Abstract

There is a large inter-subject variability in the capacity to oxidise fat (MFO) during exercise and this could have important implications for metabolic health. However, prior to this thesis the evidence in support of this statement was limited and inconsistent. Accordingly, Chapter 3 of this thesis was designed to explore the relationship between MFO with 24-h fat oxidation and insulin sensitivity as surrogate markers of long-term metabolic health. This study showed that MFO during exercise is significantly and positively associated with both 24-h fat oxidation and insulin sensitivity in young, healthy men. Such links highlighted the need for a better appreciation of the factors that influence the inter-subject variability in MFO during exercise. Therefore, Chapter 4 investigated relationships between selected plasma metabolites, hormones and overnight-fasted resting fat oxidation rates, with MFO. The results demonstrated the role of plasma lipolytic markers, plasma insulin, resting fat oxidation and aerobic capacity as important modulators of the inter-subject variability in MFO. Finally, Chapter 5 utilised the findings of Chapter 4 from a translational perspective by exploring the influence of meal timing (i.e., eating before [Fed exercise] or after [Fasted exercise]) an acute bout of aerobic exercise, on substrate utilisation, lipolytic markers and insulin, and intramuscular triglyceride (IMTG) use in obesity. This study found that Fasted- as compared with Fed-state augments exercise fat oxidation, as well as the circulating concentration of plasma glycerol and NEFA during exercise, and suggests IMTG use in obesity. In conclusion, this thesis generates new data that contributes to our understanding of the
links between MFO and metabolic risk, as well as the factors that influence the inter-subject variability in MFO during exercise. It also provides preliminary evidence of exercise-induced IMTG use in obesity, and shows clearly that fasted, as compared with fed exercise augments exercise fat oxidation, which could have meaningful implications for optimising metabolic health.
Having pursued my Bachelors and Master's degrees in Liverpool, I came to Birmingham not knowing much about the City or anyone in it. Almost four years later I see Birmingham as my second home and I have met a number of important people who have been instrumental in getting me to this position. First and foremost, I would like to acknowledge my supervisor Dr Gareth Wallis for providing me with the opportunity to complete a PhD within the School and such a respected research group. I thank you for your extensive support in improving my skills in and out of the laboratory, for the enjoyable conference trips (Miami by far the best!) and for always being on-hand to offer help and advice. Your detail-orientated and thorough approach to research is something I admire.

I extend much gratitude to the Undergraduate and Post-Graduate students who assisted with the research projects, including Dr Borut Fonda and Dr Carlijn Vernooij but in particular Gareth Fletcher who endured many early starts in the lab with me and was always on hand to offer help when needed. Of course, I cannot go without thanking the research participants who without their input this thesis would not have been possible. A huge thanks also goes to Dr Helen Bradley, Nurul Abdullah and Owen Edwards for assistance with data collection and analysis during preparation of Chapter 5 of this thesis, and (the most cheerful person in Sportex) Professor Janice Thompson for your support and guidance, and allowing me to lecture on your Public Health Nutrition
Module; your textbook was a key feature during my Undergraduate studies, so it was a real pleasure to teach on your module. Thanks also to those who have collaborated on the research projects shown in this thesis, including Dr John Hattersley, Alison Campbell, Dr Edward Chambers, Professor Gary Frost and Dr Konstantinos Manolopoulos.

My friend’s back at home in the North West definitely deserve a mention here. You have helped me through the past four years, even if you didn’t quite know exactly what I was doing! Ian, I always told you that I’d prove Miss Nelder wrong for putting me in set two for year 9 Science… perhaps this goes some way in doing so? I can’t go without thanking my girlfriend Emily for all the love and support you have provided, particularly during the latter phases of the PhD when I needed it most. You helped me immensely and I am very lucky to have you in my life.

Last but certainly not least, I thank my parents, Jane and Dean, and my sister Charlotte. You have always been there for me and have shown tremendous love and support, not only during my PhD but in all that I have done - from standing on the side line watching me play football on a cold and rainy Sunday morning to helping me fund my Masters that helped me get onto a PhD. I could not ask for a better mum, dad and sister and I am forever grateful.
Abstracts, conference communications and publications

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List of Abstracts, Conference Communications and Publications

During the period of postgraduate study, the data in the thesis also resulted in the following conference communications and awards:

American College of Sports Medicine, Integrative Physiology of Exercise Meeting 2014, Miami, September 2014 – Poster communication. Maximal Fat Oxidation during Exercise is Positively Associated with 24-Hour Fat Oxidation and Insulin Sensitivity in Young, Healthy Men.

European College of Sport Science (ECSS) Annual Conference, Malmo, Sweden, June 2015 – Oral communication. Maximal Fat Oxidation during Exercise is Positively Associated with 24-Hour Fat Oxidation and Insulin Sensitivity in Young, Healthy Men. Winner (joint 5th) of the Young Investigator Award (oral presentation).

The following papers and articles were also published during the period of postgraduate study:


**Robinson SL** and Wallis GA. Fat oxidation during exercise: an alternative perspective. The Sport and Exercise Scientist’, the quarterly magazine for the British Association of Sport and Exercise Sciences, Spring 2015.
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<td>lactate increase above baseline</td>
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<td>maximal fat oxidation</td>
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<td>magnesuim</td>
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<td>mg</td>
<td>milligram</td>
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<td>Mg²⁺</td>
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<tr>
<td>NAD⁺</td>
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<td>NEFA</td>
<td>non-esterified fatty acid</td>
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<td>NGS</td>
<td>normal goat serum</td>
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<td>NIDDM</td>
<td>non-insulin dependent diabetes mellitus</td>
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<td>Nmol</td>
<td>nanomole</td>
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<td>PAL</td>
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This thesis starts with a General Introduction (Chapter 1). Here, information is provided relating to substrate utilisation at rest and during exercise, including an outline of the most important regulators of substrate use during exercise. Thereafter, an insight into studies that have identified inter-subject differences in fat oxidation during exercise, including the Maximal capacity for Fat Oxidation (MFO), is given. This is followed by a discussion on the research that has sought to: (i) address the significance of the inter-subject variation in MFO during exercise in the context of metabolic health; and (ii) explain the determinants of this variation. The final part of this chapter explores the role of intramuscular triglyceride (IMTG) and associated lipid metabolites in the development of insulin resistance in obesity, along with insights into potential nutritional strategies to increase IMTG utilisation during exercise, which could help to improve insulin sensitivity in this population.

Next, information on the techniques used in the experimental chapters of this thesis is provided (Chapter 2). The experiments undertaken during this period of study are shown in Chapters 3-5. Presented here are studies further investigating (i) the significance of the inter-subject variability in MFO during exercise in the context of metabolic health (Chapter 3); (ii) factors which influence the inter-subject variation in MFO during exercise (Chapter 4), and; (iii) the influence of pre- or post-exercise food intake on substrate utilisation, lipolytic markers and insulin, and IMTG utilisation in obesity (Chapter 5).
Lastly, Chapter 6 summarises the key findings from the experimental chapters and discusses how these integrate and build upon the existing body of literature. It also discusses the wider implications of the thesis findings and proposes directions for future research.
CHAPTER 1

GENERAL INTRODUCTION
1.1 Introduction

At rest and during aerobic exercise, energy demands of the body are met primarily by the oxidation of the energy substrates carbohydrate and fat. During exercise, the absolute and relative contributions of each substrate to energy expenditure are influenced by a number of factors including exercise intensity (Romijn et al, 1993, Bergman & Brooks, 1999, van Loon et al. 2001), exercise duration (Romijn et al. 1993), aerobic capacity/training status (Holloszy & Coyle, 1984), nutritional status (Bergman & Brooks, 1999; Coyle et al. 2001) and sex-related differences (Tarnopolsky et al. 1990). Interestingly, there is marked inter-subject variation in the capacity to oxidise fat during exercise and it has been proposed that this variation could have important implications. From the perspective of athletic performance, an increased reliance on fat oxidation during exercise could permit an athlete to exercise for longer before becoming exhausted due to glycogen depletion, which could confer a performance advantage. Emerging evidence also suggests a link between impairments in fat oxidation and the development of unfavourable metabolic conditions such as obesity and insulin resistance, which is a precursor to type II diabetes (Zurlo et al. 1990; Kelley & Simoneau (1994). Whether a reduced fat oxidation is a cause or consequence of these adverse health outcomes is yet to be determined. Nevertheless, a better understanding of if, and why, fat oxidation during exercise is important, as well as the determinants of the apparent inter-subject variability, could help to inform novel interventions to increase fat oxidation during exercise and curtail the rise in obesity and its related disorders.
1.2 Substrate utilisation at rest and during exercise

During aerobic exercise, carbohydrate and fat are the principle substrates for oxidative phosphorylation and energy (adenosine tri-phosphate; ATP) production (Hargreaves 2012; Spriet, 2012), whilst the relative contribution of amino acids, particularly the branched chain amino acids (isoleucine, leucine and valine) is thought to be low (typically less than 5%, maximally 10%; Lemon & Mullin, 1980).

1.2.1 Carbohydrate as a fuel source

Carbohydrate is stored predominantly in skeletal muscle but also in liver as glycogen, with smaller amounts of carbohydrate (~4 g for a person weighing 70 kg; Wasserman, 2009) circulating freely in the blood stream as blood glucose.

The endogenous carbohydrate pool is small but flexible. For instance, early work that employed the liver and muscle biopsy technique demonstrated that dietary manipulation exerts a large influence glycogen reserves (Bergstrom et al. 1967; Nilsson & Hultman, 1973). Liver glycogen concentrations usually vary with the diet (range 1.4 g to 8.0 g/kg wet liver tissue in 58 healthy subjects who were studied in the post-absorptive state following a period of ad libitum diet; Nilsson, 1973) and can also vary throughout the day depending upon patterns of eating and fasting (Hultman & Nilsson 1971). Findings from muscle biopsy samples indicate that glycogen concentrations in the vastus lateralis muscle range from 60-120 mmol glycosyl residues/kg wet tissue (mean 85 mmol glycosyl residues/kg wet tissue; Hultman, 1967). If these values are extrapolated to the
whole body level, it can be estimated that a man weighing 70 kg with ~40% of his mass attributed to muscle and a liver weighing 1.8 kg, has a whole body glycogen storage capacity of ~500 g. If the highest reported values are extrapolated (Bergstrom et al. 1967; Nilsson, 1973) then this could increase to 700 g. Nevertheless, 700 g of glycogen storage correspond to only 11.7 MJ (2,800 kcal), which highlights the limited energy storage capacity of carbohydrate.

1.2.2 Fat as a fuel source

Fat is predominantly stored as triglyceride in deep visceral and subcutaneous adipose tissue, with a lesser amount (~7.8 MJ or 1,850 kcal) stored as lipid droplets inside muscle fibers as IMTG (van Loon et al. 2001). There is also some fat circulating in the bloodstream as non-esterified free fatty acids (FA), which is bound to a protein carrier (albumin). Some FAs are also found as TG incorporated in circulating lipoprotein particles (chylomicrons and very-low- (VLDL), low-, intermediate-, and high-density lipoproteins). The energy storage capacity of fat is large and markedly exceeds that of carbohydrate. For instance, a healthy, lean man of approximate body mass 70 kg can store between 9-15 kg of endogenous fat, which equates to a total energy storage of 350-586 MJ (80,000-140,000 kcal); adequate to fuel a person to walk for ~16,000 km (Jeukendrup et al. 1998).
1.2.3 Energy production at rest and during exercise

During resting conditions and after an overnight fast, a combination of carbohydrate, fat and protein are oxidised to provide the fuel necessary for basal metabolic processes. These substrate sources usually share a reciprocal relationship and the relative contribution of each can vary considerably between individuals. The contribution of each substrate to energy production at rest can vary despite a reasonably unchanged metabolic demand, predominantly arising from alterations in substrate availability.

The shift from rest to exercise induces a substantial rise in metabolic demand for energy, which can increase several-fold above that during resting conditions. Accordingly, the metabolic pathways that oxidise carbohydrate and fat must be activated simultaneously (Hargreaves, 2012), however the pattern of substrate utilisation is susceptible to many factors, including (but not exclusive to) cardiorespiratory fitness ($\dot{V}O_{2\max}$; Kiens et al. 1993; Klein et al. 1994; Phillips et al. 1996b; Coggan et al. 2000), sex (Tarnopolsky et al. 1990; Tarnopolsky et al. 1995; Tarnopolsky et al. 1997; Venables et al. 2005; Tarnopolsky et al. 2007), recent (Coyle et al. 1985; Gleeson et al. 1986; Achten & Jeukendrup, 2003) and habitual (Goedecke et al. 2000) diet, body composition (particularly fat-free mass; Venables et al. 2005) and the circulating concentration of blood-borne hormones and metabolites (Coggan et al. 2000; Horowitz et al. 1997; Horowitz et al. 1999; Achten & Jeukendrup, 2004; Moro et al. 2013). Further discussion on the influence of these factors on substrate use during exercise can be found in Chapters 3 and 4.
1.2.4 General overview of lipid metabolism during exercise

As described in Section 1.2.2, the energy storage capacity of lipids is large and it is therefore not surprising that lipids contribute to a substantial portion of energy production, particularly during low and moderate intensity exercise, as well as exercise of increasing duration (Krogh & Lindhard, 1920; Christensen & Hansen, 1939b; Romijn et al. 1990, van Loon et al. 2001; Figure 1.1). However, lipid metabolism during exercise is complex, with numerous possible sites for regulation and multiple steps to oxidation (for review see Spriet et al. 2014).

![Diagram showing energy expenditure from different sources](image)

**Figure 1.1** Maximal contribution to energy expenditure derived from glucose and FFA taken up from blood and minimal contribution of muscle triglyceride and glycogen stores.
after 30 min of exercise, expressed as a function of exercise intensity. Total amount of kilocalories (kcal) available from plasma does not change in relation to exercise intensity. Data redrawn from Romijn et al. (1993).

(i) Sources of fat as a fuel during exercise (see also Figure 1.1): Following an overnight fast, energy demands at rest are met by the oxidation of fatty acids originating predominantly from visceral adipose tissue TAGs and to a lesser extent subcutaneous adipose tissue (Arner et al. 1990). At rest, FA release from adipose tissue is ~2 times greater than FA oxidation (Klein et al. 1989) and therefore a large portion are re-esterified back into TAGs, usually by the liver. As discussed (Section 1.2.3), the shift from rest to low or moderate intensity exercise (25-65% \( \dot{V}O_2_{\text{max}} \)) causes an increase in metabolic demand and fat oxidation is elevated ~5-10 fold above resting conditions (Krogh & Lindhard, 1920) and lipolysis of adipose tissue TAGs increases ~2-3 fold (Wolf et al. 1990; Klein et al. 1994).

In addition to energy supplied from adipose tissue TAGs, there are also FA’s located within circulating triglyceride-rich lipoproteins, which can be hydrolysed by lipoprotein lipase (LPL) located on the capillary endothelium of skeletal muscle and adipose tissue. These FA’s can be taken up by skeletal muscle for oxidation, however their contribution to total fat oxidation during exercise is only very small (<10%; Havel et al. 1967).

Studies that have employed electron microscopic analysis of skeletal muscle tissue have demonstrated that IMTG droplets are situated in close proximity to the site of oxidation in
the muscle mitochondria (Hoppeler et al. 1973) and it has long been recognised that IMTG is an important contributor to overall fat oxidation during prolonged exercise (Froberg & Mossfeldt, 1971) and exercise of particular intensities (Essen et al. 1977). Indeed, plasma FA’s are the predominant contributor to overall fat oxidation during low intensity exercise in well-trained men (25% $\dot{V}O_{2max}$) but as exercise intensity increases total fat oxidation far exceeds the rate of plasma FA disappearance, such that during fasted moderate intensity exercise (65% $\dot{V}O_{2max}$) IMTG provides ~50% of the total fat oxidation, whilst its contribution declines during high intensity exercise (85% $\dot{V}O_{2max}$) (Figure 1.1). Research also demonstrates that IMTG is an important fuel source during prolonged moderate-intensity (60% $\dot{V}O_{2max}$) cycling exercise in well trained individuals (van Loon et al. 2003), and also contributes as a fuel source during resistance type exercise (Essen & Gustavsson, 1990). For a comprehensive overview of the influence of intensity, diet and training status on IMTG utilisation, the interested reader is directed to three excellent reviews (Watt et al. 2002; van Loon, 2003; Shaw et al. 2013).

1.3 Variability in the relative contribution of substrates during exercise

Although there are a number of factors that can influence substrate metabolism during exercise, it is interesting that there also seems to be substantial inter-subject variability in the contribution of carbohydrate and fat to energy expenditure during exercise even in relatively homogenous groups under standardized conditions. For example, Helge and co-workers found RER during overnight-fasted exercise performed at the same relative exercise intensity (55% $\dot{V}O_{2max}$) to range between 0.83 and 0.95 in a group of untrained,
healthy men (Helge et al. 1999). Consistent with this, but using a well-trained cohort of 61 cyclists ($\dot{V}O_{2\text{peak}}$, 56 ± 7 ml/kg/min), another study demonstrated a large degree of variation in RER at rest (0.72 – 0.93), that remained throughout exercise at three pre-determined workloads (41, 63 and 80% of $\dot{V}O_{2\text{peak}}$; Goedecke et al. 2002). Whilst these studies offered some initial insight into the apparent inter-subject variability in fat oxidation during exercise, it is noteworthy that RER considers only relative fuel utilisation and does not inform of the rate of carbohydrate and fat oxidation. Further, substrate use in these studies was assessed over only one (Helge et al. 1999) or three (Goedecke et al. 2000) pre-determined workloads, and it cannot be excluded that findings might have been different at higher or lower exercise intensities.

1.4 Inter-subject variation in absolute rates of fat oxidation during exercise

The insights obtained by the studies in Section 1.3 stimulated great interest in developing a test protocol to assess the absolute rates of carbohydrate and fat oxidation over a wide range of exercise intensities; thereby providing the resolution to more clearly identify the extent of the apparent inter-subject variation in fat oxidation during exercise. Achten and colleagues were the first to do this (Achten et al. 2002). They determined MFO and corresponding exercise intensity ($\text{Fat}_{\text{max}}$) systematically over a wide range of exercise intensities, using an incremental cycling protocol with 35 W stages of 3-minute duration, based on respiratory gaseous exchange and indirect calorimetry (Figure 1.2). The test was validated against longer duration stages and in another study its reproducibility was assessed (Achten et al. 2003). In recent years, the term Maximal Fat
Oxidation (MFO) has been increasingly used to describe an individual’s peak rate of fat oxidation and could be regarded as a useful marker of an individual’s capacity to oxidise fat during exercise.

Figure 1.2 A typical Fat\textsubscript{max} curve obtained during an incremental exercise test over a range of exercise intensities. Redrawn from Achten et al. (2002).

Interestingly, the substantial inter-subject variability in the relative contribution of fat to energy expenditure during exercise is also apparent in the maximal rates of fat oxidation that have been observed during exercise. For example, the study by Achten and co-workers showed MFO determined during cycle ergometer exercise in endurance-trained men was $0.52 \pm 0.15$ g/min (Mean $\pm$ Standard Deviation [SD]) and varied 4-fold between individuals ($0.23 - 0.91$ g/min; Achten et al. 2002). Using a more heterogeneous group of
healthy men and women, Venables and co-workers demonstrated similarly large inter-subject variability in MFO during treadmill exercise (0.46 ± 0.17 g/min; range 0.18 - 1.01 g/min; Venables et al. 2005). More recently it has been shown that there is considerable inter-subject variability in MFO in homogenous groups of overweight men (Rosenkilde et al. 2010) and normal-weight, pre-menopausal healthy women (Isacco et al. 2014).

1.5 Significance of fat oxidation in relation to metabolic health
The prevalence of obesity is rising at an alarming rate (Wang et al. 2011), which is undoubtedly a major cause for concern given the attendant inflated risk of chronic disease (WHO, 2010). Whilst much of this rise is related to genetic or epigenetic factors, as well as the increasing prevalence of sedentary lifestyles (King et al. 2005) and greater access to low cost, high energy density foods (Bell et al. 1998; Drewnowski, 2004; Monteiro et al. 2004), it is also interesting that the propensity to develop these conditions may be through disturbances in whole body and skeletal muscle lipid metabolism.

1.5.1 Links between fat oxidation and change in body mass and/or composition
One interesting observation is that an elevated 24-h respiratory quotient (RQ; reflective of tissue exchange of CO₂ produced and O₂ consumed) is commonly used in resting and calorimeter studies), which is indicative of a low relative fat to carbohydrate oxidation (an RQ of 0.7 denotes total fat oxidation, whilst an RQ of 1.0 denotes total carbohydrate oxidation), has been shown to be predictive of long-term alterations in
body mass and composition. For instance, a prospective study (Zurlo et al. 1990) measured 24-h RQ using a whole-room respiration chamber and indirect calorimetry in 152 non-diabetic Pima Indians fed a weight-maintenance diet. In 111 subjects for whom follow-up data were available (25 +/- 11 months), they found that those with an elevated 24-h RQ (90th percentile; RQ = 0.88) were predisposed to gain ≥ 5 kg in body mass compared to those with a lower 24-h RQ (10th percentile; RQ = 0.82). Importantly, this finding remained when adjusted for potential confounding variables such as energy expenditure, fat mass and fat-free mass. Moreover, the coefficient of determination of this relationship showed that 5% of the variability in body mass gain was associated with the variability in RQ, adjusted for energy balance and percent body fat.

An allied observation comes from a longitudinal study by Seidell and colleagues (Siedell et al. 1992), who followed 775 men (aged 18-98 years) for a period of approximately ten years. They measured fasting RER and resting metabolic rate (RMR) at baseline and related this to subsequent change in body mass. Following adjustment for factors such as age, body mass index, fat-free mass, and duration of follow-up, they demonstrated that RER, but not RMR, was predictive of subsequent body mass gain (P < 0.001). Furthermore, major body mass gain (from at least 5 kg to 15 kg) was associated with initial RER in non-obese white men (initial BMI < 25 kg/m²). In further analysis, non-obese white men who displayed a fasting RER of ≥ 0.85 were ~2.5 times more likely to gain ≥ 5 kg body mass compared to men with a fasting RER < 0.76. Considered collectively, these studies demonstrate that a reduced reliance on fat oxidation to daily
or resting energy expenditure may be an important metabolic predictor of body mass gain.

1.5.2 Links between fat oxidation and body mass and body fat mass re-gain

Whilst many diet and exercise interventions can effectively facilitate body mass loss, it is apparent that most individuals will re-gain body mass within 3-5 years (Weiss et al. 2007). Accordingly, a better understanding of the metabolic characteristics that predispose individuals to body mass-regain following body mass loss is an important public health challenge. It has been suggested that one such mechanism could be through impairments in daily fat oxidation, such that a high RQ predisposes individuals to re-gain body mass following a period of weight loss. This was demonstrated by Froidevaux and co-workers (Froidevaux et al. 1993) who assessed 24-h energy expenditure and 24-h RQ in a group of 10 moderately obese women on four occasions: 1) before body mass loss (body mass = 78 kg), 2) during body mass loss (64 kg), 3) after re-alimentation (63 kg), and 4) 6-15 months after the study diet with ad libitum diet (68 kg). They reported a significant and positive correlation between basal RQ and body mass re-gain following a period of diet-induced body mass loss ($R = 0.89$, $P < 0.01$) and suggested that following a period of body mass loss, an elevated postabsorptive RQ appears an important determinant of the long-term stabilization of body weight in previously obese women.
This apparent association between RQ and body mass gain has been confirmed by some (Hainer et al. 2000; Marra et al. 1998; Weyer et al. 2000; Marra et al. 2004), but not all (Weinsier et al. 1995; Weinsier et al. 2002; Weinsier et al. 2003), studies; a disparity that could be due to population differences and dietary control in study designs.

It could also be that the period of RQ measurement influences associations between daily substrate metabolism and future body mass gain. Indeed, previous studies have typically determined relationships between fasting, sleeping and 24-h RQ with subsequent alterations in body mass (Weinsier et al. 2003). However, RQ measured during fasting or sleeping conditions encompasses a period when reliance on endogenous fatty acid oxidation for fuel is high and does not account for an individual’s capacity for fat and carbohydrate oxidation across typical post-prandial periods of the day. It has been suggested that the relationship becomes stronger when RQ is measured during the non-sleeping segment of the day and is associated with changes in body mass (Ellis et al. 2010).

1.5.3 Potential mechanisms for the association between fat oxidation and alterations in body mass and composition

Mechanistically, perturbations in fat mass are underpinned by the state of fat balance (fat intake [+synthesis] – oxidation; Flatt, 1993). To reduce fat mass a negative fat balance must be reached and this can be achieved by manipulating fat intake or expenditure to a level whereby fat oxidation is greater than fat intake.
It has been suggested that a reduced fat oxidation (and thus elevated carbohydrate oxidation) impacts upon long-term body mass or composition through increased *ad libitum* food intake in free-living conditions, which may offer a behavioural explanation linking low fat oxidation to unfavourable body or fat mass development. For instance, Eckel and colleagues demonstrated in 39 young normal-weight (*n* = 23), overweight (*n* = 8), and obese (*n* = 8) men and women that those who exhibited a higher carbohydrate balance and thus lower fat balance, measured during a 24-h stay in a respiratory chamber following a 15 day isocaloric high carbohydrate diet, gained less fat mass (*P* < 0.001), percentage body fat (*P* = 0.006), and body mass (*P* = 0.024) over a 4-year follow-up period (Eckel et al. 2006). It has been proposed that the effects of substrate oxidation and balance on food intake mediate these relations (Pannacciulli et al. 2007). These authors provided 112 normoglycemic men and women with a 3-day weight-maintenance diet, following which they measured 24-h energy expenditure and substrate utilisation in a respiratory chamber and allowed participants to self-select their food intake during a subsequent 3-d period. They showed that 24-h RQ (positive association), 24-h carbohydrate oxidation (positive association), and 24-h carbohydrate balance (negative association) were significantly associated with *ad libitum* food intake. Considered collectively, these findings offer support to the glycogenostatic model devised by Flatt (1987), which proposes that those who rely less on fat oxidation for energy production (and more on carbohydrate) are likely to exhibit increased *ad libitum* food intake in an attempt maintain carbohydrate balance and replenish limited glycogen
stores, both of which are reduced according to the extent of daily carbohydrate oxidation.

As a high fat oxidation whilst physically active could make important contributions to daily fat oxidation it is not unreasonable to consider a high capacity to oxidise fat whilst physically active as advantageous for the long-term maintenance of body mass and body composition. To this author’s knowledge, there is only one study that has investigated the link between substrate utilisation during exercise and subsequent *ad libitum* feeding. Almeras and colleagues demonstrated using eleven healthy, sedentary to moderately active, young men, that those who had a reduced reliance on fat oxidation towards energy expenditure during 90 minutes of exercise at 60% $\dot{V}O_{2\text{max}}$ exhibited a higher post-exercise *ad libitum* energy intake relative to the energy cost of exercise (when meals were consumed within the laboratory [snacks ingested outside the laboratory] and energy intake covertly recorded). Furthermore, exercise RQ was positively related with post-exercise energy and lipid balance (Almeras et al. 1994). Unfortunately, this study did not associate exercise RQ with changes in body composition, but it is tempting to speculate that individuals who derive a greater proportion of energy from the oxidation of fat whilst physically active could be at a reduced risk of future body mass gain, compared with those who exhibit a lesser reliance on fat (and higher reliance on carbohydrate).
1.5.4 Links between fat oxidation and insulin sensitivity

There is evidence that the inter-subject variability in fat oxidation could be significant for the development of favourable or unfavourable metabolic phenotypes. For example, observational studies have linked impairments in fat oxidation, particularly at the level of skeletal muscle, with a reduced metabolic flexibility (defined as the capacity to adapt fuel oxidation to fuel availability) and insulin resistance, which is an early sign of the development of type II diabetes (Kelley & Simoneau, 1994; Zimmet et al. 2001).

There is also evidence that is suggestive of a link between exercise fat oxidation and insulin sensitivity. For example, one study reported a significant and positive correlation between fat oxidation during exercise at 55\% \text{VO}_{2\text{max}} \ (mg/kg/min; R = 0.42, P = 0.011) and insulin sensitivity index in a group of South Asian and European Men; an association that remained when adjusted for potential confounders such as age, BMI, fat mass and physical activity (R = 0.36, P = 0.035) (Hall et al. 2010). An allied observation was made by Abilgaard and colleagues who demonstrated a positive relationship between whole body fat oxidation measured during cycling exercise at 50\% \text{VO}_{2\text{max}} and insulin sensitivity (derived from an oral glucose tolerance test; R = 0.56, P = 0.05) in a group of healthy women (Abilgaard et al. 2013).

In line with the above-mentioned associations between a reduction in fat oxidation at rest or during exercise and insulin sensitivity, longitudinal studies show that an enhancement in fat oxidation with exercise training is associated with improvements in insulin sensitivity (Goodpaster et al. 2003). Furthermore, greater exercise training-
induced improvements in insulin sensitivity have been observed using exercise training strategies that enhance fat oxidation during acute exercise e.g., targeting training intensity $\text{Fat}_{\text{max}}$ or exercising in the overnight-fasted state (Venables et al. 2008).

From a mechanistic standpoint, the association between an elevated fat oxidation during exercise and insulin sensitivity seems plausible (for a more comprehensive overview of the etiology of insulin resistance at the potential role of fat oxidation in modulating insulin sensitivity please see Section 1.7). Briefly, it has been suggested that incomplete metabolism of fatty acids in the β-oxidation pathway induces an accumulation of acyl carnitines in the mitochondria and this is associated with insulin resistance (Koves et al. 2005). Furthermore, the accumulation of lipid metabolites such as ceramide, diacylglycerol (DAG) and long-chain fatty acyl-CoA (LCFA-CoA) has also been linked with insulin resistance in skeletal muscle (Shaw et al. 2010). Therefore, a higher fat oxidation during physical activity could help to alleviate lipotoxic stress in skeletal muscle and confer improvements in insulin sensitivity (Venables et al. 2008).

With this in mind, it seems reasonable to suggest that those who exhibit a higher MFO during exercise have better insulin sensitivity, compared with those who have a lower MFO. However, the evidence to date linking the capacity for fat oxidation during exercise to insulin sensitivity is limited and inconsistent. For example, one study observed no association between insulin sensitivity and RER at rest and during exercise (Goedecke et al. 2001), whereas a more recent study (Chu et al. 2014) showed MFO was positively correlated with insulin resistance (estimated from the homeostatic model assessment of
insulin resistance) and unrelated to whole-body insulin sensitivity index (determined from oral glucose tolerance testing).

This is in contrast to what might be expected given the above-mentioned associations between (i) reduced fat oxidation at rest (Kelley & Simoneau, 1994) and during exercise (Hall et al. 2010; Abilgaard et al. 2013) and reduced insulin sensitivity; and (ii) increased fat oxidation in response to exercise training and improvements in insulin sensitivity (Goodpaster et al. 2004; Venables et al. 2008). Of note and as previously mentioned, Goedecke and co-workers assessed RER during only three pre-determined exercise workloads (41%, 63%, and 80% \( \dot{V}O_{2\text{max}} \)). Exercise RER considers only relative fuel utilization whereas MFO, which incorporates metabolic rate, could better reflect metabolic capacity and thus be more likely to correlate with insulin sensitivity. The study population was endurance-trained individuals with high levels of insulin sensitivity and low inter-subject variation and it is unclear if similar results would be obtained in populations exhibiting larger variability in exercise substrate oxidation and insulin sensitivity. The study by Chu and colleagues was conducted in a group of normal and overweight girls and it could be that the relationship between MFO and insulin sensitivity is different in other populations.

1.5.5 Links between Maximal Fat Oxidation during exercise and metabolic health

To date there are only two studies that have investigated the association between MFO and metabolic risk factors (with the exception of insulin sensitivity). Rosenkilde et al.
(2010) demonstrated in an otherwise homogenous group of overweight young men that those with a lower MFO during exercise (and lower resting fat oxidation) had a higher clustering of metabolic syndrome risk factors than those with a higher MFO. When separated into two groups according to their MFO, half of the participants (4 out of 8) in the low MFO group (mean ± SD, 0.17 ± 0.03 g/min), compared with zero in the high MFO group (mean ± SD, 0.33 ± 0.01 g/min), displayed the metabolic syndrome. More recently, Isacco and colleagues demonstrated in healthy normal-weight pre-menopausal women that those with a lower MFO during exercise exhibited an unfavourable fat mass distribution (higher abdominal to lower body fat mass index, associated with metabolic disorders such as insulin resistance and dyslipidaemia) than those with a higher MFO (Isacco et al. 2013). This type of cross-sectional evidence affords the suggestion that an increased capacity to oxidise fat during exercise may convey protection towards developing a metabolic unhealthy cardiometabolic phenotype and metabolic syndrome.

