gene expression under a fasted state.

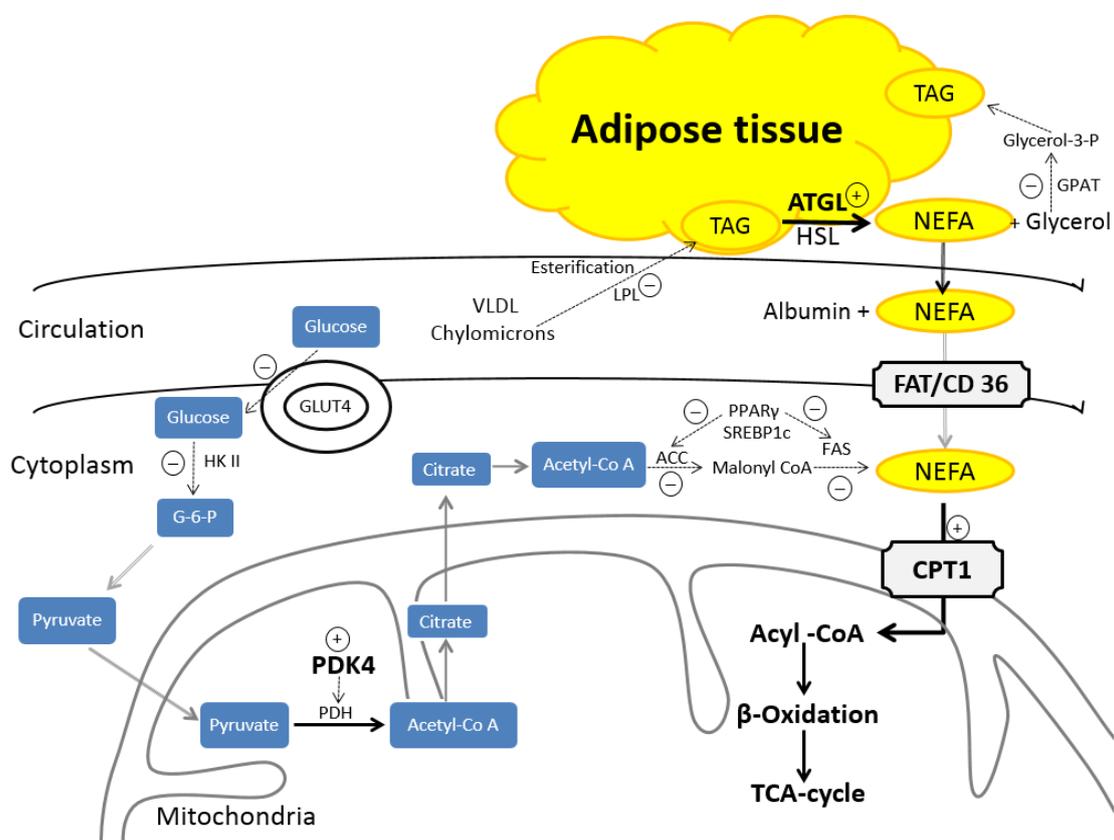


Figure 2.2 Pathways influenced by short-term fasting.

2.3 Effect of acute exercise on adipose adipokines

Over the past few decades, a number of researchers have investigated the impact of different types of exercise, including variation of intensity, duration and population, on circulating cytokines. During prolonged exercise, adipose triglycerides play a critical role of supplying energy substrates. There are different depots of adipose tissue involved including upper- and lower-body subcutaneous adipose tissue, intra-abdominal adipose tissue, plasma and intramuscular triglycerides, which all contribute diverse proportions of energy during exercise (Horowitz, 2003). Although VAT exhibits a high lipolysis rate, it is cleared by the liver so that it is not the major substrate oxidised during exercise. SCAT is the primary adipose tissue depot which contributes energy towards the overall cost of exercise, especially upper body SCAT (Horowitz, 2003). In addition to upper body SCAT, intramuscular triglycerides can be directly used and are a major fuel during exercise (Horowitz, 2003).

Cytokines can be released from diverse organs - for example skeletal muscle, liver, brain or adipose tissue. Depending on the source, these can be referred to as cytokines, myokines or adipokines. To identify the source of cytokine secretion, direct observation within tissues can help, although there has been relatively little progress in measurements such as adipokine gene expression in adipose in response to exercise in humans until relatively recently.

2.3.1 Impact of exercise on gene expression

2.3.1.1 IL-6 mRNA expression in SCAT in acute exercise

The adipokine which has received the most attention to date at the gene expression level is IL-6. At rest, adipose tissue is responsible for 15–35% of circulating IL-6 (Mohamed-Ali *et al.*, 1997b). A number of studies have investigated IL-6 mRNA in SCAT after acute prolonged exercise. During long duration exercise, circulating IL-6 is secreted primarily by contracting skeletal muscle. Subsequently, as shown in Figure 2.1, after the cessation of exercise, adipose tissue could be a major source of IL-6 if the increase in IL-6 mRNA also corresponded to an increased release of the protein (Keller *et al.*, 2003b).

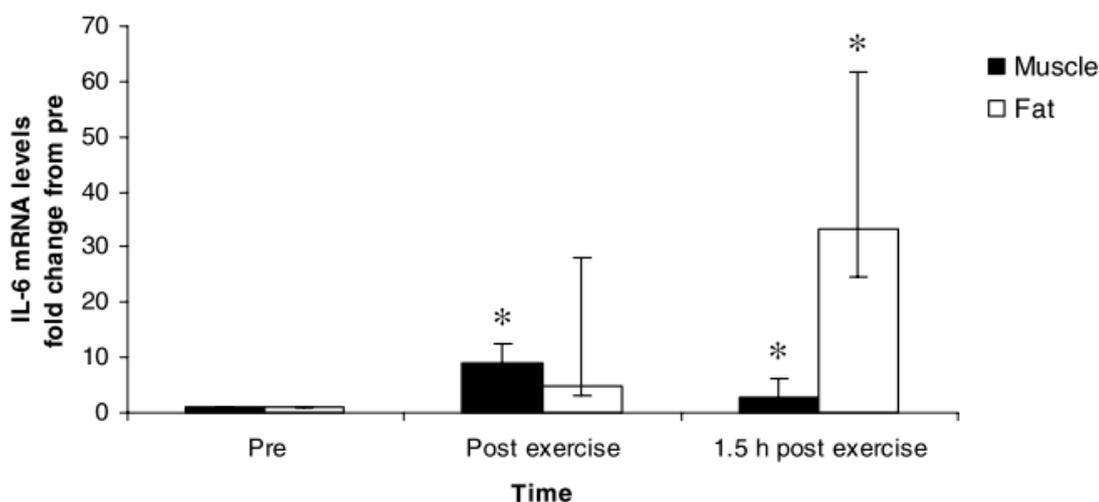


Figure 2.3 IL-6 mRNA expression in skeletal muscle and abdominal adipose tissue after 3 h of 60% $\dot{V}O_{2max}$ cycling.

* means difference over time (Keller *et al.*, 2003b)

Lyngso *et al.* (2002) used the Fick technique (arterial-venous concentration differences) in order to identify IL-6 output from abdominal subcutaneous adipose tissue and found that IL-6 concentration did not change during and immediately after 1 h exercise at 60%

$\dot{V}O_{2\max}$ in young males, but started to secrete IL-6 30 min post-exercise for the following 2.5 h and was 15-fold higher than the rest control group at this point (Figure 2.2).

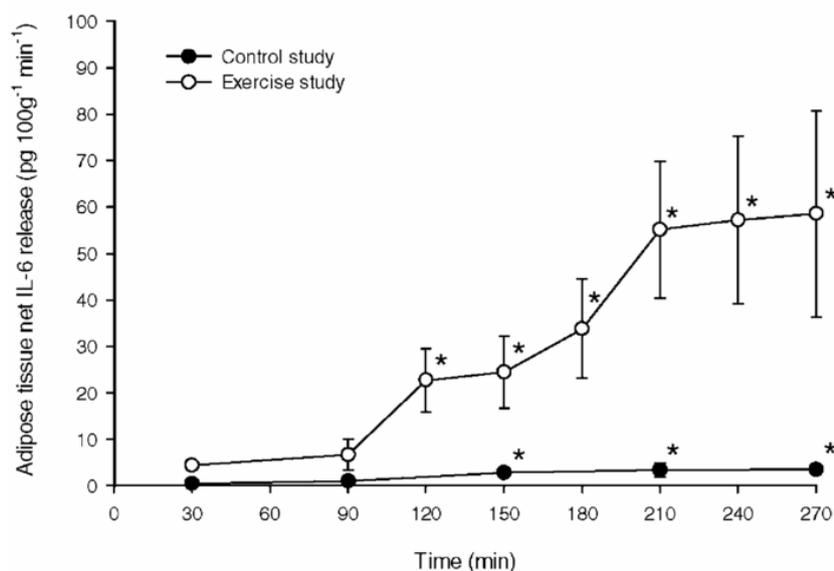


Figure 2.4: Net IL-6 production from adipose tissue during exercise.

Exercise was started at 30 min and ended at 90 min (exercise *versus* rest group).

* significant difference compared to baseline (30 min) (Lyngso *et al.*, 2002)

A similar study investigated the impact of 1 h of cycling exercise at 55% $\dot{V}O_{2\max}$ on IL-6 mRNA expression and found that IL-6 mRNA expression was enhanced in SCAT during exercise and reached a peak at 2.5 h post-exercise in both overweight and lean young males (Hojbjerre *et al.*, 2007). This study confirmed that, after cessation of exercise, IL-6 mRNA expression in SCAT remained elevated for the following 2.5 h.

One Study revealed that both overweight/obese and lean males substantially increased IL-6 mRNA in SCAT immediately after 2 h cycling at 55–60% HR_{\max} (Christiansen *et al.*, 2013a). These studies showed that IL-6 mRNA expression in SCAT increased

during prolonged exercise, but the time course of IL-6 mRNA expression in SCAT remains uncertain.

Other studies also demonstrated that IL-6 mRNA expression in SCAT was elevated immediately post-exercise after 3 h prolonged moderate intensity exercise. Keller *et al.* (2003a) indicated that after 3 h of cycling at 60% $\dot{V}O_{2max}$, IL-6 mRNA in SCAT was approximately 6.5-fold higher at the end of exercise and reached a peak of 7.5-fold at 1.5 h post-exercise – but was still raised at 3 h post-exercise in young males. Similar results can be found in other studies (Keller *et al.*, 2003b; Holmes *et al.*, 2004; Frydelund-Larsen *et al.*, 2007). Even more profoundly, one study revealed that after 3 h exercise, IL-6 mRNA in SCAT was increased at 3, 4.5, 6 and 9 h post-exercise while compared with pre-exercise (Frydelund-Larsen *et al.*, 2007). According to the above studies, it seems that IL-6 mRNA expression in SCAT might be elevated during prolonged exercise (> 2 h) and reaches a peak value in the post-exercise period. The potential mechanism of the enhanced expression and secretion of IL-6 post-exercise may be associated with providing energy by lipolysis and fatty acid mobilization from SCAT via autocrine/paracrine mechanisms to maintain energy supply (Lyngso *et al.*, 2002). It can be confirmed that lipid mobilization from adipose tissue is substantially elevated for about 1 h post-exercise and remains increased for the following 2 h (Mulla *et al.*, 2000).

In addition, IL-6 mRNA expression in SCAT could also be affected by the last acute bout of exercise. Compared to rest, obese males with a high fasting triacylglycerol concentration showed a decrease in IL-6 mRNA expression in SCAT in a fasted state 16 h after acute 60 min walking at 55% $\dot{V}O_{2max}$ (Dekker *et al.*, 2010). However, there

was no difference in IL-6 in the circulation between exercise and rest groups at this point.

2.3.1.2 Adiponectin mRNA expression in SCAT

One study has shown that, during 1 h of cycling exercise at 55% $\dot{V}O_{2max}$, subcutaneous abdominal adipose tissue interstitial adiponectin concentration increased but there was no corresponding change in plasma adiponectin in either overweight or lean men (Hojbjerre *et al.*, 2007). Surprisingly, this study found that adiponectin mRNA in SCAT *declined* in overweight and lean male groups during and post-exercise (Hojbjerre *et al.*, 2007). In contrast, other studies have found that adiponectin mRNA in SCAT increased after 2 h cycling at 55–60% maximum heart rate (HR_{max}) in lean, overweight and obese men (Christiansen *et al.*, 2013a). At present, the reason for the differences between these two studies is unclear.

2.3.1.3 Leptin mRNA expression in SCAT in acute exercise

After 3 h of cycling exercise at 60% $\dot{V}O_{2max}$, leptin mRNA in SCAT decreased over the following 5 h; although this was not statistically different to a resting control group (Keller *et al.*, 2005). Another study also found that after 1 h of cycling exercise at 55% $\dot{V}O_{2max}$, leptin mRNA in SCAT declined during and after exercise for the following 2.5 h in both overweight and lean men; even though the baseline leptin mRNA was higher in the overweight group compared to the lean group (Hojbjerre *et al.*, 2007). This result indicates that participating in at least 1 h moderate intensity exercise can decrease leptin mRNA expression in SCAT and it lasts for a period after exercise.

2.3.1.4 Other adipokines mRNA expression in SCAT in acute exercise

Circulating interleukin 18 (IL-18) concentrations positively correlate with BMI, waist-to-hip ratio and inversely with insulin sensitivity (Escobar-Morreale *et al.*, 2004). In addition, IL-18 mRNA in adipose tissue and plasma concentrations are higher in obese and metabolic syndrome populations (Leick *et al.*, 2007; Weiss *et al.*, 2011). Obesity and metabolic syndrome all have a connection with low inflammatory processes and a link to insulin resistance. As a result, this could mean that IL-18 is an inflammatory marker, and plays a role to induce metabolic-related disorders. One study found that IL-18 mRNA in SCAT was not affected in either 2 h at 60% $\dot{V}O_{2max}$ or 1.5 h exercise at 70% $\dot{V}O_{2max}$ exercise in lean individuals (Leick *et al.*, 2007). Thus, this means that IL-18 may not affect metabolic changes after acute exercise, but more research is still needed to confirm this.

After 3 h of cycling at 60% $\dot{V}O_{2max}$, visfatin mRNA expression was raised 3-fold compared with pre-exercise and increased at 3, 4.5 and 6 h post-exercise (compared with resting control group), but plasma visfatin did not change after exercise (Frydelund-Larsen *et al.*, 2007). In addition, another study showed that TNF- α mRNA increased in abdominal SCAT in overweight and lean men after acute 1 h exercise; although plasma TNF- α did not change after exercise in either the overweight or lean people (Hojbjerre *et al.*, 2007). The same study revealed that resistin mRNA in SCAT was not changed in overweight or lean men after acute exercise (Hojbjerre *et al.*, 2007). However, few studies have been done on the effect of acute exercise on adipokine gene expression.

According to the afore-mentioned adipokine gene expression studies, it appears that IL-6 mRNA in SCAT may be enhanced during prolonged exercise (at least 2 h), but it is

still unclear whether there is any change in IL-6 mRNA with shorter duration exercise (less than 1 h). IL-6 mRNA in SCAT remains increased after prolonged exercise (at least 1 h) and reaches a peak over the following hours. The increase in IL-6 mRNA in the post-exercise period might be associated with lipolytic processes. IL-6 mRNA might be decreased the next day after acute exercise in people with a high fasting TG concentration, but the potential mechanism still requires further research. The change of adiponectin mRNA in SCAT after acute exercise is still controversial. Leptin mRNA in SCAT decreases after acute exercise either in overweight or lean individuals and lasts for several hours. There have been only a few isolated studies that have examined other adipokines in adipose tissue. Thus, we need further research to build up a systematic understanding of these responses.

2.3.2 Impact of acute exercise on circulating adipokines

2.3.2.1 Circulating IL-6 in response to acute exercise

IL-6 can be released by various organs including skeletal muscle and adipose tissue and the concentration of IL-6 in overweight/obese individuals is higher than in lean ones (Christiansen *et al.*, 2013a). Circulating IL-6 can be increased after acute exercise (Mitchell *et al.*, 2011; Ostapiuk-Karolczuk *et al.*, 2012; Christiansen *et al.*, 2013a), and plays the role of both a pro- and anti-inflammatory adipokine (Pedersen, 2006). At a similar exercise intensity, IL-6 production is greater after resistance exercise compared to aerobic exercise because of greater skeletal muscle recruitment (Mitchell *et al.*, 2011). In addition, exercise intensity is another crucial determinant that affects IL-6 concentrations (Mendham *et al.*, 2011; Scott *et al.*, 2011). When exercise intensity is lower than 30% $\dot{V}O_{2max}$, circulating IL-6 is not changed (Mendham *et al.*, 2011). However, the responses of circulating IL-6 to exercise in overweight/obese and lean

individuals are different. Christiansen *et al.* (2013a) found that both overweight/obese and lean groups increased concentration of circulating IL-6 during 2 h ~55–60% of HR_{max} cycling, but that the concentration of IL-6 in the overweight/obese was higher during exercise probably due to additional body fat.

2.3.2.2 Circulating adiponectin in response to acute exercise

Adiponectin is a typical adipokine which is mainly produced by adipose tissue, and it acts as an anti-inflammatory adipokine. The level of adiponectin is normally 5–20 $\mu\text{g}\cdot\text{ml}^{-1}$ in the body (Matsuzawa, 2005). In general, adiponectin concentration has a negative correlation with body weight, BMI and waist-hip ratio (Han *et al.*, 2007). In addition, in patients with chronic diseases or obesity, adiponectin levels were lower, though regular exercise training may improve these levels (Bruun *et al.*, 2006; Kondo *et al.*, 2006). With regard to acute aerobic exercise, studies in healthy populations have reported that adiponectin is not influenced by various exercise intensities (from 50 % $\dot{V}O_{2max}$ to very strenuous intensities) and durations (from 30 min to 2 h)(Kraemer *et al.*, 2003; Ferguson *et al.*, 2004; Punyadeera *et al.*, 2005; Hojbjerg *et al.*, 2007). Despite this, one study found that adiponectin increased after 30 min strenuous running; however, adiponectin was not different after adjusting plasma volume (Kraemer *et al.*, 2003). However, the findings from studies that have investigated the effect of acute aerobic exercise on circulating adiponectin in overweight and obese individuals are inconsistent. For example, Saunders *et al.* (2012) found that the plasma adiponectin increased immediately and 30 min post-exercise after either high (75% of $\dot{V}O_{2peak}$) or moderate (50% $\dot{V}O_{2peak}$) intensity running exercise with the same energy expenditure (~400 kcal). In contrast, other studies found that adiponectin decreased or remained unchanged during 1 h cycling at 70% and 50% $\dot{V}O_{2peak}$, respectively (Numao *et al.*, 2011) or was unchanged after moderate intensity exercise (Jamurtas *et al.*, 2006;

Hojbjerre *et al.*, 2007; Bouassida *et al.*, 2010b). Thus, at the present time, the response of circulating adiponectin concentrations to acute exercise is inconsistent across studies.

2.3.2.3 Circulating leptin in response to acute exercise

In a 1994 study of mutations in the mouse ‘ob’ gene, leptin, was shown to play the role of a signalling relay in the regulation of bodyweight and energy balance (Zhang *et al.*, 1994; Campfield *et al.*, 1995; Pelleymounter *et al.*, 1995). It has been well reviewed that when exercise duration exceeds 60 min or energy expenditures greater than 800 kcal, the circulating concentration of leptin will decrease (Bouassida *et al.*, 2010a).

2.4 Impact of feeding on the acute responses to exercise

It has been suggested that molecular changes occur either in adipose tissue or skeletal muscle after short-term fasting, indicating that the alternation of energy metabolism is induced. The influence of fasting on exercise is likely to be related to its duration, but the precise definition of fasting in this context is rather vague. Broadly, the impact of feeding on the response to exercise is associated with the dietary components (carbohydrate, fat and protein proportions) and size (energy) of meals, as well as the time interval between a pre-exercise meal and exercise.

It has been widely confirmed that fasting prior to exercise increases fat utilization during exercise (Coyle *et al.*, 1985; Dohm *et al.*, 1986; Wu *et al.*, 2003; Backhouse *et al.*, 2007). Lipolysis during exercise in a fasting trial was greater than fat oxidation based on the calculation of glycerol appearance rate and there was no difference in lipolysis and fat oxidation in glucose and fructose-fed trials; however, both lipolysis and fat oxidation in the fasting trial were higher than other two trials (Horowitz *et al.*,

1997). A feeding-induced increase in insulin before exercise is likely to play a major role in suppressing lipolysis during exercise (Horowitz *et al.*, 1997).

2.4.1 Impact of feeding on exercise – in tissue level

It has been demonstrated that feeding affects skeletal muscle molecular responses to acute exercise (De Glisezinski *et al.*, 1998; Nieman *et al.*, 2003; Civitarese *et al.*, 2005; Cluberton *et al.*, 2005; De Bock *et al.*, 2005; Nieman *et al.*, 2005; Harber *et al.*, 2010; Trebak *et al.*, 2014).

Cluberton *et al.* (2005) found that glucose consumption before and during exercise altered skeletal muscle gene expression response to exercise ($\sim 75\% \dot{V}O_{2\max}$ for 1 h) in young healthy males. This study demonstrated that the expression of skeletal PDK4 and uncoupling-protein 3 (UCP3) mRNA were decreased in the feeding trial compared to an overnight fasting exercise trial. In addition, skeletal GLUT4 mRNA expression was higher (1 and 3 h after exercise) in the fasting trial compared with the feeding trial (although there was no statistical difference).

De Bock *et al.* (2005) found that feeding (carbohydrate intake (~ 150 g) before and during ($1 \text{ g (kg body weight)}^{-1}$) exercise ($\sim 75\% \dot{V}O_{2\max}$ for 2 h) decreased total skeletal muscle UCP3 mRNA expression, α -AMPK phosphorylation and phosphorylation of acetyl-CoA carboxylase- β (ACC- β) in young healthy males immediately after exercise – but that there was no difference 4 h after exercise. However, energy was provided in both trials during 4 h recovery, so this study is not well placed to reflect the molecular responses during recovery.

Civitarese *et al.* (2005) extensively investigated how skeletal muscle glucose and lipid related metabolism genes respond to exercise (cycling at 50% $\dot{V}O_{2\text{peak}}$ for 2 h) under either fed or fasted conditions in young healthy men. Muscle tissue was taken before and, 1 and 4 h after exercise. In terms of glucose metabolism genes, the expression of GLUT4 and PDK4 mRNA were all up-regulated after exercise in the fasting trial. In addition, the expression of PDK4 mRNA was further increased for the following 4 h in the fasting trial. In terms of lipid gene regulation, exercise in a fed state suppressed fatty acid translocase (FAT)/CD36, UCP3, CPT1 and AMPK α 2 mRNA expressions throughout the trial. In line with other studies (Cluberton *et al.*, 2005; De Bock *et al.*, 2005), this study demonstrated that substrate availability impacted upon glucose and lipid gene expression in skeletal muscle in response to submaximal exercise.

Taken together, feeding state prior to exercise impacted upon skeletal muscle gene expression in energy regulation (\downarrow AMPK), glucose (\downarrow PDK4, GLUT4) and lipid metabolism (\downarrow FAT/CD36, CPT1 & UCP3). Adipose tissue is one of the organs that regulates energy metabolism in response to fasting and exercise. However, surprisingly, there are no studies investigating how adipose tissue responds to exercise under fasted/fed states.

2.4.2 Impact of feeding on exercise – circulating adipokines

Only one study has investigated the impact of pre-exercise feeding *only* on circulating adipokines. Zoladz *et al.* (2005) found that there was no difference in circulating IL-6 and leptin concentrations after a short bout of exercise in either a fed or fasted state. Interestingly, IL-6 was higher in fasted than fed (2 h after breakfast) prior to exercise and this was accompanied by a high plasma norepinephrine and low respiratory exchange ratio (RER) (Zoladz *et al.*, 2005). The authors postulated that IL-6 might

serve to maintain energy supply such as modifying glucose concentrations and enhancing fat oxidation – even at rest. However, this study did not take blood samples before the meal and this could be a source of bias. In addition, the duration of exercise in this study only lasted 12 min, and this might be not long enough to understand if and how adipokines respond to exercise under different dietary conditions.

2.4.3 Impact of different feeding conditions on exercise responses at a whole body level

The effect of different feeding states prior to exercise has drawn attention over the past few decades (Coyle *et al.*, 1985; Diboll *et al.*, 1999; Wu *et al.*, 2003; Backhouse *et al.*, 2007; Gonzalez *et al.*, 2013; de Lima *et al.*, 2015). Exercise under a fasted state increases lipid oxidation owing to high lipolytic rate and increased levels of NEFA and low concentrations of circulating insulin and glucose, which is reflected by a low RER (Coyle *et al.*, 1985; Wu *et al.*, 2003; Backhouse *et al.*, 2007). The impact of different types of feeding on whole body responses to exercise is shown in Table 2.1. The results are summarized in the following section.

Glucose feeding from 25 to 312 grams between 45 min to 4 h prior to exercise reduces lipid oxidation during exercise when compared to exercise in a fasted state (Sherman *et al.*, 1989; Sherman *et al.*, 1991; Short *et al.*, 1997; Jentjens *et al.*, 2003). In addition, high glycemic index (GI), medium GI or low GI pre-exercise feeding has been observed to reduce lipid oxidation during exercise (Wu *et al.*, 2003; Backhouse *et al.*, 2007). The reasons are partly due to pre-exercise meals elevating blood glucose availability and muscle glycogen concentration (Coyle *et al.*, 1985; Wee *et al.*, 2005). Furthermore, fitness level and/or training status could affect substrate utilization during exercise (Stisen *et al.*, 2006); however, one study showed that pre-exercise meal consumption

induced the elevation of carbohydrate oxidation in both trained and untrained individuals at intensities ranging from 22% to 59% $\dot{V}O_{2\text{peak}}$, but with exercise at a vigorous intensity (75% $\dot{V}O_{2\text{peak}}$), the ratio of carbohydrate and lipid oxidation was the same irrespective of training levels (Bergman & Brooks, 1999).

The time interval between a pre-exercise feeding and exercise could critically affect substrate utilization and metabolism during exercise (Montain *et al.*, 1991; Dumortier *et al.*, 2005). Montain *et al.* (1991) compared the impact of different meal timing (2, 4, 6, 8 and 12 h) in response to exercise (70% $\dot{V}O_{2\text{max}}$ for 30 min) and found that exercise 2 and 4 h after feeding promoted carbohydrate oxidation by approximately 15% compared to an interval of 8–12 h; and that the effect disappeared by 6 h. Dumortier *et al.* (2005) found that feeding 3 h prior to exercise showed a higher fat oxidation rate compared to 1 h pre-exercise meal. In terms of meal frequency, when compared to single and multiple doses and feeding with the same calories, single or multiple feedings did not alter substrate utilizations during exercise; however, lipolysis was inhibited with either single or multiple feedings compared to fasted trials (sugar-free orange juice) (Chryssanthopoulos *et al.*, 2008).

In addition to young and healthy populations, feeding also influences how people with T2DM respond to exercise (Poirier *et al.*, 2000; Poirier *et al.*, 2001; Gaudet-Savard *et al.*, 2007; Ferland *et al.*, 2009). Poirier *et al.* (2001) found that there were no differences in FFA and RER values while exercising under a fasted (overnight fasting) or fed state (meal consumed 2 h pre-exercise) in T2DM. This could mean that T2DM might demonstrate chronic adaptation of impaired lipolytic lipases which reduces lipid metabolism during exercise. Indeed, studies have indicated that, compared to lean humans, those with overweight and obesity show a decrease in catecholamine-induced

lipolysis and HSL expression (Langin *et al.*, 2005) and the reduction of adipose ATGL and HSL in protein levels in insulin resistance humans (Jocken *et al.*, 2007). On the contrary, Ferland *et al.* (2009) compared five distinct pre-exercise meals (HGI, LGI, high fat/low carbohydrate, low-calories and overnight fasting) consumed 2 h before a 1 h 60% $\dot{V}O_{2\max}$ exercise in T2DM and found that exercise reduced circulating glucose levels between 36–51% among all meal trials compared to fasting (almost no change), but the proportion of carbohydrate and lipid oxidation is unclear due to unreported RER values. However, different levels of fasting circulating glucose (ranging from below 6 to above 8 mmol·l⁻¹) affects T2DM responses to exercise under different dietary states (Gaudet-Savard *et al.*, 2007). With this in mind, it might be hard to directly compare the effect of pre-exercise feeding on the responses of people with T2DM.

Based on the results from the aforementioned studies, most scenarios suggest that exercise under a fasted state increases lipolytic rate and lipid oxidation irrespective of the amount of energy intake, the difference of GI values, the level of fitness, time interval prior to feeding and the frequency of the meals provided. Pre-exercise feeding might affect how people with T2DM react to exercise but due to different levels of T2DM this is less clear at the present time.

