ROLE OF NUTRITIONAL INTERVENTION ON METABOLIC HEALTH AND AUTOPHAGY

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A thesis submitted by

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Summary

Obesity is an independent risk factor for a number of chronic diseases including type 2 diabetes and cardiovascular diseases. Weight loss strategies via nutritional interventions remain first line non-pharmacological therapy to mitigate the risk. The nutritional interventions include: 1) moderate caloric restriction that curbs daily food intake by 20-40%, 2) intermittent fasting that employs 1-3 days per week of fasting interspersed with ad libitum intake on the non-fasting days, or 3) time-restricted feeding that restricts daily food intake to 6-12h of the day. However, the cellular processes and mechanism governing this remains controversial in many aspects. This thesis examines the impact of these nutritional interventions at the molecular level in humans and mice, and its role in ameliorating the metabolic outcomes.

Firstly, I utilised samples from a single-centred randomised controlled trial in humans with obesity who were randomly assigned to one of the four diets: 1) CR70 (calorie restriction at 70% of baseline requirement per week), 2) IF70 (intermittent fasting at 70% of the baseline energy requirement), 3) IF100 (intermittent fasting at 100% of the baseline energy requirement per week), and 4) control (100% of baseline energy requirements). A subset of participants from CR70 and IF70 groups who had lost >5% of body weight and were not under lipid lowering medication were included for the assessment of anti-inflammatory property and cholesterol efflux capacity of high-density lipoprotein (HDL). The IF70 group displayed greater weight loss including total cholesterol and low-density lipoprotein (LDL) vs CR70, but the change in HDL was not different between groups. We did not observe within or between group effect in changes in mRNA markers of inflammation assessed including the cholesterol efflux capacity of HDL. In conclusion, the findings of the study suggest that short-term CR or

IF with clinically significant weight loss did not alter the functions of high-density lipoprotein.

I went on to explore the role of intermittent fasting in autophagy, a cellular catabolic housekeeping process, in liver of mouse and muscle of both human and mouse. Starvation is known to activate autophagy at cellular level in wide range of cells that includes HeLa, yeast, fat, muscle and ovaries cells of *Dorsophila melanogaster* and in most tissue of mouse that includes liver, fat, pancreatic β-cells, neural and muscle cells. In the mouse study, ten-weeks-old C57BL/6J male mice were fed *ad libitum* (AL) of either high-fat diet (HFD) (43% energy from fat) or chow diet for 8-weeks before randomisation to continue either AL or IF (24h fast initiated at Zeitgeber time 11) on 3 non-consecutive days/week for further 8-weeks. Tissues were collected in both fed-state and in 22h fast. Vastus lateralis muscle tissue was also collected following a 12h and 24h fast from the IF study described above and both mRNA transcripts and proteins of autophagy markers were assessed. The findings of this study show that markers of autophagy in liver, but not in skeletal muscle, were elevated in response to IF in mice. This effect was blunted in mice with obesity. In humans, there was no evidence that fasting stimulated basal levels of autophagy markers in muscle, and in fact this was reduced from baseline, likely in response to weight loss.

Circadian desynchrony in peripheral metabolic organs results from eating out of the phase of circadian rhythmicity and is associated with increased incidence of chronic diseases. Time-restricted feeding (TRF), a form of IF, limits the *ad libitum* food intake to 6-12h of the day, while maintaining the total fasting length of >12h per day. TRF improves metabolic outcomes in both humans and mouse alike. Most of the studies conducted so far have tested TRF initiated at the onset of the dark (active) phase, and

there is a lack of knowledge about the impacts of TRF in delayed setting, which may be more feasible to implement in a human population. For this study, 8-weeks-old C57BL/6J mice (n=192) were subjected to AL feeding of chow or HFD for 4-weeks before randomising them to one of the following diets: 1) continued AL feeding, 2) 10h TRF initiated at lights off (TRFe), or 3) 10h TRF initiated 4h after lights off (TRFd) for further 8-weeks. The findings of this study suggest that both forms of TRF reduced weight, fat gain, hepatosteatosis, and increased glucose tolerance, metabolic flexibility, amplitude of genes involved in circadian regulation, and markers of NAD metabolism vs AL. TRFd limited weight and fat gain benefit and induced a phase delay in body temperature, clock gene expression including markers of NAD metabolism in liver compared to TRFe, but was not different to TRFd in improving glucose tolerance and the amplitude of genes involved in circadian regulation. In conclusion, this study suggests that delaying the TRF, akin to breakfast skipping, provides equal metabolic benefits as that of TRFe.

The circadian rhythmicity of autophagy is influenced by both nutritional and clock signals and may be dampened in diet-induced obese mice. Whether TRF acts by stimulating autophagy at cellular level is unknown. We utilised the liver samples at 6 time points of mice under TRFe and TRFd to study markers autophagy. The mRNA markers of autophagy showed circadian rhythmicity. Both forms of TRF increased both the mean and amplitude of hepatic markers of autophagy vs AL in both diet groups, but no difference was observed between TRFe and TRFd. However, only TRFe increased the mean of *Tfeb* vs AL and TRFd in chow-fed mice. TRFe mice had a phase advance in *Map1lc3b* and *Tfeb* mRNA transcript vs TRFd in both diet groups. In conclusion, this study suggests that both forms of TRF are equally effective in increasing the mRNA markers of autophagy in liver.