Despite previous calls (Kelley et al. 1994; Jeukendrup & Wallis, 2005; Isacco et al. 2013), our understanding of the links between the capacity to oxidize fat during exercise and metabolic health remains uncertain. There is clearly a need for further studies to identify the extent to which inter-subject variation in fat oxidation during physical activity or exercise contributes to the observed relationships between fat oxidation and metabolic disease risk. Further understanding this could provide insights into the role of physical activity in the optimisation of metabolic health.
1.5.6 Should focus be placed upon oxidising fat or calories?

An emerging message throughout this Chapter is that increasing one's ability to utilise fat as a fuel source could have important implications from the perspective of metabolic health and future risk of weight gain, and body fat re-gain after a period of diet-induced weight loss. Accordingly, it seems logical that clinicians and practitioners who are looking to improve an individual’s metabolic health status might focus the design of their nutritional and/or training strategies towards increasing ones capacity to use fat as a fuel. However, if the goal is to reduce body fat mass then it is undoubtedly important to acknowledge that fat oxidation and fat loss are not synonymous and that the state of energy balance is more important than fat oxidation per se. Given that one must be in a state of negative energy balance to achieve a reduction in body fat mass (and the magnitude of the reduction in fat mass is dependent upon the net energy deficit), the first priority should be on the design of an intervention whereby the absolute energy expenditure is sufficient to create an exercise-induced energy deficit. This might mean prescribing an exercise regime that lies within or outside of one’s optimal ‘fat burning’ or Fat\(_{\text{max}}\) zone (±10% Fat\(_{\text{max}}\)). Indeed, exercising above this zone might be particularly appropriate if the individuals MFO occurs at a very low exercise intensity, as only prolonged exercise will create an appropriate exercise-induced energy deficit.

At present, there is no conclusive evidence to suggest that one form of exercise is more effective than another in aiding weight loss or increasing the capacity for fat oxidation during exercise. Indeed, there are many different training protocols that can do this,
ranging from prolonged endurance exercise to high intensity interval training. Accordingly, the key aim for any practitioner would be to prescribe an intervention that takes into account the individuals preferences, such that the exercise regime can be adhered to and is sustainable. As a result, regular exercise training will improve one’s aerobic capacity, which is significantly and positively associated with the capacity for fat oxidation (as described in the section below; Venables et al. 2005). This increased capacity for fat oxidation could aid progressive weight loss (Thompson et al. 2012; see Figure 8) and lead to improvements in one’s metabolic health status (Rosenkilde et al. 2010; Isacco et al. 2014).

1.6 Determinants of the inter-subject variation in fat oxidation during exercise

As exercise is a primary means to acutely increase fat oxidation and reduced fat oxidation has been linked with disorders such as obesity and insulin resistance, further understanding the factors that influence the inter-subject variability in the capacity to oxidise fat during exercise could provide important insights into strategies to increase fat oxidation and optimise metabolic health.

Prior to this thesis, there were only two published cross-sectional studies that comprehensively investigated the variability and potential determinants of fat oxidation during exercise across a range of exercise intensities. Goedecke and colleagues, who assessed substrate use using RER over a select range of exercise intensities in 61 well-trained cyclists, conducted the first (Goedecke et al. 2000). These authors observed an
~4-fold difference in fat oxidation at rest (RER 0.72-0.93) that persisted during exercise of increasing intensity. However, the determinants of this variability in fat oxidation during exercise were less uniform. Serum concentrations of metabolites (FAs and plasma lactate) were the primary drivers of the variation in RER at rest and during low intensity exercise (41% \( \dot{V}O_{2peak} \)), whereas muscle substrate concentration (glycogen and IMTG) and glycolytic enzyme activities were important at 63% \( \dot{V}O_{2peak} \), and during high intensity exercise (80% \( \dot{V}O_{2peak} \)) plasma lactate concentration contributed significantly to the variation in RER. At all exercise intensities there was no significant association between the variability in RER and \( \dot{V}O_{2max} \) (ml/kg/min), dietary carbohydrate intake (% energy), muscle fiber composition (type I or type IIa content %), body fat (%) or gender.

In another attempt to elucidate the determinants of the inter-subject variation in MFO during exercise, Venables and colleagues assessed fat oxidation over a wide range of exercise intensities using a large heterogeneous cohort (n=300) of trained and untrained, healthy men and women (Venables et al. 2005). Several factors previously identified to influence exercise substrate oxidation including aerobic fitness (\( \dot{V}O_{2max} \), ml/kg/min), self-reported physical activity level (SRPAL), fat-free mass (kg) and sex-related differences explained part (35%) of the inter-subject variation in MFO (MFO range: 0.18 - 1.01 g/min). However, when MFO was scaled relative to FFM, it was revealed that gender, physical activity level and \( \dot{V}O_{2max} \) accounted for only 12% of the observed inter-subject variability in MFO, therefore leaving a large proportion unexplained. The influence of other factors such as circulating concentrations of plasma hormones and metabolites, habitual and recent diet, and muscle fiber type was not
determined by Venables and colleagues, but could be important and remains to be comprehensively determined.

1.7 Nutritional strategies to increase fat utilisation during exercise and augment insulin sensitivity

1.7.1 Obesity and IMTG accumulation

Obesity is a condition that is partly characterized by the accumulation of IMTG and lipid metabolites such as LCFA-CoA, DAG and ceramide in skeletal muscle. The reason for this is likely to be two-fold. It could be due to an increased uptake of FAs into skeletal muscle and/or impairment in FA oxidation.

Regarding the former, FA uptake into skeletal muscle appears to be elevated in obese individuals with type II diabetes (Bonen et al. 2004) and is purported to occur as a consequence of elevated expression of proteins involved in FA uptake into skeletal muscle such as FABPpm (Simoneau et al. 1999) and/or permanent translocation of FAT/CD36; Bonen et al. 2004). A second means by which IMTG stores are increased in obesity could also be through a reduction in skeletal muscle fat oxidation. For example, several studies show that obese individuals, compared with lean individuals, display a reduced fat oxidation under postabsorptive and/or postprandial conditions (Kelley & Simoneau, 1994; Colberg et al. 1995; Kim et al. 2000; Kelley et al. 1999; Hulver et al. 2003) and this may be a result of reduced suppression in oxidative capacity. For
instance, one study demonstrated in sedentary obese individuals that the activity of CPT1, CS, and cytochrome c oxidase action (a marker of respiratory chain function) are all significantly lower, compared with lean sedentary individuals (Simoneau et al. 1999). Others have also shown that a suppression of respiratory chain activity may play a role (Kelley et al. 2002; Ritov et al. 2005; Schrauwen-Hinderling et al. 2007), whilst there is also evidence that reduced mitochondrial size and content may also be important. For instance, Kelley and co-workers observed that obese and type II diabetics had a smaller mitochondrial size and a lower NADH:02 oxidoreductase action (a marker of respiratory chain activity), compared with their lean counterparts (Kelley et al. 2002). Considered collectively these findings suggest that the reduced oxidative capacity observed in obesity is likely attributed to not one but multiple factors, including a reduction in mitochondrial function, content and size.

1.7.2 Significance of IMTG accumulation in skeletal muscle – implications for insulin resistance

Findings from studies performed over a decade ago proposed that elevations in IMTG content are a key feature underpinning the insulin resistance that is frequently observed in obesity. For example, one study demonstrated a significant and negative relationship between measured IMTG content (determined using magnetic resonance spectroscopy) and insulin sensitivity (using the hyperinsulinaemic euglycemic clamp technique) and concluded that abnormal deposition of IMTG stores may hallmark a large part of insulin resistance, even in normal healthy subjects (Phillips et al. 1996a). Indeed, this
observation is consistent with many others who investigated the association between IMTG stores and insulin resistance or type II diabetes (Goodpaster et al. 1997; Pan et al. 1997; Forouhi et al. 1999; Jacob et al. 1999; Krssak et al. 1999). In addition to observational studies, interventional studies that have employed strategies associated with improved insulin sensitivity such as body mass loss (Dengel et al. 1996; Niskanen et al. 1996; Goodpaster et al. 1999) have demonstrated a decline in IMTG content. Moreover, it has been shown in rodent models that high fat feeding leads to excessive accumulation of IMTG and this reduced their insulin sensitivity (Storlien et al. 1991).

Paradoxically, well-trained athletes exhibit high IMTG concentrations yet have muscles with high insulin sensitivity, which suggests that the expansion of the IMTG pool may accompany various metabolic purposes (Goodpaster et al. 2001). For instance, a classic adaptation of regular exercise training in well-trained individuals is the expansion of the IMTG store in an attempt to provide an additional substrate for lipid oxidation, particularly during times of increased metabolic demand i.e. during intense and/or prolonged exercise. In overweight and obese individuals, the enlargement of the IMTG pool occurs because of the abovementioned mismatch between FA supply and flux into skeletal muscle and oxidative metabolism, and it has been proposed that IMTG accumulation in obesity is an attempt to protect the muscle from excess lipotoxic metabolites and therefore insulin resistance i.e., IMTG acts as a sink to buffer excess lipid metabolites (Watt, 2009). Considered collectively, there appears to be a clear dissociation between IMTG content and insulin sensitivity.
A more contemporary viewpoint suggests that IMTG accumulation may act as a surrogate marker of insulin sensitivity, but it is the formation and accumulation of lipid metabolites such as DAG and LCFA-CoA that are important mediators of the diminishment in insulin action and can induce insulin resistance, as opposed to the inert IMTG store located within lipid droplets (Yu et al. 2002; Hegarty et al. 2003). Mechanistically, the inhibitory influence of the lipid metabolites occurs by activation of skeletal muscle pro-inflammatory/stress pathways that are associated with insulin action. For example, LCFA-CoA and DAG activate PKC, which inhibits insulin signalling by serine phosphorylation of the insulin receptor substrate-1. Ceramide induces impairments in insulin signalling by activating protein phosphatase 2A, which dephosphorylates and inactivates PKB (for review see Hulver & Dohm, 2004). Well-trained individuals are able to tolerate the enlarged IMTG pool and maintain high levels of insulin sensitivity in the face of high IMTG concentrations because they have a high IMTG turnover and muscle fat oxidation. This encourages a constant depletion and replenishment of the IMTG pool, which helps to maintain low muscle levels of ceramide, DAG and LCFA-CoA and high insulin sensitivity (Moro et al. 2008). In contrast, sedentary obese individuals have a low IMTG turnover and thus develop an inert IMTG pool encompassing expanded and inflexible lipid droplets and heightened concentrations of insulin resistance-inducing lipid metabolites.
1.7.3 Strategies to augment IMTG utilisation and improve insulin sensitivity in obesity

The positive relationship between exercise training status and high insulin sensitivity affords the suggestion that obese sedentary individuals who are insulin resistant might benefit from performing regular physical activity in an attempt to stimulate IMTG turnover and reduce the deleterious effects of excess lipid on intracellular insulin signalling pathways. To this end, longitudinal studies have reported that 12 weeks of regular exercise training does not improve insulin sensitivity in lean, obese and diabetic men, despite improvements in cardiorespiratory fitness and controlling for alterations in body mass and/or composition that may accompany exercise and impact upon insulin sensitivity (Segal et al. 1991). This finding is supported by more recent work from Ross and colleagues who demonstrated in middle-aged obese men (Ross et al. 2000) and premenopausal obese women (Ross et al. 2004) that body mass loss induced by aerobic exercise training (and in the absence of caloric restriction) improves insulin sensitivity, but exercise without body mass loss has no beneficial effect, despite improvements in cardiorespiratory fitness. Considered collectively, such findings suggest that exercise training in the absence of body mass loss does not improve long-term whole body glucose uptake and insulin sensitivity.

It could be that the timing of recent nutrition around exercise explains the failure of aerobic exercise training to induce favourable effects on peripheral insulin sensitivity in longitudinal studies on obese populations. Indeed, it has been demonstrated that a single exercise session performed in the overnight-fasted (post-absorptive; PA) state
i.e., before breakfast, substantially improves skeletal muscle insulin sensitivity within 48-h of exercise. This effect has been observed in rodents (Richter et al. 1992) and in healthy (Mikines et al. 1988; Richter et al. 1989) and insulin resistant (Devlin et al. 1987) humans and occurs through a large increase in insulin-stimulated muscle glycogen storage via improvements in insulin-stimulated glucose transport-phosphorylation (Perseghin et al. 1996). When exercise is performed in the post-prandial (PP) or fed state i.e., after breakfast, exercise appears ineffective at improving glucose homeostasis in both insulin resistant and type 2 diabetic individuals (Larsen et al. 1997; Larsen et al. 1999).

More recent work conducted in healthy, lean individuals has demonstrated that PA exercise, as compared with PP exercise, significantly increases IMTG utilisation during exercise (De Bock et al. 2005). In this study, participants performed 2-h of constant load cycling (~75% \(\dot{V}O_2\text{max}\)) either in the overnight-fasted state or in the fed-state having received carbohydrates before (~150 g) and during (1 g/kg BW/h) exercise. In both conditions, net muscle glycogen breakdown was similar, however during the subsequent 4 hours of recovery (when carbohydrate was fed), net muscle glycogen re-synthesis rate was 3-fold higher after PA compared with PP exercise. What is more, PA exercise led to a significantly higher fat oxidation rate during exercise and induced a substantial reduction in type I fibre IMTG content pre- to post-exercise (18 ± 2% to 6 ± 2%, respectively; \(P = 0.007\)). In contrast, IMTG breakdown was completely prevented in the fed-state (\(P > 0.05\)).
Interestingly, indirect evidence suggests IMTG can be utilised as a substrate source during aerobic exercise in obese individuals, at least in the PA state. For instance, Goodpaster and co-workers (Goodpaster et al. 2002), demonstrated that obese individuals had a greater reliance on fat oxidation towards energy expenditure than their lean counterparts when performing steady state exercise (50% $\dot{V}O_{2\text{max}}$) for 60 minutes, and the contribution of non-plasma FA oxidation (likely from IMTG) was significantly higher (~50%) in obese compared with lean. In this study, the exercise bout was performed in the PA state and the influence of PA versus PP exercise on IMTG use during exercise in obesity has not yet been studied. Based on the observations made from healthy, lean populations (De Bock et al. 2005; abovementioned) it is tempting to speculate that PA exercise, as compared with PP exercise, would facilitate a greater IMTG breakdown and reduction in lipotoxic stress in the skeletal muscle of obese individuals, and this could confer greater improvements in insulin sensitivity as compared with exercise performed in the PP state.

It should be noted that not all studies have reported findings that promote PA exercise for the prevention of insulin resistance. For instance, Derave and colleagues (Derave et al. 2007) used seven sedentary males with metabolic syndrome and found that although fat oxidation during exercise was higher during PA exercise, the glycemic response to a high-carbohydrate breakfast was lower following PP exercise than PA exercise. Furthermore, the timing of nutrition (either before [PA] or after [PP] exercise) did not alter glucose and insulin handling during subsequent meals throughout the day. These authors suggest that PA exercise might not be more effective than PP exercise at
improving insulin sensitivity, however they do highlight caution when extrapolating data from acute exercise to chronic exercise and training. For instance, recent studies have demonstrated that PA exercise, but not exercise with carbohydrate ingestion, promotes an increase in mRNA expression in muscle of genes involved in FA transport and oxidation such as CD36, CPT 1, uncoupling protein 3, and 5'-AMP-activated protein kinase-a (Civitarese et al. 2005). Such alterations in mRNA expression are unlikely to have an acute effect in the hours after exercise but could promote altered protein expression following chronic PA exercise. A parallel with this, a recent study conducted in lean individuals found that during a period of high fat feeding (50% kcal from fat) PA exercise was superior to PP exercise at improving glucose tolerance and promoting adaptations of muscle oxidative capacity that may confer improvements in insulin sensitivity (Van Proeyen et al. 2010). As insulin resistance appears to be caused by the excess accumulation of insulin resistance-inducing lipid metabolites in skeletal muscle, the emerging question that remains is whether chronic PA exercise, compared with PP exercise, is more effective at reducing the accumulation of these metabolites and therefore improving insulin sensitivity in obesity.

Considered collectively, it could be that the timing of nutrition in relation to the exercise bout is a critical factor in elucidating the failure of aerobic exercise training to induce favourable effects on peripheral insulin sensitivity in longitudinal studies on obese populations. Recommending obese individuals to exercise in the fasted-state, as oppose to the fed-state, could help to optimise metabolic adaptation to training and augment insulin sensitivity and a healthy metabolic phenotype.
1.8 Summary

In summary, an elevated 24-h RQ has been shown to be predictive of long-term body mass gain and regain of fat mass after diet-induced body mass loss. Furthermore, impairments in fat oxidation at the level of skeletal muscle have been associated with a reduced metabolic flexibility and insulin resistance. There is a large inter-subject variability in the capacity to oxidise fat during exercise and as a high fat oxidation during exercise could make important contributions to daily fat oxidation, it is not unreasonable to consider a high capacity to oxidise fat during exercise as advantageous for long term metabolic health. Whilst studies have begun to address this, there is clearly a need for further understanding. Accordingly, Chapter 3 aims to determine whether the capacity for fat oxidation during exercise is associated with 24-h fat oxidation and insulin sensitivity as markers for long-term metabolic health in young, healthy men. It was hypothesised that there is a significant and positive correlation between MFO and 24-h fat oxidation and between MFO and insulin sensitivity as markers for long-term metabolic health.

If this is important then a more comprehensive understanding of the factors influencing the capacity to oxidise fat during exercise is undoubtedly important. Despite appreciable efforts to elucidate the determinants of the inter-subject variability in MFO, a large proportion of the inter-subject variability remains unexplained. It could be that circulating concentrations of plasma metabolites and hormones known to influence fuel selection during exercise could be important mediators, whilst there is evidence to suggest that resting fat oxidation could also play a role, but this has not yet been investigated.
Therefore, **Chapter 4** investigates the influence of plasma lipolytic markers, insulin and resting fat oxidation on maximal fat oxidation during exercise. It was hypothesised that there would be a significant and positive relationship between MFO and plasma glycerol and NEFA concentration and a significant and negative relationship between MFO and plasma insulin concentration. Further, there would be a significant and positive relationship between MFO and resting fat oxidation.

A better insight into the factors that influence the capacity to oxidise fat during exercise could help inform of novel strategies to increase fat oxidation during exercise and promote metabolic health. An emerging body of evidence demonstrates that the accumulation of insulin resistance-inducing lipid metabolites govern the reduction in insulin sensitivity seen in obesity. Exercise training could be one means of reducing the accretion of these metabolites and improving insulin sensitivity, however longitudinal studies indicate aerobic exercise training in obese individuals in the absence of body mass loss has minimal impact on insulin resistance. At present it is not known whether the timing of recent nutrition with respect to exercise is important in explaining the inability of aerobic exercise training per se to improve peripheral insulin sensitivity in obese populations. As such, **Chapter 5** investigates the influence of meal timing i.e., eating before or after an acute exercise bout, on (1) exercise substrate utilisation, (2) the plasma hormone and metabolite response during and around exercise, and (3) IMTG utilisation in obesity. It was hypothesised that a single bout of aerobic exercise performed in the fasted- versus fed-state would (1) induce a greater reliance on fat and lesser reliance on carbohydrate towards exercise energy expenditure, (2) enhance the
availability of circulating lipolytic makers (namely, plasma glycerol and NEFA) and reduce the availability of insulin during exercise, and (3) enhance IMTG degradation.
1.9 References


• Bingley CA, Gitau R, and Lovegrove JA. Impact of menstrual cycle phase on


- **Burke LM, Angus DJ, Cox GR, Cummings NK, Febbraio MA, Gawthorn K, et al.**


- **Christensen RH and Hansen IV.** Arbeitsfahigkeit und Ehrnahrung. *Scan Arch Physiol* 81: 160-163, 1939b.


• Geerling BJ, Alles MS, Murgatroyd PR, Goldberg GR, Harding M, and Prentice


- **Hainer V, Kunesová M, Parizková J, Stich V, Mikulová R, and Slabá S.**

- **Hall, LML, Moran CN, Milne GR, Wilson J, MacFarlane NG, Forouhl NG, et al.**


- Health & Social Care Information Centre: Statistics on obesity, physical activity and
etters&utm_medium=email&utm_campaign=5400557_HMP+2015-03-
03&dm_i=21A8,37R3H,FLWRRQ,BJQNZ,1.


• **Hultman E.** Muscle glycogen content in man in the postabsorptive state. *Scand J


• Johnson NA, Stannard SR, Rowlands DS, Chapman PG, Thompson CH,


- Kjaer M, Kiens B, Hargreaves M, and Richter EA. Influence of active muscle mass


- **Krogh S.** The number and distribution of capillaries in muscles with calculations of the oxygen pressure head necessary for supplying the tissue. *J Physiol* 52: 409-415, 1919.


• **MacLaren D and Morton JP.** *Biochemistry for Sport and Exercise Metabolism.* Chichester: Wiley & Sons, 2012.


• Ross RD, Dagnone PJ, Jones H, Smith A, Paddags R, Hudson, et al. Reduction in obesity and related comorbid conditions after diet-induced weight loss or exercise-


- **Seidell JC, Muller DC, Sorkin JD, and Andres R.** Fasting respiratory exchange ratio and resting metabolic rate as predictors of weight gain: the Baltimore


2011.


• Tarnopolsky MA, Rennie CD, Robertshaw HA, Fedak-Tarnopolsky SN, Devries, MC, and Hamadeh MJ. Influence of endurance exercise training and sex on


• **van Loon LJ, Manders RJ, Koopman R, Kaastra B, Stegen JH, Gijsen AP, et al.**


• Weinsier R, Hunter G, Zuckerman P, and Darnell B. Low resting and sleeping


- **Yu CY, Chen H, Zong Y, Wang R, Bergeron JK, Kim GW, et al.** Mechanism by which fatty acids inhibit insulin activation of IRS-1 associated phosphatidylinositol 3-


CHAPTER 2

GENERAL METHODS
2.1 Introduction (see also Table 2.1)
Specific methods for each study are provided in the relevant section of each experimental chapter and will therefore not be repeated unless necessary. Rather, the purpose of this section is to overview the core principles of the major methods employed and provide a rationale for their use where appropriate.

Table 2.1 An overview of the methods / techniques used in the experimental chapters of this thesis.

<table>
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<th>Components of energy balance (EI &amp; EE)</th>
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2.2 Components of energy balance

2.2.1 Estimation of habitual energy intake
In Chapters 3 and 4, the habitual energy intake of all participants was estimated using self-reported 4-d weighed food intake. This prospective, quantitative method, alongside 24-h recalls, diet history, and food frequency questionnaires (FFQ), is common amongst experimental and large population studies. A strength of this approach lies in its capacity
to capture quantitative information, given that all foods and drinks are weighed and recorded prior to consumption. Indeed, the weighed food approach has been employed as the reference to which other dietary intake methodologies have been compared (Trabulsi & Schoeller, 2001). However, it is also accepted that this approach is subject to measurement error i.e., writing and / or processing mistakes (random error) and reporting bias such as under-reporting of true intake (systematic error) (Trabulsi & Schoeller, 2001). Regarding the latter, reporting bias can occur because the action of recording intake can influence the individuals’ food choices and alter their intake during the recording period. This bias is not usually found in retrospective methodologies (i.e. diet history and FFQs), however these are subject to error including poor recollection of past diet and underreporting of true intake. Of note, underreporting has been shown to be greater in females compared with males (32% and 13%, respectively; Goran & Poehlman, 1992) and appears positively associated with BMI, waist circumference and fat mass (Tomoyasu, Toth & Poehlman, 1999). Further, it has been demonstrated that the number of inaccuracies reported increases as the number of recording days increases (Gersovitz, Madden & Smiciklas-Wright 1978). Accordingly, a number of measures were put in place to mitigate any potential error. For instance, the participants in Chapters 3 and 4, who were all lean, healthy men, were provided with detailed instructions to enable diary completion and the investigatory team answered any questions. The chosen recording period was 4-d as any longer could increase the number of inaccuracies reported, whereas any less may not reflect the participants’ habitual diet. Further, they were given two sets of digital weighing scales; one that was portable (for use away from home) and one that was larger in size (for use at home).
Finally, each diary was checked meticulously for any cases of misreporting and to clarify any uncertainties.

2.2.2 Estimation of daily energy expenditure (EE)

Daily EE was estimated in Chapters 3 and 4 by multiplying a daily PAL value (obtained using physical activity diaries and the factorial approach; further details of this approach are provided in the experimental chapters) by an age and sex specific predictive equation (WHO, 1985). This method of assessment appears superior to the activity recall method (Conway et al. 2002) and has shown to be strongly correlated with the doubly labeled water (DLW) technique (Schulz, Westerterp & Bruck, 1989; Bratteby et al. 1997), which is considered a gold-standard tool for measuring EE. The advantage of using this approach was that it was affordable and practical, particularly in Chapter 4 when a relatively large number of participants (for physiology studies) were evaluated (n=57). However, there are also limitations of this approach such as not accounting for inter-individual differences in the energetic cost of performing a movement and there can be poor individual compliance and recording error. To mitigate this the investigatory team provided all participants with clear and detailed instructions to enable accurate completion of the diaries, any questions were answered and any potential cases of misreporting or inaccuracies were clarified. Furthermore, participants were encouraged to record physical activity instead of recall (i.e., to record activity immediately after it was performed), and to make notes on any sport or exercise performed during the 4 days such that a specific MET value could be assigned to this activity by the investigatory
team. Of note, in Chapter 3 there were no significant differences between the EE predicted using the physical activity diaries and predictive equation and that measured during the 24-h stay in the whole room calorimeter, which gives this researcher confidence that the diary approach provided an accurate estimation of habitual EE over the 4-d recording period.

In Chapter 5 daily EE was estimated using resting metabolic rate determined via indirect calorimetry (ventilated hood system) and a population specific activity factor of 1.53 (WHO, 2001). The principle of indirect calorimetry is to measure oxygen consumption and carbon dioxide production and convert it to EE using formulae, which in this Chapter was that of Weir (1949), which is commonly used in studies of exercise metabolism. Indirect calorimetry is non-invasive, very accurate and highly reproducible and is widely considered a gold standard tool for measuring EE (Volp et al. 2011). Although the procedure can be relatively complex, the use of trained personnel ensured its correct use.

### 2.3 Indirect calorimetry

The respiratory quotient (RQ; as is conventionally used in metabolic chamber studies) and respiratory exchange ratio (RER; as is conventionally used in studies of exercise metabolism) both indicate relative fuel utilisation and can be calculated by measuring the ratio of the $\dot{V}CO_2$ consumed and the $\dot{V}O_2$ produced by gaseous exchange and using
indirect calorimetry (RQ or RER = $\dot{V}CO_2 / \dot{V}O_2$). Carbohydrate, fat and protein differ in their chemical composition and in the amount of carbon dioxide produced and oxygen required for oxidation. Therefore by measuring the $\dot{V}O_2$ consumed and $\dot{V}CO_2$ produced it is possible to determine energy expenditure, as well as the absolute and relative contribution of fuels being oxidised for energy production. It is beyond the scope of this thesis to provide a detailed synopsis of indirect calorimetry, calculation of substrate oxidation and the respective strength and limitations and readers are directed to a pertinent review by Wallis and Jeukendrup (2005) should they seek this. However, a brief overview of the methods and equations used to determine energy expenditure and substrate oxidation is necessary.

2.3.1 Determination of energy expenditure and substrate oxidation at rest

Resting energy expenditure plus carbohydrate and fat oxidation (Chapters 3, 4 and 5) were calculated during a stable measurement period i.e., a deviation in $\dot{V}O_2$ of <10% of the average $\dot{V}O_2$ between minutes 20-30 according to the equations of Frayn (1983) assuming urinary nitrogen excretion to be 0.11 mg/kg/min (Flatt, Ravussin, Acheson & Jequier, 1985).

2.3.2 Determination of substrate oxidation during exercise

In Chapters 3 and 4, during the exercise test $\dot{V}O_2$ and $\dot{V}CO_2$ were averaged over the last minute of each sub-maximal exercise stage and fat and carbohydrate oxidation were calculated according to the equations of Frayn (1983), with the assumption that the
urinary nitrogen excretion rate was negligible. The last minute was chosen because participants were likely to have reached steady state of respiratory exchanges (Achten, Gleeson & Jeukendrup, 2002). Urinary nitrogen excretion rate was assumed as negligible because the quantitative contribution of amino acid oxidation to energy expenditure during exercise is usually minimal (<1%). Although it has been shown that exercising with low muscle glycogen can increase net protein degradation, the contribution to total EE remains small (Lemon & Mullin, 1980). Only when a subject partakes in prolonged exercise with no caloric intake does the contribution of amino acid oxidation to total EE increase to around 10% (Wagenmakers, 1998). Given that the graded exercise test employed in Chapters 3 and 4 was approximately 30 minutes in duration, it seems reasonable to assume that amino acid oxidation was negligible.

In Chapter 5 during the exercise test employed in visits 3 and 4, $\dot{V}O_2$ and $\dot{V}CO_2$ were measured at two time points (25-30 minutes and 55-60 minutes into exercise) and fat and carbohydrate oxidation were calculated according to the equations of Frayn (1983).

**Calculation of carbohydrate and fat oxidation (Frayn, 1983)**

$$\text{CHO oxidation (g/min)} = 4.55*\dot{V}CO_2 - 3.21* \dot{V}O_2$$

$$\text{Fat oxidation (g/min)} = 1.67* \dot{V}O_2 - 1.67* \dot{V}CO_2$$
2.3.3 Determination of Maximal Fat Oxidation (MFO) during exercise

In Chapters 3 and 4, all participants completed a graded exercise test in order to determine their MFO during exercise. The test was originally developed by Achten et al. (2002) who systematically undertook a series of studies in trained men to design a test that enables the intensity that elicits MFO, and its corresponding exercise intensity (Fat\textsubscript{max}), to be determined. The test was validated against longer duration stages (Achten, Gleeson & Jeukendrup, 2002) and its reproducibility was assessed (Achten & Jeukendrup, 2003). Achten and colleagues reported a coefficient of variation for Fat\textsubscript{max} of 9.6% in a group of 10 well-trained individuals assessed on three separate occasions and demonstrated limits of agreement that corresponded to a difference in heart rate of 9 beats per minute (range 7-14 beats per minute); concluding that the test is reliable. In contrast, Meyer and co-workers, using 21 recreationally active men and women who completed two exercise tests, reported limits of agreement that corresponded to a heart rate difference of 35 bpm, and these authors subsequently concluded that the intra-individual variability in Fat\textsubscript{max} measurements is too large (Meyer, Gassler & Kindermann, 2007). However, this last study did not control for recent activity or diet, which are both potent modulators of substrate utilisation during exercise and therefore the conclusions raised are limited to individuals tested under non-standardised conditions. Of note, these studies assessed the reliability and validity of MFO during cycling exercise. Despite a number of studies having used the treadmill approach to determine MFO during exercise (Achten, Venables & Jeukendrup, 2003; Venables et al. 2005), including those presented in Chapters 3 and 4 of this thesis (Robinson et al. 2015; Robinson et al.
2016), its reliability and validity (specific to treadmill exercise) is yet to be determined. Undoubtedly, this is something future research should seek to explore.

MFO during exercise is typically determined by one of four methods: 1) visual inspection i.e., the determination of the highest value of fat oxidation obtained during the exercise test and the corresponding exercise intensity (Achten, Gleeson & Jeukendrup, 2002; Achten & Jeukendrup, 2003a; Achten & Jeukendrup, 2003b; Venables, Achten & Jeukendrup, 2005); 2) the construction of a 3rd polynomial fitting curve (P3; Stisen et al. 2006); 3) the Sine model (SIN; Cheneviere et al. 2009); and 4) the non-protein respiratory quotient technique (Perez-Martin et al. 2001). For all participants in Chapters 3 and 4, MFO during exercise was calculated and reported using visual inspection. To ensure the main findings from these chapters were not influenced by the method employed for the determination of MFO, MFO was also calculated using the P3 method, which demonstrated a strong correlation with the visual method used in both experimental chapters (Chapter 3, $R=0.98$ and Chapter 4, $R=0.98$).