Table 2.1 Impact of feeding on whole body responses to exercise

Authors	Participants	Protocol	Meal	Main findings (During exercise)
(Allgrove <i>et al.</i> , 2009)	16 young lean healthy (8 M & 8 F) $\dot{V}O_{2max}$: M 51 ± 4 & F 40 ± 1 ml·kg ⁻¹ ·min ⁻¹	10–12 h of overnight fasting Meal 2 h before 2 h 65% $\dot{V}O_{2peak}$ cycling	Fed (~530 kcal) Fasted (~15 kcal drink)	RER (Fed > Fasted)
(Backhouse <i>et al.</i> , 2007)	6 young lean healthy F $\dot{V}O_{2max}$: 43 ± 2 ml·kg ⁻¹ ·min ⁻¹	12 h of overnight fast Meal 3 h before 1 h walk at 50% $\dot{V}O_{2max}$	Fed (~650 kcal; MGI or HGI) Fasted	NEFA & fat oxidation (all Fasted > Fed)
(Bergman & Brooks, 1999)	7 untrained & trained young M $\dot{V}O_{2max}$: untrained 39 ± 2 & trained 58 ± 2 ml·kg ⁻¹ ·min ⁻¹	12 h of overnight fasting Meal 3 h before 2 h at 22% & 40%, 1.5 h at 60%, 45 min at 80% $\dot{V}O_{2peak}$ cycling for untrained and trained	Fed (500 kcal; 53% CHO, 31% fat & 16% protein) Fasted	RER (Fed > Fasted) at 22, 40 & 59% $\dot{V}O_{2peak}$ in trained and 40 & 59% $\dot{V}O_{2max}$ in untrained
(Chryssanthopoulos <i>et al.</i> , 2008)	8 young lean healthy M $\dot{V}O_{2max}$: 52 ± 1 ml·kg ⁻¹ ·min ⁻¹	10 h of overnight fast Meal 3 h before 1 h running at 70% $\dot{V}O_{2max}$ <i>Adipose tissue biopsy taken</i>	Fed (800 kcal; single or multiple meal) Fasted (22 kcal)	RER, NEFA & glycerol (Fed > Fasted)
(Coyle <i>et al.</i> , 1985)	7 young lean healthy M $\dot{V}O_{2peak}$: 66 ± 1 ml·kg ⁻¹ ·min ⁻¹	16 h of overnight fasting Meal 4 h before 105 min 70% $\dot{V}O_{2peak}$ cycling	Fed (700 kcal; 85% CHO, 15% protein) Fasted	NEFA & glycerol (Fasted > Fed) RER (Fed > Fasted)
(de Lima <i>et al.</i> , 2015)	12 young lean healthy participants $\dot{V}O_{2max}$: 49 ± 7 ml·kg ⁻¹ ·min ⁻¹	10 h of overnight fast Meal 15 min before 36 min 65% $\dot{V}O_{2max}$ on treadmill	Fed (~349 kcal, 77% CHO, 10% fat & 13% protein) Fasted	<i>Immediately after exercise</i> Both TG ↑ (Fasted > Fed)

(Diboll <i>et al.</i> , 1999)	14 young lean healthy M $\dot{V}O_{2\text{peak}}$: $65 \pm 9 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	12 h of overnight fasting Meal 15 min before 30 min 65% $\dot{V}O_{2\text{peak}}$ on treadmill	Fed (~500 kcal, 77% CHO, 7% fat & 16% protein) Fasted	RER (Fed = Fasted)
(Dohm <i>et al.</i> , 1986)	9 young lean healthy M $\dot{V}O_{2\text{max}}$: $61 \pm 1 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	23 h of overnight fasting Meal 2-4 h before 90 min 70–75% $\dot{V}O_{2\text{max}}$ on treadmill	Fed (700 kcal; 85% CHO, 15% protein) Fasted	NEFA & glycerol (Fasted > Fed) RER (Fed > Fasted)
(Febbraio & Stewart, 1996)	6 young lean healthy M $\dot{V}O_{2\text{max}}$: $62 \pm 4 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	Overnight fasting Meal 45 min before 120 min 70% $\dot{V}O_{2\text{peak}}$ cycling	Fed (~280 kcal; HGI or LGI) Fasted (diet jelly)	NEFA (Fasted > Fed) RER & CHO oxidation (Fed > Fasted)
(Ferland <i>et al.</i> , 2009)	10 T2DM patients $\dot{V}O_{2\text{max}}$: $30 \pm 6 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	Overnight fasting Meal 2 h before 1 h 60% $\dot{V}O_{2\text{peak}}$ cycling	Fed (~450 kcal for HGI, LGI, high fat & low CHO; ~350 kcal for low-calories) Fasted	NEFA (Fasted > all Fed)
(Horowitz <i>et al.</i> , 1997)	6 young lean healthy M $\dot{V}O_{2\text{peak}}$: $53 \pm 2 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	12 h of overnight fasting Drink 1 h before 60 min 45% $\dot{V}O_{2\text{peak}}$ cycling	Fed (~240 kcal; glucose, fructose or glucose + 20% TG infusion) Fasted	Fat oxidation (Fasted > all Fed)
(Jentjens <i>et al.</i> , 2003)	9 young lean healthy M $\dot{V}O_{2\text{max}}$: $64 \pm 2 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	10-12 h of overnight fasting Drinks 45 min before 20 min 65% $\dot{V}O_{2\text{peak}}$ cycling followed by time-trial running	Fed (25, 75 & 200 g CHO) Fasted	RER (all Fed > Fasted)
(Kirwan <i>et al.</i> , 1998)	6 young lean healthy F $\dot{V}O_{2\text{max}}$: $48 \pm 3 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	Overnight fasting Meal 45min before 60% $\dot{V}O_{2\text{max}}$ cycling to exhaustion	Fed (75 g CHO; high & Low fibre) Fasted	RER (Fed > Fasted) NEFA (Fasted > Fed)
(Kirwan <i>et al.</i> , 2001a)	6 young lean healthy M $\dot{V}O_{2\text{max}}$: $54 \pm 1 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	Overnight fasting Meal 45 min before 60% $\dot{V}O_{2\text{peak}}$ cycling to exhaustion	Fed (75 g CHO; HGI or MGI) Fasted	Glycerol & RER (Fed = Fasted) NEFA (Fasted > all Fed)

(Kirwan <i>et al.</i> , 2001b)	6 young lean healthy F $\dot{V}O_{2\text{peak}}$: $47 \pm 4 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	Overnight fasting Meal 45 min before 60% $\dot{V}O_{2\text{peak}}$ cycling to exhaustion	Fed (75 g oats; MGI) Fasted	RER (Fed > Fasted) NEFA & glycerol (Fasted > Fed).
(Koivisto <i>et al.</i> , 1985)	8 young lean healthy M $\dot{V}O_{2\text{peak}}$: $57 \pm 2 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	10–12 h overnight fasting Drink 45 min before 2 h 55% $\dot{V}O_{2\text{peak}}$ cycling	Fed (75 g; glucose or fructose) Fasted	NEFA (Fasted > all Fed)
(Montain <i>et al.</i> , 1991)	9 untrained & trained young M $\dot{V}O_{2\text{peak}}$: untrained 48 ± 2 & trained $66 \pm 2 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	2, 4, 6, 8 & 12 h of fasting 30 min at 70% $\dot{V}O_{2\text{peak}}$ cycling	~500–600 kcal CHO meal	Combined untrained & trained CHO oxidation (2 & 4 h > 8 & 12 h fasting)
(Sherman <i>et al.</i> , 1989)	8 M & 2 F young lean healthy $\dot{V}O_{2\text{max}}$: $\sim 4.2 \pm 0.2 \text{ L}\cdot\text{min}^{-1}$	10 h of overnight fasting Meal 4 h before 70 min at 70% $\dot{V}O_{2\text{peak}}$ combined with 20 min at 52% $\dot{V}O_{2\text{peak}}$ cycling	Fed (312 g CHO) Fasted	RER (Fed > Fasted) Glycerol & NEFA (Fasted > Fed)
(Sherman <i>et al.</i> , 1991)	9 young lean healthy M $\dot{V}O_{2\text{max}}$: $4.1 \pm 0.2 \text{ L}\cdot\text{min}^{-1}$	10 h of overnight fasting Drink 1 h before 90 min 70% $\dot{V}O_{2\text{max}}$ followed by exercise performance test	75, 150 g CHO & placebo	<i>Only compared exercise at first 90 min</i> RER (CHO meals > placebo) NEFA (placebo > CHO meals)
(Wu <i>et al.</i> , 2003)	9 young lean healthy M $\dot{V}O_{2\text{max}}$: $58 \pm 2 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	12 h of overnight fasting Meal 3 h before a 60 min run at 65% $\dot{V}O_{2\text{max}}$ on treadmill	Fed (2 g/kg BM for a total 141 g HGI, LGI) Fasted	CHO oxidation (Fed > Fasted) NEFA & Fat oxidation (Fasted > Fed) Glycerol (Fasted > Fed)
(Zoladz <i>et al.</i> , 2005)	8 young lean healthy M $\dot{V}O_{2\text{max}}$: $3.7 \pm 0.1 \text{ L}\cdot\text{min}^{-1}$	overnight fasting Meal 2 h before incremental cycling test for 12 min from 30 W until 150 W	Fed (870 kcal) Fasted	RER (Fed > Fasted) Circulating IL-6 & leptin (Fed = Fasted)

BM, body mass; M, Male; F, Female; Ex, exercise; CHO, carbohydrate; HGI, high glycemic index; MGI, medium glycemic index; LGI, low glycemic index; NEFA, non-esterified fatty acids; RER, respiratory exchange ratio; $\dot{V}O_{2\text{max}}$, maximum oxygen uptake; $\dot{V}O_{2\text{peak}}$, peak oxygen uptake; TG, triglycerides

2.5 Breaking prolonged sitting physical activity

There is no doubt about the health benefits of participating in either prolonged exercise or accumulated physical activity (Miyashita *et al.*, 2006, 2008; Murphy *et al.*, 2009; Miyashita *et al.*, 2011, 2013). However, even though the reduction of cardiovascular-related diseases after participating in regular physical activity has been confirmed, prolonged sedentary/sitting time is still another risk factor associated with cardiovascular-related diseases and that is independent of physical activity (Edwardson *et al.*, 2012; Biswas *et al.*, 2015). It could mean that preventing prolonged sitting is another crucial strategy to keep healthy. In the last few years, breaking prolonged sitting research has been extensively investigated (Dunstan *et al.*, 2012b; Duvivier *et al.*, 2013; Howard *et al.*, 2013; van Dijk *et al.*, 2013; Thorp *et al.*, 2014; Henson *et al.*, 2016).

Table 2.2 summarises the breaking sitting research conducted in the past few years and the results are described in the following section. The first breaking prolonged sitting research was conducted by Dunstan *et al.* (2012b) and these authors found that whether 2 min light ($3.2 \text{ km}\cdot\text{h}^{-1}$) or moderate ($5.8\text{--}6.4 \text{ km}\cdot\text{h}^{-1}$) intensity physical activity every 20 min for a total 28 min walking, reduced postprandial glucose incremental area under the curve (iAUC) between 25–30% and insulin iAUC about 23% in obese middle-aged population when compared with prolonged unbroken sitting. The reduction of blood pressure was also observed during the postprandial period from the same research group (Larsen *et al.*, 2014). Other studies also demonstrated the health benefits while breaking prolonged sitting was applied (Peddie *et al.*, 2013; Bailey & Locke, 2015; Bailey *et al.*, 2016; Henson *et al.*, 2016).

Prolonged exercise and accumulated physical activity are both being suggested for exercise prescription guidelines. Small bouts of accumulated breaking sitting physical

activity has been directly compared to prolonged exercise, ranging from 1 m 40 sec to 10 min (Miyashita *et al.*, 2006; Miyashita, 2008; Miyashita *et al.*, 2008, 2011; Duvivier *et al.*, 2013; Peddie *et al.*, 2013; Saunders *et al.*, 2013; van Dijk *et al.*, 2013; Kim *et al.*, 2014). Brisk walking throughout a 9 h postprandial period for only 1 min and 40 sec (totalling 30 min) was more effective at reducing glucose and insulin iAUC when compared to a single bout of 30 min walking (Peddie *et al.*, 2013). Another study found that 4 days of breaking sitting physical activity intervention reduced fasting TG and postprandial insulin iAUC compared to prolonged sitting and prolonged sitting plus a daily bout of vigorous structured cycling (Duvivier *et al.*, 2013).

Only one study investigated the effect of breaking sitting at the tissue level, and showed that 75 skeletal muscle mRNA genes (10 genes directly involved in CHO metabolism regulation) were regulated after breaking sitting (Latouche *et al.*, 2013). More precisely, Latouche *et al.* (2013) extensively examined postprandial skeletal muscle mRNA gene expression in 8 middle-aged obese/overweight sedentary population in response to acute breaking sitting physical activity, and demonstrated that merely 2 min walking every 20 min accumulated for 28 min walking is able to regulate metabolic pathways, and cellular development, growth and proliferation of skeletal muscle mRNA genes. This study provides information showing that brief breaks of accumulated walking elicits molecular changes and to provides physiological benefits. Whether the same is true in adipose tissue is uncertain at the present time.

Collectively, breaking prolonged sedentary behaviour contributes to reduced postprandial insulin and glucose concentrations and also regulates skeletal muscle responses. But, studies have never looked into how adipose tissue responds to regular breaking sitting physical activity.

Table 2.2 The effect of breaking prolonged sitting on physiological responses

Authors	Participants	Trials	Meal	Main findings
*(Alkhajah et al., 2012)	3 M & 29 W Age: SIT (40 ± 7); BREAKS (34 ± 9) years BMI: SIT (22 ± 3); BREAKS (23 ± 3) kg·m ⁻²	<i>1 week and 3 months intervention</i> SIT normal working group (n = 14) BREAKS sit-to-stand group (n = 18)	N/A	BREAKS (fasting HDL ↑ after 3 months)
(Altenburg et al., 2013)	5 M & 6 W Age: 18-24 years BMI: 23 (20–26) kg·m ⁻²	SIT: 420 min BREAKS: sitting (372 min) + hourly 8 min (48 min) cycling at 40-60% HRR	1 st meal (843 kcal; 92 g CHO, 59 g fat & 16 g protein) + 2 nd meal (1,190 kcal; 77 g fat, 117 g CHO & 28 g protein)	TG & glucose iAUC ↔ between trials
(Bailey et al., 2016)	13 W Age: 27 ± 3 years Fat mass (%): 24 ± 8	SIT: 300 min L-BREAKS: sitting (272 min) + 2 min walking at 3.2 km/h every 20 min (28 min) M-BREAKS: sitting (272 min) + 2 min walking at 5.8-7.9 km/h every 20 min (28 min)	Drink (75 g CHO & 50 g Fat)	M-BREAKS (glucose iAUC ↓) vs. SIT & L-BREAKS Insulin iAUC ↔ among trials
(Bailey & Locke, 2015)	7 M & 3 W Age: 24 ± 3 years BMI: 27 ± 4 kg·m ⁻²	SIT: 300 min STAND: sitting (272 min) + 2 min standing every 20 min (28 min) BREAKS: sitting (272 min) + 2 min walking at 3.2 km/h every 20 min (28 min)	1 st drink (273 kcal; 75 g CHO) + 2 nd drink (467 kcal; 4 g CHO, 50 g fat & 7 g sodium)	BREAKS (glucose iAUC ↓) vs. SIT & STAND TG iAUC ↔ among trials
(Dunstan et al., 2012b)	11 M & 8 W Age: 54 ± 5 years BMI: 31 ± 4 kg·m ⁻²	SIT: 420 min L-BREAKS: sitting (392 min) + 2 min walking (3.2 km/h) every 20 min (28 min) M-BREAKS: sitting (392 min) + 2 min walking (5.8-6.4 km/h) every 20 min (28 min)	Drink (75 g CHO & 50 g fat)	L- & M-BREAKS (glucose & insulin iAUC ↓ vs. SIT)
*(Duvivier et al., 2013)	3 M & 17 W Age: 21 ± 2 years BMI: 23 ± 3 kg·m ⁻²	<i>4 days intervention</i> SIT: sitting (14 h) + standing (1 h) + walking (1 h) BREAKS: sitting (8 h) + walking (5 h) + standing (3 h) EX: sitting (13 h) + standing (1 h) + walking (1 h) + vigorous cycling (1 h)	OGTT after 16 h of last bout of cycling	BREAKS (fasting TG & insulin iAUC ↓ vs EX & SIT) Fasting glucose, HDL, LDL, insulin, & glucose & glucose iAUC ↔ among trials

(Henson <i>et al.</i>, 2016)	22 W Age: 67 ± 5 years BMI: 33 ± 5 kg·m ⁻²	SIT: 450 min STAND: sitting (390 min) + 5 min standing every 30 min (60 min) BREAKS: sitting (390 min) + 5 min walking (~3 km·h ⁻¹) every 30 min (60 min)	Breakfast: (26% CHO, 58% fat & 16% protein) and lunch (identical meal)	STAND & BREAKS (glucose, insulin & NEFA iAUC ↓; TG iAUC ↔) vs SIT
(Larsen <i>et al.</i>, 2014)	11 M & 8 W Age: 54 ± 5 years BMI: 31 ± 4 kg·m ⁻²	Protocol see (Dunstan <i>et al.</i> , 2012b)	Protocol see (Dunstan <i>et al.</i> , 2012b)	L- & M-BREAKS (average SBP, DBP ↓) vs. SIT
(Latouche <i>et al.</i>, 2013)	7 M & 1 W Age: 55 ± 6 years BMI: 31 ± 3 kg·m ⁻²	Protocol see (Dunstan <i>et al.</i> , 2012b) <i>Muscle biopsy</i>	Protocol see (Dunstan <i>et al.</i> , 2012b)	L- & M-BREAKS (insulin iAUC ↓) vs. SIT L- & M-BREAKS (75 mRNA genes were regulated) vs. SIT
(Peddie <i>et al.</i>, 2013)	38 M & 42 W Age: 26 ± 5 years BMI: 24 ± 4 kg·m ⁻²	SIT: 540 min BREAKS: sitting (510 min) + (1 min 40 sec * 18 walk every 30 min (30 min) ~45% $\dot{V}O_{2max}$ EX: Prolonged sitting (510 min) + 30 min exercise at ~60% $\dot{V}O_{2max}$	3 drinks (1.12 g CHO, 0.46 g & fat 0.54 g protein g/kg BM) Drank at 60, 240 & 420 min during trial	BREAKS (insulin, glucose iAUC ↓, mean resting RER ↑, TG iAUC ↔) vs. SIT & EX EX (TAG iAUC ↓) vs. BREAKS & SIT
(Saunders <i>et al.</i>, 2013)	11 M & 8 W Age: M (13 ± 1) & W (11 ± 1) years BMI: M (19 ± 5) & W (17 ± 3) kg·m ⁻²	SIT: 480 min BREAKS: sitting (440 min) + 2 min exercise at 30% $\dot{V}O_{2peak}$ every 20 min (40 min) EX: sitting (400 min) + 2 min exercise at 30% $\dot{V}O_{2peak}$ every 20 min (40 min) + 20 min exercise at 60% $\dot{V}O_{2peak}$ twice (40 min)	Breakfast (20% daily needs; 2,322 ± 410 kJ) + lunch (40% daily needs; 3,669 ± 799 kJ) Consumed at 0 & 4 h during trial	Insulin, glucose, HDL, LDL & TG iAUCs ↔ among trials

* Represents intervention study. M, men; W, women; BMI, body mass index; SIT, prolonged sitting; Ex, prolonged exercise; BREAKS, breaking sitting physical activity; STAND, standing. L-light intensity; M-moderate intensity; ↔ no difference between trials; ↓ decrease; ↑ increase; iAUC, postprandial incremental area under the curve; RER, respiratory exchange ratio; HDL, high-density lipoprotein; LDL; low-density lipoprotein; TG, triglycerides; CHO, carbohydrate; NEFA, non-esterified fatty acids.

2.6 Aim:

This review has demonstrated that exercise under a fasted state alters several crucial adipokines at the mRNA level such as IL-6, adiponectin and leptin. In addition, feeding prior to prolonged exercise impacts upon physiological responses including circulating parameters, energy metabolism, insulin signalling as well as skeletal muscle mRNA gene expression. Adipose tissue plays an important role in the postprandial state, during fasting and exercise. Surprisingly, studies have never examined how adipose tissue responds to exercise under different dietary conditions. On the other hand, in terms of structured exercise, participating in short bouts of physical activity has been suggested to reduce postprandial glucose, insulin concentrations, blood pressure and regulate skeletal muscle molecules responses. In the postprandial state, adipose tissue is one of the vital organs to balance the energy homeostasis. However, we are still unclear of the impact of the accumulation of short bouts of physical activity on adipose tissue responses in the postprandial state. Moreover, obesity and being overweight are the result of an energy surplus, so excess energy is stored as adipose tissue. But, we still do not understand the effect of the above-mentioned two types of physical exercise on adipogenesis. Therefore, to investigate how different types of physical activity (a single bout of structured exercise versus regular small bouts of intermittent physical activity) influences general physiological reactions and adipose tissue this will be examined during an imposed energy surplus.

Chapter 3: General methodology

3.1 Introduction

Ethical approval was received from Bristol Research Ethics Committee for each individual study. All experimental procedures were conducted in the Applied Physiology and Metabolic Research Laboratories in the Department for Health at the University of Bath, apart from Western blot analysis, which was conducted in the Department of Biology and Biochemistry, all other biochemical analysis was carried out in the biochemistry laboratory in the Department for Health. This chapter outlines the general protocols used throughout all three studies in this thesis. Methods and protocols used only in specific projects are described in each relevant chapter.

In all studies, participants provided verbal and written informed consent before taking part. In the first study (Chapter 4), participants were all overweight males aged between 18–35 years. In the second study (Chapter 5), participants were overweight males and females (postmenopausal) aged between 35–64 years. In the third study (Chapter 6), participants were all active, normal weight males aged between 18–35 years. Participants were asked to complete Physical Activity Readiness Questionnaire (PAR-Q) before any preliminary exercise testing. Smokers and participants diagnosed with any of medical conditions or taking medication that may influence metabolic or inflammatory responses were excluded in all studies.

3.2 Anthropometry

Body weight, after voiding, was measured using digital mass scales to within 0.1 kg (Inner Scan; Body Composition Monitor, model BC-543, TANITA corp. Japan). Height was measured to within 0.1 cm on a fixed stadiometer (SECA, Germany). The body mass index (BMI) was calculated using the following the formula; $BMI = \text{body mass (kg)} \cdot \text{height (m}^2\text{)}^{-1}$.

The measurement of waist and hip circumference to the nearest 0.1 cm was undertaken according to World Health Organisation guidelines (WHO, 2008) using a non-stretch tape. Waist circumference was measured at midway point between the lowest rib and the iliac crest at end of normal breath expiration – and the tape was parallel to the floor when passed around the waist. Hip circumference was measured horizontally around the widest portion of the buttocks (WHO, 2008). All of the waist and hip circumferences measurements were taken 3 times in total and then the mean value determined.

3.3 Body composition analysis

Body composition was estimated using Dual energy X-ray absorptiometry (DEXA; Discovery, Hologic, Bedford, UK) in the morning between 08:00–09:00 am after 10–12 h of fast in all three studies. The DEXA scan was performed by an individual who had completed training in ionising radiation (medical exposure) regulations (IR(ME)R). An hour prior to measurement, participants were asked to drink 1 pint of water to prevent dehydration. The results of the DEXA scan might be affected by body water (Lohman *et al.*, 2000). Therefore, before the measurement, participants voided their bladder in order to minimize variations in hydration status. Individuals were only allowed to wear lightweight shorts during analysis. Participants were asked to lie on the

DEXA scanning table (Discovery, Hologic, Bedford, UK) and the body position of participants were adjusted to the scanning area and the whole scanning process was approximately 7 min. Following completion of the scan, whole body composition analysis was performed with regions sectioned as recommended in the QDR (DEXA provided software) for Windows manual (Hologic, Bedford, UK). Abdominal subcutaneous and visceral adipose tissue (central adipose tissue) was estimated from a central region between lumbar vertebrae 1–4 (L1-L4) (Glickman *et al.*, 2004).

3.4 Physical activity assessment

As part of the preliminary measurements, for one week, participants were fitted with a combined heart rate/accelerometer monitor (Actiheart, Cambridge Neurotechnology Ltd., Cambridge, UK; Figure 3.1) attached to the left of the sternum via 2 adhesive ECG pads, as illustrated in Figure 3.1, for 24 h per day except for when showering/bathing/swimming. Participants were asked to maintain their normal life style and physical activity during the monitored week.

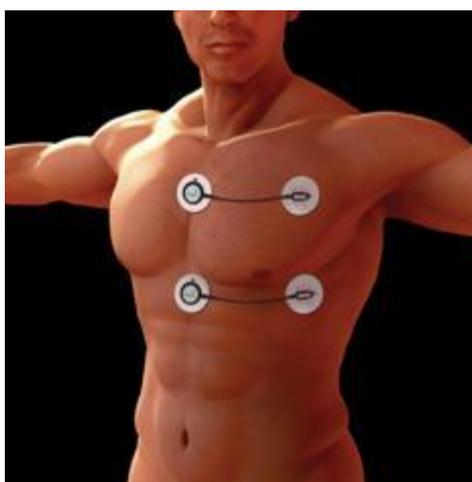


Figure 3.1 The range of locations of Actiheart monitor can be worn.

The manufacturer's software (Actiheart 2.0, Cambridge Neurotechnology Ltd., Cambridge, UK) was used to estimate energy expenditure. Physical activity level (PAL) was calculated based on 24-h period total energy expenditure divided by basal metabolic rate (BMR).

3.5 Dietary record

In order to minimize the variations of physiological conditions between main trials, in the 2 days prior to the 1st main trial in Chapter 4 and 5, participants were asked to record their food and fluid consumption and replicated the same type intake patterns in the 2 days prior to following main trial. In Chapter 6, participants were asked to complete a one week dietary record in order to prescribe their intervention diet. Further details concerning dietary intervention procedures are described in Chapter 6.

3.6 Restrictions for participant pre-trial day

In the 72 h prior to $\dot{V}O_{2\max}$ tests and main trials, participants were asked to refrain from any type of vigorous exercise. In Chapter 5 only, in the 48 h before main trial days, participants were asked to restrict their step counts under 4,000 steps in order to mimic a sedentary life style (Tudor-Locke & Bassett, 2004; Tudor-Locke *et al.*, 2013). In Chapter 6 only, participants were asked to do one, 30 min bout of moderate intensity exercise before 2 pm on the day prior to the 1st main trials in order to standardise their physical activity. In the 48 h before main trials, participants were asked to avoid alcohol and caffeine intake. In the 3 h prior to $\dot{V}O_{2\max}$ measurement, participants were asked not to consume any type of foods or drinks (except for water). Prior to each main trial, participants were asked to arrive at the laboratory after 12 h of fasting and to consume 1 pint of water upon waking on the day of main trials.

3.7 Gas collection and analysis for indirect calorimetry

One hour prior to each trial, the gas analyser was calibrated using 100% Nitrogen and mixed gas of 15.9% Oxygen and 5.005% Carbon Dioxide, and checked against atmospheric values 20.9% Oxygen and 0.04% Carbon Dioxide. The fraction of expired O₂ and CO₂ was analysed using a gas analyser (Series 1400, Servomex Ltd, Sussex, UK). In order to avoid contamination of inspired gases, during gas collections and ambient atmospheric gas samplings were undertaken with as few individuals as possible in the room.

During gas collections, participants were required to wear a nose clip and breathe into a mouthpiece with breathing valves to ensure all expired gas was collected into evacuated Douglas bags (Douglas Bags, Hans Rudolph, MO, USA). At least 30 seconds prior to gas sampling, participants were given the mouthpiece and nose clip. During each gas collection, ambient CO₂ and O₂ samples were measured to account for small changes observed throughout the day in an enclosed room, rather than assuming outside atmospheric values for inspired gas concentrations. The gas sampling tube was held within 1 meter of the participant for the final 2 min of each separate gas collection (read after 1 min 30 seconds of ambient sampling allowing sufficient time for values to stabilise). The stopwatch was started as soon as the stopcock was turned diverting expired gases into Douglas bag.

After gas sample collection was finished, gases concentrations in the expired air within the Douglas bag was sampled for 2 min at a flow rate of 2 L·min⁻¹ and were recorded after 1 min 30 seconds of sampling when values had stabilised. The Douglas bag was then completely evacuated using a dry gas meter (Harvard Apparatus, UK) and the total volume of expired air and its temperature (measured with a digital thermometer

(Checktemp 1, HANNA, UK) attached to the dry gas meter) were recorded. The ambient temperature of the laboratory was also measured and adjustments made as necessary to maintain it between 20–25 °C.

3.8 Maximal oxygen uptake test

Maximal oxygen uptake ($\dot{V}O_{2\max}$) was measured in Chapter 4 and 6 only. $\dot{V}O_{2\max}$ was determined using an incremental continuous treadmill test until the point of volitional fatigue. One minute expired air samples were collected into Douglas bags (Hans Rudolph, MO, USA), ratings of perceived exertion (RPE) using a 6–20 scale (Borg, 1973) and heart rates (HR) were measured in the final minute of each stage and also at the point of volitional exhaustion, defined as when the participant indicated that only 1 min remained until fatigue. Samples were analysed for relative expired fractions of oxygen and carbon dioxide gas (Servomex, Crowborough, UK) and the total volume within the bag was measured using a dry gas meter (Harvard Apparatus, Kent, UK). This measure was used to determine the relationship between $\dot{V}O_{2\max}$ and submaximal $\dot{V}O_{2\max}$ in order to estimate the exercise intensity required in subsequent main trials. The protocol was slightly different due to diverse population in Chapter 4 and 6.

In Chapter 4, for most participants, a treadmill speed of 4 km·h⁻¹ and gradient of 8.5% was chosen. Participants exercised at this speed and gradient with the speed increased by 1 km·h⁻¹ after every 3 min stage until volitional fatigue.

In Chapter 6, for all participants, the testing speed was chosen between 9 to 12 km·h⁻¹. The testing speed did not change until the end of test once the speed was selected. The initial gradient of treadmill was set to 2.5% and increased 2.5% every 3 min stage with fixed speed until volitional fatigue.

3.9 Determining resting metabolic rate

Participants laid on the bed for 15 min prior to the resting metabolic rate (RMR) measurement. The RMR was estimated using a minimum of 2–4 separate consecutive collections each lasting 5 min (Compher et al., 2006; Betts et al., 2011) and the values were determined using substrate oxidation under resting conditions as described (Frayn, 1983a). Participants were given the option to have a 2 min break in order to minimise any discomfort.

3.10 Blood sampling and processing

A 20-gauge 1.1 × 32 mm cannula (BD Venflon™ Pro, Becton Dickenson & Co., Sweden) was inserted into an antecubital forearm vein immediately following RMR assessment on main trial days. Blood samples were taken by using stopcock (BD connecta, Beckton Dickenson, Sweden) and syringe(s) at required time points during the all of main trials. Patency of the cannula was maintained by flushing with isotonic saline following blood sampling. Before collecting the each subsequent sample, 5 ml waste was taken from the cannula to remove the saline that would otherwise dilute the sample.

Venous blood samples were collected using syringes and dispensed into blood collection tubes (Sarstedt Ltd., Leicester, UK) containing either serum separator beads for serum collection or potassium-ethylenediaminetera acetic acid (K₃-EDTA) as an anticoagulant for plasma samples. Blood was dispensed into serum tubes first to avoid EDTA contamination which may affect some metabolite and enzyme measurements.

After gentle mixing by inversion, EDTA tubes were immediately spun at 5,000 rpm for 10 min at 4 °C (Heraeus Biofuge Primo R, Kendro Laboratory Products Plc., Bishops Stortford, UK). Venous blood in serum tubes was gently mixed by inversion and left to clot at room temperature before being centrifuged. After centrifugation, plasma and serum samples were then transferred to 1.5 ml eppendorf tubes and frozen at –80 °C for later analysis.

3.11 Blood analysis

Blood glucose, triglycerides (TG), non-esterified fatty acids (NEFA), total cholesterol, HDL-cholesterol, and alanine transaminase (ALT) were measured using commercially available assay kits (Randox, Crumlin, UK) and analyser (Daytona Rx, Crumlin, UK). Serum insulin (Merckodia, Uppsala, Sweden), and both serum and adipose tissue culture secretions of IL-6, leptin, and adiponectin (R&D systems Inc, UK) were measured using commercially available Enzyme-linked immunosorbent assay (ELISA) in Chapters 4 and 5. A SPECTROstar[®]Nano plate reader (BMG LABTECH's, Germany) was used to determine absorption at the wavelengths specified by the kit's manufacturer. Coefficients of variation and lower limits of detection for each blood parameter are shown in Table 3.1.

Table 3.1 Coefficients of variation (CV) and sensitivity data for the assay used to measure blood parameters

Parameter	Sensitivity	Intra-assay precision (CV)	Inter-assay Precision (CV)
Glucose	0.335 mmol·l ⁻¹	< 5%	< 6%
NEFA	0.072 mmol·l ⁻¹	< 5%	< 5%
Triglycerides	0.134 mmol·l ⁻¹	< 4%	< 4%
Total-Cholesterol	0.865 mmol·l ⁻¹	< 4%	< 2%
HDL-Cholesterol	0.189 mmol·l ⁻¹	< 4%	< 3%
ALT	9.70 U·l ⁻¹	< 6%	< 5%
IL-6	0.039 pg·ml ⁻¹	< 8%	< 10%
Leptin	7.8 pg·ml ⁻¹	< 4%	< 6%
Adiponectin	0.246 ng·ml ⁻¹	< 5%	< 8%
Insulin	1 mU·l ⁻¹	< 4%	< 5%

There were multiple quality controls utilised to analyse CV, the highest of these values is shown based on manufacturers values.

3.12 Adipose tissue sampling

In all three experimental chapters, adipose tissue was taken from the area around the waist, approximately 5 cm lateral to the umbilicus, using a well-established ‘needle aspiration’ technique (Walhin *et al.*, 2013; Travers *et al.*, 2015b). The area was thoroughly disinfected with videne, before injecting that anaesthetic (approximately 5 ml lignocaine hydrochloride 1%) into a small fan shaped area beneath the skin using a 27G 1¼ needle (PrecisionGlide, Beckton Dickinson, USA). Five minutes later, a larger 14G needle (Kendall Monoject, Tyco Healthcare, USA) was inserted into the subcutaneous fat tissue around the waist, approximately 5 cm lateral to the umbilicus, in order to collect approximately 0.5–1 g of adipose tissue. Sterile swabs and pressure were then applied to the area once the sample was collected and before a dressing was applied. The whole process lasted about 30 min.

Visible connective tissue and blood vessels then were removed from the adipose tissue with sterilised scissors before the remaining adipose tissue was cleaned with isotonic saline over single-use sterile gauze membrane to remove any further blood, clots and connective tissue. All re-usable equipment used for tissue handling was pre-sterilised by autoclaving.

3.13 Whole adipose tissue culture

After cleaning adipose tissue, tissue explants was minced into approximately 5–10 mg portions and directly transferred to sterile culture plates in quadruplicate in Chapter 4 and duplicate in Chapter 5 (Nunc surface, Nunc, Denmark) containing Endothelial cell Basal media (ECBM) (Promocell, Germany) supplemented with 0.1% fatty acid-free bovine serum albumin 100 U·ml⁻¹ penicillin and 0.1 mg·ml⁻¹ streptomycin (Sigma-Aldrich, Gillingham, UK). Adipose tissue explants were incubated at a final concentration of approximately 100 mg tissue per 1 ml (Fain *et al.*, 2004b). Explants were then transferred to an incubator (MCO-18A1C CO₂ incubator, SANYO) at 37 °C, 5% CO₂ and 95 ± 5% relative humidity. After 3 h of incubation, the media was removed and transferred into sterile Eppendorf's and stored at –80 °C for further analysis.

3.14 Adipose gene expression analysis

3.14.1 Adipose tissue collection

In Chapter 4 and 5, the remaining adipose tissue (approximately 200 mg) was then homogenised in 5 ml TRIzol reagent (Invitrogen, Paisley, UK) in a 15 ml RNase/DNase free sterile centrifuge tube (Invitrogen, Paisley, UK) and frozen immediately on dry ice. After the trial was completed, tissues was stored in a freezer at –80 °C until further

analysis. Ethanol and distilled water were used to wash the homogenizer between samples.

