Investigations of the Mechanisms of Diabetes Remission: A Focus on Adaptive Thermogenesis

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Abstract

Background:
The prevalence of diabetes and obesity have continued to rise over the last 30 years. In New Zealand one in three adults have obesity and one in twenty have type 2 diabetes mellitus (T2DM). Thus, there is an urgent need to implement effective and scalable treatments for both diabetes and obesity. Bariatric surgery and low-calorie diets produce weight loss and reverse the disordered glucose metabolism associated with T2DM. Both treatments create a state of negative energy balance in which the individual’s dietary energy intake is lower than their energy expenditure. However, maintaining a negative energy balance with low-calorie diets is challenging. This is partly due to a compensatory reduction in resting energy expenditure (REE), adaptive thermogenesis, that occurs during weight loss and is triggered by a period of sustained negative energy balance. Adaptive thermogenesis diminishes this negative energy balance, undermining weight loss. A better understanding of what influences adaptive thermogenesis and ways to mitigate it may inform more effective dietary interventions for the treatment of diabetes and obesity.

Objectives:
The overall aim of this thesis was to test the hypothesis that interrupting a low-calorie diet using an intermittent fast reduces adaptive thermogenesis during weight loss.

To test this hypothesis, we first needed to develop an accurate and precise method for measuring REE using indirect calorimetry. Secondly, we needed to assess the relationship between REE and body composition in a local sample population. Thirdly, we needed to assess the safety of intermittent fasting in individuals with T2DM at risk of hypoglycaemia.

Design:
In-silico validity and precision testing of indirect calorimetry was conducted using ethanol combustion and nitrogen dilution. Having determined the quality of in-silico calorimetry measurements, the test-retest reliability was assessed in two repeated measures studies of REE in human participants.
To assess the relationship between body composition and REE, a cross-sectional study of body composition and REE using indirect calorimetry was conducted, the Predictions of Resting Energy Expenditure in Māori and Pacific (PREEMPt) study. Next, the safety of intermittent fasting in individuals with T2DM was assessed in a 12-week prospective randomised control trial.

Having completed these studies, the Changes in Resting Energy Expenditure with Different Schedules of Calorie Restriction (CREEDS) study was conducted to address the primary hypothesis that intermittent fasting attenuates adaptive thermogenesis during weight loss.

Results:

Following protocol refinement, high levels of precision and accuracy were observed during the nitrogen dilution studies and the second test-retest reliability study.

The assessments of body composition and resting energy expenditure in the PREEMPt study were used to estimate the sample size required for the CREEDS study. We found that intermittent fasting increased the risk of hypoglycaemia on fasting days, this finding informed the design of the CREEDS study.

In the CREEDS study, both intermittent fasting and continuous daily restriction resulted in adaptive thermogenesis, that was not mitigated by the intermittent fasting intervention. Of note, intermittent fasting was associated with a two-fold greater reduction in fat mass compared to continuous daily restriction.

Conclusions

Intermittent fasting does not mitigate adaptive thermogenesis seen during weight loss with a low-calorie diet.
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List of Abbreviations

ADF: Alternate Day Fasting
AEBQ: Adult Eating Behaviour Questionnaire
ALP: Alkaline Phosphatase
ALT: Alaine Transaminase
ANOVA: Analysis of Variance
AST: Aspartate Transaminase
BIA: Bio-impedance Analysis
BMI: Body Mass Index
BP: Barometric Pressure
BPD: Biliopancreatic Diversion
CDR: Continuous Daily Restriction
CI: Confidence Interval
CO$_2$: Carbon Dioxide
CREEDS: The Changes in Resting Energy Expenditure with Different Schedules of Calorie Restriction.
DLW: Doubly-Labelled Water
DSP: Digital Signal Processing
DXA: Dual Energy X-ray Absorptiometry
EtOH: Ethanol
FeCO$_2$: Fractional concentration of Excurrent Carbon Dioxide
FeN$_2$: Fractional concentration of Excurrent Nitrogen.
**FeO2**: Fractional concentration of Excurrent Oxygen

**FFM**: Fat Free Mass

**FiCO2**: Fractional concentration of Incurrent Carbon Dioxide.

**FiN2**: Fractional concentration of Incurrent Nitrogen.

**FiO2**: Fractional concentration of Incurrent Oxygen

**FkN2**: Flow rate of the nitrogen validation gas.

**FM**: Fat Mass

**FR**: Flow Rate

**FRe**: Flow rate of excurrent air

**FRI**: Flow rate of incurrent air.

**FRN2**: Flow rate of nitrogen.

**FT3**: Free Tri-iodothyronine

**FT4**: Free Tetra-iodothyronine

**GGT**: Gamma- Glutamyl Transferase

**GLP-1**: Glucagon Like Peptide-1

**H2O**: Water

**HbA1C**: Glycated Haemoglobin

**HDL**: High Density Lipoprotein

**HOMA-IR**: Homeostatic Model Assessment of Insulin Resistance

**ICC**: Intra-class Correlation Coefficient

**IF**: Intermittent Fasting

**IF-Hypo Study**: The Intermittent Fasting and Hypoglycaemia Study
**IPAQ**: International Physical Activity Questionnaire.

**kPA**: Kilopascals

**LDL**: Low Density Lipoprotein

**LGB**: Laparoscopic Gastric Banding

**LSC**: Least Significant Change

**LSG**: Laparoscopic Sleeve Gastrectomy

**M**: Mean

**MET**: Metabolic Equivalent

**N₂**: Nitrogen

**NHANES**: National Health and Nutrition Examination Survey

**O₂**: Oxygen

**OR**: Odds ratio

**PAL**: Physical Activity Level:

**PREEMPt Study**: Prediction of Resting Energy Expenditure in Maori and Pacific Study

**PYY**: Peptide YY

**REE**: Resting Energy Expenditure

**RMSE**: Root Mean Squared Error

**RYGB**: Roux-en-y Gastric Bypass

**SD**: Standard Deviation.

**SEE**: Standard Error of the Estimate

**SEM**: Standard Error of the Mean

**STP**: Standard Temperature and Pressure
**T2DM**: Type 2 Diabetes

**TSH**: Thyroid Stimulating Hormone

**VBG**: Vertical Banded Gastroplasty

**VLCD**: Very Low-Calorie Diet

**WHO**: World Health Organisation

**WVP**: Water Vapour Pressure
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1 Introduction

1.1 Overview and Thesis Aims

In the past 50 years, there has been a global rise in overweight, obesity and associated obesity-related co-morbidities such as Type 2 Diabetes (T2DM) (2, 3). In New Zealand in 2015/2016, 31.6% of all individuals over the age of 15 years were obese, compared to 26.5% only five years previously, while 66.8% of all individuals were classed as either overweight or obese. Overweight and obesity are more prevalent in New Zealand Māori and Pacific ethnicities with rates of 78% and 89% respectively (4). The net result is increasing morbidity and mortality from obesity related complications and an associated increase in healthcare spending (5), (6), (7).

Of the many complications of obesity, T2DM is one of the most important. Diabetes is associated with reduced life expectancy, poorer quality of life and considerable morbidity (8). The economic cost of T2DM is substantial, and accounted for an estimated $176 billion US dollars in 2012 (9). Between 2010 and 2017 the number of individuals with diabetes registered on the New Zealand Virtual Diabetes Registry increased from 187,860 to 245,680 (10). Worldwide the global burden of T2DM is 422 million, nearly a quadrupling of the diabetes prevalence over the last 36 years. By 2030, it is estimated that diabetes will be the 7th leading cause of death (8).

Weight loss through calorie restriction can be successfully used to induce diabetes remission and treat obesity (11). Unfortunately, successful weight loss can be difficult, and weight regain following a period of weight loss is common. Therefore, the question of how best we can implement weight loss and lifestyle modifications that result in durable resolution of both obesity and T2DM, is urgent and important. Weight loss requires a sustained negative energy balance; understanding the determinants of energy balance, and factors which influence these determinants, is central to developing a better understanding of how to achieve this goal.

Therefore, the focus of this thesis is the study of human energy balance during weight loss. This introductory chapter begins with the physiology of energy balance and the aetiology of obesity and T2DM. Diet and surgical treatments that are proven to induce
remission of diabetes and successful weight loss are then discussed. The chapter concludes with a focus on the effects of diet interventions that alter schedules of energy intake. In the context of this thesis, ‘schedule’ refers to the temporal pattern of a prescribed calorie restriction.

1.2 The Physiology of Energy Homeostasis

‘Energy homeostasis’ describes biological processes that match dietary energy intake to energy expenditure (12). The goal of energy homeostasis is balance, an equal matching of dietary intake to energy expenditure (13). Conversely if energy homeostasis is not maintained, weight loss or weight gain will result. The use of the term energy homeostasis implies active regulation of body weight. An oft-cited argument for homeostatic control mechanisms is the observation that most individuals’ weight remains stable over prolonged periods despite variation in the myriad determinants of energy balance (14).

To achieve energy balance dietary intake must be matched to the sum of the components of energy expenditure, or the total energy expenditure. These components are resting energy expenditure (REE), sleeping energy expenditure, physical activity energy expenditure and diet induced thermogenesis. Resting energy expenditure, also known as resting metabolic rate, may be defined as the amount of energy used by an organism at rest while in a state of complete muscular relaxation and awake. It is the largest component of energy expenditure, accounting for 50-75% of total energy expenditure. It differs from sleeping energy expenditure by about 5-10%, the energy cost of the awake state. If taken while fasting, REE is analogous to basal metabolic rate (15). All measurements of REE in this thesis are done while fasted. Physical activity energy expenditure usually accounts for 15-40% of total energy expenditure. Physical activity energy expenditure can be further subdivided into voluntary exercise energy expenditure and non-exercise activity thermogenesis. In many developed countries physical activity accounts for only a small proportion of total energy expenditure. Non-exercise activity thermogenesis is the energy expenditure of all occupation, leisure, sitting, standing and ambulation. Non-exercise activity thermogenesis is highly variable from person to person and may account for 15-50% of total energy expenditure in individuals with very
physically active jobs (16), (17). Diet-induced thermogenesis is the increase in energy expenditure that occurs after a meal. Diet-induced thermogenesis is also known as the specific dynamic activity or the thermogenic effect of feeding, and accounts for approximately 10% of total energy expenditure (18). The main value in breaking down energy balance in this way is that each component has a definition that allows them to be measured and summed. In this way the relative contribution of each component to a state of energy excess or deficiency can be determined experimentally.

The partition of total energy expenditure in this way is useful for understanding the concept of energy homeostasis but does not offer adequate insight into how such homeostasis may be achieved. For this, a more detailed breakdown of the energy balance equation is required, that relates the components of energy balance to biological mechanisms of energy homeostasis. A proposed schema to consider energy homeostasis is presented in Fig. 1.1.

**Figure 1.1. Schema of Energy Homeostasis**
In this schema, factors that influence dietary intake and dietary energy expenditure interact with each other to produce a state of energy homeostasis. It is unlikely that this state is regulated minute to minute, but day to day, or possibly over the course of weeks, in keeping with observational studies of weight fluctuation in healthy individuals (19). Which physiological systems are involved in energy homeostasis, where they are located, how they interrelate, and what the relative importance of each system is in regulating components of energy balance, are important questions that have only been partially answered.

1.2.1 The Organisation of Energy Homoeostasis

While all tissues require energy, the key players in the regulation of energy homeostasis are; the central and peripheral nervous systems, the gastrointestinal tract, pancreas and liver, white adipose tissue, brown adipose tissue, skeletal muscle, smooth muscle, the lungs and heart (20). The peripheral nervous system and endocrine systems relay signals to and from these organs.

1.2.1.1 The Nervous System

The organisation of energy homeostasis in the central nervous system is comprised of anatomically and functionally related centres (Fig. 1.2)*. These centres are interconnected by inhibitory or excitatory neurons and receive feedback from outside the brain via sensory neurons or hormones that cross the blood brain barrier. Multiple inputs from the periphery and central nervous system are integrated and deliver a response directly or indirectly via efferent neurons (21-23).

The leptin-melanocortin pathway is one of the more completely understood pathways regulating components of energy balance (24). Following feeding, the hormone insulin is released from the pancreas and promotes leptin release from white adipocytes (25). Leptin travels via the blood stream to the blood brain barrier and is transported into the cerebrospinal fluid surrounding the brain (26, 27). From here it binds to pro-opiomelanocortin (POMC) and Neuropeptide-Y/Agouti Related Peptide (NPY/AgRP) neurons in the arcuate nucleus of the hypothalamus (Fig. 1.2). The two sets of neurons have opposite effects on appetite. POMC neurons are anorexigenic, reducing hunger. POMC is cleaved to alpha- and beta-melanocyte stimulating hormone by prohormone convertase 1 (28). These cleaved products bind melanocortin receptors in the paraventricular nucleus of the hypothalamus. This activation results in a decrease in feeding (29). Conversely, NPY/AgRP neurons are orexigenic, hunger inducing.

**NPY** = neuropeptide Y; **Pomc** = proopiomelanocortin; **AgRP** = Agouti gene-related peptide; **Arc** = arcuate nucleus; **GLP-1** = glucagon-like peptide 1. Reproduced with permission from Gautron et al.

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**Figure 1.2 Central Pathways involved in Energy Homeostasis.**
NPY/AgRP antagonises the POMC-melanocortin pathway leading to inhibition of the melanocortin receptors and an increase in feeding (22). Leptin exerts opposite effects on each system; stimulating POMC neurons and inhibiting the activity of NPY/AgRP neurons, resulting in a net inhibition of feeding.

The leptin-melanocortin system is but one example of how the central nervous system co-ordinates components of energy homeostasis, in this case appetite. Other neural pathways outside of the hypothalamus that affect components of energy regulation have been described, but a detailed discussion of these is beyond the scope of this thesis. The sensory cortex conveys food-related sensory input. The functionally-defined hedonic areas of the brain; the central amygdala, ventral tegmental area, orbitofrontal cortex, insular cortex and ventral striatum play a role in food reward and are thought to be closely intertwined with regulation of appetitive behaviour (30),(31). The spinal cord mediates parasympathetic input from the gastrointestinal tract after feeding, decreases brown adipose tissue activity (section 1.2.1.3.2) and alters resting metabolic rate via the sympathetic nervous system (23, 32),(33).

1.2.1.2 The Gastrointestinal Tract

Sitting at the entry point of dietary nutrients into the body, the gastrointestinal tract contains endocrine cells and afferent sensory nerves that relay information on nutrient intake centrally to influence meal termination and satiety. Hormones such as ghrelin, peptide YY (PYY), insulin and glucagon-like-peptide -1 (GLP-1) are released into the bloodstream or lumen of the gastrointestinal tract in response to nutrient stimulation and regulate several aspects of short term energy intake such as hunger and satiety. Though the gastrointestinal tract is home to many hormones, I will focus on those that appear to play a predominant role in energy homeostasis – the orexigenic hormone ghrelin, and the anorexigenic hormones PYY, and GLP-1.

Ghrelin

Ghrelin is a gut peptide secreted in an inactive, acylated form from the mucosa of the stomach (34). De-acylated ghrelin has an orexigenic effect when acting on the arcuate nucleus and other hypothalamic nuclei (35). It also activates hedonic or ‘pleasure/reward’ responses to food by acting on the mesolimbic pathway, a key anatomical region in the
hedonic response to eating. Ghrelin levels rise with the duration of fasting and are suppressed following a meal, in proportion to the amount of calories consumed (36, 37). This suggests a role for ghrelin as a time-dependant regulator of energy intake that prevents negative calorie balance. This observation is supported by rodent and human studies demonstrating an increase in food intake with ghrelin administration and an increase in ghrelin during calorie restriction for weight loss in humans (38),(39),(40).

**GLP-1**

GLP-1 is produced by the L-cells of the small intestine in response to nutrient ingestion (41). GLP-1 enhances insulin secretion in response to an oral glucose load, known as the incretin effect, and is used in the treatment of T2DM. In addition to GLP-1’s effects on glucose homeostasis, it reduces feeding by acting on hedonic pathways and increases satiety by enhancing satiety signals conducted by the vagus nerve from stretch receptors in the stomach (42). In rodents, GLP-1 reduces feeding by decreasing gene expression of the orexigenic neurotransmitters NPY and AgRP in the arcuate nucleus during fasting (43). In humans, endogenous GLP-1 levels are associated with activation of hedonic reward pathways during fasting (44). Exogenous GLP-1 administration is associated with increased post-prandial satiety and decreased food intake and hunger (45).

**Peptide-YY**

Like GLP-1, PYY is also released from the L cells of the small intestine. Its active and most abundant metabolite, PYY 3-36 has been shown to be a regulator of food intake, producing both orexigenic and anorexigenic signals in mice when centrally administered. Despite this duality of function Pyy knockout mice exhibit decreased satiety and express an obese phenotype that was reversed by administration of PYY, suggesting a dominant role for PYY as a mediator of satiety (46). When infused in humans, PYY reduces caloric intake and increases satiety, changes that are associated with increased activity in the hedonic reward pathways in the brain (31). PYY levels are increased following bariatric surgery and may contribute to the beneficial weight loss seen following the procedure (47, 48).
Insulin

Insulin is secreted from the pancreas, primarily in response to glucose and sympathetic signals to the pancreas (49). Insulin has a direct effect on the POMC neurons of the arcuate nucleus of the hypothalamus stimulating appetite suppression, though its primary role is in glucose homeostasis rather than overall weight maintenance. Central nervous system administration of insulin acutely suppresses appetite (50). Insulin works in concert with other centrally acting regulators of energy intake. For example, central lesions that disrupt leptin signalling and insulin signalling produce greater metabolic derangements when they occur together compared with either individual lesions alone (51). In rat models, intracerebral insulin suppresses appetite and increases REE, though these results are limited by the non-physiologic route of insulin delivery (52), (53). In the human brain, insulin acts in the hypothalamus, prefrontal cortex and occipital regions (54). It is not yet clear how obesity alters the central activity of insulin, and what its overall effect on energy homeostasis might be.

Glucose

The other class of messengers emanating from the gastrointestinal tract are nutrients. Though this discussion is focused chiefly on energy homeostasis rather than lipid, glucose or protein homeostasis, there is evidence that nutrients may act directly to modulate aspects of energy metabolism. Arguably, given its critical role in brain metabolism glucose is amongst the best studied of these. Ambient levels of glucose in the brain alter neuron firing rates in a positive, glucose excitatory, or negative, glucose-inhibitory, fashion. Many anatomical regions critical to energy homeostasis contain these glucose sensing neurons, though it remains unclear what role glucose plays in central regulation of energy homeostasis (55). Glucose has also been shown to directly suppress appetite via the arcuate nucleus in rodent models though this finding has been contradicted by several studies (56), (57).

1.2.1.3 Adipose Tissue

Adipose is both an energy storage tissue and a functionally active and adaptable endocrine tissue. Excess adiposity is the hallmark of obesity and is associated with an increased risk of impaired glucose tolerance and T2DM. Conversely, loss of fat mass
(FM) is associated with improvements in glycaemia. Adipose tissue may be white, brown or beige. Human fat stores are composed chiefly of white adipose tissue though depots of brown adipose tissue are present (58). The difference between these adipose tissue types is the quantity of uncoupling protein -1. Uncoupling protein-1 is a protein expressed on the inner mitochondrial membrane that “uncouples” substrate oxidation from ATP synthesis. Thus, rather than ATP production, energy is dissipated as heat (59, 60).

**White Adipose Tissue**

There is robust evidence for the role of white adipose tissue in energy homeostasis. Volumetrically white adipose tissue constitutes the majority of “fat mass”. Fat mass correlates with REE at weight neutrality, during weight loss, and overfeeding (61, 62). This is due to the metabolic activity of adipose tissue. Adipose tissue also secretes chemical messengers, ‘adipokines’, that act in an autocrine, paracrine or endocrine fashion (63). While over 600 adipokines have now been discovered, of these leptin and adiponectin have been the best described.

Adiponectin is a 244-amino acid, 30 kDA protein secreted exclusively from adipose tissue. It was first discovered twenty years ago in mice and was soon isolated in human plasma (64), (65). Adiponectin causes adipocyte differentiation in vivo, but it also regulates ectopic fat accumulation in the liver and beta oxidation of fatty acids in both skeletal muscle and liver (64), (66), (67). When injected intravenously adiponectin is detectable in cerebrospinal fluid. When injected into the ventricular cerebrospinal fluid of mice, adiponectin reduced weight in a dose-dependent manner over three to four days without altering energy intake. This suggests a central action of adiponectin on the regulation of energy expenditure (68). Adiponectin knockout mice models are unable to maintain their body temperature in cold environments, suggesting a central role in cold-induced energy expenditure (69). Based chiefly on these animal models, adiponectin appears to be important in adipose-brain cross talk and energy expenditure regulation. Appropriately controlled studies in mice and humans will be needed to confirm these findings.

Leptin, discovered in 1994, is the prototypic adipokine. The discovery of leptin was central to the development of an integrated view of whole body energy homeostasis, the
discovery of the melanocortin pathway and the characterisation of several leptin related disorders (70). Leptin is a 167-amino acid protein produced mainly by white adipose tissue. Its circulating levels reflect the size of individual adipocytes (71). Leptin binds to receptors located in several sites in the brain to regulate adiposity and weight gain. Leptin reduces appetite and feeding behaviour and increases in energy expenditure as a part of the leptin-melanocortin pathway (section 1.2.1.1) (24). In studies of lean participants with low levels of leptin, the administration of leptin is effective in inducing satiety (72). Conversely, in the weight-stable obese, circulating levels of leptin are typically high and leptin administration is not effective (73, 74). This is thought to be due to the development of leptin resistance in the obese. Current approaches to leptin based treatments focus on the potential to enhance leptin sensitivity using a combination of leptin and a leptin sensitizer (75). In the setting of weight loss, leptin may reduce adaptive thermogenesis normally seen in response to hypocaloric diets (76).

*Brown and Beige Adipose Tissue*

Brown adipose tissue was first discovered in non-human mammals and is important for adaptation to cold environments by facilitating heat production. In mice, control of brown and white adipose tissue thermogenesis is mediated by the sympathetic nervous system. This in turn is subject to regulation by the hypothalamus in response to hormones such as leptin, ghrelin, neuropeptide Y and insulin, forming a complete feedback loop (Fig. 1.2) (77). Brown adipose tissue was identified in humans in 1986. Until recently it was thought to be important in non-shivering thermogenesis in infants, becoming inactive or vestigial by adulthood (78). In 2007, metabolically active brown adipose tissue depots were discovered in humans, and raised the possibility that brown adipose tissue may be important to energy homeostasis in adults (79).

One of the chief arguments against a primary role of brown adipose tissue in energy homeostasis in human obesity is the very small volume of brown adipose tissue compared with total FM. In one study brown adipose tissue mass measured in five healthy individuals, were less than 67g (80). This represented less than 0.5% of total FM. Although brown adipose tissue is more metabolically active than white adipose tissue the maximum estimated contribution to TEE is in the order of 523kJ per day (81).
Furthermore, in overfeeding studies in humans, brown adipose tissue was not activated again arguing against a primary role in energy homeostasis (82).

Beige adipose tissue, which can be induced to become more “brown-like”, offers the possibility of a therapeutic role for ‘browning agents’ in the treatment of obesity (83, 84). These agents, could increase passive energy consumption by increasing metabolically active adipose tissue, thus facilitating weight loss.

1.2.1.4  The Thyroid Gland

The thyroid gland produces the thyroid hormones tri-iodothyronine (T3) and thyroxine (T4) in response to signals from the hypothalamus, pituitary and the periphery. It has long been recognised that basal metabolic rate is elevated in conditions of thyroid hormone excess (85). By contrast the role of thyroid hormone in energy balance regulation of healthy individuals is a relatively recent finding. Though the precise mechanisms are unclear, thyroid hormone is thought to regulate REE by altering substrate availability and reducing the efficiency of heat generating oxidative phosphorylation in mitochondria. This results in greater oxygen consumption and heat energy generation and stimulates heat producing intracellular processes, such as calcium release, within skeletal muscle (86), (87, 88).

1.2.2 Models of Energy Homeostasis:

A detailed understanding of the biology of energy homeostasis, is useful in understanding obesity, the overlap between diabetes and obesity, potential mechanisms of action of obesity therapies and how energy balance is regulated. However, this decomposition of energy homeostasis into component parts is unwieldy for explaining scientific or clinical observations at the whole body or whole population level. Models of energy homeostasis provide a theoretical framework within which to consider the concept of energy balance. These models were developed from engineering models of complex control systems and are a prime example of how modelling can assist scientific enquiry (89).

1.2.2.1  Single Factor Set-point Models

One of the most influential early models of energy homeostasis was the ‘lipostatic set-point hypothesis’ proposed by Kennedy in 1953 (90). This model proposed that a signal
indicating body ‘fatness’ was relayed to the hypothalamus and controlled dietary intake and thus body weight in a feedback loop. Kennedy’s model resembled several other single-factor set-point models that envisaged body weight and thus energy balance control systems as centred around a crucial physiologic parameter such as Mayer’s glucostatic model set-of food intake (91). Under this model, reductions in weight below a set-point trigger compensatory increase in energy intake and vice versa. One of the chief weaknesses of this hypothesis is its failure to account for environmental, social and psychological aspects of obesity (92),(93).

1.2.2.2 The Settling Point Hypothesis

In the 1970’s, in response to these weaknesses, Wirtshafter and Davies proposed an alternative theory known as the ‘settling-point hypothesis’(92). This hypothesis provides a framework for explaining the effect of extrinsic factors on energy homeostasis. The settling point model proposes that weight proportionally affects energy intake. As dietary energy intake exceeds energy expenditure weight gain increases. It is this gain in weight that reduces energy intake by means of satiety signals initiated by feedback signals, such as leptin, from fat stores in the periphery. This proportional relationship between the controlled parameter, in this case weight, and energy intake, means the system eventually comes to a new equilibrium that compensates for the change in input to the system. In contrast to the set-point theory, the new settling point need not be the same as before. Thus, the model can account for changes in weight over time because of environmental influences, such as a change in food availability, that alter the equilibrium. This theory does not explain the role of known physiologic control systems on aspects of both energy intake and energy expenditure and this is one of its weaknesses.

1.2.2.3 The General Intake Model

One objection to the set-point and settling point models is the assumption that all factors that alter energy homeostasis invoke a compensatory response to the perturbation. De Castro et al, 2002, argue that several factors relevant to energy balance, specifically to dietary energy intake, are not compensated for and thus are not reflected in these simpler models (94). Their assertion is based on detailed food diary records from twin studies which demonstrate several uncompensated for heritable factors that alter energy intake;
the palatability of food, social facilitation of eating, and meal times (95). In response to the perceived shortcomings of these earlier models, Castro and Plunkett proposed the “general model of intake regulation”. The general intake model proposes that dietary intake is determined by a combination of genetically determined compensated and uncompensated factors, this latter being the main point of difference from the other models. The general nature of the model means it can be applied to any type of intake; fluid, macro or micronutrient etc, and the number of factors is not pre-specified. Each factor is given a weighting which affects intake and a weighting for how intake in turn affects the compensated factors. These weightings are presumed to vary individually based on heredity and can be experimentally determined. If there is a criticism of the application of the general intake model, it is that it is concerned chiefly with the question of dietary intake and ignores the role of energy expenditure except as a factor that may influence intake.

1.2.2.4 The Dual Set-Point Hypothesis

A more flexible model is that of the ‘boundary model hypothesis’, also known as the ‘dual set-point hypothesis’. This model was originally proposed by Herman and Janet in 1984 and revisited by Speakman in 2007 (96). Briefly, they proposed that rather than a single set-point, body weight is regulated within certain “boundaries” with a lower boundary and an upper boundary. When body weight lies between these boundaries no compensatory mechanisms are active and body weight is primarily influenced by environmental factors and passive regulation as described in the settling point model. However, outside of these boundaries active regulation of energy intake and energy expenditure occurs to return body weight to a “normal” value. These boundaries may be differentially regulated, and demonstrate inter-individual variation (97),(93).

There are several advantages to this hypothesis - it accounts for inter-individual variation in the width between upper and lower boundaries in keeping with a genetic predisposition to obesity (98). It also allows for differential regulation of an upper weight boundary and lower weight boundary which is consistent with far less vigorous defence against weight gain compared with weight loss and can thus explain weight gain over the life span (99), (62). It is also compatible with both environmental influence and physiological neuroendocrine models of energy homeostasis described above that involve feed-back
control loops that are centrally mediated. Finally, in contrast to the general intake model of energy expenditure, weight, and by implication energy balance, including both energy expenditure and energy intake, can be incorporated within the framework of the model.

Thus, the dual set-point hypothesis provides the most useful framework when explaining observations about the aetiology of obesity in the context of the physiologic regulation of energy homeostasis. Were the regulation of energy expenditure completely understood, such a model would not be required. The model can be applied to adaptive thermogenesis, as a particular example of regulation of energy homeostasis that occurs during weight loss, which is itself incompletely understood.

1.2.3 Adaptive Thermogenesis

In its most general sense, adaptive thermogenesis refers to a compensatory change in energy expenditure in response to a change in some environmental factor (100). The term has been used to describe the increase in REE observed at colder temperatures or the change in physical activity related energy expenditure with imposed under- or over-feeding. The previously described regulation of energy expenditure and models of energy homeostasis may apply just as readily to states of energy excess as they do to states of energy deficiency. However, as this thesis considers not just the problem of obesity, but interventions to treat obesity, the focus will be on energy homeostasis, and REE in particular, in the setting of imposed calorie restriction. In this context adaptive thermogenesis may be defined as: “the reduction in REE, beyond that which is expected for the change in body composition, occurring during a period of dietary energy restriction”. It is this definition that will be used from here on.

In 1950 Ancel Keys published a landmark text in the field of nutrition. “The Biology of Human Starvation” is an account of semi-starvation experiments conducted from 1944-1945. During the study 34 healthy male volunteers underwent 24 weeks of semi-starvation and controlled re-feeding (101). Keys observed that during starvation, components of energy expenditure, REE, diet induced thermogenesis and physical activity energy expenditure, dropped to lower than predicted levels. This observation has been made in many subsequent studies. Saltzman et al, 1995, reviewed studies of energy expenditure during underfeeding conducted between 1985 and 1995 and found that the
decrease in REE was greater than that predicted by the change in body composition, again supporting the existence of adaptive thermogenesis (102). Similarly, Prentice et al, performed a systematic review and meta-analysis of studies of adaptive thermogenesis published until 1991 (section 1.2.3.2.1) and estimated adaptive thermogenesis as an adaption of between 5-20% beyond that expected for the degree of weight loss (103). More recently Schwartz et al, 2010, performed a recent systematic review of changes in REE during weight loss in 90 publications involving 2977 participants, confirming the presence of adaptive thermogenesis in one of the largest systematic reviews on the topic (104).

Despite having been widely studied, the assessment of adaptive thermogenesis can be challenging. The methodological requirements for assessing adaptive thermogenesis are discussed in detail in chapter 2 (section 2.3), and the characteristics of adaptive thermogenesis are presented in section 1.2.4.2.

1.2.3.1 The Purpose of Adaptive Thermogenesis

Adaptive thermogenesis is regarded as an advantageous response to conditions of environmental deprivation. In animal studies, adaptive thermogenesis occurring during periods of food deprivation helps conserve energy stores and preserve life. A lower resting metabolic rate during periods of relative food scarcity can be viewed as a positive adaptation to an environmental threat, starvation (105). In humans, such a strategy, while relevant in conditions of variable food supply, is now less relevant in many parts of the world, where obesity rather than starvation is the main threat to health. Under these circumstances adaptive thermogenesis may be seen as a maladaptive trait (106).

1.2.3.2 Characterization of adaptive thermogenesis.

Adaptive thermogenesis fits into the framework of the dual set-point hypothesis (section 1.2.3.4) as the biological response that occurs when energy intake falls below a certain threshold. According to this hypothesis, the point at which adaptive thermogenesis occurs is likely to be genetically determined and variable between individuals. The tipping of energy balance from an uncompensated level beyond this point to a compensated level is driven by environmental or external influences. If these external factors persist, the negative energy balance is maintained, and compensatory mechanisms remain in place.
Once alleviated, there is a gradual reversal of the adaptive mechanisms. The threshold for adaptive thermogenesis may be dynamic, i.e. not occurring at a fixed level of energy intake but because of the mismatch between energy intake and energy expenditure.

This schema raises some questions in relation to adaptive thermogenesis, many of which remain unanswered. For example, what is the primary regulated parameter? I have used the term energy balance or homeostasis but what is the biological correlate of this concept? During a period of negative energy balance several physiological quantities change, all of which may be potentially subject to regulation; weight, FM, leptin, thyroid hormone, fat free mass (FFM), and substrate availability (107), (108), (109), (110), (111)). What determines the strength of the adaptive thermogenesis response? Does adaptive thermogenesis depend on the degree of weight lost, the change in body composition irrespective of weight lost, the degree of calorie deprivation, or the degree of energy imbalance? How much weight can be lost, and at what rate, before adaptive thermogenesis is triggered? Finally, how is adaptive thermogenesis regulated during weight loss.

**Magnitude and Determinants of Adaptive Thermogenesis**

Rosenbaum *et al*, 2016, addressed some of these questions in a study of 17 obese individuals that underwent controlled underfeeding in an inpatient setting (108). Both total energy expenditure and REE were assessed after 10 and 20% weight loss from baseline weight. They found that the magnitude of the decline in REE, was proportional to weight loss. The decline in REE was greatest with the first 10% weight loss compared with the second 10%, providing evidence for a threshold phenomenon below which near-maximal compensation started to occur and which was not greatly increased with additional weight loss in the 10-20% range. Adaptive thermogenesis was quantified as 100 kJ per day at 10% weight loss. Finally, in this study the correlation between FM, and REE declined with weight loss but did not increase with weight gain. Apart from serving as an example of the usefulness of the dual set-point model as an inferential framework, Rosenbaum’s study adds considerably to quantifying several aspects of adaptive thermogenesis. The authors concluded that FM was a key predictor of REE but that the relationship was weakened during weight loss and unchanged with weight gain.
Prentice et al, 1991, performed a systematic review and meta-analysis of 29 studies of calorie restricted diets on metabolism up to 1991 (103). As outlined in section 1.2.3, adaptive thermogenesis was estimated to be an additional 5-20% reduction in REE beyond that expected for the degree of weight loss. Adaptive thermogenesis greater than 20% occurred only in the context of “massive” weight loss. There was a negative exponential relationship between the reduction in resting metabolic rate and percentage weight loss with most of the change occurring by 10% weight loss.

Further supporting the observation that adaptive thermogenesis is proportional to weight loss and is near maximal at approximately 10% baseline body weight loss, Siervo et al, 2015, found that the degree of adaptive thermogenesis was related to the rate of weight lost and that the severity of the diet prescription; either a six-day fast or very low-calorie diet. A more modest calorie reduction did not make a difference to the magnitude of adaptation observed. In this study adaptive thermogenesis was 7.7%. The degree of adaptation was related to the rate of weight loss only up until 10% of baseline weight but not beyond this, when the degree of adaptation plateaued (112). Knuth et al, 2014, also observed a proportional relationship between the rate of weight lost and the magnitude of adaptive thermogenesis (107).

Schwartz et al, 2010, proposed that the reduction in REE was directly proportional to the degree of weight lost and, by pooling estimates from 900 studies, estimated an average reduction in REE of approximately -64.4 kJ/kg of weight lost. In addition, they found that adaptive thermogenesis was likely to be greater with interventions of less than 6 weeks, -117 kJ/kg of weight lost, compared with those greater than 6 weeks, -54 kJ/kg of weight lost.

**Mechanisms of Adaptive Thermogenesis**

Tremblay et al, 1997, in a study of energy expenditure and body composition changes during underfeeding in monozygotic twin pairs, found a strong intrapair correlation in changes in REE after adjustment for body composition (113). REE was 9% lower than expected in the first half of the study and this decreased to 65% during the second half of the study, suggesting the attainment of a threshold level for adaptive thermogenesis half way through the study. There was a significant reduction in thyroid hormone and
noradrenaline levels with weight reduction of 5 kg, that showed a strong intrapair correlation (correlation coefficient 0.75). Weight loss in this study was almost entirely FM due to the exercise training protocol, there was no increase in lean mass. This study provides strong evidence for a genetically determined mechanism for adaptive thermogenesis, which may be mediated by sympathetic nervous system and thyroid hormone activity. It also suggests that loss of FM alone is sufficient to induce adaptive thermogenesis.

In one of the earlier studies to investigate the mechanism by which weight loss may cause a reduction in REE, Bray studied glycerol-phosphate dehydrogenase levels in subcutaneous fat in addition to REE in fourteen obese subjects undergoing weight loss. Subjects lost a mean 6.8% of body weight over one week on a calorie restricted diet (114). A reduction in REE of 15%, beyond that expected for weight loss was observed and glycerol phosphate dehydrogenase in subcutaneous adipose tissue decreased with weight loss. Glycerol phosphate dehydrogenase is a key enzyme in the provision of substrates for aerobic metabolism in the adipocyte. The reduction in glycerol phosphate observed in this study suggests regulation of enzymes involved in carbohydrate or lipid metabolism in the cell may be a means of regulating REE during conditions of energy deficiency.

In their systematic review, Prentice et al, 1991, a negative correlation between T3 and adaptive thermogenesis was noted, with a reduction in T3 of up to 70% of pre-dieting levels. This suggests that a reduction in T3 is associated with adaptive thermogenesis (103).

A study of adaptive thermogenesis in severely obese participants in the “The Biggest Loser Competition”, identified a correlation between leptin, tri-iodothyronine and metabolic adaptation during weight loss of an average 35% baseline weight (107). This has also been documented during smaller bouts of weight loss of approximately 10% baseline body weight (115).

The duration of adaptive thermogenesis

The duration of adaptive thermogenesis likely relates to an ongoing state of weight loss. The CALERIE 2 Study, detected adaptive thermogenesis occurring by three months in
free living individuals with a dietary calorie restriction of 25% from estimated baseline requirements. Other authors have confirmed adaptive thermogenesis persists out to seven months if there continues to be weight loss (107, 116).

By contrast, entering a phase of weight stability attenuates adaptive thermogenesis (117). Leibel et al., 1995, and Rosenbaum et al., 2000, performed a unique study of over and underfeeding in obese with non-obese men and women. Dynamic phases of weight change were interspersed with weight stabilisation periods over 16 months. During underfeeding to 90% of initial weight, a reduction in urine catecholamines, T3, and REE were identified (109, 117). The changes in REE, catecholamines and T3 were greater when measured during the dynamic phase of weight loss, compared to at the end of the eight-week weight-stabilisation period that followed weight loss. This suggests that measurements of REE or hormones relevant to adaptive thermogenesis are more sensitive if performed at the end of the dynamic phase of weight loss.

1.2.3.3 Is Dietary Intake the Primary Triggering Factor for Adaptive Thermogenesis Parameter

It is still not clear what triggers the onset of adaptive thermogenesis. The weight loss studies that investigate adaptive thermogenesis all induce a negative calorie balance at the outset. Adaptive thermogenesis resolves after this negative calorie balance is removed. This raises the question whether it is the diet, or the consequences of the diet that trigger adaptive thermogenesis. The correlation between FM and leptin with adaptive thermogenesis is an example of the latter (108). However, diet, whether hypocaloric or isocaloric, is itself a potent physiologic stimulus. The ingestion of food is accompanied by activation of the autonomic nervous system, the release of hormones such as insulin and GLP-1, activation of brain centres resulting in food reward, diminution of hunger, and an increase in satiety. It would be advantageous for adaptive response to a calorie deficit to trigger early, considering its obvious importance to survival. Dietary energy intake varies from hour to hour and day to day and so is subject to regulation on a shorter time scale than changes in body composition, making it a likely trigger for adaptive thermogenesis.
One way to investigate whether dietary energy intake triggers adaptive thermogenesis is to alter the schedule of calorie restriction without altering its overall magnitude. The use of different schedules of calorie restriction to investigate adaptive thermogenesis is the construct that forms the basis of the final study in this thesis, towards which the initial studies and chapters build. A handful of studies to date have studied differences in adaptive thermogenesis with different schedules of energy intake. These are discussed in section 1.6.3.4.

1.3 The Aetiology of Obesity

The World Health Organisation has defined obesity as a body mass index (BMI), a person’s weight in kg divided by their height in metres squared, of 30 kg/m$^2$ and above, and overweight as a BMI between 25 kg/m$^2$ and 30kg/m$^2$ (118). BMI provides an easily measured clinical marker of excess weight, the physiologic hallmark of obesity, and is thus a useful screening tool. However, it does have limitations both as a complete definition of obesity and as a predictor of body composition in diverse ethnicities. Ethnicity specific BMI cut-offs may be a more appropriate indicator of cardiometabolic risk, were there sufficient evidence upon which to base new cut-offs (119). There is evidence to support the use of different BMI cut-offs to indicate body composition in NZ Māori & Pacific populations. However, at present there is not sufficient evidence to support the use of different BMI cut-offs to indicate cardiovascular risk, or other clinically meaningful complications of obesity, in NZ Māori & Pacific populations. The diagnosis of obesity as a disease implies a greater risk of morbidity compared with the non-obese. However, measures of central or abdominal weight distribution such as waist to height ratio or hip to waist ratio are better predictors of obesity related complications such as diabetes, hypertension, dyslipidaemia and coronary heart disease (120, 121). This likely relates to the pathophysiology of metabolic complications of obesity. Sperrin et al, 2014, propose an alternate definition of obesity as an increase in FM sufficient to adversely affect health, reflecting the fact that not all those who have an elevated BMI have obesity related illnesses, and that FM rather than FFM is associated with the metabolic complications of obesity (122). Having considered the physiology of energy homeostasis, how does this explain the aetiology of obesity?
1.3.1 The Genetics of Obesity

1.3.1.1 The Thrifty Gene and Drifty Gene Hypotheses of Obesity

The evidence of a genetic basis for obesity is in keeping with current evolutionary perspectives on the emergence of obesity as one of today’s greatest epidemics. In 1962 Neel originally proposed his ‘thrifty gene’ hypothesis to account for, not just obesity, but the increase in rates of T2DM that were noticed at the time (106). This hypothesis suggested that we evolved to store energy, as it was evolutionarily advantageous at times of privation. However, in the 20th century access to energy-dense food meant this was no longer the case and the trait became maladaptive. An alternative view is that of Speakman’s “drifty gene hypothesis”. Speakman argues that the thrifty gene hypothesis has several weaknesses: the relatively low risk of famine, the low likelihood that famine would have a major impact on a population’s reproductive potential, the fact that contemporary hunter gatherers, such as the Namibian !Kung San, Cameroonian Pygmie and Australian Aborigine, do not become obese during periods of relative dietary excess and the partial prevalence of obesity in contemporary societies where food availability is ubiquitous (123). The alternative ‘drifty gene’ hypothesis is based on a theory of ‘predation release’. This suggests that when our ancestors were subject to predation, obese phenotypes were at risk and selected out. With the removal of this threat, random genetic mutations have occurred over the millennia that predispose to obesity. These mutations would only result in an obese phenotype in a food abundant environment. The accumulation of several genetic mutations over time would explain a polygenetic predisposition that is present in only a proportion of the population (96). The drifty gene hypothesis is not incompatible with a genetically determined thrifty phenotype - any mutation that confers an increased risk of obesity can be incorporated into the hypothesis. However, it does provide a more plausible explanation for the emergence over multiple generations of such mutations.

1.3.1.2 Evidence from Monogenic Obesity Syndromes

Until recently, evidence in favour of a genetic predisposition to obesity came largely from monogenic forms of obesity. Monogenic obesity syndromes are rare however, accounting for only 7% of severe childhood obesity and less than 0.1% of adult obesity
Nonetheless, the syndromes reveal crucial pathways in the regulation of energy expenditure and the development of obesity. To date there have been 20-30 monogenic obesity syndromes described (125). These involve mutations that affect leptin, melanocortin, pro-hormone convertase and POMC signalling as part of the leptin central melanocortin pathway (24).

1.3.1.3 Evidence from Genome Wide Association Studies

In contrast to the rare monogenic forms of obesity, heritability estimates from twin and sibling cohorts place the inherited predisposition to obesity at approximately 66% (126). Genome wide association studies (GWAS) enable whole-genome analysis for phenotype-genotype associations. In the last decade, over 50 gene loci have been identified in association with obese phenotypes (127). Two of the most significant findings from GWAS are that of the FTO and CREBRF genetic polymorphisms. While the function of the FTO gene is unclear, it is expressed in high concentrations in brain, as are many of the genetic polymorphisms implicated in obesity (98). Since its discovery in 2007, FTO has been studied in several ethnically diverse populations. Differences in the strength of the association with obesity in these groups are associated with different polymorphisms. FTO appears to be associated with increased dietary intake as its primary mechanism and is not associated with changes in physical activity, though how this effect is mediated is unclear (128). Polymorphisms of the FTO gene are common, with 43% of European populations carrying one risk allele and 20% carrying two. These FTO polymorphisms are the most prevalent globally and account for about 0.39% of the variance in BMI in European populations (129). This discrepancy between heritability estimates and variance explained by polymorphisms identified using GWAS is possibly explained by complex gene-environment interactions that are not identified by GWAS, or rare polymorphisms that have a large impact on heritability, as GWAS only identifies common polymorphisms associated with a common phenotype (126).

By contrast, the CREBRF polymorphism rs373863828, discovered in 2016, is rare globally but has a high prevalence in Samoan populations. This polymorphism is important because of its phenotypic impact and putative functional role. In those that have the polymorphism, it accounts for 1.01-1.93% of the variance in BMI in the populations studied. This is the largest effect of any polymorphism on BMI identified to
CREBF polymorphisms in a mouse model increased fat accumulation and reduced energy utilization in adipocytes compared with the wild-type protein (130). Other loci identified by GWAS are located downstream from the melanocortin 4 receptor gene and others yet are associated with genes known to be expressed in the central nervous system (127).

1.3.1.4 Evidence from Epigenetic Studies

Epigenetics describes the study of heritable changes in gene expression that are not due to changes in DNA sequence (131). Gene expression can be changed by the addition or removal of small carbon and hydrogen molecules, a process known as epigenetic regulation. For example, the addition of a methyl molecule, methylation, or removal of an acetyl molecule, de-acetylation, alters the epigenetic “marks” on DNA. Depending on the location of the mark, gene expression may be increased or decreased. Epigenetic marks have also been identified on histones, proteins that affect DNA’s folding structure and thus its accessibility to the cellular transcriptional machinery (132). In contrast to DNA, epigenetic modifications may vary over the life course, are altered by early life events and interventions for obesity, and may have a tissue specific distribution (127). This can make them challenging to study as epigenetic modification in prospective or cross-sectional studies, may be affected by events occurring many years earlier.

Epigenetic studies can adopt a hypothesis-driven target gene approach, or a broader ‘epigenome’ wide approach. The target or ‘candidate’ gene approach has identified methylation of the leptin gene (LEP) and pro-opiomelanocortin gene (POMC) in whole blood and PPARγ coactivator 1 alpha (PGC1-α) in muscle (133),(134). PPARγ coactivator 1-alpha is involved in cellular respiration and increasing mitochondrial oxidative capacity. It has also been implicated in the development of T2DM (135, 136).

The genome wide, ‘epigenome’ approach examines changes in whole genome methylation in several different regions that have been recently reviewed (24, 127, 137). Several thousand methylation sites may be identified in even one such study involving regions implicated in obesity, cellular metabolism, inflammation or indeed with no known functional associations (137). Epigenetic studies pre- and post- diet or lifestyle
intervention have been conducted that similarly demonstrated epigenetic regulation occurring at multiple functionally diverse sites.

The interpretation of such results is challenging. For any given epigenetic site, the normal variance must be known, so that the observed variance before and after an intervention can be interpreted. When an epigenetic modification is deemed significant, it is often not possible to demonstrate causality. Furthermore, interpreting the physiologic significance of tissue specific epigenetic regulation can be difficult. These significant challenges in the appropriate interpretation of epigenetic investigations notwithstanding, they remain a useful tool to supplement clinical intervention studies, develop novel hypotheses and identify new potential candidate genes in the pathogenesis of obesity.

Taken together, monogenic forms of obesity and epigenetic studies support the importance of central regulation of energy homeostasis in the aetiology of obesity. A genetically determined predisposition to obesity is consistent with the dual threshold model of energy homeostasis, with lower thresholds for compensation of positive energy balance in individuals exhibiting an obesity related polymorphism. Finally, with only approximately 2% of variance currently explained by the most common single gene polymorphism, obesity is not just polygenetic, it is a multifactorial, environmentally determined phenotype in individuals with complex genetic predisposition.

1.3.2 The Role of Environment

Human society has changed significantly over the past 50 years. Some of these changes include; a switch from recreational activity to more sedentary pursuits, the availability of technology in the home, limited pedestrian and cycling access in cities and the food environment (138, Cooper, 2000 #39).

An individual’s food environment refers to the available food that affects their diet and the systems and infrastructure through which they may obtain food. As society has changed, so have local food environments with; increased availability of cheap palatable energy dense foods such as sugar sweetened beverages, reduced access to healthy foods, the increased availability of low quality food near schools, changes in the cultural significance of food, an increased exposure to adverts for energy-dense food
compounded by the increased availability of technology in the home (139), (140), (141), (142, 143).

The impact of the environment on weight regulation can be considered as provoking or facilitating excess energy intake, or reducing energy expenditure rather than directly affecting the regulation of energy homeostasis per se. Therefore, while environmental influences are important to population levels of obesity, they don’t contribute to our understanding of energy homeostasis in the individual, which is the focus of this thesis.

1.4 T2DM and Obesity as Overlapping Diseases

Type 2 diabetes mellitus, is strongly associated with obesity. It is characterised by insulin resistance and relative insulin deficiency. Like obesity, it has a strong but incompletely understood genetic basis, possibly indicative of a heterogenous group of conditions resulting in a single common phenotype (144). Both conditions share similar predisposing factors; energy dense food, caloric excess and sedentary behaviour, though not all obese individuals will develop T2DM. It is not surprising that the rise in global obesity has been paralleled by a rise in global rates of type 2 diabetes over the past 30 years (3 ). There is strong evidence that low-calorie diets and bariatric surgery are successful treatments for both conditions. To understand how we may leverage the benefits of bariatric surgery it is useful to consider the following questions: What are the core features of T2DM and why does it occur with obesity? What is ‘remission’ of T2DM, how does it occur? Finally, how can the beneficial effects of bariatric surgery be translated into effective scalable treatments for T2DM?

1.5 Aetiology of Type 2 Diabetes in the Setting of Obesity

1.5.1 A brief overview of normal glucose homeostasis

The primary goal of glucose homeostasis is the provision of a steady supply of glucose to the tissues during both fed and fasting state. During the fasting state, blood glucose levels are largely maintained by a combination of hepatic (80%) and renal (20%) glucose production which is matched to whole body glucose uptake, resulting in a steady state concentration of blood glucose (145). Glucose production is achieved through breakdown of glycogen stores in these organs (50%) and gluconeogenesis, glucose
production from precursors such as amino acids, lactate, pyruvate and glycerol, accounting for approximately the remaining (50%). Fasting gluconeogenesis is regulated by circulating glucose, insulin, substrate availability, and non-esterified free fatty acids produced during white adipose tissue lipolysis. These fatty acids are an indirect mechanism by which insulin can regulate endogenous glucose production during the fasted state. A fall in insulin promotes white adipose tissue lipolysis increasing glycerol availability and free fatty acids which in turn increase gluconeogenesis in a pyruvate carboxylase-dependent pathway (146, 147). In the fasted state the brain accounts for most glucose utilization followed by skeletal muscle, liver, the kidney, gut and heart (148).

In the fed state, a rise in serum glucose promotes insulin release from pancreatic beta cells, which alters both glucose disposal and gluconeogenesis. The first phase insulin response is the early response to glucose stimulation that occurs within minutes. The first phase response is impaired early in the course of T2DM and is thought to be related to a reduction in the readily releasable pool of insulin from pancreatic beta cells (149), (150). There is a net suppression of hepatic gluconeogenesis by approximately 80% as insulin suppresses adipose tissue lipolysis, and thus free fatty acid mediated gluconeogenesis, and promotes glucose storage in the form of glycogen (151). Insulin also promotes increased glucose uptake in the insulin sensitive muscle, heart and adipose tissues (148). The muscle is particularly good at increasing glucose uptake in response to insulin with an eight-fold increase over basal rates during insulin exposure (152).

1.5.2 The Aetiology of T2DM

While there still exists debate about the precise evolution of T2DM, epidemiological studies suggest that, despite large heritability estimates for the inheritance of T2DM, lifestyle factors, especially excess dietary energy intake and poor-quality diet have a large effect on the aetiology of T2DM (153).

Molecular studies in animals and humans have characterised T2DM as a multi-organ disease with self-perpetuating deficits in nutrient handling. Hepatic, skeletal muscle, pancreatic, and adipose tissue insulin resistance have all been clearly documented in T2DM. The mechanistic basis for each of these observations is different and, in some cases, only partly understood. A consistent contributing factor in each of these organs is
excess lipid deposition and mitochondrial dysfunction which in turn lead to a cycle of worsening insulin resistance (154-159).

Insulin resistance in the liver appears to be mediated by the accumulation of diacylglycerol. This causes translocation of activated protein kinase Cε, to the hepatocyte membrane where it phosphorylates and thus inactivates the insulin receptor (155, 156). The accumulation of diacylglycerol in liver and muscle can be induced in animals in response to lipid infusion and in humans in response to both lipid infusion and a hypercaloric diet. In obese individuals with fatty liver, intracellular diacylglycerol is associated with hepatic insulin resistance. Similarly, intracytosolic diacylglycerol in skeletal muscle impairs glucose oxidation, glycogen synthesis and whole-body glucose disposal (157), (158), (154). In mouse models of diabetes, the development of diabetes is preceded by a progressive rise in free fatty acids, pancreatic lipid deposition and impaired glucose-stimulated insulin secretion (160). Accumulation of long chain fatty acids within the pancreatic beta cell; increases reactive oxygen species production, depletes intracellular calcium and triggers cell death (161-163). Gastaldelli et al, 2017, have examined the development of altered metabolism in the adipocyte with the onset of impaired glucose homeostasis and found a correlation between circulating free fatty acids and adipocyte insulin resistance (164). Fatty acid handling is altered in adipocytes in obese individuals, a phenomenon which is worsened by insulin resistance. Oxidation of fatty acids is downregulated, uptake of fatty acids by the adipocyte is impaired and lipolysis of storage forms of free fatty acids is increased. This is exacerbated by insulin resistance and leads to an increase in plasma free fatty acids which are taken up by the pancreas, liver and skeletal muscle (159).

Taken together, this suggests a role for intracellular lipid accumulation in non-adipose tissues during a state of energy excess as one of the key pathogenic events in the development of T2DM. Impaired storage of lipid in adipocytes and ectopic fat deposition in three of the main organs involved in glucose homeostasis leads to worsening insulin resistance and greater dysfunction of lipid handling in an ever-worsening cycle. Frayn proposed excess lipid deposition in non-adipose tissues as a key factor in the development of insulin resistance due to saturation of the storage capacity of adipocytes (165). This idea was developed in 2008 by Taylor’s “Twin Cycle” hypothesis. This
hypothesis outlines the mechanisms by which a state of calorie excess leads to the development of T2DM (166). As adiposity and whole-body insulin resistance increase, blood glucose levels rise. Excess glucose taken up by the liver is converted to lipids which are either stored in the liver, further impairing insulin sensitivity, or exported to the peripheral circulation where they contribute to intrapancreatic lipid accumulation. Increased intrapancreatic lipid impairs the beta cell response to glucose ingestion thus worsening post-prandial glycaemia and further adding to de novo lipogenesis in the liver. This hypothesis fits well with the cellular model of T2DM as a disease of disordered fatty acid metabolism leading to progressive multi-organ insulin resistance in response to maladaptive fatty acid oxidation and trafficking.

Additional evidence at the organ level for the Twin Cycle hypothesis comes from studies of weight loss with low-calorie diet or bariatric surgery. Reversal of dysglycaemia has been associated with a reduction in intrapancreatic and intrahepatic fat content visualised by MRI (167), (168). During an eight-week dietary weight-loss program in participants with diabetes, the first phase insulin response (section 1.5.1) to a glucose infusion normalised to those of the control participants without diabetes. This was associated with a reduction in intrapancreatic triglycerides (167). The observation that intrapancreatic fat decreases with weight loss, is specific to T2DM, and not simply a reflection of decreases in whole body fat generally. Obese T2DM participants who underwent bariatric surgery again demonstrated a reduction intrapancreatic fat with improvements in glucose homeostasis, that was not observed in non-diabetic controls who also underweight bariatric surgery with comparable weight loss (168). These studies provide evidence that specific deficits of glucose-stimulated insulin secretion and insulin-mediated suppression of hepatic gluconeogenesis are associated with ectopic fat deposition in the pancreas and liver respectively. They also illustrate the mechanism by which low calorie interventions may be used to improve T2DM.

