Metabolic Effects of Intermittent Fasting

By

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Abstract

Intermittent fasting describes dietary strategies in which the pattern of energy restriction (intermittent energy restriction, IER) or timing of food intake (time-restricted feeding, TRF) are altered such that individuals undergo repeated periods of “fasting”. The overarching aim of this PhD project was to investigate the metabolic health impacts of these intermittent fasting variants.

Intermittent energy restriction

Study one assessed the acute metabolic effects of substantial energy restriction (ER) in healthy, overweight/obese participants using a cross-over design. Six-hour postprandial responses were assessed the morning following one day of total 100% ER, partial 75% ER and isoenergetic intake (0% ER) via serial blood sampling and indirect calorimetry. Postprandial substrate oxidation was shifted towards fat oxidation (p=0.080) and ketogenesis (p<0.001) in an apparent dose response manner following 75-100% ER, translating to a reduction in postprandial lipaemia (p<0.001). Conversely, glucose tolerance was impaired (p=0.002).

Study two utilised similar methods to investigate the chronic effect of IER (75% ER for two days/week) on postprandial metabolism following 5% weight-loss. This was compared to matched weight-loss achieved via a “standard treatment” control of continuous ER (2510kJ/day deficit). Rates of weight-loss were similar between groups (p=0.446), despite greater reported reductions in energy intake during IER (p=0.012), which might be explained in part by an adaptive decline in resting energy expenditure (p=0.067). Both interventions comparatively (p=0.903) improved postprandial insulinaemia, whereas the relative reduction in postprandial lipaemia was greater following IER (p=0.042).

Time-restricted feeding

Study three examined the effects of a 10-week, three-hour daily shortening of the eating window on fasting metabolism and adiposity utilising a parallel-armed controlled design. In a small group of lean and overweight/obese participants, TRF led to modest reductions in adiposity (p=0.047)
and fasting glycaemia (p=0.073), possibly explained by the spontaneous reduction in energy intake observed.

Combined, these data provide novel insights into the metabolic effects of intermittent fasting. Replication and mechanistic evaluation in diverse population groups, including those with established metabolic disorders, is warranted.
Published works

Research articles


- **Antoni R**, Johnston KL, Collins AL and Robertson MD (2016) Investigation into the acute effects of total and partial energy restriction on postprandial metabolism amongst overweight/obese participants. *Br J Nutr.* 115 (6), Pg 951-959


Conference abstracts


- **Antoni R**, Johnston KL, Collins AL and Robertson MD (2015) Investigation into the acute effects of intermittent energy restriction on postprandial substrate metabolism *Proc Nutr Soc.* 75 (OCE1), E29

Declaration

This thesis and the work to which it refers are the results of my own efforts. Any ideas, data, images or text resulting from the work of others (whether published or unpublished) are fully identified as such within the work and attributed to their originator in the text, bibliography or in footnotes. This thesis has not been submitted in whole or in part for any other academic degree or professional qualification. I agree that the University has the right to submit my work to the plagiarism detection service TurnitinUK for originality checks. Whether or not drafts have been so-assessed, the University reserves the right to require an electronic version of the final document (as submitted) for assessment as above. The thesis is available for Library use on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement or consent.
Acknowledgements

I am indebted to Dr Denise Robertson and Dr Adam Collins, for believing in me enough to support me with my initial PhD application. In addition, I would also like to thank them for all of the support and encouragement they have given me during my PhD.

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This PhD would not have been possible without my participants, who selflessly gave up their time to take part in my research. Thank you also to the staff at the Surrey Clinical Research Centre, for their medical assistance during my study days, I could not have done it without you.

The biggest thanks goes to my family: to my parents, for the enormous sacrifices they made which ultimately enabled me to do a PhD. Stewart, my partner, thank you for supporting and encouraging me throughout this journey.
# Statement of Contributions

<table>
<thead>
<tr>
<th>Personnel</th>
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Postdoctoral Research Fellow, University of Surrey | Performed ELISA assay in conjunction with PhD researcher. Assisted with study days on time-restricted feeding project. |
| Research nurses  
Surrey Clinical Research Centre | Assisted with blood sampling |
| Leila Finikarides  
British Broadcasting Company | Conducted participant recruitment for time-restricted feeding project |
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<tr>
<td>3-OHB</td>
<td>3-hydroxybutyrate</td>
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<tr>
<td>ADF</td>
<td>Alternate day fasting</td>
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<td>ALP</td>
<td>Atherogenic lipoprotein phenotype</td>
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<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>ANCOVA</td>
<td>Analysis of covariance</td>
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<td>APO</td>
<td>Apolipoprotein</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<td>BMI</td>
<td>Body mass index</td>
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<td>BMR</td>
<td>Basal metabolic rate</td>
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<td>BP</td>
<td>Blood pressure</td>
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<td>CER</td>
<td>Continuous energy restriction</td>
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<tr>
<td>CETP</td>
<td>Cholesteryl ester transfer protein</td>
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<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
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<tr>
<td>CHO</td>
<td>Carbohydrate</td>
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<td>CREB</td>
<td>cAMP response element binding protein</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CV</td>
<td>Co-efficient of variation</td>
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<td>Cardiovascular disease</td>
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<td>DXA</td>
<td>Dual-energy x-ray absorptiometry</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>EMM</td>
<td>Estimated marginal means</td>
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<td>ER</td>
<td>Energy restriction</td>
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<td>ESS</td>
<td>Epworth sleepiness scale</td>
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<td>Female</td>
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<td>FAO</td>
<td>Fatty acid oxidation</td>
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<td>Fat free mass</td>
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<td>GLM</td>
<td>General linear model</td>
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<td>Glucose transporter</td>
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<td>Glucose tolerance test</td>
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<td>HbA1c</td>
<td>Glycated haemoglobin</td>
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<td>HDL</td>
<td>High density lipoprotein</td>
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<td>HEC</td>
<td>Hyperinsulinaemic euglycaemic clamp</td>
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<tr>
<td>HL</td>
<td>Hepatic lipase</td>
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<tr>
<td>HOMA-%B</td>
<td>Homeostatic model assessment of steady state β-cell function</td>
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<tr>
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<tr>
<td>HOMA-IR</td>
<td>Homeostatic model assessment of insulin resistance</td>
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<td>HSL</td>
<td>Hormone sensitive lipase</td>
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<tr>
<td>iAUC</td>
<td>Incremental area under the curve</td>
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<td>Intermediate density lipoprotein</td>
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<td>IER</td>
<td>Intermittent energy restriction</td>
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<td>IHCL</td>
<td>Intra-hepatocellular lipid</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>IR</td>
<td>Insulin resistance</td>
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<tr>
<td>ITT</td>
<td>Insulin tolerance test</td>
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<td>LDL</td>
<td>Low density lipoprotein</td>
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<td>Lipoprotein lipase</td>
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<td>LRP</td>
<td>LDL-receptor related protein</td>
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<td>M</td>
<td>Male</td>
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<tr>
<td>MAM</td>
<td>Metabolically active mass</td>
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<tr>
<td>MIT</td>
<td>Meal induced thermogenesis</td>
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<tr>
<td>mTOR</td>
<td>Mechanistic target of rapamycin</td>
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<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
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<tr>
<td>NICE</td>
<td>National institute of clinical excellence</td>
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<td>NS</td>
<td>Non-significant</td>
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<td>OGTT</td>
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<td>Plasminogen activator inhibitor-1</td>
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<td>Physical activity level</td>
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<td>Positive affect negative affect scale</td>
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<td>Power of food scale</td>
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<td>Pittsburgh sleep quality index</td>
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<td>REE</td>
<td>Resting energy expenditure</td>
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<td>RIA</td>
<td>Radioimmunoassay</td>
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<td>RQ</td>
<td>Respiratory quotient</td>
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<td>RQUICKI</td>
<td>Revised quantitative insulin sensitivity check index</td>
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<td>SAT</td>
<td>Subcutaneous adipose tissue</td>
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<tr>
<td>SCN</td>
<td>Suprachiasmatic nuclei</td>
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<td>Sd</td>
<td>Small dense</td>
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<td>SD</td>
<td>Standard deviation</td>
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<td>Standard error of the mean</td>
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<td>Sensitivity index</td>
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<td>T</td>
<td>Time point</td>
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<td>T2DM</td>
<td>Type 2 diabetes</td>
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<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
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<td>Tumour necrosis factor α</td>
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<td>TOTC</td>
<td>Total cholesterol</td>
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<td>Trend</td>
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<td>TRL</td>
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<td>Visual analogue scale</td>
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<td>Very low density lipoprotein</td>
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<td>Very low energy diet</td>
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Chapter 1 : Introduction

1.1 Brief introduction

*Obesity, type 2 diabetes and cardiovascular disease*

The worldwide prevalence of overweight (body mass index [BMI] 25-29.9 kg/m$^2$) and obesity (BMI $\geq 30$ kg/m$^2$) has more than doubled in recent decades (1). The development of overweight/obesity is closely associated with a number of inter-related metabolic complications which can in turn increase an individual’s cardiometabolic risk i.e. risk of type 2 diabetes (T2DM) and cardiovascular disease (CVD) (2), whose prevalence rates are rising in congruence with weight trends. CVD, which encompasses coronary heart disease (CHD) and stroke, is the leading cause of mortality worldwide, responsible for approximately three out of every 10 deaths (3). T2DM contributes to 1.5 million deaths per annum (4), whilst sub-optimal glycaemic control itself, even below the diagnostic threshold for diabetes, can also increase future risk of CVD (5). Dyslipidaemia frequently accompanies (pre-) diabetic dysglycaemia (6), and is considered among the most important modifiable risk factors for CVD risk (7). Dietary energy restriction (ER) and weight-management are an integral part of reducing cardiometabolic risk (8). However, when confronted with an obesogenic environment favouring sedentary lifestyles and passive around the clock food consumption, successful behaviour change becomes notoriously difficult to achieve and maintain.

*Intermittent fasting and scope of this PhD*

In recent years, several intermittent fasting variants including intermittent energy restriction (IER) and time-restricted feeding (TRF) have received considerable interest as dietary strategies for weight-management and reducing cardiometabolic risk. With these dietary strategies, the pattern of ER and/or timing of food intake are altered so that individuals undergo frequently repeated periods of fasting (*Table 1.1*). Their basic premise is that an individual may not need to energy restrict every day, or potentially at all, to attain metabolic benefits.
### Table 1.1 Overview of intermittent fasting protocols.

“Fasting” used to denote periods of substantial (≥70%) energy restriction. IER protocols involving more modest energy restriction, or infrequent energy restriction/refeed cycles not included in review.

*Abbreviations:* ADF – Alternate day fasting, TRF – Time-restricted feeding.

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The three individual studies which comprise this PhD project were designed to gain a greater understanding of the metabolic effects of these intermittent fasting protocols, with a specific emphasis on cardiometabolic risk factors.

Studies one and two predominately focused on the effects of IER on postprandial glucose and lipid metabolism, in addition to how this compares to weight-loss achieved via modest, continuous ER (CER). Factors relating to body weight regulation were also assessed. The first section of this introductory chapter therefore discusses the background relating to obesity and the development of its associated metabolic disorders, as well as the contribution of postprandial events to the pathogenesis of T2DM and CVD. This will be followed by a summary of the current IER literature relating to the research projects.

The second section of the introductory chapter presents an overview of the rodent and human TRF literature, and discusses the relative impact of fasting and ER to the already established metabolic effects of TRF. Given the scarcity of human data, Study three served as a pilot study, the findings of which will form the basis for future more detailed metabolic studies.
1.2 Development of obesity associated cardiometabolic disorders

Body weight is a function of the balance between energy intake and expenditure with prolonged imbalance between the two factors resulting in net weight gain or loss; today’s obesogenic environment has unfortunately shifted the balance in favour of the former. However, not all overweight/obese individuals succumb to its associated metabolic disorders. What determines an individual’s eventual metabolic fate involves a complex interplay between an array of genetic and environmental (diet and lifestyle) factors that we are still only beginning to unravel. One key driver appears to be the pathological response of adipose tissue to positive energy imbalance among predisposed individuals (termed “adiposopathy”), as opposed to excess accumulation of adipose tissue per se (9).

1.2.1 Adipose tissue response to positive energy imbalance

As reviewed by Bays (9), adipose tissue is not only an inert fat storage depot, but also a highly active secretory organ. Adipocytes and other cells types contained within adipose tissue, including immune cells, secrete an array of protein factors (adipokines) which are capable of influencing whole-body metabolism. In the face of positive energy balance, adipose tissue must undergo co-ordinated tissue expansion including the initial hypertrophy (increased size) of adipocytes, which is then proceeded by an increase in adipocyte number (hyperplasia). If this continues unencumbered, then increased adiposity may not lead to demonstrable adverse metabolic consequences.

Among predisposed individuals however, “pathogenic” adipose tissue growth may ensue, the manifestations of which can include: excessive adipocyte hypertrophy, reflecting a failure of adequate proliferation and/or differentiation of adipocytes; increased macrophage infiltration, resulting in low grade inflammation and altered adipokine secretion; impaired lipid storage within peripheral subcutaneous adipose tissue (SAT); and visceral adipose tissue (VAT) accumulation. The net result is the development of an unfavourable metabolic and hormonal milieu which drives the development of systemic insulin resistance (IR) and its associated disorders, ultimately
favouring atherogenesis and pancreatic β-cell dysfunction (9). A general overview of the variety of pathways leading from positive energy imbalance through to the development of T2DM and CVD is presented in Figure 1.1.
Figure 1.1 Pathways from positive energy imbalance to cardiovascular disease and type 2 diabetes

A) Pathological expansion of SAT: ↑macrophage infiltration, ↑inflammation, altered adipokine secretion (9); impaired lipid storage/clearance, leading to ↑fatty acid delivery to non-adipose tissues, driving IR (9, 11). VAT: fatty acids and inflammatory mediators drain to liver via portal vein (15). B) In the liver, IR and ↑IHCL (exacerbated by ↑VAT (15), ↑insulin* (212), ↑chylomicron remnant uptake (11)) may ↑VLDL-TAG secretion and ↑gluconeogenesis: ↑glucose levels (212) and ↑TRL, which favours formation of ALP (27) and may lead to ectopic fat deposition (212). Hepatic production of pro-inflammatory/thrombotic factors (CRP, fibrinogen) may also ↑ (9). C) Skeletal muscle IR will ↓insulin-stimulated glucose disposal. D) Four progressive stages of IR (48): i) ↑postprandial insulin*; ii) ↑postprandial insulin and glucose; iii) ↑fasting insulin and glucose; and iv) overt type 2 diabetes. E) Altered adipokine secretion, ↑glucose, ↑insulin and ALP compromise vascular health and create pro-thrombotic environment (9). Cardiovascular disease may result. F) ↑Insulin may ↑blood pressure.

Abbreviations: ALP – Atherogenic lipoprotein profile; Apo – Apolipoprotein; CRP – C-reactive protein; HDL – High density lipoprotein; IHCL – Intra-hepatocellular lipid; IL-6 – Interleukin-6; IR – Insulin resistance; LDL – Low density lipoprotein; PAI-1 – Plasminogen activator inhibitor-1; SAT – Subcutaneous adipose tissue; TAG – Triacylglycerol; TNFa – Tumour necrosis factor α; TRL – Triacylglycerol-rich lipoprotein; VAT – Visceral adipose tissue; VLDL: Very low density lipoprotein.
1.2.2 Insulin resistance: a common aetiological factor underlying cardiometabolic risk

IR can be defined as state in which a given concentration of insulin produces a less-than-expected biological effect, resulting in disruptions in glucose homeostasis that can precede the development of T2DM by many years (10). IR can occur systemically in a number of tissues including adipose tissue, skeletal muscle and the liver (10). Ectopic fat deposition which may arise from increased dietary intake, impaired adipose tissue lipid storage and/or decreased fat oxidation, is implicated as a key instigator for the development of IR in non-adipose organs as well as pancreatic β-cell dysfunction ((9-12); Figure 1.1). In addition to disrupting glucose homeostasis, IR also contributes to the development of dyslipidaemia and hypertension (Figure 1.1). Over the years, this common clustering between obesity, IR and its associated metabolic disturbances which predispose to the development of T2DM and CVD has become known by a variety of names including syndrome X, IR syndrome, metabolic syndrome and the cardiometabolic syndrome (6).

1.2.3 Subcutaneous and visceral adipose tissue depots

SAT, i.e. adipose tissue found beneath the skin, is subdivided into upper body (abdominal) and lower body (including gluteal and femoral) depots (9). Upper-body SAT is the main site of storage and release of non-esterified fatty acids (NEFA) to the systemic circulation and as such is thought to exert the greatest impact on peripheral insulin sensitivity (9, 13). Whilst elevated circulating NEFA levels are frequently implicated in the development of obesity associated IR, this may not always be a universal feature in the early stages of obesity, whereby increased adipose tissue mass may be offset by a down-regulation in lipolysis (11). Interestingly, a recent study by Mcquaid et al (11) observed a marked depression in abdominal SAT meal fat clearance, coupled with a failure to up-regulate clearance with sequential meals. This failure in appropriate lipid storage may instead provide the pathological basis for ectopic fat deposition, although, it is clear when comparing individuals at the extremes of the BMI range that the apparent down-regulation in lipolysis cannot always fully compensate for the associated increase in adipose tissue mass (14). VAT surrounds internal organs, and is composed in part of mesenteric and omental (greater and lesser) depots (9). Compared to SAT, VAT exhibits higher levels of adipocyte hypertrophy and
immune cell infiltration (9). Whilst only contributing to approximately 15% of the total systemic NEFA, its anatomical proximity and portal drainage to the liver, coupled with high metabolic (particularly lipolytic) activity implicates it as a key driver for hepatic steatosis, hepatic IR and associated dyslipidaemia (13, 15). Owed in part to its increased sensitivity to mediators of lipolysis, VAT is preferentially lost with modest weight loss although this is the effect is attenuated with greater weight-loss (16).

1.2.4 Non-lipid mediators of insulin resistance

Considerations must also be placed on other non-lipid mediators of IR (Figure 1.1). Hypertrophic adipocyte growth is associated with increased inflammatory cell infiltration and altered adipokine secretion patterns, with the development of adipocyte hypoxia proposed as one potential unifying mechanism underlying these observations (17). The production of pro-inflammatory mediators including increased interleukin-1 (IL-1), IL-6, and tumour necrosis factor alpha (TNF-α) by immune cells are increased, which are implicated causally in the development of both localised and systemic IR (9). In addition, circulating adipocyte-derived leptin levels are also elevated as adipose tissue mass increases, which exhibits pro-inflammatory and atherogenic properties (9). Conversely, expression of other adipocyte-derived adipokines such as adiponectin, which exerts insulin sensitising effects in the liver and skeletal muscle, are found to be reduced in obesity (9).

1.3 The postprandial state and relevance to disease

Whilst the clinical utility of fasting measures of glucose and lipid are undeniable, they only provide a steady-state snapshot of the metabolic situation during a relatively small proportion of the day. Based on typical Western dietary eating patterns characterized by successive meal intake, individuals spend approximately three quarters of the day in the postprandial state which is a dynamic, non-steady state condition (18). Impairments in postprandial handling of glucose and lipids can be present long before overt T2DM and CVD with the development of IR, commonly observed in the overweight/obese state, a key aetiological factor underlying both. The following section discusses the physiology and pathophysiology of postprandial glucose and lipid
metabolism, and its role in the development of T2DM and CVD.

1.3.1 Transport and tissue uptake of glucose and lipids

Numerous metabolic processes are involved in the effective transport and uptake of exogenously and endogenously derived glucose and lipids in the body. Glucose is freely transported in the aqueous plasma to target tissues, where its uptake occurs via facilitated diffusion mediated by a family of glucose transporters (GLUT) that display a tissue specific pattern of expression (19). In contrast, the transport of lipids including TAG, cholesterol esters and NEFA is relatively more complex, and requires specialised transport mechanisms owed to their hydrophobic properties (20). NEFA circulate bound to albumin and their uptake facilitated by membrane-associated fatty acid-binding proteins, whereas the transport of cholesterol and TAG occurs in specialised macromolecular structures called lipoproteins (20). Lipoproteins are comprised of a hydrophilic outer surface of amphipathic phospholipids and free cholesterol, with TAG and cholesterol esters contained within its hydrophobic core (20). Based on their major lipid composition, lipoproteins can be classified into either TAG-rich lipoproteins (TRL), which encompass chylomicrons and very low density lipoproteins (VLDL); or cholesterol-rich lipoproteins, low density lipoprotein (LDL) and high density lipoprotein (HDL) (20).

The exogenous lipid pathway (Figure 1.2A) describes the metabolism of chylomicrons and lipids (mostly TAG) sourced from the diet. The endogenous lipid pathway (Figure 1.2B) describes the re-distribution of TAG from the liver to other tissues via the secretion of VLDL (20). Within the liver, fatty acids for TAG synthesis are supplied via a number of sources: (i) hepatic uptake of TRL remnants formed during TRL lipolysis; (ii) uptake of NEFA, sourced from adipose tissue or which have “spilled over” during TRL lipolysis; (iii) TAG stored within the hepatocyte (intra-hepatocellular lipid [IHCL]); (iv) and from de novo lipogenesis (21). The relative contribution of each of these pathways varies between postprandial and post-absorptive states (21), and the overall secretion rate is determined by the balance between their oxidation and re-esterification within the liver (20).
A) The exogenous lipid pathway describes the metabolism of chylomicrons and lipids (mostly TAG) sourced from the diet. Chylomicrons are secreted into the lymphatic system, where they enter the circulation via the thoracic duct. The stepwise hydrolysis of chylomicrons by LPL, which resides on the capillary endothelium (most notably muscle and adipose tissue), leads to the formation of chylomicron remnants which are rapidly removed from the circulation by the LDL receptor or the LRP.

B) The endogenous lipid pathway describes the re-distribution of TAG from the liver to other tissues via the secretion of VLDL. VLDL particles undergo progressive hydrolysis by LPL in an analogous fashion to chylomicrons to form IDL, which can be removed from the circulation via binding to LDL receptors. The remaining IDL particles undergo further processing by LPL and HL to form cholesteryl-rich LDL particles. Approximately 70% of circulating LDL is cleared via hepatocyte LDL-receptor mediated endocytosis, with the remainder taken up by extrahepatic tissues. Figure adapted from Frayn et al (19).

Abbreviations: FA – Fatty acids; HL – Hepatic lipase; IDL – Intermediate density lipoprotein; LDL – Low density lipoprotein; LPL – Lipoprotein lipase; LRP – Low density lipoprotein related protein; TAG – Triacylglycerol; VLDL – Very low density lipoprotein.
Interactions between lipoproteins, enzymes and receptors within the lipoprotein pathways are facilitated by apolipoproteins (Apo) which are either continually exchanged between lipoproteins or (in the case of ApoB48 and Apo100) are integral to the lipoprotein particle (20). Lipoproteins are essentially lipid emulsions which permit transport of hydrophobic lipids within the aqueous plasma to sites of utilisation (e.g. for oxidation in skeletal muscle) and storage (e.g. adipose tissue) (20). This process is facilitated by lipoprotein lipase (LPL), which is considered to be the rate-limiting enzyme in TRL clearance (20). LPL is expressed in a number of tissues, most notably muscle and adipose tissue (22). LPL is produced within the cells of these tissues and subsequently exported to the capillaries, where it found bound non-covalently to glycosaminoglycan chains (20). LPL acts on TRLs as they pass through capillaries, leading to the stepwise hydrolysis of TAG contained within the lipoprotein particle. Relative levels of LPL activity differ between tissues; as will be described later, this is acutely regulated by circulating insulin concentrations (23).

The stepwise hydrolysis of chylomicrons by LPL leads to the formation of chylomicron remnants which are rapidly removed from the circulation by the liver after binding to the LDL-receptor or the LDL-receptor related protein (24). VLDL particles undergo progressive lipolysis in an analogous fashion to chylomicrons, to form intermediate-density lipoprotein (IDL), which can also be removed from the circulation via LDL-receptor mediated endocytosis. However, while the vast majority of chylomicron remnants are rapidly cleared from the circulation by the liver, only a fraction (~50%) of IDL particles are cleared. The remaining IDL particles undergo further hydrolysis and remodelling by hepatic lipase to form cholesteryl-rich LDL particles. Approximately 70% of circulating LDL is cleared via hepatocyte LDL-receptor mediated endocytosis, with the remainder taken up by extrahepatic tissues (24, 25). Some LDL may be subject to oxidative modification and can be taken up via scavenger receptors expressed by macrophages; unlike the LDL receptor, this is not subject to feedback inhibition by intra-cellular free cholesterol levels, which has important implications in relation to the atherosclerotic disease process ((22); Section 1.3.4).
1.3.2 Post absorptive state

The term post absorptive refers to a state in which “most of the food from the previous meal has been fully absorbed but not much more time has passed”, e.g. after an overnight fast (20). During this time period, glucose levels are maintained by a combination of hepatic glycogenolysis and gluconeogenesis, processes which release glucose into the systemic circulation (19). Whole body metabolism is shifted in favour of fatty acid oxidation (FAO), facilitated by increased fatty acid availability coupled with reduced circulating levels of insulin (19, 26, 27). Fatty acids mobilised by adipose tissue are the predominant substrate for FAO in tissues able to utilise them as an energy source (e.g. skeletal muscle, liver), whilst a certain proportion will be re-exported by the liver as VLDL-TAG, which transports most of the circulating TAG in the overnight fasted state (21).

1.3.3 Meal ingestion

Ingestion of a meal and consequent influx of nutrients (particularly glucose and arginine) into the circulation directly stimulates the process of insulin secretion within the pancreatic β-cell, and indirectly via the stimulation of incretin hormone release by intestinal cells (28). This rise in insulin, which is biphasic in nature, is one of the most important regulatory mechanisms for glucose and lipid metabolism in the postprandial state. Insulin (and glucose) firstly inhibits the secretion of glucagon, from pancreatic α-cells which exerts antagonistic actions to insulin (19); the consequent increase in the insulin to glucagon ratio coupled with inflow of glucose into the liver via GLUT2 stimulates glycogen synthesis whilst suppressing endogenous glucose output (i.e. release of glucose from glycogen and from gluconeogenesis) which contributes to a reduction in postprandial glycaemia (19).

Insulin also stimulates GLUT4 mediated uptake of glucose within peripheral tissues, particularly skeletal muscle and adipose tissue (19). Within skeletal muscle, glucose is preferentially oxidized over fatty acids, whilst some glucose is directed towards glycogen synthesis (20). Within adipose tissue, glucose is metabolized anaerobically, releasing lactate into the blood which is an important precursor for hepatic gluconeogenesis; newly synthesized glucose is channeled towards storage
as glycogen in the postprandial state (20). Hence, metabolism is shifted towards the use of glucose over fatty acids, and for storage of glucose as glycogen (20). Insulin contributes to this through direct stimulation of enzymes pertaining to the glycolytic and glycogen biosynthetic pathways (19), and through indirect actions such as through its stimulation of malonyl coA synthesis (via activation of acetyl-coA carboxylase) which inhibits mitochondrial fatty acid transport and thus FAO (28). Insulin also inhibits hormone sensitive lipase (HSL) mediated mobilization of fatty acids from adipose tissue, therefore alleviating competitive inhibition of glucose oxidation by fatty acids (20, 26).

At the same time, dietary lipids are digested within the gut lumen, following which they are re-assembled and incorporation into chylomicron particles within the enterocyte. Chylomicrons are subsequently secreted into the lymphatic system, where they finally enter the circulation via the thoracic duct (20). Whilst a rise in glucose concentration is usually detected within 15 minutes after the consumption of a meal (peaking by 30-60 minutes), chylomicrons by contrast enter the circulation relatively slowly, with the primary peak in circulating TAG concentrations usually occurring three to four hours after a meal (20). As well as inhibiting HSL, insulin also stimulates esterification pathways within adipose tissue which creates a favourable concentration gradient for the passage of fatty acids into adipose tissue and promotes fat storage (20). Insulin stimulates LPL activity in adipose tissue while reducing its activity (albeit fairly weakly (20)) in skeletal muscle (23). These divergent tissue-specific LPL responses essentially direct lipoprotein TAG-derived fatty acids away from muscle and to adipose tissue for storage (23), although skeletal muscle can still make a substantive contribution towards postprandial TRL clearance (29).

As well as chylomicrons, postprandial TAG also enter the circulation via the hepatic secretion of VLDL-TAG, although this is initially reduced after food ingestion as a consequence of the insulin-mediated suppression of ApoB100 coupled with the decrease in fatty acid flux to the liver (20). TRLs essentially compete for a common saturable lipolytic pathway, and so their metabolic pathways impinge on one another in the postprandial state (20). This competition between
intestinal and hepatic TRL can account for the accumulation of these particles during the postprandial period. Owing to the morphological size (20), chylomicrons are preferentially cleared so that VLDL-particles contribute up to 80% of total TRL particles in the later postprandial period (30). After hydrolysis by LPL, fatty acids derived from the TAG core of chylomicron and VLDL particles are taken up and re-esterified in the peripheral tissues. Fatty acid entrapment within adipose tissue rises to ~70-90% within 60-90 minutes of a meal (31). By four to five hours (when TAG extraction is maximal), fatty acid entrapment declines to ~50%, resulting in increased spill-over of fatty acids in the circulation which are predominantly return to the liver (31). Increased hepatic fatty acid delivery via spill-over and TRL-remnant removal pathways may stimulate VLDL-TAG secretion in the postprandial period (11, 20). By approximately eight hours after a meal, macronutrients from the meal will have been fully absorbed from the gastrointestinal tract, and it is at this point that the body would then re-enter the post absorptive state. However, in reality this post absorptive state is usually only achieved overnight as we rarely go more than eight hours between meals at other times (28).

1.3.4 Postprandial metabolism as a marker of cardiometabolic disease risk

The concept of atherosclerosis being a postprandial phenomenon was first introduced over 20 years ago by Zilversmit (32). Subsequent to this landmark paper, there has been further evidence from large prospective cohort studies which has highlighted the prognostic value of elevated non-fasting (postprandial) TAG to CVD risk (33-35). Furthermore, increased postprandial TAG has been shown to predict the presence of coronary artery disease (36) and correlates with markers of atherosclerotic progression (37). T2DM and CVD are inherently linked, and a positive relationship between post-challenge blood glucose and CVD risk exists, even among non-T2DM populations (5).

1.3.4.1 Impaired postprandial lipid metabolism

Postprandial hypertriacylglycerolaemia is a common lipid abnormality in persons with obesity and/or IR, which arises due to the increased secretion of (and hence increased competition
between) TRL and/or their impaired clearance (11, 18, 22). Impaired clearance capacity of TRL by adipose tissue may additionally provide the pathophysiological basis for ectopic fat deposition and lipotoxicity (11). Fasting TAG concentrations are typically higher in obesity, which is largely attributable to enhanced secretion of VLDL-TAG (38). Baseline TAG levels are an important determinant of the magnitude of the postprandial response as evidenced by a number of studies demonstrating correlations between the two (39, 40). Importantly however, fasting TAG only accounts for some of the variation in the postprandial TAG response. Indeed, individuals with similar fasting levels of TAG (even within the healthy range) can differ markedly in their postprandial response to a meal challenge (41, 42) which may, with repeated meal ingestion, result in an upward drift in circulating TAG levels over the course of the day. In addition, the composition as well as the secretion of VLDL-TAG also appears altered in obesity/IR. For example, one study demonstrated that insulin resistant men derive a greater proportion of fatty acids from splanchnic sources (i.e. de novo lipogenesis, hepatic TAG and VAT) in the postprandial period when compared to BMI-matched insulin sensitive controls (43).

The net result is a prolonged residence time of TRLs and their partially hydrolysed remnants within the circulation, which become progressively cholesterol enriched via the action of cholesteryl ester transfer protein (CETP). CETP is a circulating plasma protein which facilitates the neutral lipid transfer of cholesteryl esters from LDL and HDL particles to TRLs in equimolar exchange for TAG (20). TRL and their cholesteryl enriched remnants can directly contribute to atherosclerotic processes (44, 45), and their increased concentrations after a meal also lead to transient impairments in endothelial dysfunction (18). In health, CETP mediated transfer between TRLs, LDL and HDL fulfils a vital cardioprotective role as it acts as an indirect pathway for the removal of cholesterol from peripheral tissues, including arterial lesions, via hepatic uptake of these cholesteryl ester enriched TRL-remnants (i.e. reverse cholesterol transport) (20). The rate of CETP mediated transfer is proportional to the concentration of TAG, and hence exaggerated and prolonged postprandial lipaemia will accelerate CETP exchange (20). TAG enrichment of LDL and HDL particles result in morphological changes which make them more susceptible to
metabolism by hepatic lipase. HDL and LDL are subsequently remodelled into small dense (Sd) particles. SdHDL are more prone to hepatic uptake hence the availability of HDL for cholesterol clearance is reduced, whereas sdLDL are particularly atherogenic due to their enhanced ability to penetrate and be retained in the sub-endothelial space, as well as their increased susceptibility to oxidation (46, 47). As discussed in the previous section, uptake of modified LDL via scavenger receptors presented on the surface of macrophages is not subject to feedback inhibition; excessive uptake can cause macrophages to become lipid-laden foam cells which may lead to atherosclerotic development (22). This combination of lipid abnormalities has been termed the atherogenic lipoprotein phenotype (ALP) (20).

1.3.4.2 Impaired postprandial glucose metabolism

Peripheral IR (e.g. in skeletal muscle) is among the first detectable defects in the pathway to T2DM, which is thought to proceed in four progressive stages: firstly, compensatory hyperinsulinaemia in response to food; secondly, hyperinsulinaemia and hyperglycaemia in response to food; thirdly, elevated fasting insulin and glucose levels; and ultimately, the development of T2DM (48). Hence, postprandial glucose and insulin levels increase earlier and faster than their respective fasting levels. The loss of first phase insulin secretion, the earliest manifestations of pancreatic β-cell dysfunction, results in diminished suppression of glucagon secretion, inappropriate glucose production in the liver and kidneys which when coupled with inefficient glucose uptake, leads to the development of postprandial hyperglycaemia (49). This may elicit delayed and excessive production of insulin via the second phase insulin response (49). Postprandial hyperglycaemia can evoke endothelial dysfunction (50), whilst hyperinsulinaemia itself may exacerbate IR via negative feedback inhibition with the insulin receptor (49) and contributes to the development of hypertension (2). The resultant elevated fatty acid and glucose fluxes can inhibit β-cell proliferation and trigger apoptosis (glucolipotoxicity), which over a chronic time-course may result in a gradual depletion of pancreatic β-cell mass (51). Overt hyperglycaemia occurs when there is a mismatch between β-cell secretory capacity and insulin requirement, resulting in relative insulin deficiency; at diagnosis with T2DM, β-cell mass is
already depleted by 50%, and continues on a downward trajectory thereafter without intervention (52).

Put together, abnormalities in postprandial glucose and lipid metabolism can be present long before the development of overt disease, irrespective of fasting levels. Therefore, strategies capable of improving insulin sensitivity and improving postprandial glucose and lipid handling may play a significant role in slowing (or preventing) cardiometabolic disease progression.

1.4 Intermittent energy restriction

1.4.1 Weight-loss benefits and current guidelines

A moderate weight-loss of 5% or more is an often cited threshold which is adjudged to have a clinically significant health impact on overweight/obesity associated comorbidities (53, 54), although, guidance provided by the National Institute of Clinical Excellence (NICE) suggest this figure could be as low as 3%, provided it could be sustained (54). Weight-loss can improve insulin sensitivity, glycaemic control, blood pressure, fasted lipid profiles and postprandial lipid clearance, contributing to a reduction in cardiometabolic risk (53, 55). Dietary strategies for weight-loss typically entail varying degrees of CER and/or involve manipulations in macronutrient intake. NICE “best practice” guidelines recommend dieting approaches which reduce daily energy intake by 2510 kJ below estimated requirements, or diets that reduce intake of fat as a means of ER (54). However, when confronted with an obesogenic environment favouring sedentary lifestyles and passive overeating, successful weight-loss and the necessary eating restraint required for CER becomes notoriously difficult to achieve and maintain. This is further compounded by alterations in energy expenditure and appetite which accompany weight-loss, which may ultimately predispose to weight re-gain (56).

1.4.2 Intermittent energy restriction: an alternative weight-loss approach

In recent years, IER has attracted considerable interest as an alternative to “conventional” CER, its premise being that individuals need not energy restrict every day which may prove to be more
sustainable for some individuals (Table 1.1). The majority of rodent studies (57-75) and a small number of human studies (76-80) have used IER protocols that totally restrict energy intake every other day (i.e. alternate day total ER), with fasting intervals ranging from 20 to 36 hours. The earlier human studies conducted within both lean and overweight cohorts were not designed to investigate weight-loss (76-79), and it is only more recently that the application of this particular IER approach as a weight-loss strategy has been assessed (80).