3.14.2 Adipose tissue gene extraction

An RNeasy Mini Kit (Qiagen, Crawley, UK) was used to extract RNA from adipose tissue following the following protocol; after thawing and vortexing, tissue samples were centrifuged at ~4,000 rpm at 4 °C for 5 min. After centrifuging, TRIzol was transferred to a new 15 ml RNase/DNase free sterile centrifuge tube (Invitrogen, Paisley, UK) using pipette and sterile filter tips without taking up any pellet/lipid. One ml of chloroform was then added into a new TRIzol tube (200 µl chloroform per ml TRIzol) and was vigorously shaken for 15 seconds by hand, followed by a 3 min incubation. After the 3 min incubation, samples were centrifuged again at ~4,000 rpm at 4 °C for another 15 min (aqueous phase and organic phenol-chloroform phase were separated after this process). The aqueous phase (about 3 ml) was then transferred to another new 15 ml RNase/DNase free sterile centrifuge tube, mixed well with 70% ethanol (about 3 ml) in a 1:1 ratio by pipetting up and down.

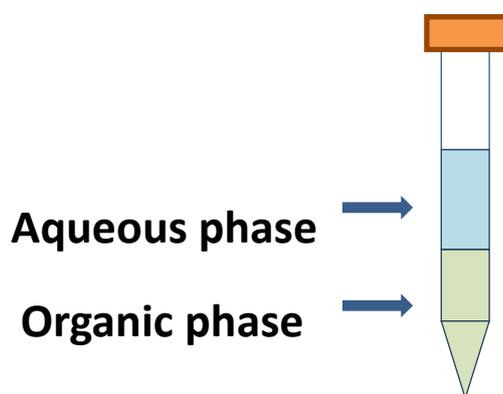


Figure 3.2. Aqueous phase and organic phenol-chloroform phase in a 15 ml RNase/DNase free sterile centrifuge tube after centrifuged.

After mixing with 3 ml of 70% ethanol, the aqueous phase was transferred onto the RNeasy Mini spin column (simplified to spin column in following content), centrifuged in a microfuge tubes until reaching top speed (the step was repeated until all the aqueous phase passed through the column). 700 μ l RW1 wash buffer was added to spin column and centrifuged for 10 seconds at top speed. 500 μ l RPE buffer was then added to the spin column and centrifuged at top speed (repeated twice with 10 seconds for the 1st spin and 2 min for the 2nd spin). All liquid was discarded after every single centrifugation.

After the 2nd spin, all ethanol was removed as many as possible. Spin columns were put onto a new Eppendorf and spun for 2 min at top speed to remove as much ethanol as possible (this processes was repeated twice). Afterwards, spin columns were transferred onto respective marked collection tubes (supplied by the kit) and left open to the air for approximately 5 min to dry. The final step of gene extraction was to add 50 μ l RNase-free water directly into the spin columns, which were then centrifuged at top speed for 1 min to elute the RNA. The organic phenol-chloroform phase from the RNA extraction was kept for further protein analysis (Western blotting analysis details is described in study 1 only). All top speeds in this gene extraction protocol refers to 13,000 rpm.

3.14.3 Adipose tissue gene quantification

In all three experimental chapters, mRNA samples were quantified using Qubit[®] RNA BR Assay kit (Life Technologies, Oregon, USA) after gene extraction. The protocol used as follows:

Master mix reagent was made up by diluting Qubit[®] RNA BR Reagent in a 1:200 ratio in Qubit[®] RNA BR Buffer. For standards, 10 μ l of standard 1 and 2 mixed up with 190

ul master mix separately, then mixed by vortexing. For samples, each individual 2 µl of mRNA sample was added to 198 µl master mix. The total volume of each individual standards and samples were 200 µl. Samples were then quantified by a Qubit[®] 2.0 Fluorometer (Life Technologies, Paisley, UK).

3.14.4 Conversion adipose mRNA to cDNA

RNA was reversed transcribed (1 µg) to cDNA using a High Capacity Reverse Transcription Kit (Applied Biosystems, Warrington, UK). The protocol is described as follows. The required amount of Diethylpyrocarbonate (DEPC)-treated Water (Fisher Scientific, USA) was added to each tube using a pipette filter tip, followed by adding the correct volume of RNA samples. Mixed RNA samples were vortex briefly, then centrifuged to top speed. Reverse transcription (RT) mix was made up with fixed volumes of 10x RT buffer 2 µl, dNTP 0.8 µl, random primers 2 µl, multiscribe (enzyme) 1 µl and water 4.2 µl for per sample. An electronic multidispensing pipette was used to dispense 10 µl RT mix per sample, followed by centrifuging samples briefly to reach maximum speed to mix. RNA samples were vortex again, and then centrifuged to mix at top speed. Afterwards, RNA samples were placed in a heating block following the steps: 25 °C for 10 min (this step was completed after RT), 37 °C for 120 min and 85 °C for 5 min. After heating cycles, samples were centrifuged to reach top speed to get all the contents to the bottom of the tube. All samples were then stored at –80 °C for further analysis.

3.14.5 RT-PCR analysis

Real-time polymerase chain reaction (RT-PCR) was performed using a StepOne (Applied Biosystems). cDNA samples were vortex briefly and centrifuged at top speed to force all contents to the bottom of the tube. Assay mix was made up in a total volume

of 18 μl (10 μl mastermix 2x, 7 μl DEPC water and 1 μl gene specific assay mix) per cDNA sample. 2 μl cDNA sample was added to a 48 well MicroAmp[®] Reaction Plate (Applied Biosystems, China) in duplicate. 18 μl assay mix (Applied Biosystems, USA) was then added to each well and the plate was spun to top speed (4,000 rpm) for 2 min (each cDNA sample and assay mix was well mixed after this process). The plate was covered with optical seal (Applied Biosystems, USA). The plate was placed on an RT-PCR analyser, with the following cycles: 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 seconds and 60 °C for 1 min. The whole process lasted approximately 2 h.

3.14.6 Adipose tissue gene expression calculations

Peptidylpropyl isomerase A (PPIA) was used as an endogenous control (Neville *et al.*, 2011) for all three studies. The comparative threshold cycle (Ct) is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold. Throughout this thesis, Ct method was used to process data where $\Delta\text{Ct} = \text{Ct target gene} - \text{Ct PPIA}$. Ct target genes were normalised to an internal calibrator (lowest ΔCt for each target gene) and baseline. Samples outside the detectable limit ($\text{Ct} > 35$) were excluded from the analysis.

Chapter 4: Feeding influences adipose tissue responses to exercise in overweight men

4.1 Introduction

It has become clear in the last 10 years or so that adipose tissue plays an active role in many physiological processes and pathological states (Weisberg *et al.*, 2003; Xu *et al.*, 2003) and dysfunction within this tissue is characterised by poor metabolic control (e.g., tissue-specific insulin resistance, local inflammation, fibrosis, and the abnormal secretion of adipokines) (Langin *et al.*, 2009). Adipose tissue secretes dozens of mediators including the archetypal adipokines, adiponectin and leptin (Fain *et al.*, 2004a; Moro *et al.*, 2007; Lehr *et al.*, 2012). Adiponectin is exclusively derived from adipose tissue and circulates in high concentrations (10–20 mg·l⁻¹). In other cases, the quantitative amounts secreted by adipose can be substantial, for example, subcutaneous adipose contributes up to a third of circulating interleukin-6 (IL-6) (Mohamed-Ali *et al.*, 1997a).

Feeding has a pronounced effect on the whole-body metabolic responses to exercise and reduces the contribution of fat towards metabolism (Coyle *et al.*, 1985; Wu *et al.*, 2003; Backhouse *et al.*, 2007; Gonzalez *et al.*, 2013). In addition, feeding influences the skeletal muscle responses to various forms of exercise (Civitarese *et al.*, 2005; Cluberton *et al.*, 2005; De Bock *et al.*, 2005; Cheng *et al.*, 2009; Harber *et al.*, 2010; Dalbo *et al.*, 2013). For example, PDK4 is significantly up-regulated in muscle with exercise in fasted but not fed conditions (Cluberton *et al.*, 2005). Other feeding-related changes in gene expression in muscle after exercise have been reported including altered expression of GLUT4, PDK4, FAT/CD36, CPT-1, UCP3 and AMPK

(Civitarese *et al.*, 2005). Collectively, therefore, there is strong evidence that feeding affects the responses to exercise in skeletal muscle. Adipose tissue and skeletal muscle both play a crucial role to regulate energy metabolism during exercise (Frayn, 2010). This led us to speculate that pre-exercise feeding may also affect the adipose tissue responses to acute exercise.

During exercise, adipose tissue plays an important role in contributing energy for working skeletal muscle (Horowitz, 2003). Exercise also initiates a number of other responses in adipose tissue such as increased blood flow and altered expression of various adipokines within abdominal subcutaneous adipose tissue (Thompson *et al.*, 2012). It is possible that these acute exercise-induced changes could be part of the mechanism through which exercise improves health (Thompson *et al.*, 2012). However, all prior studies of adipose tissue responses to exercise in humans have been conducted in the fasted state (Keller *et al.*, 2003a; Keller *et al.*, 2003b; Holmes *et al.*, 2004; Keller *et al.*, 2005; Frydelund-Larsen *et al.*, 2007; Hojbjerre *et al.*, 2007; Leick *et al.*, 2007; Christiansen *et al.*, 2013b). The effect of feeding status on the response of human adipose tissue to exercise has never been studied. This is despite the fact that feeding has a profound effect on adipose tissue (Alligier *et al.*, 2012; Johannsen *et al.*, 2014) and that we spend the vast majority (~70%) of a 24-h period in a fed state (Ruge *et al.*, 2009). With this in mind, it is important to understand whether feeding alters the response of adipose tissue to physical activity. The aim of this study was therefore to investigate whether feeding influences the adipose tissue responses to exercise.

4.2 Methods

4.2.1 Experimental design

Ten men aged 18 to 35 years with increased central adiposity were recruited via local advertisement. Participants attended the laboratory on three occasions for initial assessment of maximum oxygen uptake ($\dot{V}O_{2\max}$) and two subsequent main trials. The trial days involved walking for 60 min at 60% $\dot{V}O_{2\max}$ under either FASTED or FED conditions in a randomised, counterbalanced design separated by a 3–4 week wash-out period. Blood and adipose tissue were sampled at baseline and after exercise to examine the impact of prior feeding. The protocol was approved by Bristol Research Ethics Committee (REC reference number: 13/SW/0020). This trial is registered at ClinicalTrials.gov (ID: NCT02870075), and all participants provided verbal and written informed consent before taking part.

4.2.1.1 Inclusion and exclusion criteria

To be eligible to take part, participants were required to be overweight with a waist circumference of 94–128 cm (WHO, 2008). Participants were also required to be weight stable (no change in mass \pm 3%) (Stevens *et al.*, 2006) for at least 3 months. Participants completed a health questionnaire (Appendix 1) to exclude any existing cardiovascular and metabolic diseases and a Physical Activity Readiness Questionnaire (PAR-Q) (Appendix 2) to make sure that participants were able to exercise safely. Individuals taking any medications known to influence lipid/carbohydrate metabolism or immune function and smokers were excluded.

4.2.1.2 Participants' physical characteristics

A summary of participants' physical characteristics is shown in Table 4.1.

Table 4.1 Participant physical characteristics (n = 10)

Characteristics	Mean \pm SD
Age (years)	26 \pm 5
Body mass (kg)	102.4 \pm 10.6
Waist circumference (cm)	105 \pm 10
Hip circumference (cm)	115 \pm 6
Body mass index (kg·m ⁻²)	30.2 \pm 3.7
Fat in L1-L4 region (kg)	3.4 \pm 1.5
$\dot{V}O_{2\max}$ (ml·kg ⁻¹ ·min ⁻¹)	42.4 \pm 6.4
Systolic blood pressure (mmHg)	132 \pm 21
Diastolic blood pressure (mmHg)	73 \pm 12

4.2.2 Pre-trial assessments

4.2.2.1 $\dot{V}O_{2\max}$ measurement

An incremental continuous treadmill test until the point of volitional fatigue was used to determine $\dot{V}O_{2\max}$. For most participants, a treadmill speed of 4 km·h⁻¹ and gradient of 8.5% was appropriate. Participants were tested at this speed and gradient with the speed increased by 1 km·h⁻¹ after every 3 min stage. One minute expired air samples were collected into Douglas bags (Hans Rudolph, MO, USA) and rate of perceived exertion (RPE) and heart rate were measured in the final minute of each stage and also at the point of volitional fatigue, defined as when the participant indicated that only 1 min remained until fatigue. Samples were analysed for relative expired fractions of

oxygen and carbon dioxide gas (Servomex, Crowborough, UK) and the total volume within the bag was measured using a dry gas meter (Harvard Apparatus, Kent, UK).

4.2.2.2 Physical activity assessment

As part of the pre-trial assessments, participants wore a combined heart rate/accelerometer monitor for one week to assess their habitual physical activity energy expenditure (Actiheart, Cambridge Neurotechnology Ltd., Cambridge, UK). This was attached to the chest via 2 adhesive ECG pads for 24 h per day except for during showering/bathing/swimming (Thompson *et al.*, 2006).

4.2.2.3 Body composition analysis

Body mass was measured using digital scales following an overnight fast and post-void (TANITA corp., Tokyo, Japan). Waist and hip circumference was assessed according to World Health Organisation guidelines (WHO, 2008). Body composition was determined using Dual Energy X-ray Absorptiometry (DEXA; Discovery, Hologic, Bedford, UK). Abdominal subcutaneous and visceral adipose tissue mass was estimated from a central region between L1-L4 (Glickman *et al.*, 2004).

4.2.3 Trial days

In the 72 h prior to each main trial, participants were asked to refrain from performing any strenuous physical activity and from consuming alcohol/caffeine for 48 h prior to the main trials. A dietary record was completed 48 h before the first main trial and participants replicated this diet prior to their second main trial.

On main trial days, participants arrived at the laboratory between 08:00–09:00 am following a 12 h fast. After anthropometric measurements, participants rested on a bed

for 15 min, followed by four 5-min expired gas sample collections to determine resting metabolic rate (RMR) (Compher *et al.*, 2006; Betts *et al.*, 2011) using substrate oxidation under resting conditions (Frayn, 1983b) and during exercise (Jeukendrup & Wallis, 2005) (Figure 4.1).

After RMR assessment, a cannula was inserted into an antecubital forearm vein and a baseline venous blood sample was taken and allocated into tubes with either EDTA or serum separation beads (Sarstedt Ltd, Leicester, UK). Plasma samples were centrifuged immediately at 5,000 rpm at 4 °C for 10 min. Serum samples were left for 45 min to clot before centrifugation. Subcutaneous adipose tissue samples (~1 g) were taken under local anaesthetic (1% lidocaine) from the area around the waist approximately 5 cm lateral to the umbilicus with a 14 G needle using an aspiration technique (Walhin *et al.*, 2013) followed by adipose tissue cleaning and processing as described previously (Travers *et al.*, 2015a).

The study protocol is shown in Figure 4.1 and the protocol details are described as follows. Participants then either consumed a meal (FED) or remained fasting (FASTED) and cannula blood samples were taken every 15 min for the following 60 min. A further blood sample was collected at 120 min immediately before the walking protocol. In both the FASTED and FED treatments, participants walked on the treadmill at 60% $\dot{V}O_{2max}$ for 60 min and one minute of expired air samples, RPE and heart rate were collected at 5, 20, 40 and 60 min. After finishing exercise, another blood sample was immediately collected and participants then rested for a further 60 min. At this point, a second adipose tissue and final blood sample were taken.

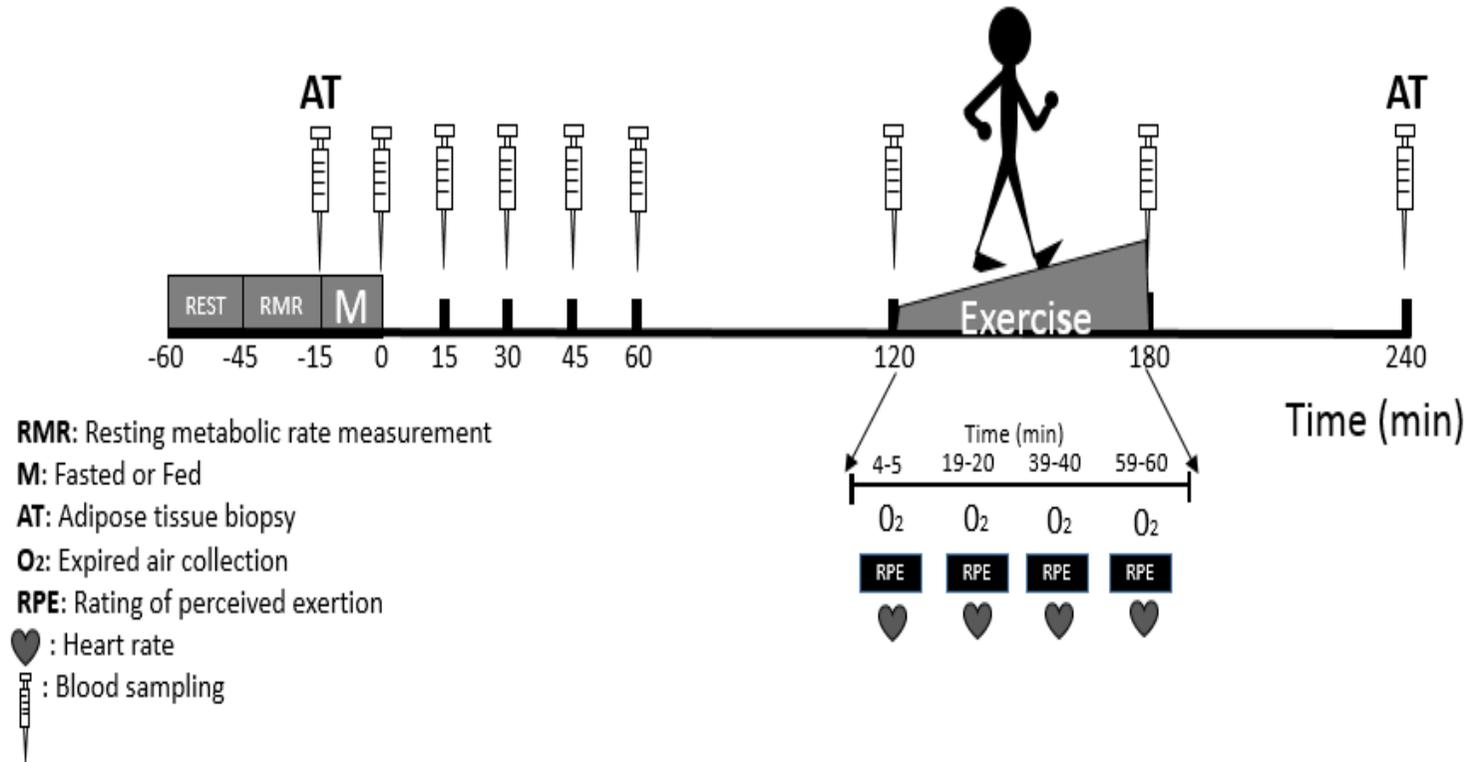


Figure 4.1 Experimental protocol

4.2.3.1 Meal

The meal in the FED trial was provided as previously described by Chowdhury *et al.* (2015). The total energy provided was 648 ± 115 kcal (CHO 120.1 ± 21.3 g, fat 12.7 ± 2.3 g and protein 20.9 ± 3.7 g) (mean \pm SD). The meal included white bread (Brace's thick white), cornflakes (Kellogg's cornflakes), semi-skimmed milk (Sainsbury; British semi skimmed milk), orange juice (Sainsbury; 100% pure squeezed smooth orange juice), spread (Unilever; I can't believe its not butter), jam (Sainsbury; strawberry jam) and sugar (Sainsbury; British white granulated sugar). Participants were asked to consume the meal within 15 min. In the FASTED trial, participants sat quietly for a 15-min period.

4.2.4 Experimental analysis

4.2.4.1 Adipose tissue culture and gene expression

After cleaning the adipose tissue biopsy, tissue was minced to approximately 5–10 mg and 400 mg was directly placed in sterile culture plates in quadruplicate (Nunc, Roskilde, Denmark) with endothelial cell basal media (ECBM) (Promocell, Germany) containing 0.1% fatty acid-free bovine serum albumin $100 \text{ U}\cdot\text{ml}^{-1}$ penicillin and $0.1 \text{ mg}\cdot\text{ml}^{-1}$ streptomycin (Sigma-Aldrich, Gillingham, UK). Adipose tissue was incubated with a final ratio of 100 mg tissue per 1 ml ECBM media for 3 h (Travers *et al.*, 2015b) at $37 \text{ }^\circ\text{C}$ in a 5% CO_2 and $95 \pm 5\%$ relative humidity incubator (MCO-18A1C CO_2 incubator; Sanyo, Osaka, Japan). After the 3-h incubation, media was transferred to sterile tubes and stored at $-80 \text{ }^\circ\text{C}$. Adipokine secretion from adipose explants was normalised to L1-L4 fat mass as described (Travers *et al.*, 2015a). The remaining adipose tissue (approximately 200 mg) was homogenised in 5 ml Trizol (Invitrogen,

Paisley, UK) in an RNase/DNase-free sterile tube (Invitrogen, Paisley, UK) and stored at -80°C before gene expression and protein analysis.

4.2.4.2 Real-time PCR

An RNeasy Mini Kit (Qiagen, Crawley, UK) was used to extract RNA from adipose tissue as described (Walhin *et al.*, 2013). Tissue samples were further quantified using a Qubit 2.0 fluorimeter (Life Technologies, Paisley, UK). RNA was reversed transcribed (1 μg) to cDNA using a High Capacity Reverse Transcription Kit (Applied Biosystems, Warrington, UK). Organic phenol-chloroform phase from the RNA extraction was kept for further protein analysis. Real-time PCR was performed using a StepOne (Applied Biosystems). Predesigned primers and probes were obtained from Applied Biosystems for the measurement of expression of interleukin 6 (IL-6) (Hs00985639_m1), adiponectin (Hs00605917_m1), leptin (Hs00174877_m1), interleukin 18 (IL-18) (Hs00155517_m1), tumour necrosis factor alpha (TNF- α) (Hs99999043_m1), monocyte chemoattractant protein-1 (MCP-1) (Hs00234140_m1), 5' AMP-activated protein kinase (AMPK) (Hs01562315_m1 and Hs00178903_m1 combined), glucose transporter type 4 (GLUT4) (Hs00168966_m1), hormone-sensitive lipase (HSL) (Hs00193510_m1), insulin receptor substrate 1 (IRS1) (Hs00178563_m1), insulin receptor substrate 2 (IRS2) (Hs00275843_s1), sterol regulatory element binding protein 1c (SREBP-1c) (Hs01088691_m1), pyruvate dehydrogenase kinase isozyme (PDK4) (Hs00176875_m1), peroxisome proliferator-activated receptor γ (PPAR γ) (Hs01115513_m1), peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 α) (Hs01016719_m1), RAC-alpha serine/threonine-protein kinase (AKT1) (Hs00178289_m1), adipose triglyceride lipase (ATGL) (Hs00386101_m1), fatty acid translocase (FAT)/CD36 (Hs00169627_m1), forkhead box protein O1 (FOXO1) (Hs01054576_m1), hexokinase 2 (HK2) (Hs00606086_m1), PI3K-85 α

(PIK3R1) (Hs00933163_m1), carnitine palmitoyltransferase 1B (CPT1B) (Hs03046298_s1), The G0/G1 switch gene 2 (G0S2) (Hs00605971_m1), peptidylpropyl isomerase A (PPIA) was used as an endogenous control (Neville *et al.*, 2011). The comparative cycle threshold (Ct) method was used to process data where $\Delta Ct = Ct \text{ target gene} - Ct \text{ PPIA}$. Ct target genes were normalised to an internal calibrator (lowest ΔCt for each target gene) and baseline. The Ct values for IL-6 (31 out of 40 samples), TNF- α (16 out of 40 samples), and IL-18 (37 out of 40 samples) were frequently over 35 and thus these results are not included.

4.2.4.3 Western blotting

The adipose tissue protein fraction was isolated from the TRIzol phenol-chloroform phase following the manufacturer's protocol (TRIzol Reagent, Life Technologies). Briefly, 1 ml of organic phase was mixed with 1.5 ml isopropanol. After mixing, the samples were incubated for 10 min at room temperature, followed by 10 min centrifugation at 12,000 g at 4 °C to pellet the protein. One millilitre of protein pellet was washed using 2 ml of 0.3 M guanidine hydrochloride in 95% ethanol for 20 min incubation followed by centrifugation at 7,500 g for 5 min at 4 °C. This process was repeated 3 times. After finishing the washing procedure, 2 ml of 100% ethanol was added to the protein pellet for a further 20 min incubation at room temperature before being centrifugation. The pellet was then left to air dry for 5–10 min. Then, 200 μl of 1% SDS was added to resuspended the pellet. The protein content of the samples was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Waltham, USA). Proteins (25 $\mu\text{g}\cdot\text{lane}^{-1}$) were separated by SDS-PAGE and transferred using a semidry electro-transfer method to a nitrocellulose membrane. Western blotting analysis was performed with the following antibodies: Akt2/PKB β (Millipore), AMPK (Cell Signalling Technology, USA), GLUT4 (Holman *et al.*, 1990), IRS1 (Millipore),

IRS2 (Millipore), PDK4 (ABGENT, San Diego, USA), HSL (Cell Signalling Technology, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Proteintech, USA). The images were acquired in an EPI Chemi II darkroom (UVP) and bands quantified using VisionWorks LS analysis software (UVP).

4.2.4.4 Biochemical analysis

Plasma glucose and non-esterified fatty acids (NEFA) were measured using commercially available assay kits and analyser (Daytona Rx; Randox, Crumlin, UK). Serum insulin (Merckodia, Uppsala, Sweden) and both serum and adipose secretion of IL-6, leptin and adiponectin (R&D systems) were measured using Enzyme-linked immunosorbent assay (ELISA).

4.2.5 Statistics

Descriptive data are presented as means \pm standard deviation (SD). All other data are presented as means \pm standard error of the mean (SEM). Incremental area under curve (iAUC) was calculated using the trapezoid method (Wolever & Jenkins, 1986). All data were checked for normality using visual checking of histograms, confirmed statistically by Shapiro-Wilk test. Non-normally distributed and ordinal data (e.g. RPE) were analysed using the equivalent non-parametric tests. The differences between trials in iAUC, gene expression and Western blotting outcomes were analysed using paired *t*-tests. Glucose, insulin, NEFA, circulating adipokines and adipose cell culture outcomes were analysed using a two-way ANOVA with repeated measures using SPSS version 22 (IBM, Armonk, NY, USA). Where significant interactions (trial \times time) were found, post hoc *t*-tests with the Holm–Bonferroni correction were used (Atkinson, 2002). Statistical significance was set at $p \leq 0.05$.

4.3 Results

4.3.1 Energy expenditure and substrate oxidation during FASTED and FED trials

There were modest feeding-induced differences between trials for relative exercise intensity ($\% \dot{V}O_{2\max}$), RPE, energy expenditure and substrate oxidation (Table 4.2). Pre-fed RMR was not different ($2,103 \pm 418$ versus $2,058 \pm 365$ kcal·d⁻¹ in FASTED and FED trials, respectively).

Table 4.2 Physiological responses during 60 min exercise

	FASTED	FED
Treadmill speed (km·h ⁻¹)	5.7 ± 0.2	5.7 ± 0.2
$\dot{V}O_{2\max}$ (%) *	59 ± 1	60 ± 1
Heart Rate (beat·min ⁻¹)	155 ± 4	155 ± 4
RPE (6–20)	12 ± 1	12 ± 1
Energy expenditure (kcal·h ⁻¹) *	746 ± 41	771 ± 43
RER ($\dot{V}CO_2:\dot{V}O_2$) *	0.93 ± 0.01	0.97 ± 0.01
Carbohydrate oxidation (g·h ⁻¹) *	147 ± 13	167 ± 12
Fat oxidation (g·h ⁻¹) *	16 ± 3	9 ± 2

Values are means ± SEM. * denotes significantly different between FASTED *versus* FED conditions ($p \leq 0.05$) (n = 10).

4.3.2 Plasma glucose, NEFA and serum insulin concentrations

Trial \times Time interaction effects were found for blood glucose, insulin and NEFA between FASTED and FED trials ($p = 0.026$, $p = 0.003$ and $p = 0.001$, respectively). As expected, iAUCs for glucose and insulin were elevated for all parameters in the FED trial ($p = 0.02$ and $p = 0.03$, respectively) (Figure 4.2b & d). Peak glucose and insulin concentrations in the FED trial were reached 15 min post-meal ($7.1 \pm 0.3 \text{ mmol}\cdot\text{l}^{-1}$ and $370 \pm 95 \text{ pmol}\cdot\text{l}^{-1}$, $p = 0.001$, Figure 4.2a; $p = 0.03$; Figure 4.2c, respectively). NEFA concentrations were lower at most time points in the FED trial (Figure 4.2e).

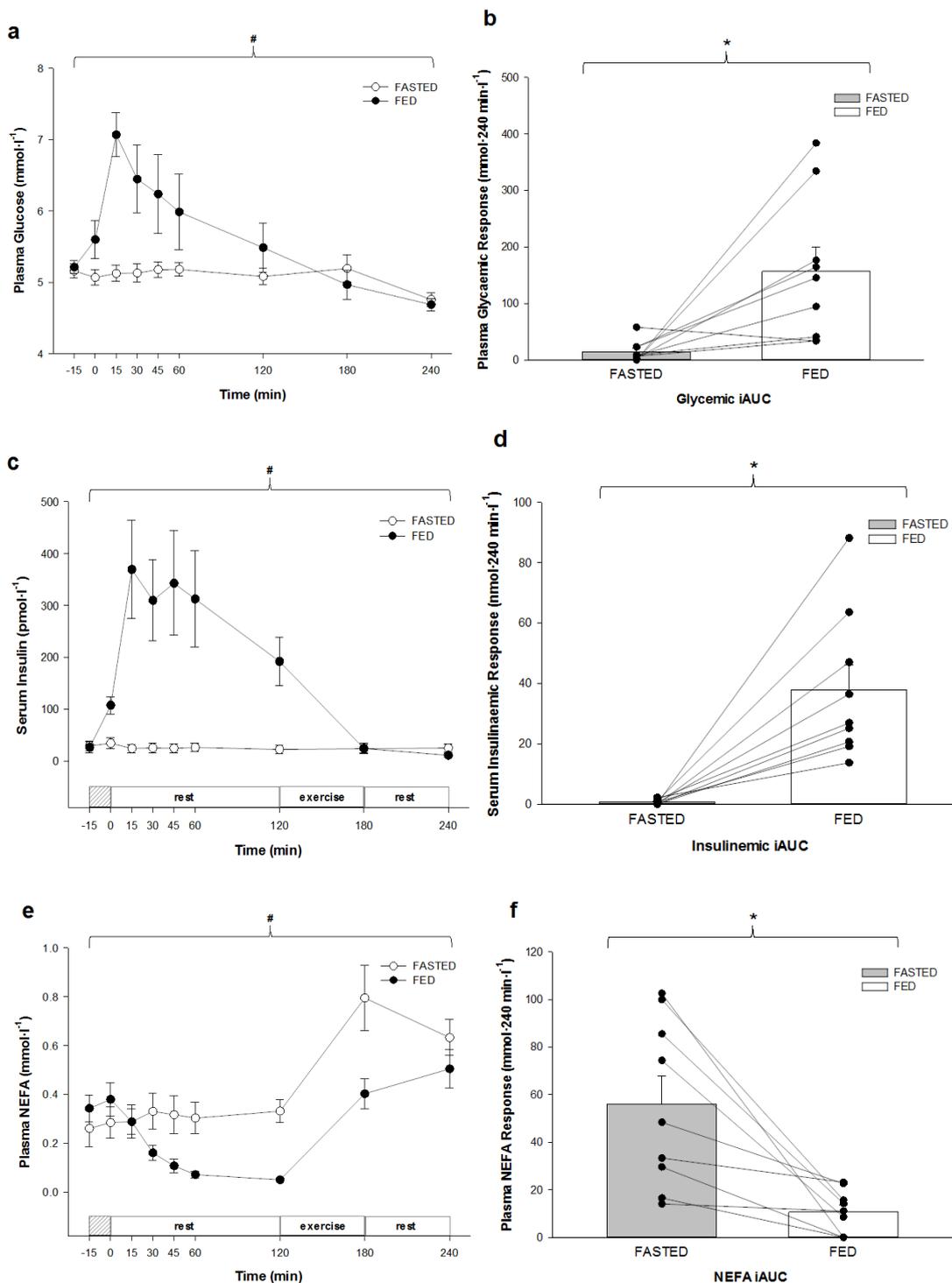


Figure 4.2 Plasma glucose, NEFA and serum insulin concentrations.