### 2.4 Assessment of body composition

Three different techniques were used across the three experimental chapters due to reasons of availability and practicality. This researcher conducted all measures of body composition performed during the studies conducted in Chapters 3, 4 and 5.
2.4.1 Dual-energy X-ray Absorptiometry (DXA)

DXA provides a quick, non-invasive three-compartment model that divides the body into bone mineral, fat mass (FM) and fat-free mass (FFM) to a high level of precision and with minimal radiation. It is widely used in osteoporosis screening and bone mineral density assessment, and is also increasingly used as a criterion measurement for body composition assessment (Pietrobelli et al. 1996).

The principle of using DXA to determine body composition is based on the different bone and soft tissue attenuation characteristics at the two-pulsed X-ray wavelengths. The DXA scanner in our laboratory sends a very thin, invisible beam of low-dose X-rays with two distinct energy peaks through the body to a detector array. These X-rays are blocked or attenuated relative to the density of the tissues they are penetrating. Bone, fat and lean tissue possess distinctive densities which can be used to calculate the relative masses of each of the three components in any given region of the body.

2.4.2 Calliper-measured skinfold thickness

Calliper-measured skinfold thickness is a two-compartment model of body composition assessment i.e., it characterizes body composition by dividing it into fat and fat-free components and assumes that the relative amounts of the FFM components (water, protein, bone mineral and non-bone mineral) are fixed. This method has been used to estimate percent body fat measures for four decades and does so by measuring skinfold thickness at specific locations on the body to determine subcutaneous fat. Results are
then inserted into equations that convert these numbers into an estimate of a subject’s percentage body fat (%BF), FM and FFM.

There are several different formulas to predict percent body fat from calliper-measured skinfold thickness, of which the most widely used are the original Durnin and Wormersely (1974) equations. However, these have recently been found to not predict percent body fat uniformly in all races or ethnicities when compared with %BF measured by DXA as the gold standard. As such, %BF, FM and FFM were calculated in Chapter using updated sex and race/ethnicity specific equations that incorporated the combinations of four skinfold sites (bicep, tricep, subscapular and suprailliac) and body height, weight, age and waist circumference (Davidson et al. 2011).

2.4.3 BODPOD

The BODPOD is an Air Displacement Plethysmograph that was first validated in the mid-1990’s (Dempster & Aitkens, 1995). It is a two-compartment model that partitions the body into two compartments of constant densities (FM = 0.9007 g/cm$^3$ and FFM = 1.100 g/cm$^3$) and assumes that the relative amounts of the FFM components (water, protein, bone mineral and non-bone mineral) are fixed. The physical design and operating principles of this system have been described elsewhere (Dempster & Aitkens, 1995). This method initially measures body mass using a highly accurate scale (with the participant outside of the BODPOD) and volume (with the participant is inside the BODPOD) using air displacement, which allows for the determination of body density
using Archimedes principle (Density = Mass/Volume). Body volume is assessed based on the physical relationship between pressure and volume. During a measurement, there are very small volume changes inside the BODPOD, which measures the pressure response to these changes. Initially, the volume of the chamber is measured whilst empty. The interior volume is then measured with the participant seated inside (IV) the chamber and subsequently the individual’s body volume is determined (body volume = EV – IV). Once the overall density of the body is known, the relative percentages of FM and FFM were determined using an equation (Siri, 1961).

2.5 Insulin sensitivity

In Chapters 3 and 5, insulin sensitivity was calculated from fasting plasma glucose and insulin concentration using the Quantitative Insulin Sensitivity Check Index (QUICKI) derived from Katz et al. (2000).

\[
\text{QUICKI} = \frac{1}{\log(I_0) + \log(G_0)}
\]

\*I_0 = fasting insulin (µIU/mL, G_0 = fasting glucose (mmol/L)

QUICKI has been validated as an accurate and reliable surrogate marker of insulin sensitivity; shown to have a strong positive correlation (R=0.78) with the gold standard hyperinsulinaemic euglycemic clamp technique. The QUICKI method was chosen as it provided a convenient measure of insulin sensitivity that was easily implemented in
these studies. The gold standard methods of determining insulin sensitivity is the
euglycemic-hyperinsulinemic clamp technique, however this is a complicated procedure
and the infusion of insulin or glucose would have affected the metabolic and hormonal
response to the GET used in Chapter 3, whilst we did not want to burden the
participants in Chapter 5 with any further invasive procedures. Nevertheless, fasting
levels of glucose and insulin have been found to contain enough information to precisely
measure insulin sensitivity in a diverse population.

2.6 Blood sampling and biochemical analysis

This researcher collected all of the blood samples obtained during the conduct of the
three experimental chapters presented in this thesis. Furthermore, he also carried out all
biochemical procedures with respect to plasma hormone and metabolite analysis, with
the exception of plasma insulin in Chapters 3 and 4, which were analysed by
collaborators at Imperial College London using the radioimmunoassay technique.

During Chapters 3, 4 and 5, each participant had a cannula placed in the forearm vein to
allow for repeated blood sampling throughout the measurement period. To allow for
repeated blood collection during the treadmill running exercise performed in Chapter 4
(without the need for changes in running speed or form), the cannula was connected to
a 150 cm polyethylene extension line, which was attached to a 3-way stopcock. When a
blood sample was required during the 24-h measurement period in the respiration
chamber, participants passed their arm through an air-tight hatch and a blood sample was obtained. On all occasions the cannula was kept patent for the duration of insertion by regular flushing of 0.9% sodium chloride.

2.6.1 Plasma metabolite analysis

Unless explicitly stated, all collected samples were analysed using enzymatic colorimetric assays for glucose (Glucose Oxidase kit, Instrumentation Laboratories, Cheshire, UK), NEFA (NEFA kit, Randox, London, UK), glycerol (GLY kit, Randox, London, UK), lactate (LAC kit, Randox, UK) and triglyceride (TG kit, Instrumentation Laboratory, Warrington, UK) using an iLAB 650 Clinical Chemistry Analyzer (Instrumentation Laboratory, Warrington, UK). This automated blood analyser is used frequently in clinical chemistry laboratories to measure plasma metabolites using spectrophotometry or turbidity. Enzyme catalysed assays were used whereby absorbance values correlate with the concentration of a particular blood metabolite. The intra-assay (inter-assay) CV for these methods in our laboratory are as follows: glucose, 1.48% (5.9%); glycerol, 2.76% (5.9%); NEFA, 3.34% (3.1%); lactate 2.04% (3.9%) and TG 1.28% (3.5%) (based on analysis of 20 duplicate plasma samples).

(i) Plasma glucose: In all experimental Chapters, the concentration of plasma glucose was quantified using glucose (oxidase) and end point analysis (trinder methodology). Here, the elevation in absorbance generated by red quinoneimine is proportional to the glucose concentration in the sample.
(ii) Plasma glycerol: A direct colorimetric procedure using a glycerol reagent (Randox, County Antrim, UK) was used for the quantification of glycerol, which included three enzymatic reactions (below). The subsequent intensity of n-(4-antipyryl)-3-chloro-5-sulphonate-pbenzoquinoneimine (ACSB: red dye) was determined and is proportional to the glycerol concentration in the sample.

\[
\begin{align*}
\beta-D\text{-glucose} + O_2 + H_2O & \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2 \\
2H_2O_2 + \text{phenol} + 4\text{-aminoantipyrine} & \xrightarrow{\text{Peroxidase}} \text{red quinoneimine} + 4H_2O
\end{align*}
\]

(iii) Plasma non-esterified fatty acids (NEFA): A direct colorimetric procedure using a NEFA reagent (Randox, County Antrim, UK) was used for the quantification of NEFA, which included three enzymatic reactions (below). The subsequent intensity of purple adduct was determined and is proportional to the NEFA concentration in the sample.

\[
\begin{align*}
\text{Glycerol} + \text{ATP} & \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP} \\
\text{Glycerol-3-phosphate} + O_2 & \xrightarrow{\text{GPO}} \text{H}_2\text{O}_2 + \text{DAP} \\
\text{H}_2\text{O}_2 + \text{DCHBS} + 4\text{-aminophenazone} & \xrightarrow{\text{POD}} \text{ACSB}
\end{align*}
\]
(iv) Plasma lactate: A direct colorimetric procedure using a lactate reagent (Randox, County Antrim, UK) was used for the quantification of lactate, which included two enzymatic reactions (below). The subsequent intensity of purple product was determined and is proportional to the lactate concentration in the sample.

\[
\text{NEFA + ATP + CoA} \xrightarrow{\text{Acyl CoA Synthase}} \text{Acyl CoA + AMP + PPI} \\
\text{Acyl CoA + O}_2 \xrightarrow{\text{Acyl CoA Oxidase}} 2,3\text{-tran-Eonyl-coA+H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 + \text{TOOS+4-AAP} \xrightarrow{\text{Peroxidase}} \text{Purple adduct + 4H}_2\text{O}
\]

(v) Plasma triglyceride \(^1\): A direct colorimetric procedure using a triglyceride reagent (Randox, County Antrim, UK) was used for the quantification of triglyceride, which included four enzymatic reactions (below). The subsequent intensity of red quinoneimine was determined and is proportional to the triglyceride concentration in the sample.

\[
\text{L-Lactate + O}_2 \xrightarrow{\text{Lactate Oxidase}} \text{Pyruvate + H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + 4\text{-aminoantipyrine + TOOS} \xrightarrow{\text{Peroxidase}} \text{Purple product + 4H}_2\text{O}
\]

\(^1\) TAG data presented in Chapter 5 of this thesis was corrected for free glycerol concentrations.
2.6.2 Plasma insulin analysis

In Chapter 3 and 4, insulin was analysed by radioimmunoassay using commercially available kits (HI-14K Human Insulin, Millipore, Billerica, MA). The intra-assay (inter-assay) CV for this method in the laboratory where analysis was conducted (Nutrition and Dietetic Research Group, Imperial College, London, United Kingdom) was 16.9% (17.1%) (based on analysis of 10 duplicate plasma samples).

The Millipore Human Insulin assay uses the double antibody/PEG technique (Morgan & Lazarow, 1963). Here, a fixed concentration of labeled tracer antigen (125I-labeled Human Insulin) is incubated with a constant dilution of antiserum (Human Insulin antiserum), which subsequently limits the concentration of antigen binding sites on the antibody. Once unlabelled antigen is added, there is competition between the labeled tracer and unlabelled antigen for the limited but constant number of binding sites on the antibody. Accordingly, the amount of tracer bound to antibody will decrease as the concentration of unlabelled antigen increases. This can be measured after separating
antibody-bound from free tracer and counting one or the other. A standard curve is constructed with increasing concentrations of standard unlabelled antigen and from this curve the amount of antigen in unknown samples can be calculated.

In Chapter 5, insulin concentrations were determined using a human insulin Enzyme Linked-Immuno-Sorbent Assay (ELISA) kit (Invitrogen, Paisley, UK) and a Biotek ELx800 analyser (Biotek Instruments, Vermont, USA). The intra-assay (inter-assay) CV for this method our laboratory was 21.0% (17.0%) (based on analysis of 10 duplicate plasma samples).

The ELISA method uses monoclonal antibodies (mAb), which are directed against distinct epitopes of insulin. Each sample is added to a small well; following which a detector mAB labeled with horseradish peroxidase is added. Following an incubation period, the enzyme–labeled antibody is removed and a substrate solution (tetramethylbenzidine – H₂O₂) is added and incubated. The reaction is stopped with HCl and the microtiter plate is read spectrophotometrically. The intensity of the colour is proportional to the insulin concentration in the sample.
2.7 Muscle sampling and analysis

In Chapter 5 Dr Gareth Wallis performed the muscle biopsy procedure and collected each of the muscle samples. This researcher was present during each of the biopsies to assist with the muscle biopsy collection. Dr Helen Bradley and myself performed the muscle sample analysis.

2.7.1 Muscle biopsy procedure

The study in Chapter 5 involved six muscle biopsies per participant (three during Visit 3 and three during Visit 4). For each, participants rested in the supine position and a muscle biopsy was obtained from the vastus lateralis using the percutaneous needle biopsy technique (Bergstrom, 1975), adapted to include suction. Prior to this, antiseptic (Videne, Ecolab, UK) was applied to the skin ~20 cm proximal to the patella, following which it was anaesthetized using 1% Lidocaine (Braun, Melsungen, Germany). When the area was numb to the touch a 4-6 mm incision was made through the skin and muscle fascia using a surgical scalpel (Swann-Morton, Sheffield, UK). A 6G biopsy needle was then inserted ~2-6 cm into the incision and a piece of muscle guillotined. A sample of ~100 mg was collected and freed from any visible non-muscle material, following which a portion was mounted in embedding Tissue Tek OCT (Sigma Aldrich, Dorset, UK) and frozen in liquid nitrogen-cooled isopentane (Sigma Aldrich, Dorset, UK) and transferred to an aluminium cryotube (Caltag Medsystems, PA6003) and transferred
to an aluminium cryotube (Caltag Medsystems, PA6003) for later immunofluorescence microscopy analysis. All samples were stored at -70°C for later analysis.

2.7.2 Analysis of muscle samples

(i) Sample Preparation and Staining: Cryosections (5 μm) were cut using a Bright 5040 (Bright Instrument Company limited, Huntingdon, England) microtome within a Bright cryostat with internal temperature kept constant at 25 °C. Sections were cut onto uncoated glass microscope slides (VWR International), with each slide containing six samples from each participant (three from visit 3 and three from visit 4 [pre-exercise, immediately post-exercise and 3-h post-exercise]) to decrease the variation in staining intensity between sections. Sections were fixed for one hour in 3.7% formaldehyde (made up in dH2O) and subsequently washed 3 times for 30 seconds in dH2O. Following this slides were incubated for 5 minutes in 0.5% triton-X100 made up in 1 x phosphate-buffered saline (PBS) at room temperature and then washed 3 times for 5 minutes with wash buffer (1 x PBS). Thereafter, slides were incubated for 2 hours in 1:25 anti-MHCI (mouse IGM, DSHB A4,840) and 1:100 anti-dystrophin (mouse IgG2b, Sigma Aldrich D8168) in 5% normal goat serum (NGS) at room temperature. After washing the slides three times for 30 seconds in 1 x PBS, slides were incubated for 30 minutes in 1:150 goat anti-mouse (GAM) IgM 633 (for MHCI) and 1:200GAM IgG2b 595 (for dystrophin) in 1 x PBS at room temperature. Incubation was followed by three 5 minutes washes (1 x PBS), after which the slides were incubated again, this time in 1:50 bodipy solution in 1 x PBS at room temperature in the dark. A final wash of the slides
was then performed twice for 3 minutes in 1 x PBS. Glass coverslips were mounted with 20 μl mowiol mounting medium (6 g glycerol [Sigma Aldrich, G5150], 2.4 g mowiol 4-88 [Fluka, 81381] and 0.026 g 1,4-diazobicyclo-[2,2,2]-octane [DABCO; Fluka, 33490] dissolved in 18 ml 0.2M Tris-buffer [pH 8.5; Sigma Aldrich, T5030]) to protect the sample and preserve the fluorescence signal. Slides were left to dry overnight before immunofluorescence visualisation.

(ii) Microscopy: All images were taken using a confocal microscope (Leica DMIRE2, Leica Microsystems) with 40x oil immersion objective (1.25 Numerical Aperture). Bodipy was excited with a 488 nm line of the argon laser and 510-652 nm emission. To detect dystrophin signal, Alexa Fluor 594 fluorophores were excited with the 594 nm line of the helium-neon laser and 668-698 nm emission. To detect myosin heavy chain type I (MHCI) signal, Alexa Fluor 633 fluorophores were excited with the 633 nm line of the helium-neon laser and 698-808 nm emission.

(iii) Image quantitation: As will be explained later, data are available for two subjects. For both, 3 slide replicates, each with a pre-exercise, immediately post-exercise and 3-h post-exercise section underwent staining, imaging and quantification. During the fasted trial and on average, 4 fibre images were captured pre-exercise, 5 fibre images were captured immediately post-exercise and 5 fibre type images were captured 3-h post-exercise. During the fed trial and on average, 5 fibre images were captured pre-exercise, 4 fibre images were captured immediately post-exercise and 4 fibre type images were captured 3-h post-exercise. The average number of type I fibres [type II fibres] analysed
across the two participants in the fasted trial was 15 [20] (pre-exercise), 24 [27] (immediately post-exercise) and 19 [44] (3-h post-exercise). The average number of type I fibres [type II fibres] analysed across the two participants in the fed trial was 32 [43] (pre-exercise), 17 [21] (immediately post-exercise) and 25 [30] (3-h post-exercise).

Bodipy spot stained area per fibre was quantified by setting uniform threshold intensity and size values to detect spots within intracellular regions of the dystrophin mask in a fibre type specific manner. A HiGauss filter was applied to bodipy images prior to bodipy signal detection. Fibre area was exported from the software based on the intracellular regions determined with the dystrophin mask. Percent area of bodipy staining of the total fibre area was calculated ([bodipy stained area [um$^2$] / area of muscle [um$^2$]]*100). Fibre area was exported from the software based on the intracellular regions determined with the dystrophin mask. The coefficient of variation for repeated measurements of IMTG content for this method in our laboratory was 35 ± 33%.
2.8 References


- **Conway JM, Seale JL, Jacobs DR, Jr., Irwin ML, and Ainsworth BE.** Comparison of energy expenditure estimates from doubly labeled water, a


• **Food and Agricultural/World Health Organisation/United Nations University.**


CHAPTER 3

MAXIMAL FAT OXIDATION DURING EXERCISE IS POSITIVELY ASSOCIATED WITH 24-HOUR FAT OXIDATION AND INSULIN SENSITIVITY IN YOUNG HEALTHY MEN

The work contained within this chapter is published:


Please note that the data presented in Chapters 3 and 4 of this thesis comes from data collected from one study.
3.1 Abstract

Disturbances in fat oxidation have been associated with an increased risk of obesity and metabolic disorders such as insulin resistance. There is large inter-subject variability in the capacity to oxidize fat when physically active although the significance of this for metabolic health is unclear. We investigated whether the maximal capacity to oxidize fat during exercise is related to 24-hour (h) fat oxidation and insulin sensitivity. Maximal Fat Oxidation (MFO; indirect calorimetry during incremental exercise) and insulin sensitivity (Quantitative Insulin Sensitivity Check Index, QUICKI) was measured in 53 young, healthy men (age 24±7 y; $\dot{V}O_{2\text{max}}$ 52±6 ml/kg/min). 24-h Fat Oxidation (24-h FO; indirect calorimetry) was assessed in 16 young, healthy men (age 26±8 y; $\dot{V}O_{2\text{max}}$ 52±6 ml/kg/min) during a 36-h stay in a whole-room respiration chamber. MFO (g/min) was positively correlated with 24-h FO (g/d) ($R=0.65$, $P=0.003$; $R=0.46$, $P=0.041$ when controlled for $\dot{V}O_{2\text{max}}$ [L/min]), 24-h % energy from FO ($R=0.58$, $P=0.009$) and insulin sensitivity ($R=0.33$, $P=0.007$). MFO (g/min) was negatively correlated with 24-h fat balance (g/d) ($R=-0.51$, $P=0.021$) but not significantly correlated with 24-h RQ ($R=-0.29$, $P=0.142$). While further investigations are needed, our data showing positive associations between maximal fat oxidation and 24-h fat oxidation, and maximal fat oxidation and insulin sensitivity, in healthy young men suggests that a high capacity to oxidize fat whilst physically active could be advantageous for the maintenance of metabolic health.
3.2 Introduction

Exercise intensity is the main determinant of fuel utilization during exercise. With increasing exercise intensity there is an obligatory rise in the contribution of carbohydrate and a decline in the contribution of fat to energy expenditure (Romijn et al. 1993; Bergstrom & Brooks, 1999; van Loon et al. 2001). The absolute rate of fat oxidation during exercise typically shows an inverted hyperbola with fat oxidation increasing to a maximum at moderate exercise intensities (45-65% maximal oxygen consumption \([\dot{V}O_{2\text{max}}]\)) and decreasing to eventually become negligible at higher exercise intensities (Pérez-Martín et al. 2001; Achten et al. 2002). Interestingly, there is substantial inter-subject variability in the relative contribution of fat to energy expenditure during exercise (Goedecke et al. 2000) and this large degree of variability is also apparent in the maximal rates of fat oxidation (MFO) that have been reported. For example, MFO determined during incremental treadmill exercise in 300 healthy men and women was 0.46±0.17 g/min (Mean±Standard Deviation [SD]) and varied ~5-fold between individuals (range: 0.18-1.01 g/min) (Venables et al. 2005). Part of the inter-subject variability can be explained by aerobic capacity/training status (Holloszy et al. 1997), nutritional status (Bergman & Brooks, 1999; Coyle et al. 2001), physical activity level (Venables et al. 2005), fat-free mass (FFM) (Venables et al. 2005) and sex-related differences (Tarnopolsky et al. 2001). Whilst appreciable efforts have been made to understand the determinants of fat oxidation during exercise (Goedecke et al. 2000; Venables et al. 2005), the significance of the observed variability in the capacity to oxidize fat during exercise for metabolic health requires clarification (Kelley & Simoneau, 1994; Jeukendrup & Wallis, 2005; Isacco et al. 2013).
Reduced fat oxidation has previously been related to long-term changes in body weight or composition. For example, increased daily (i.e., 24-h) respiratory quotient (RQ; indicative of low relative fat oxidation) has been associated with an increased risk of body mass gain (Zurlo et al. 1990) and regain of body fat mass after diet-induced weight loss (Ellis et al. 2010). As a high fat oxidation whilst physically active could make important contributions to daily fat oxidation it is not unreasonable to consider a high capacity to oxidise fat whilst physically active as advantageous for the maintenance of body mass or body composition. Mechanistically, one viewpoint is that a reduced fat oxidation (and thus elevated carbohydrate oxidation) during exercise or on a daily basis may impact upon long-term body weight or composition through increased *ad libitum* food intake in free-living conditions (Almeras et al. 1994, Pannacciulli et al. 2007). However, this is clearly an oversimplification of what is a complex area; with more recent work demonstrating that there are multiple signals involved in appetite regulation (for review see Blundell et al. 2015). Impairments in fat oxidation at the level of skeletal muscle have also been associated with reduced metabolic flexibility and insulin resistance (Kelley & Simoneau, 1994) and enhancements in fat oxidation with exercise training are related to improvements in insulin sensitivity (Goodpaster et al. 2003; Venables & Jeukendrup, 2008). However, the evidence to date linking the capacity for fat oxidation during exercise to metabolic health is limited and inconsistent. For example, a low MFO was associated with a higher clustering of metabolic syndrome risk factors in overweight men (Rosenkilde et al. 2010), yet no association between variability in Respiratory Exchange Ratio (RER) during exercise and insulin sensitivity was observed in endurance-trained men (Goedecke et al. 2001). Overall, our understanding of the
links between the capacity to oxidize fat during exercise and metabolic health remain to be comprehensively studied.

As exercise is a primary means to acutely increase fat oxidation and reduced fat oxidation has been linked to disorders such as obesity and insulin resistance, further understanding the significance of the observed inter-subject variability in the capacity to oxidize fat during exercise could provide insights into the role of physical activity in the optimization of metabolic health. Thus, the purpose of the present study was to test the hypothesis that the capacity for fat oxidation during exercise is related to 24-h fat oxidation and insulin sensitivity as markers for long-term metabolic health.
3.3 Methods

3.3.1 General design and study participants

A total of 57 young, healthy, recreationally active Caucasian men participated in the study. Their characteristics are displayed in Table 3.1. All subjects provided informed written consent and a local Research Ethics Committee in the United Kingdom approved the study. Each participant visited the laboratory on two occasions. During the first (Familiarisation) and second (Exercise Test) visits, which were separated by ~4-7 days, participants performed a graded treadmill Exercise Test to voluntary exhaustion with indirect calorimetry used to assess the MFO and $\dot{V}O_2^{\text{max}}$. Self-reported diet and physical activity was recorded via diaries in the four days immediately preceding the second visit. A resting, overnight-fasted blood sample was also collected for determination of insulin sensitivity on the second visit in 53 participants. 16 participants selected to represent a broad MFO range determined during the Exercise Test also took part in a further trial. This trial consisted of a 36-h stay in a whole-room respiration chamber to measure 24-h energy expenditure and substrate utilization by indirect calorimetry (24-h Assessment). Chamber conditions were designed to simulate participant’s habitual energy intake, percent macronutrient intake and daily energy expenditure as estimated from the food and physical activity diaries.

---

2 This study did not address the reproducibility of MFO or daily fat oxidation. However, previous reports demonstrate a coefficient of variation (CV) for Fat$_{\text{max}}$ (the exercise intensity which corresponds to MFO) of 9.6% in a group of 10 well-trained individuals (Achten & Jeukendrup, 2003) and of 0.8% for 24-h RQ and 6.7% for fat oxidation in a group of 22 healthy obese adults (Toubro, Christensen, & Astrup, 1995).
Table 3.1 Participant Characteristics

<table>
<thead>
<tr>
<th></th>
<th>24-h Assessment (n=16)</th>
<th>Insulin Sensitivity (n=53)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>26±8 (18-42)</td>
<td>24±7 (18-44)</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.81±0.05 (1.72-1.89)</td>
<td>1.80±0.07 (1.64-1.96)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>81.0±11.2 (65.5-101.7)</td>
<td>78.7±11.3 (54.7-113.0)</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>24.5±2.5 (21.3-29.7)</td>
<td>24.2±2.6 (18.9-29.9)</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>15±5 (9-30)</td>
<td>ND</td>
</tr>
<tr>
<td>$\dot{V}O_2$max (ml/kg/min)</td>
<td>52±6 (39-60)</td>
<td>52±6 (39-62)</td>
</tr>
</tbody>
</table>

Data are mean±SD (range). ND – not determined.

3.3.2 Experimental procedures

(i) Familiarisation: This visit was used for consenting, screening and to familiarize participants with the Exercise Test. Height (Stadiometer, Seca, UK) and weight (OHaus, Champ II scales, USA) were measured to calculate BMI (weight[kg]/height[m]$^2$). A treadmill-based Exercise Test, identical to that described in more detail below, was then completed on a motorized treadmill (PPS 70sport-l, Woodway, Weil am Rhein, Germany) in order to familiarize participants with the exercise testing procedures.
Participants were then given two sets of digital weighing scales (Electronic Kitchen Scale, SF 400, Zhejiang Province, China and Swees Digital Pocket Weighing Scales, Kent, UK), blank diaries and detailed instructions to enable the completion of diet and physical activity diaries. Participants were instructed to follow their normal diet and activity between the first and second laboratory visit with the exception of the day immediately preceding the second visit (see below).

For the 4 days preceding the second laboratory visit, participants were asked to weigh and record all consumed food and drink items and document activity patterns accurately and in as much detail as possible. The activity diary used was based on Bouchard et al. (Bouchard et al. 1993) and required participants to record their level of activity every 15 minutes, using a code from a 12-point scale provided for the entire 24-h of each of the four days. Participants were encouraged to make notes on any sport or exercise performed during the 4 days.

(ii) Exercise test: Participants reported to the laboratory between 07:00-08:00 h following an overnight fast from 22:00 h the evening before and having abstained from strenuous physical activity and alcohol consumption in the preceding 24-h period. Participants were asked not to perform any physical activity on the morning of testing, such as brisk walking or cycling to the laboratory, and to consume 500 ml water upon waking to promote hydration. Diet logs and physical activity diaries were checked and any potential cases of under-reporting and/or inaccuracies were clarified, after which body
weight was recorded as described above, a resting blood sample was obtained and the Exercise Test performed.

The Exercise Test was based on the protocol described previously by Achten and colleagues (Achten et al. 2003) with the starting speed and treadmill inclination set to 3.5 km/h and 1%, respectively. The treadmill speed was increased by 1 km/h every 3 minutes until the participants Respiratory Exchange Ratio (RER; as is conventionally used in studies of exercise metabolism to indicate relative fuel utilization) reached 1.00, after which the treadmill gradient was increased by 1% per minute until volitional exhaustion. Heart rate was measured continuously throughout exercise using a heart rate monitor (Polar FT-2, Finland) and recorded during the final 30 seconds of each exercise stage. Breath-by-breath measurements of oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were obtained during exercise using an online automated gas analysis system (Oxycon Pro, Jaeger, Wuerzberg, Germany). The gas analysers were calibrated with a 5.07% CO$_2$, 14.79% O$_2$, 80.14% N$_2$ gas mixture (BOC Gases, Surrey, UK), and the volume transducer was calibrated with a 3-liter calibration syringe (Jaeger, Wuerzberg, Germany). Environmental conditions were documented during the conduct of exercise testing and remained constant with humidity at 51±6%; and temperature at 22±1 °C.

(iii) Respiration chamber stay (24-h assessment): Participants (n=16 out of the 57 initially tested) were provided scanned copies of their food and activity diaries recorded in the 4-d preceding the second laboratory visit and asked to repeat these during the
four consecutive days leading up to the 24-h Assessment visit. Participants arrived at the laboratory between 18:00-19:00 h on the evening before the experimental period having abstained from alcohol and strenuous physical exercise that day. Upon arrival, height and weight were reconfirmed and body composition was assessed using air displacement plethysmography (BODPOD; Cosmed, Rome, Italy). Thereafter, participants entered and stayed in a dual-respiration whole room metabolic chamber system as described previously (Schoffelen et al. 1997) for a period of 36-h. Both chambers were occupied simultaneously to prevent feelings of isolation. Three airtight hatches were used for blood collection, the exchange of food and any urine produced. The room was ventilated with fresh air at a rate of 7 m$^3$/h during the daytime period, and 5 m$^3$/h in the evening. Environmental conditions during the 24-h chamber assessment were kept stable with humidity at 57±5% and temperature at 24±1 °C and 22±1 °C for day and night, respectively. The concentration of O$_2$ and CO$_2$ was measured using two separate sets of ABB AO2000 analysers, a paramagnetic O$_2$ analyser (Magnos 206, ABB, Germany) and CO$_2$ was measured by using infrared analysers (Uras 26, ABB, Germany). Flow was measured with dry bellows meters (G16, Meterfabriek Schlumberger, Germany). In-going air was analysed every 15 min and outgoing air once every 1 min. During the experiment the gas measurement system was calibrated every five minutes using nitrogen (N5.5) and a calibration gas (CO$_2$ 8000PPM, 18% O$_2$, balance N$_2$ ±1%). Online analysis of $\dot{V}$O$_2$ and $\dot{V}$CO$_2$ was calculated using a microcomputer (Macintosh, Apple, USA).
The first 12-h in the chamber was used to habituate the participant with the room and the 24-h Assessment commenced the following morning at 07:00 h and finished at 07:00 h the following day. A standard day in the room is outlined in Figure 3.1. This researcher provided meals and snacks throughout the measurement period which participants were asked to consume within 10-15 minutes of provision (Table 3.2; see also Appendix 6 for an example of a typical day’s food intake). Total energy and percent macronutrient intake was matched with the participants’ average daily intake (based on the 4-day weighed food intake analysed previously). Alcohol and caffeine was not provided during the 24-h Assessment. To account for alcohol, the calories consumed from alcohol during the 4-d measurement period were distributed evenly between carbohydrate, fat and protein intake during the 24-h period. The potent effect of caffeine on metabolic rate and fuel utilisation (Acheson et al. 1980) means that the lack of caffeine provision (in line with each participant’s habitual use) is a methodological limitation. Undoubtedly, it would be important for future research to consider the provision of caffeine in their study design, especially if the aim is to simulate participant’s habitual diet.

Participants performed a simple and non-exhaustive step activity at three separate time points during the 24-h Assessment period. Each exercise period lasted between 10-20 minutes and was designed to ensure that 24-h energy expenditure reflected that calculated from the 4-d self-reported activity diary. Outside the designated exercise, eating and sleeping periods, participants were not allowed to sleep or perform any additional physical activity other than light activities such as reading, watching television or working on a computer. Blood was collected routinely (10 ml per sample) via an
indwelling cannula inserted in an antecubital arm vein. Urine samples were collected for the entire 24-h measurement period. Participants were asked to empty their bladder prior to the measurement period (i.e. at 07:00 h) to ensure urine produced during the first night was not included in analyses. No alcohol, tea, coffee or caffeinated beverages were allowed but water and non-caffeinated herbal teas were available *ad libitum*.