The IER protocols used by most human studies (81-95) and by some rodent studies (62, 65, 96) permit a small amount of food intake so that energy is partially (≥70%) but not completely restricted. This is also conventionally referred to as “modified fasting”, hence the term “fasting” in the IER context denotes periods of substantial (partial or total) ER. This form of IER has predominantly been utilised as a means of weight-loss, whilst a small number of studies have also used these protocols as a weight-maintenance approach (91, 93, 95). The most well studied approaches involve either alternate days of 75% ER (“alternate day modified fasting”) or two consecutive days of 70% ER per week (“the 5:2 diet”), although other variations also exist (81, 97, 98). Intakes on “non-fasting” (or “feed”) days among these studies have ranged from ad libitum (82, 83, 85-88, 90, 92, 97), hypoenergetic (~15-30% of energy requirements) (81, 88, 95, 98), isoenergetic (84, 91, 94) or hyperenergetic (~125-175% of energy requirements) (89, 93). Some, but not all, of these studies (82, 83, 88, 94) have included a CER (standard treatment; Table 1.2) or ad libitum (no intervention) control groups, whilst others have compared two or more different IER protocols (89, 92, 93, 99). Due to the scarcity of controlled data, this chapter discusses data from both controlled and non-controlled studies to provide a broader overview of the literature.

1.4.3 Acceptability of intermittent energy restriction and effects on body weight

In overweight/obese (80-89, 91, 92, 94, 95, 97), or combined healthy/overweight (90) cohorts, the average reported weight-loss through IER has ranged between ~4-10% over periods of four to 24 weeks. A large caveat with IER is that the degree of ER is more profound, which might
prove too difficult for some individuals to tolerate and may also promote overconsumption on “feed” days. However, the latter point appears unfounded with several studies demonstrating a lack of full compensatory hyperphagia (83, 84, 91, 94), whereas, data regarding appetite are conflicting. Discrepancies between study findings, even within the same research group, likely relate to variations in the IER protocol used. For instance, participants in Harvie et al (84)’s initial study were permitted a limited variety of foods on restricted days (milk, vegetables, fruit) and consequently, a greater proportion of participants complained of significant hunger relative to those following a CER diet. In addition, fewer IER participants intended to continue with their assigned diet. In contrast, the protocol used in the group’s later study (91) permitted a larger variety of protein-rich lean meats, fish and eggs which (as well as appearing subjectively more acceptable) may have also promoted greater levels of satiety, and as such, appetite levels were similar between IER and CER groups. Among studies comparing IER (70-100% ER) to CER (Table 1.2), overall attrition rates between the two diet groups have been mostly comparable, although approximately 9-12% of prospective participants drop-out when permitted a short trial of the IER diets prior to starting the study (84, 91). On the whole, when overall prescribed ER is similar, both IER and CER lead to comparable rates of weight-loss (Table 1.2).
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<td>(80)</td>
<td>n=25(1) Male and female Obese 18-55 years</td>
<td>8 wk</td>
<td>1. IER, n=13(1): ER: Total ER on alternate days FEED: Isoenergetically (typically consumed foods) + ad libitum access to 5-7 optional 837 kJ food modules, 55% carbohydrate, 15% protein, 30% fat 2. CER, n=12(0): 1674 kJ daily deficit, typically consumed foods, 55% carbohydrate, 15% protein, 30% fat Meals: all provided (both groups)</td>
<td>Weight:</td>
<td>Fasting glucose: ↑IER (8 kg, 9%) and ↓CER (7 kg, 6%); IER &gt; CER</td>
<td>↑IER (7%, NS) CER; IER=CER</td>
<td>↑IER (19%) and ↓CER (13%); IER=CER</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fasting insulin and S: NS (both groups)</td>
<td>Truncal FM: IER=CER</td>
<td>TAG: ↓IER (~17%), NS CER; IER=CER</td>
<td>LDL: ↓IER (~23%), ↓CER (~16%); IER=CER</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FFM: ↓IER and CER; IER=CER</td>
<td>Not assessed</td>
<td>LDL particle size: ↑IER and ↑CER, NS controls; IER=CER = Control % sd LDL: ↓IER, NS CER and Controls; IER=CER=Control</td>
<td>TOTC, HDL: NS (both groups)</td>
</tr>
<tr>
<td>(86)</td>
<td>n=37(8) Male and female Overweight/obese 35-65 years</td>
<td>12 wk</td>
<td>1. IER, n=13(2): ER: 75% ER on alternate days (3d/wk, 12:00-14:00), typically consumed foods, 15% protein, 30% fat FEED: Ad libitum Meals: ER day meals provided 2. CER, n=12(3): 25% ER/d, typically consumed foods, 55% carb, 15% protein, 30% fat Meals: all meals provided 3. Control, n=12(3): Ad libitum</td>
<td>Weight: ↓IER (5%) and ↓CER (5%), NS Controls; IER=CER&gt;Control</td>
<td>Not assessed</td>
<td>LDL: ↓IER (10%) and ↓CER (8%), NS Controls; IER=CER&gt;Control</td>
<td>TOTC, HDL: NS (both groups)</td>
</tr>
</tbody>
</table>

Table 1.2 Summary table: Effects of intermittent versus continuous energy restriction on body weight and cardiometabolic risk factors

Sample size expressed as total completers (drop-outs). ↑ or ↓ – Post treatment values significantly different from baseline (p<0.05). Tr – Statistical trend (p=0.05-0.1). NS – Non significant. =<> Denotes significance of between-group comparisons. ~ Denotes that % are approximated from provided pre/post values. 1 Dual-energy X-ray absorptiometry; 2 Bioimpedance.

Abbreviations: BP – Blood pressure; CER – Continuous energy restriction; CRP – C-reactive protein; ER – Energy restriction; FM – Fat mass; FFM – Fat free mass; HbA1c – Glycated haemoglobin; HDL – High density lipoprotein; HOMA-IR – Homeostasis model assessment insulin resistance; IER – Intermittent energy restriction; IL-6 – Interleukin 6; LDL – Low density lipoprotein; REE – Resting energy expenditure; sD – Small dense; Si – Sensitivity index; TAG – Triacylglycerol; TNF-α – Tumour necrosis factor alpha; TOTC – Total cholesterol.
Ref | Participants | Study length | Dietary Interventions | Weight and anthropometry | Glycaemic control | Lipids | Other factors
--- | --- | --- | --- | --- | --- | --- | ---
(91) | n=60(17) Females Overweight/obese 20-69 years | 12 wk | 1. IER, n= 33(4): ER: 2d/wk (consecutive), 2500-2717 kJ (~70% ER), <40 g carb; 250 g protein foods, 3x dairy portions, 1x/4x low carb fruit/veg) FEED: 5d/wk isoenergetic intake, Mediterranean diet (45% carbohydrate, 25% protein, 30% fat) | Weight: ↓IER (6%) and ↓CER (5%); IER=CER | Fasting glucose, HbA1c: NS (both groups) | TOTC: ↓IER (~5%) and ↓CER (~3%); IER=CER | Systolic BP: ↓IER (~4%) and ↓CER (~8%); IER=CER
| | | | Meals: not provided (both groups) | Fasting insulin: ↓IER (~19%); NS CER; IER>CER | LDL: ↓IER (~4%) and ↓CER (~3%); IER=CER | Diastolic BP: ↓IER (~4%) and ↓CER (~8%); IER=CER
| | | | | HOMA-IR: ↓IER (~25%); NS CER; IER>CER | TAG: ↓IER (~14%) and ↓CER (~7%); IER=CER | Leptin: ↓IER (~43%) and ↓CER (~28%); IER>CER
| | | | | FFM: ↓IER (~2%) and ↓CER (~2%); IER=CER | TOTC: ↓IER (~6%) and ↓CER (~10%); IER=CER | IL-6: ↓IER (~13%) and ↓CER (~7%); IER=CER | CRP: ↓IER (~11%) and ↓CER (~22%); IER=CER
| | | | | | | Adiponectin: ↑IER (~10%); NS CER; IER>CER

(84) n=89(18) Pre-menopausal females Overweight/obese 30-45 years | 24 wk | 1. IER, n= 42(11): ER: 2d/wk (consecutive), ~75% ER (2060-2266 kJ, 50 g protein. 1.1 L semi skimmed milk, 4x vegetables, 1x fruit) FEED: 5d/wk isoenergetic intake, Mediterranean diet (45% carbohydrate, 25% protein, 30% fat) | Weight: ↓IER (8%) and ↓CER (7%); IER=CER | Fasting glucose: ↑IER (~10%); NS CER; IER=CER | TOTC: ↓IER (~6%) and ↓CER (~10%); IER=CER | Systolic BP: ↓IER (~3%) and ↓CER (~6%); IER=CER
| | | | Meals: not provided (both groups) | Fasting insulin: ↓IER (~29%) and ↓CER (~14%); IER>CER | LDL: ↓IER (~10%) and ↓CER (~10%); IER=CER | Diastolic BP: ↓IER (~6%) and ↓CER (~8%); IER=CER
| | | | | | TAG: ↓IER (~17%) and ↓CER (~23%); IER=CER | Leptin: ↓IER (~40%) and ↓CER (~36%); IER=CER
| | | | | | FFM: ↓IER (~27%) and ↓CER (~19%); IER>CER | TOTC: ↓IER (~6%) and ↓CER (~10%); IER=CER | Adiponectin: ↑IER (~10%); NS CER; IER>CER
| | | | | | | HOMA-IR: ↓IER (~27%); NS CER; IER>CER | CRP: ↓IER (~11%) and ↓CER (~22%); IER=CER
| | | | | | | HDL: NS IER, ↓CER (~6%); IER=CER

Table 1.2 Summary table: Effects of intermittent versus continuous energy restriction on body weight and cardiometabolic risk factors
Sample size expressed as total completers (drop-outs). ↓or↑ – Post treatment values significantly different from baseline (p<0.05). Tr – Statistical trend (p=0.05-0.1). NS – Non significant. =<> Denotes significance of between-group comparisons. ~ Denotes that % are approximated from provided pre/post values. 1Dual-energy X-ray absorptiometry; 2Bioimpedance. 

Abbreviations: BP – Blood pressure; CER – Continuous energy restriction; CRP – C-reactive protein; ER – Energy restriction; FM – Fat mass; FMF – Fat free mass; HbA1c – Glycated haemoglobin; HDL – High density lipoprotein; HOMA-IR – Homeostasis model assessment insulin resistance; IER – Intermittent energy restriction; IL-6 – Interleukin 6; LDL – Low density lipoprotein; REE – Resting energy expenditure; sD – Small dense; Si – Sensitivity index; TAG – Triacylglycerol; TNF-α – Tumour necrosis factor alpha; TOTC – Total cholesterol.
There is a continual debate regarding which is the “best” weight-loss approach to follow, but ultimately, it is the degree of dietary adherence and sustainability (rather than the type of dietary strategy) which will predict weight-loss outcomes. Put together, the data presented in the previous section does not necessarily support the view that IER is any “easier” to follow than CER. However, there are sufficient data which shows that it is an acceptable weight-loss option. Understanding metabolic differences between dieting approaches is nonetheless important, as it may identify potential applications of IER within certain patient subgroups.

IER essentially acts as an intermittent very low energy diet (VLED), with individuals undergoing repeated cycles of substantial ER followed by refeeding. This may have the capacity to elicit very distinct alterations in metabolic control when compared to similar weight-loss achieved via more conventional CER approaches. It is important for our understanding of the metabolic adaptation which occurs during weight-loss with IER (and how this compares to CER), that we first understand what happens acutely and independently of weight-loss i.e. during one cycle of substantial ER-refeeding. An overview of the findings from studies which have examined the acute metabolic effects of substantial ER is provided next.

### 1.4.4 Acute effects of substantial energy restriction

#### 1.4.4.1 Total energy restriction

The short-term metabolic adaptation to total starvation, which occurs and becomes maximal within the first 96 hours of total ER, has been well defined by studies conducted within both lean and overweight/obese cohorts (100). As reviewed by Soeters et al (100), the adaptive response encompasses reciprocal shifts in fuel utilisation: FAO and ketogenesis progressively predominate with extended durations, favouring the conservation of glucose (100). The obligate need for glucose are met via mobilisation of liver glycogen stores (depleted within ~24 hours) and gluconeogenesis (100). These alterations are orchestrated by changes in (neuro) endocrine homeostasis, encompassing the increased counter-regulatory (insulin-antagonising) and lipolytic activities of glucagon, growth hormone, cortisol and catecholeamines (e.g. (nor) adrenaline),
combined with a reduction in insulin levels (100). Glucose oxidation is further inhibited due to reciprocal substrate competition between glucose and fatty acids (26), driven by increased fatty acid availability. By one to two days of total ER, circulating NEFA concentrations are up to three–fold greater than that observed following a standard 12-hour overnight fast (100). The implications of elevated fatty acid mobilisation during periods of total ER might include a rise in intramuscular and/or IHCL, although again this not a consistent observation among human studies (101-104).

Following total ER, the subsequent postprandial response during refeeding is altered. On one hand peripheral insulin sensitivity (and hence glucose tolerance) is transiently impaired, whilst FAO persists as a mechanism designed to favour replenishment of glycogen stores (105-108). These impairments in glucose tolerance occur in lean and obese individuals, but appear slightly more pronounced in the former group (108). Contrastingly, lipaemic responses appear favourably altered during the subsequent refeeding period. For example, in rodents, TAG clearance is augmented following a prolonged fast (when compared to baseline ad libitum feeding conditions), due largely to enhanced LPL activity within skeletal and cardiac muscle (109). Seemingly analogous changes have been observed in humans. Based on singular morning blood samples taken over consecutive days, one human study using a mixed gender healthy cohort found that plasma TAG remained lower following 24 hours of refeeding after one day of total ER (p=0.028), although this did not reach the corrected statistical significance threshold of p<0.002 used by the study (110).

1.4.4.2 Partial ≥70% energy restriction

Data from studies in overweight individuals reveal some similarities between total and partial ER. Specifically, circulating ketone levels (3-hydroxybutyrate [3-OHB], acetoacetone) and NEFA are elevated after one to two days of partial ER relative to a standard 12-hour overnight fast (82, 84, 91), whilst insulin (84, 91) and TAG levels (84, 91) are decreased, with the latter parameter taking up to two days of refeeding to normalise (84). However, studies do reveal differences between
total and partial ER. One direct comparison in lean men reported an acute reduction in IHCL following three days of partial ER, but not total ER (102). Hepatic insulin sensitivity (84, 91), assessed via homeostasis model assessment IR (HOMA-IR) has also been shown to be acutely improved following partial ER, which is possibly a consequence of the reduction in IHCL (111)). In obese individuals with T2DM, two days of partial ER reduces endogenous glucose production (112, 113), but does not affect intra-muscular TAG (112) or whole-body glucose disposal (112, 113), although, the effects on peripheral insulin sensitivity in non-T2DM populations is not known.

Direct comparisons between these total and partial ER studies are hindered due to the considerable heterogeneity between study designs, although the limited evidence available highlights both similarities and differences between the two degrees of ER. The acute effects of partial (≥70%) ER are more relevant to the majority of human IER weight-loss studies, but are less well characterised. What is clear from these studies however, is that following acute periods of substantial ER, an individual’s metabolic and hormonal milieu differs to that observed following an overnight fast (e.g. circulating levels and utilisation of fatty acids and ketones are greater, whilst TAG and insulin levels are lower). Substrate metabolism during subsequent refeeding is also seemingly altered in favour of ongoing fatty acid utilisation. In the context of IER, these acute adaptations might have long-term implications for metabolic control when repeated over time.

1.4.4.3 Ketones: potential role in modulating cardiometabolic risk

Ketone bodies are small lipid-derived molecules that serve as a circulating energy source for tissues during fasting. Produced in the liver from fatty acids mobilised from adipocytes, they are distributed via the circulation to metabolically active tissues, such as skeletal muscle and the brain (19). Beyond this biological role as an important fuel source during fasting, emerging evidence from animal studies demonstrates that ketone bodies such as 3-OHB also have the capacity to regulate a variety of cellular functions, which may have implications for cardiometabolic disease
risk. As reviewed by Newman et al (114), 3-OHB is an endogenous inhibitor of histone deacetylases, which are a family of proteins that regulate gene expression. Usually, histone hyperacetylation (i.e. where an acetyl functional group is transferred from one molecule to another) results in increased activation of gene expression, and thus histone deacetylation suppresses gene expression. Through this inhibition of histone deacetylases, 3-OHB might contribute to improvements in cardiometabolic health in a number of ways. For example, dietary supplementation with butyrate (which is metabolised to 3-OHB) has been shown to stimulate improvements in skeletal muscle mitochondrial function and insulin sensitivity in mice via upregulation of the expression of peroxisome proliferator-activated receptor gamma co-activator 1α, a transcriptional coactivator and master regulator of mitochondrial biogenesis (115). In addition, treatment of kidney cells with 3-OHB activated the transcription of forkhead box O3, which is a transcription factor for numerous downstream targets include genes encoding antioxidant enzymes (116). Thus, 3-OHB may also protect against cardiometabolic complications by promoting cellular resistance to oxidative stress. 3-OHB is also a ligand for the free fatty acid receptor 3, a G-protein coupled receptor that is highly expressed in sympathetic ganglions (114). Antagonism of the free fatty acid receptor 3 by 3-OHB suppresses sympathetic nervous tone (117), which may have potential benefits for glucose homeostasis and the modulation of blood pressure (118). Finally, 3-OHB can also regulate cellular processes indirectly, by altering the intracellular balance of downstream metabolites (114). For example, metabolism of 3-OHB increases intracellular levels of acetyl coA (114), which serves as a substrate for histone acetyltransferases (119), and therefore functions as a secondary mechanism by which 3-OHB can alter gene expression (114). Speculatively, repeated intense stimulation of ketogenesis thus represents one potential contributory mechanism by which IER could favourably influence cardiometabolic risk.

1.4.5 Chronic effects of intermittent energy restriction
The following sections will present a literature review of the known chronic effects of IER on metabolism and physiology, in relation to cardiometabolic disease risk and body weight
regulation which are relevant to the current PhD project. For clarity, sections will begin with evidence from rodent studies before detailing evidence from human trials. This will be concluded with a summary and identification of key knowledge gaps.

1.4.5.1 Glucose and lipid metabolism

Rodent studies

Although findings from rodent studies cannot be readily extrapolated to humans, they do permit a greater level of control over dietary intakes and environmental confounders. The rodent studies described herein have used alternate day total ER protocols unless otherwise stated. Over study intervals of four weeks to one year, this form of IER (various background diets) can reduce levels of fasting glucose (57, 59, 60, 73), insulin (59, 60, 67, 69, 70, 73) and HOMA-IR (67, 69), which in turn reflects improved hepatic insulin sensitivity (120). Additionally, lipid profiles are also favourably altered in wild-type rodents (58, 61, 67), with similar observations made by one IER (85% ER on alternate days) study (96). Findings from a number of studies which have performed more dynamic assessments of glycaemic control, through use of gold-standard hyperinsulinaemic-euglycaemic glucose clamp (HEC) techniques or glucose/insulin tolerance testing (GTT/ITT), have been mixed. Several studies have reported improvements in glycaemic control in rodents fed normal or low-fat chow (64, 71), whilst some (69, 72) (but not all (67); described later) studies have shown that IER is able to protect against IR induced by a high-fat diet.

In contrast to the majority of studies, one study conducted in LDL-receptor knockout mice observed the development of marked hypercholesterolaemia after three months of IER (normal chow) when compared to ad libitum fed controls, owing to the combined effects of enhanced post-prandial secretion of VLDL-TAG and deficient clearance (and hence accumulation) of its delipidation product, LDL-cholesterol (68). These mice also displayed elevated levels of fasting and postprandial glycaemia which were found to correlate with changes in adiposity (Section 1.4.5.3) and depleted fat free mass (FFM) (a major site of glucose disposal). The effects seen,
albeit possibly unique to LDL-receptor knockout mice, were specific only to the IER eating pattern.

Further evidence for detrimental effects comes from another study in obesity-prone Sprague-Dawley rats fed a normal chow diet on alternate days, who displayed marked glucose intolerance after eight months of IER as well as elevated markers of oxidative stress (66).

In another study by Higashida et al (67), Wistar rats were assigned to either an IER-high-fat diet group, to an ad libitum high-fat group, or to an ad libitum normal chow group (the control). By 10 weeks, post-treatment levels of HOMA-IR were significantly lower in both IER and control rats, relative to ad libitum high-fat group. However, insulin-stimulated skeletal muscle glucose uptake (assessed ex vivo by measuring 2-deoxyglucose uptake in isolated skeletal muscle) and insulin sensitivity (assessed in vivo via ITT) were equally impaired in both high-fat diet groups, in direct contrast to the positive effects of the IER diet on hepatic insulin sensitivity. Corresponding with these observations, rodents in both ad libitum and IER high-fat diet groups also presented with reductions in skeletal muscle GLUT4 protein content of ~30% and ~42% (relative to control fed rodents) respectively.

Human Studies - Alternate day total energy restriction

A small number of human studies have assessed the metabolic impacts of alternate day total ER using a range of experimental protocols. The original two (76, 79) to three (77, 78) week studies conducted in small groups (n≤16) of healthy and overweight participants were not designed as weight-loss interventions, although, participants on the three-week study struggled to sustain prescribed energy intakes on feed days, and so incurred a modest weight-loss (78). No post-treatment changes in fasting levels of insulin and glucose (when measured after a “feed” day) were observed by these studies (76, 77, 79), whereas one study reported improved lipid profiles (78). Two studies used HEC techniques to assess insulin sensitivity in healthy-weight men, yielded conflicting results. Halberg et al (76) reported an increase in insulin-mediated whole-
body glucose uptake after two weeks whereas Soeters et al (79), the only group among these earlier studies to use a (cross-over) controlled study design, found no change following the same duration of time. In a three-week study by Heilbronn et al (77), conducted in a healthy/overweight cohort, significant post-treatment reductions in postprandial insulin responses to a test-meal challenge were noted among male participants, whereas there was a contrasting decline in glucose tolerance among female participants (with no change in the insulinaemic response). On closer examination of the study design, post-treatment metabolic assessments were performed following after 36 hours of fasting, whereas baseline assessments were conducted after a shorter 12-hour overnight fast. As such, the unstandardised fasting periods makes it difficult to ascribe these findings as a true chronic treatment effect, given that prolonged fasting can also acutely alter postprandial substrate responses during the subsequent refeeding period (Section 1.4.4). Ultimately, the uncontrolled study designs represent a significant limitation of most of these earlier studies. Most recently, an eight-week pilot study (n=26 obese male and female participants) reported an average weight-loss of 9%, accompanied by reductions in LDL cholesterol and fasting TAG, whereas insulin sensitivity (assessed via intravenous GTT) was unchanged (80).

**Human studies – Intermittent partial ≥70% energy restriction:**
To date, all IER studies allowing some restricted day intake have only measured fasting blood markers of cardiometabolic risk. In T2DM populations, 12-20 weeks of IER has been shown to improve levels of glycated haemoglobin (HbA1c) which is a marker of glycaemic control over the preceding three months (81, 97). By contrast the majority of the IER studies (both controlled and uncontrolled) have been conducted within heterogeneous non-T2DM populations and have failed to show any significant effect on fasting glucose levels (82, 88, 91-94) and HbA1c (91) over study durations of four to 24 weeks; unsurprising findings given that glucose levels are usually kept under tight physiological control. However, among these studies (the majority of which were “5:2” studies), improvements in other indices of insulin sensitivity have been observed, including reductions in fasting insulin (84, 91, 93, 94) and/or HOMA-IR (84, 91, 94).
IER additionally elicits favourable alterations in fasted lipid profiles (82-87, 89-92, 95) based on findings from both controlled and non-controlled studies, with inter-study variation in terms of individual lipid fractions. Among individuals with high baseline fasting TAG values (group average: ~3.1 mmol/L), one non-controlled trial reported a large ~42% reduction after eight weeks of IER (82). As previously described (Section 1.3.4), lipoproteins within each sub-fraction are not homogenous particles and exist on a spectrum, varying in size and potential atherogenicity. Several controlled and uncontrolled studies utilising alternate day 75% ER protocols (85-87, 90, 92) and one uncontrolled study using a combined protocol (one day per week 90% ER protocol, six days of 30% ER) (98) have demonstrated post-treatment shifts in LDL sub-fraction distribution and/or size towards larger less atherogenic particles, even in the absence of an overall change in total LDL (92). With regards to HDL cholesterol, results from studies have been inconsistent. The majority have found no effects on HDL cholesterol levels (83, 85, 86, 88, 90, 121, 122) whereas one controlled study combining IER and exercise noted an increase in HDL cholesterol after 12 weeks (87). Glycaemic and lipaemic outcomes do not appear to be influenced by meal timing on restricted days (92), or by dietary composition on feed days (89) although differences were observed in the latter study with regards to vascular health indices (Section 1.4.5.2).

### 1.4.5.2 Haemodynamic indices

#### Rodent studies

A small number of rodent studies have measured changes in blood pressure and markers of cardiovascular function. Three to six months of IER has been shown to lower blood pressure (60, 123-125), whilst another four-week study reported an improvement in aortic endothelium-dependent vasodilation, a marker of endothelial function (75). In contrast, evidence for harm was observed by one six-month study in Sprague-Dawley rats, which showed that IER-feeding resulted in diastolic dysfunction and myocardial fibrosis (63) providing further evidence for potential harm with long-term adoption of this form of IER.
**Human Studies - Alternate day total energy restriction**

Data are limited to one uncontrolled study which reported no effect on blood pressure after three weeks of IER, which may be a consequence of the short study duration and/or due to the healthy lean/overweight cohort studied (78).

**Human studies – Intermittent partial >70% energy restriction:**

The effects of IER have been assessed within predominantly normotensive or pre-hypertensive cohorts which may account for inconsistencies between these studies with respect to changes in blood pressure. The studies (both controlled and uncontrolled) that have found changes in systolic and/or diastolic blood pressure reported post-treatment reductions of up to ~13% in both parameters (83, 87, 88, 92, 95). In one such study by Hoddy et al (92), which did not have a control group, systolic blood pressure was only reduced when food intake on restricted days was spread over three small meals. When food intake was consumed at evening time or lunchtime only, no change in blood pressure was noted. These differences may simply reflect numerical differences between groups at baseline, with systolic blood pressure tending to be greater within the IER-small meals group. Several non-controlled studies have also measured and shown improvements in indices of arterial compliance (95) and endothelial function (89), but not when a high-fat (45% [14% saturated] vs. 25% of total energy [6%]) diet was consumed on “feed” days (89).

**1.4.5.3 Change in adiposity, fat distribution and related parameters**

Reductions in adiposity, and in particular abdominal, visceral and ectopic (IHCL, intramuscular) fat are key therapeutic targets of ER. Other functional and morphological alterations which occur during “pathological” adipose tissue expansion were discussed earlier (**Section 1.2.1**). A summary of the known effects of IER on adiposity, distribution and related parameters are discussed in the next sections.
**Rodent studies**

Rodent studies lasting between four weeks to one year have reported reductions in intra-abdominal fat (65, 96), IHCL and local inflammatory markers (69, 72), as well as favourable reductions in adipocyte size within both subcutaneous and visceral fat pads (62). Circulating levels of insulin-sensitising adiponectin have also been shown to increase following IER (65, 73). In direct contrast however, a study by Dorighello et al (68) in LDL-receptor knock out mice, found that even following 20% weight-loss, VAT, total adiposity and adipocyte size were markedly increased by 15, 72 and 68% respectively after three months of IER (normal chow), which correlated with negative glycaemic changes described previously in Section 1.4.5.1. The marked heterogeneity between these studies with respect to rodent strain, study protocols and outcomes make it difficult to draw any firm conclusions from these data.

**Human Studies - Alternate day total energy restriction**

In the one study to have used this IER protocol as a weight reduction strategy, weight-loss (~9%) was accompanied by decreases in total and truncal adiposity (assessed using dual energy X-ray absorptiometry [DXA]), as well as plasma leptin concentrations (80). The earlier human studies were not designed as weight-loss interventions. Despite the lack of change in total adiposity, one two-week study did find an improvement in adipose tissue insulin sensitivity (reflected by a reduced rate of lipolysis under HEC conditions), however this must be interpreted cautiously due to the uncontrolled study design (76). The study also measured intramuscular TAG, finding no change after the two weeks. No study to date has quantified changes in IHCL.

**Human studies – Intermittent partial ≥70% energy restriction**

In conjunction with changes in total body adiposity, IER weight-loss studies have reported reductions in VAT (92, 99) assessed via DXA, as well as beneficial changes in circulating adipokine profiles. For example, circulating leptin levels are reduced (83, 84, 90), whilst adiponectin levels are increased (83, 84, 90). Adipose tissue dysfunctions can also contribute to the development of systemic inflammation (Figure 1.1). Several uncontrolled and controlled
studies have reported reductions in IL-6, TNF-α and/or C reactive protein (84, 90, 91) (whose production is induced in the liver by IL-6 (126)). No study has examined changes in adipose tissue morphology/physiology or the localised production of inflammatory mediators within adipose tissue, nor has any human study has quantified changes within ectopic fat stores.

1.4.5.4 Energy expenditure

Total daily energy expenditure is comprised of three major components (127): i) resting energy expenditure (REE); ii) the thermic effect of feeding; and ii) the thermic effect of activity. REE constitutes 60 to 75% of daily energy expenditure and is the energy cost associated with the maintenance of major body functions. Thermic effect of feeding is the obligatory energy expenditure associated with the digestion and processing of ingested foods and constitutes approximately 10% of daily energy expenditure. Meal induced thermogenesis (MIT) refers to the thermic effect of a single test meal. The thermic effect of activity is the most variable component, accounting for 15 to 30% of daily energy expenditure. This component can be further subdivided into exercise energy expenditure and non-exercise physical activity thermogenesis. Diet-induced weight-loss typically results in a decrease in total energy expenditure, by as much as ~10% within the first two weeks (56). This can be attributed in part to accompanying reductions in metabolically active body mass, but also to a number of (neuro) endocrine alterations including declines in leptin, thyroid hormone and sympathetic nervous system activity (56). The decline in REE observed during weight-loss is said to be disproportionately greater than the associated loss in body mass, resulting in a reduction in weight-loss efficiency (i.e. the amount of weight or fat lost per unit of energy deficit) (56). Being the largest contributor to energy expenditure and thus an important determinant of energy balance, the PhD projects focused on the effects of IER on REE, for which the published evidence is limiting and conflicting.

Human Studies - Alternate day total 100% energy restriction

One uncontrolled study conducted in lean and overweight individuals reported no change in REE adjusted for fat mass (FM) and FFM after three weeks of IER (78). In contrast, another study in
lean men which used a cross-over controlled study design reported a ~247kJ decline in absolute (unadjusted) REE after two weeks, with no change in circulating levels of thyroid hormones or catecholamines (79). In obese individuals, eight weeks of this form of IER led to a ~464kJ decline in absolute REE following weight-loss as well as a reduction in leptin (80). Adjusted REE was not significantly altered, suggesting the reduction in REE was not out of proportion with the decline in body mass.

**Human studies – Intermittent partial ≥70% energy restriction:**

One uncontrolled study by Hoddy et al (92) reported a ~828 kJ decline in absolute REE, but only when food intake on restricted days was consumed as one evening meal. When food intake was spread over three small meals or at lunchtime only, no change in REE was detected. The study did not assess changes in hormonal regulators of REE whilst body compositional changes were comparable across the groups, and so the reason for such discrepancies between the IER protocols is unclear. An alternate explanation for these findings may be the numerical baseline differences, with REE tending to be greater within the IER-evening group.

**1.4.6 Does the mode of energy restriction (intermittent versus continuous) influence metabolic responses during weight-loss?**

The current section discusses the relative impacts of the IER eating pattern (independent of weight-loss) to observed metabolic changes, via comparisons with CER which represents the “standard” weight-loss approach.

Considering the rodent literature in its entirety, a large proportion of studies have reported a lack of full energy compensation on feed days irrespective of background diet (60, 61, 64, 67, 69-71) but have not included a pair-fed group, and as such, it is not possible to ascertain the relative contribution of the IER pattern and overall ER to metabolic outcomes. Rodent studies that have directly compared IER to CER have tended to demonstrate similar short-term cardiometabolic improvements (59, 66, 125), but what appears unique to IER is that an overall energy deficit is not obligatory. Data from one such study by Anson et al (59) demonstrated comparable post-
treatment reductions in fasting glucose and insulin between IER and CER (40% ER) fed rodents, whereas only IER-fed rodents exhibited an increase in circulating ketone levels. This suggests that IER facilitated a metabolic shift towards hepatic fat metabolism and ketone production, which might have the potential to prohibit IHCL accumulation. One study in diabetes prone New Zealand obese mice (69) compared the effects of IER and CER (10% ER) on IHCL, as well as markers of hepatic inflammatory cell infiltration and IR. Both groups were provided with high-fat chow. Reductions in these hepatic parameters were observed among IER-fed rodents, with similar but less marked tendencies noted in the CER group. Accordingly, insulin sensitivity was improved to a greater extent among IER-fed mice, but ultimately both groups were protected against T2DM induced by high-fat feeding. As the degree of overall ER (~27%) was greater in the IER group, the relative contribution of the IER feeding pattern and greater overall ER cannot be disentangled.

Whilst findings among rodent studies have been largely positive, a small number of rodent studies also highlight the potential for adverse health impacts which are not observed in CER-fed (or ad libitum fed (63, 68)) rodents. Specifically, in the previously described study by Cerquiera et al (66); Section 1.4.5.1) in Sprague Dawley rats, glucose tolerance was markedly impaired after eight months of IER (normal chow diet), whereas it had improved to a comparable extent as CER-fed rats at four weeks, which is suggestive of a longitudinal deterioration in glycaemic control.

Of the few human studies to have compared IER (70-100% ER) to CER ((80, 84, 86, 91); Table 1.2), the two modes of ER have been shown to be equally effective at improving most of the cardiometabolic risk factors measured by these studies. Similarly, no consistent differences have been observed between the dietary approaches with respect to changes in body weight, total and regional adiposity or circulating adipokines, with the majority of studies showing comparable between-group changes. There have been some discrepancies between IER and CER however. The two studies by Harvie’s group which have utilised the “5:2 style” protocol (i.e. 70% ER on two consecutive days) have found reductions in fasting insulin and HOMA-IR following IER that
have exceeded those of CER (25% ER) after both three months CER (91) and six months (84) on the respective diets. It is perhaps not co-incidental that the studies that have reported superior improvements in HOMA-IR have all used this two-day ER protocol, a period of time which has been shown to produce acute reductions in IHCL and hepatic IR (84, 91, 111). In another study by Varady et al (86), IER (75% ER on alternate days) led to a significant greater reduction in fasting TAG than CER (25% ER) after 12 weeks.

From the metabolic perspective, comparisons between these studies are somewhat confounded by the lack of standardisation for weight-loss between the groups. Whilst the majority of studies have reported comparable mean weight-loss following the two dietary interventions, the degree of weight-loss can vary considerably at an individual level. This is exemplified by one such study which found a proportionally greater number of IER participants tending to attain a ≥5% weight-loss (91). Without standardisation, it is difficult to interpret to what extent any underlying differences can be explained simply by the extent of weight-loss per se. This becomes particularly important when trying to address the question of whether the mode of ER (i.e. intermittent but substantial vs. continuous but modest) can influence metabolic outcomes during weight-loss. Interestingly, when changes in body weight have been statistically accounted for, evidence from one study comparing IER (100% ER on alternate days) to CER (1674 kJ daily deficit) highlighted the potential for underlying differences between the two diets in their effects on REE, with IER appearing to prevent the adaptive reduction in REE which is associated with weight-loss (80).

1.4.7 Summary and knowledge gaps relevant to PhD

In summary, there are a number of parallels between the acute and chronic literature. Specifically, short periods of substantial ER are associated with large fluxes in fatty acids contributing to transient impairments in peripheral insulin sensitivity. Meanwhile substrate utilisation is shifted in favour of FAO and ketogenesis. Consequent reductions in IHCL may underlie the acute improvements in hepatic insulin sensitivity noted by a number of studies (Section 1.4.4). Similarly, a small but not insignificant number of rodent studies (66, 67) and one (albeit
methodologically flawed) human study (77), have reported negative changes in peripheral insulin sensitivity following chronic periods of IER (100% ER on alternate days). In two of these studies, fasting indices of glycaemic control (which largely reflect of hepatic insulin sensitivity) either remained unchanged (77) or were improved (67). It should be noted however that in contrast to most rodent studies, the majority of human weight-loss studies have used IER protocols employing regular intervals of partial (but substantial) ER, which may elicit very different metabolic changes to total ER when repeated over time. However, our knowledge regarding the acute effects of substantial ER predominantly comes from studies employing prolonged total ER intervals. Our understanding of the acute effects of partial ER over durations relevant to IER are less well characterised in overweight/obese individuals.

On the whole, findings from the few human comparison studies between IER and CER have been largely positive however there is a significant shortage of studies (Table 1.2). There is some suggestion that IER may lead to greater improvements in indices reflective of hepatic insulin sensitivity and lipid metabolism (84, 86, 91), which again draws parallels with the acute literature and may originate from the repeated substantial ER/feed cycles. However, this has not been a consistent observation among all studies, with the vast majority showing comparable changes for most study outcomes.