Glucose (a), insulin (c) and NEFA (e) concentrations in FASTED and FED trials and iAUC for glucose (b), insulin (d) and NEFA (f). In both FASTED and FED trials (n = 9), due to difficulty in cannulating one participant. Values are means ± SEM. # denotes significant interaction effect under both FED and FED trials. * denotes significantly different between FASTED *versus* FED ($p \leq 0.05$). The shaded box in (a), (c) and (e) denotes meal time.

4.3.3 Adipose tissue mRNA expression

There was a difference in adipose tissue mRNA levels of PDK4, ATGL, HSL, FAT/CD36, GLUT4 and IRS2 (all $p \leq 0.05$) when exercising under FASTED vs FED conditions (Figure 4.3). Expression of PDK4 (1.68-fold), ATGL (1.19-fold), HSL (2.09-fold) and FAT/CD36 (1.19-fold) were up-regulated under FASTED conditions and expression of PDK4 (0.77-fold) and IRS2 (0.81-fold) were down-regulated under FED conditions. The expression of the remaining genes was not significantly different between trials.

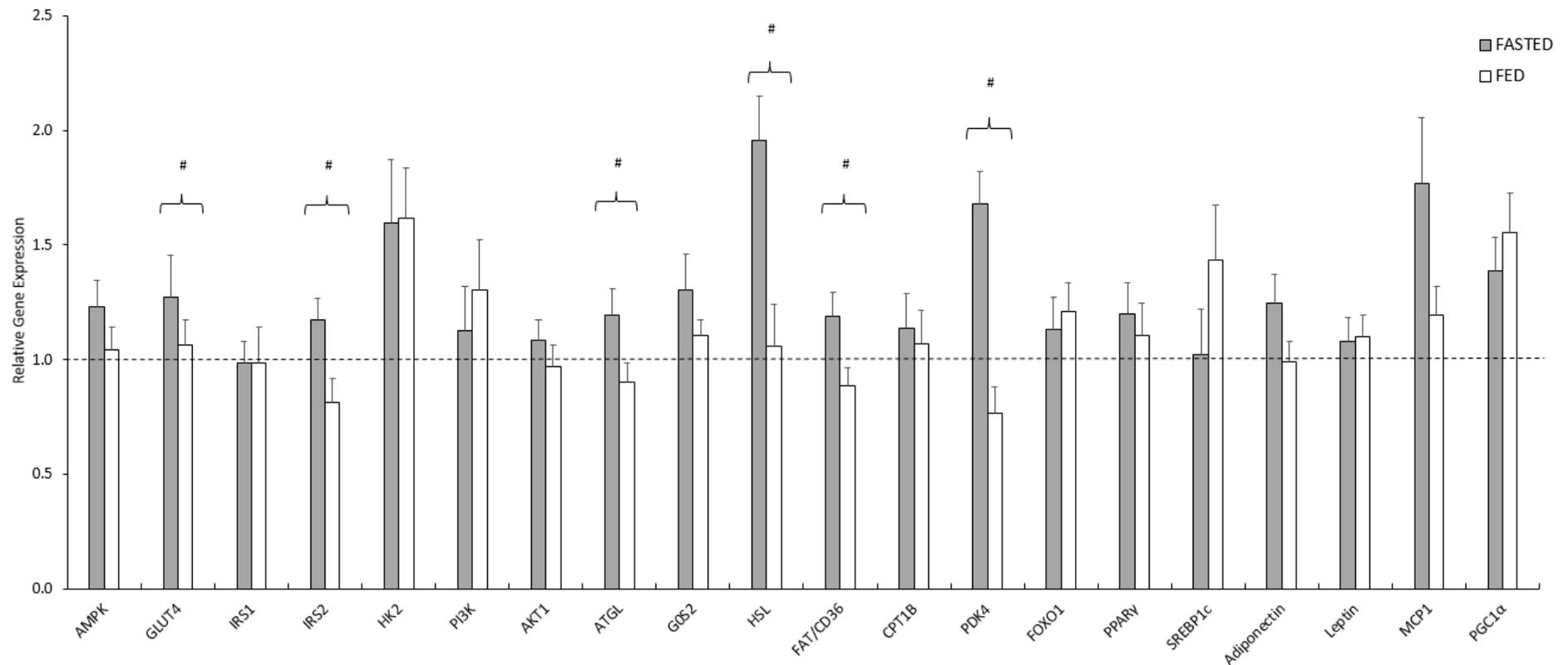


Figure 4.3 Fold changes in relative adipose tissue gene expression (mRNA levels) under FASTED and FED trial

Fold changes in relative gene expression (mRNA levels) of metabolism, insulin signaling, glucose transport and inflammatory genes in adipose tissue under FASTED and FED trials (all $n = 10$, except for HSL and GOS2 $n = 9$ owing to $Ct > 35$ for one participant). The dashed line indicates baseline. Data normalised to PPIA, internal calibrator and baseline. Samples that exceeded the detectable limit ($Ct > 35$) were excluded from the analysis. Values are means \pm SEM. # $p \leq 0.05$ between FASTED and FED trials.

4.3.4 Adipose tissue protein expression

There was a difference in IRS2 protein expression between trials ($p \leq 0.05$), showing approximately a 2-fold increase in FASTED conditions, and no change in FED conditions. The change in the expression of the remaining proteins was not statistically different between FASTED and FED conditions (Figure 4.4).

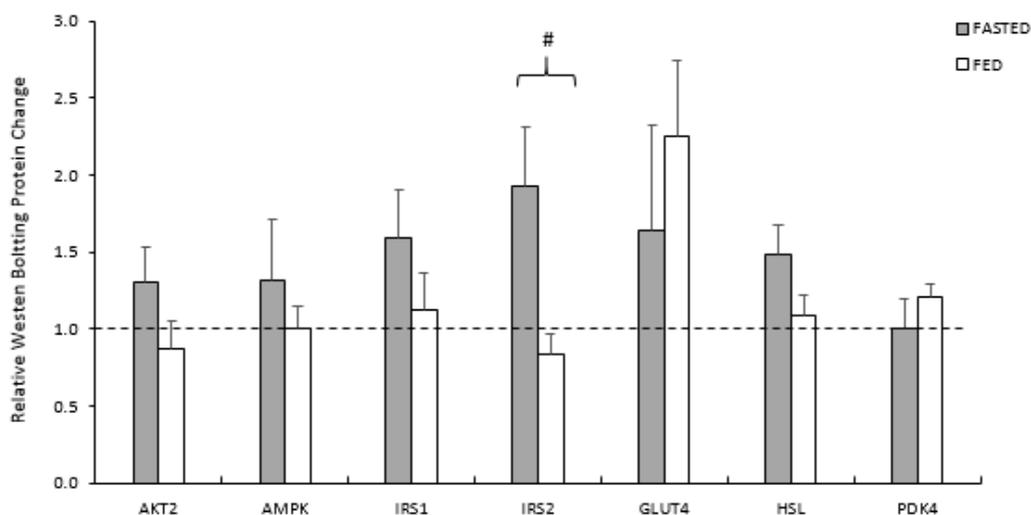


Figure 4.4 Fold changes in relative proteins levels in adipose tissue under FASTED and FED trials. The dashed line indicates baseline. Data were normalised to GAPDH. Values are means \pm SEM. # $p \leq 0.05$ between FASTED and FED trials ($n = 8$).

The representative image of Western blotting is shown on Figure 4.5 (IRS1/2, AKT2 and AMPK represents participant number 2 and 8. GLUT4, PDK4 and HSL represents participant number 3 and 4)

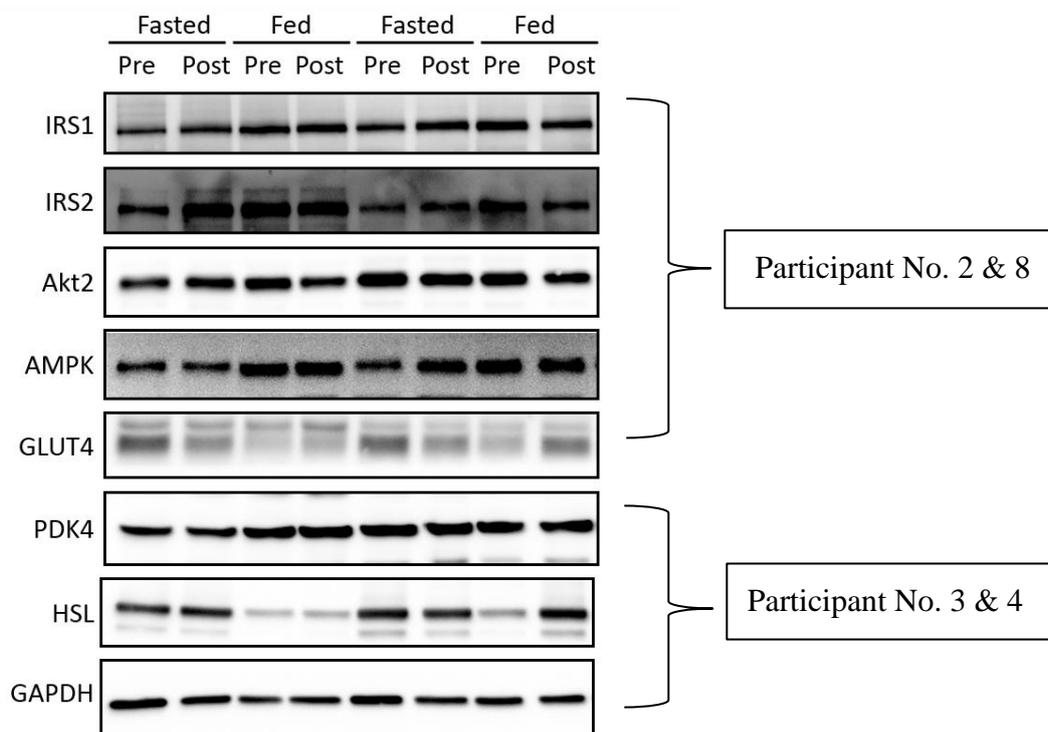


Figure 4.5 Representative images of Western blotting results in adipose tissue under FASTED and FED trials using a density scan method.

4.3.5 Serum adipokine concentrations and adipose tissue secretion *ex vivo*

Serum IL-6 increased (Figure 4.6 a) and serum leptin decreased (Figure 4.6 c) in both trials ($p < 0.05$). Time effects were also identified for adiponectin (Figure 4.6 e) ($p < 0.05$). No interaction effects were identified for IL-6, leptin and adiponectin. Furthermore, no time or time \times trial interactions were identified for *ex vivo* adipose explant secretion of IL-6, leptin and adiponectin in response to exercise under FASTED and FED trials (Figure 4.6)

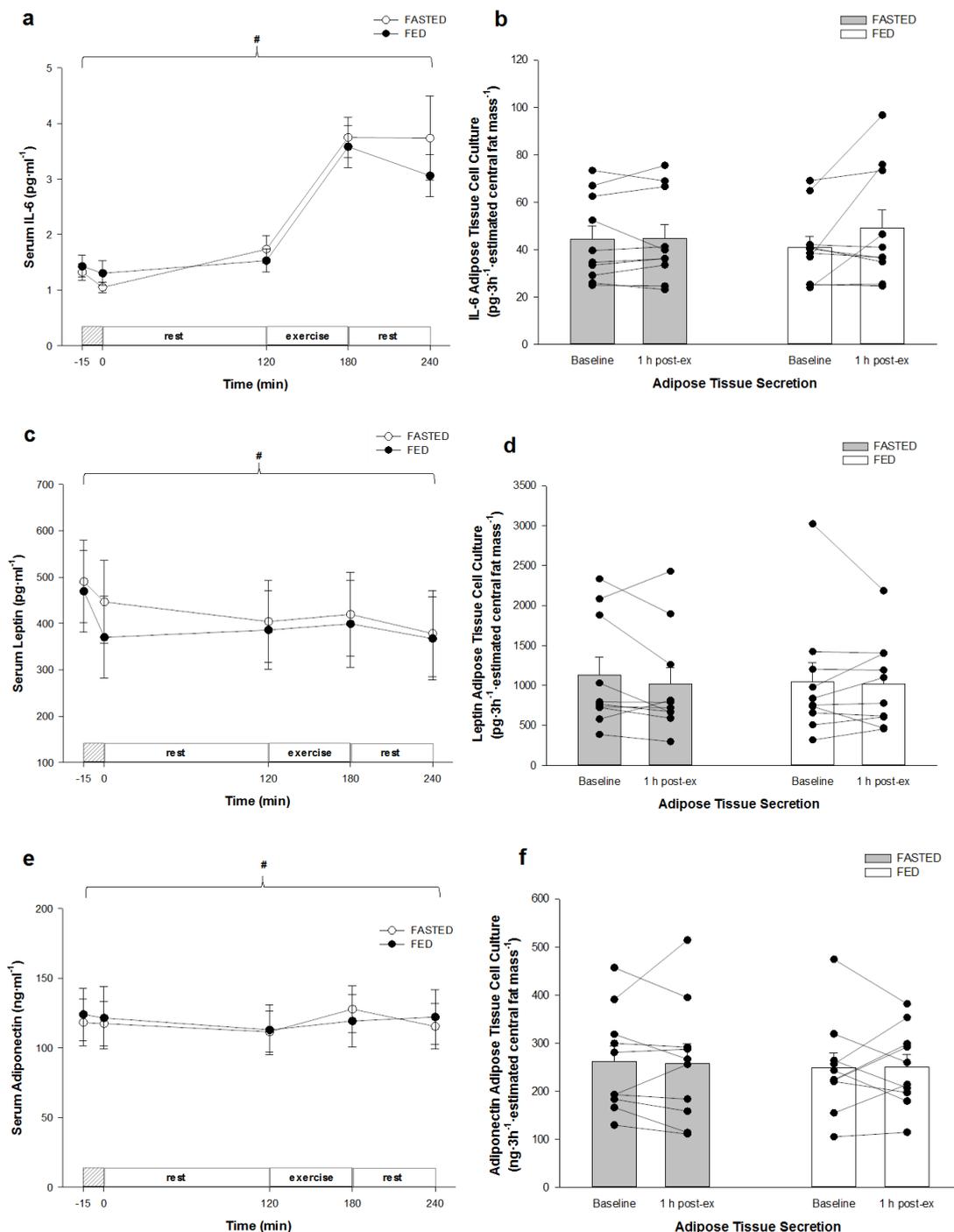


Figure 4.6 Serum adipokine concentrations and adipose tissue secretion *ex vivo*. Circulating serum IL-6 (a) leptin (c) and adiponectin (e) concentrations in FASTED and FED trials (n = 9). *Ex-vivo* adipose tissue explant protein secretion of IL-6 (b), leptin (d) and adiponectin (f) at baseline and 1 h post-exercise expressed relative to L1-L4 fat mass (n = 10). Values are means ± SEM. The shaded box in (a), (c) and (e) denotes meal time. # p ≤ 0.05 denotes time effect.

4.4 Discussion

This study presents the first evidence that feeding status alters the human adipose tissue response to exercise. Exercise in the fasted-state altered the expression of genes involved in lipid metabolism, insulin signaling and glucose transport, whereas exercise in the fed-state did not. The divergent responses to exercise performed under different feeding status may have implications for adaptation to regular exercise.

In the present study, our feeding protocol successfully manipulated systemic concentrations of glucose and insulin. In the two hours prior to exercise, there was a 168-fold and 26-fold difference in insulin and glucose iAUC, respectively. As anticipated with this study design, prior feeding increased relative carbohydrate utilisation and decreased fat oxidation during exercise (Coyle *et al.*, 1985; Wu *et al.*, 2003; Backhouse *et al.*, 2007; Gonzalez *et al.*, 2013). Thus, exercise in FED and FASTED trials was performed in a very different physiological state.

At the gene expression level, we found that adipose tissue responds differently to moderate-intensity exercise under FASTED and FED conditions, with altered expression of PDK4, ATGL, HSL, FAT/CD36, GLUT4 and IRS2. These results indicate that exercise when fasting leads to changes in adipose tissue gene expression, whereas exercise in the fed state does not elicit such changes. Over time, acute differences in skeletal muscle gene expression with exercise conducted in the fasted *versus* the fed state have been proposed to contribute to diverse physiological adaptations (De Bock *et al.*, 2008; Stannard *et al.*, 2010; Van Proeyen *et al.*, 2010; Van Proeyen *et al.*, 2011). Our data demonstrate that feeding status also alters adipose tissue responses to an acute bout of exercise and thus it is also possible that feeding status could affect the adipose tissue response to long-term exercise training.

We found a difference in adipose IRS2 protein content although the other measured proteins were not affected. This could be partly explained by the fact that our post-exercise biopsy was taken one hour after exercise had finished. Depending on the kinetics of each response, this sample timing framework will be appropriate for some outcomes and inappropriate for others. We should also highlight that our protein measurements represent only total protein content. Repeated small changes in adipose protein synthesis are likely lead to accumulated differences and functional changes in adipose phenotype over time (Booth & Neuffer, 2005; Perry *et al.*, 2010). Clearly, the only way to know if the acute changes observed in the current study translate into long term differences is to examine whether chronic training conducted in the fasted versus postprandial state leads to divergent adaptations.

4.4.1 Carbohydrate and lipid metabolism in adipose tissue

A primary function of PDK4 is to regulate glucose metabolism by inhibiting pyruvate dehydrogenase complex activity. Fasting and exercise increase PDK4 mRNA expression in skeletal muscle (Pilegaard *et al.*, 2000; Pilegaard *et al.*, 2003a) and insulin suppresses PDK4 mRNA and protein content in skeletal muscle (Majer *et al.*, 1998; Lee *et al.*, 2004). It is possible that insulin could be responsible for the lower adipose PDK4 mRNA response in the FED trial, although higher NEFA in the FASTED trial could also have increased PDK4 expression (Arkininstall *et al.*, 2004; Kiilerich *et al.*, 2010). Other feeding-related studies have shown that muscle PDK4 mRNA expression remained unchanged 1–4 h after exercise in the fed state (with lower NEFA concentrations), when compared with exercise in the fasted state (Civitarese *et al.*, 2005; Cluberton *et al.*, 2005). Thus, exercise in the fasted state appears to increase PDK4 expression in both muscle and adipose, whereas exercise in the fed state does not.

ATGL and HSL mobilise stored fat and release it into the blood (Zimmermann *et al.*, 2004; Holloway *et al.*, 2008; Lafontan & Langin, 2009). In the current study, gene expression of ATGL, HSL and FAT/CD36 in adipose were all increased in FASTED but not FED conditions. These differences were associated with higher fat oxidation during FASTED exercise (9 ± 6 versus 16 ± 8 g·h⁻¹ in FED and FASTED trials, respectively). Other studies have also shown similar responses in fasted exercise with higher fat oxidation and increased skeletal muscle FAT/CD36 gene expression (Civitarese *et al.*, 2005). Moreover, these findings are consistent with the previously observed increase in adipose HSL activity reported during cycling exercise, which is blunted with nicotinic acid ingestion (Watt *et al.*, 2004). Our results indicate that exercise in the fed state does not lead to a change in gene expression for lipid mobilisation and transport pathways in adipose tissue and, at a conceptual level, feeding seems to blunt the exercise-induced stimulus on adipose tissue.

We found higher GLUT4 mRNA and higher IRS2 mRNA and protein in adipose in the FASTED trial, compared to the FED trial. There are several explanations for this finding. One potential explanation is that glucose is required as precursor for oxaloacetic acid for lipid oxidation (Rui, 2014). Glucose is also essential for triglyceride synthesis (Nye *et al.*, 2008) and, because samples were taken one hour after exercise, it is possible that our sampling framework captures the start of this process in adipose tissue after exercise. Interestingly, our findings in adipose mirror those reported in skeletal muscle, where an increase in GLUT4 mRNA in fasted but not fed conditions has been reported after exercise (Civitarese *et al.*, 2005; Cluberton *et al.*, 2005).

PGC-1 α mRNA is a transcriptional coactivator involved in mitochondrial biogenesis (Wu *et al.*, 1999; Scarpulla *et al.*, 2012). Acute exercise increases PGC-1 α mRNA

expression in human skeletal muscle (Pilegaard *et al.*, 2003b; Psilander *et al.*, 2013; Norheim *et al.*, 2014; Gidlund *et al.*, 2015) and in rodent white adipose tissue (Sutherland *et al.*, 2009). However, the impact of feeding status and/or carbohydrate variability prior to exercise on skeletal muscle PGC-1 α mRNA is controversial. Some studies indicate that PGC-1 α mRNA expression is up-regulated whether acute exercise was in fed or fasted conditions (Cluberton *et al.*, 2005; Mathai *et al.*, 2008) but other studies show that higher carbohydrate availability prior to exercise blunts PGC1- α mRNA expression in skeletal muscle both at rest and post-exercise (Bartlett *et al.*, 2013; Psilander *et al.*, 2013). In the present study, the increase in adipose PGC-1 α mRNA expression was unaffected by feeding status. Chronic training studies indicate that adipose PGC1- α mRNA is increased in humans (Ruschke *et al.*, 2010) and rodents (Sutherland *et al.*, 2009; Trevellin *et al.*, 2014) and this has also been reported to increase PGC1- α protein content and mitochondrial biogenesis in rodents (Trevellin *et al.*, 2014). Whether the present results indicate an exercise-induced increase in mitochondrial biogenesis in human adipose tissue is unclear at the present time.

4.4.2 Adipokines response to exercise and impact of feeding status

Previous studies have shown that circulating adipokine concentrations are affected by acute exercise (Fischer, 2006; Christiansen *et al.*, 2013a) and energy consumption during exercise alters these systemic responses (Starkie *et al.*, 2001; Lancaster *et al.*, 2005; Robson-Ansley *et al.*, 2009). However, evidence from studies that have manipulated feeding status *prior to* exercise is scarce. Zoladz *et al.* (2005) found no difference in circulating IL-6 and leptin after a single bout of exercise, in a fed or fasted state; however, the duration of exercise lasted only 12 min and this might be insufficient to examine the notion that feeding status influences circulating adipokines. However, our exercise was 60 min long, and we too found no evidence that pre-exercise feeding

affects circulating IL-6, leptin and adiponectin. This may be due to the fact that moderate intensity exercise has only a modest effect on many of these parameters and thus there is little potential for feeding to interact with exercise and exert an effect (Markovitch *et al.*, 2008). Serum IL-6 was increased over time in both fasted and fed trials in the present study, which could be partly due to the effect of exercise (Petersen & Pedersen, 2005), but it is also possible that this is a product of prolonged cannulation (Dixon *et al.*, 2009).

4.4.3 Temporal and population-specific considerations

This study is the first to examine the impact of feeding on adipose tissue responses to exercise. In this study, we recruited overweight participants and this focus is a strength given that ~62% of the UK population are overweight (Health and Social Care Information Centre, 2012). Increased adiposity has a profound effect on adipose tissue function (e.g., a down regulation of GLUT 4 mRNA (Travers *et al.*, 2015a) and reduction in postprandial adipose tissue blood flow (McQuaid *et al.*, 2011) and this is an important consideration when interpreting these findings. We should also highlight that in the current study we were limited to only two adipose tissue biopsies due to potential interference from repeated sampling (Dordevic *et al.*, 2015) and so we do not have a full and complete time course. In the absence of an adipose biopsy immediately prior to exercise, it is hard to establish whether some effects in the FASTED trial are due to the modestly extended fasting period or due to exercise (or the interaction between fasting and exercise). What is apparent, however, is that exercise in FED conditions does not lead to the same changes as exercise in FASTED conditions.

4.5 Conclusion

This study provides the first evidence that the feeding status in which exercise is performed alters the response of adipose tissue in overweight humans. Feeding prior to exercise suppresses the expression of genes involved in lipid metabolism, insulin signaling and glucose transport in subcutaneous abdominal adipose tissue. Given the potential role for adipose tissue in health, regular acute changes in adipose tissue gene expression with exercise in a fasted state could potentially impact upon the accumulated long-term responses to an exercise programme.

Chapter 5: The effect of breaking prolonged sitting on metabolism and adipose tissue in middle-age overweight men and women

5.1 Introduction

In the past few decades, traditional exercise guidance has focused on the prescription of structured physical activities such as performance of prolonged moderate intensity exercise lasting longer than 30 min. This form of physical activity has been shown to improve physical fitness and decrease cardiovascular risk factors, along with increased insulin sensitivity, glucose tolerance, adiponectin concentrations (etc) (Stallknecht *et al.*, 2000; Akbarpour, 2013; Sakurai *et al.*, 2013). Unfortunately, many people struggle to undertake such structured exercise and alternative complimentary strategies are required.

In modern society, people spend much of their time using a computer, watching TV and so on causing sedentary behaviour or prolonged sitting to become increasingly common. Sedentary behaviour has been defined as step counts lower than 5,000 steps a day (Tudor-Locke & Bassett, 2004; Tudor-Locke *et al.*, 2013) or only undertaking activities where energy expenditure is less than 1.5 metabolic equivalents (METs) (Pate *et al.*, 2008; Owen *et al.*, 2010). In addition to causing a positive energy balance and weight gain, several studies have shown that increased sedentary behaviour is significantly associated with increased risk of abnormal glucose metabolism, metabolic syndrome, type 2 diabetes and cardiovascular disease (Ford *et al.*, 2005; Healy *et al.*, 2007; Wilmot *et al.*, 2012). Targeting this sedentary behaviour may be just as important for health as the use of exercise for health.

In support of this suggestion, some researchers have shown that short bouts of accumulated physical activity lasting just a few minutes spread over the course of a day has the same effect as a single bout of exercise (Miyashita, 2008; Miyashita *et al.*, 2008, 2011). Indeed, breaking prolonged sitting and sedentary time appears to offer distinct benefits to various health outcomes, including fat oxidation and postprandial glycemia and insulinemia (Dunstan *et al.*, 2012b; Ando *et al.*, 2013; Latouche *et al.*, 2013; Peddie *et al.*, 2013; Bailey & Locke, 2015; Henson *et al.*, 2016). Breaking prolonged sitting also influences skeletal muscle pathways involved in carbohydrate metabolism (Latouche *et al.*, 2013). Thus, targeting sedentary behaviour could be a very effective strategy for health.

We are learning more and more about the role of adipose tissue in health. Adipose tissue is not only an energy store, it is a complex endocrine organ that releases a number of hormones (called adipokines). These adipokines have different physiological functions and play an important role in the pathophysiology of chronic diseases such as type 2 diabetes. Acute exercise elicits various changes in adipose tissue (Horowitz, 2003; Enevoldsen *et al.*, 2004; Thompson *et al.*, 2012) but it is not clear whether breaking prolonged sedentary time also affects adipose tissue. If this strategy is effective, it would have major implications for guidance towards means of combatting the disorders associated with dysfunctional adipose tissue. Therefore, the purpose of this study is to examine the effect of breaking prolonged sitting on systemic and adipose tissue responses to feeding.

5.2 Methods

5.2.1 Experimental design

Eleven sedentary participants (7 men and 4 post-menopausal women) aged between 35 to 64 years with increased central adiposity were recruited via local advertisement. An initial screening meeting was followed by two subsequent main trials (prolonged sitting and breaking sitting). Trials were conducted in a randomised, counterbalanced design separated by a 3–4 week wash-out period. Two identical meals based on participant's total body mass were provided on the main trial days (morning and lunchtime). Blood was taken regularly during the course of each trial and adipose tissue biopsies were taken at baseline and at the end of each trial. The protocol was approved by Bristol Research Ethics Committee (REC reference number: 13/SW/0321). This trial is registered at ClinicalTrials.gov (ID: NCT02870088), and all participants provided verbal and written informed consent before taking part.

5.2.1.1 Inclusion and exclusion criteria

Participants were required to be overweight with a waist circumference of 94–128 cm (WHO, 2008) and weight stable (no change in weight \pm 3%) (Stevens *et al.*, 2006) for at least 3 months prior to taking part in this study. Participants completed a health questionnaire (Appendix 1) to exclude any existing cardiovascular and metabolic diseases and a Physical Activity Readiness Questionnaire (PAR-Q) (Appendix 2) to make sure that participants were able to walk on the treadmill safely. Smokers, pre-menopausal women and volunteers who used any medications which could influence metabolic and inflammatory responses were excluded.

5.2.1.2 A summary of participants' physical characteristics

A summary of participants' physical characteristics is shown in Table 5.1.

Table 5.1 Participants physical characteristics (n = 11)

Characteristics	Means \pm SD
Age (years)	50 \pm 5
Body mass (kg)	97.6 \pm 20.8
Waist circumference (cm)	108.8 \pm 14.0
Hip circumference (cm)	112.0 \pm 12.1
Body mass index (kg·m ⁻²)	32.5 \pm 6.7
Fat mass (%)	34.8 \pm 6.3
Fat in L1-L4 region (kg)	4.7 \pm 1.9
Systolic blood pressure (mmHg)	137 \pm 12
Diastolic blood pressure (mmHg)	89 \pm 7

5.2.2 Pre-trial assessments

5.2.2.1 Physical activity assessment

As part of the pre-trial assessments, participants wore a combined heart rate/accelerometer monitor for 7 days to assess habitual physical activity energy expenditure (Actiheart, Cambridge Neurotechnology Ltd., Cambridge, UK). This was attached to the left chest via 2 adhesive ECG pads for 24 h per day except for during showering/bathing/swimming (Thompson *et al.*, 2006).

5.2.2.2 Body composition analysis

Body mass was assessed using digital scales post-void (TANITA corp., Tokyo, Japan). Waist and hip circumference was assessed based on World Health Organisation guidelines (WHO, 2008). Body composition was determined by using Dual energy X-ray absorptiometry (Discovery, Hologic, Bedford, UK). Abdominal subcutaneous and visceral adipose tissue mass was estimated from a central region between L1-L4 (Glickman *et al.*, 2004).

5.2.3 Trial days

In the 72 h prior to each main trial, participants were asked to refrain from performing any strenuous physical activity. In the 48 h prior to each main trial, consuming alcohol/caffeine was not allowed and a weighed food and fluid record was completed. Participants were asked to replicate this diet prior to their second main trial. In addition, in the 48 h prior to each main trial, participants were asked to restrict their step counts to under 4,000 steps per day in order to mimic a sedentary lifestyle (Tudor-Locke & Bassett, 2004; Tudor-Locke *et al.*, 2013) and eliminate any acute effects from recent physical activity.

A 12 h fast was performed before arriving at the laboratory on main trial days between 08:00–09:00 am. After anthropometric measurements, participants rested on a bed for 15 min, followed by two 5 min expired gas samples collections to determine resting metabolic rate (RMR) (Compher *et al.*, 2006; Betts *et al.*, 2011) using stoichiometric equations as described by (Frayn, 1983b).

After RMR assessment, a 20-gauge 1.1 × 32 mm cannula (BD, Venflon™ Pro) was inserted into an antecubital forearm vein and a 10 ml baseline venous blood sample was taken and allocated into tubes with either EDTA or serum separation beads (Sarstedt Ltd, Leicester, UK). Plasma samples were centrifuged immediately at 5,000 rpm and 4 °C for 10 min. Serum samples were left for 45 min to clot before centrifugation. Subcutaneous adipose tissue samples (~1 g) were taken under local anaesthetic (1% lidocaine) from the area around the waist approximately 5 cm lateral to the umbilicus with a 14 G needle using an aspiration technique (Walhin *et al.*, 2013) followed by adipose tissue cleaning and processing as previously described by Travers *et al.* (2014).

The study protocol and details are shown in Figure 5.1 and details on main trial days are described as follows. Two trials were performed including one prolonged sitting and one breaking sitting trial. In the breaking trial, after consuming the 1st meal, participants walked for 2 min on a treadmill at 6.4 km·h⁻¹ (Dunstan *et al.*, 2012b) every 20 min for the following 180 min. For the remainder of the time participants sat on the chair. The 2nd meal was provided after 180 min. After finishing the 2nd meal, participants continued the 2 min walks to break up prolonged periods of sitting at the same speed every 20 min for the following 120 min. Thus, in total, participants performed 15, 2 min bouts of physical activity throughout the trial (i.e., 30 min of physical activity over 300 min). In the prolonged sitting trial, participants sat on a chair

for the whole period. Second adipose tissue and final blood sample were taken at the end of trial.

