DIETARY INTERVENTION AND TISSUE REMODELLING

A thesis submitted by

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For my wife, our son, our parents.

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Summary

Increased macrophage infiltration and extracellular matrix deposition in adipose tissue and skeletal muscle are observed in obesity and associated with insulin resistance. Daily calorie restriction (DR) and intermittent fasting (IF) are two dietary approaches to treat obesity. A handful of studies have compared the effects of DR versus IF on metabolic health in humans. However, the impact of these dietary interventions on adipose tissue and skeletal muscle remodelling are poorly investigated. This thesis focuses on the adipose tissue and skeletal muscle remodelling following 8-weeks of DR or IF in humans, and also examines metabolic characteristics and adipose tissue remodelling in lean and diet-induced obese mice following IF.

The study reported in Chapter 3 is the first randomised controlled study to compare continuous and intermittent intake patterns at two energy levels (at energy balance, or 30% energy restriction). This study showed that IF induced greater reductions in body weight, fat mass, homeostatic model assessment of insulin resistance and total cholesterol. However, the mode of dietary restriction did not impact overall insulin sensitivity by the clamp method, and fasting for 24-hours induced transient insulin resistance. In Chapter 4, weight loss by DR and IF reduced fat cell size and stimulated markers of extracellular matrix remodelling, but did not reduce markers of inflammation. In contrast, IF transiently elevated markers of inflammation in adipose tissue and muscle, which was associated with increases in non-esterified fatty acids (NEFA).

To further examine the metabolic profiles and adipose tissue remodelling in response to IF, C57BL/6J mice were fed chow or high-fat diet *ad libitum* for 8-weeks, then subjected to *ad*

libitum feeding or IF for another 8-weeks. The study in Chapter 5 suggests IF promoted fat mass loss and improved glucose tolerance in chow and high-fat diet fed mice, but decreased body weight, and visceral adipose tissue inflammation and fibrosis in high fat diet fed mice only. In contrast to humans, IF did not increase macrophages in adipose tissue in mice, despite marked increases in NEFA.

The mechanisms underlining improved metabolic phenotype in chow and high fat diet fed mice following IF was documented in Chapter 6. Our data shows that IF increased energy expenditure and promoted subcutaneous and visceral adipose tissue browning in both chow and high fat diet fed mice. However in humans, eight weeks of IF did not alter mRNA levels of uncoupling protein 1, a marker of white adipose tissue browning.

The adipose tissue and skeletal muscle remodelling in response to acute overfeeding was described in Chapter 7. This study suggests extracellular matrix remodelling in adipose tissue is an early event in response to over-nutrition, and occurs prior to altered insulin sensitivity by clamp.

In conclusion, this research highlights that energy restricted intermittent fasting promotes greater weight and fat loss, but does not induce greater improvements in insulin sensitivity by clamp versus daily calorie restriction. This thesis also suggests intermittent fasting results in favourable adipose tissue remodelling in mice, and distinct tissue adaptations versus daily calorie restriction in humans.

Declaration

I, Bo Liu, certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Name: Bo Liu

Signature: Date: 22 October 2018

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The effects of calorie restriction and intermittent fasting on metabolic health and adipose tissue remodelling. The State Scientific Meeting of Australian Society for Medical Research, Adelaide, Australia, 2016.

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List of Abbreviations

ACACA: Acetyl-CoA carboxylase alpha

ACTB/Actb: Actin beta

ADF: Alternate day fasting

Adgre1 (F4/80): Adhesion G protein-coupled receptor E1

AL: Ad libitum

ALT: Alanine aminotransferase

ANGPT2: Angiopoietin like 2

ANOVA: Analysis of variance

Arg1: Arginase 1

AST: Aspartate transaminase

AUC: Area under the curve

B2m: Beta-2-microglobulin

B-HB: Beta-hydroxybutyrate

BMI: Body mass index

CALERIE: Comprehensive Assessment of Long-Term Effects of Reducing Intake of Energy

CCL2/Ccl2: C-C motif chemokine ligand 2

CCL3/Ccl3: C-C motif chemokine ligand 3

CCR2: C-C chemokine receptor type 2

cDNA: Complementary DNA

CEBPα: CCAAT/enhancer binding protein alpha

CEBPβ: CCAAT/enhancer binding protein beta

CLS: Crown-like structure

COL1A1/Col1a1: Collagen type I alpha 1 chain

COL3A1/Col3a1: Collagen type III alpha 1 chain

COL6A1/Col6a1: Collagen type VI alpha 1 chain

CR: Calorie restriction

CRP: C-reactive protein

CT: Cycle threshold

CV: Coefficient of variation

DNA: Deoxyribonucleic acid

DR: Daily calorie restriction

DR70: Continuous energy restriction at 70% baseline energy requirements

DXA: Dual-energy X-ray absorptiometry

ECM: Extracellular matrix

EE: Energy expenditure

ELISA: Enzyme linked immunosorbent assays

FASN: Fatty acid synthase

FFM: Fat free mass

FGF21: Fibroblast growth factor 21

FM: Fat mass

Gapdh: Glyceraldehyde-3-phosphate dehydrogenase

GIR: Glucose infusion rate

GLP1: Glucagon-like peptide-1

H & E staining: Hematoxylin and eosin staining

HDL-c: High-density lipoprotein cholesterol

HFD: High fat diet

HIF1a: Hypoxia-inducible factor 1-alpha

HOMA-IR: Homeostasis model assessment of insulin resistance

Hprt: Hypoxanthine guanine phosphoribosyl transferase

Hs-CRP: High sensitive C-reactive protein

IF: intermittent fasting

IF100: Intermittent fasting diet at 100% baseline energy requirements

IF70: Intermittent fasting diet at 70% baseline energy requirements

IFNγ: Interferon gamma

IGF-1: Insulin growth factor 1

IKK: The IκB kinase

IL-10: Interleukin 10

IL-6: Interleukin 6

ingWAT: Inguinal white adipose tissue

iNOS: Inducible nitric oxide synthase

IRS-1: Insulin receptor substrate 1

Itgax (Cd11c): Integrin subunit alpha X

JNK: Jun N-terminal kinase

LDL-c: Low-density lipoprotein cholesterol

Lgals3 (Mac2): Lectin, galactoside-binding soluble 3

LIPE: Lipase E, hormone sensitive type

LPR10: LDL receptor related protein 10

LPS: Lipopolysaccharides

MCP-1: Momocyte chemoattractive protein-1

MIF: Macrophage migration inhibitory factor

MM_E macrophages: metabolically activated macrophages

MMP2/Mmp2: Matrix metallopeptidase 1

MMP9/Mmp9: Matrix metallopeptidase 1

Mrc1 (Cd206): Mannose receptor, C type 1

mRNA: Messenger ribonucleic acid

NEFA: Non-esterified fatty acids

NIA: National Institute on Ageing

OTF-2: octamer transcription factor

OGTT: Oral glucose tolerance test

PAI-1: Plasminogen activator inhibitor-1

PLIN1: Perilipin 1

PPARγ: Peroxisome proliferator-activated receptor gamma

Ppia: Peptidylprolyl isomerase A

PPIB: Peptidylprolyl isomerase B

qPCR: Quantitative polymerase chain reaction

Rn18s: 18S ribosomal RNA

RNA: Ribonucleic acid.

RQ: Respiratory quotient

SAA: Serum amyloid A

SREBF1: Sterol regulatory element-binding transcription factor 1

TC: Total cholesterol

TG: Total glycerides

TGFβ1: Transforming growth factor beta 1

TGFβ: Transforming growth factor beta

TIMP1: TIMP metallopeptidase inhibitor 1

TLR4: Toll like receptor 6

TNFα/Tnfα: Tumour necrosis factor alpha

UCP1/Ucp1: Uncoupling protein 1

VCO_2: Carbon dioxide output

VEGFα: Vascular endothelial growth factor A

VO_2: Oxygen consumption

WNPRC: Wisconsin National Primate Research Centre

Ym1(Chil3): Chitinase-like 3

ZT: Zeitgeber time

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Chapter 1: Introduction

Chapter 1

1.1 Obesity

1.1.1 Definition

Obesity is a condition in which abnormal or excessive adipose tissue accumulates in the body due to positive balance between energy intake and expenditure. Commonly, it is classified using body mass index (BMI), which is defined as a person's body weight in kilograms divided by the square of his or her height in metres (kg/m^2). According to the criteria from the World Health Organisation, for adults, obesity is a BMI greater than or equal to 30. A BMI less than 30 but greater than or equal to 25 is classified as overweight (World Health Organisation, 2018, February 16). Recently, obesity has been recognised as a chronic and preventable disease (Hruby and Hu, 2015).

1.1.2 Prevalence

The prevalence of obesity and overweight has increased rapidly worldwide and in Australia. It was estimated that the worldwide prevalence of obesity nearly tripled between 1975 and 2016. Globally, it was estimated that 39% of adults were overweight and about 13% of the world's adult population were obese in 2016, with a proportion of 11% in men and 15% in women (World Health Organisation, 2018, February 16). If this global trends continue, 18% of men and 21% of women will be obese by 2025 (N. C. D. Risk Factor Collaboration, 2016).

This situation is even more critical in Australia. The latest survey by the Australian Bureau of Statistics revealed that 63.4% of Australian adults were overweight or obese in 2014-2015, and the prevalence of obesity reached 27.9% (Australian Bureau of Statistics, 2015, December 8). A more recent study estimated that the prevalence of obesity for Australian adults would continue to climb, reaching 35% by 2025 (Hayes et al., 2017).

1.1.3 Causes

The causes of obesity are multifactorial. These include excessive calorie intake, physical inactivity, inadequate sleep, socioeconomic status, nutritional insults during intra-uterine growth, and genetic factors (Caballero, 2007, Brisbois et al., 2012, Stettler and Iotova, 2010, Goldstone and Beales, 2008, Fall, 2011, Beccuti and Pannain, 2011). For example, people with high socioeconomic status in low-income areas are more likely to be obese, whilst in high-income countries, those with high socioeconomic status are less likely to be obese (Pampel et al., 2012). Offspring from pregnant mothers who suffered from extreme food deficiency or who were obese have increased risk of adulthood obesity (Parlee and MacDougald, 2014, Liu et al., 2017c, Dabelea, 2007). Mutations in genes encoding factors regulating energy intake/expenditure such as leptin (LEP), leptin receptor (LEPR), proopiomelanocortin (POMC) and melanocortin-4 receptor (MC4R) are linked with severe early-onset obesity (Montague et al., 1997, Clément et al., 1998, Krude et al., 1998, Vaisse et al., 1998).

1.1.4 Impact

Obesity is linked with a number of health issues. Obesity increases the risks for coronary heart diseases, stroke, type 2 diabetes, pulmonary diseases, osteoarthritis and cancer etc., thus leading to increased all-cause mortality (Poirier et al., 2006, Katsiki et al., Zammit et al., 2010, Kahn et al., 2006, Basen-Engquist and Chang, 2011, Flegal et al., 2013). Additionally, obesity causes a significantly economic burden to the health system. It was estimated that obesity accounts for between 0.7% and 2.8% of a country's expenditure in healthcare annually (Withrow and Alter, 2011). But the situation in Australia is more severe. The most recent report revealed that 7% of the total health burden in Australia was due to overweight and obesity (Australian Institute of Health and Welfare, 2017, April 13). In total, 25% of the coronary heart

disease burden, 22% of the stroke burden, 19% of the cancer burden and 53% of the diabetes burden were attributed to overweight and obesity (Australian Institute of Health and Welfare, 2017, April 13).

1.1.5 Lifestyle Management

Lifestyle interventions remain the cornerstone of weight management. These interventions aim to modify eating behaviours and/or physical activity to achieve weight loss, improving health and quality of life (Heymsfield and Wadden, 2017, American College of Cardiology/American Heart Association Task Force on Practice Guidelines, 2014). The advantages of these interventions are low cost with no, or minimal side effects (Heymsfield and Wadden, 2017).

1.2 Calorie restriction

Calorie restriction (CR) is a nutritional approach characterised by reducing nutrient intake without causing malnutrition. In 1935, McCay and colleagues for the first time demonstrated that long-term CR prolonged the maximum lifespan in rats (McCay et al., 1935). Since then, this lifespan extension effect of CR has been repeatedly confirmed in a large spectrum of species, including yeast, nematode, fruit flies, mice and rats (Kapahi et al., 2017, Ingram and de Cabo, 2017). In addition to prolonged lifespan, a growing number of studies in rodents, monkeys and preliminary data in humans have shown that moderate CR can prevent and reverse age-related chronic diseases, such as obesity, insulin resistance, diabetes, and chronic heart disease (Balasubramanian et al., 2017, Ingram and de Cabo, 2017, Kapahi et al., 2017, Most et al., 2017).

1.2.1 Calorie restriction, lifespan and health-span in rodents

Rodents in moderate CR displayed lifespan extension, the degree of which depends on the onset and duration of CR. Rodents that started CR at the age of weaning (3 to 6 weeks) manifested decreased weight gain and an approximately 30-60% increase in maximum lifespan (McCay et al., 1935), and showed a more youthful metabolic phenotype and immunologic responses than the age-matched control (Masoro et al., 1980, Weindruch et al., 1979). Mice of long-lived strains that were subjected to a restricted diet in late adulthood (12-13 months) increased average mean and maximum lifespan by 10-20% compared to *ad libitum* litter mates (Weindruch and Walford, 1982). Of note, initiation of CR at an older age for rodents may not extend, and may even reduce, lifespan. Forster *et al*. reported that CR (-40% energy) in C57BL/6N mice increased lifespan when it was introduced at 4-months of age, but reduced their lifespan when initiated at 24-months of age (Forster et al., 2003). Similar findings with shorter lifespan was observed when CR was implemented in 300 day old Sprague-Dawley rats

(Ross, 1977). Further, the impacts of CR on lifespan also depend on the genotype of the rodents. In a CR study (-40% energy) which included 41 recombinant inbred strains of mice, the lifespan for the majority of the strains was not extended, and notably, nearly 27% of the strains had shortened lifespan (Liao et al., 2010).

CR also prevents the functional and structural changes in multiple organs and tissues, and improves health-span of rodents. CR inhibits spontaneous, chemical- and radiation-induced malignant tumours in several animal models of cancer (Longo and Fontana, 2010). CR can prevent or delay the incidence of chronic kidney disease and heart disease, as well as diabetes (Weindruch et al., 1986, Shimokawa et al., 1993, Masoro, 2005). Moreover, a variety of cytokines such as tumour necrosis factor α (TNFα) and leptin secreted by adipocytes were also reduced by CR (Barzilai and Gupta, 1999). Finally, neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease, Huntington's disease and cerebral stroke in mouse models can be prevented by CR, via decreased neurodegeneration and β-amyloid deposition and increased neurogenesis in the central nervous system (Cohen et al., 2009, Mattson, 2005).

It should be noted that CR may bring some detrimental effects on health-span. CR reduced the bone mineral content and bone mineral density in rodents independent of the age that CR was initiated (Huang and Ables, 2016). CR may also affect immune function causing increased risk of infection and retardation of wound healing (Gardner, 2005, Gardner et al., 2011, Ritz et al., 2008, Clinthorne et al., 2010, Harrison and Archer, 1987, Reiser et al., 1995, Otranto et al., 2009).

1.2.2 Calorie restriction, lifespan and health-span in non-human primates

Currently, there are two groups exploring the long-term impact of calorie restriction in non-human primates, one at the National Institute on Aging (NIA) (Lane et al., 1992), the other at the Wisconsin National Primate Research Centre (WNPRC) (Ramsey et al., 2000a). A large body of evidence shows that calorie restriction significantly improves health-span of non-human primates (Colman et al., 2009, Kemnitz, 2011).

Monkeys undergoing 30% food restriction showed lower body weight compared with *ad libitum* fed control monkeys, in parallel with a decrease of both subcutaneous and visceral adipose tissue measured by dual-energy x-ray absorptiometry or computer tomography (Mattison et al., 2003, Colman and Anderson, 2011, Colman et al., 1999, Ramsey et al., 2000b). Monkeys on a CR diet displayed decreased fasting insulin and glucose, improved glucose tolerance and ameliorated pancreatic β cell function (Mattison et al., 2012, Bodkin et al., 2003, Lane et al., 1995, Kemnitz et al., 1994, Anderson et al., 2009, Hansen and Bodkin, 1993, Gresl et al., 2001), suggesting that CR enhances both insulin secretion and peripheral insulin sensitivity and prevents the onset and development of diabetes in non-human primates. CR also reduces the risk of cardiovascular diseases in rhesus monkeys. Evidence shows that CR lowered blood pressure, total cholesterol and triglyceride, and improved the lipoprotein profiles by increasing high- density lipoprotein (HDL) and reducing very low-density lipoprotein (LDL) (Lane et al., 1999, Edwards et al., 1998, Rezzi et al., 2009, Verdery et al., 1997).

As the beneficial effects of CR in rhesus monkeys are in line with findings in rodents, it is reasonable to speculate that CR may also prolong lifespan of non-human primates. However, whether CR extends lifespan in monkeys is still in debate. The NIA study (Mattison et al., 2012)showed that after 20 years of intervention, CR did not improve survival outcomes in both

adult and aged monkeys. In contrast, the WNPRC study (Colman et al., 2014) reported that monkeys with long-term 30% CR showed significant improvements in age-related and age-caused survival. The authors involved in these two studies compared the distinct outcomes from two groups and pointed out that differences in study design, the source of the monkeys in each study, the age at which CR was initiated, the implementation of the diet for control monkeys, diet composition and feeding time may help explain the divergence between two studies (Mattison et al., 2017, Colman et al., 2014). In the NIA study, Food allotments for the control monkeys were based on their age and body weight in accordance with National Research Council guidelines (Mattison et al., 2012). Monkeys in the WNPRC study however were fed *ad libitum*, which mirrored human feeding habits (Colman et al., 2014). In addition, the diet in the NIA study was lower in fat, and higher in protein and fibre compared to the diet used in the WNPRC study. Thus, control monkeys in the NIA study displayed lower body weight than those in the WNPRC study, which minimised the differences between the intervention and control groups in the NIA study.

1.2.3 Calorie restriction in humans

Since CR prolongs lifespan in rodents and in one study in monkeys, questions have been raised as to whether increased longevity in humans can be achieved. So far, the most convincing evidence is from the prevalence of centenarians on the island of Okinawa in Japan (Kagawa, 1978). Okinawan adults had a 20% reduction of calorie intake along with extended life span compared to people living in mainland Japan (Suzuki et al., 2001, Japanese Ministry of Health, 2005). The mortality rates caused by cerebral vascular disease, malignancy, and heart diseases on Okinawa were significantly lower than those for the rest of Japan (Mizushima and Yamori, 1992).

The CALERIE study (Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy) was the first randomised clinical trial to investigate the long-term impacts of CR in healthy humans. In the first step, CALERIE-1 study was conducted in three centres exploring the impacts of CR after 6-12 months. A series of publications from this programme suggest that CR promoted weight and fat mass loss, and reduced the risk for type 2 diabetes and cardiovascular diseases (Heilbronn et al., 2006, Larson-Meyer et al., 2006, Redman et al., 2007, Weiss et al., 2006, Fontana et al., 2007, Villareal et al., 2006, Das et al., 2007, Pittas et al., 2006, Ahmed et al., 2009). More importantly, this study for the first time found that CR in humans reduced DNA and RNA damage in red blood cells, and increased mitochondrial DNA content in skeletal muscle (Civitarese et al., 2007, Heilbronn et al., 2006, Racette et al., 2006).

Since many of the favourable adaptations occurring in rodents by CR which are considered to contribute to the extended lifespan were not seen in humans in the CALERIE-1 study, a 2-year CALERIE-2 study was launched in multiple research centres (Ravussin et al., 2015). Currently, this study is still ongoing. Preliminary data from this trial suggest that body weight was decreased by 11.5% after 12-months of intervention and maintained at the end of 2 years (10.4%) (Ravussin et al., 2015). Two years of CR significantly reduced circulating inflammatory factors including TNFα and C-reactive protein (CRP) (Ravussin et al., 2015). Additionally, risks for cardiovascular diseases including total cholesterol, total glycerides, mean blood pressure and homeostatic model assessment of insulin resistance (HONA-IR) were significantly decreased (Ravussin et al., 2015).

There are also some non-randomised studies investigating the long-term effects of CR on human ageing. The participants in these studies were members from a Calorie Restriction Society who self-adopted a CR regimen believing this intervention would prolong their

lifespan. These individuals are very lean (BMI<20kg/m^2) and stick to CR (approximate -30%) for an average length of 15 years (Fontana et al., 2004). Compared to age- and gender-matched control individuals, these members displayed a marked decrease in risk factors for type 2 diabetes and cardiovascular diseases, including circulating levels of total cholesterol (TC), triglycerides (TC), LDL, blood pressure, carotid artery intima-media thickness, left ventricular diastolic dysfunction, glucose, insulin, HOMA-IR, CRP, TNFα and interleukin 6 (IL-6) (Fontana et al., 2004, Meyer et al., 2006, Fontana et al., 2010). However, similar to CALERIE study, circulating insulin-like growth factor 1 (IGF-1) levels, which is a key molecule linked with longevity and whose marked decrease was observed in rodent studies by CR (Sonntag et al., 1999), were not affected in these participants who underwent very long-term CR (Fontana et al., 2008).

There are some concerns about CR in humans, too. First, it is difficult to achieve and maintain the prescribed calorie intake, especially long-term. For example, in the CALERIE-1 study at Washington University, the achieved calorie restriction was only -11.5% after 12-months, which was far less than the prescribed -20% (Racette et al., 2006). Similarly, in the CALERIE-2 study, averaged CR during the first 6-months was -19.5 ± 0.8%, and -9.1 ± 0.7% over the next 18 months, which were also less than the prescribed (-25%) (Ravussin et al., 2015). Second, CR causes significant reductions in resting metabolic rate which has been confirmed by doubly-labelled water (Redman et al., 2009), metabolic chamber (Heilbronn et al., 2006) and ventilated hood system (Martin et al., 2007). This decrease in energy expenditure will compromise the expected weight loss. Third, CR decreases bone mineral density in lumbar spine, total hip and femoral neck, which may increase the risk of bone fracture (Villareal et al., 2016).

Overall, these studies show that CR improves health-span, but it is not feasible to perform a randomised controlled trial to test whether CR will prolong lifespan in humans.

1.3 Intermittent fasting

Intermittent fasting (IF) is a dietary intervention characterised by alternating between periods of eating and fasting, typically in 24 hours. In 1946, it was first reported that rats placed onto IF diets displayed prolonged lifespan (Carlson and Hoelzel, 1946).

1.3.1 Intermittent fasting, lifespan and health-span in rodents

Since the first report of prolonged lifespan in IF rats, several following-up studies have confirmed this effect, both in rats and mice (Raffoul et al., 1999). Maximum lifespan was increased by 18-80% in rats, this was consistent with a significant body weight reduction (40% to 50%) compared to *ad libitum* controls, as these rats did not gorge (Goodrick et al., 1982, Goodrick et al., 1983a, Goodrick et al., 1983b, Beauchene et al., 1986). The reasons for such a fluctuation in lifespan extension is not clear, but this is very similar in comparison with CR studies (Weindruch et al., 1988), possibly due to the study design or strain differences. In mice, maximum lifespan is increased (range 11%-56%) (Talan and Ingram, 1985, Ingram and Reynolds, 1987), while body weight changes were not significant. Talan *et al.* (Talan and Ingram, 1985) examined IF in C57BL/6J and found that the mean and maximum lifespan of IF mice was increased by 11% over *ad libitum* fed control, with the mean lifespan 29.2 months in IF and 26.4 months in control, respectively. Goodrick *et al.* (Goodrick et al., 1990) examined the effect of IF started at different ages, and found that mean and maximum lifespan of C57BL/6J mice were extended when an IF regimen was started at 1.5 or 6 months of age. Both of these two studies confirmed a smaller weight gain in IF mice compared to *ad libitum* fed control. However, some other studies reported that body weight was not different between IF and *ad libitum* fed mice (Anson et al., 2003, Varady et al., 2010, Varady et al., 2008), as mice on IF can consume sufficient food on feeding days so that there is no net energy deficit (Anson et al., 2003, Soeters et al., 2009). This phenomenon makes IF quite distinct from CR,

suggesting that fasting *per se,* instead of weight loss, can lead to favourable effects on metabolism.

In addition to lifespan, multiple effects of IF on health-span have been investigated in rodents. Similar to CR, IF delayed the onset of cancer in wild type as well as genetically modified mice which have an accelerated cancer death phenotype (Xie et al., 2017, Siegel et al., 1988). Rats bearing tumour cells that were subjected to IF displayed a longer survival period compared to *ad libitum* fed controls (Siegel et al., 1988). In addition, IF is effective to reduce the risk for diabetes. Animals subjected to an IF protocol displayed lower fasting insulin levels and glucose concentrations after administration of a glucose or insulin load (Anson et al., 2003, Xie et al., 2017, Gotthardt et al., 2016, Joslin et al., 2017). The cardio-protective effects of IF have been investigated in animal models of myocardial infarction. Compared to control animals, IF reduced myocardial infarction size and apoptotic cells, improved the survival and recovery as well as cardiac function (Ahmet et al., 2005, Godar et al., 2015, Katare et al., 2009). Further, IF has been shown to improve the brain function in animal models with neurological disorders, such as Alzheimer's disease and Parkinson's disease (Mattson et al., 2017).

To test whether 100% energy deprivation on fasting day is required for the benefits of IF in mice, modified fasting, which allows 25% food on fasting days has been tested. Modified IF resulted in similarly beneficial effects versus "true" IF that involves zero calorie intake during the fasting periods. Varady *et al.* (Varady et al., 2010, Varady et al., 2007b) found that, despite no significant weight changes, mice with 50% food restriction on fasting day for 4 weeks had reduced adipocyte size, while 75% restriction on a fasting day reduced the proportion of visceral adipose tissue and increased circulating adiponectin levels. These beneficial effects were further confirmed following human studies in which modified IF increased markers of

insulin sensitivity and decreased fat mass and blood lipids (Halberg et al., 2005, Varady et al., 2009).

Since I commenced my PhD, a number of studies have reported the effects of IF in diet induced obese mice (Gotthardt et al., 2016, Joslin et al., 2017, Liu et al., 2017b, Li et al., 2017, Kim et al., 2017). These studies are summarised in **Table 1.1** and will be discussed in **Chapter 5.**

Table 1-1: Studies examining intermittent fasting in diet-induced obese mice

First author and year	Animal and age (weeks)	Diet	Group	Trail length	Fasting protocol	Effect of IF in diet-induce obese mice			
						Weight	Adipose tissue	Glucose metabolism	Other alterations
(Kim et al., 2017)	C57BL/6J Male 8 wks	Chow HFD (45% of fat)	Chow-AL Chow-IF HFD-AL HFD-IF	16-wks IF	2 fed days followed :1 fast day	↑	Fat mass ↓ Fat cell size ↓ Inflammation ↓ M2-macrophage↑	ipGTT↑ ipITT↑	Gonadal fat browning ↑ Liver size ↓ Liver steatosis ↓
(Liu et al., 2017b)	C57BL/6J Male and female 8 wks	Chow HFD (41% of fat)	Chow-AL Chow-IF HFD-AL HFD-IF	12-wks DIO 6-wks IF	ADF	↓ in male → in female	Not assessed	ipGTT↑ ipITT→ in both sexes	Beta cell survival ↑
(Li et al., 2017)	C57BL/6N Male 7-8 wks	Chow HFD (60% of fat)	Chow-AL Chow-IF HFD-AL HFD-IF	3-month DIO 30 days IF	ADF	↓	Fat mass ↓	ipGTT ↑	Inguinal fat browning ↑ Liver steatosis ↓
(Joslin et al., 2017)	C57BL/6J Male 5 wks	Chow HFD (60% of fat)	Chow-AL HFD-AL HFD-IF	14-wks DIO 10-wks IF	ADF	↓	Not assessed	Insulin ↓ ipGTT↑	Leptin↓ Temperature (fast) ↓ Heart rate (fast) ↓
(Gotthardt et al., 2016)	C57BL/6 Male 7 wks	Chow HFD (45% of fat)	HFD-chow(AL) HFD-chow(IF) HFD-HFD(AL) HFD-HFD(IF)	8-wks DIO 4-wks IF	ADF	↓	Total fat mass ↓	Insulin ↓ oGTT → ipITT ↑	Leptin ↓ Ghrelin→

Abbreviations: Chow: rodent chow diet. HFD: high fat diet (% of calorie from fat). DIO: diet-induced obesity. ADF: alternate day fasting. ipGTT: intraperitoneal glucose tolerance test. ipITT: intraperitoneal insulin tolerance test. oGTT: oral glucose tolerance test.
↑: increased versus HFD-AL; ↓: decreased versus HFD-AL; →: no significant change versus HFD-AL.

Table 1-2: Human studies examining the effects of intermittent fasting on metabolism

First author and year	Participants	Trial length	Groups[1]	Diet	Effect of intermittent fasting			
					Weight change	Adipose tissue	Diabetes risk	Other changes
(Hoddy et al., 2014)	n=74 Obese	10 weeks	1. ADF-L 2. ADF-D 3. ADF-SM	ad libitum on feed day; 25% energy on fasting day	↓[3] (all groups)	↓[3] FM and VAT (all groups)	Unchanged	↑[3] LDL particle size; ↓[3] SBP only in ADF-SM
(Varady et al., 2013)	n=32 Nonobese	12 weeks	1. control 2. ADF	As above	↓[2]	↓[2] fat mass	Not measured	↑[2] LDL particle size, plasma adiponection ↓[2] TG, CRP, leptin
(Klempel et al., 2013c)	n=35 Obese	10 weeks	1. ADF-HF 2. ADF-LF	125% energy on feed day; 25% energy on fasting day;	↓[3] (all groups)	Not measured	Not measured	↑[3] LDL particle size (all groups); ↓[3] LDL
(Klempel et al., 2013a)	n=32 Obese	10 weeks	1. ADF-HF 2. ADF-LF	As above	↓[3] (all groups)	Not measured	Not measured	↑ adiponectin, ↓ leptin and resistin in both ADFs
(Eshghinia and Mohammad zadeh, 2013)	n=15 Overweight and obese	8 weeks	No control; 3 days fasting/week	25-30% energy on Mon, Wed, Sat); ad libitum on Friday; 1700-1800 Kcal/d on the rest 3 days	↓[3]	↓[3] fat mass	Unchanged	↓[3] WC, SBP, DBP, TC, TG, LDL
(Varady et al., 2011)	n=16 Obese	10 weeks	No control group	ad libitum on feed day; 25% energy on fasting day	↓[3]	Not measured	Not measured	↑[3] LDL particle size; ↓[4] WC, LDL and TG
(Bhutani et al., 2010)	n=16 Obese	10 weeks	No control group	As above	↓[3]	↓[3] fat mass	Not measured	↑[3] adiponectin, ↓[3] WC, LDL, TG, leptin and resistin
(Varady et al., 2009)	n=16 Obese	10 weeks	No control group	As above	↓[3]	↓[3] fat mass	Not measured	↓[3] TC,TG, LDL,SBP
(Soeters et al., 2009)	n=8 Lean	2 weeks	1. Control 2. Fast for 20-hour	Equal food intake in 2 groups	None	Not measured	Unchanged	↓[2] RMR
(Halberg et al., 2005)	n=8 Healthy	15 days	No control group	ad libitum on feed day; 20h fasting on fasting day	None	Not measured	↑[3] insulin sensitivity	↑[3] adiponectin, lipolysis
(Heilbronn et al., 2005b)	n=16 Nonobese	22 days	No control group	200% energy on feed day; energy-free food on fasting day	↓[3]	↓[3] fat mass	↓[3] fasting insulin,	↑[3] fat oxidation; ↓[3] RQ after prolonged fasting

Abbreviations : TC, total cholesterol; LDL, low density lipoprotein; TG, triglyceride; CRP, C reactive protein; SBP, systolic blood pressure; DBP, diastolic blood pressure; RMR, resting metabolic rate; RQ, respiratory quotient; FM, fat mass; VAT, visceral adipose tissue; WC, waist circumference.

[1]ADF, alternate day fasting; HF, high fat diet (45% fat); LF, low fat diet (25%); ADF-L, meal at lunch time (12pm-2pm); ADF-D, meal at dinner time (6pm-8pm); ADF-SM, three small meals. [2]P<0.05 versus control [3]P<0.05 versus baseline

1.3.2 Intermittent fasting in humans

The limited data available examining IF in humans is summarised in **Table 1.2**. In 2005, Heilbronn *et al.* (Heilbronn et al., 2005b) showed that non-obese subjects that underwent a 24-hour fast every other day for 22 days exhibited decreased body weight, as well as fat mass compared to baseline. In their study, a significant decrease in respiratory quotient was detected and both non-esterified fatty acids and blood ketones were elevated, suggesting increased fat oxidation. Meanwhile, they found that fasting insulin was also decreased compared with baseline, indicating improved insulin sensitivity in humans after IF (Heilbronn et al., 2005b). Very shortly after, Halberg *et al*. (Halberg et al., 2005) found that healthy men subjected to IF for 15 days had increased insulin-mediated glucose uptake rate, and increased lipolysis and plasma adiponectin, suggesting improved insulin sensitivity (Halberg et al., 2005). Unlike the fasting every other day protocol in Heilbronn's study, participants in Halberg's study were fasted for 20-hours on alternate days (started at 2200 and ended at 1800 the following day), thus individuals maintained their body weight (Halberg et al., 2005). Since then, several modified IF studies have been performed. Unlike the "true" IF conducted in most rodent studies, these experimental designs have allowed participants up to 25% of daily baseline energy intake on the fasting day. Modified IF resulted in significant weight loss in individuals with obesity and decreased TC, LDL, TG, leptin and resistin, and increased adiponectin (Varady et al., 2009, Bhutani et al., 2010). However, to our knowledge, there is no study examining insulin sensitivity with the gold standard method of hyperinsulinaemic-euglycaemic clamp. As long-term IF studies have not been performed, whether IF expands lifespan in humans is unknown.

1.4 Studies comparing calorie restriction versus intermittent fasting

To date, a number of mice studies have been performed comparing the effects of CR versus IF. In 1987, Ingram *et al*. (Ingram and Reynolds, 1987) examined mice undergoing CR or IF and found that IF produced greater increases in maximum lifespan (56% in IF and 36% in CR, respectively). Since then, several follow-up studies have further examined metabolic phenotypes in these two diet regimens. Anson *et al*. (Anson et al., 2003) randomised 9-week old mice to one of the following four groups: ad libitum (AL), CR (-40%), alternate day fasting (ADF) and ADF paired-fed (ADF-PF; daily food equal to the average daily intake of ADF). During the intervention, mice in ADF consumed a roughly equivalent amount of food in a 48-hour cycle as AL mice, leading to a similar body weight compared to AL. Mice in CR had a 49% smaller weight gain after 20 weeks intervention. Interestingly, both CR and ADF mice, but not ADF-PF displayed lower fasting glucose and insulin vs. AL, indicating that intermittent fasting, rather than restricted energy intake resulted in these beneficial effects. Varady *et al*. (Varady et al., 2007a, Varady et al., 2007b, Varady et al., 2008) compared CR vs. IF in mice and showed that CR and IF similarly reduced visceral fat mass, increased blood adiponectin, reduced the proliferation rates of T cells and prostate cells, and decreased blood IGF-1 levels. Of note, the "hunger hormone" ghrelin was only increased in mice following alternate day fasting. These studies, as well as modified IF mice studies are summarised in **Table 1.3**.

Table 1-3: Animal studies comparing intermittent fasting vs. calorie restriction on metabolism[1]

Reference	Animal	Trail length	Groups[2] diet	Effect of IF vs. IF			
				Weight change	Adipose tissue	Diabetes risk	Other alterations
(Varady et al., 2010)	n=30 C57BL/6J Female Age 8 weeks	4 weeks	1. AL 2. CR-25% 3. ADF-75% 4. ADF-85% 5. ADF-100%	↓[3] in CR-25%	↑[4] Subcutaneous fat ↓[4] Visceral fat (all intervention groups)	Not measured	↑[4] Adiponection, TG-glycerol synthesis and de novo lipogenesis (all intervention groups)
(Varady et al., 2008)	n=30 C57BL/6J Female Age 8 weeks	4 weeks	1. AL 2. CR-25% 3. ADF-75% 4. ADF-85% 5. ADF-100%	↓[3] in CR-25%	Not measured	Not measured	↓[4] Cell proliferation in CR-25%, ADF-85% and ADF-100%; ↓[4] IGF-1 in CR-25% and ADF-100% ↑[3] ghrelin in ADF-100%
(Varady et al., 2007b)	n=24 C57BL/6J Male Age 8 weeks	4 weeks	1. AL 2. ADF-25% 3. ADF-50% 4. ADF-100%	↓[5] in ADF-100%	↓[4] adipose size in ADF-50% and ADF-100%	Not measured	↑[3] Net lipolysis in ADF-100% ↑[4] FFA: in all ADFs
(Varady et al., 2007a)	n=24 C57BL/6J Male Age 8 weeks	4 weeks	1. AL 2. ADF-25% 3. ADF-50% 4. ADF-100%	↓[3] in ADF-100%	Not measured	Not measured	↓[4] Proliferation rates of T-cells and prostate cells in ADF-50% and ADF-100% ↓[3] IGF-1 in ADF-100%
(Anson et al., 2003)	n=32 C57BL/6 Male Age 6 weeks	20 weeks	1. AL 2. ADF 3. CR-40% 4. PF	↓[3] in CR-40%	Not measured	↓[6] glucose in CR and ADF ↓[7] insulin in CR and ADF	↑[4] IGF-1 in ADF and PF ↓[3] IGF-1in CR-40% ↑[3] β-HB in ADF ↓[3] β-HB in CR-40%

[1] DM, diabetes mellitus; FFA, free fatty acid; TG, triglyceride; IGF-1, insulin-like growth factor 1; β-HB, β-hydroxybutyrate.

[2] AL, ad libitum; ADF, alternate day fasting; CR-25%, 25% energy restriction; CR-40%, 40% energy restriction; ADF-25%,75% energy intake on fasting day; ADF-50%, 50% energy intake on fasting day; ADF-85%, 15% energy intake on fasting day; ADF-100%, no food intake on fasting day; PF, pair-fed mice (daily food allotment equal to IF);

[3] Post-intervention values of intervention groups significantly different from post-intervention values of control group, $P<0.05$.

[4] Post-intervention values of intervention groups significantly different from post-intervention values of control group, no difference between indicated intervention groups $P<0.05$.

[5] Post-intervention values of indicated group significantly different from post-intervention values of ADF-25% and ADF-50%,control group, no difference between indicated and control $P<0.05$.

[6]Post-intervention values of intervention groups significantly different from post-intervention values of control group and PF, no significant difference between indicated intervention groups $P<0.05$.

[7] Post-intervention values of intervention groups significantly different from post-intervention values of control group, significant difference between ADF and IF groups $P<0.05$.

In humans, Harvie *et al.* compared an IF diet versus CR in two separate cohorts of women over 3 or 6 months (Harvie et al., 2011, Harvie et al., 2013). The design of this diet was slightly different in that the 2 fasting days were prescribed consecutively, allowing 25% of energy requirements, followed by *ad libitum* consumption for 5 days per week. IF resulted in similar weight loss at 6-months vs. CR (Harvie et al., 2011). Of note, the IF group was assigned a meal replacement during the 2 days of severe energy restriction, whereas the CR group was prescribed a conventional food based diet. Meal replacements typically show better adherence and weight loss than conventional food based diets (Harvie et al., 2011). However, a subsequent 3-month study by this group implemented a food based program in both groups, and also reported similar weight loss following modified IF versus CR (6% vs 4%, P=0.1). A greater reduction in fat mass was observed in modified IF at 3 months, and this group continued to lose significantly more weight (-0.5kg) versus CR (-0.1kg) one month after cessation of the active weight loss phase. A slightly greater reduction in fasting insulin in the intermittent diet at 3 and 6 months was also observed (Harvie et al., 2011, Harvie et al., 2013).

Two clinical trials comparing the effects of IF versus CR have been recently published since we conducted the human study. They are summarised in **Table 1.4** and will be discussed in **Chapter 3**.

It should be noted that none of these studies examined the metabolic adaptations on both fed and fasted days (Harvie et al., 2011, Harvie et al., 2013, Trepanowski et al., 2017, Catenacci et al., 2016). It is not clear whether energy restriction is necessary for intermittent fasting to have beneficial effects on health in humans.