1.6 Treatment of Obesity and Diabetes

The rise in prevalence of obesity and T2DM over the past few decades is testament to the limited efficacy of efforts to prevent and treat obesity and its complications (3). Undoubtedly, prevention of obesity would be preferable to treatment of obesity once
established. Nevertheless, for the 33% of New Zealanders with established obesity, and for the 5% of New Zealanders with T2DM, weight loss represents the main way of reducing morbidity and extending life (4),(3).

Treatment of obesity amounts to induction of a sustained negative energy balance and thus weight loss. This can be induced pharmacologically, surgically or by means of a dietary intervention. The range of past and current pharmacotherapies and dietary therapies for weight loss is beyond the scope of this thesis. What follows is an overview of the two treatments for obesity that induce diabetes remission, bariatric surgery and calorie restriction, and a discussion of their possible mechanisms of action.

Bariatric surgery and very low-calorie diet (VLCD) interventions can produce substantial reductions in weight and improvements in glycaemia. This is in marked contrast to pharmacological treatments for T2DM which at best produce mild weight loss and at worst induce further weight gain (169). Studying how these treatments work, how they can be optimized and how they integrate with our current understanding of existing models of obesity and T2DM is a critical step in advancing treatments for obesity and diabetes.

1.6.1 The Surgical Treatment of Obesity: Bariatric Surgery

In 1967, Edward Mason proposed a gastric bypass procedure for the treatment of obesity. Surgeries which have the primary intention of promoting weight loss, are termed ‘bariatric’ surgery (170). Nowadays, the most commonly performed bariatric procedures can be grouped as purely restrictive: laparoscopic gastric banding (LGB) and laparoscopic sleeve gastrectomy (LSG), or malabsorptive: roux-en-y gastric bypass (RYGB) and biliopancreatic diversion (BPD) (Fig. 1.3)(171, 172). The anatomical and functional distinction between the procedures is relevant to the difference in efficacy and mechanism of action of each procedure.
Banding procedures involve placement of an inflatable adjustable band that encircles the stomach, thus restricting passage of food through the stomach by external compression (Fig 1.3, panel A). A sleeve gastrectomy is usually done laparoscopically (LSG) and involves surgical closure of most of the stomach volume to leave a low

A) Gastric banding, B) Sleeve gastrectomy, C) Roux-en-Y gastric bypass
D) Biliopancreatic diversion with duodenal switch

Figure 1.3: Common Bariatric Surgery Procedures
volume vertical sleeve (Fig 1.3, panel B). The vertical banded gastroplasty (VBG) involves closure of the stomach with vertical anastomosis, like LSG, followed by placement of a cuff around the residual patent portion of the stomach. Malabsorptive procedures involve anastomosing the small bowel directly to the proximal stomach to bypass the distal stomach and proximal small bowel. The Roux-en-y gastric bypass procedure (Fig 1.3, panel C), and biliopancreatic diversion (Fig 1.3, panel D) are the two most commonly performed malabsorptive procedures. The duodenal switch procedure is a variation on BPD that involves anastomosing the first portion of the duodenum to the distal small bowel.

In 1995 Walter Pories reported the normalisation of blood glucose in a cohort of 271 patients with either T2DM or impaired glucose tolerance following bariatric surgery (173). Though the effect of bariatric surgery on glucose homeostasis had been recognised much earlier, Pories’ paper was considered a landmark and stimulated research into mechanisms of diabetes remission following bariatric surgery (174). Modern bariatric surgery is now regarded as the single most effective treatment for both obesity and T2DM (175, 176). Weight loss and diabetes outcomes following either RYGB or LSG, surpass intensive medical management up to five years post-operatively (177, 178).

Bariatric procedures differ in their weight loss outcomes, the occurrence of side effects, their ability to improve glucose homeostasis and the degree of malabsorption (1, 171, 172, 179). These differences have informed the investigation of the underlying causes of diabetes remission. Comparisons between surgery types have identified which procedures are most effective at inducing weight loss and effecting diabetes remission (Table 1.1). To facilitate comparison between different bariatric procedures with respect to weight loss and diabetes outcomes, there has been a move to separating effects from different types of surgeries, standardising a definition of diabetes remission following bariatric surgery and to consider the impact of different surgical techniques for a given surgery type (1, 180, 181).
1.6.2 Bariatric Models of Diabetes Remission

The definitions of diabetes remission endorsed by the American Diabetes Association are:

“Partial remission is sub-diabetic hyperglycaemia (A1C not diagnostic of diabetes [<6.5%], fasting glucose, fasting glucose 100-125mg/dl [5.6-6.9 mmol/l] or at least one year’s duration in the absence of active pharmacologic therapy or ongoing procedures.

Complete remission is a return to “normal” measures of glucose metabolism (A1C in the normal range, fasting glucose <100mg/dl [5.6 mmol/l] of at least 1 year’s duration in the absence of active pharmacologic therapy or ongoing procedures.”

Table 1.1. Weight loss and diabetes resolution following bariatric surgery (1).

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Gastric Banding</th>
<th>Gastroplasty</th>
<th>Gastric Bypass</th>
<th>BPD/DS</th>
</tr>
</thead>
<tbody>
<tr>
<td>%EBWL</td>
<td>55.9</td>
<td>46.2</td>
<td>55.5</td>
<td>59.7</td>
<td>63.6</td>
</tr>
<tr>
<td>% Remission</td>
<td>78.1</td>
<td>56.7</td>
<td>79.7</td>
<td>80.3</td>
<td>95.1</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Remission &lt; 2 years</td>
<td>80.3</td>
<td>55.0</td>
<td>81.4</td>
<td>81.6</td>
<td>94.0</td>
</tr>
<tr>
<td>% Remission &gt; 2 years</td>
<td>74.6</td>
<td>58.3</td>
<td>77.5</td>
<td>70.9</td>
<td>95.9</td>
</tr>
</tbody>
</table>

% EBWL: Percent excess body weight loss; BPD: Biliopancreatic diversion; DS: Duodenal switch procedure
A comparison of diabetes remission rates between bariatric procedures have been documented in several large meta-analyses (172), (182), (1). Limitations of these meta-analyses include; the inclusion of heterogeneous studies - particularly with respect to different definitions of remission, the paucity of RCTs, relatively few head-to-head studies comparing treatments, incomplete reporting of the ascertainment of diabetes status and incomplete descriptions of diabetes outcome assessments(182, 183). Nonetheless, they provide a useful insight into the prevalence of diabetes remission following different surgery types.

Diabetes remission occurs most commonly following biliopancreatic diversion with duodenal switch, followed by, gastric bypass (80.3%), and gastric banding (56.7%) (Table 1.1). The likelihood of long-term remission correlates with the degree of weight loss (1). Other than weight loss, predictors of remission include: young age, absence of insulin use, the surgery type and good glycaemic control (184), (25), (185), (186). Apart from differences in the incidence of remission following different surgery, the components of glucose homeostasis that improve as well as the timing and magnitude of these changes vary between RYGB, BPD, VSG and LGB (187).

Within the first week following RYGB, before any significant weight loss, fasting plasma glucose, endogenous hepatic glucose production and fasting serum insulin decrease, and fasting insulin clearance and hepatic insulin sensitivity increase compared with preoperative levels. By three months these changes have progressed and continue to do so out to one year with ongoing weight loss (188). There are conflicting data in relation to improvements in insulin secretion following RYGB. After RYGB, some studies indicating no improvement in first phase insulin response within the first four weeks while others report the opposite (189), (190), (191, 192).

Within four weeks following BPD, reductions in fasting plasma glucose and improvements in oral but not intravenous glucose tolerance are seen (193). In one study, BPD was associated with improvements in glucose disposal, post-prandial glucose and insulin resistance by as early as day three post-operatively (194).

LSG is also associated with reductions in fasting plasma glucose and oral glucose tolerance within the first week (193). Hepatic insulin sensitivity has been shown to
improve within five days, though this has not been replicated in all studies (195). Improvements in insulin secretion following an intravenous glucose load has been reported following LSG within 72 hours (196). However, the method used for assessing this, plasma insulin concentration in response to an intravenous glucose load, is not a direct measure of assessing pancreatic beta cell function as it is a product of both insulin secretion and insulin clearance by the liver, which is altered in individuals with T2DM (197).

In one of the few studies where LGB’s effects on glucose and insulin levels in the immediate post-operative period were assessed, no significant decrease in fasting glucose was observed and none of the patients achieved diabetes remission (198). This study was chiefly limited by a very small sample size for the LGB group. By one year following LGB hepatic insulin sensitivity, measured by the surrogate marker HOMA-IR, improves modestly, though not back to a normal value (199, 200).

Together these data illustrate a common theme: early improvements in fasting glucose because of decreased endogenous hepatic glucose production and increased hepatic insulin sensitivity with resulting lowering of fasting glucose and insulin. There are also improvements in oral glucose tolerance following bypass procedures and VSG thought to be due to the incretin effect, particularly that of GLP-1 (201). There is no evidence that this occurs following LGB.

Efforts to explain the improvements in glucose homeostasis following bariatric surgery have focussed chiefly on four models; calorie restriction, alteration in gut peptides, alterations in gut microbiota and changes in bile acid metabolism (168), (202), (203), (204), (205), (206).

1.6.2.1 Calorie Restriction Improves Glucose Homeostasis Following Bariatric Surgery.

VLCD Prior to Bariatric Surgery

A low-calorie diet is simply the habitual intake of an energy content lower than the requirements of the individual. Very low-calorie diets (VLCD’s) have been defined by the National Heart Lung and Blood Institute and National Institutes of Health as diets
providing fewer than 3350 kJ/day (207). Still more restrictive, are ‘complete’ fasts, such as religious fasting that involves a total abstinence from food for a specified period.

Calorie restriction occurring in the setting of bariatric surgery usually begins pre-operatively with a VLCD (208), (209, 210). This is advocated to reduce liver volume prior to the procedure. A recent systematic review of pre-operative VLCDs highlights the variation in calorie content, macronutrient composition and duration (211). Despite this heterogeneity VLCD consistently reduces liver size, due to a reduction in liver fat content, water and glycogen (167, 212).

The Contribution of VLCD to Improvements in Glucose Homeostasis

When considering the cause of diabetes remission with VLCD alone versus bariatric surgery, the preoperative VLCD is a significant confounding variable. To what extent is the post-operative improvement in glycaemia attributable to the pre-operative intervention? The argument that glycaemia improves prior to significant weight loss post-operatively is worth considering. The inherent assumption is that weight lost, as distinct from the induction of a negative calorie balance, is primarily responsible for improvements in glycaemia seen with VLCD. VLCD induces approximately 4-5kg weight loss over two weeks (213, 214). This degree of weight loss, in patients with diabetes, is sufficient to normalise fasting plasma glucose, restore hepatic insulin sensitivity to those of non-diabetic controls, reduce hepatic triglyceride content by 30%, reduce plasma triglycerides by 50% and decrease insulin secretion by 40% all within the first week (167). Thus, by post-operative day one, if a perioperative VLCD has been employed, the patient has already experienced an intervention associated with clinically relevant improvements in glucose homeostasis.

Improvements in glucose homeostasis can be accounted for by the effect of calorie restriction: The evidence in favour.

In many studies GLP-1 secretion (section 1.2.1.2.2), measured at four days, one or three weeks post-op is clearly increased with RYGB compared with VLCD (168, 202, 215, 216). Despite the rise in GLP-1 with RYGB, many studies have failed to demonstrate a clinically meaningful difference in post-prandial glucose concentration compared with VLCD alone (168, 215, 217, 218). Where differences have been identified, it is often due
to failure to match calorie intake between the VLCD and post-RYGB days. In a more recent study, Pop et al, 2018, addressed this by measuring intake during a ten-day VLCD and providing the same intake exactly for each participant during the 10-day post RYGB period. While GLP-1 was elevated post RYGB there was no difference in glucose clearance or insulin sensitivity between treatments by post-operative day seven. Endogenous glucose production was lower in the diet-only group (217). Thus, it seems that when equivalent calorie matching within individuals is achieved, RYGB does not confer additional advantages on glucose homeostasis compared with VLCD, particularly with respect to post prandial glycaemia, the aspect of glucose homeostasis that may be expected to benefit from enhanced GLP-1 secretion.

*Improvements in glucose homeostasis can be accounted for by the effect of calorie restriction: The evidence against.*

Foo et al, 2011, studied changes in insulin resistance; six days following VLCD, six days following subsequent RYGB; and six days following a control RYGB-only group (219). Improvements in HOMA-IR attributed to improved hepatic insulin sensitivity were observed in all groups. The total reduction in HOMA-IR with VLCD and sequential RYGB was equivalent to the reduction in HOMA-IR achieved by RYGB alone in the control group. One potential explanation for this may be differing weight loss or greater calorie restriction in the RYGB-only group or a ‘saturable’ effect of calorie restriction mediated by VLCD or RYGB on HOMA-IR. The observed deterioration in whole body insulin sensitivity, measured by an insulin tolerance test in this study, may have been related to perioperative physiological stress. This has been shown to worsen peripheral insulin resistance for up to three weeks post-operatively (220).

Pournaras et al, 2016, also studied the relative contribution of VLCD, altered nutrient delivery and weight lost to diabetes remission following RYGB. Measurements were performed pre-VLCD, two weeks post-VLCD, two weeks post-operatively and at 52 weeks post-operatively. They found that the improvements in whole body insulin sensitivity were the same after two weeks of VLCD as they were two weeks after RYGB. In the same study RYGB was associated with improvements in postprandial hyperglycaemia not attributable to VLCD, while weight lost at 52 weeks was responsible for longer term improvements in whole body insulin sensitivity (221). The finding of
early improvements in peripheral insulin sensitivity, has not been observed in other studies at a similar timepoint (222). However, peripheral insulin sensitivity several months following RYGB, has been consistently shown to improve in proportion to weight lost.

*Improvements in glucose homeostasis can be accounted for by the effect of calorie restriction: Resolving the discrepancies.*

Pop et al, (section 1.6.2.1.3) addressed two aspects of matching calorie intake in the design of studies of VLCD versus RYGB in their study. First, for individuals of different sizes and activity levels, a uniform calorie prescription, will represent a different proportion of their total energy requirements, thus calorie prescriptions should be individually tailored. Secondly, the degree of calorie restriction following RYGB cannot be assumed to be the same as the protocol specified VLCD by default. Confounding factors that may alter energy intake or energy balance following RYGB include; post-operative complications such as infections or constipation, variable tolerance of a hospital diet and medication-related nausea. A third challenge in accurately calorie matching which has yet to be appropriately addressed in studies comparing VLCD and RYGB, is the increase in energy requirements caused by major abdominal surgery and the ensuing convalescence.

Basal metabolic rate is increased by at least 15-20% from the time of abdominal surgery for at least 72 hours post-operatively (223). In a 40 yr old 140 kg male with a height of 170cm, a 20% difference in REE accounts for 2150 kJ increase in daily calorie requirements approximately†. The failure to incorporate total calorie requirements is common-place in head-to-head studies of RYGB and VLCD. Determining what the calorie requirements would be for a matched VLCD is challenging given the earliest published data regarding changes in energy expenditure following RYGB are at 14 days post-operatively (224).

Laferriere et al, 2008, studied RYGB versus matched weight loss with a VLCD. Compared to VLCD, RYGB was associated with higher GLP-1 and insulin levels, a lower glycaemic response to an oral glucose challenge, and a lower fasting glucose (225).

† Harris Benedict Equation used to predict basal metabolic rate and daily calorie requirements.
A significant limitation of this study was that equivalent weight loss took three weeks longer to achieve in the RYGB group, as the calorie intake in the VLCD group was not well matched. To address this confounder, Jackness et al, 2013, performed a similar study using a 2100 kJ VLCD that produced similar weight loss in similar time. There was no difference in glycaemic outcomes in this study (226). The use of calorimetry methods pre- and post-operatively to determine daily energy requirements would provide more accurate calorie prescription for both VLCD and post RYGB, and address standardisation of proportional calorie deficit and changing energy expenditure post-operatively.

In summary, inadequate matching of induced calorie deficit in head to head studies of RYGB and VLCD has confused the relative contribution of VLCD to diabetes remission following RYGB. While a definitive study is required, those studies that have come closest to appropriate calorie matching have not demonstrated a benefit from RYGB that isn’t explained by the degree of calorie deficit. Changes in gut peptides, bile acid metabolism and gut microbiota have also been suggested to have role in diabetes remission following bariatric surgery.

1.6.2.2 Gut Peptides: GLP-1

The profile of hormones released in response to a meal changes dramatically with bariatric surgery due to more rapid nutrient delivery through the gastrointestinal tract. This is particularly true of RYGB and BPD procedures which involve bypass of the proximal small bowel, unlike LSG (227). Glucagon-like peptide 1 has been proposed as key mediators of diabetes remission following bariatric surgery(228),(229).

Relative to bile acid metabolism and gut microbiota, more is known regarding the physiologic function of the incretins in humans, and their contribution to diabetes remission following bariatric surgery. Jorgenson et al, 2012, demonstrated a ten-fold increase in GLP-1 five days following RYGB. This coincided with increased insulin secretion in response to a meal, and was sustained out to one year (230). Studies using GLP-1 blockade following RYGB demonstrate deterioration in postprandial insulin responses, providing strong evidence for a contribution of GLP-1 to improvements in glycaemia following bariatric surgery (201).
1.6.2.3 Altered Enterohepatic Cycling of Bile Acids

In obese subjects, systemic bile acids are reduced compared with their lean counterparts. Following bariatric surgery however, bile acid concentrations in the blood increase as early as six weeks post-operatively (231). This is thought to be due to disruption of enterohepatic cycling that occurs because of bypass procedures such as RYGB, VSG, duodenal-endoluminal sleeve and ileal transposition.

Bile acids have been shown in mice to; regulate the production of bile acids from cholesterol, improve glucose homeostasis, reduces lipids by increasing lipoprotein lipase, inhibiting lipogenesis, and influence GLP-1, PYY and energy metabolism (232, 233), (234),(235).

Human trials are limited to observational studies that have investigated the association between elevated bile acids and diabetes remission or weight loss. These studies have produced conflicting results and are confounded by inter-individual variation in bile acid metabolism pre-operatively. Gerhardt et al, 2013, examined over 100 patients before and after-RYGB and found no association between total bile acid concentrations and the occurrence of diabetes remission (236). Other studies have found an association between total bile acids and improved glycaemia (237),(238). Though the improvements in glycaemia occurred before the change in total bile acid concentration in one of these studies (238).

Bariatric surgery has stimulated a greater understanding of bile acid metabolism and its possible role in the resolution of obesity and diabetes. However, more work needs to be done to confirm the physiological role of bile acids in humans, determine causation and quantify the clinical significance of observed changes in bile acids following-bariatric surgery.

1.6.2.4 Gut Microbiota

The human gastrointestinal tract normally plays host to millions of bacteria. These become established in early life and remain relatively stable in most healthy individuals through adulthood. One theory by which gut microbiota may influence obesity and weight is based on the effect that microbiota have on nutrient metabolism. Other theories
propose that the post-operative gut microbiota may act indirectly to improve glycaemia by; altering bile salt metabolism, reducing inflammation and influencing central energy-homeostasis.

In mouse models of obesity, there is an observed reduction in the microbial diversity, and a reduction in some families of bacteria such as Bacteroidetes and Firmacutes (239). A comparison of lean and leptin deficient obese mouse models showed an increased extraction of short chain fatty acids from the diet, changes in whole microbiome gene expression and change in total energy extraction from the diet (240). This latter is thought to be due to more efficient extraction of energy from complex carbohydrates. In human studies, changes in the bacterial composition of the gut, including an increase in bacterial diversity, have been observed following bariatric surgery. The interpretation of these studies is challenging, as dietary intake, living environment and physical fitness, all have a bearing on the gut microbiome and are not often controlled for (205).

At this point it is not possible to draw firm conclusions in relation to the role of gut microbiota in the pathogenesis of obesity or resolution of T2DM beyond simple association. Prospective observational studies, adequately powered and controlled with a clear mechanistic basis are required before this will be possible.

In summary, there is robust evidence for the effect of calorie restriction on improvements in glycaemia. While GLP-1 may be an important contributor to early improvements in glycaemia following bariatric surgery, GLP-1 in the absence of weight loss has not been to induce diabetes remission, unlike calorie restriction. The investigation of bile acid metabolism and gut microbiota following bariatric surgery has been valuable, but to date has not been clearly shown to mediate improvements in glycaemia or diabetes remission.

1.6.3 Low-Calorie Diets in the Treatment of Obesity

Low calorie diets have been used as a treatment of obesity long-before the advent of bariatric surgery. The Greek physician Galen was one of the first to suggest low calorie foods for the treatment of obesity with his treatise: On the Slimming Diet, c.180 AD (241). This has continued through the 20th century with the development of VLCD’s as a means of losing weight quickly (242).
1.6.3.1 Features of a Calorie Restricted Diet

Apart from the degree of calorie restriction, additional characteristics of a low-calorie diet or VLCD are the schedule of dieting and the macronutrient composition. Broadly, schedules can be considered continuous or intermittent. A continuous low-calorie diet, also known as continuous daily restriction (CDR), involves prescribed calorie restriction by the same degree every day of the week. In practice this can only be approximated, as the physical activity component of total daily requirements will vary daily, outside of a supervised inpatient stay. Conversely, an intermittent fast (IF) involves a hypocaloric diet that is interrupted with days of ad libitum, isocaloric or hypercaloric intake. An example of this is alternate day fasting (ADF) which may involve VLCD’s or complete fasts on alternate days with an isocaloric or hypercaloric diet otherwise (243, 244). Another example of an IF is two days of VLCD diet per week compared with isocaloric intake otherwise, this form of IF is the intervention used in chapters six and seven. When dietary restriction is imposed over a period of hours, the schedule is known as “time-restricted feeding” (245, 246). Studies of the effect of fasting between sunrise and sundown during Ramadan, a type of religious fast, are another example of time restricted feeding.

The macronutrient composition of a diet refers to the proportion of fat, carbohydrate or protein in a diet. Macronutrient composition is conventionally expressed as the proportion of a given macronutrient, often in percent, that comprises the energy content of the diet. Each of these dietary characteristics may have a bearing on weight loss achieved or glucose homeostasis.

The roles that the magnitude of calorie restriction, macronutrient composition and schedule of calorie restriction play in treating diabetes and obesity will be examined in turn. The addition of supplements to the diet or exclusion/inclusion of specific foodstuffs represent other ways to prescribe a diet. However, the scope of the following discussion will be limited to features of a diet prescription for which there is some evidence of a role in clinically significant weight loss outcomes and/or diabetes remission.
1.6.3.2 **Magnitude of Calorie Restriction**

**The Effect of Magnitude of Calorie Restriction on Weight Loss**

In highly controlled inpatient studies of calorie restriction, the degree of weight loss proceeds in close alignment with the degree of caloric deficiency (247). Reinhardt *et al*, 2015, monitored weight loss and energy balance in response to calculated dietary energy deficiency of 50% in 12 individuals weekly for six weeks. There was a strong correlation between weight loss and calculated energy deficit, \( r = 0.75 \), though the degree of weight lost was not entirely explained by the imposed calorie deficit (247). Proportionality between the calculated energy deficit provided by a diet and the weight loss incurred has been shown elsewhere (117, 248). While the magnitude of the association in weight loss due to calorie deficit is variable between studies, this may be attributable to differences in body composition assessment methods (section 2.3.1), assumptions in relation to estimation of energy deficit, ascertainment of measures of energy expenditure, changing energy requirements during a period of sustained calorie restriction, or adherence. In a meta-analysis of six head-to-head studies of low-calorie diet (4184-6694 kJ/day) versus VLCD (1673 – 1907 kJ/day), VLCD was shown to induce greater short-term weight loss between 6-18 weeks, compared with low-calorie diet. At long-term follow-up between one and five years there was no difference in sustained weight loss between low-calorie diet and VLCD (249). This is quite likely due to changes in adherence or energy expenditure in the medium to longer term. In summary, while accurate prediction of weight loss is complex, the magnitude of weight loss is quite clearly proportional to the degree of calorie restriction.

**The Effect of Magnitude of Calorie Restriction on Glucose Homeostasis**

How VLCDs improve glucose homeostasis, particularly prior to bariatric surgery (section 1.6.2.1), has previously been discussed. That calorie restriction improves glucose homeostasis is clear. However, what is the evidence that the magnitude of such restriction influences glucose metabolism?

With respect to improved glucose homeostasis with low-calorie diets, it had been identified by Henry *et al*, 1986, that low-calorie diets were beneficial in individuals with non-insulin dependent T2DM (250, 251). Wing *et al*, 1991, studied the long-term effect
of an energy intake target of 4200-6300 kJ (low-calorie diet group) versus a very low-calorie diet of 1680 kJ (252). Both interventions lasted 20 weeks. The energy intake targets in the VLCD group were matched to those of the low-calorie diet group in the first month. This was followed by eight weeks of VLCD or low-calorie diet followed by weight maintenance again matched to the low-calorie diet group. Compared with the low-calorie diet group, VLCD was associated with a higher proportion of individuals stopping and remaining off hypoglycaemic medication, significant improvements in fasting and post prandial glucose, and insulin response to a meal test. This was despite no significant difference in weight between treatment groups by one year. The study again highlighted that weight loss between groups differed in proportion to the degree of calorie restriction. During matched dietary intake, weight loss was the same in both groups. A second study by Wing’s group compared low-calorie diet with VLCD and behavioural therapy for one year. Two periods of 12-week VLCD with a 12-week low-calorie diet between them were followed by weight maintenance with a low-calorie diet. Improvements in glycated haemoglobin in the first 12 weeks were strongly associated with the degree of weight loss, and those in the VLCD group remained off medications for longer, with a lower fasting plasma glucose. By six months, three months into the weight maintenance phase, there was no difference between low-calorie diet and VLCD in fasting insulin or fasting glucose (253), possibly due to a reduction in weight difference between both groups at this timepoint. To date, these two studies by the same group remain the only RCT’s of VLCD vs low-calorie diet on glycaemic outcomes in individuals with T2DM (254).

Based on the studies of Wing et al, it is interesting to consider whether the effect of a VLCD extends beyond simply the degree of calorie restriction. For example, do difference in food type, meal density, or meal schedule provide additional benefits on glycaemia with a VLCD compared to a low-calorie diet. While there is currently insufficient evidence to address these questions, the work of Wing et al is strong evidence that the magnitude of calorie deficiency is the primary determinant of glycaemic outcomes after a calorie restricted diet.

1.6.3.3 Macronutrient Composition

The macronutrient composition of a diet, in contrast to the schedule of dietary restriction, is an aspect of diet induced weight loss that has been comprehensively researched and
reviewed. There are a great many permutations of macronutrient composition in existence (255). When considering weight loss and glycaemic outcomes three types of macronutrient-controlled diets have been most closely studied; Low carbohydrate, low fat or high protein diets. Because fixed macronutrient diets are defined as the proportion of the energy intake that comes from a certain macronutrient, it is not possible to alter one macronutrient without reciprocal alteration of another. If changes in absolute quantities of a single macronutrient are altered in isolation, this results in change in total energy intake, which will obviously confound interpretation of the results.

There are many possible reasons why different macronutrient content may alter weight loss or glycaemic outcomes in the context of obesity and/or T2DM. These have been summarised in a recent review (256) and include both behavioural and physiological factors such as; adherence, cost, side effects, substrate utilisation, energy density and satiety.

**The Effect of Carbohydrate on Weight Loss and Glucose Homeostasis**

Foster *et al.*, 2010, conducted one of the largest and longest studies of low carbohydrate-high fat diets versus high carbohydrate-low fat diets in 307 overweight and obese men and women over two years (257). The low carbohydrate diet intervention contained less than 20% intake as carbohydrates, while the high carbohydrate diet intervention was 55% or more. The maximum weight lost was 12 kg at six months with 7 kg net weight loss by two years. There were no differences in weight loss between treatment groups at any timepoint. Glycaemic outcomes were not assessed. There was an emphasis on carbohydrate restriction in the low carbohydrate group and calorie restriction in the control group during dietary education, without strict energy intake targets in either group. No measures of adherence to dietary energy intake or macronutrient targets were reported in this study. It is thus unclear whether the planned between group difference in intake occurred.

Over fifty studies have been performed examining the effect of low carbohydrate diet interventions in individuals with T2DM. Nine separate meta-analyses of these studies have failed to reach a consensus in relation to whether low carbohydrate diets improve glycaemia in individuals with T2DM. Huntriss *et al.*, 2018, reported a reduction of -
0.28% (95% CI: -0.53 to -0.02%) or 3.3 mmol/mol (95% CI: -5.8 to -0.2 mmol/mol) in glycated haemoglobin (HbA1C) (258). By contrast Castañeda-González et al, 2011, found no consistent differences in HbA1C or fasting glucose in individuals with T2DM in studies of greater than 12 weeks duration (259).

In order to reconcile the disparities between the results of these and other meta-analyses of the effect of low carbohydrate diets on glycaemia, Van Wyk et al, 2016, performed a systematic review of ninety-two studies comprising the nine meta-analyses (260). Only randomised controlled trials in adults with T2DM were reviewed. Most studies were excluded due to; a study duration of four weeks or less, a carbohydrate content of 45% total energy intake or more in the low-carbohydrate intervention group, or no record of dietary intake during the study. Eleven of the remaining studies demonstrated no weight loss difference between a high and low carbohydrate diet. With respect to glycaemia, there were no differences in insulin sensitivity, insulin secretion or fasting plasma glucose in any of the studies. Eleven of the twelve studies demonstrated no difference in HbA1C. The assessment of glycaemia was complicated in most studies by variable medication titration protocols, and failure to incorporate changes in medication in the analysis of glycaemic outcomes.

Thus, in well-controlled RCT’s of low carbohydrate interventions longer than four weeks, there is consistency in failing to demonstrate a significant effect of a low carbohydrate diet on weight loss or glycaemia. This is in keeping with the American Diabetes Association’s emphasis on usual macronutrient distribution, rather than a focus on low carbohydrate diets, acknowledging the effects of low carbohydrate diets on glycaemia are currently unclear (261).

The Effect of Protein on Weight Loss and Glucose Homeostasis

Santesso et al performed a meta-analysis of 74 RCT’s of high versus low protein diets, 73 of these studies were in overweight or obese individuals without diabetes. While weight loss appeared to be greater with a high protein diet, this was associated with a higher baseline BMI in the high protein groups. If a fixed calorie prescription rather than proportion of intake was used, imbalance in the baseline BMI of participants may have resulted in a greater calorie deficit in the high protein group. No significant difference in
fasting glucose or HbA1C was detected. The level of evidence was graded moderate or low, and there was a high degree of heterogeneity, $I^2 > 60\%$, for most outcomes (262).

Krebs et al, 2012, conducted the largest study of high protein diet in overweight adults with T2DM. The DEWL study ran for two years and compared a high protein low carb diet containing 30% protein, 40% carbohydrate and 30% fat with a low protein high carbohydrate diet containing 15% protein, 55% carbohydrate and 30% fat. There was no difference in weight loss or HbA1C between groups. Adherence to the protein and carbohydrate targets was challenging for participants in the study, with negligible difference in macronutrient intake between treatment groups from six months onwards, emphasising the value of having incorporated a measure of dietary adherence in the study design (263).

Larsen et al, 2011, conducted a shorter study of ninety-nine overweight or obese individuals with T2DM. A three-month 30% energy restriction period was followed by a nine-month weight maintenance phase. The high protein diet contained 30% protein and the low protein diet, 15%. While there were significant differences in protein intake this amount to a difference of 7.5% between groups rather than the planned 15%. There was no difference in weight loss or HbA1C between treatments (264).

As with carbohydrate content of diet, the wide variety of study designs and variable quality limits the usefulness of meta-analyses for studies of macronutrient composition. Two of the largest well controlled RCT’s examining high protein diets have demonstrated no significant effect on weight loss or on HbA1C.

**The Effect of Fat on Weight Loss and Glycaemia**

McAuley et al, 2006, compared the effect of three diets; high carbohydrate, high fat and high protein, in insulin resistant obese women over 12 months (265, 266). Both the high protein and high carbohydrate groups had a fat content of 30% while the high fat group were prescribed a low carbohydrate diet aiming for under 20g/day for two weeks and increasing to 50g/day thereafter. The study was remarkable for the assessment of adherence, the inclusion of measures of glucose homeostasis, a low dropout rate and two lower fat dietary controls. Though the high carbohydrate diet appeared to produce more weight loss by six months, given weight loss from two to four months the authors
concluded that underreporting of energy intake was the likely explanation for this result. Adherence to the diet prescription was maintained at six months, but by 12 months, it had declined. There was no difference in weight between groups over the 12 months interval. There was no difference in fasting insulin, fasting glucose or two-hour post OGTT glucose at any time point.

Tobias et al, 2015, performed a meta-analysis and systematic review of 53 studies of RCT’s of low fat diet interventions (267). Pooled estimated for weight loss appeared to be greater with high fat interventions. As with meta-analyses of protein and carbohydrate intake, the heterogeneity was high. However, when only studies that included calorie prescriptions in both intervention and comparator groups were included there was no difference between low fat and control diets.

In conclusion, the evidence to date does not suggest an effect of macronutrient composition of a diet on weight loss or glycaemic outcomes, independent of calorie restriction. Differences in adherence to diets of differing macronutrient composition may be due to the palatability, feasibility of implementing the diet, and the cost of a diet. However, when adherence to a dietary energy prescription is achieved, the macronutrient content of a diet does not alter weight loss or glycaemia.

1.6.3.4 The Schedule of Calorie Restriction

There are many examples of diets that vary in schedule. Religious fasts, such as once weekly food abstinence or fasting during the month of Ramadan, have been around for hundreds of years. Scheduled intermittent diets have recently become more popular with the lay public. What evidence is there to support the use of diets that vary in schedule over and above a calorie restriction? Much of the evidence in favour of schedule-specific effects of dieting come from animal studies. The most studied schedules of calorie restriction are; complete ADF, IF, and time-restricted feeding.

The study of different schedules of restriction may be fruitful in two ways; first to evaluate if adaptive thermogenesis is diminished by brief periods of fasting interspersed with adequate energy intake for requirements. Secondly, interrupting fasting may improve glycaemia, lipid profile and FM loss compared to equivalent weight loss with continuous restriction, due to differences in substrate utilization.
Animal Studies of Scheduled Calorie Restriction

Animal studies enable more detailed and invasive studies than are possible in humans. In this respect they are essential and complementary to human dietary intervention studies, if done well. Unfortunately, there are serious limitations with the study of different schedules of dietary intervention in the animal literature. A detailed table of 15 RCT investigating the effect of altered feeding schedule on diverse outcomes is available in appendix A. The most pervasive methodological limitations relate to the statistical analysis, biological heterogeneity and the diet intervention.

Time Restricted Feeding Studies

Some interesting insights into possible beneficial effects of a specific schedule of feeding/fasting come from studies of time-restricted feeding in rodents. Hatori et al, 2012, studied mice for 100 days that were allowed access to food either ad libitum or only during an 8-hour light phase (245). Food intake over 24 hours was the same in the time restricted versus the ad libitum group. Time restricted feeding was associated with widespread alteration in the expression of enzymes that regulate gluconeogenesis, glycolysis, fatty acid oxidation, fatty acid desaturation, fatty acid synthesis and nucleotide biosynthesis was seen in the liver. There was also increased expression of uncoupling protein in brown adipose tissue in the time restricted mice. These changes were associated with improved glucose tolerance, reduced hepatic steatosis and less weight gain in the time restricted mice despite similar food consumption. In addition, there was an increase in nocturnal energy expenditure in the time restricted mice related to increased physical activity. Thus, it is possible that the relative difference in energy balance rather than feeding schedule may account for the difference observed above. Nonetheless, the finding that known circadian regulators of cellular metabolism were significantly altered with different schedules of feeding in mice is an important one. This provides a mechanistic basis for regulation of intermediary metabolism and energy expenditure on a circadian timescale. These findings have yet to be replicated in humans with RCT’s.
Intermittent Fasting in Animal Studies:

Complete ADF, involving alternating *ad libitum* intake and a complete fast daily, have been assessment in thirteen rodent studies (268-270),(271-278).

**Weight**

All thirteen studies reported on weight change during the diet intervention. Weight loss increased in both the *ad libitum* controls and intervention groups in eleven studies (268-272, 274-277, 279, 280). Of the other two, weight decreased by 5% from baseline in the ADF groups over a four-week period in one and by 6% in the ADF group with 33% calorie restriction on feeding days over a four-week period (273). Changes in weight were consistent with the calories provided across all studies (appendix A). In the only study that demonstrated a matched calorie intake between a CDR an IF group, weight gain of 68% was observed in both IF and CDR groups (279). Several studies noted gorging with ADF on feeding days, thus the calorie intake in an ADF group was often greater than half that of the *ad libitum* group and in two studies exceeded the intake in the *ad libitum* group (271-274, 276),(272, 274).

**Body Composition**

Body composition was assessed in three studies using MRI (273-275). Intermittent fasting was associated with a reduction in FM compared with an *ad libitum* control by Boutant *et al*, 2016. Gotthart *et al*, 2016, found lower FM and lean mass with low-fat ADF compared to high-fat *ad libitum* intake, but no difference low-fat ADF and a low-fat continuously restricted control. Thus, body composition changes relate more to calorie intake rather than schedule of feeding during weight loss.

**Assessment of Glucose Homeostasis**

Nine studies examined some aspect of glucose homeostasis(268, 270-275, 279, 280). Methods of assessment varied widely, from urine glucose measurement to intraperitoneal glucose tolerance tests (268, 270-272), (275, 279, 280), (274),(273).

Fasting insulin was no different in the ADF group compared to the *ad libitum* control in three studies and higher in one study (271, 275, 280),(279). In this study, ADF resulted in greater weight gain than *ad libitum* intake. Fasting glucose was lower following ADF
compared with continuously fed controls in three studies and no different in one study, where the IF group gained the same weight as the control group (270-272),(268).

Oral glucose tolerance tests and insulin tolerance tests were performed in the same two studies and produced discordant results (273),(275).

A hyperglycaemic euglycaemic clamp was performed by Boutant et al, 2016, after 12 weeks of either ADF or ad libitum intake and demonstrated significantly increased glucose disposal with ADF. The ad libitum mice in this study gained approximately three times more weight than the ADF group. The additional weight gain, and thus insulin resistance, in the ad libitum arm is the most likely explanation for these findings(274).

Thus, the apparent superiority of ADF over CDR could be reconciled by excessive weight gain in the control group, irrespective of the assessment method used.

Central Regulation of Energy Metabolism

Three studies measured endogenous leptin concentrations in response to different schedules of fasting (269, 273, 279). Two studies found leptin levels that were no different than continuously fasted controls with weight gain of 10% (273, 277). Bonorden et al, 2009, demonstrated that leptin was lower with intermittent fasting but weight gain and FM change was greater in this study which may accounts for the discrepant findings. Chausse et al, 2014, studied the effect of a leptin injection on food intake, and hypothalamic signalling following an ADF compared with an ad libitum fast (276). ADF rats were much more sensitive to the appetite suppressant effect of leptin and had increased expression of orexigenic neurotransmitters in the hypothalamus. Poor weight gain matching was a notable limitation of this study.

Gotthardt et al, 2016, also studied hypothalamic neurotransmitter expression in response to ADF or ad libitum diets (273). They found increased expression of orexigenic NPY, lower levels of anorexigenic POMC and no difference in AgRP in response to ADF compared with ad libitum intake. Weight gain was not matched between ADF and ad libitum groups in this study either. Nonetheless, these studies are an illustration of how animal models may further an integrated understanding of central energy homeostasis in response to dietary interventions.
Taken together, the existing data from animal studies of ADF supports the importance of the magnitude of calorie restriction but, because of study design, has not adequately addressed the question of whether differences in dietary schedule independent of calorie restriction offer any unique advantage. As a result, the metabolic differences between feeding regimens such as glycaemic responses to a glucose tolerance test, weight change, serum triglyceride content, oxidative metabolism or regulation of hepatic diacylglycerol content may be due to differences in energy intake rather than the dietary schedule (276-280),(276),(275),(278). A small number of human studies have explored the effects of different dietary feeding schedules

**Limitations of Animal Studies**

**Statistical Analysis**

Despite a well-developed hypothesis, and the prior publication of methods used to evaluate the pre-specified outcomes, no study provided either an *a priori* or *post-hoc* power-calculation for their study outcomes (245, 268-272, 274-281). Many studies assess multiple outcomes between multiple groups, without a pre-specified primary outcome or any adjustment for multiple outcomes (245, 273, 275, 277, 279, 280, 282). This increases the risk of type I error, especially without power calculations to temper confidence in the conclusions drawn. Some of the studies involved complex factorial designs (277, 278, 281), but the statistical analysis did not reflect this design. Descriptive analyses of between group differences without statistical testing of these observations is common. Rather, authors present statistics for within group change with time or between group comparisons at the end of the intervention. This method does not assess between-group difference in change from baseline (277, 278, 280).

**Biological Heterogeneity**

There is considerable heterogeneity in the biology of the animals being studied. All studies involved either mice or rats. Across the thirteen studies, seven genetically different rodent models were used. The age of the rodents at the start of the dietary intervention varied from 3 weeks to 12 weeks. As the dietary interventions lasted up to 43 weeks, the age, growth trajectory and physiology of the rodents simultaneously undergoes change (279). As in humans, these age-related changes may be associated with
the primary outcome, e.g. weight gain, prostate cancer, or the energy requirements of the rats and may lead to discordant findings between studies exploring the same intervention.

Dietary Intervention

The chief limitation in inferring an effect of schedule as distinct from the calorie restriction is in the design of the dietary interventions. No rodent studies based calorie prescription on an assessment of energy expenditure or other surrogate of energy expenditure, despite the availability of sophisticated indirect calorimetry methods for rodents. Instead prescriptions were commonly based on mean unrestricted or ad libitum intake in a control group. In every study, ad libitum intake caused weight gain. Therefore, restrictive interventions that are a proportion of the ad libitum intake, but still more than weight maintenance requirements, also caused weight gain. Thus, most rodent studies investigate the effect of reduced weight gain, rather than weight loss with different schedules of restriction (270-272, 274, 275, 279, 280).

In summary, rodent studies have described a positive effect of differing schedules of calorie restriction on glycaemia, weight loss and body composition. However, these findings should be interpreted with caution as there are significant limitations in the methodology, not least inadequate calorie matching in the continuously restricted control groups.

1.6.3.6 Human Studies of Scheduled Calorie Restriction

Time Restricted Feeding

Stote et al, 2007, and Carlson et al, 2007, compared the effect of time restricted feeding, consumption of total daily food between 17:00 and 21:00, to a calorie matched three-meals-per-day diet on weight loss, body composition and glycaemia (116, 283)(Table 1.2). They demonstrated weight loss and a reduction in FM with a single meal per day. There was also an elevation in fasting glucose and worsening of glucose tolerance on OGTT. Based on the OGTT there appeared to be a decreased first phase insulin response and a relative impairment in insulin sensitivity in the once a day meal group. A confounding aspect of this study was the substantial difference in meal size the evening prior to an OGTT or the fasting glucose sampling. The reduction in weight despite an “isocaloric” diet is also unusual, particularly as the dietary prescriptions were weight
based. This implies that adherence to an exceptionally large meal may have been overestimated. The authors comment that, given the choice, participants on the one-meal per day group would have chosen to eat less. There was no effect of meal schedule on fasted morning plasma insulin, HOMA-IR, glucagon, leptin or adiponectin.

LeCheminant et al, 2013, found that if night-time feeding is eliminated between the hours of 19:00 and 06:00 total daily energy intake was reduced compared to controls (284), suggesting an impact of altered schedule on energy intake, though REE was not measured in this study. A recent short-term study of breakfast omission indicated that morning fasting compared with a standard breakfast was not associated with a compensatory increase in eating later in the morning and was associated with a lower total energy intake over the course of the whole day (285).

In summary, time-restricted-feeding within a 24-hour period, lowers total daily energy intake compared with isocaloric controls. Time restricted feeding compared to continuously restricted diet for weight loss has not yet been studied in human trials.
Table 1.2. RCT’s of intermittent versus continuous energy restriction in humans.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Population</th>
<th>Duration (Weeks)</th>
<th>Diet Prescription</th>
<th>Sample Size</th>
<th>Basis of Diet Prescription</th>
<th>Other Outcomes</th>
<th>Weight Loss</th>
<th>Body Composition</th>
<th>Assessment of Glycaemia</th>
<th>Glycaemia</th>
<th>Energy Expenditure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trepanowski et al (286).</td>
<td>2017</td>
<td>Obese women</td>
<td>Wt. loss: 72</td>
<td>ADF: 25% required/125% required</td>
<td>ADF: 34 CDR: 35 Control: 31</td>
<td>Total Energy Expenditure by Doubly labelled Water</td>
<td>NA</td>
<td>No different</td>
<td>No different</td>
<td>Assessed by DXA at 6 months, 3 months into weight loss. No difference between groups</td>
<td>No different</td>
<td>TEE using doubly labelled water Baseline and 3 months into weight maintenance</td>
</tr>
<tr>
<td>Catenacci et al. (244)</td>
<td>2016</td>
<td>Obese</td>
<td>Wt. Loss: 8</td>
<td>ADF: AL/Complete Fast CDR: Reduction of 1675 kJ required</td>
<td>ADF: 14 CDR: 12</td>
<td>Predictive Equation: FFM calculation from DXA: 372 + 23.9xFFM.</td>
<td>No different</td>
<td>No different</td>
<td>No significant change</td>
<td>No difference in EI between ADF and CDR.</td>
<td>Fasting glucose was lower in ADF group. Difference between groups in energy intake. Fasting Insulin No difference in other measures</td>
<td>No difference</td>
</tr>
<tr>
<td>Varady et al. (243).</td>
<td>2013</td>
<td>Normal weight/overweight</td>
<td>12</td>
<td>ADF: 25% of required/AL Control: AL</td>
<td>ADF: 16 AL: 16</td>
<td>Mifflin equation</td>
<td>ADF: ↓LDL particle size/ TG/ leptin ↑ adiponectin</td>
<td>ADF: 5.2 kg weight loss AL: No change</td>
<td>DXA FM loss 3.6 kg (of 5.2 kg) in the ADF group. No change in controls</td>
<td>None</td>
<td>Not measured</td>
<td>N/A</td>
</tr>
<tr>
<td>Author</td>
<td>Year</td>
<td>Population</td>
<td>Duration (Weeks)</td>
<td>Diet Prescription</td>
<td>Sample Size</td>
<td>Basis of Diet Prescription</td>
<td>Other Outcomes</td>
<td>Weight Loss</td>
<td>Body Composition</td>
<td>Assessment of Glycaemia</td>
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<tr>
<td>Harvie et al(287)</td>
<td>2013</td>
<td>Overweight/Obese Women</td>
<td>12</td>
<td>IF-Carb: 30% required/100% required + Carb Restriction</td>
<td></td>
<td></td>
<td>Scholfield Equation and Physical Activity assessment</td>
<td></td>
<td></td>
<td>Assessed by BIA</td>
<td>Fasting insulin</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Wt. Loss: 12 Maintenance: 1</td>
<td></td>
<td>IF-PF: 30% required/100% required + AL Protein and Fat</td>
<td>IF-Carb: 37</td>
<td></td>
<td>No Difference: Leptin, adiponectin, IL-6, IGF-1, TNF-alpha, LDL, TG, BP</td>
<td></td>
<td></td>
<td>FM Reduction: IF-Carb &gt; CDR IF-PF, no different</td>
<td>Fasting glucose</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>CDR: 75% Required</td>
<td>IF-PF: 38</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HbA1C</td>
<td>HOMA-IR</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>CDR: 40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
<td>Serum insulin and HOMA improved in IF group</td>
<td>N/A</td>
</tr>
<tr>
<td>Varady et al(288)</td>
<td>2011</td>
<td>Overweight/Obese</td>
<td>12</td>
<td>ADF: 25% required/AL</td>
<td>ADF: 15</td>
<td></td>
<td>The Mifflin Equation</td>
<td></td>
<td></td>
<td>N/A</td>
<td>N/A</td>
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<td></td>
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<td></td>
<td></td>
<td>CDR: 75% required</td>
<td>CDR: 15</td>
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<td>N/A</td>
<td>N/A</td>
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<td>AL + Ex: 15</td>
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<td></td>
<td>N/A</td>
<td>N/A</td>
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<td></td>
<td></td>
<td>AL: 15</td>
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<td></td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Harvie et al(289)</td>
<td>2011</td>
<td>Pre-Menopausal Women Overweight/Obese</td>
<td>24</td>
<td>IF: 25% required 2/7 per week AL 5/7</td>
<td>IF: 53</td>
<td></td>
<td>Schofield Equation and Physical Activity assessment</td>
<td></td>
<td></td>
<td>No different</td>
<td>Fasting insulin</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CDR: 54</td>
<td></td>
<td>Comparable changes in adiponectin and IGF-1</td>
<td></td>
<td></td>
<td>HOMA-IR</td>
<td>Greater decrease in insulin, HOMA-IR with IF</td>
<td>N/A</td>
</tr>
<tr>
<td>Author</td>
<td>Year</td>
<td>Population</td>
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<tr>
<td>Ash et al (290).</td>
<td>2003</td>
<td>Men T2DM</td>
<td>Wt. Loss: 12</td>
<td>IF: Liquid Meal 4 days per week/Ad Libitum Otherwise Pre-portioned meals (PPM): 6900 kJ/day Self-selected meals (SSM): 5860-7112 kJ/day</td>
<td>IF:14</td>
<td>No different</td>
<td>N/A</td>
<td>No different</td>
<td>Assessed with DXA. Slight reduction in FM in PPM group (2.2%) compared with SSM (0.9%) but not with IF.</td>
<td>HbA1C</td>
<td>No different</td>
<td></td>
</tr>
<tr>
<td>Williams et al (291).</td>
<td>1998</td>
<td>T2DM Overweight /Obese</td>
<td>20</td>
<td>IF-1: 2092 kJ 1 day per week for 15 weeks/6280 - 7530 kJ daily otherwise IF-5: 2092 kJ 5 consecutive days every 5 weeks/6280 - 7530 kJ daily otherwise Standard Behavioural Therapy (SBT): 6280 - 7530 kJ/day</td>
<td>IF-1: 18 IF-5: 18 SBT: 18</td>
<td>Number within the 1500-1800 range picked based on initial weight</td>
<td>No Difference: TG, LDL, HDL, Tchol</td>
<td>SBT: 10.4 kg 1-day: 9.6 kg 5-days: 10.4kg</td>
<td>N/A</td>
<td>HbA1C</td>
<td>No different</td>
<td>Fasting Insulin</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Author</th>
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<th>Body Composition</th>
<th>Assessment of Glycaemia</th>
<th>Glycaemia</th>
<th>Energy Expenditure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hill et al. (292)</td>
<td>1989</td>
<td>Women Moderately Obese</td>
<td>12</td>
<td>ADF: 2510 kJ/7531 kJ No Exercise</td>
<td></td>
<td>ADF No-Ex: 10</td>
<td>Fixed Prescription Not Based on Individual Energy Requirements</td>
<td>Greater reduction in Tchol with alternating calorie regimen.</td>
<td>No different</td>
<td>By underwater weighing. No difference</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>deGroot et al. (293)</td>
<td>1989</td>
<td>Obese Women</td>
<td>9</td>
<td>IF: 50% Required/100% Required</td>
<td>IF and IF-B: 5</td>
<td>24hr EE by chamber calorimetry performed a few months before starting the diet.</td>
<td>Weight loss greatest with CDR in keeping with EI.</td>
<td>IF: 3.9kg IF-Bread: 4.9kg CDR: 5.8kg</td>
<td>By underwater weighing. No Difference in FFM</td>
<td>N/A</td>
<td>N/A</td>
<td>Sleeping Energy Expenditure Reduced in CDR group by 7% after 4 weeks</td>
</tr>
<tr>
<td>Arguin et al. (294)</td>
<td>2012</td>
<td>Obese Post-menopausal Women</td>
<td>Wt. Loss: 15 Follow up: 52</td>
<td>Daily weight and adjustment of calorie intake to ensure 1% weight loss per week.</td>
<td>IF: 12 CDR: 10</td>
<td>Based on Weight Lost and adjusted during the study.</td>
<td>No significant difference in weight lipids (HDL, LDL, Tchol, TG) or physical activity levels.</td>
<td>No different</td>
<td>Greater FFM loss in IF group over time. DXA</td>
<td>Fasting Glucose</td>
<td>No different</td>
<td>REE at baseline, 5 weeks, 15 weeks 1 year. No difference between groups.</td>
</tr>
<tr>
<td>Author</td>
<td>Year</td>
<td>Population</td>
<td>Duration (Weeks)</td>
<td>Diet Prescription</td>
<td>Sample Size</td>
<td>Basis of Diet Prescription</td>
<td>Other Outcomes</td>
<td>Weight Loss</td>
<td>Body Composition</td>
<td>Assessment of Glycaemia</td>
<td>Glycaemia</td>
<td>Energy Expenditure</td>
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<td>-------------</td>
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</tr>
<tr>
<td>Byrne et al. (295)</td>
<td>2018</td>
<td>Obese Men</td>
<td>Initial: 4</td>
<td>CDR: 16</td>
<td>26</td>
<td>CDR: 25</td>
<td>Weight Loss was declared to be greater with the intermittent energy restriction compared to the Continuous group</td>
<td>CDR: 8.5 kg</td>
<td>IF: -13.4 kg</td>
<td>N/A</td>
<td>N/A</td>
<td>REE at Baseline and monthly thereafter.</td>
</tr>
</tbody>
</table>

AL: Ad libitum; DXA: Dual energy x-ray absorptiometry; HbA1C: Glycated haemoglobin; TG: Triglycerides; HDL: High density lipoprotein; LDL: Low density lipoprotein; IGF-1: Insulin-like growth factor 1; IL-6: Interleukin-6; TNF-α: Tumour necrosis factor-α; TChol: Total Cholesterol. EI: Energy Intake. IF: Intermittent Fasting. CDR: Continuous Daily Restriction; HOMA-IR: Homeostatic model assessment of insulin resistance; BIA: Bioimpedence analysis; N/A: Not assessed
Intermittent fasting versus Continuous Daily Restriction

Intermittent fasting, including ADF, compared to continuous daily restriction (CDR) has been studied in 12 randomised controlled trials in humans (Table 1.2). The heterogeneity of these studies is both a strength and a weakness, offering an insight into the permutations of intermittent fasting that may be most effective, but with results that have not always been reproduced. These studies vary with respect to; study duration, population studied, diet prescription, type of control, estimation of calorie requirements, weight lost, energy expenditure assessment, body composition assessment outcomes, and metabolic assessment. Of these study characteristics, inadequate calorie matching between diet prescriptions in the control and intervention groups is perhaps the greatest limitation of the existing intermittent energy restriction literature so far. This is in keeping with the intermittent fasting literature in animals, and the bariatric literature concerned with diabetes remission.

Study Duration

Trials of IF versus CDR varied from nine weeks to one year(293),(286, 294). Three studies contained an initial weight stabilisation period varying from one to three weeks (287, 290, 295). The duration of active weight loss was most commonly 12 weeks in duration, but between nine weeks and six months(243, 287, 288, 290, 292), (293),(286, 289). In studies that contained a weight maintenance period following weight loss, weight regain was invariably seen.

Study Population

The studied populations varied chiefly with respect to gender, baseline weight and the presence of diabetes. As with many dietary intervention studies, there was a preponderance of women-only studies which limits the validity of these findings to REE and body composition results in men. Change in REE during the menstrual cycle, with menopause or with the oral contraceptive pill, reduces the validity and precision of assessments of energy expenditure by indirect calorimetry or doubly labelled water (296),(297),(298). None of the women-only RCT’s that assessed energy expenditure attempted to time measurements of energy expenditure with the menstrual cycle (286, 292-294).

Six studies examined obese participants, four studied overweight or obese participants, and two studied normal or overweight participants. The physiology of the established
obese, compared with overweight or normal weight participants is different. In particular, weight loss attempts in the normal weight individuals can result in preferential fat mass regain not seen in the obese, this may affect observed changes in body composition during weight loss followed by weight maintenance (110). Only two RCT’s studied individuals with T2DM. The sample size per treatment group ranged from five to 54 participants(293), (290, 291), (289).

Diet Prescription: Intermittent Fasting Group

The calorie prescription for IF involves the duration of fasting, the calorie prescription, and the non-fasting calorie prescription.

The duration of the fasting component of each cycle of an intermittent fast ranged from 24-hours to two weeks (299),(295). The proportion of time spent fasting during the intervention period varied from 14% to 50% (295),(291). It is not yet clear whether time spent in negative calorie balance, independent of degree of negative energy balance, is an important feature of intermittent energy restriction.

With regard to the fasting day prescription, one study combined a complete fast with *ad libitum* intake on alternate days (244). Six of the twelve studies used a fasting day prescription of 25-30% of requirements, while one used a fasting day prescription of 50% requirements(243, 286-289, 295). Three of the studies prescribed calorie intakes as fixed calorie counts for all participants(290-292). One study did not prescribe a fixed calorie intake but adjusted calorie intake on an individual basis to ensure 1% weight loss per week (294). Clearly these are very different diets that will produce very different degrees of weight loss. The prescription of a hypocaloric diet as a proportion of estimated daily energy requirements, rather than the use of fixed calorie prescription is preferred. It avoids variation in the degree of negative calorie balance that inevitably occurs in populations of varying size and thus total energy expenditure.