Expired air, ratings of perceived exertion (RPE) and heart rate were collected during two of the 2 min bouts of walking to determine exercise intensity, energy expenditure and energy substrate utilization as described by Jeukendrup and Wallis (2005). In addition, for both breaking sitting and prolonged sitting trials, during two 5 min periods of sitting, expired air and heart rate were taken to calculate total energy expenditure under sitting conditions as described by Frayn (1983b). While sitting, participants were only allowed to read, use a laptop or watch television but were otherwise asked to keep as still as possible throughout. In the first trial, participants were allowed to consume water *ad libitum* and the volume ingested was replicated for the 2nd trial. Expired air samples were collected into Douglas bags (Hans Rudolph, MO, USA). Samples were analysed for relative expired fractions of oxygen and carbon dioxide gas for 2 min (Servomex, Crowborough, UK) and the total volume within the bag was measured using a dry gas meter (Harvard Apparatus, Kent, UK).

In each main trial, baseline blood samples was collected before the 1st meal and hourly for the following 5 h. Additional blood samples were collected every 15 min after the first and second meal. A total of 14 blood samples were collected for each trial.

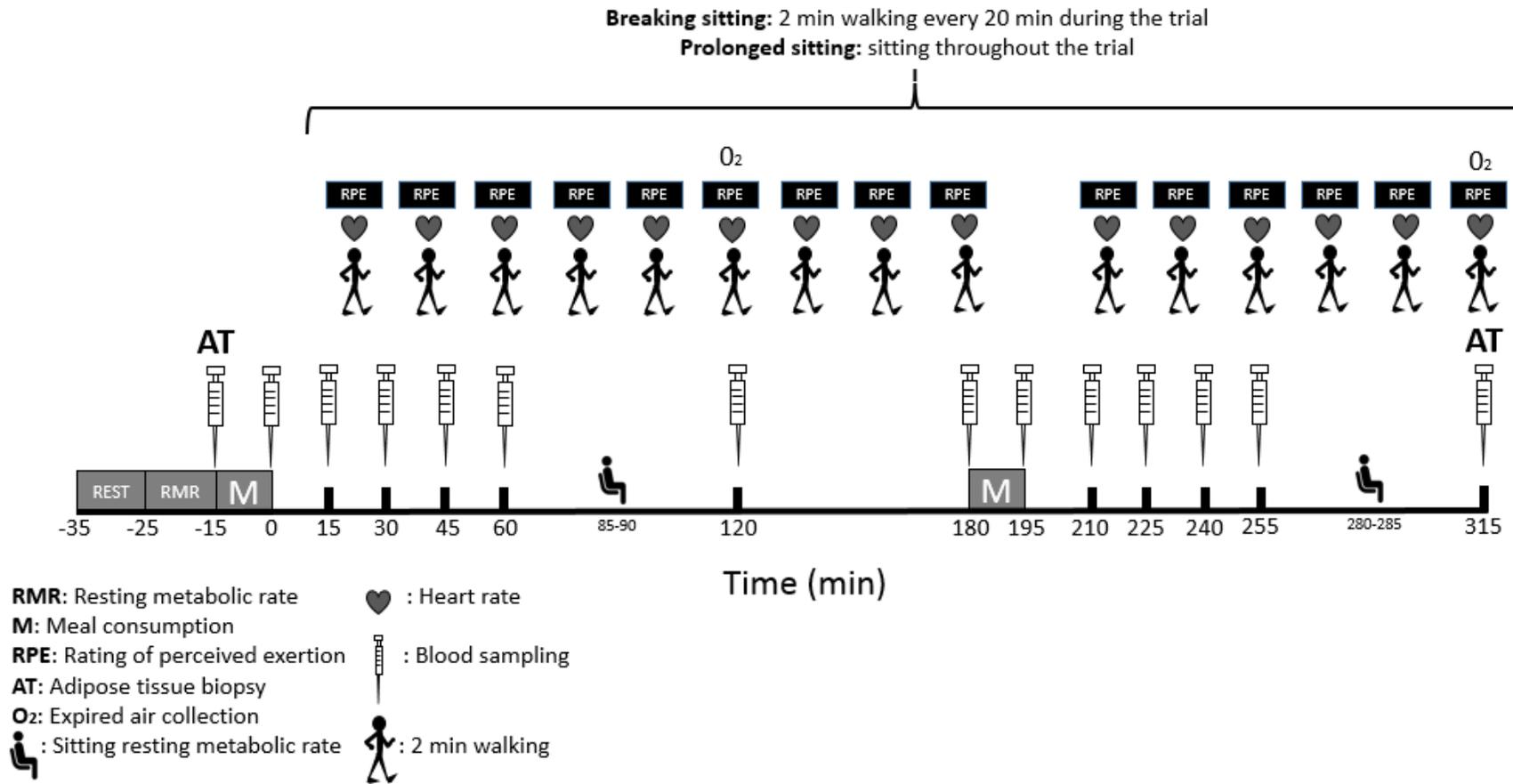


Figure 5.1 Experimental protocol

5.2.3.1 Meal

Two identical meals (breakfast and lunch) were provided and consumed within a 15 min period during each main trial. The meal was prescribed according to total body mass and provided 0.35 g fat, 1.17 g carbohydrate, 0.29 g protein and 37 kJ of total energy per kilogram body mass (Miyashita, 2008). The percentage of energy from macronutrients was 35% fat, 52% carbohydrate and 13% protein. The meal comprised white bread (Hovis; soft white bread, medium sliced), sliced cheese (Sainsbury; cheese slices, basic), butter (Unilever; I can't believe its not butter), mayonnaise (Hellmann; light mayonnaise), lettuce (Sainsbury; Iceberg lettuce), tomato (Sainsbury; tomatoes, basics), ham (Sainsbury; British honey roast), whole milk (Sainsbury; British), cocoa powder (Nesquik; cocoa powder), and yoghurt (Müller; fruit corner strawberry).

5.2.4 Experimental analysis

5.2.4.1 Adipose tissue culture and gene expression

After cleaning the adipose tissue biopsy, the tissue was minced to approximately 5–10 mg segments and 200 mg was directly placed in sterile culture plates in duplicate (Nunc, Roskilde, Denmark) with endothelial cell basal media (ECBM) (Promocell, Germany) containing 0.1% fatty acid-free bovine serum albumin 100 U·ml⁻¹ penicillin and 0.1 mg·ml⁻¹ streptomycin (Sigma-Aldrich, Gillingham, UK). Adipose tissue was incubated at a final ratio of 100 mg tissue per 1 ml ECBM media for 3 h (Travers *et al.*, 2014). A 37 °C, 5% CO₂ and 95 ± 5% relative humidity incubator was used to incubate adipose tissue explants *ex vivo* (MCO-18A1C CO₂ incubator; Sanyo, Osaka, Japan). After the 3-h incubation, media was transferred to sterile Eppendorfs and stored at –80 °C. Adipokine secretion from adipose explants was normalised to L1-L4 fat mass as described (Travers *et al.*, 2015b). The remaining adipose tissue (approximately 200 mg) was homogenised in 5 ml TRIzol (Invitrogen, Paisley, UK) in an RNase/DNase-free

sterile tube (Invitrogen, Paisley, UK) and stored at -80°C before mRNA expression analysis.

5.2.4.2 Real-time PCR

An RNeasy Mini Kit (Qiagen, Crawley, UK) was used to extract RNA from TRIzol-digested adipose tissue as described in Walhin *et al.* (2013). Tissue samples were further quantified using a Qubit 2.0 fluorimeter (Life Technologies, Paisley, UK). RNA was reversed transcribed (1 μg) to cDNA using a High Capacity Reverse Transcription Kit (Applied Biosystems, Warrington, UK). The organic phenol-chloroform phase from the RNA extraction was kept for future protein analysis. Real-time PCR was performed using a StepOne (Applied Biosystems, Warrington, UK). Predesigned primers and probes were obtained from Applied Biosystems for the measurement of expression of interleukin 6 (IL-6) (Hs00985639_m1), adiponectin (Hs00605917_m1), leptin (Hs00174877_m1), interleukin 18 (IL-18) (Hs00155517_m1), tumour necrosis factor alpha (TNF- α) (Hs99999043_m1), monocyte chemoattractant protein-1 (MCP-1) (Hs00234140_m1), 5' AMP-activated protein kinase (AMPK) (Hs01562315_m1 and Hs00178903_m1 combined), glucose transporter type 4 (GLUT4) (Hs00168966_m1), hormone-sensitive lipase (HSL) (Hs00193510_m1), insulin receptor substrate 1 (IRS1) (Hs00178563_m1), insulin receptor substrate 2 (IRS2) (Hs00275843_s1), sterol regulatory element binding protein 1c (SREBP-1c) (Hs01088691_m1), pyruvate dehydrogenase kinase isozyme (PDK4) (Hs00176875_m1), peroxisome proliferator-activated receptor γ (PPAR γ) (Hs01115513_m1), peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 α) (Hs01016719_m1), RAC-alpha serine/threonine-protein kinase (AKT1) (Hs00178289_m1), adipose triglyceride lipase (ATGL) (Hs00386101_m1), fatty acid translocase (FAT)/CD36 (Hs00169627_m1), forkhead box protein O1 (FOXO1) (Hs01054576_m1), hexokinase 2 (HK2)

(Hs00606086_m1), phosphoinositide 3-kinase 85 α (PI3K-85 α) (Hs00933163_m1), fatty acid synthase (FAS) (Hs00188012_m1), peptidylpropyl isomerase A (PPIA) was used as an endogenous control (Neville *et al.*, 2011). The comparative Ct method was used to process data where $\Delta\text{Ct} = \text{Ct target gene} - \text{Ct PPIA}$. Ct target genes were normalised to an internal calibrator (lowest ΔCt for each target gene) and baseline.

5.2.4.3 Biochemical analysis

Blood glucose, triglycerides and non-esterified fatty acids were analysed using commercially available assay kits and analyser (Daytona Rx; Randox, Crumlin, UK). Serum insulin (Merckodia, Uppsala, Sweden), and both serum and adipose secretion of IL-6, leptin, and adiponectin (R&D systems) were measured using Enzyme-linked immunosorbent assay (ELISA).

5.2.5 Statistics

Descriptive data are presented as means \pm standard deviation (SD). All other data are presented as means \pm standard error of the mean (SEM). Incremental area under curve (iAUC) was calculated using the trapezoid method (Wolever & Jenkins, 1986). All data were checked for normality using visual checking of histograms, confirmed statistically by the Shapiro-Wilk test. Differences in summative scores between trials for iAUC and mRNA expression outcomes were analysed using paired *t-tests*. Non-normally distributed data were analysed using the equivalent non-parametric tests. Serial glucose, insulin, NEFA, triglycerides, blood adipokines and adipose cell culture outcomes were determined using a two-way ANOVA with repeated measures using SPSS version 22 (IBM, Armonk, NY, USA). Where significant interactions (trial \times time) were found, a post hoc analysis was applied using *t-tests* with a Holm–Bonferroni correction (Atkinson, 2002). Statistical significance was set at $p \leq 0.05$.

5.3 Results

The average of two days of step counts prior to prolonged and breaking sitting trials was not different ($3,868 \pm 393$ versus $3,669 \pm 329$ steps·day⁻¹, respectively) and there was no difference in pre-fed RMR on the trial day between trials ($1,882 \pm 91$ versus $1,833 \pm 102$ kcal·day⁻¹, respectively).

5.3.1 Physiological response during walking in breaking sitting

During the 15 bouts of 2 min walking, the average heart rate was 135 ± 4 beats·min⁻¹ with an RPE (6–20 scale) of 10.5 ± 0.6 . The difference in energy expenditure between trials was 211 kcal (Table 5.2).

Table 5.2 Energy expenditure and substrate oxidation during each 330 min trial

	Prolonged sitting	Breaking sitting
Energy expenditure (kcal)	552 ± 27 *	763 ± 48
Carbohydrate oxidation (g)	112 ± 14 *	168 ± 15
Fat oxidation (g)	17 ± 4	18 ± 3

Values are means \pm SEM. * denotes significantly different between prolonged sitting versus breaking sitting trials ($p \leq 0.05$) (n = 11).

5.3.2 Blood glucose, insulin, triglyceride and NEFA in prolonged and breaking sitting trials

Interaction effects were found in plasma glucose concentrations between prolonged and breaking sitting trials ($p \leq 0.05$; Figure 5.2a). The glucose iAUC was attenuated significantly by breaking sitting compared to prolonged sitting trial (359 ± 117 versus 697 ± 218 $\text{mmol} \cdot 330 \text{ min} \cdot \text{l}^{-1}$, $p = 0.001$; Figure 5.2b).

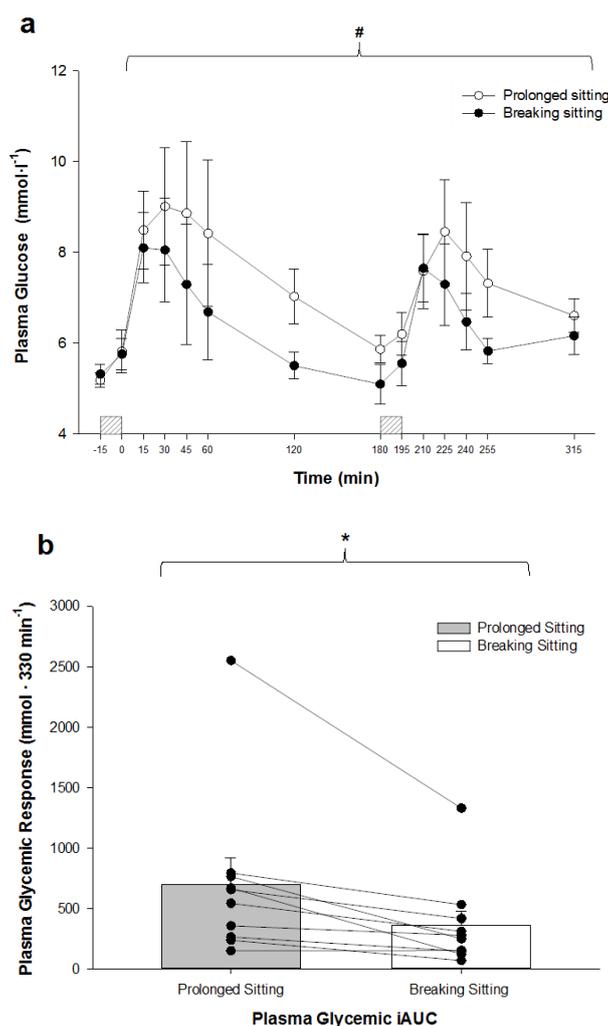


Figure 5.2 Plasma glucose concentrations (a) and glucose iAUC (b) in prolonged and breaking sitting trials. The sample size is $n = 10$ due to difficulty in cannulating one participant. Values are means \pm SEM. # denotes significant interaction effect between prolonged and breaking sitting trials ($p \leq 0.05$). * denotes significantly different between prolonged *versus* breaking sitting trials ($p \leq 0.05$). The shaded box denotes meal time.

Interaction effects were also found in serum insulin concentrations between prolonged and breaking sitting trials ($p \leq 0.05$; Figure 5.3a) The insulin iAUC were attenuated significantly by breaking sitting compared to prolonged sitting trial ($2,024 \pm 714$ versus $3,462 \pm 1,496$ $\text{nmol} \cdot 330 \text{ min} \cdot \text{L}^{-1}$, $p = 0.001$, Figure 5.3b)

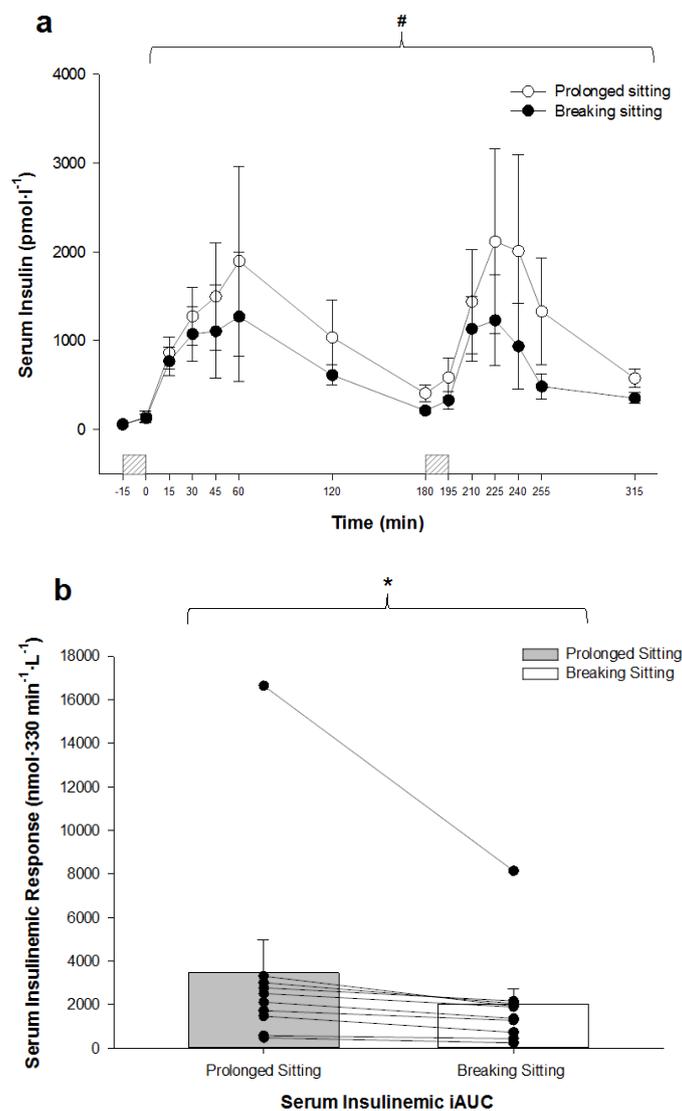


Figure 5.3 Serum insulin concentrations (a) and insulin iAUC (b) in prolonged and breaking sitting trials. The sample size is $n = 10$ due to difficulty in cannulating one participant. Values are means \pm SEM. # denotes significant interaction effect between prolonged and breaking sitting trials ($p \leq 0.05$). * denotes significantly different between prolonged *versus* breaking sitting trials ($p \leq 0.05$). The shaded box denotes meal time.

There was no interaction in blood triglyceride and NEFA in prolonged and breaking sitting trials (Figure 5.4a and c). Triglyceride iAUC was significantly higher in the breaking sitting compared to the prolonged sitting trial (283 ± 36 versus 232 ± 32 $\text{mmol} \cdot 330 \text{ min} \cdot \text{L}^{-1}$, $p = 0.002$, respectively, Figure 5.4b).

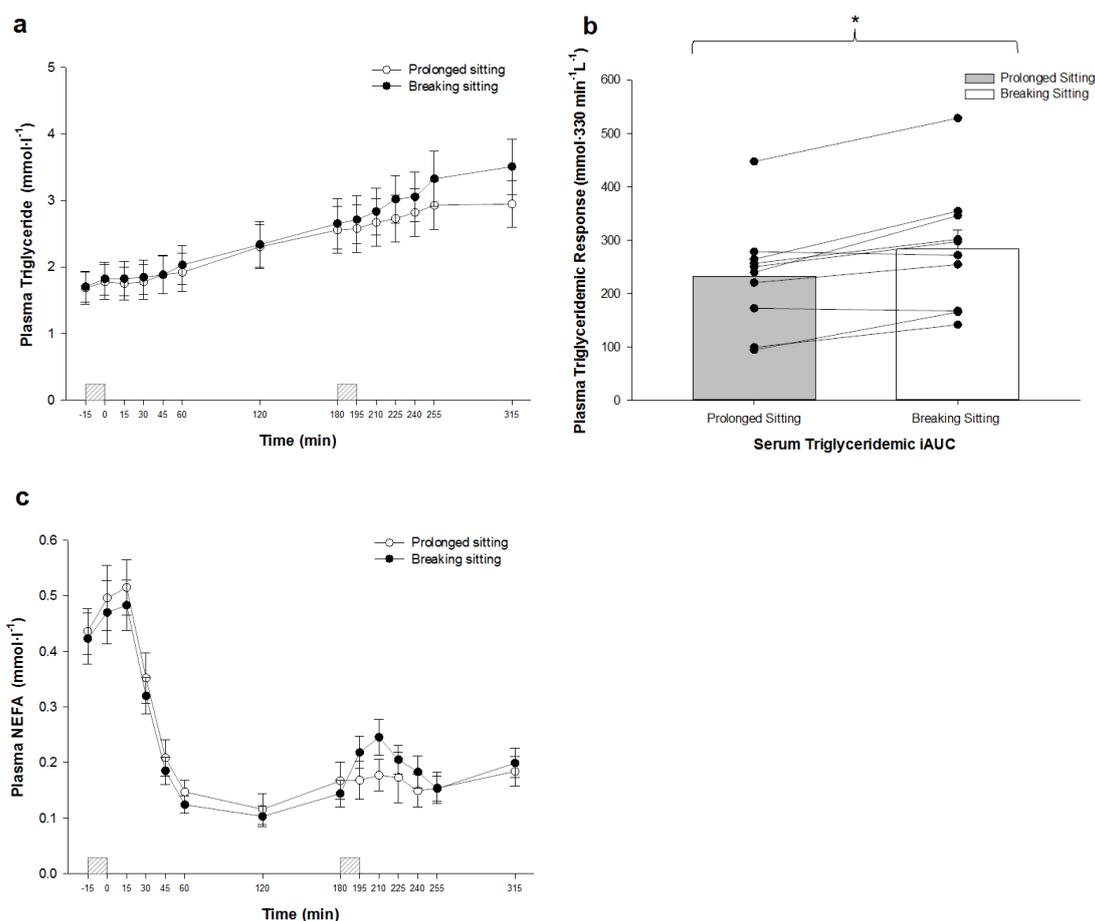


Figure 5.4 Blood triglyceride (a) and NEFA (c) concentrations in prolonged and breaking sitting trials. iAUC for triglyceride (b). The sample size is $n = 10$ due to difficulty in cannulating one participant. Values are means \pm SEM. * denotes significantly different between prolonged *versus* breaking sitting trials ($p \leq 0.05$). The shaded box denotes meal time.

Even after removing one participant who had a high glucose and insulin response compared to the other participants, a reduction in glucose and insulin iAUCs was still found in the breaking sitting trial compared to prolonged sitting trial (252 ± 48 versus 491 ± 77 $\text{mmol} \cdot 330 \text{ min} \cdot \text{l}^{-1}$ and $1,345 \pm 230$ versus $1,977 \pm 322$ $\text{nmol} \cdot 330 \text{ min} \cdot \text{l}^{-1}$, $p \leq 0.01$; respectively, Figure 5.5a and b)

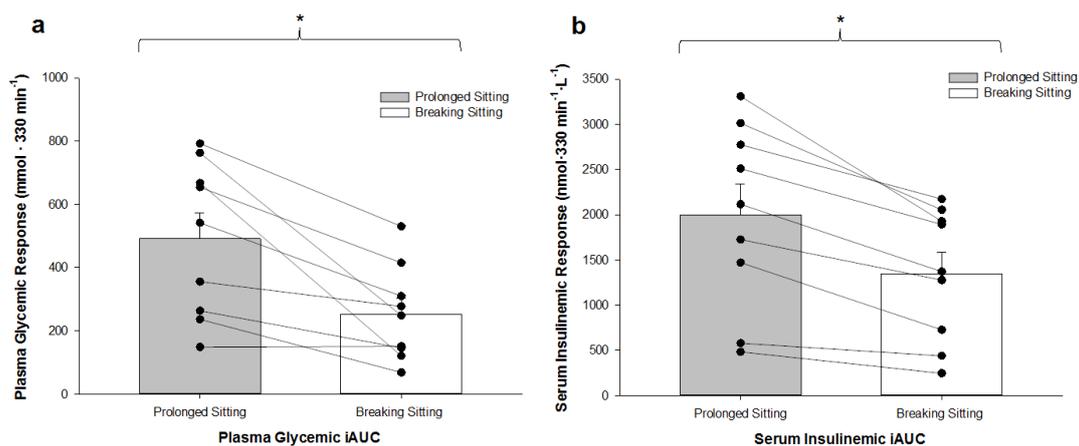


Figure 5.5 Glucose and insulin iAUC in the prolonged and breaking sitting trials.
* denotes difference between prolonged sitting *versus* breaking sitting trials ($p \leq 0.05$)
(One participant excluded, $n = 9$).

5.3.3 Adipose Tissue Gene Expression

There was no difference in adipose tissue gene expression between breaking and prolonged sitting trials for any of the energy metabolic, insulin signalling and (anti)inflammatory genes analysed (Figure 5.6).

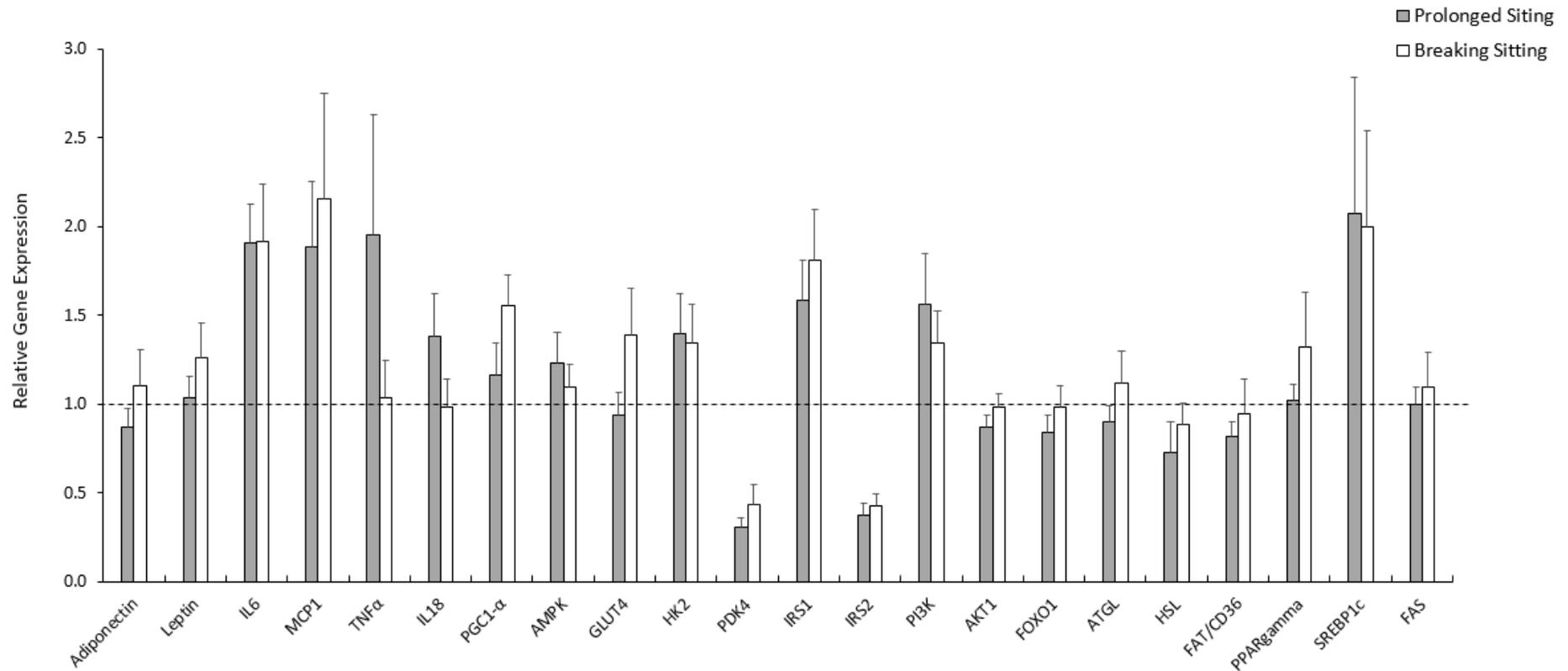


Figure 5.6 Fold changes in relative adipose tissue gene expression (mRNA levels) under prolonged sitting and breaking sitting trials. **Fold changes in relative gene expression (mRNA levels) of metabolism, insulin signaling, glucose transport and inflammatory genes in adipose tissue under prolonged sitting and breaking sitting trials** (all n = 9) due to lack of tissue samples for two participants. The dashed line indicates baseline. Data normalised to PPIA, internal calibrator and baseline sample. Samples that exceeded the detectable limit (Ct > 35) were excluded from the analysis. Values are means \pm SEM.

5.3.4 Serum Adipokine levels and Adipose Tissue Adipokine Secretion *ex vivo*

There was no difference between prolonged and breaking sitting in serum IL-6, leptin and adiponectin, or the secretion, *ex vivo*, of IL-6, leptin and adiponectin (Figure 5.7)

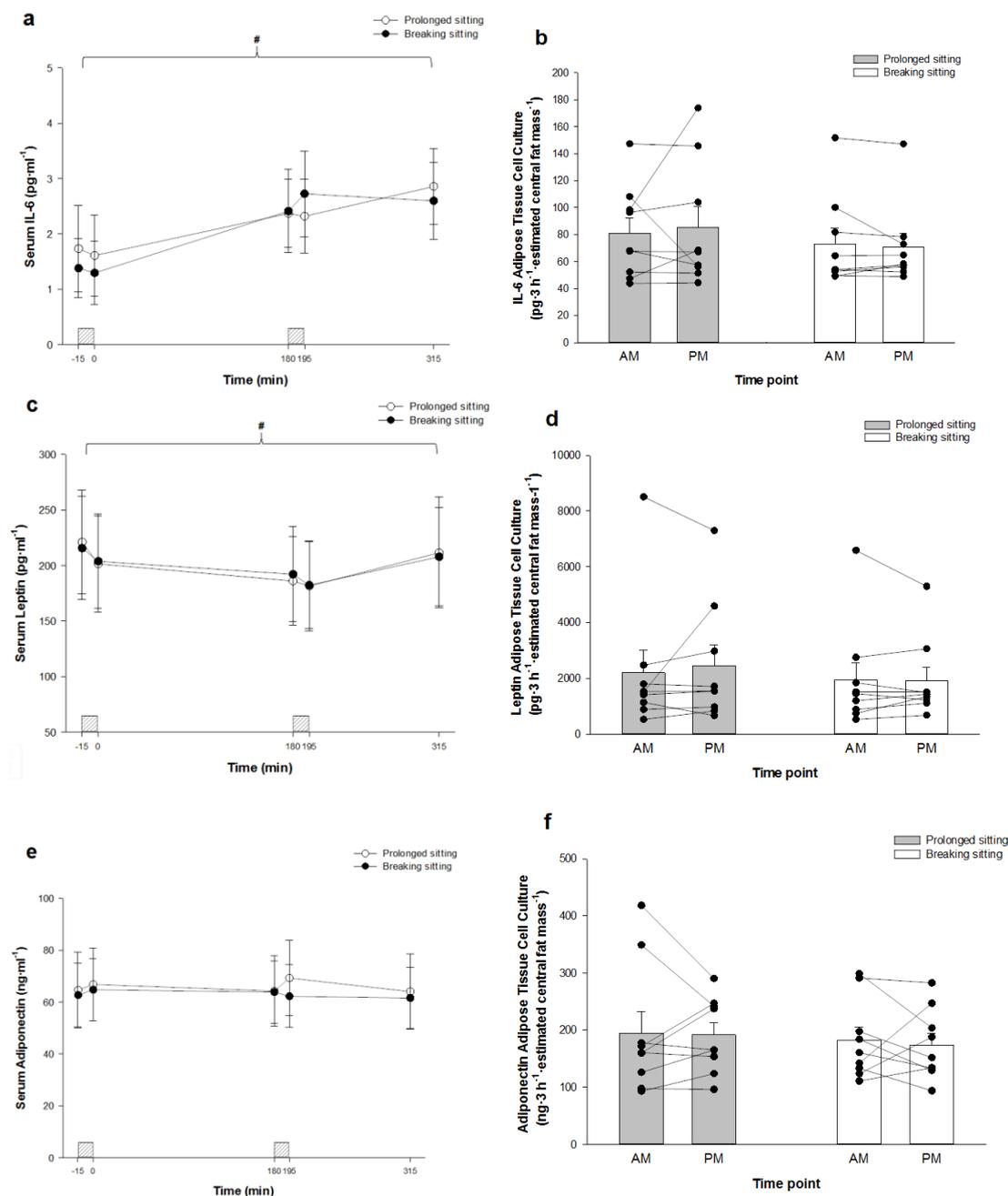


Figure 5.7 Serum adipokine levels and adipose tissue adipokine secretion *ex vivo*.

Circulating serum IL-6 (a) leptin (c) and adiponectin (e) concentrations in prolonged and breaking sitting trials. Protein secretion of IL-6 (b), leptin (d) and adiponectin (f) at baseline (AM) and at the end of trial (PM). # denotes significant time effect ($p \leq 0.05$). Values are means \pm SEM. The shaded box denotes meal time.

