METABOLIC EFFECTS OF PHYSICAL INACTIVITY AND INTERMITTENT ENERGY RESTRICTION

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ABSTRACT

The modern obesogenic environment favouring passive overfeeding and a diminished level of physical activity makes optimal health notoriously difficult to achieve. The overarching aim of this thesis was to investigate the effects of physical inactivity and 5:2 intermittent energy restriction (5:2 IER) on basal and postprandial metabolism.

Physical Inactivity (Chapter 3)

The Physical Inactivity study extended the findings of published research by evaluating the effects of two-days of physical inactivity, with (SIT=E) and without (SIT+E) a proportional decrease in energy intake, compared with a CONTROL arm (two consecutive days of standardised daily levels of moderate physical activity and matched energy intake) on basal and postprandial metabolism utilising a randomised crossover design. A physical inactivity model was used in which step counts were reduced by 80%. There was a strong tendency towards a significant difference in incremental area under the curve (iAUC) for serum inulin over 180 min postprandially between the groups (P = 0.05, η^2_p = 0.35). This was driven by the difference between the CONTROL vs. SIT+E trials. Compared with the CONTROL trial, the mean iAUC insulin was 25% higher in the SIT+E (P = 0.02) and 8% higher in the SIT=E (P = 0.39) trials. There were no differences in iAUC insulin between the SIT+E and SIT=E trials (P = 0.09). Thus, two-days of step reduction without the compensation of reduced energy intake tended to elevate postprandial insulinaemic excursion; this effect tended to be ameliorated, when accompanied by a compensatory reduction in energy intake to maintain energy balance.

5:2 Intermittent Energy Restriction (Chapters 4 and 5)

Intermittent energy restriction (IER) may overcome poor long-term adherence with continuous energy restriction (CER), for weight reduction. In a two-week randomised, parallel trial, 16 young, healthy-weight participants, were assigned to either CER (20% below estimated energy requirements (EER)) or 5:2 IER (70% below EER on two non-consecutive days; five days at EER, per week). Metabolic and appetite regulation markers were assessed before and for three hours after a liquid breakfast; followed by an *ad libitum* lunch; pre- and post-intervention. Weight loss

was similar in both groups: -2.5 (SEM 0.37) kg for 5:2 IER vs. -2.3 (SEM 0.26) kg for CER. There were no differences between groups for postprandial iAUCs for serum insulin, blood glucose or subjective appetite ratings. Compared with CER, 5:2 IER led to a reduction in fasting blood glucose concentrations (treatment-by-time interaction, $P = 0.018$, $\eta^2_p = 0.14$). Similarly, compared with CER, there were beneficial changes in fasting composite appetite scores after 5:2 IER (treatment-by-time interaction, $P = 0.0003$, $\eta^2_p = 0.35$) which may help to reduce the desire to eat.

Collectively, these findings highlight the important role of overfeeding in the negative effects of physical inactivity and provide novel insights into the metabolic effects of 5:2 IER. Replication and mechanistic assessments across different population groups and for longer periods of time are warranted.

PUBLICATIONS

Research article

Gao. Y., Tsintzas, K., Macdonald, I.A., Cordon, S.M., Taylor, M.A. Effects of intermittent (5:2) or continuous energy restriction on basal and postprandial metabolism: a randomised study in normal-weight, young participants. *Eur J Clin Nutr.* DOI: 10.1038/s41430-021-00909-2.

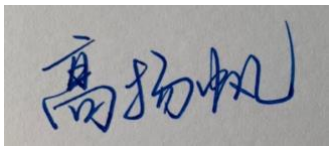
Conference abstract

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DECLARATIONS

- Sally Cordon performed all non-esterified fatty acid, triglyceride, total cholesterol, HDL-cholesterol and LDL-cholesterol analysis in all experimental chapters. In addition, to the insulin measurements in Chapter 3.
- Aline Nixon, Dr Tariq Taylor, Dr Lee Creedon, Dr Ayushman Gupta and Dr Natalie Shur assisted with cannulations (all experimental chapters).

I declare that the thesis is the result of my own work which has been undertaken during my period of registration for this degree at The University of Nottingham.

Signed: _____  _____

Date: _____14th March 2021 _____

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LIST OF ABBREVIATIONS

AA	Amino Acid
ADF	Alternate Day Fasting
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BDI	Beck Depression Inventory
BMI	Body Mass Index
BMR	Basel Metabolic Rate
BP	Blood Pressure
BW	Body Weight
CAS	Composite Appetite Score
CER	Continuous Energy Restriction
CGM	Continuous Glucose Monitoring
CHO	Carbohydrate
CIGMA	Continuous Infusion Glucose Model Assessment
CNS	Central Nervous System
COA	Coenzyme A
CONSORT	Consolidated Standards of Reporting Trials
CVD	Cardiovascular Disease
dAUC	Decremental Area Under the Curve
DIT	Diet Induced Thermogenesis
EAT-26	Eating Attitudes Test-26
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic Acid
EE	Energy Expenditure
EER	Estimated Energy Requirement
EI	Energy Intake
eTRF	Early Time-Restricted Feeding
FDA	Food and Drug Administration
F_ECO_2	Fraction of CO_2 in the Expired Air

FFA	Free-Fatty Acid
FFM	Fat Free Mass
FM	Fat Mass
GLP-1	Glucagon-like Peptide-1
GLUT2	Glucose Transporter 2
GLUT4	Glucose Transporter 4
H_2O_2	Hydrogen Peroxide
HbA1c	Glycated Haemoglobin
HDL	High Density Lipoprotein
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
HR	Heart Rate
iAUC	Incremental Area Under the Curve
IER	Intermittent Energy Restriction
IF	Intermittent Fasting
IL-6	Interleukin 6
IPAQ	International Physical Activity Questionnaire
IR	Insulin Resistance
IRS	Insulin Receptor Substrate
IVGTT	Intravenous Glucose Tolerance Test
LDL	Low Density Lipoprotein
METs	Metabolic Equivalent of Task
mL	Millilitre
MRI	Magnetic Resonance Imaging
MTT	Meal Tolerance Test
NAD^+	Nicotinamide Adenine Dinucleotide (Oxidised)
NADH	Nicotinamide Adenine Dinucleotide (Reduced)
NEFA	Non-Esterified Fatty Acid
NS	Not Significant
NCDs	Noncommunicable Diseases
OGTT	Oral Glucose Tolerance Test
PA	Physical Activity

PAEE	Physical Activity Energy Expenditure
PAL	Physical Activity Level
RBC	Red Blood Cell
RCT	Randomised Controlled Trial
REE	Resting Energy Expenditure
RER	Respiratory Exchange Ratio
RIA	Radioimmunoassay
RMR	Resting Metabolic Rate
SACN	Scientific Advisory Committee on Nutrition
SD	Standard Deviation
SEM	Standard Error of the Mean
SGU	Splanchnic Glucose Uptake
T2DM	Type 2 Diabetes Mellitus
TAG	Triacylglycerol/Triglyceride
TEE	Total Energy Expenditure
THL	Tetrahydrolipstatin
TNF-α	Tumour Necrosis Factor Alpha
TRF	Time-Restricted Feeding
VAS	Visual Analogue Scale
VAT	Visceral Adipose Tissue
V_{CO2}	Rate of Carbon Dioxide Production
V_{O2}	Rate of Oxygen Consumption
WHO	World Health Organization
YSI	YellowSprings^{TM} Glucose Analyser

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CHAPTER 1. LITERATURE REVIEW

1.1 General Background

Obesity, a state of abnormal or excessive fat accumulation in adipose tissue to the extent that health may be impaired (WHO, 2000, 2021). In light of the escalating prevalence of overweight and obesity in many countries, obesity has been proposed as a global pandemic. In 2016, 39% of adults aged 18 years and older were overweight, and 13% were obese, worldwide obesity tripled from 1975 to 2016 (WHO, 2020b). Obesity prevalence varies by population group with national statistics for England between the period of 01 April 2017 to 31 December 2018, showing obesity prevalence (including morbid obesity) appearing to be higher amongst women (30%) than men (27%); and in older age groups (in the age range 16 to 74 years) whilst lower in those above the age of 75 years. It was highest amongst men aged between 45 and 64 years (36% across these age groups), and amongst women aged between 45 and 54 years (37%) (National statistics, 2019). Obesity poses a major risk for serious diet-related non-communicable diseases (NCD), including Type 2 Diabetes Mellitus (T2DM), cardiovascular diseases (CVD), hypertension and stoke, and certain forms of cancer. Health consequences of obesity range from increased risk of premature mortality to serious chronic conditions that limit the overall quality of life (WHO, 2021a).

High availability of cheap, processed, energy-dense, and highly palatable food (Hall *et al.*, 2019) combined with physical inactivity (Brownson, Boehmer and Luke, 2005; Church *et al.*, 2011; Hruby and Hu, 2015) make the current environment potentially obesogenic. However, modest energy restriction and increased physical activity at the population level have been associated with a significant drop in body weight and the incidence, prevalence and mortality from CVD and T2DM (Look AHEAD Research Group *et al.*, 2007; Colberg *et al.*, 2010; Wing *et al.*, 2011). In the early 1990s, Cuba suffered a profound economic collapse. Severe absence of food resulted in a population-wide decrease in daily energy intake, from an average of 3,023 kcal/day between 1980-1990 to 2,452 kcal/day between 1991-1995. Concurrently, due to an international trade embargo by the US, petrol was unobtainable, and it was documented that more than one million bicycles were provided by the government to the population, resulting in a widespread increase in physical activity level (mainly attributed to walking and cycling). Both factors may have

contributed to an average weight loss per capita of 5.5 kg over the period between 1991-1995. From 1996 to 2002, there was a prominent and rapid decline in mortality from T2DM (by 50%) and coronary heart disease (by 34.4%). However, after the crisis, the prevalence of overweight or obesity rose from 33.5% in 1995 to 52.9% by 2010. A rebound in population body weight was followed by a 116% increase in T2DM prevalence and a 140% increase in T2DM incidence. In the years after 2002, T2DM mortality increased by 49% (from 9.3 deaths per 10,000 people in 2002 to 13.9 deaths per 10,000 people in 2010) (Franco *et al.*, 2013). The detrimental health effects could have been a consequence of physical inactivity, positive energy intake, or both. A greater understanding of the relative importance and interaction between these two factors would inform the design of future strategies to reduce obesity.

It has been suggested that as descendants of early *Homo sapiens*, who survived from the hunter-gatherer lifestyle over millennia, a 'Thrifty genotype' may have evolved. The 'Thrifty genotype', initially proposed by Neel (1962), being described as being highly efficient in fat storage during periods of abundance and/or utilisation of energy during periods of scarcity (Chakravarthy and Booth, 2004). This would accommodate periodic variation between feast and famine, hence ensuring substrate availability for continued hunting and spearfishing, despite intermittent availability of food. Such natural Darwinian 'positive' selection is postulated to have played a predominant role in shaping the modern human genome. However, genotype-environment interaction can determine an individual's susceptibility to becoming obese in response to their environment and determine an individual's risk of developing the serious comorbidities associated with obesity (Qi and Cho, 2008). Previous 'adaptation' may be a 'maladaptation' when food is continuously available, in plentiful supply; and sedentary behaviour is unchallenged by the need to be physically active to acquire food. Given the growing prevalence of obesity and associated metabolic disorders, the present obesogenic environment does not seem to have exerted a negative selection pressure against the 'Thrifty gene'. A recent interest in a weight reducing diet in which there are intermittent days of energy restriction (popularly known as the 5:2 diet), presents an interesting modern day parallel to the imposed intermittent dietary pattern of energy intake, when the 'Thrifty gene' was thought to provide selective advantage. In contrast to the past, when metabolic efficiency was desirable, ideally weight reducing diets, like the 5:2 diet would induce a less efficient use of energy; whilst improving metabolic health. Understanding the metabolic

consequences of intermittent energy restriction (IER) is important to ensure that such strategies are appropriate for weight loss and better health.

Having considered key concepts relating to obesity and energy metabolism, the following review will then explore the current literature relating to physical inactivity and IER, with particular reference to metabolic effects such as compromised glycaemic control.

1.2 Obesity

1.2.1 Definition and Classification of Obesity

Contrary to the modern cultural outlook, body fat has at some points been viewed as desirable, being synonymous with the success of an individual and a symbol of fertility. When fat is stored in the subcutaneous compartment, there are little deleterious effects of fat. However, the capacity to store subcutaneous fat is limited, resulting in deposition in other areas with harmful consequences. It is thus important to consider not only the quantity but also the location of fat deposition (Taylor and Holman, 2015).

Obesity is frequently characterised using the Body Mass Index (BMI, body weight in kilograms divided by the square of the height in meters), originally described as the Quetelet Index (Eknoyan, 2008). According to internationally accepted range of BMI classifications, an adult with a BMI below 18.50 kg/m^2 would be considered underweight, those with a BMI in the range of 18.50-24.99 kg/m^2 are considered normal weight. A BMI between 25.00 kg/m^2 and 29.99 kg/m^2 is classified as overweight and a BMI of 30.00 kg/m^2 and above is classified as obese. The WHO classification further categorises obesity into moderate, severe, and morbid obesity (**Table 1.1**). However, the actual cut-offs vary in different ethnic groups since disease risk in association to obesity varies with ethnicity (e.g. Caucasians and Africans vs. Southeast Asians) (Deurenberg-Yap *et al.*, 2000; Pan *et al.*, 2004; Shai *et al.*, 2006; Wen *et al.*, 2009). BMI is a simple, non-invasive, and routinely used index of body fatness at the population level. However, BMI fails to account for age, gender, growth curves in children and does not distinguish between weight associated with muscle mass and fat mass (Prentice and Jebb, 2001). BMI measures excess body weight rather than excess fat mass; conclusions based merely on BMI can be misleading at the

individual level. Some athletes may have high BMIs because of muscle bulk and are likely to be misclassified as overweight or obese (Ode *et al.*, 2007). However, in older individuals, age-related alterations in body composition due to muscle atrophy (i.e. reduced fat-free mass (FFM) and increased fat mass (FM)) and decrease in height due to kyphosis and compression of vertebral bodies may change the relationship between BMI and body fat percentage (Cook *et al.*, 2005; Villareal *et al.*, 2005).

Table 1.1 WHO classification of adults according to BMI (WHO, 2000).

Classification	BMI (kg/m^2)
Underweight	<18.50
Normal range (healthy weight)	18.50-24.99
Pre-obese (overweight)	25.00-29.99
Obese class I (moderately obese)	30.00-34.99
Obese class II (severely obese)	35.00-39.99
Obese class III (morbidly obese)	40 and above

As previously mentioned, body fat distribution is also an essential risk factor for obesity-related diseases (Klein *et al.*, 2007). Increased abdominal fat mass (i.e. abdominal, central, or visceral obesity) is related to some metabolic abnormalities, such as diminished glucose tolerance, decreased insulin sensitivity and unfavourable lipid profiles, which are risk factors for CVDs, T2DM and all-cause mortality (Després *et al.*, 2008; WHO, 2008). Waist circumference is a simple approach to evaluate abdominal adiposity (Ross *et al.*, 2020). Waist circumference is strongly associated with all-cause and cardiovascular mortality with or without BMI adjusted (Pischon *et al.*, 2008; Zhang *et al.*, 2008; Seidell, 2010; Song *et al.*, 2013; Cerhan *et al.*, 2014). Furthermore, waist circumference provides both independent and additional information to BMI for prediction of morbidity and mortality. Therefore, waist circumference is recommended as a screen tool for possible health risks related to overweight and obesity (Lean, Han and Morrison, 1995; Klein *et al.*, 2007; Siren, Eriksson and Vanhanen, 2012; Ross *et al.*, 2020). The cut-offs for waist circumference vary in different ethnic groups. Waist circumference cut-offs are 94 cm for European men and 80 cm for European women (Lean, Han and Morrison, 1995; WHO, 2008); whilst the cut-off points are lower for Asians (85 cm for men and 80 cm for women). This is because Asians tend to have an elevated metabolic risk at lower waist circumference than Europeans, explained by higher levels of body fat and abdominal adipose tissue (WHO, 2008).

1.2.2 Aetiology of Obesity: Excess Intake and/or Physical Inactivity

Although obesity is ultimately produced by a long-term positive energy balance, the causes of the imbalance between energy intake and energy expenditure are complex and potentially interrelated. Contributory factors include: genetic; behavioural (e.g. lack of physical activity, energy-dense foods ingestion, and irregular meal pattern); psychological (e.g. depression or stress may result in binge eating); physiological (e.g. ageing and pregnancy); medical factors (e.g. endocrine disorders such as hypothyroidism and polycystic ovary syndrome; and medications such as corticosteroids and antidepressants); socio economic and environmental components. Interactions between these factors are also important (Vandenbroeck, Goossens and Clemens, 2007).

It has been said that we are living in an increasingly obesogenic (obesity-inducing) environment (Swinburn and Egger, 2002). Obesogenic environments are those in which the surroundings, opportunities, and conditions of life make unhealthy choices the easy, default choice. It results from 'a multitude of genetic, biological, psychological, socio-cultural, and environmental factors that affect both sides of the energy balance equation' (Swinburn, Egger and Raza, 1999).

Obesogenic environments promote greater energy intake by impacting dietary behaviours and patterns. This is attributable to factors such as increased affordability of food, eating out and takeaway, and increased availability of convenience foods which are often energy-dense (Romieu *et al.*, 2017). Obesogenic environments also impact energy expenditure. The introduction of the car, mechanical aids (e.g. dishwashers), the computerisation of so many processes (e.g. desk jobs), and the advent of the internet have all promoted a substantial reduction in energy expenditure, by 750-1000 kcal/day (Klein *et al.*, 2004). All those factors become transformed into 'passive obesity'. The UK Foresight Programme, the government scientific think tank designed to inform strategic policy-making in light of science and technology, published a project report-Tackling Obesities: Future Choices in 2007 (Butland *et al.*, 2007). The report described passive obesity as follows: "The epidemic of 'passive obesity' is a normal response of our innate biology when confronted with modern living in 'obesogenic' environments so everyone, except the genetically very resistant, is guaranteed to put on weight except in unusual social circumstances". The report also emphasised that attempts to change behaviour at an individual level will be insufficient to produce population-

level change and that the obesogenic environment needs to be changed to create an environment where healthy choices are the easy choices (Butland *et al.*, 2007).

It has been observed that certain population are more susceptible to obesity. These include children with overweight or obese parents, socially deprived groups, ethnic minority groups, those with low levels of education, and people with learning difficulties (Vandenbroeck, Goossens and Clemens, 2007). Given the diverse range of factors that may contribute to obesity, different treatment approaches may be necessary for individuals and populations.

1.2.3 Consequences of Obesity

In 2010, 3.4 million deaths worldwide were attributable to overweight and obesity (Ng *et al.*, 2014). Globally, 44% of the diabetes burden, 23% of the ischemic heart disease, and approximately 7-41% of certain cancers in 2012 were attributed to obesity (Fried *et al.*, 2013; Frühbeck *et al.*, 2013). Main obesity co-morbidities include CVD, insulin resistance, diabetes, some cancers, obstructive sleep apnea, inflammation (e.g. atherosclerosis), gastrointestinal alterations, hypertension, pregnancy complications, psychosocial distress, osteoarthritis, and dyslipidaemia (Haslam and James, 2005; Fruh, 2017). The relative risk data for a range of diseases associated with obesity shows that people with obesity have a greater than three times likelihood of developing T2DM, gall bladder disease, dyslipidaemia, insulin resistance, breathlessness or sleep apnoea. People with obesity are two-three times more likely to develop coronary heart disease, hypertension, osteoarthritis of the knees, hyperuricaemia, or gout; and are also slightly more likely to develop certain cancers such as breast, endometrial or colon cancers, polycystic ovary syndrome, impaired fertility and back pain (WHO, 2000).

1.2.4 Treatment of Obesity and the Emerging Obesity Pharmacotherapy

Three approaches are recognised to achieve weight loss: lifestyle changes (including dietary management and physical activity), bariatric surgery, and pharmacotherapy. The principle objective of each of these is to achieve negative energy balance and reverse deleterious metabolic consequences, either directly, or secondary to weight loss. An understanding of energy balance

and the different metabolic states during feeding and fasting is essential when seeking to address obesity and its consequences.

Although the studies in this thesis mainly explore the topic of lifestyle intervention as a treatment to obesity, it is worth mentioning that the recent advent of clinical development of gut peptide analogues, which provide effective pharmacological treatments to obesity and are considered to have superior efficacy on weight loss over diet and lifestyle alone (Finer, 2021). A class of such analogues is glucagon-like peptide-1 (GLP-1) receptor agonist, which targets the human GLP1 system to exert a long-acting effect on improving appetite control to produce weight loss (Dimitriadis *et al.*, 2021). GLP-1 is a major incretin that regulates insulin and glucagon release. The combined effects of GLP-1 include inhibition of glucagon secretion, slowing of digestion, increasing satiety and reducing body weight (Nauck and Meier, 2018). The native GLP-1 releases rapidly within 15–30 min of nutrient ingestion. However, only 10–15% of secreted GLP-1 finally reaches the systemic circulation due to its restricted ~2 min half-life, which is mainly caused by a specific alanine site cleavage of GLP-1. GLP-1 agonists have a different extent of sequence homologies to native GLP-1 to ensure their affinity to GLP-1 receptors, and tend to have a substitution of the cleavage site amino acid alanine and hence provides resistance against cleavage and longer half-life (Sharma *et al.*, 2018). Initial proof of concept trials showed that participants receiving GLP-1 agonists reported lower appetite and food cravings, better control of eating and lower relative preference for fatty, energy-dense foods (Finer, 2021). More comprehensive rodent experiments and human clinical trials have shown superior clinical efficacy of several GLP-1 analogues in inducing and maintaining weight loss, which promoted Food and Drug Administration (FDA) and European Medical Association (EMA) approval of these analogues as licensed anti-obesity drugs in the US and EU market respectively (Anandhakrishnan and Korbonits, 2016). Therefore, the emerging obesity pharmacotherapy using the GLP-1 and other gut peptide analogues may contribute towards achieving successful and sustainable medical management of clinical obesity.

1.3 Energy Metabolism

1.3.1 Energy Balance

Obesity results when energy balance is positive. The first law of thermodynamics states that energy cannot be created or destroyed, it can only be transferred from one form to another – the total energy of the universe is constant. This law applies to the human body: the amount of chemical energy consumed in food and drink equals the energy output (i.e. heat, transport across membranes, mechanical work, and chemical energy used in synthesis for growth and maintenance); energy stored (i.e. high-energy phosphate bonds and other chemical bonds); and energy in waste products (e.g. in urine and faeces) (Hall *et al.*, 2012).

1.3.1.1 Energy Intake

The components of food and drink intake include micronutrients and macronutrients. Micronutrients include vitamins and minerals. Though they are not oxidised to yield energy, they are essential for facilitating the biochemical processes in the human body. Macronutrients are the primary source of dietary energy; including carbohydrate (CHO), fat and protein, with alcohol also able to contribute to energy intake. Values commonly used for the metabolisable energy content of CHO, fat, protein and alcohol are 3.75 kcal/g, 9 kcal/g, 4 kcal/g and 7 kcal/g, respectively (The Royal Society, 1972; Food Standard Agency and Public Health England, 2014). The relative contributions of the three major macronutrients to oxidation change across different metabolic states, such as fed and fasting, and at different levels of physical activity (FAO, 2001). Regulation of energy intake is discussed in section 1.5.

1.3.1.2 Energy Storage

CHO is stored as glycogen, and dietary fat is stored as triacylglycerol (TAG) in the human body. Unlike the first two, amino acids (AA) (usually consumed as dietary protein), are not stored as an energy reserve, but are used to synthesise proteins. Each protein has its function, such as structural component and enzyme. Within the human body, the amount of protein accounts for approximately 20% of the body weight, and the majority of protein exists in the skeletal muscle. AA oxidation is responsible for approximately 15% of daily oxygen consumption depending on the percentage of

total energy consumed as protein. Under nitrogen balanced conditions, daily protein intake roughly balances the daily protein oxidation. Therefore, in the typical western diet with dietary protein intake is approximately 70-100g/day, AA oxidation contributes 10-20% of the total oxidative metabolism. Body protein can be broken down to release AA for oxidation in prolonged fasting. Alcohol is not stored in the human body and remains in body water until eliminated. Alcohol oxidation begins immediately after absorption, and is ultimately entirely eliminated by oxidation (SACN, 2011; Cederbaum, 2012).

Glycogen synthesis commences with glucose 6-phosphate and includes sequential polymerisation of glucose units built upon a protein backbone (i.e. glycogenin), which dissociates from glycogen after the glycogen synthesis. Glycogen is a branched polysaccharide granule when extracted and dried; in muscle and liver, it is hydrated with approximately three times its own weight of water. Since monosaccharides are water-soluble, storing CHO in this form, or even oligosaccharides can raise osmotic problem, which may cause damages to the cells. This is overcome by synthesising a polymer from glucose, which effectively immobilises the glucose and reduces the osmotic effect. The fundamental role of glycogen stores is to maintain the blood glucose levels in a normal range. After food ingestion, the liver glycogen increases, if CHO intake is high enough then eventually some glycogen will be stored in muscle. Approximately 80% of glycogen (~ 400 g) (Taylor *et al.*, 1993) is stored in skeletal muscles and the majority of the rest is stored in the liver (~ 100 g) (Taylor *et al.*, 1996). On average, it takes 4-5 hours after meal ingestion for all the CHO to be stored away. Muscle glycogen can be utilised locally as an energy substrate for muscle, for a sudden burst of physical exertion. Since muscle lacks the enzyme glucose 6-phosphatase, skeletal muscles are incapable of liberating glucose. However, muscle glycogen can contribute to blood glucose indirectly, via glycolysis to lactate, which is then carried by the blood to the liver and made back into glucose through gluconeogenesis (Cori cycle). On the contrary, glucose 6-phosphatase is expressed in the liver, converting glucose 6-phosphate to free glucose via glycogenolysis. Therefore, glucose derived from glycogenolysis and produced by gluconeogenesis may help to maintain euglycaemia. The storage capacity of glycogen is limited, with an average male weighing 70 kg having ~ 500g glycogen contributing approximately 2000 kcal, which may be less than the energy requirement for one day (Frayn and Evans, 2019).

However, in modern society with typically three main meals per day, there is limited time for the glycogen to be utilised. Instead, the glycogen level will continue to increase after the second main meal and peaks after the third main meal in the day. When glycogen stores reach their maximum capability, the excess glucose will be transformed into fat in the liver. The newly formed fat can be used to provide energy for the liver or transported elsewhere in the human body. If the energy is not utilised within 24 hours, the fat stored in the liver will progressively accumulate. In the long run, excess energy intake will be transformed into fat (Taylor, 2008, 2013; Fabbrini, Sullivan and Klein, 2010).

TAG within adipose tissue (connective tissue consisting of lipid-rich cells called adipocytes) is the predominant form for the storage of excess energy, underpinned by the hydrophobic property of TAG molecules. TAG is an ester derived from one glycerol and three individual long-chain fatty acids. Compared with glycogen, TAG is a more efficient form for energy storage (in terms of energy content per unit weight of store). However, as a storage form for energy provision, the hydrophobic property of TAG makes it slow to be mobilised; mitochondria are necessary for its oxidation, which makes it unable to be used by erythrocytes or the renal medulla; fatty acids cannot be converted to CHO or protein, which limits metabolic flexibility; furthermore, brain metabolism does not prefer burning fatty acids to provide energy (Schönfeld and Reiser, 2013). Raised circulating free fatty acid (FFA) associated with obesity, may lead to insulin resistance by inhibiting insulin-stimulated glucose uptake and glycogen synthesis. It may also potentiate glucose-stimulated insulin secretion and activate inflammatory pathways (Frayn and Evans, 2019).

1.3.1.3 Energy Expenditure

Total energy expenditure (TEE) is the sum of basal metabolic rate (BMR), diet-induced thermogenesis (DIT), and physical activity energy expenditure (PAEE) (Westerterp, 2017).

A substantial proportion (60-70%) of daily energy expenditure is accounted for by the BMR, which supports maintenance of ion gradients across membranes, turnover of metabolic substrates and basic function such as cardiorespiratory function. Therefore, humans have evolved ways of energy storage to manage frequent periods of food shortage. BMR is measured when individuals are awake and resting after all food has been digested and absorbed, in a thermoneutral environment (SACN, 2011). BMR is determined principally by the lean body mass for both men and women.

BMR increases steadily as body weight increases and *vice versa* (Frayn and Evans, 2019). On average, men require slightly more energy per kg of the body weight than women since men tend to have more muscle. In an average adult human (70kg), the brain weighs approximately 1.3-1.4 kg, accounts for 2% of the body weight and requires a substantial amount of energy, accounting for approximately 20% of daily energy expenditure. Modern imaging techniques such as X-ray computed tomography, positron emission tomography, near-infrared spectroscopy and magnetic resonance imaging (MRI) are primary techniques available for the determination of brain metabolic rate during rest and activity (Raichle and Gusnard, 2002). Nowadays, the most widely employed technique for this purpose is the Blood Oxygen Level Dependent (BOLD) functional magnetic resonance imaging (fMRI) by measuring alterations in blood oxygenation in relation to brain activity, which was developed by Seiji Ogawa in 1990 (Ogawa *et al.*, 1990; Glover, 2011). The energy requirement of the brain is relatively constant, brain energy requirements may increase during cognitive type challenges and diminish in slow wave sleep. Glucose is the primary energy source for the human brain, except during prolonged starvation or chronic feeding of a high fat and low CHO diet (ketogenic diet) (Sokoloff *et al.*, 1955; Kety, 1957; Rolfe and Brown, 1997; Raichle and Gusnard, 2002).

The term DIT, also known as the thermic effect of food, refers to the cumulative energy expenditure required for gastrointestinal tract activity, digestion, absorption, and metabolic cost of energy storage (e.g. glycogenesis), contributing approximately 10% to TEE. Energy expenditure increases postprandially for approximately 5 hours and is principally influenced by energy content and macronutrient composition of the meal. It is typically measured by monitoring the changes in metabolic rate for several hours following the ingestion of a characterised meal of known energy content and macronutrient composition (Westerterp, 2004).

Physical activity is defined as any bodily movement produced by skeletal muscle that results in energy expenditure. Energy expenditure of physical activity accounts for 25-30% of daily energy expenditure. Physical activity is positively correlated with physical fitness. Apart from exercise, physical activity also comprises occupational, household, transportation, and other activities of daily life. Exercise is planned, structured, and repetitive bodily movement to improve or maintain physical fitness. Exercise is very positively correlated with physical fitness. PAEE is the most

variable element of TEE, independent of body size, due to the quantity, duration, type, and intensity of physical activity varying considerably (Ravussin *et al.*, 1986).

In addition to the three major components of energy expenditure, energy expenditure increases in some other circumstances. Energy expenditure for growth occurs in infants, but is negligible after the first few months of life (Butte *et al.*, 2000). Energy expenditure can be modified by environmental determinant (mainly ambient temperature). Energy expenditure increases when individuals are exposed to cold environment (through shivering) and hot (through panting) environment (Westerterp, 2017). Thermic effect of illness, such as multiple fractures, severe infection, severe burns and other pathological conditions, also lead to increased energy expenditure (FAO, 2005). Furthermore, medications such as amphetamines, bupropion and phentermine can stimulate energy expenditure (Coulter, Rebello and Greenway, 2018).

1.3.2 Metabolic States

As long ago as 1853, the renowned French physiologist Claude Bernard introduced his theory of the '*Milieu Intérieur*', which emphasised the significance of maintaining the internal environment constant for all substrates in the blood. This constancy is the state of a free and independent existence (Hoenig and Zeidel, 2014). In general, human feeding behaviour results in alternating postabsorptive and postprandial periods over each 24-hour period, with plentiful food available every day.

1.3.2.1 Postabsorptive State

The term postabsorptive state refers to the state that 'all of the last meal has been absorbed from the intestinal tract'. It typically represents the state existing after an overnight fast before breakfast starts (Frayn and Evans, 2019). During the postabsorptive state, euglycaemia is maintained by endogenous glucose production, almost exclusively from the liver, and to a lesser extent in the kidney (DeFronzo, 2004). **Figure 1.1** displays that this can be achieved by a combination of glycogen breakdown (hepatic glycogenolysis) and conversion of non-CHO substrates such as lactate, alanine (primarily from muscle), and glycerol (from adipose tissue lipolysis) (gluconeogenesis), processes that release glucose into the circulation (Rui, 2014). The ratio of these two resources mainly relies on the previous diet, which determines the amount of glycogen

stored. Glucose disposal in the postabsorptive state is ~ 2 mg of glucose per kg of body weight per minute and precisely matches the rate of endogenous glucose production, keeping the fasting blood glucose concentration tightly controlled, between 4.0 to 5.4 mmol/L (Sonksen and Sonksen, 2000). This homeostatic mechanism is vital since glucose is the primary source of energy for the brain. During this time period, approximately 50% of whole body glucose utilisation is oxidised by the brain (Grill, 1990; DeFronzo, 2004) and approximately 25% of glucose uptake happens in the splanchnic area (liver and gastrointestinal tissues) (DeFronzo *et al.*, 1985). Direct measurement of hepatic glucose uptake in humans is limited by the technical difficulty and ethical concerns, considering that the portal vein cannot be cannulated (Zheng *et al.*, 2010; Moore *et al.*, 2012). Thus, indirect methods quantifying splanchnic glucose uptake (SGU) have been developed. Currently, there are three methods used to assess SGU in humans. Hepatic vein catheterisation (HVC) allows the measurement of net hepatic glucose balance and derives total splanchnic glucose uptake by the assumption that endogenous glucose production is equal to zero (Felig, Wahren and Hendler, 1975). However, the application of the HVC technique is hampered by its time-consuming and invasive nature, by the uncertainty of the degree of gut absorption and by radiation exposure. The double-tracer method uses different tracers to differentiate the oral glucose load from the systemic glucose, and the oral glucose appearance and hepatic glucose retention can thus be determined. However, the calculation of glucose appearance rate is potentially confounded by the incorporation of glucose tracers into glycogen (Butler and Rizza, 1991). The oral glucose clamp technique was established and validated against the hepatic vein catheterisation to assess SGU noninvasively. This method starts with establishing a steady-state of glucose turnover using a hyperinsulinaemic clamp technique, that fully suppresses endogenous glucose production, and then computes the oral glucose appearance by lowering systemic glucose infusion required to maintain euglycaemia. Therefore, the oral glucose clamp and double-tracer techniques both measure initial or first-pass splanchnic glucose uptake, while the HVC technique measures the integrated glucose uptake during the process of glucose absorption, including glucose that has passed through the splanchnic area and has not been taken up by the peripheral tissues (Georg *et al.*, 2002; Zheng *et al.*, 2010). During fasting, whole-body metabolism is switched over to fatty acid oxidation. Decreased circulating levels of insulin contribute significantly to the increase in lipolysis by activating the enzyme adipose triglyceride lipase and enzyme hormone-sensitive lipase (Randle *et al.*, 1963; Frayn and Evans, 2019). The fatty acids, for the most part, leave the cells and

enter the plasma FFA pool, the sequentially elevated levels of plasma FFA promote their uptake and oxidation in tissues capable of using them as an energy source, such as the liver and skeletal muscle. The glycerol also leaves the cells, which becomes an increasingly important precursor for gluconeogenesis during fasting (Cahill, 2006).

As mentioned earlier, muscle glycogen is primarily a local energy substrate for a sudden burst of physical exertion, instead of an energy source for keeping blood glucose concentration stable during fasting. Although muscle glycogen can contribute to sustaining euglycaemia by the Cori cycle, no significant decrease in muscle glycogen content is reported during fasting in humans (Jensen *et al.*, 2011). On the contrary, the liver glycogen content experiences a rapid decrease by approximately 65% after 24-hour fasting (Magnusson *et al.*, 1992; Jensen *et al.*, 2011), and is exhausted after approximately 48 hours. During the prolonged absence of food, ketone bodies are synthesised (ketogenesis) in the liver, from acetyl-CoA derived from fatty acid β-oxidation, to compensate for the shortage of glucose. Ketone bodies can cross the blood-brain barrier and are therefore crucial to power the brain in prolonged fasting. However, ketone bodies will drop to an almost undetectably low level and account for a trivial proportion of energy metabolism in healthy people in the fed state.

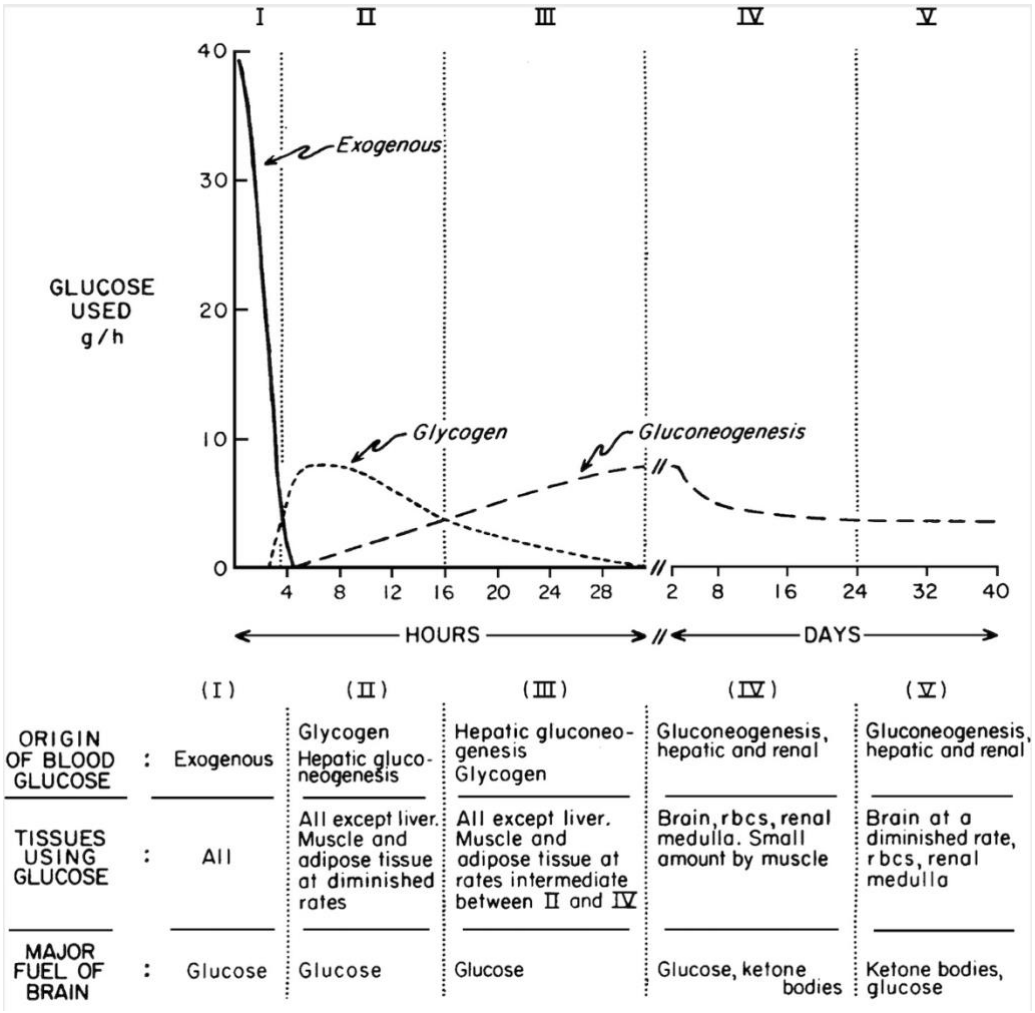


Figure 1.1 Glucose sources during fasting.
rbcs, red blood cells. Taken from Cahill (2006).

1.3.2.2 Postprandial State

The postabsorptive state is discontinued by the arrival of a meal, resulting in the switch of the predominant metabolic fuel selection from fat to CHO. The sequential influx of glucose into the circulation in response to mixed meal ingestion directly stimulates the process of insulin secretion within the β-cells of the pancreatic islets of Langerhans in a dose-response fashion (Byrne, Sturis and Polonsky, 1995), and indirectly by stimulating the release of incretin hormones, which are gut-

derived and can potentiate glucose-stimulated insulin secretion (Campbell and Drucker, 2013). Thus, there is a surge in insulin production and plasma insulin concentration rises by 10-15 fold.

Insulin is a peptide hormone, which binds with membrane receptors on its target cells. The insulin receptor has tyrosine kinase activity, such that the activated insulin receptor goes through auto-phosphorylation which leads to the phosphorylation of a group of interacting proteins known as insulin receptor substrates (IRSs). The phosphorylated IRSs initiate complex, yet not fully understood, intracellular cascades that influence cellular metabolic pathways, including inhibition of lipolysis, increased glucose transport and stimulation of glycogen synthesis. Such insulin-induced regulation on the metabolism of target cells is achieved either via direct inhibition or activation of certain enzymes by altering their catalytic activity, or, indirectly, via changing gene expression and mRNA translation through transcription factors (Petersen and Shulman, 2018).

The increase in insulin concentration has a biphasic effect on postprandial metabolism. The elevation in glucose and insulin initially suppresses the secretion of the counterregulatory peptide hormone glucagon, from pancreatic α-cells (Cooperberg and Cryer, 2010). The subsequent increase in the insulin/glucagon ratio reaching the liver, along with the inflow of glucose into hepatocytes via the glucose transporter 2 (GLUT2). The rate of GLUT2-mediated glucose transport is not responsive to insulin but determined by the relative glucose concentrations across the hepatocyte membrane. Glucose elevation outside of the hepatocyte will therefore increase hepatic glucose uptake. Meanwhile, insulin-activated hexokinase rapidly converts hepatic glucose to glucose 6-phosphate, which leads to lower intracellular glucose concentration relative to plasma concentration, which further reinforces the increase of hepatic glucose uptake (Chadt and Al-Hasani, 2020). Insulin also stimulates glycogen synthesis and endogenous glucose production suppression (i.e. release of glucose from glycogen and gluconeogenesis), which contribute to the metabolic adaptations from postabsorptive state to postprandial state (Frayn and Evans, 2019). Within approximately 30 min, endogenous glucose production by the liver is almost completely suppressed. The relatively constant energy requirement of the brain is met by the exogenous glucose from food for a few hours.

The liver receives the blood from the small intestine through the hepatic portal vein, nutrients and by-products of food digestion in the blood will be processed in the liver before continuing to the

heart. Therefore, the greatest increase in blood glucose level is observed in the liver and hepatic glucose uptake is primarily performed in an insulin-independent manner (Moore *et al.*, 2012; Frayn and Evans, 2019). In skeletal muscle and adipose tissue, glucose uptake is stimulated by the increased insulin level through translocation of glucose transporter 4 (GLUT4) to the cell membrane (Goodyear *et al.*, 1996; Frayn and Evans, 2019). In skeletal muscle which can use both fatty acids and glucose for energy, glucose is preferentially oxidised over fatty acids. Meanwhile, muscle glycogen synthase is activated by insulin, and muscle glycogen store, depleted by exercise, is replenished by glycogen synthesis. In adipose tissue, postprandial fatty acid uptake is stimulated by the increase of insulin level, glycerol 3-phosphate derived from glucose metabolism (glycolysis) is the backbone for TAG synthesis from FFA (Czech *et al.*, 2013).

1.4 Obesity, Type II Diabetes and the Role of Insulin

Diabetes is defined by elevated levels of blood glucose. In 1980, the number of adults with diabetes in the world was estimated at 108 million, since then, the numbers have crept up year by year and reached 422 million in 2014 (NCD Risk Factor Collaboration, 2016; WHO, 2020a). It is estimated that diabetes was the 7th leading cause of death in 2016 (WHO, 2020a). T2DM is considered primarily as the consequences of excess body weight and physical inactivity (WHO, 2020a). However, the accurate cause has not been ascertained (WHO, 2020a). The probability of suffering from severe complications occasioned by T2DM hinges on the age at which diabetes is diagnosed. T2DM diagnosed at a younger age is associated with a greater risk of severe problems (Deshpande, Harris-Hayes and Schootman, 2008). T2DM may result in damages to the eyes, nerves, feet, kidney, heart, and brain. However, the risk of developing these long-term disorders might be mitigated if the blood glucose level is well managed. The diagnosis of T2DM represents a point on a continuum of deterioration in glycaemic control, the severity of which is associated with degree of excess weight. A prediabetic state is defined as an intermediate state with impaired fasting glucose and/or impaired glucose tolerance but below the diabetes threshold. Prediabetes indicates an increased risk for the development of diabetes in the future (American Diabetes Association, 2015).

1.4.1 Insulin Resistance

Insulin is produced and secreted by the β-cells, within the pancreas gland. The pancreas is located in the rear of the abdominal cavity, with the head of the pancreas connected to the duodenum through the pancreatic duct. Rather than being collected into veins and taken back to the heart for recirculation, blood from the pancreas is collected into the hepatic portal vein which can carry the insulin straight to the liver. When blood glucose levels start to rise, more insulin is produced by the pancreas promptly, whilst when blood glucose levels start to fall, less insulin is produced by the pancreas. Thus, in metabolically healthy individuals, blood glucose concentrations are tightly controlled by precise changes in the plasma level of insulin. However, the sensitivity to the actions of insulin in the liver and in peripheral tissues differs from individual to individual (Frayn and Evans, 2019). A higher level of fasting plasma insulin reflects insulin-resistance since more insulin is required to maintain the level of blood glucose. From another perspective, it shows that the pancreas can compensate for an insulin-resistant liver, adipose tissue and skeletal muscle, to some extent, which further helps to keep the level of blood glucose in a reasonable range. In the liver, insulin does not stimulate glucose uptake but stimulates hepatic glycogen synthesis, TAG synthesis from glucose (*de novo* lipogenesis) (not normally of quantitative importance) and inhibits gluconeogenesis (Rui, 2014).

Liver insulin resistance will happen in people with T2DM, which shows up as an insufficient response to insulin, making the liver incapable of suppressing glucose production. This happens not only during overnight but also during the postprandial state, glucose production by the liver continues even when blood glucose levels are elevated in response to the meal. Muscle insulin resistance will happen in people prone to T2DM. After meal ingestion, a small quantity of glucose will be burned immediately for energy within hours; some will be stored as liver glycogen. Individuals with muscle insulin resistance are unable to store glucose as muscle glycogen properly; thus, more glucose will be converted into fat. Regular exercise over a prolonged period contribute to improved muscle insulin sensitivity and may delay or prevent the onset of T2DM (Colberg *et al.*, 2016).

1.4.2 Randle Cycle

It has been well established that fat and glucose metabolism follow a reciprocal relationship, but the precise regulatory mechanisms are not fully understood. In the early 1960s, the glucose-fatty acid cycle was proposed by Randle and co-workers to explain the well-reported phenomenon of lipid-induced insulin resistance (Randle *et al.*, 1963, 1965; Randle, Newsholme and Garland, 1964). This hypothesis posited the competition between glucose and fatty acids for oxidation in the mitochondria: fatty acids availability facilitates fat oxidation while suppressing glucose oxidation, and vice versa (Randle, 1998). High levels of plasma FFA concentrations would facilitate their elevated uptake and β-oxidation, which drives the accumulation of mitochondrial acetyl-CoA and nicotinamide adenine dinucleotide (NADH). The resultant increase of the acetyl-CoA/CoA and NADH/NAD^+ ratio and cytoplasmic citrate would inhibit pyruvate dehydrogenase and phosphofructokinase-1, respectively, and in turn, decrease glycolytic flux. The ensuing increase in glucose 6-phosphate concentration would in turn allosterically inhibit hexokinase and lead to an increase in intracellular glucose concentrations.

Since the proposal of the Randle Cycle hypothesis, extensive studies have provided biochemical mechanistic explanations to the inhibition of glucose utilisation by fatty acid oxidation (Randle *et al.*, 1963; Randle, Newsholme and Garland, 1964; Randle, Kerbey and Espinal, 1988; Randle, 1995, 1998), and the reciprocal aspect, namely inhibition of fatty acid oxidation by glucose (Taegtmeyer, Hems and Krebs, 1980). The inhibition of glucose uptake and glycolysis is hierarchical: pyruvate dehydrogenase inhibition is 40-60% greater than phosphofructokinase inhibition, which is 20-30% greater than glucose uptake inhibition (Frayn and Evans, 2019). More recent work discovered that the GLUT-mediated glucose transport is more regulatable than hexokinase; the Randle proposed hexokinase inhibition by glucose 6-phosphate is not very significant (Roden *et al.*, 1996; Dresner *et al.*, 1999). However, current experimental data showed that the fatty acid oxidation produced more direct interference with insulin signalling, and hence reducing insulin-stimulated glucose update (Roden *et al.*, 1996; Dresner *et al.*, 1999).

The Randle Cycle may lead to adverse consequences in situations when both glucose and FFA are elevated. These scenarios mainly happen in skeletal muscle of humans with T2DM and healthy humans without T2DM after a particularly large meal of both fat and CHO. The operation of the

Randle Cycle leads to an impairment of glucose uptake and metabolism, resulting in reduced net glucose uptake by muscles despite the spike in insulin and glucose concentration in plasma (Frayn and Evans, 2019). In short, the Randle Cycle proposes that in the face of elevated fatty acid oxidation, insulin-stimulated glucose uptake and utilisation is impaired, contributing to the onset of insulin resistance.

1.4.3 Twin Cycle Hypothesis

T2DM is regarded as a life-long disease. It has long been identified as a progressive and irreversible disorder, once onset, patients with T2DM require increasing dose of tablets, then injections and eventually insulin (Hallberg *et al.*, 2019). However, this point of view has been challenged by Roy Taylor with his Twin Cycle hypothesis (Taylor, 2008). The Twin Cycle hypothesis postulates that chronically positive energy balance allows the deposition of fat in the liver with eventual infiltration into pancreatic cells.

In 2006, Guidone reported a rapid normalisation of blood glucose within seven days after biliopancreatic diversion surgery in patients with T2DM (Guidone *et al.*, 2006). There was a rapid and profound energy restriction during this period. Since it is easier to mobilise energy from liver fat than to retrieve fat from the long-term storage depot under the skin, liver fat level might drop rapidly (Knop and Taylor, 2013). Liver fat level is highly linked to hepatic insulin sensitivity for control of glucose production (Petersen *et al.*, 2005; Ravikumar *et al.*, 2008). Thus, it is speculated that the rapid normalisation of blood glucose level may be a consequence of depletion of the excess liver fat (Knop and Taylor, 2013). This finding and resultant prediction shed substantial light on the Twin Cycles hypothesis of the etiology of T2DM.

1.4.3.1 The Liver Cycle

De novo lipogenesis will happen when individuals are in a chronically positive energy balance with a high CHO intake (Acheson *et al.*, 1988). Acetyl-CoA derived from excess CHO and/or AA relative to energy requirements is assembled into long-chain fatty acids in the liver, which are then esterified to TAG. This is exacerbated by the peripheral insulin resistance; more glucose will be taken up by the liver and converted into fat because of hyperinsulinaemia. Fat deposition in the liver gradually results in increased hepatic insulin resistance, causing attenuated insulin-mediated

suppression of hepatic glucose production and increased demand for insulin to keep euglycaemia. Increased levels of insulin concentrations will accelerate the conversion of glucose to fat in the liver. And then the whole cycle begins again (Taylor, 2008). An elevated level of hepatic fat increases the rate at which the liver exports fat into the bloodstream.