In conclusion, the findings of these studies suggest that nutritional interventions such as IF and TRF can effectively increase the hepatic autophagy in mice and improve the metabolic outcomes. However, IF does not provide a greater improvement in HDL biochemical properties compared to CR although both IF and CR can equally reduce clinically significant weight loss. This thesis indicates that although IF, TRF and CR may provide a similar metabolic outcome at physiological level, they affect differentially at cellular level.

Declaration

I, Rajesh Chaudhary, 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 institute, 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 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 institute without the prior approval of The University of Adelaide and where applicable, any partner institute responsible for the joint award of this degree.

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Name: Rajesh Chaudhary

Signature: Date: 01 April, 2021

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Conference proceedings

Rajesh Chaudhary, Bo Liu, Tim Sargeant, Amanda J Page, Gary Wittert, Amy Hutchison, Leonie K Heilbronn. Effect of intermittent fasting on autophagy in human muscle and C57BL/6J mouse muscle and liver. Joint Scientific Meeting of the Australia and New Zealand obesity society and the obesity surgery society of Australia and New Zealand, Adelaide, Australia 2018 [poster presentation].

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Rajesh Chaudhary, Joanne TM Tan, Amy Hutchison, Bo Liu, Christina A Bursill, Leonie K Heilbronn. Effect of calorie restriction and intermittent fasting on the anti-inflammatory and cholesterol efflux properties of HDL. The Austral-Asia Obesity Research Update, Australia 2020 [poster presentation]

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List of Abbreviations

AEBSF: 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride

ACTB: β-actin

ADH: Alcohol dehydrogenase

AICAR: 5-Aminoimidazole-e-carboxamide ribonucleotide

AL: Ad libitum

AMP: Adenosine monophosphate

AMPK: 5' AMP-activated protein kinase

ANOVA: Analysis of variance

APOC2/C3/C4: Apolipoprotein C2/C3/C4

ATP: Adenosine triphosphate

AUC/iAUC: Area under the curve/incremental area under the curve

AUSDRISK: Australian type 2 diabetes risk assessment tool score

B2M: β-2-microglobulin

β-HAD: β-hydroxyacyl CoA dehydrogenase

BCAA: Branched-chain amino acids

BECN1: Beclin-1

Bmal1: Brain and muscle ARNT-like 1

BMI: Body mass index

BSA: Bovine serum albumin

CCL2: C-C motif chemokine ligand 2

cDNA: Complementary deoxy-ribonucleic acid

CETP: Cholesterol ester transfer protein

CGM: Continuous glucose monitoring

CK1ε: Casein kinase epsilon

Clock: Circadian locomotor output cycles kaput

CONGA: Continuous overall net glycaemic action

CPM: Count per minute

CR: Calorie restriction

CRP: C-reactive protein

Cry1: Cryptochrome 1

CT: Cycle threshold

CVD: Cardiovascular disease

DBP: Diastolic blood pressure

DMEM: Dulbecco's Modified Eagle Medium

DPP-IV inhibitor: Dipeptidyl peptidase-IV inhibitor

EDTA: Ethylenediaminetetraaacetic acid

EE: Energy expenditure

FFA/NEFA: Free fatty acids/ non-esterified fatty acids

FFM: Fat-free mass

FM: Fat mass

FOXO1: Forkhead box protein O1

fT3: Free tri-iodothyronine

fT4: Free thyroxine

γ-GT: Gamma glutamyl transferase

GLP1: Glucagon-like peptide-1

GIP: Glucose-dependent insulinotropic peptide

GIR: Glucose infusion rate

GLUT2: Glucose transporter 2

GTT: Glucose tolerance test

OGTT: Oral glucose tolerance test

HbA1c: Glucosylated haemoglobin

HCAECS: Human Coronary Artery Endothelial Cells

HFD: High fat diet

HOMA-IR: Homeostatic model assessment of insulin resistance

HPRT1: Hypoxanthine phosphoribosyltransferase

iBMDM: Immortalised bone marrow derived macrophase

ICAM1: Intracellular Adhesion Molecule 1

IF: Intermittent fasting

IF70/IF100: Intermittent fasting with food provided at 70% or 100% of baseline energy requirement