The calorie prescription for the non-fasting components of the intermittent fasting group all have in common a calorie intake that is designed to be greater than or equal to the maintenance requirements of the individual. Seven studies controlled for the non-fasting day calorie intake, and five used *ad libitum* intake on non-fasting days(286, 287, 291-295),(243, 244, 288-290). Of those studies that prescribed energy intake, one study prescribed 125% daily energy requirements, three prescribed 100% daily energy
requirements, and the remaining three prescribed fixed calorie intakes that could not be related to participants energy requirements (286),(287, 293, 295),(290-292).

The combination of overfeeding or *ad libitum* intake with underfeeding in an intermittent fasting diet, makes interpretation of metabolic outcomes and any weight change outcomes difficult. Depending on the hypothesis, *ad libitum* intake may be more appropriate, but it does not allow for comparison with a calorie matched CDR control, being unpredictable.

**Diet Prescriptions: Control Group**

The diet prescription for the CDR control group resembled that of the IF group in terms of how it was prescribed, i.e. fixed calorie count or proportion of estimated daily energy intake, and what the estimates were based on. All but one of the studies had a CDR control, Varady *et al, 2013*, used an *ad libitum* control (243). Calorie matching between the control group and the intermittent energy restriction group was present in only four studies, when *ad libitum* prescriptions in either the IF or CDR group were excluded (286, 287, 295), (292). Assuming *ad libitum* intake represents 100% requirements an additional two studies could be considered calorie matched (288, 289).

**Estimation of Energy Requirements**

The method of determining an individual’s total daily calorie requirements is important as it is the foundation of the hypocaloric intervention (section 2.3.3). Measurement of total daily energy expenditure by doubly labelled water or REE by indirect calorimetry, combined with estimates of physical activity measures are more accurate and valid than predictive equation of energy expenditure (chapter six). Of the nine studies that tailored energy prescriptions to individuals; one study used doubly-labelled water, two studies used indirect calorimetry combined with a physical activity measure, five studies used predictive equations, one study constantly varied the diet prescriptions based on the weight loss achieved (286), (293, 295), (243, 244, 287-289), (294). This last approach quite obviously introduces a major bias in the objective assessment of differential weight loss achieved. However, the hypothesis being tested in this study was that IF would produce different changes in body composition with comparable degrees of weight loss using either IF or CDR, and so was more appropriate to addressing this question.
Study Outcomes: Weight Loss

Eight out of twelve studies identified no difference in weight loss between the IF and CDR groups (244, 286, 288-292, 294). Only one of these matched calorie intakes between groups (286). Of the four that did find a difference, two did not match calorie intake and weight loss was commensurate with the prescribed calories (243, 293). Harvie et al, 2013 found that a higher proportion of participants in the IF group achieved the pre-specified target of 5% weight loss from baseline over 12 weeks (287). In one of the IF groups, IF-Carb (Table 1.2), participants ate 68%, rather than 100% of predicted requirements on their non-fasting days and lost most weight, while the IF-PF group ate 77% rather than 100% of requirements and lost more weight that the CDR group who consumed the highest proportion of calories overall. Byrne et al, 2018, found a difference of 5.9 kg weight loss in the IF group compared to the CDR group over 16 weeks. Remarkably, despite all participants completing daily food diaries for 16 weeks, the energy intake data was not analysed, and mere diary completion was taken as a measure of dietary adherence. This recent study was published five years after that of Harvie et al, 2011. Thus, the presence of a known confounder for these studies of IF vs CDR was unaccounted for. This is the only human RCT with a calorie matched control to suggest that IF confers greater weight loss than CDR (295).

Study Outcomes: Body Composition Assessments

Whether or not IF offers advantages in terms of preferential FM, rather than lean mass, lost has been examined in all but two studies (244, 288). Underwater weighing, air displacement plethysmography, dual x-ray absorptiometry (DXA) and bio-impedance analysis were the methods used to assess body composition change. These techniques are discussed in detail in section 2.3.1. Two studies did not assess body composition, six studies found no difference in body composition (244, 286, 289, 290, 292, 293).

The studies that found no difference in body composition with IF compared to CDR, had some confounding factors. One study running for 72 weeks had a 40% drop out in the ADF group; another had only five participants per intervention group, and four studies did not match calorie intake in the IF and CDR groups (286), (293), (289), (290),(244), (292).

Four studies did find a difference in body composition change with IF (243, 287, 294, Byrne, 2018 #451), (295). Arguin found greater FFM loss with IF, as assessed by DXA
Though it is unclear if calorie intake was matched in both groups in this study, the weight loss was comparable. The absolute difference between treatment groups over the intervention period was 0.6 kg. This reduction in lean mass is of questionable clinical significance and close to the lower limit of detection for modern DXA scanners (300). Byrne et al, 2018, used air displacement plethysmography and found greater FM loss with intermittent fasting (295). Harvie et al, 2013, found greater reduction in FM using bio-impedance analysis (BIA) with the IF and carbohydrate restriction group, compared to CDR. However, this was not found with the IF and ad libitum protein and fat group, compared to CDR (Table 1.2) (287). This is likely due to differences in calorie intake between the two different IF groups. Varady et al, 2013, found greater FM loss of 3.6 kg with IF, assessed by DXA. However, in this study, the control group did not lose any weight (243).

In summary, most studies demonstrated no change in body composition with IF. One study that achieved comparable weight loss between groups demonstrated only a minor change in lean mass with IF. Studies that have demonstrated a preferential reduction in FM with IF, are mainly limited by unmatched, or unknown calorie intake between groups.

Study Outcomes: Metabolic Assessment.

Seven studies assessed at least one marker of glycaemia. These included fasting insulin, fasting glucose, an intravenous glucose tolerance test or HbA1C. Three studies identified improvements in glycaemia with intermittent fasting (244, 287, 289). Catenacci et al, 2016, found an improvement in fasting glucose, but no difference in fasting insulin or the glycaemic response to an intravenous glucose tolerance test. Harvie et al found reductions in serum insulin and HOMA-IR in two studies (287, 289). One of these studies assessed fasting insulin, HOMA-IR and triglycerides on the morning after a two-day fast, and within two days of resuming a normal diet, in a subgroup of participants. This demonstrated a reduction in insulin, HOMA-IR and triglycerides after fasting of 23%, 29% and 18% respectively in fifteen IF participants, with no significant changes in the nine CDR participants. Though only a small subgroup analysis it provides important preliminary information in support of a possible benefit of intermittent fasting. No other studies timed their assessments of glucose homeostasis in this way.
In addition to glycaemia, seven studies assessed lipid profiles (243, 244, 287, 288, 291, 292, 294). Varady et al, 2011 demonstrated a reduction in triglycerides only with IF in one study where participants had comparable weight loss (288). In a second study, the only study with a metabolic outcome as a primary end-point, IF was associated with a reduction in LDL cholesterol, triglycerides and an increase in LDL particle size (243). However, the control group did not lose any weight. Though the LDL particle size did increase significantly over 12 weeks in the IF group but not in the CDR group, the difference between groups for this change was not statistically significant. Hill et al, 1989, demonstrated a reduction in total cholesterol in the IF group, the only lipid assessed, (292), again with comparable weight loss between groups.

Leptin, adiponectin or ghrelin have been assessed in three studies. Compared to an ad libitum control without weight loss, IF reduced leptin and increased adiponectin (243). When compared to a CDR with a lower calorie intake than the IF group, there was no difference in either adiponectin or leptin (289). Catenacci et al, 2016, found no difference in ghrelin or leptin between treatments (244).

**Study Outcome: Energy Expenditure**

Differences in energy expenditure between CDR and IF have been examined in five RCTs (244, 292-295). Four used indirect calorimetry to assess REE. One used chamber calorimetry to assesses energy expenditure during sleep, cycling and sedentary activity (293). Catenacci et al, 2016, measured REE using a canopy hood indirect calorimeter before and after eight weeks of intervention. Results were adjusted using regression for FM and FFM. There was a trend toward a lower REE in the CDR group that did not achieve statistical significance. This may have been due to the relative smaller calorie deficit in the CDR group (244).

De Groot et al, 1989, studied 27 women in three studies. The design was complex (Table 1.2). Room calorimetry was conducted at baseline, week one and week four. The reduction in sleeping energy expenditure only was lower in the CDR group (10%) compared with the ADF group (3.7%) after 20 days of dieting. Due to differences in body composition, scaling by dividing sleeping energy expenditure by FFM was performed. Though this produce significant differences between groups, this method of allometric scaling is considered flawed (section 2.3.2) (293).
Arguin *et al*, 2012, examined REE in twenty five post-menopausal women randomised to IF consisting of five weeks of low-calorie diet sufficient to induce 1% weight loss per week, alternating with five weeks of weight maintenance until 15 weeks low-calorie diet was achieved. The CDR group performed 15 weeks of the low-calorie diet continuously. REE was performed after the first five weeks of low-calorie diet in both groups, and again at 15 weeks. There was no baseline measurement. Total REE for 24 hours, without adjustment for body composition, were presented. Failure to scale the energy expenditure measurements and choosing to perform the first calorimetry after five weeks of the intervention may have obscured important differences between groups (294). Hill *et al*, 1989, measured energy expenditure before and after the 12-week weight loss intervention, and adjusted REE using FFM. They found no difference in body composition between groups (292).

Within the last 12 months, Byrne *et al*, 2018, conducted a study that was powered to detect differences in REE between IF and CDR groups (295). They were the only group to study the impact of IF vs CDR on adaptive thermogenesis explicitly. This study has several strengths, the use of REE measurements to prescribe energy intake and DXA to assess body composition, repeated calorimetry assessments to adjust energy intake during the study, adequate power to detect changes in REE, calorie matched energy prescriptions, the use of a male only population to reduce variance in REE. The authors found greater reduction in energy expenditure for predicted weight in the CDR group compared to the IF group, this appears to support the concept that an interrupted fasting regimen attenuates adaptive thermogenesis occurring in the context of calorie restriction.

However, there are some substantial limitations with the calculation of adaptive thermogenesis and the dietary intake assessment methods in this study. As outlined in section 1.6.3.4.7.3, no assessment of dietary energy intake was made during the study. Adaptive thermogenesis was calculated using two different equations. The first method, used regression equation at baseline to develop an equation of predicted REE based on FFM and FM results. Though both groups appeared well matched, the authors found group allocation at baseline was a significant predictor of energy expenditure, indicating bias in group allocation at the start. Therefore, separate regression equations were developed for each treatment group prior to the start of the intervention. This clearly introduces bias, as there is no logical reason why allocation to a treatment group should alter REE prior to starting the treatment. The second method involved the application of
a single, previously published regression equation to both groups, this was developed from a different population. As outlined in chapter five, predictions of REE have been shown to have variable validity in different populations. Predicting energy expenditure from baseline measurements for use in a repeated measures design in the same individuals reduces variance and improves validity of prediction equations. The discordant results between these methods for assessing adaptive thermogenesis raise questions as to the validity of both. A more valid approach would have been the use of a single regression equation developed from all participants irrespective of randomisation status.

In summary, the few RCT’s of IF and CDR in humans demonstrate considerably heterogeneity in method. Potential advantages of intermittent fasting include, improvements in lipids, glycaemia, a reduction in adaptive thermogenesis with weight loss and preferential FM reduction. The studies to date have yielded inconsistent results with significant limitations including; use of a control group that is not calorie matched, the use of calorie prescriptions that are not individually tailored and the absence of energy expenditure assessments. Where energy expenditure measurements have been included, inappropriate allometric scaling has been a potential confounder in some studies.

1.7 Summary

The rise in diabetes in the past 50 years has paralleled the rise in obesity. Treatment of obesity with bariatric surgery has demonstrated the possibility of diabetes remission. More recently this has been shown with very low-calorie diet intervention. The discovery of leptin, pioneered a greater understanding of the hormonal contribution to human energy homeostasis. Translating all this knowledge into effective and implementable dietary interventions for the treatment of both diabetes and obesity requires carefully conducted diet intervention studies. Furthermore, the response to energy restriction and the effect of adaptive thermogenesis in modifying the expected laws of energy balance require greater understanding. Adaptive thermogenesis is triggered by a sustained reduction in energy intake, through unclear mechanisms. If the sustained reduction in energy intake were interrupted using a different schedule of energy restriction, such as intermittent fasting, would this overcome the maladaptive effects of adaptive thermogenesis on weight loss, body composition and metabolic homeostasis? This hypothesis is the fundamental question of this thesis.
1.8 Thesis Outline

Chapter two of this thesis is a methodological overview incorporating the common methods used in the studies performed during this PhD. Chapter three is an account of in silico validation and precision testing of indirect calorimetry. Chapter four outlines two studies of test-retest reliability using indirect calorimetry. Chapter five is a cross sectional study assessing the relationship between body composition and REE in New Zealand ethnicities, the Predictions of Resting Energy Expenditure in Māori and Pacific populations (PREEMPt) study. Chapter six is a randomised control study of the risk of hypoglycaemia during IF in participants with diabetes, the IF-Hypo study. Chapter seven is the culmination of the preceding work and is a randomised controlled trial of the effect of continuous versus intermittent fasting on adaptive thermogenesis, the Changes in Resting Energy Expenditure with Different Schedules of Calorie Restriction (CREEDS) study. Chapter eight is the concluding discussion chapter.
2 Methodology

2.1 Introduction

In the previous chapter, the restriction of dietary energy intake as a treatment for diabetes and obesity was discussed, the role of adaptive thermogenesis in compromising weight-loss attempts was highlighted, and the potential benefit of altering the schedule of dietary intake to reduce adaptive thermogenesis during weight loss was proposed.

The first part of this chapter discusses the principles of measuring of REE by indirect calorimetry, as this method is central to four of the studies described in this thesis. This is followed by a discussion of the methodological considerations when measuring adaptive thermogenesis during weight loss. Finally, a description of the methods used in the thesis is presented.

The validation studies and test-retest reliability studies that form chapters three and four, respectively, were methodological studies that resulted in many changes to our testing protocols for indirect calorimetry. It is the revised indirect calorimetry protocols that are discussed in this chapter. The original testing protocols and the details of their refinement are presented in chapter four. The protocol for validation testing, which differs from that used for human measurements, is presented in chapter three.

2.2 Studying Energy Balance in Humans

Calorimetry means the measurement of heat energy. In the late eighteenth-century Antoine Lavoisier and Pierre Laplace measured the heat produced by a hamster in their custom-made calorimeter. By surrounding a ventilated chamber with ice and collecting meltwater produced as the animal radiated heat, heat production was measured. Lavoisier identified that heat production and oxygen consumption were linked and so proceeded to investigate oxygen consumption in man, documenting the quantity of oxygen produced at rest and observing that it increased after a meal and with exercise (301). Thus, practical calorimetry was born. The theory of how energy balance estimates are arrived at in clinical studies of obesity is described here. The practical measurement, materials and performance characteristics of the instruments used are described in section 2.4 below.
2.2.1 Physiology of Aerobic Metabolism and Breathing

The energy required for REE is produced by metabolism of macronutrients such as fat, carbohydrate and protein. The metabolism of these macronutrients proceeds through several well-described intermediate steps and culminates in a final common pathway, the respiratory chain (302). Several important advances in physics preceded the science of indirect calorimetry.

Boyle’s Law, Charles’ Law and Dalton’s Law enable descriptions of the quantity of gas standardised for temperature and pressure. The volume of a given quantity of gas will change with temperature and pressure, however when the temperature, pressure and the partial pressure of a gas are measured, volumes can be expressed in a comparable standardised way (303, 304). Thus, all gas volumes in indirect calorimetry are expressed at a standard temperature and pressure (STP), 273 K (0 C) and 101.3 kilopascals (kPA).

Avogadro’s law allows the relationship between the volume of gas at a standard temperature and pressure to be related to the molar mass of the gas (305). It is far easier to deal in STP volumes than interconverting to molar or mass quantities in indirect calorimetry. This is because the parameter of interest, energy expenditure in kilocalories per minute is calculating using STP gas volumes per min rather than consumption of molar or mass equivalents of oxygen, for example.

Dalton’s Law, fundamental to the respirometry equations used in indirect calorimetry, states that in a mixture of gases the pressure exerted by the mixture is equal to the sum of the partial pressures of the individual components of the mixture (306). These laws have been embodied in the Ideal Gas Law (307). This law states that the volume occupied by one mole of any gas at standard temperature and pressure is 22.4l.

Hess’ Law, based on the second law of thermodynamics, states that the net energy produced or consumed in a reaction is equal to the sum of the energy produced/consumed in its intermediate steps (308). Hess’ Law allows estimation of the net energy production of complete macronutrient metabolism without needing to measure or estimate all the intermediate steps involved in the process. Carbon dioxide (CO₂), water vapour (WVP) and energy in the form of adenosine triphosphate are produced in this process, and oxygen (O₂) is consumed. Depending on the substrate being metabolised, a different amount of energy, CO₂ and O₂ will be produced by complete metabolism (309). The
measurement of CO\textsubscript{2}, O\textsubscript{2} and energy production by complete oxidation of macronutrients has been studied now for over a century. The stoichiometric relationship between energy production, CO\textsubscript{2}, O\textsubscript{2} and WVP enables extrapolation of energy expenditure based on measurement of the gases (310).

2.2.2 Types of Calorimeter

The early calorimeter developed by Lavoisier and Laplace allowed measurement of heat production from aerobic respiration based on the weight of melting ice. Knowing the latent heat of melting ice, the rate of energy produced could then be calculated. This was the first direct calorimeter. Lavoisier’s later use of oxygen volumes to measure energy production was an example of an indirect calorimeter (311).

Direct calorimeters measure heat production from an organism and are extremely costly, complex and require substantial technical expertise. This limits their widespread use. Most calorimetry studies in humans involve either indirect calorimetry or non-calorimetric methods.

Non-calorimetric methods are distinguished from direct and indirect calorimeters in that they do not measure the immediate products of respiration - heat, CO\textsubscript{2}, O\textsubscript{2} or WVP.

Indirect calorimeters do not directly measure heat but measure other products of respiration that are then used to calculate energy produced by respiration. Indirect calorimeters are now widely used in clinical and physiology research. They come in a variety of configurations and are relatively inexpensive. Indirect calorimeters may be further divided based on the volume in which the subjects’ breath is sampled and where the flow rate is generated relative to the subject. Indirect calorimetry can be conducted using any contained volume with a controlled inflow and measured outflow of air. Most commonly a canopy, a room, or a mask are used to gather samples.

In a canopy hood configuration, a ventilated transparent hood is placed over the head and neck of the participant, and resting energy expenditure may be assessed. In a mask configuration, a tight-fitting mask is worn over the face, this is more suitable for measuring energy expenditure during physical activity as the seal on the calorimeter is not disrupted by movement as it would be with a canopy hood. In a whole-room indirect calorimeter, the participant resides in a large room, often for several days, while air is
sampled from an outlet. The whole-room calorimeter can be used to measure total energy expenditure and its components.

2.2.3 Conventions and Terminology used in Indirect Calorimetry

Several conventions are used in the literature on indirect calorimetry which are observed throughout this thesis. The concentration of a single gas in a mixture of gases is expressed as a fractional concentration. For example, the fractional concentration of O\(_2\) in inspired air is 20.94\%, however fractional concentrations are more commonly expressed as a proportion relative to a maximum value of one, 0.2094. Gas volumes are expressed in STP volumes, the volume that would be occupied at standard temperature and pressure.

Barometric pressure is expressed in kilopascals (kPA) and energy expenditure is expressed in kilojoules (kJ). Resting energy expenditure is calculated from the rate of change of O\(_2\) and CO\(_2\) per unit time. These quantities are designated VO\(_2\) and VCO\(_2\) respectively. Though depleted during respiration, VO\(_2\) is always represented as a positive value.

The flow of air into the hood is known as **incoming air** and is drawn into the hood via a hole in the top surface. The air flow that flows out of the hood is altered by the depletion of O\(_2\) and enrichment with CO\(_2\) and water vapour (WVP) during respiration. The flow of air out of the hood is known as the **excurrent air**. The fractional concentration of incoming O\(_2\) is denoted FiO\(_2\), while the fractional concentration of incoming CO\(_2\) is denoted FiCO\(_2\). The fractional concentrations of these gases in excurrent air are denoted FeO\(_2\) and FeCO\(_2\), respectively (Fig 2.1). The flow rate is designated FR.

In a canopy hood pull through configuration (Fig 2.1) ambient air is “pulled” through a hood, FeO\(_2\), FeCO\(_2\), the flow rate of the excurrent air (FR), FiCO\(_2\), WVP and barometric pressure (BP) and temperature are also measured. Incurrent oxygen (FiO\(_2\)) can be measured and, once corrected for barometric pressure and water vapour pressure, it should equal 0.2094.

Pull or push configuration refers to the location of the generated flow through the volume relative to the subject. If the flow rate generator is upstream of the subject it is a push configuration. In a pull configuration, the flow rate is generated downstream of the participant. All calorimetry in this thesis is pull configuration. Thus, the controlled and
measured flow rate in a pull configuration is the excurrent flow rate. The equations required to calculate REE are dependent on a knowledge of the configuration.

Figure 2.1 Incurrent and Excurrent Air Flow During Canopy Hood Calorimetry

2.2.4 Measurement of REE by Indirect Calorimetry

The O$_2$ concentrations, CO$_2$ concentrations, water vapour pressure, barometric pressure, and excurrent flow rate can be used to calculate the VCO$_2$, VO$_2$ and thus REE from the calorimeter subject. These calculations and their derivation are well described by Lighton, 2008, and are based on a few simple principles (312). First, the summed fractional concentrations of nitrogen, oxygen and carbon dioxide in incurrent air (FiN$_2$, FiO$_2$, FiCO$_2$, respectively) must equal the summed fractional concentration of the nitrogen, oxygen and carbon dioxide in excurrent air (FeN$_2$, FeO$_2$, FeCO$_2$ respectively). This is because none of the other constituents of air are altered by respiration and so remain unchanged. Secondly, oxygen depletion concentrates CO$_2$ in expired air and this effect must be accounted for in estimating VCO$_2$ and vice-versa.

VCO$_2$ is calculated as:

Equation 2.1 \[ VCO_2 = \frac{FR \times (FeCO_2 - FiCO_2) + FiCO_2 \times (FiO_2 - FeO_2)}{(1 + FiCO_2)} \]

This equation reflects the difference in incurrent and excurrent CO$_2$ concentrations multiplied by flow rate over time. It also incorporates a correction factor. This is to account for the concentration of CO$_2$ by O$_2$ depletion. The term “FiCO$_2$* (FiO$_2$ - FeO$_2$)” results in a negative value when O$_2$ depletion occurs. This is calculated and added to the
difference in \( \text{FiCO}_2 \) and \( \text{FeCO}_2 \). A similar calculation is used to estimate \( \text{VO}_2 \), however as \( \text{VO}_2 \) is represented as a positive value \( \text{FiO}_2 \) is subtracted from \( \text{FeO}_2 \), not vice versa, and the correction factor is subtracted not added to the main term. Finally, \( \text{FiO}_2 \) can be assumed to be a constant 0.2094:

\[
\text{Equation 2.2} \quad \text{VO}_2 = \frac{\text{FR} \times (\text{FiO}_2 - \text{FeO}_2) - 0.2094 \times (\text{FeCO}_2 - \text{FiCO}_2)}{(1-0.2094)}
\]

The respiratory quotient is defined as the ratio of \( \text{VCO}_2/\text{VO}_2 \) and is calculated as:

\[
\text{Equation 2.3:} \quad RQ = \frac{\text{VCO}_2}{\text{VO}_2}
\]

Finally, REE can be calculated as described by Weir, (310):

\[
\text{Equation 2.4: Energy Expenditure} = 3.941 \times \text{VO}_2 + 1.106 \times \text{VCO}_2
\]

Thus, the measurement of REE can be achieved using equations specific to a pull-configuration indirect calorimeter. Two additional considerations in the estimation of REE using indirect calorimetry are the washout effect and the time constant of a given calorimetry configuration.

2.2.4.1 Washout Effect and the Z transformation

To calculate \( \text{VO}_2 \) and \( \text{VCO}_2 \) accurately using indirect calorimetry, the \( \text{VO}_2 \) and \( \text{VCO}_2 \) of the participant needs to be determined from air flow out of the fixed volume chamber. Time-lag correction, the shifting forward of samples in time, will address time delay in making this assessment, but it does not affect the dilution of expired gas concentrations in a fixed volume of air that is constantly being depleted and replaced by fresh air. This concept is best illustrated graphically (Fig 2.2).

A hood or room calorimeter may be considered a closed container filled with gas. The excurrent flow rate from these containers is controlled and known and determines the inflow rate. Extrapolating the change of gas occurring relies upon an understanding of the factors affecting gas concentration within the room. The production of \( \text{CO}_2 \) or depletion of \( \text{O}_2 \) per unit time from a participant within the container, are the measures of interest. As they are measured, \( \text{CO}_2 \) and \( \text{O}_2 \) are diluted by the air already in the container, in a manner proportional to the volume of the container. In addition, they are also diluted by the replacement of air in the container with atmospheric air in proportion to the flow
rate through the system. Over time these diluting effects slow the attainment of the final steady-state concentration of CO₂ and O₂.
Ink of an unknown intensity is dropped into a bath with an inflow and an outflow. The intensity of the ink is the parameter of interest. Initially there is a delay as the ink diffuses through the container and the outflow water remains clear (A). As it begins to mix with the water in the bath, the intensity of the ink is diluted and is apparently less intense as it is monitored at the outflow (B). Over time, with continued addition of ink at the same rate to the water a state of equilibrium is reached where further addition of ink produces no significant further colour change in the outflowing water (C). By this time the intensity of the ink in the outflowing water is representative of what is in the bottle.
The difficulty with the washout effect is estimating what the true VO2 and VCO2 are prior to the achievement of non-steady concentration based on the recorded measurements. In whole-room calorimetry there is a large washout effect due to the large volume of the room.

To estimate the steady-state concentrations a mathematical transformation known as the instantaneous transformation or z-transformation is applied (313). It is closely related conceptually and mathematically to the time constant. They are two different applications of the same property: the relationship of the excurrent gas concentration to time in a flow through calorimetry system. The z-transformation enables estimation of the concentration at steady-state, while the time constant is a calculation of the time required to get to steady-state.

The z transformation is given by:

Equation 2.5: \[ Z = 1 - e^{(-FR \cdot \frac{dT}{EV})} \]

It is a function of the effective volume of the chamber (EV), the flow rate through the chamber (FR) the time change between consecutive samples (dT) and the base of the natural log (e). The term EV, reflects the canopy or chamber setup and is a theoretical term that reflects not just the physical volume of the canopy, but other factors such as scrubbing column volumes, dead space, and sensor sensitivity to changing values.

2.2.4.2 The Time Constant and Choice of Flow Rate

The time taken for excurrent air flow to change with VO2 production, for example, can be calculated by dividing the volume (V) of the calorimetry system by the flow rate (FR) through the system. This gives the time constant (T).

Equation 2.6: \[ T = \frac{V}{FR} \]

T can also be used to calculate the proportion of the final value (F) of VO2 that will be achieved in an indirect calorimeter over a given period, T, after a step change in metabolic rate has occurred. This is possible by

Equation 2.7: \[ T = -\left(\frac{V}{FR}\right) \times \ln(1 - \left[\frac{100-F}{100}\right]) \]
V is the chamber volume in millilitres, T is the time in minutes required to reach F, the specified proportion of the final value in percent, FR is the flow rate in mls/min, ln is the natural logarithm (314).

A responsive system has a low time constant and can be achieved by using a low volume container, as with a canopy hood, or with a high flow rate. The downside of a high flow rate is that the washout effect is more pronounced and the change in measured concentration of gases becomes smaller for a given true change, and thus potentially harder to detect. At a minimum, the change in measured concentration of gas needs to be greater than the accuracy of the sensors, (see Table 2.2), to be detected.

Canopy hood calorimetry recordings are typically less than an hour in duration and so 24-hour REE is calculated from this shorter sample. The extrapolation to a 24-hour estimate involves certain assumptions, for example that no substance that may alter REE during the day is consumed, e.g. smoking, caffeine or medications such as methylphenidate or theophylline (315-317). Furthermore, sleeping metabolic rate is assumed to be equivalent to REE, when in fact it is approximately 5% lower (318). The practicalities of obtaining accurate measures for the components in these equations; FiCO₂, FiO₂, FeCO₂ and FR are described in section 2.4.4.

2.3 Requirements for the Study of Adaptive Thermogenesis

Adaptive thermogenesis was defined in chapter one as the reduction in REE, beyond that which is expected for the change in body composition, occurring during a period of dietary energy restriction. This definition has implications for the accurate assessment of adaptive thermogenesis.

Resting energy expenditure varies with body weight, FM and FFM. Two individuals of the same weight, one with a high proportion of muscle mass and the other with a high proportion of FM will each have a different REE due to differences in the metabolic activity of FM and FFM. During weight loss, both FM and FFM may change by different amounts. To determine adaptive thermogenesis accurately, the expected change in REE for the change in body composition must be calculated and compared to the measured change in REE. In addition, REE must be ‘scaled’ or expressed in a way that allows meaningful comparison between individuals of different sizes and body compositions.
A further requirement in the study of adaptive thermogenesis is the prescription of a standardised hypocaloric diet. There are many factors that determine daily energy expenditure (Fig 1.1), these vary between individuals. Thus, a fixed calorie intake will provide a different proportion of the total daily energy requirements for different individuals. To standardise a hypocaloric diet, individual daily energy requirements must be calculated and the diet prescription based on these. Adherence to this diet prescription must also be assessed.

In summary, to study adaptive thermogenesis during a hypocaloric diet intervention; REE and body composition need to be measured with precision and accuracy, REE needs to be normalised for inter-individual comparison, energy requirements must be estimated and adherence to the dietary prescription needs to be assessed. The principles for measurement of REE have already been discussed in section 2.2.4.

### 2.3.1 Body Composition Assessment

Resting energy expenditure is determined primarily by body composition, and FFM. There are several methods used for the assessment of body composition of which bio-impedance analysis, dual energy x-ray absorptiometry (DXA), densitometry and MRI are some of the most common. Balance methods (section 2.3.1.1.6) are discussed briefly as they are used in multi-compartment models and are relevant to the assessment of body composition with weight loss.

#### 2.3.1.1 Body Composition Assessment Methods

**Levels and Compartmental Models of Body Composition Analysis**

One of the starting questions of body composition analysis, is at what level the composition of the body will be assessed. Wang *et al*, 1992, proposed that body composition could be considered at five levels of increasing complexity; atomic, molecular, cellular, tissue-system and whole body (319). Many of the body composition assessment methods discussed below operate at the tissue-system level. While whole body MRI can be used to assess all tissues in the body, other methods assess the composition of the body by categorising them into tissue compartments of similar density (320). This requires certain assumptions to be made regarding the stability of the water content, and thus density within each compartment. DXA, for example is a two-compartmental model dividing the body into FM and FFM. Body composition can be
assessed using more complex compartmental models by combining two or more of body composition assessment methods. Examples include, a three-compartment model comprising fat, water and fat free dry mass, or a four-compartmental model comprising fat, water, mineral and protein. In these examples, whole body water can be assessed using labelled water, FM using densitometry and mineral content using whole body potassium and bone mineral content using densitometry (321). The additional cost and inconvenience of multi-compartmental methods is a disadvantage and must be weighed against the relative gain in accuracy that it provides.

**Bioelectrical Impedance Analysis**

Bioelectrical impedance analysis (BIA) involves the measurements of body composition based on resistance, more accurately called impedance, to the flow of an electrical current through tissues (322). Bioelectrical impedance analysers consist of surface electrodes that deliver a fixed current through to the body via a point of contact such as the hands, ankles or feet. They combine a weighing scale function in addition to body composition measurements. Under Ohm’s law the measured voltage across the electrodes following application of the current is inversely proportional to the impedance, which in turn is related to the tissue volume or mass. Using this principle, a quantitative measure of the size of different body compartments can be obtained (323). Multifrequency BIA involves currents of varying frequency that produce estimates of intracellular fluid, extracellular fluid, FFM, FM and total body water. In contrast to other methods such as DXA (section 2.3.1.1.3) and deuterium dilution (section 2.3.3.1), BIA is inexpensive, precise, acceptable to participants and does not require substantial technical expertise.

However, BIA relies upon several assumptions that can reduce the accuracy of the technique if they are not met. First, Ohm’s law is applicable to cylindrical conducting materials; thus, a mathematical adjustment is made to account for the non-cylindrical shape and the theoretical length of the cylinder. These adjustments may have variable accuracy depending on the shape of the individual being measured (324). Secondly, electrical conductance through human tissue, relies upon fluid volumes and electrolytes, thus hydration and a normal electrolyte balance is assumed. Fluid and electrolyte shifts may occur during weight change and violate these assumptions (325). Thirdly, as there is no theoretical basis for a relationship between FM and impedance, it is determined experimentally in sample populations of individuals. Regression equations have been
developed that plot the relationship between the electrical parameters and body fat determined by alternate methods (326). The validity of this relationship relies upon the reference method itself being accurate, and the sample population resembling the population in which the technique will be used with respect to age, gender, health status and ethnicity (323). Rush et al, 2006, evaluated BIA equations in New Zealand Asian and Fijian Indian Adults, and Māori and Pacific children and found reduced validity of equations in these ethnicities as the equations were developed in predominantly white populations (327, 328).

**DXA**

DXA measures body composition by passing x-rays of two different energy levels through the body. The energy of these rays is reduced in proportion to the depth and density of the tissue prior to hitting a detector (329). DXA is used to identify tissues of different density such as FM, Bone and FFM. DXA is quick and convenient to perform, and involves only a small dose of radiation. The coefficient of variation with DXA’s is as low as 1% for FM and FFM (300). Most commercial DXA scanners are not suitable for very obese patients due to the weight limit, the width of the scanning table or the height of the scanning arm.

**Densitometry**

Densitometry refers to body compositions assessment methods that rely on measuring body composition by assessing body weight and body volume, and thus density. These methods are distinct from DXA which relies on a different principle, x-ray attenuation, to assess body composition. Underwater weighing, or hydro-densitometry was the original method used to do this. Participants need to be submerged, often several times, to obtain an average weight and density. Residual lung volumes also need to be measured. Apart from the specialised equipment required to measure underwater weight and residual lung volume, patient burden is a significant disadvantage of this approach.

Air displacement plethysmography, another densitometry method, does not require immersion in water and performs well compared to hydro-densitometry. Participants are placed in an enclosed chamber and air displacement used to estimated body volume. A potential limitation with both densitometry methods is that the body density needs to be converted to an estimate of body composition. As with predictive equations of REE, the validity of these equations may be affected by factors such as age, body habitus or
ethnicity (330, 331). However, it is a valuable technique that can be combined with other body composition methods to develop multicompartment models (332).

**Magnetic Resonance Imaging**

Magnetic resonance imaging involves application of a strong magnetic field to the body, which excites hydrogen atoms in body tissues. These atoms gradually return to their pre-excitation state at different rates in different tissues, releasing radio-wave signals as they do so. The radio-wave signals are used to generate images that discriminate between different parts of the body. MRI can be used for whole body composition and has a high precision (332). The chief disadvantages of MRI scanners are their size and cost, being available in only a few research centres. As the scanners are narrow they may not be acceptable to participants with claustrophobia or permit measurement of very obese participants (333). Quantitative MR is an application of the MRI technique that results in a quantitative estimate of tissue, such as fat. It has been used to detect very small changes in fat deposition in specific tissues such as the liver and pancreas (167).

**Balance Methods**

Balance methods involve estimating the rate of change of FM by measuring net energy, electrolyte and/or nitrogen balance in dietary intake and excreta (334). While the composition of weight lost can be measured, the absolute body composition prior to, or following weight loss must be assessed by a different method. Balance methods are costly, labour intensive, require considerable expertise and must be performed in an inpatient setting. They involve an error of approximately 5% in the estimation of FM due to assumptions required by the method (335). While balance methods are not feasible in larger trials due to the cost and analytic work involved, they can be used for detailed metabolic studies in small cohorts and may be of use in the assessment of body composition during weight loss.

**2.3.1.2 Body Composition Assessment During Weight Loss**

Fat free mass is a convenient body composition term but is biologically heterogenous, consisting of metabolically active components, such as mitochondria rich muscle fibres, and inactive components such as water, glycogen and electrolytes. Changes in the glycogen content of muscle within the first few days of weight loss can result in secondary water loss. Electrolytes, minerals, proteins and intracellular lipid content are
also altered during weight loss (336). These changes violate the assumptions of electrolyte and fluid balance upon which many body composition analysis techniques depend. As simpler body composition methods use two compartment models, they are potentially prone to reduced accuracy during weight loss. To assess this error, they have been compared to four-compartmental models or MR.

Muller et al, 2012, evaluated the superiority of a combination of energy balance, nitrogen and fluid balance methods versus; air displacement plethysmography, quantitative MR and BIA (337). Ten participants, who lost 3.6 kg over one week were assessed for change in FM. Air displacement plethysmography significantly overestimated FM though not fat-free mass during weight loss, and the authors recommended against its use in the assessment of short term changes in FM. BIA demonstrated little systematic error but a very large interindividual variation in weight lost. DXA was not assessed in this study. Jebb et al, 1993, found little difference in mean FM loss assessed by balance methods compared with BIA during under and over feeding in three individuals (334).

Pourhassan et al, 2013, compared doubly labelled water, air-displacement plethysmography and deuterium dilution to a four-compartment model, in the assessment of body composition during weight loss (338). Overweight and obese men and women lost an average 11.2 kg over approximately two years. There were no statistically significant differences between the four-compartment model and DXA, air displacement plethysmography or deuterium during weight loss, however the mean bias for DXA was greater than the other two methods. Limitations of the study were the heterogeneity in the rate of weight lost, and non-standardized time points for body composition assessment.

The performance of DXA in this study raises an important point about using the four-compartmental model as a reference method for FM and FFM estimation. In both the Pourhassan study and in the original description by Fuller of the four-compartmental model, DXA is used to provide bone mineral density estimates not estimates of FM or FFM. Deuterium dilution and densitometry contribute to the estimates of these latter. Fuller et al, 1992, highlight this in their original paper and cite it as the reason why DXA estimates are more likely to differ from air displacement plethysmography or deuterium dilution methods (321). The four-compartmental model, thus improves overall precision, compared with DXA alone, but does not necessarily improve on criterion validity as
compared with a true independent reference method. The absence of such a method remains a major limitation in the assessment of methods of body composition analysis.

Mahon et al, 2007, also studied deuterium, air-displacement plethysmography and DXA compared to a four-compartment model during weight loss. Twenty-seven obese postmenopausal women lost a mean 9% of body weight over nine weeks. No significant differences in the assessment of FM, FFM or body fat percentage were found between groups (339).

In summary, a range of body composition assessment techniques are available, each with their own relative merits. A four-compartment method is considered optimal to mitigate potential errors with body composition assessment during weight loss. Studies comparing two compartment methods with a four-compartment reference method have not highlighted a single superior method for body composition analysis during weight loss.

2.3.2 Allometric Scaling of Resting Energy Expenditure

Allometry is the study of the relationship between size and other physiologic measures, such as head size or organ mass or energy expenditure. The term was originally coined by Teissier and Huxley in reference to the field of relative growth of limbs to body mass (340). In the study of energy expenditure, allometry is relevant to assessing how REE may vary with measures of size. As outlined in section 2.3, this is important as inter-individual changes in size may account for changes in absolute REE and confuse the detection of changes in REE that may be due to an intervention, such as an energy restricted diet. Thus, normalising REE based on some measure of size is recommended in studies of REE (341).

In the past, body surface area, calculated by incorporating height and weight, were used for normalizing REE (342). However, these methods did not fully account for variations in inter-individual REE. It was later recognised that allometric scaling using body composition, rather than total mass, could be used to explain some of this inter-individual variation (343). Thereafter body composition assessment methods have been a cornerstone of REE assessment, allowing allometric scaling. Body composition parameters such as FM and FFM, are now used to scale REE, but what is the best way to do this?
The simplest approach is to divide the REE by the body composition measure. This approach has been shown by Poehlman et al., 1995, to produce biased estimates of REE (344). Instead, using uni- or multivariate regression of REE, incorporating FFM, with other covariates such as FM, age or gender produces measures of REE that more accurately represent interindividual variations in REE. This has also been confirmed in growing children and adolescents, a group with a greater rate of growth than adults, by Zakeri et al., 2006 (345). Such regression equations used for allometric scaling are like the prediction equations described in section 2.3.3.2. The application of these equations; the comparison of REE in the context of a trial rather than the calculation of resting energy expenditure to estimate dietary energy requirements, is the chief difference.

While the use of regression equations with FM or FFM is arguably one of the most common methods for allometric scaling of REE, it is not a perfect solution, limited as it is by challenges of body composition assessment (section 2.3.1). The FM and FFM “compartments” are heterogenous and composed of small tissue compartments of varying metabolic activity. Moreover, regression equations developed in this way have a non-zero intercept. Thus, in theoretical models that represent whole body composition in terms of FM or FFM only, at zero FM and zero FFM, some energy expenditure is still expected. This is a biologically implausible scenario. With the advent of MRI for detailed body composition phenotyping, allometric scaling has been extended to include organ size. Bosy-Westphal et al., 2009, have shown that the non-zero intercept can be accounted for by differences in organ mass between individuals, assessed by MRI (346). The incorporation of organ mass, improves the explanation of variance in REE by 10%, compared to scaling with just FM and fat free-mass. The liver contributes most to REE followed by the brain, kidney and heart, in keeping with the relative size of these organs. Currently, allometric scaling using organ mass, FM and FFM is the ideal approach to allometric scaling, however this method is beyond the reach of most research centres because of the cost of the technology.

In summary, multivariate regression of REE using body composition measures is a useful method to normalise REE between individuals of different sizes and body compositions.

2.3.3 Estimating Energy Requirements

Total energy expenditure can be measured in free living individuals using the doubly labelled water method (section 2.3.1.1) or by studying participants housed in a whole-
room indirect calorimeter (section 2.2.2). If these methods are not available, total energy expenditure may be calculated from estimates of physical activity related energy expenditure and diet-induced thermogenesis combined with REE measured by indirect calorimetry. Finally, if no measurements of energy expenditure are possible, REE can be estimated using predictive energy equations. These in turn may be combined with an estimate of physical activity to obtain an approximation of total daily energy requirements.

2.3.3.1 Doubly labelled water

Doubly-labelled water (DLW) involves the administration of water containing stable hydrogen (deuterium) and oxygen isotopes. As oxygen in water is in equilibrium with the oxygen in expired carbon dioxide it can be used to trace cellular respiration and thus energy expenditure. The elimination of water through perspiration, breathing and urination confounds this assessment. The addition of the hydrogen isotope allows the elimination of oxygen due to cellular respiration to be distinguished from elimination in fluid (347). The DLW technique is an excellent method for the study of total energy expenditure in free living individuals, it may be combined with REE and measurements of diet induced thermogenesis to estimate physical activity related energy expenditure. The chief drawbacks of the method are the cost of isotope, the expertise necessary to perform the calculations required and having access to a laboratory with the equipment and expertise for the method. Deuterium dilution is a related application, that assesses the dilution of the stable isotope, deuterium, in body compartments. It is used in the assessment of body composition, rather than energy expenditure.

2.3.3.2 Predictive Energy Equations

Equations to predict energy expenditure have been developed using multivariate step-wise regression based on large datasets from different populations (348-352). By incorporating covariates relevant to REE determination in the equation, significant predictor variables are retained and coefficients determined which are incorporated in an algorithm. Depending on how they will be employed, equations may use simple anthropometric measures, such as weight and height, or body composition measures such as FM and FFM. The strongest predictor of REE is FFM, followed by FM, accounting for approximately 60-70% and 30% of the variance respectively, observed in REE in large populations. In one of the largest cohort analyses, Lazzer et al, 2007, developed
regression equations based on indirect calorimetry measurements and bio-impedance analysis of 8,780 Caucasians (350). However, the validity of a predictive equation developed in one ethnicity can be problematic when applied to other ethnicities, particularly where inter-ethnic differences in the relationship between body composition and surrogate markers of body composition, such as BMI exist. This is particularly relevant in the New Zealand population and is discussed in chapter five.

2.3.3.3 Diet Induced Thermogenesis

Diet induced thermogenesis, like REE can be measured by indirect calorimetry using a canopy hood configuration or a whole room calorimeter. Participants have fasting measurements of REE taken, with repeat energy expenditure measurements, following a meal. Diet induced thermogenesis is calculated as the difference between fasting REE and REE following a meal. It may take between four and eight hours for REE to return to its fasting level following a meal. This makes accurate assessment of REE with canopy-hood calorimetry challenging, as participants must remain stationary and awake throughout.

In the absence of measurements, diet induced thermogenesis may be estimated. Diet induced thermogenesis accounts for a small proportion of total energy expenditure, approximately 5-10% and exhibits a high intra-individual variation of up to 50% (18). Diet induced thermogenesis is proportional to total dietary energy intake and can be approximately estimated as a fixed proportion of total prescribed energy intake.

2.3.3.4 Physical Activity Assessment

The accurate assessment of physical activity in free living individuals is a challenging task. The most accurate measurements of physical activity are conducted in a chamber calorimeter or during direct observation in an inpatient metabolic ward where the intensity and duration of all activities are recorded. The disadvantage of this approach is the cost, participant burden, and the reduced validity of the assessments when applied to free-living individuals. In studies of free-living individuals, doubly labelled water (section 2.3.3.1), accelerometry and physical activity logs are amongst the most common methods used.
**Accelerometry**

Accelerometry involves equipping participants with accelerometers that register movements in space using sensors. Sensors are typically attached to the trunk or limbs in a specific configuration to register certain types of movement. This provides a surrogate marker of physical activity. These movements can be converted to energy expenditure values, using predictive equations. There are several challenges with the use of these devices for assessment of physical activity however. First, their accuracy may be reduced by interindividual variation in movement, for example different gaits with walking. Secondly, not all physical activities are measured with the same fidelity, and most accelerometers are designed to assess specific activities, such as walking, without capturing the full range of physical activities that may occur in a day (353). Thirdly, predictive equations developed in lean healthy participants may have reduced validity in the obese or in participants of a different gender (354). Plasqui et al, 2013, performed a systematic review of validation studies of accelerometry in free living individuals compared to doubly labelled water. Correlation between methods varied between 0.18 and 0.88 across a range of accelerometer types and populations. The difference between doubly labelled water and accelerometer varied from a mean underestimate of 1430 kJ/day using accelerometry, to a mean overestimate of 1674 kJ/day, with wide limits of agreement (355).

**Physical activity records**

As total daily energy requirement is a more useful measure than resting energy expenditure, when prescribing daily food intake, the REE can be combined with a record of physical activity to approximate total daily energy requirements. Physical activity records may be prospectively completed or filled-in based on recall and may capture the type and duration of physical activity over hours, days or weeks.

The energy cost of physical activities can be expressed as a ratio of the energy consumed performing that activity to resting energy expenditure - this is known as the metabolic equivalent value of the activity (MET). The MET value of a wide range of physical activities have been estimated or experimentally determined and are published in the *Compendium of Physical Activities* (356). The value of the MET is that it can allow estimation of physical activity energy expenditure when combined with the individuals weight and a record of the type and duration of the activity. Clearly aggregating all the
separate activities that make up an individual’s daily movements can be unwieldy. For simplicity the energetic cost of physical activities may be grouped into light, moderate and vigorous physical activity categories, each allocated its own MET. These estimated METs can be combined with physical activity recall methods to estimate physical activity related energy expenditure. The advantages of using physical activity records are their convenience and low cost. The chief drawbacks are recall bias and potentially low accuracy (357, 358).

In this thesis, physical activity is measured using the international physical activity questionnaire (IPAQ), one of the better studied physical activity records (359). The IPAQ assesses physical activity by asking participants to recall the duration of time spent doing activities in four different domains at three levels of intensity. Physical activities are divided into work-related, transportation-related, domestic and leisure time physical activity. In the IPAQ, physical activity intensity is assessed as walking, moderate or vigorous. Moderate physical activity is defined as activity sufficient to increase the rate of breathing but during which it is still possible to hold a conversation. Vigorous physical activity is defined as activity sufficient to increase the rate of breathing such that it is not possible to converse. The IPAQ scoring protocol multiplies the MET value of walking, moderate and vigorous physical activity by the individual’s body weight and time spent doing the activity to produce an estimate of calories spent over the previous week. The IPAQ has been showed to have good reproducibility and has been validated against doubly labelled water in a New Zealand population by Maddison et al, 2007 (360). They demonstrated good agreement between doubly labelled water and the IPAQ estimates at low levels of physical activity, ~1000 – 2000 kJ/day. The consistency of the IPAQ makes it a useful tool in repeated measures assessments.

2.3.4 Assessment of Dietary Intake

In an outpatient setting, dietary intake assessment mainly relies on subjective methods including food-records, food frequency questionnaires, 24-hour recall or focused macronutrient questionnaires (361, 362). Subjective methods such as these have several limitations, not least under-reporting of energy intake (363). Objective measures include; measuring ingested food of known composition during an inpatient stay in a metabolic research centre and digital photography/videography, which has been integrated into mobile phone based calorie counting, or food recording apps (108). These are not without
their challenges such as cost, user acceptability, participant adherence to image capture, interpretation of poor quality images and scaling of images (364, 365). A growing area of research is the use of biomarkers, such as 24-hour urinary fructose or sucrose, to adjust or calibrate reported dietary intake. While several biomarkers have been identified and validated, their reproducibility and sensitivity to change over time remains largely unknown and they are not error-free (366). In addition, the calibration process assumes that the reference method’s error is independent of the subjective intake measurement error, which is not always the case (367).

Prospectively completed food records are one of the most commonly used methods of dietary intake assessment. They are more accurate with respect to energy intake and less prone to bias, than food frequency questionnaires or methods based on recall (368-370).

However, they have some well-established limitations such as; the change in eating behaviours during a period of recording, also known as ‘reactivity’; under-reporting of energy, sodium and protein intake and increasing participant burden (363, 371-374).

The reporting bias inherent may vary depending on the populations studied. In prospectively completed food records of three to four days has been assessed by comparing recorded energy intake with measurements of total energy expenditure during weight stability. In these studies, energy intake was under reported by 12% in men compared with women and was more likely to occur in the obese (375, 376). Using prospectively filled seven day food records, dietary fat was underreported in obese men during weight stability (377).

Despite these limitations, prospectively filled food-records are less burdensome and more accurate than records of longer duration (378). They are also sufficiently long to ensure a representative sample of daily energy intake, which may vary considerable from day to day. Four-day food records were used in this thesis and are described in section 2.4.3.

2.4 Description of Methods

Having described the general principles of indirect calorimetry and outlined the methodological requirements for a study of adaptive thermogenesis during a low-calorie diet intervention, what follows is a description of the protocols used to obtain various measurements obtained during the studies described in this thesis.
2.4.1 Anthropometric Measurements:

Unless otherwise indicated, all anthropometric measurements were made in the morning between 0730 – 1030 with participants having fasted since 2200 the night before.

2.4.1.1 Height and Weight

Height was measured using a stadiometer (Leicester Height Measure, Invicta Plastics, UK). Participants were measured with their heels in contact with the backplate, weight evenly distributed, standing erect with a neutral head position. Duplicate recordings were obtained and repeated until there was concordance to within one centimetre. Weight was measured in kilograms to the nearest 0.1 kg using a digital weight scales (TBF-300, Tanita, USA) with participants dressed in a single layer of light clothing. Weight was adjusted for the weight of light clothing assuming a standard 0.2 kg for all participants.

2.4.1.2 Waist circumference and Hip circumference

Waist and hip circumference were measured in centimetres to the nearest 0.1 centimetre in accordance with the World Health Organisation recommendations (379). Waist circumference was measured at the midpoint between the lowest palpable rib and the iliac crest. Hip circumference was measured around the widest point of the buttocks. All measurements were made with the tape measure parallel to the ground, with the participant in a relaxed upright posture. Duplicate measurements were taken and if there was a difference of more than one centimetre between them they were repeated until concordant.

2.4.2 Body Composition Assessment

Body Composition was assessed using BIA and DXA (section 2.3.1).

2.4.2.1 Bioelectrical Impedance

A multifrequency tetrapolar bioimpedence analyser (TBF-300, Tanita, USA) was used to measure body composition in the intermittent fasting study and for weight in all studies. The TBF-300 has been validated against DXA and deuterium dilution (section 2.3.1) as a measure of body composition. It compares favourably with deuterium with differences in measurement of FM between BIA and deuterium ranging from -2.1% to +1.4%, depending on gender and ethnicity, in an obese adolescent population (380).
Compared to DXA for estimates of body fat percentage BIA using the TBF-300 had a correlation coefficient of 0.58 (p<0.001) (381).

2.4.2.2 DXA

**DXA Equipment and Quality Control Procedures**

A Hologic Horizon A (Hologic, USA) was used for all DXA scans. The Horizon A consists of a 196cm x 66cm bed with a weight limit of 220 kg and an arm carrying an x-ray emitter and receiver on a mechanised track. It captures body composition data in three longitudinal passes. Between each pass the x-ray beam and patient are repositioned so that the three passes form a fan beam, this virtual fan beam provides rapid data acquisition over approximately three minutes for each scan, reducing the amount of time the participants need to remain still. The Horizon comes with custom software for control of image acquisition, scan analysis and report generation. The “whole body composition” automated scanning protocol was selected for all studies.

Dual energy x-ray absorptiometry scans for the PREEMPt study (chapter five), were performed at a commercial centre, Pacific Radiology, by an accredited radiographer with experience in body composition scanning and reported by a radiologist with over 10 years’ experience in clinical densitometry. For the CREEDS study, (chapter seven) all DXA scans were conducted by me at the Centre for Translational Physiology, University of Otago, Wellington following acquisition of a new DXA scanner. I received training and certification from the Australian and New Zealand Bone and Mineral Society in the operation of a DXA scanner. Both sites were accredited to perform densitometry.

At both sites the scanner was calibrated before every recording and otherwise at least two times per week as per Australian and New Zealand Bone and Mineral Society recommendations (382). Intermittent radiographic uniformity testing, a test of the integrity of the imaging components and related signal processing, was performed approximately once weekly. Both procedures were mandatory and participant measurements could only be performed once they had been completed and passed predefined quality control standards, otherwise the system remained locked.

**Phantom precision and accuracy**

As part of the calibration process, the accuracy of the DXA machine was checked using a ‘phantom’ provided by the manufacturer. The phantom is a synthetic block for which
the weight, area and density are known. After calibration measured density, area and weight were plotted against the reference values. During the CREEDS study, compared to the reference value of 0.927g/cm², the mean density was 0.927g/cm² with a standard deviation (SD) of 0.002 g/cm².

Operator Specific In-vivo Precision

Operator inexperience is a recognised source of error when performing DXA (383). At Pacific Radiology, the radiographer and radiologist that performed the scans had recently published their rates of precision for body composition scanning using the same instrument, personnel and site as the PREEMPt study (300). These results are presented in Table 2.1.

As the DXA machine at the Centre for Translational Physiology was new, the operator-dependent precision was unknown. Therefore, while the CREEDS study was running, we conducted a DXA precision study. The lead investigator of that study was Terry O’Donnell and the co-ordinating investigator was Juliet Bergin. I performed duplicate scans in 29 lean and obese participants, the least significant change (LSC) based on these 29 scans are presented in Table 2.1, with permission, as an indicator of the operator-specific precision of DXA scanning during the CREEDS study.

The least significant change (LSC) is a marker of precision and indicates the minimum change necessary between two repeated measurements to be 95% certain that any change truly happened (384). The LSC is presented as the percentage coefficient of variation. The International Society for Clinical Densitometry (ISCD) recommends an acceptable LSC of 3%, 2% and 2% for total FM, total lean mass, and percent FM respectively these values are included for comparison. An LSC of 3% means that a change of 3% of the mean or more needs to be measured to be certain that a change truly occurred (383). Thus, for a mean FM percentage of 50%, a LSC of 2% would be a change of 1% FM percentage. Similarly, for a mean FM of 40 kg, a LSC of 3% would be a change of 1.2kg. Therefore, our data which is presented in Table 2.1 demonstrates an LSC for FFM, FM and % FM of 1.27%, 2.19% and 2.35%. These are within the acceptable range set by the ISCD, with the exception of percentage FM, which was not used for allometric scaling or estimation of adaptive thermogenesis in the PREEMPt or CREEDS studies.
Participant testing Procedure

All scans were done in the morning with the participants fasted and hydrated. Participants wore standard hospital clothing for every scan. Their height and weight were measured prior to being scanned and entered into each recording. In the PREEMPt study, which involved blood sample collection prior to scanning, a pregnancy test was performed within 24 hours of the scan for female participants and the results reviewed prior to proceeding with the scan. A form was provided to participants before every scan which contained questions relating to fracture risk, previous fractures, recent contrast containing scans, details of any prosthetics material and the date of any recent x-rays. Patients were not scanned if they had had a contrast scan within the previous two weeks. This did not affect any participants in the PREEMPt or CREEDS studies. The whole-body bone mineral density data was checked to ensure participants were not outside the normal range for their gender and age. After discussing the body composition results with a member of the research team, all participants were provided with a hard copy and pdf version of their scans.