Table 1-4: Human studies comparing intermittent fasting vs. calorie restriction

Reference	Participants (N, age, BMI)	Study duration	Group	Fasting protocol	Effects of IF vs. CR			
					Adiposity	Diabetes risk	CVD risk	Inflammation
(Trepanowski et al., 2017)	14 men 86 women 18-65 yrs 25.0-40 kg/m^2	6-month weight loss 6-month follow-up	Control CR IF	Alternate day fasting; 25% of energy requirement provided on fast day; 125% of energy requirement on fast day; Total energy matched with CR.	*6-month*			
					WL: ns FML: ns	Glu, Ins and HOMA-IR: ns	HDL: ↑ TG, LDL and TG: ns	CRP: ns
					12-month			
					ns	ns	ns	ns
(Catenacci et al., 2016)	6 men 19 women 18-55 yrs 30-52kg/m^2	8-week IF 24-week follow-up	CR IF	Alternate day fasting; Zero food on fast day; Energy not prescribed on fed days	*8-weeks*			
					WL: ns FML: ns	Glu and Ins: ns	TC, HDL, LDL and TG: ns	not assessed
					32-week			
					ns	ns	ns	not assessed
(Harvie et al., 2013)	115 women Age$_{mean}$>45yrs 24-45kg/m^2 and/or fat mass>30%	3-month weight loss 1-month weight maintenance	CR IF	2 consecutive fasting days/week, 30% of energy requirement provided on fast day. Weekly energy matched with CR (-25%) 1 fasting day/week during weight maintenance phase	*3-month*			
					WL: ns FML: ↑	Glu: ns Ins and HOMA-IR: ↑	not assessed	not assessed
					4-month			
					ns	ns	not assessed	not assessed
(Harvie et al., 2011)	107 women 30-45 yrs 24-40 kg/m^2	6-month IF	CR IF	2 consecutive fasting days per week 25% food on fast day	WL: ns FML: ns	Glu: ns Ins and HOMA-IR: ↓	TC, HDL, LDL and TG: ns	CRP: ns

Abbreviations: BMI: body mass index. CR: daily calorie restriction. IF: intermittent fasting. WL: weight loss. FML: fat mass loss. Glu: fasting glucose. Ins: fasting insulin. HOMA-IR: homeostatic model assessment of insulin resistance. CVD: cardiovascular diseases. TC: total cholesterol. LDL: low-density lipoprotein. HDL: high-density lipoprotein. TG: triglycerides. CRP: C-reactive protein.

ns: no difference in the change between IF versus CR. ↑: greater increases in IF vs. CR. ↓greater reductions in IF vs. CR.

1.5 Adipose tissue remodelling in obesity, calorie restriction and intermittent fasting.

Adipose tissue is the main site of nutrient storage in the body and accounts for most of the weight gained during obesity. It consists of both cellular and non-cellular components. Adipocytes are the main cell type in adipose tissue to store energy in the form of lipid. Immune cells including macrophages, T cells and neutrophils infiltrate into adipose tissue, playing important roles in the regulation of adipocyte function and systemic metabolism. Additionally, extracellular matrix (ECM), which is a non-cellular structure provides mechanical and nutritional support to neighbouring cells.

Adipose tissue displays great plasticity with a series of events occurring in response to nutrient availability, namely adipose tissue remodelling (Sun et al., 2011). During chronic over-nutrition, fat pads expand to store excess energy. This process is achieved through the enlargement of adipocyte size (hypertrophy) and/or new adipocyte generation (hyperplasia) (Martinez-Santibanez and Lumeng, 2014). Monocytes are rapidly recruited into adipose tissue and are polarised to classically activated M1-macrophages, which release pro-inflammatory molecules (e.g. TNFα, IL-6 and MCP1) contributing to the development of insulin resistance (Fujisaka et al., 2013, Lumeng et al., 2007a, Aron-Wisnewsky et al., 2009). Owing to the nutrient excess, the synthesis of ECM exceeds its degradation, leading to extra ECM deposition in adipose tissue (Martinez-Santibanez and Lumeng, 2014). This consequently restricts the expansion of adipocytes, causing lipid deposition in non-storage tissues such as liver and muscle, which in turn impairs insulin sensitivity (Khan et al., 2009, O'Hara et al., 2009, Li et al., 2010). Moreover, ECM may be also involved in the recruitment of macrophages into adipose tissue through ECM-macrophage crosstalk (Martinez-Santibanez and Lumeng, 2014). To catch up with the adipose tissue expansion, new blood vessels are generated to provide

nutritional support (Martinez-Santibanez and Lumeng, 2014). However, some evidence suggests this does not fully compensate the demand from adipose tissue leading to hypoxia and the activation of inflammatory cascades (Ye et al., 2007). The association between adipose tissue macrophages, extracellular matrix and insulin sensitivity will be reviewed in **Chapter 2.**

Some studies have also examined the acute effects of weight gain by overfeeding on adipose tissue remodelling in humans. Current evidence pointed out that the weight gained by 4-8 weeks of overfeeding led to impaired insulin sensitivity and increased ECM synthesis in adipose tissue, without altering macrophage accumulation in adipose tissue (Tam et al., 2010, Tam et al., 2014). However, it is not clear whether ECM remodelling in adipose tissue is an early contributor to, or a consequence of, insulin resistance in obesity.

The effects of CR on adipose tissue have been extensively studied in both animals and humans. Moderate CR decreases fat mass as well as fat cell size in both mice and humans (Varady et al., 2007b, Larson-Meyer et al., 2006). However, it appears that the decrease in fat mass is mainly contributed by the decreased fat cell size, as some evidence suggests that fat cell number remains constant following marked weight loss (Spalding et al., 2008). The adaptation of macrophages in response to CR is dependent on its length and severity. For example, mice in the early stage of CR displayed increased macrophage accumulation in adipose tissue (Kosteli et al., 2010). This phenomenon was also observed in humans on a very low calorie diet (Capel et al., 2009). Following marked weight loss, macrophages in adipose tissue were significantly reduced (Magkos et al., 2016, Zamarron et al., 2017). Interestingly, some studies have suggested that this is accompanied by a phenotype switch from inflammatory M1- to anti-inflammatory M2- profiles (Fabbiano et al., 2016). The responses in ECM are also somewhat controversial. A number of mouse and human studies have demonstrated that CR reduced the

mRNA levels of collagens and increased the mRNA of matrix metalloproteinase (MMPs) which degrade ECM (Zamarron et al., 2017, Magkos et al., 2016), however, most of these studies are limited to the measurement of gene expression. A clinical trial quantifying collagen content in subcutaneous adipose in participants following bariatric surgery showed that collagen deposition was actually increased after marked weight loss (Liu et al., 2016).

The impacts of IF on adipose tissue remodelling are less investigated and mainly limited to mice studies. Varady *et al* reported that 4-weeks of IF decreased visceral fat mass as well as fat cell size, and this change was also observed in a modified IF approach (Varady et al., 2007b, Varady et al., 2010). The effects of IF on adipose tissue inflammation is unclear. However, some studies showed that an acute 24-hour fast increased macrophage accumulation in adipose tissue in mice (Kosteli et al., 2010, Asterholm et al., 2012, Ding et al., 2016). This increase coincided with elevated NEFA levels and was proposed to buffer the lipids released from adipocytes(Kosteli et al., 2010). How IF affects ECM remodelling in adipose tissue is unclear.

1.6 Skeletal muscle remodelling in obesity, calorie restriction and intermittent fasting

Skeletal muscle contains thousands of muscle fibres which are bundled together and surrounded by a layer of extracellular matrix (Tabebordbar et al., 2013). Immune cells, including macrophages and T cells reside in muscle fibres, secrete a number of cytokines which are associated with muscular contraction, repair and regeneration (Pillon et al., 2013). Recently, it has been suggested that skeletal muscle undergoes remodelling in obesity and in response to dietary interventions (Wu and Ballantyne, 2017, Martinez-Huenchullan et al., 2017).

In mice, significant increases in macrophage and inflammation-associated markers, including *Emr1*, *Cd11c*, *Tnfα* and *Ccl2* were detected in skeletal muscle after 1-week of high-fat diet feeding, and remained higher at 10-weeks, along with impaired glucose tolerance (Fink et al., 2014). Of note, a follow up study confirmed this finding and pointed out that increased macrophages are predominantly located in extramyocellular adipose tissue, which is closely linked with insulin sensitivity (Khan et al., 2015).

Obesity also affects muscle extracellular matrix remodelling through regulating its synthesis and degradation. Compared with lean mice, diet-induced obese mice displayed higher levels of collagen genes including *Col1a1*, *Col1a2*, *Col3a1*, *Col6a1* and *integrin* which are key components of extracellular matrix (Kang et al., 2011, Inoue et al., 2013). Some evidence suggests obesity alters the expression and activity of MMPs, which belong to a zinc endopeptidase enzymatic superfamily and function to degrade extracellular matrix. Biga *et al* suggested that C57BL/6J mice fed a high fat diet (60%) for 6 weeks displayed increased mRNA levels and activity of MMP2 and MMP9 (Biga et al., 2013). In contrast, Kang *et al* demonstrated that 14-20 weeks of high fat diet feeding decreased MMP9 activity without

altering MMP2 (Kang et al., 2011). Discrepancy of these studies could be due to the diet or length of high fat diet feeding. But overall, these data suggest that obesity may cause a net increase in extracellular matrix deposition in skeletal muscle as revealed by histology (Kang et al., 2011).

How obesity affects human muscle remodelling is less known and remains controversial. Some evidence suggests that macrophage accumulation in muscle is positively linked with obesity and insulin resistance (Patsouris et al., 2014, Varma et al., 2009, Fink et al., 2014). However, there are a growing number of studies showing that muscle macrophages are not associated with obesity and insulin sensitivity (Amouzou et al., 2016, Tam et al., 2012b), or are negatively correlated with obesity and insulin resistance (Fink et al., 2013, Liu et al., 2017a). Limited evidence suggests individuals with obesity display increased extracellular matrix deposition in skeletal muscle (Martinez-Huenchullan et al., 2017, Berria et al., 2006).

Evidence that examined the impacts of CR on muscle remodelling is even scarcer. Kayo *et al* reported that 9-year CR in male rhesus monkeys down-regulated a number of genes in inflammatory responses including human leukocyte antigen DM, octamer transcription factor 2 and *iNOS* in muscle (Kayo et al., 2001). Surprisingly, genes involved in ECM synthesis were up-regulated, such as *COL1A1*, *COL1A2*, *COL3A1*, *COL6A3* and *COL8A1* (Kayo et al., 2001). Yang *et al* assessed skeletal muscle adaptations in a group of lean adults (BMI 19.2±1.1kg/m^2) from the Calorie Restriction Society who maintained a CR diet for 3-15 years (Yang et al., 2016). They found that a CR diet significantly decreased a number of factors in inflammation pathways in muscle, including nuclear factor kappa B subunit 1 (NF-κB), *TNFα*, *IL*-6, IL-8, and *iNOS* compared to age-matched control adults (BMI 25.3±2.3kg/m^2). However, the impact

of long-term CR on muscle extracellular matrix remodelling was not examined in this study. The impacts of IF on muscle remodelling is unknown.

1.7 Research questions

The general aims of this thesis are to answer the following questions:

1. Is energy restriction necessary for intermittent fasting to have beneficial effects on health in humans?
2. Does intermittent fasting increase adipose tissue and skeletal muscle inflammation in mice and humans?
3. Does intermittent fasting improve metabolic heath in chow and high fat diet fed mice?
4. Does intermittent fasting reduce adipose tissue fibrosis in mice and humans?
5. Does white adipose tissue browning contribute to improved health by intermittent fasting in mice and humans?
6. Does short-term over-nutrition trigger adipose tissue and skeletal muscle remodelling in humans?

1.8 Specific Aims and hypotheses

Study 1: Effects of intermittent versus continuous energy intakes on insulin sensitivity and metabolic risk in women who are overweight or obese

Aims:

1. To compare intermittent fasting versus continuous energy intakes at 100% or 70% of calculated energy requirements on peripheral insulin sensitivity, weight and body composition, and cardio-metabolic outcomes.
2. To explore acute metabolic changes that occur when switching between a fed (i.e. after a 12-h overnight fast) and fasted (24-h fast) state.

Hypotheses:

1. Intermittent fasting without energy restriction will improve markers of metabolic health.
2. Intermittent fasting with energy restriction may be more effective than daily calorie restriction to improve insulin sensitivity, promote weight and fat loss and reduce cardiovascular risk.
3. The "metabolic switching" between the fed and fasted states that is characteristic of IF may underlie the observed health benefits of intermittent fasting.

Study 2: Adipose tissue and skeletal muscle remodelling following eight-weeks of intermittent fasting or daily calorie restriction in women who are overweight or obese

Aim:

To compare the effects of intermittent fasting versus calorie restriction on adipose tissue and skeletal muscle remodelling.

Hypothesis:

Intermittent fasting and calorie restriction may reduce fibrosis, but intermittent fasting may increase inflammation in adipose tissue and skeletal muscle.

Study 3: Does intermittent fasting improve metabolic health in chow and high fat diet fed mice?

Aims:

1. To examine the effects of intermittent fasting on body weight and glucose tolerance.
2. To assess the effects of intermittent fasting on adipose tissue inflammation and fibrosis.

Hypotheses:

1. IF will improve glucose tolerance in chow fed mice without weight loss.
2. IF will improve glucose tolerance and promote weight loss in high fat diet fed mice.
3. IF will reduce adipose tissue fibrosis, but may increase adipose tissue inflammation.

Study 4: Does intermittent fasting promote white adipose tissue browning in mice and humans?

Aims:

1. To examine the effects of intermittent fasting on food intake and energy expenditure in chow and high fat diet fed mice.
2. To assess markers of white adipose tissue browning in mice and humans undergoing intermittent fasting.

Hypothesis:

Intermittent fasting will increase energy expenditure and promote white adipose tissue browning in mice, and will promote white adipose tissue browning in humans.

Study 5: Adipose tissue and skeletal muscle remodelling in response to acute overfeeding in young adults.

Aim:

To examine adipose tissue and skeletal muscle remodelling in response to 3 days of overfeeding in young adults.

Hypothesis:

Markers involved in adipose tissue and skeletal muscle remodelling will be sensitive to 3 days of overfeeding, and associated with markers of insulin resistance.

Chapter 2: Literature Review

Chapter 2

Do Adipose Tissue Macrophages Promote Insulin Resistance or Adipose Tissue Remodelling in Humans?

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Overall percentage (%)	30%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.		
Signature		Date	29 May 2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Drafted the manuscript		
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Chapter 2

2.1 Abstract

In diet induced and genetically obese rodent models, adipose tissue is associated with macrophage infiltration, which promotes a low grade inflammatory state and the development of insulin resistance. In humans, obesity is also closely linked with macrophage infiltration in adipose tissue, a pro-inflammatory phenotype and insulin resistance. However, whether macrophage infiltration is a direct contributor to the development of insulin resistance that occurs in response to weight gain, or is a later consequence of the obese state is unclear. There are a number of concomitant changes that occur during adipose tissue expansion, including the number and size of adipocytes, the vasculature and the extracellular matrix. In this review, we will examine evidence for and against the role of macrophage recruitment into adipose tissue in promoting the development of insulin resistance in rodents and humans, as well as discuss the emerging role of macrophages in mediating healthy adipose tissue expansion during periods of caloric excess.

Key words: adipose tissue remodelling; inflammation; insulin resistance; macrophages; obesity

2.2 Obesity is a low grade inflammatory state

The prevalence and severity of obesity is increasing rapidly in the majority of countries worldwide, and is highly associated with insulin resistance and an increased risk of developing type 2 diabetes, cardiovascular disease, and cancer. However, the precise mechanisms linking adipose tissue expansion and insulin resistance are unclear. Adipose tissue was originally considered an inert organ, whose sole function was the storage of lipid for energy. Metabolically, this is important and insufficient adipose storage has been linked to lipid storage in ectopic sites and insulin resistance (Heilbronn et al., 2004a). However, adipose tissue is now recognised to be an active endocrine organ that secretes numerous "adipokines", which have remote actions in tissues including brain, skeletal muscle and liver, as well as paracrine and autocrine actions. Many of the cytokines that are secreted from adipose tissue are pro-inflammatory, and obesity was first described as a low-grade inflammatory condition more than two decades ago. In this study, Hotamisligil *et al*. (Hotamisligil et al., 1993) showed that diet-induced obese rodents displayed elevations in circulating and adipose tissue levels of tumour necrosis factor alpha (TNFα). Since this seminal work, human and rodent obesity has been linked with altered secretion of many other inflammatory adipokines, including interleukin-6 (IL-6), plasminogen activated inhibitor (PAI-1), serum amyloid A (SAA), migration inhibitory factor (MIF), resistin, inducible nitric oxide synthase (iNOS), colony stimulating factor-1 (CSF1) and monocyte chemoattractant protein-1 (MCP-1).

Diet and lifestyle interventions that result in weight loss and improvements in insulin sensitivity reduce the circulating and local expression of pro-inflammatory cytokines (Ryan and Nicklas, 2004), which have been shown to directly inhibit the insulin signalling cascade (Krogh-Madsen et al., 2006, Lumeng et al., 2007c) via inhibitor kB Kinases (IKK), JUN N-terminal Kinase (JNK) and inhibitory serine phosphorylation of insulin receptor substrate-1 (IRS-1). In humans,

TNFα infusion also reduced insulin-stimulated glucose uptake (Krogh-Madsen et al., 2006), whilst chronic exposure to IL-6 increased hepatic and peripheral insulin resistance in mouse (Klover et al., 2003) and mice lacking TNFα are protected from obesity-induced insulin resistance (Uysal et al., 1997).

2.3 Adipose tissue macrophage infiltration and polarisation in obesity

Adipose tissue obtained from obese humans and rodents is infiltrated by an increased number of macrophages (Weisberg et al., 2003, Xu et al., 2003). This was first shown by two groups in 2003 (Weisberg et al., 2003, Xu et al., 2003), where it was identified that the stromal vascular fraction was responsible for the majority of the pro-inflammatory phenotype that is observed in obesity. Their work suggested that this increase in macrophage number was limited to adipose tissue, although other studies have since described increases in inflammatory cells in other tissues (Stanton et al., 2011, Fink et al., 2014). Macrophages are mononuclear phagocytes involved in immunological and inflammatory processes, whose function is to provide an immediate defence against foreign organisms and to clear resultant cellular debris (Fain et al., 2004, Maury et al., 2007). Macrophages can form crown-like structures (CLS) that completely surrounding necrotic adipocytes and fuse to form lipid containing giant multi-nucleated cells, that stain strongly for MAC2 (Cinti et al., 2005). Other immune cells are also present in adipose tissue, including neutrophils, mast cells, B-cells, T-cells and increased in obese animal and human models, although few T-cells were detected in subcutaneous adipose tissue collected from overweight and moderately obese individuals in our hands (Tam et al., 2010).

Whilst, the total number of adipose tissue macrophages correlates strongly with the degree of obesity and insulin resistance (Cancello et al., 2005), it has been reported that there is also a change in the type of macrophage (Morris et al., 2011). “M1” macrophages are pro-

inflammatory and release factors such as *TNF*, *IL6*, *iNOS, CD40, CD11c* and *MCP1*, whereas "M2" macrophages express an anti-inflammatory gene profile that is characterised by higher expression of *TGFβ, Ym1, arginase 1, CD206 and Il10.* Diet induced obese mice have increased expression of genes that are characteristic of M1 or "classically activated" macrophages (Fujisaka et al., 2013, Lumeng et al., 2007a). Increased M1 macrophages have also been detected in subcutaneous adipose tissue from morbidly obese *vs*. lean individuals (Aron-Wisnewsky et al., 2009). Further, surgery-induced weight loss altered the M1/M2 ratio due to a concomitant decrease in M1 and increase in M2 macrophages, which was correlated with the change in insulin sensitivity (Aron-Wisnewsky et al., 2009). However, there is poor expression of many of these markers in humans, and some have shown that obesity is associated with increased M2, as well as M1 macrophages (Bourlier et al., 2008, Fjeldborg et al., 2014, Xu et al., 2013). This controversy highlights that simple classification into "M1" and "M2" macrophages is not entirely appropriate since macrophage phenotype spans a continuum *in vivo*. Recently, Kratz *et al*. (Kratz et al., 2014) reported that cell surface markers CD274, CD38 and CD319 were classical M1 markers, since they were activated by lipopolysaccharide (LPS) and elevated in airway macrophages collected from patients with cystic fibrosis. In their hands, these cell surface markers were not elevated in subcutaneous or visceral adipose tissue collected from obese patients or rodents, even though these tissues displayed a pro-inflammatory phenotype. Kratz *et al*. (Kratz et al., 2014) went on to show that treatment of macrophages *in vitro* with metabolic activators such as glucose, insulin, palmitate and visceral adipose tissue conditioned media, increased cell surface expression of markers ABCA1, CD36 and PLIN2, but did not elevate CD274 and CD38 or M2-markers CD206 and TGF1b. This was mirrored in the subcutaneous and intraperitoneal adipose tissue depots obtained from obese mice and from obese humans. This study suggested that a distinct subset of macrophages may exist in adipose tissue, termed "metabolically activated macrophages, (MM_E)". Whether this

holds true in less obese populations, and if MM_E macrophages are differentially altered in response to weight loss or gain is currently unknown.

What causes macrophage infiltration of adipose tissue?

Numerous theories have been proposed that may explain macrophage recruitment into adipose tissue in the obese state, including increased adipose cell size and necrosis, local hypoxia and nutritional endotoxemia.

Adipocyte size and necrosis

Adipose tissue can expand through adipocyte hyperplasia or hypertrophy (Heilbronn et al., 2004a). Adipocyte size is clearly increased in obesity, and displays a strong independent association with insulin resistance and progression to type 2 diabetes (Weyer et al., 2000, Dubois et al., 2006). Adipocyte hypertrophy is also a strong predictor of the number of macrophages in adipose tissue (Weisberg et al., 2003, Tchoukalova et al., 2007). Interestingly, adiponectin overexpressing transgenic ob/ob mice have increased fat mass, but reduced adipocyte size and are protected from macrophage infiltration and inflammation in adipose tissue, and have a healthy metabolic phenotype despite massive obesity (Kim et al., 2007). The mechanisms underlying the potential relationship between adipocyte size and macrophage infiltration may be multifactorial. Larger adipocytes display an altered secretion of chemoattractant and immune-related genes that may promote macrophage infiltration (Jernas et al., 2006). Large adipocytes also have increased lipolysis (Laurencikiene et al., 2011, Michaud et al., 2014), which may stimulate macrophage infiltration as has been shown to occur in response to prolonged fasting, as well as pharmacologically (Kosteli et al., 2010). Finally, the rate of adipocyte death is also elevated in obesity (Cinti et al., 2005, Giordano et al., 2013). Immunohistochemistry shows that the CLS surround necrotic adipocytes and the phagocytic

ingestion of these adipocytes increases the lipid content of CLS, which protects against lipid leak (Altintas et al., 2011, Wu et al., 2011). This beneficial action of the macrophage may limit the continuation of the inflammatory response.

Local adipocyte hypoxia

Hypoxia occurs when oxygen availability does not match demand by the surrounding tissue. In rodent models of obesity, increased adipose tissue hypoxia is observed alongside increased expression of hypoxia-inducible factor-alpha (HIF1α) and TNFα (Ye et al., 2007). The hypoxic area in adipose tissue of obese animals coincides with macrophage infiltration (Rausch et al., 2008) and also induces M1 polarity of macrophages (Fujisaka et al., 2013). Adipocyte-specific HIF1α knockout results in improved glucose tolerance, decreased macrophage infiltration, and reduced MCP-1 and TNFα expression in adipose tissue versus wild type (Kihira et al., 2014). Suppression of HIF1α by administration of antisense oligonucleotides results in weight loss, increased energy expenditure and decreased fasting blood glucose in diet-induced obese mice (Shin et al., 2012), although this study did not examine whether infiltration of macrophages and adipose tissue inflammation was altered. Together, these data suggest that hypoxia promotes macrophage infiltration into adipose tissue, and that HIF1α may mediate this, at least in part. However, whilst adipose tissue hypoxia is established in obese mouse models (Hosogai et al., 2007, Ye et al., 2007, Rausch et al., 2008), there is conflicting evidence as to whether adipose tissue hypoxia is a feature of human obesity (Pasarica et al., 2009b, Goossens and Blaak, 2012). Pasarica et al (Pasarica et al., 2009b) showed that the partial pressure of oxygen was lower in adipose tissue from obese individuals versus lean individuals, and that this was in parallel with increased macrophage markers in adipose tissue and insulin resistance. However, Goosens et al. (Goossens and Blaak, 2012) noted that while there was low oxygen in adipose tissue, the metabolic demands of adipose tissue from obese individuals

was reduced and detected no net hypoxia in these individuals. The reason for this potential species difference is not clear, and more work is required to test whether adipose tissue in humans is hypoxic and whether localised hypoxia is a mechanism through which macrophages are recruited to adipose tissue.

Nutritional endotoxaemia

LPS activates and drives the M1 phenotype of macrophages via Toll-like receptor -4 (TLR4) and NF-κB, leading to transcription of pro-inflammatory cytokines (eg TNFα, IL-6, MCP-1) (Hoch et al., 2008, Leuwer et al., 2009). Numerous studies have examined the consequences of TLR4 deficiency, and with mixed results. Some studies have observed reductions in circulating inflammatory cytokines and macrophage infiltration of adipose tissue and improved insulin sensitivity (Shi et al., 2006, Suganami et al., 2007, Saberi et al., 2009), but this is not observed universally (Orr et al., 2012). Kratz *et al.* (Kratz et al., 2014) also recently showed that activation of "MME" macrophages was independent of TLR4. Thus, the role of TLR4 in mediating this relationship is still under debate. What is clear is that mice that are fed a high fat diet have higher LPS (Cani et al., 2007). This may be due to changes in gut microbiota, and increasing intestinal permeability by modulating the expression of genes coding for tight junction proteins (Cani et al., 2007, Cani et al., 2008, Lam et al., 2012). Interestingly, transfer of gut microbiota of obesity prone-rats increased adipose tissue macrophages, T-cell infiltration, TNFα, PAI1 and IL-6 versus transfer of gut microbiota from obesity-resistant rats (Duca et al., 2014). Treatment with antibiotics also reduced gut microbiota and activation of TLR4, and this was associated with reduced adipose tissue inflammation, as assessed by the numbers of CLS and F4/80^{+} macrophages (Cani et al., 2008, Carvalho et al., 2012). Interestingly, the mean size of adipocyte in antibiotic group was also reduced, suggesting there may be a role for gut in promoting adipose tissue remodelling (Cani et al., 2008). The

mechanisms underlying relationships between specific types of bacteria in the gut, obesity and inflammation is still in its infancy and little evidence is currently available in humans. Nonetheless, gut permeability may mediate some of the inflammatory response via TLR4 in intraperitoneal adipose tissue.

Local proliferation

Quite recent data suggests that tissue resident macrophages may also be capable of local proliferation (Jenkins et al., 2011, Hashimoto et al., 2013), and that the proliferating macrophages were M2-polarised (Bourlier et al., 2008). Haase et al (Haase et al., 2014) observed that the ki67^{+} positive proliferating macrophages were associated with CLS in human subcutaneous and visceral adipose tissue, although the absolute numbers were very low. Further work is required to understand whether local proliferation of macrophages contributes significantly to the total macrophage pool, and if this is altered in the obese state.

2.4 Are adipose tissue macrophages responsible for obesity-induced insulin resistance?

Genetic mouse models

Many genetic approaches to reduce macrophage infiltration of adipose tissue prevent the deleterious consequences of obesity. For example, RIP140 mice that have been engineered to have a reduction in the circulating monocyte populations have an altered ATM profile in white adipose tissue, with a dramatic reduction in inflammatory M1 and an expansion in M2 macrophages, which was associated with improved insulin sensitivity (Liu et al., 2014). Whole body CCL2 knockouts similarly had reduced inflammation, decreased macrophage infiltration in adipose tissue and improved insulin sensitivity when exposed to high fat diet (Lumeng et al., 2007b) and transgenic adipose tissue specific overexpressing CCL2 mice

display increased macrophage infiltration and insulin resistance (Kamei et al., 2006). This suggests that reducing the inflammatory response improves insulin sensitivity in obese mice. However, this is not universally observed (Inouye et al., 2007) and studies examining knockout of C-C motif chemokine receptor-2 (CCR2, the receptor for CCL2) have also produced mixed outcomes in terms of protection from diet induced insulin resistance (Weisberg et al., 2006, Lumeng et al., 2007a, Gutierrez et al., 2011). On the other hand, ablation of $CD11c^{+}$ cells increased insulin sensitivity in diet induced obese mice (Patsouris et al., 2008) and macrophage specific JNK1/2 knockouts were protected from macrophage infiltration of adipose tissue, and insulin resistance (Han et al., 2013). In summary, many studies using genetic manipulation to reduce macrophage infiltration of adipose tissue show an insulin sensitive phenotype. However, this is not universally observed. The reason for these discrepancies may be in the type of high fat diet imposed, the length of the overfeeding protocols and the genetic background of the mice under investigation.

Diet and Lifestyle Interventions

Substantial weight loss induced by bariatric surgery is insulin sensitising and significantly reduces the total number of macrophages in adipose tissue and induces a switch in macrophage polarisation towards an M2 phenotype, with increased staining for the anti-inflammatory cytokine *IL10* and reduced expression of *MCP1* and *HIF1*α (Cancello et al., 2005). Weight loss following a diet and lifestyle intervention also reduced adipose tissue expression of *CD14, CD68, IL-6, IL-8, and TNF*α (Bruun et al., 2006). Both of these studies examined very large changes in adiposity in morbidly obese individuals, who have a range of co-morbidities. However, studies in less overweight individuals have shown similar results (Auerbach et al., 2013). Exercise also significantly reduced circulating levels of LPS and TLR4 activation, lowered M1 macrophage infiltration, and improved insulin sensitivity in mice fed a high fat

diet (Kawanishi et al., 2013, Oliveira et al., 2013). The temporal response to caloric restriction in diet induced obese mice was recently examined (Kosteli et al., 2010). In this study, macrophage infiltration increased initially in response to caloric restriction, with the peak in adipose tissue macrophage numbers coinciding with the peak of circulating free fatty acids and adipose tissue lipolysis. Later time-points were associated with reduced macrophage infiltration. In humans, no change was observed in adipose tissue macrophage numbers after 4 weeks of very low calorie diet in moderately obese females, whereas macrophages were significantly reduced following moderate calorie restriction and weight loss over the subsequent 4 months (Kovacikova et al., 2011). These data suggest that lifestyle interventions will reduce the inflammation and macrophage infiltration in adipose tissue in obese, but that this may not occur rapidly in response to caloric restriction.

Therapeutic Interventions

Treatment with thiazolidinediones, an agonist of PPARγ, reduced macrophage number in adipose tissue, in parallel with improved insulin sensitivity by triggering apoptosis of M1 macrophages and increasing M2 macrophages (Bodles et al., 2006, Spencer et al., 2014). Administration of a recombinant adenovirus Glucagon-like peptide-1 (GLP-1) to diabetic ob/ob mice reduced fat mass, adipocyte size, M1 macrophages, and production of inflammatory cytokines (Lee et al., 2012). Dipeptidyl peptidase-4 inhibitor, which acts by inhibiting the degradation of GLP-1 or glucose-dependent insulinotropic polypeptide also reduced total macrophage numbers and M1 macrophage staining in adipose tissue as well reduced mRNA expression of inflammatory cytokines, IL-6, TNFα (Kim et al., 2005, Dobrian et al., 2011, Shirakawa et al., 2011). Angiotensin type 1 receptor (AT1R) blocker, a classical anti-hypertensive, decreased macrophage infiltration in adipose tissue of high fat diet-induced obese mice (Cole et al., 2010) and a similar response was observed in a randomised clinical trial in

humans (Goossens et al., 2012). Together, this data suggests that medications that target glucose regulation by either increasing insulin secretion and/or insulin sensitivity also reduce macrophage infiltration and inflammation in adipose tissue.

Experimental Overfeeding

There is conflicting evidence as to whether macrophage infiltration occurs early during weight gain, and thus could be responsible for the insulin resistant phenotype. A rapid induction of inflammatory gene expression was observed in adipose tissue in response to high fat diet (Chen et al., 2005, Brake et al., 2006), with significant increases in circulating levels of plasma MCP1 detected after 4-weeks (Chen et al., 2005). However, Xu *et al*. (Xu et al., 2003) quantified macrophage numbers by histology and could only detect a significant increase in macrophage infiltration after 8 weeks of high fat diet. This preceded the rise in plasma insulin in their study, indicating that macrophage infiltration occurred prior to development of insulin resistance. One elegant study examined the temporal response to high fat diet, and observed that adipocyte hypertrophy and hypoxia preceded adipocyte necrosis and macrophage infiltration, which were elevated at 8 and 16 weeks (Strissel et al., 2007). In this study, the increase in macrophage infiltration coincided with impaired responsiveness to insulin tolerance test. However, gold standard measures of insulin sensitivity, the hyperinsulinemic clamp were not performed. Temporal investigation of diet induced insulin resistance shows that whole-body insulin resistance, as measured by hyperinsulinaemic-euglycaemic clamp, is detected after 1 week but that this is due to impaired hepatic insulin sensitivity. Adipose tissue is insulin resistant after 1 week, while skeletal muscle displays insulin resistance by 3 weeks of high fat exposure (Turner et al., 2013).

Experimental overfeeding studies in humans show a much slower rate of weight gain, typically less than 2% per week (Bouchard et al., 1990). This is likely a very different stimulus to the 4-5% weight gain that occurs when susceptible mice are exposed to overfeeding diets (Strissel et al., 2007). In our hands, overfeeding for 28 days resulted in less than 5% weight gain, with significant increases in total and abdominal fat mass and liver fat deposition (Samocha-Bonet et al., 2010). In parallel, increases were observed in fasting insulin and reductions were observed in peripheral insulin sensitivity by hyperinsulinaemic clamp. Importantly, this study showed that peripheral insulin resistance occurred, without significant changes in macrophage infiltration of subcutaneous adipose tissue as demonstrated using flow cytometry, immunohistochemistry and gene expression (Tam et al., 2010). Similarly, few changes were detected in circulating markers of inflammation, with the exception of the predominately liver derived cytokines CRP and Fetuin-A (Samocha-Bonet et al., 2014). We also observed no changes in adipocyte cell size, but it should be noted this was biopsied from subcutaneous abdominal adipose tissue. Similar results have since been reported following modest weight gains in other experimental overfeeding studies in humans that also induced insulin resistance (Alligier et al., 2012, Johannsen et al., 2014). This data suggest that low-grade systemic and subcutaneous adipose tissue inflammation, macrophage infiltration and immune activation likely occurs secondary to weight gain and peripheral insulin resistance, at least in humans. However, interpretation of these findings is limited to subcutaneous abdominal adipose tissue. Visceral adipose tissue contains more macrophages potentially contributing to the well described adverse effects of visceral adiposity (Cancello et al., 2006, Harman-Boehm et al., 2007, Ortega Martinez de Victoria et al., 2009) and may be more important in this response. Tchoukalova *et al*. (Tchoukalova et al., 2010) also showed that multiple subcutaneous sites should be sampled since 4-weeks of overfeeding increased adipocyte size, but not adipocyte

number, in abdominal sites and increased adipocyte number without changes in adipocyte size in femoral sites.

2.5 Does macrophage infiltration promote healthy adipose tissue expansion?

Obesity and type 2 diabetes is associated with hypertrophic adipocytes and a reduced proportion of pre-adipocytes within adipose tissue (Tchoukalova et al., 2007). We, and others, have theorised that some individuals have reduced ability for adipogenesis, promoting adipocyte hypertrophy, ectopic lipid deposition and insulin resistance (Danforth, 2000, Heilbronn et al., 2004a). This theory however, was not supported in a recent human overfeeding study (Johannsen et al., 2014). Whilst some studies suggest that macrophages suppress adipogenesis, this evidence was produced *in vitro* (Constant et al., 2006, Hammarstedt et al., 2007). I*n vivo*, the opposite response has been observed (Sadler et al., 2005, Wernstedt Asterholm et al., 2014). This difference may be because *in vitro* there is no need for angiogenesis or remodelling of the extracellular matrix (ECM) to accommodate the increasing adipocyte mass. Mice were recently engineered to express either a dominant negative (dn) version of TNFα or dnRIDα/β, which inhibits a number of inflammatory pathways (TLR4, TNFα and IL1-β), specifically in adipose tissue (Wernstedt Asterholm et al., 2014). Both models displayed reduced adipose tissue inflammation but were glucose intolerant, had increased fibrosis and hepatic steatosis when exposed to a high fat diet. Mice also displayed similar to larger adipocytes versus controls, despite having reduced fat pad weights. This suggests that blocking the inflammatory response specifically in adipose tissue impaired the ability of this tissue to expand, and led to ectopic lipid accumulation and the development of insulin resistance. Strissel *et al*. (Strissel et al., 2007) showed significant increases in adipogenesis following 20-weeks of exposure to high fat diet, which was subsequent to

macrophage infiltration at 8-16 weeks, providing indirect evidence to suggest that macrophage infiltration promotes adipogenesis *in vivo*. Wang *et al*. (Wang et al., 2013) examined adipogenesis in various adipose depots during high fat overfeeding. Epididymal fat pads were increased by 84% and subcutaneous fat pads were increased by 163% in response to 12-weeks exposure. After 7 days, very little adipogenesis was noted and there was no change in the size of the adipocyte. After 5-weeks, adipocyte hypertrophy was observed. Adipocyte hyperplasia was observed at 12 weeks in the epididimal fat pad but was still minimal in the subcutaneous fat pads at 12-weeks. This study did not extend to 20-weeks, and macrophage infiltration was not examined.

Remodelling of adipose tissue involves not only changes to adipocyte, but also to the extracellular matrix and vasculature to accommodate the expanding mass. Morbidly obese individuals display reduced capacity for angiogenesis in subcutaneous adipose tissue versus lean (Gealekman et al., 2011), and this has been linked to impaired adipose tissue expandability (Rupnick et al., 2002, Nishimura et al., 2007). Vascular endothelial growth factor (VEGF) is a key factor in angiogenesis and is highly expressed in macrophage (Cursiefen et al., 2004, Walczak et al., 2004). Serum VEGF levels are increased in obesity, but capillarization in adipose tissue is reduced in obese humans. Recent studies utilising mouse models provide conflicting evidence as to whether VEGF-A prevents or accelerates metabolic dysfunction (Sun et al., 2012, Cao, 2013, Wu et al., 2014). In vitro, administration of anti-VEGF antibodies inhibited angiogenesis and the formation of adipogenic and angiogenic cell clusters, suggesting that this is essential for differentiation of adipocytes. Debels *et al*. (Debels et al., 2013) examined a murine tissue engineering model following addition of zymogen as an inflammatory agent, to a Matrigel matrix. In this context, macrophages were observed at 2-weeks, along with immature new blood vessels. At 4-weeks, blood vessels had matured, and

immature adipocytes were observed, with mature adipocytes observed at 6-weeks. Importantly, knockout of macrophages reduced both angiogenesis and adipogenesis, suggesting that macrophages promote healthy adipose tissue expansion, at least in this type of environment.

The extracellular matrix consists of structural and adhesion molecules such as collagens, glycoproteins and fibronectin that provide a structural lattice for cellular adherence, organisation and communication. Deposition and breakdown of the ECM is fundamental in the repair processes in response to inflammation, and is distinct from the abnormal deposition of ECM proteins, which is a process known as "fibrosis". In obesity, fibrosis in adipose tissue and other organs is clearly increased (Henegar et al., 2008, Sun et al., 2013), and some studies have shown this can persist despite massive weight loss in subcutaneous adipose tissue (Henegar et al., 2008). It is unclear what contributes to excessive ECM accumulation in obesity. *In vitro*, pro-inflammatory macrophages have been identified as one such target, contributing to fibrosis development and maintenance (Keophiphath et al., 2009). In humans, Pasarica *et al* showed increased adipose tissue expression of *COL6A3* mRNA, in parallel with increased $CD68^+$ and $CD163/MAC2^+$ macrophages and increased MIP and MCP1 expression (Pasarica et al., 2009a). Further, obese subjects with expression of COL6A3 above median had increased visceral adipose tissue mass, increased MIP1 and MCP1, and fewer smaller adipocytes. Weight loss reduced the expression of ECM genes in adipose tissue (Kolehmainen et al., 2008) and overfeeding increased expression of COL6A3 in men, suggesting that rapid changes in the ECM are needed to accommodate the changing adipose mass (Pasarica et al., 2009b). Interestingly, diet induced obese mice lacking Col6A1 have an unrestricted expansion of adipocyte size, fewer crown like structures representing regions of adipocyte death and inflammation and improved glucose control (Khan et al., 2009), suggesting that fibrosis increases the rigidity of adipose tissue and restricts adipocyte expansion. Few studies have

examined the temporal response of ECM proteins to experimental overfeeding. Alligier *et al*. examined the effects of a moderate increase in energy intake at 14 and 56 days. Increases in lipid metabolism genes were observed at 14 days, followed by increases in ECM and genes related to adipogenesis and angiogenesis at 56 days, which was supported by histological examination (Alligier et al., 2012). Similarly Tam *et al*. reported significant increases in adipose tissue expression in collagens I and II and TGFβ after 10% weight gain, although no changes were noted in expression of inflammatory genes in either study suggesting ECM remodelling was independent of macrophage infiltration (Tam et al., 2014).