5.4 Discussion

The present study investigated whether regular short bouts of walking influence circulating biomarkers, adipose gene expression and protein secretions from adipose tissue *ex vivo*. We found that regularly participating in short bouts of walking reduced postprandial glucose and insulin iAUC by 48% and 42%, respectively. However, in spite of the profound difference in postprandial insulin and glucose, subcutaneous adipose gene expression was similar in both trials. There was also no difference in adipose tissue secretions of IL-6, adiponectin or leptin.

5.4.1 The effect of breaking sitting on postprandial glycaemia and insulinaemia

The extent of changes in postprandial glucose and insulin have been associated with cardiovascular disease (Bonora & Muggeo, 2001; Cavalot *et al.*, 2006; Watanabe *et al.*, 2013) and overweight/obese populations typically show higher postprandial glucose and insulin (Perala *et al.*, 2011). Therefore, strategies to attenuate postprandial glucose and insulin would be extremely useful. Breaking prolonged sitting has been proven to reduce postprandial glucose and insulin levels in most (Dunstan *et al.*, 2012b; Peddie *et al.*, 2013; van Dijk *et al.*, 2013; Bailey & Locke, 2015; Henson *et al.*, 2016) but not all studies (Altenburg *et al.*, 2013; Saunders *et al.*, 2013). We also showed that participating in regular short bouts of walking which break up prolonged sitting attenuates postprandial glucose and insulin concentrations in sedentary middle aged overweight men and women (the glucose and insulin iAUC were attenuated by 48% and 42%, respectively). The additional energy cost of walking during the breaking sitting trial was fuelled by carbohydrate oxidation and thus we propose that the oxidation of exogenous glucose explains the relatively lower glucose concentrations observed in this trial. Thus, breaking prolonged sitting is very effective at reducing postprandial glycaemia and insulinaemia.

5.4.2 Adipose tissue responses to breaking prolonged sitting

In skeletal muscle, breaking sitting with either light or moderate intensity walking increased the expression of genes involved in glucose metabolism, including PDK4 (Latouche *et al.*, 2013). In the present study, we did not find any differences between breaking sitting and prolonged sitting trials in adipose glucose metabolism gene expression including PDK4 mRNA expression even though our walking speed, frequency and duration was similar to this previous study that have shown effects in skeletal muscle (Latouche *et al.*, 2013). This might reflect tissue-specificity with a greater effect in working skeletal muscle than adipose tissue. Skeletal muscle plays a major role in rapidly removing glucose from blood whereas adipose plays a very minor role (Im *et al.*, 2007). An alternative explanation is that the effects of the meal are so pronounced in adipose tissue, that they ‘swamp’ any effects from brief episodes of physical activity.

In the postprandial period, significant uptake of NEFA and triglyceride into adipose tissue is usually shown (Saleh *et al.*, 1998; Bickerton *et al.*, 2007; Koutsari *et al.*, 2008). We provided two mixed meals with a total $\sim 1,725 \pm 369$ (means \pm SD) ($8.83 \text{ kcal} \cdot \text{kg}^{-1}$) kcal calories which is more than 2-fold the absolute energy intake of that consumed in the single meal (~ 763 kcal) given in the study by Latouche *et al.* (2013). Given the extended timescale of the present study, $\sim 1,700$ kcal is not an unreasonable amount of energy but it is possible that the physiological pressure of processing external energy might outweigh the impact of short bouts of physical activity in adipose tissue. Thus, unlike in working skeletal muscle, breaking prolonged sitting does not seem to affect adipose tissue in spite of a pronounced effect on postprandial systemic insulin and glucose concentrations.

5.4.3 The regulation of adipose tissue gene expression after meal consumption

IRS1/2-PI3K-Akt represents one insulin signalling pathways (Cheng *et al.*, 2010). The expression of adipose IRS1/2-PI3K-Akt insulin signalling might be regulated due to different insulin concentrations, which would further regulate other downstream transcriptional molecules such as FOXO1, PGC1- α , and SREBP1c. However, in the present study, adipose IRS1/2-PI3K-Akt mRNA signalling was not different between trials, even though postprandial insulin concentrations were powerfully attenuated throughout the breaking sitting trial. This suggests that adipose IRS1/2-PI3K-Akt insulin signalling is not acutely influenced by changes in circulating insulin – or that both insulin (Dandona *et al.*, 2004) and glucose (Esposito *et al.*, 2002) influence inflammatory pathways in adipose and we speculated that differences in glycaemia and insulinaemia would translate into different inflammatory responses. However, we also saw no difference in (anti)inflammatory gene changes including IL-6, MCP-1, TNF- α , IL-18, adiponectin and leptin.

Even though we did not observe any differences in adipose tissue gene expression between breaking and prolonged sitting trials, there appear to be temporal changes in both trials that could be a response to the meal. IRS1 and IRS2 mRNA expression were consistently up-regulated and down-regulated, respectively, at the end of both trials. IRS-1 plays a central role of insulin signalling receptor (Thirone *et al.*, 2006) and IRS-2 was down-regulated in lean, overweight and first class obese populations 6 h after meal consumption (Travers *et al.*, 2015b). In addition, studies have shown that adipose IL-6 and MCP-1 mRNA expression was up-regulated in either lean, overweight or obese (Travers *et al.*, 2015b), metabolic syndrome (Meneses *et al.*, 2011), T2DM (Pietraszek *et al.*, 2011) and healthy lean populations (Dordevic *et al.*, 2015) after meal consumption. In the present study, we observed similar trends in adipose IL-6 and

MCP-1 mRNA expression. This might potentially support the notion that meal consumption enhances adipose inflammatory responses; however, based on the present results alone we cannot discount the possibility that these reflect a circadian rhythm.

5.5 Conclusions

Breaking prolonged periods of sitting acutely reduces postprandial glucose and insulin concentrations in overweight/obese populations, probably by increasing glucose oxidation. However, in spite of these differences, adipose tissue metabolic, insulin signalling and (anti)inflammatory gene expression was not affected by breaking prolonged periods of sitting, including the IRS1/2-PI3K-Akt insulin signalling pathway. Thus, whilst breaking prolonged sitting is an effective strategy for managing postprandial glycaemia and insulinaemia, this does not appear to elicit a corresponding change in adipose tissue, therefore, other strategies (e.g. longer breaking sitting walking intervention, continuous endurance exercise) might be required to elicit these responses, specifically.

Chapter 6: The effect of different physical activity strategies on short-term overfeeding in young healthy men

6.1 Introduction

A chronic positive energy imbalance leads to the excess deposition of adipose tissue that characterises obesity, and is implicated in adipose tissue hypertrophic expansion and chronic systemic low grade inflammation and ultimately results in cardiovascular diseases and type 2 diabetes. A combination of excessive energy intake and physical inactivity are the two main factors that contribute to a positive energy imbalance. Studies have consistently shown that overfeeding impairs insulin sensitivity (Wang *et al.*, 2001; Brons *et al.*, 2009; Walhin *et al.*, 2013), and alters adipose tissue energy metabolism and inflammatory gene expression (Brons *et al.*, 2009; Shea *et al.*, 2009; Alligier *et al.*, 2012; Johannsen *et al.*, 2014; Gillberg *et al.*, 2016).

Studies have shown that prolonged endurance exercise alleviates impaired metabolic responses induced by short-term overfeeding (Hagobian & Braun, 2006; Walhin *et al.*, 2013). Hagobian and Braun (2006) demonstrated that a single, 60 min bout of cycling at 60% maximum oxygen uptake ($\dot{V}O_{2\text{peak}}$) decreased insulin responsiveness to a glucose challenge after 3 days of overfeeding. Furthermore, Walhin *et al.* (2013) indicated that maintaining a 50% energy surplus over 7 days induced weight gain, impaired glucose and insulin responses to feeding but, notably, a single, daily 45 min bout of vigorous intensity exercise (70% $\dot{V}O_{2\text{max}}$) reversed impaired metabolic changes brought about by the energy surplus, even after prescribing more energy intake to offset the impact of daily exercise (i.e., to match the energy surplus). This study showed that

the expression of fatty acid and lipid synthesis genes, including sterol regulatory element-binding transcription factor 1 (SREBP-1c) and fatty acid synthesis (FAS) were blunted in the exercise group (45 min of vigorous intensity exercise alongside overfeeding and reduced physical activity (Walhin *et al.*, 2013). However, participating in structured vigorous intensity endurance exercise might not be practical for all populations. Small bouts of non-structured physical activity have been suggested to be an effective physical activity strategy (Miyashita *et al.*, 2013), but we do not know if these forms of exercise are equally effective to counteract overfeeding induced impaired metabolic adaptation.

Other studies have indicated that even small amounts of non-structured physical activity can have a profound impact on metabolic health outcomes. Small bouts of regular walking (from 1 min 40 seconds to under 10 min) to break prolonged periods of sitting attenuate postprandial insulin and glucose concentrations (Miyashita, 2008; Dunstan *et al.*, 2012b; Miyashita *et al.*, 2013; Peddie *et al.*, 2013; Kim *et al.*, 2014), and reduces resting blood pressure (Miyashita *et al.*, 2011; Larsen *et al.*, 2014). At the molecular level, both slow ($3.2 \text{ km}\cdot\text{h}^{-1}$) and brisk ($6.4 \text{ km}\cdot\text{h}^{-1}$) spells of walking to break up periods of prolonged sitting have been shown to alter carbohydrate-metabolism gene expression, including pyruvate dehydrogenase kinase 4 (PDK4), in skeletal muscle (Latouche *et al.*, 2013). One study also observed that the level of non-exercise activity thermogenesis (NEAT) could be a decisive element in determining the degree of adipose deposition after 8 weeks of $1,000 \text{ kcal}\cdot\text{day}^{-1}$ overfeeding (Levine *et al.*, 1999).

To compare prolonged exercise versus intermittent physical activity, Duvivier *et al.* (2013) compared the effect of maintaining a normal diet with prolonged sitting for 4 days to prolonged sitting with regular breaks to prolonged sitting plus a daily, 1 h bout

of vigorous cycling. These authors found that 16 h post-intervention, insulin incremental area under curve (iAUC) during an oral glucose tolerance test (OGTT) was lower in the group taking regular breaks during prolonged sitting compared to prolonged sitting with added vigorous cycling. However, this study did not compensate for the extra energy expended (~450 kcal) during the 1 h vigorous cycle, or examine the physiological adaptations to the intervention in adipose tissue. Thus, the present investigation aimed to examine the impact of two different physical activity interventions (prolonged endurance versus intermittent physical activity) on the metabolic and adipose tissue responses to overfeeding and reduced activity.

6.2 Methods

6.2.1 Experimental design

Twenty-four lean, active and healthy men aged between 18 to 35 years were recruited via local advertisement. Participants were randomised to either an overfeeding with restricted physical activity ($\leq 4,000$ steps \cdot day $^{-1}$) group (OVER), overfeeding with restricted physical activity ($\leq 4,000$ steps \cdot day $^{-1}$) plus daily 45 min endurance moderate intensity walking group (50% $\dot{V}O_{2\max}$) (OVER + EN) or overfeeding with restricted physical activity ($\leq 4,000$ steps \cdot day $^{-1}$) plus intermittent breaking sitting group (OVER + BREAKS). All groups were achieved the same overfeeding (50% of overfeeding based on their habitual diet). Participants reported to the lab on three occasions including fitness tests and two subsequent main trials (Day 1 and Day 8). The one week intervention was implemented after the 1st main trial and participants returned on the 8th day for the follow-up the 2nd main trial with identical procedures. Main trial days involved anthropometric assessments, a dual-energy x-ray absorptiometry (DEXA) scan and resting metabolic rate (RMR) measurements across 20 min, followed by a subcutaneous, abdominal adipose tissue biopsy and a 2 h oral glucose tolerance test

(OGTT). The blood pressure was measured in triplicate at the end of OGTT. The protocol was approved by the Bristol NHS Research Ethics Committee (REC reference number: 15/SW/0014). This trial is registered at ISRCTNregistry (registration number: ISRCTN18311163), and all participants provided verbal and written informed consent before taking part.

This project is being conducted in partnership with a postdoctoral researcher who is focusing on different experimental outcomes (arm cranking exercise). This other work is not included in this thesis.

6.2.1.1 Inclusion and exclusion criteria

Participants had to be weight stable (body mass) for at least 3 months ($\pm 3\%$) to be eligible for this study (Stevens *et al.*, 2006). A health questionnaire was completed to exclude those with existing cardiovascular and metabolic diseases and a Physical Activity Readiness Questionnaire (PAR-Q) (Appendix 2) was completed to make sure that participants were able to exercise safely. Individuals taking any medications known to influence lipid/carbohydrate metabolism or immune function, smokers and females were excluded.

6.2.1.2 Participant baseline physical characteristics

A summary of participants' baseline physical characteristics is outlined in Table 6.1.

Table 6.1 Participant baseline physical characteristics

	OVER	OVER + EN	OVER + BREAKS
	(n = 8)	(n = 8)	(n = 8)
Age (years)	22 ± 3	21 ± 2	22 ± 3
BMI (kg·m ⁻²)	23.8 ± 1.8	23.2 ± 1.2	23.5 ± 2.3
Body mass (kg)	73.4 ± 9.6	72.9 ± 6.8	71.5 ± 8.8
Physical activity level (PAL)	1.81 ± 0.19	1.89 ± 0.21	1.77 ± 0.13
Total energy expenditure (kcal·day ⁻¹)	3,319 ± 597	3,397 ± 395	3,178 ± 460
$\dot{V}O_{2\max}$ (ml·kg ⁻¹ ·min ⁻¹)	50.1 ± 3.4	50.7 ± 6.4	52.1 ± 6.5

Values are means ± SD.

6.2.2 Preliminary assessments

6.2.2.1 Diet and physical activity assessments

Over a period of 7 consecutive days, participants were required to record their habitual food using the weighed inventory method. Dietary records were analysed using the Nutrition Analysis Software version 1.8 (Nutritics, UK), which is based on food database in the UK. Energy intake was estimated using this software and 10% of diet induced thermogenesis (DIT) was estimated (Westerterp, 2004). During this period, a combined heart rate/accelerometer monitor (Actiheart, Cambridge Neurotechnology Ltd., Cambridge, UK) was attached at the base of the left *Pectoralis Major* via 2 adhesive ECG pads 24 h per day except when showering/bathing/swimming (Thompson *et al.*, 2006) to assess the participant's habitual physical activity energy

expenditure, and a pedometer (Yamax, Japan) was worn to record habitual daily step count.

6.2.2.2 Pre-trial fitness tests

$\dot{V}O_{2max}$ tests were performed by all participants at least one week prior to the 1st main trial. This was performed on a treadmill (Woodway, ELG70, Weiss, Germany), maintained at 9 to 12 km·h⁻¹ with an initial gradient of 2.5%. The gradient was increased 2.5% every 3 min in a stepwise manner until volitional exhaustion to determine $\dot{V}O_{2max}$. Samples were collected and analysed as described in Chapter 3.8.

6.2.2.2.1 Gradient walking test

A gradient walking test was performed by the OVER + EN and OVER + BREAKS groups to determine their 50% $\dot{V}O_{2max}$ walking speed. The gradient walking test was undertaken on a treadmill (Woodway, ELG70, Weiss, Germany) maintained at 6 to 7 km·h⁻¹ with an initial 2.5% gradient and gradient was increased by 2.5% every 5 min until gradient reached 10%. To determine energy expenditure, expired air samples were collected for 1 min at the end of each 5 min stage using the method described in Chapter 3.8.

6.2.2.2.2 Self-selected speed test and target steps calculation

In the OVER + BREAKS group, a 15 min a walking test at a self-selected speed was performed to estimate energy expenditure during a 45 min walking test at 50% $\dot{V}O_{2max}$. Participants were allowed 1 min to choose their “normal walking speed” blinded from the treadmill speedometer. Once the speed was selected, participants walked at the chosen speed for the remaining ~14 mins, and expired air was collected

for 1 min at 5 min intervals to determine energy expenditure (Jeukendrup & Wallis, 2005). Participants were then assigned a specific amount of steps to complete each day of the intervention period to match the energy expended during the 45 min walking test. To monitor this, pedometers were worn each day during the intervention period to record total step count to match the energy expended during the walking test.

6.2.3 Main trials

To standardised physical activity in the 24 h leading up to the 1st main trial, all participants conducted a bout of 30 min physical activity before 2 pm. On the morning of the 1st day main trial, participants reported to the Resting Metabolic Laboratory between 07:00–08:00 am following a 10–12 h overnight fast. Body mass was assessed to the nearest 0.1kg using digital scales (TANITA corp., Tokyo, Japan) and waist and hip circumference was measured using non-stretch tape to the nearest 0.1 cm, based on World Health Organisation guidelines (WHO, 2008), following body composition assessed using by Dual energy X-ray absorptiometry (DEXA) (Discovery, Hologic, Bedford, UK) to determine muscle mass and fat mass and percentages. Participants then rested in bed for 10 min, after which, a mouthpiece connected to a Douglas bag was inserted and four consecutive, 5 min expired gas samples were collected with the mean of at least three bags within 100 kcal was used to determine resting metabolic rate (RMR) (Compher *et al.*, 2006; Betts *et al.*, 2011) applying substrate oxidation under resting conditions as described by Frayn (1983b). Following RMR assessments, a 20 G 1.1 × 32 mm cannula (BD, Venflon™ Pro, Becton Dickenson & Co., Sweden) was inserted into an antecubital vein from which baseline 20 ml of blood was drawn through 2, 10 ml syringes and placed into serum separation beads and EDTA-containing tubes (Sarstedt Ltd, Leicester, UK). Plasma samples were centrifuged immediately at 5,000 rpm at 4 °C for 10 min. Serum samples were left to clot before centrifugation. An

adipose tissue biopsy was then performed. Adipose tissue samples (~0.5 g) were taken under local anaesthetic (1% lidocaine) from the area around the waist approximately 5 cm lateral to the umbilicus using a 14 G needle using an aspiration technique (Walhin *et al.*, 2013). Adipose tissue cleaning and processing was performed as described previously (Travers *et al.*, 2014). Following the adipose tissue biopsy, a two hour oral glucose tolerance test (OGTT) was performed following the ingestion of a commercial glucose beverage (Polycal, Nutricia, UK) with blood samples taken at 15 min intervals. The blood pressure was measured in triplicate at the end of OGTT. This protocol was replicated at the follow-up trial on day 8.

6.2.4 One week intervention description

In the OVER group, participants restricted daily physical activity to $\leq 4,000$ steps per day and were required to consume 50% more of recorded habitual diets. In the OVER + EN group, participants also restricted their daily physical activity to $\leq 4,000$ steps per day and were required to consume prescribed energy intake, plus a daily single 45 min bout of treadmill walking at 50% $\dot{V}O_{2max}$. In the OVER + BREAKS group, participants were only allowed to walk throughout the day to reach their individual target steps and were required to consume prescribed energy intake. In accordance to a similar overfeeding with restricted physical activity study, approximately 4,000 steps per day were completed in their sedentary and sedentary + daily exercise groups during the one week intervention (Walhin *et al.*, 2013); therefore, the OVER + BREAKS group were required to achieve their target steps plus an additional 4,000 steps. The accumulation of the designated daily step count for this group was requested to be performed “little and often” throughout the day (i.e., approximately every 30–40 min), each lasting ~5 min (approximately 500–600 steps). In addition, participants were asked to limit longer walks of greater than 1,500 steps to only once per day, and were asked to try and keep

this under 2,000 steps as a maximum in one go. Participants were also instructed not to sit for longer than 1 h at a time. The details of breaking sitting instructions are shown on Appendix 3.

The estimated energy expenditure during the 45 min at 50% $\dot{V}O_{2\max}$ walking test in the OVER + EN and OVER + BREAKS groups was 427 ± 54 kcal and 416 ± 89 kcal, respectively. In the OVER + EN group, treadmill speed was, on average, 6.4 ± 0.3 km·h⁻¹ with an average gradient of $6.5 \pm 2.3\%$. In the OVER + BREAKS group, the target step count was $13,771 \pm 1,778$ steps per day ($9,771 \pm 1,778$ target steps + additional 4,000 steps for each individual). During the intervention, jogging, running and weightlifting related exercise were not permitted.

Both exercise groups (OVER + EN and OVER + BREAKS) were required to consume additional energy each day to compensate for the extra energy expended during exercise interventions with an additional 5% of exercise energy expenditure to account for the excess post-exercise oxygen consumption (EPOC), respectively (Gore & Withers, 1990). DIT associated with the extra energy intake prescribed to the OVER + EN and OVER + BREAKS was also factored into calculations as an additional 10% of the energy expenditure during physical activity (including EPOC) (Westerterp, 2004). Also, RMR towards total energy expenditure during exercise was subtracted from the overfeeding calculation (Walhin *et al.*, 2013).

An example of habitual energy intake ($3,000 \text{ kcal}\cdot\text{day}^{-1}$) among three groups during overfeeding intervention is shown as Table 6.2.

Table 6.2 An example of overfeeding calculation

	OVER	OVER + EN	OVER + BREAKS
Energy intake ($\text{kcal}\cdot\text{day}^{-1}$)	3,000	3,000	3,000
50% overfeeding	+ 1,500	+ 1,500	+ 1,500
Exercise energy expenditure	-	+ 500	+ 500
EPOC	-	+ 25	+ 25
DIT (additional overfeeding)	-	+ 53	+ 53
RMR (during exercise)	-	- 54	- 54
Total energy intake prescribed	4,500 kcal	5,024 kcal	5,024 kcal
Energy surplus	1,500 kcal	1,500 kcal	1,500 kcal

6.2.5 Post intervention energy surplus calculation

The daily energy surplus was calculated by prescribed energy intake subtracted estimated energy expenditure (RMR + DIT + physical activity energy expenditure). RMR was estimated according to the average of baseline and follow-up data on the main trial days. DIT was calculated to be 10% of prescribed energy intake (Westerterp, 2004). Unstructured physical activity energy expenditure was estimated based on recorded average daily step counts (Walhin *et al.*, 2013), which was assumed stride length of 0.825 meters (Auvinet *et al.*, 2002) with consumed oxygen cost of $0.125 \text{ ml}\cdot\text{meter}^{-1}\cdot\text{kg}^{-1}$. In OVER + BREAKS groups, unstructured physical activity energy expenditure was calculated based on prescribed additional 4,000 steps as previous section described.

6.2.6 Experimental analysis

6.2.6.1 Adipose tissue gene expression

After cleaning the adipose tissue biopsy, approximately 200 mg of adipose tissue was immediately homogenised in 5 ml TRIzol (Invitrogen, Paisley, UK) in an RNase/DNase-free sterile tube (Invitrogen, Paisley, UK) and stored at -80°C until later gene expression analysis. The details of adipose tissue gene expression analysis was described in Chapter 3.14.

6.2.6.2 Real-time PCR

An RNeasy Mini Kit (Qiagen, Crawley, UK) was used to extract RNA from TRIzol-digested adipose tissue as described in Walhin *et al.* (2013). RNA was reversed transcribed (1 μg) to cDNA using a High Capacity Reverse Transcription Kit (Applied Biosystems, Warrington, UK). The organic phenol-chloroform phase from the RNA extraction was kept for future protein analysis. Real-time PCR was performed using a StepOne (Applied Biosystems, Warrington, UK). Predesigned primers and probes were obtained from Applied Biosystems for the measurement of expression of interleukin 6 (IL-6) (Hs00985639_m1), adiponectin (Hs00605917_m1), leptin (Hs00174877_m1), tumour necrosis factor alpha (TNF- α) (Hs99999043_m1), monocyte chemoattractant protein-1 (MCP-1) (Hs00234140_m1), 5' AMP-activated protein kinase (AMPK) (Hs01562315_m1 and Hs00178903_m1 combined), glucose transporter type 4 (GLUT4) (Hs00168966_m1), hormone-sensitive lipase (HSL) (Hs00193510_m1), insulin receptor substrate 2 (IRS2) (Hs00275843_s1), sterol regulatory element binding protein 1c (SREBP-1c) (Hs01088691_m1), pyruvate dehydrogenase kinase isozyme (PDK4) (Hs00176875_m1), peroxisome proliferator-activated receptor γ (PPAR γ) (Hs01115513_m1), adipose triglyceride lipase (ATGL) (Hs00386101_m1), fatty acid synthase (FAS) (Hs00188012_m1) and peptidylpropyl isomerase A (PPIA) was used

as an endogenous control (Neville *et al.*, 2011). The comparative Ct method was used to process data where $\Delta Ct = Ct \text{ target gene} - Ct \text{ PPIA}$. Ct target genes were normalised to an internal calibrator (lowest ΔCt for each target gene) and baseline. The Ct values for IL-6 (20 out of 36 samples) was frequently over 35 thus these results are not included.

6.2.6.3 Biochemical analysis

Blood glucose, triglycerides (TG), non-esterified fatty acids (NEFA), total cholesterol, high-density lipoprotein (HDL) cholesterol and alanine transaminase (ALT) were measured using commercially available assay kits and analyser (Daytona Rx; Randox, Crumlin, UK). Low-density lipoprotein (LDL) cholesterol was calculated based on the formula: $LDL = \text{total cholesterol} - HDL - (TG/2.2)$ (Friedewald *et al.*, 1972). The homeostatic model assessment for insulin resistance (HOMA-IR) was calculated based on the formula: $\text{fasting glucose (mmol}\cdot\text{l}^{-1}) \times \text{fasting insulin (mU}\cdot\text{l}^{-1})/22.5$ (Turner *et al.*, 1979). Homeostasis model assessment for β -cell function (HOMA- β) was calculated based on the formula: $\text{fasting insulin (mU}\cdot\text{l}^{-1}) \times 20/\text{fasting glucose (mmol}\cdot\text{l}^{-1}) - 3.5$ (Matthews *et al.*, 1985). Serum insulin concentrations ($\text{mU}\cdot\text{l}^{-1}$) were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Merckodia, Uppsala, Sweden).

6.2.7 Statistics

All data are presented as mean \pm standard deviation (SD). Gene expression data are presented as mean \pm standard error of the mean (SEM). Insulin and glucose iAUCs was calculated using the trapezoid method (Wolever & Jenkins, 1986). All data were checked for normality using visual checking of histograms, confirmed statistically by Shapiro-Wilk test. Where not normally distributed, data were analysed using equivalent

non-parametric tests. Gene expression data were analysed using a one-way ANOVA, pre- and post-intervention. The precise time-course of responses within and between trials were analysed using factorial 2- and 3-way mixed-model analysis of variance (group \times day & group \times day \times time, respectively), Greenhouse-Geisser corrections were utilized to intra-individual contrasts where $\varepsilon < 0.75$; for less severe asphericity the Huynh-Feldt correction was chosen (Atkinson, 2002). Where the mixed-model interaction was found, multiple t tests were used to check the location of variance both between groups at level time points and within each group between each time points relative to baseline, with both method to a Holm-Bonferroni correction (Atkinson, 2002). A main effect of the day denotes an effect of energy surplus (# denotes Day 1 versus Day 8 three groups), whereas a day \times group and day \times group \times time interaction means there was a mediating effect of exercise (* in figures and tables). A one-way ANOVA was also applied for habitual data (energy intake, steps and dietary composition). All data analysis was undertaken on SPSS version 22 software (IBM, Armonk, NY, USA). Statistical significance was set at $p \leq 0.05$.

6.3 Results

6.3.1 Induced energy surplus and energy expenditure during the intervention

The total energy intake and estimated energy expenditure in OVER, OVER + EN and OVER + BREAKS is presented in Table 6.3. There were no difference in energy surplus among three groups.

Table 6.3 Daily energy intake and expenditure

	OVER (n = 8)	OVER + EN (n = 8)	OVER + BREAKS (n = 8)
Caloric intake (kcal·day ⁻¹)	4,677 ± 1,001	5,569 ± 788	4,992 ± 978
RMR (kcal·day ⁻¹)	1,892 ± 266	1,874 ± 213	1,839 ± 203
DIT (kcal·day ⁻¹)	468 ± 100	557 ± 79	499 ± 98
Estimated physical activity energy expenditure (kcal·day ⁻¹)	111 ± 35	121 ± 28	149 ± 19
Additional exercise energy expenditure (kcal·day ⁻¹)	-	427 ± 54	416 ± 89
Energy surplus (kcal·day ⁻¹)	2,206 ± 716	2,591 ± 786	2,089 ± 589
Final energy surplus (%·day ⁻¹)	46 ± 6	46 ± 7	41 ± 4

Values are means ± SD.

6.3.2 Energy intake during habitual and the intervention

The percentage of macronutrients was not different among the three groups, and was approximately 56% carbohydrate, 17% fat and 25% protein, during habitual and intervention weeks (Table 6.4).

Table 6.4 The percentage of macronutrient intake in habitual and the intervention

	OVER (n = 8)	OVER + EN (n = 8)	OVER + BREAKS (n = 8)
Carbohydrate intake (%)	56 ± 5	58 ± 8	58 ± 7
Fat intake (%)	17 ± 5	17 ± 3	18 ± 4
Protein intake (%)	26 ± 4	24 ± 9	24 ± 8
Alcohol intake (%)	0 ± 0	0 ± 0	0 ± 0

Values are means ± SD.

There was no difference in total energy intake during the habitual week among the three groups (Table 6.5).

Table 6.5 Habitual dietary composition

	OVER (n = 8)	OVER + EN (n = 8)	OVER + BREAKS (n = 8)
Caloric intake (kcal·day ⁻¹)	3,118 ± 667	3,415 ± 510	3,038 ± 584
Carbohydrate intake (g·day ⁻¹)	366 ± 95	417 ± 87	365 ± 108
Fat intake (g·day ⁻¹)	112 ± 36	123 ± 31	107 ± 25
Protein intake (g·day ⁻¹)	167 ± 37	174 ± 66	144 ± 43
Alcohol intake (g·day ⁻¹)	3 ± 5	1 ± 2	1 ± 3

Values are means ± SD.

The total energy intake, dietary composition and overfeeding during the intervention is shown below (Table 6.6). Due to additional daily physical activity prescribed in OVER + EN and OVER + BREAKS groups, the overfeeding is different when compared to OVER group in order to achieve the same energy surplus.

Table 6.6 Dietary composition during the overfeeding intervention

	OVER (n = 8)	OVER + EN (n = 8)	OVER + BREAKS (n = 8)
Caloric intake (kcal·day ⁻¹)	4,677 ± 1,001	5,569 ± 788	4,992 ± 978
Carbohydrate intake (g·day ⁻¹)	549 ± 143	680 ± 139	601 ± 181
Fat intake (g·day ⁻¹)	251 ± 55	284 ± 113	237 ± 71
Protein intake (g·day ⁻¹)	169 ± 54	200 ± 48	176 ± 41
Alcohol intake (g·day ⁻¹)	5 ± 8	2 ± 4	2 ± 5
Overfeeding (%)	50 ± 0	63 ± 3	64 ± 2

Values are means ± SD.

6.3.3 Energy expenditure during habitual and the intervention

Habitual daily step counts were not different among three groups, nor was average energy expenditure from extra daily physical activity between OVER + EN and OVER + BREAKS (427 ± 54 versus 416 ± 89 kcal·day⁻¹).

The average habitual daily steps was decreased from $11,390 \pm 3,655$ to $2,847 \pm 821$ steps in OVER, from $10,176 \pm 2,008$ to $3,142 \pm 585$ steps in OVER + EN and from $10,388 \pm 4,752$ to prescribed 4,000 steps ($13,768 \pm 1,850$ steps subtracted from the estimated target of $9,768 \pm 1,850$ steps) in the OVER + BREAKS group (Figure 6.1).