IGF-1: Insulin like growth factor-1

IMT: Intima-media thickness

LAMP1/2: Lysosomal-associated membrane protein 1/2

LDL: Low-density lipoprotein

LPL: Lipoprotein lipase

MAGE: Mean amplitude of glycaemic excursions

Map1lc3b: Microtubule-associated proteins 1A/1B light chain 3B

MET: Metabolic equivalent

MODD: Mean of daily differences

MRI: Magnetic resonance imaging

mRNA: Messenger ribonucleic acid

mTOR: Mechanistic target of rapamycin

NAD: Nicotinamide adenine dinucleotide

NADPH: Nicotinamide adenine dinucleotide phosphate

NAMPT: Nicotinamide phosphoribosyl transferase

OxLDL: Oxidised LDL

P62: Phosphotyrosine Independent Ligand for the Lck SH2 Domain P62

PBS: Phosphate buffer saline

PCR: Polymerase chain reaction

PEG-6000: Polyethylene glycol

Per2: Period 2

PPARα/γ: Peroxisome proliferator-activated receptor alpha/gamma

PPIA: Peptidylprolyl isomerase A

pS6/S6: Phospho ribosomal protein S6

PYY: Peptide tyrosine tyrosine tyrosine

RCT: Randomised controlled trial/ reverse cholesterol transport

RELA/P65: Rel-like domain-containing proteins

Rev-ErbA: Nuclear family receptor subfamily 1, group D, member 1

rHDL: Reconstituted high-density lipoprotein

RMR: Resting metabolic rate

Rn18s: 18s ribosomal RNA

RORα: Retonic acid related orphan receptor alpha

RQ: Respiratory quotient

RT: Resistance trained

SAHMRI: South Australian Health and Medical Research Institute

SBP: Systolic blood pressure

SCN: Suprachiasmatic nucleus

SD: Standard deviation

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM: Standard error of mean

SIRT1: Sirtuin 1

SQSTM1/Sqstm1: Sequestosome-1

SREBP: Sterol regulatory element-binding protein

T2D: Type 2 diabetes

TCA cycle: Tricarboxylic acid cycle

TFEB/Tfeb: Transcription factor EB

TG: Triglycerides

TNFα:Tumor necrosis factor alpha

TRE/TRF: Time-restricted eating/feeding

TRFe/eTRF/eTRE: Early time-restricted eating/feeding

TSH: Thyroid stimulating hormone

UCP1: Uncoupling protein 1

VCAM1: Vascular cell adhesion molecule 1

VCO_2: Volume of carbon dioxide

VO_2: Volume of oxygen

WC: Waist circumference

ZT: Zeitgeber time

Outline of thesis

This thesis is organised into six chapters and this includes introduction as chapter 1 followed by four original research articles in manuscript format and a conclusion chapter.

Chapter 2 is entitled as “Intermittent fasting and caloric restriction did not affect the cholesterol efflux and anti-inflammatory property of HDL in women with obesity”. This secondary analysis compared the effects of 8 weeks of calorie restriction versus intermittent fasting diet both provided at 70% of the baseline energy requirements on the anti-inflammatory and cholesterol efflux capacity of HDL *in vitro* using HDL and serum collected from the participants who lost more than 5% of their body weight and not under any forms of lipid lowering medication. This study results highlighted that both CR and IF are effective in reducing body weight, fat mass including pro-atherogenic lipid. However, interventions had no effect on HDL anti-inflammatory or cholesterol efflux capacity.

Chapter 3 is entitled as “Intermittent fasting activates markers of autophagy in mouse liver, but not muscle form mouse and human”. This study examined the effect of 8 weeks of intermittent fasting in fed and fasted state in liver and quadriceps muscle autophagy of C57BL/6J mice and *Vastus lateralis* muscle autophagy of human. We showed that intermittent fasting activates autophagy in liver of mice and its more prominent in liver than in muscle in both mouse and human.

Chapter 4 is entitled as “Early or delayed time restricted feeding prevents the metabolic impacts of obesity in mice” and has been published in the *“Journal of Endocrinology”.* This study examined the effect of 10h time-restricted feeding initiated at lights off

(ZT12, TRFe) or 4h after lights off (ZT16, TRFd) on metabolic parameters, such as body weight, fat mass, glucose tolerance, nutrient homeostasis, hepatosteatosis, clock genes, and markers of NAD metabolism in liver. This study showed that TRFd improved metabolic phenotypes and increased the amplitude of genes involved in circadian regulation in liver despite inducing a phase delay in body temperature, and clock genes and markers of NAD metabolism in liver. However, TRFd marginally lagged behind TRFe in terms of benefits in weight and fat loss.

Chapter 5 is entitled as “Time-restricted feeding (early or delayed) transiently increases markers of autophagy in C57BL/6 mouse liver” and will be submitted for publication. This study examined the impact of early vs delayed time-restricted feeding in markers of autophagy in mouse liver. This study showed that both early and delayed time-restricted feeding transiently increases the amplitudes of the markers of autophagy in mouse liver but with a phase delay in TRFd.

Chapter 1: Introduction

1.1 Obesity

Definition and prevalence

According to the World Health Organization (WHO), obesity is defined as a body mass index (BMI) of ≥30 kg/m^2, and categorised into three classes as obese class I (BMI: 30-34.99), class II (BMI: 35-34.99), and obese class III (BMI≥40) (Organization 2020; Prospective Studies et al. 2009). Currently, 67% of Australian adults are overweight or obese (12.5 million people), up from 63% in 2014/15. The incidence of severe obesity (BMI>35), which was at 5% in 1995 is predicted to be at 13% of the Australian population by 2025 (Hayes et al. 2017).

Causes and consequences

The causes of obesity are multifactorial ranging from genetic, physiological, environmental, psychological, social, economic, to even political factors. Besides that, other contributing factors include higher accessibility to food, decreased physical activity, sleep debt, drug-induced weight gain, and endocrine disruptions (Wright & Aronne 2012). However, positive energy balance (energy intake exceeding energy expenditure) is the major driving factor (Sharma, AM & Padwal 2010).