2.6 Conclusions

Obesity is clearly associated with an increase in the total number of macrophages in adipose tissue, and increased secretion of pro-inflammatory factors and chronic low grade inflammation, all of which may contribute to the development of insulin resistance. However, we and other have shown that macrophage infiltration is not an immediate response during experimental overfeeding in humans, despite expanding adipose mass and insulin resistance. Studies in mouse suggest that macrophage infiltration occurs about 8-16 weeks after initiation of high fat diet, which is typically after the development of insulin resistance. This disconnect between macrophage infiltration and insulin resistance, is also observed in many genetic manipulation studies in mice, and raises some questions as to whether macrophages are causal in the development of the insulin resistant phenotype. Studies investigating the temporal response to exposure to high fat diet suggest that macrophage infiltration during weight gain may be transitory, and promote healthy adipose tissue expansion, at least initially (**Figure 2.1**). Moreover, blocking this response may promote unhealthy adipose tissue expansion, and ectopic lipid deposition. Further work is required to ascertain whether we can selectively

activate macrophages to promote healthy adipose tissue expansion, and therefore potentially improve metabolic health.

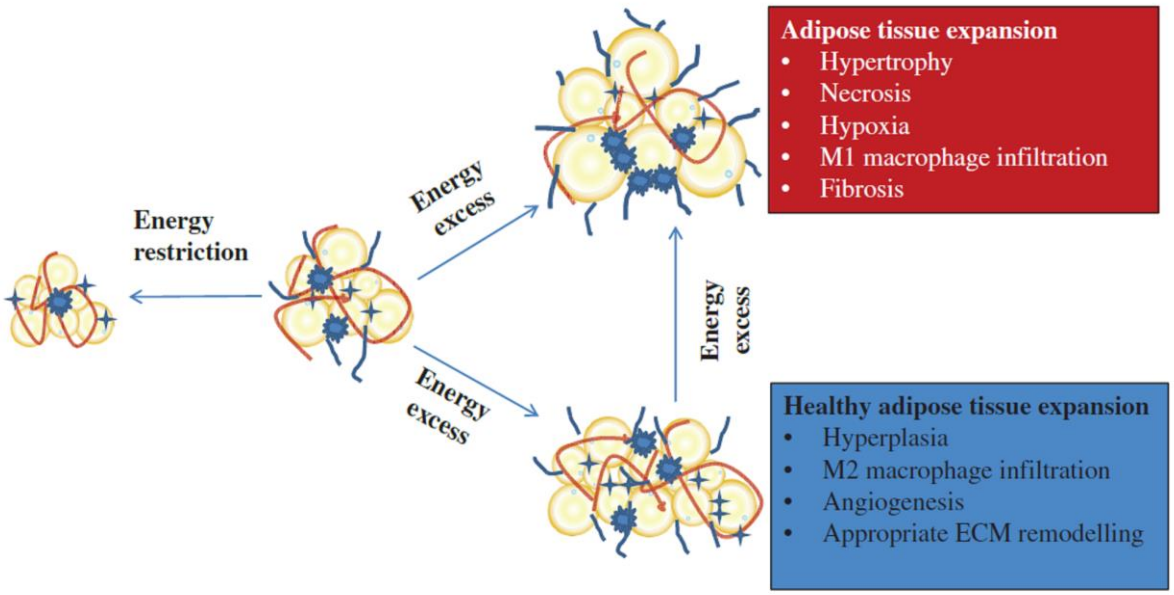

Figure 2-1: Macrophage infiltration in adipose tissue

Macrophage infiltration in adipose tissue is a dynamic process that is reduced following energy restriction, and increased upon exposure to prolonged energy excess. During weight gain, adipocyte hypertrophy, necrosis and M1 macrophage infiltration occur. Macrophage infiltration may then promote healthy adipose tissue expansion, at least initially, via adipogenesis, angiogenesis, and remodelling of the extracellular matrix, improving the metabolic milieu of adipose tissue. Failure of this process leads to unhealthy adipose tissue expansion including pro-inflammatory activation of macrophages, adipocyte hypertrophy, adipocyte hypoxia, and fibrosis (Heilbronn and Liu, 2014).

Author conflicts of interest

The authors have no conflicts to declare.

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Chapter 3: Effects of intermittent versus continuous energy intakes on insulin sensitivity and metabolic risk in women with overweight

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Name of Principal Author (Candidate)	Bo Liu		
Contribution to the Paper	Collected data, performed the metabolic visits, performed laboratory analysis, and analysed data.		
Overall percentage (%)	30%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.		
Signature		Date	11 May 2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Collected data, performed the metabolic visits, performed laboratory analysis, and analysed data.		
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Continued on next page

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Contribution to the Paper	Designed the study, provided clinical support and supervised clamps.		
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Continued on next page

Name of Co-Author	Leonie K Heilbronn		
Contribution to the Paper	Designed and supervised the study, had primary responsibility for the final manuscript		
Signature		Date	29/5/18

What is already known about this subject?

- Intermittent fasting with energy restriction produces similar reductions in body weight, and cardiovascular risk as daily energy restriction, and may have modest additional benefits to reduce markers of insulin resistance (e.g., HOMA-IR, fasting insulin).
- The acute changes in metabolic parameters during fed and fasting states, have not been reported widely, nor in this population.
- No studies to date have assessed changes in insulin sensitivity by hyperinsulinaemic-euglycaemic clamp in response to intermittent fasting after both a fed, and fasted day.

What does this study add?

- This is the first randomised controlled study to compare continuous and intermittent intake patterns at two energy levels (at energy balance, or 30% energy restriction).
- Intermittent fasting with energy restriction induced greater reductions in weight, fat mass losses, and greater reductions in total and LDL-cholesterol and non-esterified fatty acids (NEFA) compared with continuous daily restriction.
- Intermittent fasting did not impact insulin sensitivity when examined in the fed state, but was transiently impaired following a 24-hour fast.

3.1 Abstract

Objective: To compare intermittent fasting (IF) versus continuous energy intakes at 100% or 70% of calculated energy requirements on insulin sensitivity, cardiovascular risk, body weight and composition.

Methods: Women with overweight (N=88; 50.2±0.9 years, BMI 32.3±0.5 kg/m^2) were randomised to one of four diets (IF70, IF100, dietary restriction [DR70], or Control) in a 2:2:2:1 ratio for 8 weeks. IF groups fasted for 24-h after breakfast on 3 non-consecutive days/week. All foods were provided and diets matched for macronutrient composition (35% fat, 15% protein, 50% carbohydrate). Insulin sensitivity by hyperinsulinaemic-euglycaemic clamp, weight, body composition and plasma markers were assessed following a "fed" day (12-h fast), and a 24-h fast (IF only).

Results: The IF70 group displayed greater reductions in weight, fat mass, total- and low-density lipoprotein-cholesterol (LDL-cholesterol) and non-esterified fatty acids compared with DR70 and IF100 (all P ≤0.05). IF100 lost more weight and fat than Control, however fasting insulin was increased. There were no differences in insulin sensitivity by clamp, however, a 24-h fast transiently reduced insulin sensitivity.

Conclusion: When prescribed at matched energy restriction, IF reduced weight and fat mass, and improved total and LDL-cholesterol more than DR. IF prescribed in energy balance failed to improve markers of metabolic health, despite modest weight loss.

Key words: cardiovascular risk; continuous dietary restriction; insulin sensitivity; intermittent fasting; weight loss

3.2 Introduction

Continuous dietary restriction (DR) remains the cornerstone lifestyle intervention to reduce the risk of developing type 2 diabetes and cardiovascular disease in individuals who are overweight (Neter et al., 2003, Look Ahead Research Group et al., 2007, Wing et al., 2011, Diabetes Prevention Program Research, 2015, Goodpaster et al., 1999). Due to the inherent difficulty associated with long-term adherence to DR (Scheen, 2008, Moreira et al., 2011), alternative approaches are being investigated.

Intermittent fasting (IF) involves alternating periods of eating with fasting periods of up to 24 hours, for 1-4 days/week. In mice, intermittent 24-hour fasting results in favourable redistribution of adipose tissue (Varady et al., 2010), reduced fasting glucose and insulin (Anson et al., 2003), and improved cardiovascular health (Mattson and Wan, 2005). In most of these studies, the metabolic health benefits were observed with minimal weight differences (Varady et al., 2010) or versus pair fed controls (Anson et al., 2003), suggesting that fasting may be the stimulus required to improve health.

Studies in humans have shown that IF reduces weight and fat mass, total cholesterol, LDL-cholesterol, triglycerides, postprandial lipemia and fasting insulin (Heilbronn et al., 2005b, Antoni et al., 2018, Varady et al., 2009, Hoddy et al., 2014, Varady et al., 2013), while others have shown no significant improvements in metabolic health, despite weight loss (Bhutani et al., 2013, Williams et al., 1998). To our knowledge, five studies have compared an intermittent versus continuous dietary approach for 2-12 months (Catenacci et al., 2016, Harvie et al., 2011, Harvie et al., 2013, Trepanowski et al., 2017, Antoni et al., 2018). These studies have shown that IF and DR produce similar reductions in body weight and markers of cardiovascular risk. However, Harvie *et al* reported that IF reduced body fat and fasting insulin more than DR

(Harvie et al., 2011). Two studies have examined insulin sensitivity by hyperinsulinaemic-euglycaemic clamp after IF (Halberg et al., 2005, Soeters et al., 2009), but none have compared this with DR. Moreover, changes in metabolic parameters between fed and fasted states have only been examined in lean men (Halberg et al., 2005). This is important since "metabolic switching" between fed and fasting states, rather than weight loss, may underlie the health benefits of IF (Antoni et al., 2017, Anton et al., 2018).

The aims of this study were to conduct a randomised, controlled trial, in women who were classified as overweight or obese to: 1) compare the effects of intermittent versus continuous food intakes at two energy levels on peripheral insulin sensitivity, weight and body composition, and cardio-metabolic outcomes; and 2) to explore the acute metabolic changes that occur when switching between a fed (i.e., after a 12h overnight fast) and fasted (24h fast) state.

3.3 Participants and Methods

Participants

Between 1 March 2013 and 4 September 2015, 119 women were screened following advertisement in local newspapers and media to participate in this single-centre, randomised controlled trial in Adelaide, South Australia (**Fig. 3.1**). A total of 88 women were enrolled in the study. Inclusion criteria were: aged 35-70 years; BMI 25-42 kg/m^2; self-reported to be weight-stable (within 5% of their screening weight) for >6 months prior to study entry; non-diabetic; non-smoker; sedentary or lightly active (i.e., <2 moderate to high-intensity exercise sessions per week); consumed <140 g alcohol/week; no personal history of cardiovascular disease, eating disorders or major psychiatric disorders (including those taking antidepressants); not pregnant or breastfeeding; and not taking medication that may affect study outcomes (e.g., phentermine, orlistat, metformin, excluding antihypertensive/lipid lowering medication). The Royal Adelaide Hospital Research Ethics Committee approved the study, and all participants provided written, informed consent prior to their inclusion. The study was registered with Clinicaltrials.gov (NCT01769976).

Randomisation and masking

The active trial period was 10 weeks, including a 2-week lead-in, and an 8 week intervention. During the lead-in, participants consumed their normal diet and maintained their weight. Following this, participants were randomly assigned in a 2:2:2:1 ratio to one of the four diets; 1) IF70: intermittent fasting diet at 70% of calculated baseline energy requirements per week; 2) IF100: intermittent fasting diet at 100% of calculated baseline energy requirements per week (i.e., weight maintenance); 3) DR70: continuous restriction at 70% of calculated baseline energy requirements daily; 4) Control: 100% of calculated baseline energy requirements daily. Daily energy requirements were calculated by averaging predicted daily energy expenditure

from two published equations, both of which use age, gender, height and weight variables (Institute of Medicine (U.S.). Panel on Macronutrients. and Institute of Medicine (U.S.). Standing Committee on the Scientific Evaluation of Dietary Reference Intakes., 2005, Redman et al., 2009). Block randomisation (4 or 8 participants) was performed by a research officer, with stratification by BMI (<=32.9 or >=33 kg/m^2) and age (<=49.9 or >=50 years). Nine participants withdrew from the study. Seven no longer wished to participate, and two were withdrawn by the investigators; one for pre-existing bronchial issues unrelated to the study, and one due to gastrointestinal surgery that was not disclosed during the screening process (completers: DR70: n=24, IF70: n=22, IF100: n=22, Control: n=11).

On fed days, IF70 were provided with~100%, and IF100 ~145% of energy requirements. IF groups consumed breakfast before 8am on fasting days (~32% of energy requirements was given at breakfast on fasting days to IF70, and ~37% to IF100; **Appendix table 1**), then commenced a ~24 hour "fast" until 8 am the following day, on 3 non-consecutive weekdays/week. During the fast, participants were allowed water and small amounts of energy-free foods (e.g., "diet" drinks, chewing gum, mints), black coffee and/or tea, and were provided with 250 mL of a very low energy broth (20 kcal/250 mL, 2.0g protein, 0.1g fat, 3.0g carbohydrate) for lunch or dinner. All diets were matched for macronutrient composition (35% fat, 15% protein, 50% carbohydrate). Participants were free-living, and foods were delivered fortnightly to their home, excluding fresh fruit and vegetables. Portions of fruits and vegetables were standardised (1 'serve' of fruit = 150g of fresh fruit or 30g dried fruit; 1 'serve' of vegetables = 75g raw, steamed or boiled vegetables) and participants self-selected according to the number of serves specified in their individual menus.

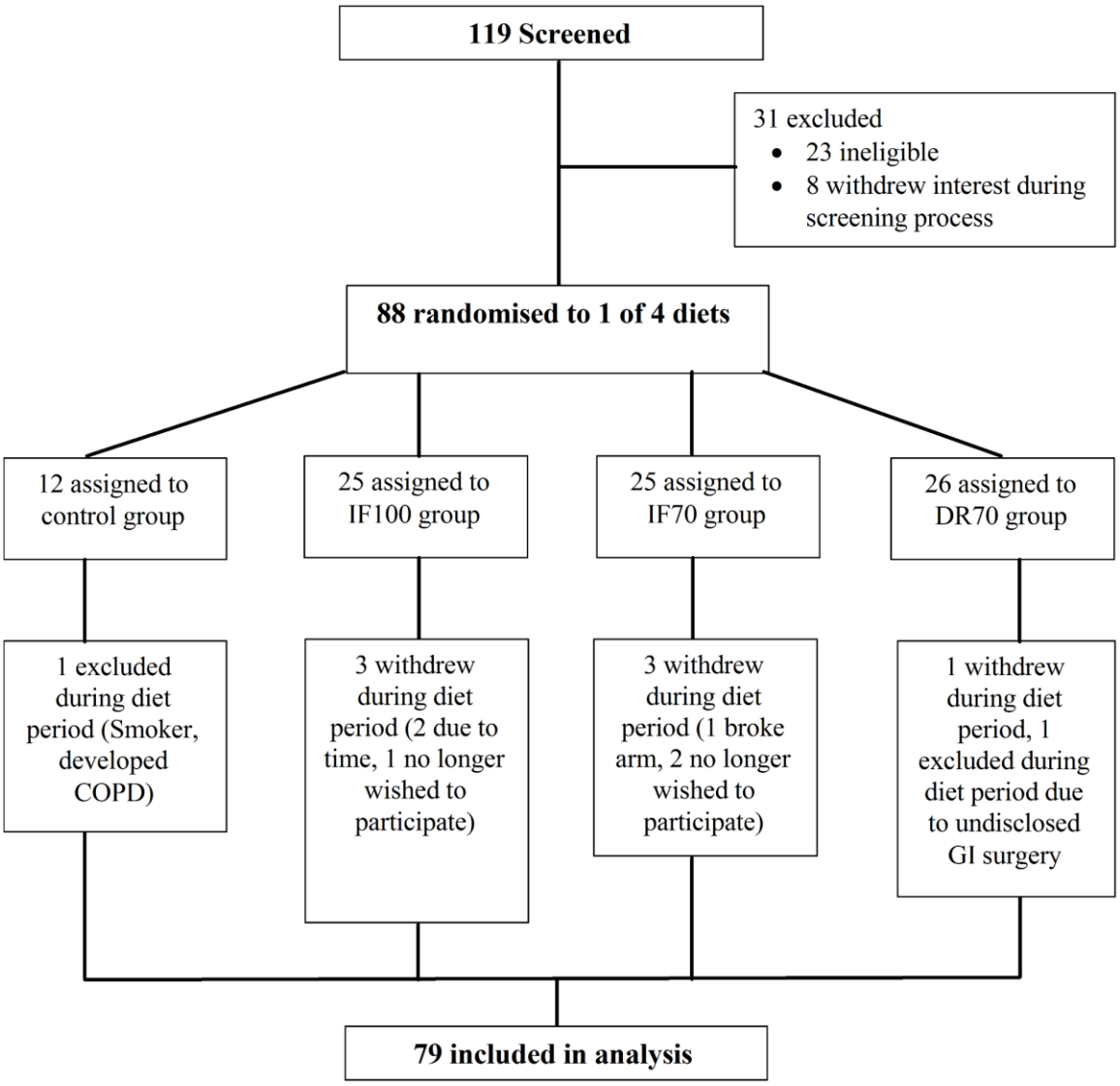

Figure 3-1: CONSORT flow diagram

Adherence and perceptions of appetite

Participants completed daily checklists to monitor adherence, and energy intake in weeks 1, 4 and 7 was calculated from 7-day food diaries using FoodWorks (version 8, Xyris Software; **Appendix table 1 and 2**). Participants attended our clinic weekly, where they returned the 7-day checklist from the previous week, were weighed (**Fig. 3.2A**), and received individual counselling to maintain compliance. Perceptions of appetite and symptoms (hunger, fullness, desire-to-eat, mental alertness, irritability and perceived difficulty adhering to the diet) were

assessed at baseline, week 1 and week 6 using validated visual analogue scales (Parker et al., 2004).

Metabolic testing

To minimize the influence of the menstrual cycle, pre-menopausal women were studied in the follicular phase. Participants consumed a standardised diet (100% of calculated energy requirements, 35% fat, 15% protein, 50% carbohydrate) for 3 days, and were instructed to avoid exercise, alcohol, and caffeine for 24 hours prior to the first metabolic testing visit ("baseline"). Participants fasted for 12 hours overnight prior to the baseline and "fed" (week 8) visits. IF groups underwent a third metabolic visit following a 24-hour fast to capture outcomes from fasting days. This visit occurred 2-7 days after the "fed" visit, depending on clinician availability (**Fig.3.2**). At all visits, participants arrived at 0730h, were weighed in a gown after voiding and waist and hip measurements were taken. Blood pressure was measured with the participant in a seated position, after 10-min of rest. Intravenous cannulae were placed, baseline samples collected and a primed 120-min hyperinsulinaemic-euglycaemic (60 mU/m^2/min) clamp commenced as previously described (Heilbronn et al., 2004b). Peripheral insulin sensitivity (M) was calculated as the mean glucose infusion rate (GIR) during steady-state (last 30 min), normalised for the estimated size of fat free mass (FFM) as described by others (GIR /kg FFM+17.7) (DeFronzo et al., 1979, Tam et al., 2012c). Steady-state insulin was significantly lower after 24-hour fasts in IF70 (P=0.002), and IF100 (P=0.05), suggesting increased insulin clearance following a prolonged fast (Heijboer et al., 2005, Soeters et al., 2008). We calculated insulin-adjusted GIR by dividing M by I, where I is the steady-state insulin concentration (mU/L),*100 (DeFronzo et al., 1979, Rebelos et al., 2011). Due to scheduling conflicts or technical issues on the day, 11 clamps were not conducted. Of these 11, 5 participants did not have baseline clamps and so subsequent clamps were not scheduled, 2

completed baseline clamps only, and 4 completed baseline and the "fed" visit only. The following n were used in the completer's analysis of the clamp data: DR70, n=22; IF70, n=17; IF100, n=19; Control, n=10. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as (fasting serum insulin (mU/L) × fasting plasma glucose (mmol/L))/22.5. Total body composition was assessed by dual-energy x-ray absorptiometry (DXA; Lunar Prodigy; GE Healthcare, NSW, Australia). All procedures were identical on study days, however no DXA was performed at the "fast" visit.

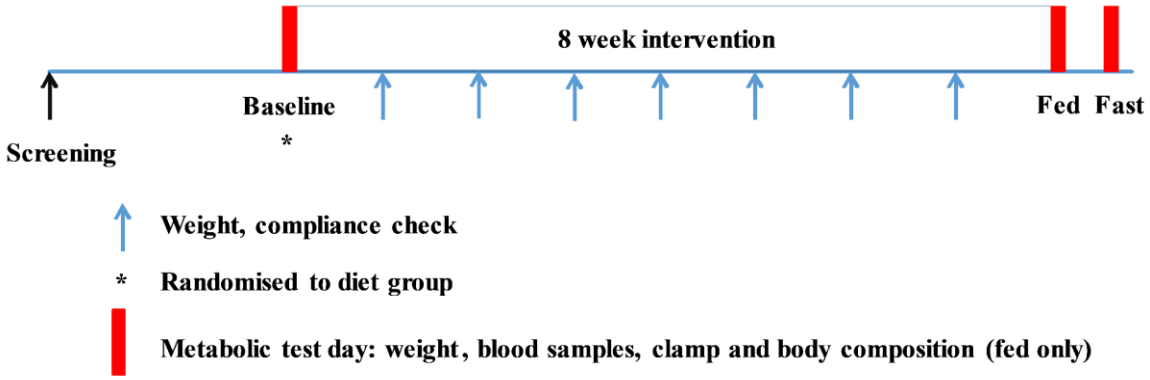

Figure 3-2: Schematic of study design

Analytical methods

Blood samples were immediately centrifuged and frozen at -80°C. Blood lipids and fasting blood glucose were examined by photometric assays in the laboratory of SA Pathology (South Australia, Australia). Serum insulin was measured by radioimmunoassay (HI-14K, Millipore; MA, USA). Serum non-esterified fatty acids (NEFA) were measured by enzymatic colorimetric assay (NEFA-HR (2), Wako Diagnostics; CA, USA). Plasma ß-hydroxybutyrate (RANBUT D-3 Hydroxybutyrate kit, Randox; Antrim, UK), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and high-sensitivity c-reactive protein (HS-CRP) were measured using commercially available enzymatic kits (Beckman Coulter Inc.; CA, USA) on a Beckman

AU480 clinical analyser (Beckman Coulter Inc.; CA, USA). Samples from each subject were analysed within the same run to reduce instrument variation. Serum fibroblast growth factor-21 was measured by ELISA (R & D Systems; MN, USA).

Statistical analysis

The number of participants was established from past studies (Samocha-Bonet et al., 2012, Heilbronn et al., 2004b, Albu et al., 2010). The primary comparison was insulin sensitivity normalised for fat free mass and steady-state insulin concentration (M/I; GIR/kg FFM+17.7/mU), between DR70 and IF70. With n=22 per group (randomised 1:1) a t-test would allow detection of a mean difference in M of 15μmol/kg FFM+17.7 between groups, based on an SD of 17, with 80% power (two-sided α=0.05). This has allowed for a 10% drop out rate, and thus we recruited a total of n=25 per group. For completeness we included an IF100 group and a non-weight loss group (Control) at half sample size.

Statistical methods

All endpoints were assessed as follows. DR70 and IF70 measured at the fed day were compared using linear regressions, adjusting for baseline levels. Thereafter we included the IF70 24-hour fast measurements and compared the three levels (DR70 vs IF70-12h vs IF70-24h) using mixed effects regressions, with a random intercept per individual and compound symmetry correlation structure. Finally, we assessed the diet comparison with and without adjusting for weight loss, which was included as an additional fixed effect. IF100 vs Control diets were compared in a similar manner using the same regression models. Finally IF70 vs IF100 were compared using linear mixed effects regressions using the same random effect structure as above, with baseline measure, time (12 hr vs 24 hr) and diet (IF70 vs IF100) as the three fixed effects. Individuals missing outcome data were excluded from each analysis, while those missing baseline data

were imputed using cohort means. After examination of residual distributions all endpoints measured from plasma (glucose, insulin, ketones and liver markers) except total-, HDL-, and LDL- cholesterol were log-transformed. Data are shown as mean ± SEM. Diet comparisons are pairwise with significance set at $P<0.05$ (two sided). Statistical analyses were performed using SPSS software (version 21.0; SPSS Inc., IBM, NY, USA) and R (version 3.3.3).

3.4 Results

A total of 88 women (mean age 50.2±0.9 years, mean BMI 32.3±0.5 kg/m2) were enrolled in the study. Baseline age, weight, BMI, percent body fat, fasting glucose, insulin, and blood lipids appeared balanced between treatment groups (**Table 3.1**). Women who were post-menopausal (n=47) were older and displayed higher levels of triglycerides, ALT and AST than those were pre-menopause (all P<0.05).

Table 3-1: Baseline characteristics of participants

	Control (n=12)	**IF100** (n=25)	**IF70** (n=25	**DR70** (n=26)
Age at enrolment (years)	49.8±2.6	51.0±1.8	49.5±2.0	50.9±1.8
Weight (kg)	83.8±4.8	84.1±2.8	89.4±2.8	88.4±2.8
BMI (kg/m^2)	30.9±1.5	31.2±0.9	32.4±0.8	32.6±1.0
Pre/Postmenopausal	6/6	10/15	13/12	11/14
Body fat (%)	44.5±2.6	47.0±1.3	48.3±1.4	48.4±1.4
Waist circumference (cm)	98.0±5.8	98.8±2.6	100.5±2.2	99.0±1.8
Hip circumference (cm)	112.1±3.5	112.1±1.9	115.1±2.1	115.7±2.4
Fasting glucose (mmol/L)	4.9±0.1	4.9±0.1	4.9±0.1	4.9±0.1
Fasting insulin (mU/L)	16.8±2.2	18.6±1.5	19.5±1.5	15.5±1.3
HOMA-IR	3.8±0.6	4.1±0.4	4.3±0.3	3.4±0.3
Total cholesterol (mmol/L)	4.5±0.4	5.0±0.2	4.8±0.1	4.9±0.1
HDL-C (mmol/L)	1.3±0.2	1.4±0.2	1.4±0.1	1.4±0.1
LDL-C (mmol/L)	2.6±0.3	3.0±0.2	2.9±0.1	3.0±0.1
Triglycerides (mmol/L)	1.4±0.3	1.5±0.1	1.2±0.1	1.3±0.1
HS-CRP (mg/dL)	2.1±0.6	2.8±0.5	2.9±0.5	2.7±0.5
ALT (U/L)	16.7±1.9	21.6±2.4	19.5±1.9	19.7±1.7
AST (U/L)	19.7±1.4	21.3±1.3	20.1±1.1	19.5±1.0
FGF-21 (mmol/L)	163.8±32.7	169.1±23.1	142.5±23.1	184.4±22.1

DR70: continuous energy restriction at 70% of baseline energy requirements: IF70: intermittent fasting diet at 70% of baseline energy requirements; IF100: intermittent fasting

diet at 100% of baseline energy requirements; Control (C): continuous food intake at 100% baseline energy requirements. Abbreviations: ALT: alanine transaminase; AST: aspartate transaminase; FGF-21: fibroblast growth factor-21; HDL-C: high-density lipoprotein cholesterol; HS-CRP: high-sensitivity C-reactive protein; HOMA-IR: homeostatic model assessment of insulin resistance; LDL-C: Low-density lipoprotein cholesterol.

Data are shown as mean ± SEM. There were no significant differences between groups at baseline in any of the outcome measures.

In addressing aim 1, total weight (P=0.03; **Fig. 3.3B**) and fat loss (P=0.05; **Fig. 3.3C**) were significantly greater in IF70 compared with DR70, and compared with IF100 (both P<0.01). Total weight and fat loss were also greater in IF100 compared with Control (both P<0.001). The reduction in FFM (**Fig. 3.3D**) was not statistically different between IF70 and DR70 (P=0.07) and IF70 and IF100 (P=0.06), but was greater in IF100 compared with Control (P=0.04). The proportion of weight lost as FFM was not significantly different between IF70 and DR70 (P=0.94), IF70 and IF100 (P=0.74) or IF100 and Control (P=0.11; **Appendix table 3**). The reduction in waist circumference was greater in IF70 compared with IF100 (P=0.04; **Appendix table 3**). There were no differences between groups for change in hip circumference (**Appendix table 3**). Given the unexpected differences in weight loss between DR70 and IF70, and IF100 and Control, we have reported comparisons below unadjusted, and adjusted for weight loss.

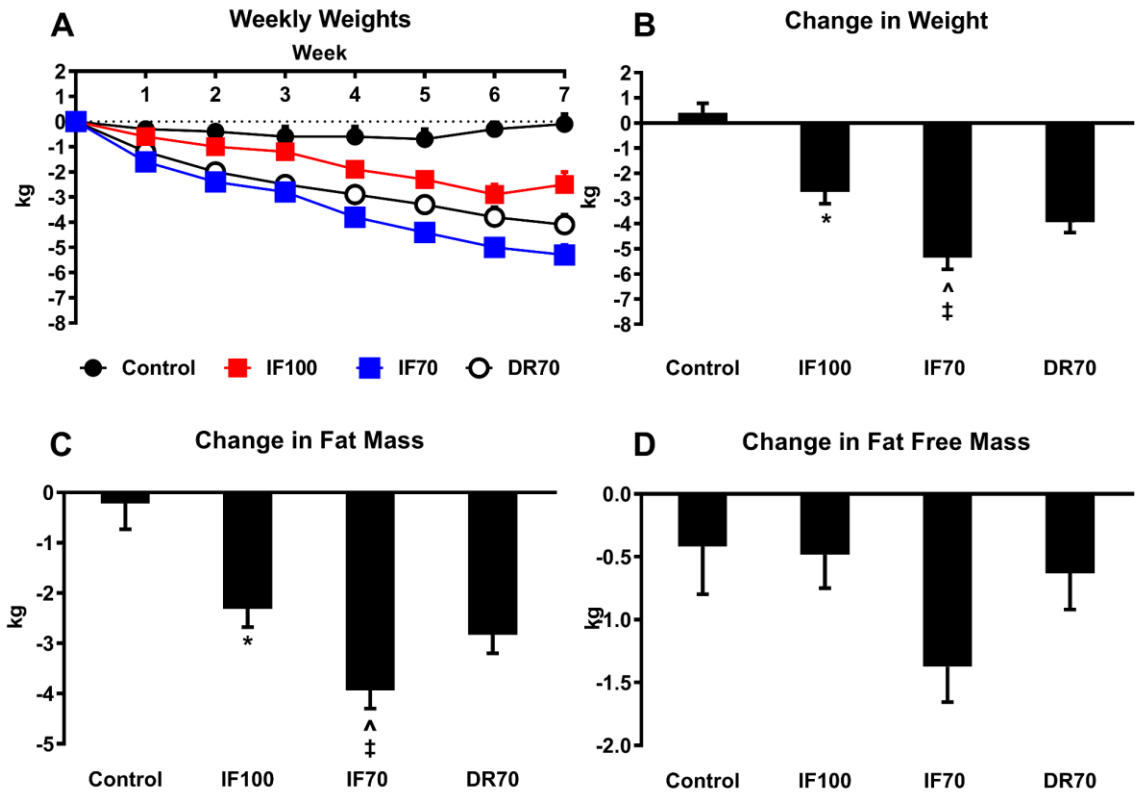

Figure 3-3: Changes in anthropometric outcomes following 8 weeks of intermittent or continuous intake at 70 and 100% of daily energy requirements

(**A**) Weekly weights; (**B**) Change in body weight; (**C**) Change in fat mass; (**D**) Change in fat free mass. Control (C): continuous energy intake at 100% of baseline energy requirements; IF100: intermittent fasting diet at 100% of baseline energy requirements; IF70: intermittent fasting diet at 70% of baseline energy requirements; DR70: continuous energy restriction at 70% of baseline energy requirements.

Data are shown as mean ± SEM. Pairwise comparisons: *P<0.05 vs Control; ^P<0.05 vs. IF100; ‡P<0.05 vs. DR70.

Self-reported energy intake was not significantly different from provided in Control (P=0.83) or DR70 (P=0.96) (**Appendix table 1**). The IF100 group reported consuming 240±336 kcal/day less than provided on fed days, and the IF70 group 188±200 kcal/day less than provided on fed days (**Appendix table 2**). This resulted in an overall average weekly deficit of ~9% and ~2% respectively. As such, energy restriction was greater in IF70 (-31±2%) compared with DR70 (-30±2%) (P=0.02), and in IF100 (-9±8%) compared with Control (0±5%; P=0.02).

Perceived difficulty adhering to the diet was higher in IF100 compared with IF70 and Control at week 1 (both P<0.05), but not at week 6 (P=0.61 compared with IF70, P=0.08 compared with Control). In week 1, self-reported feelings of hunger on a fed day were lower in IF70 compared with DR70, and higher compared with IF100. (**Appendix figure 1**).

The change in insulin sensitivity by clamp was not significantly different between IF70 and DR70 (P=0.95), IF70 and IF100 (P=0.31) or IF100 and Control (P=0.65) after a fed day (**Fig. 3.4A**). However, there was a trend for insulin sensitivity to be impaired after a fast day in IF70 compared with DR70 (P=0.08). Changes in glucose (**Fig. 3.4C**) and insulin (**Fig. 3.4D**) were significantly greater after a fast day in IF70 compared with DR70 (both P<0.05) and after a fed day compared with IF100 (both P=0.02). This translated into reduced (i.e. improved) HOMA-IR after a fast day in IF70 compared with DR70 (P=0.01; **Fig. 3.4B**) and increased HOMA-IR after a fed day in IF100 compared with IF70 after the fed day (P=0.002). Increased fasting insulin (P=0.05) in IF100 compared with Control after a fed day was also observed. There was a greater reduction in NEFA after a fed day in IF70 compared with DR70 (P=0.005), and greater increases after a fasting day (P=0.003) **Fig. 3.4E**). Adjusting for weight loss did not alter the outcomes for HOMA-IR or NEFA.

The changes in total and LDL-cholesterol were greater in IF70 compared with DR70 (both P<0.01) and IF100 (both P≤0.05, **Table 3.2**). The change in triglycerides was significantly greater in IF70 compared with DR70 (P=0.05). There were no differences between diets for the change in HDL-cholesterol (table 2), HS-CRP, ALT (**Appendix table 3**), AST (**Fig. 3.4F**) or the change in systolic and diastolic blood pressure (**Appendix table 3**). The change in FGF-21 was greater in IF70 compared with IF100 (P=0.008; **Fig. 3.4G**). After adjustment for weight

loss, differences between IF70 and DR70 for the changes in total (P=0.01) and LDL-cholesterol (P=0.04) remained.

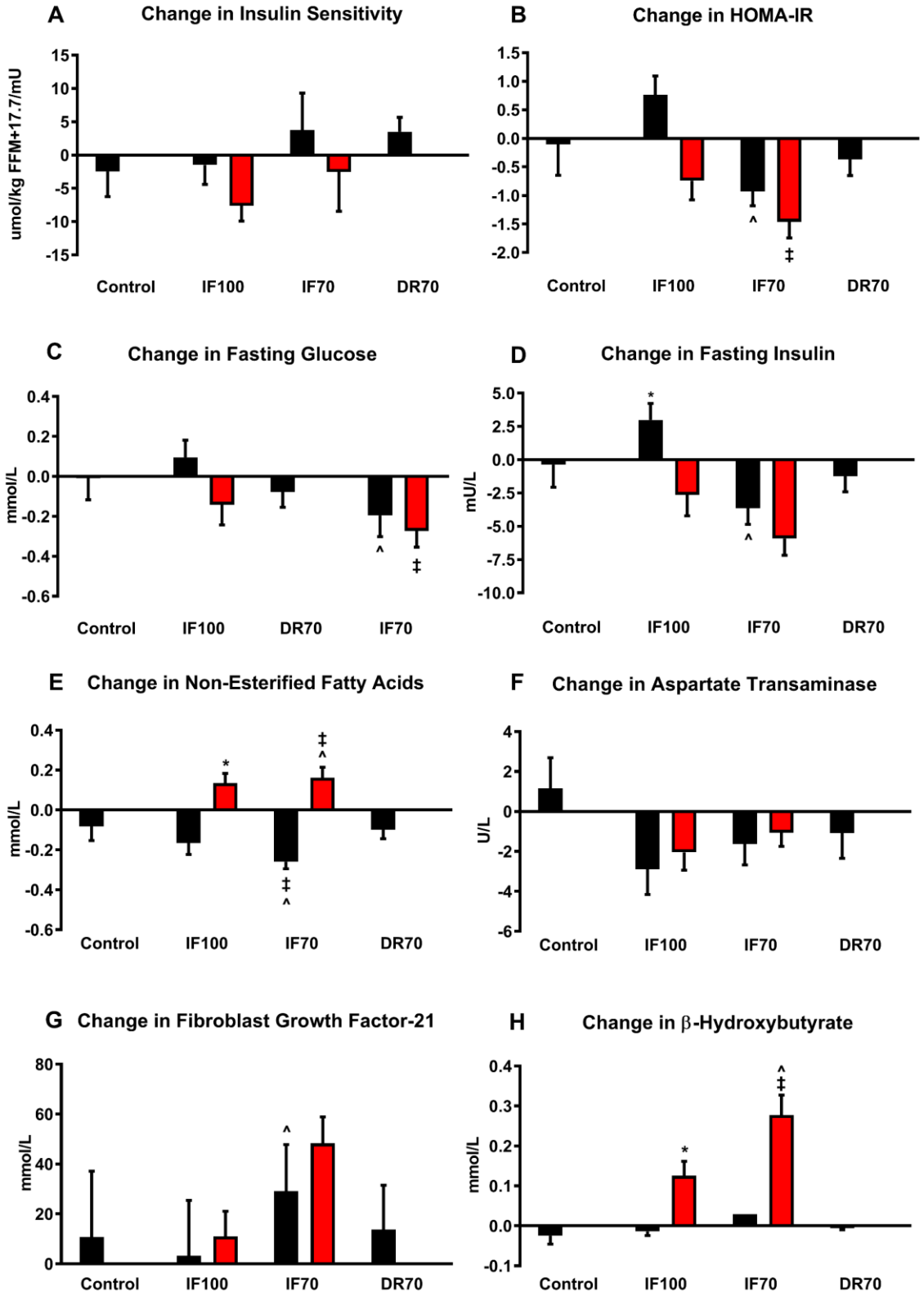

Figure 3-4: Changes in markers of insulin sensitivity and biochemical markers following 8 weeks of intermittent or continuous intake at 70 and 100% of daily energy requirements

(**A**): Change in insulin sensitivity as assessed by hyperinsulinaemic-euglycaemic clamp; completers analysis (DR70 n=22; IF70 n=18; IF100 n=19; C n=10); (**B**): Change in HOMA-IR; (**C**): Change in fasting blood glucose; (**D**): Change in fasting insulin; (**E**) Change in non-esterified fatty acids (NEFA); (**F**): Change in Alanine transaminase; (**G**): Change in fibroblast growth factor-21; (**H**): Change in β-hydroxybutyrate. Control (C): continuous energy intake at 100% of baseline energy requirements; IF100: intermittent fasting diet at 100% of baseline energy requirements; IF70: intermittent fasting diet at 70% of baseline energy requirements; DR70: continuous energy restriction at 70% of baseline energy requirements. Black bars: change from baseline to fed visit; Red bars: change from baseline to fasted visit.

Data are shown as mean ± SEM. Pairwise comparisons: *P<0.05 vs Control; ^P<0.05 vs. IF100; ‡P<0.05 vs. DR70; All end-points (excluding insulin sensitivity) were log-transformed before analysis.

With regards to aim 2, a 24-hour fast significantly impaired insulin sensitivity by clamp compared with a 12-hour fast (P=0.002; **Fig. 3.4A**, **Appendix table 4**). Contrary to this, HOMA-IR was improved by a 24-hour fast (P<0.0001, **Fig.3.4B**). Fasting glucose (**Fig. 3.4C**) and insulin (**Fig. 3.4D**) were reduced (both P=0.01) and plasma NEFA (**Fig. 3.4E**), and ketone (**Fig. 3.4H**) concentrations were increased (all P<0.001). A 24-hour fast also increased AST (P=0.01; **Fig. 3.4G**) and reduced insulin-induced suppression of NEFA (P=0.002; **Appendix table 4**).