Jewellery including piercings and glasses were removed prior to scanning and all pockets emptied. Participant were positioned according to the National Health and Nutrition Examination Survey (NHANES) method: on the scanning table lying supine, palms down and separated from the body, arms slightly angled together, and the face up with neutral chin. For comfort, ankles were not strapped but separated and inverted with the big toes slightly touching. The Hologic Horizon scanning table contains a line border

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### Table 2.1 Least Significant Change in DXA Measurements

<table>
<thead>
<tr>
<th></th>
<th>Centre for Translational Physiology /CREEDS</th>
<th>Pacific Radiology /PREEMPT</th>
<th>ISCD Standards</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fat Free Mass</strong></td>
<td>1.27%</td>
<td>1.45%</td>
<td>2.00%</td>
</tr>
<tr>
<td><strong>Fat Mass</strong></td>
<td>2.19%</td>
<td>2.15%</td>
<td>3.00%</td>
</tr>
<tr>
<td><strong>% Fat Mass</strong></td>
<td>2.35%</td>
<td>2.09%</td>
<td>2.00%</td>
</tr>
</tbody>
</table>
within which all participants were centred vertically and horizontally. Participants were instructed to lie still for the duration of the three-minute scan. Baseline scans were referred to when positioning participants for subsequent scans.

Where an individual was too wide to fit completely on the scanning bed a mirror image scan was performed according to the NHANES protocol (385). Briefly, the participant was positioned to the right of midline with the left arm moved out of the scanning field. All the body including except the left arm and forearm was imaged. During analysis, the Horizon’s mirror image protocol was used to transpose data from the right to the left arm. Only three individuals required mirror images across both the CREEDS and PREEMPt studies.
2.4.3 Dietary Intake Assessment

Four day prospectively-filled food records were used to assess differences in adherence between treatment arms to a daily energy intake prescription in the CREEDS study (chapter seven) and the IF-Hypo study (chapter six). Though many factors influence self-reported dietary intake such as BMI, prior obesity and gender, there is currently no evidence that the schedule of dieting as conducted in these studies causes systematic variation in reporting (369). In addition, following a prescribed calorie intake, a prospective food record serves to demonstrate competent calorie counting and an awareness of prescribed targets to the research team who can provide additional education if needed. It also encourages reflection on daily energy intake, which may have a positive effect on adherence compared with not keeping a record (371, 377).

Prospectively filled four-day food records were used in both the IF-Hypo study and CREEDS studies, a copy of the four-day food record used in the CREEDS study is included in appendix B. Participants were instructed in their use and completion prior to issuing. Every food record had a sample entry. Emphasis was placed on specifying the quantity of food consumed, listing all the ingredients and including brand names. Each food record was reviewed by the research team after completion for error or lack of detail and this was discussed with the participant.

Food records, were entered by a trained member of the research team into FoodWorks™ (Xyris Software, Australia) a nutritional research database. FoodWorks™ utilises the New Zealand FOODFiles database and the Australian AusFoods and AusBrands databases. The FOODFiles database was developed by the New Zealand Ministry for Health and the New Zealand Institute for Food and Plant research and is the most comprehensive food nutrient database in New Zealand. The AusFoods and AusBrands databases are based on the AusNUT 2011-2013 database developed by the Food Standards Authority Australia New Zealand.

2.4.4 Indirect Calorimetry

Indirect Calorimetry was used in all but the IF-Hypo study. As I was the first person in our institution to use the indirect calorimeter, there was no technical expertise on site, or measurement protocol. I needed a detailed knowledge of all aspects of the indirect calorimetry machine components, configuration and signal processing to; develop the
measurement and calibration protocols, train and test new operators, adapt the automated analyses to our own requirements, and perform validation and precision testing of the Promethion (chapters three and four), and most importantly to critically analyse the measured data.

2.4.4.1 The Promethion Metabolic Monitor:

The Promethion (Sable Systems, USA), is an indirect calorimetry system that comprises a gas analyser, an air flow generator, an atmospheric or “baseline” sampling unit, an interface module to communicate with the lab computer, a CO2 and WVP scrubbing column to remove CO2 and WVP from the air during calibration, a rotameter to control the flow of calibration gases, Bev-a-line™ tubing to connect the components, an air filter and a canopy hood (Fig 2.3) It may be used for both canopy hood and room calorimetry and is a pull system.
Figure 2.3. Configuration of the Promethion Indirect Calorimeter
Configuration

A schematic of the Promethion’s configuration for canopy hood studies is presented in figure 2.3. Excurrent air, after passing from the subject, through a custom air mixer and filter is subsampled by the flow rate generator, and the residual air expelled. The subsample is passed through a physical low pass filter to reduce noise or spikes caused by the sampling process. The air sample is then split in two channels as it passes into the gas analyser. The gas analyser outputs to an interface module which is connected to a desktop computer, containing Caloscreen software (Sable Systems, USA). The air mixer is not part of the standard setup and was added to the excurrent air flow prior to the main air filter, to reduce variation in the gas signals from respiration. The mixer consisted of a repurposed acrylic cylinder containing 2 offset disks with large perforation to enforce air mixing.

The Promethion performs several functions of a standard calorimeter – flow rate generation, gas measurement, data acquisition and analysis. It has a novel “dual-channel” configuration that allows it to offset sensor drift. Sensor drift occurs when gas sensors, exposed to a constant gas concentration starts to “drift” off the true value with prolonged exposure. In calorimetry this issue is circumvented by “baselining” – switching from the excurrent air flow measurement to an incurrent air flow measurement. While this prevents sensor drift, it comes with a loss of data while the excurrent air flow is left unmonitored. The Promethion gas analyser (GA-3m2, Sable Systems) contains two rows of sensors, called ‘channel one’ and ‘channel two’ each of which houses a WVP thin-film capacitative analyser, an infra-red CO2 sensor, a fuel cell O2 sensor, a barometric pressure sensor, a temperature sensor and a differential pressure flow rate sensor. The channels are embedded on a temperature-controlled plate that is set to 37°C, warms the excurrent air samples, and is continuously monitored during recordings. Baselining can be customised via Caloscreen so that channel one is monitoring excurrent air while channel two baselines and vice versa. Each channel constantly alternates between measuring excurrent air and atmospheric air during a recording. At the end of the recording all excurrent measurements and atmospheric recordings in both channels are merged (Fig 2.4). The merging process is described in more detail in appendix C. The duration of baselining and the frequency of baselining are programmable and, after experimentation during nitrogen flow studies, (chapter three) were set to a frequency of every 10 minutes for three minutes with an offset of five minutes between channel one
and channel two. This protocol allows for relatively short duration calorimetry recordings of 35 minutes in duration with an even number of baselining sequences between channels and baselining timestamps that do not interfere with the automated signal processing.

**Figure 2.4.** Baselining and Merging of Oxygen Samples from Both Channels.

Figure 2.4 shows the baselining and merging of oxygen samples from both channels.

**Components**

Performance characteristics of the Promethion components have been obtained from the manufacturer's website (386), and are presented in Table 2.2.

‘Scrubbing’ of CO2 and WVP refers to the removal of carbon dioxide and water vapour pressure from sampled air. In the Promethion system this is necessary for calibration. Ascarite and drierite were used as CO2 and WVP scrubbing chemicals, as recommended by the manufacturers.
<table>
<thead>
<tr>
<th>Sensor</th>
<th>Type</th>
<th>Range</th>
<th>Accuracy</th>
<th>Resolution</th>
<th>Drift with constant temp and N2 Flow at 50 ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO2</td>
<td>Infrared</td>
<td>0-5%</td>
<td>1% of reading</td>
<td>0.0001%</td>
<td>&lt;0.002%/hr</td>
</tr>
<tr>
<td>O2</td>
<td>Fuel-Cell</td>
<td>0-100%</td>
<td>0.1% of reading</td>
<td>0.0001%</td>
<td>&lt;0.01%/hr</td>
</tr>
<tr>
<td>WVP</td>
<td>Thin-Film Capacitative</td>
<td>0-6 kPA</td>
<td>1% of reading</td>
<td>0.0001 kPA</td>
<td>&lt;0.01 kPa/hr</td>
</tr>
<tr>
<td>Barometric Pressure</td>
<td>NA</td>
<td>NA</td>
<td>0.1% of reading</td>
<td>0.0001 kPA</td>
<td>NA</td>
</tr>
<tr>
<td>Main Flow Rate</td>
<td>Differential Pressure</td>
<td>50-250 LPM</td>
<td>2% of reading</td>
<td>.01 LPM</td>
<td>NA</td>
</tr>
<tr>
<td>Baselining Flow Rate</td>
<td>Differential Pressure</td>
<td>0-2000 ml/min</td>
<td>2% of reading</td>
<td>0.001 ml/min</td>
<td>NA</td>
</tr>
<tr>
<td>Subsampling Flow Rate</td>
<td>Differential Pressure</td>
<td>0-2000 ml/min</td>
<td>2% of reading</td>
<td>0.001 ml/min</td>
<td>NA</td>
</tr>
</tbody>
</table>
Calibration of O2, CO2 and WVP analysers.

The Promethion metabolic screening system requires intermittent calibration of its analysers. This calibration involves exposing the sensors to minimum and maximum values of their respective gases and adjusting the readings if they are not aligned with the minimum and maximum values. Calibration of the WVP, CO2 and O2 sensors were performed as per the manufacturer’s instructions.

Data Acquisition and Signal Processing

The analysis of the electronic data signals produced by indirect calorimeters necessarily requires the use of signal processing methods. Signal processing involves the analysis, creation, and modification of signals (387) created, in this case, by changing gas concentrations produced by aerobic respiration at rest. When this work is performed by a computer it is known as digital signal processing (DSP). While several DSP techniques are applied to readings from different types of calorimeter, such as the z-transformation, the actual values used to implement these techniques should be determined or at least checked experimentally for any new calorimeter setup to ensure the technique performs as expected. A detailed treatment of signal processing is beyond the scope of this thesis, but the signal processing methods that are used in the analysis of our calorimetry recordings are described in appendix C.

Sampling frequency and data selection

Calorimetry data for analysis were taken from 900 seconds into the recording until 1800 seconds into the recording, this was to allow the patient to achieve a resting state. A typical recording lasted between 2010-2030 seconds, this last four minutes enabled smoother running of the automated data processing.

Samples were collected every second for the entire recording period. Provided the protocol described in section 2.4.4.2 was adhered to and no significant movements were made during the recording, all data from 900-1800 seconds were included in the analysis.

Small movements such as moving fingers or toes are common in indirect calorimetry, such movements lasting less than three seconds were ignored. If larger movements were made or there was any doubt about the impact of certain movements, the time stamped data plus the time lag from onset to offset of physical movements were excluded from calculation of the mean VO2 and VCO2 and compared to the analysis involving all data
points to assess the impact on the recordings. If any alterations to the standard analysis were made, these were recorded with the raw data for later reference.

Application of the z-transformation & Flow Rate Selection

The time to achievement of steady-state in our canopy hood configuration is in the order of minutes, and participants are allowed to rest for the first 15 minutes of a recording there is no need for the z-transformation to compensate for washout effect with the canopy hood setup. However, it is used to compensate for sensor delay when a sensor switches from monitoring excurrent air flows to atmospheric air flows in the Promethion configuration. The z-transformation is applied to every sample recorded before any further data analysis.

In our system the canopy volume is 18.4 litres. Thus, based on equation 2.7, to reach 99.9% of the final value the response time would be:

\[ T = -\left(\frac{18400}{1000000}\right) \ln\left(1 - \frac{100 - 99.9}{100}\right) \]

I.e. a response time of 1.04 minutes to reach 99.9% of final value, close to steady-state. The true value, empirically determined by nitrogen dilution (chapter four), is slightly longer than this, underscoring the difference between effective volume and physical volume described above. During nitrogen dilution we found that a flow rate of 80 SLPM provided the ability to detect relevant changes in energy expenditure with an acceptable response time. This was used for all our studies.

2.4.4.2 Testing Procedure for Participants

Resting energy expenditure is susceptible to influence from physical activity, recent food intake, stimulants such as caffeine, nicotine, physical ailments, ambient temperature, menstrual cycle, rest time prior to starting the recording, body composition (388),(18),(389),(390), (391, 392),(393), (298), (394), (395).

To minimise variation in resting energy expenditure measurements due to these factors the following protocol was implemented; participants were asked to arrive at the testing centre by car and to avoid vigorous physical activity for 24 hours prior to the visit. Parking was available less than 200m from the testing area. No caffeine or alcohol were allowed for 24 hours prior to the visit. All visits were conducted between 0730 – 1100 in
the morning. Participants were advised to notify us prior to the visit if they were unwell or had a fever. A complete medications and medical history was taken from all participants prior to enrolment in studies using indirect calorimetry. Patients with active medical conditions or taking medications that interfered with basal metabolic rate were excluded from the studies. Current smokers were excluded from the studies as were past smokers with a history of any chronic lung disease. Indirect calorimetry recording occurred a minimum of 25 minutes after the participants arrived. Prior to the calorimetry, body composition scanning while recumbent, anthropometric measures while standing and/or questionnaire responses while sitting were obtained. Ten minutes minimum were necessary once the patient was recumbent to place the canopy hood and seal the impermeable skirting around the participant with adhesive tape to avoid air leaks.

All indirect calorimetry studies were performed with participants wearing standard hospital clothing, at temperature of 23.0 – 23.5°C with controlled relative humidity of 40% and atmospherically isolated from the operator. Participants were positioned reclining at 30-45 degrees in a reclining chair with their legs elevated for comfort. Previous research has shown that a reclining, rather than completely supine position makes little difference to the assessment of REE (396). Moreover, it is more comfortable for obese participants than lying flat. A selection of movies was offered to the participants to watch during the recording. All participants were asked to remain still and awake for the duration of the recording. Participants were constantly monitored visually through a window, if they appeared to be falling asleep the light intensity in the room was temporarily adjusted. The calorimetry room was entered via an antechamber with two sealed doors, so that if an operator needed to enter the room, contamination of the atmospheric air in the room by that outside was minimised. On the rare occasion an operator needed to enter the room, it was with breath held, for less than 30 seconds and only if necessary. All operators demonstrated competence to perform calorimetry using nitrogen dilution before being allowed to measure human participants. Participant movements, variation in environmental condition, and any other events of note during the measurement were recorded with the calorimetry recording.

Body composition assessment occurred on the same day for all studies in the CREEDS to allow appropriate allometric scaling of resting energy expenditure. For the PREEMPt studies DXA scans were performed offsite on the same day as the indirect calorimetry studies.
To minimise inter-operator variability, I was present for all but two of the 149 human calorimetry recordings carried out during the PhD. I conducted all daily -calibrations and checked weekly and monthly calibrations were up-to-date prior to every recording. I visually inspected all data channels prior to running the automated analysis to identify any errors evident in the raw data.

2.4.5 Biochemical Tests

All blood samples for biochemical tests were taken fasted between the hours of 0800 – 12:00, after calorimetry. Venous blood samples were centrifuged at 3,000 rpm for 10 minutes. The supernatant was aliquoted into cryotubes and stored at -80C until analysed.

All tests, excluding point-of-care glycated haemoglobin (HbA1C) and lipid profiles in the CREEDS, were performed by accredited commercial laboratories. Point-of-care testing (Cobas b101, Roche, Switzerland) was used for the CREEDS study. This method had correlation coefficients of greater than 0.99 compared with the equivalent measurement techniques used in the other studies (397).

Measured thyroid function tests included free thyroxine (FT4), free tri-iodothyronine (FT3) and thyroid stimulating hormone (TSH). All samples were collected in serum separator tubes. FT4 and FT3 were analysed by competitive electro-chemiluminescence immunoassay, and TSH by sandwich electro-chemiluminescence immunoassay (Cobas 602, Roche, Switzerland). Glucose was collected in fluoride-oxalate tubes, and analysed by hexokinase method (Cobas c501, Roche, Switzerland). Commercially tested lipids were collected in serum separator tubes and were analysed using enzymatic colorimetric assay for high density lipoprotein (HDL), triglycerides and total cholesterol (Cobas c501, Roche, Switzerland). Low density lipoprotein (LDL) was calculated from these values using the Friedewald formula (398):

\[
LDL = Total\ Cholesterol - (HDL + Triglycerides/2.2)
\]

Blood for lipid measurement using the point-of-care device was collected in EDTA tubes, and analysed using an enzymatic colorimetric assay (Cobas b101, Roche, Switzerland) for determination of total cholesterol, HDL and triglycerides. LDL was also calculated using the Friedewald equation. All samples for HbA1C analysis were collected in EDTA tubes. Commercially tested HbA1C was analysed using ion exchange high performance liquid chromatography (Vatiant Turbo, Bio-rad Labs, USA). Point-of-care HbA1c was
analysed using photometric transmission measurement (Cobas b101, Roche, Switzerland).

Liver function tests included; aspartate transaminase (AST), alanine transaminase (ALT), gamma glutamyl transferase (GGT), bilirubin and alkaline phosphatase (ALP). Blood was collected in serum separator tubes for liver function test measurement and analysis performed using spectrophotometric assays (Cobas, Roche, Switzerland).

2.4.6 Statistics and Data Analysis

All statistical analysis was conducted in R, a statistical computing program, except for the IF-Hypo study analysis which was carried out in SAS (SAS Institute, USA) (399).

2.5 Summary

The measurements outlined in this chapter are central to the studies described in later chapters. Of these measurements, the DXA and indirect calorimetry methods required critical assessment of performance, first as the devices were new to the research centre, and secondly because they are fundamental in the study of adaptive thermogenesis. While the theory, calculations and practical measurements of REE by indirect calorimetry are complex, it was only through an understanding of these, that the performance of the indirect calorimeter could be critically assessed and refined. This process of methodological refinement and performance assessment are described in chapters three and four.
3 Validation and Precision Testing of a Canopy Hood Indirect Calorimeter

3.1 Introduction

A major inflection-point during the work presented in this thesis was the measurement of REE in the first participant of the PREEMPt study, presented in chapter five. As the first user of a new indirect calorimeter there was no way of knowing what the quality of the recording was or what raw calorimetry data ought to look like. Consequently, the PREEMPt study was suspended until I reassured myself of the quality of the Promethion and my measurements. The first test-retest reliability study (chapter four), and the validation and precision studies presented below were essential steps in building operator experience and developing our current indirect calorimetry protocols.

3.2 Validity and Precision

Validity is defined as the degree to which a test measures the quantity it is supposed to. It is also known as accuracy or external consistency. It reflects the systematic error or bias due to the error inherent in a measurement method. Precision is the consistency of a test with repeated trials. This is also known as the reliability or internal consistency of a test. It reflects the random variability that occurs as a result of random errors associated with the measurement method rather than a change in the quantity being studied (400).

Both validity and precision are important. Precision without validity results in consistent reproducible measurements of the wrong quantity. Conversely it is not possible to have validity without precision.

As outlined in section 2.2.4, the objective of using indirect calorimetry via a ventilated hood is to estimate resting energy expenditure. The process is complex. It involves different components that need to work quickly, accurately and synchronously. Measurement error can occur at any stage of data collection, calibration, equipment maintenance or data analysis. The method requires accurate measurement of $\text{VO}_2$ and $\text{VCO}_2$, and by implication any intermediate steps required to determine these values. As this is a dynamic situation, validation of the method then must involve changing these gas concentrations. Moreover, it must be known {	extit{a priori}} what the expected change in gas concentrations will be. Apart from using a validation method that reflects the physical principles of indirect calorimetry, it is also important to validate within a range of...
physiologic values that are expected in the individuals that will be measured. Techniques that simulate VO$_2$ depletion, VCO$_2$ production or both are used based on either one of two principles; nitrogen dilution or alcohol combustion.

3.3 Validation and Precision by Nitrogen Dilution

3.3.1 The Principle of the Nitrogen Dilution Method

The nitrogen dilution technique for open circuit, indirect calorimetry was described by Fedak et al, 1981 (401). Nitrogen is inert, so does not react with other gases and thus does not alter in volume in an environment where other gases are changing. The nitrogen dilution technique involves adding a known flow rate of nitrogen to a continuously monitored flow of mixed gas, to produce a relative depletion of the other gases. Of these, O$_2$, rather than CO$_2$ is the gas of interest, as O$_2$ depletion but not CO$_2$ production, can be simulated by the nitrogen dilution method.

3.3.2 Respirometry Calculations for Nitrogen Dilution

What flow rate of nitrogen (FRn) will produce a pre-specified VO$_2$? This relationship depends on how VO$_2$ is usually calculated which in turn depends on the indirect calorimetry configuration. The appropriate choice of equation, as with calculation of VO$_2$ and VCO$_2$, is critical for developing a valid validation test. Fortunately, the relationship between VO$_2$ and the flow rate of nitrogen (FRn) can be mathematically derived. Pure nitrogen is used for validation and the flow rate is the primary determinant of VO$_2$ as alluded to above. While the mathematical derivation for the nitrogen dilution formula may appear complicated, it is a relatively straightforward set of algebraic manipulations and substitutions.

The principles of this derivation are based on those outlined by Bakken et al, 1991,(402) and Fedak et al, 1981,(401). The equations outlined in these papers cannot be directly applied to our measuring system as Bakken’s equations rely on scrubbing CO$_2$ from the incumbent air flow, while Fedak proposes equations for dealing with incumbent air that has not been scrubbed of water vapour. The Promethion system does not scrub incumbent air flows of CO$_2$ and mathematically adjusts for water vapour so that incumbent air can be considered scrubbed of water vapour. Though the derivation of equations for the Promethion system were published in 2017 (403), these were not available to me when I was preparing for the validation in 2016. Moreover, this publication outlines a set of
equations appropriate to validation with a CO₂ and N₂ mixture not N₂ alone. A precision
gas mixer is required for dual gas validation, this equipment is not currently available at
our centre. I derived equations specific for our own nitrogen-only validation as Fedak’s
equations could not be readily applied to our own system. In doing so I was kindly
assisted by Dr Thomas Foerster (Sable Systems, USA), who generously shared his
derivations for dual gas validation.

3.3.2.1 Generating the FRi term

Under normal circumstances nitrogen flows in and out of an indirect calorimeter room
or canopy hood setup without ever being altered. This can be represented algebraically.
The flow rate of nitrogen into a canopy hood in this case is given as the incurrent flow
rate, FRi, multiplied by the incurrent nitrogen concentration expressed as a fractional
concentration FiN₂. Put another way, the total incurrent volume moving per unit time
multiplied by the proportion of that total volume that is taken up by nitrogen:

\[ FRi \times FiN₂ = FRe \times FeN₂ \]

The same is true for the excurrent flow rate of nitrogen:

\[ FRN₂ = FRe \times FeN₂ \]

Thus, the unchanged nitrogen content passing through the system can be represented as:

\[ FRi \times FiN₂ = FRe \times FeN₂ \]

The addition of supplemental nitrogen to the flow through system to mimic VO₂ is:

\[ FRe \times FeN₂ = FRi \times FiN₂ + FRk \times FkN₂ \]

where k indicates the nitrogen validation gas flow. In an indirect calorimetry pull system,
the FRi term is problematic as only FRe is monitored. Rearranging equation 3.4, it can
be shown that:

\[ FRi = \frac{(FRe \times FeN₂)}{FiN₂} - \frac{(FRk \times FkN₂)}{FiN₂} \]

3.3.2.2 Generating the Fractional Nitrogen terms:

Neither FiN₂ nor FeN₂ are directly measured as there are no “nitrogen sensors” in the
Promethion. However, based on Dalton’s law of partial pressures, when gases are
expressed as fractional concentrations the sum of these gases must add to one. Given that CO₂, H₂O and O₂ and N₂ occupy >99% of air and the other components are inert the following holds:

\[ \text{Eq.3.6} \quad 1 = F_iN_2 + F_iO_2 + F_iCO_2 + F_iH_2O \]

Thus, the normal of atmospheric nitrogen into the hood can be expressed as:

\[ \text{Eq.3.7} \quad 1 - F_iO_2 - F_iCO_2 - F_iH_2O = F_iN_2. \]

The flow of nitrogen out of the hood can be expressed as:

\[ \text{Eq.3.8} \quad 1 - F_eO_2 - F_eCO_2 - F_eH_2O = F_eN_2 \]

Finally, the flow rate of supplemental nitrogen (FkN₂) as part of the nitrogen validation procedure can be expressed as:

\[ \text{Eq.3.9} \quad 1 - F_kO_2 - F_kCO_2 - F_kH_2O = F_kN_2 \]

Note as we used pure nitrogen rather than a combined H₂O, CO₂ and N₂ mixture equation 3.9 more correctly reads:

\[ \text{Eq.3.9} \quad 1 = F_kN_2 \]

For combined CO₂, and N₂ dilution an alternative equation 3.9 could be used:

\[ \text{Eq.3.9 (alt)} \quad F_kN_2 = 1 - F_kCO_2 \]

Substituting the F𝑖N₂, F𝑒N₂ and F𝑘N₂ values in equation 3.5, using equations 3.7, 3.8 and 3.9, respectively:

\[ \text{Eq.3.10} \quad F_{R_i} = \frac{F_{Re} \times (1 - F_iO_2 - F_iCO_2 - F_iH_2O)}{1 - F_iO_2 - F_iCO_2 - F_iH_2O} - \frac{F_{Rk} \times (1)}{1 - F_iO_2 - F_iCO_2 - F_iH_2O} \]

As the gases are ‘mathematically’ dried as described appendix C, the H₂O terms can be removed:

\[ \text{Eq.3.10} \quad F_{R_i} = \frac{F_{Re} \times (1 - F_eO_2 - F_eCO_2)}{1 - F_iO_2 - F_iCO_2} - \frac{F_{Rk} \times (1)}{1 - F_iO_2 - F_iCO_2} \]

3.3.2.3 Generating the VO₂ term

VO₂ is the difference between the incumbent and excurrent O₂ volumes per unit time:
Eq. 3.11 \[ VO_2 = FRi * FiO_2 - FR * FeO_2 \]

Substituting for FRi using equation 3.10 gives:

Eq.3.12: \[ VO_2 = \frac{FR(FiO_2 - FeO_2)}{1-FiO_2 - FiCO_2} - \frac{FR(1 - FiO_2 - FiCO_2)}{1-FiO_2 - FiCO_2} * FiO_2 \]

This can be simplified using the FRe term:

Eq.3.13 \[ VO_2 = FRe * \left[ \frac{FiO_2(1 - FeO_2 - FeCO_2) - FeO_2(1 - FiO_2 - FiCO_2)}{1 - FiO_2 - FiCO_2} \right] \]

The left-hand side of equation 3.13 corresponds to the calculations used by the Promethion to determine the measured VO_2. Applying this equation to a human calorimetry recording, the measured VO_2 is the true VO_2 (tVO_2) and FRk = 0, as no nitrogen is being added to the system. This can be represented as follows:

\[ FRe * \left[ \frac{FiO_2(1 - FeO_2 - FeCO_2) - FeO_2(1 - FiO_2 - FiCO_2)}{1 - FiO_2 - FiCO_2} \right] = tVO_2 = mVO_2 \]

During nitrogen validation however, this does not happen. The measured VO_2 produced by the machine is not the tVO_2, which is zero, as the nitrogen gas does not alter oxygen volume, being oxygen free. Equation 3.13 thus becomes:

\[ 0 = FRe * \left[ \frac{FiO_2(1 - FeO_2 - FeCO_2) - FeO_2(1 - FiO_2 - FiCO_2)}{1 - FiO_2 - FiCO_2} \right] \]

\[ - FRk * \left[ \frac{FiO_2 * (1 - FiO_2 - FiCO_2)}{1 - FiO_2 - FiCO_2} \right] \]

Rearranging this equation:

Eq.3.14: \[ FRe * \left[ \frac{FiO_2(1 - FeO_2 - FeCO_2) - FeO_2(1 - FiO_2 - FiCO_2)}{1 - FiO_2 - FiCO_2} \right] = FRk * \left[ \frac{FiO_2 * (1 - FiO_2 - FiCO_2)}{1 - FiO_2 - FiCO_2} \right] \]

The right-hand side of this equation is the apparent VO_2 and corresponds to a theoretical prediction of the VO_2 value the Promethion system should give for any given flow rate of nitrogen, the predicted VO_2 (pVO_2):

Eq.3.15: \[ pVO_2 = FRk * \left[ \frac{FiO_2 * (1 - FiO_2 - FiCO_2)}{1 - FiO_2 - FiCO_2} \right] \]
By comparing the mVO₂ to the pVO₂ a measure of the accuracy of the system termed “oxygen recovery” can be obtained:

Eq.3.16: \[
\text{Oxygen Recovery} = \frac{m\text{VO}_2}{p\text{VO}_2} = \frac{m\text{VO}_2(1\text{-FiO}_2\text{-FiCO}_2)}{\text{FRk}\text{\text{-FiO}}_2(1)}.
\]

The FRk required for a given pVO₂ is:

Eq.3.17: \[
\text{FRk} = p\text{VO}_2 \ast \left[\frac{1\text{-FiO}_2\text{-FiCO}_2}{\text{FiO}_2}\right] = p\text{VO}_2 \ast \left[\frac{1\text{-0.2094-FiCO}_2}{0.2094}\right]
\]

This last equation provides the means to develop a predicted flow rate required to produce the desired VO₂.

As it requires knowledge of the FiCO₂, which can only be determined after the recording, the ability to precisely set the predicted VO₂ in advance is prone to a small error. However, CO₂ values have a very small effect on the outcome particularly as the CO₂ sensors are sensitive and reliable and atmospheric CO₂ concentrations are far lower than FiO₂(404). Notwithstanding these considerations, once FiCO₂ is considered the generated pVO₂ can be calculated accurately and then compared with the measure VO₂ to produce the oxygen recovery.

### 3.3.3 The Experimental Setup for Nitrogen dilution

Performing the Nitrogen validation studies required some modification to the calorimetry setup used in human studies. The chief differences are the use of a mass flow controller for nitrogen flow control, the use of an additional computer program for nitrogen flow data capture and remote control of the mass flow meter, and the channelling of nitrogen from the gas cylinder into the canopy hood via a sealed tubing system.

#### 3.3.3.1 Mass Flow Controllers

Mass flow controllers are electronic devices used to deliver a constant mass flow of a gas. As previously noted, gas volumes change with varying temperature and pressure. Mass flow controllers vary the volumetric flow rate based on concurrently recorded temperature and pressure to ensure a constant mass flow rate. This is analogous to delivery of a constant volume of gas at a fixed temperature and pressure, the theoretical “environment” assumed by the derivations. The mass flow controllers are programmed by inputting the desired STP volumetric flow rate. They are also used in the Promethion system to generate the main flow rate and subsampling flow rates.
An Alicat mass flow controller (MC-5SLPM-D, Alicat Scientific, USA) calibrated by the manufacturer for planned nitrogen flows between 0 – 3 SLPM, was used. The accuracy of this mass flow controller was 0.8% of the reading and 0.2% of the maximum flow rate of 3SLPM. Thus, for a programmed flow rate of e.g. 800 mls/min the error would be $3000 \times 2 \times 10^{-3} + 800 \times 8 \times 10^{-3}$, 12.4 mls/min or 1.6%. Similarly, the precision is 0.2% full scale, 6mls/min or 0.75% for a flow rate of 800mls/min (405).

A gas leak is a major potential source of error. Two Clippard push-on male connectors ¼” OD to 1/8” NPT (Clippard Instrument Laboratory, USA) were used to connect the Bev-A-Line tubing to the mass flow controllers. Their threads were first wrapped in Teflon tape and the seal checked with a small amount of soapy water rubbed around the connection as per the manufacturer’s instructions. The absence of bubbles during a nitrogen flow confirmed a good seal.

### 3.3.3.2 Data Acquisition and Analysis

Data acquisition and data logging were performed using the stand-alone interface for Alicat Instruments. This is a virtual interface that allows control of all setting on the mass flow controller from a computer (406), available from: https://www.alicat.com/support/software-drivers/labview-virtual-instrument-drivers/. It also graphs in real time; the mass flow rate, volumetric flow rate, set-point flow rate and temperature. These data can be logged and the logging frequency can be customized so that information is captured every millisecond, second, minute, etc. This software has several advantages to a manual approach. First, the nitrogen input with time can be accurately recorded. Secondly, any problems with the nitrogen flow can be quickly identified visually during the recording, rather than waiting for data analysis after the study has been completed. Thirdly, changing flow rates is a matter of typing in a new volume and the change occurs within milliseconds, while manual entry requires “dialling up” to a new flow rate, which is difficult to do according to a time-specific protocol.

### 3.3.3.3 Configuration of the Equipment

To run the nitrogen dilution studies, the canopy hood was placed on pillow within the atmospherically isolated chamber. Pure nitrogen was run from a pressurised nitrogen tank via Bev-A-Line tubing into the mass flow controller gas inlet. A second tube ran from the mass flow controller gas outlet through a custom port that had been bored into
the wall of the atmospherically isolated chamber. To avoid air-leaking, a custom rubber seal was created that was fastened around the port and perforated to admit the tubing only. Once in the chamber the tubing was run into the canopy hood between the base and the pillow, the canopy skirting was wrapped around this entry point and under the pillow to prevent air leak.

The most important part of this setup was the use of a custom “diffuser”, a closed-end perforated plastic cup, sitting over the tip of the tubing sitting in the canopy hood (Fig 3.1). The addition of the diffuser, produced a dramatic improvement in validity in the order of approximately 20-30%. It works by preventing the nitrogen gas escaping via the hood air inlet after it leaves the tubing. The nitrogen enters the closed cup and exits via many tiny perforations in the sides. A schematic of the nitrogen validation setup is presented in Fig 3.1.

**Figure 3.1: Nitrogen dilution experimental setup**
3.3.4 Nitrogen Dilution Test Protocol

3.3.4.1 *Step 1: Initialising the mass flow control software.*

Once the equipment was setup as described above, the stand-alone interface for Alicat Instruments was started on the laboratory computer. The nitrogen tank was pressurised to ensure flow through the tubing. Using manual control of the mass flow controller the baud rate was set to 9600 bits on the mass flow controller, the flow rate was set to a negligible 0.003 SLPM and the control was set to RS-232 to enable remote access. The baud rate is a measure of the speed of communication between the mass flow controller and the data logging software. Both must be matched for communication to occur.

3.3.4.2 *Step 2: Setting up the calorimetry recording.*

Caloscreen was opened and the flow rate set to 80 SLPM with a baselining frequency of 10 minutes and a baselining duration of three minutes. Once the recording started, the sample number counter was used as a timestamp for all subsequent events. The start of the recording was considered timepoint zero.

3.3.4.3 *Step 3: Data logging the mass flow controller output.*

Using the stand-alone interface, a connection with the mass flow controller was established on the lab computer by specifying a baud rate of 9600 bits and the correct communications port (COM port) on the lab computer. Once this was done, the setting and real-time readings began to graph within the interface program at a rate of one sample per millisecond. The sampling frequency for the mass flow controller was reduced to one sample per second and data logging was started. The sample time at which the data logging started was recorded.

3.3.4.4 *Step 4: Altering the nitrogen flow.*

After at least 90 seconds from the start of the recording, the nitrogen flow was instantaneously increased to a flow rate of 0.757 SLPM. Assuming an FiO\textsubscript{2} of 20.95 %, this would produce a predicted VO\textsubscript{2} of 0.2011 STP L/min. The flow was monitored graphically and reduced to 0.003 SLPM at least 400 seconds after the increase in nitrogen. This was to allow sufficient time to reach steady-state and to enable smooth running of the macro. Data logging was stopped at 900 seconds and the calorimetry recording was stopped after 1450 samples.
3.3.4.5 **Step 5: Data selection and Analysis:**

All data analysis was performed in R (399). After running the automated macro as described in appendix C, markers were placed on the calorimetry recording to indicate the onset and offset of the nitrogen flow. VO\textsubscript{2}, time, nitrogen onset and offset were exported and combined with the data log produced by the Alicat standalone interface. The data log was matched to the calorimetry data by aligning the nitrogen onset marker from the calorimetry data with the change in nitrogen flow rate from 0.003 to 0.757 in the Alicat data log. For the remainder of the data analysis, the time of nitrogen onset was designated timepoint zero, all time intervals are presented in seconds.

To determine the achievement of steady-state, the first order differential of the VO\textsubscript{2} reading with respect to time was derived, dVO\textsubscript{2}/dT. This plots a graph of the rate of change of VO\textsubscript{2} over time (Fig 3.2). Using this graph, steady-state can be objectively defined as dVO\textsubscript{2}/dT = 0. This can be visually identified by graphing the data. A rolling mean technique was used to reduce noise so the time point at which the slope became zero was easier to identify. A rolling mean is a technique used in signal processing by which a prespecified “window” consisting of n samples centred on an x value, are used to calculate the mean value of f(x), for each timepoint x\textsubscript{1}, x\textsubscript{2}, x\textsubscript{3}…x\textsubscript{n} etc. A window of 10 samples was used to smooth the signal. Visual examination of these data after smoothing, identified that steady-state was achieved by 300s at a conservative estimate. Thus, the mean VO\textsubscript{2} at steady-state was calculated for all samples between 300 seconds and the time of nitrogen offset. Validity was measured using percentage oxygen recovery, which was calculated using equation 3.16:

\[
\text{Oxygen Recovery} = \frac{m \times VO_2 (1-0.2094-FiCO2)}{0.757 \times 0.2094(1)}
\]

The mean, standard error, sample number and 95% upper and lower confidence intervals were calculated for VO\textsubscript{2} and % oxygen recovery for each trial (Table 3.1). The early pilot tests, mainly numbers 1-8, contained several errors primarily due to incorrect setup which resulted in incomplete data collection. Where such an error was identified, the trial data was not analysed. Thus 12 of the 21 nitrogen dilution studies are presented here. Precision was assessed using the standard deviation of the mean VO\textsubscript{2} across all 12 trials using Pearson’s correlation coefficient.
3.3.5 Nitrogen Dilution Trial Results

Twelve trials were conducted according to the protocol. Early trials were discounted due to; incorrect baselining specification during calorimetry setup, incomplete data logging, premature software termination for the mass flow controller data log or presumed nitrogen leak from the hood prior to using a sealed cup at the nitrogen gas outlet. As a result, the trials are numbered discontinuously 9-21.

The percentage O$_2$ recovery across all trials was (M: 101%, SD: 0.5%). The fraction of inspired CO$_2$, required for calculating the predicted VO$_2$ was 0.0005% in all studies. The VO$_2$ remained consistent at steady-state as indicated by a low SD for each trial (Table 3.1). The results of the nitrogen dilution trials are presented in Table 3.1 and Fig. 3.3.
Table 3.1: Mean VO$_2$ and Oxygen Recovery from the 12 nitrogen dilution studies

<table>
<thead>
<tr>
<th>Trial No</th>
<th>Mean VO$_2$ (SLPM)</th>
<th>VO$_2$ SD (SLPM)</th>
<th>O$_2$ Recovery (%)</th>
<th>O$_2$ Recovery SD (%)</th>
<th>95% CI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>0.205</td>
<td>0.002</td>
<td>102</td>
<td>0.008</td>
<td>102.2 - 102.2</td>
</tr>
<tr>
<td>10</td>
<td>0.204</td>
<td>0.002</td>
<td>102</td>
<td>0.008</td>
<td>101.8 - 101.8</td>
</tr>
<tr>
<td>12</td>
<td>0.204</td>
<td>0.002</td>
<td>102</td>
<td>0.012</td>
<td>101.6 - 101.6</td>
</tr>
<tr>
<td>13</td>
<td>0.204</td>
<td>0.002</td>
<td>101</td>
<td>0.009</td>
<td>101.5 - 101.5</td>
</tr>
<tr>
<td>14</td>
<td>0.203</td>
<td>0.001</td>
<td>101</td>
<td>0.005</td>
<td>101.4 - 101.4</td>
</tr>
<tr>
<td>15</td>
<td>0.204</td>
<td>0.002</td>
<td>101</td>
<td>0.009</td>
<td>101.4 - 101.4</td>
</tr>
<tr>
<td>16</td>
<td>0.202</td>
<td>0.001</td>
<td>101</td>
<td>0.006</td>
<td>100.5 - 100.5</td>
</tr>
<tr>
<td>17</td>
<td>0.201</td>
<td>0.001</td>
<td>100</td>
<td>0.006</td>
<td>100.1 - 100.1</td>
</tr>
<tr>
<td>18</td>
<td>0.202</td>
<td>0.002</td>
<td>101</td>
<td>0.008</td>
<td>100.8 - 100.8</td>
</tr>
<tr>
<td>19</td>
<td>0.204</td>
<td>0.001</td>
<td>101</td>
<td>0.005</td>
<td>101.5 - 101.5</td>
</tr>
<tr>
<td>20</td>
<td>0.204</td>
<td>0.001</td>
<td>101</td>
<td>0.006</td>
<td>101.5 - 101.5</td>
</tr>
<tr>
<td>21</td>
<td>0.203</td>
<td>0.001</td>
<td>101</td>
<td>0.003</td>
<td>101.1 - 101.1</td>
</tr>
<tr>
<td>Mean</td>
<td>0.203</td>
<td>0.001</td>
<td>101</td>
<td>0.007</td>
<td>101.3 - 101.3</td>
</tr>
</tbody>
</table>
Figure 3.3:
Oxygen recovery for all 12 nitrogen dilution trials.
3.4 Validation and Precision by Alcohol Combustion

3.4.1 The Principle of Alcohol Combustion.

A more traditional and oft-used method for validation of indirect calorimetry is the alcohol combustion method (407). The complete combustion of a known quantity of ethanol produces a known quantity of carbon dioxide and consumes a known quantity of oxygen according to the following stoichiometric relationship:

\[ 1 \text{ mol } C_2H_5OH + 3 \text{ mol } O_2 \rightarrow 2 \text{ mol } CO_2 + 3 \text{ mol } H_2O \]

Ethanol can be converted to mass in grams based on its molar mass and as the volume of 1 mol of any gas at STP is 22.4 litres:

\[ 46g \text{ } C_2H_5OH + 67.2 \text{ L } O_2 \rightarrow 44.8 \text{ L } CO_2 + 67.2 \text{ L } H_2O \]

Per gram of ethanol burned, the expected O\text{2} consumption and WVP production is 1.46 l. The expected CO\text{2} production is 0.973 l CO\text{2}. Similarly, the rate of alcohol burned can be used to calculate a predicted VO\text{2} and VCO\text{2}. The RQ for ethanol is 0.6667, and the heat of combustion is 1367 kJ.

As a volatile chemical, ethanol and other alcohols tend to evaporate. This is one of the chief challenges in performing an ethanol combustion (408). A tight-fitting container cap for the ethanol, and minimising the wick surface area exposed to the air can help to reduce this, but it can be challenging to eliminate evaporation entirely.

3.4.2 Data Acquisition

As with nitrogen validation, continuous monitoring of the system input, in this case ethanol combustion, was necessary to ensure a consistent and known burn rate. PuTTY software (409), available from www.putty.org, was used to register and log continuous weight readouts from a digital weighing scale (Highland™ HCB1002, Adam Equipment, Australia) at 0.5 second intervals. This was done using PuTTY’s serial terminal interface by enabling the data logging function. The laboratory computer was connected to the digital scales via an extended RS-232 to USB connection.
3.4.3 Configuration of the Equipment

The experimental setup used in the ethanol burns is pictured in Fig 3.4. A jeweller’s alcohol lamp, consisting of a glass bottle with a brass cap was partly filled with 99.9% ethanol. A clean, trimmed 5mm diameter cotton wick was drawn out of the aperture at the top so it was protruding 1-2 cm above the surface of the brass cap. This lamp was placed on the scales which sat on a fireproof blanket within the canopy hood. To avoid the hot gas escaping out the inlet located in the top of the hood, the outlet tubing and inlet port attachments were switched, so air was drawn in the side of the canopy hood and out the top of the hood. To avoid heat damage to the acrylic canopy hood it was propped up on blocks 30 cm high placed behind and at the sides of the scales. As the canopy skirting was not large enough to cover this arrangement, a new larger skirting was created by cutting a central hole in a large vinyl sheet. This was clamped to the circumference of the table to ensure there no air leak. The larger skirting decreased the response time of the canopy hood configuration compared with the nitrogen validation approach or human calorimetry recordings. However, the steady state value achieved, and thus the estimate of validity, will not have been affected by the change in volume.

Figure 3.4: Calorimeter configuration for ethanol burn trials.
3.4.4 Ethanol Combustion Test Protocol

3.4.4.1 Step 1: Setting up the Burn Equipment
The Promethion calibrations were checked to ensure they were up to date. With the apparatus setup as in Fig 3.4, the digital scales were switched on and tared to zero and the vinyl skirting left open.

3.4.4.2 Step 2: Data Logging and Starting the Recording
The PuTTy software on the laboratory computer was started and set to logging the scale output data timestamped with the computer date and time. A calorimetry recording was initiated in Caloscreen with a flow rate of 80 SLPM and a baselining interval every 10 minutes for three minutes. The start of this recording was designated timepoint zero and all subsequent events timed in relation to this.

3.4.4.3 Step 3: Starting the Ethanol Burn
The operator entered the environmentally isolated room that housed the burn equipment, to light the ethanol lamp. At this point a stopwatch was started. The vinyl skirting was closed over and clamped to the table before leaving.

3.4.4.4 Step 4: Completing the Burn
The ethanol burn initially burns yellow, with a sooty flame due to incomplete combustion. With our apparatus, a clean consistent blue flame was achieved after three minutes. For this reason, data logging was started 200 seconds and calorimetry 300 seconds after lighting the flame. The flame was extinguished by the operator, with breath held, after 15 minutes of calorimetry exactly.

3.4.4.5 Step 5. Completing Data Logging
Calorimetry was stopped after a further 550 seconds. The PuTTy data logging was stopped simultaneously. Timestamps for the start of data logging, start of calorimetry and extinguishing of the flame and the end of calorimetry were used to collate the ethanol weight data and the calorimetry data.

3.4.5 Data Analysis
Expedata’s automated data analysis (appendix C), was performed on the raw calorimetry data. The VO$_2$, VCO$_2$, REE and sample number data were exported along with the PuTTy
data log to a comma separated value file. Both the calorimetry and weight log data were time matched, all Putty samples that preceded the start of the calorimetry recording were discarded and analysis was performed on the remaining samples. The mean VO$_2$, VCO$_2$ and RQ values were taken between 600 -900 seconds into each recording, as steady-state had been achieved by this point.

The burn data were analysed using R(399). The burn rate was calculated using linear regression of the ethanol weight readout against time. The slope of the regression line was taken as the burn rate and used to calculate the predicted VO$_2$ and VCO$_2$ based on the calculations above. Predicted RQ was 0.667. Validity was expressed as the ratio of the measured value to the predicted value for each of VO$_2$, VCO$_2$, WVP and RQ, known as the “percentage recovery” of these measures. As the ethanol burn rate is not constant between burn readings, the percentage recovery was used to assess precision by correlating readings from all burn trials, descriptive statistics for the burn rate are presented.

3.4.6 Ethanol Combustion Test Results

Ethanol combustion was extremely linear in most studies (Fig 3.5). Two outliers, trial number 14 and 15, burned slowest, produced the least linear burn and the poorest O$_2$ and CO$_2$ recovery. Linear regression produced an excellent fit, with an adjusted $R^2$ for the linear model >0.999 in all but trial number 14 and 15. The range of simulated VO$_2$ varied substantially from 0.150 - 0.412 SLPM, while VCO$_2$ varied from 0.100-0.275 SLPM. The validity as indicated by the % recovery of all burns conducted per protocol was (M:109%, SD: 15%) for VO$_2$ and (M:112%, SD: 15%) for VCO$_2$. Excluding trials 14 and 15 this improved to an accuracy for VO$_2$ of (M:103%, SD: 5.1%) and for VCO$_2$ (M:102%, SD:5.2%). It is worthwhile noting that RQ varied very little, even when there was major variation in VCO$_2$ and VO$_2$. The results of the ethanol burn trials are presented in Fig 3.5 and Table 3.2.
Figure 3.5
Ethanol burn rate for all 12 trials.
Table 3.2: \( \text{O}_2 \) and \( \text{CO}_2 \) recovery and respiratory quotient during ethanol burn tests.

<table>
<thead>
<tr>
<th>Trial No</th>
<th>Burn rate ( \text{g}_\text{min} )</th>
<th>Squared residuals ( (R^2) )</th>
<th>% ( \text{O}_2 ) Recovery</th>
<th>% ( \text{VCO}_2 ) Recovery</th>
<th>mRQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
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<td>1.0000</td>
<td>106.1</td>
<td>105.9</td>
<td>0.666</td>
</tr>
<tr>
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<td>107.6</td>
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<td>0.666</td>
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<td>12</td>
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<td>0.9997</td>
<td>103.1</td>
<td>102.2</td>
<td>0.661</td>
</tr>
<tr>
<td>13</td>
<td>0.154</td>
<td>0.9994</td>
<td>114.3</td>
<td>113.4</td>
<td>0.662</td>
</tr>
<tr>
<td>14</td>
<td>0.127</td>
<td>0.9967</td>
<td>127.2</td>
<td>127.1</td>
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</tr>
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</tr>
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<td>100.1</td>
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<td>100.2</td>
<td>0.663</td>
</tr>
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<td>0.9997</td>
<td>106.4</td>
<td>106.0</td>
<td>0.664</td>
</tr>
<tr>
<td>Mean</td>
<td>0.201</td>
<td>0.999</td>
<td>109</td>
<td>112</td>
<td>0.662</td>
</tr>
</tbody>
</table>

3.5 Characterization of the VO2 Response to Nitrogen Dilution

3.5.1 The Z-Transformation Does Not Reflect the VO2 Response Seen During Nitrogen Dilution

While performing the nitrogen dilution studies, it became apparent that change in VO2 in response to a nitrogen infusion was non-linear. Moreover, the VO2 response was not the bounded exponential curve that the z-transformation assumes. Due to the washout effect (section 2.2.4.1), a change in resting energy expenditure that is sustained for a shorter duration than it takes to achieve an approximation of steady-state, approximately 300 seconds, will never produce a resting energy expenditure measurement that reflects the true resting energy expenditure. Thus, the need to perform the z-transformation (313). If the transformation does not accurately reflect the change in VO2 over time in an indirect calorimeter system, the predicted steady-state values are called into question. The aim of characterising the VO2 response to nitrogen dilution was to determine if the VO2 response could be modelled better with a different curve. This would allow more accurate transformation of measured VO2 to steady-state values.
3.5.2 Curve-Fitting the VO₂ response:

3.5.2.1 Non-linear Regression

I characterised the time response relationship of VO₂ during nitrogen dilution by describing the response curve using a non-linear regression approach. Non-linear regression, as with linear regression, fits a line to a series of points and produces estimates of the parameters that describe the curve. Several parameters can be incorporated in a function described this way. Curve fitting enables objective and reproducible estimation of the parameters that define the curve, that is not based on graphical inspection alone. For example, rather than finding the midpoint of a curve visually, it is determined by analysing a range of possible values for the specified curve type and selecting the one that provides that best fit. The type of curve produced illustrates the physical properties of the nitrogen dilution technique and can potentially be used to predict the Promethion input based solely on the VO₂ output values.

This last point underscores a key limitation of indirect calorimetry; that the measured energy expenditure assumes the participant exists in a steady-state, or that otherwise this steady-state can be accurately predicted. Human energy expenditure does vary in time, and this variation may be of interest, as much as the averaged values that we currently use are. Just as the periodicity of the electrocardiogram or electroencephalogram can be related to pathological states or used in diagnosis, the variation in energy expenditure over time may vary with different diseases or under different environmental conditions. This is biologically interesting. However, recording this variation accurately is challenging.

3.5.2.2 Selecting the VO₂ Response Curve

An iterative process was used whereby the properties of the VO₂ curve were described and matched to a known non-linear curve, that may better represent the VO₂ response than the bounded exponential curve. These properties were, a gradual increase in VO₂ over time until a maximum value, the steady-state value, was achieved. A rate of change that initially increased exponentially until 50% of the maximum value was achieved and then decreased in an exponential fashion (Fig 3.2). These properties most closely resembled a logistic curve, frequently used to describe population growth(410). The logistic curve is given by: \[ f(x) = \frac{\theta_1}{1 + e^{-\theta_2(x-\theta_3)}}. \]
The logistic function describes a sigmoid curve that reaches a maximum value, $\theta_1$, initially exponentially until it reaches 50% of the maximum value $\theta_3$, thereafter it increases in a bounded exponential fashion. The second coefficient, $\theta_2$, modifies the steepness of the slope. For nitrogen dilution, $\theta_1$ corresponds to the steady-state VO$_2$ value, $\theta_3$, corresponds to the point at which 50% of the maximum value is achieved. $\theta_2$ is a function of the volume in which the nitrogen dilution is occurring: the larger the space the slower the rate of change of VO$_2$. It is also a function of the flow rate, FR, which increases the rate of change of VO$_2$, and a constant k.

3.5.2.3 Fitting the Logistic Curve

To test the fit of this curve to the data non-linear least squares regression (nls package, R), was used (411). This method iteratively attempts to fit the measured data from each of the trials to the logistic curve model in a stepwise fashion until a maximum fit is achieved. Unlike linear regression, starting estimates for each parameter must be provided. These are refined by the non-linear regression procedure to produce the best fit. For $\theta_1$, the predicted VO$_2$ was used. For $\theta_3$, the time point at which 50% of the max VO$_2$ was achieved was used. For $\theta_2$, a model was constructed with $\theta_1$ and $\theta_3$ specified, and $\theta_2$ set to one. As $\theta_1$ and $\theta_3$ were constrained using the estimates, the starting estimate for $\theta_2$, was found not to have a bearing on the final estimate for $\theta_2$ produced by the model.
<table>
<thead>
<tr>
<th>Trial No.</th>
<th>$\theta_1$ (SLPM)</th>
<th>SE</th>
<th>p-value</th>
<th>$\theta_2$</th>
<th>SE</th>
<th>p-value</th>
<th>$\theta_3$</th>
<th>SE</th>
<th>p-value</th>
<th>Time to Midpoint (s)</th>
<th>Model Correlation</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>&lt;2e-16</td>
<td>0.045</td>
<td>0.0004</td>
<td>&lt;2e-16</td>
<td>59</td>
<td>0.22</td>
<td>&lt;2e-16</td>
<td>0.999</td>
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</tr>
<tr>
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<td></td>
<td>&lt;2e-16</td>
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<td>0.0003</td>
<td>&lt;2e-16</td>
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<td>&lt;2e-16</td>
<td>0.996</td>
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</tr>
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<td></td>
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<td>0.063</td>
<td>0.0012</td>
<td>&lt;2e-16</td>
<td>52</td>
<td>0.35</td>
<td>&lt;2e-16</td>
<td>0.986</td>
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<td></td>
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<td>&lt;2e-16</td>
<td>50</td>
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<td>0.988</td>
<td></td>
</tr>
<tr>
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<td></td>
<td>&lt;2e-16</td>
<td>0.051</td>
<td>0.0007</td>
<td>&lt;2e-16</td>
<td>57</td>
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<td>&lt;2e-16</td>
<td>0.997</td>
<td></td>
</tr>
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<td>&lt;2e-16</td>
<td>0.052</td>
<td>0.0007</td>
<td>&lt;2e-16</td>
<td>65</td>
<td>0.32</td>
<td>&lt;2e-16</td>
<td>0.999</td>
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</tr>
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<td>0.0007</td>
<td>&lt;2e-16</td>
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<td>&lt;2e-16</td>
<td>0.052</td>
<td>0.0007</td>
<td>&lt;2e-16</td>
<td>53</td>
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<td>&lt;2e-16</td>
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<td></td>
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<td>0.0004</td>
<td>&lt;2e-16</td>
<td>62</td>
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<td>&lt;2e-16</td>
<td>0.998</td>
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<td></td>
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<td>0.0007</td>
<td>&lt;2e-16</td>
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<td>0.0005</td>
<td>&lt;2e-16</td>
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<td>&lt;2e-16</td>
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<td></td>
<td>&lt;2e-16</td>
<td>0.040</td>
<td>0.0006</td>
<td>&lt;2e-16</td>
<td>77</td>
<td>0.39</td>
<td>&lt;2e-16</td>
<td>0.986</td>
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<td>&lt;2e-16</td>
<td>0.052</td>
<td>0.0003</td>
<td>&lt;2e-16</td>
<td>59</td>
<td>0.29</td>
<td>&lt;2e-16</td>
<td>0.991</td>
<td></td>
</tr>
</tbody>
</table>
3.5.2.4 Fitting the Bounded Exponential Curve

For comparison, the bounded exponential curve described by the z-transformation was also fitted to the measured data using non-linear regression. As outlined in chapter two, the z transformation is given by,

\[ Z = 1 - e^{(-FR(dT/EV))} \]

where EV, is a theoretical quantity comprised of the actual physical volume contained by the canopy hood and tubing as well as theoretical dead space. The flow rate was 80 litres/min, or 1.333 l/sec, dT was the time elapsed since the start of the validation. As both time and flow rate are accurately measured and identical for all studies, they were incorporated as constants in the model. The remaining parameter, EV, is more difficult to estimate being comprised in part of difficult-to-measure dead space. Thus, the starting parameter used was based on the physical volume of the canopy hood calorimeter and tubing. The volume of the canopy hood is 18.4l, and the volume of the cylindrical air mixer, tubing and air filter was estimated by measuring the dimensions of each component. In total the estimated volume was 37.4 l.