At the time the PhD project was devised, no IER weight-loss study had looked beyond steady state fasting cardiometabolic risk factors; absence of differences in baseline levels does not necessarily exclude the potential for underlying changes following IER (or between IER and CER), which may only manifest when metabolically challenged (67, 77). More recently, Catenacci et al (80) found no change in insulin sensitivity (assessed via IVGTT) following eight weeks of IER (alternate days of total ER) in obese individuals which provides some reassurance that this more “extreme” form of IER may not lead to negative metabolic consequences, at least in the short/medium term. However, no study has compared the postprandial effects of IER and CER following a meal challenge, which benefits from being the more physiologically
representative non-steady state metabolic assessment method, and permits concomitant measurement of both glucose and lipid parameters.

Notwithstanding the limitations of the current data, there is some evidence of underlying differences between the two modes of ER, which require focused metabolic studies that standardise for weight-loss as opposed to the duration of the dietary intervention. Standardisation would also allow for direct comparison of proportional body compositional changes, which might also be influenced by the mode of ER. More data are also required regarding the relative effects of these diets on important aspects of body weight regulation, such as changes in REE for which evidence is particularly limited. These identified knowledge gaps and limitations form the basis of the conducted research presented in this thesis.
1.5 Meal timings and health: an overview

Over the past few years, there has been an emergence of interest in the concept of chrononutrition, i.e. the interaction between meal timing and our circadian system, which comprise self-sustained ~24 hour oscillations in physiology, metabolism and behaviour (128-130). Examples include the daily rhythms in glucose homeostasis and insulin sensitivity, which declines over the course of the day (130). These rhythms are driven by a series of molecular “clocks”. The “master” clock is located in a small brain region within the anterior hypothalamus, the suprachiasmatic nuclei (SCN). Light is the dominant entrainer (Zeitgeber) of the SCN which controls many essential physiological processes including the sleep/wake cycle and endocrine rhythms (130). Peripheral clocks are located in many tissues integral to glucose and lipid metabolism such as the liver, pancreas, skeletal muscle and adipose tissue. Feeding time acts as the major Zeitgeber of peripheral clocks, with the SCN acting as the “central conductor”, ensuring correct synchronisation between peripheral clocks (130). Possession of such rhythms permits effective co-ordination of endogenous processes in response to changes in the environment such as the daily light/dark cycle, and consequent cyclical food availability (130).

The modern day lifestyle has perturbed the human circadian system in three primary ways: the increasing use of shift work, exposure to prolonged hours of artificial light, and erratic eating patterns (compounded by around-the-clock access to energy-dense foods), which together, encourage the consumption of food outside of the appropriate phase of the endogenous circadian cycle. These perturbations are thought to play a contributory role in the chronic disease burden afflicting modern societies. Whilst much emphasis has been placed on the effect of changes in the quantity and quality of nutritional intake, it is becoming increasingly clear that alterations in the temporal pattern of food intake (i.e. the timing of eating) can also influence body weight and metabolic outcomes (128-130).

1.5.1 Time-restricted feeding

TRF is an example of a timed dietary approach, which also falls under the “intermittent fasting”
umbrella (Table 1.1). TRF involves limiting intake to a period of several hours (usually ≤12 hours), which thereby extends the length of the daily fasting interval. In contrast to IER, TRF is usually performed on a daily basis and doesn’t necessarily entail a prescribed ER, however the two approaches are not mutually exclusive, indeed, alternate day total ER can be seen as a prolonged form of TRF.

1.5.1.1 Findings from rodent studies:
Findings from a number of high profile rodent studies have attracted a lot of recent scientific interest. Being nocturnal animals, rodents typically consume ~80% of their energy during the dark/active phase when permitted ad libitum access to normal chow (131). TRF (≤12 hour) where food intake is consolidated to the dark phase, has been shown to both protect against and reverse the harmful metabolic consequences of diverse nutritional challenges including high-fat and high-sugar obesogenic diets (131, 132). TRF mice display reduced adiposity and liver steatosis, in addition to improved glucose tolerance and reduced cholesterol levels when compared to mice fed ad libitum with the same high-fat diet (131, 132). Importantly, these improvements occur in the absence of changes in energy intake or locomotor activity. An overview of the proposed mechanisms is described in the following section.

1.5.1.2 Mechanisms of time-restricted feeding: independent and additive benefits of fasting
Defined daily feeding and fasting rhythms support robust oscillation in components of the circadian clock and their output genes, but become dampened in mice models of diet-induced obesity as they start to consume a greater proportion of their food intake in the light (inactive) phase (131, 132). Rhythmicity can be restored in these rodent models through TRF (131, 132), which is made possible by the extensive cross-talk and interaction between molecular clock components and the feed/fasting responsive signals of cellular energy status. For example, fasting induces the activities of the AMP-activated protein kinase (AMPK) and cAMP response element binding protein (CREB) which promote ATP production during low energy availability. In contrast, feeding stimulates the mechanistic target of rapamycin (mTOR) pathway which
promotes anabolic processes during increased energy availability. These feed/fast responsive elements can influence the expression of peripheral clock components, and vice versa (133). In support of this, TRF has been shown to improve CREB, mTOR, and AMPK rhythms, and hence promote robust oscillations of circadian clock gene components (131, 132).

The metabolic benefits of TRF may additionally be attributed to the accompanying elongation of the daily fasting interval, which therefore increases the length of time spent in a catabolic state and promotes a metabolic shift towards fat metabolism. In the comprehensive study conducted by Chaix et al (132) which compared TRF eating windows of 9-15 hours, the magnitude of the metabolic benefits of TRF were proportional to the length of the fasting interval, with a minimum window of 12 hours required to protect mice from diet-induced obesity. On the basis of this rodent study, it appears that the length of the fasting interval may exert additive effects. However, the studies described in this chapter utilised mouse models with a predisposition to diet-induced obesity. In addition, the beneficial effects of TRF are less pronounced in mice fed normal chow who do not exhibit the same disturbances to daily feeding rhythms as mice fed ad libitum high-fat diets (131). Therefore, it is premature to begin extrapolating these findings to humans for whom, the evidence for TRF is limited and conflicting, as shall be described next.

1.5.1.3 Findings from human studies:

Observational data

Islamic religious fasting restricts food access to nocturnal hours – between sunrise and sunset. Recent meta-analyses of these Ramadan studies, which by their nature have been observational by design, have reported improvements in several cardiometabolic risk markers including fasting lipid profiles and glucose despite the nocturnal eating pattern, although, these are steadily reversed upon resumption of normal food intake (134). Also noted are modest reductions in body weight which are likely reflecting a mild ER, although this is not a universal finding (134). However, based on these observational data alone it is difficult to separate out the individual impacts of the changes in the eating window duration, the switch in feed timing and the mild ER to study
outcomes.

**Interventional data**

Table 1.3 provides an overview of the limited number of human TRF interventional studies conducted so far. In humans, TRF has been achieved by constraining all food intake to one supervised euenergetic meal a day (referred to herein as evening TRF) (135, 136), whilst the other studies permitted *ad libitum/self-selected* intake but asked participants to avoid night-time consumption (137), or to choose their own 10-11 hour shortened eating window (138). One major difference between human and rodent interventional studies is that both lean and overweight humans tend to behaviourally reduce energy intake during TRF when permitted *ad libitum/self-selected* food intake (137, 138). Consequently, modest weight-loss is incurred but unfortunately these particular studies did not take blood samples and so the metabolic impact is not known.

Only two publications, derived from the same cross-over controlled study (eight-weeks of euenergetic evening TRF compared to a three-meal-per-day control condition), have reported metabolic outcomes. The study was conducted in a small group of lean middle-aged participants. Stote *et al* (136) reported both pro-atherogenic (increasing LDL cholesterol) and anti-atherogenic (increasing HDL cholesterol and decreasing TAG) changes in lipid profiles after the eight week TRF intervention. However, baseline and post-intervention blood measurements were taken in the morning and evening respectively, and so these findings may have been confounded by normal circadian variations in these parameters. The publication by Carlson *et al* (135) reported significant elevations in fasting and postprandial glycaemia, with impairments found in both peripheral insulin sensitivity and first phase insulin secretion. Pre and post metabolic assessments in this case were both conducted in the morning, and so the post-treatment decline in glucose tolerance may reflect a metabolic adaptation to the evening eating pattern. In addition, there was no standardisation of dietary intakes on the days preceding their two metabolic assessments and so, unlike for the baseline measurement, at follow-up participants would have consumed their entire daily energy requirement the night prior to their post-intervention visit. As such, the post-
treatment increase in fasting glycaemia (relative to baseline) may reflect ongoing prandial elevation from the significantly larger preceding evening meal. Whilst these findings highlight that TRF may alter aspects of metabolism independently to overall ER, the interpretation of these studies are hindered by methodological constraints.
<table>
<thead>
<tr>
<th>Ref</th>
<th>Participants</th>
<th>Study Design</th>
<th>Dietary interventions</th>
<th>Energy intake during TRF</th>
<th>Weight and anthroscopy</th>
<th>Glycaemic control</th>
<th>Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>(135,</td>
<td>Males and females Lean 40-50 years</td>
<td>2 x 8 wk cross-over with 11 wk washout</td>
<td><strong>1. TRF:</strong> Euenergetic, 1 supervised evening meal between 17:00-21:00</td>
<td>↓272 kJ</td>
<td>Weight: ↓1.4 kg</td>
<td>Glucose tolerance (OGTT): ↓</td>
<td>TOTC: ↑12%</td>
</tr>
<tr>
<td>136)</td>
<td></td>
<td></td>
<td><strong>2. Control leg:</strong> Euenergetic intake, 3 meals/d</td>
<td></td>
<td>FM¹: ↓2.1 kg</td>
<td>Fasting glucose: ↑ (~12%)</td>
<td>LDL: ↑17%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Diet:</strong> Provided. ~2397 kJ (49% carbohydrate, 36% fat, 15% protein).</td>
<td></td>
<td>FFM¹: NS</td>
<td>Fasting insulin: NS</td>
<td>HDL: ↑8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Diets:</strong> Provided. ~2397 kJ (49% carbohydrate, 36% fat, 15% protein).</td>
<td></td>
<td></td>
<td>HOMA-IR: NS</td>
<td>TAG: ↓~9%</td>
</tr>
<tr>
<td>(137)</td>
<td>Males Lean/overweight 18-26 years</td>
<td>2 x 2 wk cross-over with 1 wk washout</td>
<td><strong>1. 13-hour TRF:</strong> Ad libitum, between 06:00-19:00 only</td>
<td>↓1000 kJ</td>
<td>Weight: ↓0.4 kg</td>
<td>Not assessed</td>
<td>Not assessed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>2. Control leg:</strong> Ad libitum</td>
<td></td>
<td>BMI: ↓0.1 kg/m²</td>
<td>Not assessed</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Diets:</strong> Self-selected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Diets:</strong> Self-selected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(138)</td>
<td>Males and females Overweight &gt;18 years</td>
<td>16 wk TRF intervention, with 3 wk baseline recording period</td>
<td><strong>1. Baseline:</strong> Ad libitum</td>
<td>↓~20%</td>
<td>Weight: ↓3.3 kg</td>
<td>Not assessed</td>
<td>Not assessed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>2. 10-11 hour TRF:</strong> Ad libitum, self-selected shortened eating window</td>
<td></td>
<td>BMI: ↓1.2 kg/m²</td>
<td>Not assessed</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Diets:</strong> Self-selected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.3 Summary table: Human time-restricted feeding interventional studies
Sample sizes expressed as total completers (drop-outs). ↓ or ↑ – Post treatment values significantly different versus control leg/baseline (p<0.05). Tr – Statistical trend (p=0.05-0.1). NS – Non significant. ~ Denotes that % are approximated from provided pre/post values.¹Bioimpedance.
Abbreviations: BMI – Body mass index; FM – Fat mass; FFM – Fat free mass; HDL – High density lipoprotein; HOMA-IR – Homeostasis model assessment insulin resistance; OGTT – Oral glucose tolerance test; TAG – Triacylglycerol; TOTC – Total cholesterol; TRF – Time-restricted feeding; Wk – Week
1.5.2 Summary and knowledge gaps relevant to PhD

Combining the evidence, TRF protects rodents from a diverse array of nutritional challenges, and does not appear to necessitate an overall ER. There is some suggestion that the length of fasting duration may exert an additive benefit, presumably by elongating the time spent in a catabolic state. However, the ability to extrapolate these findings to the human condition is questionable. Findings from human interventional studies using euenergetic TRF protocols have been contrasting with regards to metabolic outcomes (both positive and negative), but their interpretation is hindered by methodological constraints and use of extreme evening temporal restriction (135, 136). Whilst other interventional studies have imposed relatively modest constraints upon participants’ eating window, these studies did not take metabolic measurements (137, 138). They do however highlight that humans tend to behaviourally reduce energy intake when permitted *ad libitum*/self-selected intake during these restricted feeding windows, which presents another important distinction between rodent and human studies. There is a clear need for more controlled human experiments utilising protocols that reflect a realistic intervention for free-living individuals, and that assess both metabolic as well as anthropometric outcomes.
1.6 Aims and objectives of PhD project

The overarching aim of the PhD research project was to gain a greater understanding of the metabolic effects of intermittent fasting, comprising of both IER and TRF, with a specific emphasis on cardiometabolic risk factors. The literature reviews presented in this introductory chapter identified a number of distinct knowledge gaps unique to each form of intermittent fasting, and as such, the projects which comprised this thesis were based around specific objectives.

INTERMITTENT ENERGY RESTRICTION: OBJECTIVES

1. To investigate the acute (weight-loss independent) metabolic and physiological effects of substantial ER (Chapter three)

2. To compare the metabolic and physiological effects of IER versus CER following matched weight-loss (Chapter four), with a specific focus on their effects on postprandial glucose and lipid metabolism

3. Secondary objectives: To investigate the acute and chronic effects of IER on factors relating to body weight regulation including appetite, energy intake compensation and components of energy expenditure (Chapters three and four)

TIME-RESTRICTED FEEDING: OBJECTIVES

4. To investigate the metabolic effects of a realistic TRF protocol within a free-living human population (Chapter five)

5. To collect preliminary pilot and feasibility data to inform on sample size calculation for a larger clinical trial (Chapter five)

6. Secondary objectives: To examine the effects of TRF on dietary intake (Chapter five)
Chapter 2: General Methods

The following chapter describes the general methods used in the studies presented in this thesis. Please refer to Appendix A for the list of materials and apparatus used.

2.1 Body weight and composition

In all studies, body weight and composition were measured in the fasted state and in accordance with published methodological guidelines (139, 140). Participants wore light clothing and were asked to void immediately prior to the measurements. Weight, FM and FFM were assessed to the nearest 0.1kg using the Tanita BC420MA bioimpedance segmental monitor (Tanita Corp, Tokyo, Japan) in Studies one and two (Chapters three and four), and using the Tanita MC180A in Study three (Chapter five). Height was measured to the nearest 0.5 cm using a stadiometer (Seca, Birmingham, UK) with participants advised to stand up straight, with their feet together, and head positioned in the Frankfort horizontal plane. In Study two, waist and hip circumferences were measured to the nearest 0.5 cm using a stretch resistant tape (Prym, Stolberg, Germany) at the end of normal expiration. Waist circumference was measured at the midpoint between the lower margin of the least palpable rib and top of the iliac crest. Hip circumference was measured around the widest portion of the buttocks. For both measurements, participants were advised to stand with their feet close together. Each measure was repeated twice and averaged; if repeated measurements differed by >1 cm, the two measurements were repeated (140).

2.2 Assessment of energy requirements

In Studies one and two, basal metabolic rate (BMR) was calculated using the Henry predictive equation (141) as recommended by Scientific Adversary Committee on Nutrition (142). Participants were additionally asked to self-report physical activity levels (PAL), categorising their occupational and leisure activity levels as sedentary, light, moderate or heavy ((143); Table 2.1). To calculate total daily energy requirements, BMR was multiplied by the appropriate PAL for that participant.
Table 2.1 Physical activity levels used in calculation of daily energy requirements

<table>
<thead>
<tr>
<th>Non occupational activity</th>
<th>Occupational activity Light</th>
<th>Occupational activity Moderate</th>
<th>Occupational activity Heavy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men</td>
<td>Women</td>
<td>Men</td>
</tr>
<tr>
<td>Non active</td>
<td>1.4</td>
<td>1.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Moderately active</td>
<td>1.5</td>
<td>1.5</td>
<td>1.7</td>
</tr>
<tr>
<td>Very active</td>
<td>1.6</td>
<td>1.6</td>
<td>1.8</td>
</tr>
</tbody>
</table>

2.3 Assessment of energy intake

2.3.1 Diet diaries

In all studies, participants recorded food intake in validated diet diaries, which included pictorial guides to aid portion size estimations when exact weights could not be provided ((144); Appendix B). Please refer to individual chapters for the duration of dietary intake recording periods. In order to maximise accuracy, completed diet diaries were reviewed during metabolic study days and participants were asked to clarify ambiguous entries. Participants were also asked to provide food packets of any “unusual” foods as well as recipes for home cooked meals.

2.3.2 Dietary analyses

All diet diary analyses were carried out in Diet Plan Six (Study one) or Diet Plan Seven for all other studies (both Forestfield Software, Horsham, UK) using the McCance and Widdowson’s composition of foods integrated dataset. Where exact weights were not provided, typical portion weights were obtained from a published food portions book (145). Where no portion size was specified, a medium portion was assumed. Generic foods in the nutritional analysis programme were used unless specific food brands were provided, in which case nutritional composition information was manually inputted as a user added food.

2.3.2.1 Assessment of under-reporting

The revised version of the Goldberg equation was used (146) to evaluate the extent of under-reporting of energy intakes in Studies one and two and hence the validity of the dietary assessments. Briefly, to determine whether a given value of mean reported energy intake (EI_{rep}:BMR in n participants (at the population or individual level) is acceptable, the following condition must be satisfied:
\[ EI_{\text{rep}} : BMR > PAL \times \exp \left( s \times \text{min} \times \frac{S/100}{\sqrt{n}} \right) \]

Where -

- \( EI_{\text{rep}} \) = reported energy intake
- \( BMR \) = calculated using the Henry equation as specified in Section 2.2
- \( PAL \) = the mean PAL for the population under study
- \( \text{Exp} \) = the exponential function (e^x)
- \( \text{Sd min} = -2 \) for the 95\% confidence limit
- \( n \) = number of participants
- \( S \) = the factor that takes into account the variation in energy intake, BMR and energy requirements, which is given by:

\[
S = \sqrt{\frac{CV^2_{wEI}}{D} + CV^2_{wB} + CV^2_{tP}}
\]

Where -

- \( CV_{wEI} \) = the calculated day-to-day within-subject coefficient of variation in energy intake
- \( D \) = the number of days of diet assessment
- \( CV_{wB} \) = the within-subject coefficient of variation of repeated BMR measurements or the precision of estimated compared with measured BMR. A standardised value of 8.5\% was used as advised by Black (146)
- \( CV_{tP} \) = the calculated between-subject variation in PAL

Under-reporting cut offs were calculated at both individual (n=1) and study population levels.

2.4 Assessment of postprandial substrate metabolism and thermogenesis

Assessments of postprandial glucose and lipid responses to a single liquid mixed test meal challenge were conducted in Studies one and two. The test drink (400 ml Fortisip [Nutricia, Trowbridge, UK]) provided 2510 kJ, 74 g carbohydrate, 24 g protein and 23 g fat. Postprandial assessments began in a fasted state at ~08:30 with REE and respiratory quotient (RQ) measured prior to cannulation. A
diagram detailing the postprandial measurement schedule and the metabolites assessed by these studies is presented in Figure 2.1. In brief, an indwelling cannula was inserted into participants’ antecubital vein, following which an initial blood sample was taken. Participants were then instructed to consume the test drink within five minutes, with the clock started on commencement. Serial blood samples were then taken at regular intervals over a 360-minute time course. In Study one, postprandial substrate oxidation and thermogenesis were additionally assessed via indirect calorimetry over 10-minute time blocks at each time point (with the exception of time point 15). Please refer to individual study chapters for further details regarding standardisation of dietary intakes in the preceding day(s) and fasting durations prior to assessments. Participants were asked to avoid strenuous exercise for two days prior to each study day visit, and to abstain from alcohol on the preceding day (147, 148). Descriptions of and blood collection/analytical and indirect calorimetry techniques are provided in Sections 2.5 and 2.6 respectively.
Figure 2.1 Assessments of postprandial substrate metabolism and thermogenesis

Protocols used in Studies one and two. Postprandial assessments began in the morning (~08:30), with participants in a fasted state. REE and RQ were assessed via indirect calorimetry. An initial blood sample was then taken. Participants then consumed a liquid mixed test meal (400 ml Fortisip: 2510 kJ, 74 g carbohydrate, 24 g protein, 23 g fat), with the time that the participants started drinking denoted T0. Serial blood samples were taken over 360 minutes – see diagram for metabolites assessed. In Study one, MIT and substrate oxidation were measured over 10-minute time blocks at each time point (except time point 15). In Study two, C-peptide was measured. Participants remained lying down and at rest during the postprandial period.

Abbreviations: MIT – Meal induced thermogenesis; NEFA – Non-esterified fatty acids; REE – Resting energy expenditure; RQ – Respiratory quotient; T – Time point; TAG – Triacylglycerol; 3-OHB – 3-hydroxybutyrate.

2.5 Blood sample collection and analyses

2.5.1 Blood sample collection

Blood samples were collected on all studies via venepuncture or cannula. Please refer to the individual study chapters for description of blood collection methods specific to that study. Blood samples were collected into potassium EDTA tubes (for the analysis of plasma TAG, NEFA, insulin, 3-OHB, total and HDL-cholesterol) and sodium oxalate tubes (for plasma glucose analysis). For the measurement of plasma C-peptide, a separate potassium EDTA blood collection tube containing 200 kallikrein inhibiting units of aprotinin per ml of whole blood was used to prevent enzymatic degradation. Following collection, whole blood samples were kept chilled. Samples were centrifuged for 15 minutes at 2500 rpm and separated; plasma aliquots were then stored at -20°C with a subset intended for 3-OHB analysis stored at -80°C. Samples were batch analysed in duplicate, with all samples from an individual participant included on a single assay. For all assays performed, high and low concentration
quality controls were placed at the start and end. For non-automated radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) methods, quality controls were additionally placed in the middle of each assay. Intra/inter-assay coefficients of variation (CV) are reported in each individual chapter.

2.5.2 Biochemical analyses and calculations

2.5.2.1 Analysis of plasma glucose

In all studies, plasma glucose was analysed using the ILAB 650 (Instrumentation Laboratory, Milan, Italy) photometric auto-analyser, using an enzymatic colorimetric method (Kit ref: PN0018250840; Instrumentation Laboratory, Milan, Italy). In this method, glucose reacts with oxidation and water in the presence of glucose oxidase to produce gluconic acid and hydrogen peroxide. Hydrogen peroxide (H_2O_2) then reacts with phenol and 4-aminoantipyrine to produce a red quinoneimine and water, in a process catalysed by peroxidase.

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

The concentration of red quinoneimine dye produced is proportional to concentration of glucose in the sample. The limit of sensitivity for this assay is 0.1 mmol/L.

2.5.2.2 Analysis of insulin

2.5.2.2.1 Radioimmunoassay

In Studies one and two, plasma insulin was measured by RIA using a commercially available human specific insulin kit (Kit ref: HI-1AK; Merck Millipore, MA, USA). The assay uses a single labelled antigen that competes for antibody binding with the insulin contained in the plasma sample. The assay has low (<0.2%) cross-reactivity with human proinsulin, and its limit of sensitivity is 2.7 μU/mL.

2.5.2.2.2 Enzyme-linked immunosorbent assay (ELISA)

In Study three, plasma insulin was measured by sandwich ELISA using a commercially available human specific insulin kit (Kit ref: EZHI-14K; Merck Millipore, Massachusetts, US). The principles
of this method are as follows: 1) a plate is coated with a capture antibody; 2) the sample is added, and any antigen (i.e. insulin in the sample) binds to the capture antibody; 3) detecting antibody is added, which binds to the antigen; 4) an enzyme-linked secondary antibody is added, which binds to the detecting antibody; 5) substrate is added, which is converted by the enzyme to a detectable form. Enzyme activity can be measured spectrophotometrically, with absorbency directly proportional to the amount of captured antigen, and thus, concentration of insulin in the sample. The assay has a no cross-reactivity with human proinsulin in plasma, and its limit of sensitivity is 1 μU/ml.

### 2.5.2.3 Analysis of C-peptide

In Study two, plasma C-peptide was measured by RIA using a commercially available Human Specific C-Peptide Kit (Kit ref: HCP–20K; Merck Millipore, MA, USA), the principles of which were discussed in Section 2.5.2.2.1. The assay has a low (<4%) cross-reactivity with human proinsulin, and its limit of sensitivity is 0.065 ng/ml.

### 2.5.2.4 Homeostasis model assessment

In Studies two and three, HOMA-IR was calculated from basal (fasting) glucose and insulin concentrations using the HOMA2 computer model (149), which is available from the Oxford Centre for Diabetes, Endocrinology and Metabolism website (https://www.dtu.ox.ac.uk/homacalculator/). In Study two, HOMA-%B which is a marker of steady state pancreatic β-cell function, was additionally calculated from basal glucose and C-peptide utilising the same HOMA2 model.

### 2.5.2.5 Analysis of plasma triacylglycerol

In all studies, plasma TAG was analysed using the ILAB 650 (Instrumentation Laboratory, Milan, Italy) photometric auto-analyser, using an enzymatic colorimetric method (Kit ref: PN0018255640; Instrumentation Laboratory, Milan, Italy). This method involves a number of intermediary steps which ultimately yields a red coloured quinoneimine.

\[
\text{TAG} \xrightarrow{\text{lipoprotein lipase}} \text{glycerol + fatty acids}
\]

\[
\text{Glycerol + ATP} \xrightarrow{\text{glycerol kinase}} \text{glycerol-3-P + ADP}
\]
Glycerol-3-P + O₂ \xrightarrow{\text{glycerolphosphatase oxidase}} \text{dihydroxyacetone phosphate} + H₂O₂

H₂O₂ + 4-chlorophenol + 4-aminoantipyrine \xrightarrow{\text{peroxidase}} \text{red quinoneimine} + H₂O₂

The concentration of red quinoneimine produced is proportional to concentration of TAG in the sample. The limit of sensitivity for this assay is 0.02 mmol/L.

2.5.2.6 Analysis of plasma total cholesterol

In Studies two and three, fasting plasma total cholesterol was analysed using the ILAB 650 (Instrumentation Laboratory, Milan, Italy) photometric auto-analyser, using an enzymatic colorimetric method (Kit ref: PN0018250540; Instrumentation Laboratory, Milan, Italy). This method involves a series of coupled reactions that hydrolyse cholesterol esters and oxidise the 3-hydroxyl group of cholesterol. H₂O₂, produced as a by-product, reacts with 4-aminoantipyrine and phenol to produce a coloured quinoneimine compound in a peroxidase catalysed reaction.

\[
\begin{align*}
\text{Cholesterol ester} + H₂O & \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{fatty acids} \\
\text{Cholesterol} + O₂ & \xrightarrow{\text{cholesterol oxidase}} \text{cholestene-3-one} + H₂O₂ \\
2H₂O₂ + 4\text{-aminoantipyrine} + \text{phenol} & \xrightarrow{\text{peroxidase}} \text{red quinoneimine} + 4H₂O
\end{align*}
\]

The concentration of quinoneimine produced is proportional to concentration of total-cholesterol in the sample. The limit of sensitivity for this assay is 0.1 mmol/L.

2.5.2.7 Analysis of HDL cholesterol

In Studies two and three, fasting plasma HDL-cholesterol was analysed using the ILAB 650 (Instrumentation Laboratory, Milan, Italy) photometric auto-analyser, using an enzymatic colorimetric method (Kit ref: PN0018255740; Instrumentation Laboratory, Milan, Italy). In this method, antihuman β-lipoprotein antibody, which binds to lipoproteins other than HDL (i.e. VLDL, LDL and chylomicrons) is added, forming an antigen-antibody complex. This complex blocks enzyme reactions with all lipoproteins except HDL-cholesterol when a second reagent (R2) is added, which contains cholesterol esterase, cholesterol oxidase and a chromogen. H₂O₂ produced during these enzymatic reactions yields a blue coloured complex upon oxidative condensation of the chromogen. The
concentration of the blue coloured complex produced is proportional to the concentration of HDL-cholesterol. The limit of sensitivity for this assay is 0.05 mmol/L.

2.5.2.8 Analysis of LDL cholesterol

In Studies two and three, LDL-cholesterol was calculated using the Friedewald equation (150):

\[
\text{LDL cholesterol} = \text{total cholesterol} - \text{HDL cholesterol} - \left(\frac{\text{TAG}}{2.2}\right)
\]

2.5.2.9 Analysis of plasma non-esterified fatty acids

In Studies one and two, plasma NEFA were analysed using the ILAB 650 (Instrumentation Laboratory, Milan, Italy) photometric auto-analyser, using an enzymatic colorimetric method (Kit ref: FA115; Randox Laboratories Ltd, County Antrim, UK):

\[
\text{NEFA} + \text{ATP} + \text{CoA} \xrightarrow{\text{acyl coa synthetase}} \text{Acyl coA} + \text{AMP} + \text{PPI}
\]

\[
\text{Acyl CoA} + \text{O}_2 \xrightarrow{\text{acyl coa oxidase}} \text{2,3-trans-enoyl} - \text{CoA} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + \text{TOOS} + 4\text{-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{purple adduct} + 4\text{H}_2\text{O}
\]

\([\text{TOOS} = \text{N-ethyl-N-(2-hydroxy-3-sulphopropyl)-toluidine}]

The proportion of purple adduct produced is proportional to concentration of NEFA in the sample. The limit of sensitivity for this assay is 0.07 mmol/L.

2.5.2.10 Analysis of 3-hydroxybutyrate

In Studies one and two, plasma 3-OHB was analysed using the Cobas Mira (Roche Diagnostics, Massachusetts, US) photometric auto-analyser, using an enzymatic colorimetric method (Kit ref: RB1007; Randox Laboratories Ltd, County Antrim, UK). In this method, 3-OHB is oxidised to acetoacetate in the presence of the enzyme, 3-OHB dehydrogenase.

\[
3\text{-OHB} + \text{NAD}^+ \xrightarrow{3\text{-OHB dehydrogenase}} \text{acetoacetate} + \text{H}^+ + \text{NADH}
\]

This oxidation reaction is accompanied by concomitant reduction of nicotinamide adenine dinucleotide (NAD)^+ cofactor to NADH, with the associated change in absorbance proportional to the concentration of 3-OHB. The limit of sensitivity for this assay is 0.1 mmol/L.
2.5.2.11 Quantification of postprandial metabolite responses

In Studies one and two, area under the curve (AUC; for NEFA and 3-OHB) and incremental area under the curve (iAUC; for all other metabolites) were calculated to quantify the magnitude of the postprandial metabolite responses using the trapezoid method, subtracting the area below baseline for iAUC.

2.6 Indirect calorimetry

In Studies one and two, energy expenditure and substrate utilisation were calculated using data obtained from a Gaseous Exchange Monitor ISGEM319 (GEM Nutrition, Cheshire, UK), an open-circuit indirect calorimeter based on the ventilated flow-through technique (151). Prior to its use, the ISGEM319 was calibrated using two reference gases of known concentration (BOC, Guildford, UK): span gas (certified air: 20% oxygen, 1% oxygen) and zero gas (pure nitrogen). In both studies, fasted measurements of REE and RQ were taken. In brief, following a 30-minute period of rest in a supine position (and prior to cannulation) a clear plastic hood was placed over the participant’s head in order to collect expired air and calculate respiratory CO₂ and O₂ exchange. REE was measured over 20 minutes and in accordance with methodological recommendations by Compher et al (147). Readings (reported in one-minute time blocks) from the first five minutes were discounted, with the remaining 15 minutes then averaged and used to calculate the REE (Section 2.6.1) and fasting RQ, which is the ratio of VCO₂ produced to VO₂ consumed. A RQ value of 0.703 represents pure fat oxidation, a value of 1.0 or higher represents pure carbohydrate oxidation (152). Data were excluded from analyses if the CV exceeded 10% (147). In Study one, serial gaseous exchange measurements were also taken over 10-minute blocks (Figure 2.1). This enabled the calculation of postprandial substrate oxidation (Section 2.6.2) and MIT (Section 2.6.1).

2.6.1 Calculation of energy expenditure

REE and MIT were calculated using the modified Weir equation (153):

\[
kJ/\text{min} = (3.9 \, \text{VO}_2 + 1.1 \, \text{VCO}_2) \times 4.184
\]

\( \text{O}_2 \) and \( \text{VCO}_2 \) represent \( \text{O}_2 \) consumption and \( \text{CO}_2 \) production respectively, in l/min
For REE, the averaged fasting kJ/min was multiplied by 1440 to produce kJ/day. For MIT, average kJ/min values for each postprandial measurement time block were plotted over the 360-minute time course, with MIT proportional to the iAUC.

### 2.6.2 Postprandial substrate oxidation

Fasting and postprandial substrate oxidation rates were calculated using the non-protein stoichiometric equations from Frayn (152), with the assumption that protein oxidation was negligible:

\[
\text{Carbohydrate oxidation} = 4.55VCO_2 - 3.21VO_2
\]

\[
\text{Fat oxidation:} \quad 1.67VO_2 - 1.67VCO_2
\]

[V02 and VC02 represent O2 consumption and CO2 production respectively, in l/min]

Averaged substrate oxidation rates (g/min) at each postprandial time block were plotted over the 360-minute time course, with total postprandial substrate oxidation proportional to the AUC.

### 2.7 Statistical analyses

Statistical analyses were conducted using SPSS (IBM, Chicago, USA) versions 22 (Study one) and 23 (Studies two and three) with statistical significance accepted at the 5% level. Data were checked for normality prior to statistical analyses using the Shapiro Wilks normality test (154). Non-parametric data were normalised via log transformation where possible. The corresponding non-parametric tests were used where data could not be normalised. The statistical analyses used for each study are described in the methods section of the relevant chapter.
Chapter 3: Investigation into the acute effects of total and partial energy restriction on postprandial metabolism

3.1 Introduction

As introduced in Chapter one, the IER approach to weight-loss involves intermittent periods of very low (or zero) energy intake interspersed with normal eating, most commonly for two days per week, or on alternate days (Table 1.1). However, little is known about the effects of this altered eating pattern on postprandial glucose and lipid metabolism, which is pertinent given the growing evidence base implicating both as independent discriminators of CVD risk (Section 1.3). It is important for our understanding of the metabolic adaptation which occurs during weight-loss with IER, that we first establish what happens acutely and independently of weight-loss i.e. during one cycle of substantial ER and refeeding.

The short-term metabolic adaptation to total starvation, which occurs and becomes maximal within the first 96 hours of total ER, has been well defined (Section 1.4.4; (100)). Briefly, this includes an increase in circulating NEFA (predominantly sourced from adipose tissue), as well as a shift in fuel utilisation towards FAO and ketogenesis. There is also a marked decline in peripheral insulin sensitivity and hence glucose tolerance. From an evolutionary standpoint, these reciprocal adaptations in glucose and lipid metabolism serve to conserve glucose and hence limit the utilisation of protein stores. They are reversed through food reintroduction, but this may require upwards of 48 hours of refeeding.

The majority of the dietary protocols used by IER studies have allowed a small amount of food intake in an attempt to improve the tolerability and compliance to IER, such that energy intake is substantially (but not completely) restricted by at least 70% of estimated requirements. This has the potential to alter the response which has been well characterised by acute studies of total ER, and hence the subsequent postprandial response during refeeding. Acutely, this is best assessed using a within-study design which to our knowledge has not been performed in overweight/obese individuals.
3.2 Study aims, outcomes and hypothesis

3.2.1 Aims
The present cross-over study, conducted in a small sample of overweight/obese participants, aimed to characterise the acute metabolic response to varying degrees of ER (relevant to IER) by assessing fasting and postprandial responses to a liquid mixed test meal after one day of total (100%) and partial (75%) ER.

3.2.2 Outcomes
- Primary outcomes: Postprandial glycaemia.

3.2.3 Hypothesis
- Partial ER, by permitting small quantity of food intake, will attenuate the known, documented, negative impact of prolonged total ER on glycaemic control.

3.3 Participants and methods

3.3.1 Participants
Healthy, overweight or obese participants aged 18 to 60 years were recruited to the study from the Guildford and wider community via email and poster advertisement, as well as word of mouth. Participants were weight-stable (±2 kg) over the preceding three months and had no significant medical history. Health status was determined via medical questionnaire and screening blood sample. To control for the potential influence of the menstrual cycle between visits, female participants were either post-menopausal or taking oral contraceptives. Restrained eaters were identified and excluded using the Dutch eating behaviour questionnaire (cut-off: >4) as potential confounders for the secondary outcome ((155); Appendix C). The study was approved by the University of Surrey Ethics Committee (EC/2014/03/FHMS) and conducted in accordance with the guidelines laid down in the Declaration of
Helsinki. Written, informed consent was obtained from all participants.