Table 3-2: Change in plasma lipids and inflammatory marker C-reactive protein following 8 weeks of diet intervention

	C (n=11)	IF100 (n=22)	IF70 (n=22)	DR70 (n=24)
Total cholesterol (mmol/L)	-0.30±0.15	-0.37±0.15	-0.59±0.08[b,c]	-0.24±0.10
HDL-cholesterol (mmol/L)	-0.03±0.07	-0.07±0.06	-0.10±0.03	-0.05±0.02
LDL-cholesterol (mmol/L)	-0.16±0.12	-0.16±0.13	-0.37±0.07[b,c]	-0.13±0.08
Triglycerides (mmol/L)	-0.25±0.09	-0.28±0.12	-0.24±0.07[c]	-0.16±0.05
HS-CRP (mg/dL)	1.11±0.55	-0.33±0.23[a]	-0.30±0.35	0.34±0.46

Control (C): continuous energy intake at 100% of baseline energy requirements; IF100: intermittent fasting diet at 100% of baseline energy requirements; IF70: intermittent fasting diet at 70% of baseline energy requirements; DR70: continuous energy restriction at 70% of baseline energy requirements. HDL: high-density lipoprotein; LDL: Low-density lipoprotein; HS-CRP: high-sensitivity C-reactive protein.

Data are shown as mean ± SEM. [a]Significantly different from Control ($P<0.05$); [b]Significantly different from IF100 ($P<0.05$); [c]Significantly different from DR70 ($P<0.05$).

3.5 Discussion

This randomised controlled trial showed that provision of an energy restricted-IF diet led to greater loss of weight and fat mass, improvements in total and LDL-cholesterol and NEFA versus energy matched DR. There were no overall differences in insulin sensitivity by clamp between groups, although the 24-hour fast induced transient insulin resistance. When IF was prescribed in energy balance, transient increases in risk markers for type 2 diabetes were observed, despite modest weight loss. Taken together, these data suggest that IF with energy restriction improves metabolic health, while IF in energy balance does not.

One study has shown that IF led to greater weight loss over eight weeks (Catenacci et al., 2016). However, the IF group were prescribed a lower energy intake and this, rather than the mode of meal delivery, was likely responsible for this outcome. Harvie *et al* prescribed a similar energy deficit between intermittent and continuous energy-restricted groups (Harvie et al., 2011). In that study, weight loss was not statistically different between intermittent and continuous groups after 6 months (-6.4 kg [95% CI -7.9 - -4.8kg] IF compared with 5.6 kg [-6.9 - -4.4 kg] DR). Although, self-reported energy intakes were lower in the intermittent group, the intermittent group consumed meal replacements on "fasting" days, whereas the continuous group was prescribed a conventional food-based diet, daily (Harvie et al., 2011). This study was repeated with conventional food based diets prescribed to both groups for three months, and fat mass losses were greater in the intermittent versus continuous group (Harvie et al., 2013). In contrast, Trepanowski *et al*. show that weight and fat loss was not different between intermittent and continuous restriction groups at six months or one year (Trepanowski et al., 2017). The authors noted that this study was underpowered to detect weight differences.

In the current study, IF did not preserve fat free mass as has been reported previously (Varady et al., 2013, Varady et al., 2009), and resulted in significantly more weight and fat mass loss.

Whilst participants were instructed to maintain their pre-enrolment activity levels, we acknowledge that undisclosed changes in activity could have contributed to this outcome. Analysis of self-reported diet records showed excellent adherence in the Control and DR70 groups, and in the IF groups on fasting days, since reported food intake was not significantly different from prescribed. However, IF participants reported consuming less food than prescribed on "fed" days, resulting in an additional energy restriction of 2% in the IF70, and 9% in the IF100 group. A degree of spontaneous energy restriction on "fed" days has been reported previously (Harvie et al., 2013, Trepanowski et al., 2017), and appears to be a benefit of IF.

The mechanistic reason for this remains elusive, however the increase in plasma ketone concentrations may play a role. Inducing physiological ketosis reduces feelings of hunger and increases feelings of fullness in humans (Boden et al., 2005), and may also mitigate the reduction in postprandial cholecystokinin and increased ghrelin concentrations that occur in response to energy restriction (Sumithran et al., 2013). Fasting for 24 hours also reduces ghrelin concentrations (Koutkia et al., 2005). However, the impacts of IF on gut peptides are controversial (Heilbronn et al., 2005b, Catenacci et al., 2016). In the current study, we observed no differences in perceived hunger between modes of dietary restriction, but this was recorded at a single time point only each day. Previous studies report that perceived hunger on a fast day (Heilbronn et al., 2005b) or averaged across eating and fasting days (Harvie et al., 2013) was unchanged, while others report reduced hunger at the end of a fasting day (Klempel et al., 2010). The effects of IF on appetite regulation deserves further investigation.

There is controversy in the existing literature over whether IF is superior to DR to improve metabolic health, with four out of five studies reporting greater improvements in markers of

diabetes or cardiovascular risk (Catenacci et al., 2016, Harvie et al., 2013, Harvie et al., 2011, Antoni et al., 2018). In this study, we observed greater reductions in total cholesterol, LDL cholesterol and NEFA in the IF70 compared with DR70 group, and transiently lower glucose and insulin levels after the fasting day. However, the additional weight loss in the IF70 group may underlie the greater metabolic benefits observed in this study, despite our intentions to match weight loss in these groups. To account for this, we adjusted for the change in body weight, and observed that greater reductions in NEFA, total cholesterol and LDL-cholesterol levels in the IF70 group occurred independent of weight loss. The reduction in NEFA likely reflects greater improvements in adipose tissue insulin sensitivity (**Appendix figure 2**) and stimulation of fatty acid oxidation after IF.

In order to establish whether the purported health benefits of IF were attributable to weight loss, or the fasting pattern *per* se (i.e., patterns of regular feeding and fasting), as has been established in mice (Anson et al., 2003), we included the IF100 group. Women in this group were provided food at overall energy balance, which necessitated them eating at ~145% of energy balance for four days per week. Aside from modest weight and fat loss, there were no metabolic health benefits in the IF100 group. This is contrary to mouse study observations (Anson et al., 2003, Mattson and Wan, 2005, Varady et al., 2010). Further, transient increases in glucose and insulin were observed after a fed day, as we have noted previously in response to acute overfeeding (Samocha-Bonet et al., 2010). We speculate this intermittent 'overfeeding' underlies the lack of overall benefits observed in this group. The long-term impacts of these transient elevations in risk markers of type 2 diabetes are unclear. However, Trepanowski *et al*. showed that LDL concentrations were elevated by IF after 12 months (Trepanowski et al., 2017). While we cannot directly extrapolate our findings to free-living

individuals, both studies highlight the necessity of examining the safety of IF long-term, when weight loss typically slows (Ravussin et al., 2015).

In our study, we observed marked elevations in blood NEFAs and ketones, and decreases in fasting insulin and blood glucose on fasting days, reflecting the switch towards activation of adipose tissue lipolysis and fatty acid oxidation. This is similar to findings by Heilbronn *et al.* who measured samples following 3 weeks of ADF (after a 10-hour overnight fast) and again after a 34-hour fast (Heilbronn et al., 2005a). Few studies have examined the acute changes in metabolic parameters between "fed" and "fasted" states. Halberg *et al* reported that NEFA and glycerol concentrations were increased, while glucose concentrations were decreased when measured after a 20-hr fast. They observed no change in beta-hydroxybutrate, or insulin. However, samples were taken immediately before breaking a 20-hour fast (at 5pm), and compared to samples taken after an overnight fast (8am). Thus clock differences (i.e., morning vs. evening) could have contributed to this result. Nonetheless, this metabolic switching has been postulated to result in up-regulation of mitochondrial fatty acid oxidation, and may underpin the benefits to metabolic health by IF (Anton et al., 2018). In support of this, we noted that the IF70 group displayed greater reductions in NEFA following the fed days compared with DR70, which was independent of the amount of weight lost.

Transient insulin resistance was induced in response to a 24-hour fast in both IF groups, when assessed by clamp. This change was at trend level when comparing a 24-hour fast in IF70 with the fed day in the DR70 group. This may have been partly due to reduced steady state insulin concentrations, which are indicative of increased insulin clearance (Heijboer et al., 2005), despite adjustment. Nonetheless, this finding contrasts data obtained by HOMA (Harvie et al., 2013, Harvie et al., 2011) and highlights that assessing insulin sensitivity by this method is

insufficient to make inferences of "insulin sensitivity" in studies of IF. It also highlights the possibility that tissue-specific changes in insulin sensitivity may occur in response to IF, since HOMA-IR generally reflects hepatic insulin sensitivity, whilst the clamp mainly reflects muscle insulin sensitivity. To our knowledge, only two studies have previously investigated the impacts of two weeks of IF on peripheral insulin sensitivity by clamp, in lean men. In the study by Halberg *et al*, body weight was unchanged, insulin sensitivity was improved, and the authors reported an increase in insulin-induced suppression of adipose tissue lipolysis (Halberg et al., 2005). In contrast, Soeters *et al* performed a 2-step clamp to assess both hepatic and peripheral insulin sensitivity and reported no differences in either measure following 14 days of IF or a standard diet in weight-stable participants (Soeters et al., 2009). In both of these examples, insulin sensitivity was assessed solely following the "fed" day. In humans, prolonged fasting (>48h) induces insulin resistance; this is likely a protective mechanism to spare glucose for the central nervous system (Hoeks et al., 2010). Impaired glucose tolerance was observed after a 36-hour fast, and 3 weeks of alternate day fasting in women (Heilbronn et al., 2005a). Reduced insulin sensitivity has also been detected by IVGTT after a 24h fast in lean individuals (Salgin et al., 2009). This was mitigated by blocking lipolysis with acipimox, suggesting it is mediated by the increase in NEFA (Salgin et al., 2009). In light of this, further understanding of the overall effects of IF on NEFA, lipid metabolism and ectopic lipid deposition is required.

This was a short-term, highly-controlled intervention conducted solely in women. As such, this data is not translatable beyond eight weeks or to wider populations, including men, or those with established metabolic disturbances, such as type 2 diabetes. Therefore, further longer-term highly controlled interventions and in other populations are required. This study was powered to detect a 15 unit difference in M with N=22/group and thus was underpowered to detect the

primary outcome, given that we were only able to conduct clamps in N=17 from the IF70 group. The randomisation pattern of 2:1 for IF100:Control also weakens the comparisons between these two groups.

IF was more effective than DR for reducing body weight, and improving metabolic health, when prescribed with a similar energy deficit, but did not differentially impact insulin sensitivity assessed by hyperinsulinaemic-euglycaemic clamp. When IF was prescribed without energy restriction, there were transient elevations in markers of diabetes risk, and no overall beneficial effects on metabolic parameters, despite minor weight loss. This study demonstrates that IF approaches that involve repeated 24-hour fasts improve metabolic health when in energy deficit, but not when in energy balance.

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Duality of Interest

No potential conflicts of interest relevant to this article were reported.

Author Contributions

LKH and GW designed the research. ATH, BL, and REW collected data. GW and CHT provided clinical support and supervised clamps. NO'C assisted with analysis of plasma biomarkers. AV performed the statistical analysis. All authors contributed to data interpretation and preparation of the manuscript. LKH had full access to the data and had primary responsibility for the final publication.

Prior Presentation

Parts of this study were presented as an oral presentation at the Joint Scientific Meeting of The Australian and New Zealand Obesity Society and the Obesity Surgery Society of Australia and New Zealand, Adelaide, Australia, 2017

Chapter 4: Markers of Adipose Tissue Inflammation are Transiently Elevated during Intermittent Fasting in Women Who Are Overweight or Obese

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Name of Principal Author (Candidate)	Bo Liu		
Contribution to the Paper	Collected data, performed the metabolic visits and laboratory analysis, analysed data, and wrote the manuscript.		
Overall percentage (%)	50%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.		
Signature		Date	28 May 2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Continued on next page

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Signature		Date	28th May 2018

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Contribution to the Paper	Designed the study, provided clinical support and supervised clamps. Input into interpretation of the data and manuscript preparation		
Signature		Date	28/05/2018

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Contribution to the Paper	Designed and supervised the study, had full access to the data and had primary responsibility for the final publication.		
Signature		Date	29/5/18.

4.1 Abstract

Background: Increased macrophage infiltration and extracellular matrix deposition in adipose tissue and skeletal muscle occur in obesity, and are associated with insulin resistance. However, acute fasting also increases adipose tissue macrophage infiltration in mice. This study compared the effects of 8 weeks of daily calorie restriction (DR) versus intermittent fasting (IF) on markers of adipose tissue and skeletal muscle remodelling in women who were overweight and obese.

Methods: Women who were overweight or obese (N=76, BMI 25.0-42.0kg/m^2) were randomized to one of three diets and provided with all foods to meet 100% (IF100) or 70% (IF70 and DR70) of calculated energy requirements. IF groups initiated a 24-hour fast, after consuming breakfast, on 3 non-consecutive days/week. Weight, body composition, non-esterified fatty acids (NEFA), circulating inflammation markers, markers of macrophages and extracellular matrix in adipose tissue and skeletal muscle were measured at baseline and after 8-weeks of intervention following an overnight 12-hour fast (all groups) and a 24-hour fast (IF groups).

Results: IF70 resulted in greater weight and fat loss versus DR70 and IF100 ($P<0.05$). Markers of inflammation in serum, subcutaneous adipose tissue and skeletal muscle were unchanged by DR or IF, when assessed after the overnight fast. However, serum NEFA and markers of classically activated M1-macrophages in adipose tissue, and alternatively activated M2-macrophages in muscle were increased after the 24-hour fast in both IF groups (all $P<0.05$), and the changes in NEFA and *CD68* mRNA levels in adipose tissue were positively correlated ($r=0.56$, $P=0.002$). Matrix metalloproteinase-2 mRNA levels in adipose tissue were increased in IF70 and DR70, but adipose tissue collagen by histology was unchanged in any group.

Conclusions: Intermittent fasting transiently elevated markers of macrophage infiltration, which may link with stimulation of lipolysis.

Key words: adipose tissue, calorie restriction, extracellular matrix, intermittent fasting, macrophage, skeletal muscle.

4.2 Introduction

Obesity is associated with increased adipocyte size (Trayhurn et al., 1979, Salans et al., 1973), inflammation and fibrosis in adipose tissue (Xu et al., 2003, Weisberg et al., 2003, Khan et al., 2009) and skeletal muscle (Fink et al., 2014, Tam et al., 2015, Martinez-Huenchullan et al., 2017), which are linked to the development of insulin resistance (Sun et al., 2011, Martinez-Santibanez and Lumeng, 2014, Wu and Ballantyne, 2017). In humans and animal models of obesity, monocytes are recruited into adipose tissue and skeletal muscle. These macrophages are polarised towards inflammatory M1-macrophages, which release pro-inflammatory cytokines including interferon-gamma (IFN-γ), lipopolysaccharide (LPS) and tumour necrosis factor alpha (TNFα), impairing the insulin signalling cascade (Lumeng et al., 2007a, Fink et al., 2014, Martinez-Santibanez and Lumeng, 2014, Wu and Ballantyne, 2017). There is also an increase in extracellular matrix (ECM) deposition, which provides mechanical and nutritional support to neighbouring cells. Excessive accumulation of the ECM has been linked with impairments in adipocyte expansion, and ectopic lipid accumulation in liver and skeletal muscle (Sun et al., 2013, Divoux et al., 2010).

Daily calorie restriction (DR) promotes weight and fat loss, and reduces the risk of type 2 diabetes and cardiovascular diseases in individuals with obesity (Barnosky et al., 2014, Heilbronn and Ravussin, 2003). Mouse and human studies have shown that DR reduces adipocyte size (Larson-Meyer et al., 2006, Zamarron et al., 2017) and macrophage infiltration in adipose tissue and muscle (Fabbiano et al., 2016, Capel et al., 2009, Yang et al., 2016). There is also some evidence that DR may result in phenotype switching of macrophages from the inflammatory M1- to anti-inflammatory M2-macrophage profile in adipose tissue, in mice (Fabbiano et al., 2016). DR down-regulates mRNA levels of collagen genes and up-regulates

genes involved in ECM degradation in adipose tissue (Higami et al., 2006, Magkos et al., 2016), but its effects on muscle ECM remodelling are less clear.

Intermittent fasting (IF) involves intermittent periods of zero, or minimal energy intake, typically for 24 hours, followed by unlimited food access. IF reduces body weight, fat mass and improves cardiovascular and diabetes risk markers in mice and in humans (Varady et al., 2010, Gotthardt et al., 2016, Heilbronn et al., 2006, Heilbronn et al., 2005b, Trepanowski et al., 2017). The impacts of intermittent fasting on markers of adipose tissue or skeletal muscle remodelling have not been examined in humans. This is of interest since three studies in mice have reported that an acute 24-hour fast increase macrophage infiltration into adipose tissue (Asterholm et al., 2012, Ding et al., 2016, Kosteli et al., 2010).

This study compared the effects of 1) eight-week daily calorie restriction versus intermittent fasting on markers of adipose tissue and skeletal muscle remodelling in women with overweight or obesity, and 2) the acute effects of the 24-hour fast during IF on these outcomes. We hypothesised that DR and IF would differently impact markers of adipose tissue and skeletal muscle remodelling, and particularly markers of inflammation.

4.3 Research Design and Methods

Participants and Study Design

Eighty-eight women aged 35-70 years, with a BMI of 25-42 kg/m^2, were recruited. Those who were smokers, taking medications known to affect appetite, body weight, diagnosed with diabetes, cardiovascular diseases and eating disorders, pregnant or breastfeeding, or planning to become pregnant, had >5% weight change in past 6 months, took part in >2 moderate to high-intensity exercise sessions per week, consumed >140g alcohol/week were excluded. This study was approved by the Royal Adelaide Hospital Research Ethics Committee, and all participants provided written, informed consent prior to their inclusion. The study was registered as a clinical trial with Clinicaltrials.gov (NCT01769976).

This report excludes the control group (n=12), as biopsies were not obtained. Seventy-six participants were randomly assigned to one of three diets, DR70, IF70 and IF100 by BMI (<33 or >33 kg/m^2), for 8 weeks. The estimated energy requirements for each participant were calculated as described previously (Samocha-Bonet et al., 2010). DR70 were provided 70% of calculated energy requirements, daily. The IF70 group were provided foods at ~100% of energy requirements, and IF100 at ~145% of energy requirements on fed days. On fasting days, IF groups consumed breakfast before 8am (~ 32-37% of energy requirements) prior to "fasting" for 24-hours, until 8am the following day, every other week-day (3 fasting days per week). During the fasting period, participants were allowed to consume water and energy-free foods, black coffee and/or tea, and one very low calorie broth (250 ml, ~20 kcals). A total of night participants withdrew (**Fig. 4.1**).

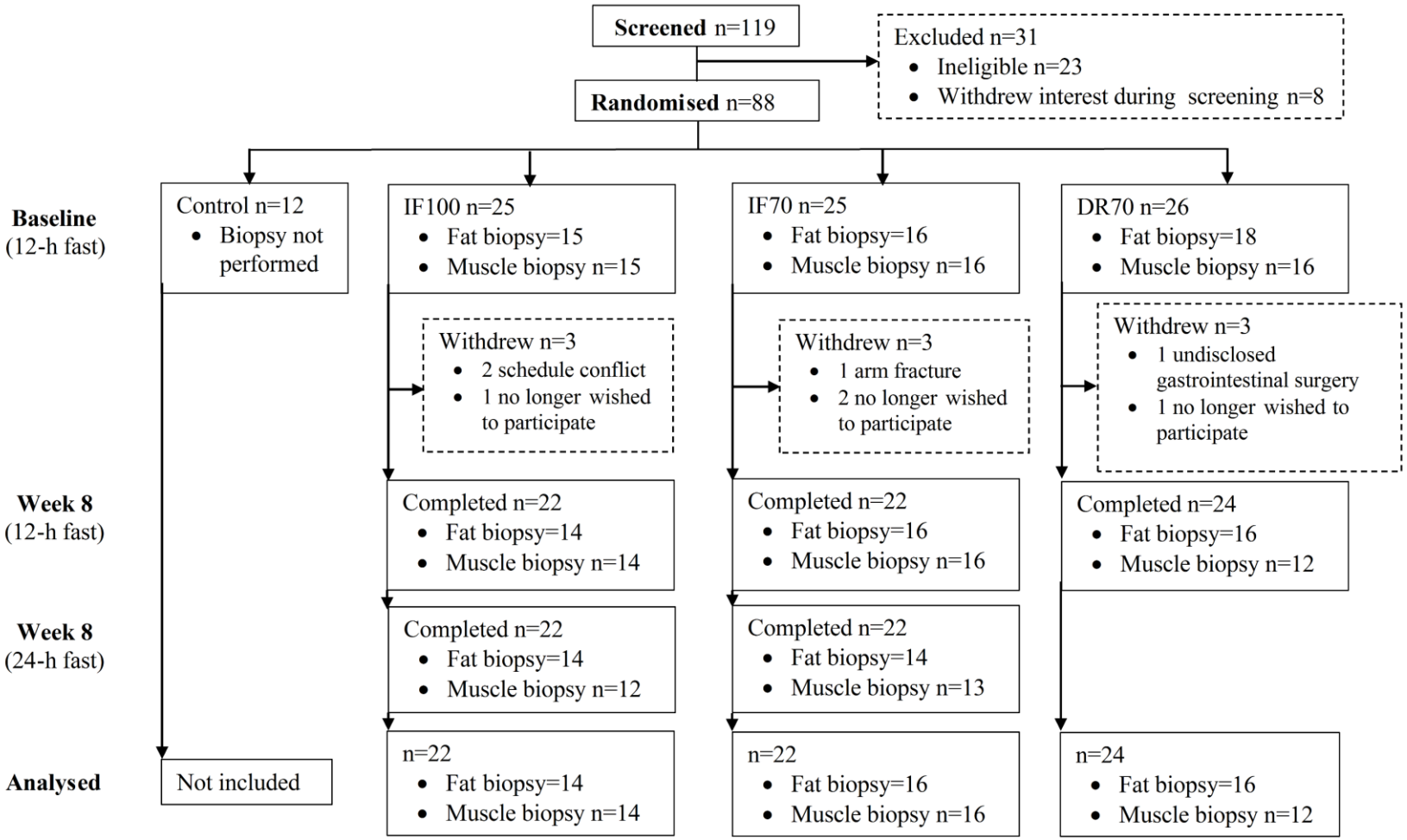

Figure 4-1: Flowchart of the study

Metabolic Testing in Participants

Participants were required to attend the research clinic at 7:30 a.m., at baseline and after 8-weeks following an overnight 12-hour fast. IF groups undertook an additional visit following a 24-hour fast. Body weight, height, waist and hip circumference were measured with the participant dressed in a hospital gown after voiding. Blood samples were obtained, and serum and plasma were separated and stored at -80°C freezer for analysis of insulin, non-esterified fatty acids (NEFA), TNFα, interleukin-6 (IL-6), interleukin-10 (IL-10) and monocyte chemoattractant protein-1 (MCP-1). Total body composition was assessed by dual-energy x-ray absorptiometry (Lunar Prodigy; GE Healthcare, NSW, Australia) at baseline and after 8-weeks following an overnight fast for all groups.

Adipose and muscle tissue biopsies

Subcutaneous abdominal adipose and vastus lateralis muscle samples were obtained by percutaneous biopsy at baseline and at week 8 after 12-hour fast (all groups) and 24-hour fast (IF groups only). Briefly, biopsy sites were located and cleaned. After anesthetizing the skin and underlying tissues with lidocaine, adipose tissue was aspirated from the periumbilical area and vastus lateralis samples were collected using the Bergstrom technique as previously described (Tam et al., 2010, Tam et al., 2017). A small piece of sample (~30mg) was fixed for histology, and the remainder (~150mg) frozen in liquid nitrogen and stored at -80°C. Not all participants consented to both adipose and skeletal muscle biopsies. Due to this, and scheduling conflicts, biopsy samples were obtained from a subset of participants (n=14-16/group for adipose tissue and n=12-16/group for muscle, **Fig. 4.1**). The baseline characteristics in those who underwent adipose tissue or skeletal muscle biopsies were not different between groups and were not different from that of those who completed 8 weeks of intervention.

Table 4-1: Taqman primers used for gene expression analysis

Gene symbol	Gene Name	Assay ID
Housekeeper		
ACTB	Actin beta	Hs01060665_g1
PPIB	Peptidylprolyl isomerase B	Hs00168719_m1
LRP10	LDL receptor related protein 10	Hs01047362_m1
Pan macrophage		
CD68	CD68 molecule	Hs02836816_g1
M1-macrophage		
CD40	CD40 molecule	Hs01002913_g1
TNF	Tumor necrosis factor	Hs01113624_g1
IL6	Interleukin 6	Hs00985639_m1
M2-macrophage		
CD163	CD163 molecule	Hs00174705_m1
IL10	Interleukin 10	Hs00961622_m1
Macrophage recruitment		
CCL2	C-C motif chemokine ligand 2	Hs00234140_m1
CCL3	C-C motif chemokine ligand 3	Hs00234142_m1
Extracellular matrix		
COL6A1	Collagen type VI alpha 1	Hs01095585_m1
MMP2	Matrix metallopeptidase 2	Hs01548727_m1
TIMP1	TIMP metallopeptidase inhibitor 1	Hs00171558_m1
Lipolysis		
LIPE	Lipase E, hormone sensitive type	Hs00193510_m1
PLIN1	Perilipin 1	Hs00160173_m1
lipogenesis		
FASN	Fatty acid synthase	Hs01005622_m1
ACACA	Acetyl-CoA carboxylase alpha	Hs01046047_m1
Adipogenesis		
CEBPb	CCAAT/enhancer binding protein beta	Hs00270923_s1
SREBF1	Sterol regulatory element binding transcription factor 1	Hs01088691_m1

Biochemical Analysis

Blood lipids and fasting blood glucose were examined by photometric assays in the laboratory of SA Pathology (Adelaide, South Australia, Australia). Serum insulin was measured by radioimmunoassay (HI-14K, Millipore, MA, USA). Plasma high-sensitivity C-reactive protein (Hs-CRP, Beckman Coulter Inc, CA, USA) was measured using commercially available enzymatic kits on a Beckman AU480 clinical analyser (Beckman Coulter Inc). Serum TNFα, IL-6 and IL-10 were analysed by multiplex bead array assays (R & D Systems, Minneapolis, USA) with MAGPIX Multiplex Reader (Luminex, Austin, TX, USA). Serum non-esterified fatty acids (NEFA) were measured by enzymatic colorimetric assay (NEFA-HR (2), Wako Diagnostics, CA, USA), and serum MCP-1 was measured using ELISA kit (BD, San Diego, CA, USA) on a VersaMax ELISA Microplate Reader (Sunnyvale, CA, USA). Samples were run in duplicate and samples from each participant were tested within the same run to reduce instrument variation. Homeostasis model of assessment-insulin resistance (HOMA-IR) was calculated as fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5.

Quantitative Real-Time PCR

As described previously (Chen et al., 2016a), total RNA was extracted from adipose (100-150mg) and muscle (30-50mg) samples using TRI Reagent (Sigma, St. Louis, USA) following manufacturer's instructions. The concentration and purity of RNA were assessed by NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, CA, USA). cDNA synthesis was conducted using T100 Thermal Cycler (Bio-Rad, CA, USA) with 1000 ng of each RNA sample using the QuantiTect reverse transcription kit (Qiagen, Valencia, CA, USA) according to kit instructions. Quantitative real-time PCR was performed using the Taqman primers for pan-macrophage (*CD68*), inflammatory M-1 macrophage (*CD40*, *TNF* and *IL6*), anti-inflammatory M2-macrophage (*CD163* and *IL10*), macrophage recruitment (*CCL2* and *CCL3*), extracellular

matrix (*COL6A1*, *MMP2* and *TIMP1*), lipolysis (*LIPE* and *PLIN1*), lipogenesis (*ACACA* and *FASN*), adipogenesis (*CEBPb* and *SREBF1*, **Table 4.1**) and Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The samples were run in duplicate on an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) with internal negative controls and a standard curve (pooled from ten participants at baseline). Relative gene expression was analysed using the $2^{-\Delta CT}$ method and normalised for the mean of *ACTB* and *PPIB* for adipose tissue, and the mean of *ACTB* and *LPR10* for skeletal muscle, which were not different at baseline, or following the intervention. *CCL3*, *TNF* and *IL6* were below detectable limits in adipose tissue and muscle.

Histological Analysis and Immunofluorescent Staining.

Adipose tissue biopsy samples were fixed in Bouin's solution (Sigma-Aldrich, HT10132), dehydrated, paraffin embedded, sectioned at 5µm and mounted on positively-charged glass slides. All slides were randomly assigned numeric codes by a research officer to blind the investigator (BL) quantifying outcomes. Hematoxylin and eosin (H&E) staining was performed using a standard protocol. Digital images were acquired using a camera (U-TV1X-2, Olympus, Tokyo, Japan) and diameters measured using cellSens Software (Olympus, Tokyo, Japan). Adipocyte diameter was measured in at least three fields of view at 20X. The mean diameter was calculated from an average of 300 cells per sample as described previously (Tam et al., 2010). Masson's trichrome staining was performed using a commercial kit (Sigma, St. Louis, MO, USA) following manufacturer's instructions. Slides were scanned using the Pannoramic 250 Flash II scanner (3DHISTECH, Budapest, Hungary) and whole sections were analysed using Masson's trichrome macro in Image J (National Institutes of Health). Particular care was taken to exclude areas which contained blood vessels, as collagen is associated with vasculature (Rhodes and Simons, 2007).

For CD40 (M1-macrophage) and CD206 (M2-macrophage) co-staining, deparaffinised and rehydrated slides were incubated with ELOXALL solution (SP-600, Vector) for 10min at room temperature to eliminate endogenous peroxidase and alkaline phosphatase. Antigen retrieval was achieved using modified citrate-based buffer (S1700, Dako) and incubation in a 95°C water bath for 20min. Slides were blocked with 5% bovine serum albumin (Sigma) in phosphate-buffered saline (PBS, Sigma) for 60min at room temperature before incubated with a mouse anti-human CD40 (1:200, MAB6321, R& D) for 90min at room temperature. Slides were then washed with PBS for 5min for 3 times prior to being incubated with a chicken anti-mouse secondary antibody (1:500, A-21200, ThermoFisher) for 60min at room temperature. Following 5min x 3 washing, goat anti-human CD206 (1:200, AF2534, R & D) and the corresponding donkey anti-goat secondary antibody (1:500, A-11057, ThermoFisher) were applied. CD40 and CD206 co-labeled slides were counterstained with ProLong Gold Antifade Mountant with DAPI (P36941, ThermoFisher). Eight to ten fields at 40X were analysed using the camera and software mentioned above. Positive cells were expressed per 100 adipocytes as described previously (Aron-Wisnewsky et al., 2009) .

Statistical analysis

Data are expressed as mean ± SEM. Individuals who withdrew from the study were not included in the analyses. Participants completing baseline and week-8 (12-hour fast) biopsies were included for gene expression analyses. All statistical analyses were performed using IBM SPSS Statistics 24 (Armonk, New York, USA). Baseline differences between groups were analysed using one-way ANOVA with Bonferroni-Holm post-hoc test. A maximum likelihood mixed effects model was employed to examine the group differences of 8-week intervention following an overnight 12-hour fast, as well as the time effects within each group. The model included fixed effects for intervention, visit and the intervention by visit interaction, and a

random effect for subject with an unstructured covariance matrix to account for the repeated visits. The effect of intervention was assessed with planned contrasts between groups in the change from baseline to week 8 (12-hour fast). Bonferroni-adjusted pairwise comparisons were also conducted within each group to assess differences over time from baseline to following a 12-hour fast (all groups) and a 24-hour fast (IF groups only). Data were log-transformed for analysis if skewness in the residuals was observed. Significance was accepted as $P < 0.05$.

4.4 Results

Anthropometrics and metabolites at baseline

Anthropometric and metabolic parameters of the participants at baseline are summarised in **Table 4.2**. There were no significant differences between groups with respect to any variables assessed at baseline. There were no significant differences between individuals completed this trial and those underwent biopsies or did not with regards to the variables listed in **Table 4.2**.

Table 4-2: Anthropometric and metabolic measures of participants at baseline

Variable	DR70	IF70	IF100	P
N	26	25	25	
Age (years)	50.9 ± 1.8	49.5 ± 2.0	51.0 ± 1.8	0.827
Pre-/post- menopause	11/16	11/14	13/12	-
Weight (kg)	88.4 ± 2.8	89.4 ± 2.8	84.1 ± 2.8	0.289
Height (cm)	164.7 ± 1.0	166.0 ± 1.7	162.2 ± 1.5	0.158
Waist(cm)	99.0 ± 1.8	100.5 ± 2.2	98.8 ± 2.6	0.859
Hip(cm)	115.7 ± 2.4	115.1 ± 2.1	112.1 ± 1.9	0.433
Waist/hip	0.86 ± 0.01	0.87 ± 0.02	0.88 ± 0.02	0.640
BMI (kg/m^2)	32.6 ± 1.0	32.4 ± 0.8	31.2 ± 0.9	0.746
TG (mmol/l)	1.3 ± 0.1	1.2 ± 0.1	1.5 ± 0.1	0.093
TC (mmol/l)	4.9 ± 0.1	4.8 ± 0.1	5.0 ± 0.2	0.256
HDL-C (mmol/l)	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.2	0.728
LDL-C (mmol/l)	3.0 ± 0.1	2.9 ± 0.1	3.0 ± 0.2	0.726
Glucose (mmol/l)	4.9 ± 0.1	4.9 ± 0.1	4.9 ± 0.1	0.956
Insulin (mU/ml)	15.5 ± 1.3	19.5 ± 1.5	18.6 ± 1.5	0.116
HOMA-IR	3.4 ± 0.3	4.3 ± 0.3	4.1 ± 0.4	0.207
Hs-CRP (mg/l)	2.7 ± 0.5	2.9 ± 0.5	2.8 ± 0.5	0.907

Data are presented as mean ± SEM. One-way ANOVA with Bonferroni-Holm post-hoc test. DR70: continuous energy restriction at 70% baseline energy requirements: IF70: intermittent fasting diet at 70% baseline energy requirements; IF100: intermittent fasting diet at 100% baseline energy requirements. BMI: body mass index; TG: triglycerides; TC: total cholesterol; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; Hs-CRP: high-sensitivity C-reactive protein

Adiposity, glucose metabolism and non-esterified fatty acids

Body weight and fat mass were reduced in all groups (all P<0.001), with greater reductions in IF70 vs. DR70 and IF100 (both P<0.05, **Fig. 4.2A** and **B**). Adipocyte size was reduced in all groups (all P≤0.01), with no difference between groups (Fig. **4.2C** and **D**). When measured following the overnight fast, NEFA levels were decreased in all groups (all P<0.05), with greater reductions in IF70 vs. DR70 (P=0.02, **Fig. 4.2E**). Fasting HOMA-IR was reduced in IF70 (P=0.01), and a group effect was observed between IF100 vs. IF70 and DR70 (both P<0.05, **Fig. 4.2F**). When measured following the 24-hour fast, NEFA levels were increased (both P<0.05, **Fig. 4.2E**) and HOMA-IR was reduced (both P≤0.01, **Fig. 4.2F**) in both IF groups. Since biopsies were performed in a subgroup of participants, an additional analysis examining the outcomes reported above was conducted. This revealed the responses to dietary intervention were not different between participants underwent biopsies and those did not. Importantly, all the within group effects reported in **Fig.4.2** held. However, the between group difference in weight and fat mass loss did not exist.

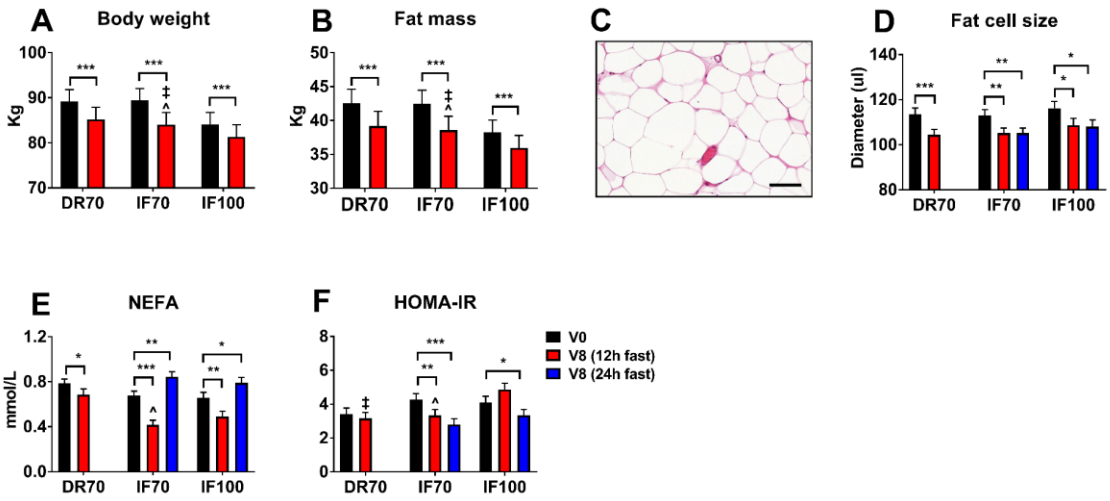

Figure 4-2: Adiposity, glucose metabolism, and non-esterified fatty acids following 8 weeks of intervention

(**A**): Body weight; (**B**): Fat mass; (**C**): Representative H&E staining of subcutaneous adipose tissue; (**D**): Fat cell size; (**E**): HOMA-IR and (**F**): NEFA. DR70: continuous energy restriction at 70% baseline energy requirements: IF70: intermittent fasting diet at 70% baseline energy requirements; IF100: intermittent fasting diet at 100% baseline energy requirements. Scale bar: 100μm.

Data presented as Mean±SEM. $^{\wedge}P<0.05$ vs. DR70 in the change from baseline to week 8 following a 12-hour fast; ‡ $P<0.05$ vs. IF100 in the change from baseline to week 8 following a 12-hour fast. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. baseline.

Systemic and tissue inflammation

When measured following the overnight fast, there were no within or between group changes in any inflammatory markers assessed in serum (**Fig. 4.3A-D**, and **Appendix figure 3**), adipose tissue (**Fig. 4.3I-K** and **Fig. 4.3M-P**) or muscle (**Fig. 4.3Q-T**, and **Appendix figure 4 and 5**), except for an increase in serum MCP-1 in IF100 (P=0.01, **Fig. 4.3D**). When measured following the 24-hour fast, serum MCP-1 (**Fig. 4.3D**), *CD40* mRNA levels (**Fig. 4.3M**), the total number of M1- and pan-macrophages in adipose tissue by histology (**Fig. 4.3I** and **K**), *CD163* mRNA levels and the *CD163*:*CD40* ratio in muscle (**Fig. 4.3R** and **T**) were increased in the IF70 group (all P<0.05). The number of M1-, M2- and pan-macrophages in adipose tissue (**Fig. 4.3I-K**), *CD163* and *CD68* mRNA levels and the *CD163*:*CD40* ratio in muscle (**Fig. 4.3R-T**) were also increased after the 24-hour fast in the IF100 group (all P<0.05). When measured following the 24 hour fast, the change in *CD68* mRNA levels in adipose tissue was positively correlated with the change in NEFA in both IF groups (r=0.56, P=0.002, **Fig. 4.3L**).