1.4.3.2 The Pancreas Cycle

Following the Liver Cycle, when the human body is exposed to a long-term high level of fat in the bloodstream, the pancreas becomes one of the susceptible tissues, which is infiltrated with fat. Fatty pancreas further drives the β-cells dysfunction (Taylor, 2008). The twin cycles interact and reinforce each other. Accumulated liver fat will drive the Pancreas Cycle; high blood glucose level caused by impaired insulin response will force up the insulin level, which further drives the Liver Cycle (Taylor, 2008). To examine the Twin Cycles hypothesis, the fat content of the liver and pancreas was measured by Magnetic Resonance Imaging (MRI). This hypothesis was firstly examined by studying the effects of very low-calorie diets comprising 600 kcal/day for eight weeks on T2DM remission in patients with T2DM with an average BMI of ~33 kg/m^2 ('The Counterpoint Study') (Lim *et al.*, 2011). This liquid formula diet with non-starchy vegetables (known as 'Newcastle Diet') produced an average of 15 kg of weight loss after an eight-week intervention, which is equivalent to 15% of their initial body weight. Dramatically, within the first seven days, fasting plasma glucose concentration had a rapid normalisation, from 9.2 to 5.9 mmol/L, with no hypoglycaemic drugs being taken during the intervention. Simultaneously, hepatic fat content decreased by 30% within seven days and continued to decline by 70% by eight weeks. Pancreatic fat content decreased by 22.5% and normal β-cells insulin secretion was restored. Hepatic insulin sensitivity returned to normal within seven days, while muscle insulin sensitivity, evaluated by the hyperinsulinaemic glucose clamp technique, did not alter over the intervention. This ground-breaking study is the initial scientific evidence that shows T2DM is reversible, at least in the short-term, but only recently diagnosed (< 4 years) patients with T2DM were studied. To examine the potential durability of the T2DM remission while eating normally, a further study ('The Counterbalance Study') replicated the meal plan of 'The Counterpoint Study' in either recently diagnosed (< 4 years) or longer term (diagnosed > 8 years) participants with T2DM (Steven *et al.*, 2016). Rapid weight loss was achieved in eight weeks after when a step return to an isoenergetic diet with normal food was introduced over two weeks. Over the following six months, an

individualised weight maintenance program was introduced. Over six months, participants kept their body weight stable, with 12 of 30 participants achieved fasting plasma glucose < 7 mmol/L (responders). Responders had a shorter duration of T2DM (3.8 ± 1.0 vs. 9.8 ± 1.6 years) and a lower initial fasting glucose level (8.9 ± 0.7 vs. 13.2 ± 0.6 mmol/L) than non-responders. This study demonstrated that the shorter duration of T2DM and lower fasting glucose levels, the greater the likelihood was of achieving T2DM remission.

1.4.4 Insulin Sensitivity and Physical Activity

In the present obesogenic environment, strategies to prevent the development of prediabetes and its progression to T2DM include the inclusion of regular physical activity into a healthy lifestyle (Colberg *et al.*, 2010). Cross-sectional studies have reported an association between regular physical activity and improved insulin sensitivity (Berman, Weigensberg and Spruijt-Metz, 2012; Roberts, Little and Thyfault, 2013). Amelioration or reversal of insulin resistance has been reported in studies involving exercise interventions (Hawley and Lessard, 2007; Conn *et al.*, 2014).

Everyday leisure-time physical activity is associated with a dose-dependent reduction in the occurrence of T2DM (Helmrich *et al.*, 1991; Hu *et al.*, 1999, 2001; Folsom, Kushi and Hong, 2000). A dose-response relationship between physical activity and the occurrence of T2DM was first uncovered by Helmrich *et al.*(1991). The occurrence of T2DM was reduced by 6% for each 500 kcal/week increase in leisure-time physical activity energy expenditure (Helmrich *et al.*, 1991). Aerobic training increased insulin sensitivity in healthy individuals (Garber *et al.*, 2011), individuals with Type 1 Diabetes Mellitus (T1DM) (Chimen *et al.*, 2012) and T2DM (Snowling and Hopkins, 2006). Alternatively, high-intensity interval training (HIIT) facilitates rapid amelioration of insulin sensitivity (Little *et al.*, 2011; Jelleyman *et al.*, 2015; Søgaard *et al.*, 2018) with the greatest effects observed in those with T2DM or metabolic syndrome (Jelleyman *et al.*, 2015). Resistance exercise promotes increased insulin sensitivity in healthy individuals (Garber *et al.*, 2011) and individuals with T2DM (Gordon *et al.*, 2009).

Acute effects of aerobic exercise include an induced elevation in muscle glucose uptake up to five-fold higher in an insulin-independent manner (Colberg *et al.*, 2016). After exercise, glucose uptake remains elevated in insulin-independent (~2 hours) and insulin-dependent (up to 48 hours)

manners if exercise is prolonged (Magkos *et al.*, 2008; Colberg *et al.*, 2016), which is related to muscle glycogen repletion (Wojtaszewski, Nielsen and Richter, 2002; Wang *et al.*, 2013). Improvement in insulin action may last for 24 hours following a single session of HIIT, involving 10x60s cycling intervals interspersed with 60s of recovery, eliciting a heart rate (HR) of ~90% HR_{max} during the intervals, in individuals with T2DM (Gillen *et al.*, 2012). Even a single session of low-intensity exercise (50% of peak oxygen uptake) for ~70 min (with 350 kcal of energy expenditure) is sufficient to enhance insulin sensitivity (using the hyperinsulinaemic euglycaemic clamp) at least into the next day in sedentary individuals with obesity (Newsom *et al.*, 2013).

Studies in the last ten years have confirmed the findings from previous research that show the efficacy of physical activity in enhancing insulin sensitivity (Bird and Hawley, 2017). This improvement may be underpinned by increased GLUT4 concentration, which is associated with changes in insulin sensitivity (Frøsig and Richter, 2009; Roberts, Little and Thyfault, 2013b; Stuart *et al.*, 2013; Mann *et al.*, 2014). Such improvement appears to be tissue-specific; since physical activity elevates skeletal muscle but not hepatic insulin sensitivity, nor insulin-stimulated glucose uptake in adipose tissue (Malin *et al.*, 2013; Reichkendler *et al.*, 2013). In addition, this improvement is underpinned by acute exercise-induced elevations in Protein Kinase B (Akt) that deactivate TBC1 domain family member 4 (TBC1D4) (also known as Akt substrate of 160 kDa, AS160), thus promoting GLUT4 translocation to the cell membrane and thereby elevating glucose uptake. This effect can last for several hours after exercise (Bird and Hawley, 2017). Furthermore, improvement in insulin sensitivity stimulated by muscle contraction is also associated with increased muscle capillary density (Roberts, Hevener and Barnard, 2013).

1.4.5 Assessment of Insulin Sensitivity/Resistance

Clinical syndromes associated with insulin resistance include T2DM, CVD, essential hypertension, polycystic ovary syndrome, non-alcoholic fatty liver disease, certain forms of cancer, and sleep apnoea (Reaven, 2004). Insulin resistance and compensatory hyperinsulinaemia related to insulin resistance are independent predictors of future CVD (Yip, Facchini and Reaven, 1998), T2DM, hypertension, coronary heart disease (Zavaroni *et al.*, 1999), a range of age-related diseases (Facchini *et al.*, 2001), and all-cause mortality (Ausk, Boyko and Ioannou, 2010) in individuals with and without T2DM. The glucose clamp technique, initially developed by DeFronzo *et al.* in

1979 (DeFronzo, Tobin and Andres, 1979), is regarded as the gold standard for directly determining insulin sensitivity. The technique consists of a hyperglycaemic clamp, which is used to quantify β-cell sensitivity in response to glucose; and hyperinsulinaemic-euglycaemic clamp, which is used to quantify peripheral tissue insulin sensitivity (DeFronzo, Tobin and Andres, 1979; Scheen *et al.*, 1994; Patarrão, Wayne Lautt and Paula Macedo, 2014). The hyperinsulinaemic-euglycaemic clamp is more commonly used (Bergman, Finegood and Ader, 1985; Scheen and Lefebvre, 1992; Scheen *et al.*, 1994). After an overnight fast, the insulin concentration is acutely raised in response to a 10-min priming insulin infusion and maintained at > 40 μU/ml in healthy individuals and > 100 μU/ml in individuals with insulin resistance by a continuous infusion of insulin. This constant exogenous insulin infusion increases insulin levels to a new steady state that is above the basal level (hyperinsulinaemic). In the meanwhile, exogenous glucose is infused at variable rates attempting to maintain euglycaemia, which is the practical meaning of "clamp". The priming insulin results in suppressed glycogenolysis, gluconeogenesis, and endogenous insulin secretion (DeFronzo, Tobin and Andres, 1979; Scheen *et al.*, 1994). When the steady state is achieved, the peripheral glucose disposal rate is equal to the glucose infusion rate assuming the hyperinsulinaemic state can thoroughly suppress hepatic glucose production and no net change in glucose concentrations, which determines the validity of insulin sensitivity measured by this technique. Under the state of hyperinsulinaemia, approximately 80% (depends on the degree of hyperinsulinaemia) of infused glucose is taken by skeletal muscle (Ferrannini, 1998); thus the glucose infusion rate mainly reflects skeletal muscle insulin sensitivity. The hyperinsulinaemic-euglycaemic clamp technology is usually combined with stable isotopic glucose tracers to quantify hepatic glucose production and whole-body glucose disposal (Rizza, Mandarino and Gerich, 1981; Campbell, Mandarino and Gerich, 1988). Whilst the glucose clamp technique is referred to as the 'gold standard' for quantifying insulin sensitivity based on its high sensitivity, it is laborious with intravenous infusion of insulin and frequent blood sampling required, which leads to the development of indirect and simplified approaches that provide indices of insulin sensitivity/resistance.

The Matsuda index is commonly used to evaluate the whole-body insulin sensitivity indices obtained from the oral glucose tolerance test (OGTT) or meal tolerance test (MTT). It employs the product of glucose and insulin obtained in the fasting state which mainly reflects hepatic insulin

sensitivity, as well as their postprandial responses during an OGTT or MTT, which mainly reflects peripheral insulin sensitivity. Therefore, the Matsuda index reflects both hepatic and peripheral tissue postprandial sensitivity to insulin (Matsuda and DeFronzo, 1999). The Matsuda index is calculated as:

Insulin sensitivity index (Matsuda) $= 10000 / \sqrt{(fasting\ G\ x\ fasting\ I)(mean\ G\ x\ mean\ I)}$, where mean G is the mean blood glucose concentration (mg/dL) during OGTT or MTT (unit conversion factor: 1 mmol/L = 18 mg/dL), mean I is the mean blood insulin concentration (mIU/L) during OGTT or MTT. The Matsuda index is typically calculated across 120 min of an OGTT. Since an MTT normally contains fat and protein in addition to CHO, the time for returning to the fasted state will be prolonged in response to the mixed MTT. Therefore, the Matsuda index is also calculated over 180 min in response to an MTT.

The homeostasis model assessment of insulin resistance (HOMA-IR) is a more simplistic index to quantify insulin sensitivity and pancreatic β-cell function, from fasting glucose and fasting insulin concentrations, first described by Matthews *et al*. (1985). The physiological principle of HOMA-IR is the feedback loop between the liver and β-cell, which sustains the balance between insulin-regulated glucose output and glucose-stimulated insulin secretion (Turner *et al.*, 1979; Wallace, Levy and Matthews, 2004). Insulin resistance is determined as: HOMA-IR = (I_0 x G_0) / 22.5, where I_0 is fasting insulin concentration (mIU/L), G_0 is fasting glucose concentration (mmol/L). The value of HOMA-IR reflects the hepatic (fasting) insulin resistance. Good correlations have been verified between insulin sensitivity estimation derived from HOMA-IR and the hyperinsulinaemic-euglycaemic clamp (Matthews *et al.*, 1985; Emoto *et al.*, 1999; Bonora *et al.*, 2000); and between HOMA-IR and the minimal model (García-Estévez *et al.*, 2003). There is also a good correlation between estimation of β-cell function using HOMA-IR and using continuous infusion glucose model assessment (CIGMA) (Hosker *et al.*, 1985; Hermans *et al.*, 1999), and the acute insulin response from the intravenous glucose tolerance test (IVGTT) (Hermans *et al.*, 1999).

Both the Matsuda index and HOMA-IR are easily adopted in epidemiological and clinical studies for diabetes development prediction in individuals without diabetes. However, their application in clinical practice is limited because of the lack of specific reference values for normal and impaired insulin sensitivity (Gutch *et al.*, 2015).

1.5 Regulation of Energy Intake

1.5.1 Appetite

Appetite can be defined as physiological and psychological regulatory processes underpinning feeding (Blundell *et al.*, 2015). It is controlled by an integrated psychobiological system that signals hunger (an irritating feeling that motivates people to approach food), satiation (terminates feeding, processes that control the size of eating episodes), and satiety (processes that control the strength of post-ingestive appetite inhibition), which further determine the energy intake (Blundell, 1991; Blundell and MacDiarmid, 1997). These processes are controlled by the episodic and tonic signals in the peripheral circulation, which are integrated by the central nervous system (CNS) (Morton *et al.*, 2006). A model that relates energy balance, energy intake, and energy expenditure was constructed (**Figure 1.2**) (Blundell *et al.*, 2012). Episodic signals arising from the gastrointestinal tract are mainly involved in meal-to-meal regulation of appetite. The diurnal oscillation of episodic signals represents the size, pattern, and frequency of meals (Blundell *et al.*, 2012). Most episodic signals are inhibitory, triggered by eating episodes, and are related to the signalling of satiety. Tonic signals arise from tissue stores and are considerably stable over days (Blundell *et al.*, 2012). High FM inhibits appetite and high FFM and resting metabolic rate (RMR) produces a drive to eat. With the FM increases, its inhibitory function weakens probably due to leptin and insulin resistance. Ultimately, the homeostatic (maintain energy balance) and the hedonic (control food choice) systems in the CNS integrate all signals and in turn determine the appetite (Blundell *et al.*, 2012).

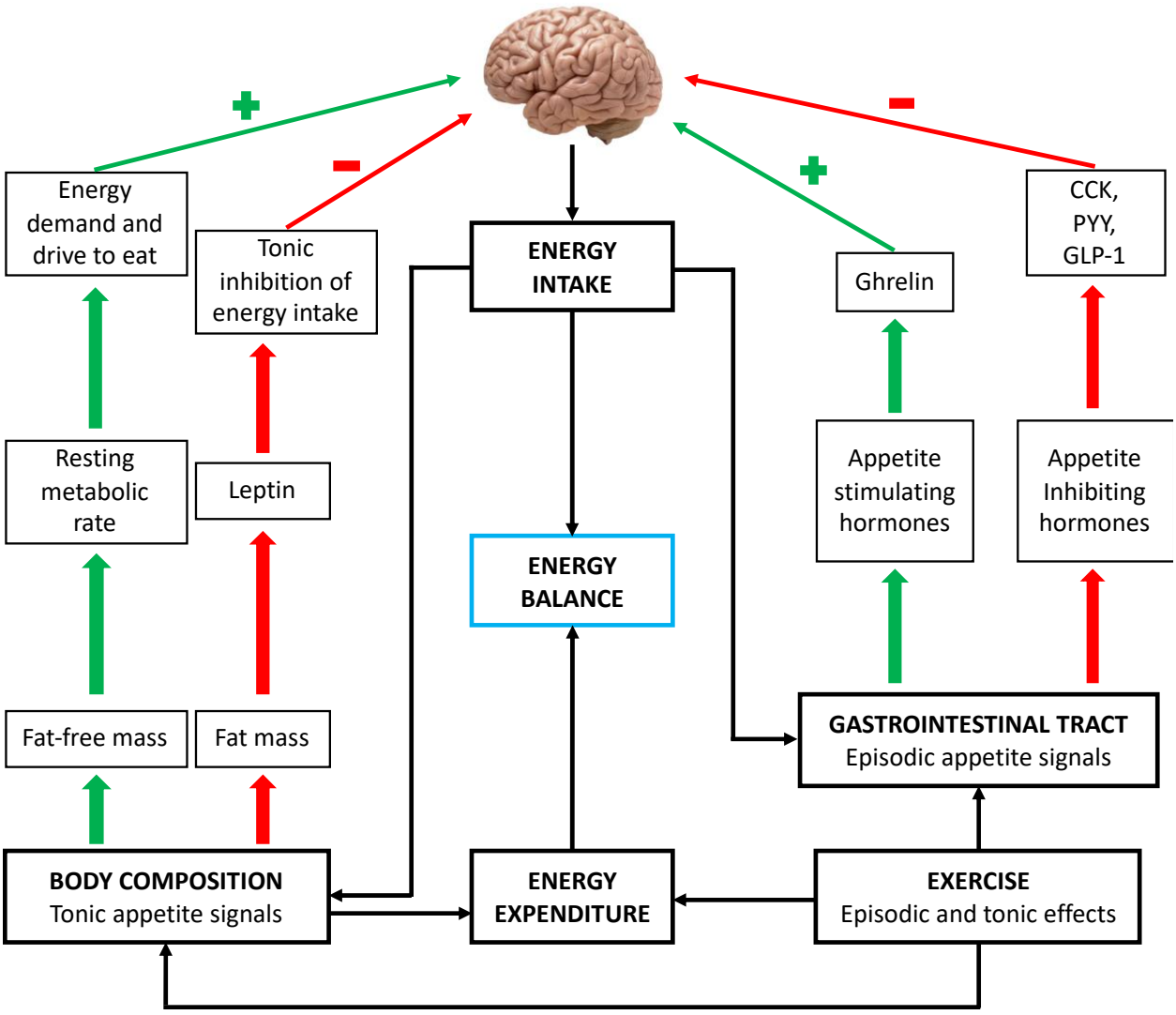


Figure 1.2 Formulation of the major influences on appetite control using an energy balance framework.

Taken from Blundell *et al.*, (2012). Green arrows indicate processes of feeding stimulation, while the red arrows indicate processes of feeding inhibition.

1.5.2 Body Composition and Energy Intake

FFM rather than FM or BMI is positively associated with energy intake in individuals who are lean and in those with overweight/obesity (Blundell *et al.*, 2012). The positive relationship between FFM and energy intake is conserved across different activity levels. Furthermore, independent observations of FFM and energy intake demonstrated a significant positive

relationship whether dietary fat content was high (> 50% of total energy) or low (< 25% of total energy). This relationship was confirmed to be robust shortly after (Weise *et al.*, 2014), and it was proposed that FFM and FM may have opposing effects on energy homeostasis. In fact, long before these findings, energy requirements had been reported to be positively associated with lean mass whereas FM added no predictive value. Energy intake appears to be a direct function of lean mass rather than adiposity (Lissner *et al.*, 1989; Cugini *et al.*, 1998). The high FFM group has a higher level of sensation of hunger and a lower level of sensation of fullness across a day, indicating that the drive to eat is reflected not just in food consumption but in the conscious sensation that motivates the eating behaviour (Blundell *et al.*, 2012). These outcomes suggest that FFM plays a functional role in the normal control of appetite, influencing the drive to eat (hunger) and daily energy intake (Blundel *et al.*, 2012). On the contrary, FM is not strongly associated with meal size, daily energy intake, nor with hunger or fullness.

1.5.3 Energy Expenditure and Energy Intake

There is no relationship between food intake and energy expenditure on one single day, but over three weeks, the correlation becomes clear. It is concluded that the differences between the food intake (of individuals) originate in the differences in energy expenditure (Edholm *et al.*, 1955, 1970). As mentioned before, FFM accounts for the large part of the variance in RMR (60-65%) whereas FM only accounts for about 7%, age accounts for 2%, and sex accounts for 0%. RMR has been found as a driver of meal size and daily energy intake, independent of the different energy density of foods (Caudwell *et al.*, 2013). This finding has been replicated in a completely independent data set where each component of energy balance was objectively quantified (Stubbs *et al.*, 2014). The regression equation between RMR and energy intake demonstrates that RMR emerges as the strongest predictor of daily energy intake. Based on these data, FM and FFM are demonstrated as not directly related to energy intake, RMR accounts entirely for the effects of FM and FFM on energy intake (Hopkins *et al.*, 2016). Both RMR and energy density jointly influence satiation and increase meal size; they are two robust but independent effects (Caudwell *et al.*, 2013; Hopkins *et al.*, 2016).

1.5.4 Physical Activity and Energy Intake

Mayer, Roy and Mitra (1956) made a great contribution in elucidating the effect of physical activity on energy intake and appetite control, based on their investigations carried out in jute mill workers in Bengal. A comparison was performed between the physical exertion and effort required by specific types of work and the recorded dietary intake. Occupations with different physical activity intensity ranging from stallholder and clerks who undertook a light physical effort to coalmen and blacksmiths whose jobs required heavy physical effort were compared. As shown in **Figure 1.3**, the right part of the curve displays a linear relationship between energy expenditure and energy intake, under conditions of higher levels of occupational physical activity. However, as displayed on the left part of the curve, under conditions of sedentary and light physical activity level, this relationship is absent with energy intake increases inversely in response to the energy expended, which consequently results in overconsumption. Besides, individuals who fall into the sedentary category were observed with higher body weight compared to those in the light to very heavy work categories (Mayer, Roy and Mitra, 1956). This model links energy intake to energy expenditure in a J- or U- shaped curve (Jacobs, 2006). However, this is a cross-sectional study so causality cannot be attributed. Therefore, the data can only be used to show an association and more mechanistic studies are needed. A similar J-shaped relationship was reported by Shook *et al.*, (2015), with energy intake (kcal/day) being measured across different physical activity groups, indicating the associations between sedentariness and attenuated homeostatic appetite control. A meta-analysis of physical activity and energy intake again reported a similar J-shaped relationship between physical activity level and energy intake. Individuals with high physical activity levels have high energy intake, displayed as a linear relationship. However, individuals with low physical activity have a higher energy intake, favouring an increase in body fat (Beaulieu *et al.*, 2016).

Physical activity induced negative energy balance will reduce adipose tissue, and will therefore reduce insulin and leptin resistance, which together enhance the inhibitory effect of adipose tissue on energy intake. Physical activity can increase FFM and RMR, which will be the drive to increased energy expenditure. Physical activity (exercise) will also change signals in the gastrointestinal tract, which may influence the episodic control of meal to meal appetite control (Blundell *et al.*, 2015).

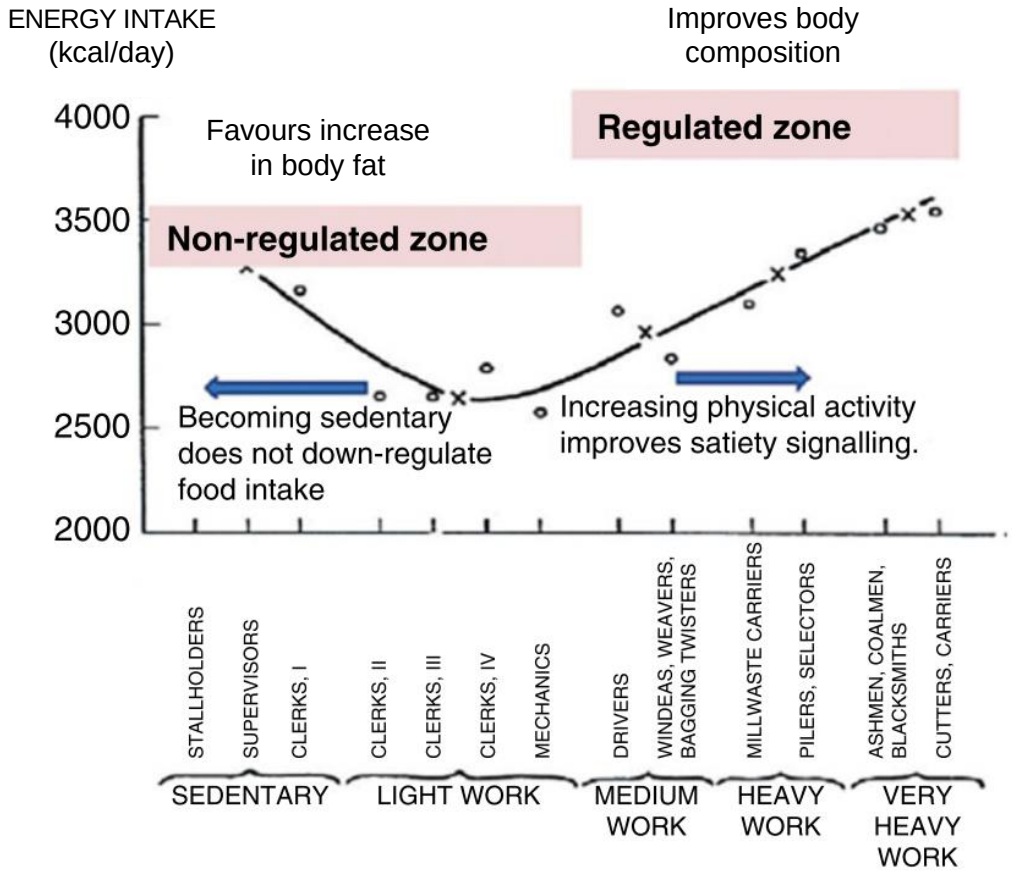


Figure 1.3 The relationship between energy expenditure and energy intake.
The relationship between energy expenditure (according to energy requirements from work) and energy intake modified based on the original figure from the study by Mayer *et al*. in 1956 (Mayer, Roy and Mitra, 1956) in Bengal jute mill workers. Adapted from Blundell (2011).

1.6 Physical Inactivity

For many decades, the leading cause of death was communicable diseases. And then step by step, it shifted towards more lifestyle-related NCD such as T2DM, obesity, hypertension, and CVD in developed countries, whilst the developing countries are still undergoing this epidemiological transition (Anjana *et al.*, 2011). Failure to reduce chronic diseases will result in heavy losses in terms of human life and economic production.

Genes play a fundamental role in this global epidemiological transition, as they will influence individual predisposition to become overweight or obese. However, they have not changed over the past centuries of decades. What has changed is our environment, transitioning from the traditional environment favouring leptogenic (leptos=lean) behaviours (i.e. promotes healthy food choices and encourages physical activity) towards a social and built environment favouring obesogenic behaviours (Swinburn, Egger and Raza, 1999). Therefore, there is a mismatch between human evolutionary history and the current environment. A physical activity transition has been observed concomitantly to this epidemiological transition. There are decreases in physical activity (**Table 1.2**) in every single dimension of our daily lives: during leisure time, transportation, or at work. It is estimated that the decrease in physical activity is equivalent to 16 km less walking per day between what we had approximately 100 years ago and nowadays (Egger, Vogels and Westerterp, 2001). There is approximately 30% of the population not reaching the physical activity targets (Guthold *et al.*, 2018). It is worth noting that this is self-reported data, when the physical activity is measured objectively with activity monitors, the physical activity of people is substantially lower (Troiano *et al.*, 2008). Furthermore, concomitantly to the increase in the prevalence of physical inactivity, we have been observing an increase in sedentary behaviour (**Table 1.2**), with the time spent sitting increasing. In the U.S, the time spent sitting increased from 5.7 hours/day between 2006-2007 to 6.4 hours/day between 2016-2017 (Du *et al.*, 2019). A remarkable reduction in step counts will exacerbate the health consequences caused by physical inactivity (Booth *et al.*, 2017).

The diminution in physical activity is considered to be one of the most significant public health challenges of the 21st century (Blair, 2007). In 2012, The Lancet published a special series about physical activity based on this alarming situation, in which physical inactivity was described as a pandemic, with far-reaching health, economic, environmental, and social consequences (Kohl *et al.*, 2012). Between 6-10% of the world's major NCDs is attributable to physical inactivity. However, by eliminating physical inactivity, more than 5.3 million deaths/year may be prevented; and there will be an increase of 0.68 years in the world's life expectancy (Lee *et al.*, 2012). However it is apparent that not only must inactivity be considered, but also the extent to which the inactive hours in the day, are actual sedentary. From a large population study (n=240819), compared with individuals who report exercise more than 7 hours/week but TV watching for <1

hour/day, individuals who report the same amount of exercise, but TV watching for more than 7 hours/day increase the risk of premature mortality by 50%. More strikingly, the risk among individuals reporting exercise more than 7 hours/week but TV watching for more than 7 hours/week is the same for the individuals who never or rarely do exercise but watch TV for <1 hour/day. Therefore, too little exercise is likely to be different from too much sitting (Matthews *et al.*, 2012).

Table 1.2 Definitions of physical activity, physical inactivity, sedentary behaviours and exercise.

Terms	Definitions
Physical activity	Any bodily movement produced by skeletal muscles that require energy expenditure. Physical activity in daily life can be categorised into occupational, sports, conditioning, household, or other activities (Caspersen, Powell and Christenson, 1985).
Physical inactivity	A term used to identify people who do not get the present recommended level of regular physical activity (Lee, Eric J Shiroma, *et al.*, 2012). "Global Recommendations on Physical Activity for Health" developed by WHO (2010): • Children and youth aged **5–17** should accumulate at least 60 min of moderate-to vigorous-intensity physical activity daily; • Adults aged **18 years and above** should do at least 150 min of moderate-intensity aerobic physical activity throughout the week or do at least 75 min of vigorous-intensity aerobic physical activity throughout the week or an equivalent combination of moderate- and vigorous-intensity activity. Aerobic activity should be performed in bouts of at least 10 minutes duration.
Sedentary behaviours	Definitions of sedentary behaviours vary from different research literature. A general definition: is any waking behaviour characterised by an energy expenditure ≤1.5 metabolic equivalents (METs), while in a sitting, reclining or lying posture (Tremblay *et al.*, 2017).
Exercise	A subcategory of physical activity that is planned, structured, repetitive, and aims to improve or maintain one or more components of physical fitness (Caspersen, Powell and Christenson, 1985).

1.6.1 The Metabolic Consequences of Physical Inactivity and Sedentary Behaviour

It has been conclusively shown that physical inactivity, with the models of bed rest, immobilisation, or sedentarism, results in a rapid muscle mass loss and a reduction in insulin sensitivity (Narici *et*

al., 2020). Step count reduction from 10,500 to 1300 steps/day for two weeks resulted in reduced muscle insulin sensitivity and lean leg mass in healthy, non-exercising young men (Krogh-Madsen *et al.*, 2010). Step count reduction from >3500 to <1500 steps/day for two weeks resulted in reduced whole-body insulin sensitivity and reduced postprandial rates of myofibrillar protein synthesis in healthy, older (mean ± SEM, 72 ± 1 years) men and women (Breen *et al.*, 2013). Introducing a short-term (7-10 days) bed-rest model in healthy individuals resulted in reduced whole-body insulin sensitivity (using glucose clamp) by 10-34% (Stuart *et al.*, 1988; Mikines *et al.*, 1991; Sonne *et al.*, 2010).

In an epidemiological study, it was observed that beyond the total time spent in sitting, the length of the sitting bouts also matters. There is a positive relationship between the total time spent being sedentary and the detrimental metabolic health factors, including cholesterol, TAG, insulin, and insulin sensitivity. On the other hand, breaks in sedentary time are negatively associated with some health outcomes, such as waist circumference, cholesterol, and biomarkers of CVD. More importantly, these associations were observed for all age, sex, race, and ethnicities, and even in individuals who were regularly exercising (Healy *et al.*, 2011). In a randomised crossover trial, a positive effect of frequent interruptions to prolonged sitting was observed. Interrupting prolonged sitting with short bouts of light or moderate-intensity walking lowers postprandial glucose (glucose iAUC) and insulin concentrations (insulin iAUC) in adults with overweight or obesity (Dunstan *et al.*, 2012). Another randomised crossover study showed that the beneficial effects are attributable to the activity-breaks rather than the increased energy expenditure. The results showed that both 30 min of moderate-intensity walking in the morning and hourly five-min moderate-intensity walking increase self-perceived energy and vigor compared to six hours of uninterrupted sitting. The two physically active conditions produce the same energy expenditure. Most importantly, short walk breaks throughout the day rather than one big walk in the morning improves mood, lower levels of fatigue, and reduces food cravings at the end of the day compared to six hours of uninterrupted sitting (Bergouignan *et al.*, 2016). However, it was merely a one-day trial; larger-scale and longer-term trials are required to examine the sustained metabolic effects of the breaks.

1.6.2 The Metabolic Consequences of Overfeeding with Physical Inactivity

Several days of sedentary lifestyle are sufficient to induce insulin resistance (principally in muscle), fat deposition and loss of muscle mass (Narici *et al.*, 2020). These metabolic perturbations are also observed with acute/short-term overfeeding (i.e., both scenarios create a positive energy balance). A single day of high-fat (68% of total energy intake) overfeeding (78% greater than daily energy requirements) reduces whole-body insulin sensitivity (Matsuda index) in healthy, young participants (Parry *et al.*, 2017). Four-days of overfeeding (50% greater than daily energy requirements) with a high-fat diet (60% of total energy intake) leads to reductions in plasma TAG concentrations and glucose clearance, and an elevation in HDL-cholesterol concentrations (Wulan, Westerterp and Plasqui, 2014). Five-days high-fat (60% of total energy intake) overfeeding (50% greater than daily energy requirements) results in increased hepatic glucose production and fasting glucose levels in young, healthy-weight men (Brøns *et al.*, 2009). Seven-days of overfeeding (50% greater than daily energy requirements) with a high-fat diet (65% of total energy intake) leads to increased postprandial glucose iAUC and insulin iAUC, and decreased whole-body insulin sensitivity (Matsuda index) in young, healthy-weight men and women (Hulston, Churnside and Venables, 2015; Parry *et al.*, 2017). Seven-days overfeeding (60% greater than daily energy requirements) results in impaired fasting (HOMA-IR) and postprandial insulin sensitivity (Matsuda index), and increased glucose-stimulated insulin secretion during OGTT in young, healthy-weight men (Lagerpusch *et al.*, 2012). Short-term studies of more than seven-days duration have found a consistent reduction in insulin sensitivity in response to overfeeding in health lean participants, as evaluated by the hyperinsulinaemic glucose clamp technique (Mott, Lillioja and Bogardus, 1986; Erdmann *et al.*, 2008), or by oral or intravenous glucose tolerance test (Olefsky *et al.*, 1975; Erdmann *et al.*, 2008; Cornford *et al.*, 2013).

A reduction in physical activity may be accompanied by the induction of positive energy balance, if energy intake is not concurrently reduced. Seven-days of physical activity level (PAL) reduction from 1.8 to 1.4 was not accompanied by a compensatory reduction in energy intake, creating a positive energy balance (Stubbs *et al.*, 2004). A dramatic step reduction (from 10,501 to 1,344 steps/day) without a proportional decrease in energy intake for two weeks resulted in a decrease in insulin action (Krogh-Madsen *et al.*, 2010). A compensatory reduction in energy intake (to

achieve energy balance) has been shown to minimise the detrimental effects of one day of prolonged sitting on insulin sensitivity (Stephens *et al.*, 2011).

However, there is a paucity of studies investigating whether reducing energy intake to match a reduction in energy expenditure will ameliorate the metabolic impact of reduced physical activity. The contribution of overfeeding to the negative effects of physical inactivity is to be elucidated.

1.7 Intermittent Energy Restriction (IER)

In modern societies, the prevalence of obesity, driven by sustained excess energy intake and prolonged sedentary behaviour, is increasing worldwide (WHO, 2020). Excess energy intake, relative to requirement, results in weight gain due to fat deposition. Over a prolonged period, increased adiposity results in obesity and associated co-morbidities. To restore a state of energy balance and promote health outcomes, an increasing number of interventions are directed at restraining energy intake and/or boosting energy expenditure (Templeman *et al.*, 2020). Modest weight reduction (5-10% of original body weight) is recommended to individuals with overweight or obesity to attain improvements in metabolic function and other health outcomes (Jensen *et al.*, 2014). However, benefits achieved from weight-loss diets are hindered by metabolic adaptations in appetite and energy utilisation (Martin *et al.*, 2011; Hall *et al.*, 2012; Müller *et al.*, 2015; Westerterp, 2018) and are further limited by poor compliance (Dansinger *et al.*, 2005; Middleton, Anton and Perri, 2013). Moreover, diets that overemphasise a single type of nutrient or food are inadvisable (Katz and Meller, 2014). Continuous energy restriction (CER) is a conventional weight management approach, which involves diets with a 2000-2500 kJ deficit each day, relative to the energy requirements. Although CER, with expert support and intensive follow-up, is recommended for sustainable weight loss for people with obesity by the National Institute for Health and Care Excellence (2014), difficulty in adhering to CER in humans is widely recognised (Varady and Hellerstein, 2007; Harvie *et al.*, 2011). In this context, intermittent fasting (IF) is becoming increasingly popular for attaining weight loss and metabolic benefits. IF is an umbrella term including different patterns of fasting (**Table 1.3**). The two most studied patterns are IER, a dietary strategy which involves shorts periods of substantial (>70%) energy restriction, interspersed with periods with the intention of meeting energy requirements; and time-restricted

feeding (TRF), daily energy restriction where food is consumed within a window between 4 and 10 hours.

Table 1.3 Types of intermittent fasting.

Type of Intermittent Fasting	Description
5:2 intermittent fasting or 5:2 intermittent energy restriction (5:2 diet)	Involves substantial energy restriction with no or little energy intake for 2 consecutive/non-consecutive days/week, interspersed with *ad libitum* energy intake, or with the intention of meeting energy requirements for the other 5 days, respectively (Patterson and Sears, 2017).
Time-restricted feeding (TRF)	Allows *ad libitum* energy intake within specific time frames (typically between 4 and 10 h), inducing regular, extended fasting intervals. The most popular is the 16-h fast/8-h eating window (Patterson and Sears, 2017).
Alternate-day fasting (ADF)	Involves a "fast day" where individuals consume 25% of their daily energy requirements, alternated with a "feed day" where individuals eat *ad libitum* (Varady and Hellerstein, 2007).
Ramadan fasting	A fast from sunrise to sunset during the holy month of Ramadan; the most common dietary practice is to consume one large meal after sunset and one lighter meal before dawn. Thus, the feast and fast periods of Ramadan are approximately 12 h in length (Patterson and Sears, 2017).

1.7.1 Intermittent Fasting: Animal Models

IER studies using alternate-day fasting (ADF) have shown acute reductions in fasting glucose (Pedersen *et al.*, 1999; Wan, Camandola and Mattson, 2003; Wan, Camandola and Mattson, 2003; Wan *et al.*, 2010) and insulin (Wan, Camandola and Mattson, 2003; Wan, Camandola and Mattson, 2003; Wan *et al.*, 2010) with improved lipid profiles (Krízová and Simek, 1996), increased insulin sensitivity (Wan, Camandola and Mattson, 2003), and improved glucose tolerance (Wan, Camandola and Mattson, 2003). However, there may be long-term consequences of the development of diastolic dysfunction with diminished cardiac reserve (Ahmet *et al.*, 2010) and impaired glucose tolerance (Cerqueira *et al.*, 2011) possible resulting from increased oxidative

stress which has been observed in some rodent studies with ADF. Conversely, TRF appears to be a beneficial approach to weight loss whilst also improving metabolic health markers, which may be the consequence of eating in alignment with circadian rhythm and fasting *per se* (Longo and Panda, 2016).

1.7.2 Intermittent Fasting: Human Studies

IF studies vary greatly in design, making it difficult to make comparisons or draw accurate conclusions. The inconsistency among previous studies may be primarily attributable to:

- Dietary patterns (e.g. 5:2 diet or ADF or TRF; eating windows for TRF, energy restriction on two consecutive days or two non-consecutive days for 5:2 diet);
- Macronutrient composition;
- Intervention duration;
- Meal timing;
- Meal frequency;
- Participant characteristics (e.g. normal weight or overweight or obese; with T2DM or not; young or old);
- Energy intake (*ad libitum* vs matching estimated energy requirements on feed days);
- Energy restriction (with no or little energy intake on energy-restricted days).

On average, IER (including 5:2 IER and ADF) results in 3-9% weight loss over a period between four weeks to one year, and it is consistently accompanied by preferential loss of FM and waist circumference but the preservation of FFM. Studies also show improvements in fasting insulin concentration, hepatic insulin sensitivity (HOMA-IR), insulin-mediated glucose uptake (using the hyperinsulinaemic euglycaemic clamp), and lipid profiles in participants without T2DM, and reduced glycated haemoglobin (HbA1c) in participants with T2DM (Patterson and Sears, 2017). Meta-analyses reveal no significant difference in weight loss compared with CER, and metabolic outcomes are mostly comparable to CER (Harris *et al.*, 2018). However, unequal weight loss between different patterns and between different study participants may confound the metabolic outcome comparisons. Moreover, the vast majority of studies have merely focused on fasting indices of risk despite 75% of our day being spent in a postprandial state.

There is limited human evidence to show the benefits of TRF due to huge variation in study design and small sample size although recent preliminary data from studies looking at early time-restricted feeding (eTRF, restricting energy intake to earlier in the day) show promising results with improved glycaemic control (Sutton *et al.*, 2018; Jamshed *et al.*, 2019), insulinaemic control (Sutton *et al.*, 2018) and improved whole-body insulin sensitivity (Jones *et al.*, 2020a). However, feedback from participants suggests that in our modern society, this diet may not be compatible with family/social life, which makes it unsustainable for many when performed on a daily basis (Antoni, Robertson, *et al.*, 2018). Furthermore, an early study shows potential harm due to impaired glycaemic control when food is limited to evenings (Carlson *et al.*, 2007). The remainder of this section will focus on IF.

1.7.3 5:2 Intermittent Fasting/5:2 Intermittent Energy Restriction

This regimen, when employing two non-consecutive energy restricted days has shown comparable reductions in body weight, FM, and FFM, as well as favourable effects on HbA1c levels and a range of fasting metabolic parameters compared with CER over 3, 6 or 12 months, in participants with overweight or obesity (Carter, Clifton and Keogh, 2016, 2018; Conley *et al.*, 2018; Schübel *et al.*, 2018; Sundfør, Svendsen and Tonstad, 2018a). One recent study has demonstrated that employing 5:2 IER with 75% energy restriction on two consecutive days/week elicits a 5% loss of body weight, decreased postprandial TAG responses when compared with matched weight loss from CER in participants with overweight or obesity. Unlike other 5:2 studies with only fasting biochemistry and physiological markers measured, both fasting and postprandial measurements were performed in this study (Antoni *et al.*, 2018). Furthermore, in a similar population, this 5:2 IER regimen facilitated greater reductions in fasting insulin concentration, insulin resistance (HOMA-IR), and FM over 12 weeks and six months at a comparable energy deficit and weight loss to CER (Harvie *et al.*, 2011; Harvie *et al.*, 2013). The differences were largely attributed to the prolonged post-absorptive times during the 5:2 IER days as a result of consuming smaller meals. 5:2 IER, compared with CER may confer greater metabolic benefit when energy restriction is performed on two consecutive days, compared with two non-consecutive days (**Table 1.4**). However, the results are inconclusive and both patterns warrant further studies.

Whilst 5:2 IER shows potential promise as a sustainable intervention to improve metabolic health, existing findings are inconsistent and have predominantly focused on assessing fasting metabolic measures (**Table 1.4**). Given that modern humans spend much of the 24 hours in a postprandial state, the assessment only of fasting measures may mask the effects on human metabolism that occur postprandially. Indeed, despite the growing trend of 5:2 IER, there is a paucity of studies that comprehensively assess postprandial metabolic responses in comparison to CER.

Table 1.4 Randomised clinical studies comparing the effects of 5:2 intermittent energy restriction versus continuous energy restriction on body weight and metabolic risk factors.

Author (year)	Sample size and Participants	Age Years	BMI Kg/m²	Intervention duration	Intervention	Comparison condition(s)	How long before measurement	Weight loss	Outcomes (Change between-groups over time unless otherwise stated)	Comments and limitations
Headland et al., (2020)	5:2 IER n=31 (18) Week-on-week-off n=38 (6) CER n= 40 (13) Overweight&obese Men and women	5:2 IER: 52 (SD 11) Week-on-week-off: 55 (SD 12) CER: 56 (SD 9)	5:2IER: 33 (SD 5) Week-on-week-off: 34 (SD 5) CER: 33 (SD 4)	12-month follow-up **12-month follow-up after completed a 12-month dietary intervention trial (See Headland et al., (2019))**	0-12 months: See below: **Headland et al., (2019)** 12-24 months: free-living period, could consume food and drink as they wished	0-12 months: See below: **Headland et al., (2019)** 12-24 months: free-living period, could consume food and drink as they wished	After an overnight fast	**Relative to baseline (pre-intervention):** ↓5:2IER: -3.5 (SD 5.1)kg ↓WOWO: -2.8 (SD 6.5)kg ↓CER: -4.5 (SD 4.9)kg	**Body weight** between the end of the 12-month weight loss trial and 12-month follow-up increased, with NS WOWO vs. CER or 5:2IER vs. CER **Total cholesterol** reduced over time and **glucose**, **HDL**, **LDL** and **TAG** were unchanged.	**Meals**: not provided
Panizza et al., (2019)	5:2 IER + Mediterranean diet (**5:2 IER +MED**) n=26 (4) Euenergetic Dietary Approaches to Stop Hypertension diet (**DASH**) n=8 (2) Men and women Overweight&obese East Asians in Hawaii	5:2 IER + MED: 48 (SD 5) DASH: 46 (SD 5)	5:2 IER + MED: 31 (SD 4) DASH: 31 (SD 3)	12 weeks	**5:2 IER + MED:** **ER**: 2d/wk (**consecutive**), ~70% ER **FEED**: 5d/wk, isoenergetic intake, Mediterranean-type diet	**DASH**: (Active comparator) euenergetic Dietary Approaches to Stop Hypertension diet 22%ER between baseline and Weeks 5-6; 16% between baseline and Weeks 11	N/A At least after an overnight fast for sure	↓5:2 IER + MED: (~7.4%): -5.9 (SEM 0.7) ↓DASH (~4.1%): -3.3 (SEM 0.6) 5:2 IER > CER	5:2 IER + MED had significantly larger reductions in DXA-measured **VAT** and **total fat mass**: −22.6 (SEM 3.6) cm^2 and −3.3 (SEM 0.4) kg, respectively vs. DASH −10.7 (SEM 3.5) cm^2 and −1.6 (SEM 0.4) kg (p = 0.02 and p = 0.005). However, after adjusting for total fat mass, change in VAT was NS between groups Improvement in **alanine transaminase** remained significantly greater for 5:2 IER+MED vs. DASH after adjusting for concurrent total fat mass	**Meals**: not provided The DASH diet is rich in fruit, vegetables, low-fat dairy products, whole grains, and limits total fat, saturated fat and sodium An active comparator was used, instead of an inactive control, because all participants were at risk of poor metabolic conditions and would likely benefit from dietary support
Headland et al., (2019)	5:2 IER n=49 (69) Week-on-week-off n=44 (66) CER n=53 (51) Overweight&obese Men and women	5:2 IER: 48 (SD 15) Week-on-week-off: 49 (SD 13) CER: 52 (SD 13)	5:2 IER: 33 (SD 5) Week-on-week-off: 34 (SD 6) CER: 33 (SD 5.0)	12 months	**5:2 IER:** **ER**: 2d/wk (**consecutive/ non-consecutive**), 2100 kJ/d for women and 2520 kJ/d for men FEED: 5d/wk, habitual eating Overall ER: 30%	**WOWO:** Week-on-week-off: alternating between the same ER as the CER for one week and one week of habitual eating Overall ER: 30% **CER:** 4200 kJ/d for women, 5040 kJ/d for men Overall ER: 30%	After an overnight fast	↓5:2 IER (~5.7%): -5 (SD 5) kg ↓WOWO(~5.6%): -5 (SD 5) kg ↓CER (~7.4%): -7 (SD 6) kg 5:2 IER = WOWO = CER	**HDL-cholesterol** rose (7%) and **TAG** decreased (13%) at 12 months with NS between groups No changes were seen for **fasting glucose** or **LDL-cholesterol** A total of 25% of participants lost ≥ 10% of their initial BW and 31% lost between 5 and 10%	**Meals**: not provided Energy restriction was not maintained as the maximum WL was at 6 months after which participants slowly regained weight

Author (year)	Sample size and Participants	Age Years	BMI Kg/m²	Intervention duration	Intervention	Comparison condition(s)	How long before measurement	Weight loss	Outcomes (Change between-groups over time unless otherwise stated)	Comments and limitations
Antoni et al., (2018)	5:2 IER n=15 (9) CER n=12 (12) Men and women Overweight/obese	5:2 IER: 42 (SEM 4) CER: 48 (SEM 3)	5:2 IER: 29.8 (SEM 0.9) CER: 30.8 (SEM 1.1)	5:2 IER: 59d (IQR 41-80) CER: 73d (IQR 48-128) To attain a 5% weight loss in both groups	**5:2 IER:** **ER**: 2d/wk (**consecutive**), 2638 kJ (~75% ER), LighterLife™ very-low energy formula-based Food Packs (38% CHO, 36% protein, 26% fat) **FEED**: 5d/wk, isoenergetic intake, Self-selected	**CER:** 23% ER/d, 2510 kJ below estimated energy requirements	Abstained from any form of ER for ≥7 d	↓5:2 IER (~5%): 88.8 (SEM 3.4) kg to 84.1 (SEM 3.2) kg ↓CER (~5%): 89.3 (SEM 4.5) kg to 84.9 (SEM 4.3) kg 5:2 IER = CER	• **FM**: ↓5:2 IER=CER • **FFM**: ↓5:2 IER=CER • **Postprandial TAG: (TAG iAUC, mmolx360min/l)**: ↓5:2 IER (~36%) and ↑CER (~11%); The relative reduction in TAG responses: 5:2 IER>CER • **Fasting glucose**: ↑5:2 IER (~4.5%), NS CER; 5:2 IER=CER • **Systolic BP**: ↓5:2 IER (~10%), NS CER; 5:2 IER>CER • **Diastolic BP**: ↓5:2 IER (~7%) and ↓CER (~7%); 5:2 IER=CER	• **Meals**: only provided for "2d" • Postprandial measurement • Only fasting Indirect calorimetry
Carter et al., (2018)	5:2 IER n=51 (19) CER n=46 (21) Men and women Overweight/obese With T2DM	5:2 IER: 61 (SD 9) CER: 61 (SD 9)	5:2 IER: 35 (SD 6) CER: 37 (SD 6)	12 months	**5:2 IER:** **ER**: 2d/wk (**mostly non-consecutive**), 2092-2510 kJ (~75% ER), **FEED**: 5d/wk, habitual eating ≥ 50g/day protein	**CER:** 7d/week, 5021-6276 kJ (30% ER) (45% CHO, 30% protein, 25% fat)	After at least 1 habitual eating day	↓5:2IER: 7.1 (SEM 1.1)kg ↓CER: 5.0 (SEM 0.8)kg 5:2 IER = CER	• **FM**: ↓5:2 IER=CER • **FFM**: ↓5:2 IER=CER • **HbA1c**: ↓5:2 IER: – 0.3 (SEM 0.1)% and ↓CER: – 0.5 (SEM 0.2)%; 5:2 IER=CER	• **Meals**: no meal replacements or foods were provided
Conley et al., (2018)	5:2 IER n=11 (1) CER n=12 (0) Obese Male war veterans	5:2 IER: 68 CER: 67	5:2 IER: 33.4 CER: 36.2	6 months	**5:2 IER:** **ER**: 2d/wk (**non-consecutive**), 2510 kJ **FEED**: 5d/wk, *ad libitum*	**CER:** 2050kJ below average requirement	After an overnight fast	↓5:2IER (~5.5%): 5.3 kg ↓CER: (~5.4%): 5.5 kg 5:2 IER = CER	• NS diastolic BP/fasting blood glucose/blood lipids	• **Meals**: not provided • Data presented as mean ± SD or mean ± SD SEM is not clarified. (Published in << Nutrition & Dietetics >>)