3.3.1.1 Sample size calculations

For the iAUC for plasma glucose, 10 participants entering a cross-over trial would give an 80% probability of detecting a treatment difference (total versus partial ER) of 122 mmol.360min.L⁻¹ at a two-sided 0.05 significance level, based on a standard deviation in response to treatment of 123 mmol.360min.L⁻¹.

3.3.2 Study protocol

The study was a three-way, randomised, cross-over study (Figure 3.1) in which participants completed three one-day dietary interventions (Day one) in a random order with a minimum one-week washout: one day of isoenergetic intake (0% ER) which served as the control; one day of total (100%) ER; and one day of partial (75%) ER. Metabolic assessments were conducted on the following day (Day two). To assess for short-term energy compensation, dietary intakes were recorded over a three-day period, which encompassed each controlled intake day (Day one), whilst participants were at the research unit (Day two) and following resumption of ad libitum intake (Days two and three). To ensure familiarity with study procedures, participants first completed a pre-study test day which was identical in design to the isoenergetic intervention.
A randomised, cross-over study. Participants completed three dietary interventions in a random order (Day one): one day of isoenergetic intake (0% ER) which served as the control; one day of total (100%) ER; and one day of partial (75%) ER. Metabolic assessments were conducted on the following day (Day two). To assess for short-term energy compensation, dietary intakes were recorded over a three-day period, which encompassed each controlled intake day (Day one), whilst participants were at the research unit (Day two) and following resumption of ad libitum intake (Days two and three). There was a minimum one-week washout period. To ensure familiarity with study procedures, participants first completed a pre-study day which was identical in design to the isoenergetic intervention.

**Abbreviations:** ER – Energy restriction; PP – Postprandial.

### 3.3.2.1 Day one: Experimental diets

Estimated requirements were calculated as previously described (Section 2.2). Participants were asked to keep to similar activity patterns and to avoid strenuous activity during each three-day intervention period. The reported range of PALs used to calculate daily energy requirements (1.4-1.6) lie within the 10th and 50th centile of the pooled data set (derived from doubly labelled water studies) used by the Scientific Advisory Committee on Nutrition in their most recent energy report (142).

#### 3.3.2.1.1 Isoenergetic control diet (0% energy restriction)

Each participant was supplied with an isoenergetic diet comprised of commonly consumed food and drink (11,040 ± 1482 kJ; ~55%, ~15% and ~30% of total energy as carbohydrate, protein and fat respectively) which provided 100% of their estimated isoenergetic needs. Hence, this trial arm served as the control, representing both energy balance and a standard overnight fast. An example of a typical
menu plan is presented in Table 3.1.

<table>
<thead>
<tr>
<th></th>
<th>Food</th>
<th>Quantity</th>
<th>Energy (kJ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breakfast (08:00)</td>
<td>Swiss muesli</td>
<td>80g</td>
<td>1278</td>
</tr>
<tr>
<td></td>
<td>Whole milk</td>
<td>150ml</td>
<td>414</td>
</tr>
<tr>
<td></td>
<td>Orange juice</td>
<td>200ml</td>
<td>306</td>
</tr>
<tr>
<td>Lunch (12:30)</td>
<td>Dolmio pasta pot</td>
<td>1 pot</td>
<td>1248</td>
</tr>
<tr>
<td></td>
<td>Celebration chocolates</td>
<td>4</td>
<td>757</td>
</tr>
<tr>
<td></td>
<td>Strawberry yogurt</td>
<td>125g</td>
<td>520</td>
</tr>
<tr>
<td>Dinner (19:00)</td>
<td>Extra lean mince</td>
<td>125g</td>
<td>682</td>
</tr>
<tr>
<td></td>
<td>Chilli con carne sauce</td>
<td>220g</td>
<td>348</td>
</tr>
<tr>
<td></td>
<td>Vegetable oil</td>
<td>20g</td>
<td>739</td>
</tr>
<tr>
<td></td>
<td>White rice</td>
<td>96g</td>
<td>1452</td>
</tr>
<tr>
<td>Snacks (10:00 and 15:00)</td>
<td>Salted peanuts</td>
<td>13g x 2</td>
<td>635</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>8379</strong></td>
</tr>
</tbody>
</table>

Table 3.1 Exemplar 8379 kJ (~2000 kcal) menu plan for isoenergetic trial
Please refer to Appendix A for brand names.

3.3.2.1.2 Total (100%) energy restriction:
Participants started their fast from 20:00 the night before their dietary intervention day until 08:00 on the morning of their study day, totalling 36 hours.

3.3.2.1.3 Partial (75%) energy restriction:
Participants consumed four commercially available LighterLife Food Packs (Essex, UK) (2638 kJ; 38%, 36% and 26% of total energy as carbohydrate, protein and fat respectively) which provided ~25% of their estimated isoenergetic needs. The degree of ER chosen is comparable to that used by previously published IER weight-loss trials (84, 87, 91). The menu plan is presented in Table 3.2.
<table>
<thead>
<tr>
<th>Food</th>
<th>Quantity</th>
<th>Energy (kJ)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breakfast (08:00)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Banana milkshake</td>
<td>1x</td>
<td>643</td>
</tr>
<tr>
<td><strong>Lunch (12:30)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vegetable soup</td>
<td>1x</td>
<td>631</td>
</tr>
<tr>
<td><strong>Dinner (19:00)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spaghetti Bolognese</td>
<td>1x</td>
<td>630</td>
</tr>
<tr>
<td><strong>Snacks (10:00 and 14:00)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nut fudge bar</td>
<td>½ x 2</td>
<td>734</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>2638</td>
</tr>
</tbody>
</table>

Table 3.2 Menu plan for partial energy restriction trial
Lighterlife (Essex, UK) meal replacement products were used.

During each controlled energy intake day, participants were advised to consume sufficient amounts of non-caloric fluids and to abstain from alcohol. On the isoenergetic and partial ER days, participants finished their last meal no later than 20:00.

3.3.2.2 Day two: Laboratory visits

Participants attended the Surrey Clinical Research Centre on the morning (08:00) after each controlled energy intake day. Body weight and composition were measured by bioimpedance (Section 2.1). Fasted measurements of energy expenditure and substrate utilisation were then taken via indirect calorimetry after participants had rested for 30 minutes. Participants then recorded baseline appetite scores using visual analogue scales (VAS). An indwelling cannula was then inserted following which the first (fasted) sample was taken. A liquid mixed test meal was given (400ml Fortisip, Nutricia, Trowbridge, UK) which provided 2510kJ, 74g carbohydrate, 24g protein and 23g fat. Serial blood samples and measurements of energy expenditure, substrate oxidation and appetite were taken at regular intervals from the start of the test-meal over the next 360 minutes. Please refer to Figure 2.1 for diagrammatic overview of the study day and sampling schedule. Participants were then presented with a large pre-weighed ad libitum homogenous pasta meal in excess of normal portions and advised to eat until comfortably full. Following this, they were instructed to resume habitual intake after leaving the unit and to keep a diet diary until midnight of the next day (Day three).
3.3.3 Experimental techniques and analyses

3.3.3.1 Indirect calorimetry:

Energy expenditure and substrate utilisation were calculated using data obtained from a Gaseous Exchange Monitor ISGEM319 (Section 2.6). Participants remained lying down and at rest during the postprandial period. Fasted measures were taken over 20 minutes and in accordance with methodological recommendations by Compher et al (147). Postprandial gaseous exchange measurements were taken over 10 minutes every 30 minutes for the first 180 minutes after the liquid mixed test meal, and then hourly thereafter until the end of the 360-minute postprandial period (Figure 2.1). REE and MIT were calculated as described in Section 2.6.1. Fasting and postprandial substrate oxidation were calculated using the non-protein stoichiometric equations from Frayn ((152); Section 2.6.2). These stoichiometric equations assume negligible contributions from gluconeogenesis, protein oxidation and ketogenesis; whilst these assumptions may not hold true following substantial ER, evidence from studies over longer (72-hour) fasting durations suggest the error introduced by not accounting for the latter two metabolic processes would be minimal (106).

3.3.3.2 Blood biochemistry

Serial fasting and postprandial blood samples were collected via cannula. Samples were batch analysed upon study completion with all samples from an individual participant included in the same assay. Metabolites were analysed using the following methods: insulin using RIA (Millipore, Billerica, USA; intra/inter-assay CVs 6% and 8%); glucose, TAG and NEFA using the ILAB 650 photometric auto-analyser (Instrumentation Laboratory, Warrington, UK; intra/inter-assay CV all <4 % and <7%); and 3-OHB using the Cobas MIRA photometric auto-analyser (Roche, Welwyn Garden City, UK; intra/inter-assay CVs 6% and 9%). Please refer to Section 2.5 for full explanation of protocols used for blood sample collection, processing, storage and the analytical techniques used. AUC (for NEFA and 3-OHB) and iAUC (for all other metabolites) were calculated as described in Section 2.5.2.11.

3.3.3.3 Dietary analyses

Nutritional intake information recorded in diet diaries (Days one, two and three), from the weighed ad
*libitum* pasta meal (Day two) and the liquid test-meal (Day two) were aggregated. This information was then used to calculate daily and cumulative three-day energy consumption, expressed as the percentage of estimated isoenergetic needs. Please refer to Section 2.3 for a full explanation of methods relating to diet diary analyses.

3.3.3.3.1 *Ad libitum* pasta meal

The *ad libitum* pasta meal was comprised of 400 g dry weight fusilli pasta, 500 g pasta sauce, 100 g mild cheddar cheese and 30 ml of vegetable oil (all Tesco, Welwyn Garden City, UK). The whole dish provided 9950 kJ (50% carbohydrate, 20% protein and 30% fat). The meal was presented to participants on a plastic serving tray alongside an empty plate, cutlery and a small glass of water. Total energy intake was calculated by multiplying energy density of the meal by the quantity eaten. The test was conducted in standardised environmental conditions, by always using the same room which was free of disturbing factors such as odours and sounds. Participants completed the test in isolation in order to avoid social interaction.

3.3.3.3.2 Assessment of under-reporting

Participants recorded habitual intake in diet diaries for three days prior to starting the study. The revised Goldberg equations were used to evaluate the potential for under-reporting of dietary intakes in this cohort (at individual and study population levels), based on reported intakes during this pre-intervention period (Section 2.3.2.1). PAL values used in this cohort ranged between 1.4 to 1.6, with the between-participant variance in reported PAL (CV_P) calculated to be 4.6%.

3.3.3.4 Appetite assessment

Appetite was assessed using validated paper-based 100 mm VAS which were comprised of a set of three questions: (1) How hungry do you feel?; (2) How full do you feel?; and (3) How much do you think you can eat? ((156); Appendix D). Participants placed a vertical mark on the 100mm horizontal line according to how they felt at the time, with the statements at the end of the lines representing the extremes of subjective appetite. Responses were measured (from left to right) using a ruler.
Participants completed the VAS every two hours (during waking hours) on their controlled energy intake day (Day one), with results presented as daily averages. On Day two, serial measurements of appetite were taken at baseline and then at regular intervals after the test drink (analogous to blood sampling protocol; Figure 2.1). Participants recorded appetite scores prior to blood measurements.

3.3.4 Statistical analyses
Data were checked for normality using the Shapiro-Wilks test, with non-normally distributed data normalised via log transformation to permit parametric testing (Section 2.7). Parametric testing was used throughout. Comparisons were made between the three experimental arms using repeated measures analysis of variance with a Sidak correction applied to post-hoc pairwise comparisons. A Sidak correction is recommended when there is concern regarding loss of power e.g. due to small sample sizes (157). For summary measures (dietary intakes, averaged daily appetite scores and fasting metabolites), dietary intervention was used as the within-participant factor. For time-course data, time point was used as an additional within-participant factor to explore main and interaction effects. All results are presented as mean ± SEM.

3.3.5 Data omissions
Indirect calorimetry data (REE, RQ, postprandial substrate oxidation) for two participants were excluded after data loss due to equipment failure, leaving n=8 for this variable. Two subjects failed to record appetite scores on one of their controlled energy intake days and so were omitted from the Day one appetite analyses, leaving n=8 for this variable.

3.4 Results
3.4.1 Participant characteristics
Fourteen (six female) participants were initially recruited to the study from the University of Surrey and wider community. Four (three female) participants did not finish the study due to non-compliance (n=1) or cannulation difficulties (n=3), culminating in ten study completers. Participant characteristics for study completers are presented in Table 3.3.
<table>
<thead>
<tr>
<th>Age (years)</th>
<th>All participants (n=10)</th>
<th>Males (n=7)</th>
<th>Females (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>36 ± 5</td>
<td>42 ± 5</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>91.2 ± 5.6</td>
<td>97.4 ± 6.5</td>
<td>76.6 ± 4.4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.1 ± 0.8</td>
<td>29.0 ± 1.1</td>
<td>29.5 ± 0.6</td>
</tr>
<tr>
<td>Body fat (%)*</td>
<td>29.8 ± 2.3</td>
<td>26.3 ± 1.9</td>
<td>37.9 ± 2.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Fasting (mmol/L)</th>
<th>iAUC (mmol.360min.L⁻¹)</th>
<th>iAUC (mmol.360.min.L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.9 ± 0.2</td>
<td>141 ± 30</td>
<td>144 ± 39</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Triacylglycerol</th>
<th>Fasting (mmol/L)</th>
<th>iAUC (mmol.360min.L⁻¹)</th>
<th>iAUC (mmol.360.min.L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.2 ± 0.1</td>
<td>95 ± 18</td>
<td>95 ± 26</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NEFA</th>
<th>Fasting (mmol/L)</th>
<th>AUC (mmol.360 min.L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.53 ± 0.07</td>
<td>126 ± 13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3-OHB</th>
<th>Fasting</th>
<th>AUC (mmol.360.min.L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05 ± 0.02</td>
<td>19 ± 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate oxidation†</th>
<th>Fat (g.360min.L⁻¹)</th>
<th>CHO (g.360min.L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 ± 5</td>
<td>59 ± 5</td>
</tr>
</tbody>
</table>

| MIT (kJ.360min.L⁻¹)† | 180 ± 34           | 215 ± 41           |

Table 3.3: Baseline characteristics for study completers
*Bioimpedance. †Indirect calorimetry. Measurements taken at pre-trial visit. Presented as mean ± SEM.

Abbreviations: AUC – Area under the curve; BMI – Body mass index, CHO – Carbohydrate; iAUC – Incremental area under the curve; MIT – Meal induced thermogenesis; NEFA – Non-esterified fatty acids; 3-OHB – 3-hydroxybutyrate.

3.4.2 Day one: Controlled energy intake day - dietary intakes and appetite

Reported dietary intakes during each controlled energy intake day were close to prescribed amounts (See later: Table 3.5). Averaged daily appetite scores are presented in Figure 3.2 A-C. There were significant main effects of dietary intervention for hunger (p=0.032), with participants tending to report greater hunger during total ER (66 ± 6 mm; p=0.084 trend) compared to when they were in energy balance (42 ± 5mm). Similar (numerical) elevations were noted during partial ER (62 ± 6 mm) compared to the day of isoenergetic intake, although, pairwise comparisons did not reach statistical significance (p=0.216). There were also main effects for prospective food consumption (p=0.001) and fullness (p<0.001). When compared to the day of isoenergetic intake (47 ± 5 mm), feelings of prospective food consumption were greater during total ER (74 ± 5 mm; p=0.024), and tended to be higher during partial ER (66 ± 5 mm; p=0.069). Fullness was lower during total (22 ± 2 mm; p=0.05) and partial (29 ± 3 mm; p=0.04) ER, relative to the day of isoenergetic intake (33 ± 3mm). Appetite scores did not differ between the two ER days (all p≥0.162).
Figure 3.2 A-C) Averaged daily appetite scores during the controlled energy intake days (Day one)
Appetite scored every two hours using 100 mm visual analogue scales then averaged
Statistics and data presentation: Repeated measures analysis of variance with Sidak correction. Main effect statistic reported in title. *Significantly different to the isoenergetic intervention (p<0.05). Presented as mean ± SEM. n=8.
Abbreviations: ER - Energy restriction.

3.4.3 Day two: Assessments conducted the day after each controlled intake day

3.4.3.1 Fasted substrate and energy metabolism

Substrate and energy metabolism were assessed in the fasted state via blood sampling and indirect calorimetry on the morning after each controlled energy intake day, with results presented in Table 3.4. Hormone and substrate levels after the day of isoenergetic intake reflect the typical metabolic situation after an overnight fast. Relative to this, fasting plasma glucose (p=0.028) and TAG (p=0.017) were significantly lower following one day of total ER. Conversely, plasma NEFA levels and 3-OHB were elevated (p=0.022 and p=0.005 respectively). Accordingly, fasting RQ was significantly lower after total ER (p=0.036), indicative of a heightened state of fat oxidation. Similar patterns were noted
for partial ER, with the exception that rise in plasma NEFA and decline in RQ did not attain significance (both \( p \geq 0.613 \) vs. isoenergetic). When comparing the two ER trials, plasma NEFA and 3-OHB were significantly higher the day after total ER (\( p=0.005 \) and \( p=0.016 \) respectively vs. partial ER). No significant differences in REE were found across the three trials (\( p=0.924 \)).

<table>
<thead>
<tr>
<th></th>
<th>Isoenergetic (0% ER)</th>
<th>Partial (75%) ER</th>
<th>Total (100%) ER</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose (mmol/L)</strong></td>
<td>4.7 ± 0.1</td>
<td>4.4 ± 0.1(^A)</td>
<td>4.3 ± 0.2(^A)</td>
</tr>
<tr>
<td><strong>Insulin (pmol/L)</strong></td>
<td>89.7 ± 9.2</td>
<td>69.1 ± 8.0</td>
<td>74.0 ± 12.4</td>
</tr>
<tr>
<td><strong>Triacylglycerol (mmol/L)</strong></td>
<td>1.5 ± 0.2</td>
<td>1.2 ± 0.2(^A)</td>
<td>1.2 ± 0.3(^A)</td>
</tr>
<tr>
<td><strong>NEFA (mmol/L)</strong></td>
<td>0.63 ± 0.07</td>
<td>0.73 ± 0.06(^B)</td>
<td>1.01 ± 0.11(^AC)</td>
</tr>
<tr>
<td><strong>3-OHB (mmol/L)</strong></td>
<td>0.05 ± 0.02</td>
<td>0.26 ± 0.07(^AB)</td>
<td>0.56 ± 0.11(^AC)</td>
</tr>
<tr>
<td><strong>REE (kJ/day)</strong></td>
<td>6169 ± 513</td>
<td>6256 ± 465</td>
<td>6154 ± 570</td>
</tr>
<tr>
<td><strong>RQ (VCO2/VO2)</strong></td>
<td>0.89 ± 0.03</td>
<td>0.84 ± 0.02</td>
<td>0.81 ± 0.03(^A)</td>
</tr>
</tbody>
</table>

* RQ: 0.703 = Pure fat oxidation; 1.0 = Pure carbohydrate oxidation.

Statistics and data presentation: Repeated measures analysis of variance with Sidak correction.

A-C Significantly different to: \(^A\) Isoenergetic, \(^B\) Total ER, \(^C\) Partial ER. Presented as mean ± SEM. n=10 (biochemistry) and n=8 (RQ, REE).

Abbreviations: ER – Energy restriction; NEFA – Non-esterified fatty acids; REE – Resting energy expenditure; RQ – Respiratory quotient; 3-OHB – 3-hydroxybutyrate.

**3.4.3.2 Postprandial glucose and insulin responses**

Postprandial glycaemic responses to the liquid mixed test meal are presented in Figure 3.3 A-B. For postprandial glucose responses, there was a main effect of dietary intervention (\( p=0.002 \)); following total ER, postprandial glucose responses were significantly greater when compared to the isoenergetic trial (402 ± 64 vs. 166 ± 35 mmol.360min.L\(^{-1}\); \( p=0.023 \)), and also tended to be higher relative to the partial ER trial (294 ± 40 mmol.360min.L\(^{-1}\); \( p=0.087 \)). A trend in favour of a greater postprandial glucose response following partial ER was also found (\( p=0.089 \) vs. isoenergetic). Time-course analyses also found a significant diet x time interaction (\( p<0.001 \)). Glucose time to peak was significantly delayed following total ER relative to both the isoenergetic (\( p=0.024 \)) and partial ER (\( p=0.018 \)) trials. There were no significant main effects of dietary intervention for postprandial plasma insulin, although, there was a trend in favour of a diet x time interaction (\( p=0.091 \)).

**3.4.3.3 Postprandial triacylglycerol responses**

For postprandial TAG responses (Figure 3.3C), time-course analyses found significant main effects
of dietary intervention and a diet x time interaction (both \( p < 0.001 \)); compared with the isoenergetic trial (131 ± 20 mmol.360min.L\(^{-1}\)), postprandial TAG was significantly lower following both total (32 ± 11 mmol.360min.L\(^{-1}\); \( p = 0.001 \)) and partial (54 ± 19 mmol.360min.L\(^{-1}\); \( p = 0.005 \)) ER. Postprandial TAG responses did not differ between the two ER trials (\( p = 0.258 \)).

### 3.4.3.4 Postprandial non-esterified fatty acids

For postprandial NEFA (Figure 3.3D), time-course analyses found significant main effects of dietary intervention (\( p = 0.008 \)) and a diet x time interaction (\( p < 0.001 \)); following total ER, levels of postprandial NEFA were greater when compared to the isoenergetic trial (169 ± 7 mmol.360min.L\(^{-1}\) vs. 138 ± 15 mmol.360min.L\(^{-1}\); \( p = 0.039 \)), and tended to be higher relative to the partial ER trial (164 ± 10 mmol.360min.L\(^{-1}\); \( p = 0.083 \)). Postprandial NEFA responses did not differ significantly between partial ER and isoenergetic trials (\( p = 0.316 \)).
Postprandial substrate oxidation and energy metabolism

Postprandial substrate oxidation and MIT responses (n=8) to the test meal challenge are presented in Figure 3. For postprandial fat oxidation, time-course analyses found a trend in favour of a main effect of dietary intervention (p=0.080), however, there were no significant pairwise differences between isoenergetic (17 ± 2 g.360min.L⁻¹), total (26 ± 4 g.360min.L⁻¹) or partial (24 ± 4 g.360min.L⁻¹) ER trials (all p≥0.184). There was a significant main effect of dietary intervention for postprandial

Figure 3.3 A-D) Postprandial glycaemic, TAG and NEFA responses, assessed after each controlled intake day
Isoenergetic intake (blue), partial 75% ER (green) and total 100% ER (red). Liquid mixed test meal provided: 2510 kJ, 74 g carbohydrate, 24 g protein and 23 g fat.

Statistics and data presentation:
(A) Glucose: There was a diet x time interaction (p<0.001).
(B) Insulin: There was a trend in favour of a diet x time interaction (p=0.091).
(C) TAG: There were main effects of diet (p<0.001) and a diet x time interaction (p<0.001). Pairwise differences: Isoenergetic vs total ER (p=0.001) and partial ER (p=0.005).
(D) NEFA: There were main effects of diet (p=0.008) and a diet x time interaction (p<0.001). Pairwise differences: Total ER vs. isoenergetic (p=0.039) and partial ER (p=0.083; non-significant trend).
Repeated measures analysis of variance with Sidak correction. Graphs A-C presented as mean change from baseline ± sem. Graph D presented as absolute concentration ± sem. n=10.

Abbreviations: ER – Energy restriction; NEFA – Non-esterified fatty acids; TAG – Triacylglycerol.

3.4.3.5 Postprandial substrate oxidation and energy metabolism

Postprandial substrate oxidation and MIT responses (n=8) to the test meal challenge are presented in Figure 3.4 A-D. For postprandial fat oxidation, time-course analyses found a trend in favour of a main effect of dietary intervention (p=0.080), however, there were no significant pairwise differences between isoenergetic (17 ± 2 g.360min.L⁻¹), total (26 ± 4 g.360min.L⁻¹) or partial (24 ± 4 g.360min.L⁻¹) ER trials (all p≥0.184). There was a significant main effect of dietary intervention for postprandial
carbohydrate oxidation (p=0.023), which tended to be lower following total ER relative to the isoenergetic trial (32 ± 6 g.360min.L⁻¹ vs. 59 ± 5 g.360min.L⁻¹; p=0.051). Postprandial carbohydrate oxidation following partial ER (43 ± 7 g.360min.L⁻¹) was not significantly different to isoenergetic or total ER trials (p≥0.113). For plasma 3-OHB responses, time-course analyses found significant main effects of dietary intervention and a diet x time interaction (both p<0.001); when compared to the isoenergetic trial (19 ± 2 mmol.360min.L⁻¹), levels of postprandial 3-OHB were significantly greater following both total (70 ± 7 mmol.360min.L⁻¹; p=0.001) and partial (60 ± 12 mmol.360min.L⁻¹ p=0.007) ER. Postprandial 3-OHB responses did not differ between the two ER trials (p=0.692). No statistically significant differences in MIT were detected across the three trials (p=0.724).
Postprandial appetite responses to the test meal challenge are presented in Figure 3.5 A-C. Postprandial hunger responses did not differ across the three dietary trials (p=0.741). For prospective food consumption, there were significant main effects of dietary intervention (p=0.008), with postprandial sensations tending to be higher following total (p=0.081) and partial (p=0.092) ER, when compared to the isoenergetic control trial. For postprandial fullness, there were significant main effects of dietary intervention (p=0.023). Pairwise differences: Isoenergetic vs total ER (59 ± 5 g.360min.L⁻¹ vs. 32 ± 6 g.360min.L⁻¹; p=0.051)
of dietary intervention (p=0.026), as well as a diet x time interaction (p=0.017). However, no significant differences in fullness were found between the three diet trials with subsequent pairwise comparisons (all p>0.05).

Figure 3.5 A-C) Postprandial appetite, assessed after each controlled intake day
Isoenergetic intake (blue), partial 75% ER (green) and total 100% ER (red). Liquid mixed test meal provided: 2510 kJ, 74 g carbohydrate, 24 g protein and 23 g fat.

Statistics and data presentation:
(A) Hunger: There were no significant main effects of diet (p=0.741).
(B) Prospective food intake: There were main effects of diet (p=0.008). Pairwise differences: Isoenergetic vs. total ER (p=0.081) and partial ER (p=0.092); both non-significant trends
(C) Fullness: There were main effects of diet (p=0.026) and a diet x time interaction (p=0.017). Repeated measures analysis of variance with Sidak correction. Presented as mean ± sem. n=10.

Abbreviations: ER – Energy restriction.

3.4.4 Days one, two and three: Cumulative dietary intakes

3.4.4.1 Energy intake

Table 3.5 displays day-by-day and cumulative energy consumption across each three-day trial.
Starting with Day two intakes, participants over-consumed by 23 ± 8% above estimated daily energy requirements (p=0.010 vs. isoenergetic) the day after total ER, and by 10 ± 6 % following partial ER (p=0.945 vs. isoenergetic). On Day three, no significant differences in energy intake were detected across the three dietary trials when expressed either in kJ or relative to isoenergetic requirement. Overall, cumulative intakes over the three-day dietary recording periods were significantly lower on both total and partial ER trials relative to the isoenergetic trial, with participants remaining in comparable net energy deficits of ~30% (both p<0.001 vs. isoenergetic). No significant differences in any dietary intake measure were noted between total and partial ER trials.

<table>
<thead>
<tr>
<th>Controlled intake day 1</th>
<th>Isoenergetic (0% ER)</th>
<th>Partial (75%) ER</th>
<th>Total (100%) ER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy intake (kJ/day)</td>
<td>11,040 ± 468</td>
<td>2688 ± 43AB</td>
<td>118 ± 73AC</td>
</tr>
<tr>
<td>% ER achieved*</td>
<td>+0.4 ± 0.3</td>
<td>-75 ± 1AB</td>
<td>-99 ± 1AC</td>
</tr>
<tr>
<td>Ad libitum meal (kJ)</td>
<td>4929 ± 354</td>
<td>5341 ± 342</td>
<td>5946 ± 534</td>
</tr>
<tr>
<td>Total 24 h intake (kJ/day)</td>
<td>11,816 ± 1057</td>
<td>12,154 ± 905</td>
<td>13,522 ± 1036</td>
</tr>
<tr>
<td>% 24 h energy balance*</td>
<td>+8 ± 9</td>
<td>+10 ± 6</td>
<td>+23 ± 3A</td>
</tr>
<tr>
<td>Day 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total 24 h intake (kJ/day)</td>
<td>9675 ± 1089</td>
<td>8428 ± 792</td>
<td>9914 ± 1026</td>
</tr>
<tr>
<td>% 24 h energy balance*</td>
<td>-13 ± 9</td>
<td>-23.1 ± 6.8</td>
<td>-9 ± 9</td>
</tr>
<tr>
<td>Day 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total daily intake (kJ/day)</td>
<td>32,532 ± 2257</td>
<td>23,026 ± 1404A</td>
<td>23,556 ± 1941A</td>
</tr>
<tr>
<td>Net 3-day energy balance*</td>
<td>-2 ± 5</td>
<td>-30 ± 3A</td>
<td>-28 ± 5A</td>
</tr>
<tr>
<td>Cumulative 3-d total</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.5 Daily and cumulative three-day energy intakes during each dietary trial

Statistics and data presentation: Repeated measures analysis of variance with Sidak correction A-C Significantly different to: A Isoenergetic, B Total ER, C Partial ER. * Expressed as the % relative to participants’ estimated daily requirement for weight maintenance. Presented as mean ± SEM. n=10.

Abbreviations: ER – Energy restriction

3.4.4.2 Macronutrient selection

Starting with Day two, fat intake was significantly higher after the day of total ER relative to the isoenergetic trial (111 ± 9g vs. 99 ± 10g respectively; p=0.012). No other significant differences in macronutrient selection was found between the three dietary trials on Days two and three. When compared to the isoenergetic trial (carbohydrate: 971 ± 74g; protein: 290 ± 18g), cumulative three day intakes of carbohydrate and protein were significantly lower on the total (carbohydrate: 710 ±90g, p=0.002; protein: 189 ± 10g, p<0.001) and partial (carbohydrate: 677 ± 56g, p<0.001; protein: 241 ± 15g, p=0.032) ER trials. Cumulative three-day intakes of protein were higher on the partial ER trial when compared to the total ER trial (p=0.013). No other statistical differences were found between the three dietary trials.
3.4.4.3 Assessment of under-reporting

Based on three day records of habitual intake completed prior to the study, the calculated Goldberg cut off at the study population level (n=10) was calculated to be 1.3. The group average $\text{EI}_{\text{rep}}:\text{BMR}$ was $1.2 \pm 0.1$, indicative of a degree of under-reporting. At an individual level (n=1), the $\text{EI}_{\text{rep}}:\text{BMR}$ cut off used to classify participants as under-reporters was calculated to be 1.1. On the basis of this cut off, half of the study cohort were deemed to be under-reporting dietary intake.

3.5 Discussion

Very few studies have quantified the metabolic and physiological changes that occur in response to varying degrees of substantial ER. These data demonstrate a number of distinct alterations to postprandial substrate metabolism, assessed on the morning after one day of total (100%) and partial (75%) ER, which were evident following both levels of ER.

Postprandial glucose metabolism

The finding, that postprandial carbohydrate oxidation and glucose tolerance were reduced after one day of total ER is in accordance with the existing research, which up until now has largely involved healthy weight participants or more prolonged (≥48 hour) total ER intervals (105-107). There was additionally a significant delay in the postprandial glucose curve which, in the context of early T2DM, is associated with impairments in pancreatic β-cell function and insulin secretion (158). Indeed, postprandial insulin profiles following both ER interventions appeared flattened and prolonged, however, without measuring C-peptide, it was not possible to assess the individual effects on insulin secretion vs. hepatic clearance.

Acute substantial ER elicits an array of metabolic and hormonal alterations which can affect substrate metabolism during subsequent refeeding (Section 1.4.4). For instance in the present study, the prolonged 36-hour period of total ER was accompanied by an elevation in plasma NEFA, which has been implicated as a key driver behind fast-associated changes in glucose tolerance; directly, peripheral tissue accumulation of NEFA and/or its derivatives (candidates include: ceramides, acyl
carnitines, diacylglycerol) are linked to disruptions in peripheral insulin signalling and/or glucose transport (100), as well as the acute insulin response (107); indirectly, the associated rise in ketosis is also associated with impaired peripheral glucose disposal (159). In addition, fatty acids competitively inhibit glucose oxidation via the Randle (glucose-fatty acid) cycle (26). Changes in circulating levels of counter-regulatory hormones, which can also affect glucose tolerance and explain study outcomes, were not measured in the current study (100). Other prolonged fasting studies have demonstrated elevated pre- and postprandial levels of some counter-regulatory hormones including growth hormone (106, 107) and glucagon (106), when compared to a standard overnight fast.

The partial (75%) ER diet was designed to place participants in a substantial negative energy balance whilst allowing some food intake. This consequently blunted the progressive rise in plasma NEFA which was noted following the prolonged total ER intervention. Accordingly, observed alterations in postprandial glucose tolerance and nutrient oxidation effectively displayed a dose-response across the two levels of substantial (75-100%) ER, with partial ER notably attenuating the impairment in glycaemic control seen after the day of total ER (in accordance with the hypothesis). However, a slight impairment remained. At the time of publication of the current study, no comparable study had been performed. Yet findings from a subsequent publication by Clayton et al (160) appear to now corroborate our initial findings. This particular two-way cross-over study compared the acute impact of one day of partial (75%) ER to a day of energy balance in a small group of overweight/obese males. The study assessed four-hour postprandial responses to a meal challenge (~3216 kJ, ~123 g carbohydrate, ~21 g protein and ~20 g fat) on the following day. Whilst the study did not find statistical differences in overall four-hour glucose iAUC, their postprandial time-course analyses did reveal a significant interaction effect, with one-hour glucose concentrations notably greater following partial ER. In addition, a depression in postprandial carbohydrate oxidation was reported. Although in the present study, changes in postprandial substrate utilisation following partial ER were not statistically different to the isoenergetic control trial, the study is likely to be underpowered to detect small differences for this specific parameter (particularly given the necessity to correct for multiple pairwise comparisons).
In an acute sense, these study findings can be viewed as an adaptive physiological response to short-term starvation, one which allows for a shift in postprandial nutrient partitioning in favour of glucose conservation and glycogen repletion (100). The premise of IER is that the repeated periods of FAO may have the potential to contribute to improvements in insulin sensitivity over time by reducing the accumulation of lipid and associated intermediaries. Compatible with this theory are a number of human studies reporting superior improvements in hepatic insulin sensitivity following IER versus CER (84, 91), as well as a suggestion of increased mitochondrial fatty acid transporter expression in skeletal muscle, reflecting long-term adaptation to the repeated elevations in NEFA/FAO (77).

Contrastingly, a number of rodent and human studies have reported impairments in glucose tolerance following ≥3 weeks of IER (66, 67, 77). Insights into the time-course of glycaemic changes were provided by one of the rodent studies, which found that despite an initial improvement after four weeks of IER, glucose tolerance then deteriorated over time (66). Interestingly one theory by Koves et al (161) suggests that it is excessive and incomplete (rather than deficient) FAO, as a consequence of fatty acid oversupply, that drives skeletal muscle IR. This can also induce oxidative stress within mitochondria, which are the primary site of endogenous reactive oxygen species production. By contrast the liver is considered relatively resilient due to its ability to shunt fatty acids towards alternative pathways such as VLDL-TAG export (or ketogenesis, which is of particular relevance to fasting).

Whilst this theory is framed in the context of obesity/high-fat feeding, IER can be thought of as an alternative model whereby metabolic tissues are repeatedly exposed to elevations in NEFA/FAO with each fast-refeeding cycle. Indeed the studies which have reported impaired glucose tolerance, all of which have used alternate day total ER protocols, have also observed increased oxidative stress markers (66), oxidative insulin receptor modification (66), reduced GLUT4 content (67), or suggestions of reduced markers of mitochondrial function (77). These data highlight the potential for harm, as well as the potential for tissue-specific responses to IER given that two of these studies
reported no change in fasting (hepatic) glycaemic indices (67, 77).

The two theories are not necessarily mutually exclusive and it could be that the alternate day total ER paradigm presents too great and/or frequent a metabolic challenge, which may over time lead to aberrant rather than beneficial changes in peripheral insulin sensitivity. To date, the effects of this form of IER in humans beyond eight-weeks (where no change in glucose tolerance was detected) are unknown (80). Acutely, data from the present study show that partial ER blunts the rise in NEFA/FAO associated with prolonged total ER. Whilst beyond the scope of this thesis, this suggests that IER protocols employing intervals of total and partial ER may differ in their long-term metabolic effects.