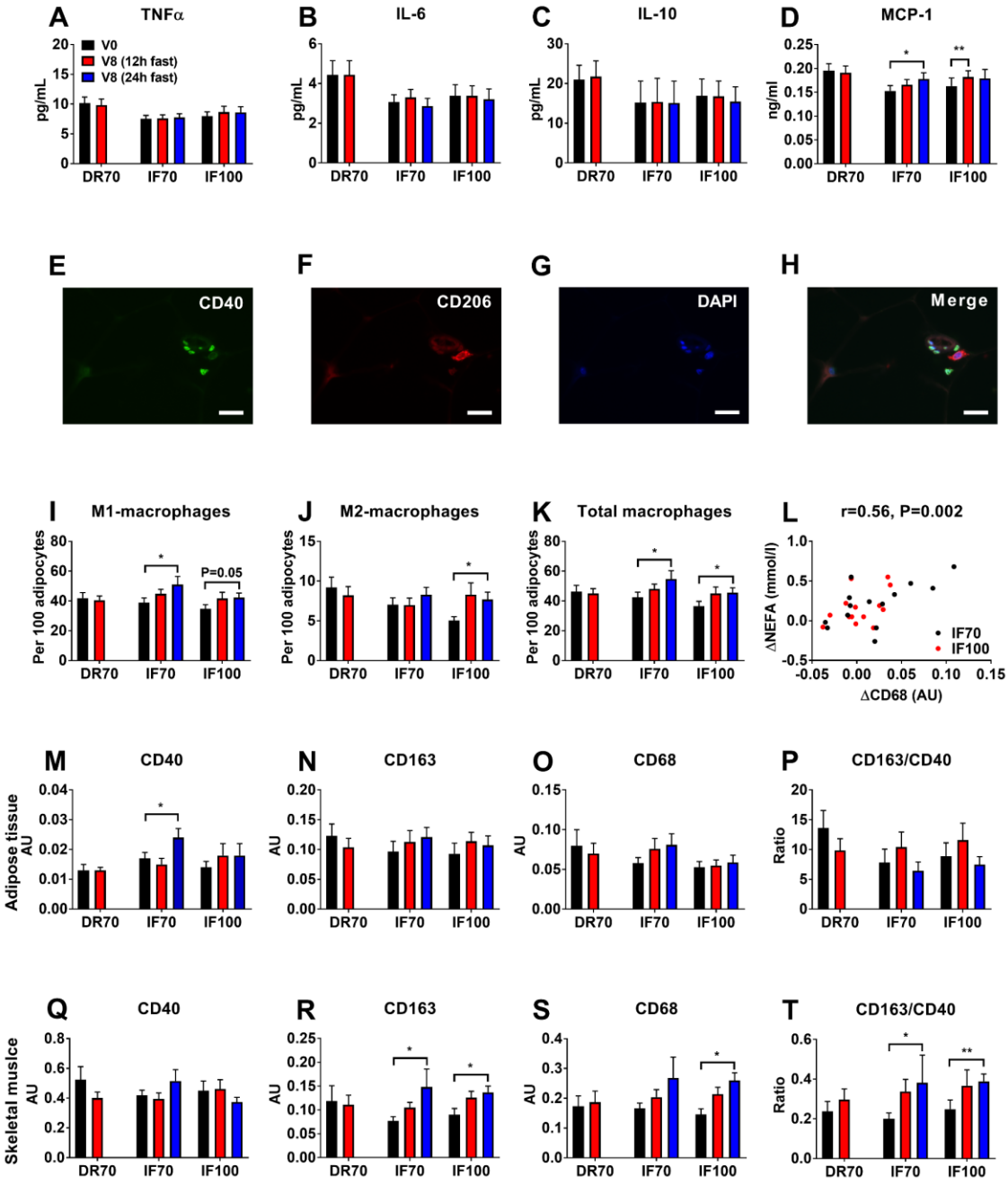

Figure 4-3: Systemic and tissue inflammation following 8 weeks of intervention

(**A-D**): Serum TNFα, IL-6, IL-10 and MCP-1; (**E-H**): Representative images of fluorescent staining for M1-macrophage (CD40), M2-macrophage (CD206), nuclei (DAPI) and layout; (**I** to **K**): Quantification of M1-, M2- and total macrophages in adipose tissue; (**L**): Correlation between changes in NEFA levels and *CD68* mRNA levels in adipose tissue after a 24-hour fast; (**M**-**O**): mRNA levels of *CD40* (M1- macrophage), *CD163* (M2- macrophage) and *CD68* (pan-macrophage) in adipose tissue; (**P**): The ratio of *CD163*:*CD40* in adipose tissue; (**Q**-**S**): mRNA levels of *CD40*, *CD163* and *CD68* in skeletal muscle and (**T**): The ratio of *CD163*:*CD40* in muscle. DR70: continuous energy restriction at 70% baseline energy requirements: IF70: intermittent fasting diet at 70% baseline energy requirements; IF100:

intermittent fasting diet at 100% baseline energy requirements. Scale bar: 20μm. AU: arbitrary unit.

Data presented as Mean±SEM. *P<0.05 and **P<0.01 vs. baseline.

Extracellular matrix remodelling in adipose tissue and muscle

When measured following the overnight fast, there were no between group differences in the change in extracellular matrix markers in adipose tissue (**Fig. 4.4A-C**) or muscle (**Fig. 4.4E-G**). However, *MMP2* mRNA levels were increased in adipose tissue in DR70 and IF70 (both P<0.01, **Fig. 4.4B**). *COL6A1* and *MMP2* mRNA levels were increased in skeletal muscle in IF100 (both P≤0.05, **Fig. 4.4E** and **F**). When measured after the 24-hour fast, *MMP2* mRNA levels remained elevated in adipose tissue in the IF70 group (P=0.002, **Fig. 4.4B**). However, the integrated density (**Fig. 4.4D**) or area (**Appendix figure 6**) of collagen in adipose tissue assess by Trichrome staining was not altered in any group.

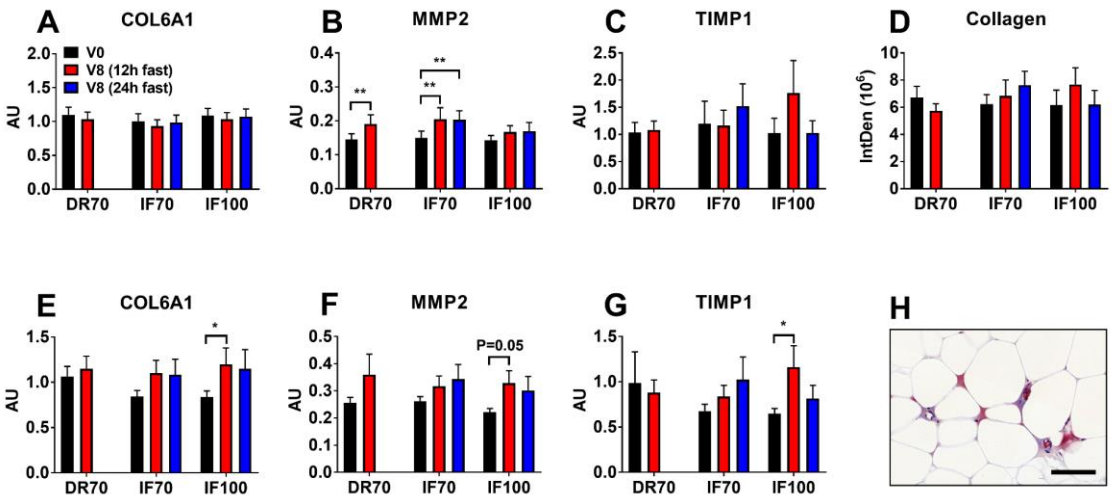

Figure 4-4: Extracellular matrix remodelling in adipose tissue and muscle following 8 weeks of intervention

(**A-C**): mRNA levels of *COL6A1*, *MMP2* and *TIMP1* in adipose tissue; (**E-G**): mRNA levels of *COL6A1*, *MMP2* and *TIMP1* in muscle; (**D** and **H**): Representative image of Masson's trichrome staining and quantification of the integrated density of collagen in adipose tissue.

DR70: continuous energy restriction at 70% baseline energy requirements: IF70: intermittent fasting diet at 70% baseline energy requirements; IF100: intermittent fasting diet at 100% baseline energy requirements. Scale bar: 100μm. AU: arbitrary unit.

Data presented as Mean±SEM. *P<0.05 and **P<0.01 vs. baseline.

Lipid metabolism and adipogenesis markers in adipose tissue

When measured following the overnight fast, *FASN* mRNA levels in adipose tissue were decreased in the IF70 group (P=0.02, **Fig. 4.5A**), and this was significantly different from the change in *FASN* mRNA levels in the IF100 group (P=0.03). *ACACA* mRNA levels in adipose tissue were tended to be reduced in the DR70 group (P=0.05, **Fig. 4.5B**). When measured following the 24-hour fast, there were no significant change in markers of lipogenesis (**Fig. 4.5A** and **B**) or lipolysis (**Fig. 4.5C** and **D**) in either IF group. mRNA levels of *CEBPb* and *SREBF1* were not altered in any group (**Fig. 4.5E** and **F**).

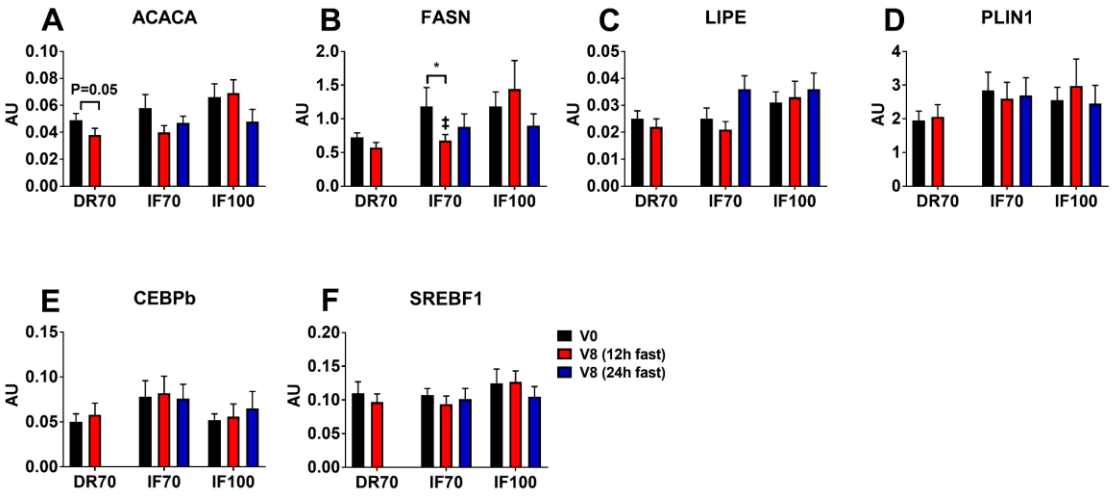

Figure 4-5: Lipogenesis, lipolysis, and adipogenesis markers in adipose tissue following 8 weeks of intervention

(**A** and **B**) mRNA levels of lipogenesis markers *ACACA* and *FASN*; (**C** and **D**) mRNA levels of lipolysis markers *LIPE* and *PLIN1*; (**E** and **F**): mRNA levels of adipogenesis markers *CEBPb* and *SREBF1*. DR70: continuous energy restriction at 70% baseline energy

requirements: IF70: intermittent fasting diet at 70% baseline energy requirements; IF100: intermittent fasting diet at 100% baseline energy requirements. AU: arbitrary unit.

Data presented as Mean±SEM. $^{‡}P<0.05$ vs. IF100 in the change from baseline to week 8 following a 12-hour fast. $^{*}P<0.05$ vs. baseline.

4.5 Discussion

Obesity is associated with increased macrophage infiltration and extracellular matrix deposition in adipose tissue, which can be at least partially reversed following weight loss by DR (Weisberg et al., 2003, Xu et al., 2003, Zamarron et al., 2017, Divoux et al., 2010, Higami et al., 2006, Magkos et al., 2016). The effects of IF, as an alternative weight loss strategy, on tissue remodelling in humans are unknown. Data obtained from mouse studies suggest that macrophage infiltration is stimulated in response to an acute 24-hour fast (Kosteli et al., 2010, Ding et al., 2016, Asterholm et al., 2012). In this study, we observed an increase in markers of macrophage infiltration in human adipose tissue in response to the 24-hour fast imposed by the intermittent fasting schedule. This was associated with increases in NEFA, a marker of adipose tissue lipolysis, as has also been shown previously in mouse models (Kosteli et al., 2010).

Macrophages are the dominant leukocyte population in adipose tissue, and can be divided into two subtypes based on their functional properties: M1- and M2- macrophages. M1, or classically activated macrophages, secrete pro-inflammatory molecules such as IFN-γ, LPS and TNFα, and contribute to obesity related morbidities. The M2- subtype represents alternatively activated anti-inflammatory macrophages (Martinez-Santibanez and Lumeng, 2014). In this study, DR did not impact any of the markers of macrophages or inflammation in adipose tissue, skeletal muscle, or systemically. This finding is in line with previous reports (Magkos et al., 2016, Tam et al., 2012a). Tam *et al* reported that 24-weeks of DR led to 10% weight loss in individuals with obesity, but did not alter systemic inflammation or markers of macrophages in adipose tissue (Tam et al., 2012a). Magkos *et al* reported that 10-15% weight loss by DR partially improved systemic inflammation, without altering mRNA levels of macrophage markers in subcutaneous adipose tissue in humans with obesity (Magkos et al., 2016). In skeletal muscle, one study reported that inflammatory markers were decreased in skeletal

muscle collected from individuals following long-term DR (3-15 yrs) versus age-matched sedentary individuals consuming western diet (Yang et al., 2016), but this was not observed after 3-months of DR (Liu et al., 2017a).

Of note, a 24-hour fast increased multiple markers of inflammation in adipose tissue and skeletal muscle, and the increase in macrophage marker in adipose tissue was associated with elevated NEFA levels. In mice, a 24-hour fast significantly increased circulating NEFA levels and macrophage infiltration in adipose tissue (Kosteli et al., 2010). The peak of adipose tissue macrophage number coincided with the peak in the circulating concentration of NEFA and glycerol released from adipose tissue, suggesting that lipolysis drives macrophage accumulation in adipose tissue (Kosteli et al., 2010). Moreover, *in vivo* and *in vitro* studies have shown that stimulation of adipocyte lipolysis increased the uptake and storage of lipids by macrophages (Caspar-Bauguil et al., 2015, Kosteli et al., 2010). Elevations in NEFA are linked with insulin resistance in liver and muscle (Delarue and Magnan, 2007), and thus macrophages may play a protective role to buffer elevated NEFA levels induced by fasting, in humans.

The phenotype of macrophages covers a continuum of functional states from inflammatory M1- to anti-inflammatory M2- profiles (Mantovani et al., 2004). The polarization of macrophages toward M1- or M2- phenotype is through distinct pathways by external stimuli. For example, lipopolysaccharides and IFNγ promotes a M1- polarization, whilst interleukin 4 and 10 yields a M2-polarization (Martinez and Gordon, 2014). Some evidence suggests macrophages can maintain their population by self-proliferation, and M2-macrophages can be transformed into M1-phenotype to promote inflammation (Zheng et al., 2013, Zamarron et al., 2017). Previous studies in mice show that an acute 24-hour fast, and intermittent fasting, also

promote M2-polarisation of macrophages in adipose tissue (Asterholm et al., 2012, Kim et al., 2017). To our knowledge, the impacts of acute or intermittent fasting on muscle macrophage phenotype have not been reported in mice or humans. In this study, we observed IF increased mRNA levels of M2- macrophages in muscle. The mechanisms by which IF promotes M2-polarization requires further study.

ECM is a non-cellular component existing in all tissues, which provides structural support to the neighbouring cells, and influences cell morphology and function through cell-matrix connections (Sun et al., 2013). The homeostasis of ECM is maintained by a balance between synthesis and degradation. Collagens are the main proteins in ECM and are degraded by matrix metalloproteinases (MMPs), which are negatively regulated by tissue inhibitors of metalloproteinases (TIMPs) (Zamarron et al., 2017). Previous studies have shown that ECM accumulation in adipose tissue and skeletal muscle is linked with obesity and poor metabolic phenotype, such as insulin resistance (Williams et al., 2015). In this study, we found that *MMP2* mRNA expression, which contributes to the degradation of ECM, was increased in adipose tissue in DR and IF70 groups. This finding suggests that weight loss, rather than the mode of dietary restriction, increases ECM degradation in adipose tissue. This is in line with a recent human study, which suggests that minor weight loss (5%) upregulates genes involved in the degradation of ECM in subcutaneous adipose tissue (Magkos et al., 2016). We did not see a reduction in collagen deposition in adipose tissue by histology, but this may be the result of the minor weight loss achieved. Substantial weight loss by bariatric surgery also did not change fibrosis in subcutaneous adipose tissue in morbidly obese patients with and without Type 2 diabetes after 6 months (Chabot et al., 2017). Interestingly, Clément's group observed increased collagen deposition in subcutaneous adipose tissue 3-12 months after bariatric surgery (Liu et al., 2016). However, this was characterised by a decrease in cross-link of matrix

fibres and increased degradation of the extracellular matrix, and was associated with an increase in M2-macrophages. This study highlights that the "structure or quality" of collagen should be taken into consideration when assessing adipose tissue fibrosis. Further studies are required to investigate the structure and quality of collagen, and the crosstalk between extracellular matrix and macrophages in adipose tissue and skeletal muscle following weight loss. Our data also supports past studies to suggest that ECM gene expression in skeletal muscle is sensitive to overfeeding (Tam et al., 2017, Tam et al., 2014), as the mRNA levels of *COL6A1* and *MMP2* were transiently increased in the IF100 group after consuming~145% of energy requirements on the fed day. This suggests both intermittent "fasting" and "overeating" in an intermittent fasting regimen are signals that could impact tissue remodelling.

This study was a short-term intervention that was limited to women who were overweight and obese. Thus, the responses in men and in individuals with normal body weight may be different. The long-term effects of intermittent fasting on adipose tissue and skeletal muscle require further study.

In conclusion, weight loss by DR or IF reduced fat cell size and stimulated markers of ECM remodelling, but did not alter markers of inflammation, when measured after an overnight fast. In response to the 24-hour fast, IF transiently elevated markers of inflammation in adipose tissue and muscle. The change in markers of macrophage infiltration in adipose tissue following the 24-hour fast was associated with the change in NEFA. The long-term effects of these transient increases in markers of inflammation in response to intermittent fasting requires further investigation.

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Duality of Interest

No potential conflicts of interest relevant to this article were reported.

Author Contributions

BL and ATH performed the study, collected and analysed the data. GAW and CHT provided clinical support. LKH and GAW designed the study. KL performed the statistical analysis. All authors contributed to data interpretation and preparation of the manuscript. LKH is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation

Parts of this study were presented as an oral presentation at the Australian & New Zealand Obesity Society 2016 Annual Scientific Meeting, Brisbane, 19-21 October, 2016, and as a poster at the 77th Scientific Sessions of the American Diabetes Association, San Diego, 9-13 June, 2017.

Chapter 5: Intermittent Fasting Improves Glucose Tolerance and Promotes Adipose Tissue Remodelling in High-fat Diet Fed Male Mice

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Publication Details	

Principal Author

Name of Principal Author (Candidate)	Bo Liu
Contribution to the Paper	Performed the study, acquired and analysed the data, and wrote the manuscript.
Overall percentage (%)	50%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.
Signature	Date 3.5.18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Supervised the study, contributed to data collection and analysis.
Signature	Date 3/5/18

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Contribution to the Paper	Contributed to data collection.
Signature	Date 3.5.18

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Miaoxin Chen		
Contribution to the Paper	Contributed to data collection and provided technical support.		
Signature		Date	3/5/18

Name of Co-Author	Gary A Wittert		
Contribution to the Paper	Interpreted the data and helped draft the manuscript.		
Signature		Date	29/5/18

Name of Co-Author	Leonie K Heilbronn		
Contribution to the Paper	Conceived, designed and supervised the study, and interpreted the data		
Signature		Date	29/5/18.

Chapter 5

5.1 Abstract

Background: Intermittent fasting (IF) promotes weight loss in diet-induced obese mice, but its effects on adipose tissue inflammation and fibrosis are unclear.

Methods: Ten-week old male C57BL/6J mice were fed a high-fat (HFD; 43% fat) or chow diet (18% fat) for 8-weeks *ad libitum* (AL), and randomised to AL or IF for an additional 8-weeks. IF was initiated one hour before lights off (Zeitgeber time 11, lights on at ZT0) for 24-hours, on 3 non-consecutive days per week. Body weight and energy intake were monitored and oral glucose tolerance (2g/kg body weight) was assessed. At the end of the study, all mice were sacrificed at ZT7-9, fed state or after a 22-hour fast. Plasma insulin and non-esterified fatty acids (NEFA) were analysed. Inguinal and gonadal fat were collected for the assessment of markers of inflammation and extracellular matrix. Fat cell size, macrophage numbers and collagen content in adipose tissue were assessed by histology. Two-way ANOVA with Bonferroni post-hoc test was used to assess the effects of diet (chow and HFD) and schedule (AL and IF) between groups. Significance was accepted as $P<0.05$.

Results: IF decreased energy intake, body weight and fat cell size in HFD fed mice, and decreased fat mass and improved glucose tolerance in chow and HFD fed mice (all $P<0.05$). IF decreased mRNA levels of *Lgals3*, *Itgax*, *Ccl2* and *Ccl3* in inguinal and gonadal fat, and adipose tissue macrophages numbers in HFD fed mice only (all $P<0.05$). IF increased mRNA levels of *Mmp9*, but reduced mRNA levels of *Col6a1* and *Timp1*, and fibrosis in gonadal fat in HFD fed mice (all $P<0.05$). The 22-hour fast elevated NEFA levels (all $P<0.05$), but did not alter inflammation markers in adipose tissue in either diet groups.

Conclusions: Intermittent fasting improved glucose tolerance in chow and high fat diet fed mice, and ameliorated the adipose tissue inflammation and fibrosis in high fat diet fed mice.

Key words: adipose tissue, extracellular matrix, glucose tolerance, intermittent fasting, macrophage

5.2 Introduction

Daily caloric restriction (DR) promotes weight loss, improves health, and extends lifespan in a variety of species. Intermittent fasting (IF) has recently gained attention as a viable alternative to DR. IF extends lifespan, reduces fat mass, and improves glucose tolerance with no, or minimal, impact on food consumption and body weight in chow fed mice (Goodrick et al., 1990, Anson et al., 2003, Varady et al., 2008, Varady et al., 2010). The impact of IF on high-fat diet fed mice are less clear, with only four animal studies conducted very recently (Gotthardt et al., 2016, Joslin et al., 2017, Li et al., 2017, Liu et al., 2017b). These studies have consistently reported that IF resulted in significant weight loss, but improvements in glucose tolerance are controversial.

White adipose tissue undergoes remodelling during weight gain, including adipocyte hypertrophy and/or hyperplasia, increases in macrophage infiltration and extracellular matrix (ECM) deposition (Sun et al., 2011, Sun et al., 2013, Martinez-Santibanez and Lumeng, 2014). Increased accumulation of macrophages and ECM in adipose tissue has been linked to development of insulin resistance (Weisberg et al., 2003, Xu et al., 2003, Olefsky and Glass, 2010, Sun et al., 2013). Macrophages may also promote ECM deposition (Spencer et al., 2010, Bourlier et al., 2012, Keophiphath et al., 2009), which could negatively impact adipocyte expansion, promote ectopic lipid deposition and impact metabolic health (Sun et al., 2013, Khan et al., 2009). Marked weight loss by DR reduces macrophage infiltration in adipose tissue (Hoevenaars et al., 2014, Zamarron et al., 2017, Kovacikova et al., 2011, Capel et al., 2009, Magkos et al., 2016), and may promote macrophage phenotype switching from a pro-inflammatory M1- towards an anti-inflammatory M2- profile (Lumeng et al., 2007a, Fabbiano et al., 2016). There is some evidence that markers of ECM synthesis in adipose tissue decrease following long-term DR in mice (Higami et al., 2006).

Paradoxically, a beneficial role of inflammation in healthy adipose tissue expansion and function is also reported (Wernstedt Asterholm et al., 2014). Increases in macrophage infiltration in adipose tissue occurs in the early phase of DR, and after 24-hours of fasting(Capel et al., 2009, Kosteli et al., 2010, Asterholm et al., 2012, Ding et al., 2016), and this increase coincides with elevated circulating non-esterified fatty acids (NEFA) (Kosteli et al., 2010). These studies have suggested that adipose tissue macrophages may play a positive role buffering lipid released from adipocytes.

We examined the effects of intermittent fasting, on both fed and fast days, in mice fed a chow or high-fat diet, on markers of adipose tissue remodelling. We hypothesised that intermittent fasting will improve metabolic phenotype and reduce adipose tissue fibrosis, but may increase adipose tissue macrophage infiltration in chow and high-fat diet fed mice.

5.3 Research Design and Methods

Ethical approval

All experimental protocols for the animal study were approved by the animal ethics committee of the South Australian Health and Medical Research Institute (SAHMRI) and the University of Adelaide, and were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Animals and diets

Forty-eight male C57BL/6J mice (Bioresources, SAHMRI, Adelaide, Australia) were housed four per cage under a 12:12h light/dark cycle, with lights on at 0700h (Zeitgeber time 0). At ten-week old, mice were fed either a lard based high-fat diet (HFD) comprising 43%, 21%, 36% of energy from fat, protein, and carbohydrate respectively (SF04-001, Specialty Feeds, WA, Australia) or a standard chow diet (Chow) comprising 18%, 24%, 58% of energy from fat, protein, and carbohydrate respectively (2018SX, Specialty Feeds, WA, Australia) for eight weeks. Mice on each diet were then randomized into *ad libitum* feeding (AL, n=8) or intermittent fasting (IF, n=16) for another eight weeks. Intermittent fasting was initiated at ZT11 (1-hour prior to lights off) for 24-hours for 3 non-consecutive days/week (**Fig. 5.1A**). Mice, cardboard tunnels and chew blocks were transferred daily between cages with or without food. *Ad libitum* fed mice were also transferred between feeding cages at the same time to standardize handling. All mice had free access to water throughout the study. Body weight and food intake were monitored at ZT11 weekly before IF was introduced, and daily after IF was implemented. At 28-week old, all mice were sacrificed at ZT7-9 with mice in IF group culled in the fed state or following a 22-hour fast. Whole blood was collected via cardiac puncture following isoflurane anaesthesia. Following cervical dislocation, inguinal and gonadal adipose tissue were collected and weighed. Another group of 10-week old male C57BL/6J mice (n=24)

were fed chow or HFD *ad libitum* for eight weeks and culled at ZT7-ZT9 in the fed state or after a 22-hour fast.

Glucose tolerance test

At twenty-four weeks of age and after 6 weeks of IF or AL feeding, mice were fasted from ZT1 for 6-hours and then challenged with an oral gavaging of glucose (2 g/kg body weight). Glucose was assessed at 0, 15, 30, 60, 90, and 120 minutes via tail vein bleeding by a glucometer (AccuChek Performa Monitor, Roche Diagnostics, Indianapolis, USA), and insulin was assessed at 0, 15, 30, and 60 minutes.

Plasma analysis

Insulin was measured using ultra-sensitive ELISA kit (Cat: 10-1249-01, Mercodia, Sweden) and Non-esterified fatty acids (NEFAs) by enzymatic colorimetric assay (NEFA-HR (2), Wako Diagnostics, CA, USA) on a VersaMax ELISA Microplate Reader (Sunnyvale, CA, USA).

Quantitative Real-Time PCR

As described previously (Chen et al., 2016a), Total RNA were extracted from gonadal and inguinal adipose tissue (100-150 mg) using TRI Reagent (T9424, Sigma, St. Louis, USA) following the manufacturer's instructions. The concentration and purity of RNA were assessed by NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, CA, USA). cDNA synthesis was conducted using T100 Thermal Cycler (Bio-Rad, CA, USA) with 1000 ng of each RNA sample using the QuantiTect reverse transcription kit (Qiagen, Valencia, CA, USA) according to kit instructions. Standard control (25ng/ul) samples were pooled from each sample. Quantitative real-time PCR was performed using Taqman primers and Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Genes for pan macrophage (*Adgre1*

and *Lgals3*), inflammatory M1-macrophage (*Itgax* and *Cd38*), anti-inflammatory M2-macrophage (*Arg1* and *Mrc1*), macrophage recruitment (*Ccl2* and *Ccl3*), ECM synthesis (*Col3a1* and *Col6a1*), ECM degradation (*Mmp2*, *Mmp9* and *Timp1*) were assessed using Taqman primers (**Table 5.1**). The samples were run in duplicate on an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) with internal negative controls and a standard curve. Six reference genes including *Rn18s, Actb, Gapdh, Hprt*, *Ppia* and *B2m* were examined, and the combination of *Actb* and *B2m* was determined as the best housekeeper using the NormFinder program as described previously (Chen et al., 2014). The relative gene expression was determined using the $2^{-\Delta CT}$ method, where $\Delta CT = (CT_{\text{target gene}} – CT_{\text{reference gene}})$.

Histological Analysis and Immunohistochemical staining

Briefly, adipose tissue samples were fixed with Bouin's solution (HT10132, Sigma, St. Louis, MO, USA), dehydrated, paraffin embedded, and sectioned at 5μm. Hematoxylin and eosin (H&E) staining was performed using a standard protocol, and Masson's Trichrome staining with a commercial kit (HT15, Sigma, St. Louis, MO, USA). For immunohistochemistry, we used F4/80 targeting to pan-macrophages and slides were counterstained with Mayer's hematoxylin. Slides were scanned using the Pannoramic 250 Flash II scanner (3DHISTECH, Budapest, Hungary). At least one thousand adipocytes were analysed for adipocyte size and collagen content using Image J built-in macros (National Institutes of Health). Crown-like structures and F4/80 positive cells were counted in ten randomly chosen areas at ×40 magnification and adjusted by adipocyte numbers as described previously (Cancello et al., 2005). Reagents used for histology are listed in **Table 5.2**.

Table 5-1: Taqman primers used for gene expression analysis

Gene symbol	Gene Name	Assay ID
Housekeeper		
Actb	Actin, beta	Mm00607939_s1
18s	Eukaryotic 18S rRNA	Mm03928990_g1
Ppia	Peptidylprolyl isomerase A	Mm02342430_g1
Hprt	Hypoxanthine guanine phosphoribosyl transferase	Mm01545399_m1
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1
B2m	Beta-2 microglobulin	Mm00437762_m1
Pan-macrophage		
Adgre1 (F4/80)	Adhesion G Protein-Coupled Receptor E1	Mm00802529_m1
Lgals3 (Mac2)	Lectin, Galactoside-Binding, Soluble, 3	Mm00802901_m1
M1-macrophage		
Itgax (Cd11c)	Integrin subunit alpha X	Mm00498701_m1
Cd38	Cluster of differentiation 3	Mm01220906_m1
M2-macrophage		
Arg1	Arginase, liver	Mm00475988_m1
Mrc1	Mannose receptor, C type 1	Mm01329362_m1
Macrophage recruitment		
Ccl2	Chemokine (C-C motif) ligand 2	Mm00441242_m1
Ccl3	Chemokine (C-C motif) ligand 3	Mm00441259_g1
Extracellular matrix		
Col3a1	Collagen, type III, alpha 1	Mm01254476_m1
Col6a1	Collagen, type VI, alpha 1	Mm00487160_m1
Mmp2	Matrix metallopeptidase 2	Mm00439498_m1
Mmp9	Matrix metallopeptidase 9	Mm00442991_m1
Timp1	Tissue inhibitor of metalloproteinase 1	Mm01341360_g1

Table 5-2: Reagents used for immunohistochemical staining

Reagent	Source	Identifier
Rat anti mouse F4/80 (1:200)	Abcam	Ab6640
Goat Anti-Rabbit HRP (IgG H&L) (1:500)	Abcam	Ab6721
Goat sera	Gibco	16210064
Endogenous Peroxidase and Alkaline Phosphatase Blocking Solution	Vector	SP-6000
DAB	Vector	SK-4105
Bovine serum albumin (BSA)	Sigma-Aldrich	A7030-10G
PBS	Sigma-Aldrich	P4417-100TAB
Triton	Sigma-Aldrich	234729

Data analysis

All data are expressed as mean ± the standard error of the mean. Data were analysed statistically with SPSS 24 (IBM, Chicago, IL, USA) and log-transformed for analysis if not normally distributed. For insulin in the fasted mice, undetectable samples (**Figure 5.3**) were input with the minimum values. Area under the curve (AUC) for glucose and insulin was calculated as mentioned previously (Matthews et al., 1990, Allison et al., 1995). Single comparisons were performed using two-way ANOVA with diet (Chow and HFD) and schedule (AL and IF) as between group factors, Bonferroni post hoc tests were performed when diet*schedule effects were present. $P<0.05$ was considered as statistically significant.

Table 5-3: Mice with insulin undetectable after the fasting day

	Eight-week intermittent fasting		Acute one day fasting	
Diet	Chow	HFD	Chow	HFD
Number	3/8	3/8	4/8	0/8

5.4 Results

IF differentially impacted on energy intake and adiposity in chow and HFD fed mice

Cumulative energy intake and final body weight was not different between chow-IF vs. chow-AL groups (**Fig. 5.1B-E**). In contrast, HFD-IF displayed reduced energy intake (-28.0%) vs. HFD-AL group and significant weight loss (-20.9%, both P<0.001). Weight loss plateaued after 5 weeks of IF in the HFD-IF group, and the final body weight was not different to the chow fed groups. Gonadal and inguinal fat mass was reduced as a result of IF in chow and HFD groups, but fat pad weights remained higher in HFD-IF vs. chow-IF (all P<0.05, **Fig. 5.1F** and **1G**). Fat cell size was decreased by IF in HFD fed mice only (all P<0.001, **Fig. 5.1H** to **1M**).

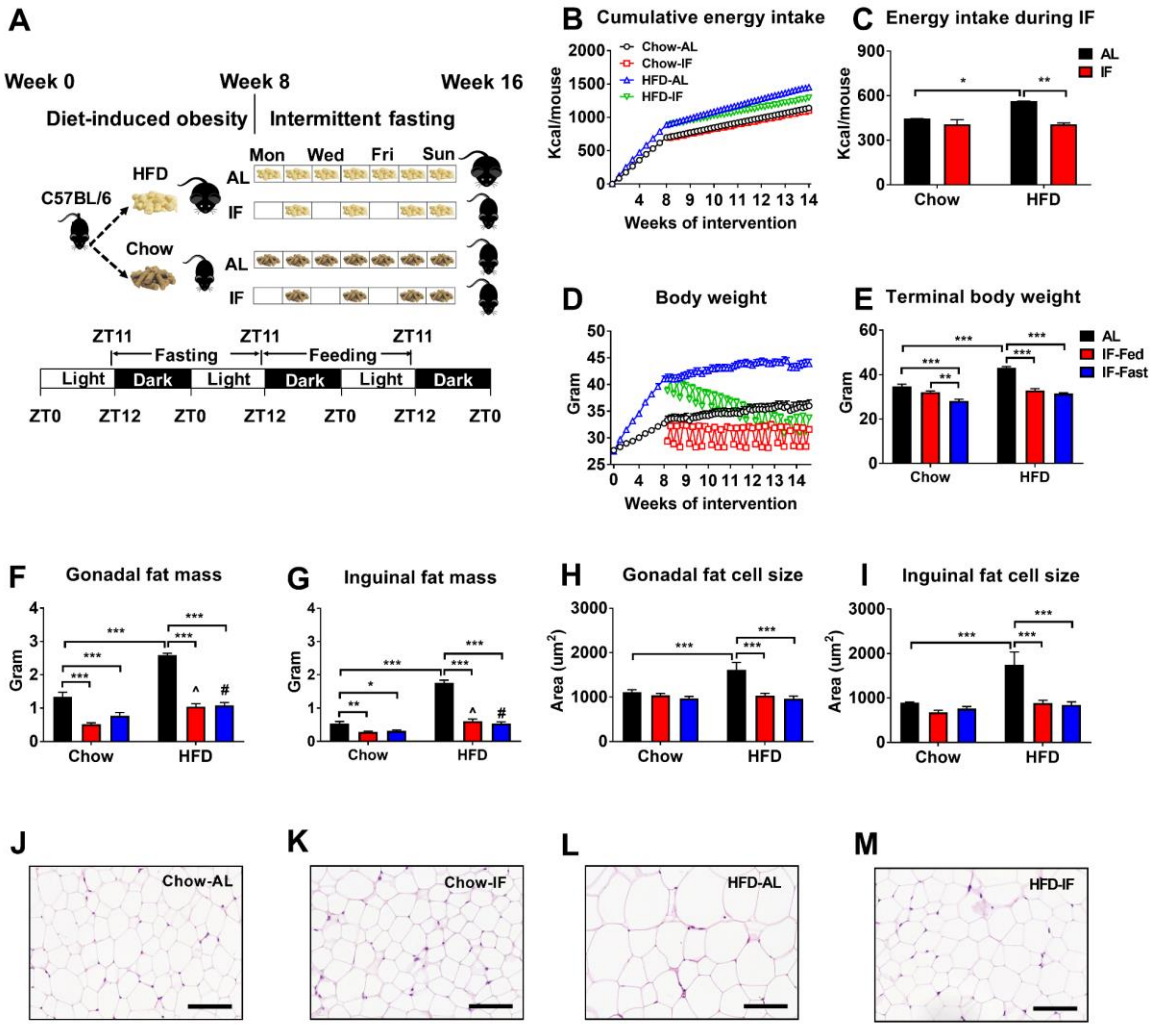

Figure 5-1: IF differentially impacted on energy intake and adiposity in chow and HFD fed mice

(**A**): Schematic outline of the intermittent fasting regimen used in this study. (**B** and **C**): Cumulative energy intake during diet-induced obesity and intermittent fasting, results were calculated based on 4 mice/cage. (**D** and **E**): Body weight during diet-induced obesity and intermittent fasting. (**F** and **G**): Gonadal and inguinal fat mass. (**L** and **M**): Gonadal and inguinal fat cell size. (**H** to **K**): Representative H & E staining of gonadal fat. Scale bar: 100μm.

Data presented as Mean±SEM. n=8 in AL and 16 in IF per diet in A-D; n=7-8/group in D-G; n=5-6 in H and I. Two-way ANOVA with Bonferroni post hoc test. Post hoc test: *P<0.05, **P<0.01, and ***P<0.001; ^P<0.01 vs. Chow-IF-Fed and $^{\#}$P<0.01 vs. Chow-IF-Fast.

IF improved glucose tolerance in chow and HFD fed mice

IF improved glucose tolerance as assessed by glucose AUC in both diet groups (all P<0.05, **Fig. 5.2A** and **2B**). Insulin AUC was reduced by IF in HFD fed mice only (P<0.001, **Fig. 5.2D** and **2E**). We additionally measured glucose tolerance in the HFD-IF group after a 20-hour fast, but this was not different from HFD-IF group that were fasted for 6-hours (**Fig. 5.2C**). In the fed state, IF reduced terminal blood glucose in both diet groups (schedule effect, P<0.01, **Fig. 5.2F**), but reduced insulin and HOMA-IR in HFD fed mice only (both P<0.05, **Fig. 5.2G** and **2H**). Fasting glucose, insulin and HOMA-IR was also reduced in fasted vs. fed state in IF groups (all P<0.01, **Fig. 5.2F** to **2H**).

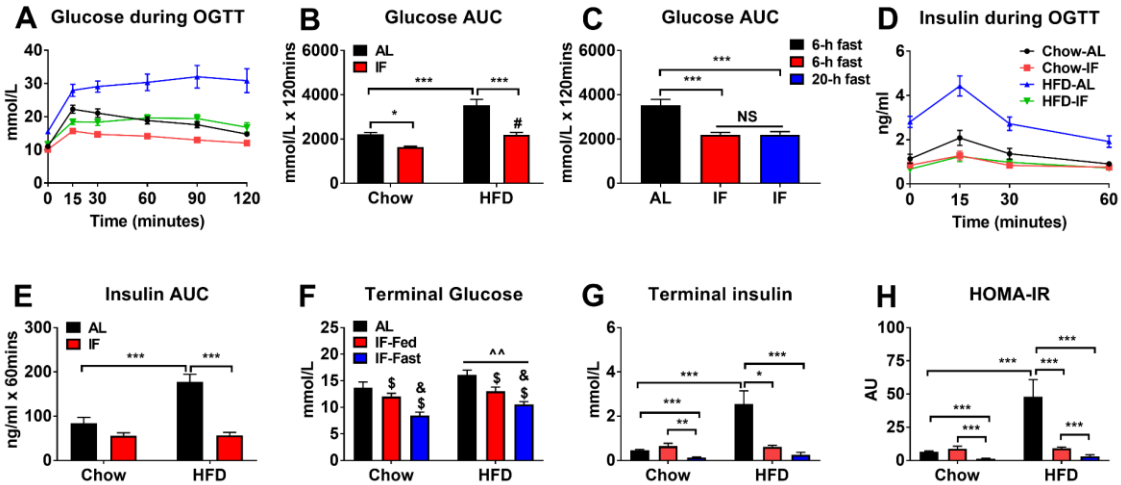

Figure 5-2: IF improved glucose tolerance in chow and HFD fed mice

(A to C): Glucose and area of glucose under the curve during OGTT. **(D** and **E):** Plasma insulin and area of insulin under the curve during GTT. **(F** and **G):** Terminal glucose and insulin. **(H):** HOMA-IR calculated from terminal glucose and insulin. AU: arbitrary unit.

Data presented as Mean±SEM, n=7-8/group. Two-way ANOVA with Bonferroni post hoc test. Diet effect: ^^P<0.01. Schedule effect: $P<0.01 vs. AL and &P<0.001 vs. IF-Fed. Post hoc test: *P<0.05, **P<0.01, and ***P<0.001; #P<0.05 vs. Chow-IF.

IF reduced adipose tissue inflammation in HFD fed mice

Markers of adipose tissue inflammation were increased in both gonadal and inguinal fat in HFD-AL vs. Chow-AL group (**Fig. 5.3A** to **3G)**. IF decreased mRNA levels of *Lgals3*, *Itgax*, and *Ccl2* in both gonadal and inguinal fat, and decreased crown-like structure and pan-macrophage numbers in gonadal fat in HFD fed mice (all P<0.05, **Fig. 5.3B**, **3D**, **3E**, **3G**, **3M**, **3O**, **3S** and **3T**). IF did not alter any markers of inflammation in chow fed mice, except for decreased *Lgals3* mRNA levels in gonadal fat (P<0.05, **Fig. 5.3B**).