Author (year)	Sample size and Participants	Age Years	BMI Kg/m^2	Intervention duration	Intervention	Comparison condition(s)	How long before measurement	Weight loss	Outcomes (Change between-groups over time unless otherwise stated)	Comments and limitations
Schübel et al., (2018) **"HELENA" trial**	5:2 IER n=45 (4) CER n=41 (8) Control n=50 (2) Men and women Overweight/obese	5:2 IER: 49 (SD 9) CER: 51 (SD 8) Con: 51 (SD 7)	5:2 IER: 32.0 (SD 3.8) CER: 31.2 (SD 4.0) Con: 31.1 (SD 3.6)	**12-wk intervention** 12-wk maintenance 26-wk follow-up	**5:2 IER:** **ER**: 2d/wk (**self-selected non-consecutive**), ~75% ER (personalized meal plans were created, in which possible choices for meal components arranged by food groups were given; participants had to select 4 food items out of the vegetable group, 2 out of the low-fat dairy product group, and 1 food item out of each of the meat/fish, CHO, and fruits groups, in combination with a minimum intake of 2 L of low-energy drinks) **FEED**: 5d/wk, isoenergetic intake,	**CER:** 20% ER/d, **Control:** no advice to restrict energy	Blood analysis excluded the participants of the 5:2 IER group who had an energy-restricted day before the blood draw (n=14)	Log_e relative weight change over 12wk: 5:2IER: -7.1 (SEM 0.7)% CER: -5.2 (SEM 0.6)% Control: -3.3 (SEM 0.6)% Significant time-by-treatment interaction across all 3 study groups; and for ICR vs. Control and CCR vs. Control, the difference between ICR vs. CCR was borderline significant (P = 0.053)	• NS **Adipose tissue transcriptome** measured in 82 preselected genes between the 5:2IER and CER • A trend toward greater **weight loss** with 5:2 IER compared to CER (log_e relative weight change −7.1 ± 0.7% vs. −5.2 ± 0.6%, p = 0.053) • A significantly stronger reduction in **fasting blood glucose** at week 12 was observed for CER (5.21 to 4.84 mmol/L) compared with 5:2IER (5.14 to 4.98). However, NS in fasting blood glucose concentrations between either CER or 5:2IER and the Control regimen, and NS at weeks 24 and 50 • NS in **weight regain** between 5:2IER and CER over follow-up periods • NS **fasting insulin** • NS **HOMA-IR**	• **Meals**: not provided • Well-designed • 3 arms • The first to investigate the effect of 5:2IER on the expression of adipose tissue genes • Drop-out rate <10% in 5:2IER and CER groups
Sundfør et al., (2018)	5:2 IER n=50 (4) CER n=55 (3) Men and women With abdominal obesity and ≥1 additional component of metabolic syndrome	5:2 IER: 50 (SD 10) CER: 48 (SD 11.6)	5:2 IER: 35 (SD 4) CER: 35 (SD 4)	**6-month intervention** 6-month maintenance	**5:2 IER:** **ER**: 2d/wk (**non-consecutive**), 1674/2510 kJ (women/men) **FEED**: 5d/wk, habitual eating Weekly ER: 28%	**CER:** 26% ER/d	After an overnight fast	↓5:2 IER (~8.4%): -9.1 (SD 5.0) ↓CER (~8.7%): -9.4 (SD 5.3) 5:2 IER = CER	• **Subjective hunger scores** 5:2 IER>CER • There were favorable improvements in **waist circumference**, **BP**, **TAG** and **HDL-cholesterol**, 5:2 IER=CER • **RMR** was reduced from baseline to 3 months, from 1917 kcal/day (SD 285) to 1824 kcal/day (SD 222) in the 5:2IER (n = 12) and from 1882 kcal/day (SD 253) to 1767 kcal/day (SD 236) in the CER (n = 12) with no between-group difference	• **Meals**: not provided • Drop-out rate <10% in both groups • Recommended ~50 g protein/day from chicken breast, lean meat, lean fish, fat free yoghurt, cottage cheese, egg or legumes and vegetables to increase satiety on fasting days
Carter et al., (2016)	5:2 IER n=26 (5) CER n=25 (7) Men and women Overweight&obese With T2DM	5:2 IER: 61 (SD 8) CER: 62 (SD 9)	5:2 IER: 35 (SD 5) CER: 36 (SD 5)	12 weeks	**5:2 IER:** **ER**: 2d/wk, 1670-2500 kJ non-/consecutive not mentioned **FEED**: 5d/wk, habitual eating	**CER:** 5000-6500 kJ/d	After an overnight fast	↓5:2 IER (~6.2%): ↓CER (~5.6%): 5:2 IER = CER	• **HbA1c**: ↓5:2 IER (~0.5%) and ↓CER (~0.6%); 5:2 IER=CER • Both groups reported comparable decrease in **appetite** and increased feelings of **fullness** and **satisfaction** • Both groups reported comparable improvements in total **FM** and **FFM**	• **Meals**: not provided • Pilot trial for Carter et al., (2018) • 45% CHO, 30% protein, 25% fat for meals

Author (year)	Sample size and Participants	Age Years	BMI Kg/m^2	Intervention duration	Intervention	Comparison condition(s)	How long before measurement	Weight loss	Outcomes (Change between-groups over time unless otherwise stated)	Comments and limitations
Harvie et al., (2013)	5:2 IER n=33 (4) CER n=27 (13) Women Overweight/obese With a family history of breast cancer	5:2 IER: 45.6 (SD 8.3) CER: 47.9 (SD 7.7)	5:2 IER: 29.6 (SD 4.1) CER: 32.2 (SD 5.6)	12 weeks	**5:2 IER:** **ER**: 2d/wk (**consecutive**), 2500-2717 kJ (~70% ER), < 40 g CHO, 250 g protein foods, 3xlow-fat dairy portions, 1x/4x low CHO fruit/veg **FEED**: 5d/wk, isoenergetic intake, Mediterranean-type diet (45% CHO, 25% protein, 30% fat)	**CER:** 25% ER/d, Mediterranean-type diet, (45% CHO, 25% protein, 30% fat)	At least 5d after the last partial ER day in 5:2 IER	↓5:2 IER (~6%): 79.4 (95%CI 74.6–84.1) kg to 74.4 (95%CI 70.0–78.9) kg ↓CER (~4%): 86 (95%CI 80.6–91.3) kg to 82.3 (95%CI 77.1–87.5) kg 5:2 IER = CER	• **FM**: ↓5:2 IER (4 kg) and ↓CER (3 kg); 5:2 IER>CER • **FFM**: ↓5:2 IER=CER • **Fasting glucose, HbA1c**: NS (both groups) • **Fasting serum insulin**: ↓5:2 IER (~19%), NS CER; 5:2 IER>CER • **Insulin resistance (HOMA-IR)**: ↓5:2 IER (~25%), NS CER; 5:2 IER>CER • **Total cholesterol**: ↓5:2 IER (~5%) and ↓CER (~3%); 5:2 IER=CER • **LDL**: ↓5:2 IER (~4%) and ↓CER (~3%); 5:2 IER=CER • **HDL**: NS (both groups) • **TAG**: ↓5:2 IER (~14%) and ↓CER (~7%); 5:2 IER=CER • **Systolic BP**: ↓5:2 IER (~4%) and ↓CER (~8%); 5:2 IER=CER • **Diastolic BP**: ↓5:2 IER (~4%) and ↓CER (~8%); 5:2 IER=CER • **Leptin**: ↓5:2 IER (~43%) and ↓CER (~28%); 5:2 IER=CER • **IL-6**: ↓5:2 IER (~13%) and ↓CER (~7%); 5:2 IER=CER • **TNF-α, adiponectin**: NS (both groups)	• **Meals**: not provided • Intention-to-treat analysis based on LOCF • Low in CHO and relatively high in protein and fat: to maximise satiety and food choice and to limit the loss of FFM within the ER period • Higher serum β-hydroxybutyrate indicates greater fat oxidation during the 2d energy restriction. • Ketones increased during the restricted periods, indicating a switch from glycogenolysis to fat oxidation • During the 1-month weight maintenance phase, 1d/week of 5:2 IER maintained the reductions in insulin resistance and body weight.

Author (year)	Sample size and Participants	Age Years	BMI Kg/m^2	Intervention duration	Intervention	Comparison condition(s)	How long before measurement	Weight loss	Outcomes (Change between-groups over time unless otherwise stated)	Comments and limitations
Harvie et al., (2011)	5:2 IER n=42 (11) CER n=47 (7) Pre-menopausal women Overweight/obese	5:2 IER: 40.1 (SD 4.1) CER: 40.0 (SD 3.9)	5:2 IER: 30.7 (SD 5.0) CER: 30.5 (SD 5.2)	6 months	**5:2 IER:** **ER**: 2d/wk (**consecutive**), 2060-2266 kJ (~75% ER), 50 g protein, 1.1 L semi skimmed milk, 4x vegetables, 1x fruit **FEED**: 5d/wk, isoenergetic intake, Mediterranean-type diet (45% CHO, 25% protein, 30% fat)	**CER:** 25% ER/d, Mediterranean-type diet, (45% CHO, 25% protein, 30% fat)	• At least 5d after the last partial ER day in 5:2 IER • In a subset of the 5:2IER (n=15), fasting blood samples were collected after 5d of normal intake, on the morning after 2d of partial ER and after 2d of normal intake • And also on these days of the week in a subset of the CER group (n =9) for comparison	↓5:2 IER (~7%): 81.5 (95%CI 77.5–85.4) kg to 75.8 (95%CI 71.4–80.2) kg ↓CER (~5.3%): 84.4 (95%CI 79.7–89.1) kg to 79.9 (95%CI 74.6–85.2) kg 5:2 IER = CER	• **FM**: ↓5:2 IER (5 kg) and ↓CER (4 kg); 5:2 IER>CER • **FFM**: ↓5:2 IER=CER • **Fasting glucose**: ↓5:2 IER (~2%), NS CER; 5:2 IER=CER • **Fasting serum insulin**: ↓5:2 IER (~29%) and ↓CER (~15%); 5:2 IER>CER • **Insulin resistance (HOMA-IR)**: ↓5:2 IER (~27%) and ↓CER (~19%); 5:2 IER>CER • **Total cholesterol**: ↓5:2 IER (~6%) and ↓CER (~10%); 5:2 IER=CER • **LDL**: ↓5:2 IER (~10%) and ↓CER (~10%); 5:2 IER=CER • **HDL**: NS 5:2 IER, ↓CER (~6%); 5:2 IER=CER • **TAG**: ↓5:2 IER (~17%) and ↓CER (~23%); 5:2 IER=CER • **Systolic BP**: ↓5:2 IER (~3%) and ↓CER (~6%); 5:2 IER=CER • **Diastolic BP**: ↓5:2 IER (~6%) and ↓CER (~8%); 5:2 IER=CER • **Leptin**: ↓5:2 IER (~40%) and ↓CER (~36%); 5:2 IER=CER • **Adiponectin**: ↑5:2 IER (~10%) and NS CER; 5:2 IER=CER	• **Meals**: not provided • A subset of participants (15 in 5:2IER and 9 in CER) provided fasting serum samples over 1 week during the study period. The 5:2IER group demonstrated acute reductions in fasting insulin (-23%), HOMA-IR (-29%) and TAG (-18%) in the morning after the 2-day restricted day, which normalized within 2 days of resuming a normal diet. There were no significant changes in the CER • May reflect a more potent influence of using two consecutive days of severe energy restriction, there were also greater reductions in energy and CHO intake in 5:2 group, which complicate the interpretation • 64% 5:2IER and 55% CER participants achieved >5% weight loss

Sample size (n) expressed as total completers (drop-outs); ↓or↑, post-intervention data significantly different from baseline data ($P < 0.05$); ~ denotes that % are approximated from provided pre- or post-intervention data.

NS, not statistically significant; *5:2 IER*, 5:2 intermittent energy restriction; *CER*, continuous energy restriction; *FM*, fat mass; *FFM*, fat-free mass; *ER*, energy restriction; *TAG*, Triacylglycerol; *BP*, blood pressure; *HbA1c*, glycated haemoglobin; *HOMA-IR*, homeostasis model assessment insulin resistance; *HDL*, high density lipoprotein; *LDL*, low density lipoprotein; *VAT*, visceral adipose tissue; *RMR*, resting metabolic rate; *IL-6*, Interleukin 6; *TNF-α*, tumour necrosis factor alpha

1.8 Aims and Objectives

With the devastating effects of long-term positive energy balance on health, lifestyle modifications through reduced energy intake and increased physical activity levels have aroused broad interest. Therefore, the overarching aim of the studies presented in this thesis was to gain a greater understanding of the metabolic effects of physical inactivity and 5:2 diet, with a specific emphasis on glycaemic control, insulin sensitivity and resting energy expenditure in response to MTT. In Chapter 3, the purpose of the study was to extend the findings of published research by evaluating the effects of two-days of physical inactivity, with and without a proportional decrease in energy intake, on insulin sensitivity, glycaemic control, appetite, and resting energy expenditure in response to food ingestion. A physical inactivity model was used in which step counts were reduced by 80%. Given the inconsistency among previous studies on the 5:2 diet presented in this introductory chapter, in Chapters 4 and 5, the aim was to compare the fasting and postprandial metabolic effects of two consecutive weeks in which either energy requirements were met on five days and restricted (70% energy restriction relative to estimated energy requirements) on two non-consecutive days each week (5:2 IER); or energy was restricted every day of the two-week period (20% daily energy restriction relative to estimated energy requirements) (CER). Both diets were thus hypoenergetic, and designed to induce the same total energy deficit over the experimental period.

Two studies designed to achieve this aim and objectives are presented in the following chapters, after Chapter 2, describing general methods.

CHAPTER 2. GENERAL METHODS

Studies 1 (Chapter 3) and 2 (Chapters 4 and 5) presented in this thesis have quite similar laboratory visit protocols and methodological procedures. The following chapter describes the general methods used in both studies. Specific methods for each study are presented in the relevant chapters.

2.1 Ethical Approvals

Studies carried out in this thesis adhere to accepted ethical standards and were approved by the University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee. All laboratory visits took place within the David Greenfield Human Physiology Unit, School of Life Sciences, Queen's Medical Centre, the University of Nottingham.

Study 1 Effects of Two Days of Physical Inactivity With and Without Energy Surplus on Basal and Postprandial Metabolism: A Randomised Crossover Study in Healthy Men and Women - medical ethics approval number I200317 (**Appendix A**). ClinicalTrials.gov PRS: NCT04172090.

Study 2 Investigating the Effects of Two Weeks of Intermittent Energy Restriction (5:2) or Continuous Energy Restriction on Basal and Postprandial Metabolism: A Randomised Study in Normal-Weight, Young Participants - medical ethics approval number 121-1809 (**Appendix B**). ClinicalTrials.gov PRS: NCT04138160.

All participants involved in these studies were informed of potential risks or inconvenience associated with research procedures that they would undergo before obtaining informed written consent. All studies were implemented in conformity to the World Medical Association (WMA) Declaration of Helsinki (DoH) (World Medical Association, 2013) and were registered at ClinicalTrials.gov.

2.2 Screening Protocol

Participants were recruited from the students and staff of the University of Nottingham and Queen's Medical Centre, Nottingham via poster advertisements on university notice boards and social media platforms.

2.2.1 Anthropometric Measurements

2.2.1.1 Height and Body Weight Measurements

Height was measured to the nearest 0.1 cm using a stadiometer (Seca, Germany) following the National Health and Nutrition Examination Survey (NHANES) Anthropometry Procedures Manual (CDC, 2007). Participants were asked to stand as tall and straight as possible with arms hanging loosely at their sides. Participants were instructed to keep their buttocks and shoulders touch the stadiometer with head in the "Frankfort plane".

In both studies, body weight was measured in the fasted state using an electronic scale (Seca, Germany) to the nearest 0.1 kg with participants in light clothing, without shoes, and with an emptied bladder. Participants were asked to stand still on the centre of the scale with their arms hanging loosely at their sides. Any heavy objects in pockets and heavy accessories on them were removed before the measurement (CDC, 2007).

2.2.1.2 BMI

Body Mass Index (BMI) was calculated from recorded body weight in kilograms, divided by the square of body height in meters (Keys *et al.*, 1972).

2.2.1.3 Circumference Measurements

Waist and hip circumferences were measured to the nearest 0.1 cm using a stretch resistant tape with participants having breathed out naturally before the measurement was taken. Waist circumference measurement was performed at the approximate midpoint between the lower margin of the last palpable rib and the top of the iliac crest (WHO, 2008). Hip circumference was measured around the widest portion of the buttocks (WHO, 2008). Both waist and hip

circumferences were measured with the tape parallel to the floor whilst participants were standing with feet close together. Each measurement was repeated twice; if the repeated measurements differed less than 1 cm, the average was calculated, otherwise the two measurements would be repeated (WHO, 2008). Waist-hip ratio was calculated as the waist circumference divide by the hip circumference, which provided additional information on body fat distribution (WHO, 2008).

2.2.1.4 Skinfold Thickness Measurements

Skinfold thickness measurements were performed to assess each participant's body fat content in both studies, at specific anatomical sites. Skinfold thickness measurement is used to measure the thickness of subcutaneous adipose tissue at different sites of the body using a skinfold caliper, which can be used to estimate the total body fat of individuals, assuming a constant relationship between subcutaneous fat and total body fat (Gibney, Vorster and Kok, 2002). Skinfold thicknesses at biceps, triceps, subscapular and suprailiac sites were measured on the right side of the body with participants standing in a relaxed state using a Harpenden skinfold calliper (CE 0120, Baty international Ltd., England). No statistical difference has been shown between measurements on either side of the body (Durnin and Womersley, 1974). Compared with other total body fat measurements, skinfold thickness measurement is less time-consuming and non-invasive with minimal quantity of instruments (Gibson, 2005). The same caliper was used throughout both studies by the same experimenter and was serviced regularly.

The body density was estimated by the logarithm of the sum of four sites skinfold thickness rather than absolute values due to a curvilinear relationship between skinfold thickness to density and a skewed skinfold distribution in the body (Durnin and Rahaman, 1967; Durnin and Womersley, 1974):

Males aged 17-19 $\quad$ **Density x 10^3 = 1.1620 - 0.0630 x Log $\sum$ (Skinfolds)**

Males aged 20-29 $\quad$ **Density x 10^3 = 1.1631 - 0.0632 x Log $\sum$ (Skinfolds)**

Males aged 30-39 $\quad$ **Density x 10^3 = 1.1422 - 0.0544 x Log $\sum$ (Skinfolds)**

Females aged 16-19 $\quad$ **Density x 10^3 = 1.1549 - 0.0678 x Log $\sum$ (Skinfolds)**

Females aged 20-29 **Density x 10^3 = 1.1599 - 0.0717 x Log ∑ (Skinfolds)**

Females aged 30-39 **Density x 10^3 = 1.1423 - 0.0632 x Log ∑ (Skinfolds)**

(Durnin and Womersley, 1974)

Body fat was estimated from density using Siri's Equation (Siri, 1956):

SIRI Equation: **% Body Fat = [4.95/(Body Density) − 4.50] x 100**

(Siri, 1956)

Skinfold thickness measurements have a number of limitations. One major drawback of this approach is that within-operator error and between-operator error are difficult to avoid. Thus, a trained and experienced operator is needed (Gibson, 2005). Another disadvantage is that when these measurements are used to assess total body fat, multiple, usually four sites skinfold measurements are required, which are more representative for the body fat distribution (Durnin and Rahaman, 1967). This method has limited utility with poor accuracy and precision in the overweight or obese subjects and more suitable for moderately overweight or thinner subjects (Wells and Fewtrell, 2006; Duren *et al.*, 2008). Moreover, the assessment requires partial skin exposure, which may hamper the employment in epidemiological studies and make certain participants uncomfortable (Gibney, Vorster and Kok, 2002).

2.2.2 Questionnaires

Participants were asked to complete the following questionnaires during the medical screening visit:

- Screening Form (including "Personal Information" and "General Health" sections) (**Appendix C**)
- Beck Depression Inventory (**Appendix D**)
- Eating Attitudes Test-26 (**Appendix E**)
- International Physical Activity Questionnaire (**Appendix F**)

2.2.2.1 Beck Depression Inventory

The Beck Depression Inventory (BDI) was used to assess the presence and severity of depression symptoms of participants in both studies (Beck, Ward and Mock, 1961). BDI is the most widely used self-rating scale for evaluating depression in the light of its high internal consistency, high content validity and validity in distinguishing between depressed and non-depressed individuals (Richter *et al.*, 1998). The BDI consists of 21 items and each item is ranked according to severity and scored from 0 to 3, with a total score ranging from 0 to 63. Total score of 0-9 indicates minimal depression, 10-18 indicates mild depression, 19-29 indicates moderate depression and 30-63 indicates severe depression. Participants with a BDI score of > 10 were excluded from the studies to minimise any potential confounding effects of depression on body weight, appetite and energy intake (Paykel, 1977).

2.2.2.2 Eating Attitudes Test-26

The Eating Attitudes Test-26 (EAT-26) was used to access the eating disorder risk of participants in both studies. The EAT was developed by Garner and Garfinkel in the form of a 40-item self-administered questionnaire, which was originally designed for patients with anorexia nervosa (Garner and Garfinkel, 1979). The EAT has been used in a variety of cultures with good psychometric properties of reliability and validity, and eating disorders sensitivity (Garner *et al.*, 1982; Garfinkel and Newman, 2001). The EAT-26 was developed by shortening the original 40-item version to a 26-item version (Garner *et al.*, 1982). Each of the 26 forced-choice Likert-scale items describes an eating disordered behaviour (except item 26), which was classified as “always”, “very often”, “often”, “sometimes”, “rarely” or “never”, with a score of 3, 2, 1, 0, 0, 0, respectively for items 1-25 and a score of 0, 0, 0, 1, 2, 3, respectively for item 26 (Garner *et al.*, 1982). Higher scores indicating greater risk of an eating disorder and scores > 20 indicates a need for further investigation by a qualified professional (Garner *et al.*, 1982). Participants with an overall score > 20 were excluded from the studies.

2.2.2.3 International Physical Activity Questionnaire

The self-administered short vision of the International Physical Activity Questionnaire (IPAQ) was used for assessing the physical activity level at screening in both studies (Hagstromer, Oja

and Sjostrom, 2006). The IPAQ was developed in the context of burgeoning global problem of physical inactivity, the requirements for population surveillance and inter-country comparisons in the late 1990s. The outcomes of the IPAQ reliability and validity study across 20 countries prove that the IPAQ can provide reliable and valid physical activity data for international study (Craig *et al.*, 2003; Mäder *et al.*, 2006; Bauman *et al.*, 2009). The IPAQ short form "last 7d" questionnaire is widely used in national and regional prevalence studies considering its administration feasibility and no differences of reliability and validity has been shown between the short and long IPAQ forms (Craig *et al.*, 2003). Physical activity level was assessed during the preceding seven consecutive days and categorised as 'low (1.4)', 'moderate (1.6)' or 'high (1.8)'.

2.2.3 Routine Blood Tests

At the end of the screening visit, an 8 mL blood sample was obtained from the median cubital vein for routine blood investigation (full blood count and urea and electrolytes) to confirm participants were in good health. Full blood count and urea and electrolytes analyses were conducted by the Clinical Chemistry and Haematology Laboratories in the Queen's Medical Centre, University of Nottingham.

2.3 Assessment of Energy Intake

2.3.1 Diet Diaries

Participants were asked to record a three-day food diary (see **Appendix G**), which was used to evaluate their habitual energy intake and food preferences. Detailed instructions with examples and graphical representations were included in the food diary. A portable digital kitchen scale (Salter Black LCD Electronic Kitchen Scales 1066, Salter housewares LTD, Tonbridge, UK) and a standby battery were provided. Participants were asked to maintain their habitual diet and return the food diaries prior to commencing the study. For the purpose of making the analysis of the diary as precise as possible, any ambiguous information entered by the participants was pointed out by the experimenter and clarified by the participants.

2.3.2 Dietary Analyses

Dietary analyses were performed using Nutritics nutrition analysis software (Nutritics Ltd, Ireland), research edition v5.096 to assess the daily energy intake and macronutrients composition for each participant. Non-branded items in Nutritics were chosen when the food brands were not reported by the participants, otherwise food nutrient values were entered manually according to nutrition labels on the back or side of the packaging and further standardised by the analysis package to the format used by the database.

2.4 Estimation of Energy Requirements for the Intervention Meal Plan

2.4.1 BMR Calculation

In Chapters 4 and 5, basal metabolic rate (BMR) was estimated using the Henry prediction equations (Henry, 2005), based on body weight and height, which were recommended by the Scientific Advisory Committee on Nutrition (SACN) in 2011 (SACN, 2011):

For men:

18-30 years: **BMR = 14.4W + 313H + 113**

30-60 years: **BMR = 11.4W + 541H – 137**

For women:

18-30 years: **BMR = 10.4W + 615H – 282**

30-60 years: **BMR = 8.18W + 502H – 11.6**

Where W is weight in kilograms, H is height in meters and BMR is basal metabolic rate in kcal/day.

2.4.2 PAL Estimation

Physical activity intensity was assessed using the IPAQ, and the results were reported in categories as low, moderate or high activity levels. To estimate the total energy requirements, BMR was multiplied by a factor appropriate to the amount of physical activity that participant does, which is known as Physical Activity Level (PAL). A PAL of 1.4 is associate with a very low level of occupational or leisure activity levels, a PAL of 1.6 represents moderate intensity activity, and a PAL of 1.8 represents high levels of physical activity (DOH, 1991; SACN, 2011). Thus, energy requirement can be predicted by multiplying the BMR with the PAL as shown below:

Estimated energy requirements (EER) = BMR x PAL

Where BMR is basal metabolic rate in kcal/day.

2.4.3 Dietary plan

In both studies, participants were proscribed dietary plans and provided with all food and beverages during the study period. In each study, the dietary plan had identical food items (same proportions but with adjusted quantities to meet individual EER and the protocol), meal times and daily distribution of energy, between groups. Participants were free-living but instructed to follow the prescribed meal plan. They were provided with a paper log and asked to note any leftovers (including amount of each left item) and any additional food ingested that had not been provided (including item name, brand if available, and amount). The log was discussed on arrival at the laboratory on the next measurement day. Participants were also asked to take photos of each with their smart phones, just before eating and send these to the experimenters in real-time so as to verify their meal time.

Total CHO and its components, including starch, total and individual sugars (glucose, fructose, sucrose, maltose, lactose) and oligosaccharides, but not fibre are expressed as CHO monosaccharide equivalents in McCance and Widdowson food database (M&W) (Roe *et al.*, 2015). **Table 2.1** shows the conversion between CHO weights and monosaccharide equivalents adapted from the introduction of M&W (Roe *et al.*, 2015). In both studies, some compositional data from food labels was taken and converted to the equivalent format used in M&W.

Table 2.1 Conversion of carbohydrate weights to monosaccharide equivalents.

Adapted from Roe *et al.* (2015).

Carbohydrate	Equivalents after hydrolysis (g/100g)	Conversion to monosaccharide equivalents
Monosaccharides e.g. glucose, fructose and galactose	100	no conversion necessary
Disaccharides e.g. sucrose, lactose and maltose	105	x 1.05
Oligosaccharides e.g.		
raffinose (trisaccharide)	107	x 1.07
stachyose (tetrasaccharide)	108	x 1.08
verbascose (pentasaccharide)	109	x 1.09
Polysaccharides e.g. starch	110	x 1.10

Energy values can be calculated from either of the following two equations according to energy conversion factors shown in **Table 2.2**:

CarbohydrateMsEq x 3.75 + Protein x 4 + Fat x 9 + Alcohol x 7 + Fiber x 2 or

Carbohydrate x 4 + Protein x 4 + Fat x 9 + Alcohol x 7 + Fiber x 2

Table 2.2 Metabolisable energy conversion factors used in meal template.

	kcal/g	kJ/g
Protein	4	17
Fat	9	37
Available carbohydrate expressed as monosaccharide	3.75	16
Alcohol (ethanol)	7	29
Fibre	2	8

In healthy individuals, metabolisable energy is the gross energy intake minus the energy lost in faeces, urine and gastrointestinal gas, which stands for the energy can be used for growth, reproduction and metabolic processes.

According to the report of a technical workshop on food energy – methods of analysis and conversion factors by FAO in 2002, the contribution of fiber (measured by the AOAC method) to energy is suggested to be taken into account with a factor of 2 kcal/g (WHO/FAO, 2003). It is noteworthy that energy percentage providing by fiber was kept relatively consistent between each meal plan. This is because dietary fiber may delay gastric emptying and further enhance satiety (Anderson *et al.*, 2009).

2.5 Measurements Made During the Intervention Periods

2.5.1 Physical Activity Monitoring

In both studies, free-living physical activity level (recorded as daily step counts) of participants were estimated using a pedometer (Omron Walking Style One 2.1, Omron, USA) worn from getting up in the morning until going to bed in the evening. In both studies, the pedometers used provided an easily accessible feedback of each participant's daily step count targets corresponding to different levels of prescribed physical activity.

2.5.2 Continuous Glucose Monitoring

2.5.2.1 *i*Pro®2

The Medtronic MiniMed *i*Pro™2 (Northridge, CA, USA) Continuous Glucose Monitoring (CGM) system was used with Medtronic Enlite™ glucose sensors inserted through the skin on the abdomen of participants to continuously monitor glucose levels in interstitial fluid and was approved by the FDA in 2016 (FDA, 2016). The *i*Pro2 consists a disposable sensor with a supply of glucose oxidase enzyme at the tip, which is inserted subcutaneously into the anterior abdominal wall with a spring-loaded device (Enlite™ Serter, Medtronic, MiniMed, Northridge, USA). Under the catalysis of glucose oxidase, a biochemical reaction happens in the presence of glucose and oxygen, which creates an electronic current. The magnitude of the current will then be converted into a glucose concentration using an algorithm and assumptions about the kinetic equilibrium of glucose concentration between interstitium and blood (Boyne *et al.*, 2003). *i*Pro2 measures and records glucose concentrations every 5 minutes, providing up to 288 readings in a 24 h period,

showing hidden glucose excursions which finger pricks may miss. Enlite™ Sensor can support a full 6 days of use and collect up to seven 24-hour periods of data. In both studies, the monitor was calibrated with four separate fingerpick capillary glucose tests each day (prior to each of the three main meals and at bedtime) using the ACCU-CHEK® Performa Blood Glucose Meter (Roche, Mannheim, Germany) which will be discussed in further detail in Section 2.5.2.2. A Unistik single use lancet (Unistik® 3 Comfort Single Use Safety Lancets, 28G gauge, 1.8mm depth, Owen Mumford Ltd., UK) was used to cut the fingertip to get enough blood applied to Accu-ChekÒ Aviva strips (Roche, Mannheim, Germany). At the end of each trial, the monitor was removed and then plugged into the docking station and the data uploaded into Medtronic CareLink™ iPro™ Therapy management software for diabetes (Boyne *et al.*, 2003) (**Figure 2.1**). Only fingerprick readings between 2.2 and 22.2 mmol/L can be used for calibration. Each fingerprick reading used for calibration is paired with the corresponding sensor glucose value generated by the calibration algorithm at the same specific time point (Damiano *et al.*, 2013, 2014; Matuleviciene *et al.*, 2014) (**Figure 2.2** is an example *i*Pro2 trace over a 24h period from 12:00 to 12:00 during 5:2 IER intervention). A control test (ACCU-CHEK® Performa Control Solution, Roche, Mannheim, Germany) for the blood glucose meter was performed before each trial for each participant and when a new test strip box was opened to ensure the glucose meter and test strips work properly and provide reliable results. The accuracy of blood glucose estimated by CGM relies on the calibration, while improper calibration performed by participants is regarded as the primary factor contributing to CGM inaccuracy (Rossetti *et al.*, 2010). A consistent time lag of 4-10 min was detected between blood glucose and interstitial glucose in subcutaneous tissues of the abdomen by using the CGM sensor, which may influence the precision of calibration (Boyne *et al.*, 2003; Steil *et al.*, 2005).

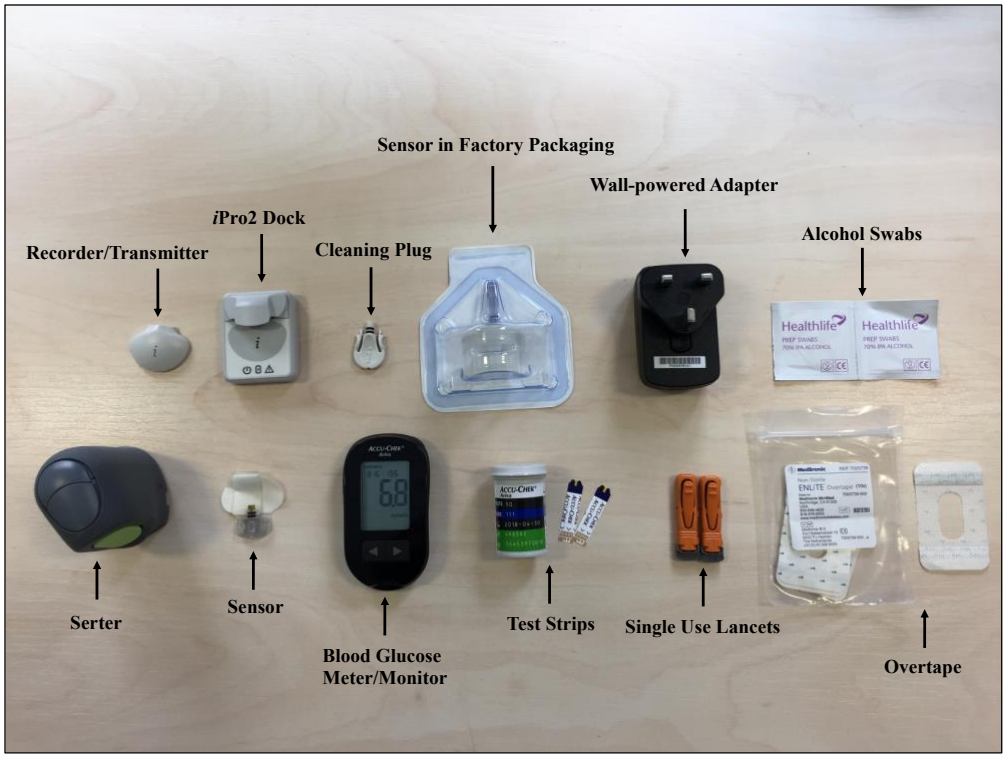


Figure 2.1 Components of Medtronic MiniMed *i*Pro™2 continuous glucose monitoring system and capillary blood glucose measurement.

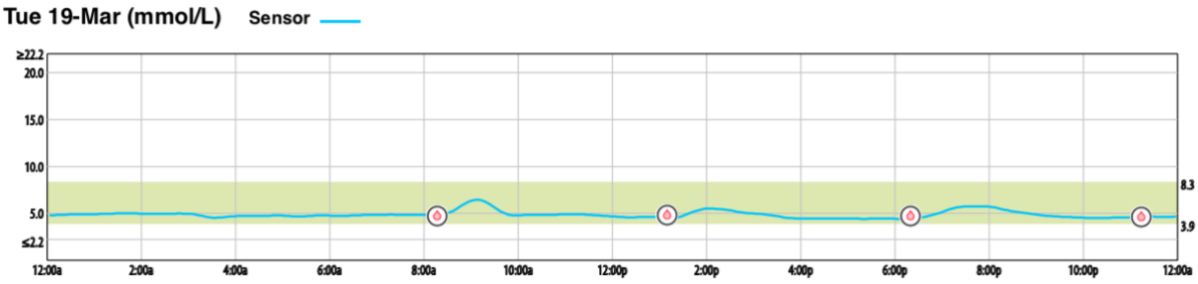


Figure 2.2 A 24h sensor data of *i*Pro®2.

The x-axis represents the time of the day and the y-axis presents glucose concentration in mmol/L. The blue line displays the continuous glucose concentration, and the blood drop displays the blood glucose concentration of finger prick tests for calibration taken by the participant.

2.5.2.2 Capillary Blood Glucose Monitoring

Standard for the practice of finger prick varies. The use of the first or the second drop of capillary blood is the most controversial one. The first drop of capillary blood may be questioned by the dilution with interstitial fluid, which may produce underestimated blood glucose values (Hortensius *et al.*, 2012). However, the different can be negligible considering the highly perfused skin of fingers (Fruhstorfer and Quarder, 2009; Palese *et al.*, 2016). Results from both non-diabetic (Fruhstorfer and Quarder, 2009) and diabetic (Li, Wang and Shan, 2014; Palese *et al.*, 2016) individuals indicated that the first drop of capillary blood was adequate for blood glucose self-monitoring. Study performed in patients with type 1 or type 2 diabetes showed that the first drop of capillary blood was closer to that from venous blood in the groups with blood glucose levels below 10 mmol/L (Li, Wang and Shan, 2014). Generally, finger prick for capillary blood glucose testing is suggested to use the first drop of blood after hands washed with soap and water, and dried properly (Baum *et al.*, 2006; Hortensius *et al.*, 2012). Hard squeezing of the finger should be refrained (Hortensius *et al.*, 2012).

In both studies, participants were provided with alcohol swabs, single use lancets, test strips, blood glucose meter, alcohol swabs and cotton balls (**Figure 2.2**). Before the skin puncture, participants were asked to ensure that the aforementioned components were ready for use, disinfect hands with soap and water, and wait until the finger is dry. If washing hand was unattainable, participants were asked to wipe the puncture site with alcohol swabs and waited until the finger was dry. For doing the skin puncture, participants were asked to hold side of finger firmly, release needle, and apply gentle pressure to digit if necessary, to obtain sufficient size drop of blood and apply to test strip for glucose reading. Used lancets were asked to be collected in a thick bag and taken to the lab on the following visit, investigators would discard the lancets in a sharps bin.

2.6 Laboratory Visit Protocol

2.6.1 Indirect Calorimetry

As one of the energy expenditure measurement approaches, direct calorimetry is termed because heat loss in humans is measured directly. The direct calorimetry method is technically demanding

and not widely used. Indirect calorimetry is more frequently used, especially in human studies, and is named 'indirect' because the energy expenditure is calculated from the respiratory exchange of O_2 and CO_2 (**Figure 2.3** illustrates the difference between direct calorimetry and indirect calorimetry). The indirect calorimetry method is based on the principle that the rate of inhaled O_2 and exhaled CO_2 exchanged in the lungs is generally equivalent to the rate of being used and released by the tissues (Kenney, Wilmore and Costil, 2015).

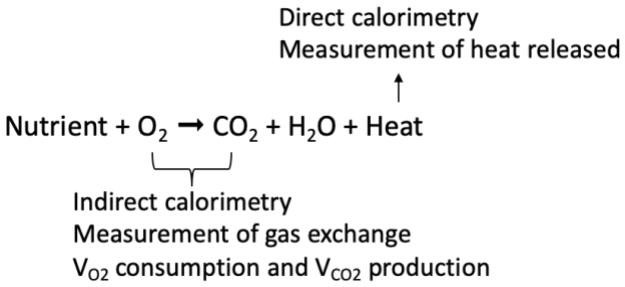


Figure 2.3. Difference between direct calorimetry and indirect calorimetry.

In both studies, resting energy expenditure and whole-body substrate oxidation rates were estimated using data obtained from a Quark CPET open-circuit metabolic cart (Cosmed, Quark PFT, Rome, Italy), which operated with the canopy dilution technique. Samples are pumped to a gas analyser for the determination of the volume and the fraction of O_2 and CO_2 of expired gas to further calculate the volume of O_2 inspired and CO_2 expired. With the canopy hood, flow rate can be measured using a digital turbine flowmeter directly (Blond *et al.*, 2011).

Both O_2 and CO_2 sensors are able to respond within 120 ms with the accuracy of 0.02%, the former is a paramagnetic sensor with a measuring range between 0 – 30% and the latter is an infrared digital sensor with a measuring range between 0 – 10%. It has been recognised that during the oxidation of CHO, fat, and proteins, the O_2 consumption and CO_2 production in the tissues happen in a ratio specific for each, which is known as respiratory quotient (RQ) (Frayn, 1983). RQ indicates the relative contribution of CHO, fat, and proteins to total energy expenditure, however, to measure the CO_2 production and O_2 consumption in the tissues requires the blood sample taken from the artery and vein, which is invasive and unfeasible. Respiratory exchange ratio (RER) is

the non-invasive alternative for the determination of substrate being oxidised, which is calculated as the ratio between the rate of CO_2 released and O_2 consumption taken through the gas exchange. An RER value between 0.707 and 1.0 suggests a mixture of both fat and CHO as the energy source, whereas a value of 1.0 indicates the exclusive use of CHO. Unlike fat and CHO, protein plays a very small part in energy metabolism under normal conditions, and its respective RER value is approximately 0.8 (Frayn, 1983). The resting energy expenditure and substrate oxidation rates can be calculated from respiratory O_2 and CO_2 exchange and the urinary nitrogen excretion rate based on the knowledge of the stoichiometry, which refers to the substrate reaction with O_2 to produce CO_2, H_2O and nitrogen end-products (Ferrannini, 1988; Simonson and DeFronzo, 1990). Non-protein equations were used in this thesis considering the contribution of protein oxidation to metabolism in non-pathological conditions is constant and any increases in protein oxidation in response to the liquid breakfast are relatively small and consistent between trials.

At the start of the study day, calibration of the flowmeter was performed using a 3 L calibration syringe, which was attached to a turbine flowmeter (18 mm turbine for resting measurement). During the flowmeter calibration, the syringe piston was moved in and out for six stokes, keeping the flow between -1 and 1 L/s, error between 1-4% was accepted. Room air calibration was conducted subsequently. Gas calibration was performed before each test using the gas calibration cylinder with known gas concentration (16% O_2, 5% CO_2, N_2 bal and pressure regulator). In both studies, following a 30-min rest in a semi-supine position in a hospital bed, a clear perspex ventilated canopy hood with a volume of 30 L was placed over the participant's head and secured around the neck in order to collect the expired air for respiratory CO_2 and O_2 exchange calculation (see **Figure 2.4**). The hood was ventilated with room air by a vacuum pump, the flow rate was monitored and regulated by the operator carefully to ensure the fraction of CO_2 in the expired air (F_ECO_2) between 0.5% to 0.6%. A bidirectional turbine flowmeter with an 18 mm diameter was used to detect the canopy ventilation rate, ranging from 0 – 80 L/min with the accuracy of 2% (Blond *et al.*, 2011). Baseline resting energy expenditure (REE) was measured in a fasted state for 20 min, which was the BMR of the participant. Readings were reported every 10 s and the first and last 2.5 min of data acquisition were excluded. The data in the mid 15 min were then averaged to calculate the REE and RER. Postprandial REE was then measured for 20 min in every hour for

three hours after the test drink consumption, which enabled the calculation of postprandial substrate oxidation and diet induced thermogenesis (DIT) of the test drink.



Figure 2.4 Indirect calorimetry was performed during the main measurement visit.
One pump was used to ventilate the hood and another pump was used to extract the ventil stream for analysis. Calculated O_2 consumption and CO_2 production were then converted using the Weir equation in Resting Metabolic Rate (Weir, 1949).

It is important to make sure participants are physically comfortable with the position they keep during the measurement and repeated measurements are in the same position (Compher *et al.*, 2006). Second, taking into considerations of the increased thermogenesis caused by agents in the environment, including nicotine in tobacco; caffeine and derivatives in coffee, tea, and chocolate; capsaicin in hot chilies, their consumption should be strictly controlled prior to measurement (Gibney, Vorster and Kok, 2002). Third, vigorous or moderate physical activities should be avoided in hours prior to measurement as they can increase the metabolism (Lopes, Chaia and Brito, 2013). Regular observation for air leaks is required because leaks will lead to artificially low BMR values (Compher *et al.*, 2006). Fifth, a steady-state is suggested to be achieved during the testing period, which is defined as a 5-minute measurement having a coefficient of variation less than 10% and the average RER changes less than 5% (Compher *et al.*, 2006). Furthermore, a critical point during the REE measurement is to ensure the F_ECO_2 lies between 0.5-0.6% by

altering the air ventilation through the hood. Therefore, combined efforts of operators' technical expertise and participants' compliance contribute to the accuracy of indirect calorimetry.

2.6.1.1 Indirect calorimetry validation

Ethanol combustion was performed every two weeks to ensure the reliability of the Cosmed results obtained (see **Figure 2.5**). The metabolic cart was warmed up 15 min prior to use. Flowmeter and gas calibration were conducted before the test as normal. Ethanol burner was filled with 99.9% (absolute) ethanol and weighed, with weight being recorded. The canopy tube was connected to the ethanol burning kit by means of a rigid plastic tube to prevent being melted. The ethanol burn was run for 20 min and repeated for 3 times in each validation test.

For ethanol, the stoichiometric equation is:

$C_2H_5OH + 3O_2 \rightarrow 2CO_2 + 3H_2O$

The ratio of V_{CO2} to V_{O2} in the burning of ethanol yields an RER of 0.667, which is one of the aims of the validation.

For O_2 recovery, the following calculations were computed:

Amount of ethanol burnt (m_{EtoH}) = Weight of burner at start – Weight of burner at end

Calculated $V_{O2} = [(m_{EtoH} / 46) \times 96)] / 1.429$

(Molar mass of C_2H_5OH is 46 g/mol; molar mass of O_2 is 32g/mol; density of O_2 at standard temperature and pressure (STP) is 1.429 g/L).

Or, simplified as: Calculated $V_{O2} = m_{EtoH} \times 1.459$

% Recovery = (Measured V_{O2}/Calculated V_{O2}) x 100

As another aim of the validation, the % recovery was expected to be above 95%.



Figure 2.5. Ethanol burn was conducted for indirect calorimetry validation.

2.6.1.2 Calculation of REE and DIT

The abbreviated Weir equation was used for REE and DIT calculation, with the assumption that the contribution of protein oxidation to metabolism in non-pathological conditions is constant and any increases in protein oxidation in response to the liquid breakfast are relatively small and consistent between trials (Weir, 1949):

kcal/min= 3.941 x (V_{O2}) + 1.1106 x (V_{CO2})

with V_{O2} and V_{CO2} in liters per minute (L/min)

For REE, the averaged kcal/min was multiplied by 1440 to produce kcal/day.

REE (kcal/day) = [3.941 x (V_{O2}) + 1.1106 x (V_{CO2})] x 1440

with V_{O2} and V_{CO2} in liters per minute (L/min)

For DIT, the trapezoidal method was adopted to calculate the iAUC of postprandial REE above the baseline REE (i.e. BMR).

2.6.1.3 Fasting and Postprandial Substrate Oxidation

Substrate oxidation rates were computed using the non-protein respiratory quotient (Péronnet and Massicotte, 1991), with the assumption that the amount of proteins oxidised is small and negligible :

Fat oxidation rate = 1.695 x V_{O2} – 1.701 x V_{CO2}

Carbohydrate oxidation rate = 4.585 x V_{CO2} – 3.226 x V_{O2}

with V_{O2} and VC_{O2} in liters per minute (L/min) and oxidation rate in grams per minute (g/min).

2.6.2 Standardised Test Drink

The standardised test drink (Ensure® Compact, Abbott Nutritional Ireland, 2.4 kcal/ml balanced nutritional supplement drink) was provided at room temperature (24 °C) in an open glass at breakfast time (see **Figure 2.6**). Participants were asked to finish the test drink within 2 min. The volume of test drink provided was varied to provide 10 kcal/kg body weight (BW). The energy distribution from the macronutrients was 47.9% CHO, 35.1% fat, and 17% protein. Therefore, participants received 1.2 g CHO/kg BW, 0.39 g fat/kg BW, and 0.43 g protein/kg BW. A volume of 10 kcal/kg BW was designed based on the average estimated daily energy requirements and average body weight of the participants. For purpose of achieving an energy balance, each participant required approximately 30 kcal/kg BW per day, which was about 10 kcal/kg BW for each meal when 30 kcal/kg BW was divided by 3. Therefore, test drink in a volume of 10 kcal/kg BW served as a breakfast was designed to stimulate reasonably enough metabolic response which would return toward baseline levels three hours postprandially.



Figure 2.6 Mixed liquid meal (Ensure® Compact) was ingested by participants at breakfast time during the main measurement visits.

2.6.3 *Ad-libitum* Test Meal

Three hours after consumption of test drink at breakfast time, a test meal of fixed macronutrient composition was served as a standardised measure of *ad libitum* food intake (**Figure 2.7**), which provided 148.2 kcal/100g with 49.8%, 15.4% and 34.8% energy provided by CHO, protein and fat, respectively. The *ad libitum* test meal was served after being thoroughly mixed and energy intake was computed from the weight of food consumed. It contained 210 g of dry weight pasta (Sainsbury's Fusilli, London, UK), which was cooked in unsalted boiling water the night before. The following morning the pasta was mixed with 38 g of mature grated white cheese (Sainsbury's), 12 g of olive oil (Napolina, UK), and 170 g of tomato and basil pasta sauce (Loyd Grossman, UK) and reheated. Participants were served portions of ~430 g in a small bowl at a temperature ≥ 82°C,

and instructed to consume as much as they wished until they felt 'comfortably full'. The bowl of pasta was repeatedly topped up, when about two thirds had been consumed, in an attempt to ensure participants were not cued to stop eating by having emptied their bowl. Participants were separated from other people in the laboratory when consuming the pasta meal, in the presence of one experimenter and no discussions associated with the meal were allowed. Energy intake was computed from the weight difference of the pasta meal before and after consumption. Duration and speed of consumption were also recorded.



Figure 2.7 *Ad libitum* pasta meal was provided to participants at lunch time during the main measurement visit.

2.6.4 Subjective Appetite Ratings

Appetite ratings were collected in both studies using visual analogue scales (VAS) (**Appendix H**) presented on single A4 paper sheets. The VAS consisted of 100 mm scales anchored at each end with extreme statements such as 'not at all full', 'totally full'. Paper based VAS are generally used to evaluate subjective appetite and to assess effects produced by an intervention (Flint *et al.*, 2000; Blundell *et al.*, 2010; Avena, Murray and Gold, 2013). VAS are in the form of horizontal lines of different length, typically 100-150 mm, with words anchored at each end, describing the most

positive rating and the most negative rating for a question (Hill, Magson and Blundell, 1984; Warwick *et al.*, 1993; Yeomans and Symes, 1999). Participants were instructed to make a vertical mark across the line according to their current feeling. Quantification of the measurement is made by measuring the distance (mm) from the left end of the horizontal line to the mark. Participants were asked about their feeling of hunger, satiety, fullness, prospective food consumption, desire to eat something fatty, salty, sweet or savoury, and palatability of the meals, in the form of text describing the extremes rating anchored at each end (Flint *et al.*, 2000). In main measurement visits, VAS was completed just before, immediately after and then every 20 min after the test drink for three hours. Further VAS was completed before and every 20 minutes for an hour after consuming the test meal in order to assess subjective appetite. Values for 'hunger', 'desire to eat', 'prospective food consumption', 'fullness' and 'satisfaction were amalgamated using the following equation to calculate a Composite Appetite Score (CAS) = [hunger + desire to eat + prospective food consumption + (100−fullness) + (100−satisfaction)]/5 (Stubbs *et al.*, 2000). A higher CAS value is indicative of greater motivation to eat or less feeling of satiety.