Postprandial triacylglycerol and fatty acid metabolism

Another interesting observation made by the present study were the marked improvements in postprandial TAG metabolism following both levels of substantial ER, which has not been shown previously in overweight/obese participants. By combining lines of evidence from this study and others, a number of contributory mechanisms could be proposed. Profound ER elicits an array of dynamic changes in substrate metabolism. Fatty acids are released from adipose tissue, and liver glycogen is converted to glucose and released into the circulation, thereby resulting in a reduction in liver glycogen stores (100). Hepatic partitioning of fatty acids is shifted towards β-oxidation and ketogenesis which would thus limit fatty acid availability for VLDL-TAG assembly (100). Accordingly, in the present study levels of fasting TAG were lower the morning after both ER interventions, although on the basis of one study employing a relatively modest ~3mJ energy deficit, these data may also reflect a decrease in VLDL-TAG (162). Additionally, the biochemical (3-OHB) and whole-body substrate oxidation data parallel one another and are suggestive of a shift in postprandial partitioning of fatty acids towards hepatic ketone body synthesis (away from VLDL-TAG secretion) and FAO (facilitating glycogen repletion). Any consequent reduction in VLDL-TAG would be expected to contribute to a reduction in the magnitude of the postprandial TAG response, by reducing the competition for clearance between chylomicrons and VLDL-TAG by LPL (27).
The pattern of the postprandial NEFA response following both ER interventions warrants attention given that a major regulator of ketogenesis in humans is the supply of fatty acids (163). Interestingly, in the partial ER trial, plasma NEFA levels over the course of the postprandial period were comparable to the isoenergetic control intervention, which suggests that these observed shifts in postprandial hepatic fuel utilisation following substantial ER were not solely driven by increased substrate availability.

On the basis of rodent data of refeeding after prolonged (24 hour) total ER (when compared to baseline ad libitum feeding conditions), these findings might also reflect an increase in LPL activity within adipose tissue, skeletal muscle and perhaps other tissues such as cardiac muscle (109). One could speculate that the sustained uptake and utilisation of circulating TAG may also contribute to the latency in the ability of skeletal muscle to oxidize carbohydrate during refeeding after substantial ER (106, 109, 164), due to ongoing reciprocal substrate competition between glucose and fatty acids (26).

**Energy intake compensation and energy expenditure**

To contextualise to a situation of weight-management, the secondary aims were to study the effects of different degrees of ER on acute compensation in energy intake and components of energy expenditure. Anecdotally, IER protocols which allow a small amount of fast day food intake are likely to be better tolerated over time, although, this has not been directly assessed. Expectedly, hunger and prospective food consumption were higher, and fullness markedly lower, during total ER (Day one) which is in accordance with previous research (165). The day after total ER, prospective food consumption during the meal challenge remained slightly higher, which translated into a greater overall intake of energy and fat by the end of Day two. This preference for high-fat food was also reported by Johnstone et al (165) in lean participants, but only at the first meal after a 24 hour fast. Similar (but less marked) trends in appetite scores were found during the day of partial ER. Overall energy intake and macronutrient selection on Day two were not significantly altered after partial ER, despite postprandial prospective food consumption being somewhat higher. These findings are mostly comparable to that of Clayton et al (160), who found little change in postprandial appetite or appetite
hormone profiles in the subsequent 24 hours after partial ER.

Cumulative three day intakes were additionally assessed, by aggregating nutritional intake information for Days one, two and three. These data showed that on both ER trials, the participants remained in negative energy balance after two days of *ad libitum* intake, which is particularly interesting given that individuals following IER often undergo repeated ER/feed cycles over the course of a week. This study is the first to show that both total and partial ER can produce comparable short-term energy intake deficits of ~30% in overweight/obese participants. No additional energy deficit was achieved during the total ER intervention, despite the fact that participants had abstained from food completely. Our data is in line with other studies who have similarly demonstrated an apparent lack of tight physiological control in day-to-day energy balance following substantial (75-100%) ER, within both lean (165, 166) and overweight/obese (160) individuals.

On the other side of the energy balance equation, we found no compensatory declines in REE or MIT after both levels of ER, which was later corroborated by Clayton *et al* (160). Overall, acute data on energy expenditure following substantial ER have been mixed (108, 160, 167-170). Whereas our assessments of energy expenditure were limited to REE and MIT responses to a single meal, others have reported ~6% reductions in 24-hour energy expenditure after acute periods of low energy intake using whole-body calorimeters (167). However, any small changes to energy expenditure are unlikely to offset the larger changes in energy intake.

**Strengths and Limitations**

A particular strength of the study was the novel use of a within-subject study design to compare individual responses to varying levels of substantial ER, whilst allowing participants to act as their own control. In addition, participants first completed a pre-study test trial, which served the purpose of familiarising participants to study procedures and minimising the risk of error on the actual study. Limitations include the small sample size (n=10) which increases the risk of type one and two error, and it is likely the study was underpowered for some measures (e.g. appetite, substrate oxidation). The
study population was heterogeneous, although this is somewhat mitigated by the within-subject study design. Dietary records are susceptible to under-reporting and (un)intentional behaviour modification (171). Based on habitual dietary records completed prior to the interventions, half of participants were adjudged to be under-reporters, highlighting the need to exercise caution when interpreting these dietary intake data. It should be noted, findings from the current study are comparable to other published work. Whilst participants were asked to maintain stable physical activity levels during each three-day period, this was not formally assessed. Therefore, the effect that substantial ER may have had on this component of energy balance is unknown. Finally, the postprandial assessments measured changes in absolute substrate concentrations after a single meal, which represents the balance but not the rate (or source) of substrate appearance or clearance.

Summary and future research directions

Put together, data from the present study demonstrate the ability of substantial (75–100%) ER to acutely alter glucose and lipid metabolism, as well as incomplete short-term energy intake compensation amongst overweight/obese participants. Partial ER (as compared with total ER) reduced the prolonged fast-associated decline in oral glucose tolerance, whilst producing a comparable three-day energy-intake deficit and improvement in postprandial TAG. Findings from the present study feed into Study two of this thesis, described in the next chapter, which seeks to establish how metabolism adapts over time to the repeated perturbations experienced during IER.
Chapter 4: Intermittent versus continuous energy restriction on postprandial metabolism following matched weight-loss

4.1 Introduction

Overweight/obesity is closely associated with the development of a number of inter-related metabolic complications including IR and dyslipidaemia, which can in turn increase an individual’s risk of developing T2DM and CVD (2). Glucose and lipid homeostasis can be improved through weight-loss, which due to current accepted dietary advice is most commonly achieved via a modest daily CER (8). However, independently of weight-loss, the regulation of glucose and lipids can also be influenced by abrupt changes in energy status. For example, the initial work presented in Study one demonstrates that short periods of substantial (75-100%) ER elicits profound shifts in fuel utilisation towards FAO and ketogenesis that can persist during subsequent refeeding. This translated to an improvement in postprandial lipaemia, whereas there was a contrasting and reciprocal decline in glucose tolerance. These cycles of substantial ER and refeeding experienced repeatedly during IER may therefore elicit very distinct effects on hepatic and peripheral glucose and lipid handling, when compared to similar weight-loss achieved via a modest CER.

Previous studies comparing the effects of IER to CER on cardiometabolic risk factors have found the two modes of ER to be equivalent for most metabolic parameters assessed (Table 1.2). Importantly, none have demonstrated negative effects of IER on glycaemic markers. There is some suggestion that IER (two consecutive days of 70% ER) may elicit greater benefits on hepatic insulin sensitivity, which may (speculatively) stem from the effects of the repeated ER/refeeding intervals on hepatic substrate utilisation and IHCL accumulation (84, 91). However, no study to date has controlled for the extent of weight-loss, which acts as a confounding factor from the perspective of metabolic comparisons (Section 1.4.6). In addition, the vast majority of these studies have conducted steady-state assessments, with blood measurements taken in the fasted state which reflect the metabolic situation during a relatively small proportion of the day. Humans spend the majority of their day in a postprandial state, which is a dynamic, non-steady state condition. As previously discussed, impairments in postprandial glucose and lipid handling are widely regarded as important independent determinants of
cardiometabolic risk (Section 1.3.4).

4.2 Study aims, outcomes and hypothesis

4.2.1 Aims
The present study conducted in a cohort of overweight/obese men and women aimed to build on earlier work by comparing the effects of IER vs. CER on postprandial glucose and lipid responses to a liquid mixed test meal challenge following matched 5% weight-loss.

4.2.2 Outcomes
- Primary outcome: Postprandial TAG.
- Secondary outcomes: Postprandial glucose, established fasting metabolic and physiological cardiometabolic disease risk factors, substrate oxidation, REE, psychological and sleep quality indices.

4.2.3 Hypothesis
- Based on observations made in Study one, the relative reduction in incremental TAG responses to the liquid mixed test meal will be greater following weight-loss via IER.

4.3 Participants and methods

4.3.1 Participants
Overweight and obese participants (BMI ≥25 kg/m²) aged 18 to 65 years were recruited to the study from the Guildford and wider community via email and poster advertisement, as well as word of mouth. Participants were weight-stable (±2 kg) over the preceding three months and had no significant medical history. Health status was determined via medical questionnaire and screening blood sample. To control for the potential influence of the menstrual cycle between visits, female participants were either post-menopausal or taking oral contraceptives. The study obtained a favourable opinion from the University of Surrey ethics committee (UEC/2014/140/FHMS) and was conducted in accordance with the guidelines laid down in the Declaration of Helsinki. Written, informed consent was obtained
from all participants.

4.3.2 Study protocol

The study was a randomised, parallel-armed, diet comparison between IER and CER. Participants were stratified by age (<42/≥42 years; the mid-point of the recruitment age range), BMI (<30/≥30kg/m²), gender, ethnicity and HOMA-IR (<1/≥1) to ensure balanced group allocation, with matched pairs randomly assigned to the two dietary interventions 1:1. The CER intervention served as the “standard treatment” control, compliant with NICE obesity management guidelines (54). Anecdotally, an earlier dietary comparison trial (ISRCTN31465600) reported difficulties with participant recruitment onto their CER group, as well as low motivation among participants following the CER diet, owing to their desire to try the more “novel” IER diet. In view of the potential bias this might introduce, participants in the present study were only informed of the comparison diet once they had completed.

To control for the degree of weight-loss, study measurements were taken at baseline and after participants had attained a 5% weight-loss, a threshold adjudged to have a clinically significant impact on cardiometabolic risk factors (53, 54). In addition, this degree of weight-loss was deemed feasible in the PhD timescale, with 37-65% of dieters expected to attain a ≥5% weight-loss by three months based on a previously published comparison study (91). The maximum duration participants were permitted to remain on the study was nine months. The study was conducted between May 2015 and August 2016.

4.3.2.1 Dietary interventions

Estimated requirements were calculated as previously described (Section 2.2). Participants were asked to maintain similar activity patterns whilst on the study. The reported range of physical activity levels used to calculate daily energy requirements (1.4-1.6) lie within the 10th and 50th centile of the pooled data set (derived from doubly labelled water studies) used by the Scientific Advisory Committee on Nutrition in their most recent energy report (142).
4.3.2.1.1 Intermittent energy restriction diet

The study utilised a commercially available IER diet by LighterLife (Essex, UK). On two consecutive days of the week, participants consumed four LighterLife Food Packs (2638 kJ: 38%, 36% and 26% of total energy as carbohydrate, protein and fat respectively) which delivered ~25% of their estimated isoenergetic needs. Participants were able to select from a range of Foodpack items, which were provided to them. An example of a typical day’s intake can be viewed in Table 3.1. In this study, no specific instructions were given on meal timing, in order to avoid imposing any additional restrictions that might otherwise adversely impact upon dietary adherence. On the remaining five days of the week (“feed days”), participants self-selected food intake but were asked to aim to consume an isoenergetic diet compliant with healthy eating guidelines (172). Healthy eating advice was provided in addition to individualised food portion lists which were tailored to their estimated requirements (Appendix E). Averaged overall prescribed ER was 22 ± 0.3%.

4.3.2.1.2 Continuous energy restriction diet

Participants assigned to the CER diet consumed a daily hypoenergetic diet of 2510 kJ below their estimated energy requirements (54). Diets were not provided but were self-selected by participants. Healthy eating advice was provided (172), and participants were given individualised food portion lists which were tailored to their energy intake target (Appendix E). No specific instructions were given on meal timings. Averaged overall prescribed ER was 23 ± 0.8%, which was comparable to the IER intervention.

4.3.2.2 Laboratory visits

All participants initially undertook a one-week baseline period during which time they were required to record habitual dietary intakes. At the end of this baseline period, participants attended the Surrey Clinical Research Centre for initial measurements. Participants were instructed to abstain from alcohol and strenuous exercise for 48 hours before the visit, and were provided with a standardised meal which they consumed before 20:00 on the preceding evening. This was based on prior knowledge that the macronutrient composition of an evening meal can affect metabolic responses on the following day.
(173). The standardised meal was a supermarket tomato and mozzarella pasta bake (Tesco stores, Welwyn Garden City, UK). Participants arrived at the SCRC following a 12-hour overnight water only fast. Body weight and composition were measured by bioimpedance (Section 2.1). After a period of rest, blood pressure measurements were taken in duplicate (UA-767; AND, San Jose, USA) and the mean recorded. Following this, fasted measurements of energy expenditure and substrate utilisation were taken via indirect calorimetry. An indwelling cannula was then inserted following which the first (fasted) sample was taken. A liquid mixed test meal was provided (400 ml Fortisip, Nutricia, Trowbridge, UK: 2510 kJ, 74 g carbohydrate, 24 g protein and 23 g fat). Serial blood samples were taken at regular intervals from the start of the test-meal over the next 360 minutes to assess postprandial changes in glucose, insulin, C-peptide, TAG, NEFA and 3-OHB. Please refer to Figure 2.1 for diagrammatic overview of the study day and sampling schedule. In between the blood sampling, participants completed a number of questionnaires related to their sleep quality, mood and eating behaviour traits. After the initial visit, both groups commenced their respective diets whilst maintaining habitual activity patterns. Within two weeks of attaining their 5% weight-loss target, participants returned to the research centre for repeated measurements. Participants in both groups abstained from any form of ER for ≥7 days prior to this post-intervention visit to mitigate the effects of acute ER on the metabolic outcomes.

4.3.2.3 Monitoring and compliance

Participants received fortnightly motivational contacts via phone, email and/or texts in addition to monthly face-to-face clinic appointments, where weight was recorded. Every two weeks, participants were sent online questionnaires which asked them to self-report their fasted weight (Appendix F). The frequency of weight monitoring increased as participants approached their 5% target. IER participants were additionally sent questionnaires which enabled them to record intakes on their ER days, and any foods consumed in addition to the prescribed food packs (Appendix G). For the purpose of this study, a compliant ER day was defined as one where energy intake was ≤3347kJ, which corresponds to the VLED threshold defined by NICE (54). All participants also completed seven-day diet diaries and self-reported physical activity levels (Table 2.1; to ensure stable activity levels) mid-way and towards the
end of the interventions.

4.3.3 Experimental techniques and analyses

4.3.3.1 Blood biochemistry

At screening, a blood sample was taken via venepuncture to measure fasting levels of glucose and insulin for the purpose of health screening and diet randomisation. On laboratory visits, serial fasting and postprandial blood samples were collected via cannula. Insulin, C-peptide and 3-OHB samples were batch analysed upon study completion. For all other metabolites, samples were analysed in three batches over the course of the study. All samples from an individual participant were included in the same assay. Metabolites were analysed using the following methods: insulin using RIA (Millipore, Billerica, USA; intra/inter-assay CVs 8% and 4%); C-peptide using RIA (Millipore, Billerica, USA; intra/inter-assay CVs 6% and 8%); glucose, TAG and NEFA using the ILAB 650 photometric auto-analyser (Instrumentation Laboratory, Warrington, UK; intra/inter-assay CV all <6% and <6%); and 3-OHB using the Cobas MIRA photometric auto-analyser (Roche, Welwyn Garden City, UK; intra/inter-assay CVs <5% and <6%). AUC (for NEFA and 3-OHB) and iAUC (for all other metabolites) were calculated as described in Section 2.5.2.11. LDL-cholesterol was calculated using the Friedewald equation (Section 2.5.2.8). HOMA-IR and HOMA-%B were calculated using the HOMA2 online calculator (Section 2.5.2.4) as proxies for hepatic insulin sensitivity and β-cell function respectively. An alternate surrogate measure of insulin sensitivity, the revised quantitative insulin sensitivity check index (RQUICKI), was additionally calculated as follows (174):

$$RQUICKI = \frac{1}{\log(fasting \ glucose \ (mg/dl)) + \log(fasting \ insulin(\mu U/ml)) + \log(fasting \ NEFA \ (mmol/l))}$$

Within pancreatic β-cells, the prohormone precursor proinsulin goes through enzymatic cleavage to produce insulin and C-peptide, which are co-secreted in equimolar amounts. Unlike insulin, C-peptide undergoes negligible extraction by the liver and constant peripheral clearance. The physiology of C-peptide hence makes it a more direct marker of insulin secretion than circulating insulin and enables evaluation of hepatic insulin extraction, which was approximated by calculating the C-peptide:insulin
AUC ratio (175). Please refer to Section 2.5 for full explanation of protocols used for blood sample collection, processing, storage and the analytical techniques used.

**4.3.3.2 Indirect calorimetry:**
REE and substrate utilisation were calculated using data obtained from a Gaseous Exchange Monitor ISGEM319 (Section 2.6). Participants rested for 30 minutes prior to this measurement. Measurements were taken over 20 minutes and in accordance with methodological recommendations by Compher *et al* (147). REE was calculated as described in Section 2.6.1 and substrate utilisation from the RQ (VC0₂/VO₂). To permit comparisons between individuals of varying body masses, REE was also normalised for metabolically active mass using the following formula (176):

\[
Adjusted\ REE = \frac{REE}{FFM + 18\ kg}
\]

This value of FFM plus a constant factor of 18 kg is thought to represent a better estimate of the metabolically active mass.

**4.3.3.3 Dietary analyses**
Participants recorded intake in diet diaries for seven days at three time points: at baseline, prior to starting their dietary intervention; once they had reached a 2.5% weight-loss milestone; and as they were approaching their 5% weight-loss target. Seven-day intakes were then averaged. Eating window also calculated in minutes from the recorded time of first and last intake of food/drink. For IER participants, intakes were additionally averaged over the five “feed” days to assess for energy compensation and altered macronutrient selection. Please refer to Section 2.3 for a full explanation of methods relating to diet diary analyses.

**4.3.3.3.1 Assessment of under-reporting**
The revised Goldberg equations were used to evaluate the potential for under-reporting of dietary intakes in this cohort (at individual and study population levels), based on reported intakes during the initial baseline (Section 2.3.2.1). PAL values used in this cohort ranged between 1.4 to 1.6, with the between-participant variance in reported PAL (CV₁₆) calculated to be 3.8%.
4.3.3.4 Mood assessment

Mood was assessed using the positive affect negative affect scale (PANAS) which consists of 20 words that describe positive and negative emotions (177); Appendix H). Participants were asked to rate to which extent they felt each emotion over the preceding week, using a scale of one (very little, or not at all) to five (extremely). The PANAS scale gives two scores, for positive and negative affect, which can each range from 10 to 50. A high score represents higher levels of positive or negative mood states. Participants additionally completed these scales every fortnight in order to calculate the averaged trait effect over the course of the study.

4.3.3.5 Eating behaviour

The Dutch eating behaviour questionnaire (DEBQ) was used to quantify eating behaviour traits ((155); (Appendix C)). The scale consists of 33 questions and three subscales designed to assess the extent of participants’ eating restraint (in order to control weight), emotional eating behaviour (eating in response to emotional arousal e.g. fear, anger, anxiety) and external eating behaviour (eating in response to environmental cues e.g. sight, smell of food). The DEBQ questions employs a scale of one (never) to five (very often), whilst a zero is scored if the question is deemed not relevant. Responses are then averaged to provide an overall score for each eating behaviour category.

4.3.3.6 Responsiveness to palatable food

The 15-item power of food scale (PFS) was used to measure participants’ hedonic wanting and responsiveness to food ((178); (Appendix I)). The motivation to consume foods beyond homeostatic need has been referred to as “hedonic hunger”, which is presumed to have evolved because the motivation to seek and consume energy contributed to survival when food sources were scarce and unpredictable. The PFS was developed to assess the psychological impact of living in food-abundant environments, as reflected in feelings of being controlled by food, independent of food consumption itself. Participants reported their responses to palatable foods at three levels of food proximity: “present but not tasted”, “available but not physically present” and “when first tasted but not consumed” using a scale of one (don’t agree at all) to five (strongly agree). Scores relating to each component were then
averaged and an aggregate domain score produced. A full category increase in aggregated PFS domain score is associated with a 1.6–2.3 increased odds of being obese.

**4.3.3.7 Self-efficacy**

The self-efficacy questionnaire was used to assess participants’ confidence in their ability to control their own weight and also their general self-confidence (Appendix J). This questionnaire uses a scale of one (not confident at all) to five (extremely confident).

**4.3.3.8 Sleep quality**

The Pittsburgh sleep quality index (PSQI) was used to assess sleep quality. The PSQI consists of 19 self-rated questions relating to seven sleep components: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbance, use of sleep medications and daytime dysfunction (179); (Appendix K). The seven component scores are combined to yield one “global score”, which can range from between zero (no difficulty) to 21 (severe difficulties in all areas).

**4.3.3.9 Sleepiness**

The Epworth sleepiness scale (ESS) was used to provide an objective measure of participants’ sleepiness (180); (Appendix L). The test comprises a list of eight situations in which participants had to rate their tendency to become sleepy on a scale of zero (no chance of dozing) to three (high chance of dozing). Scores from each component are then summed to produce an overall score. A score of 0-7 is considered to be the normal range, a score of 8-14 is suggestive of mild to moderate symptoms of daytime tiredness, and a score of 15-24 is suggestive of significant (severe) daytime tiredness. An improvement in sleep quality (PSQI) might result in a reduced ESS score.

**4.3.4 Statistical analyses**

Data were checked for normality using the Shapiro-Wilks test, with non-normally distributed data normalised via log transformation where possible to permit parametric testing (Section 2.7). The primary analysis was a one-factor analysis of covariance (ANCOVA) between the dietary intervention...
groups with post-treatment values as the dependent variable, and baseline values of each parameter as
the covariate. This is recommended statistical method (in terms of bias, precision and power) for the
analysis of continuous outcomes in randomised studies with a single post-treatment measurement
previously measured at baseline. An additional benefit of using the ANCOVA method is that it adjusts
for the presence of baseline imbalances by including baseline values in the model (181). Prior to
analyses, it was ensured that assumptions made by the ANCOVA model with regards to independence
of the covariate and treatment effect and homogeneity of regression slopes (treatment x covariate
interaction) were met (157). The Mann Whitney U test was used as the non-parametric alternative to
ANCOVA to compare change scores between intervention groups. Differences between intervention
groups at baseline were assessed using independent t-tests for continuous variables or the Chi squared
test for categorical variables. No baseline differences were found unless otherwise stated. A paired t-
test (or non-parametric Wilcoxon signed-rank test) was used to assess the change between baseline
and post intervention values within each dietary intervention group. Correlations between changes in
metabolic and dietary intake variables were explored using Pearsons (parametric) or Spearmans (non-
parametric) tests as appropriate. When significant correlations or trends were observed between two
variables, linear regression analyses were conducted to determine to what extent the variation in the
change in the variable of interest (i.e. the dependent variable) was explained by its correlate/the other
(independent) variable. Summary measures are presented as mean ± SEM (for parametric data) or
median and interquartile range (IQR, for non-parametric data).

4.3.5 Data omissions

Four participants (IER=3, CER=1) did not complete a baseline seven-day food diary and/or at least
one of their two diaries whilst dieting, and so were omitted completely from the dietary intake analyses.
Biochemical and indirect calorimetry data for one female IER participant were excluded due to the
incidental finding of a previously undiagnosed lipid disorder. The participant’s anthropometric and
dietary analyses data were not omitted. Complete removal of the participant did not alter statistical
outcomes or group balances at baseline. Plasma NEFA samples for two participants (IER=1, CER=1)
could not be analysed due to equipment failure (which could not be rectified in PhD time scale). Plasma
3-OHB samples for two participants (IER=1, CER=1) could not be analysed due to assay issues (possible interference with elevated lipids, sample haemolysis). Lastly, due to equipment failure (calibrant gas leak), indirect calorimetry could not be performed on three (IER=1, CER=2) participants during one study visit, and so their data had to be excluded from overall analyses.

4.4 Results

4.4.1 Participant baseline characteristics and attrition

Of the 41 participants (IER=24, CER=16) who started the study, 27 (IER=15, CER=12) attained their 5% weight-loss target. The consort diagram is presented in Figure 4.1.

Baseline characteristics of the 27 study completers are presented in Table 4.1. The groups were matched for age, BMI, adiposity, gender, metabolic syndrome classification and were mainly Caucasian. Twelve participants withdrew from the study due to scheduling conflicts (IER=1), bereavement (IER=1), dental problems (IER=1), problems tolerating (IER=4) or adhering to (CER=2) their diet, or were lost to follow up (IER=2, CER=1). Two CER participants were unable to attain a 5% weight-loss within the maximum timeframe and so were withdrawn from the study. Non-
completers were significantly younger than completers (27 ± 3 vs. 45 ± 3 years; p<0.001, independent t-test), no other significant differences were noted.

<table>
<thead>
<tr>
<th></th>
<th>IER (n=15)</th>
<th>CER (n=12)</th>
<th>IER vs. CER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>42 ± 4</td>
<td>48 ± 3</td>
<td>NS¹</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>7 / 8</td>
<td>6 / 6</td>
<td>NS²</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>15</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Black African</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.8 ± 0.9</td>
<td>30.8 ± 1.1</td>
<td>NS¹</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>34.8 ± 2.2</td>
<td>37.5 ± 2.0</td>
<td>NS¹</td>
</tr>
<tr>
<td>Men</td>
<td>29.3 ± 1.7</td>
<td>33.2 ± 2.4</td>
<td>NS¹</td>
</tr>
<tr>
<td>Women</td>
<td>41.4 ± 1.6</td>
<td>41.8 ± 1.9</td>
<td>NS¹</td>
</tr>
<tr>
<td>Metabolic Syndrome²</td>
<td>4 / 15</td>
<td>2 / 12</td>
<td>NS²</td>
</tr>
</tbody>
</table>

Table 4.1 Baseline characteristics for study completers
*Bioimpedance. ¹International Diabetes Federation criteria (182).
Statistics and data presentation: ¹Unpaired t-test, ²Chi squared. NS – Non-significant. Presented as mean ± SEM.
Abbreviations: BMI – Body mass index; CER – Continuous energy restriction; F – Female; IER – Intermittent energy restriction; M – Male.

4.4.2 Body weight and body composition

Mean percentage weight-loss was 5.3 ± 0.3% in the IER group and 5.0 ± 0.3% in the CER group (p=0.446, ANCOVA). The accompanying changes in body composition were also comparable between the groups (p≥0.430, ANCOVA). Body weight and composition measured before and after 5% weight-loss are reported in Table 4.2.

<table>
<thead>
<tr>
<th></th>
<th>IER (n=15)</th>
<th>CER (n=12)</th>
<th>IER vs. CER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>88.8 ± 3.4</td>
<td>84.1 ± 3.2</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.8 ± 0.9</td>
<td>28.2 ± 0.8</td>
<td>NS</td>
</tr>
<tr>
<td>Fat mass (kg)*</td>
<td>30.8 ± 2.3</td>
<td>27.1 ± 2.3</td>
<td>NS</td>
</tr>
<tr>
<td>Fat free mass (kg)*</td>
<td>58.0 ± 3.1</td>
<td>57.0 ± 3.0</td>
<td>NS</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>102 ± 3</td>
<td>98 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Hip (cm)</td>
<td>113 ± 2</td>
<td>109 ± 2</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 4.2 Body weight and composition before and after 5% weight-loss via IER and CER
*Bioimpedance.
Statistics and data presentation: ¹Analysis of covariance. *Significant within-group change (p<0.05, paired t-test). NS – Non-significant. Presented as mean ± SEM.
Abbreviations: BMI – Body mass index; CER – Continuous energy restriction; IER – Intermittent energy restriction.

It took IER participants a median of 59 days (IQR: 41, 80) to attain their 5% weight-loss target and
CER participants 73 days (IQR: 48, 128), which was statistically comparable between groups (p=0.246, Mann Whitney U test). The time-course of weight changes is depicted in Figure 4.2.

Figure 4.2 Weight-loss time-courses during the IER and CER dietary interventions
IER (black) and CER (grey)
Statistics and data presentation: Presented as median (interquartile range). n=27 (IER=15, CER=12)
Abbreviations: CER – Continuous energy restriction; IER – Intermittent energy restriction.

4.4.3 Dietary intakes and physical activity

Dietary intakes during the study for the 23 (IER=12, CER=11) participants who completed a baseline seven-day food diary and at least one of two diaries whilst dieting (mid-way [2.5% weight-loss] and towards end [5% weight-loss] of intervention) are reported in Table 4.3. At baseline, dietary intakes did not differ between IER and CER groups (p≤0.330, unpaired t-test), whilst eating window tended to be slightly longer in the CER group (669 ± 24 vs. 730 ± 18 minutes; p=0.058, unpaired t-test).

Both groups reported reductions in averaged weekly intakes of energy and most macronutrients, however, by the end of the intervention the reductions in energy intake were significantly greater in the IER group (mean difference: 1081 kJ [95% confidence intervals: -1900, -263]; p=0.012, ANCOVA), with a similar tendency noted for total carbohydrate intake (mean difference: -28 g [-57, 1]; p=0.054, ANCOVA). In contrast, when expressed relative to estimated energy requirements, the overall weekly ER between IER and CER diets at the end of the study was comparable, at -42 ± 8%
and -39 ± 3% respectively (p=0.773, unpaired t-test), however, there is a high probability of under-reporting (Section 4.3.3.3.1). The percentage contribution of protein to total energy intake increased comparably in both IER (p<0.001, paired t-test) and CER (p=0.05, paired t-test) groups relative to baseline (p=0.302, ANCOVA). Eating windows and physical activity levels remained stable in both groups across the study.

Dietary adherence to intermittent energy restriction protocol and energy compensation
Adherence to the IER protocol (i.e. two substantial ER days/week) was high (93 ± 4%), and were most frequently completed on consecutive days (86 ± 7%). The averaged reported intake on substantial ER days was 2771 ± 50 kJ/day, equating to a 75 ± 1% ER. “Feed day” intake data revealed a lack of dietary over-compensation, with averaged five-day intakes of energy and most macronutrients lower relative to baseline intakes by the end of the intervention (all p≤0.081, paired t-test). Relative to estimated energy requirements, participants were under-eating by 12 ± 21% at the mid-way point, and by 31 ± 10% by the end of the study (p=0.183, paired t-test). Relative to baseline intakes, the respective values were -7 ± 5% and -21 ± 6% (p=0.118, paired t-test).

Post-intervention
At the end of the study 13 (87%) IER participants and 12 (100%) CER participants planned to continue dieting, whilst the remaining two IER participants intended to maintain their weight through periodic substantial ER (e.g. one day/week). Of the IER participants who intended to continue dieting, nine (75%) planned to continue their assigned diet.
<table>
<thead>
<tr>
<th></th>
<th>IER</th>
<th>CER</th>
<th>IER vs. CER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline (n=12)</td>
<td>Midway - 2.5% weight-loss (n=8)</td>
<td>End -5% weight-loss (n=12)</td>
</tr>
<tr>
<td>Energy intake (kJ/day)</td>
<td>8057 ± 432</td>
<td>5771 ± 358(^A)</td>
<td>5199 ± 319(^A)</td>
</tr>
<tr>
<td>Feed days only</td>
<td>-</td>
<td>(7077 ± 479)</td>
<td>(6236 ± 447)</td>
</tr>
<tr>
<td>Carbohydrate (g/day)</td>
<td>224 ± 20</td>
<td>147 ± 10(^A)</td>
<td>141 ± 9(^A)</td>
</tr>
<tr>
<td>Feed days only</td>
<td>-</td>
<td>(183 ± 13)</td>
<td>(170 ± 14)(^A)</td>
</tr>
<tr>
<td>Fibre (g/day)</td>
<td>19 ± 1</td>
<td>18 ± 1</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>Feed days only</td>
<td>-</td>
<td>(19 ± 2)</td>
<td>(18 ± 1)</td>
</tr>
<tr>
<td>Sugars (g/day)</td>
<td>84 ± 14</td>
<td>48 ± 8(^A)</td>
<td>52 ± 8(^A)</td>
</tr>
<tr>
<td>Feed days only</td>
<td>-</td>
<td>(62 ± 11)</td>
<td>(62 ± 11)(^A)</td>
</tr>
<tr>
<td>Fat (g/day)</td>
<td>74 ± 5</td>
<td>52 ± 5(^A)</td>
<td>45 ± 15(^A)</td>
</tr>
<tr>
<td>Feed days only</td>
<td>-</td>
<td>(66 ± 9)(^A)</td>
<td>(56 ± 6)(^A)</td>
</tr>
<tr>
<td>Saturated fat (g/day)</td>
<td>29 ± 3</td>
<td>19 ± 2(^A)</td>
<td>16 ± 2(^A)</td>
</tr>
<tr>
<td>Feed days only</td>
<td>-</td>
<td>(25 ± 4)</td>
<td>(20 ± 3)(^A)</td>
</tr>
<tr>
<td>Protein (g/day)</td>
<td>83 ± 5</td>
<td>73 ± 3(^A)</td>
<td>67 ± 5(^A)</td>
</tr>
<tr>
<td>Feed days only</td>
<td>-</td>
<td>(78 ± 4)(^A)</td>
<td>(63 ± 7)(^A)</td>
</tr>
<tr>
<td>Alcohol (g/day)</td>
<td>10 ± 3</td>
<td>8 ± 4(^A)</td>
<td>11 ± 5(^A)</td>
</tr>
<tr>
<td>Salt (g/day)</td>
<td>5.4 ± 0.6</td>
<td>4.9 ± 0.5</td>
<td>4.0 ± 1.3</td>
</tr>
<tr>
<td>Feed days only</td>
<td>-</td>
<td>(4.4 ± 0.5)(^A)</td>
<td>(3.6 ± 0.3)(^A)</td>
</tr>
<tr>
<td>Carbohydrate (% total energy)</td>
<td>43 ± 2</td>
<td>40 ± 2</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>Feed days only</td>
<td>-</td>
<td>(40 ± 2)</td>
<td>(43 ± 2)</td>
</tr>
<tr>
<td>Fat (% total energy)</td>
<td>34 ± 1</td>
<td>33 ± 2</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>Feed days only</td>
<td>-</td>
<td>(35 ± 3)</td>
<td>(33 ± 2)</td>
</tr>
<tr>
<td>Protein (% total energy)</td>
<td>18 ± 1</td>
<td>21 ± 2(^a)</td>
<td>22 ± 1(^a)</td>
</tr>
<tr>
<td>Feed days only</td>
<td>-</td>
<td>(19 ± 2)</td>
<td>(19 ± 2)</td>
</tr>
<tr>
<td>Alcohol (% total energy)</td>
<td>4 ± 1</td>
<td>4 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Eating window (minutes)</td>
<td>669 ± 24</td>
<td>670 ± 30</td>
<td>660 ± 27</td>
</tr>
<tr>
<td>Physical activity levels(^1)</td>
<td>1.56 ± 0.02</td>
<td>1.57 ± 0.01</td>
<td>1.57 ± 0.01</td>
</tr>
</tbody>
</table>

Table 4.3 Dietary intakes and physical activity levels at baseline, midway through and at the end of the IER and CER dietary interventions

Statistics and data presentation: Data expressed as an average of seven days or five feed days. \(^1\)Analysis of covariance. \(^A\)Significant within-group change: \(^A\) vs. baseline or \(^B\) between mid-way and end time points (p<0.05, paired t-test). \(^A\)(b) Within-group statistical trend (p=0.05-0.1). NS – Not significant. Presented as mean ± SEM. \(^1\)n=27 (IER=15, CER=12). Abbreviations: CER – Continuous energy restriction; IER – Intermittent energy restriction.
4.4.3.1 Assessment of under-reporting

Based on three day records of habitual intake completed prior to the study, the calculated Goldberg cut off at the study population level (n=23) was calculated to be 1.5. The overall group average EIrep:BMR was 0.7 ± 0.1, highly indicative of under-reporting. The IER and CER group averages were also 0.7 ± 0.1 (p=0.899, ANCOVA). At an individual level (n=1), the EIrep:BMR cut off used to classify participants as under-reporters was calculated to be 1.2. On the basis of this cut off, all participants were classified as under-reporters.

4.4.4 Fasting biochemistry and physiological markers

Fasting biochemistry and physiological markers measured before and after the dietary interventions are reported in Table 4.4.