For each nitrogen validation recording, the VO₂ predicted by the bounded exponential model output, its correlation with the measured result, and correlation with the VO₂ predicted by the logistic model are presented in Table 3.4. The predicted VO₂ for the logistic model and bounded exponential model, and measured VO₂ are pictured in Fig 3.6.

As an additional step, the EV estimate was iteratively changed from the starting estimate of 37.4 l to see if the prediction of VO₂ could be improved beyond that provided by the non-linear regression procedures.
Table 3.4: Non-linear regression and manually calculated model estimates for the bounded exponential curve

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>NLR - EV (L)</th>
<th>SE</th>
<th>p-value</th>
<th>NLR VO2/ Measured VO2 Correlation</th>
<th>MAN- EV (L)</th>
<th>MAN VO2/ Measured VO2 Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>272</td>
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<td>&lt;2x10^{-16}</td>
<td>0.78</td>
<td>75</td>
<td>0.984</td>
</tr>
<tr>
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<td>304</td>
<td>6</td>
<td>&lt;2x10^{-16}</td>
<td>0.81</td>
<td>90</td>
<td>0.987</td>
</tr>
<tr>
<td>12</td>
<td>205</td>
<td>4</td>
<td>&lt;2x10^{-16}</td>
<td>0.84</td>
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<td>0.989</td>
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<tr>
<td>13</td>
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<td>0.83</td>
<td>64</td>
<td>0.993</td>
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<td>6</td>
<td>&lt;2x10^{-16}</td>
<td>0.87</td>
<td>75</td>
<td>0.990</td>
</tr>
<tr>
<td>15</td>
<td>226</td>
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<td>&lt;2x10^{-16}</td>
<td>0.89</td>
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<tr>
<td>16</td>
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<td>&lt;2x10^{-16}</td>
<td>0.80</td>
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<tr>
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<tr>
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<td>21</td>
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<td>&lt;2x10^{-16}</td>
<td>0.91</td>
<td>97</td>
<td>0.987</td>
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<tr>
<td>Mean</td>
<td>229</td>
<td>6</td>
<td>&lt;2x10^{-16}</td>
<td>0.85</td>
<td>75</td>
<td>0.987</td>
</tr>
</tbody>
</table>
Figure 3.6: Change in Predicted $\text{VO}_2$ with Time for Logistic and Bounded Exponential Models and Experimentally Measured $\text{VO}_2$
3.5.2.5 Comparing Logistic and Bounded Exponential Models

The logistic models correlated well with the measured data, with a mean correlation of 0.99 between the observed data and that predicted by each model. The model parameters across all tests (Table 3.2) were; \( \theta_1 \) (M:0.2009, SLPM, SD:0.0023 SLPM); \( \theta_2 \) (M:0.05, SD:0.01); \( \theta_3 \) (M:59 sec, SD:8 sec).

By contrast the estimates produced by the non-linear regression procedure for the bounded exponential model correlated poorly with the measured VO\(_2\) (\( r: 0.85 \)). Using the manually calculated value for EV in the bounded exponential model, the correlation improved to \( r: 0.987 \).

3.6 Discussion

Validation and precision testing using two methods, nitrogen dilution and ethanol burn, were conducted to assess the in-silico validity and precision of the Promethion indirect calorimeter. Finally, the nitrogen validation data were used to investigate whether modelled VO\(_2\) using a logistic curve, rather than a bounded exponential curve produced by the z-transformation, would be more accurate.

3.6.1 Comparison of Nitrogen Dilution and Ethanol Combustion Methods

3.6.1.1 Nitrogen Dilution Precision and Accuracy

The nitrogen dilution studies demonstrated a mean O\(_2\) recovery of 101% (95%CI: 99.6 to 102.4%) indicating a high in-silico precision and a high degree of accuracy. Both VO\(_2\) and VCO\(_2\) are involved in the nitrogen dilution calculations. However, VCO\(_2\) is not robustly assessed using nitrogen dilution only, as the very low concentrations observed during the nitrogen dilution studies are not in the physiological range of expired air. Nonetheless, accurate barometric pressure, flow rate, temperature and water vapour detection were necessary to contribute to the validation and precision results seen with the nitrogen dilution technique.

A mixed N\(_2\) and CO\(_2\) gas dilution such as that conducted by Rising et al, 2017(403), is a better assessment of REE, as it involves a more accurate simulation of VCO\(_2\). A precision gas mixer is required for this technique which could otherwise be easily conducted using; the equations outlined by Rising et al, a second mass flow controller, calibrated for physiologic human ranges, and Alicat Software modified to monitor two mass flow
controllers simultaneously. With this setup, it would be possible to use the combined gas flow to mimic REE variation, such as that seen with human REE measurements. This would enhance validity of the method, as it would bear more resemblance to in-vivo indirect calorimetry measurements.

3.6.1.2 Ethanol Burn Precision and Accuracy

The results of the ethanol burn validation differed from those suggested by the nitrogen dilution method. Both the O₂ recovery and VCO₂ recovery were greater than expected at 103% (95%CI: 93 to 113%) and 102% (SD:92 to 112%) respectively, while the RQ was 0.662, close to the expected 0.667 expected value. Compared to the nitrogen dilution study the accuracy was reduced and the measured precision was poorer as indicated by the wide 95% confidence intervals.

There are several potential reasons for these results. Firstly, the ethanol burn method assumes complete combustion of ethanol. While the hood is well supplied with oxygen, there is the possibility that intermediary products form and are combusted on the cotton wick during the 30-minute burn. During studies 14 and 15, the ethanol reservoir was lower than for the other studies, with the jeweller’s lamp approximately half filled. It was replenished after study 15. Evaporation from the wick within the enclosed lamp may have led to erratic ethanol capillary flow from the reservoir to the flame accounting for the non-linear burn rate during these studies, a violation of the assumptions of the method. While the burn rate appeared stable for the other studies, the substantial errors produced by tests 14 and 15 were reflected in ostensibly minor reductions in the squared residuals and minor fluctuations in the measured burn rate during the test (Fig 3.5). It is possible that lesser degrees of erratic combustion in the other tests may have contributed to the reduced validity and precision compared with the nitrogen validation.

Secondly, evaporation from the wick outside the lamp may also have reduced accuracy but one would expect that the VO₂ consumption would be less than predicted were this the case as O₂ is not altered with evaporation.

3.6.1.3 VO₂ and VCO₂ values estimated by Nitrogen and Validation Methods

While VO₂ for the nitrogen dilution studies was calculated to be a physiological 0.201 SLPM, the simulated VO₂ for the ethanol burn studies ranged from 0.154 – 0.413 SLPM, VCO₂ value ranged from 0.100 to 0.275 SLPM in the ethanol burn study, these are
relatively physiologic, values. The VCO\(_2\) value for the nitrogen dilution study were a fixed 0.0005% which is below a physiologic range, unsurprising given the method is designed to test VO\(_2\) consumption only.

3.6.1.4 Should RQ alone be used for Validation of Indirect Calorimetry?
RQ alone does not detect errors that affect both VCO\(_2\) and VO\(_2\) equally. Assuming the gases are well mixed the potential errors that fall into this category are many: air leaks, inaccurate barometric pressure/temperature/water vapour sensors, unstable flow rate generation or inaccurate flow rate detection. The advantage of using the RQ is that it does not rely on weighing the ethanol to estimate burn rate, however this seems a small inconvenience relative to the enhanced detection of error using O\(_2\) and CO\(_2\) recovery.

In summary, the assessment of the validity and precision with nitrogen dilution was considered more reliable than that produced by ethanol combustion. This was chiefly due to the violation of the assumption of a constant burn rate, and the error introduced by a high rate of water vapour production using the ethanol combustion method.

3.6.2 Models of the VO\(_2\) response to Nitrogen Dilution
3.6.2.1 The Logistic Model predicts VO\(_2\) better than the Bounded Exponential Model
The observation during the nitrogen dilution trial that the measured VO\(_2\) curves did not resemble the bounded exponential curve that the z-transformation is based on was supported by two pieces of evidence. First, the first order differential of the nitrogen dilution data (Fig 3.2) is not an exponential curve. One of the mathematical properties of an exponential curve is that its derivative is also an exponential. Fig 3.2 illustrates that the first order differential consists of a rate of change that increase to a point, then decreases. Integrating a graph of this type produces an exponentially increasing graph, to a point, followed by an exponentially decreasing graph. I.e. a sigmoid curve, rather than an exponential.

Secondly, the specification of a logistic curve in the non-linear regression produced a curve that correlated more closely with the measured data than either the non-linear regression bounded exponential curve, or the manually calculated bounded exponential curve.
Why did the non-linear bounded exponential curve perform correlate so poorly? The non-linear regression method is not without error. Potential sources of error may be in the selection of data – though this does not appear to have been a problem in this case, as the same starting value was used for both the manually calculated and the non-linear regression method. A more plausible explanation is that there were insufficient data at the start and the end of the change in VO\textsubscript{2}. These are the regions that differentiate the models most, and as they are of relatively short duration there may not have been sufficient samples to inform an adequate model fit.

The slope of the resulting non-linear regression curves appeared inappropriately shallow (Fig 3.6), and this informed the decision to proceed with manually altering the EV parameter iteratively until a maximum fit was achieved for each curve. This greatly improved the correlation with measured data but was still less than that seen with the logistic curve.

3.6.2.2 Why is VO\textsubscript{2}/VCO\textsubscript{2} Production Not Predicted by the Bounded Exponential Model

Christensen \textit{et al}, 1946, first describe the response to chemical production of CO\textsubscript{2} vapour in a closed calorimetry system as an exponential function of time involving the volume of the measurement chamber, the flow rate from the chamber as well as the amount of CO\textsubscript{2} produced. The equation that relates these factors is a bounded exponential one. In this experiment, VCO\textsubscript{2} was simulated by dropping sulphuric acid at a fixed rate onto sodium bicarbonate.

This simulation method does differ from the nitrogen validation method described here in two ways. Firstly, the production of a simulated VCO\textsubscript{2} using sodium bicarbonate cannot be instantaneously induced as with a mass flow controller. Also, the rate of production of VCO\textsubscript{2} from a constant addition of sulphuric acid to bicarbonate is not linear, but as with any chemical reaction is itself an exponential function of time. This does not serve well as a step function for evaluating response times. The z-transformation operates on the basis that changes in energy expenditure occur instantaneously. Naturally, Christensen’s paper and Bartholomew’s later paper predated modern mass flow controllers but it does beg the question whether the transformation derived from these older methods requires re-evaluation.
3.6.2.3 Current and Possible Uses of the Z-Transformation

The z-transformation is currently used in the Promethion to reduce the time lag when gas analyser channels are switched. The sensor lag associated with switching expired air for atmospheric air can be reduced this way, with a small z-transformation.

A second application of the z-transformation is in room calorimetry. The relationship between a gas entering the hood calorimeter and being detected is complex. For one, the detection of the true value is delayed until it reaches “steady-state” after about three minutes. Once a participant has started the recording, variations from the mean VO$_2$, VCO$_2$ or REE that are not sustained longer than three minutes will never have their true value registered on the indirect calorimeter. This problem is much greater in indirect room calorimetry for two reasons. First, changes in energy expenditure, not merely averaged values, are often under investigation in a larger room calorimeter as participants are free to move. Second, the room volume is so much larger than a hood calorimeter that it may take several hours to come to steady-state. The z-transformation applied to room calorimetry helps calculate steady-state values before they have been achieved.

The z-transformation has relatively minor role in indirect hood calorimetry. With both the logistic models and the bounded exponential models, steady-state is achieved after six minutes. For all the indirect calorimetry recordings data is taken from 900 samples onward, thus the z-transformation does not need to be used.

The purpose of examining the bounded exponential model more closely relates to potential future applications to hood calorimetry. If changes in VO$_2$, VCO$_2$ and REE are accurately modelled, as with the logistic curve, then the true values can be extrapolated from the measured values, second by second. Human resting energy expenditure measurement during hood calorimetry may thus be extended from the simple mean, currently in use, to a measure of true physiologic variation in energy expenditure.

Though outside the scope of this thesis, a complementary approach would be to describe, mathematically, the relationship between the input to the system (‘true VO$_2$’) and the synchronous output of the system (‘measured VO$_2$’), this is known as the impulse response. Once obtained, the impulse response can be used to calculate an output from any possible input and vice versa. This is possible using a branch of signal processing.
known as systems identification to determine the calorimetry’s impulse response. This work has already been completed as an extension of the work in this thesis.

3.7 Conclusion

In summary, validation and precision assessments of the Promethion indirect calorimeter canopy hood configuration were conducted using nitrogen dilution and ethanol combustion. A high level of accuracy and precision, M: 101% (SD: 0.5%), were demonstrated using nitrogen dilution. The ethanol combustion method demonstrated lower accuracy and precision, M:103% (SD: 5.1%), even after exclusion of two studies with obviously inconsistent burn rates. The development of models to predict measured VO$_2$ and VCO$_2$ in the presence of a varying input, such as a nitrogen gas flow, may be of potential use in assessing variability in VO$_2$, VCO$_2$ and REE.
4 Calorimeter Performance in Human Subjects

4.1 Introduction

This chapter contains results of the first and second test-retest reliability studies. The in-silico studies presented in the preceding chapters were performed between the first and second test-retest reliability studies following several protocol revisions discussed in this chapter. The follow-up test-retest reliability study was performed on duplicate measure data that were being collected for another study. These data were kindly provided by Terry O Donnell using the protocol I developed (section 2.4.4). I was not involved in the calorimetry data collection but completed the data analysis for this second study.

Test-retest reliability is a measure of precision. In the case of indirect calorimetry this measure indicates how well the calorimeter can detect changes in energy expenditure over time. A high precision means small changes can be detected with confidence. In preparation for a repeated measures study of adaptive thermogenesis, CREEDS study (chapter six), it was important to establish that the calorimeter was reliable had high precision during repeated measure human studies. I also wanted to quantify this reliability in terms of absolute energy expenditure change to help with planning for the CREEDS study and any other future studies of energy expenditure. Both aims were achieved with the second test retest reliability study, having incorporated lessons learned from the first study.

4.1.1 How is test-retest reliability quantified?

Test retest reliability is a function of variance. In any series of measurements there will be the mean and the variance from the mean. Every measurement can be expressed in terms of its deviation from the mean. Squaring the deviation eliminates directionality. The mean of the sum of the squares gives the variance of the data, the average dispersion of points from the mean.

The nitrogen dilution and ethanol burn procedures assessed error due to measurement by the calorimeter in quantities that had very little variance, as the burn rates and nitrogen inputs were reasonably consistent across studies. Isolating measurement error in this way is useful as it allows problems with the equipment and process to be identified more easily than if other sources of variation, such as biological variation, were present. However, this reliability assessment cannot be used to determine how well the
calorimeter would perform with repeated measurements in human participants with natural biological variation. To do this, a more complete analysis of precision is required.

Despite being a fundamental aspect of any instrument, there is no uniformly observed method for measurements of test-retest reliability. A recent meta-analysis found the most common tests for reliability analysis were t-tests, the correlation coefficient, Bland-Altman plots and the intra-class correlation coefficient (414). T-tests evaluate group average changes over two time-points, but do not incorporate variation at the individual level. Pearson’s correlation coefficient evaluates the strength of an association between a first and second reading but does not incorporate systematic error. Neither Bland Altman limits of agreement, t-tests or Pearson’s correlation coefficient allow for more than two measurements per subject in an assessment of reliability. By contrast, the intra-class correlation coefficient (ICC) does account for individual variation, systematic and random error, and can be used to assess many repeated measurements. The use of the ICC to assess reliability is supported by several authors (384, 414-417).

4.1.2 The Intra-class Correlation Coefficient (ICC)

The intra-class correlation coefficient (ICC) can be generally expressed as the ratio of the between subject variation in a measurement to the between subject variation plus the error (equation 4.1) (415).

\[
\text{Eq 4.1. } ICC = \frac{\text{between subject variance}}{\text{between subject variance} + \text{error}}
\]

Thus, if the error in the measurement is large the ICC will be low, and vice-versa.

This ICC definition has multiple permutations as outlined by Shrout and Fleiss and McGraw and Wong (417, 418). Different calculations for the ICC are used depending on the design of the reliability analysis. In selecting the appropriate calculation for the ICC the following factors were taken into consideration (416). First, the test-retest reliability study was a fully crossed design – all subjects were measured at all time-points and the testing time points were considered fixed effects, being representative of any repeated testing that may occur in future studies, thus a two-way ICC model was chosen. Secondly, an average measure ICC was used. This is appropriate when the outcome of interest is the within subject variation across all time-points, rather than the variation of measures at a single timepoint. Thirdly, as the outcome variables, VO2, VCO2 and REE
are continuous variables, the ICC was chosen to reflect the absolute agreement between repeated tests rather than the consistency, which is more appropriate for ranked variables.

The between subject variance, between test variance, and residual variance can be calculated using a repeated measure analysis of variance (ANOVA). If a significant effect of repeated tests is present when the measurement of interest should not be changing, this indicates systematic error. In this way the subject variance, systematic error (test variance), and random error (residual variance) can be partitioned.

\[
\text{Eq. 4.2} \quad ICC(2,1) = \frac{\text{Mean Square Subject} - \text{Mean Square Error}}{\text{Mean Square Subject} + (k-1)\text{Mean Square Error} + \frac{k(\text{Mean Square Trials} - \text{Mean Square Error})}{n}}
\]

where, k is the number of trials for each subject and n is the number of subjects (417).

4.1.3 Standard Error of the Mean and Least Significant Change

The ICC is a relative measure of precision. If the variance in energy expenditure within a group of individuals is high and the total error associated with that measurement is also high this will produce the same ICC as a group with proportionately low variance in energy expenditure and low total error variance of the measurement. Clearly this latter scenario is desirable as a small systematic change, for example due to a drug treatment, will be easier to detect. This simply reflects the distinction between ICC, a property of an instrument, and sensitivity to change which is a property of both the instrument but also the population being tested. Absolute measures of precision are more useful for determining a minimum detectable effect size between measurements. The standard error of the mean (SEM) can be calculated from the ICC and the standard deviation of all measurements to provide an absolute measure of precision (384):

Equation 4.3: \( SEM = SD \sqrt{1-ICC} \)

The SEM can be used to calculate the least significant change (LSC). The LSC is the minimum change in a measurement that has a less than five percent probability of being due to chance alone (equation 4.4).

\[
\text{Eq. 4.4:} \quad LSC = SEM * \sqrt{2} * 1.96
\]
4.2 The First Test Re-Test Reliability Study

4.2.1 Objectives

To assess the test-retest reliability of VO₂, VCO₂ and REE in lean healthy individuals using the Promethion indirect calorimeter when tested on three separate days in the same week.

4.2.2 Study Population

Participants were recruited by advertising within the university and local hospital staff mailing lists. Inclusion criteria were age 18-65yrs, and the absence of any active current medical conditions. Exclusion criteria were: a diagnosis of obstructive sleep apnoea, or an Epworth sleepiness score greater than nine; current smoking; weight change greater than five kg in the preceding three months; pregnant or breastfeeding; and the use of oral steroid medications. Each participant provided written informed consent.

Under the terms of the New Zealand Health and Disability Ethics Committee’s guidance, a formal application to the committee was not required for this study as it was regarded as a minimal risk observational study (419).

4.2.3 Methods

4.2.3.1 Anthropometry

Weight and height were measured for all participants. FM and FFM were measured by bio-impedance analysis. Anthropometry was conducted as outlined in section 2.4.1.

4.2.3.2 Indirect Calorimetry

All visits were conducted at the Centre for Translational Physiology, University of Otago Wellington. Indirect calorimetry was performed as described in section 2.4.4 with some exceptions. First, there was no low pass filter for the atmospheric sampling inlet or between the hood and air filter, as this was a modification added after the first test re-test reliability study. Secondly, calorimetry was conducted in a clinic room approximately 20 metres square in size. Thirdly, two operators and computers were co-located with the participant in the room throughout the recording. Fourthly, the recording duration was 46 minutes with a baselining interval of 60 seconds and a baselining frequency of once every six and a half minutes. Calorimetry data were taken from 15 until 40 minutes into...
the recording. Finally, as the testing room did not contain a temperature or humidity control, temperature was recorded using a room thermometer/hygrometer at the start and end of the calorimetry recording.

4.2.3.3 Statistical Analysis

The mean VO$_2$, VCO$_2$ and REE between 15 and 40 minutes were taken for each recording. This was used to calculate the ICC, SEM and LSC as described in section 4.1. Two-way ANCOVA specifying the participants as a random effect and the testing occasion as a fixed effect was used to generate the variances. Pairwise t-tests were used for change in environmental conditions during the recordings and change in weight and body composition from visit one to visit three.

Two measures of intra-recording variability were used. The mean standard deviation for VO$_2$ was calculated by obtaining the standard deviation for VO$_2$ within each recording. These values were then averaged across all participants. For example, if the standard deviations of VO$_2$ within each recording for three participants were 0.001, 0.007 and 0.010, the mean standard deviation for VO$_2$ would be 0.006. Similarly, the percentage mean standard deviation of VO$_2$ was calculated by expressing the standard deviation of VO$_2$ within each recording as a percentage of the mean VO$_2$ of each recording. These were then averaged across all recordings. The mean standard deviation and percentage mean standard deviation were calculated in the same way for VCO$_2$ and REE.

4.2.4 Results

Three female and seven male participants aged 18-32 years were recruited. Nine participants were New Zealand European and one was Indian. Participants completed all three visits between three and nine days after the first visit. The average interval between the first and last visit was six days. Two participants took nine days to complete the study due to work commitments.

There was a reduction in mean weight (M: -0.5kg; 95% CI: -0.14 to -0.87 kg) and FFM (M:+1.38 kg; 95%CI: 0.1 – 2.6 kg) from visit one to visit three. By contrast, there no significant change in FM (M:+0.92kg; 95%CI: -0.4 to +2.3kg). The change in FFM was driven largely by a single outlier (fig 4.1). When this participant was excluded from the analysis the change in FFM (M: -1.0; 95%CI: -1.99 to + 0.01) and FM (M:+0.46kg; 95%CI: -0.6 to + 1.5 kg) was non-significant. The weight change was unaffected (M:-
0.57kg; 95%CI: -0.96 to -0.2kg). Two participants showed a large shift in fat mass, one of whom also had a shift in fat free mass, in the absence of significant weight change. This was probably due to a measurement error, such as a change in hydration status between visits, or failure to remove jewellery during the visit on one occasion.

**Fig 4.1:** Change in weight, fat free mass and fat mass.

The output from the analysis of variance, the reliability measures calculated from these data, and the measures of intra recording variability for VO\(_2\), VCO\(_2\) and REE are presented in table 4.1.

The ANOVA output showed that the variance with repeated visits was small and similar to the residual error. For VO\(_2\), between-subject variation accounted for 93.7% (p < 0.001), the study visit accounted for 3.4% (p = 0.27) and residual error accounted for 2.6% of the variance. For VCO\(_2\), between-subject variation accounted for 94% (p < 0.001), the study visit accounted for 1.7% (p = 0.68) and residual error accounted for 4.4% of the variance. For REE, between subject variation account for 94.2% (p <0.001), the study visit accounted for 2.9% (p = 0.38), and residual error accounted for 2.9% of the variance.
The least significant change, as a proportion of the mean VO$_2$ VCO$_2$ and REE respectively were 7.6%, 8.3% and 7.8%. The intra recording variation, calculated as the mean standard deviations, expressed as a percent of the mean value, for VO$_2$, VCO$_2$ and REE were, 20.3%, 22.2% and 20.1%, respectively.

Table 4.1: Dispersion, variances and reliability measures for VO$_2$, VCO$_2$ and 24 hr REE from the 1st Test-Retest Reliability Study.

<table>
<thead>
<tr>
<th></th>
<th>VO$_2$ (SLPM)</th>
<th>VCO$_2$ (SLPM)</th>
<th>REE (kJ/24hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>0.2240</td>
<td>0.1910</td>
<td>6590</td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td>0.0357</td>
<td>0.0329</td>
<td>1063</td>
</tr>
</tbody>
</table>

| Variance from ANOVA |  |  |  |
|---------------------|-------------------------------|
| **Subject** | 0.003879 | 0.003184 | 813361 |
| **Visit**   | 0.000151 | 0.000061 | 25329  |
| **Residuals** | 0.000107 | 0.000151 | 24677  |

| Reliability Measures |  |  |  |
|----------------------|-------------------------------|
| **ICC**              | 0.97 | 0.96 | 0.97 |
| **SEM**              | 0.0062 | 0.0057 | 188 |
| **LSC**              | 0.0172 | 0.0158 | 519 |

| Intra-recording Variability |  |  |  |
|-----------------------------|-------------------------------|
| **Mean SD**                 | 0.0454 | 0.0423 | 1318 |
| **Mean %SD**                | 20.3 | 22.2 | 20.1 |

Thus, a relatively low variance seen with repeated testing, suggesting a high precision however the high intra-recording variation raised the suspicion of poor validity in the calorimetry recordings. This was confirmed during the early nitrogen validation studies and informed the development of the calorimetry protocol described in chapter two.
4.3 The Second Test-Retest Reliability Study

The second test re-test reliability study was based on calorimetry recordings from participants who underwent two calorimetry visits within a week as part of an environmental exposure study. Though this study was designed to examine differences between cold and warm exposure in an environmental chamber, the first 30 minutes was at controlled thermoneutral conditions. The precision test was repeated in view of the change in the measurement protocol following the first test re-test reliability study.

4.3.1 Objectives

To re-assess the test-retest reliability of VO$_2$, VCO$_2$ and energy expenditure in non-obese healthy individuals in the recently validated Promethion indirect calorimeter when tested on two separate days in the same week, using the modified protocol developed from the results of the first test re-test reliability study.

4.3.2 Study Population

Participants were recruited by advertising within the university and local hospital staff mailing lists. Inclusion criteria were age 18-60yrs, with a BMI of 18-30 kg/m$^2$. Exclusion criteria were; major cardiovascular disease; BMI>30; pregnancy; diabetes; any medications which may affect autonomic function. Each participant provided written informed consent. The overall trial was registered with the Australia New Zealand Clinical Trials Registry, trial number, ACTRN12616001459415. As the trial from which the second test retest reliability data were drawn also involved an intervention, ethics approval was obtained from the central region Health, Disability and Ethics Committee ref. 15/CEN/177.

4.3.3 Methods:

Weight, DXA scanning and indirect calorimetry were performed as outlined in chapter two. FM and FFM were assessed by DXA which was conducted only at baseline of the main study. Two days washout was allowed between visits to mitigate short term changes in REE due to the environmental intervention.

All raw data channels were visually inspected for error prior to analysis. The mean VO$_2$, VCO$_2$, and REE were calculated between 15-30 minutes into the recording. Mean and standard deviations for all recordings are presented. Two-way ANOVA, using subjects
as random effects and visits as fixed effects were used to generate the variances. ICC, SEM, LSC were calculated as outlined in section 4.1. The mean standard deviation and percentage mean standard deviation for VO$_2$, VCO$_2$ and REE were calculated as outlined in section 4.2.3.3.

4.3.4 Results

Thirty-two participants were recruited. Of these; four attended for one visit only; one calorimetry recording was unusable due to an incorrect baselining algorithm, and one participant attended their second visit 16 days after the first visit. The data for these six participants were therefore not used in the analysis.

Of the remaining 26 participants, 13 were female and 13 were male. 18 participants identified as New Zealand European, four Asian and four Māori. Baseline age was (M: 25 yr; SD: 7 yr). The mean interval between visit one and visit two was three days. Baseline weight was (M: 72 kg; SD: 13 kg). One participant took 8 days to complete the second study visit, the others all completed both visits within a week.

There was no difference in weight between visit one and visit two (M: 0.22 kg; 95% CI: -0.07 to + 0.5 kg), (fig 4.2). The mean, variances, ICC, SEM and LSC are presented in table 4.2. For VO$_2$, between-subject variation accounted for 95.9\% (p < 0.001), the study visit accounted for 0.2\% and residual error accounted for 3.9\% (p = 0.81) of the variance. For VCO$_2$, between-subject variation accounted for 93.9\% (p < 0.81), the study visit accounted for 0.3\% (p = 0.81) and residual error accounted for 5.8\% of the variance. For REE, between subject variation account for 95.7\% (p < 0.001), the study visit accounted for 0.3\% (p = 0.81), and residual error accounted for 4.0\% of the variance. Thus, in the repeat, there was also not a significant impact of the repeated measurement, on the determination of either VO$_2$, VCO$_2$ or REE.

The least significant change, as a proportion of the mean VO$_2$ VCO$_2$ and REE respectively were 7.4\%, 10\%, 7.7\%.
Fig 4.2: Change in weight during the 2\textsuperscript{nd} Test Re-Test Reliability Study
Table 4.2: Dispersion, variances and reliability measures for VO₂, VCO₂ and 24 hr REE from the 2nd Test-Retest Reliability Study.

<table>
<thead>
<tr>
<th></th>
<th>VO₂</th>
<th>VCO₂</th>
<th>REE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(SLPM)</td>
<td>(SLPM)</td>
<td>(kJ/24hr)</td>
</tr>
<tr>
<td>Mean</td>
<td>0.2470</td>
<td>0.2120</td>
<td>7280</td>
</tr>
<tr>
<td>SD</td>
<td>0.0172</td>
<td>0.0188</td>
<td>494</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Subject</th>
<th>Visit</th>
<th>Residuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variance</td>
<td>0.003973</td>
<td>0.003263</td>
<td>836813</td>
</tr>
<tr>
<td></td>
<td>0.000009</td>
<td>0.000011</td>
<td>2326</td>
</tr>
<tr>
<td></td>
<td>0.000160</td>
<td>0.000202</td>
<td>35275</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reliability Measures</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ICC</td>
<td>0.98</td>
<td>0.97</td>
<td>0.98</td>
</tr>
<tr>
<td>SEM</td>
<td>0.0066</td>
<td>0.0076</td>
<td>201</td>
</tr>
<tr>
<td>LSC</td>
<td>0.0184</td>
<td>0.0209</td>
<td>561</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intra-recording Variability</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean SD</td>
<td>0.0172</td>
<td>0.0188</td>
<td>498</td>
</tr>
<tr>
<td>Mean %SD</td>
<td>5.7</td>
<td>6.8</td>
<td>5.8</td>
</tr>
</tbody>
</table>

4.4 Discussion

Comparing the first and second reliability studies (table 4.3), the ICC, LSC and SEM values for VO₂, VCO₂ and REE are very similar. This was despite the identification of several sources of error that led to revision of the measurement protocol.
Table 4.3: Comparison of the dispersion, variance and reliability measures for both test-retest reliability studies.

<table>
<thead>
<tr>
<th></th>
<th>VO₂ (SLPM)</th>
<th>VCO₂ (SLPM)</th>
<th>REE (kJ/24hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TRT1</td>
<td>TRT2</td>
<td>TRT1</td>
</tr>
<tr>
<td>Mean</td>
<td>0.2240</td>
<td>0.2470</td>
<td>0.1910</td>
</tr>
<tr>
<td>SD</td>
<td>0.0357</td>
<td>0.0172</td>
<td>0.0329</td>
</tr>
<tr>
<td>Variance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subject</td>
<td>0.003879</td>
<td>0.003973</td>
<td>0.003184</td>
</tr>
<tr>
<td>Visit</td>
<td>0.000151</td>
<td>0.000009</td>
<td>0.000061</td>
</tr>
<tr>
<td>Residuals</td>
<td>0.000107</td>
<td>0.000160</td>
<td>0.000151</td>
</tr>
<tr>
<td>Reliability Measures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICC</td>
<td>0.97</td>
<td>0.98</td>
<td>0.96</td>
</tr>
<tr>
<td>SEM</td>
<td>0.0062</td>
<td>0.0066</td>
<td>0.0057</td>
</tr>
<tr>
<td>LSC</td>
<td>0.0172</td>
<td>0.0184</td>
<td>0.0158</td>
</tr>
<tr>
<td>Intra-recording Variability</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean SD</td>
<td>0.0454</td>
<td>0.0172</td>
<td>0.0423</td>
</tr>
<tr>
<td>Mean %SD</td>
<td>20.3</td>
<td>5.7</td>
<td>22.2</td>
</tr>
</tbody>
</table>
4.4.1 Source of Error During the First Test Retest Reliability Study

During the first test-retest reliability the calorimetry data contained large intra-recording variation for VCO₂, VO₂ and REE (fig.4.4). The main reason for this ‘noisy’ data was contamination of the atmospheric sampling inlet and the indirect calorimetry hood inlet by the operators. A key assumption of the calorimetry recordings is that the hood air inlet, which is not directly sampled, has the same gas concentration as the atmospheric sample inlet. Were CO₂-enriched and O₂-depleted air from an operator to enter the canopy hood inlet but not the atmospheric inlet, this assumption would be violated, and reduce the validity of the recording. This problem was easily solved by isolating participants and the atmospheric inlet for the second study. The atmospheric inlet was also placed close to the hood air inlet so samples passing into it would be representative of the incurrent air flow.

A second source of error was inadequate air mixing. This is best understood by considering the air flows as inhomogeneous mixes of different gases. If such a mixture reaches the sensor chains directly there may be spikes in VO₂ or VCO₂ that do not accurately reflect the average values in the air volume at that time. This is reduced by mixing. A low pass filter is a digital signal filter that permits signals below a certain frequency to pass while blocking higher frequency ‘spikes’. It is used to improve noisy signals. The physical low pass filters that were added to the calorimeter were simply additional volume within which the travelling gas mixtures from the atmospheric sample or the canopy hood were mixed.

The final major change to the experimental protocol between studies was a change in the baseline frequency and duration. During the first study, the atmospheric sampling duration and frequency was relatively short at one minute per channel on a six-and-a-half-minute cycle. Using this baselining programme, the atmosphere was being monitored less than one third of the time. The baselining frequency was changed to three minutes per channel every ten minutes, sixty percent of the time. The first purpose of baselining, as outlined in chapter two, is to prevent sensor drift (section 2.4.4.1.1). Given the Promethion has a dual chain analyser setup this does not come at a cost of monitoring the excurrent air flows, as one sensor can do this while the other monitors the atmosphere. However, the baselining process is also crucial for monitoring incurrent gas flows. The shorter the baselining coverage the less likely the baselining values are to be...
representative of what is entering the hood. Ideally, the sensors would alternate between
inurrent and out-current flows so that both were constantly measured. However, there
will always be a time-lag when switching between inurrent and excurrent air flow occurs
as the sensor adjusts to the new readings. In addition, to copy from channel 1 to channel
2 as outlined in appendix C, the baselining section for any one channel needs to be
flanked by non-baselined segments (fig 4.3). By trial and error, it was determined that
baselining for three minutes per channel on a ten-minute cycle is the closest to
“continuous” baselining that can be achieved with the Promethion.
Oxygen tracings from channel 1 are pictured in red and tracing from channel 2 in blue. The interval between baselining sequences (←→) is required to allow copying across data channels.
4.4.2 Measures of Quality during Human Indirect Calorimetry.

The main differences between the first and second reliability studies is illustrated in fig. 4.4. The final calorimetry recordings conducted for both studies are presented. A reduction in the variability in REE was seen following implementation of the newer calorimetry protocol. The reduction in ‘noise’ between the first and second test retest reliability studies was the clearest indicator of improvements in the quality of the measurement protocol. There was a small improvement in precision between studies as indicated by a rise in the ICC and a reduction in the variance due to the study visit in the second re-test reliability study (fig 4.3). However, these relatively small differences in precision suggest a consistent application of a protocol that chiefly compromised validity in the first test-retest reliability study.
Fig 4.4: Resting energy expenditure variability during the first (TRT1) and second (TRT2) test retest reliability studies.
4.4.2.1 Validity

One of the challenges in assessing the quality of live subject indirect calorimetry recordings, is the inability to assess validity. As alluded to in chapter three, there is no way of knowing a priori what any one individual’s energy expenditure ought to be, and so no way to assess validity in a human subject. A potential solution to this issue is to cross validate REE measurements using alternate calorimetry methods such as direct calorimetry or doubly labelled water. However, doubly labelled water measures total energy expenditure and is prohibitively costly for large studies, and direct calorimetry is not widely available. Instead, the nitrogen gas and ethanol burn studies outlined in chapter three were carried out to determine the validity of measurements using the new protocol. This provides a measure of validity for the system and measuring protocol that is assumed to be maintained during human indirect calorimetry recordings.

4.4.2.2 Intra-Recording Variability as a Proxy of Validity

A second challenge in assessing the quality of a human calorimetry recording is the elimination of variation during a recording that must occur when mean values of VO₂, VCO₂ and REE are used. These are the key parameters used in studies of REE and ought to be the focus of a calorimetry precision study. However, they do not reflect the recording quality. As validity is untestable during human recordings, the within-recording variability can serve as a proxy for a valid recording. This is based on the understanding that REE should not vary by up to two or three-fold during a recording, as was observed during the first test-retest reliability study. This variability is best captured by a measure of within-recording variability such as the mean SD for all recordings.

Within recording variability as a measure of validity has been used by Haugen et al, 2003, during precision testing of an indirect calorimetry hood (420). Though this was not a primary outcome measure, a variation in VO₂ of less than 15% over 15 minutes was used as a basis for selecting valid data for analysis. Such approaches are common and provide a reproducible measure of recording quality. One limitation of selecting ‘valid’ data in this way is the inherent assumption that all errors in measuring energy expenditure increase intra-recording variability rather than decrease it. Were human energy expenditure to vary at an average of 10% of the mean, a variability of less than 5% or greater than 15% would be equally invalid. Nonetheless, it remains one of the few available objective measure of recording quality.
The intra-recording variability for the ethanol and nitrogen validation studies, both test re-test reliability studies and the study by Haughen et al, 2003, are presented in table 4.4 for comparison (420).

Table 4.4: Intra recording variability expressed as the percentage mean standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>VO2</th>
<th>VCO2</th>
<th>REE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRT-1</td>
<td>20.3</td>
<td>22.2</td>
<td>20.1</td>
</tr>
<tr>
<td>Nitrogen Dilution</td>
<td>0.8</td>
<td>2.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Ethanol Burn</td>
<td>0.8</td>
<td>2.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Haughen et al</td>
<td>&lt;15</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>TRT-2</td>
<td>5.7</td>
<td>6.8</td>
<td>5.8</td>
</tr>
</tbody>
</table>

Both validation methods show minimal variation in the recording over time. This is unsurprising given they are both intended to produce constant values of VO$_2$, VCO$_2$ and REE over the measurement period that the mean SD was calculated. Thus, these values are an indicator of the within recording variation attributable to the measurement method.

By comparison, the intra-recording variability during human studies for VO$_2$, VCO$_2$ and REE are several-fold higher. Using the <15% variability proposed used by Haughen, all recordings obtained during TRT2 study would have been considered eligible whereas only 43% of recordings in the first test-retest reliability study would have been eligible. Only one recording had an intra-recording variability greater than 10% during the second Test Retest Reliability study. An intra-recording variability of less than 10% may be used as a more stringent validity criterion during human studies.

4.4.2.3 Precision

Both studies indicate that the precision was satisfactory with an LSC of 519 kJ/24 hours for the first test-retest reliability study and 560 kJ/24 hours for the second. The least significant change in the second study was higher due to the greater between participant variability, as a larger more heterogenous population was studied. Weight did not change
significantly during either study, and what variation was detected in the first TRT1 study was likely due to error in the BIA measurement rather than true weight change. Though the variance in REE attributable to the visit was small, it is worth noting that it fell from 3% of total variance during the first study, to 0.3% during the second study.

This consistency in precision between studies appears to contradict the assertion that the measurement performance was better with the second test-retest study. However, the sources of error outlined in section 4.4.1 need not have affected precision provided they occurred at all visits.

The consistent precision and lack of a significant effect of repeated visit, indicates no ‘training effect’. A training effect occurs when a subject responds differently to repeated measurements. This may occur with indirect calorimetry if first-time participants are anxious lying in the enclosed hood but become used to it by the second or subsequent visits. The 15-minute acclimation period at the start of every calorimetry visit allows participants to become used to the hood prior to the first recording. This suggests that an initial training calorimetry test is not required with prospective studies of indirect calorimetry.

### 4.5 Conclusion

We conducted studies of test retest reliability of the Promethion indirect calorimeter before and after several modifications to the testing protocol. While these modifications did not have a significant improvement on the precision of the recordings, the validity of the recordings as reflected in the improved intra-recording variability was substantially better. An intra recording variability of <10% is suggested as a proxy of validity for human recordings.
5 The PREEMPt Study

5.1 Introduction

The preceding two chapters outline the development of a valid and precise method of measuring human REE. The predictions of REE in Māori and Pacific populations (PREEMPt) pilot study, was designed to assess the variance in energy expenditure in overweight and obese Māori and Pacific populations. The rationale for the study was two-fold. First, the test-retest reliability studies in chapter four were conducted in predominantly New Zealand European lean individuals, most of whom were in their twenties. By contrast, my overall objective was to study adaptive thermogenesis in overweight and obese individuals undergoing weight loss. Based on New Zealand BMI prevalence data, these individuals were more likely to be in their 30’s, 40’s and 50’s. Thus, an overweight and obese local population would provide more accurate samples size estimates for the planned weight loss intervention study, the CREEDS study, described in chapter seven.

Secondly, predictive equations can be used to prescribe energy intake in the absence of directly measured energy expenditure. However, the accuracy of these equations is unknown in New Zealand cohorts, and specifically in Māori and Pacific populations. Therefore, the PREEMPt study provides the data to estimate the sample size required to assess the validity of predictive energy equations in a New Zealand population.

5.1.1 Predictive Energy Equations

5.1.2 The Calculation & Applications of Predictive Energy Equations

As outlined in chapter two (section 2.3.3.2) predictive energy equations allow calculation of REE and using physical and demographic information such as weight, height, age and gender. The Mifflin St Jeor Equations (equations 5.1 and 5.2) are examples of predictive energy equations:

Eq.5.1:

\[ \text{Men: } \text{REE (kcal)} = 10 \times \text{weight (kg)} + 6.25 \times \text{height (cm)} - 5 \times \text{age (y)} + 5 \]

Eq.5.2:

\[ \text{Women: } \text{REE (kcal)} = 10 \times \text{weight (kg)} + 6.25 \times \text{height (cm)} - 5 \times \text{age (yr)} - 161 \]
Predictive equations are used in national guidelines for daily recommended energy intake, food labelling guidelines, websites, smartphone apps, gym equipment and wearable technology. In effect, they are the only way an individual without access to measurements of energy expenditure can determine their daily energy intake. Increasingly, predictive energy equations are used as a personal indicator of healthy dietary calorie intake. As such, their accuracy deserves careful consideration when used in an obese New Zealand population.

Predictive equations are developed using multiple regression which provides an estimate of the relationship between a predictor variable, such as weight, age or height, and resting energy expenditure. As with any statistical test, these estimates contain a component of error, represented numerically by the standard error of the estimate (SEE). The SEE is similar to the SEM, discussed in chapter four. Once a regression equation has been developed it is typically applied to a validation sample from which the SEE can be calculated as follows.

\[
SEE = \frac{\sum (\text{Measured EE} - \text{Predicted EE})^2}{(\text{Total No Measurements} - 1) \sqrt{\text{Number of Measurements}}}
\]

Error is an intrinsic part of predictive equations. When used in the same populations in which they were derived error is minimised and validity increased, as the relationships between the predictor variables and energy expenditure are likely to be similar. For example, body weight and height are incorporated as proxy measures of combined FM and FFM in predictive equations. However, the contribution of FM and FFM to resting energy expenditure can vary by ethnicity (421).

The validity of predictive equations in diverse populations is not a new concern. The joint FAO/WHO/UNU that provide guidance on the development of national recommendations for dietary energy intake, recommend that each country develop their own predictive equations for estimating REE (422). As a result, many predictive equations have been developed in the past 20 years. Below I briefly review four of the most commonly used predictive equations and the populations in which they were developed.
5.1.3 Commonly used Predictive Energy Equations

5.1.3.1 The Harris-Benedict Equations

In 1918, Harris & Benedict developed some of the earliest predictive equations of human energy expenditure. The Harris-Benedict equations (equations 5.4 & 5.5) were derived by regression from measurements of 136 men, 103 women and 94 infants carried out at the Nutrition Laboratory, Carnegie Institute of Washington (348). Participants were aged 21-70 years, weight 25-124.9kg and height of 151-200 centimetres.

Eq.5.4: Men: REE (kcals)
\[ = 66.5 + 5.0 \times (\text{height}) + 13.8 \times (\text{weight}) - 6.8 \times (\text{age}) \]

Eq.5.5: Women: REE (kcals)
\[ = 655.1 + 1.8 \times (\text{height}) + 9.6 \times (\text{weight}) - 4.7 \times (\text{age}) \]

The equations were updated in 1984 (equations 5.6 and 5.7) by Roza and Shizgal, 1984, who incorporated 94 additional patients studied by Benedict after 1918 that represented a wider age range, (16 – 62 yr) than the original equation, (16 – 45 yr) (423). Though there is no mention of ethnicity in the original papers containing these equations, the census data for Washington state in 1910 indicate a prevalence of “whites” of 96.9%, with the remainder comprised of other ethnicities (424).

Eq.5.6: Men: REE (kcal) = 88.4 + 4.8 \times (\text{height}) + 13.4 \times (\text{weight}) - 5.7 \times (\text{age})

Eq.5.7: Women: REE (kcal)
\[ = 447.6 + 3.1 \times (\text{height}) + 9.3 \times (\text{weight}) - 4.3 \times (\text{age}) \]

5.1.3.2 The Schofield or WHO/FAO/UNU Equations

In 1981, the World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO), commissioned a review of human energy requirements with the intention of producing accurate predictive equations of REE. This was not the first such commission but developed previous estimates of human energy expenditure by addressing concerns regarding the quality and methodological rigor of earlier similar analyses. These concerns included the use of unreliable calorimetry devices, or measurement protocols and the inclusion of duplicate values (425).
The new analysis reported by Schofield was based on calorimetry recordings from 4937 men and 2612 women taken during the 1930’s and 1940’s (352) and were cross-validated in an independent cohort of 3874 subjects. Subjects were almost exclusively from Italy or North America, except for 322 subjects from India. When the predictive equations were derived from the Indian subgroup only, they forecasted lower resting metabolic rate per unit mass. As a result, Schofield suggested that the validity of the equations may be comprised when used in populations that differ from the original North American and Italian cohorts. Subsequent analyses have demonstrated that the Schofield Equations consistently overestimate REE in ethnicities native to both tropical and temperate regions (426).

The Schofield equations combine gender, age and weight to predict basal metabolic rate. They are still used to provide estimates of daily energy intake for the Australian and New Zealand nutritional guidelines (427, 428).

5.1.3.3 The Mifflin-St Jeor Equation

Mifflin et al, 1990, developed a predictive regression equation based on a sample of 247 women with a mean age of 45 years, and 251 men, with a mean age of 44 years, from Reno, Nevada (429). Once again, no ethnicity data was reported in this study. However, the ethnic distribution of Washoe County at the time of enrolment for the study was 88% White, 4% Asian and Pacific Island, 2% Black and 2% Native American (430). The rationale for this work was the change in body weight, body composition, physical activity and diet characteristics of the population that had occurred in the intervening 70 years since the Harris-Benedict equations were developed. In the cohort of women and men respectively, the mean body weight was 70.2 kg and 87.5 kg, and the mean BMI was 26.2 kg/m² and 27.5 kg/m². Twenty-eight years later, the mean BMI in the New Zealand adult was similar was 28.0 kg/m² and 28.1 kg/m² respectively (4). This suggests that the Mifflin St. Jeor equation may be more valid for use in New Zealand populations than the Harris Benedict equation, which was developed in a cohort that was lighter and almost certainly had a different body composition to the average New Zealand adult.

5.1.3.4 The Oxford Equations

In 2005 Henry published the Oxford equations alongside a critique of the Schofield equations (431). To address the limitations of the Schofield equations, he performed an
updated systematic review of 166 published studies of REE comprised of 10552 individual measurements of REE. Recordings from 5794 men and 4702 women were included in this analysis. Notably, the Italian cohort that were included in the Schofield equations were excluded from the analysis on the basis that their REE was “unusually” high and raised concerns in relation to validity (425). The Oxford equations incorporated more studies based in tropical regions, accounting for 38% of the total cohort. An additional point of difference between the Schofield and Oxford equations was the exclusion of studies that reported only the lowest value of REE obtained during a recording from the Oxford analysis. This approach was considered biased compared with simply taking the mean REE (425).

5.1.3.5 Which equations to use?

The Schofield and Oxford equations are a set of age, weight and gender specific equations. The performance of any one equation from within these sets is likely to vary as not all age and weight categories were equally represented in the data pool from which they were derived. This is reflected in the different standard errors for each equation. While the Schofield and Oxford equations are derived from a larger database of measurements than the other equations, these data are drawn from many studies that used diverse energy expenditure measurement techniques that will not have been cross calibrated. In addition, these databases incorporate studies that are now more than 100 years old. The Mifflin St Jeor equations, by contrast, use contemporary measurement methods and all data were collected prospectively using the same measurement protocol.

In one of the most recent and comprehensive reviews on this topic, Frankenfield et al, 2005, examined the performance of the Mifflin-St Jeor, Schofield and Harris Benedict Equations. This work formed part of an evidence analysis report commissioned by the American Dietetic Association in 2005 (432). They reviewed the original studies from which each equation was derived and calculated the accuracy of each of the equations using individual-level data from each of these studies, rather than group means. The proportion of participants that had a predicted REE within 10% of the measured REE was used as a measure of accuracy. The Schofield equations only referenced group mean data and so could not be analysed using this metric. This work preceded the publication containing the Oxford equations, which included studies that did report individual level data.
The findings of the working group found a paucity of data amongst all equations reviewed for US-residing ethnic minorities, particularly; Pacific Islander, Asian, African-American, American Indian, Alaskan Native or Hispanic populations. Elderly individuals, particularly overweight or obese elderly were also under-represented. The Mifflin St Jeor equation performed best, with predictions of within 10% of measured values for 82% of non-obese and 70% of obese participants. The Harris Benedict was accurate in 45-80% of non-obese and 38-64% of obese individuals. Two limitations of this analysis were the use of a definition of accuracy that excluded an equation developed from the largest pool of measurements, the Schofield equation, and the focus on predominantly US populations. Nevertheless, the American Dietetic Association has adopted the Mifflin St. Jeor equation as the most valid for a US population on the strength of the working group’s report.

Ideally, a New Zealand specific predictive equation would be developed using a standardised, validated measurement protocol in the New Zealand populations for which it is being developed. In the absence of a New Zealand specific equation, validation of several existing equations against measurements of energy expenditure may identify an equation that is most accurate. This work is yet to be done, and the PREEMPt study was planned to lay the groundwork by providing data for sample size estimation.

5.1.4 Sample Size Estimation for Measurement of Resting Energy Expenditure

The aim of the PREEMPt study was to estimate the sample size requirements for a definitive study of the accuracy of predictive energy equations in Pacific and New Zealand Māori adults. A second application of the PREEMPt data was to provide sample size estimates for the CREEDS study (chapter seven). As the CREEDS study is designed to look at changes in resting energy expenditure beyond that expected for weight loss, it does not use total REE as an outcome variable. Rather, allometrically-scaled REE data using either FFM or a combination of FM and FFM (chapter two, section 2.3.2) were planned as the outcome variables.

The accuracy and precision of either REE or allometrically-scaled REE measurement varies depending on the method of body composition assessment. DXA, though superior to BIA as an estimate of body composition is also considerably more expensive. Thus, sample size estimates using the data from both DXA and BIA were conducted.
5.2 The PREEMPt Study

5.2.1 Outcomes

1. The primary outcome was the difference between measured and predicted REE using the Schofield, Harris-Benedict, Mifflin-St. Jeor and Oxford equations.

Secondary outcome variables included:

2. The difference between body composition measured by DXA and BIA.

3. Weight, waist circumference, height, BMI and body fat composition measured by BIA and DXA.

4. HbA1c, fasting plasma glucose, high density lipoprotein (HDL), low density lipoprotein, triglycerides (TG), total cholesterol and cholesterol:hdL ratio.

In addition to these outcomes, sample size requirements for a validation study of resting energy expenditure using either DXA or BIA, and sample size requirements for the CREEDS study using either DXA or BIA were performed.

5.2.2 Study Population

Participants were recruited from advertisements in general diabetes and endocrine clinics, local GP practices, the regional hospital and university staff mailing lists, advertisements disseminated via regional community noticeboards and local Pacific health networks.

Inclusion criteria were; a BMI $\geq 25$ kg/m$^2$, self-identification as Māori or Pacific Island ethnicity and age 20-65yrs.

Individuals were excluded if; they had a weight change of greater than five kg over the previous three months; were pregnant or breast-feeding; had a current medical illness or were taking medications that could influence resting metabolic rate; were a current smoker, or had a weight >220kg, the maximum weight limit on the DXA machine.

Participants with prosthetic cardiac valves, cardiac pacemakers, joint-pins or metal plates were excluded from BIA measurement.
5.2.3 Methods

5.2.3.1 Ethics Approval, Māori Consultation and Funding

Consultation with Māori was conducted during protocol development with Wellington Regional Hospital’s Research Advisory Group – Māori. Ethics approval was obtained from the Northern B, Health, Disability and Ethics Committee, ref. 15/NTB/175. Funding for the commercial DXA scans used in this study was provided by the Wellington Medical Research Foundation Ltd., registered charity no CC10659.

5.2.3.2 Study Procedures

Eligible participants attended the Centre for Translational Physiology, University of Otago, Wellington for one visit. All participants provided written informed consent. Women of child bearing age had a pregnancy test performed with consent, prior to having the DXA.

At the visit, questionnaires, anthropometry, phlebotomy and indirect calorimetry were completed. Participants arrived by car having fasted from 2200h the night before and remained fasting until the DXA scan was completed.

DXA, BIA, biochemical analysis of blood samples, and indirect calorimetry were performed as outlined in chapter two. The DXA scan was conducted on the same day as the indirect calorimetry.

5.2.3.3 Statistical Analysis

The sample size for the study was 20 participants, 10 Māori & 10 Pacific. This number was chosen to provide an adequate sample of variation in resting energy expenditure. No subgroup analysis was performed.

Mean, standard deviation and standard error of the mean were calculated for anthropometric, biochemical and calorimetric data. The difference between predicted and measured REE was performed using paired t-tests after log transformation to correct for skewed data.

Bland Altman plots were used to present comparisons of predicted versus measured energy expenditure and BIA vs DXA for body composition analysis. BIA estimates of FM, FFM and body fat percent were calculated using the in-built regression equations on
the Tanita TBF-300 scales. Pearson’s correlation coefficient, $r$, was used to quantify the relationship between measured and predicted energy expenditure.

Allometric scaling of resting energy expenditure was conducted using linear regression, incorporating either FFM or both FFM and FM as predictors. Linear regression using body composition for BIA and DXA are presented separately.

Power calculations were performed in G*Power 3 (433). For a definitive study of the accuracy of REE prediction, the mean, standard deviation and correlation of the measured and predicted REE were used. The planned analysis was a two-tailed, paired t-test. Power calculations for the CREEDS study were based on allometric scaling by linear regression of REE from the PREEMPt study. The root mean squared error (RMSE) of the regression model was used as the standard deviation of REE in both groups of the CREEDS study. For conservative and relaxed estimates, the upper and lower 95% confidence limits of the RMSE were also used to calculate sample size. The mean REE in the PREEMPt study was used as the anticipated mean REE in the control group of the CREEDS study. The mean intervention group REE was set at 95%, 90% and 85% of that of the control group. A one-way ANOVA with fixed effects was specified. Power was set at 0.80, alpha was set at 0.05 for all power calculations.

5.2.4 Results

5.2.4.1 Demographic Characteristics

Twenty participants, 10 New Zealand Māori & 10 Pacific Island were recruited. The Pacific participants consisted of seven Samoan, two Tokelauan, one Solomon Island and one Cook Island Māori. One participant identified as both Tokelauan and Samoan. Five Māori participants were male, the other five and all Pacific participants were female. The mean age was 44 years with a range of 24-60 yrs. Three participants had T2DM, controlled with tablets only. Hypoglycaemic medications were withheld on the morning of the visit until all assessments were completed, to avoid hypoglycaemia while fasted. Other medications, such as anti-hypertensives were continued.
5.2.4.2 Body Composition

Anthropometric measurements are presented in table 5.1. Bland-Altman plots of BIA versus DXA for assessment of lean mass, FFM and body fat percentage are presented in fig 5.1. There was a significant difference in FM predicted by Tanita’s inbuilt BIA equations compared with DXA, with BIA overestimating FM (M:1.76kg, 95%CI:0.03 to 3.50; p:0.047). There was a trend toward overestimation of body fat percentage with BIA (M:2.13%, 95%CI: -0.3 to 4.5; p:0.07). There was no difference in lean mass estimation (M: -0.3, 95%CI: -6.3 to 5.7; p:0.91).