2.6.5 Blood Sampling and Processing

2.6.5.1 Arterialised Venous Blood

Placement of a cannula in systemic arteries on healthy subjects has been proven to be complicated, potentially dangerous and provides risk and discomfort to the subjects especially for routine clinical research studies (Gallen and Macdonald, 1990). In current research studies, the arterialized venous blood from heated dorsal hand veins has been widely used and especially in metabolic studies (McGuire *et al.*, 1976; Ferrannini *et al.*, 1983; Maggs *et al.*, 1998; Toft-Nielsen *et al.*, 2001; Bajaj *et al.*, 2005). Blood flow through the digital arteries increases by heating the hand, thus produces the arterialisation of venous blood in the superficial dorsal hand veins (McGuire *et al.*, 1976; Gallen and Macdonald, 1990). When a blood sample is obtained for the purpose of measuring metabolite concentrations in the whole body, arterialised venous blood can be used as it provides no significant differences in concentrations of glucose (McGuire *et al.*, 1976), lactate

(Forster *et al.*, 1972), ketone (Sonnenberg and Keller, 1982), several amino acids (Abumrad *et al.*, 1981), FFA, insulin, and glucagon (Zello *et al.*, 1990; Morris *et al.*, 1997).

In both studies, arterialised venous blood was obtained by inserting a 20-G cannula (pink Venflon, Ohmeda, Sweden) into a dorsal hand vein in a retrograde manner (see **Figure 2.8**). Before cannulating, the hand was kept in some warm water for 5 min before a small amount of local anaesthetic (1 % lignocaine: B.Braun Melsungen AG, Melsungen, Germany) was applied, which made the area become numb and thus to avoid any discomfort. 0.9 % saline infusion (Baxter Healthcare Ltd. Thetford, UK) was infused slowly (about 120 mL/h) through the cannula throughout the sampling period to avoid the blood clotting in the cannula. The hand was warmed in a warm-air box with a constant air temperature of 55 °C, so as to minimise inter- and intra- visit variability. In practice, participants may require to leave the warm-air box for toilet breaks, hands should be kept in the box for at least 10 min before the next sample is taken.



Figure 2.8 A participant having his left hand warmed in the warm-air box during the main measurement visit.

2.6.5.2 Blood Processing

Blood samples were drawn by employing a 3-way tap. The first 2mL of each sample was discarded into a clinical waste bin as it was a mixture of blood and saline. Two baseline blood samples were obtained with 5 min apart just before the standardised test drink ingestion to evaluate the mean of fasting blood glucose, serum insulin, plasma FFA, serum TAG, total cholesterol, HDL-cholesterol, LDL-cholesterol and gut hormones (such as ghrelin). Blood samples were also obtained every 10 min for the analysis of blood glucose and every 20 min for the determination of insulin, FFA, TAG and gut hormones concentrations for three hours after the test drink. A final blood sample was taken at one hour after the ingestion of the *ad libitum* pasta meal for determination of blood glucose and gut hormones.

Immediately after each sampling, the syringe with blood sample was sampled by the YSI directly for the assessment of blood glucose. A 2mL blood sample was then dispensed into a Lithium Heparin vacutainer containing 15 μL EGTA-Glutathione for plasma FFA determination, 1 mL blood into a Ethylenediaminetetraacetic acid (EDTA) vacutainer containing 50 μL aprotinin for total ghrelin determination, and 2 mL blood (for serum) into a gold-top vacutainer containing spray-coated silica and a polymer gel for insulin, TAG, total cholesterol, HDL-cholesterol and LDL-cholesterol determinations. The serum tube was allowed to clot for 30 min at room temperature before centrifugation. All samples were centrifuged (5702R, Eppendorf, Germany) for 10 min at 4400 (x g) at 4 °C. The supernatant from the Lithium Heparin vacutainer was aliquoted into microcentrifuge tubes containing 10 μL tetrahydrolipstatin (THL) for FFA assessment. The supernatant from other vacutainers was transferred into empty microcentrifuge tubes with corresponding labels. Serum and plasma samples were clearly labelled and were stored at -80 °C for blood analysis.

2.7 Blood Samples Analysis

2.7.1 Serum Insulin

Serum aliquots were stored at -80°C to retain full immunoreactivity, and multiple (>5) freeze/thaw cycles were avoided. The level of insulin in serum were determined by the commercially available

human radioimmunoassay (RIA) kit (Kit catalogue number: HI-14K; Merck Millipore, MA, USA) using ^{125}I-labelled human insulin and a human insulin antiserum (YALOW and BERSON, 1960). The cross-reactivity with Human Proinsulin of this RIA is less than 0.2%. The limit of sensitivity for this human insulin RIA is 2.715 μU/ml (100 μL sample size). Duplicated start-of-run and end-of-run quality controls were placed before and after the samples to check for assay drift. The average intra- and inter-assay coefficient of variation was 7.8% and 13.6%, respectively.

2.7.2 Plasma Total Ghrelin

In Chapter 3, the level of total ghrelin (sum of the ghrelin in the forms of acylated and unacylated) were determined by the commercially available human RIA kit (Kit catalogue number: GHRT-89HK; EMD Millipore, Billerica, MA, USA) using a HPLC purified ^{125}I-Ghrelin and a rabbit anti-Ghrelin serum. EDTA coated vacutainers containing aprotinin (Nordic Pharma, Reading, UK) 50 μL/mL whole blood were used to collect samples for the total ghrelin assay. The coefficient of variation between duplicate sample counts have been shown to be ≤ 5%, so samples were analysed in single. The average intra- and inter-assay coefficient of variation was 5.0% and 6.2%, respectively. Considering the variation occurs on the RIA techniques, complete samples for a participant were analysed on the same assay to produce the optimal data.

2.7.3 FFA

Plasma non-esterified fatty acid (NEFA) or free fatty acid (FFA) concentrations were quantitatively determined using coupled enzymatic colorimetry (ABX Pentra 400, Horiba Ltd., France), using a commercially available kit (NEFA-HR-2, Wako, Germany):

$$\text{NEFA} + \text{ATP} + \text{CoA-SH} \xrightarrow{acyl\ coa\ synthetase} \text{Acyl-CoA} + \text{AMP} + \text{PPi}$$

$$\text{Acyl-CoA} + 0_2 \xrightarrow{acyl\ coa\ oxidase} \text{2,3-trans-Enoyl-CoA} + H_2O_2$$

$$2\ H_2O_2 + \text{4-AA} + \text{MEHA} \xrightarrow{peroxidase} \text{Quinoneimine-colour} + 4\ H_2O$$

FFA in the sample is converted to Acyl-CoA, AMP and pyrophosphoric acid (PPi) catalysed by the action of Acyl-CoA synthetase (ACS), in the presence of coenzyme A (CoA) and ATP. The

Acyl-CoA produced is oxidised by Acyl-CoA oxidase (ACOD) to yield hydrogen peroxide (H_2O_2), which reacts with 4-aminoantipyrine (4-AA) and MEHA as a violet colour agent to produce a quinonimine chromophore. Absorbance of the blue purple colour at 546nm is proportional to the concentration of FFA in the sample. Lithium Heparin tubes were used to collect blood with additional preservative ethylene glycol tetraacetic acid (EGTA)-Glutathione 7.5 µL/mL whole blood. 10 µL/mL plasma of THL was added to microcentrifuge tubes to limit *in vitro* lipolysis and prevent the overestimation of plasma FFA, and therefore allowing more accurate determination of plasma FFA concentrations (Krebs *et al.*, 2000). THL was in ethanol solution, so it was required to be added to the plasma rather than whole blood, in which case haemolysis would occur. The average intra- and inter-assay coefficient of variation was 1.78% and 2.08%, respectively.

2.7.4 Lipids

2.7.4.1 Triglycerides

Serum TAG concentrations were analysed by coupled enzymatic colorimetry using a photometric auto-analyser (ABX Pentra 400, Horiba Ltd., France). Enzymatic determination of triglycerides involves the following reactions and yields a red coloured quinoneimine ultimately:

$$\text{Triglycerides} + H_2O \xrightarrow{Lipoprotein\ lipase} \text{Glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{Glycerokinase} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-Phosphate} + O_2 \xrightarrow{Glycerol-3-phosphate\ Oxidase} \text{Dihydroxyacetone phosphate} + H_2O_2$$

$$2H_2O_2 + \text{4-aminoantipyrine} + \text{p-Chlorophenol} \xrightarrow{Peroxidase} \text{red quinoneimine} + 4\ H_2O$$

TAG in the serum sample is converted to glycerol and fatty acids by the action of lipoprotein lipase (LPL). ATP phosphorylates glycerol to produce Glycerol-3-phosphate (G3P) and ADP catalysed by glycerokinase. The G3P produced is oxidised and yields dihydroxyacetone phosphate and H_2O_2 in the presence of G3P oxidase. The H_2O_2 then reacts with 4-aminoantipyrine and p-Chlorophenol to form a quinonimine chromophore by the action of peroxidase. The absorbance of the

chromophore is proportional to the concentration of TAG in the sample (Bucolo and David, 1973). The average intra- and inter-assay coefficient of variation was 1.86% and 2.66%, respectively.

2.7.4.2 Total Cholesterol, HDL-cholesterol and LDL-cholesterol

Serum total cholesterol, HDL-cholesterol and LDL-cholesterol concentrations were analysed with the photometric auto-analyser (ABX Pentra 400, Horiba Ltd., France), using an enzymatic photometric method. All samples were evaluated in the same run to avoid inter-assay variation. Three types of cholesterol were analysed in the same run to prevent errors caused by different numbers of freeze/thaw cycles for different cholesterol types. The average intra-assay coefficient of variations for total cholesterol, HDL-cholesterol and LDL-cholesterol were 1.05%, 0.86% and 1.79%, respectively. The average inter-assay coefficient of variation for total cholesterol, HDL-cholesterol and LDL-cholesterol was 1.14%, 1.77% and 2.03%, respectively.

2.7.5 Glucose

Blood glucose was measured immediately using a YellowSprings™ glucose and lactate analyser (YSI) (YSI 2300 STAT PLUS, Yellow Springs Inc., Ohio., USA). The YSI STAT PLUS 2300 utilises an enzyme-based, amperometric biosensor for determining glucose concentrations.

$$\text{D-Glucose} + O_2 \xrightarrow{glucose\ oxidase} \text{gluconolactone} + H_2O_2$$

$$H_2O_2 \rightarrow 2H^+ + 2e^- + O_2$$

$$4H + O_2 \rightarrow 2H_2O + 4e^-$$

In this method, the biosensor employs a glucose oxidase-containing membrane for catalysing the oxidation of glucose to gluconolactone and H_2O_2. The H_2O_2 is then oxidised at the platinum anode by an H_2O_2 sensitive electrode, producing electrons. The electron flow is linearly proportional to the steady state hydrogen peroxide concentration, which is in turn proportional to the concentration of glucose in the sample (Chua and Tan, 1978). The YSI Sipper Pump Piston retracts and draws in 25 μL of sample and measures glucose with a detection range limit of 0-30 mmol/L. The YSI is capable of providing good within batch and between-batch precision. Compared to other glucose

determination methods, YSI has superior analyse speed, simple procedure and only small sample required (Chua and Tan, 1978).

2.8 Statistical Analyses

GraphPad Prism 8.4.2 (GraphPad Software Inc., San Diego, CA, USA) was used for data entry and analyses with statistical significance accepted as the 5% level for all statistical tests. Values presented in text, figures and tables are presented as mean ± standard deviation (SD) unless otherwise stated. Data were tested for normality using the D'Agostino-Pearson normality test to determine whether a parametric or nonparametric analysis should be conducted.

Values for the AUCs of postprandial REE, glucose, insulin, FFA, TAG, ghrelin and CAS were calculated by applying the trapezoid rule to both positive and negative increments to distinguish between fasting and postprandial changes.

In Chapter 3, one-factor repeated-measure ANOVAs were used to detect differences between the three trials (fasting blood measurements, postprandial AUC for blood measurements over 180 min after consumption of the liquid breakfast, HOMA-IR, MATSUDA-180min, fasting REE and DIT). When significant intervention or treatment x time interaction effects were observed, post-hoc comparisons (where relevant) were explored using Fisher's LSD test. Other variables were analysed using a two-way repeated measures ANOVA (intervention x time). When significant intervention or intervention x time effects were identified, post-hoc multiple comparisons were undertaken using Bonferroni corrections to reduce the chance of type I error. Effect size was estimated as partial eta squared (η^2_p).

In Chapters 4 and 5, two-factor Mixed Model ANOVA (between groups factor: treatment, 5:2 IER vs. CER; within groups factor: sampling time, pre- vs. post- each two-week energy restricted intervention) evaluated intervention effects. When significant treatment, time or treatment x time interaction effects were observed, post-hoc comparisons (where relevant) were explored using paired or unpaired student's t-test. Unpaired student's t-tests were performed to compare single data points between two groups (e.g., weight loss). Effect size was estimated as η^2_p, to determine the proportion of variance associated with each treatment.

CHAPTER 3. EFFECTS OF TWO DAYS OF PHYSICAL INACTIVITY WITH AND WITHOUT ENERGY SURPLUS ON BASAL AND POSTPRANDIAL METABOLISM: A RANDOMISED CROSSOVER STUDY IN HEALTHY MEN AND WOMEN

3.1 Introduction

Physical inactivity is a global pandemic and is estimated to have caused 6% of coronary heart disease cases, 7% of T2DM cases, 10% of breast cancer cases and 10% of colon cancer cases (Lee *et al.*, 2012). Physical inactivity was identified as being responsible for 9% of premature deaths and physical inactivity linked deaths were a total of 5.3 million out of the total 57 million deaths that occurred worldwide in 2008 (Lee *et al.*, 2012) and has been proposed to be the 4th leading risk factor for mortality in the world (WHO, 2009).

An early study of London bus drivers, who spent many hours of sitting on their shift, and conductors, who spent many hours walking up and down stairs in the double-floor buses per working day, was the first to link physical inactivity to higher rates of coronary heart disease in middle age men (Morris *et al.*, 1953). From then on, numerous observational studies have conclusively shown that physical inactivity is closely associated with morbidity and reduced life expectancy (Paffenbarger *et al.*, 1986; Wen *et al.*, 2011; Lee *et al.*, 2012). Furthermore, a systematic review and meta-analysis conducted by Wilmot *et al.* (2012) concluded that a higher level of sedentary behaviour is associated with an increased risk of diabetes, cardiovascular disease, and of cardiovascular and all-cause mortality.

It has been reported that every 2,000 extra footsteps/day for a year could reduce the cardiovascular diseases risk (e.g. myocardial infraction and stroke) by 8-10% in individuals with impaired glucose tolerance, regardless of sex, age, ethnicity, body weight or initial level of activity (Yates *et al.*,

2014). Alternating prolonged sitting with even short but frequent bouts of standing or light-intensity walking may attenuate postprandial glycaemic and insulinaemic excursion (Dunstan *et al.*, 2012; Buckley *et al.*, 2014; Thorp *et al.*, 2014). A meta-analysis of data from over one million participants concluded that approximately 60-75 min of moderate intensity physical activity per day may eliminate the increased risk of mortality related to high sitting time. In contrast, the similar physical activity level may attenuate, but not eliminate the increased risk associated with TV-viewing for more than five hours per day (Ekelund *et al.*, 2016). This difference is partly attributed to the potential combination of TV-viewing with snack ingestion (Frydenlund *et al.*, 2012) or altered eating behaviour in response to food advertisements on TV (Harris, Bargh and Brownell, 2009). Moreover, this difference may also be explained by the detrimental effects of prolonged postprandial sedentary time on glucose and lipid metabolism, considering that TV-viewing typically occurs in the evenings (Benatti and Ried-Larsen, 2015; Ekelund *et al.*, 2016).

Whilst some research and associated literature reviews have been carried out on the effects or associations of physical inactivity or sedentary behaviour with health markers, only a small number of intervention studies have attempted to investigate the short-term effects of reduced physical activity. An intervention study to reduce the step counts from 10,501 to 1,344 per day for two weeks in healthy, non-exercising, free-living young men reported lower insulin action and decreases in VO_{2max} and leg lean mass. However, participants in this study still maintained their habitual energy intake whilst having a dramatic decrease in step counts, which may result in a state of positive energy balance (Krogh-Madsen *et al.*, 2010). Stubbs *et al.* (2004) investigated the effects of seven-days of PAL reduction from 1.8 to 1.4 on energy balance, appetite and *ad libitum* energy intake. Using whole-body continuous indirect calorimetry and *ad libitum* access to meals, results showed no compensatory reduction in energy intake in response to a decreased PAL, creating a positive energy balance, which over a longer time would lead to an increase in fat deposits. To better understand the acute effects of physical inactivity, Stephens *et al.* (2011) assessed the insulin sensitivity response to a 24-hour period of physical inactivity achieved by prolonging sitting time, with or without reduction of energy intake, using a glucose infusion technique. They observed that 24 hours of sitting dramatically reduced insulin sensitivity. This effect was minimised, but not eliminated, when accompanied with a compensatory reduction in energy intake to maintain energy balance, compared with a control trial, where participants

remained active and avoided prolonged periods of sitting. Collectively, these studies suggest that both relative overfeeding and a reduction in energy expenditure contribute to the deleterious effects of physical inactivity on human metabolism.

The present study aims to extend the findings of previous studies by evaluating the effects of two-days of physical inactivity, with and without a proportional decrease in energy intake, on insulin sensitivity, glycaemic control, appetite and resting energy expenditure (REE) in response to food ingestion; in those who are usually more physically active. A physical inactivity model was used in which the participants' usual step counts were reduced by 80%. It was hypothesised that a compensatory decrease in energy intake to maintain energy balance during physical inactivity would attenuate the adverse effects on insulin sensitivity.

3.2 Methods

3.2.1 Participants

Eight young (between the ages of 18-35 years), healthy, men and women with a BMI between 18-27 kg/m^2 and a waist circumference < 94 cm for men and < 80 cm for women were recruited from the students and staff of the University of Nottingham and Queen's Medical Centre. All participants were habitually moderately active or active with a PAL value of 1.70-1.99, whilst sedentary or light active lifestyle is characterised by a PAL value of 1.40-1.69 and a vigorously active lifestyle by a PAL between 2.00-2.40 according to the FAO/WHO/UNU report (FAO, 2001).

Exclusion criteria included:

- Smoking
- Any metabolic (e.g. diabetes), endocrine (e.g. hyperthyroidism) or cardiovascular (e.g. heart or blood) abnormalities including hypertension or heart failure;
- Taking any medication other than over-the-counter drugs;
- Clinically significant abnormalities on screening including ECG abnormalities;
- High alcohol consumption (routinely >4 units/d for men; >3 units/d for women);
- Women who were pregnant, lactating or with irregular menstrual cycles;

- Self-reported allergy, intolerance or strong dislike of foods or drinks to be offered for the test breakfast, pasta meal or during the intervention period;
- BDI or EAT-26 scores providing evidence of depression (BDI score > 10) or eating disorder (EAT-26 score > 20);
- Regularly skipping breakfast;
- Significant weight changes in the previous three months (> ± 2 kg);
- Shift workers;
- On an energy-restricted diet.

Participants were asked to attend the laboratory for a total of eight occasions, including a medical screening visit, a preliminary visit and three main experimental trials (two visits each, including a short visit before each trial). The experimental design is summarised in **Figure 3.1**.

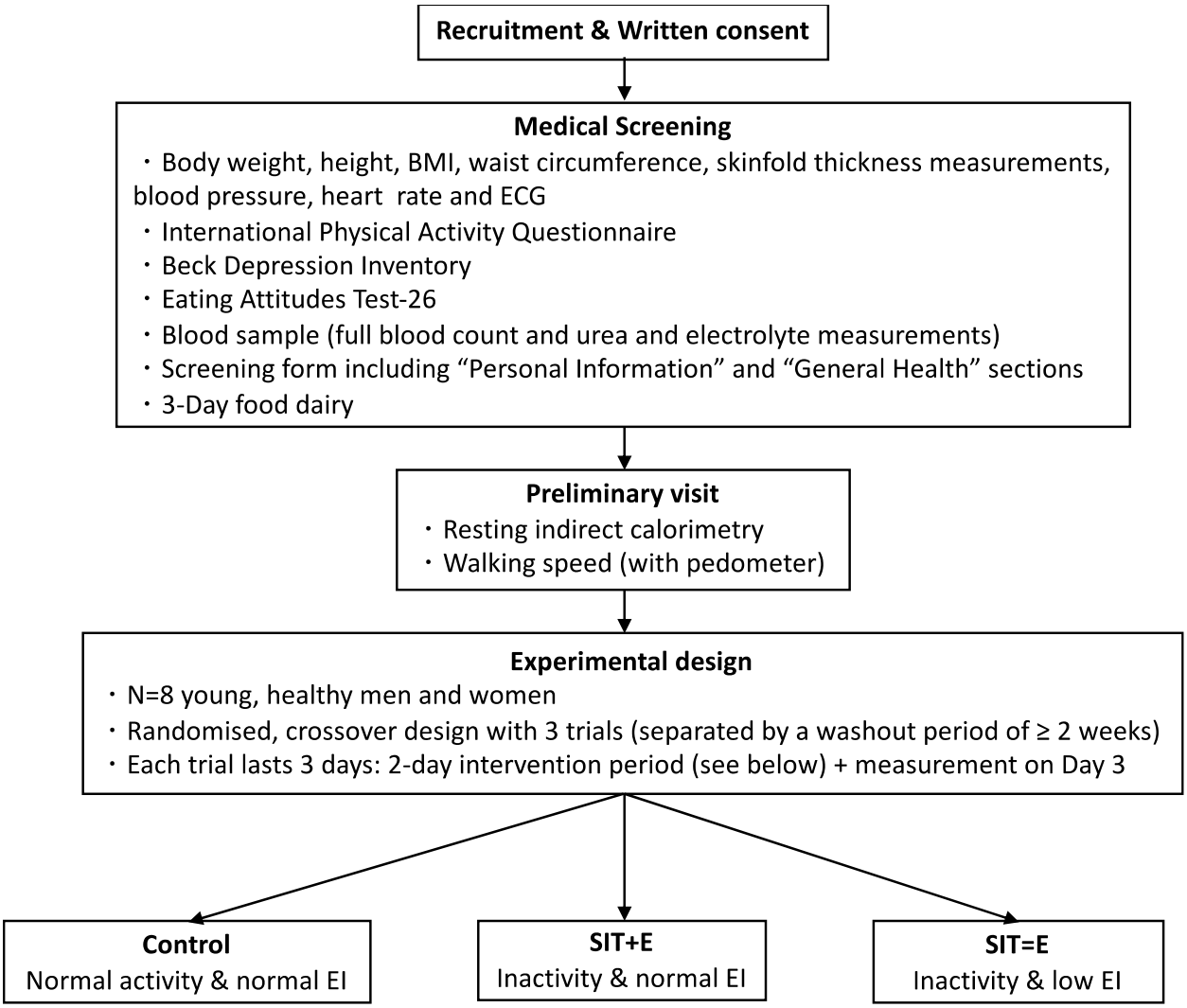


Figure 3.1 Schematic of experimental design.

3.2.2 Screening

Once participants consented to being screened, they reported to the laboratory for a medical screening to check their suitability for the study. This involved having body weight, height, waist circumference, a 12 lead ECG measured and an 8 mL blood sample obtained from the median cubital vein via venepuncture for routine blood investigation (full blood count and urea and electrolyte measurements). Skinfold thicknesses at biceps, triceps, subscapular and suprailiac sites

were measured using a skinfold calliper to estimate body fat percentage (see Section 2.2.1.4). Two BP and HR measurements were taken with participants in a supine position. A further three measurements were taken when participants were sitting, standing for 1 min and standing for 3 min to evaluate orthostatic hypotension. Participants were required to complete a screening form including "Personal Information" and "General Health" sections (see **Appendix C**); the BDI, which was used to assess the presence and severity of depression symptoms of participants (Beck, Ward and Mock, 1961) (see **Appendix D**); the EAT-26, which was used to access the eating disorder risk of participants (Garner *et al.*, 1982) (see **Appendix E**) as well as the IPAQ (Craig *et al.*, 2003) (see **Appendix F**) (see Section 2.2.2).

3.2.3 Experimental Design

If the medical screening result was satisfactory, participants were asked to complete a weighed three-day food record including two week-days and one weekend day, which was used to evaluate their habitual energy intake and temporal distribution of energy intake throughout the day (see **Appendix G**). The quality of food record completion was an initial indication of their adherence prior to starting the main trials, and was returned on the following preliminary visit. During that visit, participants were asked to attend the laboratory after an overnight fast (~12 h) and initially rest semi-supine in a hospital bed for 30 min with the ambient temperature maintained at 23-24°C. REE and whole-body substrate oxidation rates were measured in a fasted state for 20 min using indirect calorimetry. At this visit, walking pattern (i.e. cadence), in terms of footsteps per minute, was recorded using a pedometer. Participants were asked to walk for three 5-min bouts at their normal walking paces, with a 2-min standing rest between bouts. Step counts produced in each bout were divided by 5-min to calculate cadence in steps/min.

The study followed a randomised, crossover design with three different interventions lasting three consecutive days each (two days of intervention plus one day of assessment) and separated by a washout period of not less than two weeks (four weeks for women who were tested in the first week of the follicular phase only). Participants were instructed to adhere to their habitual physical activity and diets during the washout period, which was designed to eliminate an interaction between two adjacent interventions. **Figure 3.2** shows a schematic diagram of the sequence of measurements conducted during each trial.

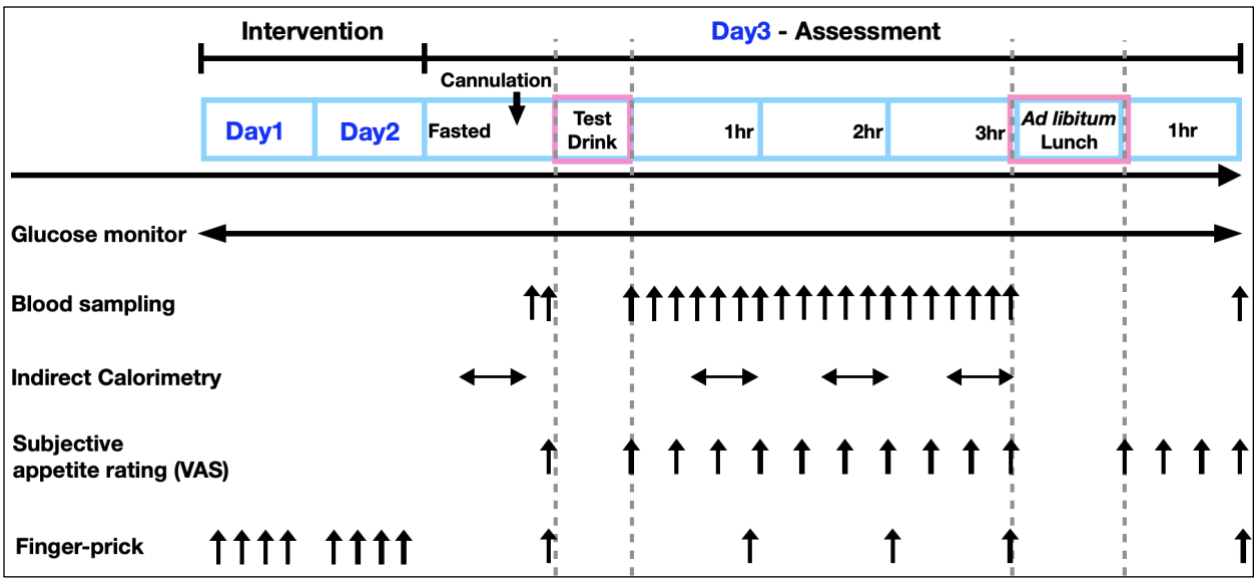


Figure 3.2 Schematic diagram of the sequence of measurements conducted during each trial.

Before each trial, participants were asked to continue with their normal daily physical activities (occupational, recreational, domestic and commuting) and refrain from alcohol consumption for the previous two days and caffeine consumption on the day of the trial. The three interventions included:

(A) (**CONTROL**): two consecutive days of standardised daily levels of moderate physical activity (PAL=1.85), and matched energy intake;

(B) (**SIT+E**): two consecutive days of reduced physical activity induced by prolonged periods of sitting and reduced step counts (PAL=1.4) whilst maintaining the level of energy intake prescribed in the CONTROL trial, thus creating a positive energy balance;

(C) (**SIT=E**): two consecutive days of isoenergetic diet matched to the reduced physical activity induced by prolonged periods of sitting and reduced step counts (PAL=1.4) to achieve an energy balance.

3.2.3.1 Estimated Energy Requirements

Using basal metabolic rate (BMR) obtained from the indirect calorimetry measurement conducted at the preliminary visit and the PALs prescribed in this study (i.e. PAL=1.85 or 1.4), the daily energy requirements were estimated for each participant and each condition using PAL times BMR per 24 hours.

3.2.3.2 Activity Schedule

An activity schedule, resulting in the required PAL value (i.e. PAL=1.85 or 1.4), listed the main daily activities was tailored and prescribed to each participant (see **Appendix I**). Physical activity ratio (PAR) is the energy expenditure of a given activity per unit of time (e.g. minute or hour) expressed as a multiple of BMR. Thus, PAR can be calculated as energy expenditure for an activity/BMR, for the selected time unit. All PAR values used in this study were referenced according to the Report of a Joint FAO/WHO/UNU Expert Consultation, Human Energy Requirements (FAO, 2001). Thus, certain daily activities with durations were prescribed to participants having utilised the following equation to design the schedule:

$$PAL = \frac{\sum_{n=1}^{N}(PAR_n \times Duration_n \times BMR/24)}{BMR}$$

Where Duration is in hours and BMR is in kcal/day.

In the CONTROL trial, sitting time was interspersed with a large amount of 'walking at varying paces without a load' with an associated PAR of 3.2. In the SIT+E and SIT=E trials, the daily schedule introduced prolonged periods of sitting whilst decreasing the time of walking dramatically. Using total duration (min) for walking on the prescribed activity schedule and the walking pattern (steps/min) measured at the preliminary visit, the daily step count target was prescribed for each participant corresponding to the different levels of prescribed physical activity. Overall, reduced physical activity was accomplished by reducing the normal daily step count by 80% on both days with verification by using a pedometer. In summary, during Days 1 and 2 of each experimental treatment, participants were prescribed with an activity schedule to follow. A pedometer provided an easily accessible feedback of each participant's daily step targets corresponding to different level of prescribed physical activity.

3.2.4 Intervention Periods

Participants were asked to report to the laboratory the day before Day 1 of each intervention period. On those visits, a subcutaneous glucose monitoring device (the Medtronic MiniMed iPro^{TM}2 CGM system) was placed on the abdominal area to allow for estimation of continuous 24h blood glucose profile over the duration of each trial. Participants were then given an activity schedule to follow during Days 1 and 2 of each intervention period. A pedometer was also supplied to provide an easily recognisable feedback of each participant's daily step count targets corresponding to different levels of physical activity. Three main standardised meals (breakfast, lunch, and dinner) and snacks for each two-day intervention were also prepared for each participant to take home. Daily energy intake was of mixed composition providing ~50% energy from CHO, ~35% from fat, and ~15% from protein. To avoid potential confounding by altering the meal time, instructions were given to consume breakfast between 9 am - 9:30 am, Snack 1 between 10:45 am – 11 am, lunch between 1 pm – 1:30 pm, Snack 2 between 2:45 pm – 3 pm, dinner between 5 pm – 5:30 pm and Snack 3 between 7 pm – 7:15 pm. Any incidents of leftover food or consumption of additional food not provided were asked to be reported.

3.2.5 Laboratory-Visit Protocol and Procedures

On Day 3 of each experimental trial, the participants were asked to report to the unit at 8:30 am following an overnight fast of ~12 h to undergo a series of appetite and metabolic measurements before and in response to a standardised liquid breakfast (meal tolerance test, MTT) and a subsequent *ad libitum* lunch to assess the effect of each intervention. All anthropometric measurements described in the screening section were repeated at each laboratory visit. Once body weight measurement was completed, participants were asked to rest in a semi-supine position, in a hospital bed for 30 min. Baseline REE and whole-body substrate oxidation rates were measured in a fasted state for 20 min using indirect calorimetry. Readings were reported every 10 s and the first and last 2.5 min of data acquisition were excluded.

An intravenous cannula was then inserted into a dorsal hand vein in a retrograde manner for arterialised venous blood sampling with the hand of the participant warmed in a warm-air box with a constant air temperature of 55°C (described in Section 2.6.5). Two baseline blood samples were

obtained, 5 min apart, just before ingestion of the standardised liquid breakfast, to evaluate mean fasting whole blood glucose, serum insulin, plasma FFA, serum TAG, and plasma total ghrelin. Blood samples were obtained subsequently every 10 min for the analysis of blood glucose, every 20 min for the determination of insulin, FFA, and TAG concentrations and every 60 min for the determination of total ghrelin concentration, for a duration of three hours after the liquid breakfast. VASs (see **Appendix H**) were completed just before, immediately after and then every 20 min after the liquid breakfast for three hours. The standardised test drink (described in Section 2.6.2) was provided at approximately 9:30 am at room temperature (24°C) in an open cup. Based on body weight on the day of the visit, 10 kcal was provided per kg of body weight. Participants were asked to consume the drink within 2 min. Three hours after consumption of breakfast, an *ad libitum* pasta meal of fixed macronutrient composition was provided (described in Section 2.6.3). Further VAS was completed just after and every 20 min for an hour after consuming the *ad libitum* test meal in order to assess subjective appetite. Upon the completion of the measurement, the cannula was removed before the participant was allowed to leave the laboratory. Blood samples analysis for the present study was explained in Section 2.7.

3.2.6 Statistical Power

In a study conducted by Stephens *et al.* (2011), it was shown that one day of physical inactivity by prolonging the sitting periods with energy surplus resulted in a 39% ($P < 0.001$) decrease in insulin sensitivity when compared to one day of moderate physical activity (isoenergetic). If a similar magnitude of difference is expected in the present study following two days of physical inactivity with positive energy balance, it should be detectable using eight participants with a power of 80% at a significance level of $P < 0.05$.

3.2.7 Statistical Analysis

GraphPad Prism 8.4.2 (GraphPad Software Inc., San Diego, CA, USA) was used for data entry and analyses with statistical significance accepted as $P < 0.05$. Data presented in text, figures and tables are presented as mean and SD unless otherwise stated. One-factor repeated-measure ANOVAs were used to detect differences between the three trials (fasting blood measurements, postprandial area under the curve for blood measurements over 180 min after consumption of the

liquid breakfast, HOMA-IR, Matsuda-180min, fasting REE and DIT). Other variables were analysed using a two-way repeated measures ANOVA (intervention x time). When significant intervention or intervention x time effects were identified, post-hoc multiple comparisons were undertaken using Bonferroni corrections to reduce the chance of type I error. More details of the statistical methods were explained in Section 2.8.

3.3 Results

3.3.1 Participant Characteristics

Of the 16 participants who initially expressed interest, 10 fulfilled the inclusion criteria and were recruited to the study. Eight participants completed the study (CONSORT diagram in **Figure 3.3**).

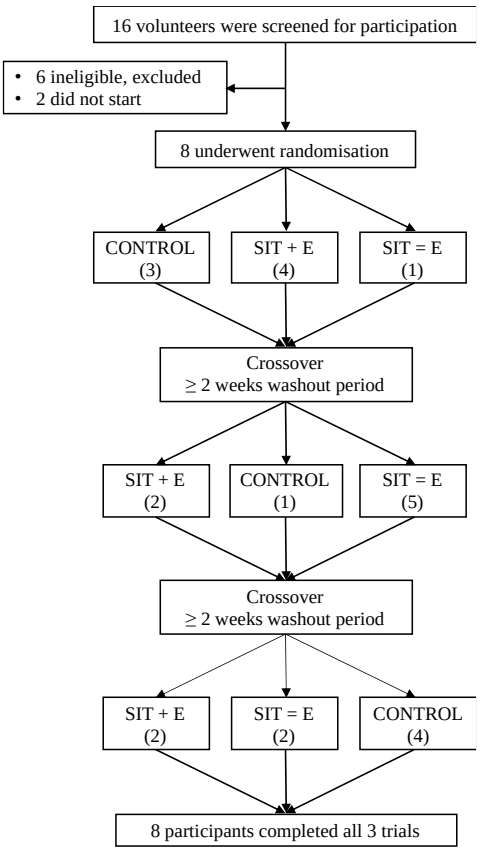


Figure 3.3 CONSORT (Consolidated Standards of Reporting Trials) diagram.

Participant characteristics at baseline are detailed in **Table 3.1.** Body weight in three main visits is reported in **Table 3.2**. There was no significant difference in body weight for the three main visits. Participants were instructed to follow the activity plan and achieve the daily step count targets they had been given. Reported daily step counts were 23841 (SD 3312), 5282 (SD 1485), and 4726 (SD 791) steps/day during CONTROL, SIT+E and SIT=E interventions, respectively. Daily step count reduced by 78% during the SIT+E intervention and by 80% during the SIT=E intervention compared with the daily step count during the CONTROL trial. Energy intake and macronutrients composition of the MTT in the three main visits are detailed in **Table 3.3**.

Table 3.1 Descriptive characteristics for completers at screening.

Variables (*n* 8)	Mean ± SD
Sex, *n*	
Male	4
Female	4
Age, years	23 ± 3
Body weight, kg	63.5 ± 10.9
BMI, kg/m^2	22.0 ± 2.2
Body fat, % [a]	21.6 ± 6.6
Waist circumference, cm	72.4 ± 7.6

[a] Estimated using skinfold thickness values

Table 3.2 Body weight and fasting blood measurements at the end of each intervention.

	CONTROL	SIT + E	SIT = E	
	Mean ± SD	Mean ± SD	Mean ± SD	*P* [a]
Body weight, kg	63.6 ± 11.3	63.5 ± 11.3	63.5 ± 11.2	0.94
Fasting glucose, mmol/L	4.20 ± 0.42	4.45 ± 0.30	4.38 ± 0.28	0.16
Fasting insulin, mIU/L	9.15 ± 2.93	9.57 ± 4.51	9.54 ± 3.57	0.90
Fasting FFA, mmol/L	0.49 ± 0.09	0.44 ± 0.11	0.43 ± 0.11	0.51
Fasting TAG, mmol/L	0.79 ± 0.27	0.74 ± 0.17	0.72 ± 0.22	0.58
Fasting Ghrelin, ρg/mL	1048 ± 422	924 ± 250	987 ± 279	0.26

[a] One-factor repeated-measures ANOVA

Table 3.3 Energy intake and macronutrients composition at the MTT.

	CONTROL	SIT + E	SIT = E
	Mean ± SD	Mean ± SD	Mean ± SD
Energy intake, kJ	2662.1 ± 473.6	2657.4 ± 472.3	2657.9 ± 469.4
Carbohydrate, g	76.2 ± 13.6	76.1 ± 13.5	76.1 ± 13.4
Fat, g	24.8 ± 4.4	24.8 ± 4.4	24.8 ± 4.4
Protein, g	27.0 ± 4.8	27.0 ± 4.8	27.0 ± 4.8

3.3.2 Fasting Blood Measurements

Results for fasting whole blood glucose, serum insulin, plasma FFA, serum TAG, and plasma total ghrelin concentrations are shown in **Table 3.2**. There were no significant differences in fasting blood measures between the three trials.

3.3.3 Postprandial Blood Measurements

3.3.3.1 Postprandial Serum Insulin Responses

In all trials, serum insulin rapidly elevated after the MTT and peaked after 60-80 min, before steadily declining, but did not reach fasting concentrations within 180 min (**Figure 3.4**). No significant interaction or main effect of intervention were found between the three trials (2-way ANOVA). There was a strong tendency to a significant difference in iAUC for serum insulin over 180 min postprandially between the three trials (P = 0.05, one-way ANOVA, η^2_p = 0.35) (**Table 3.4**). This is driven by the difference between the CONTROL vs. SIT+E trials. Compared with the CONTROL trial, the mean iAUC insulin was 25% higher in the SIT+E (P = 0.02, Fisher's LSD test) and 8% higher in the SIT=E (P = 0.39, Fisher's LSD test) trials. There were no differences in iAUC insulin between the SIT+E and SIT=E trials (P = 0.09, Fisher's LSD test).

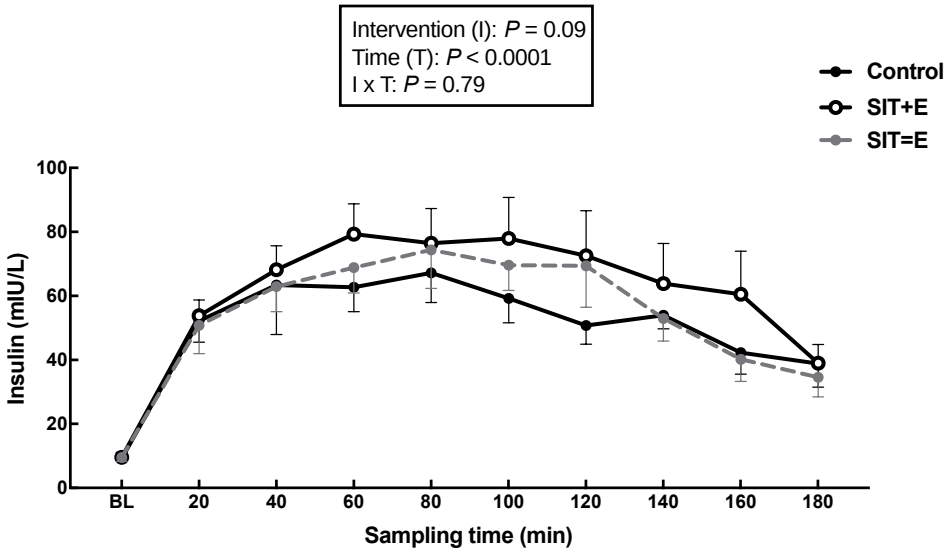


Figure 3.4 Serum insulin following the liquid breakfast.
Values are means with their standard errors. *n*=8.

Table 3.4 Postprandial blood measurements over the study.[a]

	CONTROL	SIT + E	SIT = E	
	Mean ± SD	Mean ± SD	Mean ± SD	*P* [b]
Insulin iAUC, mIU/Lx180 min	7868 ± 2031	9817 ± 3134	8509 ± 2847	0.05
Glucose iAUC, mmol/Lx180 min	242 ± 49	231 ± 68	233 ± 45	0.86
TAG iAUC, mmol/Lx180 min	36 ± 26	33 ± 20	34 ± 29	0.94
FFA dAUC, mmol/Lx180 min	-64 ± 16	-55 ± 14	-56 ± 14	0.42
Ghrelin dAUC, ρg/mLx180 min	-62544 ± 45135	-48844 ± 20838	-58731 ± 28676	0.28

iAUC incremental area under the curve, *dAUC* decremental area under the curve
[a] Postprandial incremental area under the curve (Glucose, Insulin and TAG) and decremental area under the curve (FFA and Ghrelin) for blood measurements over 180 min after consumption of the liquid breakfast
[b] One-factor repeated-measures ANOVA

3.3.3.2 Postprandial Whole Blood Glucose Responses

Whole blood glucose concentrations increased rapidly after the MTT and remained above fasting levels for 180 min, but no significant interaction or main effect of intervention were found between the three trials (2-way ANOVA) (**Figure 3.5**). There was no significant difference in iAUC for whole blood glucose between the three trials (**Table 3.4**).

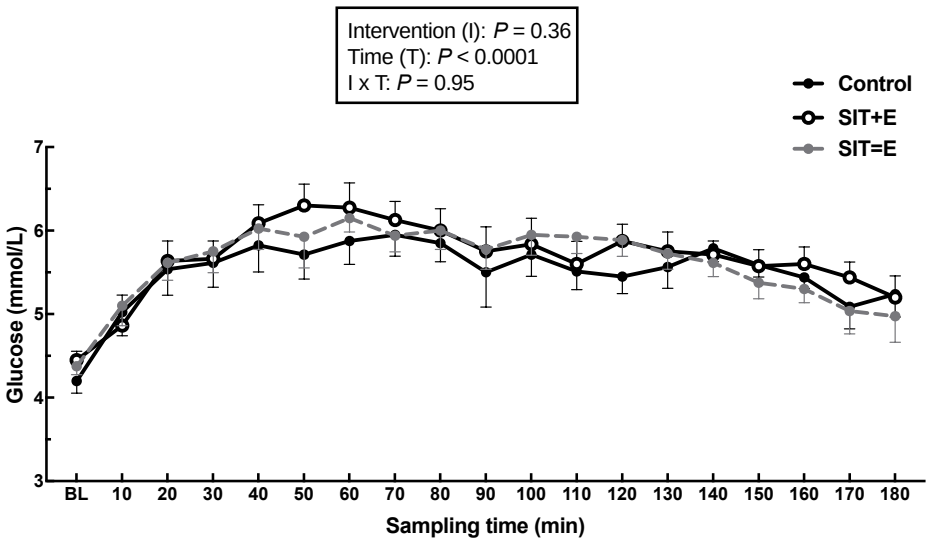


Figure 3.5 Whole blood glucose following the liquid breakfast.
Values are means with their standard errors. *n*=8.

3.3.3.3 Postprandial FFA and TAG Responses

Plasma FFA tended to follow an opposite response pattern to insulin. Upon ingestion of a mixed test drink after a 12-hour fasting, the predominant fuel mobilisation for energy metabolism shifted from fat to CHO which coincided with a reduced plasma FFA concentration (**Figure 3.6**). FFA concentrations fell below 0.1 mmol/L by 40 min after the MTT, which were maintained for the next two hours and started to rise again during the last 40 min of the postprandial measurement period, but no significant interaction or main effect of intervention were found between the three trials (2-way ANOVA). In contrast, TAG concentrations steadily increased after the MTT, but no significant interaction or main effect of intervention were found between the groups (2-way ANOVA) (**Figure 3.7**). There were no significant differences in AUCs for plasma FFA or serum TAG between the three trials (**Table 3.4**).

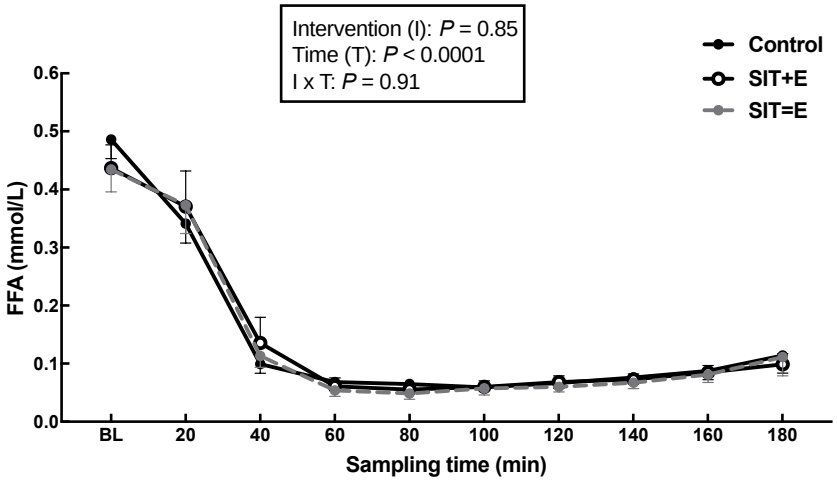


Figure 3.6 Plasma FFA following the liquid breakfast.
FFA, free fatty acid. Values are means with their standard errors. *n*=8.

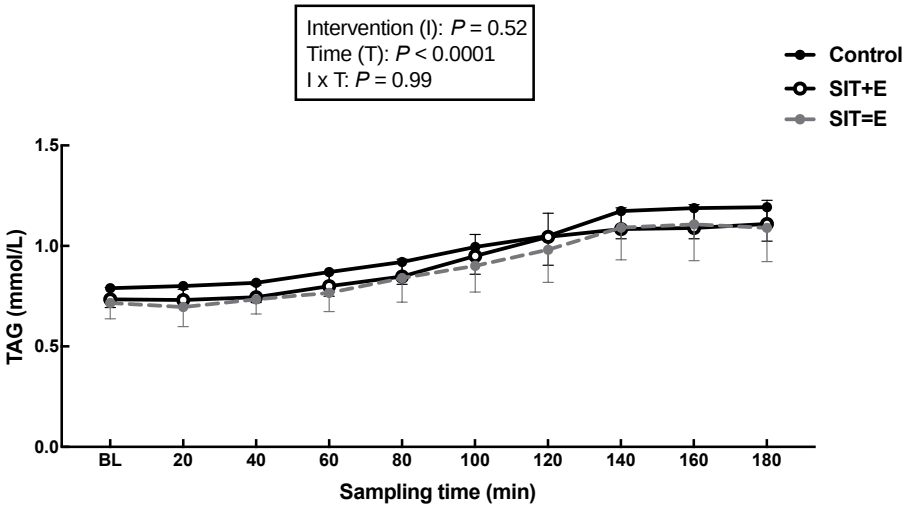


Figure 3.7 Serum TAG following the liquid breakfast.
TAG, triglycerides. Values are means with their standard errors. *n*=8.

3.3.3.4 Postprandial Plasma Total Ghrelin Responses

Following the MTT, plasma concentrations of total ghrelin decreased in all visits (**Figure 3.8**), but no significant interaction or main effect of intervention were found between the three trials (2-way ANOVA). There was no significant difference in dAUC for plasma total ghrelin between the three trials (**Table 3.4**).

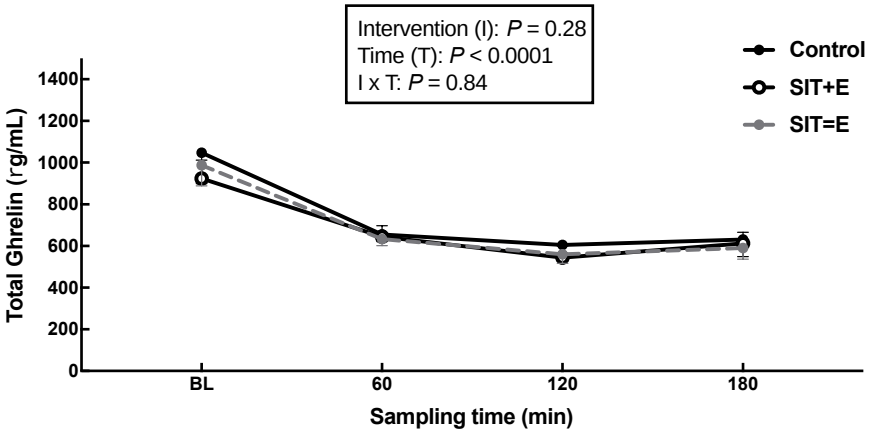


Figure 3.8 Plasma total ghrelin following the liquid breakfast.
Values are means with their standard errors. *n*=8.

3.3.4 Whole-Body Insulin Sensitivity

Whole-body insulin sensitivity evaluated using the HOMA-IR and Matsuda index-180min are presented in **Figure 3.9** and **Figure 3.10**, respectively. Matsuda index-180 min was calculated considering the time for returning to the fasted state was prolonged in response to the mixed MTT. This is supported by serum insulin concentrations remaining approximately six times higher than baseline levels at 120 min (**Figure 3.4**). There were no significant differences in HOMA-IR or Matsuda index-180 min between the three trials (one-way ANOVA).