**Figure 3.1** A schematic overview of proceedings during the 36-h stay in the respiration chamber. E = exercise, M = meal, S = snack, BP = body composition assessment, **blood draw**, **= urine collection.**
3.3.3 Self-report diet and activity analysis

Food diaries were analysed using dietary analysis software (Dietplan Version 6.7, Forestfield Software, Horsham, UK). Daily energy intake (EI; kcal/d), absolute macronutrient intake (g/d) and percentage contribution of macronutrients to daily energy intake were established for each participant. Daily energy expenditure was estimated using the factorial approach (Manore et al. 2009). Here, each of the 12 codes, which had a corresponding metabolic equivalent (MET) value, was assigned a Physical Activity Level (PAL; Recommended Daily Allowances, 1989) and a daily PAL was determined by multiplying each of the 12 codes by the total amount of time spent at the activity level. Where participants had made notes on a specific sport and exercise activity undertaken during the 4 days, the Compendium of Physical Activities (Ainsworth et al. 2011) was used to calculate the specific PAL value. Daily energy expenditure was then estimated by multiplying the daily PAL value by the age and sex specific resting metabolic rate (RMR; kcal/d) using height, weight and the World Health Organization (WHO) equation (WHO, 1985):

Energy Expenditure (kcal/d; men < 30 years): Daily PAL value * RMR (15.4 * body mass [kg]) - (27.0 * height [m]) + 717

Energy expenditure (kcal/d; men > 30 years): Daily PAL value * RMR (11.3 * body mass [kg]) - (16.0 * height [m]) + 901
3.3.4 Plasma and urine analyses

Whole blood was collected into EDTA-containing Vacutainers (BD, New Jersey, USA). Samples were immediately centrifuged at 3000 revolutions per minute for 15 minutes at 4°C. Aliquots containing plasma were stored at -80°C until analysed. All collected samples were analysed using enzymatic colorimetric assays for glucose (Glucose Oxidase kit, Instrumentation Laboratories, Cheshire, UK), non-esterified fatty acids (NEFA; NEFA kit, Randox, London, UK), glycerol (GLY kit, Randox, London, UK) and lactate (LAC kit, Randox, London, UK) using an ILAB 650 Clinical Chemistry Analyser (Instrumentation Laboratory, Warrington, UK). Insulin was analysed by radioimmunoassay using a commercially available kit (HI-14K Human Insulin, Millipore, Billerica, MA). Collected urine volume was measured using digital weighing scales (Model 323, Salter, Kent, UK) and analysed enzymatically for urea (UREA kit, Roche, Mannheim, Germany) and creatinine (CREA kit, Roche, Mannheim, Germany) using an automated clinical chemistry analyser (Roche Cobas c702 Analyser, Roche, Mannheim, Germany).

3.3.5 Calculations

Insulin sensitivity was calculated using the Quantitative Insulin Sensitivity Check Index (QUICKI; as described in Chapter 2, Section 2.5), which has been validated against and shown to have a strong positive correlation ($R = 0.78$) with insulin sensitivity measured using the gold standard hyperinsulinaemic euglycemic clamp technique (Katz et al. 2000). During the exercise test $\dot{V}O_2$ and $\dot{V}CO_2$ were averaged over the last minute of
each sub-maximal exercise stage with fat and carbohydrate oxidation were calculated according to the equations of Frayn (Frayn et al. 1983), with the assumption that the urinary nitrogen excretion rate was negligible. $\dot{V}O_{2\text{max}}$ was calculated as the highest rolling 60 second average $\dot{V}O_2$ for participants displaying two of the three following criteria: an RER >1.1; heart rate within 10 beats of predicted maximum (calculated as 220-age; Fox et al. 1971) or; an increase of < 2 ml/kg/min in $\dot{V}O_2$ with a further increase in workload. $\dot{V}O_2$ peak (highest rolling 30 seconds average) was calculated for participants who failed to demonstrate at least two of the above criteria. For each participant, two previously characterized (Achten et al. 2002) features were established: 1) MFO, obtained by visual inspection as originally described (Achten et al. 2002); 2) Fat$_{\text{max}}$, defined as the exercise intensity ($\%\dot{V}O_{2\text{max}}$) that elicits MFO.

For the 24-h Assessment $\dot{V}O_2$ and $\dot{V}CO_2$ were initially averaged in 30 minute intervals and extrapolated to 24-h values and used to calculate 24-h Respiratory Quotient (RQ = $\dot{V}CO_2 / \dot{V}O_2$; as the expression of relative fuel utilization conventionally used in metabolic chamber studies), 24-h EE (Weir, 1949), 24-h carbohydrate and 24-h fat oxidation (Frayn, 1983) with urinary nitrogen correction where appropriate. Urinary nitrogen content was estimated by correcting urinary urea and creatinine by 1.11 to account for non-measured nitrogen sources (e.g., ammonia, urate) (Bingham et al. 1988). 24-h protein oxidation (g/d) was calculated by multiplying total N (g/d) by 6.25. 24-h fat balance was calculated as dietary 24-h fat intake (g/day) – 24-h fat oxidation (g/day). The food quotient (FQ) for the chamber diet was calculated as previously described (Tourbo et al. 1998). RQ and substrate utilization was also estimated for non-
sleeping (encompassing 07:00-23:00 h) and sleeping (Sleep; encompassing 01:45-04:45 h, [Schoffelen & Westerterp, 2008]) periods. As with the 24-h determinations, $\dot{V}O_2$ and $\dot{V}CO_2$ was averaged in 30-minute intervals and extrapolated to the corresponding period duration in order to calculate RQ and substrate utilization (Frayn, 1983). We corrected substrate oxidation rates using 24-h urinary nitrogen excretion values rather than using excretion values specific to the non-sleeping and sleeping periods and we acknowledge this as a methodological limitation.

### 3.3.6 Statistical analyses

Data were analysed using SPSS (SPSS Version 21, Chicago, IL). All data were normally distributed according to the Shapiro-Wilk test of normality and are presented as Mean±SD (and range where appropriate). Statistical significance was accepted at $P < 0.05$. Paired samples $t$-tests were used to compare differences in habitual and chamber 24-h energy intake (kcal/day), 24-h energy expenditure (kcal/d), 24-h energy balance (kcal/d), and plasma hormone and substrate responses over time. Bivariate correlations were used to explore relationships between MFO and: 1) 24-h fat oxidation; 2) other potential correlates of 24-h fat oxidation (e.g., $\dot{V}O_{2\text{max}}$, blood metabolite/hormone responses assessed as 24-h area under the curve [trapezoid method]); and 3) insulin sensitivity. Partial correlations were used where applicable to adjust for potential relevant confounders.
3.4 Results

Results for the 24-h assessment are presented for 16 participants with the exception of the blood data, which due to difficulty in blood collection, is presented for 15 participants. Associations between MFO and insulin sensitivity are based on 53 participants.

3.4.1 Diet and energy expenditure

As can be seen from Table 3.2 and as planned there were no significant differences between the participants’ energy and macronutrient intake, 24-h energy expenditure and 24-h energy balance estimated from 4-day food and physical activity diaries and that measured during the 24-h Assessment in the respiration chamber.
Table 3.2 Comparison between 4-d self-report and chamber food intake, energy expenditure and energy balance.

<table>
<thead>
<tr>
<th></th>
<th>4-d</th>
<th>24-h Assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy intake (kcal/d)</td>
<td>2779±652</td>
<td>2773±657</td>
</tr>
<tr>
<td>Energy Expenditure (kcal/d)</td>
<td>2705±219</td>
<td>2621±203</td>
</tr>
<tr>
<td>Energy balance (kcal/d)</td>
<td>74±638</td>
<td>153±601</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>42±10</td>
<td>43±11</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>34±7</td>
<td>36±6</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>20±7</td>
<td>20±7</td>
</tr>
<tr>
<td>Alcohol (%)</td>
<td>4±5</td>
<td>n/a</td>
</tr>
<tr>
<td>FQ</td>
<td>0.84±0.03</td>
<td>0.84±0.03</td>
</tr>
</tbody>
</table>

3.4.2 MFO and 24-h substrate oxidation

The MFO for the 16 participants who undertook the 24-h Assessment was 0.59±0.21 g/min (range 0.30-1.02 g/min). In these participants Fat\(_{\text{max}}\) was 56±17 % \(\dot{V}O_{2\text{max}}\) and ranged from 30-78 % \(\dot{V}O_{2\text{max}}\). During the 24-h Assessment, 24-h RQ was 0.86±0.03 (range 0.81-0.90). 24-h fat, carbohydrate and protein oxidation was 81±30 g/d (range 27-135 g/d), 346±75 g/d (range 153-470 g/d) and 120±54 g/d (range 25-204 g/d), respectively. The relative contributions of fat, carbohydrate and protein oxidation to total energy expenditure throughout the 24-h Assessment are shown in Figure 3.2 and this
corresponded to 28±10, 53±10 and 19±9 %, respectively. 24-h fat balance was 31±46 g/d. Sleeping RQ (01:45-04:45 h) was significantly lower than non-sleeping RQ (07:00-23:00 h) (0.82±0.02 and 0.88±0.03, respectively, \( P < 0.001 \)). As can be seen in Figure 2, the relative contributions of each macronutrient to 24-h energy expenditure differed between sleeping and non-sleeping (fat 36±10 % and 25±10 %; carbohydrate 28±6 % and 59±12 %; protein 29±11 % and 16±8 %; all \( P < 0.001 \)).

**Figure 3.2** Percentage contribution of carbohydrate, fat and protein to energy expenditure for \( n = 16 \). E = exercise, M = meal, S = snack.
MFO (g/min) was significantly and positively correlated with 24-h fat oxidation (24-h FO, g/d), 24-h % energy from fat oxidation (%EnFO) and negatively correlated with 24-h fat balance (Figure 3.3) but was not significantly associated with 24-h RQ ($R=-0.29$, $P=0.142$). $\dot{V}O_{2max}$ (L/min) was positively correlated with MFO (g/min) and 24-h FO (g/d) ($R=0.72$, $P=0.001$ and $R=0.51$, $P=0.022$, respectively). MFO (g/min) remained positively correlated with 24-h FO (g/d) when controlled for $\dot{V}O_{2max}$ as a potential confounder (L/min) ($R=0.46$, $P=0.041$). The findings were similar when MFO expressed relative to FFM (mg/kg FFM/min) was correlated with 24-h FO (g/kg FFM/d, $R=0.70$, $P=0.001$) and 24-h %EnFO ($R=0.60$, $P=0.007$) and 24-h RQ ($R=0.31$, $P=0.12$). $\dot{V}O_{2max}$ (L/min) was positively correlated with MFO (mg/kg FFM/min; $R=0.58$, $P=0.009$) but not 24-h FO (g/kg FFM/d, $R=0.35$, $P=0.093$); therefore partial correlations between MFO and 24-h FO (with both expressed relative to FFM) controlled for $\dot{V}O_{2max}$ were deemed unnecessary.
Figure 3.3 Correlations between MFO (g/min) and (A) 24-h FO (g/d), (B) 24-h FO (%EnFO) and (C) 24-h fat balance (g/d).
When the data was separated into Sleep (01:45-04:45 h) and non-sleeping (07:00-23:00 h) periods, MFO (g/min) was significantly negatively correlated with sleeping RQ ($R=-0.51$, $P=0.022$) but was not significantly correlated with non-sleeping RQ ($R=-0.24$, $P=0.187$). MFO (g/min) was positively correlated with fat oxidation during both the Sleep and non-sleeping periods (g: $R=0.71$, $P=0.001$ and $R=0.58$, $P=0.01$, respectively; %EnFO: $R=0.59$, $P=0.008$ and $R=0.51$, $P=0.021$, respectively). Similar findings prevailed when MFO expressed relative to FFM (g/kg FFM/min) was correlated with sleeping and non-sleeping FO (g/kg FFM: $R=0.73$, $P=0.001$ and $R=0.63$, $P=0.004$, respectively).

24-h carbohydrate intake (g/d) and 24-h energy balance (kcal/d) were significantly negatively correlated with 24-h FO (g/d, $R=-0.59$, $P=0.008$ and $R=-0.53$, $P=0.017$, respectively), 24-h %EnFO ($R=-0.62$, $P=0.003$ and $R=-0.56$, $P=0.013$, respectively) and 24-h FO relative to FFM (g/kg FFM/d, $R=-0.62$, $P=0.005$ and $R=-0.58$, $P=0.009$, respectively). 24-h glycerol concentration AUC (38.6±10.6 mmol/L/d) was positively correlated with 24-h FO (g/kg FFM/d, $R=0.44$, $P=0.048$) and showed a tendency for a positive correlation with 24-h FO when expressed otherwise (g/d, $R=0.40$, $P=0.071$ or %EnFO, $R=0.40$, $P=0.069$). 24-h AUC for the other measured plasma metabolite and hormone responses did not correlate with any expression 24-h of fat oxidation.
3.4.3 Plasma metabolites and hormones

Plasma metabolite and hormone responses to the different activities performed in the respiratory chamber are shown in Table 3. Plasma glycerol concentration increased significantly pre- to post-exercise in the fasted state whereas plasma glucose, insulin and NEFA showed no change. In response to feeding, plasma insulin and glucose concentrations increased, whereas plasma glycerol and NEFA concentrations remained similar. Overnight, plasma insulin concentration decreased and plasma glycerol and NEFA concentrations both increased, whereas plasma glucose showed no change.

Table 3.3 Plasma hormone and metabolite responses to different activities performed within the respiratory chamber.

<table>
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<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Insulin (µIU/mL)</td>
<td>15±6</td>
<td>13±6</td>
<td>15±7</td>
<td>59±47</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.2±0.5</td>
<td>5.4±0.7</td>
<td>5.1±0.9</td>
<td>6.1±1.9</td>
</tr>
<tr>
<td>Glycerol (µmol/L)</td>
<td>30±16</td>
<td>51±24</td>
<td>16±4</td>
<td>18±7</td>
</tr>
<tr>
<td>NEFA (mmol/L)</td>
<td>0.32±0.19</td>
<td>0.38±0.17</td>
<td>0.26±0.17</td>
<td>0.34±0.22</td>
</tr>
</tbody>
</table>

* and ** denote significance change in concentration pre- to post-sample at the \( P<0.05 \) and \( P<0.01 \) level, respectively.
3.4.4 Insulin sensitivity

MFO assessed in the larger cohort of participants was 0.60±0.18 g/min (range 0.30-1.02 g/min) and this corresponded to an exercise intensity of 58±17 % \(\dot{V}O_{2\text{max}}\) (range 21-83 % \(\dot{V}O_{2\text{max}}\)). Resting plasma glucose and insulin concentration was 5.0±0.4 mmol/L and 11±4 µIU/mL, respectively. Insulin sensitivity (IS) as estimated using the QUICKI was 0.34±0.02 (range 0.31-0.40; arbitrary units, AU). MFO (g/min) was significantly positively correlated with IS (\(R=0.33\), \(P=0.007\)). \(\dot{V}O_{2\text{max}}\) (L/min) was significantly positively correlated with MFO (g/min) but not with IS (\(R=0.72\), \(P=0.001\) and \(R=0.11\), \(P=0.223\), respectively), therefore partial correlations between MFO and IS controlled for \(\dot{V}O_{2\text{max}}\) were deemed unnecessary.
3.5 Discussion

The purpose of the present study was to investigate the hypothesis that the capacity for fat oxidation during exercise is related to daily fat oxidation and insulin sensitivity as markers for long-term metabolic health. Accordingly, for the first time we report significant and positive relationships between the maximal capacity for fat oxidation during exercise (MFO) and 24-h fat oxidation, and between MFO and insulin sensitivity in healthy young men.

In seeking to explain the observed association between MFO and 24-h FO we explored the influence of a variety of factors on these two variables. We found that $\dot{V}O_{2\text{max}}$ was a strong correlate of 24-h FO and as $\dot{V}O_{2\text{max}}$ was also correlated with MFO it suggests that aerobic capacity could be a driver of the association between MFO and 24-h FO. The findings of the present study support those of Venables and colleagues (Venables et al. 2005) who showed $\dot{V}O_{2\text{max}}$ to be a significant predictor of MFO assessed in 300 healthy men and women and are consistent with the well-established effect of endurance exercise training to increase $\dot{V}O_{2\text{max}}$ and the capacity to oxidize fat during exercise (Kiens et al. 1993; Martin et al. 1993). A parallel with our finding can be drawn from a prior report showing $\dot{V}O_{2\text{max}}$ to explain the major (76%) proportion of variance in adaptation of 24-h fat balance to increased dietary fat intake (Smith et al. 2000), suggesting aerobic capacity can exert an important influence over 24-h fat oxidation under these conditions. However, and in contrast to what might be expected, cross-sectional studies do not report higher 24-h fat oxidation in endurance trained individuals as compared to their sedentary counterparts (Lanzi et al. 2014), suggesting a high
aerobic capacity may not be a necessary pre-requisite for high daily fat oxidation. In support of this, the present study showed that MFO was still significantly and positively related to 24-h fat oxidation when controlled for $\dot{V}O_{2\text{max}}$, which suggests an independent relationship. Overall, while we reveal a correlation between MFO and 24-h FO the present study highlights the need for longitudinal training studies to delineate the relative role of changes in maximal aerobic capacity and the capacity to oxidize fat during exercise on daily patterns of substrate oxidation.

It could be argued that variables other than $\dot{V}O_{2\text{max}}$ may also be contributing to our observations relating MFO to 24-h FO. Previous research shows that carbohydrate intake and the state of energy balance (Jequier & Schutz, 1983) can influence fuel utilization. In the present study carbohydrate intake and energy balance were significantly and negatively associated with 24-h FO, although they were not associated with MFO (data not shown). The lack of association of carbohydrate intake with MFO is consistent with the findings of Goedecke et al. (Goedecke et al. 2000) who found no association between dietary carbohydrate intake and inter-subject variability in exercising RER over a range of pre-determined workloads. Moreover, Rosenkilde (Rosenkilde et al. 2010) found no differences in habitual carbohydrate intake or energy balance between two groups of moderately overweight men who differed markedly in their MFO during exercise. Thus, while it is well established from intervention studies that nutritional status can exert large effects on the balance of fuels oxidized during exercise (Bergman & Brooks, 1999; Coyle et al. 2001), we do not believe this to be underlying the associations between MFO and 24-h FO in the present study.
Regular blood draws were taken throughout the 24-h Assessment in an attempt to explore relationships between plasma metabolic responses and 24-h FO. While the majority of these were uninformative, including NEFA concentration AUC (correlation with 24-h FO: g/d, $R=0.22$, $P=0.220$; g/kg FFM/d, $R=0.25$, $P=0.185$), glycerol concentration AUC was significantly and positively associated with 24-h FO (correlation with 24-h FO: g/kg FFM/d, $R=0.44$, $P=0.048$). Our glycerol data reflects static concentration measurements and we cannot infer whether these glycerol concentrations are driven by alterations in lipolytic rate or glycerol clearance. Furthermore, as the relationship between MFO and 24-h FO did not alter substantially ($R=0.61$, $P=0.011$) when glycerol AUC was included as a partial correlate the observation should be treated with caution. Nonetheless, previous work suggests a link between lipolytic rate and fat oxidation. For example, Horowitz et al. (Horowitz et al. 1997) found that a reduced lipolytic rate was associated with a suppression of fat oxidation, whereas Coggan et al. (Coggan et al. 2000) observed a higher lipolytic rate in trained compared with untrained subjects, and this was associated with a higher fat oxidation during 30 minutes of exercise at 75% to 80% maximal oxygen consumption ($\dot{V}O_{2\text{max}}$). More recently, Isacco et al. (Isacco et al. 2013) found that women with a preferential body fat distribution (a lower abdominal to lower body fat mass ratio) oxidized more fat during a 45-minute cycling exercise compared with women who had a less preferential body fat distribution (higher abdominal to lower body fat mass ratio) and this was associated with increased glycerol concentrations. The use of isotopic tracers of lipid metabolism would be needed to fully
investigate the potential involvement of lipolytic rate in the observed association between MFO and 24-h FO.

We chose to look at relationships between MFO with 24-h FO, as the latter may be more reflective of long-term susceptibility to obesity. For example, previous work links high daily RQ, which is indicative of a low relative fat oxidation, with an increased risk of body mass gain (Zurlo et al. 1990) and regain of body fat mass after diet-induced weight loss (Ellis et al. 2010). While we did not observe a significant association between MFO and 24-h RQ the correlation was directionally consistent ($R=-0.29$) with what would be predicted. Nonetheless, our data did reveal a significant and negative correlation between MFO and sleeping RQ, which could suggest that the relationship becomes stronger under conditions where the suppressive effects of insulin on lipid metabolism may be less apparent. Indeed, previous studies have demonstrated that as compared to non-exercise control conditions fat oxidation in response to acute aerobic exercise was higher during the sleeping but not the non-sleeping period (Jequier & Schutz, 1983; Hawkins et al. 2012). Collectively, this suggests the sleeping period may be particularly sensitive to alterations in lipid metabolism in response to physiological perturbations. While we did not see a significant correlation between MFO and 24-h RQ, we reiterate that we report positive associations between MFO and 24-h absolute fat oxidation, and between MFO and 24-h fat oxidation as a proportion of daily energy expenditure. These relationships, which incorporate energy expenditure, are likely more relevant than RQ as they will contribute ultimately as determinants of absolute, and relative substrate balances, respectively. The negative association between MFO and fat balance
observed in the present study reinforces this concept. In additional analysis, we explored relationships between MFO and fat oxidation measured during the various activities performed in the respiratory chamber (data not shown). This analysis indicated that the relationship between MFO and 24-h FO appears to reflect an overall elevated oxidation of fat rather than being restricted to a particular time period or to certain activities. Thus, MFO could reflect an overall capacity for daily whole body fat oxidation and our interpretation of the present data is that MFO could be a marker for long-term regulation of body mass or composition, although we accept further work is necessary to test this concept.

We also explored relationships between MFO and insulin sensitivity using a surrogate marker of insulin sensitivity (QUICKI). In contrast with Goedecke et al. (Goedecke et al. 2001) who observed no association with exercising RER we found a small to moderate correlation between MFO and insulin sensitivity. The exact reason for this discrepancy is unclear, but could be explained by methodological differences. For instance, in the present study rates of fat oxidation were determined over a wide range of exercise intensities providing the resolution to identify the maximal capacity for fat oxidation during exercise whereas the study by Goedecke and colleagues assessed RER during three pre-determined exercise workloads. Also, exercise RER considers only relative fuel utilization whereas MFO, which incorporates metabolic rate, could better reflect metabolic capacity and thus be more likely to correlate with insulin sensitivity. However, this suggestion is not consistent with the observations of Chu and colleagues who recently showed MFO to be positively correlated with insulin resistance (estimated from
the homeostatic model assessment of insulin resistance) and unrelated to whole-body insulin sensitivity index (determined from oral glucose tolerance testing) (Chu et al. 2013). However, this was observed in a group of normal and overweight girls, which is quite different to the study population tested herein.

The data from the present study, which is suggestive of a link between MFO and insulin sensitivity in young, healthy men is consistent with observational studies showing that impairments in fat metabolism at the level of skeletal muscle are associated with reduced metabolic flexibility and insulin resistance (Kelley & Simoneau, 1994) and longitudinal studies showing enhancement in fat oxidation with exercise training is associated with improvements in insulin sensitivity (Goodpaster et al. 2003; Venables & Jeukendrup, 2008). Furthermore, greater exercise training-induced improvements in insulin sensitivity have been observed using exercise training strategies that enhance fat oxidation during acute exercise (e.g., targeting training intensity $\text{Fat}_{\max}$ or exercising in the overnight-fasted state (Venables & Jeukendrup, 2008). From a mechanistic standpoint, this association between MFO and insulin sensitivity seems plausible. It has been suggested that incomplete metabolism of fatty acids in the $\beta$-oxidation pathway induces an accumulation of acyl carnitines in the mitochondria and this is associated with insulin resistance (Koves et al. 2005). Furthermore, the accumulation of lipid metabolites such as ceramide, diacylglycerol and long-chain fatty acyl-CoA has also been linked with insulin resistance in skeletal muscle (Shaw et al. 2013). We acknowledge that we have not studied insulin sensitivity using direct methods (e.g., hyperinsulinaemic euglycemic clamp technique) but our observations could imply that a
higher capacity for fat oxidation during physical activity could alleviate lipotoxic stress in skeletal muscle and confer improvements in insulin sensitivity.

Finally, we believe there are two further aspects of our study that warrant consideration in discussion. Firstly, we purposely chose to feed subjects their habitual diet and to replicate activity levels in the metabolic chamber trial in order to reflect variation between individuals under conditions that mimicked their daily lives and we were successful in achieving this. It would be important in future work to confirm these observations under conditions of standardised dietary and physical activity levels. Secondly, our findings linking MFO to 24-h FO advance a potential role for MFO as a determinant of long-term regulation of body mass or composition. However, it is notable that obese individuals do not always have a lower MFO compared with their lean counterparts (Ara et al. 2011; Lanzi et al. 2014), even when matched for cardiorespiratory fitness (Croci et al. 2014). Nonetheless, Rosenkilde and colleagues found that even within a relatively homogenous group of overweight men those with a lower MFO exhibited a higher clustering of metabolic syndrome risk factors than those with a higher MFO (Rosenkilde et al. 2014). Therefore, we believe that the inter-subject variability in MFO should be considered as a potential marker of risk for metabolic disorders within defined population groups (e.g., normal weight, overweight).

In conclusion, we observed a significant and positive association between maximal fat oxidation during exercise and 24-h fat oxidation, and maximal fat oxidation during exercise and insulin sensitivity in healthy young men. Future work should seek to
establish if this is a causal relationship and address in a variety of subject populations the long-term implications of the apparent inter-subject variation in maximal fat oxidation during exercise and risk for disorders associated with disturbances in fat metabolism, such as obesity, insulin resistance and type II diabetes.
3.6 References


• **Kozey S, Lyden K, Staudenmayer J, and Freedson P.** Errors in MET estimates of physical activities using 3.5 ml·kg⁻¹·min⁻¹ as the baseline oxygen consumption. *J of Phys Act Health* 7: 508-516, 2010.


• Melanson EL, Gozansky WS, Barry DW, MacLean PS, Grunwald GK, and Hill JO. When energy balance is maintained, exercise does not induce negative fat balance in lean sedentary, obese sedentary, or lean endurance-trained individuals. *J Appl Physiol* 107: 1847-1856, 2009.


• **Rising R.** Twenty-four hour metabolic rate measurements utilized as a reference to evaluate several prediction equations for calculating energy requirements in healthy infants. *Nutr* 10: 10-14, 2011.


CHAPTER 4

LIPOLYTIC MARKERS, INSULIN AND RESTING FAT OXIDATION ARE ASSOCIATED WITH MAXIMAL FAT OXIDATION

The work contained within this chapter is published:


Please note that the data presented in Chapters 3 and 4 of this thesis comes from data collected from one study.
4.1 Abstract

The maximal capacity to oxidise fat during exercise (MFO) is associated with 24-h fat balance and insulin sensitivity. Understanding the factors that influence MFO could have implications for metabolic health. We investigated relationships between selected plasma metabolites and hormones, and resting fat oxidation with MFO. Resting fat oxidation and MFO was measured in 57 young, healthy men and venous blood was collected at rest and during incremental exercise. Plasma glycerol (R=0.39, P=0.033), non-esterified fatty acids (NEFA: R=0.27, P=0.030) and insulin (R=-0.36, P=0.007) measured at MFO correlated with MFO; only glycerol remained correlated when controlled for resting concentrations (R=0.36, P=0.008). The change in glycerol from rest to MFO correlated with exercise-induced fat oxidation (R=0.32, P=0.012). \( \dot{V}O_2 \text{max} \) was correlated with both resting fat oxidation (R=0.44, P=0.001) and MFO (R=0.52, P<0.001). Resting fat oxidation also correlated with MFO (R=0.55, P<0.001); this relationship remained when controlled for \( \dot{V}O_2 \text{max} \) (R=0.41, P=0.001). This study reports new relationships between plasma lipolytic markers, insulin and resting fat oxidation with MFO and shows the plasma glycerol response to uniquely reflect exercise-induced fat oxidation. \( \dot{V}O_2 \text{max} \) is important for MFO but the relationship can be dissociated, which could have implications for physical activity interventions aimed at increasing fat oxidation to optimise metabolic health.
4.2 Introduction

Impairments in fat oxidation are associated with the development of cardio-metabolic risk factors. For example, an elevated daily (i.e. 24-h) respiratory quotient (RQ; indicative of a low relative fat oxidation) has been shown to be predictive of body mass gain (Zurlo et al. 1990) and regain of body fat mass after diet-induced weight loss (Ellis et al. 2010). Furthermore, a reduced fat oxidation under resting conditions at the level of skeletal muscle is associated with impaired insulin sensitivity and metabolic flexibility (Kelly & Simoneau, 1994). We have recently demonstrated that the maximal attainable rate of fat oxidation during exercise (maximal fat oxidation, MFO) is associated with 24-h fat oxidation, 24-h fat balance and insulin sensitivity as markers of long-term metabolic health (Robinson et al. 2015). A greater understanding of the factors that influence MFO could therefore provide insights into how to increase fat oxidation and optimise metabolic health.

The circulating metabolic and hormonal milieu has been shown to influence fat oxidation during fixed intensity exercise. For example, an increase in plasma glucose or insulin typically suppresses fat oxidation during exercise, whereas elevations in non-esterified fatty acid (NEFA) concentrations increase it (Costill et al. 1977; Vukovich et al. 1993). However, the independent and relative influence of these plasma markers on MFO as a marker of the maximal capacity for fat oxidation has not been comprehensively studied. Rosenkilde and colleagues reported a significant and positive relationship between resting NEFA concentration and MFO, although as NEFA concentration was not measured during exercise the significance of this observation is unclear (Rosenkilde et
al. 2010). Interestingly, Lanzi and colleagues demonstrated markedly higher circulating levels of NEFA, glycerol and insulin across a range of exercise intensities in obese as compared to lean individuals yet reported no group differences in MFO (Lanzi et al. 2014). This indicates that the plasma lipolytic and insulin response to exercise may not be important in modulating MFO during exercise although the confounding presence of obesity could alter expected relationships between these plasma markers and MFO.

Previously, in a large cross-sectional study, indicators of physical fitness ($\dot{V}O_{2\text{max}}$, physical activity level), body composition (fat free mass, fat mass) and sex-related metabolic differences have been identified as significant and independent determinants of MFO during exercise (Venables et al. 2005). Whilst informative, the study did not consider the influence of resting substrate oxidation on MFO. Studies of metabolic flexibility have shown that dietary nutrient intake and energy balance can affect baseline substrate oxidation and subsequent responses to metabolic challenges (Galgani et al. 2008) and thus it is plausible that baseline fat oxidation could influence the substrate oxidation response to a subsequent bout of exercise. Indeed, fasting RER has been shown to predict RER during exercise in trained athletes and MFO in overweight men (Goedecke et al. 2000; Rosenkilde et al. 2010). However, fasting RER does not provide information on rates of substrate oxidation and as such the influence of resting rates of fat oxidation on MFO is not known. This is important to describe in order to inform the necessity to consider resting fat oxidation rates in future studies using MFO as a marker of fat oxidation capacity during exercise.
In summary, the evidence to date linking circulating levels of plasma hormones and metabolites as potential correlates of MFO is limited and inconsistent and requires further clarification. Further, the links between the actual rate of resting fat oxidation and MFO has not yet been established. Therefore, the purpose of the present study was to investigate the influence of selected plasma metabolites and hormones and resting fat oxidation on maximal fat oxidation during exercise.
4.3 Methods

4.3.1 Participants
57 young, healthy, recreationally active Caucasian men were recruited for the study (age 24±7 y; height 1.80±0.07 m; weight 78.7±11.3 kg; \(\dot{V}O_{2\text{max}}\) 52±6 ml/kg/min; Body Mass Index [BMI] 24.2±2.6 kg/m\(^2\)). Each participant provided written informed consent and a local Research Ethics Committee in the United Kingdom approved the study. The data from 53 of the participants used in this study has been presented elsewhere (Robinson et al. 2015), however the principle aims of that study were different to those of the current study, therefore allowing us to present novel interpretations of the data herein.