Obesity is associated with insulin resistance, metabolic syndrome, cardiovascular diseases, and increased risk of developing type 2 diabetes (T2D). AusDiab study estimated that the prevalence of T2D increases from 3.9% in individual without obesity (BMI<25 kg/m^2) to 16% in individual with obesity (BMI>25 kg/m^2) (Marks 2001). Similarly, the Framingham Heart Study shows that the rise of BMI by 1 kg/m^2 increases the risk of heart failure by 5% and 7% in men and women, respectively (Kenchaiah et

al. 2002). And, that 32-49% of people with obesity or 31-40% of people with overweight suffer from heart failure.

Obesity, dyslipidaemia, and related consequences

Dyslipidaemia is characterised by elevated level of plasma lipids, such as low-density lipoprotein (LDL), very-low density lipoprotein (VLDL), triglycerides (TG) or reduced level of high-density lipoprotein (HDL) or combination of both (Pirillo et al. 2021). Dyslipidaemia is one of the consequences of obesity determined by either the genetic or environmental factors including unhealthy lifestyle and is associated with cardiovascular events in individuals with central obesity (Akil & Ahmad 2011). HDL has long been considered as the good cholesterol in relation to cardiovascular health. Framingham study in 1980's found a strong positive association between coronary heart disease and low HDL (Castelli et al. 1992), possibly due to its role in reverse cholesterol transport (RCT) and/or cholesterol efflux capacity (CEC). In brief, RCT is a cellular process where HDL accepts excess cholesterols from peripheral tissues moves it to liver for either metabolism and eliminating via gall bladder or redistribution to other tissues (Marques et al. 2018). CEC is an integral part of RCT process where accumulated cholesterol in the subintimal of the vessel wall is removed by ATP-binding membrane cassette transporter A1 (ABCA1) or by alternative mechanisms such as passive diffusion or via scavenger receptor B1 vaveolins and sterol 27-hydroxylase (Ohashi et al. 2005).

HDL, Cholesterol efflux and cardiovascular disease

Epidemiological studies suggest that HDL is cardioprotective in nature due to its role in cholesterol metabolism and having anti-inflammatory properties (Fotakis et al. 2019). However, this casual inverse relationship between the plasma level of HDL and

cardiovascular relationship is challenged by the large clinical trials such as Copenhagen General Population Study (Mortensen et al. 2015) and Mendelian randomisation studies (Haase et al. 2010; Voight et al. 2012). These findings, thus, diverted attention towards understanding the in-depth molecular and chemical properties of HDL such as its cholesterol efflux capacity, which is considered as the determining factor for cardiovascular health.

Reverse cholesterol transport (RCT) reduces the atherosclerotic burden by removing the cholesterol from extrahepatic cells and tissues to the liver for excretion (**Figure 1. 1**). Cholesterol being highly hydrophobic in nature, it requires a carrier molecule to be transported in the highly hydrophilic environment of circulatory system. The major players in RCT process include molecules such as: HDL, apoA1, lecithin: cholesterol acyltransferase (LCAT), phospholipid transfer protein (PLTP), hepatic lipase (HL) and cholesterol ester transfer protein (CETP) (Ohashi et al. 2005). The mechanism behind the cholesterol efflux capacity of HDL has already been discussed in detail (Ohashi et al. 2005). Briefly, cholesterol efflux is the first step in RCT, where macrophages within the vessel wall secret cholesterol outside cells to the cholesterol acceptor such as HDL via pathways such as: aqueous diffusion, ABCA1 (ATP-binding cassette transporter A1), ABCG1 (ATP-binding cassette transporter G1), and SR-B1 (scavenger receptor class B type 1) (Anastasius et al. 2016; Heinecke 2012).