Adgre1, *Mrc1*, *Arg1* and *Ccl2* mRNA levels were lower in fasted vs. fed states in gonadal fat in IF groups (P<0.01, **Fig. 5.3A**, **3I**, **3J** and **3M**). Crown-like structure and pan-macrophage numbers were not different between fed vs. fasted states in both diets (**Fig. 5.3S** and **3T**). NEFA levels were increased in fasted vs. fed state in both diets (both P<0.05, **Fig. 5.3P**), but there was no relationships between the changes in NEFA and any marker of inflammation.

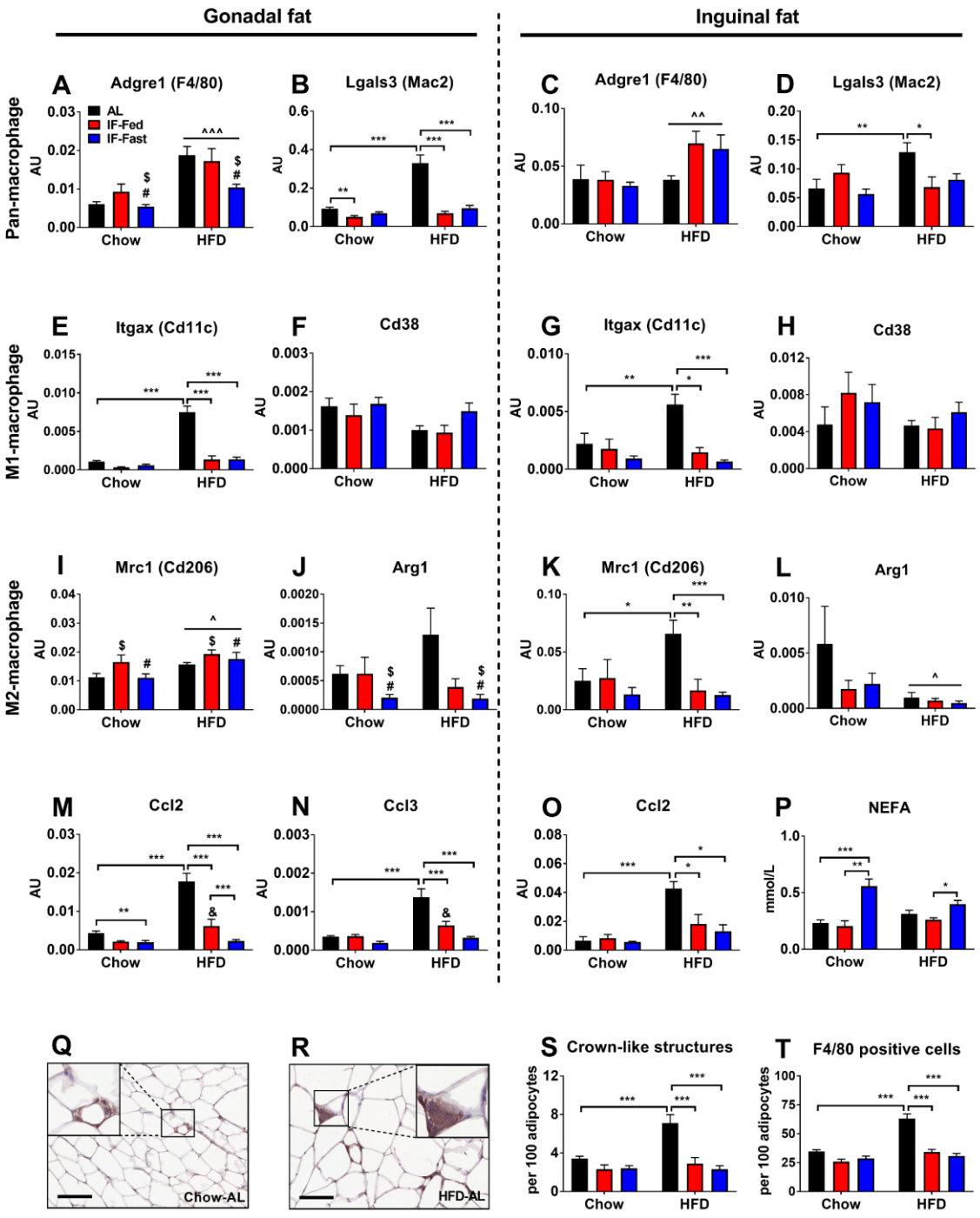

Figure 5-3: **IF reduced adipose tissue inflammation HFD fed mice**

(**A** to **D**): mRNA levels of pan-macrophage marker *Adgre1* and *Lgals3*. (**E** to **H**): mRNA levels of M1-macrophage marker *Itgax* and *Cd38*. **(I** to **L):** mRNA levels of M2-macrophage marker *Mrc1* and *Arg1*. **(M** and **N):** mRNA levels of macrophage recruitment marker *Ccl2* and *Ccl3* in gonadal fat. **(O):** mRNA levels of macrophage recruitment marker *Ccl2* ininguinal fat, *Ccl3* was undetectable in inguinal fat. **(P):** Plasma NEFA. (**Q** and **R**): Representative images of immunohistochemical staining for F4/80 (pan-macrophage) in gonadal fat in chow and HFD *ad libitum* fed mice. (**S** and **T**): Quantification of Crown-like structures and F4/80 positive cells in gonadal fat, results were adjusted adipocyte numbers. Scale bar: 100µm. AU: arbitrary unit.

Data presented as Mean±SEM. n=7-8/group for A to P, n=5/group for S and T. Two-way ANOVA with Bonferroni post hoc test. Diet effect: $^{\wedge}$P<0.05, $^{\wedge\wedge}$P<0.01 and $^{\wedge\wedge\wedge}$P<0.001 vs. chow. Schedule effect: $^{\$}$P<0.05 vs. AL and $^{\#}$P<0.05 vs. IF-Fed. Post hoc test: *P<0.05, **P<0.01 and ***P<0.001; $^{\&}$P<0.05 vs. Chow-IF-Fed.

IF reduced fibrosis in gonadal adipose tissue in HFD fed mice

IF increased *Col3a1* mRNA levels in gonadal and inguinal fat (schedule effect, both P<0.05, **Fig. 5.4A** and **4C**), but decreased *Col6a1* mRNA levels in gonadal fat (schedule effect, P<0.01, **Fig. 5.4B**). IF increased *Mmp9* mRNA levels and decreased *Timp1* mRNA levels in gonadal fat in HFD fed mice only (all P<0.001, **Fig. 5.4F** and **4I**). Collagen deposition assessed by histology was reduced in gonadal fat in HFD-IF vs. HFD-AL group (P=0.05, **Fig. 5.4K** and **4L**), but was increased in inguinal fat in Chow-IF vs. Chow-AL group (P<0.001, **Fig. 5.4M** and **4N**). Decreased *Col3a1*mRNA levels, but increased *Col6a1* and *Mmp2* mRNA levels in gonadal fat were observed in the fasted vs. the fed state in both IF groups (schedule effect, all P<0.05, **Fig. 5.4A**, **4B** and **4E**).

Responses to a single acute fast in chow and HFD fed mice

Recent studies have shown that acute fasting for 24-hours increased markers of inflammation in adipose tissue (Kosteli et al., 2010, Ding et al., 2016, Asterholm et al., 2012). We did not detect this in response to IF, but habituation to fasting may have occurred. Thus, a group of mice were examined after the first exposure to an intermittent fasting diet. In response to one acute 22-hour fast, blood levels of NEFA were increased and insulin levels were decreased in both diet groups (schedule effect, all P<0.001, **Fig. 5.5A** and **5B**). Fasting did not increase the mRNA levels of any inflammation markers examined in gonadal fat or inguinal fat (**Fig. 5.5C** to **5J**, inguinal fat data not shown). These results were confirmed by histology (**Fig. 5.5K** and

5L). *Col6a1* and *Timp1* mRNA levels were decreased after one acute fast (schedule effect, all P<0.05, **Fig. 5.5N** and **5Q**).

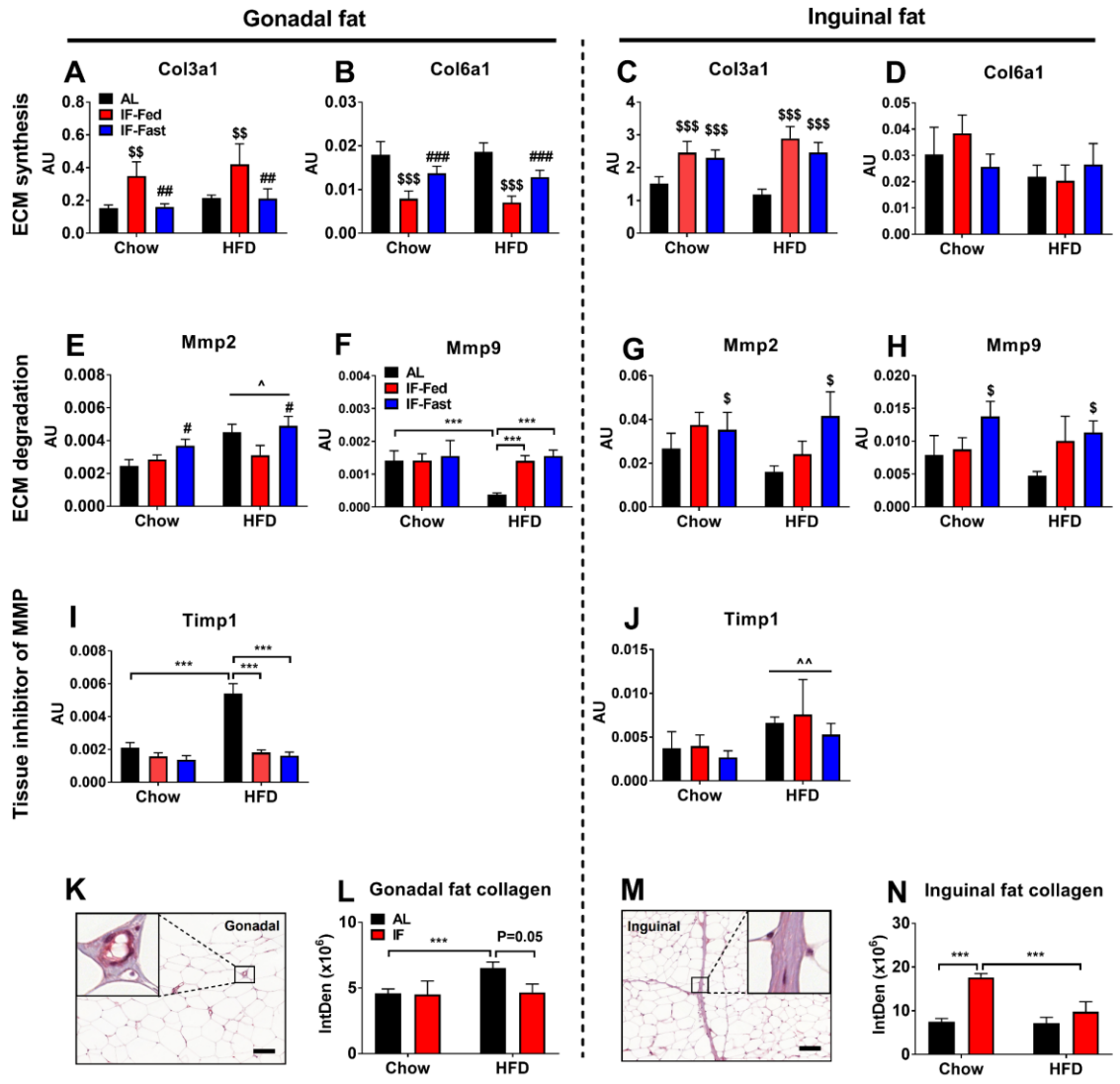

Figure 5-4: IF reduced fibrosis in gonadal fat in HFD fed mice

(A to **D):** mRNA levels of ECM synthesis marker *Col3a1* and *Col6a1*. **(E** to **J):** mRNA levels of ECM degradation marker *Mmp2*, *Mmp9* and *Timp1*. (**K** and **M**): Representative images of Masson's trichrome staining for gonadal and inguinal fat. (**L** and **N**): Quantification of collagen integrated density in gonadal and inguinal fat. Scar bar: 100µm. AU: arbitrary unit.

Data presented as Mean±SEM. n=7-8/group for A to J and n=5-6/group for L and N. Two-way ANOVA with Bonferroni post hoc test. Diet effect: $^{\wedge}$P<0.05, $^{\wedge\wedge}$P<0.01 and $^{\wedge\wedge\wedge}$P<0.001 vs. chow. Schedule effect: $^{\$}$P<0.05, $^{\$\$}$p<0.01 and $^{\$\$\$}$P<0.001 vs. AL; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. IF-Fed. Post hoc test: ***P<0.001.

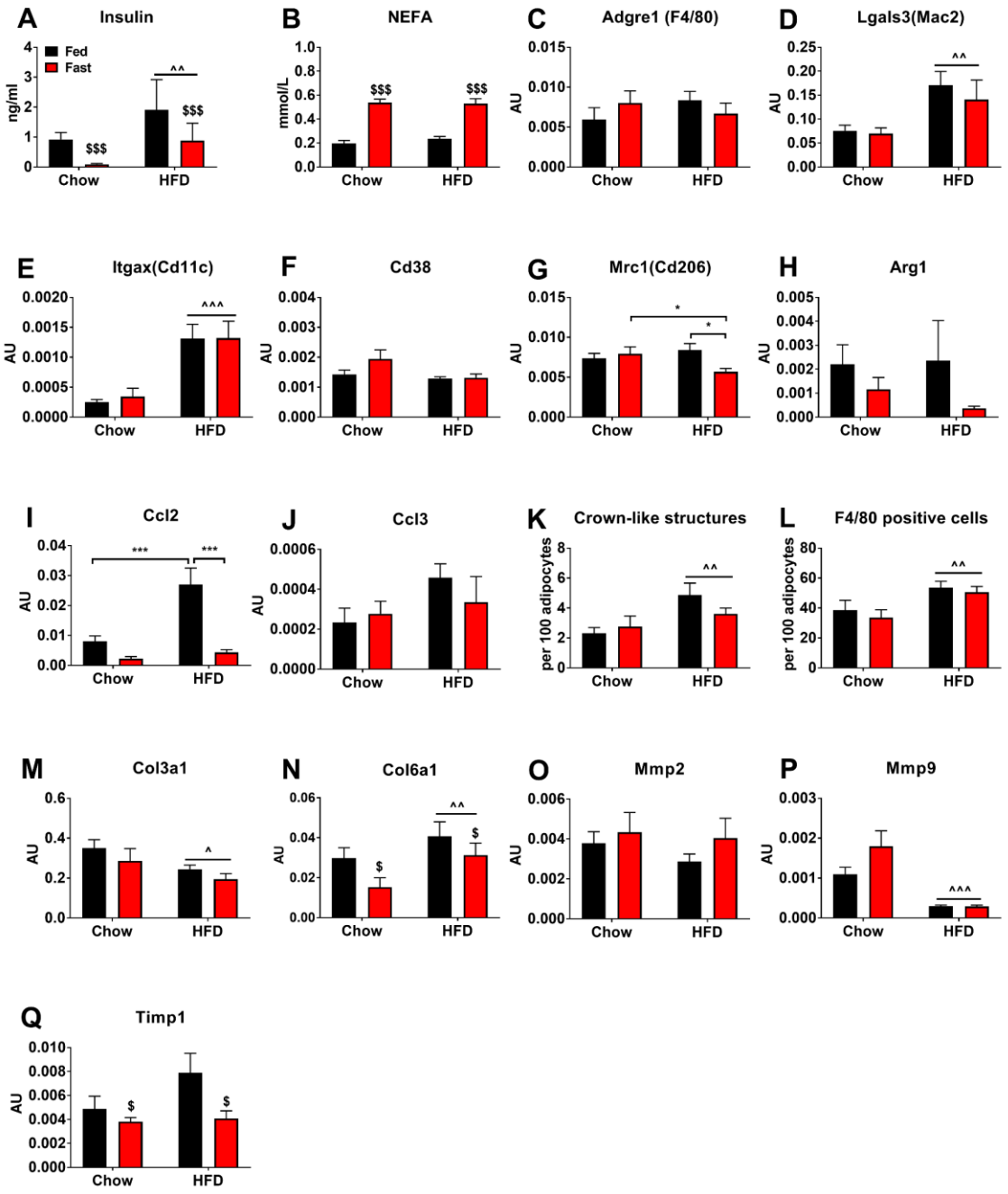

Figure 5-5: Responses to a single acute fast in chow and HFD fed mice

(A and **B):** Plasma insulin and NEFA levels. (**C** and **D**): mRNA levels of pan-macrophage marker *Adgre1* and *Lgals3*. (**E** and **F**): mRNA levels of M1-macrophage marker *Itgax* and *Cd38*. **(G** to **H):** mRNA levels of M2-macrophage marker *Mrc1* and *Arg1*. **(I** and **J):** mRNA levels of macrophage recruitment marker *Ccl2* and *Ccl3*. **(K** and **L):** Crown-like structures and F4/80 positive cells, results adjusted adipocyte numbers. **(M** and **N):** mRNA levels of ECM synthesis marker *Col3a1* and *Col6a1*. **(O** to **Q):** mRNA levels of ECM degradation marker *Mmp2*, *Mmp9* and Timp1. AU: arbitrary unit.

Data presented as Mean±SEM. n=6/group. Two-way ANOVA with Bonferroni post hoc test. Diet effect: $^{\wedge}$P<0.05, $^{\wedge\wedge}$P<0.01 and $^{\wedge\wedge\wedge}$P<0.001 vs. chow. Schedule effect: $^{\$}$P<0.05 and $^{\$\$\$}$P<0.001 vs. AL. Post hoc test: *P<0.05 and ***P<0.001.

5.5 Discussion

Inflammation and fibrosis of adipose tissue occurs in obese animals, and in humans, and is associated with impaired glucose tolerance and insulin resistance (Weisberg et al., 2003, Xu et al., 2003, Lumeng et al., 2007a, Sun et al., 2013, Pasarica et al., 2009a). Caloric restriction improves this phenotype (Zamarron et al., 2017, Higami et al., 2006, Anson et al., 2003, Argentino et al., 2005). IF promotes weight loss and may improve glucose tolerance in diet-induced obese mice (Joslin et al., 2017, Li et al., 2017, Liu et al., 2017b). However, the effects of IF on adipose tissue remodelling is unclear. Our results suggest that IF improves glucose tolerance in both lean and obese mice, and reduced adipose tissue inflammation and fibrosis in obese mice.

In this study, chow fed mice were able to consume sufficient energy during feeding days to compensate for the fasting days, and maintain body weight. However, mice that were fed a high-fat diet were unable to compensate entirely, resulting in weight loss. This is consistent with recent studies (Liu et al., 2017b, Joslin et al., 2017). In our study, HFD-IF mice presented with greater inguinal and gonadal fat mass compared to chow-IF mice. This is in agreement with previous studies which showed formerly diet-induced obese mice retained greater adiposity compared to lean controls (Wang et al., 2011, Guo et al., 2009). Gonadal and inguinal fat pad weight was also lower in chow-IF versus chow-AL mice, although these mice did not display overall weight differences. This could indicate increased lean mass after IF. Indeed, increased lean mass was reported in diet-induced obese mice subjected to chow diet with IF (Gotthardt et al., 2016). Preservation of lean mass was also observed in humans who underwent a modified IF intervention, where they were allowed to consume 25% of energy requirement on each fasting day (Klempel et al., 2013b), but was not observed in a recent study by our

group whom consumed ~30% of requirements at breakfast prior to initiating a 24-hour fast (**Chapter 3**)

In this study, we observed that oral glucose tolerance was improved by IF in both diet groups. In diet-induced obese mice, this finding is controversial. Gotthardt *et al* (Gotthardt et al., 2016) observed no change in oral glucose tolerance using the same dose applied in this study. However, glucose tolerance was improved in diet-induced obese mice when given at 1g/kg orally or by intraperitoneal injection (Liu et al., 2017b, Joslin et al., 2017, Kim et al., 2017). There is also some evidence that glucose tolerance is impaired on fasted vs. fed days in IF mice that were fed a HFD (Joslin et al., 2017). We have also shown transient insulin resistance by clamp following a fasting day in humans with obesity after 8 weeks of IF (**Chapter 3**). In our hands, fasting did not alter oral glucose tolerance in mice fed a high-fat diet. Since a group of mice that were paired-fed to the HFD-IF group was not included, and thus results cannot be distinguished between caloric restriction and weight loss, and IF *per se*. In mouse studies, insulin sensitivity is reported to be improved (Kim et al., 2017, Gotthardt et al., 2016) and unchanged (Liu et al., 2017b) when it was assessed by insulin tolerance test (Liu et al., 2017b, Kim et al., 2017, Gotthardt et al., 2016). No studies have examined measures of insulin sensitivity by tracers, or hyperinsulinaemic-euglycaemic clamp in response to intermittent fasting.

Obesity is a low-grade inflammatory state with increased macrophage accumulation in white adipose tissue (Xu et al., 2003, Weisberg et al., 2003). In mice, a large body of evidence has suggested that weight loss reduces adipose tissue macrophages and improves insulin sensitivity (Hoevenaars et al., 2014, Zamarron et al., 2017, Kovacikova et al., 2011, Magkos et al., 2016). Weight loss may also promote the phenotype switching of macrophages from inflammatory

M1- to anti-inflammatory M-2 profile (Lumeng et al., 2007a, Cancello et al., 2005, Fabbiano et al., 2016). In this study, HFD increased markers of adipose tissue macrophages and inflammation, which was rescued by IF. This is in agreement with a recent study by Kim *et al* (Kim et al., 2017), which showed that a modified IF regimen (which was comprised of 2 feeding days followed by 1 fasting day) reduced inflammation-related genes in gonadal fat in HFD fed mice (Kim et al., 2017). However, we did not observe M2-polarisation of macrophages following IF as reported by Kim *et al* (Kim et al., 2017). Of note, there was no change in markers of macrophages in IF mice that were fed a chow diet, despite improvements in glucose tolerance.

Increased macrophage accumulation in adipose tissue has also been reported during the early stage of weight loss by calorie restriction, and after a 24-hour fast (Kosteli et al., 2010, Ding et al., 2016, Asterholm et al., 2012, Ebke et al., 2014). Further studies show that adipose tissue macrophages take up and store lipids (Caspar-Bauguil et al., 2015, Kosteli et al., 2010). Contrary to studies that have reported increased macrophage infiltration in response to an acute 24-hour fast(Kosteli et al., 2010, Asterholm et al., 2012, Ding et al., 2016), there was no change in adipose tissue macrophages in either IF group, after the fasting day, despite marked elevation in NEFA. We theorized this could be due to habituation to intermittent fasting and therefore examined the response to the first day of IF in a separate group of mice fed chow or HFD. Under these conditions, fasting did not alter the mRNA levels of any inflammatory genes or macrophage numbers as assessed by histology. This discrepancy could be due to differences in the mice strains, ages or diet composition, or the clock time that the tissues were collected. Indeed, in one study (Kosteli et al., 2010), the time they collected tissue from fasted mice was at ZT2, which was 7-8 hours earlier than the time tissue was collected in our study. This could be of importance, since macrophages and cytokines levels are under circadian control (Keller

et al., 2009). We should also consider that mice were fasted for 22 hours rather than 24 hours, and that the "fed mice" in our study had likely not eaten since lights on at ZT0, potentially elevating the baseline comparison level. However, the single reference gene used to normalized gene expression results in previous studies (Kosteli et al., 2010, Ding et al., 2016) was reduced by acute fasting in our hands. Additionally, that study adjusted the macrophage count by total nuclei, or white blood cells, in adipose tissue. Fasting reduces white blood cells, and monocyte counts in blood in mice and humans (Walrand et al., 2001, Brandhorst et al., 2015, Cheng et al., 2014, Choi et al., 2016), which if occurring in adipose tissue, could artificially elevate the number of macrophages detected.

Increased fibrosis in adipose tissue is linked with inflammation and insulin resistance (Sun et al., 2013, Guglielmi et al., 2015). The homeostasis of ECM is a balance between collagen synthesis, and degradation by matrix metalloproteinases (MMPs), and tissue inhibitor of metalloproteinases (TIMPs) that negatively regulate the enzyme activity of matrix metalloproteinase, to avoid excessive degradation of the ECM (Bonnans et al., 2014, Sun et al., 2013, Martinez-Santibanez and Lumeng, 2014). Our results show that IF reduced ECM synthesis and promoted ECM degradation in gonadal fat in mice that were fed a high-fat diet. This result was supported by histology. In contrast, mRNA levels of collagen, and collagen content, were increased in inguinal adipose tissue by IF. This could be due to the different nature of collagen in gonadal and inguinal fat. In gonadal fat, collagen presents a peri-adipocyte property, surrounding adipocytes and dominantly locating in crown-like structures area (Sun et al., 2013, Zamarron et al., 2017). In inguinal fat, however, large fibre bundles are frequently presented in adipose tissue through which subcutaneous fat pads attach to the skin. We speculate that IF promotes fat pad loss, but may have less impact on large fibre bundles than peri-cellular collagens. This highlights that not only the "quantity" but also the "structure or

quality" of collagen may be important when assessing tissue fibrosis. Further studies are required to investigate the structure or stiffness or adipose tissue following IF.

In conclusion, IF promoted fat mass loss and improved glucose tolerance in mice fed a chow or high-fat diet. Adipose tissue inflammation and fibrosis was also improved as a result of intermittent fasting in mice fed a high-fat diet.

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Duality of Interest

No potential conflicts of interest relevant to this article were reported.

Author contributions

BL performed the study, acquired and analysed the data, and wrote the manuscript. AJP supervised the study, collected and interpreted the data. GH and MC performed the study and acquired the data. GAW interpreted the data and helped draft the manuscript. LKH conceived, designed and supervised the study and interpreted the results. All authors reviewed and approved the final manuscript. LKH is the guarantor of this work and, as such, had full access

to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation

Parts of this study were presented as an oral presentation at the Joint Scientific Meeting of The Australian and New Zealand Obesity Society and the Obesity Surgery Society of Australia and New Zealand, Adelaide, Australia, 2017.

Chapter 6: Intermittent Fasting Increases Energy Expenditure and Promotes White Adipose Tissue Browning in Mice, but Not in Humans

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Publication Details	

Principal Author

Name of Principal Author (Candidate)	Bo Liu		
Contribution to the Paper	Performed the study, collected and analysed the data, and wrote the manuscript.		
Overall percentage (%)	50%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.		
Signature		Date	18 May 2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Amanda J Page		
Contribution to the Paper	Supervised the mouse study, contributed to data collection and analysis for mouse study.		
Signature		Date	18/5/18

Continued on the next page

Name of Co-Author	Amy T Hutchison		
Contribution to the Paper	Performed the human study, collected and analysed the data for human study.		
Signature		Date	21.5.2018

Name of Co-Author	Gary A Wittert		
Contribution to the Paper	Contributed to study design, clinical support to the human study and manuscript preparation.		
Signature		Date	18/05/18

Name of Co-Author	Leonie K Heilbronn		
Contribution to the Paper	Conceived, designed and supervised the mouse and human studies.		
Signature		Date	29/5/18

6.1 Abstract

Background: Intermittent fasting (IF) may limit metabolic adaptations that reduce energy expenditure, potentially by stimulating white adipose tissue browning. This study examined the effects of 8 weeks of intermittent fasting on energy metabolism and markers of white adipose tissue browning in lean and diet-induced obese mice and in women who were overweight or obese.

Methods: Ten-week old male C57BL/6J mice were fed a high-fat (43% fat) or chow diet (18% fat) for 8 weeks *ad libitum*, then randomized to *ad libitum* or IF for 8 weeks. A 24-hour fast was initiated one hour before lights off, on 3 non-consecutive days per week (Mon, Wed, Fri). At 27 weeks of age, meal size and number, energy intake and energy expenditure were measured for 3 days in a metabolic cage (2 fed and one fast day), followed by collection of inguinal and gonadal fat pads in the fed state, or after a 22-hour fast (IF mice only). Uncoupling protein 1 (UCP1) in fat was assessed by qPCR and immunohistochemistry in mice. *UCP1* mRNA levels were also examined in subcutaneous adipose tissue at baseline, and after 8-weeks of IF (in the fed state, and after a 24-hour fast), in women who were overweight or obese (BMI 25.0-42.0kg/m^2, aged 35-70 years).

Results: IF reduced body weight and energy intake in high-fat, but not chow diet, fed mice. Gonadal and inguinal fat pad weights were reduced by IF in both diet groups (all $P<0.05$). Energy expenditure and meal numbers were increased in both IF diet groups on fed days ($P<0.05$). IF increased *Ucp1* mRNA levels in both inguinal and gonadal fat depots, and UCP1 protein in inguinal fat (schedule effect, all $P<0.05$). In women, IF reduced body weight and fat mass (-4.0±0.4kg and -3.1±0.3kg [Mean± SEM], respectively, both $P<0.001$), but had no effect

on *UCP1* mRNA levels in subcutaneous adipose tissue, irrespective of whether it was sampled in the fed or fasted state.

Conclusions: Intermittent fasting increased energy expenditure and promoted white adipose tissue browning in chow and high-fat diet fed mice. However, IF did not alter *UCP1* mRNA levels in subcutaneous adipose tissue in women.

Key words: adipose tissue, browning, energy expenditure, intermittent fasting

6.2 Introduction

Intermittent fasting (IF) is a dietary intervention that involves periods of minimal or no caloric intake followed by periods, typically 24-hours, of unrestricted eating. IF is effective to extend lifespan (Arum et al., 2009, Xie et al., 2017, Goodrick et al., 1990), reduce fat mass (Gotthardt et al., 2016, Barquissau et al., 2018, Li et al., 2017), improve glucose tolerance and insulin sensitivity (Anson et al., 2003, Gotthardt et al., 2016, Joslin et al., 2017), and reduce the risk for cardiovascular diseases (Krizova and Simek, 1996, Wan et al., 2003) and cancer (Xie et al., 2017, Chen et al., 2016b) in chow fed rodents. However, few studies have investigated the response in obese animals (Gotthardt et al., 2016, Joslin et al., 2017, Liu et al., 2017b, Li et al., 2017, Kim et al., 2017). In humans, most studies of IF have been conducted in individuals who are overweight or obese. In these studies, IF promotes weight and fat mass loss (Heilbronn et al., 2005b, Klempel et al., 2013b, Trepanowski et al., 2017, Byrne et al., 2018), reduces total cholesterol, low-density lipoprotein cholesterol, triglycerides, systolic blood pressure (Varady et al., 2009, Trepanowski et al., 2017), and improves markers of insulin sensitivity (Heilbronn et al., 2005b).

Brown adipose tissue plays a critical role in energy homeostasis and thermogenesis (Sidossis and Kajimura, 2015). In addition to the classical brown adipocytes residing in brown fat, brown-like adipocytes located within white adipose tissue also have thermogenic properties (Sidossis and Kajimura, 2015). A range of external cues, such as cold exposure (Barbatelli et al., 2010, Lim et al., 2012, Fisher et al., 2012), exercise (Bostrom et al., 2012, Xu et al., 2011, Stanford et al., 2015) and pharmaceutical treatment (Wu et al., 2013, Cypess et al., 2015), promote the development of beige adipocytes in white adipose tissue in mice. This is known as “browning” of white adipose tissue. Recently, three animal studies have shown that both daily calorie restriction and IF promote white adipose tissue browning (Fabbiano et al., 2016,

Li et al., 2017, Kim et al., 2017), and suggest this could be linked with the alternative activation of macrophages (Kim et al., 2017, Li et al., 2017).

The aim of this study was to examine the effects of intermittent fasting on food intake and energy expenditure and adipose tissue browning in mice fed chow or high-fat diet in the fed and fasted state. We also assessed the effects of IF on expression of uncoupling protein 1 (UCP1) in subcutaneous adipose tissue in women who were overweight or obese.

6.3 Research Design and Methods

Animals and diets

This study was approved by the animal ethics committees of the South Australian Health and Medical Research Institute (SAHMRI) and the University of Adelaide, and was performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Mice used in this study were the same as the mice used in **Chapter 5**. Briefly, ten-week old male C57BL/6J mice (Bioresources, SAHMRI, Australia) were housed four per cage in the Bioresources Facility at SAHMRI under a 12:12h light/dark cycle, with lights on at 7am (Zeitgeber time 0, ZT0) and temperature at 21±3°C. Mice were fed either a lard based high-fat diet (HFD, 43% fat, SF04-001, Specialty Feeds, Australia) or a chow diet (chow, 18% fat, 2018SX, Envigo, United States) *ad libitum* for eight weeks prior to randomizing mice on each diet into *ad libitum* feeding (AL, n=8) or intermittent fasting (IF, n=16) for another eight weeks. Intermittent fasting was initiated at ZT11 for 24-hours for 3 non-consecutive days/week. Food access was controlled by transferring mice daily between cages with or without food. Mice fed *ad libitum* were also transferred between feeding cages at the same time to standardize handling. All mice had free access to water throughout the study. Body weight and food intake were monitored at ZT11 weekly before IF was introduced, and daily after IF was implemented. At 28-weeks old, all mice were sacrificed at ZT7-ZT9 with mice in IF groups culled after feeding or 22-hours fasting (n=7-8/group). Inguinal and gonadal adipose tissue were collected.

At twenty-seven weeks of age, indirect calorimetry (n=7-8/group) was assessed. Mice were acclimated to the metabolic cages for 24-hours prior to data collection (Promethium, Sable Systems, Las Vegas, NV). Three days of data were collected after acclimation. This was two consecutive feeding days, followed by a fasting day. Food access for mice in IF groups was controlled using gates connected to the food hoppers. Food spilled by mice and dropped into

bedding was carefully removed using forceps before the commencement of data recording, and prior to the fasting day, to improve the accuracy of food consumption monitoring. Four mice in each HFD group dragged food from the hopper during monitoring periods, this data was not included in the analysis and an additional 4 mice were measured to repeat metabolic monitoring. However, this behaviour was repeated in some mice and finally n=4-7/group were included for the analysis of food related parameters including meal size, meal number and energy intake. Data analysis of oxygen consumption (VO_2), carbon dioxide expired (VCO_2), respiratory quotient (RQ), energy expenditure (EE) and activity was performed in n=7-8/group. VO_2 and VCO_2 were measured at 5 minutes intervals for 1 minute to calculate RQ and EE as described previously (Kaiyala et al., 2012). VO_2, VCO_2 and EE were adjusted by a modified body mass which was determined by subtracting collected inguinal and gonadal fat mass from body mass, lessening the weight bias from this metabolically less active tissue (Tschop et al., 2011). We also normalized EE by other ways including body weight, body weight raised to the power 2/3 or 3/4, or analysis of covariance as mentioned previously (Tschop et al., 2011). For ambulatory activity, consecutive adjacent infrared beam breaks in x-, y- and z-axes as an activity count were recorded every 5 min as previously described (Kaiyala et al., 2012). Data acquisition and food access control were coordinated by MetaScreen v.2.3.4 and raw data were extracted using ExpeData v.1.6.4 (Sable Systems) with built-in macros. All metabolic data were expressed in two ways: hourly and daily. Hourly value was calculated by the real-time output in one hour. Daily value was determined by the sum of hourly value on each day. Weekly values for energy intake and energy expenditure were calculated using (Day 1+ Day 2+ Day 3)/3*7 for AL groups, and (Day 1 *3+Day 2+Day3*3) for IF groups.

Quantitative real-time PCR

The extraction of total RNA, the determination of RNA concentration and purity, and the synthesis of cDNA from RNA were performed as previously reported (Chen et al., 2014). Quantitative real-time PCR was performed using the Taqman Ucp1 primer (Mm01244861_m1), and Fast Universal PCR Master Mix (Cat: 4352042, Applied Biosystems, Foster City, CA, USA). The samples were run in duplicate on an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) with corresponding internal negative controls and standard curve (pooled from ten participants at baseline, and from individual mouse, respectively). Relative gene expression was analysed using the $2^{-\Delta CT}$ method, where $\Delta CT = (CT_{\text{target gene}} - CT_{\text{reference gene}})$. *Actb* (Mm00607939_s1) and *B2m* (Mm00437762_m1) were selected out from 6 housekeepers (*18s*, *Actb*, *Gapdh*, *Hprt*, *Ppia* and *B2m*) using NormFinder as the most stable reference for mice.

Immunohistochemistry

Inguinal adipose tissue from mice (n=6/group) were fixed in Bouin's solution (HT10132, Sigma-Aldrich), dehydrated, paraffin embedded, sectioned at 5um and mounted on positively-charged glass slides. De paraffinised and rehydrated slides were incubated with ELOXALL solution (SP-600, Vector) for 10 minutes at room temperature to eliminate endogenous peroxidase and alkaline phosphatase. Antigen retrieval was achieved using modified citrate-based buffer (S1700, Dako) and incubation in a 95°C water bath for 20 minutes. Slides were incubated with a rabbit anti-UCP1 (1:400, Ab10983, Abcam) overnight and then goat anti-rabbit secondary antibody (1:500, Ab6721, Abcam) for 1-hour at room temperature. Immunohistochemical detection was performed using 3, 3′-diaminobenzidine (DAB, SK-4105, Vector) and slides were counterstained with Mayer's haematoxylin, followed by dehydrating and mounting. All slides were randomly assigned numeric codes by a research officer to blind the investigator (BL) quantifying outcomes. Slides were scanned using the Pannoramic 250

Flash II scanner (3DHISTECH, Budapest, Hungary). UCP1 positive areas was analysed in 10 randomly selected fields at 40X using Image J built-in macros (National Institutes of Health).

Human study

This human study was registered as a clinical trial with Clinicaltrials.gov (NCT0176997) and approved by the Research Ethics Committee of the Royal Adelaide Hospital and the University of Adelaide. All participants provided written, informed consent prior to their inclusion. The design of the human study and baseline characteristics of the participants have been reported in **Chapter 3**. Briefly, fifty healthy women who were overweight and obese, aged 35-70 years, BMI 25.0-42.0 kg/m^2 were randomly assigned to one of two IF groups for 8 weeks. Participants were provided with ~30% of their daily energy requirements for breakfast, and then initiated a 24-hours fast on 3 non-consecutive days/week. On fed days, one group was provided foods at ~100% of energy requirements, to achieve an overall 30% energy deficit (IF70). The other group was provided foods at ~145% of their daily energy requirements on fed days, without overall energy restriction (IF100). Periumbilical subcutaneous adipose was obtained by percutaneous biopsy at baseline and after 8 weeks of the intervention diet, after a 12-hour overnight fast and 24-hour fast as described previously (Chen et al., 2016a). Twenty-two participants in each group completed the intervention. Due to the unwillingness of some participants to undergo biopsies and scheduling conflicts, biopsy samples were obtained from all 3 visits from only fourteen individuals in each group. For the purpose of this study, the two IF groups were combined together to assess *UCP1* mRNA levels in subcutaneous adipose tissue. *UCP1* mRNA levels (Hs00222453_m1) in human subcutaneous adipose tissue were normalized by *ACTB* (Hs01060665_g1) and *PPIB* (Hs00168719_m1), which was not different at baseline and following the intervention.

Statistical analysis

Data are shown as mean ± SEM. All statistical analysis was performed using IBM SPSS Statistics 24 (Armonk, New York, USA). The normality of data distribution was assessed by Shapiro-Wilk test, and data were log10 transformed if not normally distributed. Single comparisons in mouse study were performed using two-way ANOVA with diet (chow and HFD) and schedule (AL and IF) as between group factors, Bonferroni post-hoc tests were performed when diet by schedule effects were presented. Time effects within group in mouse and human studies were examined by repeated measures ANOVA with Bonferroni post-hoc test or paired *t* test (for energy intake and meal numbers in IF mice on two consecutive feeding days only). Significance was accepted as $P < 0.05$.

6.4 Results

As reported in **Chapter 5**, final body weight was increased in HFD-AL (43.0±0.8g) vs. HFD-IF (32.9±0.8g), chow-AL (34.7±1.0g) and chow-IF (32.2±0.5g, all P<0.05), and was not different between Chow-AL, Chow-IF and HFD-IF groups. Gonadal and inguinal fat mass was reduced by IF in both diet groups, but was greater in HFD-IF vs. chow-IF groups (all P<0.05).