4.3.2 General design
This cross-sectional study involved two laboratory visits each separated by ~4-7 days (Figure 4.1). The first (Familiarization) and second (Main Experimental Trial) visit had participants perform a laboratory-based graded treadmill exercise test to volitional exhaustion to allow for the determination of MFO and \(\dot{V}O_{2\text{max}}\) by indirect calorimetry. The second visit also involved an assessment of body composition (via dual-energy x-ray absorptiometry [DXA] or skinfolds), resting fat oxidation and blood sample collection at rest and during exercise. Self-reported diet and physical activity were documented using diaries in the 4-d immediately preceding the second visit.
4.3.3 Experimental procedures

(i) Familiarisation: Please refer to Chapter 3, Section 3.3.2 (i).

(ii) Main experimental trial: Participants arrived at the laboratory at 08:00 h following an overnight fast from 22:00 h the evening before and having abstained from alcohol consumption and strenuous physical activity in the preceding 24-hour period. Participants were asked not to perform any physical activity on the morning of testing, such as brisk walking or cycling to the laboratory, and to consume 500 ml water upon waking to promote hydration. The researcher checked diet and physical activity diaries meticulously and any potential cases of misreporting were discussed and clarified, following which body weight was recorded and body composition assessed. For the first 29 participants, body fat percentage, lean-tissue mass and fat mass were calculated using DXA (QDR software, Hologic Inc., Bedford, MA). For the remaining 28 participants, these features of body composition were determined using 4-site skinfolds and the updated sex and race/ethnicity specific equations (Davidson et al. 2011). All measurements were taken by an International Society for the Advancement of
Kinanthropometry (ISAK) Level 1 Accredited Anthropometrist and followed International Standards for Anthropometric Assessment.

An indwelling cannula (20g IV catheter, BD Venflon, Helsingborg, Sweden) was inserted into an antecubital arm vein and connected to a 150 cm polyethylene extension line (V-Green I.V. Extension Line, Vygon, Swindon, UK). This was attached to a 3-way stopcock (BD Connecta, Vygon, Swindon, UK) to allow for repeated blood collection during treadmill running exercise without the need for changes in running speed or form. The cannula was kept patent for the duration of insertion by regular flushing of 0.9 % sodium chloride (B Braun, Melsungen, Germany). Participants then rested for 30 minutes in the supine position in a dimly lit, thermo-neutral, quiet room with indirect calorimetry used to assess resting energy expenditure and fat oxidation (see below). Participants were instructed to refrain from any movement during this period. Upon completion a resting blood sample (10 ml) was obtained and ~15 minutes later the exercise test was performed.

The procedure for the exercise test is described in Chapter 3, Section 3.3.2 ii. As well, a venous blood sample (5 ml) was obtained prior to the start of exercise (with the participant standing still on the treadmill) and during the final 30 seconds of each exercise stage until RER reached 1.00. A final blood sample (10 ml) was drawn at $\dot{V}O_2^{\text{max}}$. 
Breath-by-breath measurements of oxygen consumption (\(\dot{V}O_2\)) and carbon dioxide production (\(\dot{V}CO_2\)) were collected during rest and exercise using an online automated gas analysis system (Oxycon Pro, Jaeger, Wuerzberg, Germany). The gas analysers were calibrated with a 5.07 % CO\(_2\), 14.79 % O\(_2\), 80.14 % N\(_2\) gas mixture (BOC Gases, Surrey, UK), and the volume transducer was calibrated with a 3-liter calibration syringe (Jaeger, Wuerzberg, Germany). Environmental conditions during testing were: humidity 50±5 %; temperature 22±2 °C.

### 4.3.4 Self-report diet and activity analysis

Please refer to Chapter 3, Section 3.3.3.

### 4.3.5 Plasma analyses

Please refer to Chapter 3, Section 3.3.4 for details regarding the analysis of plasma glucose, glycerol, NEFA and insulin concentrations. Leptin (rest only) was analysed by radioimmunoassay using a commercially available kit (HL-81K Human Leptin; Millipore, Billerica, MA).

### 4.3.6 Calculations

Resting energy expenditure and fat oxidation were calculated during a stable measurement period (i.e., a deviation in \(\dot{V}O_2\) of <10% of the average \(\dot{V}O_2\) between minutes 20-30; mean±SD recording period was 5±2 minutes) according to the equations of Frayn (Frayn, 1983) with a protein correction factor of 0.11 mg/kg/min, as used
previously (Flatt et al, 1985; Hall et al. 2010). Carbohydrate and fat oxidation, $\dot{V}O_{2\text{max}}$ / $\dot{V}O_{2\text{peak}}$, MFO and $\text{Fat}_{\text{max}}$ were determined according to the procedures outlined in Chapter 3, Section 3.3.5.

4.3.7 Statistical analyses

Data were analysed using SPSS (SPSS Version 21, Chicago, IL). All data were normally distributed according to the Shapiro-Wilk test of normality and are presented as Mean±SD (and range where appropriate). A One-Way Repeated Measures ANOVA was used to investigate the temporal differences in plasma hormone and metabolite concentrations with post hoc paired samples t-tests (using the Bonferroni correction) applied where necessary for multiple comparisons. Bivariate correlations were used to investigate relationships between MFO and: 1) metabolite/hormone concentrations and their response to exercise; and 2) other potential correlates of MFO (e.g., resting fat oxidation, $\dot{V}O_{2\text{max}}$, FFM). Partial correlations were used where applicable to adjust for potential relevant confounders. A paired samples t-test was employed to compare the mean exercise intensities at MFO and LIAB. Statistical significance was accepted at $P < 0.05$. 
4.4 Results

The data described below represents 57 participants, with the exception of resting fat oxidation whereby we report data for 51 participants and blood data where we report data for between 45-57 participants due to difficulty withdrawing blood from some participants at certain time-points.

4.4.1 Diet and activity

Self-reported energy intake, energy expenditure and energy balance were 2854±604 kcal/d, 2808±384 kcal/d and 45±613 kcal/d, respectively. The contribution of carbohydrate, fat, protein, fiber and alcohol to daily energy intake was 42±9 % (4.00±1.71 g/kg), 34±8 % (1.30±0.52 g/kg), 18±6 % (1.58±0.64 g/kg), 2±3%, and 4±5%, respectively.

4.4.2 Resting and exercise substrate oxidation

Resting energy expenditure was 1.37±0.28 kcal/min (range 0.81-2.03 kcal/min). Fasted (resting) RER, fat oxidation (g/min) and (% energy from FO [%EnFO]) was 0.80±0.04 (range 0.73-0.87), 0.08±0.03 g/min (0.02-0.15 g/min) and 53±16% (range 14-80%), respectively. The MFO arising from the exercise test was 0.60±0.18 g/min (range 0.30-1.02 g/min) or 9.18±2.65 mg/kg FFM/min (range 4.13-15.98 mg/kg FFM/min) and this corresponded to an exercise intensity of 58±17 % $\dot{V}O_{2\text{max}}$ (range 21-83 % $\dot{V}O_{2\text{max}}$).
4.4.3 Plasma metabolite and hormonal responses

Plasma metabolite responses are presented in Table 4.1.

Table 4.1 Plasma metabolite responses from rest to MFO to $\dot{V}O_{2\text{max}}$.

<table>
<thead>
<tr>
<th></th>
<th>Rest</th>
<th>MFO</th>
<th>$\dot{V}O_{2\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.0±0.4</td>
<td>5.1±0.5</td>
<td>5.8±0.1** ^</td>
</tr>
<tr>
<td>Glycerol (µmol/L)</td>
<td>40.0±19.8</td>
<td>80.2±53.5*</td>
<td>151.8±85.2** ^</td>
</tr>
<tr>
<td>NEFA (mmol/L)</td>
<td>0.57±0.19</td>
<td>0.81±0.45*</td>
<td>0.66±0.37</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td>0.8±0.2</td>
<td>2.1±1.4*</td>
<td>10.2±3.3** ^</td>
</tr>
</tbody>
</table>

Values are mean ± SD. * denotes a significant change from rest to MFO, ** from MFO to $\dot{V}O_{2\text{max}}$, and ^ from rest to $\dot{V}O_{2\text{max}}$

In response to exercise, plasma glycerol ($P=0.001$), NEFA ($P=0.002$) and lactate ($P=0.011$) concentration increased significantly from rest to MFO, whereas glucose did not change. The change from MFO to $\dot{V}O_{2\text{max}}$ resulted in a significant rise in plasma glucose ($P<0.001$), glycerol ($P<0.001$) and lactate ($P<0.001$), however plasma concentration of NEFA was unaltered. Plasma glucose ($P<0.001$), glycerol ($P<0.001$) and lactate ($P<0.001$) were all significantly higher at $\dot{V}O_{2\text{max}}$ than under resting conditions. Plasma insulin did not change from rest to MFO (10.9±3.5 µIU/mL and 8.6±3.2 µIU/mL, respectively; $P>0.05$) or from MFO to $\dot{V}O_{2\text{max}}$ (8.6±3.2 µIU/mL and 8.3±6.9 µIU/mL, respectively; $P>0.05$) but was significantly lower at $\dot{V}O_{2\text{max}}$ than at rest.
(10.9±3.5 μIU/mL and 8.3±6.9 μIU/mL, respectively; \(P=0.026\)). Resting leptin concentration was 3.5±3.6 ng/mL.

### 4.4.4 Correlational analysis

For clarity, correlations of specific variables with fat oxidation rates are presented with oxidation rates expressed in absolute terms (i.e., g/min). Unless explicitly stated, expressing fat oxidation relative to lean body mass does not change the outcomes.

(i) **Resting fat oxidation:** \(\dot{V}O_{2\text{max}}\) (L/min, \(R=0.44, P=0.001\)), FFM (kg; \(R=0.39, P=0.002\)) and energy expenditure (kcal/d; \(R=0.34, P=0.009\)) were significantly and positively associated with resting fat oxidation. No measured plasma hormones or metabolites were significantly associated with resting fat oxidation, with the exception of resting plasma NEFA concentration which was significantly and positively correlated with resting fat oxidation expressed relative to lean body mass (\(R=0.29, P=0.019\)).

(ii) **Maximal Fat Oxidation during exercise:** Fasting RER (\(R=-0.47, P<0.001\)), resting fat oxidation (\(R=0.55, P<0.001\); Figure 4.2), \(\dot{V}O_{2\text{max}}\) (L/min; \(R=0.52, P<0.001\)), body mass (kg; \(R=0.24, P=0.037\)), FFM (kg; \(R=0.28, P=0.018\)) and BMI (kg/m\(^2\); \(R=0.25, P=0.031\)) were all significantly correlated with MFO. Resting fat oxidation (g/min) remained positively correlated with MFO when controlled for \(\dot{V}O_{2\text{max}}\) as a potential confounder.

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3 This researcher accepts that there is potential for the issue that so many correlations run the risk of multiple co-linearity. One way around this is to perform multiple regression analysis to look at independent predictors. However, it was felt that the sample size was insufficient to perform this form of analysis, so it is acknowledged that the weak to moderate, albeit significant, individual correlations should be interpreted with caution.
Fat mass, energy intake, energy expenditure, energy balance and macronutrient intakes were not significantly correlated with MFO ($P>0.05$).

While resting glycerol concentration was not significantly correlated with MFO, glycerol concentration at MFO was significantly and positively associated with MFO (Figure 4.3), and this correlation remained when controlled for potential confounding variables (i.e., glycerol concentration at rest, $R=0.36$, $P=0.008$; $\dot{V}O_{2\text{max}}$, $R=0.51$, $P<0.001$). Furthermore, the change in glycerol concentration from rest to MFO correlated with the change in fat oxidation from rest to MFO ($R=0.32$, $P=0.012$).
The concentration of NEFA at rest and at MFO was significantly and positively associated with MFO ($R=0.36$, $P=0.003$ and $R=0.27$, $P=0.030$, respectively), however the significant relationship between NEFA concentration at MFO and MFO was lost when resting NEFA concentration was considered as a potential confounding variable ($R=0.14$, $P=0.162$). Furthermore, the change in NEFA concentration from rest to MFO

**Figure 4.3** Correlations between (A) plasma glycerol concentration at rest and MFO; and (B) plasma glycerol concentration at MFO and MFO.
did not correlate with the change in fat oxidation from rest to MFO ($R=0.10$, $P=0.236$). Glucose and lactate concentration at rest and at MFO were not significantly associated with MFO.

Insulin concentration at rest and at MFO was significantly and negatively associated with MFO ($R=-0.29$, $P=0.016$ and $R=-0.36$, $P=0.007$, respectively), and the relationship between insulin concentration at MFO and MFO showed a trend for significance when resting insulin concentration was included as a partial correlate ($R=-0.23$, $P=0.060$). The change in insulin concentration from rest to MFO did not correlate with the change in fat oxidation from rest to MFO ($R=-0.115$, $P=0.224$). Resting plasma leptin concentration was not associated with resting fat oxidation or MFO but was significantly and positively associated with the following parameters of body composition: body weight (kg; $R=0.24$, $P=0.048$), BMI ($R=0.33$, $P=0.011$), waist circumference ($R=0.40$, $P=0.002$) and % body fat ($R=0.59$, $P<0.001$).
4.5 Discussion

The purpose of the present study was to investigate the influence of selected plasma metabolites and hormones and resting fat oxidation on MFO during exercise. Accordingly, we report significant relationships between circulating concentrations of NEFA, glycerol and insulin measured during exercise and MFO. We also show that resting fat oxidation is significantly and positively associated with MFO and provide new insights into the role of $\dot{V}O_{2\text{max}}$ as a determinant of fat oxidation across a range of physiological contexts.

The first main set of observations in the current study was that plasma NEFA or glycerol concentrations and plasma insulin concentrations measured during exercise at the point of MFO, were positively and negatively associated with MFO, respectively. To the best of our knowledge, such associations specific to MFO have not previously been reported. The findings are however consistent with what might be predicted based on the observations of others. For example, Goedecke and colleagues demonstrated that plasma NEFA concentration measured during exercise was a significant determinant of the inter-subject variability in RER during low (~41% $\dot{V}O_{2\text{peak}}$) and moderate (~63% $\dot{V}O_{2\text{peak}}$) intensity exercise in trained cyclists (Goedecke et al. 2000). Furthermore, as compared to untrained individuals, endurance-trained men exhibited elevated plasma glycerol concentrations and rates of fat oxidation during 30 minutes of exercise at 75-80% $\dot{V}O_{2\text{max}}$ (Coggan et al. 2007). Finally, Horowitz and co-workers showed that elevations in plasma insulin concentration suppress fat oxidation during low (25-45% $\dot{V}O_{2\text{max}}$) and moderate (68% $\dot{V}O_{2\text{max}}$) intensity exercise (Horowitz et al. 1997, Horowitz et
Collectively, the results of the present study extend support for the integrated influence of lipolysis (as reflected by plasma glycerol concentration), the anti-lipolytic action of insulin, and plasma NEFA availability as determinants of fat oxidation during exercise.

Interestingly, Lanzi and colleagues demonstrated that MFO was similar in lean and obese individuals, yet the obese group displayed markedly higher circulating levels of NEFA, glycerol and insulin across a range of exercise intensities (Lanzi et al. 2014). While it should be noted that the lean group were considerably fitter than the obese group (\(\dot{V}O_{2\text{peak}}\): 44.9±1.3 versus 53.8±1.7 ml/kg FFM/min), the findings do contrast our current observations and would indicate that these plasma responses are not important modulators of MFO. In the study of Lanzi and co-workers, fat oxidation rates were higher in the obese group at lower exercise intensities (20-30% \(\dot{V}O_{2\text{peak}}\)) and higher in the lean group at higher exercise intensities (65-85% \(\dot{V}O_{2\text{peak}}\)). Furthermore, studies comparing fitness-matched lean and obese subjects have shown higher rates of fat oxidation in obesity despite the presence of hyperinsulinemia and similar or elevated plasma NEFA concentration during exercise (Horowitz & Klein, 2000; Goodpaster et al. 2002). Therefore, the role of circulating levels of NEFA, glycerol, and insulin in determining fat oxidation during exercise may be altered as a function of obesity.

We also explored associations between the resting concentrations of glycerol, NEFA, insulin and leptin with MFO. In line with Rosenkilde and colleagues (Rosenkilde et al. 2010) we observed a significant and positive relationship between resting NEFA
concentration and MFO. We also identified previously undocumented significant positive and inverse correlations between resting concentrations of glycerol and insulin with MFO, respectively. Interestingly, the relationships between the concentrations of NEFA and insulin during exercise with MFO were lost when controlled for their resting concentrations using partial correlations. Moreover, the exercise-induced changes in NEFA and insulin were not correlated with the exercise-induced change in fat oxidation (i.e., resting to MFO). This suggests that while plasma NEFA and insulin concentrations at rest and during exercise are generally correlated with MFO, they may not be the prominent drivers of the exercise-induced increase in fat oxidation. While leptin has been shown to promote fat oxidation in muscle (Minokoshi et al. 2012), we did not observe a significant relationship between resting plasma leptin concentration and whole-body fat oxidation. Nevertheless, our data is consistent with previous studies that show leptin concentration is positively associated with body mass and body fat (Ostlund et al. 2013).

By contrast, the significant and positive relationship between plasma glycerol concentration during exercise and MFO remained when controlled for resting glycerol concentration and the exercise-induced change in glycerol was significantly and positively correlated with the exercise-induced change in fat oxidation. Moro and colleagues have recently demonstrated that 33% of the variation in fat oxidation observed during 90 minutes exercise at 70% $\dot{V}O_{2\max}$ could be explained by the subcutaneous abdominal adipose tissue dialysate glycerol concentration (as a marker of lipolysis) area under the curve during the exercise period (Moro et al. 2013).
Additionally, previous studies have reported that changes in lipolytic rate (i.e., rate of appearance of whole-body glycerol) coincide temporarily with changes in fat oxidation during exercise (Coggan et al. 2007, Horowitz et al. 1997). In this light, we interpret our observations to indicate that plasma glycerol concentrations uniquely reflect the metabolic processes underlying the regulation of fat oxidation during exercise. Nonetheless, we acknowledge that further work is necessary to fully delineate the influence of lipolytic rate and plasma NEFA kinetics as determinants of the inter-subject variability in MFO. From a practical standpoint, these findings suggest that previously established interventions that help maximise the lipolytic response could assist with increasing ones capacity to oxidise fat during exercise, i.e., conducting fasted- as oppose to fed-state exercise (de Bock et al. 2005) or strategically manipulating the composition of the pre-exercise diet (Thomas, Brotherhood & Brand, 1991; Stevenson et al. 2009).

Consistent with the findings of others we report that $\dot{V}O_{2max}$ (Achten & Jeukendrup, 2002; Venables et al. 2005) and FFM (Venables et al. 2005) are significantly and positively correlated with MFO. However, these previous reports did not consider the role of resting fat oxidation as a potential correlate of MFO. Indeed, fasting RER has been shown to predict both RER during low-moderate exercise intensities in trained athletes and MFO in overweight men (Goedcke et al. 2000; Rosenkilde et al. 2010). Accordingly, we confirm the observation of Rosenkilde and colleagues (Rosenkilde et al. 2010) by showing fasting RER was negatively correlated to MFO and extend this to demonstrate that the actual rate of resting fat oxidation is significantly and positively...
correlated with MFO. Our further analysis shows that $\dot{V}O_{2\text{max}}$ is positively correlated with both resting fat oxidation and MFO. We previously reported significant and positive correlations between $\dot{V}O_{2\text{max}}$ and 24-hour fat oxidation (Robinson et al. 2015) and collectively this implicates $\dot{V}O_{2\text{max}}$ as an important determinant of fat oxidation under a range of physiological conditions. Interestingly, the significant relationships between resting fat oxidation and MFO reported herein and MFO and 24-hour fat oxidation reported previously (Robinson et al. 2015) remain when controlled for the influence of $\dot{V}O_{2\text{max}}$. This suggests that metabolic factors either directly or indirectly associated with $\dot{V}O_{2\text{max}}$ such as skeletal muscle fibre type, capillary density and/or muscle oxidative capacity are also important determinants of an individual’s overall capacity to oxidize fat.

In conclusion, we report new associations between plasma glycerol, NEFA and insulin concentration and resting fat oxidation with MFO and identify the plasma glycerol response to exercise to uniquely reflect exercise-induced fat oxidation. In line with previous suggestions, our results indicate that resting fat oxidation should be considered in studies investigating exercise-induced fat oxidation (Goedecke et al. 2000). That is, it would be important for such studies to account for baseline (resting) fat oxidation in explaining differences between individuals (e.g., whether differences in MFO are exercise specific or reflect more generalised differences in fat oxidation) or in studies designed to assess the impact of an intervention, for example on exercise fat oxidation (i.e., does an intervention affect exercise fat oxidation per se or fat oxidation more generally). We also identify that $\dot{V}O_{2\text{max}}$ is related to fat oxidation at rest and during exercise, which aligns with our previous work showing that $\dot{V}O_{2\text{max}}$ is related to fat
oxidation over a 24-h period (Robinson et al. 2015). Nonetheless, resting fat oxidation also correlated with exercise fat oxidation and exercise fat oxidation correlated with 24-h fat oxidation even when controlled for the influence of $\dot{V}O_{2\text{max}}$. Our finding that $\dot{V}O_{2\text{max}}$ and fat oxidation could be dissociated implies that improvements in cardio-respiratory fitness (i.e., $\dot{V}O_{2\text{max}}$) per se would increase fat oxidation but is not essential for an increase in fat oxidation. This is consistent with recent work showing exercise training-induced improvements in insulin sensitivity to be unrelated to changes in $\dot{V}O_{2\text{max}}$ (Brennan et al. 2014) and could have implications, perhaps through a focus on improving metabolic capacity, for the design of physical activity interventions aimed at optimising metabolic health.
4.6 References


• Stevenson EJ, Thelwall PE, Thomas K, Smith F, Brand-Miller J, and Trenell MI. Dietary glycemic index influences lipid oxidation but not muscle or liver


CHAPTER 5

INFLUENCE OF PRE- AND POST-EXERCISE FEEDING ON SUBSTRATE UTILISATION, LIPOLYTIC MARKERS AND INSULIN, AND INTRAMUSCULAR TRIGLYCERIDE UTILISATION IN OBESITY
5.1 Abstract

In the absence of weight loss exercise does not appear to improve insulin sensitivity in obese populations, which is surprising given that regular exercise training has been shown to augment IMTG turnover and reduce the accretion of insulin-resistance inducing lipid metabolites. It could be that the timing of nutrition around exercise influences this response. We aimed to investigate the effects of a single exercise bout performed either before (Fasted exercise) or after breakfast (Fed exercise) on exercise substrate utilisation, lipolytic markers and insulin, and intramuscular triglyceride (IMTG) utilisation in obesity. Using a randomized, crossover design, six overweight or obese but otherwise healthy men performed 1-h of constant load bicycle exercise (65% $\dot{V}O_{2\text{max}}$) followed by 3-h of controlled recovery. On one occasion, exercise was performed after an overnight fast (Fasted) and breakfast was provided 30 minutes following exercise, and on the other they received a standardised breakfast 90 minutes before exercise (Fed). Indirect calorimetry was used during exercise to measure whole body substrate utilisation, and venous blood was collected routinely before, during and after exercise to assess the plasma hormone and metabolite response ($n=6$). Fibre type-specific relative IMTG content was determined by immunofluorescence staining in needle biopsies from the vastus lateralis before, immediately after and 3-h after exercise ($n=2$). During exercise, fat oxidation was significantly higher in Fasted vs. Fed (Fasted, 0.64±0.13 g/min; Fed, 0.41±0.09 g/min, $P<0.01$). The concentration of circulating plasma glycerol (25-30 min: Fasted 67±30 μmol/l, Fed 28±9 μmol/l, $P=0.01$; 55-60 minute: Fasted 122±61 μmol/l, Fed 45±20 μmol/l; $P=0.02$) and NEFA (25-30 min: Fasted 0.39±0.35 mmol/l, Fed 0.12±0.08 mmol/l; $P=0.047$; 55-60 minute: Fasted 0.61±0.56 mmol/l, Fed
0.15±0.10 mmol/l; P=0.04) were significantly higher during exercise in the Fasted- vs. Fed-state. The influence of exercise, and the timing of nutrition around exercise, in modulating IMTG degradation could not be firmly established based upon the low sample size and divergent responses reported. This study shows that an acute bout of aerobic exercise performed in the Fasted- as compared with Fed-state augments exercise fat oxidation, as well as the circulating concentration of plasma glycerol and NEFA during exercise in obesity. Further work is required to establish the efficacy of exercise, and the timing of nutrition around exercise, on IMTG utilisation in obesity.
5.2 Introduction

The increasing prevalence of obesity is undoubtedly a major public health concern, largely because obesity is closely associated with a cluster of clinical symptoms of which includes insulin resistance; a precursor to cardiovascular disease and type II diabetes (Haffner & Miettinen, 1997). Weight loss via caloric restriction and increasing levels of physical activity are two primary strategies used in the treatment of obesity. Weight loss can improve insulin sensitivity in obesity (Golay et al. 1985; Webber et al. 1994), but maintaining weight loss often proves to be difficult, with most individuals re-gaining body mass within 3-5 years (Weiss et al. 2007). Regular physical activity can aid weight maintenance following weight loss (Wing & Hill, 2001) and it can also enhance aerobic capacity, which is a key determinant of exercise and daily fat oxidation (Chapter 3, Robinson et al. 2015; Chapter 4, Robinson et al. 2016).

Chapter 3 of this thesis, coupled with the work of others, demonstrates that enhancing exercise and/or 24-h fat oxidation could be beneficial for improving insulin sensitivity (Robinson et al. 2015) and optimising metabolic health (Zurlo et al. 1990; Ellis et al. 2010; Rosenkilde et al. 2010; Isacco et al. 2014; Robinson et al. 2015) and thus it seems logical to expect that regular physical activity should be a mainstay strategy for those seeking to optimise metabolic health.

Indeed, the skeletal muscle of highly trained individuals typically exhibits high peripheral insulin sensitivity. This is because regular exercise training ensures high oxidative capacity and constant stimulation of intramuscular triglyceride (IMTG) turnover, which
lessens the accumulation of insulin resistance-inducing lipid metabolites in skeletal muscle such as ceramides, diacylglycerols and fatty acyl CoA (Shaw, 2010). The positive relationships between (i) exercise training status and (ii) exercise fat oxidation, with insulin sensitivity, affords the suggestion that obese sedentary individuals who are insulin resistant might benefit from performing regular physical activity in an attempt to stimulate IMTG turnover and reduce the deleterious effects of excess lipid on intracellular insulin signalling pathways. Yet surprisingly, carefully controlled longitudinal exercise training studies demonstrate that exercise training in the absence of weight loss has no or at best modest impact on peripheral insulin resistance in obesity (Ross et al. 2000; Ross et al. 2004).

Interestingly, previous work conducted in lean, healthy individuals suggests the timing of recent nutrition around exercise could be important in mediating IMTG utilisation during exercise. For instance, De Bock and colleagues (De Bock et al. 2005) demonstrated that 2-h of constant load cycling (75% $\dot{V}O_{2\text{max}}$) performed in the overnight fasted state induced a significantly higher fat oxidation rate during exercise, which was paralleled with a substantial reduction in type I fibre IMTG content pre- to post-exercise (18±2% to 6±2%, respectively; $P=0.007$). In contrast, IMTG breakdown was completely prevented when exercise was performed in the fed state i.e., following breakfast ($P>0.05$).

There is also evidence to suggest that overnight fasted exercise could help optimise metabolic adaptation to training in lean individuals, which could induce long-term benefits for improved insulin sensitivity. For instance, Civitarese and colleagues
(Civitarese et al. 2005) demonstrated in healthy, lean men that an acute bout of fasted aerobic exercise was significantly more potent than fed exercise at augmenting mRNA expression in muscle of genes that are pivotal players in FA transport and oxidation such as CD36, CPT 1, uncoupling protein 3, and 5'-AMP-activated protein kinase-a. Encouragingly, studies performed in lean individuals comparing the effects of chronic exercise training performed in the fasted- vs. fed-state demonstrate a similar pattern of response (Van Proeyen et al. 2010; Van Proeyen et al. 2011).

The influence of fasted vs. fed exercise on IMTG utilisation and muscle oxidative adaptation has not yet been studied in obesity, however there is indirect evidence from a stable isotope tracer study which suggests that IMTG can be used as a substrate source during fasted exercise in this population (Goodpaster et al. 2002). In this study, relative to their lean counterparts, obese individuals exhibited a greater reliance on fat oxidation towards energy expenditure during 60-minutes of aerobic exercise performed in the overnight fasted (post-absorptive) state (i.e., before breakfast), with the contribution of non-plasma fatty acid oxidation (likely from IMTG) being ~50% higher. Considered collectively, it could be speculated that the timing of recent nutrition around exercise is a critical factor in explaining the failure of aerobic exercise training to induce favourable effects on peripheral insulin sensitivity in longitudinal studies on obese populations.

The emerging question is whether chronic exercise performed in the fasted state is superior to exercise performed in the fed state at promoting oxidative adaptation and IMTG turnover, reducing the accretion of lipid metabolites in muscle and, therefore,
improving insulin sensitivity in obesity. This could have important implications regarding nutritional strategies to improve insulin sensitivity through aerobic exercise training in these populations. Accordingly, it is first necessary to demonstrate improvements in the metabolic responses to an acute exercise bout, which ultimately is likely to underpin long-term metabolic health benefits.

The current study aimed to explore the influence of fasted- versus fed-state exercise on (1) exercise substrate utilisation, (2) the plasma hormone and metabolite response during and around exercise and (3) IMTG utilisation in obesity. It was hypothesised that a single bout of aerobic exercise performed in the fasted- versus fed-state would (1) induce a greater reliance on fat and lesser reliance on carbohydrate towards exercise energy expenditure, (2) enhance the availability of circulating lipolytic makers (namely, plasma glycerol and NEFA) and reduce the availability of insulin during exercise (metabolites and hormones previously identified as important correlates of exercise fat oxidation; Chapter 4, Robinson et al. 2016), and (3) enhance IMTG degradation.
5.3 Methods

5.3.1 Participants

A total of six sedentary, overweight men (white European, BMI 25-29.9 kg/m\(^2\) or Asian, BMI 23-27.4 kg/m\(^2\)) with a high waist circumference (white European ≥ 94 cm [37 inches], Asian > 90 cm [35 inches]) or Class I obese men (white European men, BMI 30-34.9 kg/m\(^2\) or Asian BMI 27.5-35 kg/m\(^2\)) (n=6; 5 white European men, 1 Asian man) were recruited for the study based on body composition criteria that placed them at risk for cardiovascular disease and type II diabetes (NICE, 2014). Their characteristics are shown in Table 5.1. They gave written informed consent to participate in the study, which was approved by the National Research Ethics Committee West Midlands The Black Country (Ref: 15/WM/0128).
Table 5.1 Participant Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Men, ( n=6 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>32±12</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.80±0.04</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>103±12</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>32±4</td>
</tr>
<tr>
<td>Waist/hip circumference (cm)</td>
<td>113±11/ 113±7</td>
</tr>
<tr>
<td>Blood pressure (systolic/diastolic; mm Hg)</td>
<td>130±6/79±6</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>33±6</td>
</tr>
<tr>
<td>( \dot{V}O_2 \text{max} ) (ml/kg/min)</td>
<td>29±4</td>
</tr>
<tr>
<td>Resting metabolic rate (kcal/d)</td>
<td>1744±322</td>
</tr>
<tr>
<td>Estimated energy expenditure (kcal/d)</td>
<td>2670±492</td>
</tr>
</tbody>
</table>

5.3.2 General design

This study involved four laboratory visits. On the first (Screening), participants had their Body Mass Index (BMI) determined and waist and hip circumference measured, following which they had their resting blood pressure checked and a 12-lead electrocardiogram (ECG) was conducted.
The second visit (Pre-Study Evaluation) was used to determine the participant’s body composition (via Dual-Energy X-Ray Absorptiometry [DXA]), resting metabolic rate (using a ventilated hood), maximal aerobic capacity (\(\dot{V}O_{2\text{max}}\), using a short incremental cycling exercise test performed to exhaustion) and to confirm work intensity for the main experimental trials.