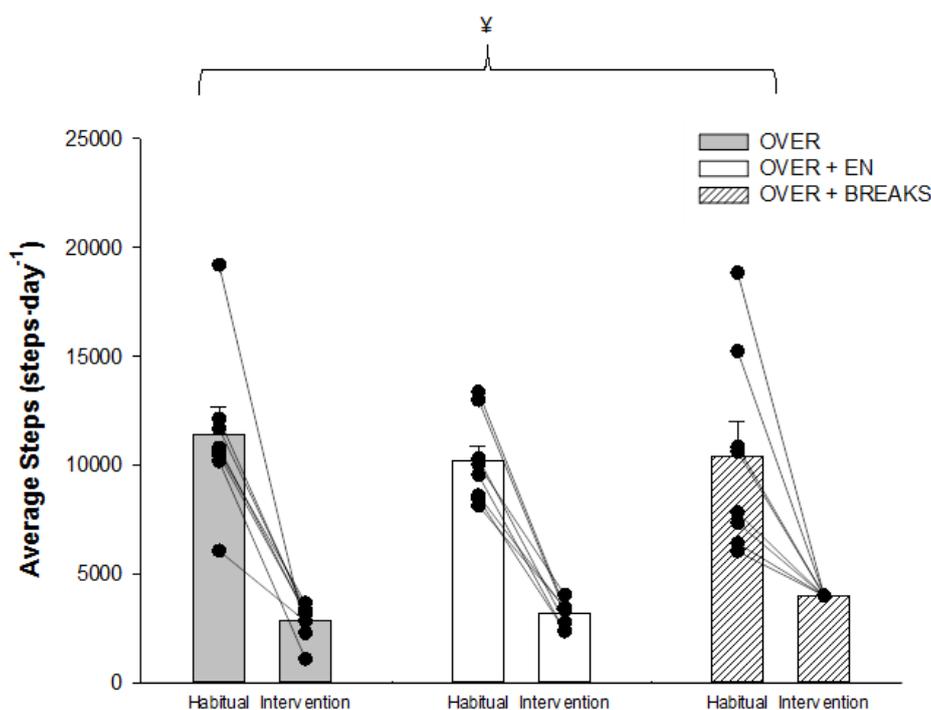


Figure 6.1 The change in average daily step counts at baseline (Habitual) and follow-up (intervention) in OVER, OVER + EN and OVER + BREAKS groups. All $n = 8$; these show the data outside the intervention (i.e., not including the steps taken during moderate intensity exercise or activity breaks in OVER + EN and OVER + BREAKS, respectively). Values are means \pm SEM. ¥ The difference between habitual versus intervention ($p \leq 0.05$).

6.3.4 Physical characteristics measures after the intervention

There was no interaction for all physical characteristics among three groups after intervention, except for lean mass which approached significance ($p = 0.06$) (Table 6.7). A day effect (Day 1 *versus* Day 8, the average of three groups; $p \leq 0.05$) was, however, apparent for several parameters after overfeeding (Table 6.7), including resting metabolic rate (RMR) and respiratory exchange ratio (RER) for most of participants as shown in Figure 6.2.

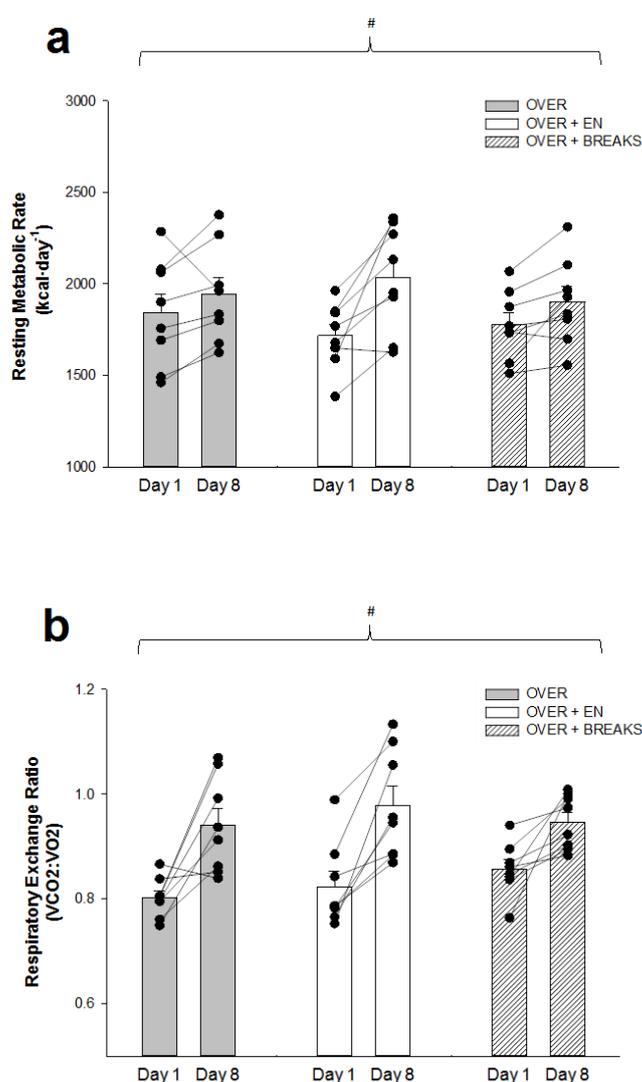


Figure 6.2 Resting metabolic rate (a) and respiratory exchange ratio (b) at baseline (Day 1) and follow-up (Day 8) in OVER, OVER + EN and OVER + BREAKS groups (all $n = 8$). Values are means \pm SEM. # Day effect (Day 1 *versus* Day 8, the average of three groups; $p \leq 0.05$).

Table 6.7 Participant physical characteristics at baseline (Day 1) and after the intervention (Day 8)

	OVER (n = 8)			OVER + EN (n = 8)			OVER + BREAKS (n = 8)		
	Day 1	Day 8	Δ (95% CI)	Day 1	Day 8	Δ (95% CI)	Day 1	Day 8	Δ (95% CI)
Body mass (kg; scale) #	74.3 ± 9.8	76.1 ± 9.9	1.8 (1.1–2.6)	72.8 ± 6.9	75.4 ± 7.8	2.7 (1.7–3.7)	71.5 ± 8.8	73.2 ± 9.4	1.7 (1.0–2.3)
Body mass (kg) #	73.8 ± 9.7	75.6 ± 9.8	1.9 (1.1–2.6)	72.3 ± 6.8	74.9 ± 7.8	2.6 (1.4–3.8)	71.1 ± 8.8	72.7 ± 9.3	1.6 (1.0–2.2)
Lean mass (kg) #	59.4 ± 8.3	60.4 ± 8.4	1.0 (0.5–1.6)	58.8 ± 7.4	61.2 ± 8.6	2.4 (1.2–3.6)	57.8 ± 7.2	58.8 ± 7.4	1.0 (0.2–1.8)
Fat mass (kg) #	11.5 ± 4.5	12.4 ± 4.3	0.8 (0.2–1.5)	10.6 ± 4.4	10.8 ± 4.0	0.2 (-0.3 to 0.7)	10.3 ± 3.9	11.0 ± 4.3	0.6 (0.1–1.1)
Fat mass (%)	15.5 ± 5.3	16.2 ± 5.0	0.8 (-0.1 to 1.6)	14.7 ± 6.3	14.5 ± 5.9	-0.1 (-0.8 to 0.5)	14.4 ± 4.4	14.9 ± 4.7	0.5 (-0.1 to 1.1)
WC (cm) #	83.4 ± 7.1	85.0 ± 6.5	1.6 (0.7–1.0)	81.7 ± 5.2	83.9 ± 5.1	2.2 (1.0–3.3)	81.7 ± 7.6	82.6 ± 6.9	1.0 (-0.1 to 2.0)
HC (cm) #	95.7 ± 6.0	96.9 ± 5.9	1.3 (0.8–1.7)	93.7 ± 3.3	94.8 ± 3.9	1.1 (0.2–2.1)	92.9 ± 4.2	93.4 ± 4.8	0.5 (-0.3 to 1.3)
RMR (Kcal·d ⁻¹) #	1,841 ± 294	1,942 ± 268	101 (-29 to 230)	1,716 ± 181	2,033 ± 292	337 (181–492)	1,776 ± 188	1,901 ± 236	122 (33–210)
RER ($\dot{V}CO_2:\dot{V}O_2$) #	0.80 ± 0.04	0.94 ± 0.09	0.15 (0.08–0.22)	0.82 ± 0.08	0.94 ± 0.12	0.16 (0.06–0.26)	0.86 ± 0.05	0.95 ± 0.05	0.09 (0.05–0.14)
SBP (mmHg) #	122 ± 8	125 ± 8	3 (-0.8 to 7.2)	118 ± 7	127 ± 7	8 (3–14)	123 ± 8	123 ± 8	1 (-4 to 5)
DBP (mmHg) #	71 ± 5	73 ± 6	3 (-2 to 7)	69 ± 7	76 ± 4	7 (2–12)	71 ± 9	70 ± 7	1 (-5 to 4)

SBP, systolic blood pressure; DBP, diastolic blood pressure; RMR, resting metabolic rate; RER, resting metabolic rate; WC, waist circumference; HC, hip circumference. Values are means ± SD, with fold changes (Day 8 / Day 1) after intervention. Change scores with 95% confidence interval (CI) for each group. # Day effect (Day 1 *versus* Day 8, the average of three groups; $p \leq 0.05$).

6.3.5 Blood parameters measures after the intervention

A significant group \times day \times time interaction was found in serum insulin responses to the 2 h OGTT ($p \leq 0.05$). This might be due to the OVER group showing higher serum insulin concentrations from 75 min to the end of OGTT test (Figure 6.4). There was no interaction for plasma glucose, serum insulin iAUCs (Figure 6.5), plasma glucose responses to the 2 h OGTT test (Figure 6.3) and other blood parameters (Table 6.8) among three groups. However, a day effect (Day 1 *versus* Day 8, the average of three groups; $p \leq 0.05$) was found with a 1.5-fold increase in OVER, 1.8-fold increase in OVER + EN and 1.7-fold increase in OVER + BREAKS group in insulin iAUC as a result of the intervention (Figure 6.5 b).

In addition, a group \times day interaction was found in white blood cell (WBC) counts ($p \leq 0.05$) among three groups and when comparing baseline (Day 1) to follow-up (Day 8) trials, WBC counts were increased in OVER (from 5.3 ± 1.3 to 7.3 ± 3.0 ; $p = 0.06$) and OVER + EN (from 5.3 ± 1.3 to 5.9 ± 1.1 ; $p \leq 0.05$) but not in OVER + BREAKS (from 5.7 ± 1.9 to 5.6 ± 1.5) (Table 6.8).

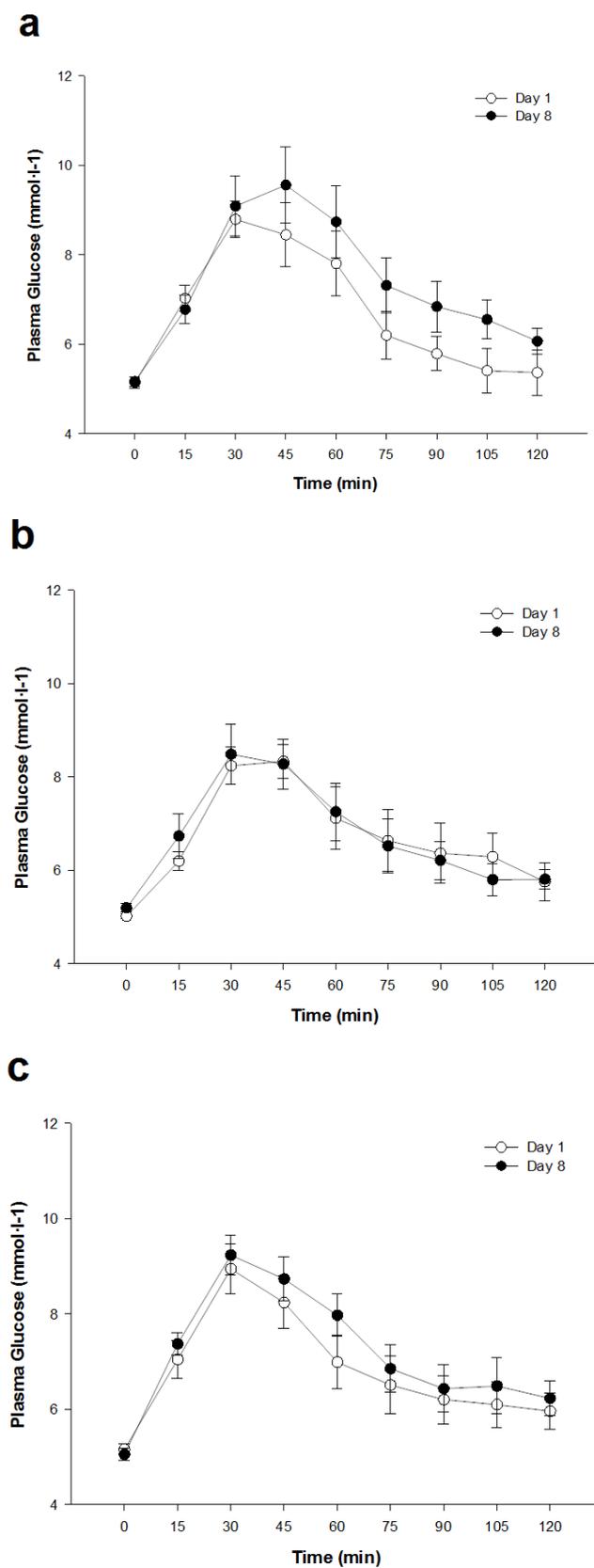


Figure 6.3 Plasma glucose responses to a 2 h OGTT at baseline (Day 1) and follow-up (Day 8) in OVER (a), OVER + EN (b) and OVER + BREAKS (c) groups (all $n = 8$). Values are means \pm SEM.

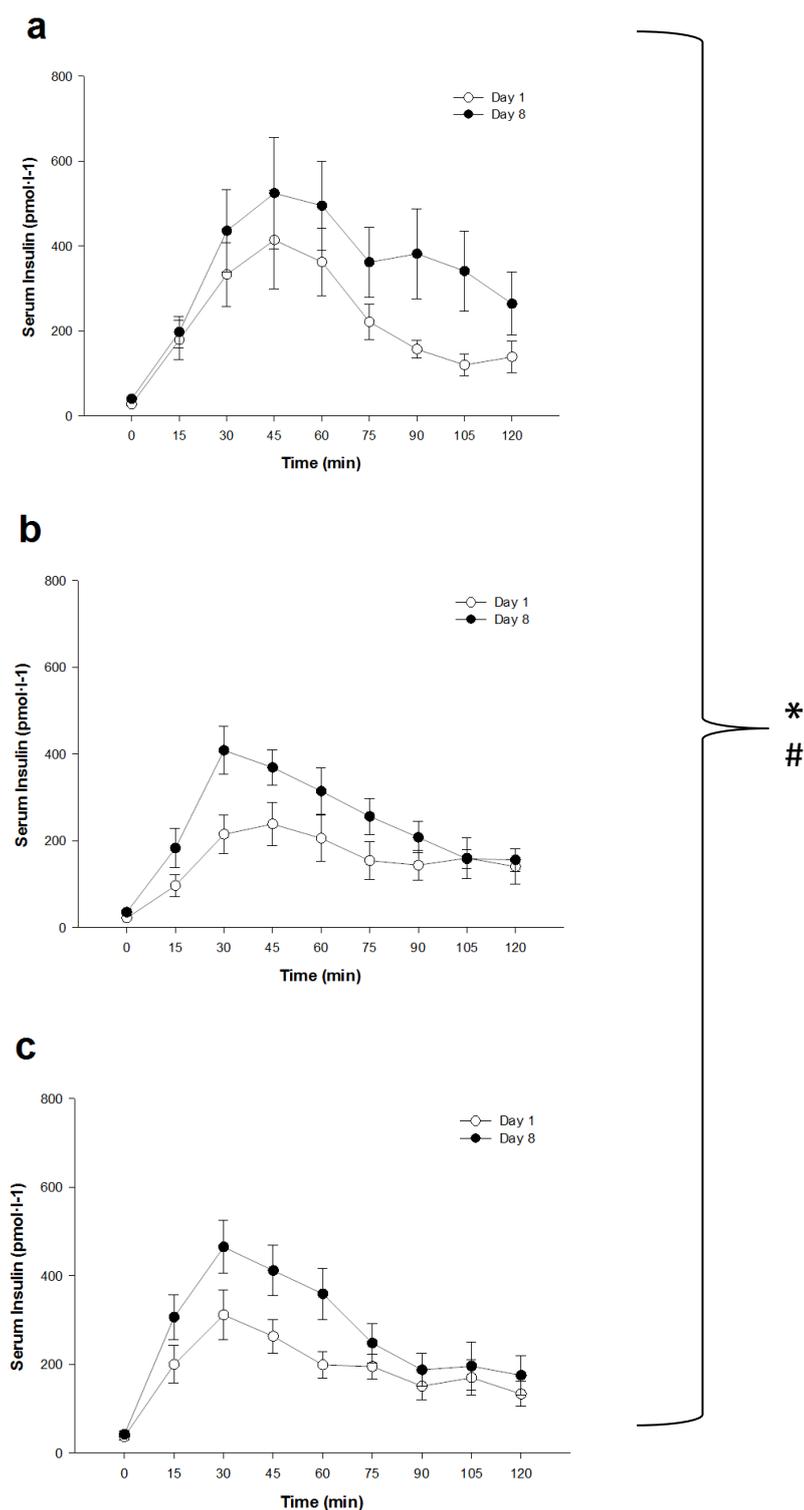


Figure 6.4 Serum insulin responses to a 2 h OGTT at baseline (Day 1) and follow-up (Day 8) in OVER (a), OVER + EN (b) and OVER + BREAKS (c) groups (all $n = 8$). Values are means \pm SEM. * indicates interaction effect (group \times day \times time; $p \leq 0.05$). # indicates time effect (day \times time, Day 1 *versus* Day 8, the average of three groups; $p \leq 0.05$).

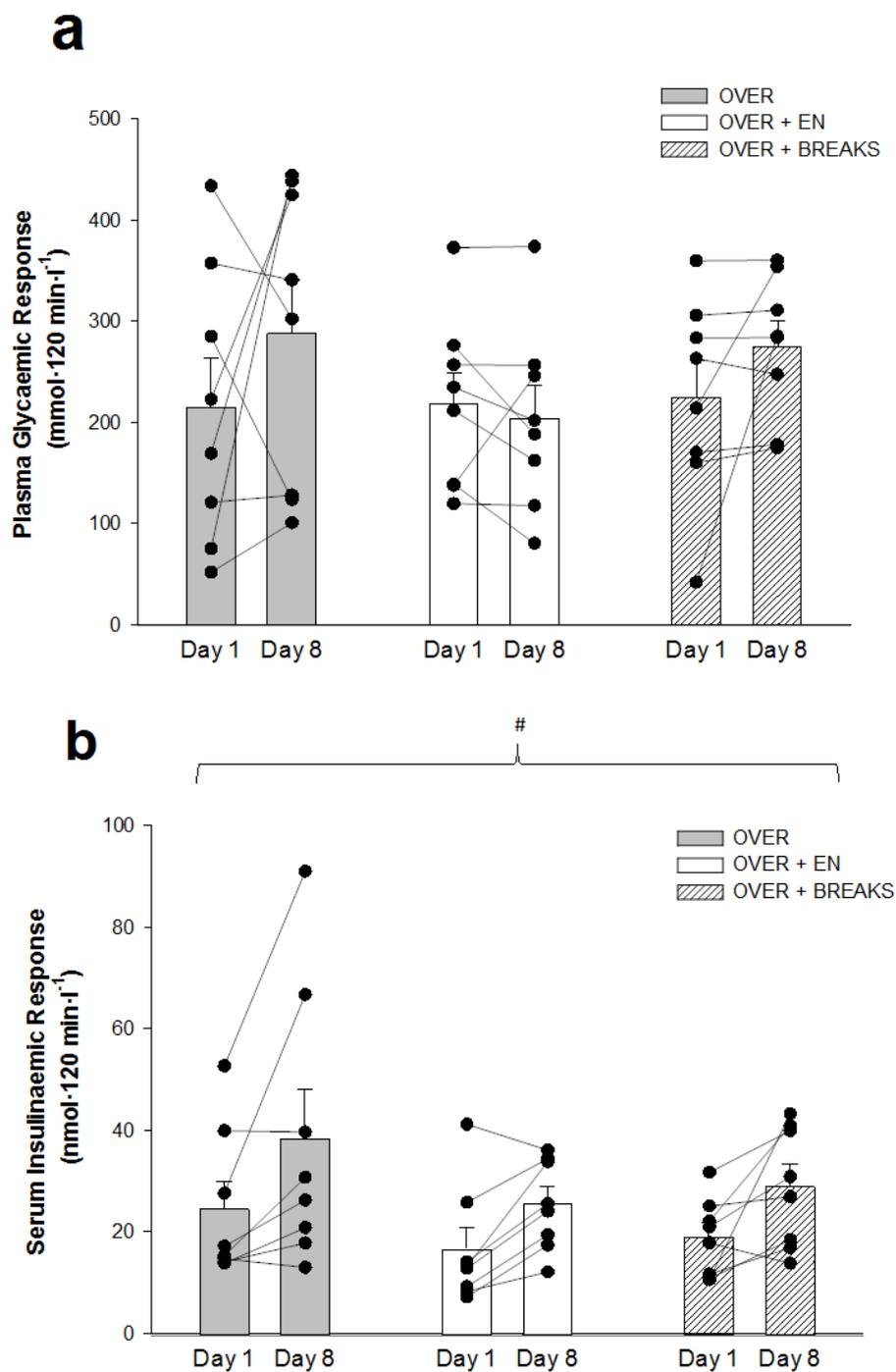


Figure 6.5 Plasma glucose (a) and serum insulin (b) iAUCs in response to a 2 h OGTT at baseline (Day 1) and follow-up (Day 8) in OVER, OVER + EN, and OVER + BREAKS groups (all $n = 8$). Values are means \pm SEM. # Day effect (Day 1 versus Day 8, the average of three groups; $p \leq 0.05$).

Table 6.8 HOMA-IR, HOMA- β and blood measures at baseline (Day 1) and after intervention (Day 8)

	OVER (n = 8)				OVER + EN (n = 8)				OVER + BREAKS (n = 8)			
	Day 1	Day 8	Fold changes	Δ (95% CI)	Day 1	Day 8	Fold changes	Δ (95% CI)	Day 1	Day 8	Fold changes	Δ (95% CI)
Fasting glucose (mmol·l ⁻¹)	5.1 ± 0.3	5.2 ± 0.3	1.0 ± 0.1	0.1 (-0.2 to 0.2)	5.0 ± 0.2	5.2 ± 0.3	1.0 ± 0.1	0.2 (0.0 to 0.3)	5.2 ± 0.3	5.1 ± 0.4	1.0 ± 0.1	-0.1 (-0.3 to 0.0)
Fasting insulin (pmol·l ⁻¹) #	27.9 ± 8.6	40.4 ± 16.2	1.5 ± 0.4	13 (5–20)	22.0 ± 3.9	35.5 ± 12.7	1.7 ± 0.7	14 (4–23)	37.1 ± 21.9	42.4 ± 17.2	1.3 ± 0.6	5 (-11 to 22)
HOMA-IR #	1.06 ± 0.34	1.56 ± 0.69	1.5 ± 0.4	0.5 (0.1–0.8)	0.81 ± 0.14	1.37 ± 0.51	1.7 ± 0.8	0.6 (0.2–0.9)	1.41 ± 0.83	1.59 ± 0.73	1.3 ± 0.6	0.2 (-0.5 to 0.8)
HOMA- β (%) #	60 ± 27	81 ± 29	1.4 ± 0.4	21 (8–35)	49 ± 12	72 ± 29	1.5 ± 0.6	23 (4–41)	78 ± 47	95 ± 41	1.4 ± 0.6	17 (-17 to 52)
TG (mmol·l ⁻¹) #	0.7 ± 0.2	1.3 ± 0.7	1.8 ± 0.2	0.6 (0.2–1.0)	0.6 ± 0.2	0.9 ± 0.3	1.6 ± 0.7	0.3 (0.1–0.5)	0.8 ± 0.2	1.0 ± 0.4	1.3 ± 0.4	0.3 (0.0–0.5)
NEFAs (mmol·l ⁻¹) #	0.5 ± 0.3	0.2 ± 0.1	0.6 ± 0.5	-0.3 (-0.6 to -0.1)	0.4 ± 0.2	0.1 ± 0.1	0.3 ± 0.2	-0.3 (-0.4 to -0.1)	0.8 ± 0.7	0.2 ± 0.1	0.3 ± 0.2	-0.6 (-1.1 to 1.02)
Total cholesterol (mmol·l ⁻¹)	3.9 ± 0.7	4.0 ± 0.7	1.0 ± 0.1	0.1 (-0.2 to 0.3)	3.7 ± 0.6	3.8 ± 0.3	1.0 ± 0.1	0.1 (-0.2 to 0.4)	4.0 ± 0.6	4.1 ± 0.5	1.0 ± 0.1	0.1 (-0.2 to 0.4)
HDL cholesterol (mmol·l ⁻¹)	1.1 ± 0.3	1.1 ± 0.3	1.0 ± 0.1	0.1 (-0.1 to 0.1)	1.1 ± 0.3	1.1 ± 0.3	1.0 ± 0.1	0.1 (-0.1 to 0.1)	1.3 ± 0.2	1.3 ± 0.2	1.0 ± 0.1	0.1 (-0.1 to 0.1)
LDL cholesterol (mmol·l ⁻¹)	2.5 ± 0.5	2.2 ± 0.4	0.9 ± 0.1	-0.2 (-0.4 to -0.1)	2.4 ± 0.5	2.3 ± 0.3	1.0 ± 0.2	-0.1 (-0.3 to 0.1)	2.3 ± 0.4	2.3 ± 0.3	1.0 ± 0.2	-0.1 (-0.3 to 0.3)
ALT (U·l ⁻¹) #	23 ± 7	29 ± 13	1.2 ± 0.5	6 (-1 to 13)	23 ± 11	36 ± 11	1.8 ± 0.7	13 (5–20)	24 ± 7	32 ± 12	1.4 ± 0.6	8 (0 to 16)
Whole blood WBC (×10 ⁹ ·l ⁻¹) *	5.3 ± 1.3	7.3 ± 3.0	1.4 ± 0.5	2.0 (0.3–3.8)	5.3 ± 1.3	5.9 ± 1.1	1.1 ± 0.1	0.6 (0.3–1.0) ¥	5.7 ± 1.9	5.6 ± 1.5	1.0 ± 0.1	-0.1 (-0.7 to 0.5)

HOMA-IR, homeostatic model assessment for insulin resistance; HOMA- β , homeostatic model assessment for β -cell function; ALT, alanine transaminase; TG, triglycerides; NEFA, non-esterified fatty acids; HDL, high-density lipoprotein; LDL, low-density lipoprotein; WBC, while blood cell. Values are means ± SD, with fold changes (Day 8 / Day 1) after intervention. Change scores with 95% confidence interval (CI) for each group. * Interaction effect (group × day; $p \leq 0.05$); # Day effect (Day 1 *versus* Day 8, the average of three groups; $p \leq 0.05$). ¥ Day effect (Day 1 *versus* Day 8 within group; $p \leq 0.05$).

6.3.6 Adipose Tissue mRNA Expression

As shown in Figure 6.6, adipose tissue SREBP-1c gene expression was up-regulated in all three groups and a difference was found among three groups following the intervention ($p \leq 0.05$). Post hoc analysis revealed that the change in adipose SREBP-1c mRNA expression in OVER + EN was higher than OVER and OVER + BREAKS groups ($p \leq 0.05$), though no difference was found between OVER and OVER + BREAKS groups.

Adipose tissue FAS mRNA expression was also up-regulated in all three groups after intervention and the difference approached statistical difference among three groups ($p = 0.06$). The expression of the remaining adipose genes were not different but there were systematic changes in all three groups. The expression of AMPK, adiponectin, PDK4, HSL and IRS2 were all down-regulated and GLUT4 was up-regulated after the one week energy surplus in all three groups, independent of the intervention applied.

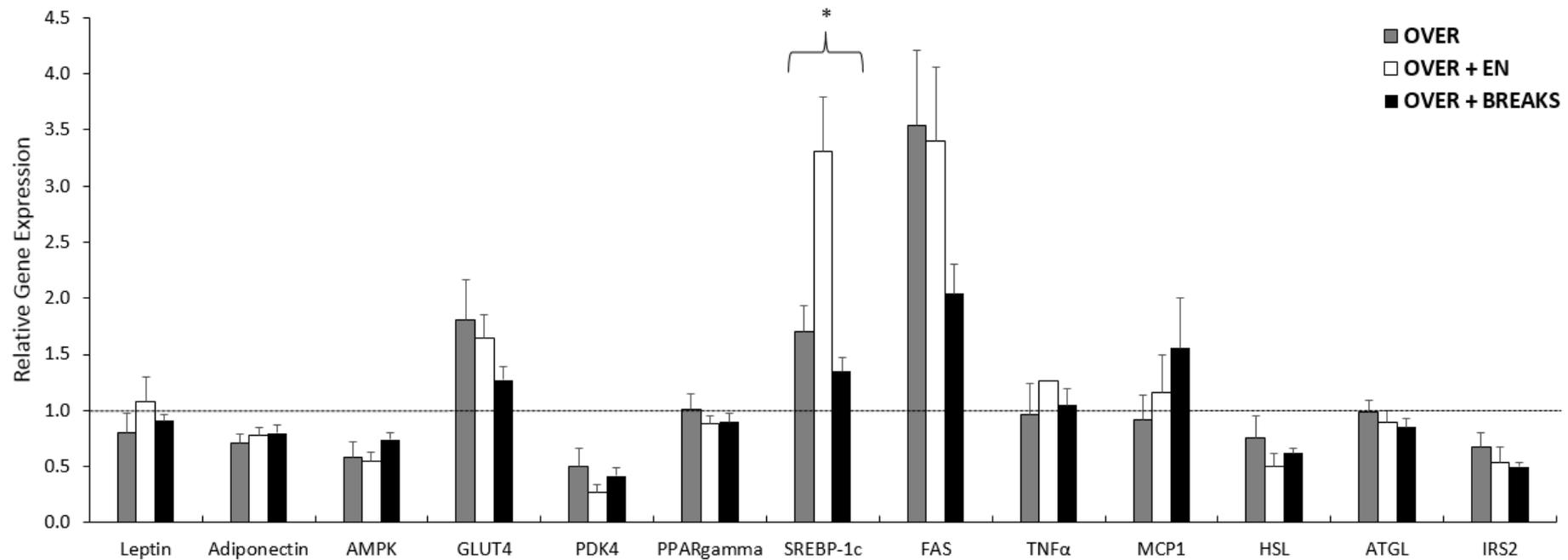


Figure 6.6 Fold changes in relative adipose tissue gene expression (mRNA levels)

Fold changes (Day 8/Day1) in relative gene expression (mRNA levels) of metabolic, insulin signaling, glucose transport and inflammatory genes in adipose tissue in OVER and OVER + EN (both $n = 5$), and OVER + BREAKS ($n = 8$) groups, except for TNF- α ($n = 4$ in OVER, $n = 1$ in OVER + EN and $n = 5$ in OVER + BREAKS). The dashed line indicates baseline (Day 1). Data normalised to PPIA, internal calibrator and baseline. Samples that exceeded the detectable limit ($Ct > 35$) were excluded from the analysis. Values are means \pm SEM. * $p \leq 0.05$ OVER + EN greater than OVER and OVER + BREAKS.

6.4 Discussion

This is the first study to investigate the effect of different forms of physical activities on the response to overfeeding. We demonstrated that short-term overfeeding combined with restricted physical activity resulted in the reduction of insulin sensitivity and altered the expression of genes related to carbohydrate and lipid metabolism, lipid synthesis and insulin regulation pathway expression in adipose tissue. In adipose lipid synthesis genes, the expression of SREBP-1c and FAS mRNA were up-regulated in all three groups after the intervention; however, the expression of these two genes was relatively lower in OVER + BREAKS when compared to OVER and OVER + EN groups. In addition, we also found that intermittent physical activity (constantly breaking prolonged sitting) is capable of alleviating the energy surplus-induced circulating inflammatory response (i.e., it reduced WBC counts).

6.4.1 Circulating blood measures responses after the intervention

Only one other study has shown that a single, daily bout of vigorous intensity exercise (70% $\dot{V}O_{2max}$) was capable of reversing impairments to insulin sensitivity induced by overfeeding (Walhin *et al.*, 2013). Our results demonstrated that insulin iAUCs were increased in all three groups after one week of overfeeding, irrespective of the type of physical activity assessed. One reason for this discrepancy could be due to the exercise intensity, as for exercise intensities above 70% $\dot{V}O_{2max}$, up to 70% of energy is derived from carbohydrate (Kang *et al.*, 2007), which might translate to approximately 50 g·day⁻¹ of negative carbohydrate storage (Walhin *et al.*, 2013). However, the exercise intensity in OVER + EN was 50% $\dot{V}O_{2max}$, reducing the capabilities of the body to utilize carbohydrate at this intensity (Venables *et al.*, 2005; Kang *et al.*, 2007). This might

explain why the insulin response to an OGTT might not have been affected in the current study.

As was also observed in the only other study to investigate the effects of exercise and overfeeding (Walhin *et al.*, 2013), in the present study, TAG and ALT were increased and NEFA was decreased after one week overfeeding.