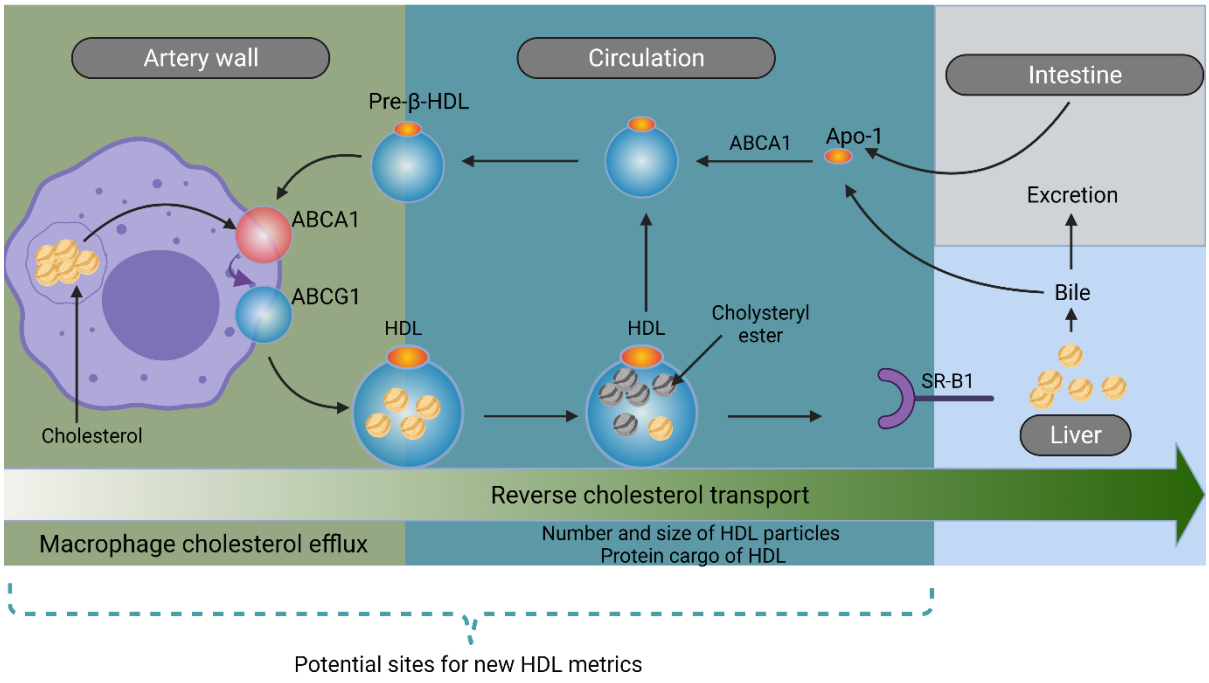

Figure 1. 1 Overview of reverse cholesterol transport by HDL. Adapted with permission from Heinecke, JW 2012, 'The not-so-simple HDL story: A new era for quantifying HDL and cardiovascular risk?', *Nat Med*, vol. 18, no. 9, Sep, pp. 1346-1347. Created with BioRender.com

Pre-β-HDL, lipid poor HDL and small HDL particle. Cholesterol transporters: ATP-binding cassette transporter A1 (ABCA1), ATP-binding cassette transporter G1 (ABCAG1), scavenger receptor class B type 1 (SR-B1). Accumulation of cholesterol in macrophage activates the membrane transporters ABCA1 and ABCG1. Next, free cholesterol in nascent HDL is converted to cholesterol ester by lecithin-cholestero acyltransferase (LCAT). Cholesterol ester from HDL is then removed by hepatocytes membrane-bound protein SR-B1 which is then converted to cholesterol and then to bile for excretion.

Prevention and management

Obesity is the result of multifactorial determinants of weight gain, and, health benefits can be derived through weight loss, by pharmacotherapy but also through nutritional

interventions such as caloric restriction (Makris & Foster 2011; Park et al. 2018). Fasting and calorie restriction has been practiced for millennia, and are considered safe and effective strategies to lose weight and improve health (Longo & Mattson 2014). Fasting is categorically subdivided into different sub-forms: 1) Intermittent fasting (IF), also known as Alternate Day Fasting (ADF) or periodic fasting (PF), 2) Inter-meal fasting (two short eating windows within 24h), and 3) Time-restricted feeding (TRF), which will be discussed in detail in their respective headings below.

1.2 Dietary interventions

Calorie restriction

Calorie restriction (CR) is a non-pharmacological dietary intervention which is achieved by limiting the calorie intake by 10-40% of ad libitum (AL), typically on a daily sustained basis (Bordone & Guarente 2005; Fontana et al. 2004; Heilbronn & Ravussin 2003; Meydani et al. 2016). CR imparts a wide range of physiological and metabolic health benefits that ranges from lowering body weight and fat mass, blood pressure, blood glucose and cholesterol (Heilbronn & Ravussin 2003; Verdery & Walford 1998; Walford, Harris & Gunion 1992) to extending life-span in model organisms (Fontana, Partridge & Longo 2010; Languille et al. 2012; Mattison et al. 2017).

CR has profound metabolic health benefits in rodents

Clear evidence shows that CR extends lifespan in lower model organisms, such as fruit flies (Bruce et al. 2013; Grandison et al. 2009; Min & Tatar 2006), nematodes (Uno et al. 2013), yeast (Jiang et al. 2000; Lin et al. 2002; Wu, Liu & Huang 2013) and also in rodents (McCay, Crowell & Maynard 1989), although the evidence in non-human primates is controversial (Colman et al. 2009; Kemnitz et al. 1994; Mattison et al. 2012).

Mice placed under 30% CR in conjunction with HFD (compared to ad libitum HFD) had a reduced weight gain, reduced serum level of cholesterol and leptin with increased glucose tolerance and serum adiponectin levels (Duivenvoorde et al. 2011). CR also reversed β-cell dysfunction, insulin resistance, and restored glucose homeostasis in diet-induced obese mice (Gao et al. 2015; McCay, Crowell & Maynard 1989).

CR also provides cardioprotective benefits. In one previous study, 15% CR for 18 weeks reduced blood pressure, delayed onset of cachexia, lowered fasting hyperlipidemia, cardiac, renal and lung weight, reduced plasma IL-6 and TNF-α and cardiac oxidative damage, and improved diastolic chamber function and cardiac index in Dahl salt-sensitive rats (that are prone to gradual hypertensive-associated diastolic dysfunction) (Seymour et al. 2006). CR-mediated cardio-protection is imparted by several mechanisms at the cellular level, of which modulation of autophagy, reduced inflammation, attenuation of oxidative stress and mitochondrial dysfunctions are some of the major contributors.