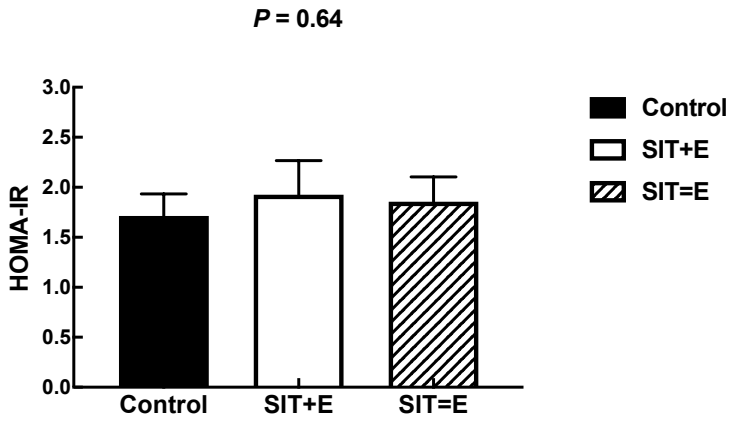


Figure 3.9 Insulin sensitivity assessed using the HOMA-IR.
HOMA-IR, homeostasis model assessment of insulin resistance. Values are means with their standard errors. *n*=8.

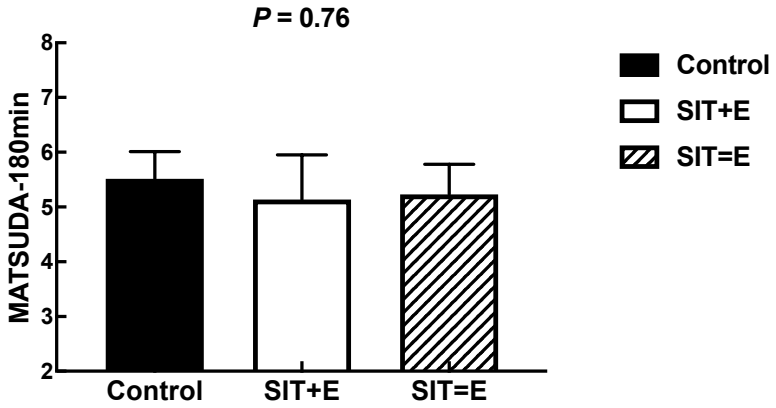


Figure 3.10 Insulin sensitivity assessed using Matsuda index.
Values are means with their standard errors. *n*=8.

3.3.5 Free-living Continuous Glucose Monitoring

Whole-day glycaemic profiles, with each time point representing the mean of two consecutive intervention days per participant, averaged over the participants, by treatment, are presented in **Figure 3.11**. Error bands (SEM) indicate the inter-individual variation for the mean of two days. Data were analysed per 24 h, during the day hours (07:30 to 23:59) and night hours (24:00 to 07:29), with the calculation of the mean, maximum, minimum, and postprandial iAUC of glucose

concentration. Furthermore, SD and %CV of each time period were calculated to describe the glycaemic variability within a day or during the day/night hours. There was a sharp rise in interstitial glucose level in response to each main meal consumption. There was a significant intervention effect for whole-day glycaemic profiles (P = 0.04, main effect of intervention, 2-way ANOVA), with higher interstitial glucose concentrations during the SIT+E trial, which was primarily attributed to higher levels in SIT+E vs. SIT=E in the morning, higher levels in SIT+E vs. CONTROL in the afternoon and higher levels in SIT+E vs. SIT=E at night.

When considered across the full 24 hours, there was a significant difference in mean glucose concentrations between the three trials (P = 0.04, one-way ANOVA) (**Table 3.5**), with higher mean glucose concentrations in the SIT+E trial than in the CONTROL (P = 0.04, Fisher's LSD test) and SIT=E trials (P = 0.02, Fisher's LSD test). Between 24:00 to 07:29, there was a significant difference in mean glucose concentrations between the groups (P = 0.02, one-way ANOVA), with higher mean glucose concentrations in the SIT+E trial than in the SIT=E trial (P = 0.007, Fisher's LSD test). The minimum glucose concentrations for the full 24 h (P = 0.04), during the day hours (P = 0.04) and night hours (P = 0.02) were significantly different between the three trials (**Table 3.5**). No significant differences were observed in the other CGM parameters.

The first day of physical inactivity seemed to have a more profound effect on interstitial glucose concentrations (**Figure 3.12 a**), while this effect was attenuated on intervention Day 2 (**Figure 3.12 b**). Further analysis was performed by comparing the whole-day glycaemic profiles between intervention Day 1 and intervention Days 1 and 2 (**Table 3.6**). When considered across the full 24 hours during intervention Day 1, there was a significant difference in mean glucose concentrations between the three trials (P = 0.02, one-way ANOVA), with higher mean glucose concentrations in the SIT+E trial than in the CONTROL (P = 0.009, Fisher's LSD test) and SIT=E trials (P = 0.04, Fisher's LSD test). Between 07:30 to 23:59, there was a significant difference in mean glucose concentrations between the three trials (P = 0.005, one-way ANOVA), with higher mean glucose concentrations in the SIT+E trial than in the CONTROL (P = 0.002, Fisher's LSD test) and SIT=E trials (P = 0.03, Fisher's LSD test). The minimum glucose concentrations for the full 24 h (P = 0.048) and during the day hours (P = 0.004) were significantly different between the three trials (**Table 3.6**). Compared to intervention Day 1, effects on interstitial glucose concentrations were attenuated over intervention Days 1 and 2.

Further analysis of CGM data was performed to tease out potential differences by splitting the day into day hours (07:30 to 23:59) and night hours (24:00 to 07:29) to clarify the differences. There was a significant intervention effect for higher interstitial glucose levels in the SIT+E trial during intervention Day 1 day hours (P = 0.0076, main effect of intervention, 2-way ANOVA) (**Figure 3.13 a**), but not the intervention Day 2 day hours (**Figure 3.13 b**). There was no significant difference in interstitial glucose levels during intervention Day 1 night hours between the three trials (**Figure 3.13 c**), but there was a significant intervention effect for higher interstitial glucose levels in the SIT+E and CONTROL trials during intervention Day 2 night hours (P = 0.02, main effect of intervention, 2-way ANOVA) (**Figure 3.13 d**).

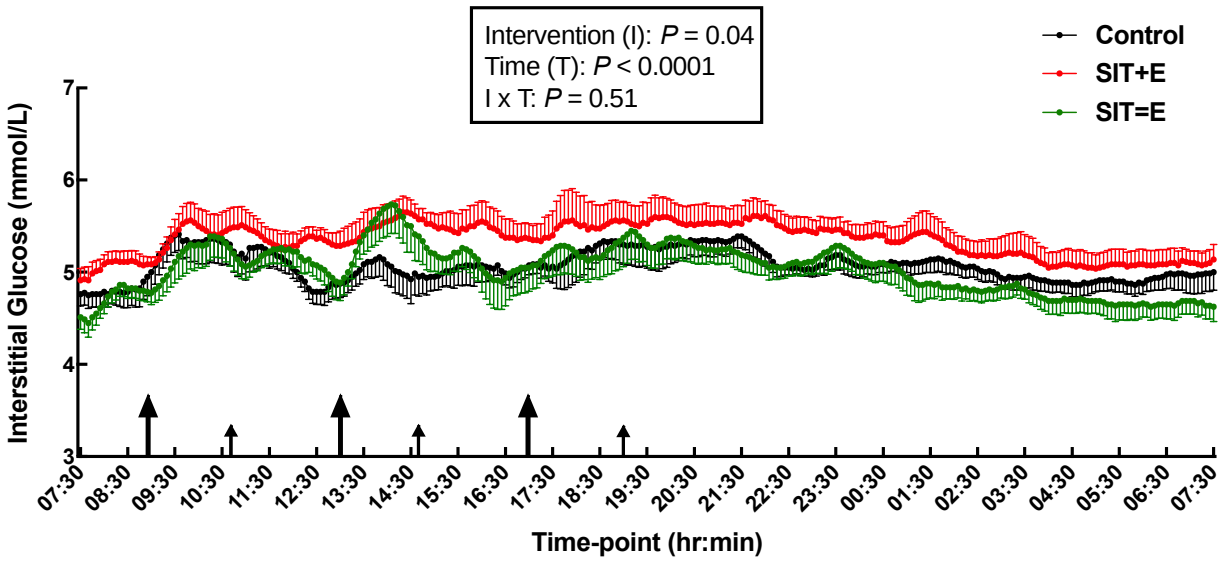


Figure 3.11 Whole-day glycaemic profiles calculated as mean interstitial glucose concentrations of two consecutive intervention days for eight participants.
Values are means with their standard errors. n=8. Longer black arrows at 09:00, 13:00 and 17:00 indicate when the breakfast, lunch and dinner started, respectively. Shorter black arrows at 10:45, 14:45 and 19:00 indicate when snacks started.

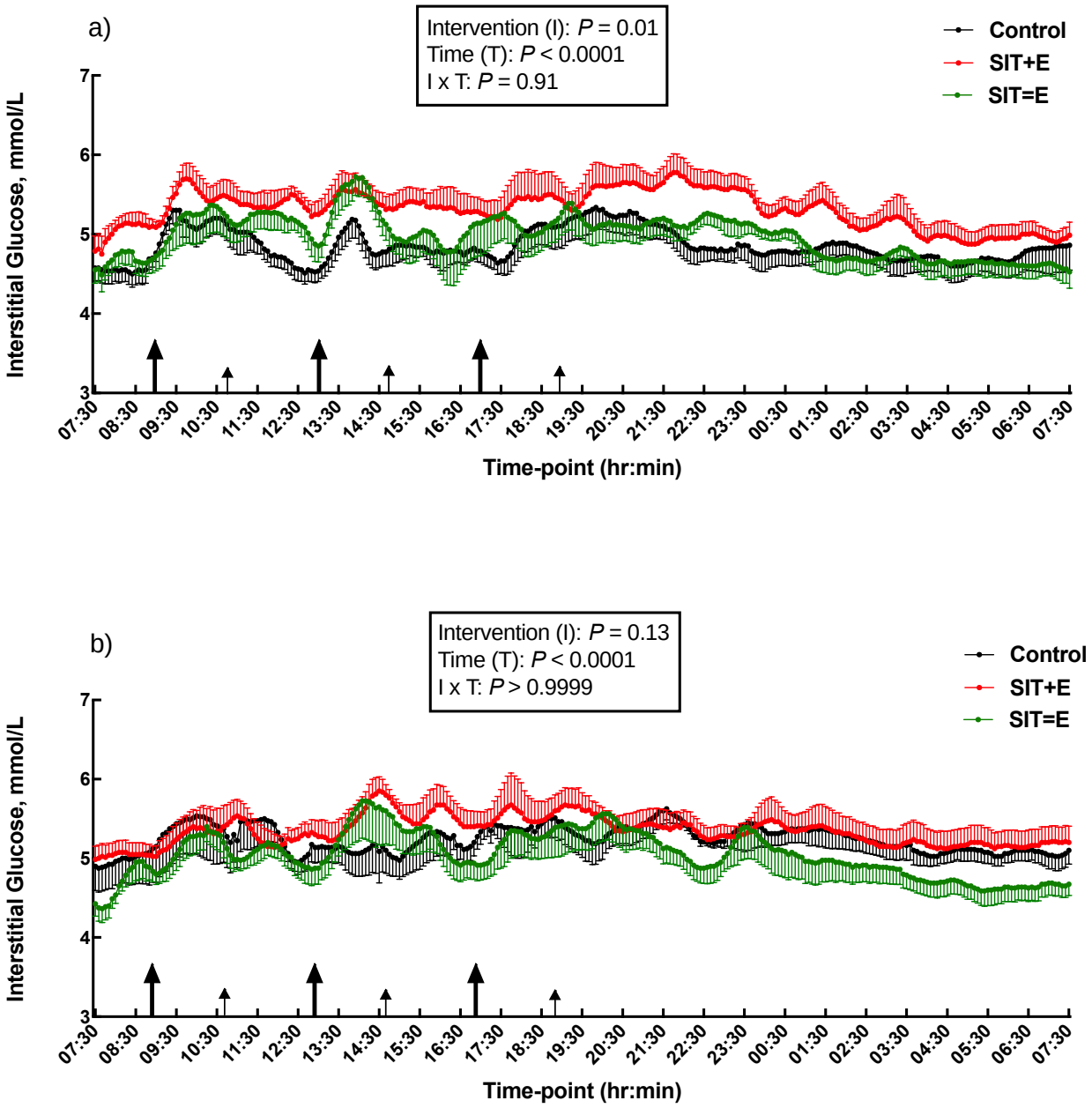
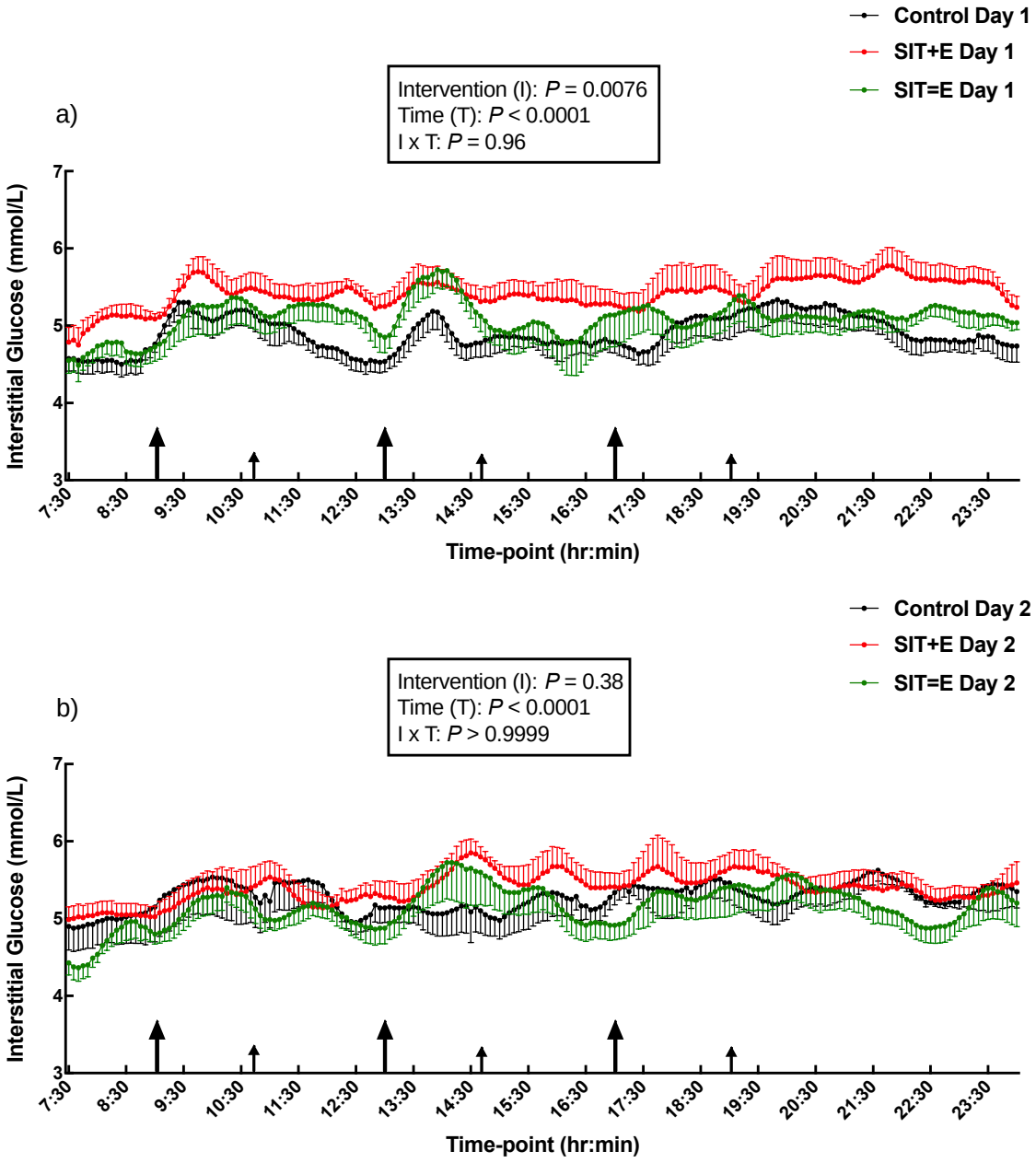


Figure 3.12 Whole-day glycaemic profiles calculated as mean interstitial glucose concentrations of intervention Day 1 (a) and Day 2 (b) for eight participants.
Values are means with their standard errors. *n*=8. Longer black arrows at 09:00, 13:00 and 17:00 indicate when the breakfast, lunch and dinner started, respectively. Shorter black arrows at 10:45, 14:45 and 19:00 indicate when snacks started.


a)
Control Day 1
SIT+E Day 1
SIT=E Day 1
Intervention (I): P = 0.0076
Time (T): P < 0.0001
I x T: P = 0.96
Interstitial Glucose (mmol/L)
Time-point (hr:min)
b)
Control Day 2
SIT+E Day 2
SIT=E Day 2
Intervention (I): P = 0.38
Time (T): P < 0.0001
I x T: P > 0.9999
Interstitial Glucose (mmol/L)
Time-point (hr:min)

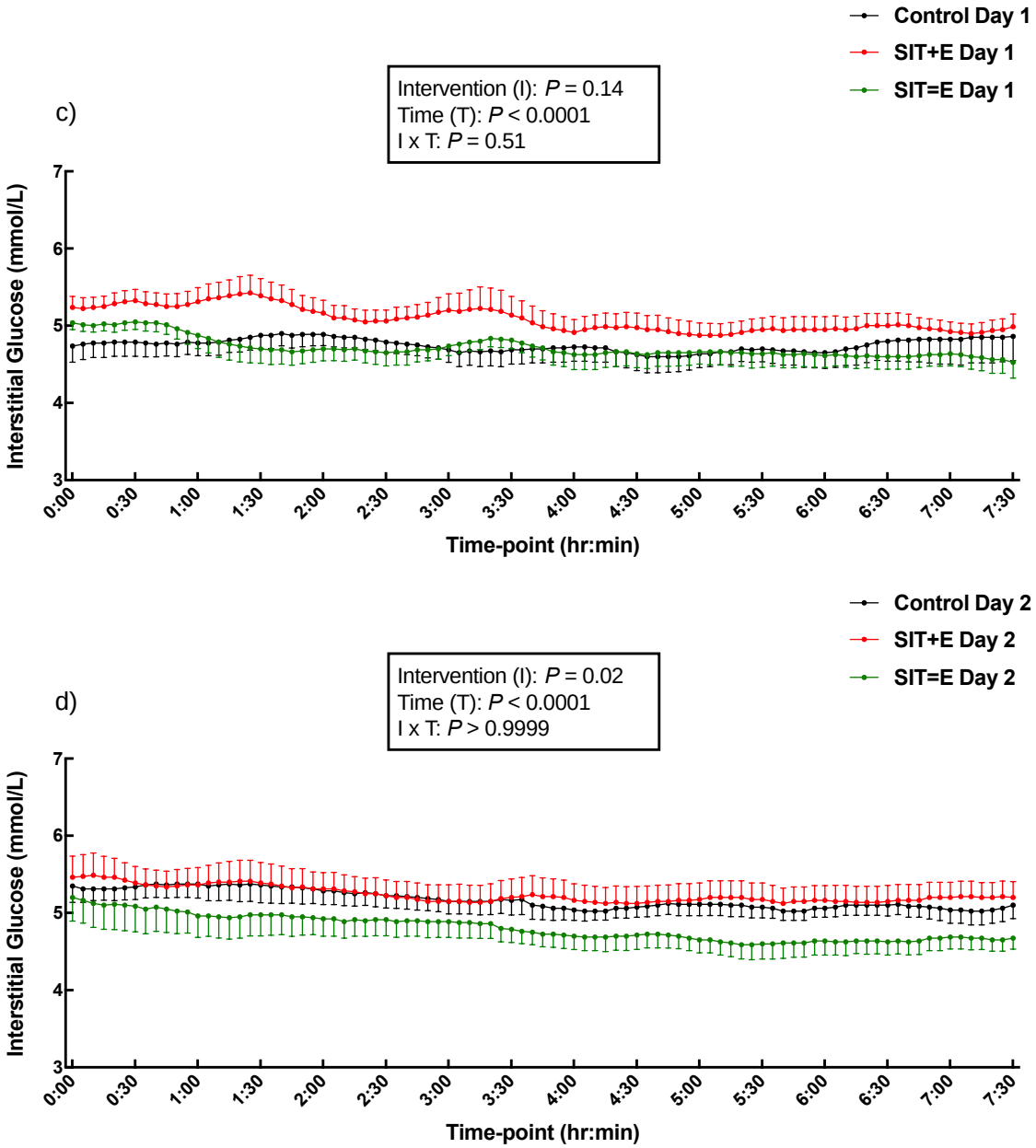


Figure 3.13. Glycaemic profiles calculated as mean interstitial glucose concentrations of intervention Day 1 day hours (a), intervention Day 2 day hours (b), intervention Day 1 night hours (c) and intervention Day 2 night hours (d) for eight participants.

Values are means with their standard errors. *n*=8. Longer black arrows at 09:00, 13:00 and 17:00 indicate when the breakfast, lunch and dinner started, respectively. Shorter black arrows at 10:45, 14:45 and 19:00 indicate when snacks started.

Table 3.5 Indices derived from 24 hours continuous glucose monitoring during two-day interventions.

	Full 24h				Day hours (0730 to 2359)				Night hour (2400 to 0729)			
	CONTROL	SIT + E	SIT = E	*P* [a]	CONTROL	SIT + E	SIT = E	*P* [a]	CONTROL	SIT + E	SIT = E	*P* [a]
	Mean ± SD	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	Mean ± SD	
Mean glucose, mmol/L	5.1 ± 0.3	5.3 ± 0.4	5.0 ± 0.3	0.04	5.1 ± 0.3	5.4 ± 0.4	5.2 ± 0.4	0.06	5.0 ± 0.3	5.2 ± 0.5	4.8 ± 0.3	0.02
Maximum, mmol/L	6.1 ± 0.7	6.1 ± 0.6	6.3 ± 0.9	0.53	6.1 ± 0.7	6.1 ± 0.6	6.3 ± 0.9	0.53	5.3 ± 0.4	5.6 ± 0.5	5.2 ± 0.5	0.14
Minimum, mmol/L	4.4 ± 0.3	4.7 ± 0.3	4.4 ± 0.4	0.04	4.4 ± 0.3	4.7 ± 0.3	4.4 ± 0.4	0.04	4.7 ± 0.3	4.9 ± 0.4	4.5 ± 0.4	0.02
SD	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	0.31	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	0.27	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.86
% CV	7.0 ± 2.0	6.1 ± 0.9	8.4 ± 4.7	0.19	7.4 ± 2.7	5.9 ± 1.4	8.2 ± 4.2	0.15	3.2 ± 1.6	3.5 ± 2.2	4.2 ± 4.8	0.80
	Breakfast (0900)				Lunch (1300)				Dinner (1700)			
	CONTROL	SIT + E	SIT = E	*P* [a]	CONTROL	SIT + E	SIT = E	*P* [a]	CONTROL	SIT + E	SIT = E	*P* [a]
iAUC, mmol/L x 60 min	18 ± 11	19 ± 11	20 ± 22	0.96	11 ± 11	10 ± 10	30 ± 41	0.17	8 ± 19	10 ± 14	14 ± 29	0.73
iAUC, mmol/L x 120 min	38 ± 27	45 ± 34	52 ± 47	0.44	21 ± 16	31 ± 20	68 ± 98	0.21	26 ± 38	23 ± 34	35 ± 50	0.59
iAUC, mmol/L x 180 min	55 ± 44	64 ± 45	77 ± 60	0.36	34 ± 22	45 ± 27	90 ± 128	0.27	45 ± 55	37 ± 38	65 ± 83	0.36
iAUC, mmol/L x 240 min	62 ± 49	80 ± 51	98 ± 70	0.24	47 ± 31	58 ± 42	100 ± 127	0.32	63 ± 69	49 ± 46	86 ± 109	0.40

% CV percentage coefficient of variation, *iAUC* incremental area under the curve

[a] One-factor repeated-measures ANOVA

Table 3.6 Indices derived from 24 hours continuous glucose monitoring during intervention Day 1.

	Full 24h				Day hours (0730 to 2359)				Night hour (2400 to 0729)			
	CONTROL	SIT + E	SIT = E	*P* [a]	CONTROL	SIT + E	SIT = E	*P* [a]	CONTROL	SIT + E	SIT = E	*P* [a]
	Mean ± SD	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	Mean ± SD	
Mean glucose, mmol/L	4.9 ± 0.3	5.3 ± 0.4	5.0 ± 0.3	0.02	4.9 ± 0.3	5.4 ± 0.5	5.1 ± 0.4	0.005	4.7 ± 0.4	5.1 ± 0.4	4.7 ± 0.4	0.11
Maximum, mmol/L	6.2 ± 0.6	6.4 ± 0.6	6.5 ± 0.8	0.60	6.1 ± 0.6	6.4 ± 0.6	6.5 ± 0.8	0.20	5.3 ± 0.8	5.7 ± 0.6	5.1 ± 0.3	0.15
Minimum, mmol/L	4.0 ± 0.3	4.4 ± 0.5	4.1 ± 0.5	0.048	4.0 ± 0.3	4.5 ± 0.6	4.5 ± 0.6	0.004	4.4 ± 0.4	4.7 ± 0.4	4.4 ± 0.6	0.20
SD	0.5 ± 0.1	0.4 ± 0.0	0.5 ± 0.2	0.25	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	0.07	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.2	0.62
% CV	9.6 ± 2.9	7.6 ± 0.7	10.3 ± 5.1	0.15	9.2 ± 2.7	7.2 ± 1.6	10.7 ± 4.4	0.03	5.7 ± 4.8	4.8 ± 2.2	4.3 ± 3.6	0.68
	Breakfast (0900)				Lunch (1300)				Dinner (1700)			
	CONTROL	SIT + E	SIT = E	*P* [a]	CONTROL	SIT + E	SIT = E	*P* [a]	CONTROL	SIT + E	SIT = E	*P* [a]
iAUC, mmol/L x 60 min	22 ± 17	26 ± 15	24 ± 35	0.96	23 ± 18	14 ± 12	14 ± 12	0.20	14 ± 27	9 ± 10	14 ± 27	0.61
iAUC, mmol/L x 120 min	46 ± 30	55 ± 34	62 ± 71	0.73	42 ± 24	28 ± 21	67 ± 79	0.24	30 ± 39	32 ± 32	32 ± 47	0.96
iAUC, mmol/L x 180 min	57 ± 38	76 ± 45	98 ± 96	0.36	61 ± 30	40 ± 24	82 ± 84	0.26	62 ± 64	50 ± 43	59 ± 75	0.76
iAUC, mmol/L x 240 min	59 ± 39	96 ± 57	128 ± 109	0.15	81 ± 33	56 ± 35	100 ± 84	0.24	94 ± 84	76 ± 63	78 ± 95	0.74

% CV percentage coefficient of variation, *iAUC* incremental area under the curve

[a] One-factor repeated-measures ANOVA

3.3.6 Resting Energy Expenditure (Indirect Calorimetry Data)

Hourly values for REE following the MTT are presented in **Figure 3.14**. Fasting REE and DIT are presented in **Figure 3.15** and **Figure 3.16**, respectively. The REE increased above the fasted levels after the MTT in all three visits. There were no significant differences in fasting REE or DIT (iAUC for the entire postprandial REE response) between the three trials. Hourly values for RER following the MTT are presented in **Figure 3.17**. Neither fasting nor postprandial RER responses differed between the three trials.

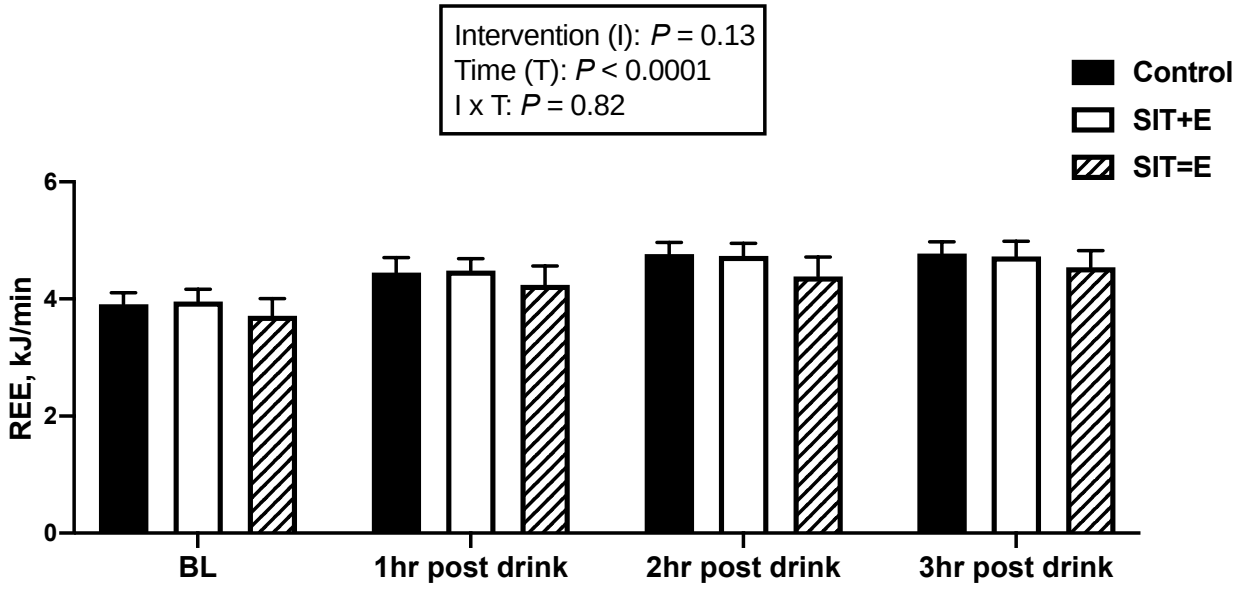


Figure 3.14 Resting energy expenditure.
Values are means with their standard errors. *n*=8.

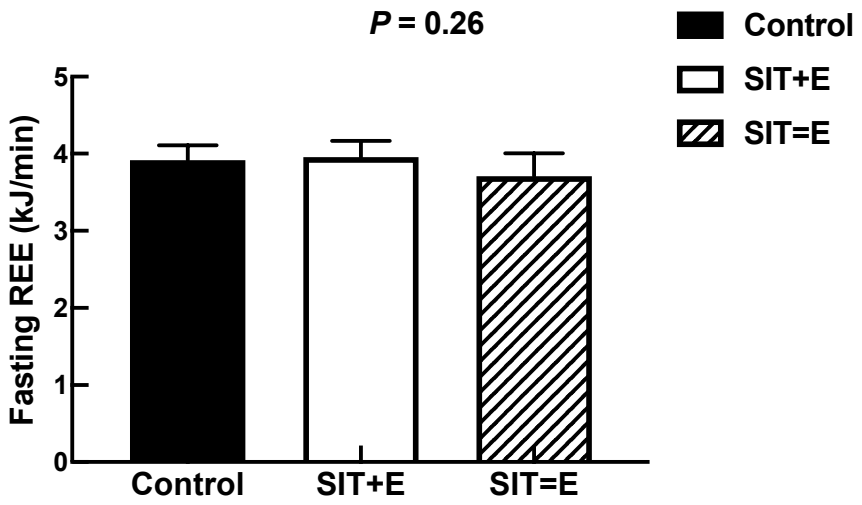


Figure 3.15 Resting energy expenditure (REE).
Values are means with their standard errors. *n*=8.

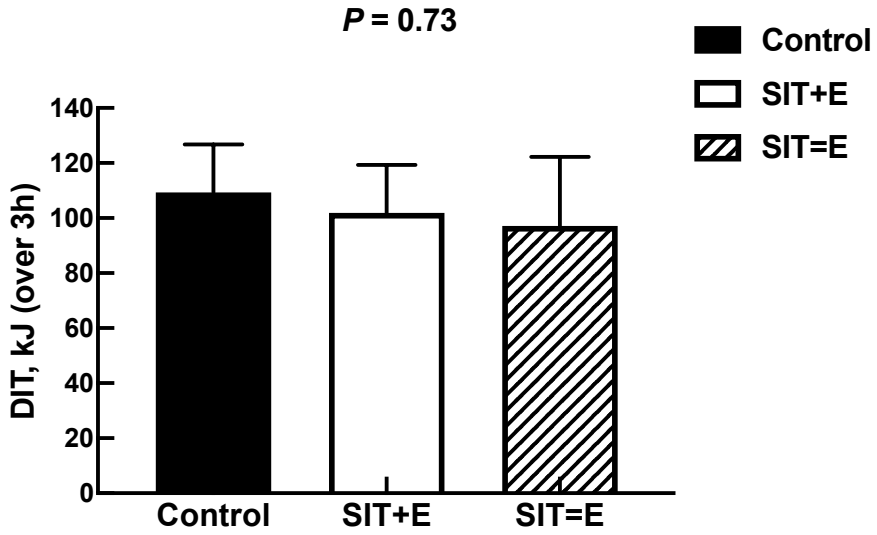


Figure 3.16 Diet induced thermogenesis (DIT).
DIT was calculated by the incremental area under the curve for the resting energy expenditure above the fasting value, using trapezoidal method. Values are means with their standard errors. *n*=8.

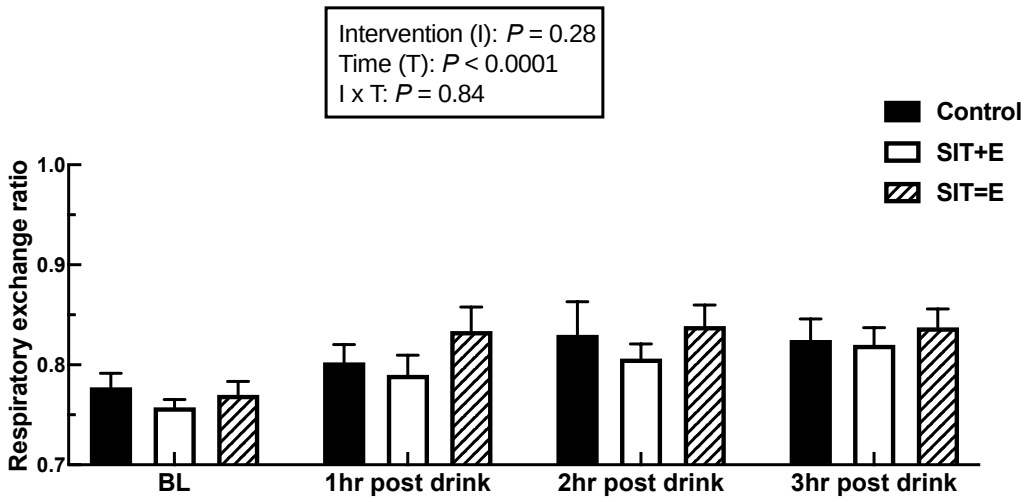


Figure 3.17 Respiratory exchange ratio.
Values are means with their standard errors. *n*=8.

3.3.7 Subjective Appetite Ratings

Fasting subjective appetite ratings (VAS) over the study are reported in **Table 3.7**. Postprandial iAUCs (and dAUCs when indicated) for subjective appetite ratings (VAS) following both the MTT and the *ad-libitum* meal are presented in **Table 3.8**. Mean CAS following the MTT and the *ad-libitum* meal is shown below in **Figure 3.18**. There were no significant differences in fasting or postprandial values for VAS or CAS appetite ratings between the three trials.

Table 3.7 Fasting subjective appetite ratings over the study.

	CONTROL	SIT + E	SIT = E	
	Mean ± SD	Mean ± SD	Mean ± SD	*P* [a]
Hunger (mm)	70 ± 15	80 ± 16	66 ± 15	0.23
Satiety (mm)	27 ± 18	24 ± 19	25 ± 15	0.95
Fullness (mm)	20 ± 16	15 ± 10	23 ± 13	0.41
Desire to eat (mm)	74 ± 17	80 ± 13	67 ± 18	0.30
Prospective food consumption (mm)	72 ± 13	76 ± 12	67 ± 11	0.11
Fasting CAS (mm)	74 ± 15	80 ± 13	70 ± 11	0.33

CAS composite appetite score

[a] One-factor repeated-measures ANOVA

Table 3.8 Incremental area under the curve (iAUCs for Satiety and Fullness) or decremental area under the curve (dAUCs for Hunger, Desire to eat, Prospective food consumption and CAS) for subjective appetite ratings following the liquid breakfast and the *ad-libitum* meal over the study.

		CONTROL	SIT + E	SIT = E	
		Mean ± SD	Mean ± SD	Mean ± SD	*P* [a]
Hunger	Test drink (dAUC, mm over 3h)	-4677 ± 3647	-5272 ± 2994	-3607 ± 2889	0.46
	Ad-libitum meal (dAUC, mm over 1h)	-4803 ± 1068	-5091 ± 1132	-4629 ± 1540	0.51
Satiety	Test drink (iAUC, mm over 3h)	5471 ± 3956	4532 ± 3570	4579 ± 3279	0.75
	Ad-libitum meal (iAUC, mm over 1h)	4694 ± 1241	4718 ± 1289	4561 ± 1335	0.87
Fullness	Test drink (iAUC, mm over 3h)	6348 ± 3746	6065 ± 2372	4557 ± 3303	0.36
	Ad-libitum meal (iAUC, mm over 1h)	4863 ± 1008	4565 ± 1365	4256 ± 1647	0.46
Desire to eat	Test drink (dAUC, mm over 3h)	-5053 ± 3381	-5010 ± 2628	-3841 ± 3325	0.55
	Ad-libitum meal (dAUC, mm over 1h)	-4893 ± 912	-4814 ± 1576	-4734 ± 1102	0.82
Prospective food consumption	Test drink (dAUC, mm over 3h)	-5068 ± 3128	-4643 ± 2553	-3808 ± 2943	0.41
	Ad-libitum meal (dAUC, mm over 1h)	-4466 ± 1684	-4311 ± 1918	-4307 ± 1294	0.88
CAS	Test drink (dAUC, mm over 3h)	-5267 ± 3442	-5024 ± 2757	-3960 ± 3033	0.52
	Ad-libitum meal (dAUC, mm over 1h)	-4744 ± 1109	-4700 ± 1339	-4497 ± 1316	0.68

CAS composite appetite score

[a] One-factor repeated-measures ANOVA

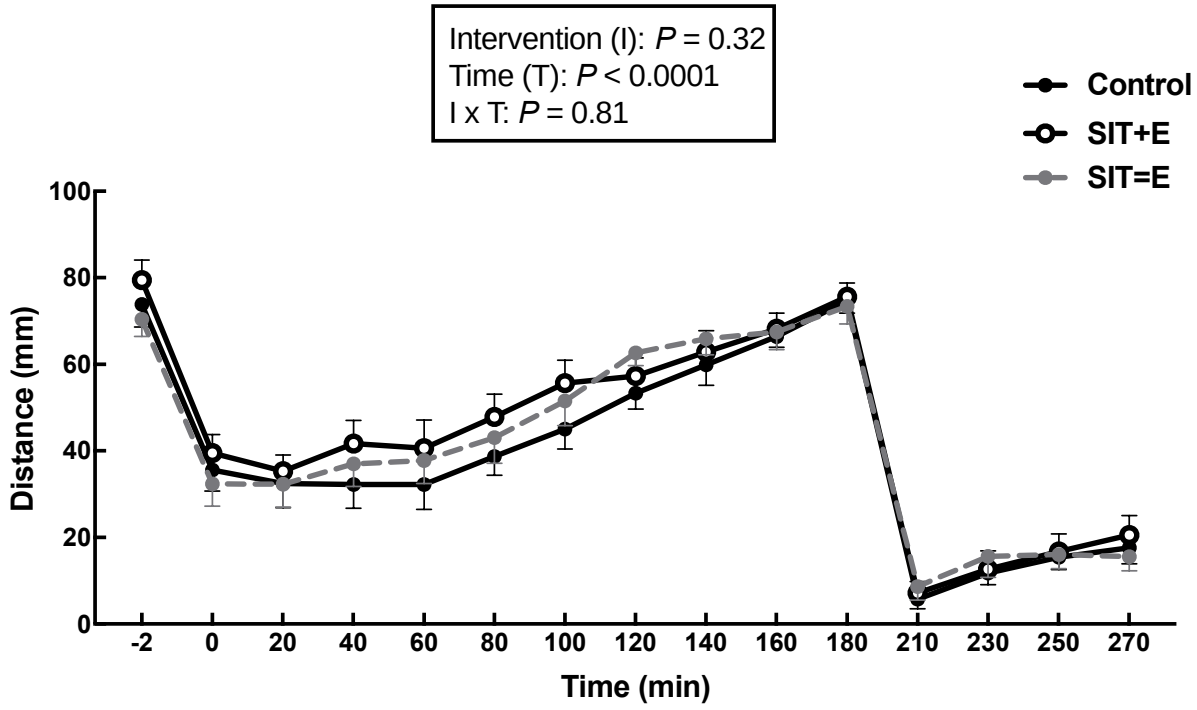


Figure 3.18. **Mean composite appetite score (CAS).**
Values are means with their standard errors. *n*=8.

3.3.8 Energy Intake of the *Ad libitum* Test Meal

There was no significant difference for energy intake of the *ad libitum* test meal between the three trials: 2292 (SD 553), 1990 (SD 587), and 1991 (SD 430) kJ for the CONTROL, SIT+E and SIT=E trials, respectively. No significant difference for the eating duration was identified between the three trials: 13 (SD 3), 13 (SD 2), and 12 (SD 1) min for the CONTROL, SIT+E and SIT=E trials, respectively.

3.4 Discussion

To elucidate the contribution of energy surplus to the detrimental effects of physical inactivity, the effects of two days of physical inactivity with and without energy surplus on basal and postprandial metabolism in normal-weight, young participants, were examined in the present study. The main finding of this study was that two days of step reduction (positive energy balance) showed a strong

tendency towards a significantly elevated postprandial insulinaemic excursion. There was a significant intervention effect for whole-day glycaemic profile, with higher interstitial glucose concentrations during the SIT+E trial throughout the two-day intervention period.

Physical inactivity leads to reductions in glucose tolerance and insulin sensitivity (Bergouignan *et al.*, 2011). Physical inactivity may also result in an energy surplus unless energy intake is reduced to achieve the energy balance (Stephens *et al.*, 2011). Metabolic perturbations resulting from physical inactivity are also observed with overfeeding. Two (Hagobian and Braun, 2006) and three (Cornier *et al.*, 2006) days of energy surplus reduce insulin sensitivity. Therefore, the effects attributed to reduced daily energy expenditure may be explained by the concurrent positive energy balance. In the current study, the effect of two days of physical inactivity (by reducing step count) with and without a proportional decrease in energy intake, insulin sensitivity, glycaemic control, appetite, and REE in response to food ingestion were evaluated. The contribution of energy surplus to the metabolic responses to physical inactivity can thus be identified. The present study found that a prescribed compensatory decrease in energy intake to maintain energy balance during physical inactivity attenuated the adverse effects on insulin sensitivity. Insulin sensitivity was derived from the insulinaemic response to the liquid test meal. There was a strong tendency towards a significant difference in iAUC for serum inulin over 180 min postprandially between the three trials (P = 0.05, η^2_p = 0.35) (with large effect size). This is driven by the difference between the CONTROL vs. SIT+E trials. Compared with the CONTROL trial, the mean iAUC insulin was 25% higher in the SIT+E (P = 0.02) and 8% higher in the SIT=E (P = 0.39) trials. iAUC insulin was comparable between SIT+E and SIT=E (P = 0.09). Two days of step reduction without a prescribed compensatory reduction in energy intake, tended to elevate postprandial insulinaemic excursion; this effect tended to be ameliorated, when accompanied by a prescribed compensatory reduction in energy intake to maintain energy balance. Therefore, this finding may underpin the pivotal role of energy balance in the negative effects of physical inactivity. This is consistent with the findings of a previous study, where a significant reduction in insulin action, determined from the glucose infusion technique, was reported after one-day of prolonged sitting in young, healthy men and women. This effect was minimised, but not eliminated, when energy intake was reduced to achieve an energy balance (Stephens *et al.*, 2011). The promising trend for two-day step reduction to affect insulin sensitivity markers in the present study warrants further

investigation to confirm insulin sensitivity using more sensitive and dynamic assessments, such as the hyperinsulinaemic-euglycaemic clamp, which would also allow the differentiation between hepatic and peripheral insulin sensitivity.

Across the lifespan, physical activity level decreases, and time spent sitting increases. Older individuals tend to engage in less physical activity than younger individuals (Trost *et al.*, 2002) with a significant decrease in levels of leisure-time physical activity (King *et al.*, 2000; Sasidharan *et al.*, 2006; Willey *et al.*, 2010). The higher possibility to be hospitalised exacerbates the problem (Kortebein *et al.*, 2007). The detrimental effects of physical inactivity with different models of inactivity in old individuals have been well investigated (Hirsch *et al.*, 1990; Paddon-Jones *et al.*, 2006; Fisher *et al.*, 2011; Breen *et al.*, 2013; Rezende *et al.*, 2014; Srikanthan and Karlamangla, 2014; Churchward-Venne *et al.*, 2016; Pišot *et al.*, 2016; McGlory *et al.*, 2018; Ekelund *et al.*, 2019). We live in an environment that impedes physical activity and requires prolonged sitting, which is a common behaviour while at home, at work, travelling, or in our communities (Hill *et al.*, 2003). People from different age groups experience this lifestyle. Therefore, the physical inactivity paradigm with reduced step count and increased time spent sitting which was prescribed to participants in this study reflects the real-world situations. Several human studies have reported potentially detrimental effects of physical inactivity on metabolism including insulin sensitivity (Lipman *et al.*, 1972; Stuart *et al.*, 1988; Mikines *et al.*, 1991; Blanc, 2000; Hamilton, Hamilton and Zderic, 2007; Krogh-Madsen *et al.*, 2010). However, there is a paucity of studies investigating how fast these alterations occur. Significant reduction in whole-body insulin action (using the glucose infusion technique) was observed after a 24-hour period of physical inactivity achieved by prolonging sitting time (Stephens *et al.*, 2011). Compared with the present study, the Stephens *et al.* study was better controlled; participants were asked to stay in the laboratory over the intervention period with meal consumption monitored. Knudsen *et al.* (2012) investigated the metabolic effects of step reduction by 85% (from 10,000 to 1,500 steps/day) in healthy, young men. They observed an increase in insulin iAUC to OGTT at days 7 and 14, and a decline in insulin sensitivity, estimated using the Matsuda index at days 3 and 7. In addition, glucose infusion rates decreased by approximately 44% during the hyperinsulinaemic-euglycaemic clamp after a 14-day step reduction. Similar findings were reported in a shorter-term trial, increased insulin iAUC to an OGTT was reported after three-day step reduction (from ~13,000 to ~4300) in young, healthy men

and women (Mikus *et al.*, 2012). Therefore, reduced indices of insulin sensitivity appear to be an early response to physical inactivity.

The outcomes of the present study emphasise the contribution of energy surplus to the negative effects of physical inactivity; this remains a less explored area (Stephens *et al.*, 2011). In the present study, to avoid potential confounding effects caused by different meal frequencies and macronutrient distribution, three main meals and snacks with a macronutrient distribution of ~50 % energy from CHO, ~35 % from fat and ~15 % from protein were designed for all three interventions. In addition, meal time was also consistent across all three conditions to avoid potential confounding effects by altering the meal time. Reducing energy intake to maintain the energy balance during physical inactivity was shown to reduce the insulinaemic response by approximately 2/3, which is comparable to a similar study in a laboratory setting (Stephens *et al.*, 2011). These findings suggest that overfeeding plays a crucial role in the acute metabolic responses to physical inactivity. However, there is a paucity of studies which control all components of energy balance in free-living humans. The associations between physical inactivity and dysregulated appetite control have been consistently demonstrated (Stubbs *et al.*, 2004; Myers *et al.*, 2017; Panahi and Tremblay, 2018), except in bedridden sick patients and astronauts (Narici *et al.*, 2020). However, in the present study, the absence of changes in appetite ratings and energy intake at the *ad libitum* lunch indicate that perhaps longer-term interventions are required to elicit pronounced changes in appetite control. It has been suggested that diet-induced weight loss is associated with elevated ghrelin (the only known orexigenic hormone) levels, potentially reflecting adaptive responses to energy restriction (Wisse *et al.*, 2001; Cummings *et al.*, 2002). In contrast, there is a trend towards decreased ghrelin levels in individuals who experienced weight gain through overfeeding (Ravussin *et al.*, 2001). In the present study, there were no significant differences in fasting or dAUC for plasma total ghrelin between the three trials after two-days overfeeding, which may indicate that ghrelin is associated with metabolic adaptation to longer-term changes in energy intake. Physical inactivity is associated with a reduction in energy expenditure, which in the absence of compensatory changes in appetite or energy intake, induces a positive energy balance (Murgatroyd *et al.*, 1999; Shook *et al.*, 2015; Beaulieu *et al.*, 2016). Thus, it appears reasonable to assert that energy surplus and physical inactivity may usually coexist. In the present study, the absence of change in HOMA-IR (index derived from fasting glucose and

insulin concentrations), compared with a strong tendency towards a significant difference in insulinaemic response, suggests that skeletal muscle may underpin the detrimental effects of physical inactivity with energy surplus. This is consistent with the findings of other physical inactivity studies, which have demonstrated that physical inactivity resulted in a specific reduction in muscle insulin sensitivity without altering the hepatic insulin sensitivity (Stuart *et al.*, 1988; Mikines *et al.*, 1991; Blanc, 2000; Krogh-Madsen *et al.*, 2010). Together, these findings underscore the crucial role of skeletal muscle in inactivity-induced insulin resistance (Narici *et al.*, 2020).

A particular strength of the present study was that the effects of physical inactivity *per se* were dissociated by adding an experimental arm that seeks to compensate for the low energy expenditure by reducing energy intake. Normal-weight, young participants were recruited to examine the effects on metabolism and appetite in people who were unlikely to have alterations in these variables typically associated with excess body weight. Adherence to the eating schedule and physical activity plan was high, supported by the significant differences in mean 24-h glucose levels, mean glucose levels during the day (strong trend), mean glucose levels during the night and minimum glucose levels for the full 24 h, during the day and the night. Furthermore, data from CGM showed excursions in daily glucose profiles, which coincided with the prescribed meal consumption times, also reflecting good compliance during the interventions. To my knowledge, this is the first study with CGM used throughout the interventions to monitor adherence and allow insight into the effects of physical inactivity on whole-day glycaemic profiles. The main limitation is that interventions took place in a free-living setting. Although daily step count targets were achieved in all three trials, accelerometer/heart rate data during this time to corroborate the intensity levels of the physical activities undertaken by the participants was not collected. It is not possible to evaluate the frequency, intensity, or duration of the physical activity, nor to capture the occurrences of resistance exercise, bicycling, or cycling from monitoring the step count (Bassett *et al.*, 2017). Therefore, in the present study, the possibility of participants doing more exercise without altering step count cannot be excluded.