The third and fourth visits (Main Experimental Trials) were separated by a minimum of seven days and conducted in a randomized order. These trials were preceded by a 2-day standardized weight-maintaining diet based on individual energy requirements. On one occasion a standardized breakfast was consumed, followed one hour later by the collection of a skeletal muscle biopsy sample and then a further 30 minutes by the performance of a 60-minute steady-state moderate intensity cycling exercise bout. Immediately, and at 3-h after exercise further skeletal muscle biopsy samples were collected. Expired breath collected using indirect calorimetry was recorded at intervals during exercise testing to quantify whole body fat oxidation during each trial. On the other occasion, participants underwent the same procedures but the standardized breakfast was consumed 30 minutes following cessation of exercise.

5.3.3 Experimental procedures

(i) Visit 1 - Screening: The purpose of this visit was to obtain consent and screen participants. The participants arrived at the laboratory in the morning (08:00 h). On arrival, measures of height (Stadiometer, Seca, UK) and body weight (OHaus Champ II
Scales, USA) were taken to determine Body Mass Index (BMI; weight [kg]/height² [m]). Waist and hip circumference to the nearest mm and resting blood pressure (Omron M6, The Netherlands) were also determined (those with a resting blood pressure ≥140/90 mmHg were not included in the study). If participants successfully completed the Screening Form and met the eligibility requirements with respect to BMI/waist circumference/blood pressure they were asked to undertake a 12-lead electrocardiogram (ECG) examination in order to detect any potential cardiac issues (Oxycon Pro, Jaeger, Wuerzberg, Germany).

(ii) Visit 2 - Pre-study evaluation⁴: Participants reported to the laboratory between 07:00-17:00 h at least one week before the Main Experimental Trials. Participants were weighed in light clothing following which body composition was determined using DXA (QDR software, Hologic Inc., Bedford, MA). Thereafter, participants rested for 30 minutes in the supine position in a dimly lit, thermo-neutral, quiet room with a ventilated hood system (GEM; GEM Nutrition, Cheshire, UK) and indirect calorimetry used to assess resting energy expenditure (see below). Participants were instructed to refrain from any movement during this period. Approximately 15 minutes later an incremental exercise test on an electronically braked cycle ergometer (Lode Sport Excalibur, Groningen, The Netherlands) was performed to determine the participants level of aerobic fitness (\(\dot{V}O_{2\text{max}}\)). For this, participants started cycling at 35 W and the work rate was increased 35 W every 3 min until volitional exhaustion. Heart rate was measured

⁴ It is acknowledged as a limitation of the present study that activity and food intake was not controlled on the day preceding the measurement of resting energy expenditure.
continuously during exercise using a heart rate monitor (Polar FT-2, Finland) and recorded during the final 30 seconds of each exercise stage. Breath-by-breath measurements of oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were collected during exercise using an online automated gas analysis system (Oxycon Pro, Jaeger, Wuerzberg, Germany). The gas analysers were calibrated with a 5.07 % CO$_2$, 14.79 % O$_2$, 80.14 % N$_2$ gas mixture (BOC Gases, Surrey, UK), and the volume transducer was calibrated with a 3-liter calibration syringe (Jaeger, Wuerzberg, Germany). Environmental conditions during testing were: humidity $56\pm4$ %; temperature $21\pm2$ °C. After an ~30 minute rest period participants undertook a familiarisation exercise bout that had them perform a ‘practice’ cycling exercise bout (30 minutes at a sub-maximal steady-state intensity [$65\% \dot{V}O_{2\text{max}}$]) to accustom them with the exercise procedures to be employed during Visits 3 and 4.

(iii) Visits 3 and 4 - Main experimental trials (Figure 5.1): These visits were separated by at least 7 days and each was preceded for two days by a standardized weight maintaining diet (50% carbohydrate, 35% fat, 15% protein) based on estimated individual energy requirements (see below). Participants arrived in the morning (~08:15 h) following an overnight abstinence from food and drink (from 10pm, except water) and having performed no strenuous physical activity and abstained from alcohol and caffeine consumption for the 2 days before. On the morning of these visits participants were asked to consume ~1 pint of tap water upon waking to ensure they were adequately hydrated on arrival at the laboratory. Participants had a cannula placed in a forearm vein to allow for a baseline and then repeated blood sampling during the experimental
session. On one occasion (15 minutes after arrival) a standardised breakfast was consumed within 15 minutes (25% daily energy intake; 65%, 20%, 15% energy from carbohydrate, fat and protein, respectively), followed one hour later by the collection of a skeletal muscle percutaneous needle muscle biopsy sample (B1) (biopsy procedure and analysis described below). Participants then performed a 60-minute steady-state moderate intensity (65% $\dot{V}O_{2\text{max}}$) cycling exercise bout, as this has previously been shown to induce IMTG degradation in lean individuals (Watt et al. 2002; Shepherd et al. 2012). Immediately (B2), and at 3-h after exercise (B3) further skeletal muscle biopsy samples were collected. On the other occasion, participants underwent the same procedures but the standardised breakfast was consumed 30 minutes following cessation of exercise. Expired breath collected using indirect calorimetry was recorded twice during exercise to determine rates of oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) to quantify whole body fat oxidation. Heart rate was monitored continuously during exercise and recorded every 10 minutes (Polar FT-2, Finland), alongside self-rated perceived exertion using the standard Borg scale (Borg, 1970). Environmental conditions were documented during the conduct of exercise testing. They were kept stable and did not differ between the two trials with humidity at 51±3 % and 52±4 %; temperature 22±0 °C and 22±1 °C for fasted and fed conditions, respectively.
Figure 5.1 A schematic representation of the Main Experimental Trials. EB = collection of expired breath. * = blood collection.

5.3.4 Blood sampling and analysis

Venous blood was sampled from an antecubital vein and collected into either EDTA- or serum containing Vacutainers (BD, New Jersey, USA) and immediately centrifuged at 3,500 revolutions per minute for 15 minutes at 4°C. Aliquots containing plasma were stored at -80°C until analysed. Sample analysis was performed using enzymatic colorimetric assays for glucose (Glucose Oxidase, Instrumentation Laboratories, Cheshire, UK), glycerol (GLY, Randox, London, UK), NEFA (NEFA, Randox, London, UK) and triglyceride (TG kit, Instrumentation Laboratory, Warrington, UK) using an ILAB 650 Clinical Chemistry Analyser (Instrumentation Laboratory, Warrington, UK). Plasma insulin was analysed using a human insulin Enzyme Linked-Immuno-Sorbent Assay (ELISA) kit (Invitrogen, Paisley, UK) and a Biotek ELx800 analyser (Biotek Instruments, Vermont, USA).
5.3.5 Muscle

(i) Muscle sampling: In this study all six participants received six muscle biopsies (three during Visit 3 and three during Visit 4). For each, participants rested in the supine position and a muscle biopsy was obtained from the vastus lateralis using the percutaneous needle biopsy technique (Bergstrom, 1975), adapted to include suction. Prior to this, antiseptic (Videne, Ecolab, UK) was applied to the skin ~20 cm proximal to the patella, following which skin and muscle fascia was anaesthetized using 1% Lidocaine (Braun, Melsungen, Germany). When the area was numb to the touch a 4-6 mm incision was made through the skin and muscle fascia using a surgical scalpel (Swann-Morton, Sheffield, UK). A 6G biopsy needle was then inserted ~2-6 cm into the incision and a piece of muscle guillotined. A sample of ~100 mg was collected and freed from any visible non-muscle material, following which a portion was mounted in embedding Tissue Tek OCT (Sigma Aldrich, Dorset, UK) and frozen in liquid nitrogen-cooled isopentane (Sigma Aldrich, Dorset, UK) and transferred to an aluminium cryotube (Caltag Medsystems, PA6003) for later immunofluorescence microscopy analysis. All samples were stored at -70°C for later analysis.

Unfortunately, due to freezer failure, muscle samples from 4 of the 6 participants were of insufficient quality to undergo immunofluorescence analysis. Muscle from these participants appeared to have thawed and re-frozen during its period of storage in the -70 freezer. Upon opening the aluminium tube, the muscle and Tissue Tek mounting medium was moulded to the shape of the tube (Figure 5.2). Accordingly, it was not possible to detect any myosin heavy chain type I (MHCI; used to determine fibre type) or
dystrophin signal (a plasma membrane marker) once these muscles were stained appropriately (Figure 5.3; staining protocol described below). It is suspected this was because the thaw and re-freeze damaged the muscle architecture and / or the proteins such that the antibodies were unable to bind appropriately. Therefore all muscle data is presented on two participants.

![Figure 5.2](image)

**Figure 5.2** Example images showing muscle and Tissue-Tek mounting in a sample that had thawed and re-frozen (of insufficient quality to undergo analysis; A) and muscle that had not thawed and re-frozen (of sufficient quality to undergo analysis; B). As can be seen, Tissue-Tek in (A) is moulded differently on top of the cork than in (B). During the period of removal from the freezer, the Tissue Tek in (A) thawed and subsequently expanded towards and over the edge of the cork, whereas in (B) the Tissue-Tek retained its shape that was apparent upon initial freezer storage.
Figure 5.3 Representative images comparing muscle samples that were unable (A) and able (B) to undergo immunofluorescence analysis. As can be seen, the muscle architecture in image A is clearly distorted with several of the fibres appearing to have disjoined. There is also an absence of MHC1 staining preventing the identification of fibre type. Yellow bars represent 50 μm.

(ii) Sample preparation and staining: Cryosections (5 μm) were cut using a Bright 5040 (Bright Instrument Company limited, Huntingdon, England) microtome within a Bright cryostat with internal temperature kept constant at -25 °C. Sections were cut onto uncoated glass microscope slides (VWR International), with each slide containing six samples from each participant (three from visit 3 and three from visit 4 [pre-exercise, immediately post-exercise and 3-h post-exercise]) to decrease the variation in staining intensity between triplicate sections. Sections were fixed for one hour in 3.7%
formaldehyde (made up in dH₂O) and subsequently washed 3 times for 30 seconds in dH₂O. Following this, slides were incubated for 5 minutes in 0.5% triton-X100 made up in 1 x phosphate-buffered saline (PBS) at room temperature and then washed 3 times for 5 minutes with wash buffer (1 x PBS). Thereafter, slides were incubated for 2 hours in 1:25 anti-MHCI (mouse IgM, DSHB A4,840) and 1:100 anti-dystrophin (mouse IgG2b, Sigma Aldrich D8168) in 5% normal goat serum (NGS) at room temperature. After washing the slides three times for 5 minutes in 1 x PBS, slides were incubated for 30 minutes in 1:150 goat anti-mouse (GAM) IgM conjugated to AlexFluor 633 (for MHCI) and 1:200 GAM IgG2b 594 (for dystrophin) in 1 x PBS at room temperature. Incubation was followed by three 5 minutes washes (1 x PBS), after which the slides were incubated again, this time in 1:50 bodipy solution in 1 x PBS at room temperature in the dark. A final wash of the slides was then performed twice for 3 minutes in 1 x PBS. Glass coverslips were mounted with 20 μl mowiol mounting medium (6 g glycerol (Sigma Aldrich, G5150), 2.4 g mowiol 4-88 (Fluka, 81381) and 0.026 g 1,4-diazobicyclo-[2,2,2]-octane (DABCO) (Fluka, 33490) dissolved in 18 ml 0.2M Tris-buffer (pH 8.5) (Sigma Aldrich, T5030) to protect the sample and preserve the fluorescence signal. Slides were left to dry overnight before immunofluorescence visualisation.

(iii) Microscopy: All images were taken using a confocal microscope (Leica DMIRE2, Leica Microsystems) with 40x oil immersion objective (1.25 Numerical Aperture). Bodipy was excited with a 488 nm line of the argon laser and 510-652 nm emission. To detect dystrophin signal, Alexa Fluor 594 fluorophores were excited with the 594 nm line of the helium-neon laser and 668-698 nm emission. To detect MHCI signal, Alexa Fluor 633
fluorophores were excited with the 633 nm line of the helium-neon laser and 698-808 nm emission.

(iv) Image quantitation: For both subjects, 3 slide replicates, each with a pre-exercise (B1), immediately post-exercise (B2) and 3-h post-exercise section for both trials underwent staining, imaging and quantification.

For the fasted trial, the average number of type I fibres [type II fibres] analysed across the two participants was 15 [20] (pre-exercise), 24 [27] (immediately post-exercise) and 19 [44] (3-h post-exercise). For the fasted trial, the average number of type I fibres [type II fibres] analysed across the two participants was 32 [43] (pre-exercise), 17 [21] (immediately post-exercise) and 25 [30] (3-h post-exercise).

Bodipy spot stained area per fibre (for assessment of IMTG) was quantified by setting uniform threshold intensity and size values to detect spots within intracellular regions of the dystrophin mask in a fibre type specific manner. A HiGauss filter was applied to bodipy images prior to bodipy signal detection. Fibre area was exported from the software based on the intracellular regions determined with the dystrophin mask. Percent area of bodipy staining of the total fibre area was calculated ([bodipy stained area $[\text{um}^2]$ / area of muscle $[\text{um}^2]$]*100).
5.3.6 Calculations

Insulin sensitivity was calculated using the Quantitative Insulin Sensitivity Check Index (QUICKI; Katz et al. 2000). For each of the six participants, baseline plasma glucose and insulin concentrations were averaged over the two trials, following which this average was inputted into the equation (as described in Chapter 2, Section 2.5). Upon reflection, this researcher accepts that a more accurate indice of insulin sensitivity would have been derived from an Oral Glucose Tolerance Test, which should have been performed during the Screening Visit.

Resting metabolic rate (RMR) was calculated during a stable measurement period (i.e., a deviation in $\dot{V}O_2$ of <10% of the average $\dot{V}O_2$ between minutes 20-30; mean±SD recording period was 6±4 minutes) and using the equation of Weir (1949). Daily energy expenditure (DEE) was then estimated by multiplying RMR by a population specific activity factor (1.53; WHO, 2001).

\[
RMR = 3.9*\dot{V}O_2 + 1.1*\dot{V}CO_2
\]

\[
DEE = RMR*1.53
\]

During the incremental exercise test $\dot{V}O_2$ was considered as maximal when two of the following three criteria were met; an RER >1.1, heart rate within 10 beats of predicted maximum (calculated as 220-age; Fox, Naughton & Haskell, 1971), or an increase of < 2ml/kg/min in $\dot{V}O_2$ with a further increase in workload. $\dot{V}O_{2\text{max}}$ was calculated as the highest rolling 60 second average $\dot{V}O_2$. 

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For the Main Experimental Trials, carbohydrate and fat oxidation were quantified according to the equations of Frayn (1983), with the assumption that the urinary nitrogen excretion rate was negligible.

\[
\text{Carbohydrate oxidation (g/min)} = 4.55\times \dot{V}\text{CO}_2 - 3.21\times \dot{V}\text{O}_2
\]

\[
\text{Fat oxidation (g/min)} = 1.67\times \dot{V}\text{O}_2 - 1.67\times \dot{V}\text{CO}_2
\]

5.3.7 Statistical analyses

Data were analysed using SPSS software (version 21; Chicago, IL). All data were normally distributed according to the Shapiro-Wilk test of normality and are presented as means±SD unless explicitly stated (and ranges where appropriate). Statistical significance was positioned at \( P<0.05 \). Incremental areas under the curve (iAUC) for glucose, glycerol, insulin, NEFA and TG were calculated for each trial using the trapezoid method. Paired-sample \( t \)-tests were used to compare differences in heart rate, RPE, \( \dot{V}\text{O}_2 \) uptake, \( \dot{V}\text{O}_{2\text{max}} \), \( \text{CO}_2 \) production, RER, fat oxidation (g/min and % energy from fat oxidation [%EnFO]), carbohydrate oxidation (g/min and % energy from carbohydrate oxidation [%EnCO]) and iAUC for plasma insulin and metabolites between trials. A two-way analysis of variance (condition and time) was used to assess metabolic differences between conditions. Where a significant interaction effect was observed, a post-hoc Bonferroni pairwise comparison was performed to locate the variance. Due to low sample size muscle data was not analysed statistically but is instead described
5.4 Results

5.4.1 Pulmonary gas exchange and substrate oxidation during exercise (Table 5.2).

There were no significant differences in heart rate or \( \dot{V}O_2 \) during exercise between Fasted and Fed conditions, respectively. However, \( \dot{V}CO_2 \) was significantly higher and, accordingly, exercise RER was significantly lower during Fasted exercise as compared with Fed. A parallel with this, the calculated fat oxidation rate and %EnFO was significantly higher, whereas the carbohydrate oxidation rate and %EnCO was significantly lower in Fasted relative to Fed. Subjective rating of RPE was significantly lower in the Fasted condition.
Table 5.2 Pulmonary gas exchange and substrate oxidation rates during exercise (values are mean±SD).

<table>
<thead>
<tr>
<th></th>
<th>Fasted Exercise</th>
<th>Fed Exercise</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>126±12</td>
<td>127±16</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>RPE</td>
<td>12±2</td>
<td>13±2</td>
<td>P=0.44</td>
</tr>
<tr>
<td></td>
<td>(12 = between 'light [11]' and 'fairly hard [13]'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO2, ml/min (%VO2max)</td>
<td>1990±176 (65±1)</td>
<td>2017±159 (65±2)</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>VCO2, ml/min</td>
<td>1786±129</td>
<td>1889±178</td>
<td>P=0.02</td>
</tr>
<tr>
<td>RER</td>
<td>0.90±0.02</td>
<td>0.94±0.02</td>
<td>P=0.01</td>
</tr>
<tr>
<td>Fat oxidation, g/min (%EnFO)</td>
<td>0.64±0.13 (31±5)</td>
<td>0.41±0.09 (21±5)</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Carbohydrate oxidation, g/min (%EnCO)</td>
<td>3.45±0.14 (69±5)</td>
<td>4.22±0.58 (79±5)</td>
<td>P=0.01</td>
</tr>
</tbody>
</table>

5.4.2 Metabolic and hormonal response to feeding and exercise (Figure 5.4).
There were no significant differences in plasma glucose, glycerol, NEFA and triglyceride concentrations at Baseline between Fasted and Fed conditions (P>0.05). Plasma insulin was significantly higher in the Fasted trial at Baseline (P=0.03), however this is unlikely
to be physiologically relevant as the difference in plasma insulin concentration between Fasted and Fed conditions was minor (27.8±9.1 µIU/mL and 22.9±11.2 µIU/mL, respectively; Figure 5.4 C).

(i) Plasma glucose concentration (Figure 5.4 A): Glucose differed significantly between conditions during the post-exercise period i.e., at 230 minutes (a parallel with ingestion of the post-exercise meal provided in the Fasted condition) and at 290 minutes. No significant differences in glucose concentration were observed between conditions at any other time points (note: a trend towards a significantly higher glucose availability was observed at 75 min in Fed [P=0.055]). In the Fasted condition, glucose concentration was stable before and during exercise but increased markedly following ingestion of the post-exercise meal. Glucose returned close to its baseline value at 350 min. In the Fed condition, glucose concentration increased following ingestion of the pre-exercise meal but returned towards baseline values immediately before exercise where it remained stable until the end of the assessment period. iAUC for glucose was significantly higher in Fasted vs. Fed (2490±481 mmol/L x min and 2201±436 mmol/L x min, respectively; P=0.03).

(ii) Plasma glycerol concentration (Figure 5.4 B): Glycerol was significantly higher prior to and during exercise in Fasted, whilst it was higher at all time points during the post-exercise period in Fed. In both conditions, glycerol concentration decreased marginally between the baseline and pre-exercise measurement, but increased during exercise, with this increase being greater in Fasted than Fed. In the Fasted condition, the post-
exercise meal reduced glycerol concentration. In contrast, glycerol concentration in the Fed condition significantly decreased during the first 65 minutes post-exercise, but increased thereafter. Glycerol iAUC did not significantly differ between conditions (Fasted 15,036±4,682 μmol/l x min; Fed 12,359±4,018 μmol/l x min).

(iii) Plasma insulin concentration (Figure 5.4 C): Insulin was significantly higher at baseline and during the post-exercise period in Fasted as compared with Fed, however no significant differences were observed between conditions at any other time point. In Fasted, insulin remained stable throughout the pre-exercise and exercise period, but showed a marked increase following the post-exercise meal. Insulin remained high throughout the post-exercise period. In Fed, the pre-exercise meal increased insulin concentration, which thereafter declined over the remaining course of stay, except during the first thirty minutes of exercise when it increased from immediately pre-exercise. There was a trend for iAUC to be higher in the Fasted as compared with Fed condition (29,511±27,939 μIU/mL x min and 20,681±19,931 μIU/mL x min, respectively; P=0.051).

(iv) Plasma NEFA concentration (Figure 5.4 D): NEFA availability was significantly higher in Fasted than Fed immediately before and during the exercise bout, however no significant differences were observed at any other time point between conditions (note: a trend towards significantly higher NEFA availability in Fed was observed at 350 min [P=0.053]). In the Fasted condition, NEFA concentration remained stable prior to exercise, however 30 minutes into exercise it was lower than pre-exercise levels, but
increased above pre-exercise levels at the end of exercise. The post-exercise meal induced a significant decline in NEFA concentration throughout the post-exercise period. In the Fed condition, the pre-exercise meal caused a significant reduction in NEFA availability throughout exercise; during which it remained stable. Only after exercise did NEFA concentration show a significant rise back towards, and then above, its baseline value. A trend towards a significantly higher NEFA iAUC was observed in Fasted vs. Fed (151±95 and 112±51, respectively; \( P=0.063 \)).

(v) Plasma triglyceride concentration (Figure 5.4 E): Triglyceride concentration did not differ significantly at any time point between conditions. iAUC for triglyceride showed a trend towards being higher in Fasted vs. Fed (689±460 mmol/L x min and 682±303 mmol/L x min, respectively; \( P=0.050 \)).
Figure 5.4 Circulating plasma glucose (A), glycerol (B), insulin (C), NEFA (D), and triglyceride (E) concentration over the 350-minute stay. Double headed arrows represent the exercise period. * Significant difference between Fasted and Fed trials ($P<0.05$). † Significant difference at Baseline between conditions ($P<0.05$). Values are Mean±SEM.
5.4.3 QUICKI

Insulin sensitivity estimated using the QUICKI was 0.29±0.02 (range 0.27-0.32). Of note, this score, obtained in an overweight / obese population of men, is lower than that found in Chapter 3 (0.34±0.02; range 0.31-0.40), which employed a cohort of healthy, lean men and similar to that previously reported in diabetic subjects (0.30±0.01; Katz et al. 2010). This implies that this study employed a population with potentially compromised insulin sensitivity.

5.4.4 IMTG lipid content

As mentioned previously, muscle data is provided for two participants. Accordingly, it is acknowledged that the sample size is insufficient to make strong conclusions. Descriptions are provided for both participants separately in light of the divergent responses reported between individuals.

(i) Participant 1 (Figure 5.5): Pre-exercise IMTG content was higher in type I fibres compared with type II fibres in both Fasted and Fed conditions (type I Fasted 13%, type I Fed 5%; type I Fed 5%, type 2 Fed 2%).

Cycling at 65% \( \dot{V}O_2_{\text{max}} \) for 60 minutes induced a reduction in IMTG content in type I fibres between B1 and B2 in Fasted (-56%), however there was an increase in Fed (+42%). In both conditions there was a reduction in IMTG content between B2 and B3, however this reduction was greater in Fasted (-68%) than Fed (-22%). Overall, i.e.,
between B1 and B3, there was a reduction in IMTG content in both conditions, with this being greater in Fasted (-86%) as compared with Fed (-18%).

In type II fibres, there was a reduction in IMTG content between B1 and B2 in Fasted (-36%), but an increase in Fed (+29%). In both conditions, IMTG content decreased between B2 and B3, as well as between B1 and B3, however the magnitude of this decrease was greater in Fasted than in Fed (B2 and B3: Fasted -79%, Fed 35%; B1 and B3: Fasted 86%, Fed 15%).
Figure 5.5 Comparison of exercise in the Fasted- vs. Fed-state on IMTG lipid content. Data are presented as means±SEM (n=1; participant 1).
Participant 2 (Figure 5.6): Pre-exercise IMTG content was higher in type I fibres compared to type II fibres in both Fasted and fed conditions (type I Fasted 10%, type II Fasted 3%; type I Fed 9%, type 2 Fed 2%).

In both conditions, cycling at 65% \( \dot{V}O_2 \text{max} \) for 60 minutes induced a reduction in IMTG content in type I fibres between B1 and B2, however this reduction was greater in fed (-51%) than in fasted (-32%). In both conditions there was an increase in IMTG content between B2 and B3, however this increase was greater in fasted (+75%) than fed (+16%). Overall i.e., between B1 and B3, there was an increase in IMTG content in fasted (+19%) and a reduction in fed (-43%).

In type II fibres, there was a greater reduction in IMTG content between B1 and B2 in fed (-41%) than in fasted (-9%). In both conditions, between B2 and B3 there was an increase in IMTG content, with this rise being slightly greater in fasted relative to fed (+25% and +21%, respectively). IMTG content increased between B1 and B3 in fasted (+25%) but was reduced in fed (-29%).
**Figure 5.6** Comparison of exercise in the Fasted- vs. Fed-state on IMTG lipid content. Data are presented as means±SEM (n=1; participant 2).
5.5 Discussion

The aim of the current study was to investigate the influence of Fasted- versus Fed-state exercise on (1) exercise substrate utilisation, (2) the plasma hormone and metabolite response during and around exercise and (3) IMTG utilisation, in an obese group of men identified as at risk for cardiovascular disease and type II diabetes on the basis of select anthropometrical measurements (NICE, 2014).

5.5.1 Whole body substrate utilisation during exercise

By using indirect calorimetry we were able to quantify the influence of feeding on whole body substrate utilisation during exercise. From this, we found that exercise fat oxidation was ~35% higher and subsequently carbohydrate oxidation was ~17% lower during exercise performed in the Fasted- as compared with Fed-state. This finding is consistent with previous reports using healthy (de Bock et al. 2005) and obese (Derave et al. 2007) men and accordingly it is acknowledged that this is not an entirely novel finding. Nevertheless, this data further demonstrates the potency of fasted as compared with fed exercise in augmenting exercise fat oxidation, which is an effect that could have important implications. For instance, it is becoming progressively apparent that increasing exercise fat oxidation could help to optimise metabolic health. For example, in Chapter 3 of this thesis it was demonstrated, using healthy lean men, that the Maximal rate of Fat Oxidation during exercise is significantly and positively associated with insulin sensitivity (Robinson et al. 2015). What is more, work in overweight men shows that a lower MFO is associated with a higher clustering of metabolic syndrome risk factors,
when compared with those who exhibit a higher MFO, whereas Isacco and colleagues 
(Isacco et al. 2014) have shown that a lower MFO is associated with a more 
unfavourable body fat mass distribution in post-menopausal women.

Chapter 3 of this thesis also revealed a significant and positive relationship between 
MFO and 24-h fat oxidation (Robinson et al. 2015), with the latter being previously linked 
with body mass gain (Zurlo et al. 1990) and body mass re-gain after diet-induced weight 
loss (Ellis et al. 2010). Interestingly, recent work conducted in young, non-obese men 
has shown under energy-balanced conditions increased 24-hour fat oxidation following 1 
hour of aerobic exercise performed in the overnight fasted state, with this response 
being completely blunted when exercise was performed postprandial (Iwayama et al. 
2015). Considered collectively, these findings (Iwayama et al. 2015; Robinson et al. 
2015) suggest that the increased fat oxidation observed during Fasted vs. Fed exercise 
in this study may extend to 24-h, and that Fasted- as compared with Fed-exercise could 
be a more viable strategy to improve metabolic health in an at risk population. 
Nevertheless, it is accepted as a limitation of the present study that, due to reasons of 
equipment availability, substrate utilisation was not assessed around the exercise 
period. Thus we must concede that the influence of Fasted versus Fed exercise on post-
exercise substrate utilisation could not be established. This is important information to 
know, given that the influence of exercise on substrate metabolism is not confined to the 
period of physical activity itself, and the impact of exercise on lipid metabolism can 
persist hours into recovery (Kuo et al. 2005; Henderson et al. 2007). The insights gained 
by Iwayama and colleagues (Iwayama et al. 2015) suggest that Fasted exercise could
be superior to Fed exercise at augmenting daily fat utilisation (independent of energy balance), at least in young, non-obese men. The next logical step would be to establish whether this effect persists in obesity and, if so, to then perform carefully controlled longitudinal studies investigating the influence of regular aerobic exercise training performed in either the fasted- or fed-state on exercise and daily fat oxidation, whilst also considering potential subsequent effects on metabolic health.

5.5.2 Plasma hormone and metabolite response to exercise and feeding

The greater contribution of fat towards exercise energy expenditure observed in the Fasted condition was paralleled with a greater circulating concentration of plasma glycerol and NEFA during exercise, compared with Fed. This corroborates with our recent report showing that the plasma lipolytic and insulin response to exercise is a key regulator of exercise fat oxidation in lean, healthy men (Chapter 4; Robinson et al. 2016). Our finding also aligns with De Bock and colleagues (De Bock et al. 2005) who reported markedly higher NEFA and glycerol concentrations throughout fasted exercise compared with fed. Interestingly, no significant differences were observed in plasma insulin concentration during exercise between conditions, which is surprising given the anti-lipolytic nature of insulin (and previous observed negative associations between maximal fat oxidation during exercise and circulating insulin concentration; Chapter 3, Robinson et al. 2016). However, it is notable that insulin was higher at all time points during the exercise bout in Fed and it is likely that greater statistical power would have yielded significant differences between conditions.
As well as during exercise, we explored the plasma hormone and metabolite response before and after exercise. From this we observed a higher glucose iAUC for the overall intervention (time 0-350 min) when exercise was performed fasted. Although we did not assess the glycaemic response to breakfast across conditions, we did observe a greater rise in glucose following ingestion of breakfast in Fasted vs. Fed. Indeed, others have demonstrated a greater glycaemic response to a standardised breakfast when exercise was performed in the Fasted as compared with Fed state (Derave et al. 2007) and type II diabetes (Larsen et al. 1997; Poirer et al. 2001). Derave and colleagues speculated that the blunted response in Fed may be attributed to an exercise-induced transient suppression of the meal-induced elevation in glucose, which is subsequently used as a substrate to fuel muscle contraction. Interestingly, the present study shows this may not be the entire case, as although glucose decreased during exercise in Fed, it declined to a much greater extent during the period between the meal and the onset of exercise (minutes 75 to 110). This could be due to a synergistic effect of muscle contraction coupled with a concomitant elevation in insulin reduced hepatic glucose production following the ingestion of breakfast before exercise. The marked increase in glucose concentration following breakfast in the Fasted trial is likely explained by adaptations in splanchnic tissues following exercise (Rose et al. 2001). Specifically, prior exercise has been shown to reduce hepatic glucose uptake and increase intestinal glucose absorption and we believe it is these alterations that are likely the primary contributing factors towards the overall higher iAUC for glucose observed in Fasted.
Finally, it should be noted that the measurement period following exercise was likely too short to entirely capture the various plasma hormone and metabolite responses to exercise. This is particularly pertinent to plasma triglyceride concentrations, which have been shown to take several hours to rise and recover (Davitt et al. 2013). As such, we acknowledge this as a limitation of the present study and would ask future research to be cognisant of this when designing similar research herein.

5.5.3 Influence of exercise and the timing of feeding on IMTG utilisation

One hypothesis for this study was to investigate the influence of an acute bout of exercise performed in either the Fasted or Fed state on IMTG utilisation in obesity. Unfortunately due to reasons previously documented, muscle from only two individuals was of sufficient quality to undergo analysis. Accordingly, we acknowledge that the strength of the interpretations made is greatly limited by the low sample size, and we ask readers to be cognisant of this herein.