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>101.0</td>
<td>10.9</td>
<td>(83.1-123.6)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>168</td>
<td>8</td>
<td>(156 – 189)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>35.7</td>
<td>4.0</td>
<td>(30.3-44.3)</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>58.2</td>
<td>9.6</td>
<td>(46.0-83.8)</td>
</tr>
<tr>
<td>FM (kg)</td>
<td>41.0</td>
<td>6.9</td>
<td>(29.7-55.2)</td>
</tr>
<tr>
<td>% Fat</td>
<td>40.5</td>
<td>6.0</td>
<td>(28.0-47.7)</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>57.9</td>
<td>10.8</td>
<td>(46.3-87.1)</td>
</tr>
<tr>
<td>FM (kg)</td>
<td>42.8</td>
<td>8.5</td>
<td>(33.8-63.5)</td>
</tr>
<tr>
<td>% Fat</td>
<td>42.6</td>
<td>7.2</td>
<td>(29.5-55.2)</td>
</tr>
</tbody>
</table>

Table 5.1: Body composition values for PREEMPt participants.
Fig 5.1: Bland Altman analysis of body composition measured by DXA and BIA.

Fat Free Mass (kg)

Fat Mass (kg)

Fat Mass (%)

DXA - BIA (kg)
5.2.4.3 Measured Resting Energy Expenditure

The mean resting energy expenditure for all participants was 7590 kJ/24 hrs (6188 – 10108 kJ/24 hrs). The standard deviation was 1100 kJ/24 hours. Estimates of REE scaled for FFM and FM using DXA and BIA are presented in table 5.2. A comparison of fits for the regression models is presented in table 5.3.

Table 5.2: Allometric scaling of REE by linear regression using FM & FFM from BIA and DXA.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>SEE</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DXA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Model 1</strong></td>
<td>FFM (kJ/kg)</td>
<td>100</td>
<td>11</td>
<td>(80 to 121)</td>
</tr>
<tr>
<td>Intercept (kJ)</td>
<td>1736</td>
<td>661</td>
<td>(-42 to 3514)</td>
<td>0.0175</td>
</tr>
<tr>
<td><strong>Model 2</strong></td>
<td>FFM (kJ/kg)</td>
<td>100</td>
<td>12</td>
<td>(77 to 123)</td>
</tr>
<tr>
<td>FM (kJ/kg)</td>
<td>-5</td>
<td>16</td>
<td>(-16 to 5)</td>
<td>0.74</td>
</tr>
<tr>
<td>Intercept (kJ)</td>
<td>2013</td>
<td>1067</td>
<td>(-80 to 4104)</td>
<td>0.076</td>
</tr>
<tr>
<td><strong>BIA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Model 3</strong></td>
<td>FFM (kJ/kg)</td>
<td>32</td>
<td>22</td>
<td>(-10 to 75)</td>
</tr>
<tr>
<td>Intercept (kJ)</td>
<td>5740</td>
<td>1310</td>
<td>(3172 to 8309)</td>
<td>3.58 x 10^{-4}</td>
</tr>
<tr>
<td><strong>Model 4</strong></td>
<td>FFM (kJ/kg)</td>
<td>25</td>
<td>25</td>
<td>(-24 to 73)</td>
</tr>
<tr>
<td>FM (kJ/kg)</td>
<td>-22.6</td>
<td>31</td>
<td>(-71 to 38)</td>
<td>0.47</td>
</tr>
<tr>
<td>Intercept (kJ)</td>
<td>7137</td>
<td>2322</td>
<td>(2586 to 11690)</td>
<td>7x10^{-3}</td>
</tr>
</tbody>
</table>
Table 5.3: Comparison of fit for regression models 1-4.

<table>
<thead>
<tr>
<th>Method</th>
<th>F-Statistic</th>
<th>Adjusted R²</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>DXA</td>
<td>79.8</td>
<td>0.81</td>
</tr>
<tr>
<td>Model 2</td>
<td>DXA</td>
<td>37.9</td>
<td>0.80</td>
</tr>
<tr>
<td>Model 3</td>
<td>BIA</td>
<td>2.1</td>
<td>0.05</td>
</tr>
<tr>
<td>Model 4</td>
<td>BIA</td>
<td>1.3</td>
<td>0.03</td>
</tr>
</tbody>
</table>

5.2.4.4 Predicted versus Measured Resting Energy Expenditure

Predicted resting energy expenditure using the Schofield, Harris-Benedict, Mifflin St. Jeor and Oxford Equations are presented in table 5.4. Bland Altman plots of measured versus predicted resting energy expenditure are presented in Fig.5.2. As indicated by the mean difference between measured and predicted REE, all four equations underestimated REE, though the greatest bias was seen with the Mifflin St. Jeor equation. The limits of agreement were narrowest for the Schofield, followed by the Oxford, Harris- Benedict and Mifflin-St. Jeor equations.
Table 5.4: Difference between measured and predicted REE (kJ/24hrs)

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>Measured:Predicted (95% CI)</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Measured REE</strong></td>
<td>7590</td>
<td>1100</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Schofield</strong></td>
<td>7477</td>
<td>950</td>
<td>1.01 (0.97 to 1.06)</td>
<td>0.62</td>
<td>0.54</td>
</tr>
<tr>
<td><strong>Harris-Benedict</strong></td>
<td>7434</td>
<td>736</td>
<td>1.02 (0.97 to 1.07)</td>
<td>0.65</td>
<td>0.52</td>
</tr>
<tr>
<td><strong>Mifflin-St. Jeor</strong></td>
<td>7197</td>
<td>686</td>
<td>1.05 (0.99 to 1.11)</td>
<td>1.82</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Oxford</strong></td>
<td>7427</td>
<td>883</td>
<td>1.02 (0.97 to 1.07)</td>
<td>0.86</td>
<td>0.40</td>
</tr>
</tbody>
</table>
Fig 5.2: Bland-Altman plots of measured versus resting energy expenditure (kJ/24 hours).

- **Schofield**: $r = 0.72$
- **Harris-Benedict**: $r = 0.62$
- **Mifflin-St.Jeor**: $r = 0.51$
- **Oxford**: $r = 0.69$
5.2.4.5  *HbA1c, Glucose, Lipids*

All female participants had a negative pregnancy test. HbA1c, glucose and lipids are presented in table 5.5.

**Table 5.5: HbA1C, fasting glucose and lipids.**

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HbA1C (mmol/mol)</strong></td>
<td>38</td>
<td>13</td>
<td>28 to 70</td>
</tr>
<tr>
<td><strong>HbA1C (%)</strong></td>
<td>5.6</td>
<td>1.2</td>
<td>4.7 to 8.6</td>
</tr>
<tr>
<td><strong>Glucose (mmol/l)</strong></td>
<td>5.0</td>
<td>0.6</td>
<td>4.2 to 6.8</td>
</tr>
<tr>
<td><strong>Total Cholesterol (mmol/l)</strong></td>
<td>4.7</td>
<td>0.7</td>
<td>3.9 to 6.0</td>
</tr>
<tr>
<td><strong>HDL (mmol/l)</strong></td>
<td>1.3</td>
<td>0.3</td>
<td>0.93 to 1.84</td>
</tr>
<tr>
<td><strong>LDL (mmol/l)</strong></td>
<td>2.8</td>
<td>0.7</td>
<td>1.9 to 4.3</td>
</tr>
<tr>
<td><strong>Triglycerides (mmol/l)</strong></td>
<td>1.4</td>
<td>0.9</td>
<td>0.5 to 3.7</td>
</tr>
<tr>
<td><strong>Chol:HDL ratio</strong></td>
<td>3.8</td>
<td>0.9</td>
<td>4.2 to 6.9</td>
</tr>
</tbody>
</table>

5.2.4.6  *Power Calculations for the CREEDS Study*

Using resting energy expenditure scaled for lean mass, the mean resting energy expenditure was 7590 kJ/day. The root mean squared error for model 1 was 460 kJ/day, the root mean squared error for model 2 was also 460 kJ/day. As model fit did not improve significantly with model 2, model 1 was used for all power calculations. The 95% confidence interval for the RMSE was 318 to 603 kJ/day. The sample size estimates are presented in Table 5.6.
### Table 5.6: Sample size estimates for the CREEDS Study

<table>
<thead>
<tr>
<th>Variance (kJ/day)</th>
<th>Difference in REE (%)</th>
<th>Difference in REE (kJ/day)</th>
<th>Sample Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper 95% CI RMSE</td>
<td>5</td>
<td>381</td>
<td>82</td>
</tr>
<tr>
<td>602 kJ/day</td>
<td>10</td>
<td>757</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1138</td>
<td>12</td>
</tr>
<tr>
<td>RMSE</td>
<td>5</td>
<td>381</td>
<td>48</td>
</tr>
<tr>
<td>460 kJ/day</td>
<td>10</td>
<td>757</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1138</td>
<td>8</td>
</tr>
<tr>
<td>Lower 95% CI RMSE</td>
<td>5</td>
<td>381</td>
<td>24</td>
</tr>
<tr>
<td>318 kJ/day</td>
<td>10</td>
<td>757</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1138</td>
<td>6</td>
</tr>
</tbody>
</table>

Multiple sample size estimates were calculated based on; the RMSE, and an anticipated difference of either 5%, 10%, or 15% in REE between treatment groups for the CREEDS study.

#### 5.2.4.7 Power Calculations for a Definitive Study of Predictive Energy Equations

For the Schofield, Harris-Benedict, Mifflin St. Jeor, Oxford equations the correlation coefficients were 0.72, 0.62, 0.51, 0.69 respectively. The mean and standard deviations are presented in Table 5.4. To confirm the observed difference in calculated versus predicted energy expenditure a sample size of; 376, 248, 50, 194, would be required for each of the above equations. This is partly due to the small mean bias between the Schofield equation and the measured REE, which was in the order of 1-2%.
5.2.5 Discussion

This study demonstrated differences in the performance of four commonly-used predictive equations in estimating REE when applied to an obese Māori and Pacific cohort; confirmed the poor performance of BIA for allometric scaling of REE, and informed sample size estimation for the CREEDS study (chapter seven)

5.2.5.1 Cohort Profile

The pool of participants recruited were almost exclusively in the 30-60 yr age group. Three quarters of the cohort were female. By contrast, only 36% of the Schofield cohort were female. The updated Harris Benedict equation, Mifflin St.-Jeor and Oxford equations were more balanced with a female cohort of 50%, 50%, 45% respectively. All four predictive equations include gender status in the estimation of REE, however these predictions may perform differently in men than women, thus affecting the validity of the sample size estimate for a planned cohort study with a different gender distribution. The under-representation of male participants in this study highlights the challenge in recruiting overweight and obese male Māori & Pacific participants.

All recruited individuals were obese, despite including overweight in the eligibility criteria. This may reflect bias in perceived weight, such that potential overweight participants may not have considered themselves eligible (434). For a definitive study to derive a prediction equation for REE in Māori & Pacific, it would be important to overcome both of these issues and recruit similar numbers of men and women, across a wider age and BMI range.

5.2.5.2 Accuracy of BIA compared to DXA

BIA estimates of FM, FFM and percentage FM compared poorly to those produced by DXA. The lowest correlation was for FFM. This may reflect the susceptibility of FFM estimation to fluctuations in hydration. However, all participants were given the same instructions and encouraged to keep hydrated prior to the study visit.

Another possible reason for the poor performance of BIA compared to DXA is the validity of the algorithms used by the BIA method to relate height, weight and bioimpedence to body composition. The conversion of bioimpedence values to body composition is itself a prediction equation derived from multivariate regression, with the same issues of validity as predictions of REE. Swinburn et al, 1999, evaluated the
accuracy of FM assessment by BIA compared with DXA in Māori and Pacific New Zealanders, and developed a new equation for estimation of FM in this population. BIA overestimated FM particularly at a BMI of greater than 30 kg/m² (435). These are consistent with our findings. It would have been useful to compare the Tanita’s inbuilt FM estimates with those of Swinburn’s equation. However, this was developed using a different bio-impedance analyser, and the resistance values are not the same as those produced by the Tanita.

5.2.5.3 Assessing Performance of Predictive Equations

All predictive equations were biased, underestimating REE by between 1-5% on average. The confidence intervals were wide with an upper limit of 6-11% overestimation. In this small sample, the Schofield, followed by the Oxford equations, performed best with the greatest correlation to measured REE and the least bias. As the Schofield equation is used in New Zealand a definitive study of the validity of predictive equations in NZ populations must be powered to assess its effectiveness. Based on the sample data presented here, 376 participants would be required to confirm a difference of 1%, between measured and predicted REE. To derive new predictive equations, a similarly large sample would be required, the size depending on the complexity of the proposed prediction model. Rush et al., 2006, used a cohort of 140 participants to develop, and 70 participants to validate predictive equations in an Asian Indian cohort using BIA and DXA (327). Their equation performed better than those developed in predominantly European cohorts.

McLay-Cooke et al., 2017, conducted a study investigating the performance of predictive equations in New Zealand participants of unspecified ethnicity (436). This study was published after completion of the PREEMPt, and so did not influence protocol development. The Schofield, Mifflin St. Jeor and Oxford equations were found to overestimate REE in individuals with a measured REE up to 7950 kJ, with underestimation of RMR above this level. By contrast in the PREEMPt study, we found systematic underestimation of resting energy expenditure. Rush et al., 2003, have previously demonstrated greater lean mass in Pacific individuals compared to European individuals at the same BMI (437). As none of the predictive equations assessed include body composition, it is possible that a greater degree of lean mass in our Polynesian cohort may have accounted for the underestimation of resting energy expenditure. Assuming a
sample representative of Dunedin’s ethnic distribution was recruited by McLay-Cooke *et al*, approximately 90% of the sample would be New Zealand European, 8% Māori and 2% Pacific (438).

5.2.5.4 *Use of BIA for Allometric Scaling of Resting Energy Expenditure*

The difference in body composition with BIA and DXA has implications for assessment of REE. BIA does not have the same validity that DXA does for the assessment of body composition. This inaccuracy is indicated by the SEE, the $R^2$ & F statistics, and the significance of the estimates using BIA. The SEEs were 2-fold greater in the BIA models than those that used DXA estimates of lean and FM. In the BIA models the FFM estimate did not achieve statistical significance, despite being very strongly associated with REE in the DXA-based models. Notwithstanding the bias introduced by the measurement method, the cost of the reduced accuracy compared with DXA is significant. The sample size estimate to detect a 10% difference between groups in the CREEDS study is 14, assuming the RMSE is correct. Using BIA, 60 participants would be required to detect the same effect, making it less cost effective than using DXA.

5.2.5.5 *Power Calculations for the CREEDS Study*

Multiple sample sizes were estimated based on the variance in the resting energy expenditure. Adaptive thermogenesis has previously been quantified in the range of 5-10%. A sample size of 32, excluding dropouts, would provide 90% power to detect a difference of 7% in resting energy expenditure between intervention groups. Some caveats apply to this estimate. First, the PREEMPt study was a cross sectional design, whereas the CREEDS study was planned to be a mixed, repeated measures design. The repeated measures in the CREEDS study design should increase power by reducing the within-participant variation. Secondly, this estimate is based off the RMSE rather than the 95% upper confidence limit of the RMSE. Assuming the 95% upper confidence limit, a sample size of 32 would give >90% power to detect a difference of 10%, while a sample size of 58 would be required to detect a difference of 7%. The possible impact of menstrual cycle on resting energy expenditure was not assessed in this study. Resting energy expenditure is more variable in menstruating women, with an approximate two-fold increase in variability over a month compared with men (296). Finally, the CREEDS study is not restricted to Māori & Pacific ethnicities, in choosing to use only this ethnicity, it may have reduced the validity of the power calculations.
5.2.6 Conclusion

Based on this pilot sample of 20 obese Māori and Pacific participants, a sample size of 32 was chosen for the CREEDS study. It was determined that for reasons of validity and efficiency, DXA rather than BIA would be the most appropriate body composition assessment method. An estimate sample size of approximately 376 participants would be required to confirm the bias in the estimate of REE produced by the currently used Schofield equation.
6 The Intermittent Fasting Hypoglycaemia Study

Fig 6.1: Title-page for the intermittent fasting hypoglycaemia study

Research: Treatment

Intermittent fasting in Type 2 diabetes mellitus and the risk of hypoglycaemia: a randomized controlled trial

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Accepted 29 January 2018

Abstract

Aims To establish whether the risk of hypoglycaemia is greater with 2 consecutive days of very-low-calorie diet compared with 2 non-consecutive days of very-low-calorie diet in people with Type 2 diabetes.

Methods This was a non-blinded randomized parallel group interventional trial of intermittent fasting in adults. The participants had a BMI of 30–45 kg/m², Type 2 diabetes treated with metformin and/or hypoglycaemic medications and an HbA1c concentration of 7–9 mmol/mol (57–104%). The participants followed a 2092–2510 kJ diet on 2 days per week for 12 weeks. A total of 41 participants were randomized 1:1 to consecutive (n=19) or non-consecutive (n=22) day fasts, of whom 37 (n=18 and n=19, respectively) were included in the final analysis. The primary outcome was difference in the rate of hypoglycaemia between the two study arms. Secondary outcomes included change in diet, quality of life, weight, lipid, glucose and HbA1c levels, and liver function.

Results The mean hypoglycaemia rate was 1.4 events over 12 weeks. Fasting increased the rate of hypoglycaemia despite medication reduction (RR 2.05, 95% CI 1.17 to 3.52). There was no difference between fasting on consecutive days and fasting on non-consecutive days (RR 1.54, 95% CI 0.35 to 6.11). Improvements in weight, HbA1c, fasting glucose and quality of life were experienced by participants in both arms.

Conclusions In individuals with Type 2 diabetes on hypoglycaemic medications, fasting of any type increased the rate of hypoglycaemia. With education and medication reduction, fewer than expected hypoglycaemic events occurred. Although it was not possible to determine whether fasting on consecutive days increased the risk of hypoglycaemia, an acceptable rate was observed in both arms.

Diabet. Med. 35, 588–594 2018

Introduction

With rising rates of obesity and Type 2 diabetes mellitus worldwide [1,2], there is a need for accessible, safe and cost-effective treatments for both conditions. A very-low-calorie diet can facilitate weight loss and improve glucose homeostasis [3,4]; however, in those taking hypoglycaemic medication, such caloric restriction increases the risk of hypoglycaemia and the best way to avoid this remains unclear.

One form of very-low-calorie diet is ‘intermittent fasting’. This describes a schedule of caloric restriction on some days combined with ad libitum calorie intake on others. The degree of caloric restriction may vary from partial to complete restriction. The schedule may involve restriction for several hours a day, alternate days or several days per week [5,6]. A systematic review, but without meta-analysis, of studies comparing intermittent fasting with daily energy restriction reported the same degree of weight loss between the two treatment options in nine out of 12 studies (75%). The individual study results, however, were more variable with regard to differences in HbA1c levels, fasting glucose levels and markers of insulin sensitivity for intermittent fasting compared with daily energy restriction [7]. Some of this variation may have been attributable to differences in the prescribed diet on fasting days and the schedule of fasting. An issue yet to be investigated regarding intermittent fasting is the risk of hypoglycaemia during intermittent fasting in patients using hypoglycaemic medication. There have been few studies to date of intermittent fasting in participants with Type 2 diabetes [8–10,11] and in only two of these were participants taking medication with the potential to cause hypoglycaemia during the intervention period [8,9].
6.1 Published Abstract

**Aims:** To establish whether the risk of hypoglycaemia is greater with two days’ consecutive compared with non-consecutive very low-calorie diet in individuals with type 2 diabetes (Type 2 DM).

**Methods:** This was an un-blinded randomized parallel group interventional trial of intermittent fasting in obese adult participants with Type 2 DM treated with dietary modification and/or any combination of hypoglycaemic medications in Wellington, New Zealand. Participants followed a 2092 – 2510 kJ diet two days per week for 12 weeks. Forty-one participants were randomised 1:1 to consecutive (n=19) or non-consecutive (n=22) day fasts, 37 (n=18 and n=19) were included in the final analysis. The primary outcome was difference in the rate of hypoglycaemia between treatment arms. Secondary outcomes included; change in diet composition, quality of life, weight, lipids, glucose, glycated haemoglobin and liver function tests.

**Results:** The mean hypoglycaemia rate was 1.4 events over 12 weeks. Fasting (RR 2.05, 1.16 to 3.6), but not allocation to consecutive day fasting (RR1.54, -2.87 to 6.11), increased hypoglycaemia risk despite medication reduction. Significant reduction in weight, HbA1C, fasting glucose, and improvement in quality of life occurred in both arms.

**Conclusions:** In individuals with Type 2 DM on various hypoglycaemic medications, intermittent fasting with weekly supervision, hypoglycaemia education and structured medication reduction, is associated with increased hypoglycaemia on fasting days but a low overall risk of hypoglycaemia.

**Registration & Funding:**

This study was funded by a grant from the New Zealand Society for the Study of Diabetes.

Australia New Zealand Clinical Trial Registry Number: ACTRN12614000402640
6.2 Introduction

An unanswered question regarding altered schedules of calorie restriction, is the risk of hypoglycaemia during IF in patients using hypoglycaemic medication. There have been four studies to date of IF in participants with T2DM and in only two of these were participants taking medication with the potential to cause hypoglycaemia during the intervention period (290, 291, 439, 440).

The popular IF diet was chosen as a model for schedule restriction for this study. This involved a very low-calorie diet (VLCD), 2092 kJ fast in women and 2510 kJ in men, for two days per week, with ad libitum intake on the other five days. The significant difference in energy intake between the fasting and non-fasting days creates an obvious risk for hypoglycaemia on fasting days in people with T2DM taking insulin or oral agents which promote endogenous insulin release. The best approach to medication adjustment for fasting days in those with T2DM undertaking IF, and the risk of hypoglycaemia for an approach of consecutive or non-consecutive fasting are unknown.

This study aimed to test the hypothesis that during a 5:2 IF diet with medication adjustment, non-consecutive day caloric restriction reduces the overall risk of hypoglycaemia, compared to consecutive days of calorie restriction.

The intermittent fasting hypoglycaemia (IF-Hypo) study was published in Diabetic Medicine (fig 6.1) (441). The accepted manuscript has been reproduced for use in this chapter, consistent with Diabetic Medicine’s copyright transfer agreement (442). It has been modified to ensure consistency with the format of this thesis. The introduction presented here differs from that of the article to avoid repetition of information presented earlier in the thesis. However, the abstract, methods, results, discussion and conclusion are the same.

6.3 Methods

6.3.1 Participants

Participants older than 18 years with T2DM and an HbA1C 50-85 mmol/mol (6.7 to 9.9%) with a body mass index 30 - 45 kg/m2 were recruited from secondary care diabetes clinics, local community networks, and primary care practices. Exclusion criteria were: weight change of >5 kg in the preceding three months, a diagnosis of an eating disorder,
pregnant or planning pregnancy, blood pressure > 180/100 mmHg despite medical therapy, or previous bariatric surgery. Each participant gave written informed consent. The study was approved by the New Zealand Health and Disability Ethics Committee (14/NTB/33/AM03), was performed in accordance with the Declaration of Helsinki, and the Australia New Zealand Clinical Trial Registry number was: ACTRN12614000402640.

6.3.2 Study Visits & Randomisation

All participants attended the Centre for Endocrine, Diabetes & Obesity Research; Wellington Hospital, New Zealand on three occasions over 12 weeks; at baseline, six weeks and 12 weeks. At baseline participants were randomised, by a computer based process, in a 1:1 ratio to either consecutive or non-consecutive days of fasting two days per week. Participants were free to choose which day of the week to fast, this could vary from week to week to allow flexibility and improve adherence.

Treatment allocations were printed and placed in sequentially numbered sealed envelopes by a member of the research team prior to enrolment of the first participant in the study. The allocation was blinded from the staff member conducting enrolment until un-blinding at the first study visit.

6.3.3 Dietary Intervention & Medication Adjustment

Participants were given nine days of written sample recipes. These were developed by a research dietician and structured as two small snacks and one light meal amounting to between 2092 and 2510 kJ per day. Each participant was provided with written and verbal information about symptoms, management and common causes of hypoglycaemia. The importance of capillary glucose monitoring and hypoglycaemia prevention was emphasized. Hypoglycaemic medication was adjusted as follows: Sulfonylureas and NPH Insulin was reduced by 50% on fasting days. Humalog; Novorapid & Apidra was reduced by 70% on fasting days; Mixed Insulins were reduced by 25% on the night before a fast and 50% on the day of a fast, Lantus was reduced by 50% on the morning of a fasting day and/or by 50% on the evening before a fasting day. The dose of metformin or other medication not resulting in hypoglycaemia was unchanged.
6.3.4 Hypoglycaemic Events

The primary outcome was the total number of hypoglycaemic events during 12-weeks observation. Participants were contacted weekly by phone or email during the study. Days of the week spent fasting, change in medication, the date, time, circumstances, severity, and capillary blood glucose concentration of any hypoglycaemic events were recorded. Severe hypoglycaemia was defined according to the American Diabetes Association guidelines as an event requiring assistance of another person to actively administer carbohydrate, glucagon, or other resuscitative actions (443). If a hypoglycaemia event was avoidable, e.g. due to a missed meal, appropriate advice was given to prevent a further occurrence and hypoglycaemic medications were not adjusted. If there was no clear cause, or if hypoglycaemia events were recurrent despite appropriate advice, the participants’ medication was reduced further.

6.3.5 Anthropometry, Dietary Composition, Quality of Life, Continuous Glucose Monitoring

Anthropometric measurements & food intake were assessed at all visits and included; height, weight, waist circumference, body fat composition by tetra-polar bioimpedence analysis (TBF-300, Tanita) and blood pressure (Flexiport, Welch Allyn). Participants completed a four day food diary to record calorie intake at baseline, six & 12 weeks (444). Baseline diaries contained non-fasting entries only. At six and 12 weeks, each day of the food diary was coded fasting or non-fasting. Quality of life and biochemical assessments were conducted at baseline and 12 weeks.

The Audit of Diabetes-Dependent Quality of Life 19 (ADDQOL) Questionnaire was used for quality of life measurement at baseline and 12 weeks (445, 446). The ADDQOL contains an overall quality of life assessment score ranked between +3 (excellent) to -3 (extremely bad); and a measure of the impact of diabetes on quality of life in general. It also measures the impact in 19 different domains. For each domain participants are asked to rate how their life would be if they did not have diabetes. The impact scales for each domain range from “very much better” (-3) to “worse” (+1) and are multiplied by an importance score from 0 to +3 which reflects the importance of that domain to the participant. Lower scores reflect poorer quality of life. A mean weighted impact score is calculated as a summary score across all domains.
Continuous glucose monitoring (Guardian REAL CGMS devices, Medtronic) was performed between week six and week 12. The target monitoring period was four days including at least one fasting and one non-fasting day.

6.3.6 Glycated haemoglobin, fasting lipids, thyroid stimulating hormone, free thyroxine, liver function, renal function, and fasting glucose

Fasting venous blood was collected and analysed for; lipids, glucose, liver function & renal function (Cobas c-501, Roche); free thyroid hormone and thyroid stimulating hormone (FT4, TSH, Cobas e601, Roche); full blood count (XS-1000i, Sysmex); and glycated haemoglobin (HbA1C, D-10, Biorad).

6.3.7 Statistical Analysis

The relative rate of hypoglycaemia events was estimated using a generalized linear mixed model. Co-variates in the model were: baseline insulin use, baseline sulfonylurea use and randomised dietary intervention, all as dichotomous variables. Repeated measures on the same participants were accounted for by incorporating them as random effects. A Poisson distribution for count data was used with the logarithm of the number of days’ fasting as the offset variable. Anthropometric & biochemical outcomes were assessed using a general linear mixed model that incorporated participants as random effects and treatment group and timepoint (baseline, 12 weeks) as fixed effects (Table 2). Quality of life data were analysed using two-way ANCOVA incorporating treatment group as a predictor variable and baseline scores as a covariate in the model. For glucose profiles on fasting and non-fasting days, only recordings that covered >85% of day and contained a minimum of three calibrations per day were used. SAS version 9.4 was used.

6.3.8 Sample size

The sample size was estimated by simulation from random Poisson variables. Past research suggested a hypoglycaemia rate of 4.4 per 12 weeks (447). We felt that detecting an increased rate of hypoglycaemia of 2.2 per 12 weeks is clinically meaningful. By simulation a sample size of 18 in each group; 36 in total, has 80% power with a two-sided type I error rate of 5% to detect this size difference.
6.4 Results

Baseline demographic data and medication use are shown in table 6.1. The flow of participants is shown in fig 6.2.

**Table 6.1: Baseline demographic profile and medication use**

<table>
<thead>
<tr>
<th></th>
<th>Non-Consecutive</th>
<th>Consecutive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 19</td>
<td>n = 18</td>
</tr>
<tr>
<td><strong>Mean (Min to Max)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>58 (42 to 74)</td>
<td>62 (44 to 77)</td>
</tr>
<tr>
<td><strong>n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>8 (42)</td>
<td>7 (39)</td>
</tr>
<tr>
<td>Maori</td>
<td>2 (10)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>NZ European</td>
<td>13 (68)</td>
<td>12 (67)</td>
</tr>
<tr>
<td>Insulin Use</td>
<td>9 (47)</td>
<td>12 (67)</td>
</tr>
<tr>
<td>Sulfonylurea Use</td>
<td>11 (58)</td>
<td>10 (57)</td>
</tr>
<tr>
<td>Metformin Use</td>
<td>18 (95)</td>
<td>15 (83)</td>
</tr>
</tbody>
</table>
6.4.1 Hypoglycaemia

Overall there were 53 hypoglycaemic events during 84 days of observation affecting 15 participants. Twenty-two participants (59%) had no hypoglycaemic events. Twenty-three hypoglycaemic events occurred over 851 fasting days, a crude rate of 1 event per 37 days of fasting, and 30 over 2257 non-fasting days, a crude rate of 1 event per 75 participant days of non-fasting. Thirty-five hypoglycaemic events occurred in 7 of eighteen participants in the consecutive fasting group with 1,512 participant days of observation for a crude rate of 1 event per 43 days. Twenty events occurred in eight of 19 participants in the non-consecutive fasting group with 1,596 participant days of observation for a crude rate of 1 events per 80 days. There were no reported severe hypoglycaemic events.

The risk of having a hypoglycaemic event was two-fold greater with fasting, relative rate (95% CI): 2.05 (1.16 to 3.6), p=0.013. The risk of having a hypoglycaemic event was not different between treatment arms 1.54 (-2.87 to 6.11), p = 0.51. Although incorporated
as co-variates, we did not detect a significant effect of baseline sulfonylurea use \(-1.59\ (\text{-}9.20 \text{ to } 3.28), \ p = 0.56\) or baseline insulin use: \(2.16 \ (\text{-}2.64 \text{ to } 3.29)\ p=0.141\) and the risk of hypoglycaemia. Over 12 weeks, further medication adjustments were required in response to hypoglycaemia in \(9/37\ (24\%)\). Of these, seven had their medications adjusted in the first two weeks, one at three weeks and one at five weeks. Six of these participants required one medication adjustment, two participants required two medication adjustments and one participant required three medication adjustments. The three participants that dropped out or were lost to follow up had no reported hypoglycaemia prior to drop out.

6.4.2 Continuous Glucose Monitor

In twenty-seven participants, a glucose monitoring sensor was used during the study resulting in 786 non-fasting and 425 fasting readings. There were seven hypoglycaemic events in five participants while wearing the monitor. Two occurred on fasting days and five occurred on non-fasting days. Mean (SD) subcutaneous glucose reading on fasting days was \(8.34 \ (2.18)\ mmol/L\) and on non-fasting days was \(8.93 \ (2.59)\ mmol/L\).

6.4.3 Diet Composition

Baseline, six-week and 12-week food diaries were completed by \(33 \ (89\%)\), \(25 \ (68\%)\) and \(33 \ (89\%)\) of participants respectively. The mean (SD) total energy intake was \(3430 \ (1129)\ kJ\) on fasting days and \(7472 \ (2418)\ kJ\) on non-fasting days. There was a sustained reduction in total calorie intake after six and 12 weeks compared to baseline (fig 6.3). There was no significant difference in proportional macronutrient composition between fasting and non-fasting days at any timepoint. After six and 12 weeks, self-reported adherence with the calorie target of \(2510\ kJ\) on fasting days was \(20\%\) and \(24.2\%\) respectively.
Table 6.2. Changes in anthropometric and biochemical parameters from baseline to week 12

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline Mean (SD)</th>
<th>12 Weeks Mean (SD)</th>
<th>Change from Baseline to 12 Weeks, between group difference. Mean (95%CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>109.8 (20.3)</td>
<td>108.7 (20.4)</td>
<td>-0.8 (-13.8, 12.0)</td>
<td>0.89</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>36.8 (5.2)</td>
<td>36.6 (5.3)</td>
<td>0.6 (-3.0, 4.2)</td>
<td>0.75</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>41.2 (6.7)</td>
<td>38.6 (7.2)</td>
<td>-1.5 (-6.2, 3.2)</td>
<td>0.53</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>122.5 (13.6)</td>
<td>120.4 (17.0)</td>
<td>-0.4 (-10.2, 9.4)</td>
<td>0.93</td>
</tr>
<tr>
<td>HbA1C (mmol/mol)</td>
<td>66 (7)</td>
<td>68 (10)</td>
<td>2.1 (-3.3, 7.6)</td>
<td>0.44</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>8.2 (1.3)</td>
<td>8.4 (1.8)</td>
<td>0.2 (-0.3, 0.7)</td>
<td>0.77</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/L)</td>
<td>9.0 (0.5)</td>
<td>8.2 (2.8)</td>
<td>-0.8 (-2.0, 0.3)</td>
<td>0.17</td>
</tr>
<tr>
<td>TSH (mU/L)</td>
<td>1.6 (0.8)</td>
<td>2.1 (1.9)</td>
<td>0.3 (-0.4, 1.0)</td>
<td>0.81</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.2 (1.0)</td>
<td>3.9 (0.8)</td>
<td>-0.1 (-0.6, 0.5)</td>
<td>0.81</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>1.1 (0.2)</td>
<td>1.0 (0.2)</td>
<td>-0.1 (-0.2, 0.1)</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>Baseline Mean (SD)</td>
<td>12 Weeks Mean (SD)</td>
<td>Change from Baseline to 12 Weeks, between group difference. Mean (95%CI)</td>
<td>p-value</td>
</tr>
<tr>
<td>------------------------------</td>
<td>--------------------</td>
<td>--------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>--------</td>
</tr>
<tr>
<td></td>
<td>Non-Consecutive (n=19)</td>
<td>Consecutive (n=18)</td>
<td>Non-Consecutive (n=19)</td>
<td>Consecutive (n=18)</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>2.1 (0.8)</td>
<td>2.1 (0.8)</td>
<td>2.0 (0.7)</td>
<td>2.25 (0.81)</td>
</tr>
<tr>
<td>Cholesterol:HDL ratio</td>
<td>3.8 (1.0)</td>
<td>3.9 (0.9)</td>
<td>3.5 (0.9)</td>
<td>3.9 (1.0)</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.8 (0.6)</td>
<td>1.8 (0.7)</td>
<td>1.7 (0.4)</td>
<td>1.7 (0.8)</td>
</tr>
<tr>
<td>Creatinine (umol/L)</td>
<td>79 (20)</td>
<td>100 (44)</td>
<td>82 (20)</td>
<td>93 (35)</td>
</tr>
<tr>
<td>Estimated GFR (ml.min⁻¹.1.73m²⁻¹)</td>
<td>74 (17)</td>
<td>59 (20)</td>
<td>73 (17)</td>
<td>63 (20)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>133 (15)</td>
<td>132 (12)</td>
<td>129 (12)</td>
<td>129 (16)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>78 (11)</td>
<td>74 (11)</td>
<td>75 (10)</td>
<td>72 (10)</td>
</tr>
</tbody>
</table>
There were no statistically significant differences between consecutive or non-consecutive arms in secondary outcomes (table 6.2). Weight, waist circumference, FM, HbA1C & fasting glucose improved from baseline to 12 weeks. Reductions in alanine transaminase, alkaline phosphatase & aspartate transaminase achieved statistical significance but were small and not considered clinically significant. In 6/37 (16%) HbA1C rose between 1 and 4 mmol/mol.

6.4.5 Quality of Life

Treatment group had no significant impact on any quality of life or diabetes impact score in any domain. However, in all participants there was a small but statistically significant improvement in the global quality of life rating between baseline and week 12 (0.66, 95% CI [0.48, 0.85] p = 0.020). The effect was an improvement of 0.66 on a scale where the
maximum achievable difference is 6.0. This was offset by a small, statistically significant increase in the global impact of diabetes on quality of life between baseline and week 12 (0.70, 95% CI [0.34,1.04] p <0.001). The effect was an increase in the negative impact of diabetes on quality of life of 0.70 units, on a scale where 4 units’ difference is the maximum. There was no significant change in the impact of diabetes on any of the 19 specific domains or the average weighted score based on these domains.

6.5 Discussion

In this study, we found that despite hypoglycaemic education, a proactive standardized medication reduction and weekly contact, IF was associated with a two-fold increase in hypoglycaemia on fasting days. However, the overall risk of hypoglycaemia on both fasting and non-fasting days was lower than expected, there were no episodes of severe hypoglycaemia and most participants did not experience hypoglycaemia. These observations suggest that the risk of hypoglycaemia appears to be more dependent on individual characteristics than on the pattern of fasting. Continuous glucose monitor recordings supported the reported absence of severe hypoglycaemia and the relatively low reported rate of hypoglycaemia.

This is one of few studies examining the risk of hypoglycaemia in people with T2DM following an intermittent calorie restriction(290, 440). Ash et al studied 51 overweight or obese men with T2DM on oral hypoglycaemia medication randomised to intermittent energy restriction, pre-portioned meals or self-selected meals. The intermittent energy restriction was four consecutive days of a 4184 kJ liquid meal for 12 weeks. While a comparable reduction in HbA1C was observed, medication adjustment or hypoglycaemic events were not reported(290). More recently Carter et al. studied a medication reduction protocol based on baseline HbA1C. This was altered during the study period due to excess hypoglycaemia in those taking sulfonylureas. The reported hypoglycaemia rate occurring in insulin users was 4.3 +/- 3.8 events over the 12-week study period(440). This compares with an overall crude rate of 1.4+/-.1 events (n=37) in our study over the same period despite a greater proportion of insulin & sulfonylurea users at baseline (56%) with only two participants not on hypoglycaemic medication. This rate is substantially lower compared to recent published rates of hypoglycaemia in comparable populations (447-449). This is most likely explained by the combination of hypoglycaemic education, a proactive reduction in hypoglycaemic medication, and
weekly contact to discuss hypoglycaemia. Most importantly HbA1C did not deteriorate because of medication reduction at baseline.

Consistent with other studies of IF there was a clinically relevant reduction in weight, HbA1C and fasting glucose despite the relatively mild dietary intervention and short duration of the study.

We observed a difference in adherence to the target calorie intake at six weeks and 12 weeks, this may be due to the difference in response rate, as a larger proportion of participants failed to return the six-week dietary record.

We found that participant’s perceptions of the negative impact of diabetes on their quality of life increased slightly during the study. This may have resulted from the dietary constraints, requirement for regular testing and the emphasis on body shape and weight during a period of calorie restriction. Despite this there was a small improvement in their global quality of life rating.

6.5.1 Limitations

Our primary outcome was reliant on self-reported hypoglycaemia during weekly contact. While spontaneously self-reported hypoglycaemia rates are low in individuals with T2DM (448), self-reported hypoglycaemia in response to questionnaires is widely used to measure of the true burden of hypoglycaemia in population studies (448, 449). In retrospect, it would have been helpful to have collected data on rates of hypoglycaemia prior to the commencement of the intervention.

Generalized linear mixed models for count measures are prone to bias depending on the estimation methods used and the specified distribution of the outcome variable, in this case hypoglycaemia (450). To test for this, we ran the procedure in both R (399) & SAS (SAS Institute, USA) and found no difference in the estimates. For our analysis, we categorised insulin use as a dichotomous variable which may have obscured a dose related increase in hypoglycaemia (448, 449).

The lower than anticipated hypoglycaemia rate meant we were underpowered to detect a difference in hypoglycaemia between arms. However, as we were looking for a clinically relevant increase in hypoglycaemia compared with published averages, the finding of a lower than anticipated hypoglycaemia event rate in both arms is meaningful.
Systematic underreporting of food intake in self-reported food diaries is well recognised (444). In this study, there may have been greater risk of underreporting on fasting days. However, the within-participant comparison between fasting and non-fasting days may have attenuated any one individual’s tendency to under-report their intake. Systematic underreporting would not be expected to differ between consecutive and non-consecutive arms.

6.6 Conclusion

The principle finding of this study was that IF was associated with a lower than expected overall risk of hypoglycaemia when combined with weekly supervision, hypoglycaemia education, & medication reduction at baseline. Although fasting days were associated with a two-fold increase in rate of hypoglycaemia, whether the two days were consecutive or non-consecutive did not have a significant effect. The intervention did result in weight loss, reduced HbA1c and a small improvement in quality of life. Our study protocol can be adopted for the longer-term studies that will be required to assess the tolerability and sustained efficacy of an intermittent fast.
7 The CREEDS Study

7.1 Introduction

As described in chapter one, dietary energy restriction is the mainstay of treatment for obesity and the resultant weight loss is the only proven way to induce remission of T2DM. Unfortunately, durable weight loss is challenging to maintain and is accompanied by a maladaptive reduction in REE. Adaptive thermogenesis lowers the daily calorie intake required to maintain weight loss during dietary restriction and is thought to be a product of incompletely understood energy homeostatic control mechanisms.

The Changes in Resting Energy Expenditure with Different Schedules of calorie restriction (CREEDS) study aimed to investigate whether the magnitude of adaptive thermogenesis is attenuated with intermittent fasting (IF) compared with continuous daily restriction (CDR). Interrupting calorie restriction with periods of isocaloric intake, may reveal the time-scale over which adaptive thermogenesis is regulated. The CREEDS study was also designed with a clinical dietary intervention in mind. In effect, we wanted to see not just if adaptive thermogenesis could be mitigated, but also whether the impact of an altered schedule of calorie restriction would inform the implementation of a diet that is both feasible and acceptable in the wider population.

The indirect calorimetry and DXA techniques used in the assessment of adaptive thermogenesis were of known precision and validity following the testing outlined in chapters two, three and four. In addition, the power calculations for the study were drawn from studies of a local overweight and obese population sample using these same techniques to maximise validity for the study as outlined in chapter five. Whether IF provides additional glycaemic benefits over and above CDR was of interest in designing the CREEDS study. However, in view of the increased rate of hypoglycaemia on fasting days, outlined in chapter six, it was decided to exclude individuals with T2DM on hypoglycaemic medications.

Therefore, the aim of the CREEDS study was to compare changes in energy expenditure over six weeks between IF and CDR in a NZ population of overweight and obese individuals.
7.2 The CREEDS Study

7.3 Study Population

Participants were recruited from advertisements in general diabetes and endocrine clinics, local GP practices, the regional hospital and university staff mailing lists, advertisements disseminated via regional community noticeboards.

Inclusion criteria were

- BMI $\geq 30$ kg/m$^2$
- Male
- Aged 18-65 years

Exclusion criteria were:

- Weight change $> 5$ kg over the previous three months
- Current medical illness or medications that may, influence REE.
- Current smoker
- Weight $> 220$ kg, the maximum weight limit on the DXA machine.
- A history of disordered eating.
- History of T2DM requiring treatment other than metformin or diet OR poorly controlled T2DM with a HbA1C $> 64$ mmol/mol (8.0%) OR a fasting plasma glucose $> 10$ mmol/l.

Male participants were chosen to avoid variation in REE due to changes in the menstrual cycle observed in women (296), particularly given the three-week interval between study visits (section 7.5.3). Poorly-controlled T2DM has been associated with an elevation in the REE, and was therefore also an exclusion (451). Individuals with T2DM on hypoglycaemic medication were excluded due to the increased risk of hypoglycaemia with fasting day intake as outlined in chapter six.
7.4 Outcomes

The CREEDS study was a six-week non-blinded randomised parallel group, dietary intervention study of IF compared to CDR.

1. The primary outcome was the change in REE over six weeks comparing participants undergoing daily calorie restriction versus an intermittent calorie restriction, providing the same net energy deficit per week.

The secondary outcomes were:

1. Adherence to the prescribed calorie restriction.
2. Change in macronutrient composition of the diet.
3. Change in weight, FM, FFM, waist and hip circumference with CDR compared to IF.
4. Changes in fasting glucose, HbA1C, total cholesterol, triglycerides, high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol.
5. Changes in REE on fasting compared with non-fasting days.
7. Changes in physical activity levels.
8. Change in thyroid function, leptin, total ghrelin, active ghrelin and adiponectin between the IF group and the CDR group. *

*Analysis of thyroid function, fasting glucose, adiponectin, leptin and ghrelin is planned. These will be included in the CREEDS manuscript but are outside the scope of this thesis.

7.5 Methods

7.5.1 Diet Intervention

Participants in the CDR group were prescribed 80% of estimated total daily calorie requirements every day. Participants in the IF group were prescribed 25% of estimated total daily calorie intake on two non-consecutive days per week and 100% of estimated daily requirements on the other five days. Thus, both groups were required to calorie
count daily throughout the study. Diet prescriptions were calculated by combining REE assessed by indirect calorimetry, Physical activity energy expenditure assessed by IPAQ and by assuming DIT accounted for 10% of TEE. Physical activity estimates were obtained from the IPAQ using the method outlined in chapter two, section 2.3.3.4.2. Diet prescriptions were calculated for all participants at each study visit. The diet duration was six weeks.

7.5.1.1 Diet Education

Dietary education was provided during a one-hour individual education session with the lead researcher and research dietitian during the first study visit. The first half of the session was spent discussing accurate food diary completion, usual diet and food preferences, and the components of a healthy diet as outlined by the New Zealand Ministry of Health (428). The second half was spent discussing calorie counting, portion size estimation, converting between kilocalories and kilojoules, label reading, demonstrating smart-phone apps and websites to assist with calorie counting, and reviewing the written resources.

A guide to calorie counting was prepared for the CREEDS study (appendix D). Briefly, this included; tips for sticking to a low-calorie diet, a two-page quick reference guide containing calorie counts for common food and drink items, sample meal-plans for fasting day intakes in the IF group and recipe cards for low calorie meals. The guide also contained; a list of recipe websites that provide calorie counts, links to online-calorie counters, the names of android and iOS compatible calorie counting apps and calorie counting or IF recipe books. The Easy Diet Diary (Xyris software) app for iphone and the Calorie King website (www.calorieking.com.au) were recommended for calorie counting, as they are based on the food composition databases of the Food Standards Authority Australia New Zealand.

7.5.1.2 Four-day food records

Participants completed four-day food records prior to their first study visit and during the first, third, and sixth weeks of the diet intervention. Template food records were provided in hardcopy and softcopy format (see appendix A). The baseline food record was used to educate participants about completing food diaries accurately, to provide an indication of their diet habits and food preferences, and to help with tailoring the dietary education and advice on adhering to a low-calorie diet. They were not used in the study analysis.
The remaining food records were used to assess adherence to the diet prescription and assess macronutrient composition of the diet at week one, week three and week six. Several participants indicated a preference for using spreadsheets or apps to log their food intake. Any method that facilitated accurate food records recording was encouraged. Irrespective of the method of recording food intake, participants were asked: to complete at least four days of food records at each time-point; to record all items consumed including beverages; to complete records at least once daily; to record the day and the time-point of all food records; and, in the IF group, to note if it was a fasting or a non-fasting day. All records were checked and any omissions discussed during the next study visit or by telephone contact, whichever occurred first. Food records were analysed using Food Works 9 (Xyris software), diet analysis software.

7.5.1.3 Weekly telephone contact

Between visits to the study centre, participants were contacted weekly by telephone. Using a standard list of questions, each participant was asked about: any barriers to adherence; the number of days successful calorie restriction that week; whether they had any question about calorie counting or the diet prescription; whether there had been any change in medications or their health; and whether they had completed and returned their food record, if applicable.

7.5.2 Randomisation

Participants were randomly allocated 1:1 to CDR or IF. Randomisation was performed using a web-based application (www.RANDOM.org, Randomness and Integrity Services Ltd.). Randomised allocations were concealed in sequentially numbered envelopes. Investigators were blinded to the allocations until the participant’s first visit.

7.5.3 Study Visits

The study visit structure is presented in Table 7.1. Participants attended the centre three times over six weeks. Visits were scheduled between 0700 – 11:00 in the morning. The IF group attended for a fourth visit to measure REE on a fasting day to compare fasting with non-fasting days.
Table 7.1: Schedule of procedures for the CREEDS Study

<table>
<thead>
<tr>
<th>Procedures</th>
<th>Visit 1 (Week 0)</th>
<th>Visit 2 (Week 3)</th>
<th>Visit 3 (Week 6)</th>
<th>Visit 4 (Week6+1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eligibility screen</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Consent</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Randomisation</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phlebotomy</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Anthropometry</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>DXA</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Physical Activity Assessment</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Indirect calorimetry</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Structured Diet Education</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet Prescription</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Eating Behaviour Questionnaire</td>
<td>✓</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Food Record review</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Visits two and three were scheduled within 21 days +/- 3 days from the previous visit. Visit four was scheduled within 24 hours of visit three.
7.5.4 Body composition and Energy Expenditure

Body composition and energy expenditure were measured by standard anthropometry, DXA and indirect calorimetry as outlined in chapter two, section 2.4.

7.5.5 Biochemical Tests

HbA1C and lipids, were collected and analysed using a point of care device as outlined in chapter two, section 2.4.5.

7.5.6 The International Physical Activity Questionnaire

The long-form IPAQ was used to assess physical activity at every study visit (section 2.3.3.4.2) (359). The same interviewer carried out all IPAQ interviews during the study. Physical activity related energy expenditure was expressed as a percentage of total daily estimated energy expenditure to account for differences in body weight.

7.5.7 The Adult Eating Behaviour Questionnaire

The adult eating behaviour questionnaire (AEBQ) is a 35 item-instrument that evaluates participants appetitive eating behaviours (452). Items are presented as a series of statements and participants are asked to select from five possible responses; “strongly disagree”, “disagree”, “neither agree nor disagree”, “agree”, “strongly agree. Each item belongs to one of seven appetitive behaviour domains or an eighth domain that represents hunger. The seven domains are; enjoyment of food, emotional over-eating, emotional under-eating, food fussiness, food responsiveness, slowness in eating and satiety responsiveness. Participants self-administered the AEBQ at baseline and six weeks. Answers for questions within each domain were averaged.

7.5.8 Data Management

All study data were recorded in REDCAP, a secure online data management tool hosted at the University of Otago Wellington (453). A tailored REDCAP database was developed for the CREEDS Study. To minimise data entry error and to ensure no data were omitted, validation rules were created for each field. This involved specifying the number of decimal points, format and expected range for all measured variables, highlighting essential missing values, automating calculation of fields such as BMI and age, and auto-completing fields in one instrument with data entered in another to reduce errors from entry of duplicate values. Where appropriate drop-down menus or radio-
buttons were used to avoid data entry errors. Data were entered contemporaneously at each visit by the research team.

7.5.9 Statistical Analysis

Statistical analysis was performed in R (399). For the primary endpoint a general linear mixed model was used, incorporating individuals as random effects and treatment, timepoint, FFM and FM as fixed effects. Timepoint by treatment was included as an interaction term. The restricted estimates of maximum likelihood method was used as it is associated with less bias for smaller samples. For secondary outcomes, changes from baseline to six weeks were evaluated using ANCOVA incorporating baseline values as a covariate and treatment as a fixed effect. Comparison of macronutrient composition and physical activity between groups was conducted using student t-tests at each time point, with Bonferroni correction for multiple comparisons. Differences indirect calorimetry recordings following a fast-day compared with a non-fasting day were assessed with a paired student’s t-test. As outlined in chapter five (section 5.2.5.5), a sample size of 32, excluding dropouts, was estimated to provide 90% power to detect a difference of 7% in REE between intervention arms.

7.5.10 Ethics Approval, Maori Consultation and Funding

Consultation with Maori was conducted during protocol development with Wellington Regional Hospital’s Research Advisory Group – Māori. Ethics approval was obtained from the Northern A, Health, Disability and Ethics Committee, ref. 16/NTA/208. A grant-in-aid for this study was received from the Maurice and Phyllis Paykel Trust, registered charity number CC27541. All study visits were conducted at the Centre for Translational Physiology, University of Otago Wellington.

7.6 Results

7.6.1 Participant Recruitment and Retention

A flow chart for participant recruitment is presented in Fig. 7.1. Fifty-two individuals expressed an interest in the study and thirty-six were eligible for inclusion. Sixteen participants were not eligible for inclusion; twelve had a BMI < 30kg/m2; one had a diagnosis of obstructive sleep apnoea; one had a mitochondrial disorder; one had T2DM
requiring oral hypoglycaemic medications, and one was enrolled in a pharmacological weight loss study.

**Figure 7.1**: Participant Flow Chart for the CREEDS Study.

7.6.2 Baseline Characteristics

Of the 34 included participants 26 identified as New Zealand European, seven New Zealand Māori, two European, one Asian and one Australian. Three participants identified with more than one ethnic group. The median age was 50 with an interquartile range of 41 to 57 years. Baseline anthropometric, biochemical and body composition data are presented in Table 7.2.
Table 7.2: Anthropometric, body composition and biochemical data at baseline and six weeks for the IF and CDR intervention arms.

<table>
<thead>
<tr>
<th></th>
<th>Baseline Mean (SD)</th>
<th>6 Weeks Mean (SD)</th>
<th>Change from Baseline Effect of Treatment</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CDR (n= 16)</td>
<td>IF (n = 16)</td>
<td>CDR (n= 16)</td>
<td>IF (n = 16)</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>111 (23)</td>
<td>107 (18)</td>
<td>107(24)</td>
<td>101 (18)</td>
<td>2.9</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>35 (5)</td>
<td>34 (5)</td>
<td>34(5)</td>
<td>33(6)</td>
<td>2.7</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>114 (14)</td>
<td>112 (11)</td>
<td>109 (15)</td>
<td>109 (12)</td>
<td>0.47</td>
</tr>
<tr>
<td>Hip Circumference (cm)</td>
<td>116 (9)</td>
<td>114 (11)</td>
<td>114(10)</td>
<td>112(13)</td>
<td>0.40</td>
</tr>
<tr>
<td>Fat Free Mass (kg)</td>
<td>71 (10)</td>
<td>67 (6)</td>
<td>69(10)</td>
<td>64(6)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fat Mass (kg)</td>
<td>39 (13)</td>
<td>39 (12)</td>
<td>37 (14)</td>
<td>35 (12)</td>
<td>4.8</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>34 (5)</td>
<td>35 (5)</td>
<td>34 (5)</td>
<td>34 (5)</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>Baseline Mean (SD)</td>
<td>6 Weeks Mean (SD)</td>
<td>Change from Baseline Effect of Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>--------------------</td>
<td>-------------------</td>
<td>------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CDR (n= 16)</td>
<td>IF (n = 16)</td>
<td>F-value</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>121 (12)</td>
<td>125 (10)</td>
<td>1.25</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>78(8)</td>
<td>82(11)</td>
<td>2.0</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>HbA1C (mmol/mol)</td>
<td>38 (8)</td>
<td>39(8)</td>
<td>2.1</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>5.6 (0.7)</td>
<td>5.7 (0.7)</td>
<td>2.1</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.1 (1.5)</td>
<td>4.6 (1.4)</td>
<td>0.30</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>2.8 (1.1)</td>
<td>3.32 (1.0)</td>
<td>0.39</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.2 (0.6)</td>
<td>1.1 (0.3)</td>
<td>1.19</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Baseline Mean (SD)</td>
<td>6 Weeks Mean (SD)</td>
<td>Change from Baseline Effect of Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------------</td>
<td>-------------------</td>
<td>------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CDR (n= 16)</td>
<td>IF (n = 16)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol:HDL ratio</td>
<td>4.6 (1.5)</td>
<td>5.0 (1.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.2 (1.2)</td>
<td>4.5 (1.0)</td>
<td>0.28</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.1 (1.4)</td>
<td>1.9 (1.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4 (0.9)</td>
<td>1.5 (0.7)</td>
<td>0.27</td>
<td>0.61</td>
<td></td>
</tr>
</tbody>
</table>
7.6.3 Adaptive Thermogenesis

The general linear mixed models are presented in Table 7.3. Model one contained the time by treatment interaction. This was not significant and thus dropped for model two. There was minimal loss of fit (Table 7.3) with removal of the interaction terms. Subjects, incorporated as random effects, had a SD of 760 kJ/24 hours in REE and a residual of 335 kJ/24 hours indicating significant between subject variation not accounted for by fixed effects in model one. Thus, subjects were retained as a random effect for model two. Plots of the standardized residuals for both models are presented in Fig. 7.2

**Fig 7.2**: GLM Model Fit with (model 1) and without (model 2) the time by treatment interaction
Table 7.3: General linear mixed models for 24 hr REE.