6.4.2 Adipose tissue mRNA gene expression after the intervention

AMPK regulates cellular energy homeostasis by becoming activated in the face of high AMP/ATP and ADP/ATP ratios, which stimulates energy synthesis and inhibits lipogenesis and gluconeogenesis (O'Neill, 2013). The expression of adipose AMPK mRNA was down-regulated after short-term overfeeding with a single daily bout of vigorous intensity exercise (Walhin *et al.*, 2013). Similarly, we found that even though different forms of physical activity was undertaken, adipose AMPK mRNA expression was still down-regulated in all three groups. This implies that gluconeogenesis and lipogenesis might be elevated due to an extra 50% energy surplus. However, compared to the other two groups, the reduction of AMPK mRNA expression in OVER + BREAKS was relatively lower (albeit not significantly). Although far from certain at this stage, one explanation for this finding could be that regular body movements as a consequence of breaks to prolonged periods of sitting constantly stimulated AMPK activation.

In addition to the reduced energy synthesis as a result of the down-regulation of AMPK mRNA expression, a reduction in glycolysis and lipolysis might also play a role in the regulation of energy metabolism. The expression of adipose PDK4 and HSL mRNA

were both down-regulated in all three groups in current study. The main role of PDK4 is to regulate glucose metabolism by inhibiting the pyruvate dehydrogenase (PDH) complex and the activated PDK4, switching energy metabolism preference from glucose to lipid utilisation. Similar to other overfeeding studies (Shea *et al.*, 2009; Walhin *et al.*, 2013), we also found that the expression of PDK4 was down-regulated after one week's overfeeding irrespective of the combination with different types of physical activity. The reduction of PDK4 would increase glucose utilisation which evidenced here by an increased RER in all three groups (0.80 ± 0.04 to 0.94 ± 0.09 in OVER; 0.82 ± 0.08 to 0.94 ± 0.12 in OVER + EN and 0.86 ± 0.05 to 0.95 ± 0.05 in OVER + BREAKS).

ATGL and HSL are the two most important lipolytic enzymes which are activated during fasting and exercise and suppressed with feeding. Surprisingly, only HSL was down-regulated in OVER, OVER + EN and OVER + BREAKS. Adipose HSL gene expression was also found to decrease in response to overfeeding in combination with vigorous intensity exercise in a previous study (Walhin *et al.*, 2013). Taken together, the down-regulation of adipose AMPK, PDK4 and HSL mRNA gene expression might potentially decrease glycolysis, lipolysis as well as energy synthesis.

From an energy metabolism perspective, the proportion of substrate utilization in OVER + EN and OVER + BREAKS may differ. In OVER + EN group (continuous exercise), adipose and intramuscular triglycerides, and circulating NEFA are the main energy substrates (Horowitz, 2003). Therefore, the original stored lipid might provide most of the energy expenditure, so that most of the consumed energy needs to be processed and stored. On the contrary, the preference for energy substrate utilized in

OVER + BREAKS (intermittent exercise) might have been towards carbohydrate (based on the result of the Chapter 5) and consumed carbohydrate might constantly be utilized during walking. Accordingly, even though physical activity induced energy expenditure was very similar (approximately 420 kcal per day) between OVER + EN and OVER + BREAKS groups; it could potentially result in very different types of substrate utilization throughout the intervention. As a result, this might influence energy storage and relevant gene regulation differently.

SREBP-1c is a transcriptional factor, which is induced by feeding/insulin and plays a role to regulate the expression of several genes involved in lipid synthesis. FAS is one of the lipogenic enzymes regulated by SREBP-1c (Gosmain *et al.*, 2005) and its main function is to catalyse malonyl-CoA into palmitoyl-CoA. The expression of adipose SREBP-1c and FAS mRNA has been shown to be up-regulated in response to overfeeding (Minehira *et al.*, 2004; Walhin *et al.*, 2013). However, Walhin *et al.* (2013) found that a daily 45 min bout of vigorous intensity exercise (70% $\dot{V}O_{2max}$) blunted both genes in response to overfeeding. Similarly, we found that the expression of SREBP-1c and FAS was up-regulated in all three groups after overfeeding but surprisingly their expression was blunted in OVER + BREAKS rather than in OVER + EN group. There are several possible explanations for this occurrence: Insulin has been shown to affect SREBP-1c mRNA expression, demonstrating 3–4 fold changes in human adipose SREBP-1c mRNA expression after 4 h insulin infusion (Boden *et al.*, 2013). In the current study, the OVER + EN group demonstrated the highest fasting insulin levels, possibly explaining why SREBP-1c mRNA expression was significantly higher than the other two groups. SREBP-1c has also been linked with *de novo* lipogenesis (DNL). The lipid stored in adipose tissue can be from dietary lipid, from carbohydrate via DNL,

or from a mixture of the two. Indeed, to lend support to this concept, DNL has been shown to significantly increase in response to high carbohydrate overfeeding interventions (Minehira *et al.*, 2004; Acheson K, 1998). The short-term energy imbalance-induced weight gain might increase glycogen content alongside water storage via DNL (Walhin *et al.*, 2013). As lean mass increased in the OVER (1.0 ± 0.8 kg), OVER + EN (2.4 ± 1.7 kg) and OVER + BREAKS (1.0 ± 1.2 kg) groups, this could be demonstrative of increased glycogen and water storage as a result of DNL.

The translocation of adipose GLUT4 from intracellular compartments to the cell surface is stimulated by insulin signalling pathways (e.g., in postprandial period) (Huang & Czech, 2007) and adipose tissue is one of the tissues which disposes of postprandial glucose. In agreement with other studies (Walhin *et al.*, 2013; Gillberg *et al.*, 2016), the expression of GLUT4 mRNA here was up-regulated in response to overfeeding. There are several possible explanations for this occurrence. First, the increase in fasting insulin levels in all three groups might result in the higher expression of adipose GLUT4 at baseline level, and there was a correlation for these parameters ($r^2 = 0.55$; $p = 0.01$). Adipose GLUT4 mRNA expression was found to be associated with insulin resistance (Kouidhi *et al.*, 2013; Travers *et al.*, 2015b). Another potential explanation could be that a short-term energy surplus resulted in GLUT4 insensitivity, resulting in high baseline GLUT4 expression to overcome this insensitivity.

IRS2 is one of insulin receptors which mediates the initial stage of insulin signalling. In rodent studies, disruption of IRS2 (e.g., IRS2 deficient or knockout mice) impaired glucose homeostasis by a deterioration in peripheral insulin signalling and pancreatic β -cell function (Withers *et al.*, 1998; Kubota *et al.*, 2000; Kubota *et al.*, 2004).

Moreover, chronic low levels of IRS2 mRNA have been found in insulin resistant humans (Travers *et al.*, 2015b). It has been consistently shown that both short-term high carbohydrate and high-fat overfeeding reduces the expression of adipose IRS2 mRNA (Shea *et al.*, 2009; Walhin *et al.*, 2013; Gillberg *et al.*, 2016). The reduction of adipose IRS2 mRNA expression in the present study might reflect the process of impaired insulin sensitivity in response to overfeeding. Interestingly, vigorous intensity exercise was found to prevent the overfeeding-induced down-regulation of IRS2 mRNA expression (Walhin *et al.*, 2013), implying that exercise intensity has the capacity to regulate IRS2 mRNA expression even in the face of short-term energy surplus. Collectively, the expression of specific insulin signalling genes were systematically altered after overfeeding (i.e., increased GLUT4 and decreased IRS2 mRNA) which might be due to impaired insulin sensitivity, and this seems independent of the different types of physical activity involved.

6.5 Conclusion

This study demonstrated that short-term overfeeding combined with restricted physical activity resulted in the impairment of insulin sensitivity independent of the different type of physical activity involved and also altered the expression of genes related to carbohydrate and lipid metabolism, lipid synthesis and insulin regulation pathways in adipose tissue. However, constantly breaking prolonged sitting physical activity was able to reduce overfeeding induced circulating inflammatory response (i.e., reducing WBC counts) and reduced adipose lipid synthesis gene expression, suggesting that constant short bouts of walking might be considered useful for general populations and also applied for future exercise prescription.

Chapter 7: General discussion

7.1 Overview

Throughout the conducted three experimental projects, I have intended to explore the impact of different types of physical activity via structured prolonged exercise and NEAT-type physical activity on physiological responses – especially focusing on adipose tissue. Chapter 4 intended to understand how diet interacts with the responses to acute exercise. Chapter 5 investigated the effect of NEAT-type physical activity and Chapter 6 examined how different type of physical activity (a single bout of daily moderate intensity exercise *versus* NEAT-type physical activity) impacts upon the response to short-term energy surplus. The findings and conclusions in each chapter are summarised as follows:

7.1.1 Chapter 4: Feeding influences adipose tissue responses to exercise in overweight men

1. Overnight fasting prior to exercise impacted upon metabolism during moderate intensity exercise (60% $\dot{V}O_{2max}$ for 1 h), increasing fat oxidation and decreasing carbohydrate oxidation.
2. Exercise in a fasted state influenced adipose tissue responses to exercise including an up-regulation of adipose lipolytic enzymes (ATGL and HSL), lipid transporter (FAT/CD36), glucose transporter (GLUT4), glycolytic inhibitor enzyme (PDK4) and insulin signalling receptor (IRS2) mRNA expression when compared to exercise in a fed state.

3. Circulating and adipose *ex-vivo* adipokine secretion (IL-6, adiponectin and leptin) responses were not affected with exercise either under a fasted or fed state.

7.1.2 Chapter 5: The effect of breaking prolonged sitting on metabolism and adipose tissue in middle-age overweight men and women

1. Participating in regular short bouts of walking (merely 243 ± 66 kcal energy expenditure) reduces postprandial glucose and insulin concentrations in overweight and obese people, possibly via an increase in carbohydrate oxidation during walking.
2. Even though there was a reduction of postprandial glucose and insulin concentrations with breaking sedentary behaviour, this did not influence adipose tissue metabolic, insulin signalling and inflammatory gene expression.
3. Several adipose genes were systematically changed in both prolonged sitting and breaking sitting trials. This could reflect meal-induced adipose tissue responses and these might outweigh the small impact from regular walking.
4. Accumulating short bouts of physical activity did not affect circulating and adipose *ex-vivo* adipokine secretion (IL-6, adiponectin and leptin).

7.1.3 Chapter 6: The effect of alternative physical activity strategies on short-term overfeeding in young healthy men

1. Short-term energy surplus (50% overfeeding of habitual diet) impaired insulin sensitivity irrespective of the type of physical activity.

2. NEAT-type physical activity (OVER + BREAKS) possibly alleviated overfeeding-induced inflammation, with no change in white blood cell (WBC) counts in comparison to both other groups.
3. OVER + BREAKS blunted overfeeding induced lipogenic signals via a reduction in adipose SREPB-1c and FAS mRNA expression.

The studies in this thesis used a variety of different physical activity interventions and meal/diet based challenges. A summary of energy intake and expenditure on trial days are shown as below (Table 7.1).

Table 7.1 Energy intake and energy expenditure in each experimental trial

Trial types	Chapter 4		Chapter 5		Chapter 6		
	Fasted exercise	Fed exercise	Prolonged sitting	Breaking sitting	OVER	OVER + EN	OVER + BREAKS
Exercise intensity	60% $\dot{V}O_{2max}$	60% $\dot{V}O_{2max}$	-	6.4 km·h ⁻¹ walking	-	50% $\dot{V}O_{2max}$	4.9 km·h ⁻¹ walking
Energy expenditure during exercise (kcal·day ⁻¹ ·trial ⁻¹)	746 ± 129	771 ± 135	-	243 ± 66	-	427 ± 54	416 ± 89
Meal type (CHO %)	-	70	52	52	56 ± 5	58 ± 8	58 ± 7
Total energy intake (kcal·day ⁻¹ ·trial ⁻¹)	-	648 ± 115	1,724 ± 369	1,730 ± 366	4,677 ± 1,001	5,567 ± 788	4,992 ± 978
Overfeeding level (%)	-	-	-	-	50 ± 0	63 ± 3	64 ± 2
Overfeeding-induced energy surplus (%·day ⁻¹)	-	-	-	-	46 ± 6	46 ± 7	41 ± 4

Values are means ± SD.

7.2 The effect of the energy intake on adipose tissue

Apart from providing energy during prolonged exercise, another main task of adipose tissue is to process and/or store consumed energy (Romanski *et al.*, 2000; Summers *et al.*, 2001). The results indicated that SREPB-1c, IRS2 and PDK4 were consistently altered throughout all three projects. Indeed, it seems that fasting and feeding effectively regulate these pathways. For instance, high levels of hepatic IRS2 mRNA expression was significantly reduced by feeding (Ide *et al.*, 2004), and the expression of adipose and hepatic SREPB-1c mRNA was up-regulated after feeding (Palou *et al.*, 2008). Inverse correlations have been found between various nutritional conditions and SREPB-1c as well as IRS2 mRNA (Ide *et al.*, 2004). In a rodent study, in liver, SREBP was found to *directly* repress transcription of IRS2 and this further inhibited hepatic insulin signaling with a switch from glycogen synthesis to lipogenesis (Ide *et al.*, 2004).

In this thesis, the expression of lipogenesis-related transcriptional molecules SREPB-1c were all increased with energy intake in all trials (Figure 7.1). In Chapter 4 and 5, the expression of IRS2 mRNA was decreased after energy intake independent of the type of meal and total energy intake. In Chapter 6, overfeeding induced energy surplus down-regulated adipose IRS2 mRNA in OVER, OVER + EN and OVER + BREAKS (Figure 7.2). Taken together, over the three projects, and similar to rodents, the results demonstrate that *human adipose SREPB-1c and IRS2* mRNA respond very clearly to feeding. Other studies also show similar findings, indicating that a short-term energy surplus resulted in increased adipose SREPB-1c and a decrease in IRS2 (Walhin *et al.*, 2013). Consistent with previous studies, the expression of adipose IRS2 mRNA is decreased whether after a single meal in lean, overweight and obese men (Travers *et al.*, 2015b), or short-term energy surplus (Shea *et al.*, 2009; Walhin *et al.*, 2013).

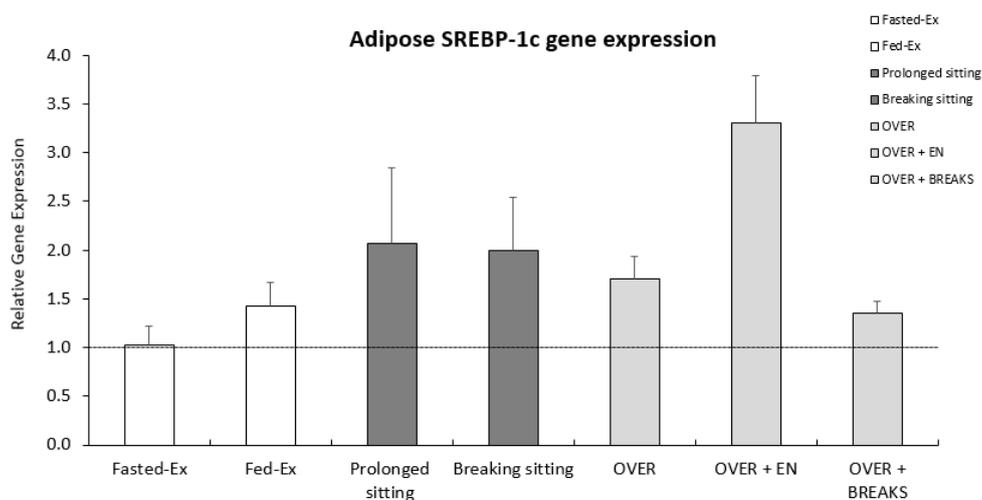


Figure 7.1 The expression of adipose SREBP-1c responses throughout this thesis

Fasted-Ex and Fed-Ex (all $n = 10$), Prolonged sitting and Breaking sitting (all $n = 9$), OVER and OVER + EN (both $n = 5$) and OVER + BREAKS ($n = 8$). Values are means \pm SEM. The dashed line indicates baseline.



Figure 7.2 The expression of adipose IRS2 responses throughout this thesis.

Fasted-Ex and Fed-Ex (all $n = 10$), Prolonged sitting and Breaking sitting (all $n = 9$), OVER and OVER + EN (both $n = 5$) and OVER + BREAKS ($n = 8$). Values are means \pm SEM. The dashed line indicates baseline.

PDK4 mainly regulates glucose metabolism and inhibits glycolysis when activated. Fasting elevated the expression of skeletal muscle and hepatic PDK4 mRNA (Spriet *et al.*, 2004; Palou *et al.*, 2008), and refeeding decreased hepatic PDK4 mRNA expression after 8 h of fasting (Palou *et al.*, 2008). Consistent with previous findings, adipose PDK4 mRNA was up-regulated in Chapter 4 with exercise under a fasted state, and all other feeding trials consistently found that adipose PDK4 mRNA was down-regulated (Figure 7.3). A decrease in PDK4 elevates glycolysis and decreases lipolysis, which corresponds to the findings of an increase in lipogenesis-with SREPB-1c discussed above.

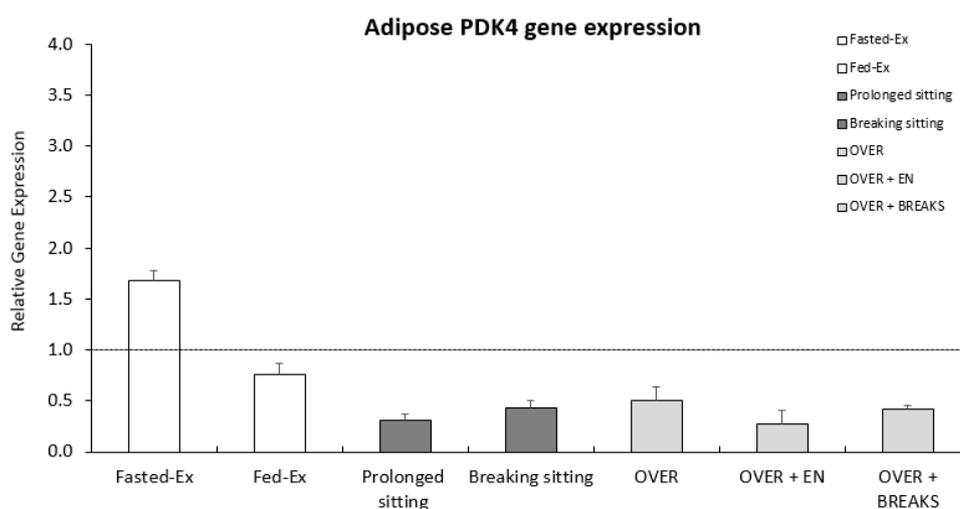


Figure 7.3 The expression of adipose PDK4 responses throughout this thesis.

Fasted-Ex and Fed-Ex (all $n = 10$), Prolonged sitting and Breaking sitting (all $n = 9$), OVER and OVER + EN (both $n = 5$) and OVER + BREAKS ($n = 8$). Values are means \pm SEM. The dashed line indicates baseline.

The expression of IRS2 adipose mRNA is down-regulated in obese and overweight populations compared to lean people (Travers *et al.*, 2015b). In addition, IRS2 mRNA expression is down-regulated after one-week overfeeding plus sedentary life style (Walhin *et al.*, 2013). Taken together, this demonstrates that the decrease in adipose

IRS2 mRNA could be a result of inflammatory processes. In addition, a previous study showed that feeding acutely down-regulated adipose IRS2 mRNA expression across obese, overweight and lean people (Travers *et al.*, 2015b). Therefore, collectively, diet-induced acute down-regulation of adipose IRS2 mRNA expression might potentially link inflammatory processes with a role of the insulin receptor. But, the expression of adipose IRS2 mRNA was increased only in the trial whilst exercising under fasted status in Chapter 4. The up-regulation in adipose mRNA might indicate that potential health benefits while exercise is undertaken under a fasted state. However, this hypothesis is not certain at this stage and would need results from long-term training studies.

SREPB-1c is a transcription factor which regulates fatty acid synthesis when energy is consumed. It is reasonable to observe up-regulation of adipose SREPB-1c mRNA in Chapter 5 and 6 due to energy intake outweighing energy expenditure. Interestingly, in Chapter 4, while exercise under a fed condition, even though the overall exercise expenditure and energy intake was similar, the expression of adipose SREPB-1c mRNA was still increased 4 h after meal consumption. The increase of adipose SREPB-1c mRNA in this study might be due to an increased external energy intake, as a physiological response to store the consumed energy. On the contrary, whilst exercising under a fasted state in Chapter 4, even after a negative energy balance was induced by 1 h of moderate intensity exercise, the expression of adipose SREPB-1c was not changed. Taken together, the result might demonstrate that whilst energy intake and expenditure were similar, dietary modifications could be the main factor influencing adipose SREPB-1c mRNA expression rather than exercise, *per se*.

Collectively, the changes in these two adipose molecules (IRS2 and SREPB-1c) emphasise how adipose tissue plays an active role in responding to feeding and physical activity.

7.3 Physical activity in the regulation of insulin and glucose levels

It is well established that a feeding-induced increase in insulin can be alleviated by acute structured exercise. Unsurprisingly, in Chapter 4, exercise at 60% $\dot{V}O_{2\max}$ for 60 min reduced insulin concentrations in overweight people. In Chapter 5, participating in merely 2 min light intensity exercise every 20 min throughout two meals reduced circulating glucose and insulin levels in overweight middle-age men and women. This finding suggests that NEAT-type physical activity can be used as a strategy to regulate circulating glucose and insulin – and this could be useful because structured exercise is not feasible and practical for all populations. In addition, although this study is not fully complete, NEAT-type physical activity seemed to reduce overfeeding-induced inflammation (as indicated by white blood cell count). Therefore, for some outcomes, it appears that this could be an effective physical activity strategy for health and this challenges the notion that physical activity needs to be accumulated in bouts of 10 min or more.

7.4 Limitations

There are some limitations in the experimental studies in this thesis. In Chapter 4, while exercising under different dietary status (fasted *versus* fed), due to the potential effect from repeated biopsies inducing adipose tissue inflammation (Dordevic *et al.*, 2015),

an immediate pre-exercise adipose biopsy was not taken. Therefore, it is hard to elucidate whether the magnitude of adipose responses whilst exercising under a fasted state is caused by overnight fasting, exercise or interaction of both conditions. In Chapter 5, a resting trial was not included (i.e., without meal intake) and owing to the nature of adipose circadian rhythms (Johnston *et al.*, 2009; Johnston, 2012), the regulation of gene expression in adipose tissue (i.e., meal or circadian rhythm effect) is still unclear. In Chapter 6, protein measures (i.e., Western Blotting) are missing and the study is also relatively short – thus it is difficult to extrapolate to longer term energy surpluses.

Apart from the limitations in each experimental study, it is also important to highlight that any attempt to compare between the studies in this thesis is also limited. Firstly, the recruited participants' characteristics were different, owing to the different research purpose of each project, which could induce a diverse adipose tissue and general physiological responsiveness to meals and exercise/physical activity. Secondly, the exercise/physical activity intensity, exercise/physical induced energy expenditure, the proportion of the macronutrients, absolute energy intake and the trial length are different among these three projects and this can also limit comparisons between studies.

7.5 Future work

Based on the results of Chapter 4, given that adipose tissue had a very different acute response while exercising under different dietary conditions, it is possible that adipose tissue adaptation might differ when exercise training under diverse feeding states. This would need to be tested in an intervention study. In Chapter 5, in both prolonged sitting and breaking sitting trials, adipose tissue responded in a broadly-similar manner.

It is not clear whether this is a meal or circadian rhythm effect. Studies have reported circadian rhythms in adipose tissue (Johnston *et al.*, 2009; Johnston, 2012). Therefore, studies could further examine this question to better understand the effect from feeding. The study described in Chapter 6 showed that at mRNA gene expression level, adipose tissue responsiveness to different physical activity interventions alongside overfeeding is different (especially in lipogenic signals). Western blotting analysis can be considered to further identify adipose tissue responsiveness at the protein level.

7.6 Conclusion

The results from this thesis emphasise the various ways in which physical activity can impact upon adipose tissue and metabolic health. This includes a clear effect on energy balance and changes in pathways in adipose tissue associated with a reduction in adipose tissue masses. However, this thesis also includes effects from physical activity that appear independent and not necessarily associated with a (profound) reduction in adipose tissue masses. This notwithstanding, the effects of feeding on adipose tissue are also profound and clearly more studies are required to understand the relationship between the effects of these two potentially contrasting stimuli (i.e., feeding and physical activity).

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APPENDICES

9.1 Appendix 1: Health Screen

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continued well-being and (ii) to avoid the possibility of introducing bias into the study outcomes.

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

1. Age.....
2. Do you smoke Yes No
3. 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
4. In the past two years, have you had any illness which require you to:
 - (a) consult your GP Yes No
 - (b) attend a hospital outpatient department Yes No
 - (c) be admitted to hospital Yes No
5. Have you ever had any of the following:
 - (a) Convulsions/epilepsy Yes No
 - (b) Asthma Yes No
 - (c) Eczema Yes No

Appendix 1: Heath Screen

- (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) Breathing problems Yes No
- (j) Problems with bones or joints Yes No
- (k) Disturbance of balance/coordination Yes No
- (l) Numbness in hands or feet..... Yes No
- (m) Disturbance of vision Yes No
- (n) Ear / hearing problems Yes No
- (o) Thyroid problems..... Yes No
- (p) Kidney or liver problems Yes No
- (q) Problems with immune system Yes
.....No
- (r) Allergies to any anaesthetics Yes No

Appendix 1: Heath Screen

- (s) Porphyria Yes No
- (t) Myasthenia gravis Yes No
- (u) Adam's-Stokes Syndrome Yes No
- (v) Wolff-Parkinson-White Syndrome Yes No

The local anaesthetic we use is called lidocaine, this is the anaesthetic dentists typically use. Have you ever had this before to know if you have any allergies to this anaesthetic (or any other anaesthetics) or have you ever been told that you shouldn't have this?..... Yes

No

If YES to any question, or if there is any other information you think we should know, please describe briefly (eg to confirm problem was/is short-lived, insignificant or well controlled)

.....
6. Has anyone in your family been diagnosed with any of the above? If so, please specify
.....

7. During a typical **7-Day period** (a week), how many times on the average do you do the following kinds of exercise for **more than 15 minutes** during your free time (write on each line the appropriate number –e.g. 2 hours total of moderate intensity exercise per week = 8 x 15 minutes, so write 8):

a) STRENUOUS EXERCISE (HEART BEATS RAPIDLY)

.....
(e.g., running, jogging, hockey, football, soccer, squash, basketball, judo, roller skating, vigorous swimming, vigorous long distance bicycling)

b) MODERATE EXERCISE (NOT EXHAUSTING)

.....
(e.g., fast walking, baseball, tennis, easy bicycling, volleyball, badminton, easy swimming, popular and folk dancing)

c) MILD EXERCISE (MINIMAL EFFORT)

.....
(e.g., yoga, archery, fishing from river bank, bowling, golf, easy walking)

9.2 Appendix 2: PAR-Q questionnaire

Physical Activity Readiness
Questionnaire - PAR-Q
(revised 2002)

PAR-Q & YOU

(A Questionnaire for People Aged 15 to 69)

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly: check YES or NO.

YES	NO	
<input type="checkbox"/>	<input type="checkbox"/>	1. Has your doctor ever said that you have a heart condition <u>and</u> that you should only do physical activity recommended by a doctor?
<input type="checkbox"/>	<input type="checkbox"/>	2. Do you feel pain in your chest when you do physical activity?
<input type="checkbox"/>	<input type="checkbox"/>	3. In the past month, have you had chest pain when you were not doing physical activity?
<input type="checkbox"/>	<input type="checkbox"/>	4. Do you lose your balance because of dizziness or do you ever lose consciousness?
<input type="checkbox"/>	<input type="checkbox"/>	5. Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?
<input type="checkbox"/>	<input type="checkbox"/>	6. Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?
<input type="checkbox"/>	<input type="checkbox"/>	7. Do you know of <u>any other reason</u> why you should not do physical activity?

If
you
answered

YES to one or more questions

Talk with your doctor by phone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

- You may be able to do any activity you want — as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.
- Find out which community programs are safe and helpful for you.

NO to all questions

If you answered NO honestly to all PAR-Q questions, you can be reasonably sure that you can:

- start becoming much more physically active — begin slowly and build up gradually. This is the safest and easiest way to go.
- take part in a fitness appraisal — this is an excellent way to determine your basic fitness so that you can plan the best way for you to live actively. It is also highly recommended that you have your blood pressure evaluated. If your reading is over 144/94, talk with your doctor before you start becoming much more physically active.

DELAY BECOMING MUCH MORE ACTIVE:

- if you are not feeling well because of a temporary illness such as a cold or a fever — wait until you feel better; or
- if you are or may be pregnant — talk to your doctor before you start becoming more active.

PLEASE NOTE: If your health changes so that you then answer YES to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

Informed Use of the PAR-Q: The Canadian Society for Exercise Physiology, Health Canada, and their agents assume no liability for persons who undertake physical activity, and if in doubt after completing this questionnaire, consult your doctor prior to physical activity.

No changes permitted. You are encouraged to photocopy the PAR-Q but only if you use the entire form.

NOTE: If the PAR-Q is being given to a person before he or she participates in a physical activity program or a fitness appraisal, this section may be used for legal or administrative purposes.

"I have read, understood and completed this questionnaire. Any questions I had were answered to my full satisfaction."

NAME _____

SIGNATURE _____

DATE _____

SIGNATURE OF PARENT
or GUARDIAN (for participants under the age of majority) _____

WITNESS _____

Note: This physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if your condition changes so that you would answer YES to any of the seven questions.



Health Santé

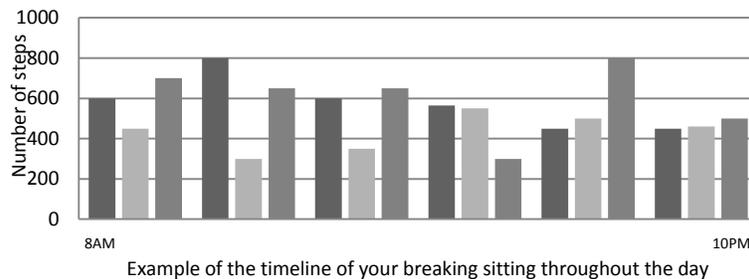
9.3 Appendix 3: Instructions for the breaking sitting time

Instructions for breaking sitting time

Your target number of steps is _____ steps per day

Overview

You have been allocated to a group where the main aim is to engage in short bouts of activity throughout the day. This also means that there will only be a few periods of prolonged sedentary (e.g., sitting) time. So, from a conceptual perspective, we would like to ensure that you spread your target steps throughout the day – a bit like in the graph below:



Although this means you will be able to move freely during this study, this might be challenging at times and so we have come up with a guide below to help you achieve your target steps spread throughout the day:

1. You should try to take 15-25 walks throughout the day (i.e., approximately every 30-40min), each lasting ~5 minutes to ensure you achieve your target steps (at your normal pace). This will make sure you reach your target number of steps easily.
2. You should try to make sure that you never spend more than 1 h sat in one place.
3. Plan your day to make sure you do not exceed your target number of steps.
4. Aim to **reach half your target number of steps** _____ **before 2-3pm.**
5. Wear the pedometer on your waist every day but remember to re-set it to zero first thing in the morning.
6. Please record the timing of the activity breaks and the number of steps in the booklet provided.
7. Ideally, we would like to do activity 'little and often' and preferably you would not do any long walks. Most of time, you should plan approximately 500-600 steps to meet your daily target number of steps little by little. Try to limit longer walks greater than 1,500 steps to only **once per day**, and try to keep this under 2000 steps as a maximum in one go.
8. Try to do any weekly tasks on the days before the main trial starts (i.e. weekly shopping). Otherwise, you will exceed 1,500 steps in one go.
9. If you have a lecture, please try to add to your steps during any breaks.

Appendix 3: Instructions for breaking prolonged sitting

10. It does not matter if you have more activity breaks and these can be shorter than 5 minutes if this is helpful or useful - as long as the target number of steps is reached each day.

General strategies for reaching the step target

- Find a place near where you spend most of your time which is roughly 500-600 steps walking distance.
- You might want to set up an alarm to remind you to have a walk every 30-40min.
- Plan short walks to increase your steps alongside other activities (e.g., when going to the toilet).
- Plan 500-600 steps to grab your breakfast, lunch and dinner (you can accumulate 1,500-1,800 steps by doing this).
- Do 500-600 steps after finishing your breakfast, lunch and dinner (you can accumulate 1,500-1,800 steps by doing this).
- If possible, try to accumulate your steps on flat ground as much as possible (e.g. avoid increasing your steps by going upstairs and downstairs).
- If you have a long meeting or lecture without a break, please plan a 1,000 steps walk (it takes approximately 10 min) before and afterwards. This can help you to accumulate your target number of steps easily.