<table>
<thead>
<tr>
<th></th>
<th>IER (n=14)</th>
<th>CER (n=12)</th>
<th>IER vs. CER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Post</td>
<td>Baseline</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.4 ± 0.1</td>
<td>4.6 ± 0.1^A</td>
<td>4.4 ±0.2</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>78 ± 8</td>
<td>71 ± 5</td>
<td>64 ± 8</td>
</tr>
<tr>
<td>C-Peptide (pmol/L)</td>
<td>527 ± 36</td>
<td>504 ± 38</td>
<td>504 ± 45</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.6 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>HOMA-%B</td>
<td>139 ± 10</td>
<td>126 ± 8</td>
<td>138 ± 12</td>
</tr>
<tr>
<td>RQUICKI^†</td>
<td>0.36 ± 0.01</td>
<td>0.38 ± 0.01</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>TOTC (mmol/L)</td>
<td>4.2 ± 0.3</td>
<td>4.0 ± 0.2</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.6 ± 0.3</td>
<td>2.4 ± 0.2</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>TAG (mmol/L)</td>
<td>1.1 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>NEFA (µmol/L)^2</td>
<td>637 ± 63</td>
<td>491 ± 50^A</td>
<td>517 ± 56</td>
</tr>
<tr>
<td>3-ΟΗΒ (µmol/L)^2</td>
<td>144 ± 37</td>
<td>112 ± 33</td>
<td>84 ± 19</td>
</tr>
<tr>
<td>Systolic BP (mm/Hg)</td>
<td>123 ± 3</td>
<td>111 ± 3^B</td>
<td>115 ± 3</td>
</tr>
<tr>
<td>Diastolic BP (mm/Hg)</td>
<td>74 ± 3</td>
<td>69 ± 3^B</td>
<td>75 ± 3</td>
</tr>
<tr>
<td>REE (kJ/day)</td>
<td>6617 ± 257</td>
<td>6139 ± 256^A</td>
<td>6190 ± 309</td>
</tr>
<tr>
<td>REE (kJ/kg MAM/day)</td>
<td>87 ± 4</td>
<td>81 ± 2^A</td>
<td>83 ± 2</td>
</tr>
<tr>
<td>RQ (VO2/VO2)</td>
<td>0.86[0.84,0.88]</td>
<td>0.83[0.77,0.89]^A</td>
<td>0.87[0.84,0.9]</td>
</tr>
</tbody>
</table>

Table 4.4 Fasting biochemistry and physiological markers measured before and after 5% weight-loss via IER and CER

Statistics and data presentation: ^1 Analysis of covariance or ^2 Mann Whitney U test. ^A Significant within-group change (p<0.05, paired t-test or Wilcoxon signed ranks). (a) Within-group statistical trend (p=0.05-0.1). NS – Non-significant. RQ presented as median (interquartile range), all other data are mean ± SEM. *n=24 (IER=13, CER=11). ^ns=23 (IER=13, CER=10).

Abbreviations: BP – Blood pressure; CER – Continuous energy restriction; HDL – High density lipoprotein; HOMA – Homeostasis model assessment; %B – β-cell function and IR – insulin resistance; IER – Intermittent energy restriction; LDL – Low density lipoprotein; MAM – Metabolically active mass; NEFA – Non-esterified fatty acids; REE – Resting energy expenditure; RQ – Respiratory quotient; RQUICKI – Revised quantitative insulin sensitivity check index; TAG – Triacylglycerol; TOTC – Total cholesterol; 3-ΟΗΒ – 3-hydroxybutyrate.

Within the IER group, there was a small 3 ± 1% increase in fasting glucose (p=0.008, paired t-test), whilst a trend in favour of reduced plasma NEFA (18 ± 8%) was also observed (p=0.056, paired t-
test); however, no significant between-group differences were noted for either variable (p≥0.158, ANCOVA). No other significant within-group changes (all p≥0.115, paired t-test), or between-group differences (all p≥0.147, ANCOVA) were found between IER and CER groups.

Systolic blood pressure was significantly reducing following IER (9 ± 2% (p≤0.001, paired t-test) whereas there was little change in the CER group (2 ± 1%; p=0.154, paired t-test). The relative change between groups was significant (p=0.020, ANCOVA). A relationship between the changes in energy intake and systolic blood pressure was found (r=0.461, p=0.047). Linear regression analyses found that the change in energy intake accounted for 21% of the variation in the change in systolic blood pressure with an adjusted $R^2$ of 17%, a medium size effect according to Cohen (183).

For diastolic blood pressure, reductions were observed in the CER group (9 ± 4%; p=0.047, paired t-test) with a similar tendency in the IER group (-6 ± 3%; p=0.054, paired t-test), with no difference between the groups (p=0.691, ANCOVA).

For REE, a trend in favour of a 7 ± 4% reduction in REE was observed following IER (p=0.058 paired t-test), whilst there was little change in the CER group (2 ± 3%; p=0.766, paired t-test), however, the relative change was not significantly different between groups (p=0.205, ANCOVA). Similar within-group trends were noted when REE was normalised for metabolically active mass, whereas the between-group differences were strengthened (p=0.067, ANCOVA).

A significant within-group decline in fasting RQ (indicative of greater FAO) was noted in the IER group (-6% [-8, 1] p=0.045, Wilcoxon signed ranks test), but not the CER group (-1% [-5,6]; p=0.787, Wilcoxon signed ranks test), although, the relative change did attain significance between groups (p=0.148, Mann Whitney U test).

4.4.5 Postprandial glucose metabolism

Postprandial glycaemic responses to the liquid mixed test meal, assessed before and after the dietary interventions are presented in Figure 4.3 A-I. For postprandial glucose responses, no significant
between-group differences (p=0.226, ANCOVA) or within-group changes were observed in the IER (p=0.943, paired t-test) and CER (p=0.252, paired t-test) groups. Conversely, incremental insulin responses were reduced following weight-loss via IER (by 14 ± 7%; p=0.048, paired t-test) and tended to be reduced following CER (by 13 ± 7%; p=0.083, paired t-test). This reduction in postprandial insulinaemia was comparable between groups (p=0.903, ANCOVA). A suggestion of a between-group difference was found for postprandial C-peptide responses (p=0.057). Incremental responses were lowered by 16 ± 7% in the IER group (p=0.032, paired t-test), whilst there was little change in the CER group (2 ± 6%; p=0.991, paired t-test). There were no significant between-group differences (p=0.227, ANCOVA) or within-group changes in the C-peptide:insulin AUC ratio between IER (+5 ± 1%; p=0.356, paired t-test) and CER groups (+18 ± 1%; p=0.129, paired t-tests), which was used as a proxy of insulin clearance.
Figure 4.3. A-I) Postprandial glycaemic indices before and after 5% weight-loss via IER and CER

For postprandial graphs: Baseline (blue) and post-treatment (red). Figures C, F, I: IER (filled circles), CER (filled squares). Liquid test meal provided: 2510 kJ, 74 g carbohydrate, 24 g protein and 23 g fat.

Statistics and data presentation: ¹Paired t-tests used to assess within-group change in iAUC. ²Analysis of covariance. Presented as mean ± SEM. n=26 (IER=14, CER=12).

Abbreviations: CER – Continuous energy restriction; iAUC – Incremental area under curve; IER – Intermittent energy restriction.
4.4.6 Postprandial lipid metabolism

Postprandial TAG, NEFA and 3-OHB responses to the liquid mixed test meal, assessed before and after the dietary interventions, are presented in Figure 4.4 A-I. The relative changes in postprandial TAG were significantly different between the two diets (p=0.045, ANCOVA), with incremental responses reduced by ~40% following IER, but slightly increased (by ~10%) following CER. A strong trend in favour of a positive relationship between decreases in incremental TAG and RQ was found (r=0.34, p=0.06). Linear regression analyses found that the change in RQ accounted for 16% of the variation in the change in incremental TAG with an adjusted $R^2$ value of 12.0%, a medium size effect according to Cohen (183). For postprandial NEFA, there were no significant between-group differences (p=0.410, Mann-Whitney U test), although, a tendency for reduced NEFA AUC was observed within the CER group (median -25% [IQR: -38, -3]; p=0.059, Wilcoxon signed ranks test), which was not present in the IER group (-9% [IQR -33, 14]; p=0.239, Wilcoxon signed ranks test). No significant within-group changes (p=0.618, ANCOVA) or between-group differences (p≥0.248, paired t-tests) in postprandial 3-OHB responses were found.

4.4.6.1.1 Power calculation for primary outcome variable

For the iAUC for plasma TAG, retrospective power calculations determined that at a two-sided 0.05 significance level, the study had 50% power to detect a mean difference of 59 mmol.360min.L⁻¹ between treatment groups (IER vs. CER), based on a standard deviation of 73 mmol.360min.L⁻¹. To attain 80% power utilising an analogous parallel-armed study design, 26 participants would be required per group.
Figure 4.4 A-I) Postprandial lipid indices before and after 5% weight-loss via IER and CER

For postprandial graphs: Baseline (blue) and post-treatment (red). Figures C, F, I: IER (filled circles), CER (filled squares). Liquid test meal provided: 2510 kJ, 74 g carbohydrate, 24 g protein and 23 g fat.

Statistics and data presentation: 1Paired t-tests or 2Wilcoxon signed ranks test used to assess within-group change in (i)AUC. 3Analysis of covariance or 4Mann Whitney U test. Figure F presented as median (interquartile range), all other data as mean ± SEM. TAG: n=26 (IER=14, CER=12). NEFA and 3-OHB: n=24 (IER=13, CER=11).

Abbreviations: CER – Continuous energy restriction; (i)AUC – (Incremental) area under curve; IER – Intermittent energy restriction; NEFA – Non-esterified fatty acids; 3-OHB – 3-hydroxybutyrate.
4.4.7 Questionnaires

Psychological and sleep quality indices measured before and after the dietary interventions are reported in Table 4.5.

<table>
<thead>
<tr>
<th></th>
<th>IER (n=15)</th>
<th>CER (n=12)</th>
<th>IER vs. CER&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eating behaviour (1-5)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emotional eating</td>
<td>2.5 ± 0.3</td>
<td>2.4 ± 0.3</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>External eating</td>
<td>3.1 ± 0.2</td>
<td>2.9 ± 0.2</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>Eating restraint</td>
<td>2.9 ± 0.1</td>
<td>3.5 ± 0.2&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>Mood (10-50)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive affect</td>
<td>34 ± 2</td>
<td>36 ± 1</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>Negative affect</td>
<td>15 ± 2</td>
<td>13 ± 2</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>Self-efficacy (1-5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight control</td>
<td>2.5 ± 0.2</td>
<td>3.2 ± 0.2&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>Self-confidence</td>
<td>3.0 ± 0.3</td>
<td>3.2 ± 0.2</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>Power of food (1-5)</td>
<td>2.6 ± 0.2</td>
<td>2.2 ± 0.2&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Sleepiness (0-24)</td>
<td>8.3 ± 1.0</td>
<td>6.8 ± 1.1&lt;sup&gt;(a)&lt;/sup&gt;</td>
<td>7.0 ± 0.9</td>
</tr>
<tr>
<td>Sleep quality (0-21)</td>
<td>5.0 ± 0.7</td>
<td>3.9 ± 0.4</td>
<td>5.0 ± 0.6</td>
</tr>
</tbody>
</table>

Table 4.5 Mood, sleep quality and eating behaviour traits before and after 5% weight-loss via IER and CER

Questionnaires completed before and after dietary interventions

Statistics and data presentation: <sup>1</sup> Analysis of covariance. <sup>A</sup> Significant within-group change (p<0.05, paired t-test). <sup>(a)</sup> Within-group statistical trend (p=0.05-0.1). NS – Non-significant. Presented as mean ± SEM.

Questionnaires: Eating behaviour – Appendix C; Mood – Appendix H; Self-efficacy – Appendix J; Sleep quality – Appendix K; Sleepiness – Appendix L.

Abbreviation: CER – Continuous energy restriction; IER – Intermittent energy restriction.

There were no significant between-group differences for any parameter assessed (p≥0.103, ANCOVA). Participants in both IER (p=0.002, paired t-test) and CER (p=0.008, paired t-test) groups exhibited greater levels of eating restraint by the end of the study. Mood scores were not significantly altered following either diet (p≥0.234, paired t-test). Averaged mood trait scores over the course of the study were comparable between IER (positive: 34 ± 2, negative: 14 ± 3) and CER (positive: 33 ± 4, negative: 17 ± 3) groups (p≥0.234, unpaired t-test). Participants’ confidence in their ability to control their weight was increased following both IER (p=0.002, paired t-test) and CER (p=0.005, paired t-test) diets. Aggregate PFS score declined comparatively within both groups (p=0.639, ANCOVA). CER participants reported greater levels of sleepiness by the end of the diet (p=0.012, paired t-test), whereas sleepiness tended to get better during the IER diet (p=0.062, paired t-test).
4.5 Discussion

Novel findings from the present study highlight underlying differences between IER and CER with respect to their effects on postprandial glucose and lipid handling, with IER eliciting similar, if not greater improvements, than CER following matched 5% weight-loss. This was despite few changes in fasting biochemistry, which highlights the importance of looking beyond fasting indices of cardiometabolic risk. In addition, there were some variations between the diets in their effects on a blood pressure and (suggestively) basal substrate utilisation. The time taken to achieve 5% weight-loss and changes in body composition were comparable between groups, although, the IER group reported greater relative reductions in energy (~1081 kJ/day) driven by under-consumption on “feed” days (where an isoenergetic diet was prescribed). This lack of energy-intake compensation is in accordance with previous research (83, 84, 91) although an element of under-reporting is highly probable. Discrepancies between the dietary intake data and weight-loss trajectories may also relate in part to adaptive changes in REE. These key findings within and between the IER and CER groups shall now be discussed further, with consideration given to the possible contribution of the between-group differences in dietary intakes to metabolic outcomes.

Postprandial glucose metabolism

No changes in postprandial glucose responses were found after either diet which is perhaps unsurprising given the normoglycaemic study population. Conversely, insulinaemic meal challenge responses were improved comparatively following weight-loss via both IER and CER; hence, glucose levels were maintained at a relatively lower level of insulin concentration, suggestive of improved insulin sensitivity. It is important to note that only ~22% of the study cohort met the metabolic syndrome criterion, comprised of a cluster of cardiometabolic risk factors for which IR is implicated as a common causative factor (182). Therefore, improvements in glycaemic indices were never likely to be pronounced. Nonetheless, one could argue that even modest improvements in insulin sensitivity could be of benefit to long-term risk of T2DM.
The absolute insulin concentration in the circulation is a function of its rate of appearance and clearance (175). Evidence from longitudinal studies in animals (184) and humans (185) suggest that reductions in hepatic insulin clearance occur as an early adaptive phenomenon in the development of IR during hyperenergetic feeding, perhaps serving to maintain normoglycaemia whilst preserving pancreatic β-cell function. To ascertain the relative contribution of changes in insulin appearance and clearance to study observations, changes in postprandial C-peptide were also measured, a more direct marker of insulin secretion than circulating insulin (175). In contrast to the insulin data, postprandial C-peptide was reduced following IER, but not CER, indicative of a reduction in pancreatic β-cell demand. The discrepancies between the C-peptide and insulin data point to differences between the diets in their effects on hepatic insulin clearance. Indeed, there was a suggestive increase in clearance within the CER group, and although these numerical trends did not attain statistical significance, the study was not specifically powered to detect small but potentially influential changes in this parameter (See also: Limitations). It is not possible to fully explain the underlying differences between the diets. It may be that these data are reflecting subtle baseline differences between the groups with respect to their individual longitudinal progression from IR to dysglycaemia, despite participants being matched at baseline for all insulin sensitivity indices.

Postprandial lipid metabolism

The second important observation made by the study were the differences between the two diets on their effects on postprandial lipid handling, with the relative reduction in incremental TAG responses to the meal challenge significantly greater following IER. This is in accordance with the study hypothesis. Acutely, one day of substantial 75% ER reduced incremental TAG responses by ~60% (Chapter 3). Chronically, the present study found a ~40% reduction in incremental responses following IER. Participants in both groups had abstained from ER for a minimum of seven days prior to metabolic assessments, in order to mitigate the acute effects associated with ER. Whether these metabolic changes can be retained following more prolonged periods of weight-stabilisation is unknown and requires further investigation. There is
considerable variation between postprandial studies with regards to methodological techniques; many studies use a fairly large (≥40 g) lipid loads in order to sufficiently overwhelm metabolic capacity (148). Hence the relatively modest lipid load (~23 g) employed in the present study can be considered a weakness, but equally a strength, given that the dose used is representative of the typical fat content of a meal.

Evidence from large prospective cohort studies highlight a strong independent link between elevated non-fasting (postprandial) TAG and CVD risk (33-35). Furthermore, the magnitude of incremental TAG responses have been shown to predict the presence of coronary artery disease (36), and to positively correlate with markers of atherosclerotic progression (37). The mechanisms underlying these associations were discussed in Section 1.3.4. On this basis, interventions which can improve postprandial lipid handling could play a role in delaying the atherosclerotic disease process. Findings from the present study are novel and as such, there is no comparative data in the literature. There are some indirect (albeit limited) data highlighting how potential differences between IER and CER in their effects on postprandial lipid metabolism might translate with regards to inter-related CVD risk markers such as LDL particle size distribution. Specifically, Varady et al (86) have previously shown that following a comparable average weight-loss of 5%, IER (75% ER on alternate days), but not CER (25% ER/day) or exercise (three times/week, 75% heart rate max), led to a reduction in the proportion of atherogenic sdLDL. However, this did not attain between-group significance. In addition, the effects of the “5:2” IER pattern used by the present study (where the ER-refeed cycles are less frequent) on lipoprotein particle size distribution are unknown but is worthy of future investigation.

The precise mechanisms are unclear but these findings could reflect both a reduction in the rate of postprandial TRL-TAG appearance and/or an increase in the rate of TRL clearance. Insulin, which is raised in the postprandial state, suppresses lipolysis from adipose tissue, whilst stimulating adipose tissue LPL (27). Hence, weight-loss per se can theoretically improve postprandial lipid handling through its associated improvements in these insulin-sensitive
pathways. However, there were no significant differences between the dietary groups in the various insulin sensitivity indices used by the study, or in changes in postprandial NEFA which might have otherwise helped to explain these findings. Whilst reductions in total adiposity and waist circumference were also comparable between IER and CER groups, changes within visceral and IHCL stores were not measured. Excessive accumulation of fat within these depots are linked to increased VLDL-TAG production, and so reductions following weight loss would be expected to favourably impact upon postprandial lipaemia (15, 186). Both IHCL and VAT are acutely sensitive to periods of profound negative energy balance (16, 187), and it could therefore be speculated that the acute periods of substantial ER may preferentially mobilise fat from these stores, perhaps more so than CER over a chronic time-course. This remains to be seen as human studies have yet to directly measure this.

**Substrate utilisation**

Parameters relating to whole-body and hepatic substrate oxidation were additionally assessed. Postprandial 3-OHB responses were not significantly altered following either diet, suggesting hepatic fatty acid partitioning was unchanged. Interestingly however, a within-group decline in basal RQ (reflective of increased whole-body FAO) was observed following IER but not CER. Further exploratory analyses also found a positive relationship between changes in postprandial TAG responses and substrate oxidation; i.e. the greater the increase in basal fat oxidation, the greater the post-treatment improvement in postprandial TAG. It is speculated that activation of AMPK might mediate some of the beneficial effects of ER and, in the context of the present study, presents one possible mechanism that could explain this correlation (188).

AMPK is a key nutrient sensor with the ability to regulate whole-body metabolism. AMPK is activated upon an increase in the AMP/ATP ratio, reflective of low cellular energy status (e.g. during fasting, or ER). Upon activation, AMPK turns on catabolic pathways to restore ATP levels, both in the short-term (e.g. by promoting FAO), and long-term (e.g. by increasing mitochondrial content and capacity for FAO) in tissues such as skeletal muscle. AMPK achieves this through its
interactions with sirtuin 1, which subsequently activates a number of transcriptional regulators (e.g. peroxisome proliferator-activated receptor gamma co-activator 1α, forkhead box protein O1) (188). The expression of skeletal muscle LPL mRNA is also induced by activation of AMPK and its associated pathways (189), hence providing the mechanistic link underlying the correlations found between changes in FAO and postprandial TAG.

The fact that FAO was increased following IER but not CER, is perhaps due to more potent stimulation of AMPK and its associated pathway, owing both to the substantive periods of ER coupled with mild ER on “feed” days. The relative contribution of the greater overall reduction in energy intake during IER (versus the IER eating pattern per se) to study observations cannot be ascertained. It must also be stressed that between-group differences in the changes in FAO did not achieve significance (p=0.148), but this was likely given the sample size of the study. Notwithstanding, it is perhaps not unreasonable to speculate that IER may have stimulated skeletal muscle adaptations, that could in turn explain the augmented basal FAO and may have led to increased TAG clearance.

Unfortunately, few IER studies have assessed changes in substrate utilisation or gene expression at the tissue level. Following two to three weeks of IER, modest increases in sirtuins and mitochondrial fatty acid transporter gene expression have been observed in non-obese individuals (77, 93), but the two previous studies that have assessed substrate oxidation both reported no change (76, 79). However, all of these studies are limited by their short study durations and lack a CER comparison group. In addition, these studies also used protocols that promoted alternating days of ER and hyperphagia, which may have a very different metabolic impact to the IER protocol used in the present study.

Resting energy expenditure

Dieting success and maintenance rates are notoriously poor (190). Alongside numerous environmental and behavior confounders, weight-loss is typically accompanied by a decline in
energy expenditure which is owed in part to reductions in metabolically active tissue coupled with numerous (neuro)endocrine alterations, which serve to hinder dieting efforts and subsequent weight-maintenance ((191); Section 1.4.5.4). In the present study however, absolute REE was reduced by ~7% (~477 kJ) following IER, but not CER; although not significant between groups, it remains noteworthy given that the study is likely under-powered to detect between-group differences in REE of <10-15% (192). When REE was normalised for metabolically active mass, the between-group differences became more pronounced. Our findings contradict that of the sole study published to date to have directly compared IER (alternate day total ER) to CER (1674 kJ/day deficit), which reported comparable reductions in absolute REE in obese individuals following eight-weeks of both diets (80). By contrast, REE adjusted for FM and FFM declined solely in the CER group only. Findings from previous studies which have not featured a CER comparison arm have been mixed, with some reporting reductions (~247-828 kJ; (79, 92)) and one reporting no change (78) in REE following 2-8 weeks of IER. However, the substantial heterogeneity between studies in terms of study cohorts, methodology and dietary protocols do not permit direct comparisons to the present study.

The study did not compare changes in circulating hormonal regulators of energy metabolism such as adipocyte-derived leptin or thyroid hormones between the groups, which might have otherwise provided mechanistic insights. Previously conducted studies have not yielded consistent differences between the diets with respect to changes in leptin levels, although the comparative effects on other regulators of energy metabolism are unknown (Table 1.2). Body composition is another influential factor in energy expenditure. FFM makes a substantive contribution to overall REE, explaining between 60-95% of the variability between individuals (193). However, the composition of weight-loss was comparable between IER and CER groups, with both groups losing ~80% of body weight as body fat and the rest as FFM (consistent with previous research (84)).

The underlying mechanism(s) for why REE was reduced following IER but not CER is therefore
not immediately apparent. These data contrast with recent speculation that IER, through its fluctuating periods of ER and energy balance may mitigate the adaptive physiological reductions in energy expenditure that occur during weight-loss, which are typically greater than expected due to changes in body composition alone (80, 194). The important distinction here is that participants under-consumed on “feed” days so most probably rarely attained energy balance. There is some suggestion that the greater the deficit between energy requirements and intake, the greater the magnitude of adaptive changes in REE which occur during weight-loss (194). Perhaps the greater relative decline in energy intake reported by IER participants may help to explain these findings.

**Blood pressure**

The blood pressure data confirm findings from numerous studies that show modest weight-loss, regardless of method, can lead to beneficial reductions in blood pressure although there were some differences between the groups (53). At baseline, approximately half of IER participants were either pre-hypertensive or hypertensive. Following the dietary intervention period, all but one IER participants became normotensive. In contrast, the proportion of participants who were (pre) hypertensive (~30%) did not change significantly following the CER diet, although diastolic blood pressure was reduced. The shift observed in the IER group was largely driven by a substantive reduction in systolic blood pressure which was not significantly altered by CER. To date, previous comparison studies have found no significant differences between the two diets (Table 1.2), and so these findings were unexpected. Numerical (but not statistical) trends in favour of higher baseline blood pressures within the IER group, however this would have been adjusted for by the ANCOVA model. The sometimes transient nature in which blood pressure is improved following weight-loss also highlights the role of dietary ER/negative energy balance in mediating this improvement (195), and in the present study, positive correlations were found between changes in systolic blood pressure and the degree of change in energy intake.

Speculatively, repeated intense stimulation of ketogenesis presents an alternative blood pressure
lowering mechanism underlying IER (Section 1.4.4.3). Specifically, ketone bodies such as 3-OHB have the capacity to bind to and antagonise the free fatty acid receptor 3, a G protein-coupled receptor present in sympathetic ganglia. Antagonism of the free fatty acid receptor 3 by 3-OHB suppresses sympathetic nervous tone (117), which may have potential benefits for the modulation of blood pressure (118), and may hence underlie the apparent superiority of IER at lowering systolic blood pressure.

Acceptability and attrition to intermittent energy restriction protocol

Whilst compliance to the IER protocol among completers was (expectedly) high, there were some important caveats with IER. A greater proportion of IER participants dropped out of the study, with 44% citing inability to tolerate the diet (both fasting per se and the meal replacement products) as the reason for drop-out. Study dropouts were younger, reflecting the fact that many were students as study recruitment was largely campus based, and peak dropouts (regardless of reasoning) tended to occur during exam times. These findings contrast from the literature which, when taken as a whole, which show statistically comparable dropout rates between IER and CER diets (Table 1.2). When compared specifically to the studies from Harvie’s group (84, 91), who used a similar “5:2” IER pattern, an important difference is that Harvie’s studies permitted participants to complete a short trial of the IER diets prior to starting the study. 9-12% of prospective participants were unable to tolerate the trial and hence decided to drop-out prior to starting the study. In addition, participants in these studies consumed “real food” products on energy restricted days, which are likely to be more appealing than meal replacement products. Indeed, a large proportion of IER participants intended to continue with their assigned diet either continuously or periodically, using a “real food” approach. Whilst participants on the present study had the opportunity to taste the meal replacement products prior to starting the study, it was decided to not conduct similar pre-study test in order to get a true reflection of likely dropouts for individuals embarking on the diet, and because of prior knowledge that many potential negative side effects (e.g. hunger) during IER often habituate over time, based both on published literature (91, 196) and anecdotal reporting. Overall dropout rates were 34% in the study cohort as a whole,
which exceeds that of previous 5:2 studies where dropout rates have ranged from 21-23%. The addition to the reasons stated previously, inflated dropout rate is likely a product of the relative sample size and study design, whereby participants were assigned to the diet until a weight-loss target was achieved rather than fixed duration of time. The proportion of participants achieving 5% weight-loss milestones in Harvie’s studies ranged between 37-65% over study durations of three to six months.

**Psychological and sleep quality indices**

Participants completed a number of psychological and sleep quality questionnaires before and after completing the study in order to gain a more holistic insight into the effects the dietary interventions. Based on published research, ER is expected to lead to improvements in psychological well-being, mood and sleep quality (197). On the whole, both IER and CER had mostly positive or neutral effects with no overt differences between the groups. Mood remained stable over the course of the intervention. By the end of the intervention, participants in both groups felt increasingly confident in their ability to manage their weight. Predictably, both groups exhibited greater levels of eating restraint which corresponded with a decline in hedonic drive to consume palatable foods, reflecting high motivation to maintain positive health behaviours. Previous studies have reported that a small proportion of participants following IER (<10%) exhibit difficulties staying asleep (198), but there were no such indications of sleep disturbances based on data from the sleep questionnaires. Neither group saw improvements in sleep quality, however this likely reflects the fact that neither group exhibited difficulties in any sleep component at baseline. There was a suggestion of differences with respect to daytime sleepiness which tended to increase in the CER group but decline in the IER group. The reason for the contrasting effects is unknown but the changes were not ultimately significant between groups.

**Strengths and Limitations**

The main strengths of the study were that weight-loss was controlled for, and that dynamic, concurrent, assessments of postprandial glucose and lipid metabolism were conducted in addition
to static, steady state (fasting) measurements. Limitations include the small sample size which increases the risk of type one and two error, and it is likely the study was underpowered for some outcomes (e.g. hepatic insulin clearance, energy expenditure and substrate utilisation). Participants were only informed of diet assignment after screening, and were only informed of the comparison diet once they had completed the study. A large proportion of participants assigned to the CER diet declined to participate in part due to lack of “novelty”, which negatively impacted upon the sample size of the study. The study cohort was heterogeneous, although importantly, participants were matched at baseline. Hormonal and inflammatory regulators of metabolism, appetite and energy expenditure were not assessed (although the literature review of previously conducted IER versus CER comparison studies did not reveal any consistent differences, Table 1.2).

Next, dietary records are known to be susceptible to (un) intentional behaviour modification and under-reporting, with average under-reporting levels of 20-40% noted among obese individuals (199, 200). Based on habitual dietary records completed prior to the interventions, all participants in the present study were classified as under-reporters, necessitating cautious interpretation of the dietary intake data. Although a potential source of discrepancy between the dietary intake and weight-loss trajectory data, under-reporting was present in both intervention groups.

Whilst participants were asked to maintain stable physical activity levels during each three-day period, in order to minimise measurement fatigue, physical activity levels were only assessed via the factorial method, which is insensitive to small changes in activity and is unable to differentiate between the various components of energy expenditure (Section 1.4.5.4). Next, the use of bioimpedance which, whilst validated against more robust anthropometrical techniques such as dual-energy X-ray absorptiometry (DXA), systematically underestimates body fat with increasing adiposity (201). Finally, the postprandial assessments only measured changes in absolute substrate concentrations after a single meal, which represents the balance but not the rate (or source) of substrate appearance or clearance. By the end of the six-hour postprandial blood
sampling period, substrate levels had not always returned to baseline which led to an under-
estimation of the true magnitude of postprandial responses, and introduced error in the calculation
of hepatic insulin extraction (175).

**Summary**

Put together, our data is suggestive that mode of ER (intermittent but severe vs. modest
continuous) may have different cardiometabolic effects in which may be important to long term
disease risk. Despite the fact that participants were normoglycaemic and the majority were
normolipaemic (at least on the basis of fasting levels), differences were observed between the
diets, particularly with regards to postprandial lipid handling which was improved to a greater
extent following IER (in accordance with the study hypothesis). Contrastingly, the IER diet was
associated with greater declines in REE and rates of attrition than the CER diet, but for those
participants who were able to tolerate the diet, the vast majority intended to continue a similar
eating pattern. The longer-term impacts of IER on metabolism and body-weight regulation are
unknown.
Chapter 5: Investigation into the effects of time-restricted feeding on human dietary intake, body fat and metabolic physiology

The research project featured on the television programme: “Trust me I’m a Doctor” (British Broadcasting Company). Date aired: 13th January 2016.

5.1 Introduction

Many aspects of mammalian metabolism exhibit daily variation driven by an endogenous circadian timing system (Section 1.5). The concept of chrononutrition reflects the basic idea that in addition to the amount and composition of food intake, ensuring its correct timing in coordination with the body’s daily rhythms, is important to health. There is a growing interest in the effects of temporal food restriction, with findings from recent rodent studies demonstrating that changes to the duration of the daily feeding period can greatly influence metabolism and body weight. Specifically, these studies (Section 1.5.1.1) have shown that the restriction of food intake to within a ≤12-hour window, termed TRF, can both protect against and reverse the adverse consequences of obesogenic diets even when overall food intake is not decreased (131, 132), whilst some benefits (albeit less pronounced) are also seen in rodents fed normal-chow (131). The precise mechanisms underlying the effects of TRF are not fully understood, but it is thought that the defined feeding and fasting rhythms this dietary pattern promotes, help to improve the oscillations of peripheral clock components (which become dampened in rodent models of diet induced obesity), whilst also ensuring that food intake is limited to the appropriate phase of the endogenous circadian cycle (Section 1.5.1.2; (131, 132)). In addition, the beneficial effects of TRF may simply be attributed to the accompanying elongation of the daily fasting interval. Consequently, TRF can be considered another form of intermittent fasting (Table 1.1).

Findings from the few human studies (Table 1.3) that have attempted to translate these rodent data have been inconsistent, but are limited either by their use of extreme temporal restriction (135, 136) or by their lack of metabolic measurements (137, 138). There is therefore a clear need for more controlled human experiments utilising realistic protocols that can be easily implemented by free-living individuals.
5.2 Aims, outcomes and hypothesis

5.2.1 Aims
The present 10-week study aimed to assess the feasibility of a TRF protocol in reducing the food intake window, in addition to the effect size and variability in changes in primary and secondary outcomes. Due to the lack of comparable TRF experiments in humans (to have investigated metabolic outcomes), this work was conducted in the first instance as a pilot study using a controlled study design.

5.2.2 Outcomes
- Primary: Changes in eating window duration.
- Secondary: Attrition rates, changes in dietary intake, body weight, body composition and fasting cardiometabolic risk markers.

5.2.3 Hypothesis
- The study outcomes were chosen to mirror those of earlier animal studies. As a first-in-human investigation of the metabolic effects of TRF (where the number of meals is not reduced), the study was designed to be hypothesis generating rather than driven.

5.3 Participants and methods

5.3.1 Participants
Healthy participants (BMI $\geq 20 \text{ kg/m}^2$) aged 35 to 60 years were recruited to the study from the Guildford and wider community. Recruitment was conducted externally by the British Broadcasting company, for a television show entitled “Trust me I’m a Doctor” (date aired: 13th January 2016), via website and social media advertising. Participants were weight-stable ($\pm 2 \text{ kg}$) over the preceding three months and had no significant medical history as confirmed by pre-study health questionnaire. Participants additionally completed a number of questionnaires relating to sleep quality (PSQI (179); Appendix K) and daytime sleepiness (ESS (180); Appendix L) at screening. Participants were excluded if they scored $>5$ on the PSQI questionnaire which
classifies individuals as “poor sleepers”; >9 on the ESS questionnaire indicative of high daytime sleepiness; if they had travelled across more than two time zones within the month preceding the study; if they had habitual irregular sleep patterns on more than two nights per week (bed time outside 22:00-01:00h and wake up time outside 6:00-9:00h) or a sleep duration on more than two nights per week of <7 or >9 hours; or if they had participated in rotating or night shift work for more than six months prior to the study. The study obtained a favourable opinion from the University of Surrey Ethics Committee (UEC/2015/076/FHMS) and was conducted in accordance with the guidelines laid down in the Declaration of Helsinki. Written, informed consent was obtained from all participants.

5.3.1.1 Sample size
As the study was conducted in the first instance as a pilot study, no a priori sample size was selected. Data obtained from the present study was used to inform power calculations, with the view to conducting a fully powered, larger, study in the future.

5.3.2 Study design and protocol overview
The study was a randomised, controlled study, comprised of two parallel dietary intervention groups: a TRF intervention group (three-hour contraction of habitual eating window) and a control (no intervention) group. The groups were balanced for age, BMI and gender. The dietary intervention period was 10 weeks, with study measurements (anthropometry and biochemistry) taken before and after. Food diaries were completed at baseline and final (tenth) week of the intervention. The study was conducted between September and November 2015.

5.3.2.1 Study design considerations
Due to inter-individual variation in habitual eating duration, participants were asked to decrease the duration of their eating window by a set amount relative to their habitual intake. A modest three-hour total temporal restriction was chosen to ensure acceptability of the TRF intervention, and because it was likely to have some effect based on findings from a previous TRF intervention
study which assessed anthropometric outcomes. This study found that a ~3-4 hour contraction in habitual eating window (comparable to the present study) led to a 3.3 kg reduction in body weight after 16 weeks in overweight/obese individuals (138).

5.3.2.2 Dietary interventions

5.3.2.2.1 Time restricted feeding intervention

Participants assigned to the TRF intervention were asked to alter their daily feed-fast cycle by delaying their first energy intake of the day and advancing their last energy intake of the day, each by 1.5 hours. This symmetrical compression of feeding duration avoided any bias of the intervention to one end of the day compared to the other. TRF participants were asked to maintain their usual physical activity patterns throughout the intervention period.

5.3.2.2.2 Control intervention

Participants assigned to the control intervention were asked to maintain their usual dietary and physical activity patterns throughout the intervention period.

5.3.2.3 Clinical visits

All participants initially undertook a one-week baseline period, during which they were required to maintain habitual feed-fast cycles. The timing and composition of energy intake was recorded in diet diaries over the final four days. At the end of the baseline period, participants attended the Clinical Investigation Unit (University of Surrey) for initial measurements. Participants were instructed to abstain from alcohol and strenuous exercise for 24 hours before the visit, and consumed their preceding evening meal before 20:00. Participants arrived at the unit at 08:00 following a 12-hour overnight water only fast. Body weight and composition were measured via bioimpedance (Section 2.1). Following this, a fasted blood sample was taken via venepuncture which was used to measure fasting glucose, insulin and lipid profiles. After the initial clinical visit, both groups commenced their respective dietary interventions whilst maintaining habitual activity patterns. At the end of the 10-week intervention period, participants returned to the
Clinical Investigations Unit for repeated measurements (pre- and post-intervention clinical visits were identical). Before the second clinical visit, participants received the same instructions given before the initial visit regarding alcohol and exercise restrictions. In addition, participants consumed the same evening meal as they did prior to the first clinical visit, as it has previously been shown that the macronutrient composition of an evening meal can affect metabolic responses on the following day (173).