<table>
<thead>
<tr>
<th>Model 1</th>
<th>Intercept</th>
<th>Estimate (95% CI)</th>
<th>SEE</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat Free Mass</td>
<td></td>
<td>14.7</td>
<td>4.5</td>
<td>3.2</td>
<td>0.002</td>
</tr>
<tr>
<td>Fat Mass</td>
<td></td>
<td>14.0</td>
<td>3.1</td>
<td>4.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IF</td>
<td></td>
<td>-25.1</td>
<td>70.4</td>
<td>-0.4</td>
<td>0.723</td>
</tr>
<tr>
<td>Visit 2</td>
<td></td>
<td>-67.7</td>
<td>30.2</td>
<td>-2.24</td>
<td>0.0293</td>
</tr>
<tr>
<td>Visit 3</td>
<td></td>
<td>-8.6</td>
<td>31.5</td>
<td>-0.27</td>
<td>0.785</td>
</tr>
<tr>
<td>IF:Visit 2</td>
<td></td>
<td>30.5</td>
<td>41.9</td>
<td>0.73</td>
<td>0.470</td>
</tr>
<tr>
<td>IF:Visit 3</td>
<td></td>
<td>-12.5</td>
<td>43.2</td>
<td>-0.29</td>
<td>0.774</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Model 2</th>
<th>Intercept</th>
<th>Estimate (95% CI)</th>
<th>SEE</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat Free Mass</td>
<td></td>
<td>15.2</td>
<td>4.5</td>
<td>3.4</td>
<td>0.001</td>
</tr>
<tr>
<td>Fat Mass</td>
<td></td>
<td>13.2</td>
<td>3.0</td>
<td>4.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IF</td>
<td></td>
<td>-27.2</td>
<td>66</td>
<td>-0.4</td>
<td>0.684</td>
</tr>
<tr>
<td>Visit 2</td>
<td></td>
<td>-53.0</td>
<td>21.7</td>
<td>-2.4</td>
<td>0.0179</td>
</tr>
<tr>
<td>Visit 3</td>
<td></td>
<td>-13.4</td>
<td>26.5</td>
<td>-0.5</td>
<td>0.616</td>
</tr>
</tbody>
</table>
Adherence to Dietary Prescription

Food diaries were completed and returned by 91%, 84% and 63% of participants at weeks one, three and six respectively. Across all timepoints the average return was 77% in the CDR group and 81% in the IF group. A total of 323 days of food diary entries were collected and analysed, 168 in the IF group and 155 in the CDR group. In the CDR group, the average recorded energy intake as a proportion of estimated requirements was 68% across the whole study period, compared with a diet prescription of 80%. In the IF group, non-fasting day energy intake was 74% of requirements rather than 100%, and fasting day intake was 30% rather than the prescribed 25% requirements. Averaged over the week, the mean proportionate energy intake in the IF group was 61% compared with the 80% prescribed. Food record returns and energy intake at each timepoint are presented in table 7.4.

Table 7.4: Food Diary Returns and Energy Intake as a Proportion of Estimated Requirements

<table>
<thead>
<tr>
<th></th>
<th>Week 1</th>
<th>Week 3</th>
<th>Week 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Returns (%)</td>
<td>EI (SD) (%)</td>
<td>Returns (%)</td>
</tr>
<tr>
<td>CDR</td>
<td>100</td>
<td>62 (24)</td>
<td>75</td>
</tr>
<tr>
<td>IF&lt;sub&gt;Avg&lt;/sub&gt;</td>
<td>81</td>
<td>58 (23)</td>
<td>94</td>
</tr>
<tr>
<td>IF&lt;sub&gt;F&lt;/sub&gt;</td>
<td>81</td>
<td>30 (8)</td>
<td>94</td>
</tr>
<tr>
<td>IF&lt;sub&gt;NF&lt;/sub&gt;</td>
<td>81</td>
<td>69 (17)</td>
<td>94</td>
</tr>
</tbody>
</table>

IF group non-fasting day (IF<sub>NF</sub>); IF group fasting day (IF<sub>F</sub>); IF group average weekly intake (IF<sub>Avg</sub>). All IF recordings contained both fasting and non-fasting day entries. The target calorie intake for CDR and IF<sub>Avg</sub> was 80%.
7.6.5 Difference in Macronutrient Composition at 1, 3 and 6 weeks

The proportion of daily energy intake consumed as fat was lower in the IF group at week one, 22% (SD: 7%), compared with the CDR group 28% (SD: 4%). This difference in macronutrient intake was driven by fasting day fat intake of 19% (SD: 7%) of total energy, with no significant difference between the IF non-fasting macronutrient intake, 25% (SD: 5%) of total energy, compared to the CDR group, 28% (SD: 4%) of total energy intake. Macronutrient intakes were not different between groups at any other timepoint (table 7.5). However, there was a trend toward lower fat and higher protein intake in the IF group compared with the CDR group at all timepoints, particularly on fasting days. Protein fell below the recommended intake of 20% only in the CDR group, at week three only.

7.6.6 Intra-recording variability in REE, VO2, VCO2, RQ.

As a marker of the quality of calorimetry recordings, the intra-recording variability was calculated, for REE, VO2, VCO2 and RQ. The SD expressed as a percentage of the mean was averaged across all recordings as a marker of variability, as outlined in chapter four, section 4.4.2.2. The mean SDs for REE, VO2, VCO2 and RQ respectively were; 5.6%, 5.5%, 7.0% and 3.5%.
Table 7.5: Difference in macronutrient composition at one, three and six weeks.

<table>
<thead>
<tr>
<th></th>
<th>CDR</th>
<th>IF\textsubscript{NF}</th>
<th>t</th>
<th>p-value</th>
<th>IF\textsubscript{F}</th>
<th>t</th>
<th>p-value</th>
<th>IF\textsubscript{AVG}</th>
<th>t</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Week 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Protein (%)</strong></td>
<td>20</td>
<td>20</td>
<td>0.07</td>
<td>0.941</td>
<td>23</td>
<td>-1.3</td>
<td>0.227</td>
<td>22</td>
<td>-0.8</td>
<td>0.433</td>
</tr>
<tr>
<td><strong>Fat (%)</strong></td>
<td>28</td>
<td>25</td>
<td>1.4</td>
<td>0.183</td>
<td>19</td>
<td>3.5</td>
<td>0.004</td>
<td>22</td>
<td>3.0</td>
<td>0.004</td>
</tr>
<tr>
<td><strong>Carbohydrate (%)</strong></td>
<td>42</td>
<td>45</td>
<td>-1.1</td>
<td>0.273</td>
<td>47</td>
<td>-1.3</td>
<td>0.232</td>
<td>46</td>
<td>-1.5</td>
<td>0.152</td>
</tr>
<tr>
<td><strong>Week 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Protein (%)</strong></td>
<td>18</td>
<td>21</td>
<td>-1.2</td>
<td>0.250</td>
<td>28</td>
<td>-2.4</td>
<td>0.032</td>
<td>24</td>
<td>-2.4</td>
<td>0.022</td>
</tr>
<tr>
<td><strong>Fat (%)</strong></td>
<td>28</td>
<td>27</td>
<td>0.8</td>
<td>0.450</td>
<td>24</td>
<td>1.6</td>
<td>0.120</td>
<td>25</td>
<td>1.4</td>
<td>0.165</td>
</tr>
<tr>
<td><strong>Carbohydrate (%)</strong></td>
<td>43</td>
<td>43</td>
<td>0.1</td>
<td>0.953</td>
<td>38</td>
<td>1.2</td>
<td>0.248</td>
<td>43</td>
<td>0.967</td>
<td>0.340</td>
</tr>
<tr>
<td><strong>Week 6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Protein (%)</strong></td>
<td>20</td>
<td>21</td>
<td>-0.2</td>
<td>0.836</td>
<td>24</td>
<td>-1.3</td>
<td>0.206</td>
<td>22</td>
<td>-1.0</td>
<td>0.333</td>
</tr>
<tr>
<td><strong>Fat (%)</strong></td>
<td>32</td>
<td>25</td>
<td>2.1</td>
<td>0.053</td>
<td>23</td>
<td>2.3</td>
<td>0.031</td>
<td>24</td>
<td>2.5</td>
<td>0.024</td>
</tr>
<tr>
<td><strong>Carbohydrate (%)</strong></td>
<td>38</td>
<td>44</td>
<td>1.8</td>
<td>0.07</td>
<td>42</td>
<td>-1.0</td>
<td>0.333</td>
<td>43</td>
<td>-1.6</td>
<td>0.120</td>
</tr>
</tbody>
</table>

The IF group diet composition is presented by non-fasting day (IF\textsubscript{NF}), fasting day (IF\textsubscript{F}) and average intake (IF\textsubscript{AVG}).
7.6.7 Changes in Weight and Body Composition

Changes in weight and body composition at visit one, two and three are presented in table 7.6.

7.6.7.1 Weight Change

At week three participants in the CDR and IF groups, participants lost 2.5 kg (SD:1.6 kg) and 4.1 kg (SD:1.8 kg), respectively. This was 2.3% (SD:1.4%) and 3.8% (SD:1.8%) of the baseline weight.

By week six participants in the CDR group lost 4.4kg (SD: 2.5kg) and in the IF group 5.9kg (SD:2.2kg). This represented 4.2% (SD: 2.2%) and 5.6% (SD: 2.0%) of baseline weight respectively.

7.6.7.2 Body Composition Change

At three weeks in the CDR group FFM loss was 0.9 kg (SD: 2.1 kg), and FM loss was 1.0 kg (SD:2.8 kg), a change of 1.4% (SD: 3.0%) and 2.7% (SD: 6.8%), respectively. In the IF group FFM loss was 2.4kg (SD: 1.8kg) and the FM loss was 2.4 kg (SD: 1.8kg), a change of 2.3% (SD: 2.5%) and 6.3% (SD: 4.5%), respectively.

At six weeks in the CDR arm FFM loss was 2.3 kg (SD: 1.8kg) and FM loss was 1.8 kg (SD: 2.4), representing a change of 3.3% (SD: 2.7%) and 5.1% (SD:5.4%) respectively. In the IF arm, FFM fell by 2.4 kg (SD:0.89 kg); and FM by 3.6 kg (SD: 2.0 kg) reflected a change of 3.6% (SD:1.5%) and 9.4% (SD:5.3%), respectively.

There was a significant effect of treatment on FM loss ($\beta$: -1.73kg; 95% CI: -3.4 to –0.11kg, p= 0.04), but not FFM or overall weight loss (Table 7.2).
Table 7.6: Weight, fat free mass and fat mass loss relative to baseline.

<table>
<thead>
<tr>
<th></th>
<th>Week 3 kg (SD)</th>
<th>Week 3 % (SD)</th>
<th>Week 6 kg (SD)</th>
<th>Week 6 % (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>CDR 2.5 (1.6)</td>
<td>2.3 (1.4)</td>
<td>4.4 (2.5)</td>
<td>4.2 (2.2)</td>
</tr>
<tr>
<td></td>
<td>IF 4.1 (1.8)</td>
<td>3.8 (1.8)</td>
<td>5.9 (2.2)</td>
<td>5.6 (2.0)</td>
</tr>
<tr>
<td>Fat Free Mass</td>
<td>CDR 0.9 (2.1)</td>
<td>1.4 (3.0)</td>
<td>2.3 (1.8)</td>
<td>3.3 (2.7)</td>
</tr>
<tr>
<td></td>
<td>IF 2.4 (1.8)</td>
<td>2.3 (2.5)</td>
<td>2.4 (0.89)</td>
<td>3.6 (1.5)</td>
</tr>
<tr>
<td>Fat Mass</td>
<td>CDR 1.0 (2.8)</td>
<td>2.7 (6.8)</td>
<td>1.8 (2.4)</td>
<td>5.1 (5.4)</td>
</tr>
<tr>
<td></td>
<td>IF 2.4 (1.8)</td>
<td>6.3 (4.5)</td>
<td>3.6 (2.0)</td>
<td>9.4 (5.3)</td>
</tr>
</tbody>
</table>

7.6.8 Change in Cardiovascular Risk Factors

7.6.8.1 Lipids

Changes in lipids are presented in Table 7.2. There was no statistically significant effect of the diet intervention on lipid profile.

7.6.8.2 HbA1C

HbA1C rose by 0.02 mmol/mol (SD: 0.11 mmol/mol) in the CDR group and decreased by 0.03 mmol/mol (SD: 0.18 mmol/mol) in the IF group.

There was no statistically or clinically significant effects of the diet intervention on HbA1c or lipid profile (Table 7.2).
7.6.8.3 Blood Pressure

There was no significant effect of IF, compared with CDR on the change in either systolic or diastolic blood pressure (Table 7.2).

7.6.9 Changes in REE measured following a Fasting versus Non-Fasting Days

In the IF group, 15 of 16 participants completed the fourth study visit. Unfortunately, two participants did not observe fasting day calorie intake during the day prior to this visit and these were excluded from the analysis. REE following a non-fasting day (M: 7954 kJ/24hr; SD: 1234 kJ/24hr) was not significantly different from that following a fasting day (M: 8096 kJ/24hr; SD: 1360 kJ/24hr): (M: 67 kJ/24hrs; 95%CI: -201 to 343 kJ/24hours; t: 0.55; p = 0.59). Given the small sample size (n=13) and minimal observed difference between groups, a post hoc sensitivity analysis was conducted in G*Power (433). With a difference in REE of 1.6% between consecutive measurements, such as we observed, the power was estimated to be ~20%, indicating an unacceptably high risk of type II error. However, when a 5% difference was chosen as the minimal clinically relevant difference between REE measurements, there was >80% power to detect a change of this magnitude.

7.6.10 Changes in Adult Eating Behaviour Questionnaire

The effect of treatment group on each of the seven eating behaviour domains is presented in table 7.7. Possible domain scores vary on a five-point scale from -2, strongly disagree to +2, strongly agree, and reflect agreement with statements relating to the eating behaviour domain. There was no significant effect of diet intervention on eating behaviour. Changes in eating behaviour changed little over the six-week diet intervention period.

7.6.11 Changes in Physical Activity Levels

At baseline there was no difference between physical activity energy expenditure in the CDR group (M: 7%, SD: 7%) and the IF group (M: 3%, SD: 5%). This was also evident at visit two, CDR (M: 6%, SD: 6%), IF (M: 4%, SD: 7%) (t = 1.0, p = 0.316); and visit three, CDR (M: 8%, SD: 8%), IF (M: 5%, SD: 5%), (t = 1.4, p = 0.18).
Table 7.7: Scores by treatment group from the Adult Eating Behaviour Questionnaire

<table>
<thead>
<tr>
<th></th>
<th>Baseline Mean (SD)</th>
<th>6 Weeks Mean (SD)</th>
<th>Change from Baseline Effect of Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CDR (n= 16)</td>
<td>IF (n = 16)</td>
<td></td>
</tr>
<tr>
<td>Enjoyment of Food</td>
<td>1.5 (0.5)</td>
<td>1.3 (0.6)</td>
<td></td>
</tr>
<tr>
<td>Emotional Over-Eating</td>
<td>0.1 (1.1)</td>
<td>-0.3 (0.9)</td>
<td></td>
</tr>
<tr>
<td>Emotional Under-Eating</td>
<td>-0.8 (0.5)</td>
<td>-0.8 (0.6)</td>
<td></td>
</tr>
<tr>
<td>Food Fussiness</td>
<td>-1.2 (0.6)</td>
<td>-0.9 (0.6)</td>
<td></td>
</tr>
<tr>
<td>Food Responsiveness</td>
<td>0.2 (0.6)</td>
<td>0.2 (0.6)</td>
<td></td>
</tr>
<tr>
<td>Slowness in Eating</td>
<td>-0.8 (0.9)</td>
<td>-0.4 (0.9)</td>
<td></td>
</tr>
<tr>
<td>Hunger</td>
<td>-0.3 (0.7)</td>
<td>-0.3 (0.6)</td>
<td></td>
</tr>
<tr>
<td>Satiety Responsiveness</td>
<td>-1.2 (0.5)</td>
<td>-0.9 (0.6)</td>
<td></td>
</tr>
</tbody>
</table>

F-value and p-value for each comparison.
7.7 Discussion

The CREEDS study, was designed to investigate the hypothesis that IF would reduce adaptive thermogenesis during weight loss, compared with a conventional continuous energy restricted dieting schedule, CDR. We found no significant effect of IF on adaptive thermogenesis. IF was associated with preferential FM loss, but no difference in cardiovascular risk factors after six weeks. Fat mass loss, and dietary fat intake at week one was greater with IF. No other differences were found in cardiovascular risk factors, adult eating behaviour, or physical activity levels between groups.

7.7.1 Adaptive thermogenesis

Adaptive thermogenesis was assessed using a general linear mixed model. The advantage of such a model is that it incorporates body composition changes and repeated measures of REE while enabling between group comparisons. In the context of this model adaptive thermogenesis is interpreted as the change in REE beyond that which is predicted by changes in body composition.

Visit two, occurring at three weeks was a significant predictor of REE after accounting for body composition change with estimated adaptive thermogenesis of -222 kJ/24 hrs (95%CI: -402 to -42 kJ/24 hours). While statistically significant this is a very small effect, accounting for <5% of mean REE observed at visit one. By visit three, the estimate of adaptive thermogenesis was a reduction in REE of – 54 kJ/ 24 hours.

The detection of adaptive thermogenesis is challenging and may be influenced by; the method used for allometric scaling, the assessment of body composition with weight loss, the magnitude of adaptive thermogenesis and the accuracy of REE measurements. These factors are discussed below. The achievement of weight loss, another determinant of adaptive thermogenesis, and an important outcome, independent of adaptive thermogenesis, is discussed separately in section 7.7.2.

7.7.1.1 Allometric Scaling of REE using Fat Free Mass and Fat Mass

As expected, FFM and FM were significant predictors of REE. The estimates for both FFM and FM were 63 kJ/kg (95%CI: 26 to 100 kJ/kg) and 54 kJ/kg (95% CI: 29 to 80 kJ/kg) respectively, with a regression intercept of 2059 kJ/day. While this differs slightly from the PREEMPt study estimates of 100 kJ/kg (95%CI: 75 to 121 kJ/kg) and -5 kJ/kg
(95%CI: -16 to 5) for FM and FFM, the 95% confidence intervals for the FFM estimates from both the CREEDS study and the PREEMPt study overlap. The effect of FM on REE is smaller than for FFM and may not have been detected in the smaller PREEMPt sample.

Lazzer et al, 2010, in a study of 7,368 obese Italian adults, used regression to model the relationship between FFM, FM and REE (351). They produced estimates for FFM of 50 kJ/kg and FM of 41 kJ/kg, with an intercept of 3,270 kJ/day. Thus, the FFM and FM estimates produced in the CREEDS study, while not identical, are in keeping with expected values for an obese cohort. This provides an additional measure of assurance regarding the validity of the DXA and indirect calorimetry methods used during the study.

7.7.1.2 Errors in Body Composition Assessment with Weight Loss

As outlined in chapter two, section 2.3.1.2, the accuracy of body composition analysis may be compromised during weight loss. Muller et al, 2012, suggest that balance methods should be used to assess the composition of weight loss as they are more accurate, particularly for smaller degrees of weight loss (337).

Should balance methods to measure body composition have been used for the CREEDS study?

There are several problems with using balance methods to assess body composition as it might apply to the CREEDS study, notwithstanding the challenges of using balance methods outlined previously. First, Yang et al, 1977, who performed one of the earliest analyses of various types of balance methods in the assessment of body composition during weight loss identified that the changes in ratio of water and nitrogen loss violate assumptions of balance methods, and suggested that they not be used during a period of dynamic weight change (335).

Secondly, the validity of balance methods is assumed in the studies of Muller and Jebb, rather than compared to a truly valid reference method. This is perhaps because no such method has been developed for assessing body composition during weight loss (334, 337). It is unlikely that balance methods are entirely unbiased or without random error. It follows that comparison of other body composition assessment methods will undoubtedly appear worse when balance methods are assumed as a reference criterion.
Thirdly, the studies of Jebb and Muller assessed FM change only, not whole-body composition. For the CREEDS study a measure of whole body composition is required to scale REE. Muller et al suggest using a combination of balance methods and an alternate body composition method to circumvent this issue, but this would mean waiting for weight stabilisation to measure both REE and body composition. As the presumed biological basis for adaptive thermogenesis is conservation of energy during a period of weight loss, what is required is a method of measuring body composition that is valid in this dynamic phase.

Fourth, the rapid weight loss induced in these studies, approximately three kilograms in one week, is likely to produce a greater flux in water, protein and electrolytes than the weight loss observed in the CREEDS study in which a mean of three kilograms was lost over three weeks. Finally, though Muller and others’ data indicate that the standard error of the measurements using balance methods is less than in-vivo methods, the sample sizes for these studies are very small with six participants in two studies (334, 335) and 10 participants in a third (337), which limit the generalizability of their findings.

Assessment of Body Composition During Weight Loss in the CREEDS Study

In the CREEDS study, a total of 96 DXA scans were conducted over nine months and the single operator precision was evaluated using the DXA scanner at our test lab as discussed in chapter two, section 2.4.2.2. For reasons of cost, time and manpower this would not have been possible with balance methods. This does leave the question of the validity of DXA measurements during weight loss. As discussed in section 2.3.1.2, the validity of DXA is compromised during weight loss. However, it is not clear precisely how significant an impact this might have on the estimate of adaptive thermogenesis at visit two and visit three. The least significant change for obese participants assessed by the DXA operator and machine used for the CREEDS study was 1.27% for FFM and 2.19% FM during weight stability. By comparison, the smallest observed changes in the CREEDS study were 1.4% for FFM and 2.7% for FM, both in the CDR arm. Therefore, if dynamic weight change resulted in a modest additional loss of precision it may not have compromised the accuracy of the weight loss detected. However, an increase in the LSC of 25% or more would certainly have had an impact on the accuracy of the detected body composition change.
The additional loss of precision accompanying weight loss may have compromised the body composition assessment at visit two. However, it is less likely that it had an impact at visit three given the minimum FM and FFM changes were approximately three-fold greater than the LSC values for the method established during weight stability. The ANCOVA for the effect of treatment on body composition incorporated baseline and visit three values only, not visit two. However, the primary outcome analysis did incorporate repeated measures at baseline, visit two and three, and so may have been affected by the presumed loss of precision with dynamic weight change.

Using a mixed design, the analysis of adaptive thermogenesis allowed for estimates in the relationship between FFM and FM and REE, across visits, while assessing the treatment effect between groups. While this approach doesn’t solve the question of whether the reduction in REE at visit two was due to adaptive thermogenesis or errors in body composition assessment with weight loss, it allows us to answer the question of whether IF had any bearing on this observed effect. As weight loss was similar in both groups and occurred at a similar rate, it is expected that any error in body composition assessment during weight loss would have been similar between groups, and any difference between groups would have been due to adaptive thermogenesis.

### 7.7.1.3 The Magnitude of Adaptive Thermogenesis

Assuming the reduction in REE compared to that expected for the change in body composition was due to adaptive thermogenesis and not the assessment of body composition, how do these findings compare with other similar studies of adaptive thermogenesis? Liebel *et al.*, 1995, reported a 10-15% percent reduction in REE with controlled underfeeding to 90% of initial body weight over six to 14 weeks, in 17 participants in an inpatient setting (117). As outlined in chapter one, Prentice *et al.*, 2014, estimated adaptive thermogenesis of 5-25% in response to 'slimming diets'(103). Though only one study directly compared a VLCD with less severe forms of dietary energy restriction, the authors noted greater adaptive thermogenesis, measured REE less than 15% predicted, in studies with a daily energy intake of under 3000 kJ compared to adaptive thermogenesis less than 5% REE in studies with a prescribed energy intake of under 5000kJ.

Several studies have reported changes in adaptive thermogenesis comparable to those seen in the CREEDS study with comparable weight loss. In the CALERIE-2 study, a
25% restricted low-calorie diet compared to a non-dieting control resulted in a significant 377 kJ/24 hr reduction in REE by six months (454). In the MATADOR study, participants were randomised to either low-calorie diet alternating with isocaloric intake in eight two-week blocks or matched continuous calorie restriction. By four weeks, the drop in REE below predicted, i.e. adaptive thermogenesis, was ~ 105 kJ in the continuous group and ~ 209 kJ in the IF group (Fig 3, Panel C and D), this was even less by eight weeks. Adaptive thermogenesis was not statistically significant at either timepoint (295). The rate of weight loss for this study, 7-10 kg over 16 weeks, compared to the CREEDS study with weight loss of 4.5-6 kg over six weeks. In the Biosphere 2 study, normal and overweight participants were underfed 10% of their habitual intake and achieved a weight loss of 9.1 kg over two years (455). By this time there was no significant adaptive thermogenesis detected, with a mean reduction in SEE of -573 kJ (SD: 1276 kJ), the study sample was comprised of eight individuals, which may account for these results. Camps et al, 2013, detected a difference of 4% in predicted versus measured REE after an average weight loss of 9.6kg (SD 4.1 kg) achieved with a VLCD over eight weeks in 91 individuals (456).

In practical terms these are small differences, between 3 – 5% of daily REE, though this may be sufficient during gradual sustained weight loss, to have a significant clinical impact on weight loss over months.

7.7.1.4 The Quality of Indirect Calorimetry Recordings

As discussed in chapter four, the assessment of human indirect calorimetry recording quality can be challenging as there is no reference criterion against which to evaluate the method (section 4.4.2.1). Haughen et al, 2003, have previously used an intra-recording variability of 15% as an indicator of quality of REE recordings (420). An acceptable threshold of 10% was suggested based on the low intra recording variability observed during the second test-retest reliability study. In the CREEDS study the mean intra-recording variability for REE was well below this threshold, at 5.6%.

7.7.1.5 IF Does Not Attenuate Adaptive Thermogenesis

In the final model, IF was associated with a mean reduction in REE of 113 kJ/day (95%CI: -674 to + 745 kJ/day) compared to CDR and was thus not clinically significant. The CREEDS study is the only study of adaptive thermogenesis using this schedule of
IF. As outlined in chapter one, there have been several studies of various intermittent schedules of fasting versus CDR however only one study prior to the CREEDS study IF vs CDR using calorie matched controls with calorie prescriptions based on individually calculated energy requirements.

The MATADOR study was published in February 2018, after completion of the CREEDS study. It was a 16-week RCT of CDR versus IF in obese men. The IF group was structured as two weeks of energy balance alternating with two weeks of calorie restriction. The net energy intake in both groups was 67% as compared with 79% in the CREEDS study. Body composition analysis was performed by air displacement plethysmography and REE by indirect calorimetry. Diet prescriptions were based on REE multiplied by physical activity level, as in the CREEDS study. The authors found that IF was associated with a 377kJ reduction compared to the continuous restriction group. They concluded that IF attenuated the reduction in REE seen with fasting and suggested,

“... these findings provide preliminary support for the model as a superior alternative to continuous ER”

The MATADOR study addresses many of the limitations with studies of IF vs CDR to date (chapter one, section 1.6.3.4.7) however there are some significant remaining limitations. First, though participants were randomised to the treatment group, allometric scaling was done separately for each group at baseline or using an equation developed in a separate population of obese men. There was not a substantial difference between these two methods. However, using separate regression equations for the randomised groups at baseline risked introducing significant bias as the method used for calculating predicted REE, and thus adaptive thermogenesis, was different before the intervention even began. The use of a regression equation developed in a separate population also raises legitimate concerns regarding the validity of the estimates, as outlined in chapter five.

Secondly, the authors performed repeated measures at four, eight, twelve and sixteen weeks. The difference in adaptive thermogenesis was only significant at 16 weeks. Prior to this it appeared that IF produced greater adaptive thermogenesis (non-significant), a trend which was reversed between week 12 and 16 weeks. As the superiority of IF in
attenuating adaptive thermogenesis was offered as an explanation for superior weight loss, one would expect that the attenuation of adaptive thermogenesis ought to have been present throughout the study, not simply at the final timepoint.

Thirdly, IF participants were issued scales and contacted during the weight maintenance periods by phone. They were advised to alter their diet if they were losing or gaining weight consistently for three days. While energy requirements do vary during a diet intervention study, the primary outcome for the MATADOR study was weight loss. A procedure that directly interfered with weight loss or gain depending on the response of the individual during the intervention was another source of significant bias.

Finally, there was no measurement of adherence to diet prescription performed in the MATADOR study, despite participants completing diet records.

Thus, based on the findings of both the MATADOR study and the CREEDS there does not appear to be compelling evidence of a significant effect of IF on adaptive thermogenesis.

7.7.2 Significant Weight Loss Was Achieved with IF and CDR.

Weight loss in both groups of the study was approximately 5% over the six weeks intervention period. Based on self-reported dietary intake, participants in both groups under-ate relative to the diet prescription, however they remained well matched in terms of net calorie intake with a mean 7% deficit in the IF group compared with the CDR group.

Thomas et al, 2011, have developed a tool that enables prediction of expected weight loss during diet intervention studies (457). The model predicts REE based on age, gender and anthropometry; assumes mild to moderate physical activity; contains an estimate of DIT, and incorporates a parameter, α, to model adaptive thermogenesis. The model was developed from obese North American participants. The weight loss predicted by this model for each participant was computed and compared to the observed weight loss in the CREEDS study. Assuming adherence to a 79% calorie restriction, the mean weight loss predicted for the entire study cohort was 5.76kg (SD: 1.37kg). In the event, the observed weight loss was 5.27 kg (SD: 2.4kg), only 0.5kg less (t = 1.12, p = 0.27). Bearing in mind the reported energy intake was not 79% but 65% on average across the
entire cohort, this suggests that underreporting of intake probably did occur, but that participant energy intake was close to the prescribed target of 79%.

7.7.3 Preferential Fat Mass Loss was Observed with Intermittent Fasting

Of the secondary outcomes assessed during the CREEDS study, the preferential FM loss with IF was the chief significant finding. While the CREEDS study was not powered to detect differences in FM, the two-fold greater FM loss in the IF group compared to the CDR group, is an interesting one. Baseline FM and weight were similar between groups (table 7.2).

The challenges in assessment of body composition during weight loss are obviously relevant to a correct interpretation of the preferential FM loss with IF. However, only the difference between visit three and visit one was used to assess body composition change, and the difference in FM loss between groups relative was sufficiently large to make it unlikely, albeit not impossible, that the observed difference was attributable to measurement error alone.

How can this observed preferential loss of FM with IF be explained? Differences in body composition during weight loss may be related to the extent of weight loss, the macronutrient content of the diet, the degree of physical activity or the schedule of energy restriction.

7.7.3.1 Did the Rate of Weight Loss Affect Fat Mass Loss?

Heymsfield et al, 2012, have described phases of weight loss during a calorie restricted diet associated with different body composition loss (458). The early phase of weight loss is associated with greater loss from the FFM compartment compare, between 6-24 weeks of a hypocaloric diet, there appears to be a greater contribution of FM to the weight lost. It is possible that the IF group, having lost more weight at week three and week six, were losing more FM than the CDR group because of this effect. However, the weight loss difference was 0.5 kg between groups and seems insufficient to explain a 1.8kg difference in FM loss, particularly as the difference in FM loss with the IF group was evident by week three (table 7.6).
7.7.3.2 Did Macronutrient Composition of the Diet Affect Body Composition?

The POUNDS LOST Study assessed four calorie restricted diets of different macronutrient composition on changes in body composition with weight loss by six months and two years in 424 overweight or obese men and women (459). The diets were low fat/average-protein, low-fat/high-protein, high-fat/average protein and high-fat/high-protein. There was no effect of diet on FM loss found. By contrast Clifton et al, 2014, performed recent systematic review and meta-analysis on the effect of low-carbohydrate/high protein diets on body composition change (460). No fixed protein or carbohydrate thresholds were set as inclusion criteria, the protein content needed to be higher, and the carbohydrate lower than the control group. Studies were a minimum one year in duration. The high protein/low carbohydrate diet was associated with an additional reduction of 0.44 kg in FM compared with the control group. This dropped to 0.3kg if the difference in protein was <5% by the end of the intervention. In the CREEDS study, the only significant difference in macronutrient composition was a higher fat intake at week one in the CDR arm. Therefore, it is not likely that macronutrient intake accounted for the preferential FM reduction seen during the study.

7.7.3.3 Did Changes in Physical Activity Affect Body Composition?

Physical activity reported in the study was very low as a proportion of total daily energy expenditure. In the CREEDS study, two participants at visit one, three participants at visit two and no participants at visit three had a daily physical activity energy expenditure greater than 2000 kJ/day. More importantly, there were no significant differences in physical activity between groups to explain the difference in body composition.

7.7.3.4 Does the Intermittent Fasting Schedule Affect Body Composition?

As outlined in chapter one (section 1.6.3.4.7.7) body composition changes have been assessed during several intermittent schedules of fasting. In the MATADOR study, the IF group lost an extra 2.7 kg FM compared with the CDR group. The weight difference between groups was 3.5 kg in that study, and the proportion of weight lost as fat was >80% in both groups. The excess weight loss alone may account for the extra FM lost in the IF group. As percentage FM lost was not reported, this cannot be ruled out. By contrast in the CREEDS study the weight loss difference was only 0.5kg greater in the IF group, while the difference in FM loss was 1.8 kg between groups. Nonetheless given the similarity of the MATADOR study population, the diet prescription and the body
composition outcomes, it does raise the possibility of the schedule of fasting having an
effect on change in body composition.

Harvie et al., 2011, using a similar schedule of intermittent energy restriction as the
CREEDS study found no difference in the change in body composition between groups
(289). The diet prescription in this study was 25% on fasting days and ad-libitum
otherwise. Thus, the relative calorie deficit between groups may have been less than in
the CREEDS study. Furthermore, the body composition method used was BIA which
may underestimate changes in FM with weight loss in obese individuals (461).

In a second study by Harvie et al., 2013, IF combined with carbohydrate restriction was
associated with a reduction in FM, also assessed by BIA. This was not observed when IF
was combined with protein and fat restriction or in the calorie-matched CDR control
(287). This suggests that macronutrient intake rather than the fasting schedule may have
had a bearing on composition of weight loss. However, as outlined above, the observed
differences in macronutrient composition between groups in the CREEDS study were
small and not sustained throughout the six-week intervention.

Arguin et al., 2012, detected a reduction in FFM, assessed by DXA, in obese women
undergoing IF compared with CDR. In this study IF consisted of five-week blocks of
weight loss, alternating with five weeks of weight stabilization. The calorie prescription
in this study was adjusted according to weight loss. Thus, both groups were not calorie
matched which may have confounded these findings (294). Arguin and Harvie’s studies
were conducted in women only, thus there may be a gender-specific of IF on body
composition.

Though a secondary outcome, the preferential loss of FM in the IF arm may represent an
ancillary benefit with this type of fasting. However, the finding does need to be confirmed
in a longer-term study, designed and powered to assess body composition change as the
primary outcome. An examination of the gender specific effects of IF on body
composition would be of great interest in such a study.

7.7.4 A Fast Day Prior to Indirect Calorimetry does not Affect REE

To establish if the preceding days energy intake would affect the indirect calorimetry
precision for the IF arm, we studied participants on consecutive days. The mean
difference between measurements was almost negligible at 67 kJ/day. Not only does this
alleviate any concerns about the impact of the intervention on precision, it reproduced the high level of precision that was demonstrated in the second test retest reliability study (chapter four). This finding will be of use when planning future studies of energy expenditure during IF.

7.8 Conclusion

In conclusion, we conducted a randomised controlled trial of IF, consisting of a five-day isocaloric and two-day hypocaloric diet compared with seven days of continuous restriction, with matched total energy prescription between groups. While other authors have investigating this schedule of IF previously, this is the first study that has looked at adaptive thermogenesis using this schedule. Using this schedule, IF does not appear to attenuate adaptive thermogenesis compared with CDR after three or six weeks. We had good adherence, high participant retention rates and significant weight loss. The magnitude of adaptive thermogenesis observed was smaller than expected in this study and this may have reduced our ability to detect a difference in the mitigation of adaptive thermogenesis between groups. Fasting the day before an indirect calorimetry recording was found not to affect REE measurements. IF was associated with preferential FM reduction after six weeks. This observation warrants confirmation with an appropriately powered larger study including women and over a longer period.
8 Discussion

8.1 Evolution of the Work Presented

The principle area of interest of our research group is in the association between obesity and T2DM. This thesis was conceived to further understand the mechanisms that mediate the disruption of glucose metabolism in obesity and how these can be reversed with weight loss.

Bariatric surgery is one model for understanding these mechanisms and has been studied for over 25 years as a model for weight loss and improvement in glycaemia. This literature has been very fruitful, contributing to a greater understanding of gut peptide physiology, intestinal microbiota, bile acid metabolism and diabetes pathophysiology. However, most studies investigating the mechanisms of diabetes remission in the bariatric literature do not control for calorie restriction.

Dietary calorie restriction offers another model for the study of mechanism of diabetes remission, however prescribed weight loss in diet intervention studies does not always result in the expected weight loss. Upon reviewing the reasons for this, it became clear that the occurrence of adaptive thermogenesis during weight loss was a contributing factor to this failure to lose weight and is incompletely understood. This led to the hypothesis that adaptive thermogenesis may be triggered by the degree or pattern of energy restriction and that altering the schedule of intake may mitigate adaptive thermogenesis. The intermittent fasting schedule was appealing both as a research tool, to investigate the regulation of metabolism and energy expenditure, and potentially as a diet intervention. However, there were only a handful of studies in humans and many of these had limitations, particularly with the prescription of dietary energy. The Changes in Resting Energy Expenditure with Different Schedules of calorie restriction (CREEDS) study was planned to address these limitations. To conduct such a study, reference methods for measurement of body composition and energy expenditure are required. Our research group was instrumental in the collaborative effort to fund and install an indirect calorimeter and DXA scanner, which coincided with the conception of this thesis. Before the CREEDS study could be conducted, several methodological issues with these tools and protocols needed to be addressed. This then became an integral component of the methodological development of this thesis.
8.2 Overview of the Thesis

The principal aim of this thesis was to evaluate the effect of intermittent fasting on adaptive thermogenesis during weight loss. To achieve this, I required the ability to accurately and precisely measure energy expenditure and body composition. First, I developed and tested the validity of the indirect calorimetry protocols as described in chapter three. In chapter four I assessed the test-retest reliability of the indirect calorimeter and DXA protocols in human subjects. In chapter five I investigated the relationship between body composition and REE in an obese cohort using the methods that I had validated in chapter two. This allowed me to plan the sample size for the CREEDS study and provided preliminary data on the validity of the assumptions of commonly used predictive energy equations. To determine if intermittent fasting could be studied safely and accurately in individuals with T2DM, it was necessary to assess the risk of hypoglycaemia with intermittent fasting in individuals taking oral hypoglycaemic medications, as described in chapter six. Chapter seven describes the final CREEDS study, that investigated the effects of intermittent fasting compared with continuous daily restriction on adaptive thermogenesis.

This provided the framework for the studies that form this thesis. The first test-retest reliability recordings of the new indirect calorimetry system exhibited an intra-recording variability in REE of 20.1%. This was clearly unacceptable and raised concerns about the validity of the indirect calorimetry recordings.

Because of the first test-retest reliability study, the indirect calorimetry measurement protocols were refined. Between the first test-retest reliability study and the later accurate nitrogen validation studies lay a steep learning curve. This was particularly true in the absence of on-site expertise or a user manual for the Promethion. I needed to become familiar with all aspects of the indirect calorimetry technique to identify sources of error. Much of the technical knowledge in chapters two and three, and the appendix were acquired during this period of protocol refinement.

Prior to testing the new protocols in human participants, in-silico studies with nitrogen dilution and ethanol combustion were performed. It was unclear to me which method would be superior in the assessment of validity, so I performed both. The consistency of the ethanol burn was inferior to that of the nitrogen validation study and the large water
vapour production meant the ethanol burn was less physiological as an assessment of validity than the nitrogen dilution method. The nitrogen dilution results (section 3.3.5) demonstrated a mean O$_2$ recovery of 101%, with a high degree of consistency between dilution trials (Table 3.1). These results indicated that I could proceed to indirect calorimetry measurements in human participants, confident in the quality of my indirect calorimetry methods.

When I assessed the precision of indirect calorimetry in the second test-retest reliability studies it was similar to that of the first test-retest reliability study, but the quality of the measurements was superior, as indicated by an intra recording variability of 5.8%, compared to >20% in the first study. The intra class correlation coefficient was established as 0.98 and the least significant change in resting energy expenditure was established as 561 kJ (section 4.3.4) in the second test re-test reliability. This value for the least significant change was rather high, considering the precision represented by the intra class correlation coefficient measure and the precision of approximately 99%, from the nitrogen dilution study, and was due to the variance in resting energy expenditure in the cohort used to generate the least significant change value. The intra class correlation coefficient value, which is a ratio of within subject variation to within subject variance plus error, was a more useful marker in extrapolating the precision observed in the second test retest reliability study to the CREEDS study, as it is not population dependent. This was considered more than satisfactory as an indicator of the precision of the indirect calorimetry with the new protocol.

To generate sample size estimates for the CREEDS study using allometrically scaled resting energy expenditure, the Prediction of Resting Energy Expenditure in Māori and Pacific (PREEMPt) study (chapter five) was conducted. Based on the variance in resting energy expenditure observed in this study, a sample size of 32 was determined appropriate to detect a between group difference of 7% in the intermittent fasting group versus the continuous daily restriction group of the CREEDS study. There were some important differences in the populations studied for the PREEMPt and CREEDS studies. First, in the PREEMPt study the baseline REE was 7586 kJ/day, compared with 8437 kJ/day in the CREEDS study. This was due to a lower mean weight in the PREEMPt study, 101 kg, compared to 109 kg in the CREEDS study. Body composition was also different, the mean FM percentage in the PREEMPt study was higher at 40% compared with 35% in the CREEDS study. The difference in body composition between studies
was likely due to selection of men only for the CREEDS study but should not have affected the ability to detect a difference in adaptive thermogenesis in the CREEDS study.

Having formed the research question and determined the sample size required for the CREEDS study I also considered whether I would include participants with established T2DM on medication. The IF-Hypo study was conducted to determine the risk of hypoglycaemia with intermittent fasting in those with T2DM on hypoglycaemic medication, which was unknown up to that point. The IF-Hypo study demonstrated a small increased risk of hypoglycaemia despite a carefully constructed and tested protocol for medication adjustment. As the primary research question in CREEDS was not specifically about glucose metabolism, but rather adaptive thermogenesis, we therefore adopted a conservative approach in excluding participants on hypoglycaemic medications from the CREEDS study.

The final CREEDS study did not demonstrate a difference in adaptive thermogenesis during weight loss with intermittent fasting compared to continuous daily restriction but did identify a preferential reduction in FM of 3.6 kg in the intermittent fasting group compared to 1.8 kg in the continuous daily restriction group.

8.3 Strengths

A large component of this thesis was the development and assessment of indirect calorimetry methods of a high standard. We have demonstrated a high degree of precision and validity in our recordings and have developed a robust method for conducting indirect calorimetry at our centre. Knowing the accuracy and precision for both our DXA and indirect calorimetry methods, reassured us during the interpretation of the CREEDS study that the lower than anticipated adaptive thermogenesis was not due to measurement error.

A second strength of the work in this thesis was the use of local population variance in REE to predict sample size estimates for the CREEDS study. To the best of my knowledge, none of the previous studies of adaptive thermogenesis discussed in this thesis have performed sample size estimation based on allometric scaling of REE in a pilot sample.
An important strength of this thesis was the acknowledgment of uncertainty with allometric scaling of REE in both the PREEMPt and CREEDS study. Allometric scaling is superior to any alternative methods of normalising REE. However, it does come at a cost. The use of a regression model which incorporates FM or FFM will account for 60-70% of the variance in REE at best. The regression model used in the CREEDS study accounted for 66% of the variance in REE. The unexplained variance contributes to uncertainty in the estimate of the relationship between REE and body composition. This uncertainty must temper the interpretation of the presence and magnitude of adaptive thermogenesis.

This uncertainty in allometric scaling by regression has two implications. First, were an allometric scaling model to explain more of the variance in REE, e.g. by identifying additional covariates, or refining body composition analysis, it would greatly improve the ability to detect adaptive thermogenesis. The second implication is that calculation of adaptive thermogenesis is most valid when the error in the estimates of allometrically scaled REE are incorporated in the statistical assessment. These variances are incorporated in a linear mixed model such as that in the CREEDS study and were reflected in my sample size estimates produced by the PREEMPt study, when I incorporated the 95% confidence intervals of the root mean square error in the sample size calculations. Catenacci et al, 2003, used a similar statistical approach in a study of alternate day fasting versus continuous daily restriction. However, it is more common to find adaptive thermogenesis calculated by subtracting measured values of REE from predicted values of REE. This concept is illustrated in fig. 8.1 using data from the CREEDS study. The FM and FFM estimates obtained from regression of the entire cohort at baseline were compared to the CDR arm. The 95% confidence intervals for the estimates were calculated using the RMSE of the regression equation and plotted.

Were the regression model only used without any consideration of the error in the model, it would be easy to conclude that there was indeed adaptive thermogenesis identified. Examining the 95% confidence limits, this assertion needs to be seriously questioned. In the analysis that was conducted for the CREEDS study the linear mixed model controlled for inter-individual variations in REE, and individual estimates incorporated three measurements per participant in the model. This would have reduced the variance in the model, as shown by the statistically significant finding of adaptive thermogenesis at visit two, despite the inclusion of the variance in the regression estimates.
Fig 8.1 Estimating Adaptive Thermogenesis using Baseline Regression Values

Lines in red represent the 95% confidence intervals for the predicted REE.

8.4 Limitations

While the nitrogen dilution quality control testing that was completed as part of this thesis is a strength of our current calorimetry setup, it does not incorporate CO$_2$ gas flows or a fluctuating REE simulation such as that seen in human recordings. Ideally a second method such as doubly labelled water could be used to cross validate the indirect calorimetry measurements in obese subject recordings. Unfortunately, doubly labelled water isn’t currently available at our centre.
A second limitation of this work was the small degree of adaptive thermogenesis seen in the CREEDS study. This was below the 7% estimate that was used to power the study. One of the challenges with planning studies of adaptive thermogenesis is the wide range of published findings on the magnitude of the effect of adaptive thermogenesis. In planning the CREEDS study a trade-off between the power of the study and the cost, and thus feasibility of the study needed to be achieved. Dulloo et al., 2013, have summarised the challenges of measuring adaptive thermogenesis and suggest that it may be a theoretical rather than a measurable entity, a question that remains controversial (462). Many of the challenges with measuring adaptive thermogenesis raised in Dulloo’s paper such as the use of statistical models that incorporated inter-individual variability, and ensuring high levels of methodological precision and validity with body composition and calorimetry methods, were addressed in preparation for the CREEDS study. For the CREEDS study itself, we opted for conservative inclusion criteria such as male gender, the exclusion of individuals on hypoglycaemic medications, frequent contact, one to one diet education, and regular REE measurements to maximise our ability to detect adaptive thermogenesis. This approach appeared to pay off with good weight loss observed and excellent participant retention. A study of this intensity would not have been possible in the time available with the large cohort required to detect a difference of 2-3% in adaptive thermogenesis.

While the CREEDS study answered the question of whether there is likely to be an important impact of intermittent fasting on adaptive thermogenesis during weight loss in an outpatient setting, it revealed less about the time scale of regulation of adaptive thermogenesis or the effect of intermittent fasting on components of energy expenditure other than REE. While analysis of ghrelin, adiponectin, leptin, and thyroid hormones from the CREEDS study is planned, a more detailed study of intermittent fasting in an inpatient setting may provide different insights into the effects of intermittent fasting on metabolism. Such a study could include objectively measured dietary intake with whole room calorimetry, and examine change in diet induced thermogenesis, physical activity related energy expenditure and sleeping energy expenditure and may determine whether there are differences in intermittent fasting compared with conventional dietary restriction. This work would be challenging to accomplish due to; inter-individual variability in energy expenditure, increasing the sample size requirements; the requirements to be admitted for a week or more until weight loss is achieved.
8.5 Future Work

The work in this thesis has laid the groundwork for future studies of dietary energy balance and metabolism. During the PhD a total of 224 indirect calorimetry recordings were conducted, 164 of these had paired body composition scans. The foundation of a body composition and REE data archive would be a useful resource to advance the investigation of the relationship between body composition and energy expenditure and to develop predictions of resting energy expenditure in New Zealand populations and in Pacific and Māori ethnic groups, in particular.

Adaptive thermogenesis could be assessed using different schedules than the ones examined in this thesis. For example, rather than varying the energy intake over the week, it could be varied over a day with altered meal allocation, that vary on circadian time scales. Expression of circadian regulators, discussed briefly in (section 1.6.3.4.2), are entrained in mice. It would be of interest to see if genes involved in intermediary metabolism can be similarly entrained in humans to circadian variations in energy intake. It would also be of interest to see if adaptive thermogenesis during weight loss was altered by conditions of thyroid under- or over-activity.

The gas validation method should be extended to a dual gas analysis, by incorporating CO2 using a high precision gas mixer. An additional extension of the gas validation method would be to programme variable flows, not simply constant flow rates, to emulate the variation in REE that is seen in human participants to assess if this affects the validity of the recording. This application is possible with modification of our current software and to my knowledge has never previously been done.

There is the opportunity to analyse REE without relying exclusively on a mean REE value during a recording. One approach to this is to analyse REE measurements using deconvolution, the decomposition of waveforms into component frequencies. Changes in these components during exposure to different environmental conditions, in different disease states, or in response to different medications, may help to reveal the time scale over which REE is regulated. While deconvolution methods have been used in other fields they have not been applied in this way to canopy hood indirect calorimetry. Component frequencies common to individuals, in response to environmental stimuli,
medications or disease states, may offer insights into the regulation of basal metabolism that would otherwise be missed if the mean REE alone were used.

Using our current body composition and calorimetry methods we could examine the effect of weight loss on improvements in glycaemia with bariatric surgery compared to a calorie matched very low-calorie diet intervention. An accurately matched calorie prescription could be achieved using serial measures of resting energy expenditure in the bariatric cohort in the immediate post-operative period. Measurements of calorie requirements prior to the 14th post-operative day are not available in the published literature. A study of this type would clarify the relative contribution of a hypocaloric diet to the improvements in glycaemia following bariatric surgery.
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Glossary of Terms

**Accelerometry**: The measurement of movement. In energy balance studies this often involves measurement of limb movement to estimate physical activity related energy expenditure.

**Ad libitum intake**: Unrestricted Dietary energy intake

**Adaptive thermogenesis**: the reduction in REE, beyond that which is expected for the change in body composition, occurring during a period of dietary energy restriction

**Adipokine**: Hormones secreted by adipose tissue

**Allometry**: The study of the relationship between body size and other physiological measures.

**Anorexigenic**: Producing a decrease in appetite.

**Arcuate nucleus**: A nucleus of the hypothalamus that contains POMC and AgRP/NPY neurones involved in the regulates of appetite.

**Autocrine**: Relating to a cell-produced substance that has an effect on the cell by which it is secreted.

**Bariatric surgery**: Surgeries with the primary intention of promoting weight loss.

**Endocrine**: Relating to or denoting glands which secrete hormones or other products directly into the blood.

**Energy homeostasis**: The biological processes that match dietary energy intake to energy expenditure.

**Epigenetics**: the study of heritable changes in gene expression that are not due to changes in DNA sequence

**First-phase insulin response**: The early insulin response to a glucose load, occurring within minutes of nutrient ingestion.

**Glycated haemoglobin**: A form of haemoglobin that is used a marker of glycaemia.

**Gut microbiome**: The bacterial composition of the gut
**Histones**: Proteins that influence a gene’s folding structure

**Lipolysis**: The breakdown of fats and other lipids by hydrolysis to release fatty acids

**Locus** (pl. loci): the position of a gene or mutation on a chromosome.

**Macro**: A single instruction given to a computer that produces a set of instructions for the computer to perform a particular piece of work

**Metabolic equivalent**: the energy cost of physical activity expressed as a ratio to a standard energy expenditure of 4.184 kJ per kilo of body weight per hour of activity performed

**Non-exercise activity** thermogenesis: the energy expenditure of all occupation, leisure, sitting, standing and ambulation

**Obese**: A body mass index 30kg/m² and above

**Orexigenic**: Producing an increase in appetite

**Overweight**: A body mass index of between 25 kg/m² and 30kg/m².

**Paracrine**: Relating to or denoting a hormone which has effect only near the gland secreting it.

**Resting Energy Expenditure**: The amount of energy used by an organism at rest while in a state of complete muscular relaxation and awake

**Satiety**: The feeling of fullness after eating

**Sensor drift**: The tendency of a gas sensor, when exposed to a stable gas concentration starts to “drift” off the true value with prolonged exposure.