In summary, reduced step count with positive energy balance tended to elevate postprandial insulinaemic excursion; the prescribed compensatory reduction in energy intake to achieve energy balance may stop that from occurring in healthy participants. A physical inactivity model was used

in which step counts were reduced by 80%, and participants were prescribed with prolonged periods of sitting. The present study was designed to extend upon a similar study (24-hour prolonged sitting) by evaluating the effects of two-days of physical inactivity on insulin sensitivity. Larger-scale RCTs with insulin sensitivity measured using a more accurate method and with all components of energy balance well-controlled are needed to establish the cellular and molecular mechanisms behind the effects of physical inactivity on metabolism.

CHAPTER 4. EFFECTS OF TWO WEEKS OF 5:2 INTERMITTENT ENERGY RESTRICTION ON CIRCULATING INSULIN, GLUCOSE AND LIPIDS IN HEALTHY-WEIGHT YOUNG PARTICIPANTS

4.1 Introduction

In modern societies, the prevalence of obesity, driven by sustained excess energy intake and prolonged sedentary behaviour, is increasing worldwide (WHO, 2020). Excess energy intake, relative to requirement, results in weight gain due to fat deposition. Over a prolonged period, increasing adiposity results in obesity, which is one of the leading global risk factors for all-cause mortality (National Center for Chronic Disease Prevention and Health Promotion, 2019). Obesity greatly increases the pathogenesis and progression of cardiovascular diseases (Lavie, Milani and Ventura, 2009), T2DM (Kahn, Hull and Utzschneider, 2006), dyslipidemia (Klop, Elte and Cabezas, 2013), dementia (Kivipelto *et al.*, 2005), certain cancers (Bhaskaran *et al.*, 2014) and contributes to premature mortality (Angelantonio *et al.*, 2016). To restore a state of energy balance, or create negative energy balance, and promote health outcomes, an increasing number of interventions are directed at restraining energy intake and/or increasing energy expenditure (Templeman *et al.*, 2020). Modest weight reduction (5-10% of original body weight) is recommended to individuals with overweight or obesity to attain improvements in metabolic function and other health outcomes (Jensen *et al.*, 2014). However, benefits achieved from weight-loss diets are hindered by metabolic adaptations in appetite and energy utilisation (Martin *et al.*, 2011; Hall *et al.*, 2012; Müller *et al.*, 2015; Westerterp, 2018) and are further limited by poor compliance (Dansinger *et al.*, 2005; Middleton, Anton and Perri, 2013). Moreover, diets that overemphasise a single type of nutrient or food are inadvisable (Katz and Meller, 2014). Continuous energy restriction (CER) is a conventional weight management approach, which involves diets with a 2000-2500 kJ deficit each day, relative to energy requirements. Although CER, with expert support and intensive follow-up, is recommended for sustainable weight loss for people with obesity by the National Institute for Health and Care Excellence (National Institute

for Health and Care Excellence, 2014), difficulty in adhering to CER in humans is widely recognised (Varady and Hellerstein, 2007; Harvie *et al.*, 2011). Against this context, intermittent fasting (IF) or intermittent energy restriction (IER) are becoming increasingly popular for achieving weight loss and metabolic benefits. Both IF and IER are dietary strategies that involve short periods of substantial energy restriction with no or little energy intake, interspersed with periods without intentional energy restriction, or with the intention of meeting energy requirements, respectively (Mattson, Longo and Harvie, 2017).

One popular IER regimen is 5:2 IER. This regimen, when employing two non-consecutive energy restricted days has shown comparable reductions in body weight, fat mass, and fat-free mass, as well as favourable effects on glycated Hb levels and a range of fasting metabolic parameters compared with CER, in participants with overweight or obesity over 3, 6 or 12 months (Carter, Clifton and Keogh, 2016, 2018; Conley *et al.*, 2018; Schübel *et al.*, 2018; Sundfør, Svendsen and Tonstad, 2018). One recent study has demonstrated that employing 5:2 IER with 75% energy restriction on two consecutive days/week elicits a 5% loss of body weight, decreased postprandial TAG responses when compared with matched weight loss from CER in participants with overweight or obesity (Antoni *et al.*, 2018). Furthermore, in a similar population, this 5:2 IER regimen facilitated greater reductions in fasting insulin concentration, insulin resistance (HOMA-IR), and fat mass over 12 weeks and six months at a comparable energy deficit and weight loss to CER (Harvie *et al.*, 2011; Harvie *et al.*, 2013). The differences were largely attributed to the prolonged post-absorptive times during the 5:2 IER days as a result of consuming smaller meals. 5:2 IER, compared with CER may confer greater metabolic benefit when energy restriction is performed on two consecutive days, compared with two non-consecutive days, but the results are inconclusive, and both patterns warrant further study.

Whilst 5:2 IER shows great promise as a sustainable intervention to improve metabolic health, existing findings are inconsistent and have predominantly focused on assessing fasting metabolic measures. Given that modern humans spend much of the 24-hour period in a postprandial state, the sole assessment of fasting measures may mask the effects on human metabolism that occur postprandially. Indeed, despite the growing trend of 5:2 IER, there is a paucity of studies that comprehensively assess postprandial metabolic responses in comparison to CER.

This study thus aims to compare the fasting and postprandial metabolic effects of two consecutive weeks in which either estimated energy requirements (EER) were met on five days and restricted on two non-consecutive days each week (70% energy restriction relative to EER) (5:2 IER); or energy was restricted every day of the two-week period (20% daily energy restriction relative to EER) (CER). Both diets were thus hypoenergetic, and designed to induce the same total energy deficit over the experimental period. Healthy, young, normal-weight participants were chosen to minimise the confounding effects of any intrinsic metabolic defects associated with overweight and obesity, which may interfere with the intervention efficacy. The outcomes of this study may provide a reference for the application of 5:2 IER in other population groups. All food and beverages were provided to improve compliance. The main objective of this study was to compare the iAUC for serum insulin, concurrently with whole blood glucose, plasma FFA and serum TAG responses, for three hours after a standardised liquid breakfast following two weeks of 5:2 IER and CER.

4.2 Methods

4.2.1 Participants

Sixteen young (between the ages of 20 – 35 years), healthy men and women with a BMI between 20 and 25 kg/m^2 and a waist circumference < 94 cm for men and < 80 cm for women were recruited from the students and staff of the University of Nottingham and Queen's Medical Centre. All participants were moderately physically active according to self-assessment using the IPAQ (Section 2.2.2.3) with a stable weight (± 2 kg) over the previous three months. Exclusion criteria included: smoking; regularly skipping breakfast; any metabolic, endocrine or cardiovascular abnormalities; taking any medication other than over-the-counter drugs; individuals with high-intensity physical training; shift workers; on an energy-restricted diet; high alcohol consumption (≥ 3 units/d for men; ≥ 2 units/d for women); women who were pregnant, lactating or with irregular menstrual cycles; self-reported allergy, intolerance or strong dislike of foods or drinks to be offered during the intervention; BDI (Section 2.2.2.1) or EAT-26 (Section 2.2.2.2) scores providing evidence of depression (BDI score > 10) or eating disorder (EAT-26 score > 20). Participants were asked to visit the David Greenfield Human Physiology Unit at the Queens Medical Centre

Nottingham for a medical screening visit, a preliminary information session, two main experimental visits, and a mid-intervention visit from November 2018 to May 2019 (**Figure 4.1**).

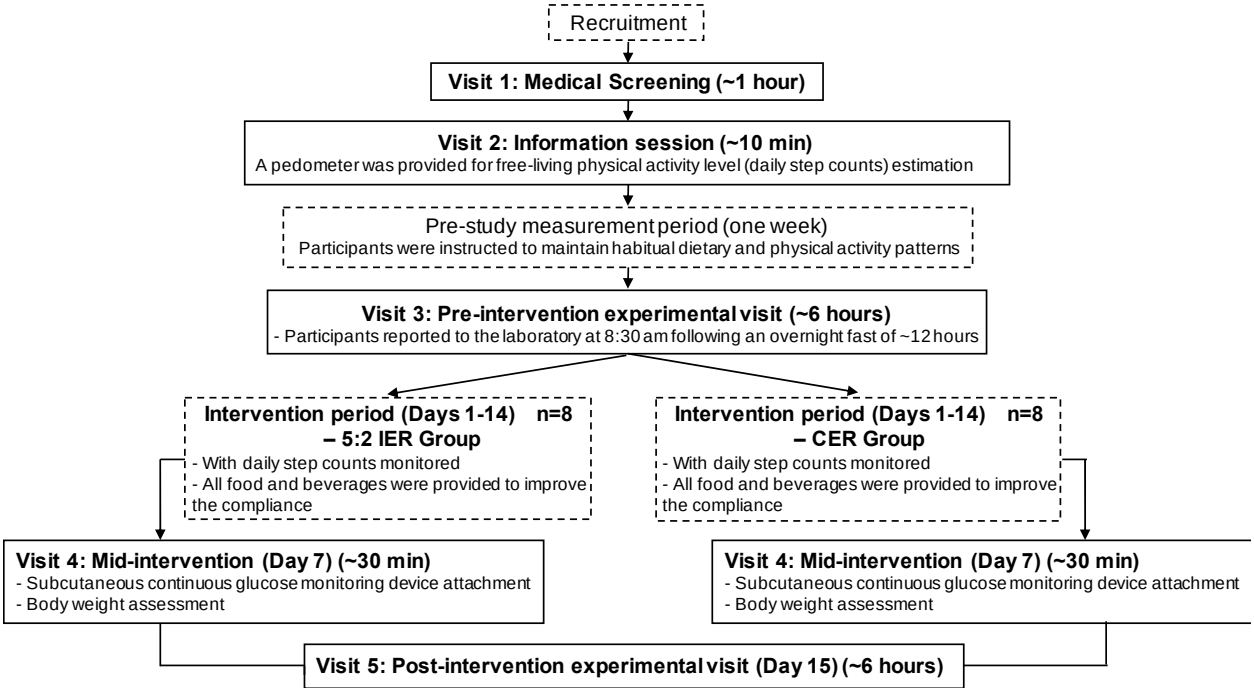


Figure 4.1 Schematic diagram of experimental protocol.

4.2.2 Screening

Once participants consented to being screened, they reported to the laboratory for a medical screening to check their suitability for the study. This involved having body weight (see Section 2.2.1.1), height (see Section 2.2.1.1), BMI (see Section 2.2.1.2), waist and hip circumferences (see Section 2.2.1.3), skinfold thicknesses at biceps, triceps, subscapular and suprailiac sites measured (see Section 2.2.1.4), and an 8 mL blood sample obtained from the median cubital vein via venepuncture for routine blood investigation (full blood count and urea and electrolyte measurements) (see Section 2.2.3). Two BP and HR measurements were taken with participants in a supine position. A further three measurements were taken when participants were sitting, standing for 1 min, and standing for 3 min to evaluate orthostatic hypotension. Participants were required to complete a screening form including "Personal Information" and "General Health"

sections (see **Appendix C**); the BDI, which was used to assess the presence and severity of depression symptoms of participants (Beck, 1961) (see **Appendix D**); the EAT-26, which was used to access the eating disorder risk of participants (Garner *et al.*, 1982) (see **Appendix E**) as well as the IPAQ (Craig *et al.*, 2003) (see **Appendix F**) (see Section 2.2.2).

4.2.3 Experimental Design

Participants were required to sign a witnessed consent form to join the study and attended the laboratory on a total of four further occasions after the screening visit (**Figure 4.1**). This study followed a randomised, parallel-armed design, comparing a 5:2 IER diet with a CER diet, each for a period of two weeks. During each week of the 5:2 IER diet, food was provided for five days at participants' EER and for two non-consecutive days per week on an energy restricted diet (70% energy restriction relative to EER). In the CER diet, energy intake was restricted on all days (20% energy restriction relative to EER), resulting in the same overall energy restriction in the two diets. Participants were asked to undergo a one-week pre-study measurement period, in which they maintained their normal dietary and physical activity patterns. Free-living physical activity level (recorded as daily step counts) of participants was also estimated using a pedometer (Omron Walking Style One 2.1, Omron, USA) worn during the waking day. The pre-study measurement period started, for women, at the beginning of the second week of the follicular phase of their menstrual cycle.

Participants then attended a pre-intervention lab visit at the end of the pre-study measurement period, when they were just at the end of the follicular phase. Seven days after the pre-intervention lab visit, participants began the randomly assigned two-week intervention. Randomisation was done using an online computer programme (https://www.randomizer.org/) by the experimenter. Participants were aware of their randomisation because of the obvious difference in meal patterns. The seven-day gap between the pre-intervention lab visit and the start of the intervention ensured that the post-intervention lab visit, at the end of the two-week intervention, was also conducted during the second week of the follicular phase (in the next menstrual cycle). This was to minimise any potential effects of menstrual stage difference on metabolic outcomes (Valdes and Elkind-Hirsch, 1991; Mumford *et al.*, 2011). On Day 7 of each two-week intervention period, participants reported to the laboratory in the morning after an overnight fast (~12 h) for the assessment of body

weight. At this visit, a subcutaneous continuous glucose monitoring (CGM) device was attached to the participants to allow for estimation of continuous blood glucose over Days 8-13 of each intervention period (Section 2.5.2).

Using data recorded by the pedometer during the first week of the intervention, compliance with the required activity level (based on activity during the pre-study measurement period) was checked.

4.2.4 Dietary Intervention Periods

The types of food and drinks provided to participants as well as the total energy restriction over the two weeks (i.e. 20% restriction relative to EER), were the same for both arms of the trial. Using estimated BMR obtained using the Oxford-Henry prediction equations (Henry, 2005) and the individual daily PAL, estimated from the IPAQ, the daily energy requirements were estimated for each participant using BMR x PAL. The IPAQ results were reported in categories as low, moderate, or high activity levels. A PAL of 1.4 was associated with a low level of occupational and leisure activity, a PAL of 1.6 represented a moderate level, and a PAL of 1.8 represented a high level (Department of Health, 1991; SACN, 2011). Thus, energy intake was tailored to each participant's specific EER. The two non-consecutive 70% energy restriction days per week were fixed on Day 1, Day 4, Day 8, and Day 11 of the 5:2 IER intervention. The diet pattern throughout the interventions in 5:2 IER and CER groups is presented in **Table 4.1**. Therefore, post-intervention measurements for participants following the 5:2 IER were conducted after three-days of non-restricted diet, to eliminate any potential acute effects that the more severe 70% energy restriction (relative to CER) might have on metabolic outcomes.

Table 4.1 The diet pattern throughout the interventions in 5:2 IER and CER groups.

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Day of menu cycle	A	B	C	A	B	C	A	B	C	A	B	C	A	B
5:2 IER Semi fasting day (30% EER)	30% EER			30% EER				30% EER			30% EER			
5:2 IER Non fasting day (100% EER)		100% EER	100% EER		100% EER	100% EER	100% EER		100% EER	100% EER		100% EER	100% EER	100% EER
CER (80% EER)	80% EER	80% EER	80% EER	80% EER	80% EER	80% EER	80% EER	80% EER	80% EER	80% EER	80% EER	80% EER	80% EER	80% EER

5:2 IER 5:2 intermittent energy restriction, *CER* continuous energy restriction, *EER* estimated energy requirements.

The meals on the intervention days were based on a three-day sequence of menus to avoid boredom but to be practical regarding the provision of foods (**Table 4.2**). Each menu provided three meals/day to avoid a potential confounding effect caused by different meal frequencies in the two interventions. Three meals/day (and no snacks) was selected to make each eating incident acceptable, with respect to energy content, on the most restricted days. Three meals with a macronutrient distribution of ~50% energy from CHO, ~31% from fat, and ~19% from protein were prescribed. This maintained the same absolute amount of protein as would be provided by a non-restricted diet in which 15% of energy was from protein. The distribution of energy across the day was 25% of daily energy requirements from breakfast, 37.5% from lunch, and 37.5% from evening meals (You, One, 2018). To avoid potential confounding by altering the meal time, instructions were given to consume breakfast between 8 am - 8:30 am; lunch between 1 pm - 1:30 pm, and evening meal between 6 pm - 6:30 pm. Food composition was matched on a "meal-by-meal" basis in and between both groups to eliminate any confounding effects caused by macronutrient difference. Food items were identical between groups across the intervention to eliminate any confounding effects caused by textural and visual differences. The menu was designed so that the energy provided per meal could be proportionally increased and decreased depending on the intervention requirements and was informed by the likely availability of cooking facilities and to optimising food safety. Participants were also asked to refrain from alcohol and caffeine consumption. Home deliveries or participant collection from the laboratory occurred weekly. Participants were free-living but instructed to follow the prescribed meal plan. Submission to the experimenter, in real-time, of a photograph of each meal, was required prior to eating; leftovers, or additional food eaten, were logged.

Table 4.2 Examples of menus A, B, C throughout the interventions.

(For **8368 kJ or 2000 kcal** of total intake)

Menu A	**Menu B**	**Menu C**
Breakfast	**Breakfast**	**Breakfast**
Cheerios Cereal, 30g British Semi Skimmed Milk, 200g Pancakes, 56g Peanut Butter Smooth, 14g Cashew Kernels, 10g	Cheerios Cereal, 30g British Semi Skimmed Milk, 200g Pancakes, 56g Peanut Butter Smooth, 14g Cashew Kernels, 10g	Cheerios Cereal, 30g British Semi Skimmed Milk, 200g Pancakes, 56g Peanut Butter Smooth, 14g Cashew Kernels, 10g
Lunch	**Lunch**	**Lunch**
Slow Cooked Spaghetti Bolognese, 400g Gala Apples, 225g No Added Sugar 0% Fat Peach Yogurt, 125g	Chicken & Bacon Pasta Bake, 400g Easy Peelers, 70g Chocolate Chip Muffins, 30g No Added Sugar 0% Fat Strawberry Yogurt, 125g	Classic Shepherd's Pie, 525g Chocolate Chip Muffins, 30g Gala Apples, 130g No Added Sugar 0% Fat Peach Yogurt, 250g
Dinner	**Dinner**	**Dinner**
Mixed Vegetables, 220g Indian Chicken Tikka Masala, 180g Rice Egg Fried, 197g Easy Peelers, 110g	Mixed Vegetables, 178g Chilli Con Carne, 450g Chocolate Chip Muffins, 50g Easy Peelers, 58g	Mixed Vegetables, 115g Spaghetti Carbonara, 400g Chocolate Chip Muffins, 30g Easy Peelers, 55g

(For **2510 kJ or 600 kcal** of total intake, with 70% energy restriction)

Menu A	**Menu B**	**Menu C**
Breakfast	**Breakfast**	**Breakfast**
Cheerios Cereal, 9g British Semi Skimmed Milk, 60g Pancakes, 16.7g Peanut Butter Smooth, 4.2g Cashew Kernels, 3g	Cheerios Cereal, 9g British Semi Skimmed Milk, 60g Pancakes, 16.7g Peanut Butter Smooth, 4.2g Cashew Kernels, 3g	Cheerios Cereal, 9g British Semi Skimmed Milk, 60g Pancakes, 16.7g Peanut Butter Smooth, 4.2g Cashew Kernels, 3g
Lunch	**Lunch**	**Lunch**
Slow Cooked Spaghetti Bolognese, 120g Gala Apples, 67.5g No Added Sugar 0% Fat Peach Yogurt, 37.5g	Chicken & Bacon Pasta Bake, 120g Easy Peelers, 21g Chocolate Chip Muffins, 9g No Added Sugar 0% Fat Strawberry Yogurt, 37.5g	Classic Shepherd's Pie, 157.5g Chocolate Chip Muffins, 9g Gala Apples, 39g No Added Sugar 0% Fat Peach Yogurt, 75g
Dinner	**Dinner**	**Dinner**
Mixed Vegetables, 66g Indian Chicken Tikka Masala, 54g Rice Egg Fried, 59g Easy Peelers, 33g	Mixed Vegetables, 53.4g Chilli Con Carne, 135g Chocolate Chip Muffins, 15g Easy Peelers, 17.4g	Mixed Vegetables, 34.5g Spaghetti Carbonara, 120g Chocolate Chip Muffins, 9g Easy Peelers, 16.5g

4.2.5 Measurements Made During Intervention Periods

4.2.5.1 Daily Step Count Measurement

Participants were instructed to maintain their average daily step count at the same level as that recorded during the pre-study measurement period. A pedometer provided easily accessible feedback to the participant on their compliance with the target they had been given.

4.2.5.2 Continuous Glucose Monitoring

The Medtronic MiniMed *i*Pro™2 (Northridge, CA, USA) CGM system was used with Medtronic Enlite™ glucose sensors inserted through the skin on the abdomen of participants, to continuously monitor glucose levels in the interstitial fluid (see Section 2.5.2.1). The CGM was attached on Day 7 and removed during the post-intervention measurement visit of each trial (Day 15). The monitor was calibrated with four separate finger-prick capillary glucose tests each day (prior to each of the three main meals and at bedtime) using the ACCU-CHEK® Performa Blood Glucose Meter (Roche, Mannheim, Germany). At the end of each trial, the monitor was removed and then plugged into the docking station; and the data uploaded into Medtronic CareLink™ iPro™ Therapy management software. Whole-day glycaemic profiles (from 00:00 to 24:00) were analysed using data obtained from Day 8 to Day 13 of the intervention. Data were analysed per 24 h, during the day hours (07:00 to 23:59) and night hours (00:00 to 06:59), with the calculation of the mean, maximum, minimum, and five-hour postprandial iAUC of glucose concentration. Furthermore, SD and %CV of each time period were calculated to describe the glycaemic variability within a day. Whole-day glycaemic profile was plotted by calculating the mean of each of the six days of measurement (Days 8-13) at a timepoint, for a participant, and then calculating the mean at the timepoint for the participants in each treatment group. The pattern of glucose excursions was also used to verify the meal times.

4.2.6 Laboratory-Visit Protocol Pre- and Post-Intervention

Participants reported to the laboratory at 8:30 am following an overnight fast of ~12 h to undergo a series of metabolic measurements in response to a standardised liquid breakfast (meal tolerance

test, MTT) and a subsequent *ad libitum* lunch. All anthropometric measurements described in the screening section were repeated at each laboratory visit. Once the anthropometric measurements were completed, participants were asked to rest in a semi-supine position, in a hospital bed for 30 min. Resting HR and BP were measured in triplicate after at least 20 min at rest. **Figure 4.2** shows a schematic diagram of the sequence of measurements conducted during each trial.

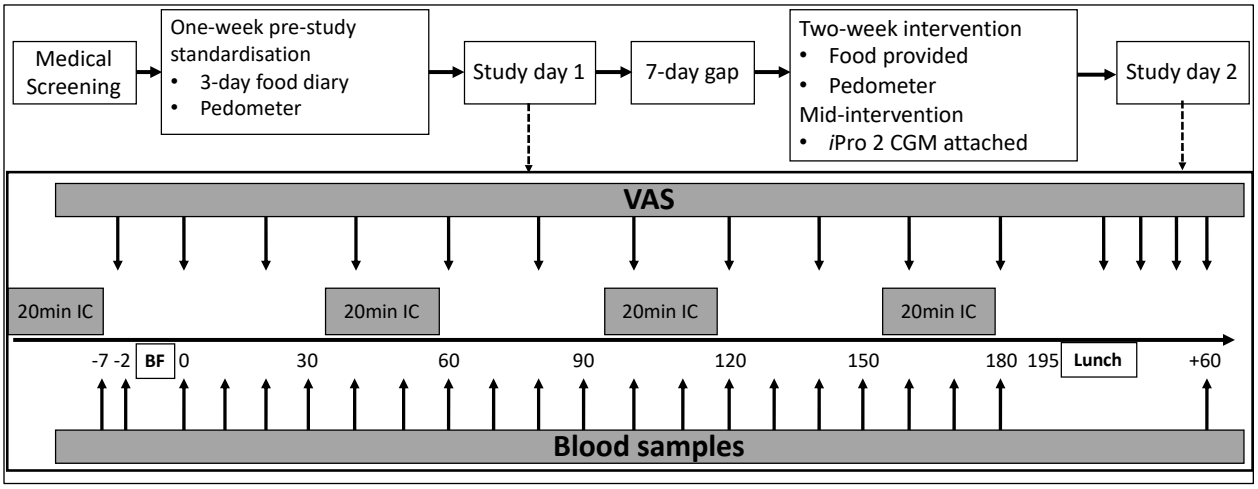


Figure 4.2 Schematic diagram of the sequence of measurements conducted during each trial.
IC, Indirect Calorimetry.

4.2.6.1 Blood Sampling and Analysis

Arterialised venous blood was obtained by inserting a 20-G cannula (pink Venflon, Ohmeda, Sweden) into a dorsal hand vein in a retrograde manner with the hand of the participant warmed in a warm-air box with a constant air temperature of 55°C (McGuire *et al.*, 1976). Before cannulating, the hand was kept in warm water for 5 min before a small amount of local anaesthetic (1% lignocaine: B.Braun Melsungen AG, Melsungen, Germany) was injected subcutaneously, prior to insertion of the cannula. 0.9% saline infusion (Baxter Healthcare Ltd. Thetford, UK) was infused slowly (approximately 120 mL/h) throughout the sampling period to avoid blood clotting in the cannula (described in Section 2.6.5).

Blood samples were drawn using a three-way tap, discarding the first 2 mL that may comprise a mixture of blood and saline. Two baseline blood samples were obtained, with 5 min apart, just before the MTT, to evaluate mean fasting whole blood glucose, serum insulin, FFA, serum TAG, total cholesterol, HDL-cholesterol, and LDL-cholesterol. Blood samples were obtained subsequently every 10 min for the analysis of blood glucose and every 20 min for the determination of insulin, FFA, and TAG concentrations, for a duration of three hours after the MTT. A final blood sample was taken one hour after the ingestion of the *ad libitum* test meal, for determination of whole blood glucose. Blood samples analysis for the present study was explained in Section 2.7.

4.2.6.2 Indices of Insulin Sensitivity

Assessment of insulin sensitivity using the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) and the Matsuda Insulin Sensitivity Index described in Section 1.4.4 was applied here.

4.2.6.3 Test-Drink Consumption

The standardised test drink (Ensure® Compact, Abbott Nutritional Ireland, 10 kJ/mL balanced nutritional supplement drink with vanilla flavour) was provided at approximately 9:30 am at room temperature (24°C). Based on body weight on the day of the visit, 42 kJ was provided per kg of body weight. The energy distribution of the test drink was 47.9% CHO, 35.1% fat, and 17% protein. Therefore, participants received 1.2 g CHO/kg body weight, 0.39 g fat/kg body weight, and 0.43 g protein/kg body weight. Participants were asked to consume the drink within 2 min. The test drink, served as the first food of the day (a breakfast), was designed to stimulate a measurable metabolic response that would return towards baseline levels three hours postprandially (described in Section 2.6.2).

4.2.7 Statistical Analyses

Prism 8 software (GraphPad Software Inc., CA, USA) was used for data entry and analyses (statistical significance accepted as $P < 0.05$). Values in the text, figures and tables are mean and SD unless otherwise stated. Data were tested for normality using the D'Agostino-Pearson normality test. iAUC and dAUC of the postprandial measurements were calculated using the trapezoid rule. Unpaired student's t-tests compared single data points between the two groups.

Two-factor Mixed Model ANOVA (between groups factor: treatment, 5:2 IER vs. CER; within groups factor: sampling time; pre- vs. post- each two-week energy restricted intervention) evaluated intervention effects; When significant treatment, time or treatment x time interaction effects were observed, post-hoc comparisons (where relevant) were explored using paired or unpaired student's t-test. Effect size was estimated as partial eta squared (η^2_p), which is the standard method giving the proportion of variance associated with 2-way ANOVA analysis.

In response to limited previous studies on postprandial responses to 5:2 IER or 5:2 intermittent fasting, the power analysis was based on an intermittent fasting study using glucose disposal as an index of postprandial insulin sensitivity (Halberg *et al.*, 2005). The statistical power analysis indicated that eight participants were required per group to detect a 16% improvement in postprandial whole-body insulin sensitivity with a power of 80% at a significance level of $P < 0.05$.

The primary outcome of this study was the iAUC for insulin for three hours after a standardised liquid breakfast. Secondary outcomes were changes in postprandial glucose, FFA, TAG, Matsuda index and whole-day glycaemic profiles for the average of six consecutive days. Exploratory outcome was fasting blood glucose.

4.3 Results

4.3.1 Participant Characteristics

Of the 27 participants who initially expressed interest, 18 fulfilled the inclusion criteria and were recruited to the study. Two participants withdrew because of an inability to adhere to the finger prick test and because of personal reasons, respectively (11% attrition rate). Eight participants in each group completed the study (CONSORT diagram in **Figure 4.3**). Two participants in each group were excluded from the analysis of free-living continuous glucose monitoring due to incomplete data collection.

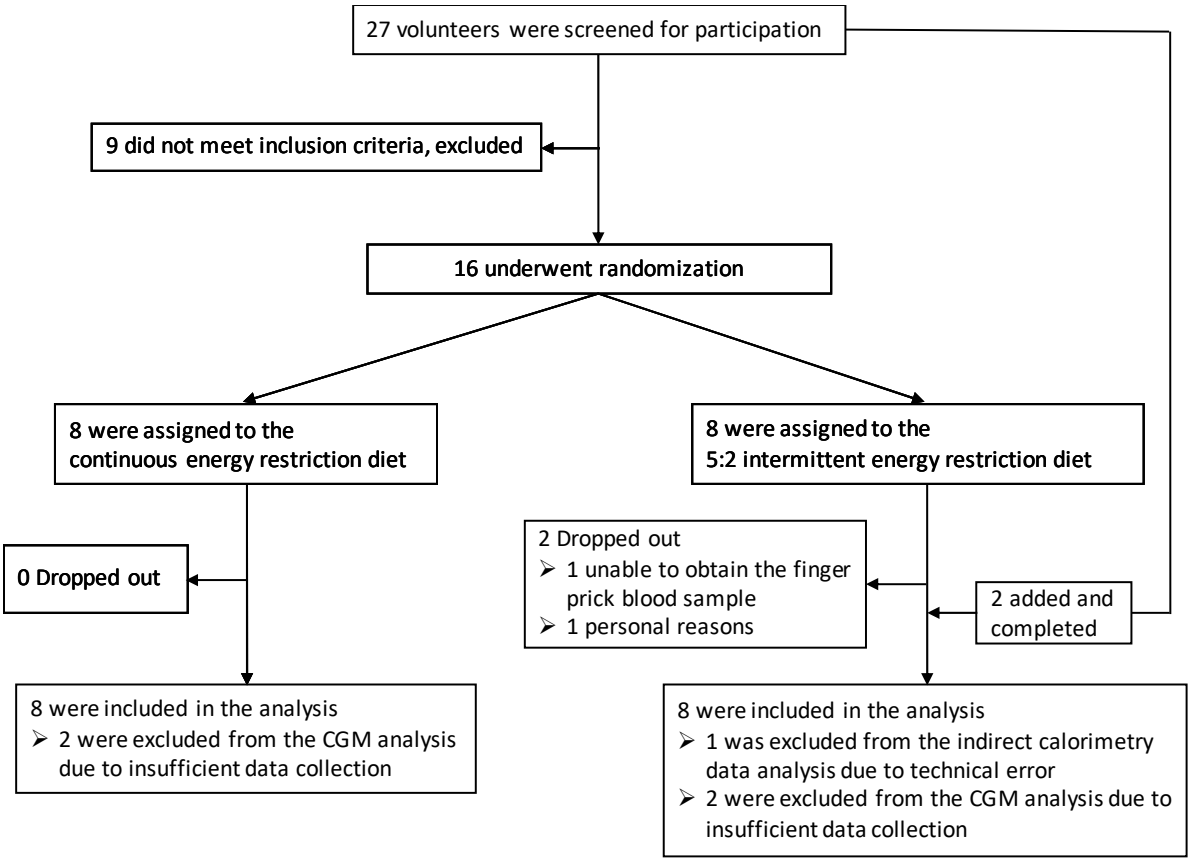


Figure 4.3 CONSORT (Consolidated Standards of Reporting Trials) diagram.
CGM, continuous glucose monitoring.

Upon recruitment to the study, the 5:2 IER and CER groups were matched for age, sex, and anthropometrics. Participant characteristics at baseline are detailed in **Table 4.3**. Changes in participant fasting anthropometric and physiological characteristics over the course of each intervention are reported in **Table 4.4**. Both 5:2 IER and CER were equally effective for weight loss, mean weight change was -2.5 (SD 1.0) kg 5:2 IER, -2.3 (SD 0.7) kg CER after two-weeks intervention; -1.3 (SD 1.2) kg 5:2 IER, -1.1 (SD 0.5) kg CER after one-week intervention, with no significant differences between groups. Both groups experienced comparable reductions in waist and hip circumferences. There was a significant treatment-by-time interaction for fasting HR ($P < 0.05$, 2-way ANOVA). Neither group exhibited a change in baseline (fasting) blood pressure. Over

the course of the two-week intervention, the daily step count did not change from habitual levels in either group and was not different between groups.

Table 4.3 Descriptive characteristics for completers at screening.

	5:2 IER (*n* 8)	CER (*n* 8)	
	Mean ± SD	Mean ± SD	5:2 IER v. CER [a]
Sex (*n*)			
Male	4	4	
Female	4	4	
Age (years)	23 ± 4	26 ± 5	0.21
Body weight (kg)	63.4 ± 14	66.1 ± 11	0.68
BMI (kg/m^2)	21.7 ± 2.4	22.7 ± 1.6	0.34
Body fat (%) [b]	20.2 ± 5.7	22.1 ± 7.6	0.57

[a] Unpaired student's t-test

[b] Estimated using skinfold thickness values

Table 4.4 Anthropometric, physiological characteristics, and fasting blood measurements before (pre-) and after (post-) each intervention.

	5:2 IER (*n* 8)		CER (*n* 8)		
	Pre	Post	Pre	Post	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	5:2 IER v. CER [a]
Body weight, kg	63.4 ± 14.0	60.9 ± 13.2	66.1 ± 11.2	63.9 ± 11.0	0.61
Waist, cm	71.1 ± 8.7	68.7 ± 8.1	75.2 ± 6.7	70.8 ± 5.2	0.11
Hip, cm	96.3 ± 4.7	93.8 ± 6.1	96.7 ± 7.2	93.6 ± 7.1	0.52
Heart rate, BPM	68 ± 6	62 ± 6	60 ± 8	60 ± 7	0.02
DBP, mmHg	65 ± 2	65 ± 5	63 ± 8	63 ± 5	0.87
SBP, mmHg	110 ± 11	108 ± 11	104 ± 13	103 ± 10	0.77
Fasting glucose, mmol/L	4.39 ± 0.28	4.07 ± 0.23	4.42 ± 0.16	4.43 ± 0.17	0.02
Fasting insulin, mIU/L	9.40 ± 3.06	7.78 ± 2.87	7.98 ± 1.54	6.43 ± 1.78	0.97
Fasting FFA, mmol/L	0.46 ± 0.20	0.43 ± 0.10	0.43 ± 0.16	0.45 ± 0.11	0.57
Fasting TAG, mmol/L	0.80 ± 0.37	0.64 ± 0.28	0.68 ± 0.26	0.60 ± 0.16	0.59
Fasting total cholesterol, mmol/L	3.64 ± 0.37	3.35 ± 0.45	3.76 ± 0.63	3.62 ± 0.54	0.46
Fasting LDL-C, mmol/L	2.03 ± 0.23	1.87 ± 0.41	2.15 ± 0.42	2.06 ± 0.43	0.63
Fasting HDL-C, mmol/L	1.17 ± 0.30	1.06 ± 0.25	1.20 ± 0.21	1.16 ± 0.16	0.23

5:2 IER 5:2 intermittent energy restriction, *CER* continuous energy restriction, *DBP* diastolic blood pressure, *SBP* systolic blood pressure

[a] 2-way ANOVA, treatment-by-time interaction

4.3.2 Fasting Blood Measurements

Results for fasting whole blood glucose, serum insulin, plasma FFA, serum TAG, total cholesterol, HDL-cholesterol, and LDL-cholesterol concentrations are shown in **Table 4.4**. There were no significant treatment-by-time interactions or main effects of treatment or time in fasting insulin, FFA, TAG, total cholesterol, or LDL-cholesterol concentrations. As an exploratory finding, there was a significant treatment-by-time interaction between the 5:2 IER and CER regimens for fasting blood glucose ($P = 0.02$, 2-way ANOVA, $\eta^2_p = 0.14$); with post-intervention values significantly lower after 5:2 IER than CER ($P < 0.01$). For fasting HDL-cholesterol, there was no treatment-by time interaction, or main effect of treatment, but there was a main effect of time ($P < 0.05$). HDL-cholesterol decreased across the study.

4.3.3 Postprandial Blood Measurements

4.3.3.1 Postprandial Serum Insulin Responses

Serum insulin rapidly increased and peaked after 20-40 min, before steadily declining, but did not return to fasting levels within 180 min (**Figure 4.4**). There were no significant treatment-by-time interactions ($P = 0.30$, 2-way ANOVA, $\eta^2_p = 0.01$) or main effects of treatment or time in iAUC for insulin over 180 min postprandially (**Table 4.5**).

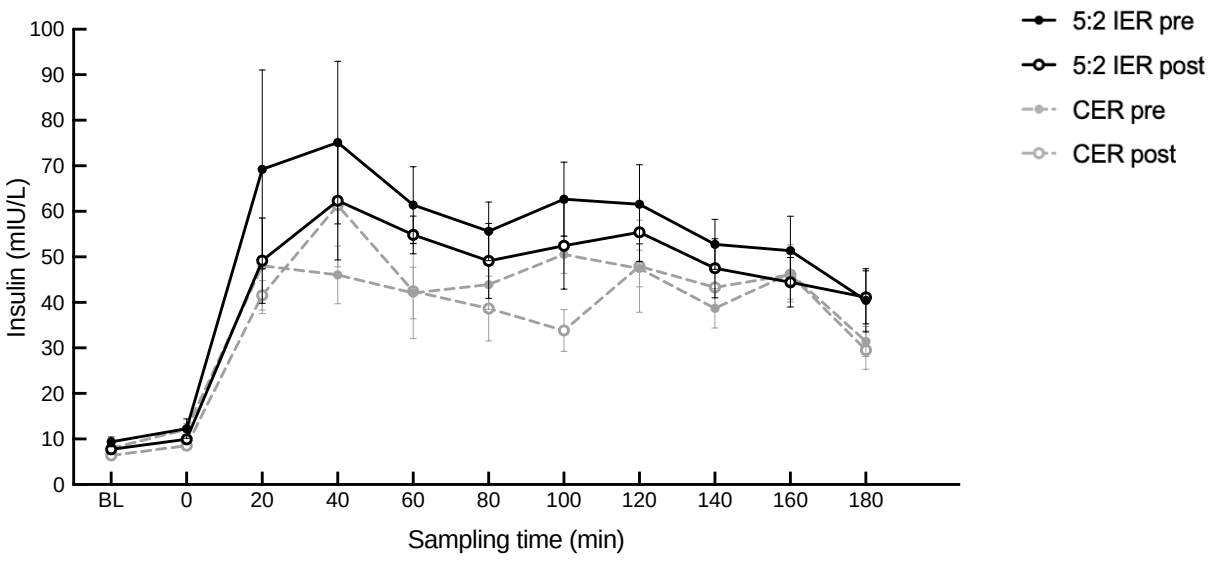


Figure 4.4 Serum insulin following the liquid breakfast.
Pre, pre-intervention measurement; Post, post-intervention measurement; 5:2 IER, 5:2 intermittent energy restriction; CER, continuous energy restriction. Values are means with their standard errors. *n*=8 per group.

Table 4.5 Postprandial blood measurements before (pre-) and after (post-) each intervention[a].

	5:2 IER (*n* 8)		CER (*n* 8)		
	Pre	Post	Pre	Post	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	5:2 IER v. CER [b]
Insulin iAUC, mIU/Lx180 min	8633 ± 4016	7419 ± 2983	6270 ± 1721	6330 ± 2436	0.30
Glucose iAUC, mmol/Lx180 min	261 ± 124	334 ± 173	222 ± 76	247 ± 65	0.44
TAG iAUC, mmol/Lx180 min	62 ± 60	36 ± 22	42 ± 32	29 ± 22	0.51
FFA dAUC, mmol/Lx180 min	-54 ± 30	-46 ± 10	-52 ± 22	-53 ± 17	0.54

5:2 IER 5:2 intermittent energy restriction, *CER* continuous energy restriction, *iAUC* incremental area under the curve, *dAUC* decremental area under the curve

[a] Blood measurements include fasting blood measurements, postprandial incremental area under the curve (Glucose, Insulin and TAG) and decremental area under the curve (FFA) for blood measurements over 180 min after consumption of the liquid breakfast

[b] 2-way ANOVA, treatment-by-time interaction.

4.3.3.2 Postprandial Whole Blood Glucose Responses

Whole blood glucose concentrations increased rapidly after the MTT and remained above fasting levels for 180 min (**Figure 4.5**). There were no significant treatment-by-time interactions or main effects of treatment or time in iAUC for whole blood glucose (**Table 4.5**).

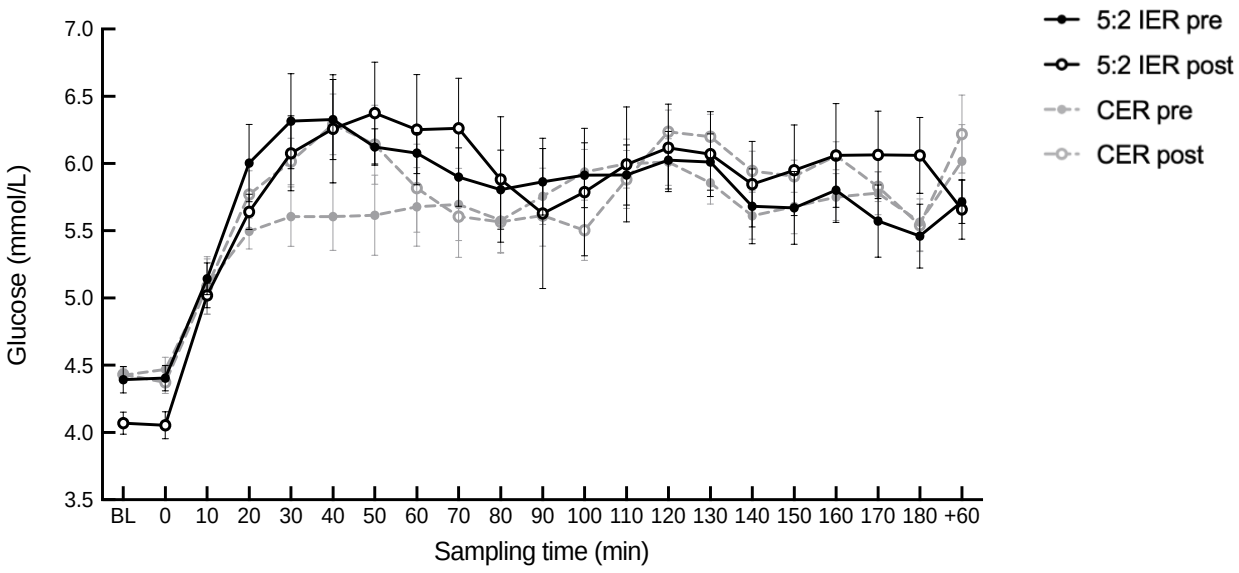


Figure 4.5 Whole blood glucose following the liquid breakfast and the *ad-libitum* meal.
Pre, pre-intervention measurement; Post, post-intervention measurement; 5:2 IER, 5:2 intermittent energy restriction; CER, continuous energy restriction. Values are means with their standard errors. *n*=8 per group.

4.3.3.3 Postprandial FFA and TAG Responses

Plasma FFA tended to follow an opposite response pattern to insulin. There was steady reduction in plasma FFA concentrations following the MTT (**Figure 4.6**). In contrast, TAG concentrations steadily increased after the MTT (**Figure 4.7**). There were no significant treatment-by-time interactions or main effects of treatment or time in AUCs for plasma FFA and serum TAG (**Table 4.5**).

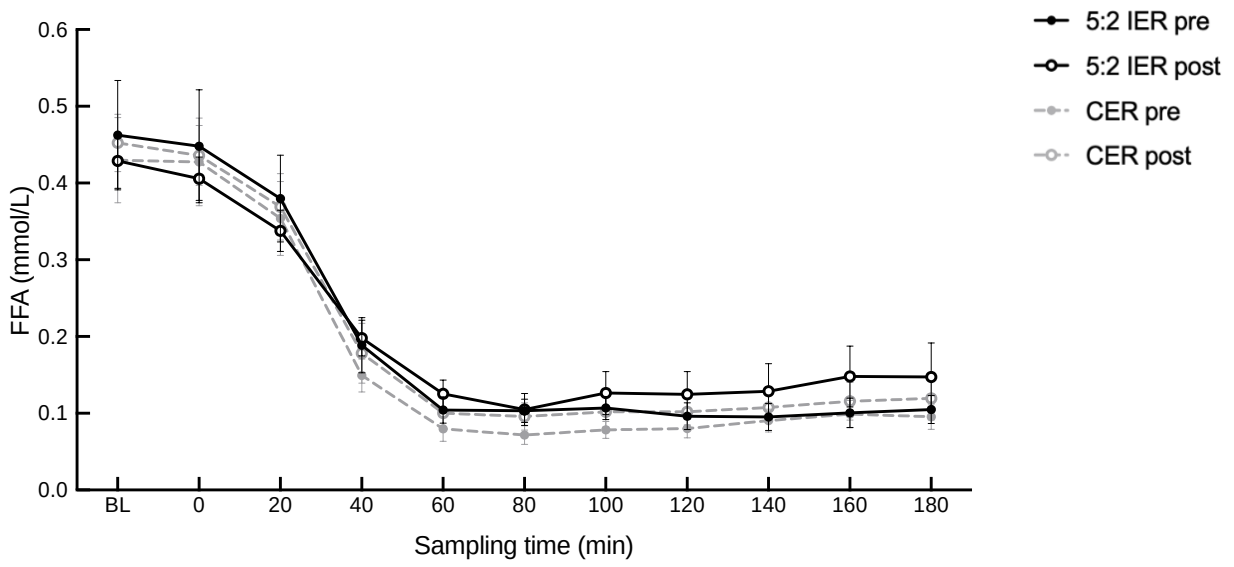


Figure 4.6 Plasma FFA following the liquid breakfast.

Pre, pre-intervention measurement; Post, post-intervention measurement; 5:2 IER, 5:2 intermittent energy restriction; CER, continuous energy restriction; FFA, free fatty acid. Values are means with their standard errors. *n*=8 per group.

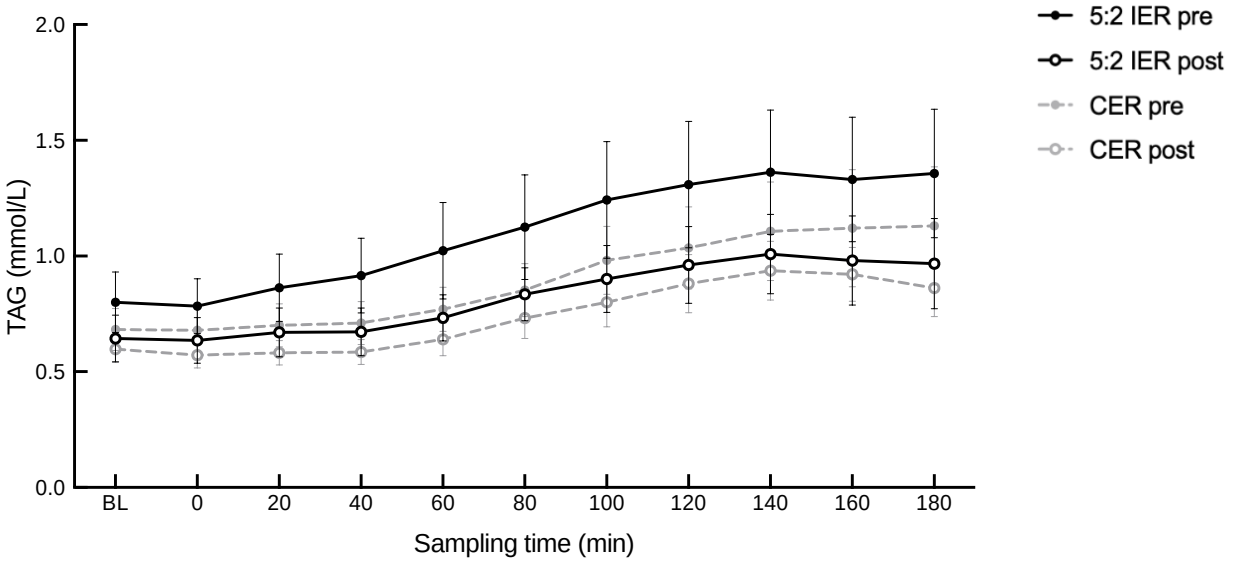


Figure 4.7 Serum TAG following the liquid breakfast.

Pre, pre-intervention measurement; Post, post-intervention measurement; 5:2 IER, 5:2 intermittent energy restriction; CER, continuous energy restriction; TAG, triglycerides. Values are means with their standard errors. *n*=8 per group.

4.3.4 Whole-Body Insulin Sensitivity

Whole-body insulin sensitivity evaluated using the HOMA-IR and Matsuda index are presented in **Figure 4.8** and **Figure 4.9**, respectively. No significant treatment-by-time interaction was detected in HOMA-IR. However, there was a tendency to a decrease in both groups over the two-week intervention period (P = 0.052, main effect of time, 2-way ANOVA, η^2_p = 0.15). Similarly, no significant treatment-by-time interaction between groups was detected in the Matsuda index, calculated over 180 min. However, the Matsuda index increased in both groups over the two-week interventions (P < 0.05, main effect of time, 2-way ANOVA, η^2_p = 0.08), indicating improvement in whole-body insulin sensitivity.

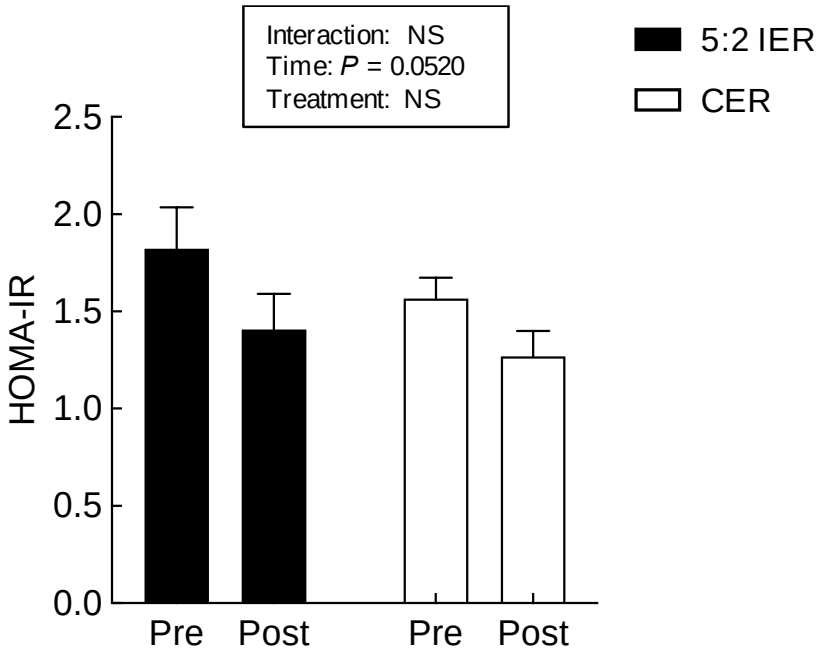


Figure 4.8 Insulin sensitivity assessed using the HOMA-IR.
Pre, pre-intervention measurement; Post, post-intervention measurement; 5:2 IER, 5:2 intermittent energy restriction; CER, continuous energy restriction; HOMA-IR, homeostasis model assessment of insulin resistance. Values are means with their standard errors. *n*=8 per group.