5.3.3 Experimental techniques and analyses

5.3.3.1 Blood biochemistry

Blood samples were taken at each clinical visit via venepuncture. Please refer to Sections 2.5 for full description of blood collection, processing and laboratory techniques. Plasma samples were batch analysed upon study completion with all samples from an individual participant included in the same assay. Metabolites were analysed using the following methods: insulin using ELISA (Millipore, Billerica, MA, USA; intra-assay CV 5%); glucose, TAG, total and HDL cholesterol using commercially available kits (Instrumentation Laboratory, Warrington, UK) for the ILAB650 (Instrumentation Laboratory, Warrington, UK; intra/inter-assay CV all <10%). LDL-cholesterol was calculated using the Friedewald equation (Section 2.5.2.8). HOMA-IR was additionally assessed as a proxy for hepatic insulin sensitivity (Section 2.5.2.4).

5.3.3.2 Dietary analyses

Participants recorded intake in diet diaries for four days at two time points: at baseline (prior to starting their dietary intervention) and in the final (tenth) week of the intervention. Four-day intakes were then averaged. Recorded time of first and last energy containing food/drink was used to calculate each participant’s eating window. Target eating window was calculated by subtracting 180 minutes from an individual participant’s baseline eating window. Compliance in the final week of the intervention was assessed by comparing the reported eating window (recorded in the second diet diary) to the individual’s prescribed eating window. Please refer to Section 2.3 for a full explanation of methods relating to diet diary analyses.
5.3.4 Statistical analyses

Data were checked for normality using the Shapiro-Wilks test, with non-normally distributed data normalised where possible via log transformation to permit parametric testing (Section 2.7). The primary analysis was a one-factor ANCOVA between the dietary intervention groups with post-treatment values as the dependent variable, and baseline values of each parameter as the covariate. This is recommended statistical method (in terms of bias, precision and power) for the analysis of continuous outcomes in randomised studies with a single post-treatment measurement previously measured at baseline. An additional benefit of using the ANCOVA method is that it adjusts for the presence of baseline imbalances by including baseline values in the model (181).

Prior to analyses, it was ensured that assumptions made by the ANCOVA model with regards to independence of the covariate and treatment effect and homogeneity of regression slopes (treatment x covariate interaction) were met (157). A significant treatment x covariate interaction existed for plasma glucose responses, and so a general linear model (GLM) was created which incorporated this interaction term. Using this GLM, further exploratory analyses were conducted to explore this interaction, and to predict treatment responses (estimated marginal means [EMM]) to the TRF or control interventions at the varying baseline levels of fasting glucose observed within the cohort. This analysis was conducted as the study was a pilot designed to inform future work, potentially within pre-T2DM and T2DM populations.

To construct this model and obtain the EMMs, the following SPSS syntax was inputted, with rx representing treatment groups:

```
Unianova change by rx with baseline/print=parameter/emmeans=tables(rx)/
   emmeans=tables(rx) with (baseline=3.5) / emmeans=tables(rx) with (baseline=4) /
   emmeans=tables(rx) with (baseline=4.5)/emmeans=tables(rx) with baseline=5) /
   emmeans=tables(rx) with (baseline=5.5) /
```

The Mann Whitney U test was used as the non-parametric alternative to ANCOVA to compare change scores between intervention groups. Differences between intervention groups at baseline were assessed using independent t-tests for continuous variables or the Chi squared test for categorical variables. No significant baseline differences were found. A paired t-test or non-parametric Wilcoxon signed-rank test were used to assess the change between baseline and post-
intervention values within each dietary intervention group. Results are presented as mean ± SEM.

5.3.5 Data omissions

One control participant did not complete their second diet diary, so dietary intake data are presented for the remaining 12 participants who completed both diaries.

5.4 Results

Of the 16 participants initially recruited into the study, three participants (TRF=2, Control=1) did not complete the study. Reasons for dropouts included faintness during blood collection at the first clinical visit (Control=1), travel across multiple time zones as well as change to habitual diet through participation in another research study (protocol violation, TRF=1), or loss of contact (TRF=1). The consort diagram is presented in Figure 5.1.

![Consort diagram](image)

**Figure 5.1 Consort diagram for the time-restricted feeding study**

Baseline characteristics of the 13 study completers (TRF=7, Control=6) are reported in Table 5.1. The groups were matched for age, BMI, adiposity, gender and were all Caucasian.
### Table 5.1 Baseline characteristics for study completers

*Bioimpedance.

**Statistics and data presentation:** ¹ Unpaired t-test, ² Chi squared. NS – Non-significant. Presented as mean ± SEM.

**Abbreviations:** BMI – Body mass index; F – Female; M – Male; TRF – Time-restricted feeding.

<table>
<thead>
<tr>
<th></th>
<th>TRF (n=7)</th>
<th>Control (n=6)</th>
<th>TRF vs. Control</th>
</tr>
</thead>
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<tr>
<td><strong>Age (years)</strong></td>
<td>47 ± 3</td>
<td>45 ± 4</td>
<td>NS¹</td>
</tr>
<tr>
<td><strong>Gender (M/F)</strong></td>
<td>1 / 6</td>
<td>0 / 6</td>
<td>NS²</td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td>86.2 ± 5.2</td>
<td>77.8 ± 7.6</td>
<td>NS¹</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>29.0 ± 1.7</td>
<td>28.6 ± 2.8</td>
<td>NS¹</td>
</tr>
<tr>
<td><strong>Lean</strong></td>
<td>1 / 7</td>
<td>3 / 6</td>
<td></td>
</tr>
<tr>
<td><strong>Overweight</strong></td>
<td>3 / 7</td>
<td>1 / 6</td>
<td>NS²</td>
</tr>
<tr>
<td><strong>Obese</strong></td>
<td>3 / 7</td>
<td>2 / 6</td>
<td></td>
</tr>
<tr>
<td><strong>Body fat (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>36.0 ± 2.9</td>
<td>34.6 ± 3.5</td>
<td>NS¹</td>
</tr>
<tr>
<td>Males</td>
<td>21.9</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Females</td>
<td>38.4 ± 1.9</td>
<td>34.6 ± 3.5</td>
<td>NS¹</td>
</tr>
</tbody>
</table>

5.4.1 Eating window and dietary intakes

Reported eating windows and dietary at baseline and the final (tenth) week of the intervention are presented in Table 5.2. Eating window and dietary intakes did not differ between TRF and control participants at baseline (p≥0.251, unpaired t-test). Within the control group, no significant changes in any dietary parameters were noted between baseline and week 10 diaries, apart from a small increase in the percentage contribution of protein to total energy intake (p=0.012, paired t-test). Eating window was significantly reduced in the TRF group (p<0.001; paired t-test and ANCOVA) by 264 ± 48 minutes, which exceeded the 180-minute target set out in the design. TRF participants achieved (or exceeded) their target eating window on ~2.5 ± 1 out of four potential days. Relative to the control group (ANCOVA), this decrease in eating window duration noted among TRF participants was accompanied by reductions in total intakes of energy (p<0.001), carbohydrate (p=0.06 strong trend), protein (p=0.010) as well as the percentage energy contribution from alcohol (p=0.030); intakes of each dietary parameter were significantly reduced by 24 ± 13%, 26 ± 6%, 23 ± 5% and 62 ± 7% respectively (all p≤0.010, paired t-tests). Within-group comparisons also found significant improvements in some indices of diet quality including reductions in saturated fat (22 ± 4%; p=0.002) and sugar (24 ± 10%; p=0.057 strong trend), however, observed reductions were not significantly greater relative to the control group (all p≥0.234, ANCOVA). The percentage contributions of carbohydrate, fibre, protein and fat to total energy intake did not change significantly in the TRF group, suggesting that reductions in intakes
of these macronutrients (in g/day) occurred in proportion to the observed decrease in total energy.

<table>
<thead>
<tr>
<th></th>
<th>TRF (n=7)</th>
<th>Control (n=5)</th>
<th>TRF vs. Control¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Week 10</td>
<td>Baseline</td>
</tr>
<tr>
<td><strong>Eating window min</strong></td>
<td>743 ± 32</td>
<td>517 ± 22A</td>
<td>652 ± 50</td>
</tr>
<tr>
<td><strong>Energy intake kJ/day</strong></td>
<td>8733 ± 415</td>
<td>6493 ± 218A</td>
<td>7917 ± 1381</td>
</tr>
<tr>
<td><strong>Carbohydrate g/day</strong></td>
<td>237 ± 16</td>
<td>174 ± 16A</td>
<td>220 ± 44</td>
</tr>
<tr>
<td>(% total energy)</td>
<td>(42 ± 2)</td>
<td>(41 ± 3)</td>
<td>(44 ± 4)</td>
</tr>
<tr>
<td><strong>Sugars g/day</strong></td>
<td>99 ± 9</td>
<td>75 ± 12A</td>
<td>100 ± 23</td>
</tr>
<tr>
<td><strong>Fibre g/day</strong></td>
<td>20 ± 2</td>
<td>15 ± 2A</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>(% total energy)</td>
<td>1.9 ± 1</td>
<td>1.9 ± 1</td>
<td>2.1 ± 1</td>
</tr>
<tr>
<td><strong>Fat g/day</strong></td>
<td>81 ± 5</td>
<td>65 ± 3A</td>
<td>78 ± 17</td>
</tr>
<tr>
<td>(% total energy)</td>
<td>(35 ± 1)</td>
<td>(37 ± 2)</td>
<td>(35 ± 4)</td>
</tr>
<tr>
<td><strong>Saturated fat g/day</strong></td>
<td>33 ± 1</td>
<td>25 ± 2A</td>
<td>26 ± 7</td>
</tr>
<tr>
<td><strong>Protein g/day</strong></td>
<td>86 ± 3</td>
<td>66 ± 3A</td>
<td>73 ± 11</td>
</tr>
<tr>
<td>(% total energy)</td>
<td>(16 ± 1)</td>
<td>(17 ± 1)</td>
<td>(15 ± 1)</td>
</tr>
<tr>
<td><strong>Alcohol units/day</strong></td>
<td>2.3 ± 0.8</td>
<td>0.9 ± 0.6A</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td>(% total energy)</td>
<td>(5 ± 2)</td>
<td>(2 ± 1)A</td>
<td>(4 ± 2)</td>
</tr>
</tbody>
</table>

*Table 5.2 Eating window and dietary intakes at baseline and the final (tenth) week of the TRF and control (habitual diet) interventions*

**Statistics and data presentation:** Data expressed as an average of four days. ¹ Analysis of covariance. ² Significant within-group change vs. baseline (p<0.05 paired t-test). ³ Within-group statistical trend (p=0.05-0.1). **Non-significant.** Presented as mean ± SEM.

**Abbreviation:** TRF – Time-restricted feeding.

### 5.4.2 Body weight and body composition

Weight was not significantly changed after either the control (77.8 ± 7.5 to 77.3 ± 7.7 kg; p=0.114, paired t-test) or TRF (86.2 ± 5.2 to 85.5 ± 5.2 kg; p=0.374, paired t-test) interventions, and this was comparable between groups (p=0.716, ANCOVA). Similarly, BMI was not significantly changed after either the control (28.6 ± 3 to 28.4 ± 3 kg/m²; p=0.233, paired t-test) or TRF (29.0 ± 2 to 28.7 ± 2 kg/m²; p=0.335, paired t-test) interventions, and this was comparable between groups (p=0.716, ANCOVA). On the other hand, there was a significant effect of the TRF intervention on total adiposity (Figure 5.2; p=0.047, Mann Whitney U test¹); mean reduction in body fat was 1.9 ± 0.3 percentage points after TRF (p=0.001, paired t-test), whereas it was not significantly changed in the control group (-0.8 ± 0.7 percentage points; p=0.305, Wilcoxon signed ranks test).

¹Non-parametric test used due to presence of outlier in control group who lost substantive body fat, as non-parametric tests are more resilient to outlier effects (157). The participant was not an outlier for other study outcomes.
5.4.3 Fasting cardiometabolic risk markers

Fasting cardiometabolic risk markers measured before and after 10 weeks of TRF or continued habitual diet and lifestyle (control) are presented in Table 5.3. There was a trend in favour of a relative difference between treatment groups for fasting plasma glucose (p=0.073, GLM); glucose levels rose significantly by 14 ± 3.6% amongst control participants (p=0.012, paired t-test), whilst there was little change in the TRF group (-1 ± 3%; p=0.485, paired t-test). See also Figure 5.3 A-C for further exploratory analyses and individual fasting glucose responses. There was a statistical trend in favour of an effect of the TRF intervention on fasting total cholesterol (p=0.057); fasting levels rose by 14 ± 6.2% amongst control participants (p=0.083 trend, paired t-test), whilst there was little change in the TRF group (-2 ± 3%; p=0.395, paired t-test). This seemed mostly driven
by changes in LDL cholesterol, however no between group differences were found following baseline adjustment (p=0.110, ANCOVA). There was a trend in favour of increased fasting TAG in the control group (14 ± 6%; p=0.057, paired-test) but no differences between groups (p=0.524, ANCOVA). There were no effects of the TRF intervention on any other blood marker, either between groups (p≥0.490, ANCOVA) or within-groups (p≥0.208, paired-tests).

![Table 5.3 Fasting biochemistry before and after 10 weeks of TRF and control (habitual diet) interventions](image)

Statistics and data presentation: 1 Analysis of covariance. 2 General linear model. 3 Significant within-group change (p<0.05, paired t-test). 4 Within-group statistical trend (p=0.05-0.1). NS – Non-significant. Presented as mean ± SEM.

*Abbreviations:* HDL – High density lipoprotein; HOMA-IR – Homeostasis model assessment insulin resistance; LDL – Low density lipoprotein; TAG – Triacylglycerol; TOTC – Total cholesterol; TRF – Time-restricted feeding

5.4.4 Fasting plasma glucose: further exploratory analyses

5.4.4.1 Predicted treatment responses

Further exploratory analyses were conducted for changes in fasting glucose. Figure 5.3A displays predicted treated responses (EMMs) across the observed range of fasting glucose levels, with individual treatment responses plotted in Figure 5.3 B-C. These data show that the greater the initial fasting glucose level, the greater the predicted reduction in fasting glycaemia in response to TRF. In contrast, fasting glycaemia consistently deteriorated among controls.
Figure 5.3 A-C) Further analyses on effects of TRF on fasting glycaemia

A) Predicted treatment responses to TRF or no intervention (habitual diet) across varying levels of baseline fasting glucose. Figures B-C) Individual changes in fasting glucose following TRF (B) or control intervention (C).

Statistics and data presentation: General linear model. The regression equations for this model, used to predict treatment responses (estimated marginal means), were as follows:

- Controls: 3.759 – 3.692 + (0.121 * baseline in mmol/L)
- TRF: 3.759 + (– 0.819 * baseline in mmol/L)

Figure A) Results presented as mean (95% confidence intervals) with line of equality (---) representing no treatment effect. n=7 (TRF) and n=6 (controls). NB: there are two overlapping lines in Figure C hence only four lines appear.

Abbreviations: TRF – Time-restricted feeding.

5.4.4.2 Power calculation

In view of the novel findings made by the present study, power calculations were conducted based on these data to inform the sample size for future metabolic investigations into the effects of TRF on fasting glycaemia. It was determined that to attain 80% power at the two-sided 0.05
significance level, utilising a two-treatment parallel-armed control study designed, a total of 20 participants would be required if the true difference between treatments is 0.69 mmol/L. This is based on the assumption that the standard deviation of the response variable is 0.52 mmol/L, and that the effect size and variability found in the pilot study would be representative.

5.5 Discussion

This 10-week pilot study provides initial evidence that within a free-living population, a three-hour contraction of the eating window is achievable, and has the capacity elicit favourable changes in dietary intakes, body composition and influences fasting glycaemia. The following section provides further discussion of the key findings from the study.

Acceptability, attrition rates, recruitment

Unlike previous human studies, the TRF protocol used in this study entailed a modest symmetrical restriction of the daily eating window. Participants were advised to delay and advance the timing of their first and last meal respectively by 1.5 hours, with no restrictions placed on meal frequency or overall energy intake. In fact on average, the total eating window was reduced by ~4.5 hours (30%), to ~8.5 hours, based on comparisons between four day dietary records kept at baseline and the final week of the intervention. Therefore, the TRF intervention was achievable and sustainable over this time-frame. Attrition rates to the TRF intervention were low, with seven of the nine participants successfully completing the 10-week study. One participant was excluded as they had participated in another research project which resulted in a change to their habitual diet (therefore the reason for drop-out was not due to difficulties with adhering to the TRF intervention). The second participant was lost to follow up and so the reason for drop-out is unknown. Due to narrow deadlines (the project was scheduled to feature in a television documentary), recruitment was conducted over a very short time-frame (<2 weeks). Therefore, the small sample size was due to time constraints as opposed to difficulties with recruitment per se.
**Effects on dietary intakes and body composition**

Whilst participants were advised to maintain their habitual diets, TRF led to a spontaneous ~2240kJ (24%) reduction in energy intake, with proportional decreases in carbohydrate, protein and fat. In addition, there were improvements in several indices of dietary quality, particularly a reduction in alcohol consumption which is suggestive of behavioural modifications. Coinciding with these changes in dietary intake, were significant reductions in total adiposity, with percentage body fat decreased on average by two percentage points. Importantly, control participants maintained their usual dietary habits and eating window, whilst body composition was similarly unchanged.

Our dietary intake findings are comparable to those of a number of previously published human TRF studies. For example, in a recent study by Gill and Panda (138), overweight participants with habitual eating windows of >14 hours were asked to restrict food intake to within a self-selected 10-11 hour period for 16 weeks. Energy intake was reduced by ~20%, culminating in a 3.3 kg body-weight reduction which was equivalent to 1.2 kg/m² units. In contrast to the present study, Gill and Panda’s study did not feature a control group, nor did they assess changes in adiposity. We did not find any significant changes in BMI following TRF (-0.2 ± 2kg/m²), which may be due to our use of a combined healthy/overweight cohort, the shorter study duration and it should also be noted that we did not measure changes in FFM. In an earlier cross-over study by LeCheminant *et al* (137) in healthy adult males (24.4 [s.d.] 2.5 kg/m²), food was restricted between 19:00 to 06:00, thus equating to a 13-hour daytime eating window. Compared to the *ad libitum* control condition, two weeks of this daytime TRF led to a ~1000 kJ reduction in energy intake, as well as a significant (albeit modest) 0.1 kg/m² decline in BMI, although, the effects on body composition were not assessed. Another cross-over study, this time in exclusively healthy weight middle aged individuals, restricted intake to one euenergetic evening meal consumed within a four-hour supervised evening period (17:00 to 21:00) for eight weeks (“evening TRF”) (136). This was compared to an isoenergetic three meals/day control intervention of equal length. The study found significant reductions in body-weight and adiposity (assessed via bioimpedance)
following the evening TRF leg, of 1.4 kg and 2.1 kg respectively, reflective of a slight increase in FFM. Also observed was a modest (~272 kJ/day) decrease in energy intake, however this is unlikely to account fully for the anthropometrical changes noted by this particular study.

**Metabolic effects of TRF**

The current study also measured a number of cardiometabolic risk indices, and found suggestive differences in the relative change in fasting glucose levels between the treatment groups. This was largely driven by a significant increase among the controls, however, as the only differences between the groups from the outset was eating duration, one could argue that this was still a TRF-driven effect. Similar between-group observations were made for LDL-cholesterol, however these were not significant when numerical baseline differences were accounted for using the ANCOVA model. With regards to individual glucose responses, **Figure 5.3 B-C** show that fasting glycaemia rose consistently among control participants (whose responses symbolise the expected response in the absence of intervention) but not consistently among TRF participants. Whilst the control group exhibited a numerical increase in HOMA-IR (a measure of hepatic IR), these changes were neither significant within or between groups. Hence, both the physiological and behavioural reason underlying the negative metabolic changes seen in the control group are unclear. The study was conducted during the months of September to November, so our findings may reflect behavioural changes (e.g. in the run up to Christmas). There were minimal changes in reported dietary intakes in the control group which is suggestive of alterations in other unknown, uncontrolled, variable(s) and/or possibly reflects the limitations of the dietary intake assessments employed by the study (See: Limitations).

Due to interest in the potential applications of TRF in (pre)T2DM populations, exploratory analyses were conducted by predicting treatment changes in fasting glycaemia across an array of baseline levels. Visual inspection of **Figure 5.3A** demonstrates a consistent deterioration in fasting glycaemia among control participants irrespective of baseline values, whereas the predicted treatment reduction through TRF became greater with increasing baseline levels. It must be conceded that this interaction between baseline and treatment responses to TRF was largely
influenced by one participant, warranting replication of this study within larger and more diverse cohorts. Power calculations which could be used to inform future studies were presented in Section 5.4.4.2.

Rodents maintained on TRF exhibit an array of improvements including reductions in adiposity, liver steatosis, as well as improvements in glycaemic markers and total cholesterol (131, 132). Interestingly, these changes have also been shown to occur in the absence of ER (131, 132) and appear most pronounced when a high-fat diet is administered (131). These present two important distinctions between the animal data and the present study. From a metabolic perspective, the alterations in dietary intakes (both quantity and quality) noted among TRF participants confounds the interpretation of our biochemical and anthropometric data. It is therefore not possible to say whether the TRF eating pattern could have exerted any independent or additive effects on our study outcomes, over and above the mild ER (and positive dietary changes) it promoted. The importance of these ‘unintentional’ dietary modifications cannot be disregarded in the context of our obesogenic environment, however, participation in the study did affect social eating/drinking opportunities in the evening. In view of the potential implications this might have on long-term compliance, it would be of interest to explore whether similar metabolic benefits can be gained when TRF is performed on a more intermittent basis.

Prior to this study, only the evening TRF study (described earlier) had previously conducted metabolic investigations, finding both pro-atherogenic (increasing LDL cholesterol) and anti-atherogenic (increasing HDL cholesterol and decreasing TAG) changes after eight weeks. The group also reported significant elevations in fasting and postprandial glycaemia (135). Owing to the vastly different study protocol, a direct comparison with the present study is not possible. Whilst subject to some methodological limitations (Section 1.5.1.3), findings from that particular study do however highlight potential negative cardiometabolic implications of evening TRF (in the absence of energy deficit).
Strengths and Limitations

The particular strengths of the present study include the controlled study design and modest but achievable TRF protocol. This pilot study had a number of limitations which should be discussed. The study was conducted in a small group of well-motivated, predominantly female, participants within a high-income region in Surrey. This limits generalisability of the findings to other population groups. Limitations include the small sample size which increases the risk of type one and two error, as well as the methodology used to assess changes in body composition and food intake. Whilst anthropometric measurements were taken using standardised methodology to minimise sources of measurement error (Section 2.1), the study did not control or account for the natural hormonal fluctuations (e.g. the menstrual cycle) which can affect body composition, as well as glucose and lipid metabolism (139, 202, 203). In addition, bioimpedance cannot measure regional changes in fat distribution or ectopic fat deposition. Dietary records are susceptible to under-reporting and (un)intentional behaviour modification. Two participants were away from home during dietary recording periods and so reported atypical eating patterns. In addition, changes in diet during the intervention periods, i.e. in between the two measurements, were not factored in and it is unknown how compliance to TRF changed over time. Finally, whilst participants were asked to maintain habitual activity levels, no formal monitoring of physical activity was conducted. It is therefore unknown whether TRF led to compensatory changes in physical activity, which would also influence overall energy balance.

Conclusions

Put together, findings from this pilot study demonstrate that a three-hour contraction of the eating window is achievable among free-living humans, and have also laid the foundations for future metabolic investigations. 10 weeks of TRF led to improvements in body composition and positively influenced fasting glycaemia. These findings are likely to be largely explained by the spontaneous reduction in energy intake observed among TRF participants. The potential influence of the TRF eating pattern on these findings, independent of its effects on energy intake, cannot be ascertained. Nonetheless, this unintentional reduction in energy intake highlights the potential
utility of TRF as another ‘small changes’ strategy for body weight maintenance and/or loss. Further metabolic studies, utilising larger and more diverse study cohorts are required.
Chapter 6 : General Discussion

6.1 Summary

Overweight/obesity is becoming an increasingly prevalent threat to the health and wealth of modern day societies; related health complications including T2DM and CVD now account for a considerable proportion of yearly deaths (3, 4). Weight-management is considered the cornerstone in preventing these obesity-associated cardiometabolic diseases, and yet our increasingly obesogenic environment makes sustaining positive health behaviours difficult to achieve. There is therefore an increasing need to develop an array of preventative dietary strategies for weight-loss and/or maintenance which can be offered to patients. At the same time, understanding the metabolic differences between different dietary approaches may help identify potentially applications of a given diet for specific metabolic disorders. The three experimental pieces described in this thesis were primarily designed to investigate the metabolic impacts of IER and TRF, both of which fall under the “intermittent fasting” umbrella. Their purported premise is that individuals either need not restrict food intake every day or (potentially) at all to attain metabolic benefit, an appealing premise in the context of today’s obesogenic environment. The overview of the findings from the studies which comprise this thesis will be discussed in the next section, alongside their potential implications and limitations.

6.1.1 Intermittent energy restriction

As discussed in Chapter one, overweight/obesity and the pathogenesis of its associated metabolic disorders are complex, however it is clear that aberrations in glucose and lipid metabolism in the postprandial period play a key role in the development of CVD and T2DM. The studies which comprised this portion of the PhD therefore placed particular emphasis on evaluating the effects of IER on postprandial glucose and lipid regulation. Previous research assessing the metabolic effects of IER in rodents were conflicting, and the considerable variability between studies with respect to rodent strains and study protocols made drawing meaningful conclusions from these data difficult. Notwithstanding, a small but not insignificant number of studies highlighted the
potential for adverse health consequences which appear unique to the IER eating pattern, particularly with regards to effects on glycaemic control (67, 68, 204). In some cases, the metabolic abnormalities only manifested when metabolically challenged (67), highlighting the importance of looking beyond simple fasting indices of cardiometabolic risk.

On the whole, findings from published human weight-loss studies conducted prior to the PhD project had been promising (importantly none had shown negative health consequences), but relatively few had compared IER to modest (e.g. ~2510 kJ) CER, which on the basis of current NICE guidance is considered the best practice approach for weight-loss (54). Of the comparison studies that had been conducted, most had demonstrated comparable changes for most of the outcomes assessed (some differences were apparent, but these will be discussed later in the context of the findings from the present study). However, most studies had solely collected fasted blood samples, and none of these studies controlled for weight-loss. This poses a significant confounding factor when attempting to address whether IER offers any distinct metabolic advantage or disadvantage over CER.

With these points in mind, the two studies which comprise this portion of the PhD took a stepwise approach to further our understanding around the metabolic impacts of IER. Study one (Chapter three) addressed the acute (weight-loss independent) impact of substantive ER on metabolism. Assessments of postprandial glucose and lipid responses to a liquid mixed test meal were conducted the morning immediately following one day of total (100%) and partial (75%) ER in conjunction with measurements of substrate metabolism and aspects relating to body weight regulation (appetite, energy intake compensation, energy expenditure). Comparisons were made between the two one-day ER interventions and a third isoenergetic (control) leg, which represented both energy balance and a standard overnight fast. This led into Study two which investigated the chronic effect of IER (two days of 75% ER/week) following 5% weight-loss and how this compared to a matched weight-loss following CER (2510kJ/day daily deficit). The averaged overall prescribed ER was ~22-23% for both dietary interventions over the course of
the week. This study design removed the confounding influence of uneven weight-loss and also allowed assessment of proportional changes in body composition. Both the acute and chronic studies utilised similar methodological techniques and overweight/obese (but otherwise healthy) study cohorts, which permitted direct comparisons to be made across the studies. Although it was not possible to distinguish between lipoprotein fractions, postprandial measures of circulating 3-OHB were additionally taken with the intention that this might provide some insights into potential alterations in hepatic fatty acid partitioning. The key findings and implications from the studies will be discussed.

Postprandial lipid metabolism and substrate oxidation

Among the most interesting observations made by the acute study were the shifts in postprandial substrate metabolism following both levels of substantial (75-100%) ER, and subsequent effects this had on postprandial lipaemia. Specifically, following both one-day ER trials, postprandial substrate oxidation was shifted towards FAO and hepatic ketogenesis, which occurred in an apparent dose response manner. This translated to a ~60-75% reduction in incremental postprandial TAG responses following partial and total ER respectively, whilst carbohydrate oxidation was reciprocally depressed. Although this latter aspect was not statistically significant following partial ER, this may largely reflect a low study power for this outcome measure, as the study employed a relatively modest sample size of 10 participants. Use of the incremental method of quantifying postprandial responses meant that these observations were not solely explained by changes in baseline levels of TAG, albeit these were also reduced following both levels of ER. As 3-OHB concentrations were markedly elevated in the postprandial period following both ER trials, one could theorise that fatty acid availability for VLDL-TAG production and export would be reduced, contributing to this reduction in lipaemia. These findings likely also reflect an increase in TRL clearance, on the basis of observations made by rodent studies (109). These data are novel, in that no previous human study has compared postprandial TAG responses following varying levels of substantial ER. These apparent acute intervals of intense FAO/ketogenesis (which seemingly persists during refeeding) could hypothetically exert a prohibitive effect on
IHCL accumulation over time, via a number of potential mechanisms:

a) IHCL may be mobilised during each ER period (as has been observed by others (102)), and the extent of IHCL accrual that occurs during the initial refeeding interval might also be limited whilst FAO/ketogenesis persists.

b) Repeated intervals of ER and/or reduced carbohydrate intake might lead to upregulation of hepatic FAO/ketogenic capacity via altered gene regulation over time (205).

c) Although not directly assessed, circulating TAG and insulin levels would presumably be reduced over the entire course of these ER periods relative to a standard day of unrestricted intake. Elevated insulin levels exert anabolic effects, by inhibiting FAO/ketogenesis, whilst favouring TAG synthesis and DNL (28), that might (with a background of IR) favour IHCL accumulation. Hence, repeated, sustained, periods of reduced circulating insulin concentrations may contribute to a reduction in IHCL. Similarly, reducing the duration of time spent in a “fed” / lipaemic state may also alleviate the “hepatic TAG burden” by reducing the extent of hepatic TRL remnant uptake by the liver.

As no human study has yet measured changes in IHCL, this hypothesis remains to be tested, however, previous IER studies have demonstrated superior reductions in HOMA-IR (which correlates with IHCL (206)) when compared to CER, and so appears to corroborate this theory. I theorised at this point that chronic reductions in IHCL and/or alterations in hepatic substrate partitioning as a consequence of these intense repeated ER cycles might impact upon fasting and/or postprandial VLDL-TAG secretion (primarily VLDL₁ which is associated with liver fat (207)), perhaps more so than modest CER. As such, differences between the dieting approaches in terms of their effects on postprandial incremental TAG became the primary metabolic outcome in Study two. Although IHCL could not be directly assessed, Study two included a number of measures including HOMA-IR and fasting TAG, which would be expected to be reduced following IER according to the study hypothesis. However, unexpectedly neither IER nor CER resulted in appreciable changes in any fasting biochemical markers. On reflection, use of a predominantly metabolically healthy cohort (at least on the basis of metabolic syndrome
classification), who were normoglycaemic and predominately normolipaemic, likely explains these findings. At this point, it might have led one to conclude that the diets had failed to produce any significant cardiometabolic health benefits, despite achieving what is deemed clinically significant weight-loss (54).

Nevertheless, the study did add weight to the apparent value of postprandial assessments, in that metabolic differences between the two dieting approaches only became apparent in the postprandial period, with incremental TAG responses reduced by ~40% following IER, whereas there was little change following CER (in accordance with the hypothesis). Again, the mechanism underlying these observations are unclear as to whether they reflect reduced TRL appearance and/or increased TRL clearance. Postprandial 3-OHB was not significantly altered following IER suggesting that, unlike in the acute study, there was no longer lasting shift in hepatic fatty acid partitioning, which might have otherwise explained these observations. It is currently not clear why the reduction following IER was greater than following CER on the basis of the data available. Although changes in waist circumference were comparable between groups, this measure is unable to distinguish between SAT and VAT depots which is an important limitation of this study. As described earlier, the anatomic proximity and portal drainage of VAT implicates as a key driver of dyslipidaemia (Section 1.2.3).

Interestingly, there was a notable shift in whole-body substrate oxidation towards greater FAO in the IER group only which, was found to relate to the reductions in incremental postprandial TAG. Discussion was provided in Chapter four regarding how alterations in skeletal muscle gene transcription (through ER-induced activation of AMPK/sirtuin responsive transcriptional regulators) could have facilitated the shift in substrate oxidation and possibly augmented TRL clearance. Although suggestive, the within-group shift in FAO was ultimately not significant between IER and CER groups, due to the study likely being underpowered for this outcome measure. Nonetheless, this could be a worthwhile area of further exploration by studies employing larger study cohorts.
Postprandial glucose metabolism

Review of the literature suggests that longer term adoption of IER (total ER on alternate days) has the potential to adversely impact glycaemia as a consequence of decreased peripheral insulin sensitivity (67, 204). This may relate to the repeated exposure of peripheral tissues to elevated fatty acid fluxes. The important distinction here between human and rodent studies is that most human studies have used partial ER protocols. Acutely, prolonged periods of total ER were known to impair glucose tolerance in humans, but the effects following partial ER in overweight/obese individuals were less well characterised, and comparisons between studies were made difficult owing to heterogenous designs and study cohorts. Study one benefited from the use of a within-participant study design which enabled each participant to act as their own control. What was observed, akin to the lipid data, was essentially a dose response effect. Postprandial glycaemia was increased following total ER whereas partial 75% ER (as compared with total ER) reduced the fast-associated decline in oral glucose tolerance (in accordance with the hypothesis). This likely relates to attenuation of the fatty acid fluxes by allowing some ER-day intake. Also observed was a suggestion of a change in the insulin secretion dynamics – whilst there was no overall change in the incremental response, the secretion profile following total and partial ER were more flattened and delayed, suggestive perhaps of an attenuation of the first phase insulin response. However, it is not possible to determine how changes in hepatic insulin extraction may have contributed to this difference.

This informed the methodological design of the weight-loss study (Study two), which measured both postprandial insulin and C-peptide concentrations. The physiology of C-peptide makes it a more direct marker of insulin secretion, whilst also providing an approximation of hepatic clearance (175). Importantly, no impairment in glycaemia was observed following IER, which is in accordance with a recently published eight-week IER study in obese individuals employing an IVGTT (80). Instead, Study two observed modest reductions in postprandial insulinaemia following weight-loss through IER, reflective perhaps of an improvement in insulin sensitivity which would thus reduce pancreatic β-cell secretory demand (corroborated by a reduced
postprandial C-peptide response). Postprandial insulinaemia was also reduced following CER although the mechanisms appeared slightly different, with the reduction in the latter group possibly mediated by an increase in hepatic clearance.

The mechanisms underlying the improvements in insulin sensitivity may relate to the favourable alterations in body composition encompassing reductions in total and regional (central) adiposity which would be expected to reduce fatty acid delivery to peripheral tissues and thus ectopic fat deposition (12). Speculatively, observed shifts in substrate oxidation towards FAO may have also exerted additive benefits on ectopic fat in the IER group, for instance in skeletal muscle, the major site of glucose disposal (12). Unfortunately, changes in intramuscular lipid were not measured in Study two, nor indeed have they been measured by any other IER versus CER comparison studies. These potential mechanisms remain purely hypothetical, but may form the basis of future work. In addition, the study did not assess changes in adipokines and inflammatory markers although, again, no consistent differences have been observed by previous studies. On the whole the changes in postprandial insulinaemia in Study two were modest, with no change in other glycaemic control indexes (HOMA – IR and %B) noted, but this is not unexpected considering the predominately metabolically healthy cohort. Nonetheless, postprandial hyperinsulinaemia represents step one of Kendrick’s four step progression from IR to T2DM (48), therefore the study observations may have important implications for T2DM risk.

Weight regulation: effects of intermittent energy restriction on components of energy balance

Studies one and two also investigated aspects pertaining to weight regulation including components of energy expenditure, appetite and food intake. In Study one, appetite levels were elevated during the ER interventions (as would be expected), however these did not translate into marked overcompensation during refeeding. In fact, when expressed over a three-day period (encompassing the ER day and two subsequent days of ad libitum feeding), participants still remained in a net energy deficit of ~30%, with no additional benefit derived from fasting completely. Similarly, during the weight-loss intervention, participants neither over-compensated
on “feed days” nor significantly altered their macronutrient consumption when expressed as a proportion of total energy intake. Both the acute and chronic data are in accordance with previous research examining the effects of acute substantive ER and IER on energy compensation (83, 84, 91, 165).

As a consequence, this lack of compensatory hyperphagia facilitated a greater overall energy reduction in IER when compared to CER participants; although, this did not translate into a greater rate of weight-loss with IER. This might well reflect an element of under-reporting within diet diaries, an important limitation across all the studies in this thesis. The majority of participants taking part in Studies one and two (with the exception of only five participants) were adjudged to be under-reporters on the basis of calculated Goldberg cut offs. Conversely, this discrepancy between energy deficit and weight-loss might reflect compensatory changes in other components of energy balance. Acutely, substantial ER does not appear to effect REE or MIT, however following weight-loss, a reduction in absolute (unadjusted) REE was observed. This is not unexpected, and is a common feature of weight-loss (56), yet surprisingly a within-group reduction in REE was observed in the IER group only. Although no statistical differences were observed between IER and CER groups (p=0.205), this might reflect study power for this secondary outcome measure. When REE was normalised for metabolically active mass, there was a trend towards between-group differences (p=0.067) and further implies that weight-loss efficiency was reduced (i.e. the amount of weight or fat lost per unit of energy deficit) in the IER group. It is unclear why a reduction in REE (absolute and adjusted) was observed following IER, but not CER. These data contradict findings from the only other comparison study published to date (80), whilst reported changes in REE from previously conducted non-CER controlled studies have been inconsistent (Section 1.4.5.4). Hormonal mediators of energy homeostasis such as leptin were not assessed during the studies and so their contribution to these findings are unknown.