Calorie restriction improves metabolic health in human

Low calorie intake during is associated with life-long low body mass index (BMI) and low risk of mortality from age-related diseases (Willcox et al. 2007). An assessment of long-term CR in humans without obesity was recently conducted in the CALERIE2 study (Comprehensive Assessment of Long-Term Effects of Reducing Calorie Intake) (Kraus et al. 2019). In that study, 25% CR reduced weight and measures of cardiovascular risk factors, namely total cholesterol (TC), low-density lipoprotein (LDL), including systolic and diastolic blood pressure and improved insulin sensitivity.

High-density lipoprotein (HDL) is ubiquitously regarded as a good cholesterol for having inverse association with cardiovascular risk based on the epidemiological studies (Filippatos & Elisaf 2013; Gordon et al. 1977). However, a large number of cardiovascular trials have failed to translate substantial increase in HDL level (20-50%) to cardiovascular benefits (Abourbih et al. 2009; Arsenault et al. 2012; Barter et al. 2007; Investigators et al. 2011). Recent studies conclude that, it is the cholesterol efflux capacity (CEC) of HDL (the first step in reverse cholesterol transport mechanism) that may determine cardiovascular health, rather than absolute serum HDL level (Ebtehaj et al. 2019; Qiu et al. 2017), and this is because cholesterol efflux capacity is independent of HDL concentration (Agarwala et al. 2015). Moreover, efflux capacity of HDL from macrophage, independent of HDL concentration, was strongly inversely associated with intima-media thickness and likelihood of angiographic coronary artery disease (Khera et al. 2011). The effects of nutritional intervention such as CR on efflux capacity of HDL is unclear. A one-year lifestyle modification program conducted in 149 males (age 30-65 years) that included moderate calorie restriction (~500 kCal/day) with healthy eating habit and 160 min of weekly aerobic activity increased HDL and apolipoprotein A-1 level by >13% and increased the HDL-CEC by 14% (Boyer et al. 2018). However, other studies in individuals with obesity and type 2 diabetes have failed to raise HDL-CEC capacity either with CR or diet-induced weight loss (Stein et al. 2003; Talbot et al. 2018; Wang, Y et al. 2011).

Intermittent fasting

Intermittent fasting (IF) is a novel dietary intervention that involves periods of fasting for 1-3 days per week with ad libitum consumption on non-fasting days (Barnosky et al. 2014). A myriad of studies suggest there are metabolic benefits of IF in both human (Bhutani et al. 2010; Harney et al. 2019; Heilbronn, Civitarese, et al. 2005; Heilbronn,

Smith, et al. 2005; Klempel, Kroeger & Varady 2013) and rodents alike (Ahmet et al. 2005; Delahaye et al. 2018; Godar et al. 2015; Gotthardt et al. 2016; Kim, KH et al. 2017; Kim, YH et al. 2019; Liu, B, Page, Hatzinikolas, et al. 2019; Marinho et al. 2019; Wan et al. 2010). These benefits are produced even under modified form of IF where a very low-calorie diet (25-30% of baseline energy requirement) was consumed during the fast day (Eshghinia & Mohammadzadeh 2013).

IF improves life span and metabolic health benefits in rodents

IF has now been studied extensively in rodents (Gotthardt et al. 2016; Kim, YH et al. 2019; Liu, B, Page, Hutchison, et al. 2019; Mattson, Longo & Harvie 2017). The evidence suggests that IF is effective in increasing the lifespan in both mouse (Xie et al. 2017) and rat (Carlson & Hoelzel 1946) alike (Swindell 2012). A study by Goodrick et al., on the effect of IF on body weight and lifespan suggests that the beneficial effects of IF varies based on strain and the age of initiation (Goodrick et al. 1990). In that study, IF modestly reduced body weight and increased both mean and maximum lifespan in C57BL/6J mice when initiated at young age (1.5 to 6 months) but has no effect in mean lifespan when initiated at ten months of age. Moreover, AJ mice displayed no change in body weight, irrespective of age of initiation (either 1.5, 6 or 10 months), although IF was able to increase both mean and maximum lifespan when initiated at one and half to six months of age.

Rodents maintained on alternate day fasting (ADF), irrespective of low-fat diet or HFD assignment, had reduced body weight and increased lean mass following IF (Gotthardt et al. 2016; Varady et al. 2007). This was accompanied by an increase in glucose tolerance in all diet groups except HFD-fed mice under IF and reduction in area under the curve (AUC) for insulin tolerance in all diet groups (Gotthardt et al. 2016). A study

by Anson et al., (Anson et al. 2003) revealed that ADF mice had similar body weight as that of AL fed mice but exhibited a significant improvement in glucose metabolism characterised by reduced level of both insulin and glucose along with increased β-hydroxybutyrate levels, suggesting beneficial effect of IF independent of weight loss. Our previous finding has also shown that 8-weeks of IF improves glucose tolerance in both chow and HFD-fed mice (Liu, B, Page, Hatzinikolas, et al. 2019), and increased energy expenditure and adipose tissue browning (Liu, B, Page, Hutchison, et al. 2019).