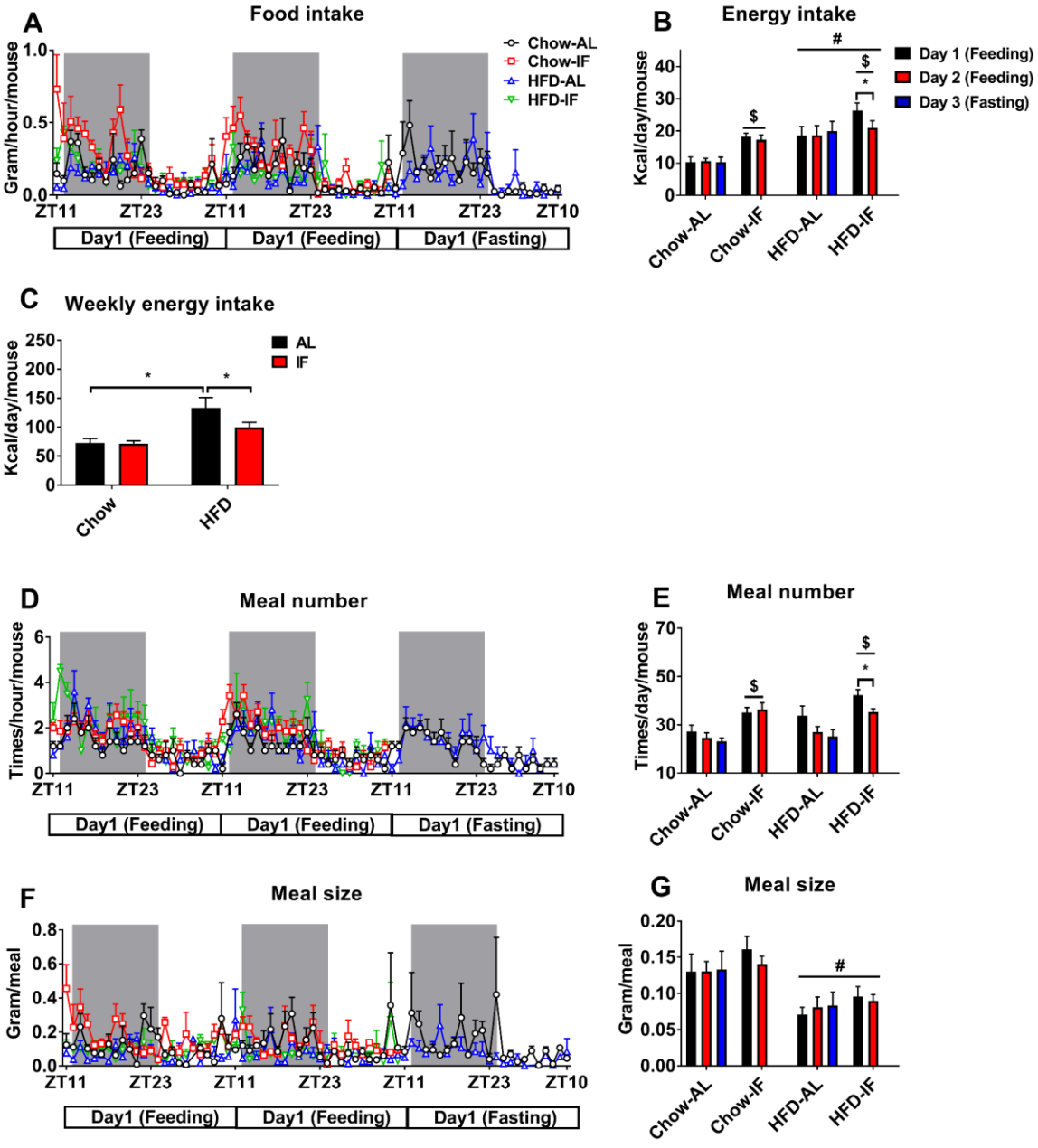

Figure 6-1: Energy intake, meal size and meal number in metabolic cages

(A-C): hourly, daily and calculated weekly food intake. **(D-E):** hourly and daily meal numbers. **(F-G):** hourly and daily meal size. Day 1 and 2 were two consecutive feeding days, followed by a fasting day (day 3). Effects of diet and schedule on each day or in a week (energy intake only) were analysed by two-way ANOVA with Bonferroni post hoc tests. Within group effects over days were analysed using repeated measures ANOVA with Boferroni post hoc test (AL groups) or paired *t* test (IF groups).

Data presented as Mean±SEM. N=4-7/group. Diet effect: $^{\#}P<0.05$ vs. chow on day 1, day 2 and day 3. Schedule effect: $^{\$}P<0.05$ vs. AL on day 1 and day 2. Post hoc test: $^{*}P<0.05$.

There were significant diet and schedule effects observed for energy intake measured in the metabolic chamber on two refeeding days, with increased energy intake in HFD vs. chow fed mice, and in IF vs. AL mice (all P<0.05, **Fig. 6.1A** and **1B**). Meal number was increased by IF on refeeding days (schedule effect, both P<0.05, **Fig. 6.1D** and **1E**). Of note, chow-IF mice maintained similar energy intake and meal numbers on both refeeding days (both P>0.05). However, HFD-IF mice displayed decreased energy intake and meal number on the second vs. first refeeding day (both P<0.05). Mathematical extrapolation of this to a weekly value suggested that overall energy intake was not different in chow-IF vs. chow-AL mice (-1.4%, P=0.94), but was 25% lower in HFD-IF vs. HFD-AL mice (P<0.05, **Fig. 6.1C**). This data supports the weighed food intake data that we reported in **Chapter 5**. Meal size was not altered by IF, but was smaller in HFD vs. chow fed mice (all P<0.05, **Fig. 6.1F** and **1G**).

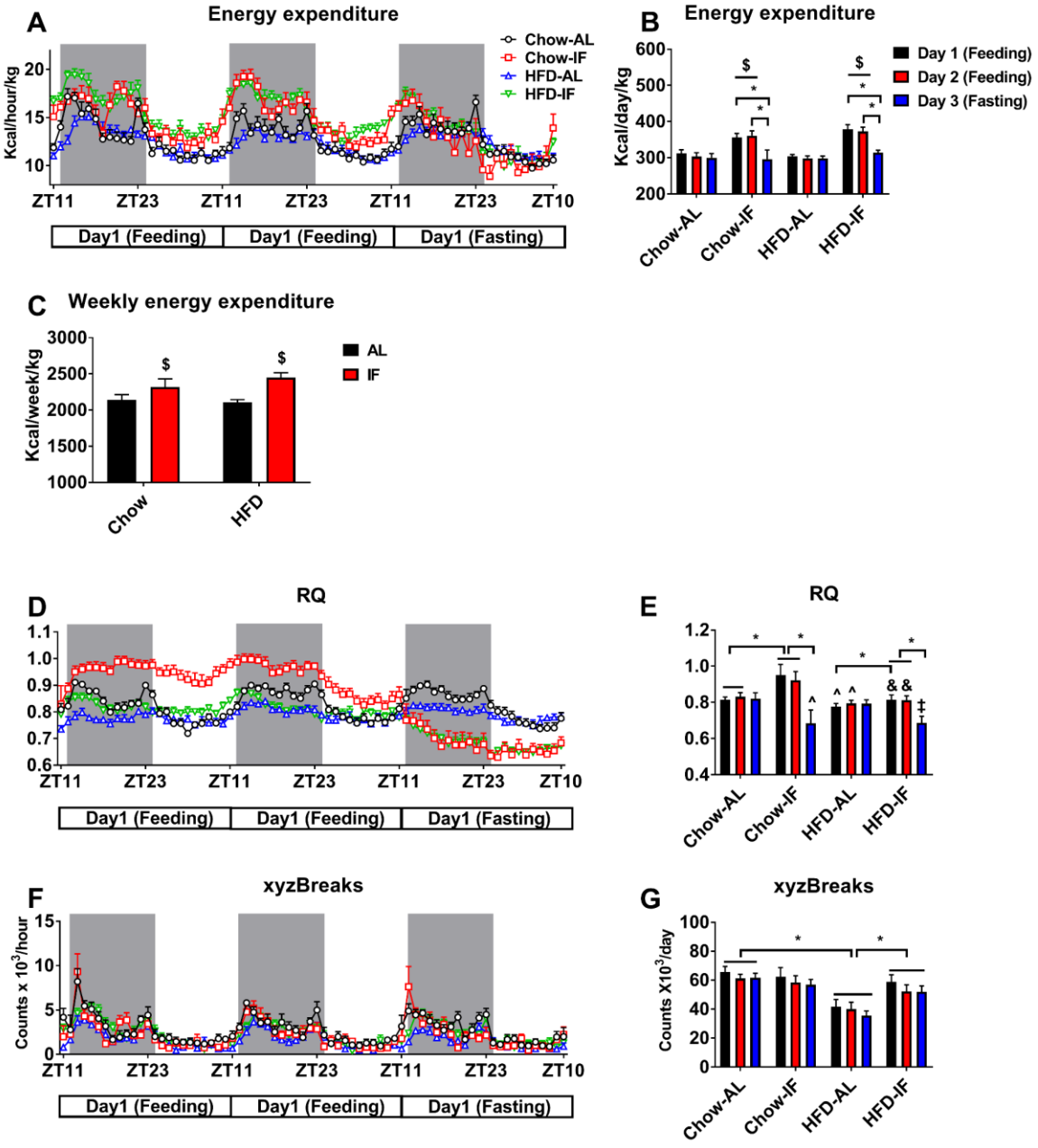

Figure 6-2: Energy expenditure, respiratory quotient and activity

(A-C): Hourly, daily and calculated weekly energy expenditure. **(D-E)**: Hourly and daily RQ. **(F-G)**: Hourly and daily activity. Day 1 and 2 were two consecutive feeding days, followed by a fasting day (day 3). Effects of diet and schedule on each day, or in a week (energy expenditure only) were analysed by two-way ANOVA with Bonferroni post hoc tests. Within group effects over three days were analysed using repeated measures ANOVA with Boferroni post hoc test.

Data presented as Mean±SEM. N=7-8/group. Schedule effect: $^{\$}P<0.05$ vs. AL on day 1 and day 2. Post hoc test: $^{*}P<0.05$; $^{\wedge}P<0.05$ vs. chow-AL; $^{\&}P<0.05$ vs. chow-IF and $^{\ddagger}P<0.05$ vs. HFD-AL.

There were significant schedule effects for energy expenditure on the two refeeding days, with increased energy expenditure in IF vs. AL mice (schedule effect, both P<0.001, **Fig. 6.2A** and **2B**). Energy expenditure on a fasting day was not different between IF and AL mice, but was lower versus a fed day (schedule effect, P<0.05, **Fig. 6.2B**). Mathematical extrapolation of this showed that calculated weekly energy expenditure was higher in IF vs. AL mice (schedule effect, P<0.001, **Fig. 6.2C**). This result held when energy expenditure was normalized against modified body mass, raw body weight, or body weight raised to the power 2/3 or 3/4, or by analysis of covariance with body weight as the covariate (Tschop et al., 2011). There were significant diet by schedule effects for averaged RQ on the two refeeding days (all P<0.01, **Fig. 6.2D** and **2E**). RQ was increased in chow-IF vs. chow-AL and HFD-IF mice on both refeeding days (all P<0.05), but was increased in HFD-IF vs. HFD-AL mice on the first refeeding day only (P<0.05). As expected, RQ was lower on fasted vs. fed days in IF groups (schedule effect, P<0.001), and vs. AL mice (both P<0.05). There were significant diet by schedule effects for activity, with reduced activity in HFD-AL vs. chow-AL and HFD-IF mice (all P<0.05, **Fig. 6.2F and 2G**). There was no difference in activity between fed and fasted days in IF mice.

Ucp1 mRNA levels in gonadal fat were higher in HFD vs. chow fed mice (diet effect, P<0.05, **Fig. 6.3A**). After a fed day, *Ucp1* mRNA levels in inguinal and gonadal fat and UCP1 protein in inguinal fat were increased in IF vs. AL mice (schedule effect, all P<0.05, **Fig. 6.3A** to **3E**). *Ucp1* mRNA levels in both fat pads were lower on a fasted vs. fed day in both IF groups (schedule effect, both P<0.05), but was not different from AL mice.

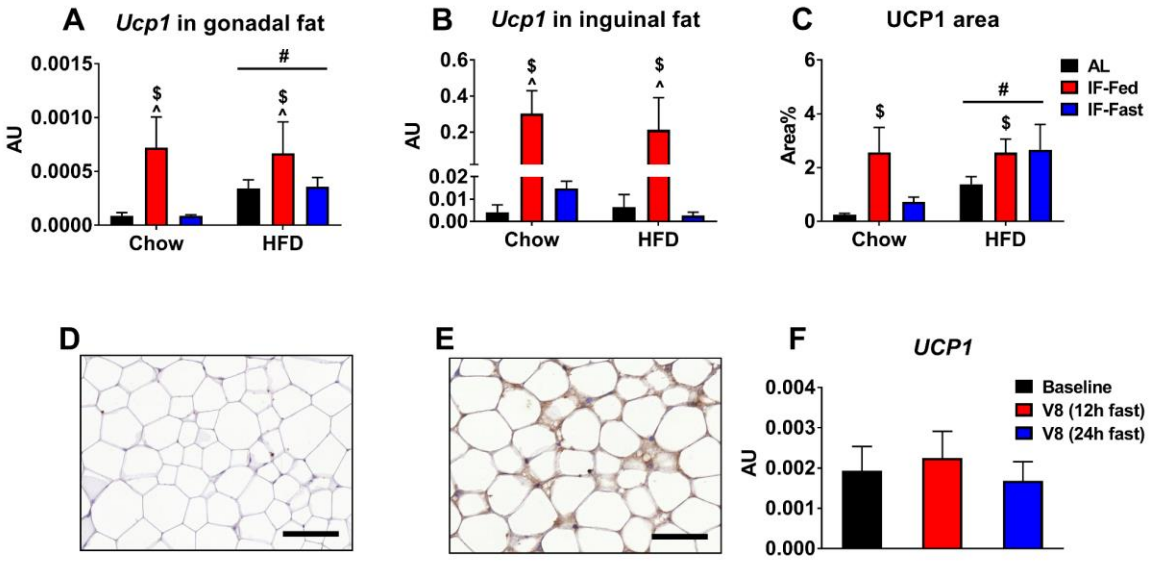

Figure 6-3: IF promoted white adipose tissue browning in mice but not in humans

(A and **B):** *Ucp1* mRNA levels in gonadal and inguinal fat in mice. **(C):** UCP1 positive area in inguinal fat in mice. **(D** and **E):** Representative images of immunohistochemical staining of Ucp1 in chow-AL (**D**) and chow-IF mice (**E**). **(F):** *UCP1* mRNA levels in human subcutaneous adipose tissue. Effects of diet and schedule (Figure 3A-3C) were analysed using two-way ANOVA with Bonferroni post hoc test. Time effects within group (Figure 3F) were analysed using repeated measures ANOVA with Bonferroni post hoc test. Scale bar: 100μm. AU: arbitrary unit.

Data presented as Mean±SEM. N=7-8/group for Figure 3A and 3B, N=6/group for Figure 3C. N=28 for Figure 3F. Diet effect: $^{\#}P<0.05$ vs. chow; Schedule effect: $^{\$}P<0.05$ vs. AL and $^{\wedge}P<0.05$ vs. IF-Fast.

In humans, 8-weeks of intermittent fasting reduced body weight and fat mass (-4.0±0.4kg and 3.1±0.3kg, respectively, both P<0.001). *UCP1* mRNA levels in subcutaneous adipose tissue were detectable in 19 out of 28 participants who consented to biopsy (IF100/IF70=10/9). There was no difference with regards to any assessed baseline characteristics in participants with *UCP1* mRNA detectable or undetectable. There were no changes in the *UCP1* mRNA levels following 8-weeks of IF, either when measured in the morning following a 12-hour overnight fast (after a fed day), or after a 24-hour fast (**Fig. 6.3F**). The change in *UCP1* mRNA levels following intermittent fasting was not associated with any clinical outcomes reported in **Chapter 3** and **4**.

6.5 Discussion

Intermittent fasting reduces adiposity, improves glucose tolerance and markers of insulin sensitivity in rodent models (Varady et al., 2010, Gotthardt et al., 2016, Anson et al., 2003, Joslin et al., 2017, Heilbronn et al., 2005b, Harvie et al., 2011). Recent evidence suggests that browning of white adipose tissue may contribute to this phenotype (Kim et al., 2017, Li et al., 2017). Our study confirms and extends this, showing that IF promoted visceral and subcutaneous adipose tissue browning in chow and HFD fed mice. However, *UCP1* mRNA level was not altered in subcutaneous adipose tissue in women with overweight or obesity who underwent intermittent fasting for 8-weeks.

Total daily energy expenditure consists of resting energy expenditure and thermoregulation, food-induced thermogenesis, and physical activity (Speakman, 2013), and increasing this is one approach to tackle obesity (Hill et al., 2012). In this study, energy expenditure was increased by intermittent fasting. In chow fed mice, this was observed without a change in activity. Whilst increases in food-induced thermogenesis on refeeding days may have partially contributed to increased EE, energy expenditure was not different when measured on a fasting day between chow-IF and chow-AL groups. Physical activity was lower in mice fed a high-fat diet *ad libitum*, and likely contributed to some of the difference in energy expenditure between HFD groups. These results support recent findings that intermittent fasting increases energy expenditure in mice (Kim et al., 2017, Li et al., 2017). Similar to previous studies (Goodrick et al., 1990, Anson et al., 2003, Gotthardt et al., 2016, Liu et al., 2017b, Kim et al., 2017), our study confirmed that chow-IF mice compensate for intermittent food deprivation by overeating on refeeding days, and do not lose weight. This is in contrast to IF mice fed a high-fat diet, where calculated food intake was lower and activity and energy expenditure was higher, resulting in weight loss. Further analysis using metabolic cage revealed that upon refeeding,

chow-IF started to consume food as soon as food was provided even prior to dark phase, but HFD-IF mice fasted for an additional hour and initiated eating in the dark phase. Additionally, chow-IF mice displayed hyperphagia on two consecutive refeeding days, but HFD-IF mice lost hyperphagia after 24-h refeeding consuming similar food as HFD-AL mice. However, the signal that is preventing overconsumption on a HFD is unclear.

Intermittent fasting increased adipose tissue browning in inguinal and subcutaneous adipose tissue. This is consistent with two recent reports (Kim et al., 2017, Li et al., 2017). We further show that the increases in *Ucp1* mRNA levels in both inguinal and gonadal adipose tissue were higher after the refeeding day. This data suggests that "intermittent overfeeding", rather than "intermittent fasting" promotes white adipose tissue browning. Acute fasting (24-48 hours) decreases *Ucp1* expression in both brown and white adipose tissue (Tang et al., 2017, Ding et al., 2016, Sivitz et al., 1999, Champigny and Ricquier, 1990), whilst refeeding increased *Ucp1* expression (Li et al., 2017, Champigny and Ricquier, 1990). Further, Ding et al. demonstrated that an acute 24-hour fasting suppressed white adipose tissue browning via microRNA 149-3p and PR domain containing 16 pathway (Ding et al., 2016). Interestingly, recent work by Fabbiano *et al* suggested that daily calorie restriction (-40%) also led to white adipose tissue browning in both subcutaneous and visceral adipose tissue. This was mediated by increased eosinophil infiltration, type 2 cytokine signalling and alternative activation of macrophage in fat (Fabbiano et al., 2016). In our hands, intermittent fasting did not promote alternate activation of macrophages in white adipose tissue in mice, or in humans who underwent 8-weeks of IF (**Chapter 3** and **4**)

In humans, white adipose tissue browning occurs following burn injury, and administration of β3- adrenergic receptor agonists (Cypess et al., 2015, Sidossis et al., 2015, Patsouris et al.,

2015). However, mild to moderate external stimuli such as cold exposure, exercise and daily calorie restriction that promote visceral and subcutaneous adipose tissue browning in mice (Barbatelli et al., 2010, Lim et al., 2012, Fisher et al., 2012, Bostrom et al., 2012, Xu et al., 2011, Stanford et al., 2015, Fabbiano et al., 2016), do not induce subcutaneous adipose tissue browning in humans (van der Lans et al., 2013, Nakhuda et al., 2016, Norheim et al., 2014, Barquissau et al., 2018). We did not see changes in *UCP1* mRNA levels in subcutaneous adipose tissue following IF. This is in line with a previous study which suggested diet-induced weight and fat mass loss is independent of subcutaneous adipose tissue browning in people with obesity (Barquissau et al., 2018). It should also be noted that while brown-like adipocytes are found in both visceral and subcutaneous adipose tissue (Zuriaga et al., 2017), their distribution patterns are different in mice and humans. As shown in our study, and previously, mice have higher levels of brown-like adipocytes in subcutaneous adipose tissue, whereas these are higher in visceral fat depots in humans (Zuriaga et al., 2017). This indicates there is likely to be a greater potential for visceral than subcutaneous adipose tissue to brown in response to external stimuli in humans. However, it was not possible to obtain visceral fat samples in this study.

The clock time that food is withdrawn and introduced during IF is likely important in the overall phenotype (Albrecht, 2017, Damiola et al., 2000). In this study, we returned food at ZT11, half an hour before the twilight when mice start to become active. This is different from most of the previous studies where the time to start fasting/feeding was set 2-7 hours after lights on (9am to 1pm), which could have induced circadian desynchrony (Goodrick et al., 1990, Varady et al., 2010, Anson et al., 2003, Gotthardt et al., 2016, Liu et al., 2017b, Xie et al., 2017). Further studies are necessary to examine the importance of this in mice and in humans.

In this study, only male mice were examined, and we recruited females in the human study, and gender rather than species differences could explain the discrepancy in results. There is one study examined the effect of IF in chow and HFD fed female mice. Unlike male mice, these female mice were resistant to HFD-induced weight gain and IF-induced weight loss (Liu et al., 2017b). Secondly, both trials were of short duration, limiting interpretation.

In conclusion, IF increased energy expenditure and promoted white adipose tissue browning in both chow and HFD fed mice, but did not alter *UCP1* mRNA levels in human subcutaneous adipose tissue.

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Duality of Interest

No potential conflicts of interest relevant to this article were reported.

Author contributions

BL performed the study, acquired and analysed the data, and wrote the manuscript. AJP supervised the animal study and assisted the data analysis. ATH performed the human study. GAW provided clinical support to the human study. LKH conceived, designed and supervised the study. All authors reviewed and approved the final manuscript. LKH is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation

Parts of this study were presented as an oral presentation at the Joint Scientific Meeting of The Australian and New Zealand Obesity Society and the Obesity Surgery Society of Australia and New Zealand, Adelaide, Australia, 2017.

Chapter 7: Adipose Tissue and Skeletal Muscle Remodelling in Response to Acute Overfeeding in Young Adults

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Publication Details	

Principal Author

Name of Principal Author (Candidate)	Bo Liu		
Contribution to the Paper	Performed the study, acquired and analysed the data, and wrote the manuscript.		
Overall percentage (%)	50%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.		
Signature		Date	28 May 2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Miaoxin Chen		
Contribution to the Paper	Performed the study, acquired and analysed the data.		
Signature		Date	28/05/2018

Continued on the next page

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Contribution to the Paper	Provided clinical support and supervised clamps.		
Signature		Date	28th MAY 2018

Name of Co-Author	Gary A Wittert		
Contribution to the Paper	Provided clinical support and supervised clamps.		
Signature		Date	28/05/2018

Name of Co-Author	Leonie K Heilbronn		
Contribution to the Paper	Conceived, designed and supervised the study, and interpreted the data.		
Signature		Date	29/5/18

7.1 Abstract

Background: Adipose tissue and skeletal muscle undergo remodelling in obesity characterised by increased macrophage and extracellular matrix accumulation. The aim of this study was to examine the acute effects of 3 days of overfeeding on markers of adipose tissue and skeletal muscle remodelling in young adults, and the association with insulin sensitivity.

Methods: Thirty-four young healthy adults (24 women and 10 men; age: 18-27 yrs; BMI: 17.1-35.1 kg/m^2) were recruited. Metabolic assessments were performed at baseline after 3 days energy balanced diet (30% fat), and 3 days after overfeeding (+1,250 kcal/day, 45% fat) including body weight, fasting glucose, insulin, serum C-reactive protein (CRP), monocyte chemoattractant protein 1 (MCP-1) and insulin sensitivity by a hyperinsulinaemic-euglycaemic clamp (80mU/m^2/min). Adipose tissue and skeletal muscle biopsies were obtained from a subgroup of participants at baseline and after overfeeding to assess mRNA levels of makers of macrophage, extracellular matrix, adipogenesis and angiogenesis.

Results: Overfeeding increased body weight (1.1±0.2%), fasting glucose and insulin (all $P<0.05$)), but did not alter peripheral insulin sensitivity assessed by clamp, or serum CRP or MCP-1. In adipose tissue, mRNA levels of markers involved in extracellular matrix synthesis (*COL1A1*, *COL3A1*) and adipogenesis (*SREBF1*) were increased (all $P<0.01$), but macrophage and angiogenesis markers were not altered. There were no changes in any of the markers assessed in muscle.

Conclusion: Extracellular matrix remodelling in adipose tissue occurs rapidly in response to acute over-nutrition and weight gain in young adults.

Key words: adipose tissue, extracellular matrix, inflammation, overfeeding, skeletal muscle, young adults

7.2 Introduction

Adipose tissue and skeletal muscle are key organs involved in development of insulin resistance, and undergo marked alterations in response to obesity (Wu and Ballantyne, 2017, Sun et al., 2011, Petersen et al., 2007). In animals, this includes increases in adipose tissue mass through enlargement in fat cell size (hypertrophy) and generating new adipocytes (hyperplasia) (Sun et al., 2011, Strissel et al., 2007). This is associated with an increase in macrophages (Weisberg et al., 2003, Xu et al., 2003, Lumeng et al., 2007a, Fink et al., 2014, Wu and Ballantyne, 2017) and deposition of extracellular matrix muscle (Sun et al., 2013, Kang et al., 2011), which provides mechanical and nutritional support to neighbouring cells, in adipose tissue and skeletal muscle (Sun et al., 2013, Kang et al., 2011). Lastly, there is an increase in new blood vessel formation (angiogenesis) (Cao, 2010, Silvennoinen et al., 2013), as an adaptive response to meet expanded tissue requirements.

In humans, increased markers of inflammation, extracellular matrix and angiogenesis have been reported in adipose tissue and skeletal muscle in individuals with obesity (Xu et al., 2003, Weisberg et al., 2003, Wu and Ballantyne, 2017, Sun et al., 2013, Martinez-Huenchullan et al., 2017, Cao, 2010), and were observed in some short-term (28-56 days) overfeeding studies (Tam et al., 2014, Tam et al., 2017, Alligier et al., 2012). Since these studies also observed an impairment in insulin sensitivity assessed by hyperinsulinaemic-euglycaemic clamp (Tam et al., 2014, Tam et al., 2017, Alligier et al., 2012), but whether the remodelling occurring in adipose tissue and muscle in response to overfeeding are a cause to or consequence of insulin resistance is unclear.

In this study, we examined adipose tissue and skeletal muscle remodelling in response to 3 days of overfeeding in young adults. We hypothesised that markers involved in macrophage,

extracellular matrix, adipogenesis and angiogenesis in adipose tissue and muscle would be sensitive to acute changes in energy intake, and that this would be associated with changes in markers of insulin sensitivity.

7.3 Research design and methods

Ethics

The study was approved by the Research Ethics Committee of the Royal Adelaide Hospital and the Human Research and Ethics Committee of the University of Adelaide, and was registered as a clinical trial at clinicaltrials.gov, registration number NCT01230632. All participants provided written, informed consent prior to the commencement of the study.

Study design and participants

The study design and protocol have been described previously (Chen et al., 2016a, Chen et al., 2014). Briefly, 34 young adults with age from 18 to 27 yrs and BMI from 17.1 to 35.1 kg/m^2 were recruited. Thirty-one individuals (10 men and 21 women) completed the study and were used for the data analysis. Individuals were excluded if they were born premature (<37 weeks) or with low birthweight (<2500g), displayed any significant medical conditions, took medications affecting glucose or lipid metabolism, smoked cigarettes or drank alcohol (>140g/week), and had first-degree relatives with type 2 diabetes or cardiovascular disease. Female participants were tested in their follicular phase of menstrual cycle.

Participants were provided with a standardised diet calculated as previously described (Chen et al., 2014) (100% of calculated energy requirements, 35% fat, 15% protein, 50% carbohydrate) for 3 days prior to the metabolic testing (**Fig. 7.1**). After baseline assessment, individuals were instructed with an overfeeding diet (+1,250 kcal/day, 45% fat, 15% protein, and 40% carbohydrates) for 3 days before metabolic assessments were repeated. Participants were provided with all foods and completed daily food checklists.

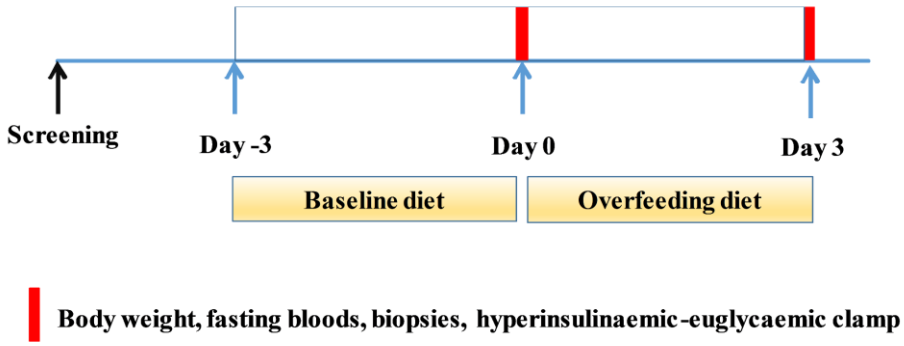

Figure 7-1: Scheduling of the overfeeding study

Participants attended the clinic at 8am after a 12-hour overnight fast at baseline and after 3-days of overfeeding. The tests at two visits were identical except that body composition was measured at baseline only using dual energy X-ray absorptiometry (Lunar DPX-Lunar Radiation, Madison, Wis., USA). Weight and height were measured in a hospital gown after voiding. Intravenous cannulae were inserted and fasting blood samples were collected. Following this, a 2-hour hyperinsulinaemic-euglycaemic clamp (80 mU/m^2 body surface/min) was performed. Glucose was infused at a variable rate to maintain its level at 5.0 mmol/L. Peripheral insulin sensitivity was calculated as the glucose infusion rate during steady-stage (90-120min), and normalised to fat-free mass as described previously (Chen et al., 2016a).

Adipose tissue and muscle biopsies

Subcutaneous abdominal adipose tissue and vastus lateralis muscle tissue were obtained by percutaneous biopsy as mentioned previously (Tam et al., 2017, Tam et al., 2010) before clamp was conducted. Briefly, biopsy sites were located and cleaned. After anesthetising the skin and underlying tissues with lidocaine, adipose tissue (~150mg) and vastus lateralis (~100mg) samples were collected using the biopsy needles, and were snap frozen in liquid nitrogen and

stored at -80°C for future analysis. Adipose tissue and muscle biopsies were available from 15 patients at baseline, and 12 and 8 individuals, respectively, after overfeeding.

Table 7-1: Taqman primers used for gene expression analysis

Category	Gene name	Assay ID
Macrophage		
CD68	CD68 molecule	Hs02836816_g1
CD40	CD40 molecule	Hs01002913_g1
CD163	CD163 molecule	Hs00174705_m1
Macrophage recruitment		
CCL2	C-C motif chemokine ligand 2	Hs00234140_m1
CCL3	C-C motif chemokine ligand 3	Hs00234142_m1
Extracellular matrix		
COL1A1	Collagen type I alpha 1 chain	Hs00164004_m1
COL3A1	Collagen type III alpha 1 chain	Hs00943809_m1
MMP2	Matrix metallopeptidase 2	Hs01548727_m1
MMP9	Matrix metallopeptidase 9	Hs00234579_m1
TIMP1	TIMP metallopeptidase inhibitor 1	Hs00171558_m1
Adipogenesis		
CEBPa	CCAAT/enhancer binding protein alpha	Hs00269972_s1
SREBGF1	Sterol regulatory element binding transcription factor 1	Hs01088691_m1
Angiogenesis		
VEGFa	Vascular endothelial growth factor A	Hs00900055_m1
ANGPT2	Angiopoietin 2	Hs01048042_m1
Housekeeper		
ACTB	Actin beta	Hs01060665_g1

Quantitative real-time PCR

As described previously (Chen et al., 2016a), total RNA was extracted from adipose tissue (100-150 mg) and skeletal muscle (~30mg) using Trizol (Invitrogen, USA) following

manufacturer's instructions. The concentration and purity of RNA were determined by NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, CA, USA). cDNA synthesis was conducted using T100 Thermal Cycler (Bio-Rad, CA, USA) with 800 ng of each RNA sample using the QuantiTect reverse transcription kit (Qiagen, Valencia, CA, USA) consistent with the manufacturer's protocol. Quantitative real-time PCR of genes involved in macrophage (*CD68*, *CD40* and *CD163*), macrophage recruitment (*CCL2* and *CCL3*), extracellular matrix (*COL3A1*, *COL6A1*, *MMP2*, *MMP9* and *TIMP1*), adipogenesis (*CEBPα* and *SREBF1*) and angiogenesis (*VEGFa* and *ANGPT2*) was performed using Taqman primers (**Table 7.1**) and Fast Universal PCR Master Mix on an ABI 7500 sequence detection system (all from Applied Biosystems, Foster City, CA). Samples were run in duplicate with internal negative control and a standard curve for which standard samples were pooled from ten participants at baseline. Relative gene expression was analysed using the $2^{-\Delta CT}$ method, and normalised by *ACTB* which was not different before and after overfeeding.

Biochemical analysis

Glucose was analysed using a glucose dehydrogenase method (YSI Life Sciences, Ohio, USA), and serum insulin by radioimmunoassay (Millipore, St Charles, MO, USA). Homeostasis model of assessment - insulin resistance (HOMA-IR) was calculated as fasting glucose (mmol/l) x fasting insulin (mU/L)/22.5. Plasma monocyte chemotactic protein-1 (MCP-1) and C-reactive protein (CRP) were assessed by Quantikine® ELISA (R&D Systems Europe, Ltd., Abingdon, UK) on a Beckman AU480 clinical analyser (Beckman Coulter Inc), with intra- and inter-assay CVs of MCP-1 <8% and <7%, and CRP <9% and <8%, respectively.

Statistics

Data are shown as mean ± SEM. All statistical analyses were performed using IBM SPSS Statistics 24 (SPSS, Chicago, IL, USA). Data were log10 transformed if not normally distributed. Response to overfeeding was analysed using paired *t*-test. Correlations were calculated using Pearson Correlation Coefficients. Significance was accepted as $P < 0.05$.

7.4 Results

Clinical characteristics of participants at baseline and 3-day after overfeeding are summarised in **Table 7.2.** Fat mass was positively correlated with HOMA-IR (r=0.43, P=0.01), CRP (r=0.40, P=0.02), and mRNA levels of *CD68* (r=0.58, P=0.01) in adipose tissue. Clinical characteristics of the participants who completed 3 days of overfeeding were not different from that of those underwent adipose tissue or skeletal muscle biopsies. However, the baseline correlations between fat mass, HOMA-IR and CRP did not exist when this was analysed using the subgroup of participants underwent biopsies.

Table 7-2: Clinical characteristics and metabolic measures before and after 3 days of overfeeding

Variable	Baseline	Overfeeding	P value
Number	10/21 (M/F)	10/21(M/F)	
Age, years	21.0±0.5	-	
Waist/hip Ratio	0.81±0.01	Not assessed	
Fat mass, %	35.6±2.0	Not assessed	
Weight, kg	67.5±2.5	68.2±2.5	<0.001
BMI	23.0±0.8	23.2±0.8	<0.001
Fasting glucose, mmol/L	4.13±0.05	4.23±0.04	0.006
Fasting insulin, μU/mL	12.44±0.78	13.52±0.86	0.017
HOMA-IR, AU	2.33±0.17	2.55±0.18	0.008
GIR, umol/kg FFM/min	99.35±4.24	96.79±4.05	0.306
MCP-1, ng/L	144.43±10.60	145.48±12.81	0.912
CRP, mg/L	1.36±0.33	1.43±0.39	0.751

BMI: body mass index; HOMA-IR: homeostasis model of assessment-insulin resistance; GIR: glucose infusion rate; FFM: fat free mass; MCP-1: monocyte chemotactic protein-1; CRP: C-reactive protein.

Data presented as Mean ± SEM; Statistics were performed by paired *t* test.

As reported previously (Chen et al., 2016a), 3-days of overfeeding resulted in increased body weight (1.1±0.2%), fasting glucose and insulin, and thus HOMA-IR. However, peripheral insulin sensitivity assessed by clamp was not changed by acute overfeeding (P=0.3) and there was no significant changes in serum levels of MCP-1 and CRP. These results held when the analysis was performed in those who underwent adipose tissue or skeletal muscle biopsies (**Table 7.3**).

Table 7-3: Comparison of clinical outcomes between participants completed overfeeding and underwent adipose tissue or skeletal muscle biopsies

Parameters	**Completers**	**Fat biopsies**	**Muscle biopsies**
N	31	12	8
Body weight	↑ P<0.001	↑ P<0.001	↑ P<0.001
Fasting glucose	↑ P=0.006	↑ P=0.037	↑ P=0.044
Fasting insulin	↑ P=0.017	↑ P=0.050	↑ P=0.050
HOMA-IR	↑ P=0.014	↑ P=0.045	↑ P=0.042
GIR	↔ P=0.306	↔ P=0.586	↔ P=0.596
MCP-1	↔ P=0.912	↔ P=0.409	↔ P=0.350
CRP	↔ P=0.751	↔ P=0.520	↔ P=0.712

↑: increased vs. baseline; ↓: decreased vs. baseline and ↔ no change vs. baseline.

In adipose tissue, mRNA levels of *COL1A1*, *COL3A1* and *SREBF1* were increased by overfeeding (all P<0.01, **Fig. 7.2**), while *CD68*, *CD40*, *CD163*, *CCL2*, *CCL3*, *VEGFa*, *ANGPT2*, *MMP2*, *MMP9*, *TIMP1*, and the ratio of *MMPs* to *TIMP1*(not shown) which represents the activity of extracellular matrix degradation were not changed. We did not observe any changes in the mRNA levels of these genes in skeletal muscle. The change in *CD40* expression in adipose tissue was positively associated with fasting glucose (r=0.67,

P=0.03, **Fig. 7.3**). These correlations remained significant after adjustment for BMI, gender and age.

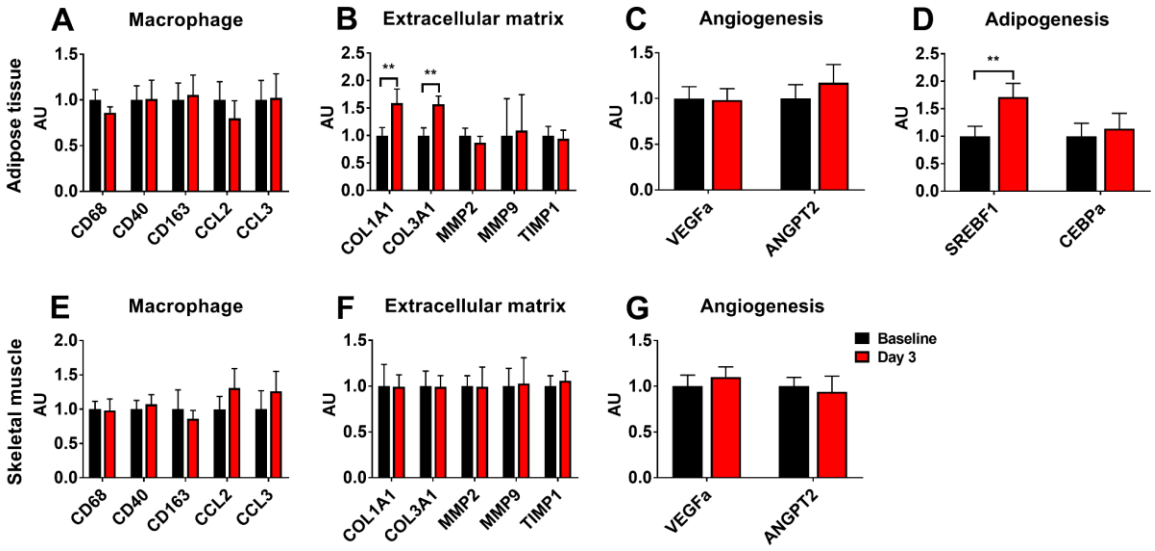

Figure 7-2: Tissue remodelling genes in adipose tissue and skeletal muscle before and after 3 days of overfeeding.