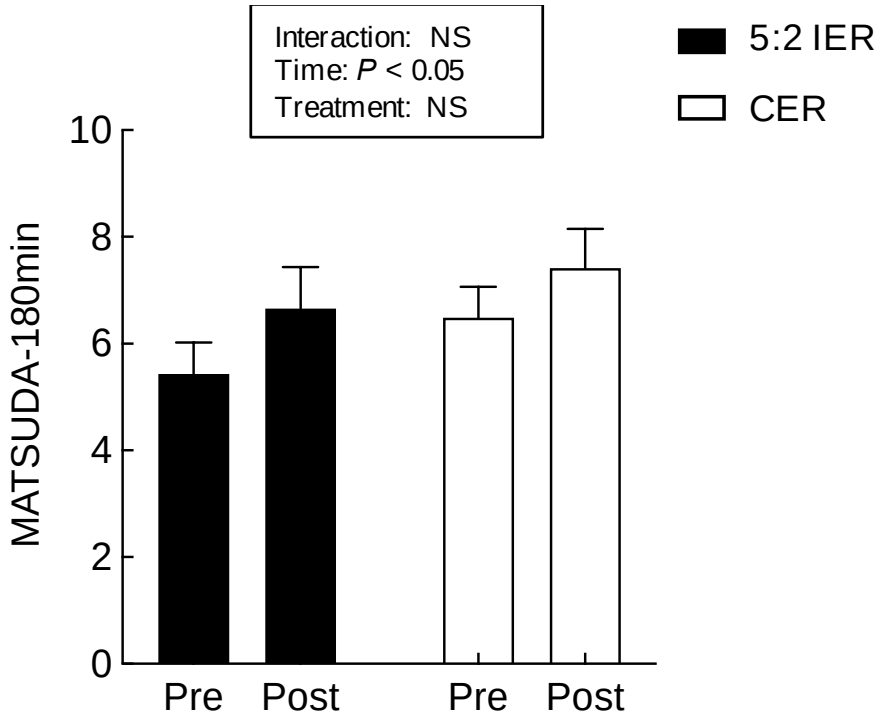


Figure 4.9 Insulin sensitivity assessed using Matsuda index.
Pre, pre-intervention measurement; Post, post-intervention measurement; 5:2 IER, 5:2 intermittent energy restriction; CER, continuous energy restriction. Values are means with their standard errors. *n*=8 per group.

4.3.5 Free-living Continuous Glucose Monitoring

Whole-day glycaemic profiles, with each time point representing the mean of six consecutive days (Days 8-13) per participant, averaged over the participants, by treatment, are presented in **Figure 4.10** (restricted intake on Days 8 and 11 for the 5:2 IER group). Shaded error bands (SEM) indicate the inter-individual variation for the mean of six days. Both groups exhibit sharp elevations in interstitial glucose concentrations following consumption of the three main meals, peaking after approximately one hour and returning to within the fasting range before the next meal. Results were similar between the two groups, except for glucose iAUC for five hours after lunch, with the glucose value being lower during 5:2 IER than CER ($P < 0.05$, Unpaired student's t-test) (**Table 4.6**).

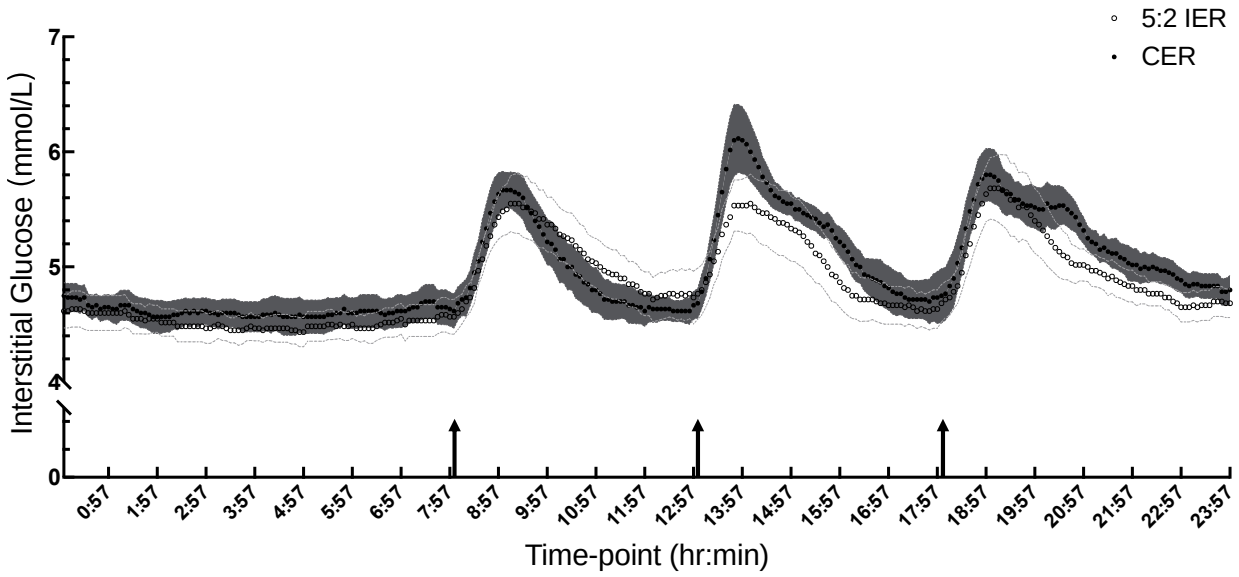


Figure 4.10. Whole-day glycaemic profiles calculated as mean interstitial glucose concentrations of 6 consecutive days (Days 8-13) for 6 participants of the 5:2 IER (○) and CER (●).

SEM for the 5:2 IER is the light shading and for the CER is the dark shading. 5:2 IER, 5:2 intermittent energy restriction; CER, continuous energy restriction. Values are means with their standard errors. *n*=6 per group. Black arrows at 08:00, 13:00 and 18:00 indicate when the breakfast, lunch and dinner started, respectively.

Table 4.6 Indices derived from 24 hours continuous glucose monitoring during Day 8 to Day 13 of both interventions.

	Full 24h			Day hours (0700 to 2359)			Night hour (2400 to 0659)		
	5:2 IER (*n* 6)	CER (*n* 6)		5:2 IER (*n* 6)	CER (*n* 6)		5:2 IER (*n* 6)	CER (*n* 6)	
Valid days	Day 8 - 13	Day 8 - 13		Day 8 - 13	Day 8 - 13		Day 8 - 13	Day 8 - 13	
	Mean ± SD	Mean ± SD	*P* [a]	Mean ± SD	Mean ± SD	*P* [a]	Mean ± SD	Mean ± SD	*P* [a]
Mean glucose, mmol/L	4.8 ± 0.3	5.0 ± 0.3	0.55	5.0 ± 0.4	5.1 ± 0.3	0.58	4.5 ± 0.3	4.6 ± 0.3	0.53
Maximum, mmol/L	6.0 ± 0.6	6.3 ± 0.6	0.52	6.0 ± 0.6	6.3 ± 0.6	0.52	4.7 ± 0.4	4.8 ± 0.3	0.55
Minimum, mmol/L	4.3 ± 0.4	4.4 ± 0.4	0.76	4.4 ± 0.4	4.4 ± 0.4	0.82	4.4 ± 0.3	4.5 ± 0.3	0.60
SD	0.4 ± 0.2	0.5 ± 0.1	0.57	0.4 ± 0.2	0.5 ± 0.2	0.57	0.1 ± 0.0	0.1 ± 0.0	0.85
% CV	8.4 ± 3.2	9.4 ± 3.1	0.61	8.0 ± 3.0	9.1 ± 3.8	0.60	1.6 ± 0.6	1.6 ± 0.6	0.85
	Breakfast (0800)			Lunch (1300)			Dinner (1800)		
	5:2 IER (*n* 6)	CER (*n* 6)	*P* [a]	5:2 IER (*n* 6)	CER (*n* 6)	*P* [a]	5:2 IER (*n* 6)	CER (*n* 6)	*P* [a]
iAUC, mmol/Lx300 min	146.4 ± 70.1	107.0 ± 45.3	0.28	76.1 ± 98.6	174.6 ± 42.7	0.048	122.6 ± 83.7	163.0 ± 114.0	0.50

5:2 IER 5:2 intermittent energy restriction, *CER* continuous energy restriction, *% CV* percentage coefficient of variation,

iAUC incremental area under the curve

[a] Unpaired student's t-test

To further explore the changes in whole-day glycaemic profiles during 5:2 IER, comparisons between Day 8 (with 70% energy restriction) vs. Day 11 (with 70% energy restriction) (**Figure 4.11 a**) and Day 9 (with 0% energy restriction) vs. Day 13 (with 0% energy restriction) (**Figure 4.11 b**) during 5:2 IER were conducted. There were no differences in glucose iAUC for 90 min after three main meals or any reported indices of glucose variability, shown in **Table 4.7**. However, there was a significant difference in mean 24-hour glucose concentrations between Day 8 vs. Day 11 ($P < 0.05$, Paired student's t-test), with higher glucose concentrations during Day 11 (the fourth energy restricted day) than during Day 8 (the third energy restricted day).

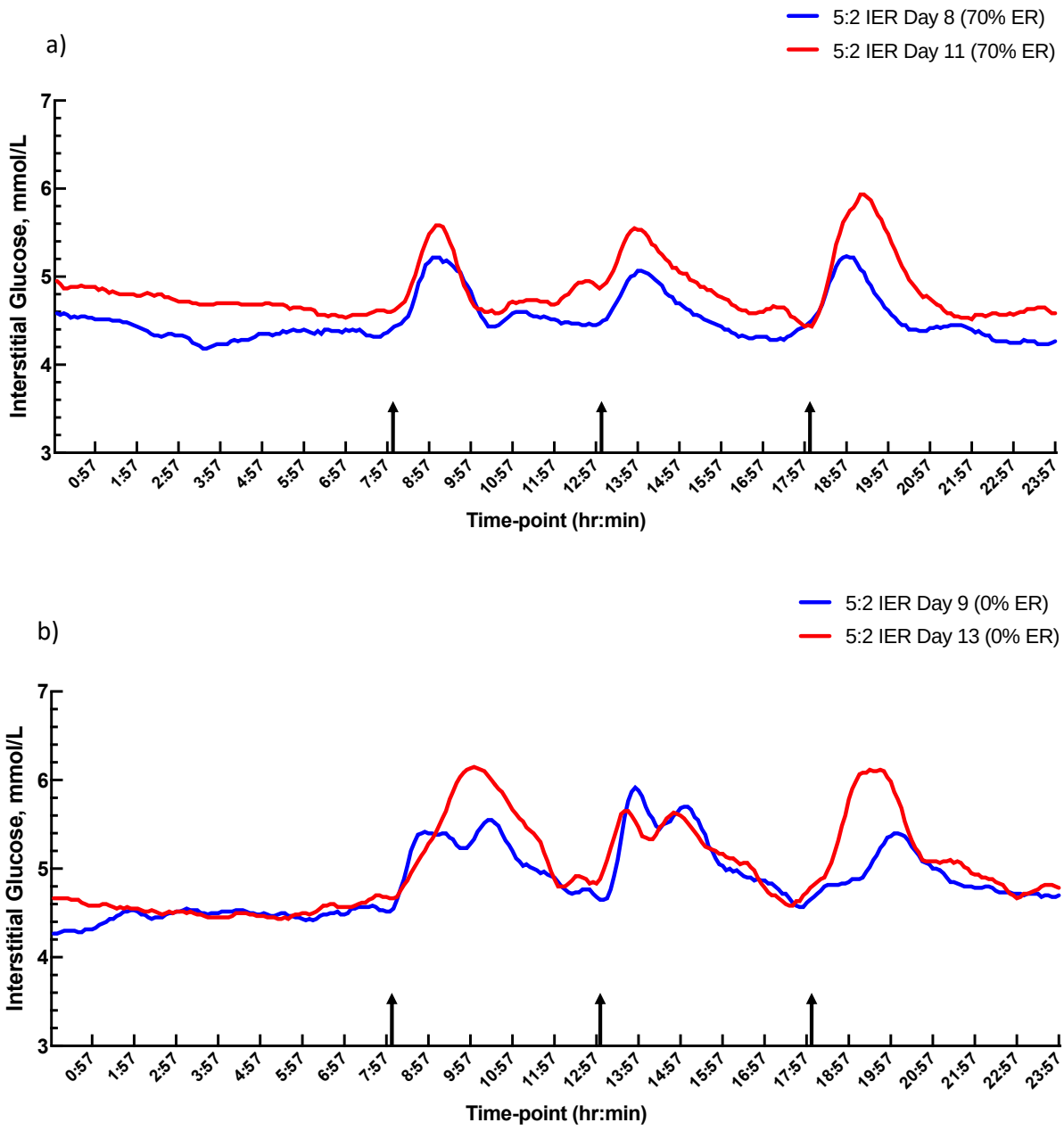


Figure 4.11 Whole-day glycaemic profiles calculated as mean interstitial glucose concentrations of intervention Day 8 vs. Day 11 (a) and Day 9 vs. Day 13 (b) of 5:2 IER for 6 participants.

5:2 IER, 5:2 intermittent energy restriction; ER, energy restriction. Values are means with their standard errors. *n*=6 per group. Black arrows at 08:00, 13:00 and 18:00 indicate when the breakfast, lunch and dinner started, respectively.

Table 4.7 Indices derived from 24 hours continuous glucose monitoring during Day 8 vs. Day 11 and Day 9 vs. Day 13 of 5:2 IER.

	5:2 IER, full 24h					
	5:2 IER (*n* 6) Day 8 (70% ER)	5:2 IER (*n* 6) Day 11 (70% ER)		5:2 IER (*n* 6) Day 9 (0% ER)	5:2 IER (*n* 6) Day 13 (0% ER)	
	Mean ± SD	Mean ± SD	*P* [a]	Mean ± SD	Mean ± SD	*P* [a]
Mean glucose, mmol/L	4.5 ± 0.3	4.8 ± 0.5	0.047	4.9 ± 0.4	5.0 ± 0.4	0.44
Maximum, mmol/L	5.9 ± 0.7	6.4 ± 1.2	0.07	6.4 ± 0.7	6.8 ± 0.9	0.29
Minimum, mmol/L	3.9 ± 0.4	4.2 ± 0.6	0.24	4.1 ± 0.4	4.0 ± 0.4	0.52
SD	0.4 ± 0.2	0.5 ± 0.2	0.37	0.5 ± 0.2	0.7 ± 0.2	0.11
% CV	8.7 ± 4.3	9.3 ± 3.1	0.65	11.2 ± 4.4	13.3 ± 3.0	0.10
iAUC (+90 min Breakfast), mmol/Lx90 min	44.0 ± 26.4	49.9 ± 27.2	0.57	59.6 ± 59.6	44.8 ± 33.3	0.67
iAUC (+90 min Lunch), mmol/Lx90 min	34.9 ± 9.9	38.7 ± 64.1	0.89	67.3 ± 67.6	46.0 ± 35.5	0.29
iAUC (+90 min Dinner), mmol/Lx90 min	43.8 ± 40.5	77.9 ± 32.0	0.11	17.2 ± 13.4	62.2 ± 57.2	0.17

5:2 IER 5:2 intermittent energy restriction, *ER* energy restriction, *% CV* percentage coefficient of variation, *iAUC* incremental area under the curve

[a] Paired student's t-test

4.4 Discussion

The present study demonstrates that under free-living conditions, two-weeks of 5:2 IER fails to improve postprandial insulin iAUC response (primary outcome) compared with CER in healthy young men and women. Nor were postprandial glucose, FFA, or TAG (secondary outcomes) different. 5:2 IER resulted in a decrease in fasting glucose compared with CER (exploratory outcome). Participants in both groups lost 3-4% of original body weight, with men losing 4.3% and women losing 3.0% on average.

The absence of differences in postprandial insulinaemic response (with small effect size) and postprandial glycaemic, FFA, and TAG responses to MTT suggest that metabolic differences (e.g. fasting insulin and insulin resistance) observed between these diets in previous, longer trials (Harvie *et al.*, 2011; Harvie *et al.*, 2013) cannot be explained by differences in postprandial responses. However, lower fasting glucose following 5:2 IER in the present study (with large effect size) has been observed previously (Cho *et al.*, 2019).

Whole-body postprandial insulin sensitivity (Matsuda index) improved equally (with intermediate effect size), along with a strong tendency for increased fasting insulin sensitivity (HOMA-IR) (with large effect size) after two-week interventions in both groups, which may primarily be attributed to the weight loss incurred. Greater reductions in fasting insulin concentration and insulin resistance (HOMA-IR) were reported following 5:2 IER compared with CER in two similar studies (Harvie *et al.*, 2011; Harvie *et al.*, 2013), however two-day energy restriction was on consecutive days and participants had overweight or obesity thus possibly insulin-resistance, increasing the potential to detect significant differences between treatments. Moreover, as noted by Borai *et al.* (2011), there is a potential that someone is insulin resistant but without showing fasting hyperinsulinaemia. Therefore, the limitation of using HOMA-IR only could not be overlooked. Importantly, as was the case in the present study, the 5:2 IER groups in those previous studies were assessed at least three days after the last energy-restricted day, yet exhibited the greatest changes in insulin measures, suggesting the favourable metabolic effects of 5:2 IER are not the result of an acute negative energy balance.

There were no significant differences in postprandial TAG responses reported in the present study. However, Antoni *et al.* (2018) demonstrated that postprandial TAG decreased with 5:2IER but increased with CER, which is consistent with the findings of a previous study, which reported enhanced suppression of adipose tissue lipolysis following an ADF regimen (Halberg *et al.*, 2005).

Three major excursions in daily glucose profiles, were seen in the CGM, coinciding with prescribed meal times, reflecting good dietary adherence, facilitated by food provision. Interestingly, all indices of CGM were similar between the two treatment groups with the exception of the five-hour postprandial iAUC for glucose after lunch that was lower following 5:2 IER than CER. The reduction in fasting blood glucose following 5:2 IER may in part be mediated by the attenuated postprandial glycaemic responses on the two days/week that 70% energy restriction was applied and the reduced postprandial glycaemia after lunch over the entire treatment period (mean of restricted and non-restricted days), collectively resulting in the estimated improved hepatic insulin sensitivity. The promising results of 5:2 IER on glucose and insulin sensitivity markers in the present study warrants further investigation to confirm insulin sensitisation using the hyperinsulinaemic-euglycaemic clamp and the determination of body composition changes to further elucidate the mechanisms underlying these effects.

To the best of my knowledge, this is the first study to examine the effects of 5:2 IER in normal-weight, healthy participants. The present study extends the investigations of intermittent fasting (Halberg *et al.*, 2005; Jones *et al.*, 2020b) and other metabolic challenges (Krug *et al.*, 2012; Alhussain, Macdonald and Taylor, 2016) by considering both fasting and postprandial state in young, normal-weight participants in whom effects on metabolism and appetite are not confounded by the metabolic abnormalities associated with excess body weight.

Well-controlled energy intake is pivotal for clinical trials investigating the effects of different dietary regimens on weight loss and metabolic benefits. Considering the magnitude of underreporting, self-reported food diaries are not recommended as a measure of energy intake (Subar *et al.*, 2015), however, a robust standard for evaluating free-living energy intake is yet to be established (Rynders *et al.*, 2019). Among the existing RCTs comparing the 5:2 IER and CER regimens, none provided food over the entire intervention period, however one study did provide food for only the energy-restricted days (Antoni *et al.*, 2018). Instead, previous studies have

prescribed dietary instructions to participants, who were then asked to source the food themselves, which may result in excessive energy intake and differences in macronutrient composition. It could be speculated that human intervention studies in which all food is provided may facilitate compliance with the prescribed diet and improve the validity and reliability of results. To that effect, consistent eating windows, eating frequencies, and identical foods were prescribed to each participant in the present study, and CGM was employed to monitor compliance. This counters the potential limitation of the participant being free-living (Subar *et al.*, 2015; Rynders *et al.*, 2019), as did monitoring activity by using step counts, promoting confidence in the comparisons between the two regimens. Furthermore, since humans spend considerable amounts of time in the postprandial state, both fasting and postprandial measurements were performed to best capture the metabolic effects of the different regimens. The balanced sex ratio of participants may help to offset any sex bias, hence making this study more representative of a more general population.

Notably, in the present study, pre-intervention metabolic measurements may be confounded by the dinner consumed the evening before. A prescribed standardised dinner, prior to each measurement, may help to minimise the effects of diverse dietary characteristics of the dinner on fasting and postprandial substrates metabolism the day after (Robertson *et al.*, 2002). Furthermore, this study is limited by the short-term nature of the intervention and cannot be extrapolated with respect to the long-term feasibility of the 5:2 IER regimen compared with that of CER.

In conclusion, this study shows that two weeks of 5:2 IER is not superior in improving postprandial insulin and glucose responses compared with CER, suggesting that changes in postprandial responses are not key to metabolic differences in longer-term intervention studies. Interestingly, the 5:2 IER regimen led to potentially beneficial changes in fasting blood glucose, which was independent of weight loss, over a two-week period. This was consistent with similar studies conducted in participants with overweight or obesity. However, it is still difficult to reach a consensus on the effects of the 5:2 IER regimen throughout the literature. The inconsistency among previous studies may be primarily attributable to dietary patterns (e.g. energy restriction on two consecutive days or two non-consecutive days), participant characteristics (e.g. normal weight or obese; with T2DM or not), energy intake (e.g. *ad libitum* or matching EER on the other five days), meal frequency and intervention duration. It would be intriguing to compare 5:2 IER with two consecutive energy-restricted days to that with two non-consecutive days. Furthermore, the

combined effects of 5:2 IER and exercise remain to be investigated. Sundfør *et al.* (2018) proposed that a sustainable diet practice should refrain from macronutrient deficiencies and dehydration, should be satiating and satisfactory so as to prevent overconsumption on normal eating days, as well as facilitate FFM preservation and FM loss. To this effect, the 5:2 IER regimen appears to fulfil these criteria, however larger-scale, longer-term randomised controlled human trials are required to establish mechanisms of action and to examine the long-term adherence, safety, and efficacy for improving health outcomes.

CHAPTER 5. EFFECTS OF TWO WEEKS OF 5:2 INTERMITTENT ENERGY RESTRICTION ON ENERGY BALANCE AND APPETITE REGULATION IN HEALTHY-WEIGHT YOUNG PARTICIPANTS

5.1 Introduction

To remedy the metabolic dysfunction as a consequence of long-term positive energy balance, dietary pattern manipulations attempt to restore the underlying energy imbalance by decreasing energy intake and/or increasing energy expenditure, which can contribute to weight loss and improved health outcomes (Most *et al.*, 2017). However, these improvements are hindered by a compensatory decrease in resting metabolic rate (RMR) and an increase in hunger. Diet-induced weight loss is associated with compensatory decreases in RMR and non-resting energy expenditure (mainly in the form of physical activity) in individuals with obesity and normal weight (Leibel, Rosenbaum and Hirsch, 1995). Diet-induced weight loss is also associated with a decrease in leptin (fullness hormone) in participants with and without obesity (Weigle *et al.*, 1997). Participants with obesity following a six-month diet-induced weight loss diet experienced a 24% elevation in plasma ghrelin (hunger hormone) levels (Cummings *et al.*, 2002). A study in women with normal weight found almost doubled ghrelin levels from 770 to 1322 ρmol/L after a three-month weight-loss diet (Leidy *et al.*, 2004).

Intermittent fasting (IF) is periods of voluntary abstinence from energy intake, and has become a popular strategy for achieving weight loss and metabolic benefits. It is of great importance to know whether compensatory adjustments mentioned above would be invoked following IF.

Findings on the effects of IF or intermittent energy restriction (IER) on appetite have been scarce and inconsistent. Unaltered (Varady *et al.*, 2013; Hoddy *et al.*, 2016; Coutinho *et al.*, 2018), decreased (Klempel *et al.*, 2013; Kroeger *et al.*, 2018), and increased (Sundfør, Svendsen and

Tonstad, 2018a) subjective hunger levels have been reported. Decreased leptin level has been reported in participants with or without obesity following 12-weeks of alternate day fasting (ADF) compared to daily *ad libitum* energy intake. Participants achieving the highest weight loss following a 12-month ADF diet demonstrated decreased hunger and increased fullness (Kroeger *et al.*, 2018). However, in a 6-month 5:2 IER study comparing 20% of EER for two non-consecutive days and five-day *ad libitum* energy intake based on participants' habitual eating against CER in participants with obesity, higher subjective hunger levels were reported compared with those following a CER diet (Sundfør, Svendsen and Tonstad, 2018a).

Findings on the effects of ADF on RMR have been inconsistent. Both Heilbronn *et al.* (2005) and Catenacci *et al.* (2016) reported no significant changes in RMR following complete ADF; whilst a study employing 20 hours fasting every other day demonstrated a decrease in RMR of 247 kJ/day (Soeters *et al.*, 2009). Ramadan fasting is a form of time-restricted feeding (TRF) performed as a religious practice. It leads to approximately 12 hours of abstention from food and drink (from dawn to sunset). No significant changes in RMR or total energy expenditure were reported with Ramadan fasting (Lessan and Ali, 2019). In the 5:2 IER study described above, both groups experienced comparable reductions in RMR from baseline to three months (Sundfør, Svendsen and Tonstad, 2018a). However, as highlighted by Dhurandhar *et al.* (2015), measurements of all aspects of the energy balance equation in a well-controlled and reliable manner are warranted, to achieve an accurate determination of energy balance. IF or IER may blunt the compensatory responses to a negative energy balance, however, insufficient information about individual factors of energy expenditure (i.e. RMR, DIT, and physical activity expenditure) impedes reliable conclusions being drawn.

This study thus aims to compare both the fasting and postprandial resting energy expenditure rate (REE) and appetitive effects of two weeks of 5:2 IER (with non-consecutive energy restricted days) and CER. Participants were healthy, young, and of normal-weight to minimise the potential impact on treatment differences, of metabolic defects associated with overweight and obesity. All food and beverages were provided to improve compliance.

5.2 Methods

5.2.1 Participants

The study presented in this chapter was conducted on the same participants reported in Chapter 4 at the same time.

Sixteen healthy men and women (20–35 years, BMI 20-25 kg/m^2, waist circumference < 94 cm for men and < 80 cm for women) attended five visits (1x10 min, 1x30 min, 1x1 hour, and 2x6 hours) from November 2018 to May 2019. Inclusion and exclusion criteria described in section 4.2.1 were applied here. The study was approved by the University of Nottingham, Faculty of Medicine and Health Sciences Research Ethics Committee (Reference number 121-1809), conformed to the World Medical Association Declaration of Helsinki, and was registered at ClinicalTrials.gov PRS: NCT04138160. A schematic diagram of the experimental protocol is presented in **Figure 4.1**.

5.2.2 Screening

The screening protocol described in Section 2.2 and Section 4.2.2 were applied here.

5.2.3 Experimental Design

The experimental design of the study detailed in Section 4.2.3 was applied here.

5.2.4 Laboratory-Visit Protocol Pre- and Post-Intervention

Figure 4.2 shows a schematic diagram of the sequence of measurements conducted during each trial.

5.2.4.1 Test-Drink Consumption

Postprandial appetite and RMR were assessed for three hours, following a standardised test drink (MTT, as described in Section 4.2.6.3).

5.2.4.2 Resting Energy Expenditure Measurement

REE was estimated using data obtained from a Quark CPET open-circuit metabolic cart (Cosmed, Quark CPFT, Rome, Italy), which operated with the canopy dilution technique. Flowmeter calibration and air calibration were carried out at the start of each laboratory visit. Gas calibration was performed before each test using the gas calibration cylinder with known gas concentration. The canopy hood was fitted over the participant's head following 30-min resting in a semi-supine position. The hood was ventilated with room air by a vacuum pump, the flow rate was monitored and regulated by the operator carefully to ensure the fraction of CO_2 in the expired air remained fixed between 0.5% to 0.6%. Baseline REE (i.e. RMR or basal metabolic rate) was measured in a fasted state for 20 min. Readings were reported every 10 s and the first and last 2.5 min of data acquisition were excluded. The data points in the mid 15 min were averaged to calculate the REE. The abbreviated Weir equation (WEIR, 1949) was used for REE: REE (kJ/min) = [3.941 x (V_{O2}) + 1.1106 x (V_{CO2})] x 4.184, with V_{O2} and V_{CO2} in litres per minute (L/min), with the assumption that the contribution of protein oxidation to metabolism in non-pathological conditions is constant and any increases in protein oxidation in response to the MTT are relatively small and consistent between trials. Postprandial REE was then measured for 20 min in every hour for three hours after the MTT, enabling the calculation of DIT (expressed as iAUC over the entire three-hours postprandial period) in response to the MTT.

5.2.4.3 *Ad libitum* Test Meal

Three hours after consumption of the MTT, a test meal of fixed macronutrient composition, designed to provide a consistent macronutrient composition between each spoonful, was served as a standardised measure of *ad libitum* food intake, which provided 620 kJ/100g with 49.8%, 15.4% and 34.8% energy provided by CHO, protein and fat, respectively (described in Section 2.6.3). The *ad libitum* test meal was served after being thoroughly mixed and energy intake was computed from the weight of food consumed. Participants were served portions of ~430 g in a small bowl at a temperature ≥ 82°C, and instructed to consume as much as they wished until they felt 'comfortably full'. The bowl of pasta was repeatedly topped up, when about two-thirds had been consumed, in an attempt to ensure participants were not cued to stop eating by having emptied their bowl. Participants were separated from other people in the laboratory when consuming the

pasta meal, in the presence of one experimenter and no discussions associated with the meal were allowed. Energy intake was computed from the weight difference of the pasta meal before and after consumption. Duration and speed of consumption were also recorded.

5.2.4.4 Subjective Appetite Ratings

Subjective appetite ratings were obtained using VAS (see Section 2.6.4). VASs were completed just before, immediately after and then every 20 min after the MTT for three hours. Further VAS was completed before, and every 20 min for an hour after consuming the *ad libitum* test meal in order to assess subjective appetite. Values for 'hunger', 'desire to eat', 'prospective food consumption', 'fullness' and 'satisfaction were amalgamated using the following equation to calculate a Composite Appetite Score (CAS) = [hunger + desire to eat + prospective food consumption + (100−fullness) + (100−satisfaction)]/5 (Stubbs *et al.*, 2000). A higher CAS value is indicative of greater motivation to eat or less feeling of satiety.

5.2.5 Statistical Analyses

Prism 8 software (GraphPad Software Inc., CA, USA) was used for data entry and analyses with statistical significance accepted as $P < 0.05$ for all statistical tests. Statistical analyses detailed in Section 4.2.7 was applied here.

Data reported in this chapter are secondary outcomes (i.e. changes in postprandial CAS and energy intake of the *ad libitum* test meal). The power calculation was based on the primary outcome (changes in postprandial insulin), which was explained in Section 4.2.7. Exploratory outcomes reported in this chapter included fasting and postprandial ratings of subjective appetite, fasting REE, DIT and fasting and postprandial RER responses.

5.3 Results

5.3.1 Participant Characteristics

Eight participants in each group completed the study (CONSORT diagram in **Figure 4.3**). Due to technical difficulties, indirect calorimetry data was not obtained from one participant in the 5:2 IER group.

Participant characteristics at baseline (**Table 4.3)** and changes in participant fasting anthropometric and physiological characteristics over the course of each intervention (**Table 4.4**) are described in Section 4.2.1.

5.3.2 Resting Energy Expenditure (Indirect Calorimetry Data)

Fasting REE is presented in **Figure 5.1**. A significant main effect of time was observed for fasting REE ($P < 0.05$, main effect of time, 2-way ANOVA), which was reduced by approximately 0.26 kJ/min in 5:2 IER and by 0.08 kJ/min in CER, however there was no significant difference between groups. Following the MTT, for all visits (pre- and post- for each intervention), REE increased above the fasting values. REE at baseline, one hour, two hours and three hours showed no visit-by-time interaction, or main effect of visit, but did show a main effect of time ($P < 0.001$, 2-way ANOVA). However, when DIT was calculated as the iAUC for the entire postprandial REE response, there was no significant interaction or treatment or time effect between diet groups (2-way ANOVA) (**Figure 5.2**). Hourly values for RER following the MTT is presented in **Figure 5.3**. Following the MTT, for all visits, RER increased above the fasting values. RER at baseline, one hour, two hours and three hours showed no visit-by-time interaction, or main effect of visit, but did show a main effect of time ($P < 0.001$, 2-way ANOVA). Neither fasting nor postprandial RER responses differed between groups.

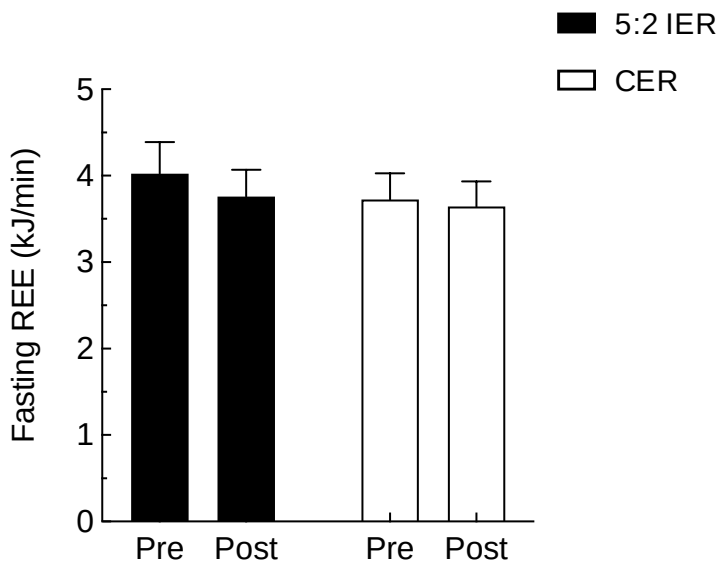


Figure 5.1 Fasting resting energy expenditure (REE).

Pre, pre-intervention measurement; Post, post-intervention measurement; 5:2 IER, 5:2 intermittent energy restriction; CER, continuous energy restriction. Values are means with their standard errors. *n*=7 for 5:2 IER, *n*=8 for CER.

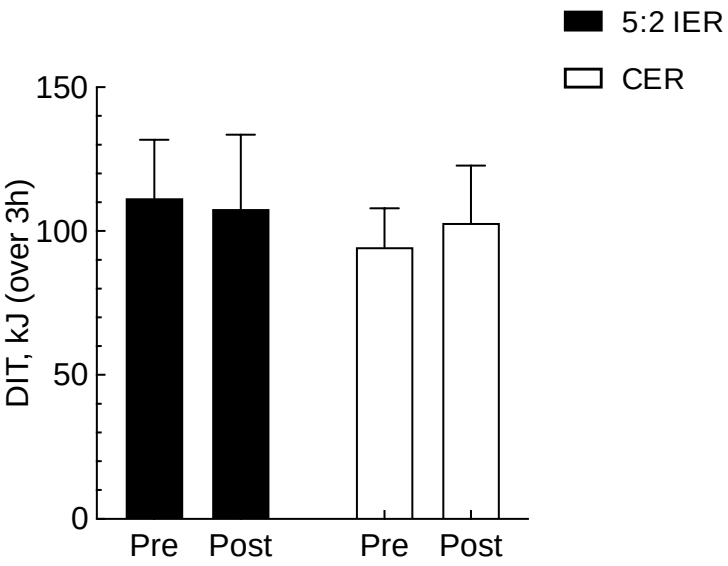


Figure 5.2 Diet induced thermogenesis (DIT).

DIT was calculated by the incremental area under the curve for the resting energy expenditure above the fasting value, using trapezoidal method. Pre, pre-intervention measurement; Post, post-intervention measurement; 5:2 IER, 5:2 intermittent energy restriction; CER, continuous energy restriction. Values are means with their standard errors. *n*=7 for 5:2 IER, *n*=8 for CER.

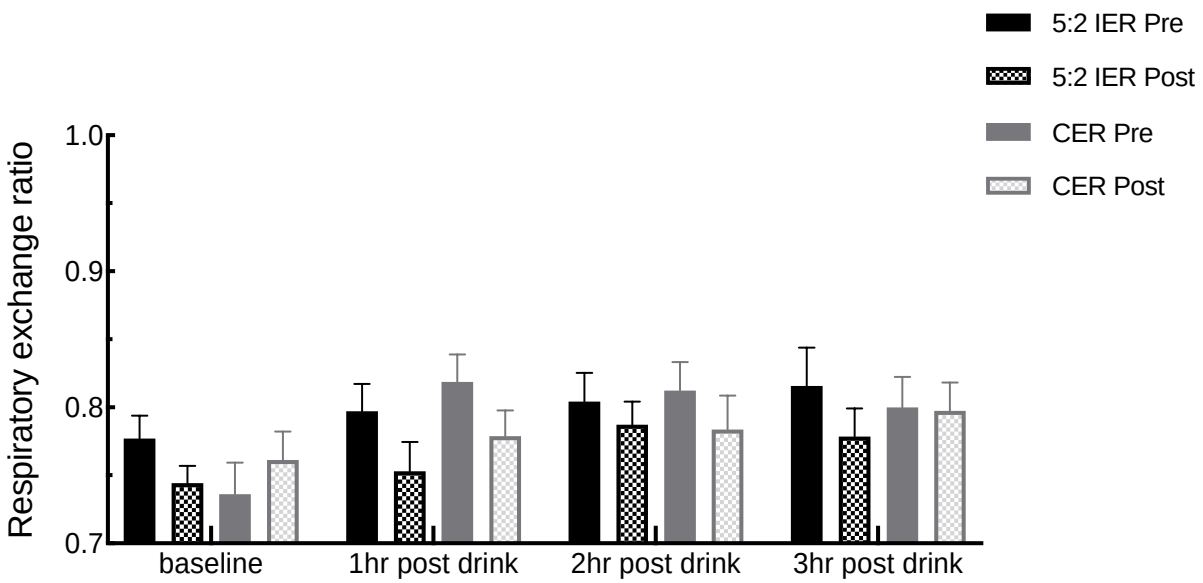


Figure 5.3 Respiratory exchange ratio (RER).
Pre, pre-intervention measurement; Post, post-intervention measurement; 5:2 IER, 5:2 intermittent energy restriction; CER, continuous energy restriction. Values are means with their standard errors. *n*=7 for 5:2 IER, *n*=8 for CER.

5.3.3 Subjective Appetite Ratings

Fasting subjective appetite ratings over the study are reported in **Table 5.1**. Postprandial iAUCs (and dAUCs when indicated) for subjective appetite ratings following both the MTT and the *ad-libitum* meal are presented in **Table 5.2**. There were significant treatment-by-time interactions between diet patterns for fasting subjective ratings of hunger, satiety, fullness, desire to eat and prospective food consumption ($P < 0.001$, $P < 0.01$, $P < 0.01$, $P < 0.05$, and $P < 0.001$, respectively). After 5:2 IER, fasting ratings of subjective hunger ($P < 0.001$), prospective food consumption ($P < 0.001$) and desire to eat ($P > 0.05$) decreased, whilst subjective satiety and fullness increased ($P < 0.01$). The opposite was seen with CER (treatment-by-time interaction, $P < 0.05$). There were no significant differences for iAUCs or dAUCs for individual subjective appetite ratings during the postprandial period after the MTT. Responses of subjective hunger and satiety ratings during the one-hour postprandial period following the *ad libitum* test meal showed significant treatment-by-time interactions between groups ($P < 0.05$, 2-way ANOVA). Higher postprandial responses

for hunger and lower responses for satiety were observed in the 5:2 IER group compared with the CER group for one hour after the *ad libitum* test meal.

Table 5.1 Fasting subjective appetite ratings over the study.

	5:2 IER (*n* 8)		CER (*n* 8)		
	Pre	Post	Pre	Post	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	5:2 IER v. CER [a]
Hunger (mm)	83 ± 11	57 ± 16	65 ± 15	81 ± 17	0.0001
Satiety (mm)	17 ± 7	34 ± 16	27 ± 20	14 ± 14	0.0018
Fullness (mm)	11 ± 7	30 ± 14	16 ± 10	11 ± 13	0.0018
Desire to eat (mm)	76 ± 7	63 ± 23	72 ± 12	83 ± 18	0.0313
Prospective food consumption (mm)	82 ± 10	61 ± 10	69 ± 12	78 ± 11	0.0002
Fasting CAS (mm)	83 ± 4	63 ± 14	73 ± 11	83 ± 12	0.0003

5:2 IER 5:2 intermittent energy restriction, *CER* continuous energy restriction, *CAS* composite appetite score

[a] 2-way ANOVA, treatment-by-time interaction

Table 5.2 Incremental area under the curve (iAUCs for Satiety and Fullness) or decremental area under the curve (dAUCs for Hunger, Desire to eat, Prospective food consumption and CAS) for subjective appetite ratings following the liquid breakfast and the *ad-libitum* meal over the study.

		5:2 IER (*n* 8)				CER (*n* 8)				
		Pre		Post		Pre		Post		
		Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD		5:2 IER v. CER [a]
Hunger	Test drink (dAUC, mm over 3h)	-5910	1384	-5763	1707	-5269	2307	-6243	2169	0.16
	Ad-libitum meal (dAUC, mm over 1h)	-4849	878	-3545	1132	-4547	1095	-4618	1339	0.04
Satiety	Test drink (iAUC, mm over 3h)	5664	3141	6293	2434	5317	2868	6078	3039	0.71
	Ad-libitum meal (iAUC, mm over 1h)	4983	1455	3804	1483	4582	1156	4811	1142	0.04
Fullness	Test drink (iAUC, mm over 3h)	6418	3312	7002	1908	6769	2510	7112	3182	0.95
	Ad-libitum meal (iAUC, mm over 1h)	5006	1348	4219	1585	4726	1009	4946	1061	0.15
Desire to eat	Test drink (dAUC, mm over 3h)	-5032	3635	-5966	3548	-5316	2574	-6172	2824	0.99
	Ad-libitum meal (dAUC, mm over 1h)	-4934	1262	-3778	1898	-4375	1267	-4393	1649	0.16
Prospective food consumption	Test drink (dAUC, mm over 3h)	-5152	2812	-4650	2282	-4441	2064	-5068	1823	0.13
	Ad-libitum meal (dAUC, mm over 1h)	-4948	1569	-3904	1902	-4131	1247	-4133	1454	0.16
CAS	Test drink (dAUC, mm over 3h)	-5570	2549	-5853	1593	-5355	2094	-6112	2241	0.66
	Ad-libitum meal (dAUC, mm over 1h)	-4944	1248	-3850	1546	-4472	1079	-4580	1274	0.08

5:2 IER 5:2 intermittent energy restriction, *CER* continuous energy restriction, *CAS* composite appetite score

[a] 2-way ANOVA, treatment-by-time interaction.

Mean CAS is displayed in **Figure 5.4**. Fasting CAS scores showed significant treatment-by-time interaction between the 5:2 IER and CER groups ($P < 0.001$). Fasting CAS was significantly lower after 5:2 IER than before ($P < 0.001$) but was significantly higher after CER than before ($P < 0.05$). Following the MTT, CAS declined before steadily returning to near fasting levels within three hours before consumption of the *ad libitum* test meal in all conditions. There were no significant treatment-by-time interactions in CAS dAUCs over three hours after the MTT or one hour after the *ad libitum* test meal between the two groups.

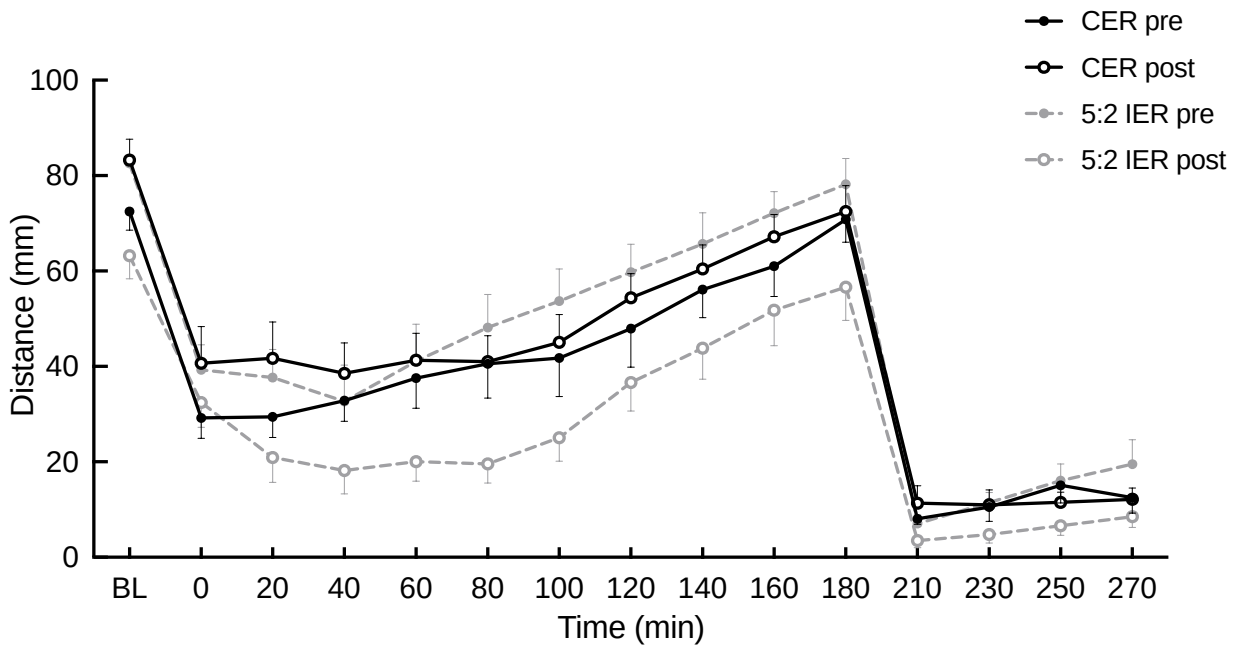


Figure 5.4 Mean composite appetite score (CAS).
5:2 IER pre/post, 5:2 intermittent energy restriction pre-intervention/post-intervention measurement; CER pre/post, continuous energy restriction pre-intervention/post-intervention measurement. Values are means with their standard errors. *n*=8 per group.

5.3.4 Energy Intake of the *Ad libitum* Test Meal

There was no treatment-by-time interaction or a main effect of treatment or time for energy intake of the *ad libitum* test meal: 2253 (SEM 338), 1798 (SEM 249), 1898 (SEM 163), and 1883 (SEM 303) kJ in the pre- and post- 5:2 IER and the pre- and post- CER visits, respectively. When compared the change in CAS from just before to immediately after consuming the *ad libitum* test

meal, there was a significant treatment-by-time interaction between the 5:2 IER and CER regimens ($P < 0.05$, 2-way ANOVA). The difference fell significantly after 5:2 IER ($P < 0.01$), with no changes after CER. These were consistent with the numerical changes in energy intake of the *ad libitum* test meal. No interaction or main effects of eating duration were identified: 14.0 (SEM 1.7), 11.3 (SEM 1.2), 12.4 (SEM 0.6), and 10.9 (SEM 1.2) min pre- and post- 5:2 IER and CER interventions, respectively. Comparable eating speed was also reported: 42.3 (SEM 8.1), 39.9 (SEM 6.1), 36.7 (SEM 2.3), and 40.6 (SEM 4.0) g/min pre- and post- 5:2 IER and CER interventions, respectively.

5.4 Discussion

The present study demonstrates that two-weeks of 5:2 IER fails to improve postprandial CAS or energy intake at the *ad libitum* test meal (secondary outcomes) compared with CER in healthy young men and women. However, 5:2 IER resulted in a decrease in fasting subjective hunger ratings compared with CER (exploratory outcome) in participants with normal weight; concurrent weight loss was comparable (3-4% of original body weight).

Although no significant differences in postprandial CAS response were observed between interventions, potentially beneficial changes in fasting CAS and individual subjective appetite and satiety ratings were observed in the 5:2 IER but not the CER group. These perceived changes may reduce the desire to eat, which could help to facilitate long-term compliance and subsequent weight loss with IF paradigms. In contrast, previous studies reported no changes in subjective hunger rating in normal-weight participants following 12-weeks of ADF, while 8-12 weeks of ADF actually diminished hunger in participants with obesity (Varady *et al.*, 2013). In a 12-week study comparing 1670-2500 kJ/day energy restriction for two non-consecutive days per week against CER in participants with T2DM and overweight or obesity, both groups reported a comparable reduction in appetite and increased feelings of fullness and satisfaction (Carter, Clifton and Keogh, 2016). However, participants were taking hypoglycaemic drugs and therefore the effect of the medication on glycaemia and appetite cannot be excluded. Differences between the findings were even reported within the same research group, which were probably attributable to variations in the dietary design. In a study utilising a 5:2 IER protocol, participants were asked to restrict food

intake on two-consecutive energy restricted days to only milk, vegetables, and fruit. As a consequence, a greater proportion of participants reported significant hunger in the 5:2 IER group compared with those following a CER diet. Moreover, fewer participants in the 5:2 IER group intended to continue with the prescribed regimen (Harvie *et al.*, 2011). On the contrary, when the same protocol was applied in the same type of group permitted a greater variety of protein foods including lean meat, fish, eggs, subjective appetite ratings were comparable between the two groups (Harvie *et al.*, 2013).

While appetite-related hormones (e.g. leptin, ghrelin, and PYY) were not measured in the present study, decreased subjective hunger ratings did not translate to significantly decreased energy intake at the *ad libitum* lunch, although a numerical decrease was observed in the 5:2 IER group. Energy intake at the *ad libitum* lunch was approximately 2 MJ in both groups, equating to 20% of their estimated daily energy requirements. This finding indicated that they were not excessively hyperphagic, which was in line with the findings of several studies showing a lack of complete compensatory hyperphagia (Varady *et al.*, 2009; Harvie *et al.*, 2011; Harvie *et al.*, 2013, 2016). Perhaps longer periods of intervention are required to promote adaptations in homeostatic appetite regulation. Furthermore, a greater accumulated difference in total energy intake might occur across all meals of the day. However, subjective and objective measures of appetite may not always align (Blundell *et al.*, 2010), especially in relatively short-term interventions.

The present study found no significant difference between groups in fasting RMR although a significant main effect of time was observed, which showed a reduction by approximately 0.26 kJ/min in 5:2 IER and by 0.08 kJ/min in CER. Reduction in fasting RMR in the 5:2 IER group was consistent with a 5:2 IER study conducted in participants with obesity, which showed a reduction by approximately 0.27 kJ/min in 5:2 IER and by 0.33 kJ/min in CER (Sundfør, Svendsen and Tonstad, 2018). Comparable decreases in fasting RMR between the two groups were probably attributable to equivalent weight loss. The reduction in fasting RMR did not hinder the weight-loss progress in either group, however, this physiological adaptation to weight loss may predispose to weight regain if a healthy, lower energy diet is not maintained.