IF is also protective against development and progression of cardiovascular, cancer and neurological disorders. Adult rat maintained under ADF regime for 2-4 months developed resistance of hippocampal neurons to excitotoxin-induced degeneration and resistance against kainite-induced deficits in performance in water-maze learning and memory tasks (Bruce-Keller et al. 1999). Moreover, ADF delayed huntingtin mutant mice from onset of neurodegeneration and motor dysfunction when initiated prior to onset of motor dysfunction (Duan et al. 2003). IF increased cardiac function and reduced left ventricular volume and diameter in rats with induce heart failure compared to their AL fed counterpart (de Lucia et al. 2018). Similarly, rats maintained under three months of ADF prior to myocardial infarction (MI) exhibited reduced myocardial infract size and apoptotic cells in the penumbra compared to AL fed mice (Ahmet et al. 2005).

IF improves metabolic health benefits in humans

In humans, IF has physiological and clinical health benefits that includes reduction in body weight, body fat, fasting glucose and insulin, plasma cholesterol, LDL-cholesterol, triglycerides and potentially reduces risk of CVD (Horne, Muhlestein & Anderson 2015; Johnson et al. 2007; Tinsley & La Bounty 2015; Varady et al. 2010; Varady et al. 2013)

(Heilbronn, Smith, et al. 2005; Johnson et al. 2007; Varady et al. 2011). Despite significant changes in the pro-atherogenic lipids in human, majority of studies showed no change in HDL, except two studies that showed increase in HDL (Heilbronn, Smith, et al. 2005; Johnson et al. 2007).

A comparative assessment of IF vs CR in a previous study conducted in subjects with obesity for a period of two years, reported increased weight loss during CR period compared to IF period (Aksungar et al. 2017). A comparative assessment of CR vs IF showed that both CR and IF equally reduce visceral fat, proliferation of T cells and prostrate cells and decreases insulin-like growth factor-1 (IGF-1) levels while increases blood adiponectin level (Varady et al. 2010). Although IF has several benefits, there are some reports of some side effects of IF in human. For example, there are reports of participants experiencing headache (n = 2) and constipation (n = 1) (Varady et al. 2013). Similarly, Hutchison et al., (Hutchison, Liu, et al. 2019) reported a trend towards impairment in insulin sensitivity in IF70 group (intermittent fasting participants who were provided with 70% of their baseline energy requirements per week on their fasting days) vs DR70 (daily calorie restricted group who were provided with 70% of their baseline energy requirement in every single day throughout the study duration). Liu et al., (Liu, B, Hutchison, et al. 2019) reported a transient increase in M1 markers of inflammation in adipose tissue on fasting days. A small number of participants (generally <15%) have reported feeling cold, irritable, low energy and hunger (Harvie, M et al. 2013; Harvie, MN et al. 2011). Similarly, a longer average menstruation was observed in women with obesity after six months of intermittent CR (two consecutive days of 25% of weekly energy restriction) compared to continuous 25% CR group (Harvie, MN et al. 2011). However, these findings come from a limited number of studies with short intervention duration and larger trials are recommended.

Circadian rhythm and regulation

Most life forms exhibit and anticipate change in pattern of their environment and their physiology based on light-and-dark cycle known as circadian rhythm (Patke, Young & Axelrod 2020). The term circadian stems from the Latin word "*circa diem*" meaning "about a day". These 24h physiological rhythms are governed by the intrinsic genes known as CLOCK genes via a transcription-translation feedback loop which is controlled by the central pacemaker located in the suprachiasmatic nuclei (SCN) of hypothalamus and crosstalk between the central and peripheral clocks (Patton, AP & Hastings 2018). The molecular mechanism behind the regulation of circadian clock system is already discussed elsewhere (Patke, Young & Axelrod 2020). In brief, CLOCK: BMAL heterodimer interact with E-box response element in the promoter region of the target genes that drives transcription-translation oscillating (TTO) loop. These two target genes encode circadian proteins Period (Per1, Per2 and Per3) and Cryptochrome (Cry1 and Cry2) followed by translocation of Cry and Per to the nucleus and inhibit CLOCK and BMAL1 genes leading to transcription repression. In addition to this, REV-ERBα and REV-ERBβ, the two targets of CLOCK and BMAL1, along with retinoid-related orphan receptor (ROR$\alpha/\beta/\gamma$) forms a second loop and regulates the rhythmic expression of BMAL1.

Circadian system is regulated by both the internal and external signal that ranges from hormonal signals such as melatonin and cortisol to body temperature, external light and feeding and fasting cycles (Longo & Panda 2016; Mistlberger 2009; Serin & Acar Tek 2019). Feeding-fasting patterns are one of the most robust signals that influences daily biological rhythm and erratic pattern of eating, for example eating during the inactive phase or that misaligns with the daily clock time, leads to chronic diseases including development and progression of metabolic syndrome (Manoogian & Panda

2017). Food-anticipatory circadian rhythm in mice and rats is already proven where they behaviourally anticipate daily mealtime by entrainment of circadian oscillator known as food-entertainable oscillators. The circadian organisation is lost in the event of ad libitum food availability with ablated suprachiasmatic nucleus (SCN), but the food anticipatory activity rhythms and circadian rhythms of physiology are restored when food is provided once every 24h in a reduced amount (Patton, DF & Mistlberger 2013). Time-restricted eating (TRE, also known as time-restricted feeding in animal (TRF)) limits daily food intake to a shorter and specific time of the day that aligns with the circadian timing of the day has been found to improve metabolic parameters and attenuates harmful effect of circadian disruption (Adamovich et al. 2014; Chaix et al. 2014; Challet 2019; Sutton et al. 2018). The details on the impacts of TRF at both metabolic and associated circadian rhythmicity is discussed further in their specific section.