(**A** and **B**): mRNA levels of macrophage (*CD68*, *CD40* and *CD163*) and macrophage recruitment (*CCL2* and *CCL3*) in adipose tissue and muscle. (**C** and **D**): mRNA levels of extracellular matrix genes (*COL3A1*, *COL6A1*, *MMP2*, *MMP9* and *TIMP1*) in adipose tissue and muscle**.** (**E** and **F**): mRNA levels of adipogenesis (*SREBF1* and *CEBPa*) and angiogenesis (*VEGFa* and *ANGPT2*) genes in adipose tissue and muscle**.** *SREBF1* and *CEBPa* were not assessed in muscle.

Data presented as mean ± SEM, N=12 for adipose tissue and n=8 for muscle. Relative mRNA expression was calibrated by baseline value. AU: arbitrary unit. Paired *t* test, **P<0.01 vs. baseline.

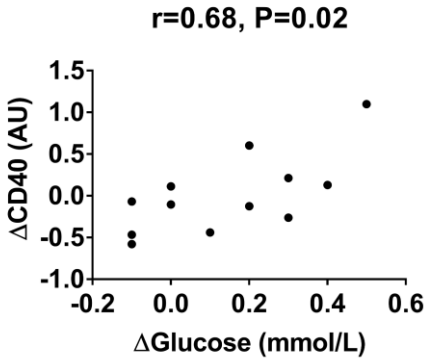

Figure 7-3: Correlation between the change in fasting glucose and CD40 mRNA levels in adipose tissue

N=12. Correlations were calculated using Pearson Correlation Coefficients. AU: arbitrary unit.

7.5 Discussion

Adipose tissue and skeletal muscle undergo remodelling in obesity which is linked with impaired insulin sensitivity (Sun et al., 2011, Wu and Ballantyne, 2017, Petersen et al., 2007). However, it is not clear whether adipose tissue and/or skeletal muscle remodelling is an early contributor to, or a consequence of, insulin resistance in obesity. Our results show that changes in the extracellular matrix in adipose tissue is an early event in response to acute over-nutrition and may contribute to the onset of insulin resistance during overfeeding.

As we have previously reported (Chen et al., 2016a), 3 days of overfeeding increased body weight (~1.1%). Since body composition was not assessed after overfeeding and it is likely that minor change in fat mass after 3 days of overfeeding cannot be differeciated by a DEXA, it is not clear that the weight gain achieved is attributed to increased fat mass or glycogen synthesis or both. That 3 days of overfeeding increased fasting glucose, insulin and thus HOMA-IR is consistent with previous studies of short-term overfeeding (3-day) by our group and others (Samocha-Bonet et al., 2010, Tam et al., 2017, Jordy et al., 2014), which likely represents induction of hepatic insulin resistance (Singh and Saxena, 2010). Of note, peripheral insulin sensitivity as assessed by hyperinsulinaemic-euglycaemic clamp, a gold-standard technique, was unchanged. The clamp reflects whole body glucose disposal, and primarily skeletal muscle since >80% of the glucose infused during clamp is taken up by skeletal muscle (DeFronzo and Tripathy, 2009). This result is in line with a previous study that observed elevated HOMA-IR index in the absence of impaired insulin sensitivity assessed by clamp following 5 days of overfeeding (Brons et al., 2009).

Compared to normal weight, people with obesity exhibit chronic low-grade inflammation including increases in circulating pro-inflammatory mediators, such as tumour necrosis factor

α (TNFα), interleukin 6 (IL-6), CRP and MCP-1 *etc*. (Rodriguez-Hernandez et al., 2013), which directly inhibit the insulin signalling cascade (Shoelson et al., 2006). In this study, we did not see changes in CRP and MCP-1 levels following short-term 3 days overfeeding, which is in agreement with a previous work by Adochio and colleagues (Adochio et al., 2009). However, our past work did show a significant increase in CRP levels after 3 days of overfeeding (Tam et al., 2010). This discrepancy could be because the participants recruited in that cohort were more overweight (BMI: 26.0 vs. 23.0 kg/m^2), older (age: 37 vs. 21 yrs) or since that cohort was enriched for people with a familial risk of type 2 diabetes (Tam et al., 2010). We also saw no change in the mRNA levels of macrophage and macrophage-recruitment genes in adipose tissue and skeletal muscle after 3 days of overfeeding. These data are in line with previous studies by our group and others (Tam et al., 2017, Tam et al., 2014, Tam et al., 2010, Alligier et al., 2012). Previous studies by our group have also reported that 28 days of overfeeding did not alter markers of adipose tissue and muscle inflammation, despite inducing insulin resistance (Tam et al., 2010, Tam et al., 2017). Similarly, moderate weight loss of 5-10% improves insulin sensitivity without altering adipose tissue inflammation (Magkos et al., 2016). Our work contributes to growing body of evidence highlighting that adipose tissue and skeletal muscle inflammation is a consequence of insulin resistance in obese state.

Excessive collagen deposition, a hallmark of fibrosis, in adipose tissue and skeletal muscle also occurs in obesity and insulin resistance (Sun et al., 2013, Williams et al., 2015). Collagens are the main structural proteins in extracellular matrix, which are cleaved by matrix metalloproteinases (MMPs) (Bonnans et al., 2014). The enzyme activity of MMPs is negatively regulated by tissue inhibitor of metalloproteinases (TIMPs) to avoid excessive degradation of extracellular matrix. Herein, we observed increased mRNA levels of *COL1A1* and *COL3A1* in

adipose tissue after 3 days of overfeeding, which encode fibrillary collagens. We did not see changes in mRNA levels of *MMP2*, *MMP9*, *TIMP1*, or the ratio of *MMPs* to *TIMP1*, which may suggest an increase in extracellular matrix accumulation in adipose tissue. These results are supported by previous longer term overfeeding studies (28-56 days) which showed that a range of collagen markers including *COL1A1*, *COL3A1*, and *COL6A3* were increased in adipose tissue (Pasarica et al., 2009a, Tam et al., 2014, Alligier et al., 2012), and extend these studies by showing this process is a very early event.

Up-regulation of extracellular matrix genes including *COL1A1*, *COL3A1*, *COL4A1*, *COL5A1*, *COL6A1*, *COL6A3* and *SPARC* have also been reported in skeletal muscle in men after 56 days of overfeeding (Tam et al., 2014). In this study, we did not see changes in mRNA levels of extracellular matrix genes in skeletal muscle after 3 days of overfeeding. This result is in agreement with a recent study by our group where detectable changes in extracellular matrix markers appeared at day 28 of overfeeding with no change at day 3 (Tam et al., 2017). Together, these data suggest that extracellular matrix in adipose tissue is sensitive to over-nutrition, but more substantial weight gain is required to trigger extracellular matrix remodelling in skeletal muscle.

The formation of mature adipocyte from its precursor cells is finely regulated by a group of transcriptional factors including CCAAT/enhancer-binding proteins (CEBP) and sterol regulatory element-binding transcription factors (SREBF) (Tang and Lane, 2012). Whether nutrient manipulations alters adipocyte turnover, particularly in humans, is debated. Approximately 10% of adipocytes are renewal annually in adults (Rosen and MacDougald, 2006, Spalding et al., 2008). Some evidence suggests that adipocyte death or generation rates are not changed in adults with obesity (Spalding et al., 2008). However, increased adipocyte

numbers are observed in rodent models of obesity, occurring when adipocytes reach a crucial cell size (Cleary et al., 1979, Johnson and Hirsch, 1972, Strissel et al., 2007). In this study, *SREBF1* mRNA levels were increased in adipose tissue after 3 days of overfeeding, suggesting a possibility of increased adipogenesis. However, further studies are required to address whether nutritional changes impacts adipogenesis, particularly in humans.

Angiogenesis is the physiological process of the formation of new capillaries from pre-existing blood vessels, and plays an important roles in tissue growth, expansion and repair (Cao, 2010). Tissue angiogenesis is regulated by multiple factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor (TGF) and angiopoietins (Angs) (Cao, 2007). In adipose tissue, the rapid expansion of fat mass through hypertrophy and/or hyperplasia during energy excess depends on an increase in vascular perfusion to provide oxygen and nutrients (Cao, 2010). However, this is controversial, with some studies suggesting that compensatory angiogenesis in adipose tissue is insufficient (Ye et al., 2007), but others showing adipose tissue hypoxia is not present in individuals with obesity (Vink et al., 2017). In skeletal muscle, one animal study suggests obesity increases angiogenesis makers and capillary density in skeletal muscle (Silvennoinen et al., 2013). We investigated markers of this in both tissues, but observed no changes in response to acute overfeeding.

In conclusion, our study shows that 3 days of overfeeding did not alter insulin sensitivity or inflammation in adipose tissue and skeletal muscle, however, increased markers of extracellular matrix synthesis in adipose tissue. This highlights that extracellular matrix remodelling in adipose tissue is an early event in response to over nutrition, and occurs prior to altered insulin sensitivity.

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Duality of Interest

No potential conflicts of interest relevant to this article were reported.

Author Contributions

BL performed the study, acquired and analysed the data, and wrote the manuscript. MC performed the study, and acquired and analysed the data. GAW and CHT provided clinical support and supervised clamps. LKH conceived, designed and supervised the study. All authors reviewed and approved the final manuscript. LKH is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Chapter 8: Conclusions and Future Directions

8.1 Conclusions

The mouse study highlighted that IF reduced energy intake, body and fat mass, visceral adipose tissue inflammation and fibrosis in high fat diet fed mice only, but improved glucose tolerance in both chow and high fat diet fed mice. Results in mice demonstrate that improved glucose tolerance by IF in chow fed mice was independent of weight loss, since they fully compensated for food deprivation by overeating on refeeding days. The mice work further reveals that IF increased energy expenditure and promoted white adipose tissue browning in both chow and high fat diet fed mice. This could be one mechanism to explain the improved metabolic phenotype in chow and high fat diet fed mice.

The clinical study is the first randomised controlled trail to compare the effects of intermittent versus continuous food intakes at two energy levels (at energy balance, or 30% energy restriction). This study demonstrates that provision of an energy restricted IF led to greater loss of weight and fat mass, improvements in homeostasis model assessment of insulin resistance, total cholesterol and non-esterified fatty acids versus provision of a matched continuous energy restriction diet. However, there were no overall differences in insulin sensitivity by clamp. When IF was prescribed in energy balance, transient increases in risk markers for type 2 diabetes were observed, despite modest weight loss. Taken together, these data suggest that energy restriction is required for beneficial effects on markers of metabolic health in humans.

My results show that intermittent fasting promotes different responses in adipose tissue and in muscle collected from humans and mice. The human study demonstrated that a prolonged 24-hour fast increased circulating, adipose tissue and muscle inflammation. Moreover, increased macrophages in adipose tissue after a 24-hour fast were positively correlated with serum non-esterified fatty acids levels. This data suggests that infiltrated macrophages in adipose tissue

could play a physiological role to buffer the excessive release of non-esterified fatty acids. However, results from the animal study do not support these findings. Despite increases in the blood non-esterified fatty acids levels, prolonged fasting did not increase markers of subcutaneous and visceral adipose tissue inflammation in chow and high fat diet fed mice. My results also show that intermittent fasting did not alter collagen content in subcutaneous adipose tissue in humans, however, this was increased in subcutaneous adipose tissue in mice and reduced in visceral fat fibrosis in high fat diet fed mice. Lastly, intermittent fasting promoted white adipose tissue browning in mice, but this does not appear to occur in humans at least in subcutaneous adipose tissue. My work highlights the limited translation that may be made from mouse studies.

My work also supports previous human studies that weight change by calorie restriction or short-term overfeeding did not alter adipose tissue and skeletal muscle inflammation, but markers of extracellular matrix in adipose tissue were sensitive to short-term over-nutrition.

In conclusion, this research highlights that energy restricted intermittent fasting may lead to additional health benefits compared to calorie restriction in humans. This study also suggests intermittent fasting results in distinct tissue adaptations versus daily calorie restriction in humans, and special attention should be paid to the species difference in the study of obesity (**Table 8.1**).

Table 8-1: Summary of the thesis

Dietary intervention		**IF**				**DR**
Animals/humans		**Male mice**		**Women**		**Women**
Diet	**Status**	**Chow**	**HFD**	**IF100**	**IF70**	**DR70**
Energy intake	Fed	↔	↓↓	↓	↓↓	↓↓
Body weight	Fed	↔	↓↓	↓	↓↓	↓
Fat mass	Fed	↓	↓↓	↓	↓↓	↓
Glucose tolerance	Fed	↑	↑↑	NA	NA	NA
	Fasted	NA	↑↑	NA	NA	NA
HOMA-IR	Fed	↔	↓↓	↔	↓	↔
	Fasted	↓↓	↓↓↓	↓	↓↓	NA
GIR	Fed	NA	NA	↔	↔	↔
	Fasted	NA	NA	↓	↓	NA
Fat cell size	Fed	↓	↓↓	↓	↓	↓
	Fasted	↓	↓↓	↓	↓	↓
Adipose tissue inflammation	Fed	↔	↓	↔	↔	↔
	Fasted	↔	↓	↑	↑	↔
Adipose tissue fibrosis	Fed	↔	↓	↔	↔	↔
	Fasted	↔	↓	↔	↔	↔
Adipose tissue browning	Fed	↑↑	↑↑	↔	↔	↔
	Fasted	↑↑	↑↑	↔	↔	↔

IF: intermittent fasting; DR: daily calorie restriction; HFD: high-fat diet; IF100: intermittent fasting diet at 100% of baseline energy requirements; IF70: intermittent fasting diet at 70% of baseline energy requirements; DR70: continuous energy restriction at 70% of baseline energy

requirements; HOMA-IR: homeostatic model assessment of insulin resistance; GIR: glucose infusion rate during hyperinsulinaemic-euglycaemic clamp; NA: not assessed.

8.2 Future directions

What are the long-term effects of IF in humans?

IF is effective to improve health markers and prolong lifespan in rats and mice (Goodrick et al., 1982, Goodrick et al., 1983a, Goodrick et al., 1990, Xie et al., 2017). So far, the longest IF study in humans is one year duration. This study suggests IF was as effective for weight loss, weight maintenance, and cardio-protection as daily calorie restriction (Trepanowski et al., 2017). Whether IF alters key molecules implicated in longevity in humans is unknown.

Does gender differentially impact the response to IF?

Male mice are more susceptible to diet induced obesity and alterations in glucose metabolism (Hwang et al., 2010). It is possible that males and females may display different responses to intermittent fasting. In humans, we observed increased adipose tissue macrophages after 24-hour fast which was linked with elevated non-esterified fatty acids, but this was not supported by mice study. Similarly, the browning phenomenon was detected in mice but not in humans. Since only women were recruited for the clinical study, and male mice were used for the animal work. It is currently unclear whether the discrepancy in these results is gender or species dependent. A study including both genders would help clarify this point.

Does the increased macrophages after a fast in human adipose tissue buffer excess lipids released from adipocytes?

Acute 24-hour fast increased macrophage infiltration in adipose tissue which was positively associated with elevated NEFA levels in mice (Kosteli et al., 2010). *In vitro* and *in vivo* studies suggest macrophages store excess lipids releases from adipocytes (Caspar-Bauguil et al., 2015). We observed increased macrophages filtration in adipose tissue after a 24-hour fast along with elevated non-esterified fatty acids, but whether these macrophages are responsible

for the uptake of the released lipids from lipolysis by a fast in humans was not tested, as there was insufficient sample. An *in vitro* study treating macrophage cell line by fatty acids or serum collected from the participants may help test this hypothesis.

Does IF affect appetite regulation?

Recent evidence suggests that individuals following an IF regimen are not able to compensate the deprived food on fasting days by overeating on feeding days in a free-living environment (Catenacci et al., 2016, Trepanowski et al., 2017). Our human study also highlights that participants in the IF groups did not consume all prescribed food on fed days. From metabolic cage data, we observed delayed food uptake when food was returned and a significant reductions in cumulative energy intake in high fat diet fed mice. Therefore, the sense of hunger and satiety may be altered by IF. The effect of IF on appetite regulation requires further investigation, and is subject of a new study being run in our laboratory in 2018.

Does IF improve insulin sensitivity in mice?

A number of animal studies have assessed insulin sensitivity in chow and high fat diet fed mice by insulin tolerance test (Gotthardt et al., 2016, Kim et al., 2017), but the results are controversial. Peripheral insulin sensitivity by gold standard hyperinsulinaemic-euglycaemic clamp has not been reported. Further, as reported by our group and others that prolonged fasting impairs peripheral insulin sensitivity (van der Crabben et al., 2008, Vendelbo et al., 2012). It is unclear how IF affect insulin sensitivity by clamp and whether insulin sensitivity alters between the fed and fasted states in mice.

Does IF impact the circadian rhythm?

The physical and behaviour activities of mammals display circadian rhythm which are coordinated by internal clock genes. These genes are finely regulated by the light and dark cycle as well as the pattern of feeding and fasting playing important roles in the maintenance of homeostasis(Manoogian and Panda, 2017). Synchronising the eating activity with the internal clock such as restricting the time window for eating to a certain period promotes weight loss and improves health in rodents and in humans (Chaix et al., 2014, Hatori et al., 2012, Gill and Panda, 2015). It's unclear whether IF impacts circadian rhythm in mice and humans. Additionally, since internal clock has been found in macrophages (Keller et al., 2009). Whether the responses of peripheral macrophages to IF display circadian rhythm is unknown.

What mechanism triggers adipose tissue browning following IF in mice?

We, and others, have shown that IF promotes white adipose tissue browning (Kim et al., 2017, Li et al., 2017). By comparing the responses between the fed and fasted states, our data highlight that the *Ucp1* mRNA levels in both visceral and subcutaneous adipose tissue mirrored the food access during intermittent fasting, suggesting intermittent overfeeding is the trigger for this to occur. Recent works demonstrated that brown-like adipocytes in adipose tissue were derived from their precursor cells as well as existing mature white adipocytes. This process is regulated by both transcriptional and post-transcriptional factors, such as AMP-activated protein kinase, co-regulator PR domain containing 16, DNA and histone methylation, histone acetylation and non-coding RNAs (Sambeat et al., 2017, Kajimura et al., 2015, Mottillo et al., 2016). Our future work will focus on AMP-activated protein kinase and histone acetylation pathway, since its activity is regulated by fasting and feeding (Drazic et al., 2016, Lage et al., 2008).

Does intermittent fasting affect skeletal muscle inflammation?

Obesity is characterised by macrophage infiltration into adipose tissue and weight loss partially reverses this phenotype. This has been documented in both humans and animals (Xu et al., 2003, Weisberg et al., 2003, Zamarron et al., 2017, Lumeng et al., 2007a, Aron-Wisnewsky et al., 2009). Whether this phenomenon exists in skeletal muscle, a key organ involved in energy metabolism, remains unclear. In our hands, intermittent fasting appears to trigger distinct polarisation of macrophages in adipose tissue and skeletal muscle in humans in which a 24-hour fast shifts the macrophage population towards a classically activated M1-phenotype in fat while alternatively activated M2-phenotype in muscle. Additionally, the response to intermittent fasting could be of difference in skeletal muscle from mouse to human, since a ~24-hour fast failed to provoke a M1- phenotype in both gonadal and inguinal adipose tissue in mice. Further studies are required to examine the population and polarisation of macrophages in skeletal muscle in response to intermittent fasting.

Is skeletal muscle involved in white adipose tissue browning in mice following intermittent fasting?

Skeletal muscle and brown adipocytes have been closely linked, not only because of the detection of ectopic brown adipocytes in skeletal muscle but the crosstalk (Almind et al., 2007, Rodríguez et al., 2017). Brown adipose tissue has been shown to regulate skeletal muscle function such as exercise capacity via the secretion of factor myostatin, and *vice versa* (Kong et al., 2018, Shan et al., 2013). Myokines such as interleukin 6 and irisin that are released during exercise have been reported to promote the browning of white adipose tissue in animals (Kim and Plutzky, 2016). A recent study suggests that intermittent fasting increased lean mass in mice (Gotthardt et al., 2016), whether this is linked to the browning of white adipose tissue could be an interesting direction in the future.

Appendices

Appendix table 1: Calculated energy and macronutrient intakes based on self-reported diet checklists

	Provided	Week 1					Week 4					Week 7				
	kcal/day	kcal/day consumed	protein (g/day)	Total fat (g/day)	Saturated fat (g/day)	Carbohydrate (g/day)	kcal/day consumed	protein (g/day)	Total fat (g/day)	Saturated fat (g/day)	Carbohydrate (g/day)	kcal/day consumed	protein (g/day)	Total fat (g/day)	Saturated fat (g/day)	Carbohydrate (g/day)
Control	2357±98	2363±82	102±4	91±4	29±1	258±9	2338±84	104±4	90±4	29±1	257±9	2377±87	106±4	92±4	30±1	257±9
IF100	2366±66	2131±68[d,e]	86±3	82±3	27±1	243±8	2139±76[d,e]	88±3	83±4	28±1	242±9	2111±67[d,e]	87±2	82±3	28±1	239±8
IF70	1702±49	1657±62[d,f]	72±3	58±2	20±1	193±8	1602±64[d,f]	69±3	56±3	19±1	189±8	1630±61[d,f]	71±2	58±2	19±1	187±8
DR70	1725±44	1694±134	92±2	66±2	22±1	208±7	1748±136	95±2	68±2	23±1	211±7	1699±134	102±7	67±2	23±1	201±5

Data are mean ±SEM. DR70: continuous energy restriction at 70% of baseline energy requirements: IF70: intermittent fasting diet at 70% of baseline energy requirements; IF100: intermittent fasting diet at 100% of baseline energy requirements; Control (C): continuous energy intake at 100% of baseline energy requirements. [d]P<0.05 compared with kcal provided. [e]P<0.05 compared with Control. [f]P<0.05 compared with DR70.

Appendix table 2: Calculated energy intakes on fed and fasted days, and averaged in intermittent fasting groups based on self-reported diet checklists

		Week 1			Week 4			Week 7		
	Prescribed Kcal/day	Average kcal/day consumed	Average kcal/fasting day	Average kcal fed/day	Average kcal/day consumed	Average kcal/fasting day	Average kcal/fed days	Average kcal/day consumed	Average kcal/fasting day	Average kcal/fed day
IF100	2366±56	2131±68	873±34	3074±105	2139±76	882±34	3082±114	2111±67	859±31	2863±93
IF70	1702±49	1657±62	697±22	2345±93	1602±64	732±33	2246±95	1630±31	680±24	2316±93

Data are mean ±SEM. DR70: continuous energy restriction at 70% of baseline energy requirements: IF70: intermittent fasting diet at 70% of baseline energy requirements; IF100: intermittent fasting diet at 100% of baseline energy requirements; Control (C): continuous energy intake at 100% of baseline energy requirements.

Appendix table 3: Changes in outcome parameters following 8 weeks of dietary intervention[a,b]

	C (n=11)	IF100 (n=22)	P value C vs IF100	IF70 (n=22)	P value IF100 vs IF70	DR70 (n=24)	P value IF70 vs DR70
Weight (kg)	0.4±0.4	-2.7±0.5[c]	**0.000**	-5.4±0.5[d,e]	**0.000**	-3.9±0.4	**0.03**
Fat mass (kg)	-0.2±0.5	-2.3±0.4[c]	**0.001**	-3.9±0.4[d,e]	**0.004**	-2.8±0.4	**0.05**
Fat free mass (kg)	-0.4±0.4	-0.5±0.3	0.07	-1.4±0.3	0.06	-0.6±0.3	0.07
% of weight lost as fat free mass	72±45	14±13	0.11	19±7	0.74	19±6	0.94
Waist circumference (cm)	-1.4±1.7	-4.3±1.0	0.19	-7.6±1.2[d]	**0.04**	-5.2±1.0	0.14
Hip circumference (cm)	-0.3±1.3	-3.6±0.7	0.07	-5.3±0.8	0.11	-4.4±1.0	0.45
M/I (μmol/min/FFM+17.7 kg/mU)	-2.4±3.7	-1.5±2.9	0.65	3.7±5.5	0.31	3.5±2.1	0.95
HOMA-IR	-0.1±0.5	0.8±0.3	0.08	-0.9±0.3[d]	**0.002**	-0.4±0.3	0.38
Fasting glucose (mmol/L)	0.01±0.1	0.1±0.1	0.37	-0.2±0.1[d]	**0.02**	0.1±0.1	0.22
Fasting insulin (mU/L)	-0.4±1.9	2.9±1.4[c]	**0.05**	-3.6±1.0[d]	**0.02**	-1.2±1.1	0.70
Fasting NEFA (mmol/L)	-0.08±0.07	-0.16±0.06	0.22	-0.26±0.03[e]	0.38	-0.10±0.04	**0.005**
Total cholesterol (mmol/L)	-0.30±0.15	-0.37±0.15	0.88	-0.59±0.08[d,e]	**0.05**	-0.24±0.10	**0.005**
HDL-cholesterol (mmol/L)	-0.03±0.07	-0.07±0.06	0.35	-0.10±0.03	0.30	-0.05±0.02	0.20
LDL-cholesterol (mmol/L)	-0.16±0.12	-0.16±0.13	0.88	-0.37±0.07[d,e]	**0.03**	-0.13±0.08	**0.001**
Triglycerides (mmol/L)	-0.25±0.09	-0.28±0.12	0.62	-0.24±0.07[e]	0.32	-0.16±0.05	**0.05**
HS-CRP (mg/dL)	1.11±0.55	-0.33±0.23[c]	**0.008**	-0.30±0.35	0.17	0.34±0.46	0.53
ALT (mmol/L)	1.5±2.2	-1.3±1.6	0.60	-3.4±1.6	0.26	-3.0±1.7	0.39
AST (mmol/L)	1.2±1.5	-2.9±1.5	0.23	-1.6±1.0	0.55	1.0±1.3	0.81
FGF-21 (mmol/L)	10.8±26.3	3.2±22.2	0.66	29.2±18.7[d]	**0.008**	13.7±17.9	0.42
β-hydroxybutyrate (mmol/L)	-0.02±0.02	-0.01±0.01	0.42	0.03±0.02[d]	**0.001**	-0.00±0.01	0.25
Systolic blood pressure (mmHg)	1.5±1.7	-5.6±3.4	0.14	-0.6±3.2	0.23	-4.2±2.9	0.34

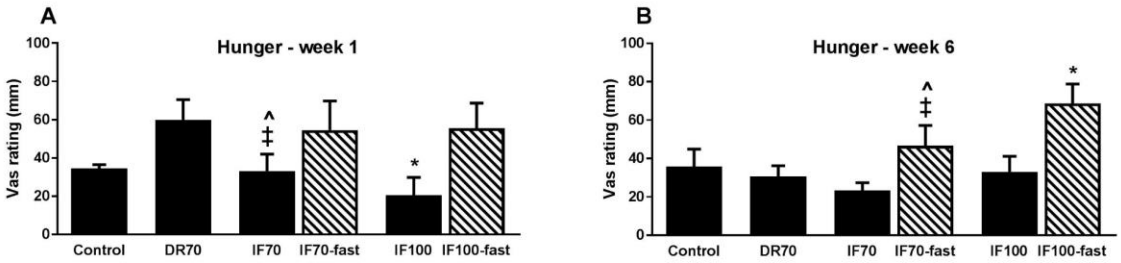
Diastolic blood pressure(mmHg)	-1.5±2.0	-2.5±1.4	0.68	-0.4±1.0`	0.23	-0.1±1.5	0.87

[a]Control (C): continuous energy intake at 100% of baseline energy requirements; IF100: intermittent fasting diet at 100% of baseline energy requirements; IF70: intermittent fasting diet at 70% of baseline energy requirements; DR70: continuous energy restriction at 70% of baseline energy requirements. [b]Pairwise comparisons between intermittent and continuous energy intakes at each energy level (i.e., IF100 vs control, DR70 vs IF70), and between fasting diets (IF100 vs IF70), measured after a fed day. Data are shown as mean ± SEM. [c]Significantly different from control ($P<0.05$); [d]Significantly different from IF100 ($P<0.05$); [e]Significantly different from DR70 ($P<0.05$). HDL: high-density lipoprotein; LDL: Low-density lipoprotein; HS-CRP: high-sensitivity C-reactive protein.

Appendix table 4: Changes in outcome parameters after a fed day (Control or DR70) or fasted day (IF70 and IF100), following 8 weeks of intermittent fasting[a,b]

	Control Fed	IF100 Fasted	IF70 Fasted	DR70 Fed
M/I (µmol/min/FFM+17.7 kg/mU)	-2.4±3.7	-7.6±2.2	-2.5±5.9[f]	3.5±2.1
HOMA-IR	-0.1±0.5	-0.7±0.3	-1.5±0.3[e]	-0.4±0.3
Fasting glucose (mmol/L)	0.01±0.1	-0.1±0.1	-0.3±0.1[e]	0.1±0.1
Fasting insulin (mU/L)	-0.4±1.9	-2.6±1.5	-5.9±1.2[e]	-1.2±1.1
Fasting NEFA (mmol/L)	0.08±0.07	0.13±0.04[c]	0.16±0.05[d,e]	0.10±0.04
Fasting triglycerides (mmol/L)	-0.25±0.09	-0.25±0.08	-0.16±0.06	-0.16±0.05
HS-CRP (mg/dL)	1.11±0.55	-0.02±0.34	0.08±0.37	0.34±0.46
ALT (mmol/L)	1.5±2.2	-4.2±3.0	0.8±0.3	-3.0±1.7
AST (mmol/L)	1.2±1.5	-2.0±0.9	0.1±0.1	1.0±1.3
FGF-21 (mmol/L)	10.8±26.3	11.0±10.1	48.3±10.6[f]	13.7±17.9
β-hydroxybutyrate (mmol/L)	-0.02±0.02	0.12±0.03[c]	0.27±0.05[d,e]	-0.00±0.01

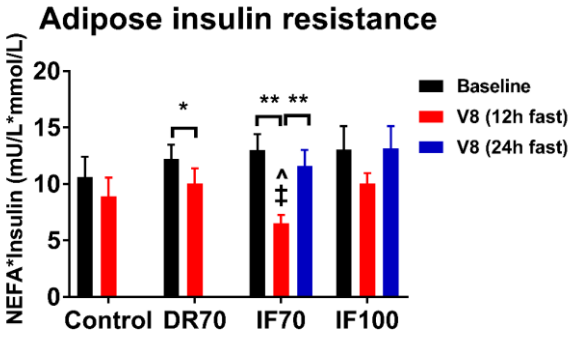
[a]Control (C): continuous energy intake at 100% of baseline energy requirements; IF100: intermittent fasting diet at 100% of baseline energy requirements; IF70: intermittent fasting diet at 70% of baseline energy requirements; DR70: continuous energy restriction at 70% of baseline energy requirements. [b]Pairwise comparisons between intermittent and continuous energy intakes at each energy level (i.e., IF100 vs control, DR70 vs IF70), and between fasting diets (IF100 vs IF70). Data are shown as mean ± SEM. HS-CRP: high-sensitivity C-reactive protein, NEFA: non-esterified fatty acids. [c]Significantly different from control (P<0.05) [d]Significantly different from IF100 (P<0.05).[e]Significantly different from DR70 (P<0.05).[f]trend compared with DR70 (P<0.08).



Appendix figure 1: Ratings of perceived hunger following 1 and 6 weeks of intervention

Control (C): continuous energy intake at 100% of baseline energy requirements; IF100: intermittent fasting diet at 100% of baseline energy requirements; IF70: intermittent fasting diet at 70% of baseline energy requirements; DR70: continuous energy restriction at 70% of baseline energy requirements.

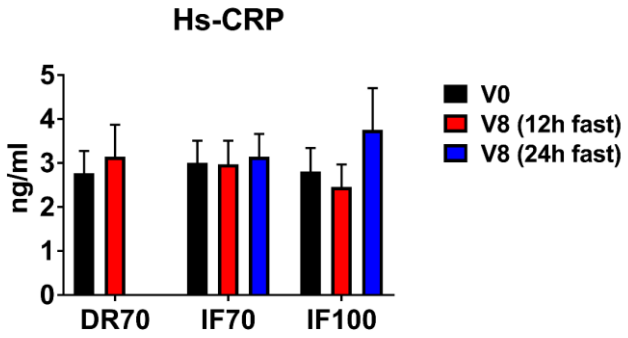
(**A**) ratings of hunger in week 1; black bars denote fed days, striped bars denote fasted days in IF groups; (**B**) ratings of hunger in week 6; black bars denote fed days, striped bars denote fasted days in IF groups; Pairwise comparisons: * P<0.05 vs control; ^ P<0.05 vs. IF100; ‡ P<0.05 vs. DR70.



Appendix figure 2: Adipose tissue insulin resistance following 8 weeks of intervention

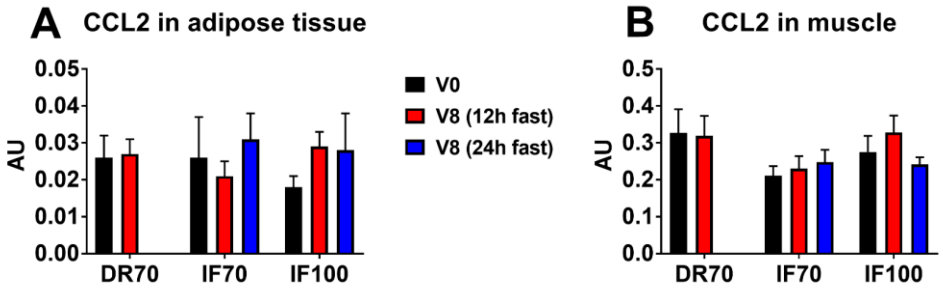
Control (C): continuous energy intake at 100% of baseline energy requirements; IF100: intermittent fasting diet at 100% of baseline energy requirements; IF70: intermittent fasting diet at 70% of baseline energy requirements; DR70: continuous energy restriction at 70% of baseline energy requirements.

Data presented as Mean±SEM. ^P<0.05 vs. DR70 in the change from baseline to week 8 following a 12-hour fast; ‡ P<0.05 vs. IF100 in the change from baseline to week 8 following a 12-hour fast. *P<0.05 and **P<0.01 vs. baseline.



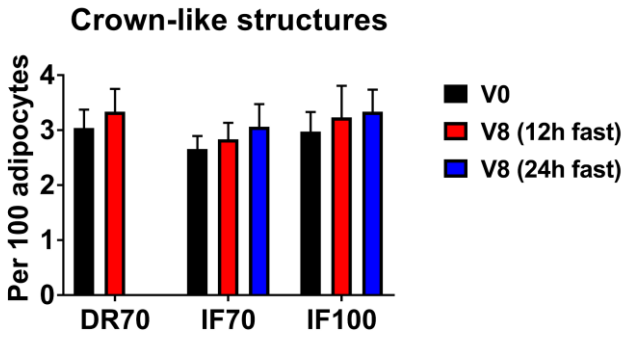
Appendix figure 3: High sensitive C-reactive protein following 8 weeks of intervention

DR70: continuous energy restriction at 70% baseline energy requirements: IF70: intermittent fasting diet at 70% baseline energy requirements; IF100: intermittent fasting diet at 100% baseline energy requirements.



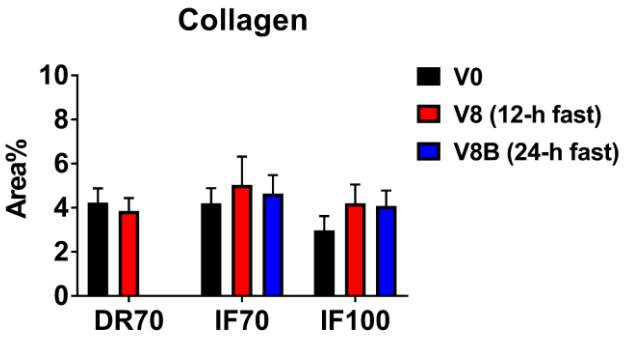
Appendix figure 4: mRNA levels of *CCL2* in adipose tissue and skeletal muscle after 8 weeks of intervention

DR70: continuous energy restriction at 70% baseline energy requirements: IF70: intermittent fasting diet at 70% baseline energy requirements; IF100: intermittent fasting diet at 100% baseline energy requirements.



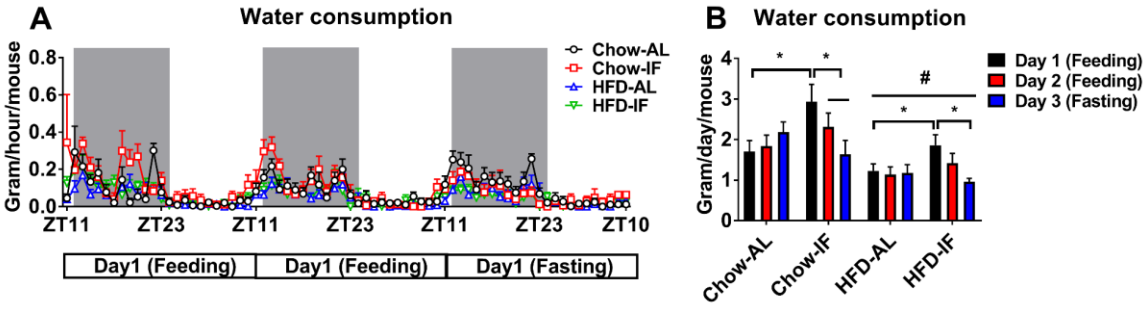
Appendix figure 5: Crown-like structure numbers in adipose tissue after 8 weeks of intervention in women

DR70: continuous energy restriction at 70% baseline energy requirements: IF70: intermittent fasting diet at 70% baseline energy requirements; IF100: intermittent fasting diet at 100% baseline energy requirements



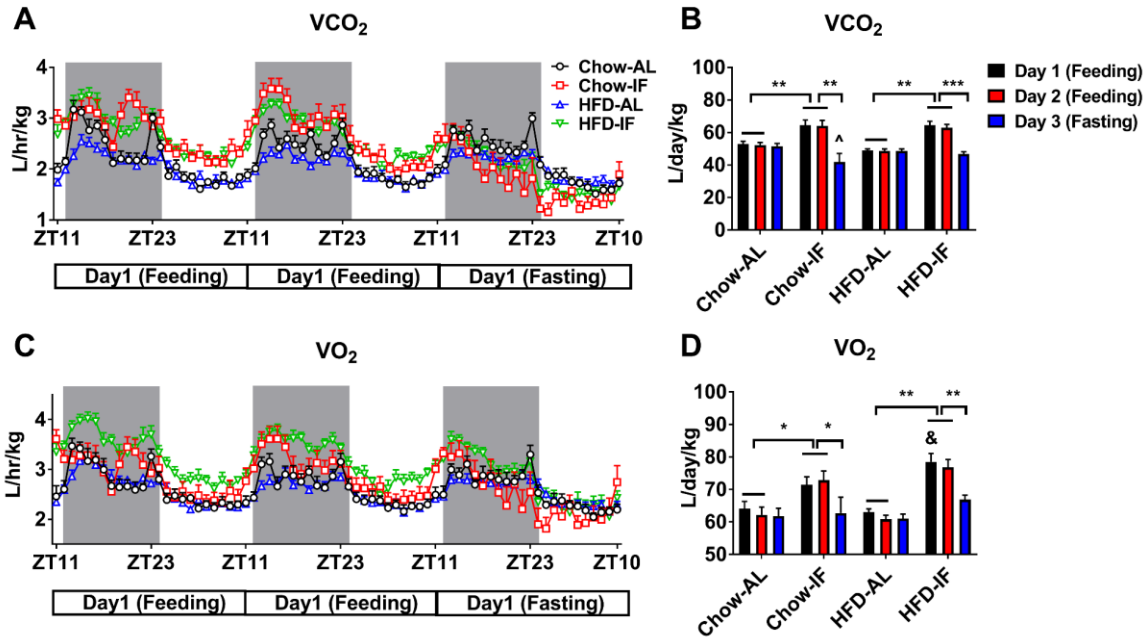
Appendix figure 6: Quantification of the collagen area in adipose tissue

DR70: continuous energy restriction at 70% baseline energy requirements: IF70: intermittent fasting diet at 70% baseline energy requirements; IF100: intermittent fasting diet at 100% baseline energy requirements.



Appendix figure 7: Water consumption of mice measured by metabolic cages

(A): Hourly water consumption and **(B)**: Daily water consumption.



Appendix figure 8: VCO_2 and VO_2 of mice measured by metabolic cages

(A and **B)**: Hourly and daily VCO^2 and **(C** and **D)**: Hourly and daily VO^2.

Mean±SEM, n=7-8/group. Effects of diet and schedule on each day were analysed by two-way ANOVA with Bonferroni post-hoc tests. Within group effects over three days were analysed using repeated measures ANOVA with Boferroni post-hoc test. Day 1 and 2 were two consecutive feeding days, followed by a fasting day (day 3). Diet effect: $^{\#}P<0.05$ on each day. $^{*}P<0.05$; $^{**}P<0.01$ and $^{***}P<0.001$; $^{\wedge}P<0.05$ vs. chow-AL on day 3; $^{\&}P<0.05$ vs. chow-IF on day 1.

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