Consistent with previous reports, and supporting the accuracy of the assay used in this study, the data demonstrates that IMTG content is up to 4-fold higher in type I compared with type II fibres (Steffensen et al. 2002; De Bock et al. 2005). Although limited by a low sample size, the data presented suggests that both type I and type II fibre IMTG can be utilised during acute exercise in obesity. With regards to type I fibre degradation, our finding aligns with previous reports showing a reduction in type I fibre IMTG content during acute fasted exercise in trained lean individuals (van Loon et al. 2003; de Bock et
al. 2005) and untrained lean individuals (Shepherd et al. 2012). It also lends support to the work of Goodpaster and colleagues (Goodpaster et al. 2002) who provided indirect evidence which suggested that IMTG could contribute to exercise fat oxidation in obesity, at least when exercise is performed in the overnight fasted state. We extend this suggestion by demonstrating type I fibre IMTG degradation following fasted but also fed exercise. Interestingly, our finding contradicts those of Ipavec-Levasseur and colleagues (Ipavec-Levasseur et al. 2015) who reported no IMTG degradation in obesity following 60 minutes of steady-state cycling exercise, and with Nellemann and colleagues who observed no alteration in IMTG content after 90 minutes of cycling at 50% $\dot{V}O_{2\text{max}}$ using 8 overweight but otherwise healthy, untrained men (Nellemen et al. 2014).

Discrepancies observed between studies could be due to differences in study methodology. For example, Ipavec-Levasseur and colleagues assessed IMTG content using the non-invasive magnetic resonance spectroscopy (MRS) technique, which despite being shown to have good test-retest reliability (Larson-Meyer et al. 2006) is not without its limitations. Firstly, from the perspective of its technique there can be difficulty in arranging the muscle volume of importance to minimise the involvement of vasculature and subcutaneous adipose tissue, and to ensure orientation of the muscle fibres along the magnetic field. The presence of extramuscular lipids could cross-contaminate the IMTG signal and confound the measurement, whereas histochemical determination of IMTGs (as used in this study) allows direct quantification of lipid within muscle fibres. Furthermore, one MRS scan takes at minimum 15 minutes, meaning that post-exercise measurement of IMTG may have been influenced by post-exercise IMTG
utilisation during the period of preparation. Biopsy samples in this study were taken immediately (i.e., within a few minutes) post-exercise to mitigate this potential issue. Lastly, Ipavec-Levasseur and colleagues chose to analyse the soleus muscle group, which has less involvement during cycling exercise than the quadriceps muscle (as analysed in this study). In the study by Nellemann and colleagues, the lack of IMTG degradation might be because they used a mixed muscle enzyme assay and therefore the presence of extra-muscular triglyceride may have cross-contaminated the IMTG signal and confounded the IMTG measurement (Watt et al. 2002). One strength of the present study is that individual muscle fibers were dissected from the muscle sample and then analysed, which permitted direct quantification of lipid within muscle fibres. Nevertheless, we would emphasise that our finding of a reduction in type I IMTG content following an acute bout of endurance exercise in obesity is preliminary and it would undoubtedly be of virtue to further investigate this response using a larger population sample so that a firmer conclusion can be established.

The observed exercise-induced degradation of type II fibre IMTGs in obesity is consistent with Shepherd and colleagues (Shepherd et al. 2012) who reported a reduction in type II fibres (mean±SD reduction was 37±5%) following 60 minutes of resistance exercise in sedentary men. However, our finding contrasts with others who observed no significant reduction in type II fibre IMTG content during prolonged moderate-intensity cycling exercise using young, active men (De Bock et al. 2005) and endurance-trained men (van Loon et al. 2003). Of note, these three studies utilised oil red O (ORO) staining to image IMTG whereas the current study used bodipy, which has
been suggested to be the preferred dye of choice due to its narrower emission spectra and lack of influence on light transmission in comparison with ORO (Prats et al. 2013). Even so, it is clear that further work is needed to clarify the influence of exercise, as well as the timing of nutrition around exercise, on type II fibre IMTG degradation in obesity and it is to be reiterated that the low sample size with respect to muscle analysis in this study greatly limits the strength of our interpretations.

Indeed, the low sample size and divergent responses reported with respect to muscle analysis meant that the influence of the timing of nutrition around exercise on IMTG utilisation could not be clearly established. Nevertheless, insights from indirect calorimetry and blood measurements permit some speculation. For instance, our finding of a significantly higher exercise fat oxidation and circulating concentration of glycerol and NEFA during exercise in Fasted than Fed is consistent with De Bock and colleagues (De Bock et al. 2005). Interestingly, these changes in the metabolic milieu during exercise were paralleled with an elevated degradation of type I fibre IMTG. Accordingly, it could be that the lower exercise-induced fat oxidation in Fed vs. Fasted in the present study is due to a suppression of adipose and muscle tissue lipolysis, coupled with increased glucose utilisation through greater availability in the presence of increased insulin in the Fed state (Kiens, 2006). This suggestion is supported by the observed decline in glucose in Fed but not Fasted throughout exercise (Figure 5.4 A). Whilst we cannot elucidate the influence of feeding on modulating potential alterations in IMTG degradation during exercise, it is hoped that these initial insights and speculations will encourage future research to explore the influence using a larger sample size.
5.5.4 Practical considerations

In this study, each participant successfully performed 60 minutes of steady-state (65% \( \dot{V}O_{2\text{max}} \)) cycling exercise on two separate occasions. This duration of exercise was selected because it has previously been shown to induce IMTG degradation in lean individuals (Watt et al. 2002; Shepherd et al. 2012) and is recommended by the Institute of Medicine (IOM 2002) and the International Association for Obesity (Saris et al. 2003) to mitigate unhealthy body mass gain or regain after a period of weight loss in obesity. However, this researcher would like to point out that there was a large inter-subject response regarding the ease at which each participant completed the exercise sessions. Specifically, the mean±SD (range) RPE for the Fasted and Fed session was 12±2 (10-16; 10 = between ‘very light [9]’ and ‘light [11]’, 16 = between ‘hard [15]’ and ‘very hard [17]’) and 13±2 (10-15), respectively. This demonstrates that some subjects found the exercise bout considerably more difficult than did others, which could impact upon the adherence of this type of exercise in overweight and obese populations (both over the short- and long-term).

Interestingly, subjective RPE was (on average) significantly lower during the Fasted as compared with Fed condition. Specifically, 50% of participants (3 out of 6) reported a lower RPE during the Fasted bout whilst the other 50% reported no difference between conditions. Whilst speculative, this effect could be because feeding in close proximity to exercise caused some degree of GI distress during exercise. It is noteworthy that the
sample size in this study was low and therefore these findings can by no means be generalised to the entire population. While some people may prefer to exercise in the Fasted as compared with Fed state, others may prefer vice-versa and so recommending Fasted exercise to those who find it more difficult to perform could be an additional ‘barrier’ to exercise. Also interesting is that Fasted as compared with Fed exercise increased exercise fat utilisation, which could have potential implications for daily fat utilisation and fat balance (Iawayama et al. 2015). As such, performing exercise in the Fasted state could be a means of increasing the efficiency of exercise training, which would translate into greater clinical benefits with equal effort by the individual; an effect that could increase exercise adherence (Hansen, Strijcker & Calders, 2016).

In light of the above it would be important when designing and implementing exercise interventions aimed at improving one's metabolic health status that clinicians and practitioner's consider the viability of the exercise load and the nutritional state by which one commences exercise on a case-by-case basis, particularly if the aim is to prescribe an exercise regime that is enjoyable and, importantly, sustainable.

In conclusion, this study shows that an acute bout of aerobic exercise performed in the Fasted- as compared with Fed-state augments exercise fat oxidation, as well as the circulating concentration of plasma glycerol and NEFA during exercise in obesity. The data is suggestive of IMTG utilisation during aerobic exercise in this population, however the low sample size with respect to muscle analysis greatly limits any interpretation. The
influence of the timing of nutrition around exercise could not be established and is something future research should seek to explore.
5.5.5 References


- **Food and Agricultural/World Health Organisation/United Nations University.**


• Kiens B. Skeletal muscle lipid metabolism in exercise and insulin resistance. Physiol Rev 86: 205-243, 206.


Van Proeyen KV, Szlufik K, Nielens H, Ramaekers M, and Hespel P. Beneficial metabolic adaptation to endurance exercise training in the fasted state. *J Appl*


CHAPTER 6

GENERAL DISCUSSION
6.1 Summary of key findings

Prior to this thesis the links between the inter-subject variability in the capacity to oxidise fat (MFO) during exercise and metabolic health were only partially understood. Accordingly, the first aim of this thesis was to offer better resolution on the links between MFO and metabolic risk by determining the relationship between MFO and daily (i.e., 24-hour) fat oxidation, and MFO and insulin sensitivity, as surrogate markers for long-term metabolic health in healthy young men (Chapter 3). The first novel finding from this investigation was that there was a significant and positive association between MFO and 24-h fat oxidation (expressed as both g/min and %EnFO) and a significant and negative correlation between MFO and daily fat balance (g/d). A second original finding was that there was a significant and positive association between MFO and insulin sensitivity as determined using a surrogate marker (QUICKI). Considered collectively, these findings build upon the existing body of literature showing that one’s capacity to oxidise fat during exercise or whilst physically active could have important implications for metabolic health.

The new findings presented in Chapter 3 extend the need for a more comprehensive understanding of the factors that influence the inter-subject variability in MFO during exercise. This information is important as it could be used in the design of physical activity strategies to optimize fat oxidation and improve metabolic health. Prior to this thesis, the evidence linking plasma hormone and metabolite concentrations as correlates of MFO were limited and inconsistent, whereas the links between resting fat oxidation rate had not been established. Therefore, a second aim of this thesis was to
investigate the influence of selected plasma hormones and metabolites and resting fat oxidation on MFO during exercise. The study presented in Chapter 4 revealed novel relationships between circulating concentrations of NEFA, glycerol and insulin measured during exercise and MFO. Of particular note, the plasma glycerol response to exercise could uniquely reflect exercise-induced fat oxidation (or the metabolic processes underlying the regulation of fat oxidation during exercise). This study also demonstrated, for the first time, that resting fat oxidation was significantly and positively associated with MFO.

A better understanding of the factors that influence the capacity to oxidise fat during exercise could help to inform novel strategies to increase fat oxidation during exercise and subsequently exert a favourable influence on one’s metabolic health. At the start of this PhD project it was not known whether the timing of recent nutrition with respect to exercise is important in explaining the inability of aerobic exercise training per se to improve peripheral insulin sensitivity in obese populations. Accordingly, Chapter 5 was designed to investigate the influence of meal timing i.e., eating before or after an acute exercise bout, on fuel utilisation, the plasma hormone and metabolic response, and IMTG use during exercise in overweight and obese men. This study demonstrated that fasted, as compared with fed, exercise significantly increases exercise fat oxidation and suppresses carbohydrate oxidation; a response that was paralleled with a greater circulating concentration of plasma glycerol and NEFA during exercise in fasted vs. fed. Data also suggests type I and type II fibre IMTG degradation in obesity, however the power of this finding and the influence of feeding in modulating this response is limited.
by the low sample size (n=2) and divergent responses reported. Nevertheless, it is hoped that the insights provided from this study will prompt future research to explore the overall response to the timing of nutrition around exercise in a larger sample, as this could have important implications from the perspective of optimising metabolic health.

6.2 Maximal fat oxidation during exercise is positively associated with 24-hour fat oxidation and insulin sensitivity in young, healthy men.

This first study was designed to explore the relationship between MFO and 24-h fat oxidation, as the latter could be reflective of long-term susceptibility to perturbations in body mass (Zurlo et al. 1990) and/or composition (Ellis et al. 2010). The study reports, for the first time, significant and positive relationships between MFO (g/min) and 24-hour fat oxidation (expressed as g/d and %EnFO) and a significant and negative relationship between MFO and 24-h fat balance. It therefore builds upon the existing body of literature and lends support to previous suggestions that a high capacity for fat oxidation during exercise could be advantageous for metabolic health (Rosenkilde et al. 2010; Isacco et al. 2013).

Such findings linking MFO to 24-h FO advance a potential role for MFO as a marker of long-term body mass and/or composition. However, this researcher acknowledges that there are a number of important considerations that must first be brought to attention before any definitive conclusions can be made. Firstly, from the perspective of study
design it should be noted that the cohort demographics of the participants used in this study (i.e., young, healthy men) limit extrapolation to the general population. Whilst there is good evidence showing that the inter-subject variability in MFO is apparent across a range of populations i.e., obese (Croci et al. 2014: MFO 0.38 ± 0.19 g/min), sedentary overweight (Rosenkilde et al. 2010: mean±SD MFO in low-RER group 0.33±0.10 g/min, high-RER group 0.17±0.03 g/min), normal weight and healthy (Robinson et al. 2015: MFO 0.59±0.21 g/min), highly-trained individuals (Achten & Jeukendrup, 2003a: 0.52±0.19 g/min) and children (Chu et al. 2014) we restricted this study to a very specific population (young, healthy, recreationally active men) and it is not yet known whether the links between MFO and 24-h fat oxidation extend to other populations such as children, teenagers, women etc. Following up this type of work in other populations, particularly those at risk for disorders associated with disturbances in fat metabolism such as obesity, insulin resistance and type II diabetes, would be an important way forward in this area and this is something that future research should seek to explore. It is also important to re-emphasise (in light of the point made in the Discussion of Chapter 3) that it would be important in future work to confirm these observations under conditions of standardized dietary and physical activity levels.

The presumption that MFO could be a useful marker for the long-term regulation of body mass and/or composition is based on the correlational data presented (Chapter 3) and an important question raised from this study is whether MFO is associated with long-term changes in body mass and/or composition. Indeed, these findings could help to provide new insights into why there are responders and non-responders to exercise for
weight loss. For example, whilst this is clearly a complex issue, there is some evidence that a reduced fat oxidation (or alternatively, a high carbohydrate oxidation) during exercise drives higher ad libitum food intake in free-living conditions (Almeras et al. 1994). However, these authors measured exercise substrate utilisation using RQ and did not monitor short- and/or long-term changes in participants’ body mass or composition. Accordingly, they were unable to formulate a potential link between exercise fat oxidation, ad libitum food intake, and body mass and/or composition. To this researchers knowledge, the metabolic link to the compensatory changes in diet or activity that can occur in individuals who do not respond to exercise for weight loss has not been comprehensively studied and is at present a grey area.

Although it is beyond the scope of this thesis, it would be interesting to follow-up with the subjects studied in Chapter 3 (~every 5 years) to determine whether the absolute contribution of fat towards exercise energy expenditure is related to long-term changes in body mass and/or composition. This information could help offer a metabolic link explaining whether a lower contribution of fat towards exercise energy expenditure predicts changes in body mass and/or composition. If MFO is predictive of long-term changes in body mass and/or composition then a better appreciation of the mechanisms that underpin this association, as well as establishing viable nutrition intervention and/or exercise training strategies aimed at optimising MFO, would be important.

The data presented in Chapter 3 suggests that the measurement of MFO during exercise could have value in exercise prescription. For example, exercise training at the
intensity that elicits MFO (Fat_{max}) has been shown to be superior to moderate intensity interval training at increasing MFO during exercise and insulin sensitivity (Venables & Jeukendrup, 2008). Therefore, training at Fat_{max} could be recommended in order to optimise the rate of fat oxidation during exercise, which could confer improvements in ones metabolic health status. However, it is notable that for some individuals their MFO could occur at a very low exercise intensity (Fat_{max} for 19% [11 out of 57] of the participants employed in Chapter 4 occurred at an exercise intensity <35% \dot{V}O_{2\text{max}} meaning that in the absence of extremely prolonged exercise the absolute energy expenditure would likely be insufficient if their goal was to create an exercise induced energy deficit. Accordingly, this researcher believes that it is important the individual’s goals are accounted for before exercise and/or training strategies are recommended based upon an individual’s MFO.

A second novel finding from this study was the observed significant and positive relationship between MFO and insulin sensitivity (QUICKI), which affords the suggestion that a higher fat oxidation during physical activity could confer improvements in insulin sensitivity. From a mechanistic perspective, a higher fat oxidation during exercise could help to alleviate lipotoxic stress in skeletal muscle by reducing the accumulation of insulin resistance-inducing lipid metabolites such as ceramide, DAG and LCFA-CoA (Shaw et al. 2010). However, this is merely a speculation at present. The novel findings presented in Chapter 3 pave way for future research to investigate the role of changes in MFO on insulin sensitivity and, if a relationship exists, to explore the mechanistic basis
for this by using skeletal muscle sampling to delineate the influence of markers of lipotoxic stress on this response.

In an attempt to explain the association between MFO and 24-h FO, we explored the influence of a variety of factors on these variables. The data presented in Chapter 3 showed clearly that aerobic fitness ($\dot{V}O_{2\text{max}}$) was the primary driver of both MFO and 24-h fat oxidation, which is consistent with others who employed a more heterogeneous cohort (Venables et al. 2005). This finding would suggest that promoting both physical activity and physical fitness are important strategies to increase one’s capacity to oxidise fat during exercise.

Whilst it is clear that aerobic capacity is an important driver of MFO during exercise, it is interesting that cross-sectional studies demonstrate no relationship between aerobic training status and 24-h fat oxidation, which is reinforced by our additional analysis that showed the relationship between MFO and 24-hour fat oxidation remained when controlled for $\dot{V}O_{2\text{max}}$ as a potential confounding variable and suggests an independent relationship. Indeed, to date this work, and the work of others, have been mainly cross-sectional in nature. From a physiological perspective there is a need to perform carefully controlled longitudinal training studies of exercise training to establish if some of the relationships observed in Chapter 3, such as the links between MFO and $\dot{V}O_{2\text{max}}$, MFO and 24-h FO, and MFO and insulin sensitivity are indeed causal, as well as the investigation of underlying mechanisms and their significance for their long-term control of body weight, composition and metabolic health. The use of longitudinal training
studies could be used to delineate the relative role of changes in maximal aerobic capacity and the capacity to oxidize fat during exercise on daily patterns of substrate oxidation. This would help inform whether physical activity interventions designed to increase MFO should focus on improving one’s \( \dot{V}O_{2\text{max}} \) or instead concentrate on enhancing metabolic factors either directly or indirectly associated with \( \dot{V}O_{2\text{max}} \) (e.g., skeletal muscle fibre type, capillary density and/or oxidative capacity).

6.3 Plasma lipolytic markers, insulin and resting fat oxidation are associated with maximal fat oxidation during exercise

Appreciable efforts have been made by multiple research teams to uncover the factors that influence the inter-subject variability in fat oxidation during exercise (Goedecke et al. 2000; Venables et al. 2005; Rosenkilde et al. 2010; Isacco et al. 2014), however prior to this thesis a large proportion of this variation was unexplained and, in particular, the influence of selected plasma hormones and metabolites had not been established. This is important information to know, as it could help to inform the most favourable hormonal and metabolic milieu to optimise exercise fat oxidation, with implications thus for strategies to manipulate this milieu with overall potential benefits for metabolic health. Accordingly, the purpose of Chapter 4 was to investigate the relationships between various regulators and indices of fat oxidation during the transition through maximal fat oxidation with incremental exercise.
This chapter provides new insights into previously unidentified factors that influence MFO during exercise. Firstly, the study revealed significant relationships between plasma NEFA (positive relationship), glycerol (positive relationship) and insulin (negative relationship) concentrations at MFO with MFO. These relationships extend support for the integrated influence of lipolysis (as reflected by plasma glycerol concentration), the anti-lipolytic action of insulin and plasma NEFA availability as factors that influence one’s capacity to oxidise fat during exercise. In line with this, resting concentrations of NEFA and insulin were also significantly positively and negatively correlated with MFO, respectively. Interestingly, the significant associations between concentrations of NEFA and insulin at MFO with MFO were lost when their resting concentrations were included as a partial correlate. Moreover, the exercise-induced changes in NEFA and insulin were not correlated with the exercise-induced change in fat oxidation (i.e., resting to MFO). This suggests that NEFA and insulin are generally associated with MFO, but they are likely not the prominent drivers of the exercise-induced increase in fat oxidation.

The significant and positive relationship between plasma glycerol concentration during exercise and MFO remained when controlled for resting glycerol concentration and the exercise-induced change in glycerol was significantly and positively correlated with the exercise-induced change in fat oxidation. Coupled with previous work that demonstrates associations between lipolytic rate (i.e., rate of appearance of whole-body glycerol) with changes in fat oxidation, this novel finding suggests that plasma glycerol concentrations uniquely reflect the metabolic processes underlying the regulation of fat oxidation during exercise. However, as stated above plasma NEFA concentration did not correlate with
MFO when resting NEFA concentration was considered as a potential confounding variable. This makes it tempting to presume that a higher MFO is associated with a greater utilisation of IMTG, as opposed to a higher utilisation of adipose tissue derived fatty acids. This seems plausible given that glycerol released following IMTG degradation is not re-utilised within muscle, but is instead released into the circulation. However, it could also be due to greater re-esterification of NEFA in adipose tissue, as the source of glycerol for triglyceride synthesis in fat tissue is glucose (Holloszy & Kohrt, 1996).

Nevertheless, previous work shows that the absolute contribution of other fat sources (i.e., IMTG) to energy expenditure during exercise increases from 40% $W_{\text{max}}$ to 55% $W_{\text{max}}$ (0.29 ± 0.05 to 0.39 ± 0.03, respectively), whereas the contribution of free fatty acids remains similar (0.39 ± 0.04 to 0.41 ± 0.04, respectively; van Loon et al. 2001). Whilst speculative, this suggestion lends support to the findings in Chapter 3, which identified a higher MFO was associated with greater insulin sensitivity. As mentioned above, insulin resistance appears to be associated with increased accumulation of lipid metabolites known to interfere with insulin signalling pathways i.e. ceramide, diacylglycerol and LCFA CoA. If an elevated MFO induces a higher turnover of IMTG, this could help alleviate lipotoxic stress and augment insulin sensitivity. To this end, further investigation is necessary to fully delineate the influence of lipolytic rate and plasma NEFA kinetics as determinants of the inter-subject variability in MFO and determine the exact location of the fuel source.
Another interesting observation from this study is that there was significant and positive association between resting fat oxidation and MFO during exercise. This was found to be the case when resting fat oxidation was expressed both in relative (i.e., RER) and absolute (i.e., g/min) terms. This latter relationship is novel and builds upon the existing research in this area by offering a more resolute comparison between resting fat oxidation and MFO. This could be important information to know, particularly in relation to the role of accounting for resting (or ‘baseline’) fat oxidation in future studies i.e., in explaining differences between individuals (e.g., whether differences in MFO are exercise specific or reflect more generalized differences in fat oxidation) or in studies designed to assess the impact of an intervention on exercise fat oxidation (i.e., does an intervention affect exercise fat oxidation per se or more so general fat oxidation).

In additional analysis (the results of which are not presented in the experimental chapters of this thesis), this researcher explored the relationship between resting fat oxidation (g/min) and 24-h fat oxidation (g/d) for the 16 people who took part in both experiments presented in Chapters 3 and 4 (Figure 6.1). Whilst no significant relationship was observed (R=0.34 P=0.098), the correlation was directionally consistent with what might be anticipated and it is reasonable to assume that greater statistical power might have yielded a statistically significant difference. Future work may wish to explore the relationship between resting fat oxidation (g/min) with both MFO and 24-h fat oxidation across a range of populations. Knowing this could add value to the measurement of resting fat oxidation in research and/or clinical settings for predicting metabolic risk and/or risk of unfavourable perturbations in body composition. Indeed, the
links between MFO and resting fat oxidation (identified in Chapter 4 of this thesis) coupled with the links between MFO and 24-h fat oxidation and insulin sensitivity as surrogate markers of metabolic health (identified in Chapter 5 of this thesis) are suggestive of a role for resting fat oxidation as a useful tool for predicting metabolic risk. Reinforcing this supposition are two studies, which demonstrate a high RQ at rest is related to increases in fat mass (Shook et al. 2015) and exercise-induced changes in RQ from rest predict exercise-induced fat loss (Barewell et al. 2009). From the perspective of application, the measurement of resting fat oxidation is likely to be more easily applicable in the real world setting than measures of substrate utilisation during exercise or over a day (i.e., 24-h), in that it takes less time to conduct, places minor burden on the individual, and can be performed by a wider range of individuals (including those who are unable to perform treadmill exercise to exhaustion such as the elderly or disabled).

Consistent with the findings presented in Chapter 3, this study reported a significant and positive relationship between \( \dot{V}O_{2\text{max}} \) and MFO, but also between \( \dot{V}O_{2\text{max}} \) and resting fat oxidation. As Chapter 3 also demonstrated a positive relationship between \( \dot{V}O_{2\text{max}} \) and 24-h fat oxidation, these findings collectively imply that \( \dot{V}O_{2\text{max}} \) is an important determinant of fat oxidation under a range of physiological conditions (Figure 6.1). Nevertheless, these parameters remained significantly and positively correlated with MFO when controlled for \( \dot{V}O_{2\text{max}} \) as a potentially confounding variable, which adds further weight to above mentioned suggestion that other metabolic factors, either directly
or indirectly associated with $\dot{V}O_{2\text{max}}$, are also important drivers of an individual’s capacity to oxidise fat.

**Figure 6.1** The relationship between resting fat oxidation (FO), MFO and 24-h FO and the influence of cardiorespiratory fitness ($\dot{V}O_{2\text{max}}$) on these parameters. Parentheses denote correlation and significance when $\dot{V}O_{2\text{max}}$ is controlled for as a potential confounding variable.

The primary purpose of the study presented in Chapter 4 was to provide greater resolution on the factors contributing to the inter-subject variability in MFO during exercise. However, it is noteworthy that the full spectrum of potential influencers was not explored. For example, it is well established from intervention studies that nutritional
status can exert a large influence on the balance of substrates utilised during exercise, yet the contribution of habitual diet to the inter-subject variability was not explored in this study. Nevertheless, during the preparation of this thesis our research team performed a large-scale cross-sectional study (150 men, 155 women; age 25±6 yrs.; BMI 23±2 kg/m²; \( \dot{V}O_{2\text{max}} \) 50±8 ml/kg/min [data are mean ± SD]) that aimed to identify if habitual dietary intake independently predicts MFO in healthy men and women (Fletcher et al. manuscript in review). Briefly, we have demonstrated that habitual dietary intake makes a small (2.6%) but significant contribution to the observed inter-subject variation in MFO. Specifically, dietary intake of fat and/or carbohydrate can make significant and independent contributions to the individual variability in MFO. As well as habitual diet, it is known that other factors such as skeletal muscle fiber type (Wade et al. 1990; Goedecke et al. 2000) and genetic predisposition (Zurlo et al. 1990; Tourbo et al. 1998; Wu et al. 2015) can influence the contribution of fat towards energy expenditure. However, their contribution to explaining the observed inter-subject variability in MFO during exercise is currently unknown and is something that future research should seek to explore.

A limitation of the studies presented in Chapters 3 and 4 of this thesis (in addition to those already presented in the respective Chapters) is that a 24-h recall was not used to validate self-reported dietary intake and physical activity. Whilst this is something that future work in this area should consider, this researcher would like to point out that he performed internal validation of the self-report energy intake and expenditure measures and observed excellent consistency with energy balance calculated using the methods
described in this manuscript (4-d food and physical activity diaries) and objectively measured energy balance using whole-room calorimetry ($r=0.943, p<0.001, n=16$; unpublished observations based on data from Chapter 3; Robinson et al. 2016). As such, this researcher feels confident with the data presented in this thesis.

6.4 Influence of pre- or post-exercise feeding on substrate utilisation, lipolytic markers and insulin, and intramuscular triglyceride utilisation in obesity.

A recurring message throughout this thesis is that increasing one’s capacity to oxidise fat (MFO) whilst physically active could be beneficial for long-term metabolic health (Rosenkilde et al. 2010; Isacco et al. 2013; Robinson et al. 2015 [Chapter 3]). Accordingly, Chapter 4 provided further resolution on the key factors that influence MFO during exercise, in an attempt to inform strategies (e.g., dietary and/or training) aimed at optimising exercise fat oxidation, which ultimately could confer improvements in metabolic health. The next logical step for this thesis was to utilise these findings from a translational perspective and, as such, Chapter 5 provided a feeding strategy that attempted to offer the necessary modulation to test the significance of increasing exercise fat oxidation in a population at risk for cardiovascular disease and type II diabetes.

A key finding from this investigation, and one that is consistent with others (de Bock et al. 2005; Derave et al. 2007), was that fasted exercise induced a significantly greater
reliance on fat and lesser reliance on carbohydrate towards exercise energy expenditure, as compared with fed exercise. As anticipated based upon the data presented in Chapter 4 of this thesis (Robinson et al. 2016), circulating concentrations of plasma glycerol and NEFA were significantly higher in fasted than fed, with insulin being higher, albeit not significantly (probably due to a lack of statistical power), at both measurement time-points during exercise in the fed condition. These findings demonstrate that we were successful in creating a hormonal and metabolic milieu that favours greater fat utilisation during exercise in obesity.

Unfortunately, the low sample size and divergent responses reported with respect to muscle analysis meant the influence of these differences on IMTG degradation could not be established. Whilst the data suggests exercise-induced type I and type II IMTG degradation in obesity, this finding must be observed with caution in light of the low sample size and divergent responses reported. Indeed, other research has failed to show exercise-induced degradation of IMTGs in obese (Nellemann et al. 2014) and overweight populations (Larson-Meyer et al. 2006). Differences in study methodology, including between-study differences of the techniques used to measure IMTG content, could potentially explain these discrepancies. As such, it is this researcher’s belief that future work should strive to adopt a similar experimental set-up (i.e., similar exercise intensity, duration and type) such that a fair comparison can be made between studies and that the set-up is one that is practically applicable to the population at hand. For instance, in Chapter 5 we purposely chose to exercise participants for 1-h at a steady-state (65% \(\dot{V}O_{2\text{max}}\)) as this has previously been shown to induce IMTG degradation in
lean individuals (Shepherd et al. 2012; Watt et al. 2002). A study design that incorporates longer duration exercise (e.g. 1.5-3-h) may offer more resolution on the potential differences in fibre-specific IMTG use between conditions (i.e., fasted vs. fed), as this has been show to lead to greater reductions in IMTG content in lean individuals (Watt et al. 2002). However, it is unlikely that such a prolonged exercise bout is clinically translatable, whereas 60 minutes of exercise as used in this study is recommended by the Institute of Medicine (IOM 2002) and the International Association for Obesity (Saris et al. 2003) to mitigate unhealthy body mass gain or regain after a period of weight loss in obesity. The identification of strategies that augment IMTG turnover, and subsequently reduce the accretion of such metabolites, in this population is undoubtedly important. The next logical step would be to assess this response using a large and more diverse population sample and explore the influence of recent nutrition with respect to exercise in mediating alterations in IMTG content following an acute bout of aerobic exercise.

Interestingly, recent work by Ross and co-workers suggests there could still be merit to fasted exercise even if meal timing around exercise has negligible influence on IMTG content. These researchers demonstrated that reduced fat mass, particularly from visceral adipose tissue, is linked with improvements in insulin sensitivity (Brennan et al. 2014). Undoubtedly, fat balance (fat intake [+ synthesis] – oxidation) is the master regulator of fat loss and therefore increasing fat oxidation during exercise or whilst physically active could be a more influential means of creating a negative fat balance. In this respect, it is acknowledged as a limitation of the study presented in Chapter 5 that
(due to reasons of equipment availability) substrate utilisation around the exercise bout i.e., before and after exercise, was not assessed, and this is something future research should strive to determine such that a more complete picture of substrate utilisation during and around the exercise bout can be obtained. Nevertheless, the influence of fasted- versus fed-exercise on daily fat utilisation was recently addressed by Iwayama and colleagues (Iwayama et al. 2016) who demonstrated that, under energy-balanced conditions, 60 minutes of fasted exercise (50% \( \overline{VO}_{2\text{max}} \)) was superior to fed exercise at increasing daily fat utilisation; an effect that was independent of energy balance and could be due to transient carbohydrate deficits (Iwayama et al. 2015). As such, it is anticipated that the Fasted-exercise performed in Chapter 5 induced a greater reliance on fat oxidation during the entire period of stay, however it is accepted that this is merely speculation. It would be interesting to follow-up the work by Iwayama and colleagues by determining the contribution of fat sources (i.e., IMTG, subcutaneous and visceral adipose tissue derived FAs) in mediating the observed effect and to assess the long-term influence of Fasted- versus Fed-exercise on body fat mass and metabolic health, as well as its viability across and within a range of populations.

6.5 Final conclusions and practical application

The work contained within this thesis has developed new insights in the understanding of the role of the maximal capacity for fat oxidation (MFO) during exercise as a marker of metabolic health. In this respect, evidence demonstrating that 24-h fat oxidation and
insulin sensitivity is significantly and positively associated with MFO in young, healthy men is reported for the first time. From a translational perspective, this would suggest that to improve an individual’s metabolic health status it would be favourable to target a physical activity / exercise intervention that is aimed at increasing one’s capacity to oxidise fat during exercise (MFO). Though, this researcher would like to reiterate that further work is required to confirm these associations across a range of populations, including (and importantly) those at risk for adverse metabolic health outcomes (i.e., overweight / obese and/or insulin resistant individuals).