Matched weight loss between the two groups indicates that the 5:2 IER regimen *per se* promoted several beneficial changes or differences in indices of metabolic health and subjective appetite

scores, independent of the weight loss. Human trials implementing larger sample sizes and longer intervention durations are necessary to examine the sustainability of 5:2 IER before definitive conclusions can be drawn.

As a consequence of modern lifestyles, potentially much of the 24-hour period is typified by a postprandial and lipogenic state (Ruge *et al.*, 2009; McQuaid *et al.*, 2011), which predispose to fat accretion (Frayn, 2016; Travers *et al.*, 2017). Therefore, energy restriction regimens attempting to decrease the amplitude of postprandial excursion can ultimately improve the liberation and utilisation of endogenous lipid reservoirs. However, CER cannot redress the imbalance between the feeding and fasting windows (Soeters *et al.*, 2012; Anton *et al.*, 2018). Periods of severe energy restriction or omission of energy intake prescribed by IF or IER may lower the postprandial excursions, which further leads to metabolic benefits independent of energy deficit (Halberg *et al.*, 2005; Patterson and Sears, 2017; Anton *et al.*, 2018). However, there are very limited data on the impact of 5:2 IER on postprandial metabolism.

Whether the potential beneficial effects of IF or IER are mainly attributable to regularly prolonged and uninterrupted post-absorptive periods, or merely to the energy deficit indirectly elicited by any pattern of energy restriction warrant further exploration in larger-scale and longer-term RCTs. Moreover, it would be interesting to examine whether the metabolic benefits of IF or IER can be sustained across longer periods of weight stabilisation. In summary, a lack of consensus on the beneficial effects of IF or IER within the literature makes drawing firm conclusions from these data difficult. Further human RCTs with appropriate controls are warranted.

CHAPTER 6. GENERAL DISCUSSION

6.1 Summary

Eating pattern, including dietary composition and temporal eating patterns, is considered to be one of the most fundamentally important factors for optimal health (Hu, 2002, 2003, 2009; Sacks and Katan, 2002; Giugliano, Ceriello and Esposito, 2006; Swain *et al.*, 2008; Ford *et al.*, 2009). Historically, dietary recommendations were mainly aimed at preventing deficiency diseases (Mozaffarian, Rosenberg and Uauy, 2018). The first Recommended Dietary Allowances (RDAs) for protein, total energy, minerals and specific vitamins were produced in 1941, in the context of the World War II, where war had led to widespread malnutrition or starvation (Murphy *et al.*, 2016). Afterwards, although maintaining energy intake and selected micronutrients was still crucial (Mozaffarian, Rosenberg and Uauy, 2018), in developed countries the main objectives of nutritional recommendations and nutrition research have shifted towards dealing with growing challenges of overweight/obesity, non-communicable diseases (NCDs) and ageing (Shao *et al.*, 2017; Mozaffarian, Rosenberg and Uauy, 2018). Nowadays, there is an emergence of interest in specific diets for weight-management; and managing, reversing, or even curing diseases. The priority for modern nutrition has shifted towards investigating the dietary contribution to achieve optimal health and prevent metabolic abnormalities (Biesalski *et al.*, 2011; Georgiou, Garssen and Witkamp, 2011; Heaney, 2012) and includes considering dietary composition and most recently, temporal eating patterns (Alhussain, Macdonald and Taylor, 2016). In the context of the current obesogenic environment, unhealthy diet (including both overconsumption of energy and some nutrients and underconsumption of others) and physical inactivity have become the leading causes of the major NCDs (WHO, 2004). This situation emphasises the important role of lifestyle (e.g. increasing physical activity, reducing sedentary behaviour and avoiding physical inactivity), in addition to diet, for optimising health. The ultimate goal is to identify the optimal combination of dietary behaviour (composition and temporal pattern) and physical activity, by investigating the complementary and interactive relationships between the two, to achieve optimal health (Baranowski, 2004). In fact, the importance of the combinations of diet and exercise to maintain health had already been acknowledged by the ancient Greeks. As stated by Hippocrates (460–377

BC): '*If we could give every individual the right amount of nourishment and exercise, not too little and not too much, we would have found the safest way to health*'.

The overarching aim of this PhD projects was to gain a greater understanding of the metabolic effects of physical inactivity and 5:2 diet in metabolically healthy participants, with a specific emphasis on glycaemic control, insulin sensitivity and resting energy expenditure in response to meal tolerance test (MTT). In Chapter 3, the purpose of the study was to extend the findings of published research by evaluating the effects of physical inactivity over a two-day period, with and without a proportional decrease in energy intake, on insulin sensitivity, glycaemic control, appetite, and resting energy expenditure in response to MTT. A physical inactivity model was used in which step counts were reduced by 80%. In Chapters 4 and 5, the aim was to compare the fasting and postprandial metabolic effects of two consecutive weeks in which either energy requirements were met on five days and restricted (70% energy restriction relative to estimated energy requirements) on two non-consecutive days each week (5:2 IER); or energy was restricted every day of the two-week period (20% daily energy restriction relative to estimated energy requirements) (CER). Both diets were thus hypoenergetic, and designed to induce the same total energy deficit over the experimental period.

Given that modern humans spend much of the 24-hour period in a postprandial state, which is a dynamic and non-steady-state, both fasting and postprandial measurements are believed to be important dimensions when establishing the metabolic effects of the different regimens. Postprandial hyperglycaemia and hypertriacylglycerolaemia have been shown as independent risk factors for cardiovascular diseases (CVDs) and T2DM (Cavalot *et al.*, 2006; O'Keefe and Bell, 2007). Prospective cohort studies have confirmed that postprandial hyperinsulinaemia was a main underlying abnormality associated with CVDs (Ginsberg, 2000). Therefore, Chapters 3, 4 and 5 placed particular emphasis on examining the effects of physical inactivity/5:2 IER on postprandial insulin, glucose and lipid levels.

In this chapter, following a general discussion of the approaches selected, each of the studies will be considered separately, with particular reference to future work.

6.2 General Discussion of the Approach Taken to Address the Research Questions

6.2.1 Compliance and Free-Living Setting

In both cases, a randomised intervention study was undertaken as this would enable attribution of cause and effect (Sibbald and Roland, 1998). A key requirement for successfully execution of each study, was compliance with the dietary intervention. Therefore, all food and beverages were provided in both cases, which appeared to be a successful strategy, based on data from the CGM (Chapters 3, 4 and 5) and similarities in weight change (Chapters 4 and 5). During the intervention periods, participants were permitted to live freely, other than following the dietary and physical activity prescriptions. The advantages of this over participants remaining on a metabolic unit were that it provided more flexibility for the participants and would improve recruitment; and the disadvantages were its less precision and accuracy (Gibbons *et al.*, 2014). In future studies, devices such as Actiheart (a device that combines accelerometery with heart rate monitoring) would enable better monitoring of activity compliance and partitioning of sedentary behaviour, as opposed to physical inactivity. Moreover, these studies have demonstrated the feasibility of techniques such as CGM within these protocols.

6.2.2 Metabolically Healthy Participants with Healthy Body Weight

In both studies, participants were healthy and of healthy body weight. The justification for this was that the results would not be impacted on by underlying metabolic abnormalities. The next step will thus be to expand on the current work, that was best suited to explore the proof of concept, into broader populations, for example, older individuals who are inactive, or those who are obese or with prediabetes.

6.3 Physical Inactivity and Overfeeding

It is widely recognised that high levels of physical activity are associated with improved health outcomes and reduced disease risks (Warburton, Nicol and Bredin, 2006). In contrast, physical

inactivity is a major threat to optimal health (Blair, 2009) and has been shown to be associated with increased risks of depression, T2DM, cancer, CVDs, mortality and poor metabolic health (Ekelund *et al.*, 2016; Patterson *et al.*, 2018; Stamatakis *et al.*, 2019; Huang *et al.*, 2020). Furthermore, as a reduction in activity-associated energy expenditure may concurrently occur with overfeeding, this may amplify the deleterious effects of physical inactivity on health. Overfeeding will occur if energy intake is not decreased in line with the decreased energy expenditure, associated with physical inactivity, due to the absence of efficient compensatory changes in appetite regulation or maladaptive behaviour (Panahi and Tremblay, 2018). No compensatory reduction in *ad libitum* energy intake was shown in response to a seven-days intervention with decreased PAL (from 1.8-1.4) (Stubbs *et al.*, 2004). The combination of energy surplus with physical inactivity results in insulin resistance (Stuart *et al.*, 1988; Blanc *et al.*, 1998), fat accumulation especially visceral adipose tissue mass (Olsen *et al.*, 2008), and lean body mass catabolism (Krebs *et al.*, 1990; Gretebeck *et al.*, 1995; Ferrando *et al.*, 1996; Barbe *et al.*, 1999; Lovejoy *et al.*, 1999; Stein *et al.*, 1999; Blanc *et al.*, 2000; Scheld *et al.*, 2001; Shackelford *et al.*, 2004). Energy surplus, physical inactivity and accumulation of adipose tissue also stimulate a low-grade inflammatory response and increase oxidative stress (Schäffler *et al.*, 2006; Van Guilder *et al.*, 2006).

However, little is known about the undesirable effects on metabolism that occur with physical inactivity, when energy intake is rigorously controlled; with or without overfeeding for a longer time period than 24 hours. The acute transition (two weeks) of healthy young men from higher (~10,000) to lower (~1300) daily step counts induced a significant increase in postprandial plasma insulin iAUC but did not include an experimental arm that seeks to match the energy intake with decreased energy expenditure (Olsen *et al.*, 2008). Plasma insulin iAUC increased significantly after seven-days of step reduction (10,000 to 1,500 steps/day) and coexisting overfeeding (+50% kcal) in healthy young men (Knudsen *et al.*, 2012). Similarly, the insulin response to the OGTT increased after three-days of step reduction (from $\geqslant$ 10,000 to < 5000 steps/day) without compensatory decline in energy intake in healthy, free-living men and women (Mikus *et al.*, 2012). Stephens *et al.* (2011) considered only 24 hours of prolonged sitting which dramatically reduced insulin sensitivity (using a glucose infusion technique) but did include both an overfeeding and energy intake and expenditure matched arm. The effect was attenuated, but not eliminated, when

accompanied by a compensatory reduction in energy intake to maintain energy balance. Therefore in Chapter 3, the one-day physical inactivity study by Stephens *et al.* (2011) was extended by evaluating the effects of two-days of physical inactivity (with step counts reduced by 80%) tested both with (SIT+E) and without (SIT=E) energy surplus, compared with a CONTROL arm (two consecutive days of standardised daily levels of moderate physical activity and matched energy intake) on basal and postprandial metabolism. Under these conditions, there was a strong trend towards a significant difference in iAUC for serum inulin over 180 min postprandially between the groups ($P = 0.05$, $\eta^2_p = 0.35$) (with large effect size). This was driven by the difference between the CONTROL and the SIT+E trials. Compared with the CONTROL trial, the mean iAUC insulin was 25% higher in the SIT+E but only 8% higher in the SIT=E trials. The results were in accordance with the hypothesis and consistent with the findings of the aforementioned one-day physical inactivity study. Across the lifespan, physical activity level decreases, with older individuals tending to engage in less physical activity than younger individuals; concurrently, energy requirement decreases with loss of lean body mass. Therefore, findings from Chapter 3 suggest that it is important to maintain physical activity and reduce energy intake to minimise the deleterious effects of physical inactivity that accompanies the ageing process on metabolic health. Differences in glycaemic profiles captured by CGM during Day1 and Day 2, and during day hours and night hours, extended the insight into the acute effect of physical inactivity on glycaemic control, which enabled the identification of early processes involved in the development of metabolic diseases induced by physical inactivity. Although step count reduction is a semi-quantitative model to achieve reduced levels of physical activity, it is a natural element of real-life rather than particular interventions (e.g. dry immersion model and unilateral limb suspension model) (Silva, Jayawardana and Meyer, 2018).

A particular strength of Chapter 3 was that the effects of physical inactivity *per se* were dissociated by adding an experimental arm designed to compensate for the low energy expenditure by reducing energy intake unlike in many of the previous studies. CGM was used to maximally explore the impact of the interventions on 24 hours interstitial glucose, as well as to monitor adherence throughout the interventions. All food and beverages were provided to optimise dietary control. Although recruitment of young and normal-weight participants in this study can help to minimise the potential impact on treatment differences, of metabolic defects associated with overweight and

obesity, this may limit the generalisability of the findings to other populations for whom the work may be particularly relevant.

Physical inactivity has acute effects on insulin action and postprandial insulinaemic excursion; however, the cellular and molecular mechanisms mediating the direct effect of physical inactivity are unclear. Future studies should address this by combining hyperinsulinaemic euglycaemic clamp technique with muscle/adipose tissue biopsies, to fully elucidate the mechanisms underlying the detrimental effects of physical inactivity. Careful consideration will need to be given to the ethics of overfeeding with physical inactivity over a longer period. Larger-scale RCTs, conducted in diverse population groups, with all components of energy balance well controlled, are warranted. Results from Chapter 3 highlight the importance of maintaining energy balance during step reduction; therefore, public health strategies that focus on restricting physical inactivity behaviours and achieving dietary control may play a significant role in reducing metabolic disease risk. Finding strategies to achieve dietary control is essential, and one potential intervention, the 5:2 diet was investigated in Chapters 4 and 5.

6.4 5:2 Intermittent Energy Restriction

5:2 IER and alternate day fasting have been studied as an alternative dietary approach, to a traditional continuous daily energy restriction, for weight loss and to improve metabolic health; whereas for time-restricted feeding, there is not necessarily a prescribed energy deficit and it has not been studied as a weight-loss approach, but as an unintentional consequence of restricted length of the daily eating window, people tend to reduce the energy intake involuntarily and therefore experience weight loss (LeCheminant *et al.*, 2013; Tinsley *et al.*, 2017; Antoni *et al.*, 2018; Gabel *et al.*, 2018; Jones *et al.*, 2020b). It was argued that the metabolic benefits occurring during intermittent fasting (IF)/intermittent energy restriction (IER) were merely attributed to the diet-induced weight loss. To address this issue, in Chapters 4 and 5, 5:2 IER was compared with a CER arm under isoenergetic conditions over two weeks. The element of weight loss was therefore controlled, permitting direct comparisons between the two groups. Contrary to the hypotheses, the postprandial insulin iAUC response was comparable between the two groups in healthy, young men and women (Chapter 4). Nor were postprandial glucose, FFA, TAG, CAS or energy intake at

the *ad libitum* test meal different (Chapters 4 and 5). This suggests that metabolic differences (e.g. fasting insulin and insulin resistance) observed between these diets in published, longer trials (Harvie *et al.*, 2011; Harvie *et al.*, 2013) cannot be explained by changes in postprandial responses.

The lower fasting glucose concentrations following 5:2 IER, compared with CER, described in Chapter 4 have been observed previously (Cho *et al.*, 2019). Further investigation of improved hepatic insulin sensitivity is warranted, to elucidate the mechanisms underlying the observed differences in fasting blood glucose levels. Insulin sensitivity offers a useful index of metabolic health and predicts the development of CVDs even in healthy individuals (Yip, Facchini and Reaven, 1998; Facchini *et al.*, 2001). In Chapter 4, whole-body postprandial insulin sensitivity (Matsuda index) improved equally, along with a strong tendency for increased fasting insulin sensitivity (HOMA-IR) in both groups, which were probably attributable to equivalent weight loss. Greater reductions in fasting insulin concentration and insulin resistance (HOMA-IR) were reported following 5:2 IER compared with CER in two previous similar studies (Harvie *et al.*, 2011; Harvie *et al.*, 2013), however two-days energy restriction was on consecutive days and participants had overweight or obesity. It seems reasonable to speculate that individuals with a lower insulin sensitivity at baseline may be more likely to achieve improvement after 5:2 IER than metabolically healthy individuals (Harvie *et al.*, 2011; Harvie *et al.*, 2013; Antoni *et al.*, 2018). In Chapters 4 and 5, is described a lower mean value (Days 8-13) of the five-hour lunchtime postprandial glucose iAUC during 5:2 IER compared with CER. Abnormality in postprandial glucose may be present long before the development of diseases (American Diabetes Association, 2009); thus, improved postprandial glucose responses may play an important role in slowing or preventing cardiometabolic disease progression.

It has been consistently demonstrated that prolonged periods of CER elicit an adaptive decline in REE and increase in appetite that preserve body fat stores and hinder dieting effort and subsequent weight-maintenance (Spiegelman and Flier, 2001; Schwartz *et al.*, 2003; Heilbronn *et al.*, 2006; Rosenbaum *et al.*, 2008; Redman *et al.*, 2009; Martin *et al.*, 2011; Kissileff *et al.*, 2012; Betts *et al.*, 2014; Chowdhury *et al.*, 2016). However, adaptive physiological changes in REE and appetite that occur during weight-loss achieved via IF have been inconsistent. Therefore, the precise alterations that occur in all components of energy balance (REE, physical activity thermogenesis, DIT, and energy intake) warrant further investigation. In Chapter 5, REE was assessed by indirect

calorimetry in the fasted state and in response to the MTT, further enabling the calculation of DIT. In contrast to this study, study conducted by Alhussain, Macdonald and Taylor (2016) showed that irregular meal pattern resulted in a reduced DIT. The 5:2 IER is in effect an irregular meal pattern, as participant did not consistently eat in the same pattern every day. This inconsistency may be explained by the degree of irregularity, as in a seven-day period, five of the days had a comparable pattern, relative to the two days that differed.

Beneficial changes in fasting CAS and individual subjective appetite and satiety ratings which may help to reduce the desire to eat were observed in the 5:2 IER but not the CER group. These differences might be expected to support long-term adherence to the 5:2 IER eating schedule and are in common with a study in participants with obesity; but in contrast to another considering participants with normal weight (Klempel *et al.*, 2010; Varady *et al.*, 2013). In participants with T2DM and overweight or obesity, both 5:2 IER and CER groups reported comparable reductions in appetite and increased feelings of fullness and satisfaction (Carter, Clifton and Keogh, 2016). However, participants were taking hypoglycaemic drugs, potentially impacting on glycaemia and appetite. Although decreased fasting CAS after 5:2 IER did not lead to a significantly reduced energy intake at the *ad libitum* lunch, there was a numerical reduction after the post-5:2 IER intervention. However, the assessment of energy intake was limited to a single meal; an accumulatively greater difference in total energy intake might occur across all meals of the day. Compensatory reductions in REE were found after both regimens, which may hamper the degree of negative energy balance elicited by energy-restricted regimens, indicating that timely adjustment of energy intake to match the decreased REE is required during a weight-loss diet.

Metabolic effects of different eating patterns can vary considerably due to the complexities of the causal pathway (Katz and Meller, 2014). With regards to weight-loss elicited by IF regimens, the average weight loss is largely dependent on the duration of intervention. There is currently a lack of consensus favouring one diet; comparability is hindered by the absence of rigorous and long-term RCTs directly comparing the diverse range of dietary patterns. Therefore, the claim about a diet that is best for health or weight loss may be overstated (Katz and Meller, 2014). Ultimately, the optimal dieting approach is dependent on the individuals. It is of importance to make a

reasonable plan before starting a diet regimen, including meal timing, meal frequency and macronutrient composition on energy-restricted days (Paoli *et al.*, 2019). Whether meal replacement products should be used on the two energy-restricted days of the 5:2 diet is a controversial issue. Using meal replacement products is simple and time-saving; however, it does little to educate people about their eating habits or change their behaviours; and is associated with a risk of getting weight regain once people stop using the meal replacement products (American Diabetes Association, 2008).

The main strength of Chapters 4 and 5 was the similarity of weight-loss elicited by both diets in a free-living setting in two matched groups, considering unequal weight-loss may confound the metabolic comparisons between IER and CER dieting approaches, this was facilitated by the carefully designed dietary interventions to which participants adhered well.

The dynamic measurements of postprandial glucose and lipid metabolism were conducted in addition to the static and steady-state (fasting) assessments. Chapters 4 and 5 extended the investigations of IF (Halberg *et al.*, 2005; Jones *et al.*, 2020b) and other metabolic challenges (Krug *et al.*, 2012; Alhussain, Macdonald and Taylor, 2016) by considering both fasting and postprandial states in young, normal-weight participants in whom effects on metabolism and appetite are not confounded by the metabolic abnormalities associated with excess body weight. Published research was largely limited to fasting indices of risk, however, people spend the majority of a 24-hour day in a postprandial state. Importantly when studying meal pattern and metabolism, and in contrast to previous studies, food was provided over the intervention period and compliance monitored by CGM. Both groups were prescribed with matched meal frequency, macronutrient distribution, energy distribution across the day, meal time and food items. Three major excursions in daily glucose profiles were seen in the CGM, coinciding with prescribed meal times, reflecting good dietary adherence, facilitated by food provision. This counters the potential limitation of the participants being free-living (Subar *et al.*, 2015; Rynders *et al.*, 2019), as did monitoring activity by using step counts. Other strengths of these two chapters include the balanced sex ratio of participants. Considering the metabolic responses to energy restriction may differ between men and women (Soeters *et al.*, 2007; Mauvais-Jarvis, 2018; Tramunt *et al.*, 2020), Chapters 4 and 5 benefited from including both men and women with a balanced sex ratio, which may help to offset any sex bias, hence making this study more representative of the general

population. The utilisation of CGM not only provided an insight into the impact of the interventions on glycaemic control but also enabled the adherence throughout the interventions to be monitored. Furthermore, assessments in Chapters 4 and 5 were made at least three days after the last energy-restricted day with 5:2 IER to minimise the confounding effects of acute energy restriction; favourable metabolic effects of 5:2 IER appear not to be due to an acute carry-over effect of greater negative energy balance during the last energy-restricted day with 5:2 IER. Limitations included the short-term nature of the study duration, which limited the understanding of the long-term feasibility of 5:2 IER. Pre-intervention metabolic measurements may be confounded by the dinner consumed the evening before. A standardised dinner, prior to each measurement day, may help to minimise the effects of diverse dietary characteristics of the dinner on fasting and postprandial substrates metabolism (Robertson *et al.*, 2002). Furthermore, due to the healthy cohort studied, who do not necessarily have an established impairment, the findings from Chapters 4 and 5 cannot be readily extrapolated to other populations.

6.5 Future Research Directions

- Physical inactivity has acute effects on insulin action and postprandial insulinaemic excursion; however, the mechanisms are unclear. By combining more sensitive measurements of insulin sensitivity (e.g. hyperinsulinaemic euglycaemic clamp) with muscle/adipose tissue biopsies, the mechanisms underlying the negative effects of physical inactivity may be fully elucidated.

- Whilst IER appears to be a promising approach for weight maintenance following weight loss (Harvie *et al.*, 2013; Zuo *et al.*, 2016; Schübel *et al.*, 2018; Sundfør, Svendsen and Tonstad, 2018b), the long-term applications of IER as a weight maintenance approach for individuals with overweight or obesity following weight loss warrants further investigation.

- 5:2 IER has a potential application as an uncomplicated and feasible dietary strategy to achieve weight loss and improve metabolic health. However, it is unclear whether this regimen can deliver sustainable weight loss and metabolic health improvement in the long-term since studies have seldom been conducted beyond a period of a year. Studies with a

total duration (weight loss plus weight maintenance) more than one year may be of practical value and likely to reveal novel mechanistic findings.

- A further dimension of the 5:2 IER work is to explore what would happen if participants choose their own energy intake.

- There is a paucity of 5:2 IER studies including equal (or nearly equal) numbers of men and women. Given the metabolic responses to energy restriction may differ between men and women (Soeters *et al.*, 2007; Mauvais-Jarvis, 2018; Tramunt *et al.*, 2020), it would be interesting to directly compare the metabolic effects of 5:2 IER between men and women.

- With regards to the mechanistic evaluation of the effects of 5:2 IER on glycaemic control, results from different studies have been inconclusive. Using a hyperinsulinaemic euglycaemic clamp can not only directly assess insulin sensitivity but allow the differentiation between peripheral and hepatic insulin sensitivity which may be significantly altered by 5:2 IER.

- As suggested by Anton *et al.* (2018), future RCTs of IF are expected to use biomarkers of the metabolic switch such as plasma ketone levels to assess adherence and the magnitude of energy deficit during the energy-restricted period.

- Understanding the differential metabolic effects elicited by different IF protocols may be conducive to identify the potential application of a given IF protocol for particular metabolic disorders.

6.6 Conclusions

This thesis provides an insight into the effects of physical inactivity and 5:2 IER on basal and postprandial metabolism in normal-weight, young participants.

The outcomes of the physical inactivity study (Chapter 3) emphasise the important role of overfeeding in the negative effects of physical inactivity. Physical inactivity is about more than insulin resistance and obesity, it is about reduced physical independence and mobility and accelerated unhealthy ageing. It was characterised as a global pandemic in 2012 (Kohl *et al.*, 2012). The current COVID-19 pandemic is an unprecedented health challenge, which makes the physical inactivity situation even worse, due to the lockdown, as entire populations have been asked to restrict their social interactions, isolate or live in home confinement.

Data from 5:2 IER study (Chapters 4 and 5) provide novel insights into the metabolic effects of 5:2 IER. 5:2 IER is not superior in improving postprandial insulin and glucose responses compared with CER, suggesting that these are not key to metabolic differences in longer-term intervention studies. Interestingly, the 5:2 IER regimen led to potentially beneficial changes in fasting blood glucose and fasting subjective appetite scores, which were independent of weight loss, over a two-week period. More mechanistic and long-term safety RCTs are required, within a variety of population groups (e.g. those with established metabolic disorders) to establish mechanisms of action and examine long-term adherence, safety and efficacy of 5:2 IER. However, the number of literature reviews and diet books is much greater than the number of RCTs examining the safety and metabolic effects of IER (Patterson and Sears, 2017).

In a world characterised by a remarkable increase in the prevalence of overweight/obesity and associated metabolic perturbations, as a consequence of sedentary behaviour and excessive intake of energy-dense food; personalised physical activity and diet prescription are considered a promising strategy in preventing and managing obesity-related metabolic syndromes. This work furthers our understanding of the impact of inactivity, and offers a potential dietary regimen that is an alternative to traditional continuous energy restriction.

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APPENDIX

Appendix A. Medical Ethics Approval – Physical Inactivity Study

University of Nottingham
UK | CHINA | MALAYSIA

Email: FMHS-ResearchEthics@nottingham.ac.uk

Faculty of Medicine & Health Sciences
Research Ethics Committee
c/o Faculty PVC Office
School of Medicine Education Centre
B Floor, Medical School
Queen's Medical Centre Campus
Nottingham University Hospitals
Nottingham, NG7 2UH

16 April 2018

Dr Kostas Tsintzas
Associate Professor
Room D62a, D Floor Medical School
School of Life Sciences
QMC Campus
Nottingham University Hospitals
NG7 2UH

Dear Dr Tsintzas

Ethics Reference No: I200317 **– please always quote**	
Study Title: Short-term effects of physical inactivity on insulin sensitivity, appetite, energy balance, and cardiovascular responses in healthy humans.	
Chief Investigator/Supervisor: Dr Kostas Tsintzas, Associate Prof, School of Life Sciences, University of Nottingham, PhD, Prof. Ian Macdonald, School of Life Sciences, University of Nottingham, PhD, Dr Moira Taylor, Associate Prof, School of Life Sciences, University of Nottingham, PhD.	
Lead Investigators/student: Miss Yangfan Gao, PhD student, Miss Eman Al Sultan, PhD student, School of Life Sciences.	
Other Key Investigators: Mrs Julie Littlewood, Research Associate, School of Life Sciences.	
Type of Study: Research Project, PhD	
Proposed Start Date: 01/04/17	**Proposed End Date:** 30/04/2019 24mths
No of Subjects: 10	**Age:** 18-35 years
School: Life Sciences	

Thank you for notifying the Committee of amendment no 2: 11.04.2018 in summary as follows:

- Time extension of 1 year with a new end date of 30 April 2019.
- Change 2-day intervention period from being carried out in the laboratory to self-directed at home as part of participant's every day routine. This provides more flexibility for the participant and will improve recruitment.

and the following revised documents were received:

- Participant Information Sheet, version 2.0: 11.04.2018
- Protocol, version 2.0: 11.04.2018

These have been reviewed and the study amendment no 2: 11.04.2018 is approved.

Yours sincerely

Professor Ravi Mahajan
Chair, Faculty of Medicine & Health Sciences Research Ethics Committee

Appendix B. Medical Ethics Approval – 5:2 IER Study

University of Nottingham
UK | CHINA | MALAYSIA

Email: FMHS-ResearchEthics@nottingham.ac.uk

Faculty of Medicine & Health Sciences
Research Ethics Committee
c/o Faculty PVC Office
School of Medicine Education Centre
B Floor, Medical School
Queen's Medical Centre Campus
Nottingham University Hospitals
Nottingham, NG7 2UH

23 October 2018

Dr Moira Taylor
Associate Professor of Human Nutrition (Dietetics)
Room E89, E Floor, Medical School
School of Life Sciences
QMC Campus
Nottingham University Hospitals
NG7 2UH

Dear Dr Taylor

Ethics Reference No: 121-1809 – **please always quote**	
Study Title: Investigating the effects of two weeks of 5:2 intermittent energy restriction or continuous energ restriction on basal and postprandial metabolism: a randomized study in twenty young normal-weight participants.	
Chief Investigator/Supervisor: Professor Ian Macdonald, Professor of Metabolic Physiology, School of Life Sciences.	
Lead Investigators/student: Ms Yangfan Gao, PhD Student, School of Life Sciences	
Other Key Investigators: Dr Moira Taylor, Associate Professor, Human Nutrition (Dietetics), Dr Kostas Tsintzas, Associate Professor, Human Physiology, School of Life Sciences	
Type of Study: PhD, basic science, physiology	
Proposed Start Date: 01.09.2018	**Proposed End Date:** 30.09.2019 12mths
No of Subjects: 20	**Age:** 18+years

The Committee considered this application at its meeting on 28 September 2018 and the following documents were received:

- FMHS REC Application form and supporting documents version 1.0: 02.09.2018

These have been reviewed and are satisfactory and the study has been given a favourable opinion.

A favourable opinion has been given on the understanding that:

1. Human tissue taken under this ethical approval will fall under the licence if stored for more than seven days, if this is the case please speak to your local person designate or HTMG quality manager, details can be found on the HTA workspace page: https://workspace.nottingham.ac.uk/display/HTA/HTMG+Person%27s+Designate It is not considered "end user exception".
2. The protocol agreed is followed and the Committee is informed of any changes using a notice of amendment form (please request a form).
3. The Chair is informed of any serious or unexpected event.
4. An End of Project Progress Report is completed and returned when the study has finished (Please request a form).

Yours sincerely

Professor Ravi Mahajan
Chair, Faculty of Medicine & Health Sciences Research Ethics Committee

Appendix C. Screening Form



Screening Form

School of Life Sciences
Medical School
Queen's Medical Centre
Nottingham
NG7 2UH

PERSONAL INFORMATION

Participant Code _____ Date ___/____/___

1. **Name** ……..

2. **Contact Address** ……

……..

……..

3. **Contact Telephone number** ……

4**.** **Date of Birth** ……./……./ 19…….

GENERAL HEALTH

1. In general would you say your health is;

Please tick one response

a. ☐ *Excellent*
b. ☐ *Very good*
c. ☐ *Good*
d ☐ *Fair*
e. ☐ *Poor*

2. Compared to one year ago, how would you rate your health in general now?

Please tick one response

a. ☐ *Much better than a year ago*
b. ☐ *Somewhat better than a year ago*
c. ☐ *About the same*
d. ☐ *Somewhat worse than a year ago*
e. ☐ *Much worse than a year ago*

3. The following questions are concerned with any medical conditions you might have.

Do you suffer from any of the following?

Please tick one box on each line

	No	*Yes*	*Don't Know*
Diabetes			
Epilepsy			
Depression			
Hayfever			
Asthma			
Eczema			
High blood pressure			
Heart problems			
Breathing problems *			
Indigestion / heartburn			
Irritable bowel syndrome			
Any other medical condition not listed above (please detail below)			

*** other than asthma**

Please detail any other medical condition/s

……
……

If you answered '*yes*' to any of the above, when did you first start having the problem and what medication (if any) do you use?

……
……
……

4. Have you had any operations in the last year?

   If 'Yes', please detail:

……

……

5. Have you ever had any operations on your stomach/abdomen?

   If 'Yes', please detail:

……

……

6. **Do you have a tendency to faint in certain situations?** ☐ ***Yes*** ☐ ***No*** ☐ ***Don't Know***

   e.g. on standing, in a warm room, after fasting

7. **Smoking** On average, how many cigarettes do you smoke a day? ……..

   (If a non-smoker, please put '*none*')

DIET AND HEALTH

The following questions relate to your diet and your health

Please tick one box on each line

a. Would you consider yourself a **vegetarian**? i.e. eat no meat or fish, but eat dairy products ☐ ***Yes*** ☐ ***No*** ☐ ***Don't know***

b. Would you consider yourself a **vegan**? i.e. eat no meat or fish or dairy products ☐ ***Yes*** ☐ ***No*** ☐ ***Don't Know***

c. **Are you on a medically prescribed diet?** e.g. diabetic, gluten-free etc ☐ ***Yes*** ☐ ***No*** ☐ ***Don't Know***

If '*yes*', what diet are you following? …….

…….

d. Do you suffer from any **food allergies**? e.g. nuts, wheat, milk ☐ ***Yes*** ☐ ***No*** ☐ ***Don't Know***

If '*yes*', which foods are you allergic to?

…….

…….

e. Do you regularly (at least once a week) take **vitamin** and / or **mineral** supplements ☐ ***Yes*** ☐ ***No*** ☐ ***Don't Know***

If '*yes*', which supplements do you take?

…….

…….

f. Do you regularly (at least once a week) take any other supplements for your health? E.g.. Creatine, Carnitine, Echinacea, St John's Wort, etc

☐ ***Yes*** ☐ ***No*** ☐ ***Don't Know***

If '*yes*', which supplements do you take?

……

……

g. **Alcohol** On average, how many units of alcohol do you drink in a week?

…….Units

(pint of beer/lager =3 units, pub measure of wine / spirits =1 unit)

h. Are you currently 'dieting' to control your weight?

☐ ***Yes*** ☐ ***No*** ☐ ***Don't Know***

PHYSIOLOGICAL MEASUREMENTS

1. **Height ……cm**

2. **Weight ……kg**

3. **BMI …….kg/m^2 (ref. 20-27 kg/m^2)**

4. **Waist circumference ……cm**

   Hip circumference ……cm

5. **Skinfold test:**

   Biceps:…… Triceps:…… Subscapular:……

   Suprailiac:…… % body fat:……

6. **BP ……. /……. Lying**
   ……. /……. Standing (1 min)
   ……. /……. Standing (3 min)

7. **HR: ……/……../……**

8. **Have you taken part, or do you intend to take part in any other trials within the three months prior to or following this particular study?** ☐ ***Yes*** ☐ ***No***

9. **Suitable for blood sample to be taken?** ☐ ***Yes*** ☐ ***No***

10. **Blood sample taken?** ☐ ***Yes*** ☐ ***No***

Appendix D. Beck Depression Inventory (Beck *et al*., 1961)

Please read each group of statements carefully and pick out the one statement that best describes the way you have been feeling during the past two weeks, including today. Circle the number besides the statement you have picked. If several statements in the group seem to apply equally well, circle the highest number for that group.

A. Mood

0 I do not feel sad
1 I feel blue or sad
2a I am blue or sad all the time and I can't snap out of it
2b I am so sad or unhappy that it is very painful
3 I am so sad or unhappy that I can't stand it

B. Pessimism

0 I am not particularly pessimistic or discouraged about the future
1a I feel discouraged about the future
2a I feel I have nothing to look forward to
2b I feel that I won't ever get over my troubles
3 I feel that the future is hopeless and that things cannot improve

C. Sense of Failure

0 I do not feel like a failure
1 I feel I have failed more than the average person
2a I feel I have accomplished very little that is worthwhile or that means anything
2b As I look back on my life all I can see is a lot of failures
3 I feel I am a complete failure as a person (parent, husband, wife)

D. Lack of Satisfaction

0 I am not particularly dissatisfied
1a I feel bored most of the time
1b I don't enjoy things the way I used to
2 I don't get satisfaction out of anything any more
3 I am dissatisfied with everything

E. Guilty Feeling

0 I don't feel particularly guilty
1 1 feel bad or unworthy a good part of the time
2a I feel quite guilty
2a I feel bad or unworthy practically all the time now
3 I feel as though I am very bad or worthless

F. Sense of Punishment

0 I don't feel I am being punished
1 I have a feeling that something bad may happen to me
2 I feel I am being punished or will be punished
3a I feel I deserve to be punished
3b I want to be punished

G. Self-Hate

0 I don't feel disappointed in myself
1a I am disappointed in myself
1b I don't like myself
2 1 am disgusted with myself
3 I hate myself

H. Self- Accusations

0 I don't feel I am any worse than anybody else
1 I am very critical of myself for weaknesses or mistakes
2a I blame myself for everything that goes wrong
2b I feel I have many bad faults

I. Self- Punitive Wishes

0 I don't have any thoughts of harming myself
1 I have thoughts of harming myself but I would not carry them out
2a I feel I would be better off dead
2b I have definite plans about committing suicide
2c I feel my family would be better off if 1 were dead
3 I would kill myself if I could

J. Crying Spells

0 I don’t cry any more than usual
1 I cry more now than I used to
2 I cry all the time now. I can't stop it
3 I used to be able to cry but now I can't cry at all even though 1 want to

K. Irritability

0 I am no more irritated now than I ever am
1 I get annoyed or irritated more easily than I used to
2 I feel irritated all the time
3 I don't get irritated at all at the things that used to irritate me

L. Social Withdrawal

0 I have not lost interest in other people
1 I am less interested in other people now than I used to be
2 I have lost most of my interest in other people and have little feeling for them
3 I have lost all my interest in other people and don't care about them at all

M. Indecisiveness

0 I make decisions about as well as ever
1 I am less sure of myself now and try to put off making decisions
2 I can't make decisions any more without help
3 I can't make any decisions at all any more

N. Body Image

0 I don't feel I look any worse than I used to
1 I am worried that I am looking old or unattractive
2 I feel that there are permanent changes in my appearance and they make me look unattractive

3 I feel that I am ugly or repulsive looking

O. Work Inhibition

0 I can work about as well as before
1a It takes extra effort to get started at doing something
1b I don't work as well as I used to
2 I have to push myself very hard to do anything
3 I cannot do any work at all

P. Sleep Disturbance

0 I can sleep as well as usual
1 I wake up more tired in the morning than I used to
2 I wake up 1-2 hours earlier than usual and find it hard to get back to sleep
3 I wake up early every day and can't get more than 5 hours sleep

Q. Fatigability

0 I don't get any more tired than usual
1 I get tired more easily than I used to
2 I get tired from doing anything
3 I get too tired to do anything

R. Loss of Appetite

0 My appetite is no worse than usual
1 My appetite is not as good as it used to be
2 My appetite is much worse now
3 I have no appetite at all any more

S. Weight Loss

0 I haven't lost much weight, if any, lately
1 I have lost more than 5 pounds
2 I have lost more than 10 pounds
3 I have lost more than 15 pounds

T. Somatic Preoccupation

0 I am no more concerned about my health than usual
1 I am concerned about aches and pains or upset stomach or constipation or other unpleasant feelings in my body
2 I am so concerned with how I feel or what I feel that it's hard to think of much else
3 I am completely absorbed in what I feel

U. Loss of Libido

0 I have not noticed any recent change in my interest in sex
1 I am less interested in sex than I used to be
2 I am much less interested in sex now
3 I have lost interest in sex completely

Thank you...
All information provided will be kept strictly confidential and used only for the purpose of this study

Appendix E. Eating Attitudes Test-26 (Garner *et al.*, 1982)

Please choose one response by marking a check to the right for each of the following statements:		**Always**	**Very often**	**Often**	**Sometimes**	**Rarely**	**Never**
1	Am terrified about being overweight						
2	Avoid eating when I am hungry						
3	Find myself preoccupied with food						
4	Have gone on eating binges where I feel that I may not be able to stop.						
5	Cut my food into small pieces.						
6	Aware of the calorie content of foods that I eat						
7	Particularly avoid food with a high carbohydrate content (i.e. bread, rice, potatoes, etc.)						
8	Feel that others would prefer if I ate more.						
9	Vomit after I have eaten.						
10	Feel extremely guilty after eating.						
11	Am occupied with a desire to be thinner						
12	Think about burning up calories when I exercise						
13	Other people think that I am too thin.						
14	Am preoccupied with the thought of having fat on my body						
15	Take longer than others to eat my meals						
16	Avoid foods with sugar in them.						
17	Eat diet foods.						
18	Feel that food controls my life						
19	Display self-control around food.						
20	Feel that others pressure me to eat						
21	Give too much time and thought to food						
22	Feel uncomfortable after eating sweets						
23	Engage in dieting behaviour.						
24	Like my stomach to be empty.						
25	Have the impulse to vomit after meals.						
26	Enjoy trying new rich foods.						

Appendix F. International Physical Activity Questionnaire

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (August 2002)

SHORT LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health–related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is supported to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an ***International Physical Activity Prevalence Study*** is in progress. For further information see the IPAQ website.

More Information

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective*. Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

   _____ **days per week**

   ☐ No vigorous physical activities → ***Skip to question 3***

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?

   _____ **hours per day**

   _____ **minutes per day**

   ☐ Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

   _____ **days per week**

   ☐ No moderate physical activities → ***Skip to question 5***

SHORT LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised August 2002.

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

_____ **days per week**

☐ No walking → ***Skip to question 7***

6. How much time did you usually spend **walking** on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

This is the end of the questionnaire, thank you for participating.

SHORT LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised August 2002.

Appendix G. Three-Day Food Diary

3-Day Food Diary

(Household Measures)

Centre: David Greenfield Human Physiology Unit (B Floor, Medical School)

Participant ID: _____

Group: ˙**5:2 IER** ˙**CER** (please circle)

Please complete your diary on the following days:

Day 1……(e.g. Thursday) Date……

Day 2…….. Date……

Day 3…….. Date……..

Please complete the dairy and bring it back on the next visit

Please try to consume your regular diet on the days you complete the diary!

It is really important that you try to accurately report the time and quantity of food + drink consumption, as these are essential components of our study.

For office use only

Diary checked by:	**Date:**
Diary coded by:	**Date:**
Diary entered by:	**Date:**
Computer software used:	**Version of software:**

INSTRUCTIONS FOR FILLING IN YOUR FOOD DIARY

- Please keep a food diary for the 3-day period shown on the front cover. Please include **two consecutive week days plus a weekend day** (e.g. Thursday, Friday Saturday OR Sunday, Monday, Tuesday). The record should include as much detail as possible. Please start a new page each day (ensure the day and date is recorded at the top of the page). Please record everything at the time, **not** from memory at the end of the day.

- Fill in the food diary each time you consume a drink, a meal or a snack. Please note the time, and describe everything you eat or drink. Use a further page if you need more space. (Please remember to record the day and date at the top of the new page).

- When you record a food or drink, please give a thorough description and include any Brand names. You cannot give us too much detail! For example:-
  - Was the bread white/wholemeal/granary
  - Did you have a baguette/cob/pitta/slice?
  - Did you use butter or margarine, was the margarine made with olive/sunflower oil?
  - Was the product a diet product- e.g. sugar-free lemonade?
  - Was anything added- e.g. sugar to tea, butter to toast, tomato ketchup or mayonnaise etc?
  - Do you use full fat/semi-skimmed/skimmed milk?
  - How did you cook your food – eg. boiled/fried/baked/raw?
  - How was the food processed – eg. fresh/tinned/frozen?
  - Was the product Heinz/Tesco's own etc.?

- The amounts you have should be described **as fully as possible** using household measures
  - Use kitchen equipment – a measuring jug, a mug (of know volume), a teaspoon (see below) and a tablespoon


a level teaspoon


a rounded teaspoon


a heaped teaspoon

  - **Look on cans and packets to see whether the weight/volume is given and describe how much of the packet you had (eg. Half a 400g can of Heinz tomato soup)**
  - Record the number of sausages/fish fingers/bacon rashers etc. that you ate
  - Describe the size of a piece of food – eg. egg sized potato, small matchbox sized piece of cheese

- Please record each item of food separately. If you drink white tea with sugar, please record the type and amount of tea, milk and sugar separately.

- If you had a packed lunch, each part must be recorded in the diary.

- It is important that you complete the **'leftovers'** section with anything you did not actually eat. Please give us as much detail as possible

- For homemade recipes, the **raw ingredients** should be recorded in the back of the diary in the 'Recipes' section. Please tell us what the meal is composed of and how many it served. You can then put an entry in the diary such as "one quarter of the spaghetti Bolognese made with recipe 1".

We understand that it will take you some time to complete this diary carefully. However, it is essential that you record everything as precisely as possible.

AN EXAMPLE OF HOW TO COMPLETE YOUR FOOD DIARY AND RECIPE PAGE IS SHOWN ON THE NEXT PAGE

Participant ID -Example Day – 1, Wednesday Date - 01/10/13

Time	Type of food or drink with brand names and cooking method	Amount	Food or drink left over and amount	Office use only: code and weight
7.30am	Beans on toast:			
	Heinz baked beans (reduced sugar and salt)	3 heaped tablespoons		
	White toast (Warburtons)	2 thick slices		
	Flora spread	2 heaped teaspoons		
	Cup of tea:			
	PG tips tea	300ml		
	Semi-skimmed milk	50ml		
	Sugar	1 teaspoon		
10.30am	Dairy Milk chocolate	40g bar		
	Diet coke	330ml can		
12.45pm	Ham sandwich:			
	Ham (Tesco Honey Roast)	3 thin slices	¼ of a slice	
	White bread (Warburtons)	2 thick slices		
	Flora spread	2 heaped teaspoons		
	Tomato	½ beef tomato		
	Cup of tea (as above)	300ml		
	Walkers Salt & Vinegar crisps	30g bag		
	Granny Smith apple	medium		
7.15pm	1/4 of spaghetti Bolognese made using recipe 1			
	John Smiths beer	2 pints		
	Mr Kipling Bramley Apple pie	2 individual pies		
9.00pm	Hot chocolate made with semi- skim milk	2 teaspoons chocolate/ ½ pint of milk		

Participant ID……Example……

Recipe no: 1	**Dates used: …01. 10.13 ……..**	**Serves: 4** **Please also indicate on food diary the proportion of recipe you had on each occaision**	**Name of recipe: Spaghetti Bolognese**
Ingredient (with brand names and cooking method)		**Amount**	**Comments**
Garlic		1 clove	Fried in the sunflower oil
Onion		1 medium	Fried in sunflower oil
Sunflower oil		2 teaspoons	
Sainsbury's organic lean minced beef (uncooked weight)		500g	
Ragu original smooth bolognese sauce		454g jar	
Dried spaghetti		250g	

Participant ID	Day – ,	Date -		
Time	**Type of food or drink with brand names and cooking method**	**Amount**	**Food or drink left over and amount**	**Office use only: code and weight**

Participant ID……

Recipe no: 1	Dates used: …….. ……...	Serves: Please also indicate on food diary the proportion of recipe you had on each occaision	Name of recipe:
Ingredient (with brand names and cooking method)		Amount	Comments

Appendix H. Visual Analogue Scales (VAS)

Participant code _____ **Date** _____/_____/20____
Visit number _____ **Time point** _____

Please make a vertical mark through the horizontal line to show how you feel at the moment.
Left and right extremes represent minimum and maximum values respectively.

How hungry do you feel?

I am not hungry at all _____ I have never been more hungry

How satisfied do you feel?

I am completely empty _____ I cannot eat another bite

How full do you feel?

Not at all _____ Totally full

How strong is your desire to eat?

Very weak _____ Very strong

How much do you think you could eat?

Nothing at all _____ A lot

Appendix I. Physical Activity Plan

PAL=1.85

These are activity schedule examples with PAL values of 1.85 and 1.4 for a participant with a BMR value of 1647 kcal/day. All participants followed the same physical activity schedule but were prescribed with different daily step count targets due to the different walking patterns.

Time	Activity	Duration(h)	PAR	Duration*PAR	BMR: 1647kcal/ day = 68.6kcal/ hr
23:30-07:30	Sleeping	8.00	1.00	8.0	548.8
07:30-08:00	Personal care	0.50	2.30	1.2	78.9
08:00-08:30	Walking	0.50	3.20	1.6	109.8
08:30-09:00	Sitting	0.50	1.45	0.7	49.7
09:00-09:30	Breakfast	0.50	1.50	0.8	51.5
09:30-10:45	Sitting	1.25	1.45	1.8	124.3
10:45-11:00	Snack	0.25	1.50	0.4	25.7
11:00-13:00	Walking	2.00	3.20	6.4	439.0
13:00-13:30	Lunch	0.50	1.50	0.8	51.5
13:30-14:45	Sitting	1.25	1.45	1.8	124.3
14:45-15:00	Snack	0.25	1.50	0.4	25.7
15:00-16:30	Sitting	1.50	1.45	2.2	149.2
16:30-17:00	Walking	0.50	3.20	1.6	109.8
17:00-17:30	Dinner	0.50	1.50	0.8	51.5
17:30-19:00	Walking	1.50	3.20	4.8	329.3
19:00-19:15	Snack	0.25	1.50	0.4	25.7
19:15-19:30	Sitting	0.25	1.45	0.4	24.9
19:30-22:00	Walking	2.50	3.20	8.0	548.8
22:00-23:00	Sitting	1.00	1.45	1.5	99.5
23:00-23:30	Personal care	0.50	2.30	1.2	78.9
			Total	44.4	3046.7
			PAL	1.85	1.85

PAL=1.4

Time	Activity	Duration(h)	PAR	Duration*PAR	BMR: 1647kcal/ day = 68.6kcal/ hr
23:30-07:30	Sleeping	8.00	1.00	8.0	548.8
07:30-08:00	Personal care	0.50	2.30	1.2	78.9
08:00-08:30	Public transportation (Taxi, tram or bus)	0.50	1.20	0.6	41.2
08:30-09:00	Sitting	0.50	1.45	0.7	49.7
09:00-09:30	Breakfast	0.50	1.50	0.8	51.5
09:30-10:45	Sitting	1.25	1.45	1.8	124.3
10:45-11:00	Snack	0.25	1.50	0.4	25.7
11:00-13:00	Sitting	2.00	1.45	2.9	198.9
13:00-13:30	Lunch	0.50	1.50	0.8	51.5
13:30-14:45	Sitting	1.25	1.45	1.8	124.3
14:45-15:00	Snack	0.25	1.50	0.4	25.7
15:00-16:30	Sitting	1.50	1.45	2.2	149.2
16:30-17:00	Walking	0.50	3.20	1.6	109.8
17:00-17:30	Dinner	0.50	1.50	0.8	51.5
17:30-19:00	Sitting	1.50	1.45	2.2	149.2
19:00-19:15	Snack	0.25	1.50	0.4	25.7
19:15-19:30	Sitting	0.25	1.45	0.4	24.9
19:30-19:50	Walking	0.33	3.20	1.1	73.2
19:50-23:00	Sitting	3.17	1.45	4.6	314.9
23:00-23:30	Personal care	0.50	2.30	1.2	78.9
			Total	33.5	2297.7
			PAL	1.40	1.40