Another methodological issue common to both studies is that neither conducted formal
assessment of physical activity levels beyond self-reports of habitual leisurely/occupational activity level, which will have introduced error in the calculations of both energy requirements and energy balance. In addition, the factorial approach used to quantify physical activity levels by the studies (Table 2.1) is insensitive to small changes in day-to-day exercise and non-exercise activity (Section 1.4.5.4). Therefore, although Study two observed no overall changes in physical activity between IER and CER groups, this does not exclude the possibility that underlying differences between the two groups in terms of physical activity levels may have contributed to the study outcomes. For example, extended morning fasting (i.e. breakfast skipping) has been shown to decrease spontaneous light-intensity physical activity thermogenesis (208). Speculatively, a subtle decline in this component of energy expenditure during the intervals of substantial ER (“fasting”) may help to explain why rates of weight-loss were comparable between IER and CER groups, despite IER participants reporting a greater overall decrease in energy intake. Alternatively, if IER participants increased activity levels to a greater extent than CER participants, this may have contributed to the superiority of IER at improving postprandial lipaemia (209). However, this is debatable as participants in Study two had abstained from exercise for two days prior to metabolic assessments, and it is argued that the low levels of postprandial lipaemia exhibited by endurance-trained individuals are largely due to the TAG-lowering effect of individual exercise sessions as opposed to long-term training adaptations (209).

Of the previous comparison studies of IER and CER, two have similarly reported no overall changes in physical activity (assessed via physical activity questionnaire) (84, 91), whilst the remaining two studies did not measure changes in physical activity (80, 87). It is clear that more data are required on the effects of IER on energy expenditure.

Acceptability of the intermittent energy restriction diet

An important caveat of IER revealed by Study two was the higher attrition rate within the IER group, which contradicts findings from most previous studies Table 1.2. Part of this might relate to the study designs (discussed Section 4.5), but also the use and acceptability of the meal
replacement products in Study two. Studies have varied greatly with regards to dietary protocols, however few have compared the impact of different protocols on acceptability and metabolism. In contrast to our findings, Klempel et al (98) noted higher levels of dietary compliance to IER utilising meal replacement products (versus “real food”). Harvie et al (91) showed that an intermittent ad libitum low carbohydrate high fat/protein diet for two days of the week produced comparable weight-loss as IER (two days 70% ER) although only IER led to improvements in HOMA-IR. Others have shown that despite weight-loss, having a high-fat background diet attenuates the benefits of IER, and may slightly impair vascular health (89). At present there is insufficient data to make any conclusions regarding the ‘optimal’ IER diet composition with regards to tolerability and metabolic effects, as very few studies have directly assessed this.

6.1.2 Time-restricted feeding

During the PhD project there was an opportunity to partake in pilot research on TRF, a relation of IER in that both fall under the intermittent fasting umbrella. In contrast to IER, TRF is usually performed on a daily basis although alternate day total ER could be viewed as an extended form of TRF. Pervasive rodent data exists demonstrating the metabolic benefits of TRF even in absence of energy deficit (131, 132). One of these rodent studies demonstrated that the magnitude of the benefits appeared to be proportional to the fasting interval, suggesting that simply spending less time in a fed, anabolic state is of benefit to metabolic health (132). However, very few human studies had been conducted and so highlighting the value for more controlled human experiments incorporating basic biochemical measurements which may form the basis for future studies. For this reason, Study three (Chapter five) examined the effects of a modest, symmetrical, three-hour daily contraction of the eating window on basic blood biomarkers and adiposity. The study was novel in that it employed a controlled parallel armed study design. Whilst originally intended to be in overweight/obese individuals, the recruitment criteria had to be widened to ensure adequate recruitment into the study, nevertheless the resultant small study cohort (n=13) remains a limiting factor for the study.
After 10 weeks of TRF modest improvements in adiposity were observed and a suggestion of a beneficial effect of TRF on fasting glycaemia, with the magnitude of the benefit seemingly proportional to baseline glycaemia. Much like other human studies allowing ad libitum/self-selected food intake, energy intake was overall reduced when following TRF and so favourable effects may well be attributed to this as opposed to a distinct benefit of the TRF eating pattern per se. The TRF protocol was achievable and well tolerated over the course of the study, however, participation in the study did infringe on social eating and drinking opportunities. Similar to Studies one and two, limitations to Study three included reliance on self-reported dietary intakes and lack of robust assessment of energy expenditure. Another limitation of this study in particular is that it did not conduct regular assessments of compliance to the prescribed eating window.

6.2 Conclusions

Intermittent energy restriction

- Acute periods of substantial ER had contrasting effects on postprandial glucose and lipid metabolism, impairing glucose tolerance whilst improving postprandial lipaemia in healthy overweight/obese individuals. This likely reflects a normal transient response designed to conserve and replenish glycogen stores lost during acute periods of food deprivation, by shifting postprandial substrate oxidation in favour of FAO.

- In the short/medium term, IER (two consecutive days of 70% ER/week) did not lead to adverse effects on glucose tolerance, despite likely repeated exposure of peripheral tissues to elevated NEFA fluxes. Modest 5% weight-loss through IER led to improvements in postprandial glycaemic control that was comparable to CER, although the mechanisms underlying these improvements may differ between the two dieting approaches.

- Modest 5% weight-loss through IER led to superior improvements in incremental TAG responses than matched weight-loss achieved via CER, however the mechanisms are unclear.

- IER was not more tolerable than CER, and a higher attrition rate was observed in the IER
dietary intervention group. Of those able to tolerate IER, and successfully lose weight, the majority saw IER as a potential long-term approach for weight-loss and/or maintenance.

- IER was not associated with energy intake over-compensation, either acutely or chronically, based on self-reported intakes. The contribution of the greater overall ER it seemingly facilitated (relative to CER) to study outcomes is unknown.

- Weight-loss through IER may lead to a reduction in REE. This does not hinder weight-loss, which occurs at a comparable rate to CER, but may predispose to weight regain if healthy eating behaviours are not maintained.

**Time-restricted feeding**

- Small contractions of the daily eating window among free-living healthy and overweight/obese individuals is achievable, and leads to modest reductions in body fat, energy intake and positively influences fasting glycaemia.

- The ER independent contribution of the TRF pattern to observed metabolic and anthropometric improvements in the study cannot currently be ascertained.

- TRF likely infringes on social eating/drinking opportunities which might pose a barrier for its long-term uptake, at least on a daily basis.

**6.3 Future research directions**

The results from the studies which comprised the PhD project were interesting, promising and informative, and have yielded multiple avenues for future research:

**Intermittent energy restriction**

- Findings from the present study now require replication in larger study cohorts, and following greater degrees of weight-loss. In addition, assessments following a longer period of weight stabilisation are required to establish whether the metabolic benefits of IER can be retained.
Mechanistic evaluation of the effects of IER on lipid and lipoprotein metabolism: The postprandial studies solely measured changes in absolute concentrations of TAG over time and as such it is not possible to ascertain the exact mechanisms underlying the study findings, based on the available data. Use of gold standard stable isotope techniques would permit more robust assessments of changes in fatty acid flux and oxidation, hepatic fatty acid synthesis as well as lipoprotein metabolism and may hence provide mechanistic insights behind study observations (210). Assessments of lipoprotein particle size would also prove valuable, given that rapid remodelling of lipoprotein particles occurs in the postprandial state (18).

Mechanistic evaluation of the effects of IER on glycaemic control: Use of HEC techniques would also be of particular value in the IER field. In addition to being the gold standard technique in the evaluation of insulin sensitivity (211), use of this technique would permit differentiation between peripheral and hepatic insulin sensitivity which might be distinctly altered by IER.

Measurements of changes in skeletal muscle expression of genes involved in fatty acid transport, oxidation and mitochondrial biogenesis could help provide mechanistic insights into the comparative effects of the two modes of ER on substrate utilisation as well as peripheral glucose and lipid handling.

Is there an optimal IER regimen? It would be of interest to examine the metabolic impacts of alterations in ER frequency, severity and macronutrient composition on both ER and non-restricted days.

Regional and ectopic adiposity, adipocyte characteristics: The majority of studies that have compared IER to CER including the study presented in this thesis have used BIA and waist circumferences to measure changes in total and regional adiposity. Mechanistic studies evaluating comparing the effects of IER vs. CER must also include evaluation of changes in VAT and ectopic fat deposition (IHCL, intramuscular) which may be preferentially reduced by IER. VAT and IHCL are particularly sensitive to acute periods of negative energy balance (16, 212) and could be evaluated for example through use of
magnetic resonance imaging and magnetic resonance spectroscopy. In addition, as described in Section 1.2.1, “pathogenic” growth of adipose tissue encompasses hypertrophic adipocyte growth, IR, and enhanced inflammatory cell infiltration which driver localised IR, and may contribute to systemic IR. Rodent studies suggest IER can favourably modulate some aspects of adipose morphology such as adipocyte cell size (62), however, the effect in humans remains unknown.

- Application of IER in certain disease states: Results from this thesis indicate that IER can attenuate postprandial lipoaemia in mostly normolipidaemic participants. One could argue that the treatment benefits may be greater among dyslipidaemic cohorts and this warrants evaluation.

- Weight maintenance: The applications of IER as a weight-maintenance strategy following weight-loss, and among healthy weight participants, requires further evaluation. Whilst there is some data highlighting successful application of IER as a weight-maintenance strategy following weight loss (91, 95), there has been very little research conducted in healthy weight individuals.

- Long-term safety: a number of rodent studies have demonstrated the potential for harmful consequences of IER for peripheral insulin sensitivity and also heart function (63, 66-68). Whilst the present study detected no evidence of harm in the short/medium term on glycaemic control, more human long-term safety data on the use of IER as a long-term weight-loss (or maintenance) strategy is required.

- Physical activity: More rigorous assessments of all components of energy expenditure (in conjunction with hormonal regulators of energy metabolism) are required utilising more robust techniques such as the doubly labelled water method.

**Time-restricted feeding**

Given that IER and TRF are likely to share some common mechanisms, many of the future research directions discussed in the preceding section can also apply to TRF. However, in view of the relative lack of human TRF data, the following suggestions could perhaps be viewed as key
research priorities:

- Larger, controlled, long term studies are now required to establish whether findings from the present study can be replicated, encompassing individuals who are both healthy and/or are overweight/obese who might both benefit from TRF.

- Mechanistic evaluation: Metabolic investigations of TRF examining time of day and length of fasting interval effects on metabolic outcomes could be performed to establish the relative contribution of changes in meal timing and fasting on metabolic outcomes, as well as how this might impact on circadian rhythms. Future studies must control for energy intake, preferably through provision of experimental diets and/or through more frequent monitoring of food intake to establish whether there are any ER independent benefits.

- Application of IER in certain disease states: Data highlighted by the general linear modelling highlights potential applications of TRF as a strategy among individuals with (pre)T2DM which warrants further study.

- Body composition: More stringent measures of body composition (including changes in fat distribution and/or ectopic fat), which might be preferentially mobilised by extended periods of fasting are required.

- Intermittent TRF: Anecdotally, participants reported that TRF affected their participation in social events, which might impact on compliance over time and can account for some of the observed reduction in energy and alcohol intake. The rodent study by Chaix et al (132) demonstrated the efficacy of an intermittent TRF, for five days per week, at improving metabolic outcomes. It would be of interest to explore whether similar metabolic benefits can be gained when reducing frequency of TRF days per week in free-living humans. This intermittent approach is likely to be more acceptable in the long term.

- Physical activity: More rigorous assessments of changes in physical activity are required (see preceding section).
6.4 Broader impact of findings and dissemination to stakeholders

Considerations must be placed on the broader impact of these study findings and strategies for the dissemination of study findings to key stakeholders.

The broader impact of study findings and key messages from the research projects are as follows:

- IER represents a novel and acceptable alternative approach to weight-loss for members of the public, pertinent in view of the current obesity epidemic.
- IER represents a potential therapeutic option for individuals with impairments in postprandial TAG metabolism, an important modifiable risk factor for CVD (Section 1.3.4.1). In clinical practice, clinicians are unlikely to have access to sophisticated metabolic assessment techniques so when conveying this message, it important to provide tools to help identify the most at risk patient groups. For instance, a patient in clinic may present with an elevated level of non-fasting TAG and/or other components of atherogenic dyslipidaemia (Figure 1.1).
- TRF represents a “minimally invasive” strategy for improving body composition and metabolic health that does not require “drastic” changes to diet and lifestyle
- On the basis of findings from these metabolic investigations, there are multiple potential avenues for future research (Section 6.3), which may yet reveal mechanistic insights and potential applications of intermittent fasting in other population groups
- Data from these research projects also highlight the importance of looking beyond fasting indices of cardiometabolic risk, an important consideration for example when reviewing the therapeutic progress of a patient, or when evaluating dietary research studies.

Dissemination channels:

- General public: public engagement events, mass media, inter-personal communication.
- Clinical nutritionists, dietitians and other health care professionals: Scientific publications and conferences, presentations to colleagues in the work place, inter-personal communications, publication of news articles within specialist magazines.
• Research scientists: Scientific publications, conferences and research seminars, inter-
personal communications.

• Weight-loss industry: such as Lighterlife, who are key stakeholders as funders of the 
work, in informing outreach activity, lay publications and training of program facilitators.
References


80. Catenacci V, Pan Z, Ostendorf D, et al. (2016) A randomized pilot study comparing zero-
calorie alternate-day fasting to daily caloric restriction in adults with obesity. *Obesity*. 24, 1874–1883.


Appendix A. List of Materials

Apparatus, equipment and analysis software
- BOC calibration gases (BOC, Guildford, UK)
- Cannula, BD Venflon (Becton infusion therapy, Helsingborg, Sweden)
- Cobas MIRA (Roche Diagnostics, Massachusetts, US)
- Cryobox (Starlab, Hamburg, Germany)
- Diet Plan 6 (Forestfield Software, Horsham, UK)
- Diet Plan 7 (Forestfield Software, Horsham, UK)
- Gamma counter-Wizard 1470 Multicalc Level 4 Software (Wallac Int, Turku, Finland)
- GEM ISGEM319 (GEM Nutrition, Cheshire, UK)
- ILAB 650 (Instrumentation Laboratory, Milan, Italy)
- Microtubes (Alphalab, Eastleigh, UK)
- Potassium EDTA tubes (Teklab, County Durham, UK)
- Sodium oxalate tubes (Teklab, County Durham, UK)
- SPSS version 22 (IBM, Chicago, USA)
- SPSS version 23 (IBM, Chicago, USA)
- Stadiometer - Seca (Seca, Birmingham, UK)
- Stretch-resistant measuring tape (Prym, Stolberg, Germany)
- Tanita MC180A bioimpedance segmental monitor (Tanita Corp, Tokyo, Japan)
- Tanita BC420MA bioimpedance segmental monitor (Tanita Corp, Tokyo, Japan)

Chemicals, re-agents and assay kits
- Aprotinin from bovine lung lyophilized powder 3-8 TIU/mg solid (Ref- A1153, Sigma Chemical Company Limited, Poole, UK)
- Instrumental laboratory kits (Instrumental laboratory Milan, Italy)
  - Glucose oxidase (Kit ref - PN0018250840)
  - TAG (Kit ref – PN0018255640)
  - Total cholesterol (Kit ref - PN0018250540)
  - HDL cholesterol (Kit ref - PN0018255740)
- Merck Millipore Kits (Merck, Massachusetts, US)
  - Human Insulin specific radioimmunoassay (Kit ref – HI-14K)
  - Human Insulin Enzyme-linked immunosorbent assay (Kit ref – EZHI-14K)
  - Human C-peptide radioimmunoassay (Kit ref - HCP–20K)
- Randox kits (Randox Laboratories Ltd, County Antrim, UK)
  - 3-hydroxybutyrate (Kit ref - RB1007)
Non-esterified fatty acids (Kit ref – FA115)

Food

- Alpen Swiss muesli original (Weetabix, Kettering, UK)
- Celebrations chocolate flavours (Mars Food UK, Dublin, Ireland)
- Danone Activia Yogurt 125g pot – Strawberry (Danone UK, Trowbridge, UK)
- Dolmio pasta pot – assortment of flavours (Mars Food UK, Dublin, Ireland)
- Every day value chilli con carne sauce (Tesco stores, Welwyn Garden City, UK)
- Every day value long grain rice (Tesco stores, Welwyn Garden City, UK)
- Every day value orange juice carton 200ml (Tesco)
- Every day value salted peanuts (Tesco stores, Welwyn Garden City, UK)
- Extra lean mince (Tesco stores, Welwyn Garden City, UK)
- Fortisip Chocolate 200ml (Nutricia, Trowbridge, UK)
- Fusilli Pasta twists (Tesco stores, Welwyn Garden City, UK)
- LighterLife Food Pack range for very low energy diets – assortment of flavours (Lighterlife, Essex, UK)
- Italian tomato and mozzarella pasta bake (Tesco stores, Welwyn Garden City, UK)
- Mild cheddar cheese (Tesco stores, Welwyn Garden City, UK)
- Onion and garlic pasta sauce (Tesco stores, Welwyn Garden City, UK)
- Pure vegetable oil (Tesco stores, Welwyn Garden City, UK)
- Whole milk (Tesco stores, Welwyn Garden City, UK)
Appendix B. Food Diary

Example page (1/2) from the food diary given to participants.

Example diary page

What did you eat?

Date _______2/3/2010____ Day of the Week ___________ Tuesday ___________

<table>
<thead>
<tr>
<th>Time of day</th>
<th>Food &amp; Drink (Please describe in detail, including brand names where appropriate)</th>
<th>Amount Eaten</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.00am</td>
<td>Museli - Sainsbury's - no added sugar</td>
<td>1/2 bowl</td>
</tr>
<tr>
<td></td>
<td>Semi skimmed milk</td>
<td>200ml</td>
</tr>
<tr>
<td></td>
<td>Brown sugar</td>
<td>1 heaped tsp</td>
</tr>
<tr>
<td></td>
<td>Toast - Granary bread - thick sliced</td>
<td>2 slices</td>
</tr>
<tr>
<td></td>
<td>margarine (Floral)</td>
<td>thinly spread</td>
</tr>
<tr>
<td></td>
<td>honey</td>
<td>1 tablespoon</td>
</tr>
<tr>
<td></td>
<td>tea</td>
<td>1 mug</td>
</tr>
<tr>
<td></td>
<td>semi skimmed milk (in tea)</td>
<td>Splash - 1 tblsp</td>
</tr>
<tr>
<td></td>
<td>white sugar (in tea)</td>
<td>1 heaped tsp</td>
</tr>
<tr>
<td>10.30am</td>
<td>Coffee with whole milk (in coffee)</td>
<td>1 mug</td>
</tr>
<tr>
<td></td>
<td>packet of crisps (Salt and Vinegar - Walkers)</td>
<td>34.5g</td>
</tr>
<tr>
<td>1.00pm</td>
<td>Lunch from canteen: cheese sandwich (white bread)</td>
<td>2 medium slices</td>
</tr>
<tr>
<td></td>
<td>margarine (brand unknown)</td>
<td>thick spread</td>
</tr>
<tr>
<td></td>
<td>Cheddar cheese</td>
<td>4 slices</td>
</tr>
<tr>
<td></td>
<td>cucumber</td>
<td>6 slices</td>
</tr>
<tr>
<td></td>
<td>Blackberry and apple pie</td>
<td>large slice</td>
</tr>
<tr>
<td></td>
<td>Custard</td>
<td>3/4 bowl</td>
</tr>
<tr>
<td></td>
<td>orange juice</td>
<td>250ml</td>
</tr>
<tr>
<td>5.30pm</td>
<td>Tea with whole milk &amp; white sugar</td>
<td>1 mug (as before)</td>
</tr>
<tr>
<td></td>
<td>Twix</td>
<td>2 finger</td>
</tr>
<tr>
<td>6.00pm</td>
<td>Tea with whole milk &amp; white sugar</td>
<td>Mug (as before)</td>
</tr>
<tr>
<td></td>
<td>Boiled brown rice</td>
<td>large (1/4 plate)</td>
</tr>
<tr>
<td></td>
<td>chicken curry (see recipe)</td>
<td>large (1/4 recipe)</td>
</tr>
<tr>
<td></td>
<td>mineral water (Sainsbury's)</td>
<td>1/2 pint</td>
</tr>
<tr>
<td></td>
<td>Strawberry yoghurt (Sainsbury's low fat)</td>
<td>150g</td>
</tr>
<tr>
<td>10.30pm</td>
<td>Lager (brand unknown)</td>
<td>330ml bottle</td>
</tr>
</tbody>
</table>

Notes/comments/recipes:

Chicken curry - 70g chicken, 1 med onion, 1 tblsp polyunsaturated margarine, 1 tblsp curry powder, 3/4 pint chicken stock, 1 tblsp mango chutney, 3 tblsp sultanas, 1/4 pint low fat yoghurt, 1 tblsp plain flour.

Reference 144.
Example page (2/2) from the food diary given to participants.

**Food portion sizes (10 inch plate)**

<table>
<thead>
<tr>
<th>SMALL</th>
<th>MEDIUM</th>
<th>LARGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suitable for vegetable or meat stews (not meat without vegetables)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SMALL</th>
<th>MEDIUM</th>
<th>LARGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suitable for fish in breadcrumbs or batter (not for chops or steaks)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SMALL</th>
<th>MEDIUM</th>
<th>LARGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suitable for chips only</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SMALL</th>
<th>MEDIUM</th>
<th>LARGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suitable for boiled rice and rice dishes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix C. Dutch eating behaviour questionnaire

Please answer the following questions as carefully and honestly as possible. Read each question and simply fill in the column which best applies to you.

<table>
<thead>
<tr>
<th>Question</th>
<th>Never</th>
<th>Seldom</th>
<th>Sometimes</th>
<th>Often</th>
<th>Very often</th>
<th>Not relevant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. If you have put on weight, do you eat less than you usually do?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Do you have a desire to eat when you are irritated?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. If food tastes good to you, do you eat more than you usually do?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Do you try to eat less at meal times than you would like to eat?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Do you have a desire to eat when you have nothing to do?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Do you have a desire to eat when you are depressed or discouraged?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. If food smells and looks good, do you eat more than you usually eat?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. How often do you refuse food or drink offered because you are concerned about your weight?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. Do you have a desire to eat when you are feeling lonely?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. If you see or smell something delicious, do you have a desire to eat it?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. Do you watch exactly what you eat?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12. Do you have a desire to eat when somebody lets you down?</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>13. If you have something delicious to eat, do you eat it straight away?</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>14. Do you deliberately eat foods that are slimming?</td>
<td></td>
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</tr>
<tr>
<td>15. Do you have a desire to eat when you are cross?</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>16. Do you have a desire to eat when you are approaching something unpleasant to happen?</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>17. If you walk past the baker do you have a desire to buy something delicious?</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>18. When you have eaten too much, do you eat less than usual the following days?</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>19. Do you get a desire to eat when you are anxious, worried or tense?</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>20. If you walk past a snack bar or café, do you have a desire to buy something delicious?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21. Do you deliberately eat less in order not to become heavier?</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>22. Do you have a desire to eat when things are going against you, or things have gone wrong?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23. If you see others eating, do you have also the desire to eat?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24. How often do you try not to eat between meals because you are watching your weight?</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>25. Do you have a desire to eat when you are frightened?</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>26. Can you resist eating delicious food?</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>27. How often in the evening do you try not to eat because you are watching your weight?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28. Do you have a desire to eat when you are disappointed?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29. Do you eat more than usual when you see other eating?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30. Do you take your weight into account when you eat?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31. Do you have a desire to eat when you are emotionally upset?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32. When preparing a meal are you inclined to eat something?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33. Do you have a desire to eat when you are bored or restless?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The coloured asterisks indicate the questions relating to external eating (green), emotional eating (blue) and restrained eating (red). Reference 155.
Appendix D. Visual analogue scales

<table>
<thead>
<tr>
<th>How hungry do you feel?</th>
<th>I am not hungry at all</th>
<th>I have never been more hungry</th>
</tr>
</thead>
<tbody>
<tr>
<td>How much do you think you can eat?</td>
<td>Nothing at all</td>
<td>A lot</td>
</tr>
<tr>
<td>How full do you feel?</td>
<td>Not at all full</td>
<td>Extremely full</td>
</tr>
</tbody>
</table>

Reference 156.
Appendix E. Food portions guide

Food portions guide provided to participants in Study two (1/2). Taken from Weight-loss you can see booklet produced by Nutrition and diet resources (NDR; Glasgow, Scotland).

<table>
<thead>
<tr>
<th>Food group</th>
<th>Calorie per portion</th>
<th>Recommended portions per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bread, rice, potatoes, pasta and other starchy foods</td>
<td>80</td>
<td>8</td>
</tr>
<tr>
<td>Fruit and vegetables</td>
<td>40</td>
<td>7</td>
</tr>
<tr>
<td>Milk and dairy foods</td>
<td>90</td>
<td>3</td>
</tr>
<tr>
<td>Meat, fish, eggs, beans and other non-dairy sources of protein</td>
<td>140</td>
<td>2</td>
</tr>
<tr>
<td>Fats</td>
<td>50</td>
<td>3</td>
</tr>
<tr>
<td>Extras (sweet foods, extra portions, desserts, alcohol, crisps)</td>
<td></td>
<td>180 calories</td>
</tr>
</tbody>
</table>

After talking about her eating habits with her dietitian, Kate set herself a few realistic goals to help with her weight loss plan.

Kate’s Goals

1. Swap snacks of crisps and biscuits in the morning and afternoon for fruit.

2. Cut down on pasta serving at evening meal.

3. Swap large unmeasured glass of wine for a measured 125ml glass of wine topped up with soda water.

4. Walk for 30 minutes at lunchtime rather than sitting at desk.
Food portions guide provided to participants in Study two (2/2).

**Bread, rice, potatoes, pasta and other starchy foods: 80 calorie portions**

- Fusilli pasta, uncooked - 24g or 2 dspns; cooked - 50g or 3 dspns
- Spaghetti, uncooked - 23g; cooked - 77g
- Egg noodles, uncooked - ½ slab; cooked - 20g
- Cous cous, uncooked - 22 or 2 dspns; cooked - 50g or 3 dspns
- Potato, baked - 104g or ½ a medium potato
- Oven chips (chunky), frozen and baked - 49g or 4 large chips
- Potato boiled and mashed with milk - 135g or 3 heaped dspns
- New potatoes, boiled - 121g or 3⅓ egg-sized potatoes
- Sweet potato, boiled - 95g
- Bran flakes - 24g or 3 dspns
Appendix F.  Online questionnaire: weight record

What was your weight this morning? Please state whether you have weighed yourself in stones, pounds or kg.
Please weigh yourself in light clothing and before breakfast.

Example answer: 85 kg
Appendix G. Online questionnaire: energy restriction compliance

Over the last fortnight, on which days did you fast?

- Week 1 Monday
- Week 1 Tuesday
- Week 1 Wednesday
- Week 1 Thursday
- Week 1 Friday
- Week 1 Saturday
- Week 1 Sunday
- Week 2 Monday
- Week 2 Tuesday
- Week 2 Wednesday
- Week 2 Thursday
- Week 2 Friday
- Week 2 Saturday
- Week 2 Sunday

What did you eat on week one, fast day 1?
Example answer: 1x nut fudge bar, 1x veg soup, 1x porridge, 1x chocolate shake
If you ate something other than the food packs, or drank something other than water then please describe what you had in as much detail as possible.

...
Appendix H. Positive affect negative affect scale

The following scale consists of a number of words that describe different feelings and emotions. Read each item and then mark the appropriate answer in the space next to that word. **Indicate to what extent you have felt this way during the past week.** Use the following scale.

<table>
<thead>
<tr>
<th>1 Very slightly or not at all</th>
<th>2 A little</th>
<th>3 Moderately</th>
<th>4 Quite a bit</th>
<th>5 Extremely</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interested</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distressed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excited</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upset</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strong</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guilty</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scared</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hostile</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enthusiastic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proud</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irritable</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alert</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ashamed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inspired</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nervous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Determined</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Attentive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jittery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Afraid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Scoring instructions:**
Positive Affect Score: Add the scores on items 1, 3, 5, 9, 10, 12, 14, 16, 17, and 19. Scores can range from 10 – 50, with higher scores representing higher levels of positive affect.
Negative Affect Score: Add the scores on items 2, 4, 6, 7, 8, 11, 13, 15, 18, and 20. Scores can range from 10 – 50, with lower scores representing lower levels of negative affect. Ref 177.
Appendix I. Power of food scale

Please indicate the extent to which you agree that the following items describe you. Use the following 1-5 scale for your responses.

1. I find myself thinking about food even when I’m not physically hungry. ___
2. I get more pleasure from eating than I do from almost anything else. ___
3. If I see or smell a food I like, I get a powerful urge to have some. ___
4. When I’m around a fattening food I love, it’s hard to stop myself from at least tasting it. ___
5. It is scary to think of the power that food has over me. ___
6. When I know a delicious food is available, I can’t help myself from thinking about having some. ___
7. I love the taste of certain foods so much that I can’t avoid eating them even if they’re bad for me. ___
8. Just before I taste a favourite food, I feel intense anticipation. ___
9. When I eat delicious food I focus a lot on how good it tastes. ___
10. Sometimes, when I’m doing everyday activities, I get an urge to eat “out of the blue” (for no apparent reason). ___
11. I think I enjoy eating a lot more than most other people. ___
12. Hearing someone describe a great meal makes me really want to have something to eat. ___
13. It seems like I have food on my mind a lot. ___
14. It is very important to me that the foods I eat are as delicious as possible. ___
15. Before I eat a favourite food my mouth tends to flood with saliva. ___

Scoring instructions:
Factor 1 (Food Available) = Average of items 1, 2, 5, 10, 11 and 13
Factor 2 (Food Present) = Average of items 3, 4, 6, and 7
Factor 3 (Food Tasted) = Average of items 8, 9, 12, 14, and 15

Reference 178.
**Appendix J. Self-efficacy questionnaire**

Please indicate on the below scale how confident you are in your ability to be able to control your own weight.

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not at all confident</td>
<td>Slightly confident</td>
<td>Reasonably confident</td>
<td>Very confident</td>
<td>Extremely confident</td>
</tr>
</tbody>
</table>

Please indicate on the below scale how confident you are in yourself generally.

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not at all confident</td>
<td>Slightly confident</td>
<td>Reasonably confident</td>
<td>Very confident</td>
<td>Extremely confident</td>
</tr>
</tbody>
</table>
Appendix K. Pittsburgh sleep quality index

Instructions: the following questions relate to your usual sleep habits during the past month only. Your answers should indicate the most accurate reply for the majority of days and nights in the past month. **Please answer all questions.**

1. During the past month, when have you usually gone to bed at night?
   Usual bed time: ………

2. During the past month, how long (in minutes) has it usually taken you to fall asleep each night?
   Number of minutes: ………

3. During the past month, when have you usually gotten up in the morning?
   Usual getting up time: ………

4. During the past month, how many hours of actual sleep did you get at night?
   Hours of sleep per night: ………

For the remaining questions, circle the one best response. **Please answer ALL questions.**

5. During the past month, how often have you had trouble sleeping because you…..

   (a) **Cannot get to sleep within 15 minutes**
      Not during the past month  Less than once a week  Once or twice a week  Three or more times a week
   (b) **Wake up in the middle of the night or early morning**
      Not during the past month  Less than once a week  Once or twice a week  Three or more times a week
   (c) **Have to get up to use the bathroom**
      Not during the past month  Less than once a week  Once or twice a week  Three or more times a week
   (d) **Cannot breathe properly**
      Not during the past month  Less than once a week  Once or twice a week  Three or more times a week
   (e) **Cough or snore loudly**
      Not during the past month  Less than once a week  Once or twice a week  Three or more times a week
   (f) **Feel too cold**
      Not during the past month  Less than once a week  Once or twice a week  Three or more times a week
   (g) **Feel too hot**
      Not during the past month  Less than once a week  Once or twice a week  Three or more times a week
   (h) **Had bad dreams**
      Not during the past month  Less than once a week  Once or twice a week  Three or more times a week
(i) Have pain
Not during the past month
Less than once a week
Once or twice a week
Three or more times a week

(j) Other reason(s), please describe
Not during the past month
Less than once a week
Once or twice a week
Three or more times a week

How often during the past month have you had trouble sleeping because of this?
Not during the past month
Less than once a week
Once or twice a week
Three or more times a week

6. During the past month how would you rate your sleep quality overall
Very good
Fairly good
Fairly bad
Very bad

7. During the past month, how often have you taken medicine to help you sleep?
Not during the past month
Less than once a week
Once or twice a week
Three or more times a week

8. During the past month, how often have you had trouble staying awake while driving, eating meals or engaging in social activity?
Not during the past month
Less than once a week
Once or twice a week
Three or more times a week

9. During the past month, how much of a problem has it been for you to sleep up enough enthusiasm to get things done?
Not a problem
Only a very slight problem
Somewhat of a problem
A very big problem

Scoring instructions:
Component 1: Subjective sleep quality
Examine question 6 and assign a score as follows
Very good – 0
Fairly good – 1
Fairly bad – 2
Very bad – 3

Component 2: Sleep latency
Examine question 2 and assign a score as follows
≤ 15 minutes – 0
16 – 30 minutes – 1
31 – 60 minutes – 2
> 60 minutes – 3

Examine question 5a and assign a score as follows
Not during the past month – 0
Less than once a week – 1
Once or twice a week – 2
Three or more times a week – 3
Add question 2 and 5a scores together
Assign component 2 score as follows
0 – 0
1-2 – 1
3-4 – 2
5-6 – 3

Component 3: Sleep duration
Examine question 4 and assign a score as follows
>7 hours – 0
6-7 hours – 1
5-6 hours – 2
<5 hours – 3

Component 4: Habitual sleep efficiency (HSE)
HSE = number of hours slept / number of hours spent in bed x 100

Examine question 4 and assign a score as follows
HSE (%) =
>85% – 0
75 – 84% – 1
65 – 74% – 2
<64% – 3

Component 5: Sleep disturbances
Examine questions 5b to j and assign scores for each question as follows
Not during the past month – 0
Less than once a week – 1
Once or twice a week – 2
Three or more times a week – 3

Add scores for questions 5b-j together
Assign component 5 score as follows
0 – 0
1 – 9 – 1
10 – 18 – 2
19 – 27 – 3

Component 6: Sleep medications
Examine question 7 and assign a score as follows
Not during the past month – 0
Less than once a week – 1
Once or twice a week – 2
Three or more times a week – 3

Component 7: Daytime dysfunction
Examine question 8 and assign a score as follows
Not during the past month – 0
Once or twice – 1
Once or twice a week – 2
Three or more times a week – 3

Examine question 9 and assign a score as follows
No problem at all – 0
Only a very slight problem – 1
Somewhat of a problem – 2
A very big problem – 3
*Add the scores for questions 8 and 9 together*
*Assign component 7 score as follows*

0 – 0  
1 – 2 – 1  
3 – 4 – 2  
5 – 6 – 3

**Global PSQI score:**  
Add seven score components together to obtain global score.

Reference 179.
Appendix L. Epworth sleepiness scale

How likely are you to doze off or fall asleep in the following situations, in contrast to just feeling tired? This refers to your usual way of life in recent times. Even if you haven't done some of these things recently, try to work out how they would have affected you. Use the following scale to choose the most appropriate number for each situation:

0 = Would never doze  
1 = Slight chance of dozing  
2 = Moderate chance of dozing  
3 = High chance of dozing

<table>
<thead>
<tr>
<th>Situation</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sitting and reading</td>
<td></td>
</tr>
<tr>
<td>Watching TV</td>
<td></td>
</tr>
<tr>
<td>Sitting inactive in a public place (e.g. a theatre or meeting)</td>
<td></td>
</tr>
<tr>
<td>As a passenger in a car for an hour without a break</td>
<td></td>
</tr>
<tr>
<td>Lying down to rest in the afternoon when circumstances permit</td>
<td></td>
</tr>
<tr>
<td>Sitting and talking to someone</td>
<td></td>
</tr>
<tr>
<td>Sitting quietly after lunch without alcohol</td>
<td></td>
</tr>
<tr>
<td>In a car, while stopped for a few minutes in traffic</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
</tr>
</tbody>
</table>

Scoring instructions
The ESS is made up of 8 questions which the subject is required to answer by rating each item on a scale of 0 to 3. When scoring the test, the sum of all the responses to each article gives the overall ESS score. A score of 0-7 is considered to be within the normal range. A score of 8-14 is suggestive of mild to moderate symptoms of daytime tiredness, and a score of 15-24 is suggestive of significant (severe) symptoms of daytime tiredness.

Reference 180.