This thesis also offers new and supporting knowledge concerning the regulators and indices of fat oxidation across a range of physiological contexts i.e., at rest, during incremental exercise and over 24-h. This information could be useful in the design of physical activity interventions aimed towards increasing fat oxidation and improving metabolic health. Specifically, and consistent with others (Venables et al. 2005), the findings presented within Chapters 3 and 4 of this thesis demonstrate that $\dot{V}O_2\text{max}$ is an important determinant of MFO during exercise. However, the data also shows that the relationship between $\dot{V}O_2\text{max}$ and MFO, $\dot{V}O_2\text{max}$ and resting fat oxidation, and $\dot{V}O_2\text{max}$ and 24-h fat oxidation, can be dissociated. Accordingly, from a practical standpoint it would be important that clinicians / practitioners who are seeking to increase an individual’s overall capacity to oxidise fat focus the design of their physical activity / exercise interventions towards increasing one’s aerobic capacity ($\dot{V}O_2\text{max}$), but also metabolic factors either directly or indirectly associated with $\dot{V}O_2\text{max}$ such as skeletal muscle fiber type, capillary density and/ or muscle oxidative capacity.
There is no definitive evidence showing that one form of exercise is superior to another at increasing an individual’s capacity to oxidise fat during exercise and there are various forms of exercise that can increase \( \dot{V}\text{O}_2\text{max} \) and above-mentioned metabolic factors.

Therefore, it is this researchers belief that in order to increase ones capacity to oxidise fat during exercise (with potential favorable implications for metabolic health) focus should be placed on designing a physical activity / exercise regime that is practically suitable and sustainable by the individual - with its viability considered on a case-by-case basis. Indeed, this should also be the focus if a reduction in body fat is sought after and in this respect this researcher would like to reiterate that fat oxidation and fat loss are not synonymous, and ultimately it is the state of energy balance that will determine alterations in body composition. The recommendation for an individual to exercise in their optimal ‘fat burning’ zone (±10% Fat\(_\text{max}\)) may not be entirely appropriate, especially if this zone occurs at a low exercise intensity meaning that the overall energy expenditure incurred by exercise is likely to be insufficient to create an appropriate exercise-induced energy deficit. It may also not be applicable given that expensive technical equipment and skilled personnel are required to appropriately assess an individual’s MFO and Fat\(_{\text{max}}\). There is a low level of access to these tests for the general public as their availability is often (but not always) limited to a University laboratory setting, and so prescribing this type of exercise prescription could prove to be very difficult.
Chapter 4 also identified weak-to-moderate, albeit significant, relationships between plasma lipolytic markers, insulin and resting overnight-fasted fat oxidation with MFO and showed the plasma glycerol response to uniquely reflect exercise-induced fat oxidation. Accordingly, the next logical step for this thesis was to utilise these findings from a translational perspective and, as such, Chapter 5 provided a feeding strategy that attempted to offer the necessary modulation to test the significance of increasing exercise fat oxidation in a population at risk for cardiovascular disease and type II diabetes. Unfortunately, for reasons previously documented (Section 5.3.5), the influence of meal timing on IMTG degradation could not be established. However, the data shows clearly that fasted, as compared with fed, exercise augments exercise fat oxidation. Accordingly, recommending individuals perform select exercise sessions in the fasted-state will likely increase exercise fat oxidation, which could confer improvements in metabolic health and encourage a reduction in body fat mass (Iwayama et al. 2015). Though this recommendation should not come at the cost of reducing one’s likelihood of performing exercise itself; which could occur if an individual does not enjoy or have the appropriate knowledge to perform fasted exercise.

It is expected that the work contained within this thesis will encourage future research to (i) advance the potential role of MFO as a marker of metabolic disease risk across a wide range of populations, (ii) to further identify the factors that underpin the large inter-subject differences in MFO during exercise, and (iii) to explore the influence of recent nutrition with respect to exercise on fat and IMTG utilisation in obesity, with potential...
implications for the design of strategies (training and / or nutrition) aimed towards optimising metabolic health in obesity.
6.6 References


Appendix 1  Informed consent form: chapters 3 and 4

**Informed Consent Form**

Determining the capacity for fat oxidation during physical activity

**Location(s):**
Human Performance Laboratory, School of Sport, Exercise & Rehabilitation Sciences, University of Birmingham
Humans Metabolism Research Unit (HMRU), University Hospital, Coventry

**Investigators:**
Dr Gareth Wallis, Dr John Hattersley, Dr Victoria Burns, Scott Robinson

**Contact details:**
Scott Robinson, School of Sport, Exercise & Rehabilitation Sciences, The University of Birmingham, Edgbaston, Birmingham, B15 2TT. Tel: [Redacted]. E-mail: [Redacted]

Dr Gareth Wallis, School of Sport, Exercise & Rehabilitation Sciences, The University of Birmingham, Edgbaston, Birmingham, B15 2TT Tel: [Redacted]. E-mail: [Redacted]

**Name:**

**Address:**

**D.O.B:**

I have read the Participant Information Sheet related to this study discussed the investigation with ....................... who has explained the procedures to my satisfaction. I am willing to undergo the following procedures or assessments (please initial);

- General Health Questionnaire
- Height and Weight Measurement
- Treadmill Exercise Testing
- Blood collection
- Respiration Chamber Stay
  (if eligible for Visit 3)

I am willing to undergo the investigation but understand that I am free to withdraw at any time.

Signed………………………………
Appendix 2  General health and screening questionnaire: chapters 3 and 4

The University of Birmingham
School of Sport and Exercise Sciences

General Health and Screening Questionnaire

Screening No:

Name:  ..........................................................................................................................
Address: ....................................................................................................................
............................................................................................................................
............................................................................................................................
Phone/Email: ...........................................................................................................

Name of the responsible investigator for the study:
............................................................................................................................

Please answer the following questions. If you have any doubts or difficulty with the questions, please ask the investigator for guidance. Your answers will be kept strictly confidential.

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<td>1</td>
<td>You are.......</td>
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<td>2</td>
<td>What is your exact date of birth?</td>
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<td>Day........ Month........ Year..19.......</td>
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<td>So your age is......................... Years</td>
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<td>3</td>
<td>When did you last see your doctor?</td>
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<td></td>
<td>Year.............. More than a year.......</td>
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<td>Question</td>
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<td>4. Are you currently taking any medication?</td>
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<td>5. Has your doctor ever advised you not to take vigorous exercise?</td>
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<td>6. Has your doctor ever said you have “heart trouble”?</td>
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<td>7. Has your doctor ever said you have high blood pressure?</td>
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<td>8. Have you ever taken medication for blood pressure or your heart?</td>
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<td>9. Do you feel pain in your chest when you undertake physical activity?</td>
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<td>10. In the last month have you had pains in your chest when not doing any physical activity?</td>
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<td>11. Has your doctor (or anyone else) said that you have raised blood cholesterol?</td>
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<td>12. Have you had a cold or feverish illness in the last month?</td>
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<td>13. Do you ever lose balance because of dizziness, or do you ever lose consciousness?</td>
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| 14. a) Do you suffer from back pain  
   b) if so, does it ever prevent you from exercising?                                                   |     |    |
| 15. Do you suffer from asthma?                                                                                                               |     |    |
| 16. Do you have any joint or bone problems which may be made worse by exercise?                                                              |     |    |
| 17. Has your doctor ever said you have diabetes?                                                                                             |     |    |
| 18. Have you ever had viral hepatitis?                                                                                                        |     |    |
| 19. Do you know of any reason, not mentioned above, why you should not exercise?                                                             |     |    |
| 20. Are you completely sedentary in your lifestyle?                                                                                            |     |    |
| 21. Are you an exercise-trained athlete?                                                                                                      |     |    |
| 22. Are you accustomed to vigorous exercise (~1 h/week)                                                                                       |     |    |

I have completed the questionnaire to the best of my knowledge and any questions I had have been answered to my full satisfaction.

Signed:.............................................................................

Date:.............................................................................

Research team use only:

Height (m) =  
Weight (kg) =  
BMI (kg/m^2) =  

Visit 1 eligibility check

<table>
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<tr>
<th>Criteria</th>
<th>Tick = eligible, cross = ineligible</th>
<th>Comment from consenting and screening investigator</th>
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245
Consent form signed
Sex (male)
Age (18-49 years)
Health questionnaire (NO for Q4-21, YES for Q22)
BMI (18.5-30 kg/m²)

This participant is eligible / ineligible (circle as appropriate) to participate in this study

Signed: ___________________ (Scott Robinson, PI)       Date: ________

Appendix 3 Graded exercise Test Data Sheet: Chapters 3 and 4

Graded Exercise Test:

Date:                Subject Identification Number:

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<th>Time</th>
<th>Speed (km/h)</th>
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NOTES:

246
We would like you to keep this diary of everything you eat and drink over 4 consecutive days. Please include all food consumed at home and outside the home e.g. work, college or restaurants. It is very important that you do not change what you normally eat and drink just because you are keeping this record. **Please eat as you normally would do.**

**Day and Date**
Please circle the study day (1,2,3,4) and write the date at the top of the page each time you start a new day of recording.

**Time Slots**
Please note the time of each eating occasion into the space provided.

**What did you eat?**
Please describe the foods you eat in as much detail as possible. Be as specific as you can. The example day shows the level of detail needed such as –
Cooking methods: (fried, grilled, baked, micro-waved etc).
Any additions (sugar/sweeteners, sauces, pepper, salt, mustard, etc).
Type and amount of fat / oil used for cooking, e.g. teaspoon peanut oil, 15g butter.
What brands, e.g. Kellogg’s corn flakes, places food bought, fresh, frozen, tinned, raw.
What variety – e.g. semi skimmed milk, low fat pro-biotic yogurt, reduced salt, reduced sugar?
Whether soft drinks were low calorie (diet) or decaffeinated?
Were fruit juices UHT, pasteurised or freshly squeezed, made from concentrate
Products such as cheese, fish and meat were they smoked or not

E.g. a cheese sandwich is really 3 foods – Bread, Margarine and Cheese. What type / amount of bread? Type / amount of spread? Type / amount of cheese?

Remember to record all snacks and drinks throughout the day.

**Portion sizes**
Please use the kitchen weighing scales provided to weigh every item of food / drink consumed – Remember to Zero after each item of food, and check it’s in grams. Eat the amount you would normally eat – don’t use the scales to determine when to stop! When weighing is not possible, food quantities can be described using:
- household measures, e.g. one teaspoon (tsp) of sugar, two thick slices of bread, 4 tablespoons (tbsp) of peas, ½ cup of gravy, large portion of takeaway chips. Be careful when describing amounts in spoons that you are referring to the correct spoon size.
- use weights from labels, e.g. 4oz steak, 420g tin of baked beans, 125g pot of Yoghurt – but only if everything is eaten – did you eat the fat/skin/bones?
- number of items, e.g. 4 fish fingers, 1 Rich Tea biscuit, 1 king size mars bar
With foods such as fruit, remember to record the weight of the skin/core/stone in the weight leftover column

For drinks, quantity can be described using:
- weight (grams), the size of glass, cup etc (e.g. large glass) or the volume (e.g. 300ml, 1 pint).
- volumes from labels (e.g. 330ml can of fizzy drink, 500ml strawberry Innocent smoothie).

We would like to know the amount that was actually eaten which means taking into account leftovers. You can do this in two ways:
1. Record what was served and note what was not eaten e.g. 30g of peas, only 12g eaten; 1 Weetabix, ate ½
2. Only record the amount actually eaten i.e. 18g of peas; ½ Weetabix

Homemade dishes
If you have eaten any homemade dishes e.g. chicken casserole, please record the name of the recipe, ingredients with amounts (including water or other fluids) for the whole recipe, the number of people the recipe serves, and the cooking methods used. Write this down in the recipe section at the end of the diary. Record how much of the whole recipe you have eaten in the portion size column.

Take-aways and eating out
If you have eaten a take-aways or eaten dishes not prepared at home such as at a restaurant or a friend’s house, please record as much detail about the ingredients as you can e.g. vegetable curry containing chickpeas, aubergine, onion and tomato. Please also record the name of the restaurant, takeaway, and the name of the meal, e.g. Domino’s pizza, 16inch meat feast, thin base.

Brand name
Please note the brand name (if known). Most packed foods will list a brand name, e.g. Bird’s eye, Hovis, or Supermarket own brands. For ready-made meals or for less well known brands, please keep the packet’s nutritional information in the bag provided.

Supplements / Medications
Please also provide information about any supplements you took. Please record the brand name, full name of supplement, strength and the amount taken should be recorded, e.g. Maximuscle cyclone powder – 40g (2 scoops), Holland and Barret Cod Liver Oil and Glucosamine Capsules (500mg) – 1 capsule. If you take any new medicines or stop taking ones we know about please record it here.

Was it a typical day?
After each day of recording please tell us whether this was a typical day or whether there were any reasons why you ate and drank more or less than usual. E.g. Drank 4 pints of Guinness as it was St Patricks day, day 2 ate very little as not feeling well.

When to fill in the diary
Please record your eating as you go, not from memory at the end of the day. Use written notes if you forget to take your diary with you and fill out your diary ASAP. Each diary day covers a 24hr period, so please include any food or drinks that you may have had during the night. Remember to include foods and drinks between meals (snacks) including water.

Overleaf you can see an example day that have been filled in. These examples show you how we would like you to record your food and drink, and how to record a homemade dish.

Please document what you ate & drank in as much detail as possible, Remember If it has passed your lips record it! 😊

It only takes a few minutes for each eating occasion!

We thank you for your efforts in filling out this diary.
Recipes / Takeaways
Write in recipes of ingredients of homemade dishes or take-aways

<table>
<thead>
<tr>
<th>Name of Dish</th>
<th>Fairy Cakes</th>
<th>Serves: makes 20 cakes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ingredients – Description, Brand,</strong></td>
<td><strong>Amounts</strong></td>
<td></td>
</tr>
<tr>
<td>Tate &amp; Lyle caster sugar</td>
<td>175g</td>
<td></td>
</tr>
<tr>
<td>Anchor butter, unsalted</td>
<td>175g</td>
<td></td>
</tr>
<tr>
<td>ASDA, free range eggs</td>
<td>3 eggs, 172g (no shell)</td>
<td></td>
</tr>
<tr>
<td>ASDA Self raising flour</td>
<td>175g</td>
<td></td>
</tr>
<tr>
<td>Co-op Baking powder</td>
<td>1 teaspoon</td>
<td></td>
</tr>
<tr>
<td>Silver Spoon Icing Sugar</td>
<td>140g</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>10ml</td>
<td></td>
</tr>
</tbody>
</table>
Description of cooking method

Mix all ingredients (1-5) together, then separate into 20 equal portions in cup-cases, cook in oven for 15mins

Mix ingredients 6 and 7, pour on top of individual cakes after they are cooked and cooled.

<table>
<thead>
<tr>
<th>Name of Dish</th>
<th>Big Joe’s 16” Meat Feast pizza</th>
<th>Serves: 2 equal portions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients</td>
<td></td>
<td>Amounts</td>
</tr>
<tr>
<td>Deep pan pizza base</td>
<td>16 inch, weight unknown</td>
<td>Tomato Sauce based</td>
</tr>
<tr>
<td>Green peppers</td>
<td>Half green bell pepper</td>
<td>Spicy salami</td>
</tr>
<tr>
<td>Pepperoni</td>
<td>~ 20 small slices</td>
<td>Tandoori chicken pieces</td>
</tr>
<tr>
<td>Beef meatballs</td>
<td>6 small meatballs</td>
<td>Onion</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time</th>
<th>Item / Description</th>
<th>Brand / variety</th>
<th>Preparation</th>
<th>Amount</th>
<th>Left-overs</th>
</tr>
</thead>
<tbody>
<tr>
<td>07.00</td>
<td>Coco pops</td>
<td>Asda</td>
<td></td>
<td>82g</td>
<td></td>
</tr>
<tr>
<td>07.15</td>
<td>Raisins</td>
<td>Tesco value</td>
<td></td>
<td>27g</td>
<td></td>
</tr>
<tr>
<td>07.15</td>
<td>Skimmed Milk</td>
<td>Asda</td>
<td></td>
<td>322g</td>
<td></td>
</tr>
<tr>
<td>07.50</td>
<td>Pure Orange Juice, not from concentrate</td>
<td>Tropicana – with the bits</td>
<td>1 large Glass - 457g</td>
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<td></td>
</tr>
<tr>
<td>09.15</td>
<td>Crunchy Granola Oats and Honey</td>
<td>Nature Valley</td>
<td></td>
<td>1 bar – 42g</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>Meal/Drink</td>
<td>Brand/Description</td>
<td>Ingredients</td>
<td>Amount</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>------------------------------------------------</td>
<td>--------------------------------------------</td>
<td>----------------------------------------------------------------------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>10.30</td>
<td>Apple</td>
<td>Golden Delicious</td>
<td>Raw</td>
<td>120g</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17g core</td>
<td></td>
</tr>
<tr>
<td>12.00</td>
<td>Hummus Sandwich</td>
<td>Home made</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bread (Tesco the finest)</td>
<td>2 slices</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tomato</td>
<td>84g</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lettuce</td>
<td>35g</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chicken Sandwich</td>
<td>105g</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Butter</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Cucumber</td>
<td>47g</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Water</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Mug of strong tea – Same as above</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.30</td>
<td>Can of Coca-Cola zero</td>
<td>Coca-Cola</td>
<td>1 can 330ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.45</td>
<td>Grande Latte</td>
<td>Starbucks</td>
<td>Extra shot of coffee + vanilla syrup</td>
<td>Grande size</td>
<td></td>
</tr>
<tr>
<td>15:45</td>
<td>Chocolate Brownie</td>
<td>Starbucks</td>
<td></td>
<td>154g</td>
<td></td>
</tr>
<tr>
<td>16.30</td>
<td>Water</td>
<td>Tap</td>
<td></td>
<td>1 pint</td>
<td></td>
</tr>
<tr>
<td>18.30</td>
<td>Pizza - meat feast</td>
<td>Big Joe’s – see recipe</td>
<td>Takeaway</td>
<td>Half of 16 inch</td>
<td></td>
</tr>
<tr>
<td>18.45</td>
<td>Diet Lemonade</td>
<td>Aldi</td>
<td></td>
<td>1.5pints</td>
<td></td>
</tr>
<tr>
<td>20.00</td>
<td>Beer normal strength (5%)</td>
<td>Carlsberg</td>
<td></td>
<td>1 pint</td>
<td></td>
</tr>
<tr>
<td>20.20</td>
<td>Beer normal strength (5%)</td>
<td>Carlsberg</td>
<td></td>
<td>1 pint</td>
<td></td>
</tr>
<tr>
<td>20.45</td>
<td>Beer normal strength (5%)</td>
<td>Carlsberg</td>
<td></td>
<td>1 pint</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>Item / Description</td>
<td>Brand / variety</td>
<td>Preparation</td>
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<td>Left-overs</td>
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<tr>
<td>21.20</td>
<td>Beer normal strength</td>
<td>Carlsberg (5%)</td>
<td></td>
<td>1 pint</td>
<td></td>
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<tr>
<td>22.00</td>
<td>Red-bull, normal (not diet)</td>
<td></td>
<td></td>
<td>1 glass</td>
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<tr>
<td></td>
<td>Vodka</td>
<td>Smirnoff</td>
<td>Double measure</td>
<td></td>
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<tr>
<td>22.30</td>
<td>Toast (Tesco the finest)</td>
<td>Tesco, sliced, multigrain</td>
<td>toast</td>
<td>2 slice</td>
<td></td>
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<tr>
<td></td>
<td>Baked beans – reduced sugar.</td>
<td>Tesco</td>
<td>Microwave</td>
<td>420g</td>
<td></td>
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<tr>
<td></td>
<td>Chilli Sauce</td>
<td>Nandos extra hot peri peri sauce</td>
<td></td>
<td>3 desert spoons</td>
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<tr>
<td>22.45</td>
<td>Water</td>
<td>Tap water</td>
<td></td>
<td>1 pint</td>
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</table>

Notes: This day I drank more alcohol than normal as it was a colleagues birthday.
<table>
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<tr>
<th>Time</th>
<th>Item / Description</th>
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<td>Preparation</td>
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<td>Leftovers</td>
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Notes

Date __/__/__
<table>
<thead>
<tr>
<th>Time</th>
<th>Item / Description</th>
<th>Brand / variety</th>
<th>Preparation</th>
<th>Amount</th>
<th>Left-overs</th>
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</table>

Notes

Date __/__/__
Write in recipes of ingredients of homemade dishes or take-aways

<table>
<thead>
<tr>
<th>Name of Dish</th>
<th>Serves:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients – Description, Brand,</td>
<td>Amounts</td>
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<tr>
<td>Description of cooking method</td>
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<tr>
<td>Name of Dish</td>
<td>Serves:</td>
</tr>
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<td>--------------</td>
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<tr>
<td>Ingredients – Description, Brand,</td>
<td>Amounts</td>
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</tbody>
</table>

Description of cooking method
Appendix 5  24-h Physical Activity Diary: Chapters 3 and 4

**Twenty-Four Hour Activity Diary**

We would like to find out about your normal physical activity levels.

Please fill in this diary continuously throughout the day, starting from when you get out of bed in the morning to when you go back to bed at night. The diary is split into the hours of the day, and then four fifteen-minute periods for that hour. In each box, write the code number (see below) which corresponds to the activity which you have carried out during this fifteen minute period. Please fill in all the boxes.

Make any notes you feel appropriate during the hours or at the bottom.

The first page is an example which has been done for you.

Please do not wait until the end of the day to fill this out – fill out as you go!

<table>
<thead>
<tr>
<th>Code Number</th>
<th>Description and Activity examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sleeping, resting in bed, or lying down.</td>
</tr>
<tr>
<td>2.</td>
<td>Resting in bed, lying down (still awake)</td>
</tr>
<tr>
<td>3.</td>
<td>Sitting, eating, watching TV, reading, listening to music, writing, sewing, talking on phone etc.</td>
</tr>
<tr>
<td>4.</td>
<td>Standing fairly still for example in a Que, or showering, driving a car</td>
</tr>
<tr>
<td>5.</td>
<td>Standing tasks – dish washing, cooking, vacuuming, ironing, playing a musical instrument, dressing, yoga, slow walk &lt;2.5mph</td>
</tr>
<tr>
<td>6.</td>
<td>Walking at a moderate pace (2.8 – 3.5mph), playing with children (moving), brushing/cleaning, gardening, washing windows.</td>
</tr>
<tr>
<td>7.</td>
<td>Golf, bowling, darts, very gentle cycling, juggling, non-competitive volleyball, table tennis, tai chi, archery, yoga</td>
</tr>
<tr>
<td>8.</td>
<td>Digging, sawing, mowing lawn, walking 3mph whilst carrying 10kg, cycling ~10mph</td>
</tr>
<tr>
<td>9.</td>
<td>Cycling &gt;10mph, Heavy Resistance (weight) training, hard exercise class, ballet, basketball, volleyball, wrestling, Tennis (doubles), general dancing</td>
</tr>
<tr>
<td>10.</td>
<td>Hockey, fencing, cricket, badminton (competitive), squash, tennis (singles), gym cross-trainer, rock climbing, circuit class, intense aerobics, rowing, recreational football, lacrosse, running 5mph</td>
</tr>
<tr>
<td>Time</td>
<td>0 - 15 mins</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>00:00-01:00</td>
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<tr>
<td>14:00-15:00</td>
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<tr>
<td>15:00-16:00</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>Time</td>
<td>0 - 15 mins</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>22:00-23:00</td>
<td></td>
</tr>
<tr>
<td>23:00-24:00</td>
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</table>

Date __/__/__
<table>
<thead>
<tr>
<th>Time</th>
<th>0 - 15 mins</th>
<th>16 - 30 mins</th>
<th>31 - 45 mins</th>
<th>46 - 60 mins</th>
<th>Activity / Intensity</th>
<th>Notes</th>
</tr>
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<tbody>
<tr>
<td>23:00-24:00</td>
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<td></td>
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</table>


Appendix 6  An example of a day’s food intake whilst in the respiratory chamber, including timings of meal and snack provision: Chapter 3.

<table>
<thead>
<tr>
<th>Meal component (time of intake)</th>
<th>Portion (g)</th>
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</thead>
<tbody>
<tr>
<td><strong>Meal 1 – Breakfast (07:45-08:00)</strong></td>
<td></td>
</tr>
<tr>
<td>wholemeal toast</td>
<td>120</td>
</tr>
<tr>
<td>margarine</td>
<td>20</td>
</tr>
<tr>
<td><strong>Snack (10:15-10:30)</strong></td>
<td></td>
</tr>
<tr>
<td>digestive biscuit</td>
<td>15</td>
</tr>
<tr>
<td>chargrilled chicken slices</td>
<td>160</td>
</tr>
<tr>
<td><strong>Meal 2 – Lunch (12:15-12:30)</strong></td>
<td></td>
</tr>
<tr>
<td>soup</td>
<td>400</td>
</tr>
<tr>
<td>brazil nuts</td>
<td>30</td>
</tr>
<tr>
<td>ciabatta roll</td>
<td>90</td>
</tr>
<tr>
<td><strong>Meal 3 – Dinner (17:15-17:30)</strong></td>
<td></td>
</tr>
<tr>
<td>baked potato</td>
<td>400</td>
</tr>
<tr>
<td>baked beans</td>
<td>200</td>
</tr>
<tr>
<td>chargrilled chicken slices</td>
<td>120</td>
</tr>
<tr>
<td>sports drink</td>
<td>125</td>
</tr>
<tr>
<td><strong>Snack (20:00-20:15)</strong></td>
<td></td>
</tr>
<tr>
<td>digestive biscuit</td>
<td>30</td>
</tr>
<tr>
<td><strong>Energy and macronutrient intake</strong></td>
<td></td>
</tr>
<tr>
<td>energy (kcal)</td>
<td>2207</td>
</tr>
<tr>
<td>carbohydrate (g)</td>
<td>273</td>
</tr>
<tr>
<td>fat (g)</td>
<td>67</td>
</tr>
<tr>
<td>protein (g)</td>
<td>124</td>
</tr>
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</table>
Appendix 7  Written Consent Form: Chapter 5

CONSENT FORM

Title of Project: Influence of pre- or post-exercised food intake on muscle metabolism in obesity

Name of Researchers: Mr Scott Robinson, Dr Gareth Wallis, Dr Konstantinos Manolopoulos

Please initial all boxes

1. I confirm that I have read and understand the information sheet dated 24/JAN/2015 (Version 1) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

3. I understand that relevant sections of my data collected during the study, may be looked at by individuals from the University of Birmingham. I give permission for these individuals to have access to my records.

4. I agree to take part in the above study.

____________________  ____________________  ____________________
Name of Participant  Date  Signature

____________________  ____________________
Address of Participant  

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### Appendix 8 General Health Questionnaire: Chapter 5

#### GENERAL HEALTH QUESTIONNAIRE

**Title of Project:** Influence of pre- or post-exercised food intake on muscle metabolism in obesity

Please answer the following questions. If you have any doubts or difficulty with the questions, please ask the investigator for guidance. Your answers will be kept strictly confidential.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Man</th>
<th>Woman</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>You are a.......</td>
<td>Man</td>
<td>Woman</td>
</tr>
</tbody>
</table>
| 2. | What is your exact date of birth?  
Day........... Month..........Year..19.......  
So your age is....................... Years | YES | NO |
| 3. | How would you describe your ethnicity? ............  
Please refer to ethnicity codes and enter a code. | YES | NO |
| 4. | When did you last see your doctor?  
In the:  
Last week............ Last month.......... Last six months..........  
Year............... More than a year.......... | YES | NO |
| 5. | Are you currently taking any prescription or non-prescription medication? | YES | NO |
| 6. | Has your doctor ever advised you not to perform vigorous exercise? | YES | NO |
| 7. | Has your doctor ever said you have “heart trouble”? | YES | NO |
| 8. | Has your doctor ever said you have high blood pressure? | YES | NO |
| 9. | Have you ever taken medication for blood pressure or your heart? | YES | NO |
| 10. | Do you feel pain in your chest when you undertake physical activity? | YES | NO |
| 11. | In the last month have you had pains in your chest when not doing any physical activity? | YES | NO |
| 12. | Has your doctor (or anyone else) said that you have raised blood cholesterol? | YES | NO |
13. Have you had a cold or feverish illness in the last month?  
   - YES  
   - NO

14. Do you ever lose balance because of dizziness, or do you ever lose consciousness?  
   - YES  
   - NO

15. a) Do you suffer from back pain  
   b) If so, does it ever prevent you from exercising?  
   - YES  
   - NO  
   - YES  
   - NO

16. Do you suffer from asthma?  
   - YES  
   - NO

17. Do you have any joint or bone problems that may be made worse by exercise?  
   - YES  
   - NO

18. Has your doctor ever said you have diabetes?  
   - YES  
   - NO

19. Have you ever had viral hepatitis?  
   - YES  
   - NO

20. Are you currently participating in another clinical study?  
   - YES  
   - NO

21. Are you aware of any known allergies to local anesthetic (such as lidocaine)?  
   - YES  
   - NO

22. Are you a current or recent smoker (last 30 days)  
   - YES  
   - NO

23. Have you previously (within 5 years of the present study) had 4 or more muscle biopsies obtained from the thigh quadriceps region?  
   - YES  
   - NO

24. Have you previously donated blood (last 12 weeks)?  
   - YES  
   - NO

27. If you are a woman, are you pregnant?  
   - YES  
   - NO

29. If you are woman, are you breastfeeding?  
   - YES  
   - NO

28. Have you gained/lost ≥2kg’s body mass in the past 3 months?  
   - YES  
   - NO

29. Do you know of any reason, not mentioned above, why you should not exercise?  
   - YES  
   - NO

30. Do you perform regular physical activity?  
   - YES  
   - NO

**Ethnicity codes**

1. White – British  
2. White – Irish  
3. Other – White background  
4. Black or Black British-Caribbean  
5. Black or Black British-African  
6. Other black background  
7. Asian or Asian British Indian  
8. Asian or Asian British Pakistani  
9. Asian or Asian British-Bangladeshi  
10. Chinese  
11. Other Asian background
12. Mixed – White and Black Caribbean
14. Mixed – White and Asian
15. Other mixed background
16. Other ethnic background
17. Other – please specify

Appendix 9 Screening Form: Chapter 5

SCREENING FORM (completed by investigator)

Title of Project: Influence of pre- or post-exercised food intake on muscle metabolism in obesity

Consent Form signed?  Yes □ No □

From General Health Questionnaire, is participant eligible to continue? To be eligible participants will have indicate 'No' to questions 5-30
  Yes □ No □

If No, inform participant they are not eligible. If Yes, continue with Screening Procedures

Demographic Details:
1. Sex  M/W (Circle)
2. Ethnicity Code
3. DOB ___/____/___
4. Age
5. Body Weight ____________ (kg)
6. Height _________________ (m)
7. BMI _________________  BMI = \( \frac{\text{mass (kg)}}{\text{height (m)}^2} \)
8. Waist Circumference (measured midway between top of the iliac crest and lowest rib) _______________ (cm)
9. Hip circumference (measured at the widest part of the buttocks, perpendicular to the trunk)
   _______________ (cm)
10. Blood Pressure ________________ mmHg
11. ECG administered □ Yes □ No □
   (This should now be sent to Dr Konstantinos Manolopolous)

Eligibility Check

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Tick = eligible, cross = ineligible</th>
<th>Comment from consenting and screening investigator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consent form signed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (18-49 years)</td>
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<td></td>
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<tr>
<td>Health questionnaire (NO for Q5-30)</td>
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<tr>
<td>At risk for cardio-metabolic disease</td>
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<tr>
<td>(anthropometric criteria)</td>
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<td></td>
</tr>
<tr>
<td>Resting blood pressure (&lt;140/90 mmHg)</td>
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<td></td>
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<tr>
<td>ECG outcome</td>
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</tr>
</tbody>
</table>

This participant is eligible / ineligible (circle as appropriate) to participate in this study

Name of Person conducting eligibility check  Date  Signature