**The mesolimbic pathway**: Part of the limbic system involved in the hedonic response to food.

**Very low-calorie diets**: Diets providing fewer than 3350 kJ/day.

**Appendices**
Appendix A: Table of RCT’s of Intermittent Fasting in Animals

A.1 Table of RCT’s of Intermittent Fasting in Animals

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Animal Model</th>
<th>Age at start of Intervention</th>
<th>Duration</th>
<th>Diet Prescription</th>
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<th>Sample Size</th>
<th>Basis for Sample Size</th>
<th>Basis of Energy Prescription</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatori et al</td>
<td>2012</td>
<td>Obese mouse model, C57/BL6 mice</td>
<td>3 months</td>
<td>18 weeks</td>
<td>NF: Nocturnal AL Feeding</td>
<td>Absolute quantity the same in NF &amp; AL groups. When normalised for weight, NF mice ate more</td>
<td>NF: 6</td>
<td>Not given</td>
<td>Nocturnal feeding: AL, 8 hrs, lights off.</td>
</tr>
<tr>
<td>Belkacemi et al</td>
<td>2010</td>
<td>Wild Psammomys Obesus Rats</td>
<td>Unknown</td>
<td>4 weeks of TRF</td>
<td>All Chow animals feed 58 kcals on feeding days. All vegetal animals fed 0.42 kcals/g body weight Time restricted feeding(TRF): Fasting 1700-0800 each day. Vegetarian Diet Control: Veg-CR Vegetarian Diet TRF: Veg-TRF Non-Diabetic Chow: Chow-CR Non-Diabetic Chow TRF: Chow-TRF Diabetic Chow: DM-Chow-CR Diabetic Chow TRF: DM-Chow-TRF</td>
<td>Veg-CR: 34.6 kcal/day Veg-TRF: 14.1 kcal/day Chow-CR: 48 kcal/day Chow-TRF: 29.6 kcal/day DM-Chow-CR: 47.3 kcal/day DM-Chow-TRF: 28.1 kcal/day</td>
<td>Veg-AL: 10</td>
<td>Veg-AL: 10</td>
<td>Vegetarian group based on weight of rats. The chow group were given a standard prescription throughout</td>
</tr>
<tr>
<td>Author</td>
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<tr>
<td>Mager et al (268)</td>
<td>2006</td>
<td>Sprague Dawley Rats</td>
<td>2.5 months</td>
<td>3 wks AL +16 weeks Intervention</td>
<td>ADF: AL/Complete Fast CDR: 40% baseline AL intake Standard Chow</td>
<td>Not reported</td>
<td>ADF: 6</td>
<td>CDR: 6</td>
<td>ADF: Complete restriction &amp; AL intake CDR: Baseline AL intake</td>
</tr>
<tr>
<td>Ahmet et al (269)</td>
<td>2005</td>
<td>Sprague Dawley Rats</td>
<td>2 months</td>
<td>12 weeks</td>
<td>ADF: AL/Complete Fast CDR: AL daily</td>
<td>Not reported</td>
<td>ADF: 30</td>
<td>CDR: 30</td>
<td>Not given</td>
</tr>
<tr>
<td>Pedersen et al (270)</td>
<td>1999</td>
<td>BB Rats</td>
<td>3 weeks</td>
<td>26 weeks</td>
<td>AL: Control IF-1: Complete Fast 1 day weekly/AL 6 days ADF:Complete Fast/AL all days</td>
<td>Not reported</td>
<td>AL: 74</td>
<td>IF-1: 40 CDR: 44</td>
<td>Not given</td>
</tr>
<tr>
<td>Anson et al (271)</td>
<td>2003</td>
<td>C57BL/6 Mice</td>
<td>9 weeks</td>
<td>29 weeks</td>
<td>AL:Ad libitum ADF:Complete fast/AL CDR: 60% AL Animal Intake PF: Food allotment matched to ADF</td>
<td>Exact figures not given: ADF=AL=PF CDR = 60% AL</td>
<td>Not given</td>
<td></td>
<td>Not given</td>
</tr>
<tr>
<td>Author</td>
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</tbody>
</table>
| Gotthardt et al (273) | 2016 | C57BL/6 Mice          | 7 weeks                     | 4 weeks  | 8 weeks AL high fat diet phase + 4 weeks  
HF-AL: High Fat Ad Libitum: HF-AL  
LF-AL: Low Fat Ad Libitum: LF-AL  
HF-ADF: AL High Fat/Fast  
LF-ADF: AL Low Fat/Fast | Cumulative food intake (kcal)  
HF-AL: 700  
LF-AL: 600  
HF-ADF: 600  
LF-ADF: 550 | HF-AL: 8  
LF-AL: 8  
HF-ADF: 8  
LF-ADF: 8 | Not given | N/A |
| Boutant et al (274) | 2016 | SIRT-1 Transgenic Mice (tg)  
Wild Type Mice (wt) | 12 weeks                    | 12 weeks | ADF: AL/Complete Fast  
AL: Ad Libitum       | Average dietary intake/day  
ADF: ~3.8g  
AL: ~2.6g | Exact figures not given: Approx 16-24 for each group. | Not given | N/A |
| Baumeier et al (275) | 2015 | New Zealand Obese (NZO) | 3 weeks                     | 10 weeks | All were fed high fat diet  
AL: Ad Libitum  
CDR: 90% of AL intake  
ADF: AL/Complete Fast | Daily Calorie Intake on Feeding Days (Relative to AL)  
AL: 4.8g (100%)  
CDR: 4.3g (90%)  
ADF: 6.9g/0g (73%) | AL: 14  
CR: 14  
ADF: 14 | Not given | N/A |
| Chausse et al (282) | 2014 | Male Sprague Dawley Rats | 8 weeks                     | 3 weeks  | AL: Ad Libitum  
ADF: Ad libitum/Complete Fast | Cumulative intake relative to AL:  
AL: 100%  
ADF: 80%  
Gorging pattern in ADF mice noted on fast days. Not | ADF: 12  
AL: 12 | Not given | N/A |
<table>
<thead>
<tr>
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<tr>
<td>Varady et al (277)</td>
<td>2010</td>
<td>C57BL/6J Female Mice</td>
<td>7 weeks</td>
<td>4 weeks</td>
<td>1-week AL prior to randomization</td>
<td>Mean Daily Food Intake (g/day):</td>
<td>CDR-25%: 6</td>
<td>Not stated</td>
<td>Based on assumption that AL consumption at baseline = 100% requirements</td>
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<td>CDR-25%: 75% AL intake</td>
<td>CDR-25%: 2.04</td>
<td>ADF-75%: 6</td>
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<td>ADF-75%: 25% AL Intake/AL</td>
<td>ADF-75%: 2.23</td>
<td>ADF-85%: 6</td>
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<td>ADF-85%: 15% AL Intake/AL</td>
<td>ADF-85%: 2.21</td>
<td>ADF-100%: 6</td>
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<td>ADF-100%: Complete Fast/AL</td>
<td>ADF-100%: 2.21</td>
<td>AL: 6</td>
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<td>AL: Ad libitum</td>
<td>AL: 2.57</td>
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<td>AL: 2.57</td>
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<tr>
<td>Rusli et al (280)</td>
<td>2015</td>
<td>C57BL/6J</td>
<td>9 weeks</td>
<td>39 weeks</td>
<td>AL: Ad libitum CR-30%: 30% Calorie Restricted</td>
<td>Presented as proportion of AL Control*</td>
<td>AL: 89</td>
<td>Not clear</td>
<td>Calorie restriction as a proportion of the ad-libitum group intake calculated only at baseline and 24 weeks.</td>
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<td>MF-AL: 25% Fat Ad Libitum</td>
<td>Relative proportions only verified at baseline &amp; 6 months:</td>
<td>CR: 117</td>
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<td></td>
<td>IF: 40% Cal Res. 1 week/MF-AL 1 week</td>
<td>AL: 100%</td>
<td>MF-AL: 127</td>
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<td></td>
<td></td>
<td>CR: 60%</td>
<td>IF: 87.9%</td>
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<td></td>
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<td>MF: 115%</td>
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<tr>
<td>Hsieh et al(281)</td>
<td>2005</td>
<td>C57BL/6J female mice</td>
<td>7 week</td>
<td>4 weeks</td>
<td>AL: Ad libitum</td>
<td>AL: 22g week</td>
<td>AL: 6</td>
<td>Not given</td>
<td>AL intake</td>
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<tr>
<td></td>
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<td></td>
<td>CR-5%-DF: 95% AL.Daily Feed.</td>
<td>Other arms not measured</td>
<td>CR-5%-DF: 6</td>
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<td></td>
<td>CR-5%-PF: 95% AL.Continuous.</td>
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<td>CR-5%-PF: 6</td>
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<td></td>
<td>CR-5%-ADF: 95% AL.3x per wk</td>
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<td>CR-33%-ADF: 6</td>
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<td></td>
<td>CR-33%-DF: 67% AL.Daily Feed.</td>
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<td>CR-33%-DF: 6</td>
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<td>CR-33%-PF: 67% AL.Continuous.</td>
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<td>CR-33%-PF: 6</td>
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<td></td>
<td>CR-33%-ADF: 67% AL. 3x per wk</td>
<td></td>
<td>CR-33%-ADF: 6</td>
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<tr>
<td>Bonorden et al(279)</td>
<td>2009</td>
<td>C57BL/6J mice Transgenic prostate adenocarcinoma model</td>
<td>7 weeks</td>
<td>43 weeks</td>
<td>AL: Ad libitum</td>
<td>Relative to AL intake: AL: 100% CR: ~75% IF: ~75%</td>
<td>AL: 39</td>
<td>Unclear</td>
<td>Age matched ad-libitum intake</td>
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<td></td>
<td></td>
<td>CR-25%: 75% AL intake</td>
<td></td>
<td>CR: 75</td>
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<td></td>
<td>IF: 2 week 50%AL/100%AL</td>
<td></td>
<td>IF: 96</td>
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</table>

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A.2 Table of RCT’s of Intermittent Fasting in Animals (ctd.)

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Other Outcomes</th>
<th>Weight Change</th>
<th>Body Composition</th>
<th>Assessment of Glycaemia</th>
<th>Glycaemic Changes</th>
<th>REE Assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatori et al(245)</td>
<td>2012</td>
<td>NF-HF:</td>
<td>NF gained 5g-7g</td>
<td>MRI. Less Fat Mass accumulation in NF-HF group.</td>
<td>↑ Intraperitoneal glucose tolerance</td>
<td>VO2 assessed. No difference in physical activity.</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Fatty Acid Oxidation ↑ Fatty Acid Synthesis ↓ ↓ adipocyte hypertrophy</td>
<td>AL gained 9g - 22g</td>
<td></td>
<td>↑ Expression pyruvate carboxylase │</td>
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<tr>
<td></td>
<td></td>
<td>altered expression of circadian regulator genes</td>
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<td>↑ Expression Glucose 6 phosphatase</td>
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<td></td>
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<td>↓ hepatic intracellular lipids</td>
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<td></td>
<td></td>
<td>↑ mitochondrial</td>
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<td></td>
<td></td>
<td>↓ hepatic Steatosis</td>
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<td></td>
<td></td>
<td>↑ rhythmic UCP-1 expression in BAT</td>
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<tr>
<td></td>
<td></td>
<td>↓ Inflammtory markers</td>
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<tr>
<td>Belkacemi et al(278)</td>
<td>2010</td>
<td>No difference between Veg &amp; Chow diets</td>
<td></td>
<td></td>
<td>2 hour post prandial glucose tolerance test</td>
<td>IPGTT with intermittent fasting worse in CR groups, unchanged with TRF (Not compared statistically).</td>
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<td></td>
<td></td>
<td>No difference by diabetes status</td>
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<td></td>
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<td>Weight loss with</td>
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<td>Veg-CR: + 18.8g (17.2%), n =4</td>
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<td>Veg-TRF: - 20.0g (15.3%), n=4</td>
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<td>Chow-TRF(pooled): - 18.4g</td>
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<td></td>
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<td>Chow-CR(pooled): +21.1g</td>
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<tr>
<td>Mager et al(268)</td>
<td>2006</td>
<td>ADF: ↓ Circadian Activity</td>
<td>Weight gain approximately 20% (no figures given), both arms</td>
<td>N/A</td>
<td>Fasting glucose</td>
<td>No difference between arms</td>
<td>No</td>
</tr>
<tr>
<td>Ahmet et al(269)</td>
<td>2005</td>
<td>Cardiac Damage with coronary artery ligation.</td>
<td>IF: 49 g weight gain AL:125g weight gain</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>No</td>
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<tr>
<td>Pedersen et al(270)</td>
<td>1999</td>
<td>No difference in the degree of insulitis</td>
<td>Exact figures not given. Approx:</td>
<td>N/A</td>
<td>Diabetes Diagnosed</td>
<td>Cumulative Incidence DM: AL: 80% IF-1: 50% ADF: 52% Lower blood glucose levels in non-diabetic controls: IF-1&lt;ADF&lt;AL</td>
<td>No</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>AL: +345g</td>
<td>N/A</td>
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<td></td>
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<td>IF-1: +300g</td>
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<td></td>
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<td>ADF: + 245g</td>
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<tr>
<td>Anson et al(271)</td>
<td>2003</td>
<td>Neuronal survival in response to chemical injection: ADF &amp; PF &gt;AL</td>
<td>AL: +17 g</td>
<td>N/A</td>
<td>Fasting venous glucose</td>
<td>ADF vs PF: decrease in glucose and insulin.</td>
<td>No</td>
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<td></td>
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<td>ADF: +10 g</td>
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<td>Fasting insulin</td>
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<td>CR: +4 g</td>
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<td>PF: +12 g</td>
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<td>Cerqueira et al(272)</td>
<td>2011</td>
<td>Adiponectin: CR &gt; IF &amp; AL</td>
<td>Exact figures not given:</td>
<td>N/A</td>
<td>Fasting glucose</td>
<td>Response to IPGTT: IF &gt; CR &gt; AL</td>
<td>No</td>
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<td>Approximate Intake (g):</td>
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<td>Weighted intra-abdominal fat deposits: IF &gt; CR &amp; AL</td>
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<td>Intraperitoneal glucose tolerance test.</td>
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<td>Gotthardt et al(273)</td>
<td>2016</td>
<td>Leptin: Lower in all groups compared to HF-AL. Ghrelin: Unaffected Neurotransmitter concentrations: Norepinephrine concentration ADF-diets: Greater concentrations in the antero-medial hypothalamus compared with AL controls ADF-LF: Greater concentration in the in the posterior hypothalamus. Dopamine Concentration: IMF-LFD &gt; other arms NPY&amp;POMC mRNA expression: NPY expression greater with ADF. POMC lower in all groups compared to HF-AL AgRP: No difference GHSR expression greater with ADF</td>
<td>AL: +700 CR:+500 IF: +550</td>
<td>Weighted Skeletal Muscle: IF &lt;CR &amp; AL</td>
<td>MRI. 2g difference in Absolute fat mass(g) greater in HF-AL compared with all other groups. Lean mass approx between LF-ADF &amp; other arms. Significant. Relative proportion not expressed.</td>
<td>OGTT: Lower AUC in IMF-LF group compared to others ITT: IMF-LF was lower than HF-AL, but higher than other higher calorie groups.</td>
<td>Indirect calorimetry. VO2, VCO2, RER assessed. Body weight used as a covariate, not body composition. All groups had lower RER compared to HFD.</td>
</tr>
<tr>
<td>Boutant et al(274)</td>
<td>2016</td>
<td>ADF-wt: + 1g ADF-tg: +2g</td>
<td>MRI. Exact values not given:</td>
<td>Intraperitoneal Tolerance Test</td>
<td>ADF: ↑ Hepatic insulin Sensitivity ↑ Peripheral</td>
<td></td>
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</tr>
<tr>
<td>Author</td>
<td>Year</td>
<td>Other Outcomes</td>
<td>Weight Change</td>
<td>Body Composition</td>
<td>Assessment of Glycaemia</td>
<td>Glycaemic Changes</td>
<td>REE Assessed</td>
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<tr>
<td>Baumeier et al(275)</td>
<td>2015</td>
<td>Beta agonist stimulated lipolysis: NEFA, TG, Glycerol Analysis of Hepatic Lipid Droplet Proteins Liver &amp; Skeletal Muscle TG content: ADF ↓ muscle DAG &amp; TG content, Increases muscle FA oxidation</td>
<td>AL: +36 CR: + 32 ADF: +31</td>
<td>MRI. Fat Mass/Lean Mass AL: +15g /+15g CR: + 17g /+12g ADF: +10g /+13g</td>
<td>Plasma Insulin Oral glucose tolerance Insulin tolerance test</td>
<td>Fasting insulin higher in CR but not AL compared with ADF No difference in IVTT or OGTT between groups</td>
<td>No. RQ only. Indirect Calorimetry</td>
</tr>
<tr>
<td>Author</td>
<td>Year</td>
<td>Other Outcomes</td>
<td>Weight Change</td>
<td>Body Composition</td>
<td>Assessment of Glycaemia</td>
<td>Glycaemic Changes</td>
<td>REE Assessed</td>
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| Chausse et al(282) | 2014 | **Spontaneous Physical Activity:**  
No difference during light phase:  
During dark phase, fasting ADF mice moved less.  
**Higher Temp & VO2** in ADF mice on feeding days (unsurprising - TEF).  
**Effect of IP Leptin on Food Intake:**  
Decreased intake in ADF mice but not AL mice  
**Effect of intra-abdominal leptin on hypothalamic signalling:**  
Increased  
**Skeletal muscle mitochondria:**  
No change in mass or function  
**Hypothalamic Neurotransmitter mRNA**  
↑ ADF: AGRP & NPY feeding & fasting. Orexin, fasting days only.  
↓ ADF: TRH, fasting days only  
**Unchanged:** CRH, POMC, Melanin Concentrating.  
**Daily fluctuation** in overall weight and liver mass:  
The store depletion is unlikely to be similar to that of human subjects over a similar time course. | ADF: +22.5g  
AL: + 50.3g | Not Assessed | Not Assessed | Not Assessed | VO2 & RER measured by indirect calorimetry.  
Higher VO2 on Fed days in ADF rats  
Higher RER in ADF rats on fasting days indicating lipid oxidation on fast days |
| Varady et al(277) | 2010 | **TG-Synthesis assessed by Deuterium:**  
Unaffected in ScAT and VAT  
**Net Lipolysis & Lipogenesis:**  
ScAT not VAT: Denovo lipogenesis greater in all arms compared with AL.  
No significant difference in weight between any groups excepts CR-25% and AL. |  |  |  |  | No |
<table>
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<tr>
<th>Author</th>
<th>Year</th>
<th>Other Outcomes</th>
<th>Weight Change</th>
<th>Body Composition</th>
<th>Assessment of Glycaemia</th>
<th>Glycaemic Changes</th>
<th>REE Assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rusli et al(280)</td>
<td>2015</td>
<td>No difference in lipolysis. No difference in lipogenic or lipolytic gene expression</td>
<td>Absolute wt gain (% baseline)</td>
<td>ScAT/VAT(% total): AL:41/59 CR-25%:59/41 ADF-75%:68/32 ADF-85%:55/45 ADF-100%:65/35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsieh et al(281)</td>
<td>2005</td>
<td>ALT: Lowest in IF group AST: Lowest in IF group Liver Histology: Cirrhosis in MF-AL &amp; AL groups Hepatic TG deposition: Lower in IF &amp; CR groups. Genes assoc with Liver Droplet formation:</td>
<td>No exact figures given Weight gain (%baseline wt)</td>
<td>Not assessed</td>
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</table>

Weight gain (% baseline wt) AL: +14g (63.6%) CR-30: -2g (9%) MF-AL: +20g (91%) IF: +6g (27%)
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<tr>
<th>Author</th>
<th>Year</th>
<th>Other Outcomes</th>
<th>Weight Change</th>
<th>Body Composition</th>
<th>Assessment of Glycaemia</th>
<th>Glycaemic Changes</th>
<th>REE Assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bonorden et al(279)</td>
<td>2009</td>
<td>Estrus Cycle: Not examined for IF Cell Proliferation: reduced epithelial, T-Cell &amp; Epidermal proliferation in CR-5%-ADF compared with AL intake</td>
<td>Lowest in INT group. Liver weight: MF&gt;AL&gt;INT&gt;CR</td>
<td>Exact figures not given: No SEM or SD's given Approximate Intake (g): AL: +2g (11%) CR-5%-DF: -3.5g (20%) CR-5%-PF: 1.5g (9%) CR-5%-ADF: 0.5g (3%) CR-33%-DF: -3 (15%) CR-33%-PF: -2.5g (13%) CR-33%-ADF: -1g (6%)</td>
<td>Not assessed</td>
<td>Not assessed</td>
<td>Not assessed</td>
</tr>
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</table>
Appendix B: Four Day Food Record

Diabetes Research
Capital and Coast Health
4 Day Food Record

Change in Resting Energy Expenditure with Different Schedules of Caloric Restriction: The CREEDS study

1. Complete & Bring With You to Your Next Appointment
2. Please Tick One of the Following:
   - [ ] Before Week 1
   - [ ] Week 1
   - [ ] Week 3
   - [ ] Week 6
How To Fill Out The Four Day Food Record Sheet -

Please eat whatever you usually would, don't make any changes.

1. For each day, please write down everything you eat and drink in that day, including tea and coffee and snacks.
   - If you eat something outside the meal spaces suggested, just add it in the nearest time space – e.g. if you eat a banana at Sam, put it under breakfast.
   - If you do not eat at a particular time, for example, during the morning, just put a line through the space.

2. Please also write down all the ingredients, for example – Bread Roll - chicken, lettuce and tomato, round white roll. Toast – 2 x toast slice, wholemeal bread, marmite
   - If you can, give the brand name or type of food, e.g. whole milk, weetbix, Wattie's baked beans.
   - Please also write down how something is cooked, e.g. 2 toast potatoes in canola oil
   - If you eat a dish or product that is made of lots of ingredients, like a muffin or a stir-fry with rice, just be as specific as you can, for example
     1 blueberry muffin
     ½ cup cooked rice with 1 cup veges – zucchini, broccoli, onion and red pepper

3. For everything you eat please estimate how much it is, using measures like cups, teaspoons or grams. For example:
   - 1 cup tea with milk and 2 sugars
   - 1 cup muesli with ½ a cup milk
   - ½ cup broccoli
   - 1 teaspoon honey
   - If you eat a packet of something, write down the weight of the packet – e.g. one 55 gram dark chocolate bar, ½ a 425g can of peaches.
   - If you are having trouble measuring something, compare it to something else, e.g. an egg, a matchbox.

<table>
<thead>
<tr>
<th>Meal</th>
<th>Amount Eaten</th>
<th>Food and Drink</th>
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<tbody>
<tr>
<td>Breakfast</td>
<td>2 slices</td>
<td>Yogels bread</td>
</tr>
<tr>
<td></td>
<td>2 tsp</td>
<td>Margarine</td>
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<tr>
<td></td>
<td>2 tsp</td>
<td>Marmite</td>
</tr>
<tr>
<td></td>
<td>1 cup</td>
<td>Tea with milk, no sugar</td>
</tr>
<tr>
<td>During Morning</td>
<td>1</td>
<td>Plain muffin</td>
</tr>
<tr>
<td>Meal</td>
<td>Amount Eaten</td>
<td>Food and Drink</td>
</tr>
<tr>
<td>----------------------</td>
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</tr>
<tr>
<td>Breakfast</td>
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<td>During Morning</td>
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<td>Lunch</td>
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<td>During Afternoon</td>
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<tr>
<td>Dinner</td>
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<tr>
<td>Dessert</td>
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<tr>
<td>During Evening</td>
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Day Two:  Mon / Tues / Wed / Thur / Fri / Sat / Sun (please omit)

<table>
<thead>
<tr>
<th>Meal</th>
<th>Amount Eaten</th>
<th>Food and Drink</th>
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<tbody>
<tr>
<td>Breakfast</td>
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<td>During Morning</td>
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<tr>
<td>Lunch</td>
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<tr>
<td>During Afternoon</td>
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<tr>
<td>Dinner</td>
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<tr>
<td>Dessert</td>
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<tr>
<td>During Evening</td>
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</table>
Day Three: Mon / Tues / Wed / Thur / Fri / Sat / Sun (please circle)

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<tr>
<th>Meal</th>
<th>Amount Eaten</th>
<th>Food and Drink</th>
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<tbody>
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<td>Breakfast</td>
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<tr>
<td>During Morning</td>
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<td>Lunch</td>
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<td>During Afternoon</td>
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<td>Dinner</td>
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<td>Dessert</td>
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<td>During Evening</td>
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</table>
Day Four: Mon / Tues / Wed / Thur / Fri / Sat / Sun (please circle)

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<thead>
<tr>
<th>Meal</th>
<th>Amount Eaten</th>
<th>Food and Drink</th>
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<tbody>
<tr>
<td>Breakfast</td>
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<tr>
<td>During Morning</td>
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<tr>
<td>Lunch</td>
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<td>During Afternoon</td>
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<tr>
<td>Diner</td>
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<tr>
<td>Dessert</td>
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<tr>
<td>During Evening</td>
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Appendix C: Promethion Signal Processing Algorithm Used to Calculate REE from Indirect Calorimetry

Macros, sets of instructions that allowed batch processing and automation of most of the data processing, were provided with the Promethion and executed in Expedata (Sable Systems, USA). These macros were Promethion-specific but their validity for our unique calorimetry setup was unknown at the outset. I adjusted the code for these macros in Expedata so that they occurred in a stepwise, graphically illustrated fashion. This allowed me to visually inspect every step of the data processing and to identify if an error in the data was amplified by application of the standard macro. This stepwise macro was used during the data processing of studies where; the raw data differed from expected absolute values, showed a larger than expected variability, when sensors from different channels differed significantly or when unexpected outputs were produced by the automated data processing.

**Correction of Temporal Lag**

The measurement of excurrent O₂ and CO₂ is assumed to occur simultaneously. In our setup, the O₂ and CO₂ analysers are placed in series in each channel and there is a temporal lag between the recordings of these gases. There is also a temporal lag when a channel switches from measuring excurrent to incurrent gas flows. The issue of temporal lag is a common one in signal processing and is easily corrected by shifting the data samples backward with respect to time. A different correction is applied to the O₂ and CO₂ channels to temporally align these sensors. The switch from excurrent to incurrent air is “marked” in the raw data and the adequacy of the temporal lag correction is possible to check by visual inspection of the data to see that the change in gas values align with the channel switch as expected.

**Smoothing Barometric Pressure**

Smoothing is another signal transformation that reduces noise in signal processing. This can be achieved by several statistical techniques (Wood GA, 1982). Nearest neighbour smoothing is a form of smoothing that bases an estimation, y, of a given sample point x, on the average of a pre-specified set of “nearest neighbour values” i.e. the set of values adjacent to the point in question. This is repeated for every point on the line. For example, we use the 599 samples on either side of any given sample to smooth the samples.
Barometric pressure is slow to change, and thus smoothing over a ten-minute interval reduces noise that would otherwise introduce inaccuracies in the correction for barometric pressure.

**Correction of O<sub>2</sub>, CO<sub>2</sub> and Flow Rate for WVP and Barometric Pressure**

The barometric pressure of the measured gas mixture is inversely proportional to the volume of the gases in that mixture, as mentioned in section 2.1.1. To adjust for this, the following equation can be used:

Equation C.1: \[ \text{Corrected } O_2 = \frac{\text{Measured } O_2 \times 101.325}{\text{Measured BP}} \]

Thus, when barometric pressure is lower than the standard pressure of 101.325 kPA of oxygen the gases are multiplied by a factor <1 and their STP value is, appropriately, less than the measured value at a lower barometric pressure. The converse is also true.

When water vapour increases in a mixed volume of gases it reduces the partial pressure of that gas, as the calorimetry equation assumes dry air at CTP, the effect of water vapour pressure on the “dilution” of CO<sub>2</sub> and O<sub>2</sub> must be corrected for. This is done by modifying the above equation as follows:

Equation C.2: \[ \text{Corrected } O_2 = \frac{\text{Measured } O_2 \times 101.325}{\text{Measured BP} - \text{Measured WVP}} \]

It is this equation that is employed in our standard macro, a similar equation is used for CO<sub>2</sub> correction. Thus, at a fixed atmospheric pressure, water vapour pressure diminishes the denominator, thus increasing the factor by which the O<sub>2</sub> or CO<sub>2</sub> is corrected. Simply put, the higher the WVP, the greater the correction factor applied. This is analogous to mathematically ‘drying’ the measured air in addition to adjusting for barometric pressure in one step.

A related correction must be applied to flow-rate estimation. Barometric pressure alters gas volumes traversing a flow sensor per unit time, i.e. flow rate, in the same way that it affects gas volumes. By contrast WVP “inflates”, rather than dilutes, the flow rate compared to dry air at STP. It does this because it represents an additional volume of gas passing along the flow sensor. The equation to correct this produces a correction factor less than one, for any WVP > 0 kPA, by which the flow rate is multiplied. As with the
previous equation this adjustment can be performed simultaneously with an adjustment for barometric pressure:

Equation C.3: \[ \text{Corrected } FR = \frac{FR \cdot (\text{Measured BP} - \text{Measured WVP})}{\text{Measured BP}} \]

**Calculating \( \text{FiO}_2 \) and \( \text{FiCO}_2 \).**

The Promethion baselining process for any given channel results in a timestamped mix of excurrent and incumbent samples. To calculate \( \text{VCO}_2 \) and \( \text{VO}_2 \) the \( \text{FeO}_2 \) and \( \text{FeCO}_2 \) values for the measurement period need to be “separated” from the \( \text{FiCO}_2 \) and \( \text{FiO}_2 \) vectors. The \( \text{O}_2 \) value of atmospheric air should be 0.2094. The most level consecutive baseline samples in the oxygen channels are adjusted to this level, if they are not already there, and all other samples are adjusted by the same degree. This correction calibrates all oxygen samples. It does rely on accurate WVP calibration, which is why this is performed daily. The \( \text{FiO}_2 \) values at STP can now be treated as a constant, 0.2094, and the decrement from this value to the measured \( \text{FeO}_2 \) is the delta \( \text{O}_2 \).

Generation of the \( \text{FiCO}_2 \) vector involves linear interpolation, this technique creates a line based on a set of data points. Once again, the most level set of data points in the baselining segments in each channel are used. This involves the assumption that CO2 change slowly in the atmosphere surrounding the hood calorimeter and that for any given channel the atmospheric CO2 does not change when it is not being monitored. In practice, the amount of time that the baseline is not being monitored by either channel is 360 seconds out of 1800 seconds, consisting of 3 intervals of 120 seconds each. The baselining frequency was deliberately set to minimise these unmonitored intervals while maximising the number of baselining instances and maintaining compatibility with our macro. Non-contiguous baseline segments of \( \text{CO}_2 \) are thus replaced by a single line of data points that can be treated as the \( \text{FiCO}_2 \), this is done for both channels. \( \text{FiCO}_2 \) and \( \text{FiO}_2 \) are used to calculate \( \text{VCO}_2 \) and \( \text{VO}_2 \) for incorporation in the Weir equation (equation 2.4, section 2.2.4.1).

**Calculation of the delta \( \text{CO}_2 \) and delta \( \text{O}_2 \).**

Having determined the \( \text{FiCO}_2 \), \( \text{FiO}_2 \), \( \text{FeO}_2 \), \( \text{FeCO}_2 \) at each sampling point, the \( \text{CO}_2 \) production and \( \text{O}_2 \) depletion can be calculated. These correspond to the (\( \text{FeCO}_2-\text{FiCO}_2 \)) term and the (\( \text{FiO}_2-\text{FeO}_2 \)) term in the \( \text{VCO}_2 \) and \( \text{VO}_2 \) equations (equation 2.1 and 2.2,
section 2.2.4.1) and are termed delta CO$_2$ and delta O$_2$ respectively. All samples in each O$_2$ channel are subtracted from 0.2094 – the FiO$_2$ at STP. This produces two delta O$_2$ vectors of 1800 samples each specifying the reduction in O$_2$ from baseline at each time point. Similarly, each CO$_2$ channel is subtracted from a level section of the corresponding baseline CO$_2$ value to produce two delta CO$_2$ vectors.

The delta vectors are used to calculate VO$_2$ and VCO$_2$ using; flow rate, and a correction for the effect of changing CO$_2$ on O$_2$ and vice versa.

**Merging FiCO2, O2, CO2 Channels**

Having generated values for FiO$_2$, FiCO$_2$, delta CO$_2$ and delta O$_2$ for both channel one and channel two, these paired vectors must now be combined. At this point, the FiO$_2$ vector is considered a constant value 0.2094 and there are two FiCO$_2$ vectors that represent incurrent CO$_2$ values across all 1800 timepoints. These two vectors are combined by averaging them to create a single FiCO$_2$ vector.

Once calculated, within each delta CO$_2$ channel and each delta O$_2$ channel are excurrent segments containing the delta values interspersed with baseline samples containing the incurrent values (fig 2.3). For each gas, each channel baselining timing is offset with respect to the other channel so that when one channel is measuring baseline samples, the opposite channel is measuring excurrent air. In contrast to the baselining measurements, which miss 360 samples during the usual setup, the excurrent air samples are continuously acquired by one or the other channel. For each channel, its baseline readings are replaced using a merging function that copies the alternate channels excurrent readings across. This is done for WVP, CO$_2$ and O$_2$ channels. Once this is done, both vectors for each gas are combined and then divided by two to produce a single averaged reading for each of WVP, CO$_2$ and O$_2$.

**Calculating VO$_2$, VCO$_2$, RQ and REE**

Finally, the combined, baseline-free delta CO$_2$ and O$_2$ vectors are incorporated in equation 1 and equation 2, (section 2.2.4.1) to calculate VO$_2$ and VCO$_2$ respectively:

Equation 2.1: $VCO_2 = \frac{FR\times(1+FiCO_2)}{FiCO_2}\times(FeCO_2-FiCO_2+FiCO_2\times(FeO_2-FiO_2))$.

Equation 2.2 $VO_2 = \frac{FR\times(1-0.2094)\times(FeO_2-FiO_2)-0.2094\times(FeCO_2-FiCO_2)}{(1-0.2094)}$.
VCO2 and VO2 are then incorporated in the equation 4 to calculate REE:

Equation 2.4: \( REE = 3.941 \times VO_2 + 1.106 \times VCO_2 \).
Appendix D: Guide to Calorie Counting Used for the CREEDS Study

The CREEDS Study Calorie Counting Resources

1. Tips for Sticking to the Calorie Count
2. Calorie Counting Resources
3. Lunches/Snacks
4. Dinner
5. Breakfast/Dessert
6. Two Page Calorie Counter Guide
7. Sample Fast Day Intakes for a 500-600kcal day
8. Weekly Meal Planner
9. Appendix: Your Preferred Meals
Tips For Sticking to the Calorie Count

- Keep the range of meals simple and easy to prepare.
- Prepare Lunches and Meals in Bulk and Refrigerate/Freeze them where possible.
- Take care to watch the portion size, divide the portions evenly as per the recipe portion guide.
- Black Coffee, herbal tea & water are all calorie free.
- Watch out for low fat but high sugar alternatives e.g. yoghurts, ice cream etc.
- Protein such as nuts & eggs can increase your feeling of fullness after a meal.
- It is easier to plan your meals at the start of the week and ensure all of your ingredients are in the house for when you need them.
- Anticipated hunger between meals and plan to have a snack available.
- Schedule your meal preparation time once or twice per week.
- It is easier to fight with two. If you have a partner or friend who wants to participate get them to join in, they can attend for a weigh in at 3 weeks and 6 weeks.

Measuring Energy in Calories
1. A calorie is also known as a Kilocalorie or Cal or Kcal. They are interchangeable.
2. Kilojoules (kJ) are also used to measure energy but it is not the same as a calorie.
3. To convert from Kilojoules to Kilocalories:
   - Multiply by 0.239 or divide by 4.
   - E.g. 100 kilojoules is 23.9 calories. 500 kilojoules is 125 calories.

Eating out at Restaurants:
1. Restaurant food is very hard to estimate unless it’s plain or unless the calorie count is listed (e.g. Subway & Pita Pit).
2. The calorie king website contains some Australian and NZ restaurant outlets.
3. The website of restaurant chains such as Domino’s will contain some of this information too.
4. If you know what you usually order check before you go.
5. Watch the portion sizes in a restaurant when estimating calories.

Calorie Counting Resources

- Nutrient Labelling Resources (Food Safety Authority Australia, New Zealand)
Calorie Counting Applications For Smart Devices:
These apps are NZ specific and all use the same reporting standards, there is other software available but it is not NZ specific:
Calorie Counter NZ, Xyris Software – Easy Diet Diary. For apple devices only, data will required to download but not require otherwise.

For google & android devices
Save the following website link to your device: [www.calorieking.com.au](http://www.calorieking.com.au). Data will required to access.

Non-NZ standardized:
My Fitness Pal,
MyFatsecret.co.nz – this relies on user nutrient labelling and is not considered accurate.

Books:
The 5:2 Diet and 5:2 Diet Cookbook (both by Kate Harrison; Calorie Counts contained within).
The New Zealand Calorie Counter – Penguin books: An NZ specific calorie counter

Websites: Calories Counting Recipes Online
http://www.goodtoknow.co.uk/recipes/5383117/5-2-diet-meal-plans-what-to-eat-for-500-calorie-fast-days
https://www.bbcgoodfood.com/recipes/category/healthy
[www.cfrecipes.com](http://www.cfrecipes.com)
### Lunches/Snacks: 100 - 200 calories

#### Ciabatta Roll & Banana

| Time: 2 min | No. Serves: 1 | Calories per Serve: 333 |

**Ingredients:**
- 1 Ciabatta Roll (80g)
- 1 tsp Margarine (thin spread)
- 1 Banana – Medium (170g with skin)

**Notes**

#### Roasted Broccoli with Lemon & Garlic

| Time: 25 mins | No. Serves: 6 | Calories per Serve: 47 |

**Ingredients:**
- 2 heads Broccoli
- 2 tbsps Extra Virgin Olive Oil
- 1 tsp Sea Salt
- ½ tsp Ground black pepper
- 1 clove Garlic
- ½ tsp Lemon juice

**Directions:**
1. Preheat the oven to 200 degrees C (400 degrees F).
2. In a large bowl, toss broccoli florets with the extra virgin olive oil, sea salt, pepper and garlic. Spread the broccoli out in an even layer on a baking sheet.
3. Bake in the preheated oven until florets are tender enough to pierce the stems with a fork; 15 to 20 minutes. Remove and transfer to a serving platter. Squeeze lemon juice over the broccoli before serving.

**Notes**

#### Beetroot & Lentil Salad with Mustard

| Time: 25 min | No. Serves: 5 | Calories per Serve: 148 |

**Ingredients:**
- 200 g Puy Lentils

**Notes**
1 1/3 tbsp. Extra Virgin Olive Oil
300g 300g pack cooked beetroot (not in vinegar), sliced
Large handful Leafy greens [e.g. Rocket, lettuce, Tarragon]

**Directions:**

1. If not using pre-cooked lentils, cook the lentils following pack instructions, drain and leave to cool. Meanwhile, combine the mustard, oil and some seasoning to make a dressing.
2. Tip the lentils into a bowl, pour over the dressing and mix well. Stir through the beetroot, tarragon and some seasoning, then serve.

**Notes:** Vegetarian

---

**Chicken, Lettuce & Avocado Sandwich**

**Time:** 5 mins  
**No. Serves:** 1  
**Calories per Serve:** 727

**Ingredients:**

- 2 slices Sandwich cut (medium) wholegrain bread  
- 202 leaves Lettuce  
- 3 pieces Crumber chicken  
- 1 cup Hot chips  
- 1 tsp Butter (hiny spread – double if thick)  
- 1/2 small Avocado

**Directions:**

N/A

**Notes** Without chips: Total is 504 calories  
Without chips: Total is 504 calories
### Dinner: 300 - 500 calories

#### Egg & Rocket Pizzas

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Calories per Serve</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Wraps, medium (the width of your hand from tip to wrist)</td>
<td>435 cal</td>
</tr>
<tr>
<td>1 Red Pepper (optional)</td>
<td>77 cal</td>
</tr>
<tr>
<td>2 Tomatoes</td>
<td>46 cal</td>
</tr>
<tr>
<td>2 tbsp Tomato Purée</td>
<td>8 cal</td>
</tr>
<tr>
<td>1 tbsp Dried Oregano</td>
<td>21 cal</td>
</tr>
<tr>
<td>2 Eggs</td>
<td>114 cal</td>
</tr>
<tr>
<td>65g Rocket or Baby Spinach</td>
<td>21 cal</td>
</tr>
<tr>
<td>⅛ Brown Onion, finely sliced</td>
<td>19 cal</td>
</tr>
<tr>
<td>2 tbsp Parsley or dried Basil</td>
<td>0 cal</td>
</tr>
</tbody>
</table>

#### Directions:
- Lay the tortillas on two baking sheets, brush sparingly with the oil then bake for 3 mins.
- Meanwhile chop the pepper and tomatoes and mix with the tomato purée, seasoning and herbs.
- Turn the tortillas over and spread with the tomato mixture, leaving the center free from any large pieces of pepper or tomato.
- Break an egg into the center then return to the oven for 10 mins or until the egg is just set and the tortilla is crispy round the edges. Serve scattered with the rocket and onion.

#### Notes: Substitute your preferred toppings but check the calorie count before you do.

---

#### One Pot Chicken & Rice

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Calories per Serve</th>
</tr>
</thead>
<tbody>
<tr>
<td>400g Skinless Chicken (Thighs)</td>
<td>454 cal</td>
</tr>
<tr>
<td>2 tbsp Canola Oil</td>
<td></td>
</tr>
<tr>
<td>1 Large Onion</td>
<td></td>
</tr>
<tr>
<td>3 cloves Garlic</td>
<td></td>
</tr>
<tr>
<td>1 tsp Turmeric</td>
<td></td>
</tr>
<tr>
<td>1 tsp Ground Cumin</td>
<td></td>
</tr>
<tr>
<td>1 ⅓ cups Long grain rice</td>
<td></td>
</tr>
<tr>
<td>1 can Chopped tomatoes</td>
<td></td>
</tr>
<tr>
<td>1 ⅓ cups Water</td>
<td></td>
</tr>
<tr>
<td>3 Carrots Sliced</td>
<td></td>
</tr>
<tr>
<td>1 cup Frozen peas</td>
<td></td>
</tr>
<tr>
<td>1 cup Frozen/canned corn</td>
<td></td>
</tr>
</tbody>
</table>

---

289
Directions:
1. Slice chicken into bite-sized pieces.
2. Heat oil in a large saucepan over a medium heat. Add chicken and cook for two minutes.
3. Add onion, garlic, spices and rice. Cook for a further two minutes.
4. Add canned tomatoes, water and carrots.
5. Cover and simmer on a low heat for around 20 minutes. Stir regularly so it doesn't stick to the bottom of the saucepan.
6. Add frozen peas and corn. Cook for another 5 minutes.

Notes:

Pesto Pasta with Chicken

| Time: 30mins | No. Serves: 8 | Calories per Serve: 328 |

Ingredients:
- 440g Bowtie Pasta
- 1 tsp Olive Oil
- 2 cloves Garlic
- 2 Boneless, Skinless Chicken Breasts cut into bite sized pieces
- 1 tsp Crushed Red Pepper Flakes
- 1/3 cup Oil-packed sun-dried tomatoes, drained & cut into strips
- 1/2 cup Pesto sauce

Directions:
1. Bring a large pot of lightly salted water to a boil. Add pasta and cook for 8 to 10 minutes or until al dente; drain.
2. Heat oil in a large skillet over medium heat. Sauté garlic until tender, then stir in chicken. Season with red pepper flakes. Cook until chicken is golden, and cooked through.
3. In a large bowl, combine pasta, chicken, sun-dried tomatoes and pesto. Toss to coat evenly.

Notes
# Mushroom, spinach & potato pie

<table>
<thead>
<tr>
<th>Time:</th>
<th>No. Serves:</th>
<th>Calories per Serve:</th>
</tr>
</thead>
<tbody>
<tr>
<td>45 mins</td>
<td>4</td>
<td>269</td>
</tr>
</tbody>
</table>

**Ingredients:**
- 400g Baby Spinach
- 1 tbsp Olive Oil
- 500g Mushroom
- 2 Garlic Cloves
- 250 ml Salt Reduced Vegetable Stock
- 300g Cooked New Potatoes
- 1 tbsp Grain mustard
- 1 tsp Freshly grated nutmeg
- 2 tbsp Single cream
- 3 sheets Filo Pastry
- 300g Broccoli, steamed
- 300g Green Bean, steamed

**Directions:**
Heat oven to 200C/180C fan/gas 6. Will spinach in a colander by pouring a kettleful of hot water over it.

Heat half the oil in a large non-stick pan and fry mushrooms on a high heat until golden. Add garlic and cook for 1 min, then tip in stock, mustard, nutmeg and potatoes, bubble for a few mins until reduced. Season, then remove from the heat; add single cream and spinach. Pour into a pie dish and allow to cool for a few mins.

Brush filo with remaining oil, quarter sheets then loosely scrunch up and lay on top of pie filling. Bake for 20-25 mins until golden.

Meanwhile steam the green beans and broccoli. Serve with vegetables.

**Notes:** Broccoli can be steamed or boiled.
Chicken & Broccoli Pasta Bake

Time: 40 mins  
No. Serves: 4  
Calories per Serve: 919

Ingredients:
- 350g Pasta Shells
- 200g Broccoli cut into small florets
- 2 tbsp Olive Oil
- 300g Boneless, Skinless Chicken Breasts
- 175g Mushrooms
- 4 tbsp Tomato Paste
- 284mls Carton Single Cream
- 4 Spring Onions
- 85g Mature Cheddar
- 1 Garlic Clove
- 50g Flaked Almonds

Directions:
1. Preheat the oven to 190°C/gas 5/fan170°C. Bring a large pan of salted water to the boil. Throw in the pasta, stir well and return to the boil. Cook for 6 minutes, then add the broccoli and cook for 5-6 minutes more until the pasta is just cooked. Drain well, then return to the pan.
2. Heat the oil in a wide pan, add the chicken pieces and fry until lightly browned. Tip in the mushrooms and stir fry for 1 minute, then stir in the tomato paste and cream. Gently simmer, stirring for 15 minutes to thicken the sauce. Season with salt and pepper.
3. Pour the sauce over the pasta, stirring gently until coated, then tip into a shallow ovenproof dish (about 1.7 litre capacity) and level the top.
4. Mix the spring onions, cheddar, garlic and almonds for the topping and sprinkle over the pasta. Bake for 20 minutes until golden.

Notes
Red Lentil & Carrot Soup

Time: 25 mins  
No. Serves: 2  
Calories per Serve: 258

Ingredients:
1 Onion  
2 tsp Olive Oil  
3 Garlic Cloves  
2 Carrots  
85g Red Lentils  
1 Salt Reduced Vegetable Stock Cube  
1 Sprig Parsley

Directions:
1. Put the kettle on to boil while you finely dice the onion.  
2. Heat the oil in a medium pan, add the onion and fry for 2 mins while you slice the garlic and dice the carrots. Add them to the pan, and cook briefly over the heat.  
3. Pour in 1.5 litres of the boiling water from the kettle, stir in the lentils and stock cube, then cover the pan and cook over a medium heat for 15 mins until the lentils are tender.  
4. Take off the heat and stir in the parsley. Ladle into bowls, and scatter with extra parsley leaves, if you like.

Notes: Parsley is optional and can be replaced with any Pepper, Chili etc

Indian Chickpea & Vegetable Soup

Time: 25 mins  
No. Serves: 4  
Calories per Serve: 200

Ingredients:
1 tbsp Vegetable Oil  
1 large Onion  
1 tsp Finely grated fresh ginger  
1 tbsp Garam masala  
850mls Low Salt vegetable stock  
2 large Carrots, quartered lengthways and chopped  
400g can Chickpea drained  
100g Green Beans, chopped

Directions:
Heat the oil in a medium saucepan, then add the onion, ginger and garlic. Fry for 2 mins, then add the garam masala, give it 1 min more, then add the stock and carrots. Simmer for 10 mins, then add the chickpeas. Use a stick blender to whizz the soup a little. Stir in the beans and simmer for 3 mins. Pack into a flask or, if you've got a microwave at work, chill and heat up for lunch. Great with naan bread.
Breakfast: < 300 Calories

Banana Yoghurt Pots

<table>
<thead>
<tr>
<th>Time:</th>
<th>No. Serves:</th>
<th>Calories per Serve:</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mins</td>
<td>1</td>
<td>236</td>
</tr>
</tbody>
</table>

**Ingredients:**
- 7 tbsp Unsweetened greek style yoghurt
- 1 pinch Cinnamon (ground)
- 2 tbsp Raisins
- 1 tbsp Walnuts, chopped & toasted on a pan

**Directions:**
Dollop about 1 tbsp yogurt into the bottom of a small glass. Add a layer of banana, then some more yogurt. Repeat the layers until the glasses are full. Scatter over the raisins and nuts.

**Notes:**

Butter or Jam or Marmite on Toast

<table>
<thead>
<tr>
<th>Time:</th>
<th>No. Serves:</th>
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</tr>
</thead>
<tbody>
<tr>
<td>5 mins</td>
<td>1</td>
<td>222/197/171</td>
</tr>
</tbody>
</table>

**Ingredients:**
- 2 Medium Thickness Bread (White) 153
- 2 tsp Marmite Spread 18
- 2 tsp Jam 44
- 2 tsp Butter 69

**Directions:**
(type recipe directions here)

**Notes:** Avoid laying spreads on too thick.
### Fruit Salad with Yoghurt

<table>
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<tr>
<th>Time:</th>
<th>No. Serves:</th>
<th>Calories per Serve:</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mins</td>
<td>2</td>
<td>178</td>
</tr>
</tbody>
</table>

**Ingredients:**
- 1 Apple
- 1 Banana
- 1 Kiwifruit
- 100g Yoghurt
- 1 pinch Cinnamon

**Directions:**
- Peel & slice the fruit
- Top with yoghurt & cinnamon

### Weetbix with Milk

<table>
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<tr>
<th>Time:</th>
<th>No. Serves:</th>
<th>Calories per Serve:</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mins</td>
<td>x</td>
<td>166</td>
</tr>
</tbody>
</table>

**Ingredients:**
- 3 biscuits Weetabix
- 150mls Light Blue Milk

**Directions:**
- N/A

**Notes:** Top with a small banana (76 cals), a tablespoon of almonds (90 cals), or a tablespoon of honey (85 cals)
# Brown Toast, Vegemite & Honey

<table>
<thead>
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<th>Time:</th>
<th>No. Serves:</th>
<th>Calories per Serve</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mins</td>
<td>1</td>
<td>317</td>
</tr>
</tbody>
</table>

**Ingredients:**
- 2 slices Brown Bread/Toast
- 1 tsp Vegemite
- 1.5 tsp Honey
- 2 tbsp Butter

**Directions:**
N/A

**Notes:**
## Quick Reference Guide

This is for a quick estimate only. Where possible, check using the Calorie King app or website as these are more accurate.

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</tr>
<tr>
<td>White - Med Slice</td>
<td>29g</td>
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<td>69</td>
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<tr>
<td>White - Thick Slice</td>
<td>37g</td>
<td>1 slice</td>
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<tr>
<td>Multigrain Light Bread</td>
<td>37g</td>
<td>1 slice</td>
<td>89</td>
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<tr>
<td>Long White Roll</td>
<td>27g</td>
<td>1 roll (medium)</td>
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<tr>
<td>Muffin - sweet with fruit</td>
<td>124g</td>
<td>1</td>
<td>314</td>
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<tr>
<td>Cream Crackers</td>
<td>1g</td>
<td>1</td>
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<tr>
<td>Filled Ham Roll</td>
<td>109g</td>
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<td>Bagel</td>
<td>74g</td>
<td>1</td>
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<td>Croissant</td>
<td>75g</td>
<td>1 large</td>
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<tr>
<td>Focaccia</td>
<td>50g</td>
<td>1/8 loaf</td>
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<td>Spreads</td>
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<tr>
<td>Marmite</td>
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<tr>
<td>Butter Margarine</td>
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<td>Canola Oil</td>
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<td>Honey</td>
<td>21g</td>
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<tr>
<td>Jam - Berrythul</td>
<td>16g</td>
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<td>Cereals</td>
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<td>Cornflakes</td>
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<tr>
<td>Weetbix</td>
<td>17g</td>
<td>1 biscuit</td>
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<td>Muesli natural</td>
<td>107g</td>
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<td>Muesli toasted, sweetened</td>
<td>110g</td>
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<td>All-Bran</td>
<td>60g</td>
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<td>Pasta &amp; Rice</td>
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<tr>
<td>Rice, white, boiled</td>
<td>144g</td>
<td>1 cup</td>
<td>21</td>
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<tr>
<td>Spaghetti (canned in sauce)</td>
<td>268g</td>
<td>1 cup</td>
<td>161</td>
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<td>Baked Beans</td>
<td>283g</td>
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<tr>
<td>Pasta (Cooked)</td>
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<td>179</td>
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<td>275g</td>
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<tr>
<td>Gravy (homemade)</td>
<td>260g</td>
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<td>Vegetables</td>
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<tr>
<td>Potato mashed with butter &amp; milk</td>
<td>209g</td>
<td>1 cup</td>
<td>199</td>
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<tr>
<td>Pumpkin, baked</td>
<td>217g</td>
<td>1 cup</td>
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<tr>
<td>Broccoli</td>
<td>164g</td>
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<tr>
<td>Fruits</td>
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<tr>
<td>Raw-apple</td>
<td>130g</td>
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<tr>
<td>Dried-cherries</td>
<td>35g</td>
<td>10 halves</td>
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<tr>
<td>Peaches in canned syrup, drained</td>
<td>208g</td>
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<tr>
<td>Milk - Standard</td>
<td>258g</td>
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<td>Just Juice</td>
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<td>Milo</td>
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<tr>
<td><strong>Meat, Poultry, Dairy etc</strong></td>
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<tr>
<td>Chicken – fish, cooked</td>
<td>135g</td>
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<tr>
<td>Roast Slinn</td>
<td>42g</td>
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<tr>
<td>White Fish, baked</td>
<td>140g</td>
<td>1 fillet</td>
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<td>Tuna in Oil, oil drained (canned)</td>
<td>180g</td>
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<td>Mussels, marinated</td>
<td>160g</td>
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<tr>
<td>Beef Lasagna</td>
<td>370g</td>
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<tr>
<td>Lamb Chops</td>
<td>32g</td>
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<td>Sausages – grilled</td>
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<tr>
<td>Cheese Cheddar</td>
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<tr>
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<tr>
<td>Egg, boiled</td>
<td>50g</td>
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<tr>
<td><strong>Desserts</strong></td>
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<td>Yoghurt – low fat</td>
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<td>Icecream – standard</td>
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<td>Jelly</td>
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<tr>
<td>Cream</td>
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<tr>
<td><strong>Beverages</strong></td>
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<tr>
<td>Long Black</td>
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<td>Flat white, skimmed milk</td>
<td>220 ml</td>
<td>1 Cup</td>
<td>68</td>
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<td>Mocha, skimmed milk</td>
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<td>1 Cup</td>
<td>110</td>
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<tr>
<td>Mocha whole milk</td>
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<td>1 Cup</td>
<td>128</td>
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<tr>
<td>Cappuccino, skimmed milk</td>
<td>220 ml</td>
<td>1 Cup</td>
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</tr>
<tr>
<td>Cappuccino, whole milk</td>
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<tr>
<td>Tea, Herbal</td>
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<tr>
<td>Tea, skimmed milk</td>
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<tr>
<td>Sugar</td>
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<td>1 Teaspoon</td>
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<tr>
<td>White Wine</td>
<td>160 ml</td>
<td>1 Medium Glass</td>
<td>109</td>
</tr>
<tr>
<td>Red Wine</td>
<td>160 ml</td>
<td>1 Medium Glass</td>
<td>94</td>
</tr>
<tr>
<td>Beer (4.9%)</td>
<td>200 ml</td>
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<td>70</td>
</tr>
<tr>
<td>Light Beer (2.7%)</td>
<td>200 ml</td>
<td>1 Glass</td>
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<tr>
<td>Stella Artois (5%)</td>
<td>330ml</td>
<td>1 Bottle</td>
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</tr>
<tr>
<td>Bourbon Whiskey</td>
<td>30ml</td>
<td>1 Measure</td>
<td>62</td>
</tr>
<tr>
<td>Coca Cola</td>
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<td>86</td>
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<tr>
<td>Diet Coke</td>
<td>200ml</td>
<td>1 Glass</td>
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</table>
Sample fast day intakes for a 500 - 600 kcals Target

Each sample is structured as Light Breakfast, a Main Meal and a Small snack (e.g. fruit)

1. 3 weetbix + 150ml milk = 201 kcal
   1 apple = 50 kcal
   1 cup stir fried vegetables + 150g chicken breast = 330 kcal
   = 581 kcal

2. 1 cup porridge (1/2 cup oats) made with water + 50ml milk + 2 tspn honey = 174 kcal
   2 mandarins = 82 kcal
   1 slice blaken bread, 1 egg scrambled with 1 chopped tomato and ½ cup mushrooms and 1 T milk = 218 kcal
   = 497 kcal

3. Smoothie made from 100ml yoghurt, ½ cup milk, ½ banana = 196 kcal
   1 slice bread with ½ cup baked beans + 1 thin slice cheese = 234 kcal
   1 banana = 49 kcal
   = 480 kcal

4. 2 egg omelette with 1 tablespoon grated cheese, ¾ diced onion and 5 cherry tomatoes = 216 kcal
   1 orange = 51 kcal
   1 small tin lentils tuna + ½ cup cooked rice + ½ cup cooked frozen vegetables = 235 kcal
   = 502 kcal

5. 100g steak + coleslaw (1/2 cup cabbage, 1 tbl carrot, 1 tbl oil, 2 tbl vinegar) = 222 kcal
   1 pear = 53 kcal
   2 slices bread, 2 tbl marmite, 2 tbl jam = 229 kcal
   = 504 kcal

6. Pita bread pizza – 1 small pita bread + 2 tsp tomato paste + 50g ham + 2 slices tinned pineapple = 137 kcal
   1 whole banana (small) = 133 kcal
   5 cashew nuts, 5 dried apricots, 1 dessertspoon sunflower seeds = 155 kcal
   = 525 kcal

7. 1 slice multigrain bread (sandwich size) 74 kcal
   26g Panni hummus = 45 kcal
   Wattles condensed tomato soup – 1 tin made with water = 105 kcal
   1 slice whole grain bread (sandwich size) = 74 kcal
   1 cup chopped red pepper = 39 kcal
   100g ham steak grilled = 105 kcal
   1 cup blueberries = 60 kcal
   = 502 kcal
### Sample Meal Planner

<table>
<thead>
<tr>
<th>Day</th>
<th>Breakfast</th>
<th>Morning T</th>
<th>Lunch</th>
<th>Afternoon T</th>
<th>Dinner</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td>Monday</td>
<td>3 Weetabix &amp; Milk + Long Black</td>
<td>Pear</td>
<td>2 slices bread, 2 tsp marmite, 2 tsp jam</td>
<td>None</td>
<td>Red Lentil &amp; Carrot Soup</td>
<td>728</td>
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<tr>
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</tbody>
</table>

Note: The Easy Diet Diary App also contains a Meal planner with an inbuilt calorie counter.
## Your Preferred Meals

### Ciabatta Roll & Banana

- **Time:** 2 min
- **No. Serve:** 1
- **Calories per Serve:** 333

#### Ingredients:
1. Ciabatta Roll (80g) - 206
2. Margarine (thin spread) - 28
3. Banana - Medium (170g with skin) - 99

#### Notes
## Chicken & Broccoli Pasta Bake

| Time:  | 40 mins | No. Serves: | 4 | Calories per Serve: | 919 |

**Ingredients:**
- 350g Pasta Shells
- 200g Broccoli cut into small florets
- 2 tbsp Olive Oil
- 350g Boneless, Skinless Chicken Breasts
- 175g Mushrooms
- 4 tbsp Tomato Paste
- 284ml Can Jon Single Cream
- 4 Spring Onions
- 85g Mature Cheddar
- 1 Garlic Clove
- 50g Flaked Almonds

**Directions:**
1. Preheat the oven to 190C (gas mark 170C). Bring a large pan of salted water to the boil. Throw in the pasta, stir well and return to the boil. Cook for 5 minutes, then add the broccoli and cook for 5-6 minutes more until the pasta is just cooked. Drain well, then return to the pan.
2. Heat the oil in a wide pan, add the chicken pieces and fry until lightly browned. Tip in the mushrooms and stir fry for 1 minute, then stir in the tomato paste and cream. Gently simmer, stirring for 15 minutes to thicken the sauce. Season with salt and pepper.
3. Pour the sauce over the pasta, stirring gently until coated, then tip into a shallow ovenproof dish (about 1.7 litre capacity) and level the top.
4. Mix the spring onions, cheddar, garlic and almonds for the topping and sprinkle over the pasta.
5. Bake for 20 minutes until golden.

## Chicken, Lettuce & Avocado Sandwich

| Time:  | 5 mins | No. Serves: | 1 | Calories per Serve: | 727 |

**Ingredients:**
- 2 slices Sandwich cut (medium) wholegrain bread
- 202
- 202 leaves Lettuce
- 1
- 3 pieces Crumber chicken
- 163
- 1 cup Hot chips
- 223
- 1 tsp Butter (thickly spread - double if thick)
- 35
- 1/2 small Avocado
- 104

**Directions:**
N/A

**Notes:** Without chips: Total is 504 calories Without chips: Total is 504 calories