Time-restricted feeding (TRF) improves metabolic outcomes in mice

Time-restricted feeding (TRF) (also known as time-restricted eating (TRE) in humans) is a modified form of IF that restricts the eating window between 6-12 hours during the biological active phase without affecting the quantity or quality of nutrient intake (Chaix et al. 2014; Cienfuegos et al. 2020; de Goede et al. 2019; Gill et al. 2015; Hatori et al. 2012). TRF provides metabolic benefits and help to prevent and manage chronic metabolic diseases in both animal and human alike. TRF provides metabolic benefits in wide range of species from *Dorsophila melanogaster* to mouse and human. TRF (4h allotted eating window for 18-weeks) reduced body weight and cholesterol by 12% and 21%, respectively, and lowered HOMA-IR by 1.4-fold in diet-induced obese mice fed HFD (Sherman et al. 2012). In another study, the TRF (3h eating window) group lost 9% of their body weight compared to AL group, despite consuming 95% of AL calories

(Sherman et al. 2011). Similarly, daily TRF of 9-15h protected mice from HFD-induced obesity with a dose dependent effect (Chaix et al. 2014). In that study, the percentage of weight and fat mass gained by the mice was linearly related to the eating windows (i.e. 9h TRF mice fed HFD had 26% weight gain, 15h TRF HFD mice gained 43% body weight, and AL mice gained 65% body weight). Similarly, mice under longest TRF window had lowest reduction of body fat by TRF when fed HFD.

The health benefits of TRF in rodents is not limited to the body weight, fat mass and glucose metabolism. Mice under TRF (3h eating window for 4 months) possessed robust circadian rhythmicity in the majority of clock genes that were assessed in both liver and jejunum, namely *Clock*, *Bmal1*, *Cry1*, *Per1*, and *Per2* and had reduced mRNA expression of inflammatory markers, namely *Il-6*, *Tnf-*α, *Nf-*κ*B* (Sherman et al. 2011). In another similar study, biological active phase TRF mice (12h restricted eating for four months) had enhanced immune function compared to TRF mice fed during the inactive phase (Cisse et al. 2018). Moreover, TRF also prevented from both age and HFD-induced reduction in cardiac contractile function (Gill & Panda 2015; Tsai et al. 2013).

The metabolic benefit imparted by the TRF is most pronounced when aligned to the circadian timing system (i.e. feeding during the active phase). A study conducted by Goede et al., (de Goede et al. 2019) showed that the TRF mice (12h feeding window, 3-4 weeks, active phase) had lower insulin level in line with the glucose clearance which was not evident in the mice subjected to TRF schedule during the inactive phase. The TRF-mediated metabolic benefits emerge from circadian regulation of metabolic intermediates. In liver, TRF reduced the amplitude of pyruvate carboxylase, glucose-6-phosphatase and increased that of glucokinase during the active phase (Chaix et al.

2014; Hatori et al. 2012), potentially indicating reduced hepatic glucose production while increasing glucose utilisation. At the molecular front, TRF also increased the amplitude of AMPK and mTOR (Hatori et al. 2012; Sherman et al. 2012) including ribosomal phospho-S6 in skeletal muscle during active phase. This suggests an increased mTOR and a reduced autophagy after feeding (Chaix et al. 2014). The more details are discussed later on.

Time-restricted eating (TRE) improves metabolic outcomes in human

In humans, TRE improves metabolic functions by reducing body weight and fat mass, improving glucose tolerance and reducing fasting glucose, fasting insulin, HOMA-IR and blood pressure, especially in individuals with obesity (Gabel et al. 2018; Gill & Panda 2015; Hutchison, Regmi, et al. 2019; Jamshed et al. 2019; Sutton et al. 2018). TRE also lowered BMI, both the systolic or diastolic blood pressure including total cholesterol and LDL-cholesterol (Wilkinson et al. 2019). To date, the majority of TRE studies are pilot, short-term trials (lasting 4-days to 16-weeks) and have been conducted on a limited number of participants (n=100) (Cienfuegos et al. 2020), and accumulating evidence suggest that it is metabolically healthy form of dietary intervention (Anton et al. 2019; Hutchison, Regmi, et al. 2019; Jamshed et al. 2019; Ravussin et al. 2019; Tinsley et al. 2017).

Many physiological processes are under 24h rhythm known as circadian rhythmicity. This includes sleep-wake cycle, hormone release, and fluctuation of body temperature that are controlled by the peripheral clocks (Mohawk, Green & Takahashi 2012; Richards & Gumz 2012). Circadian desynchrony is associated with an increased risk of obesity and cardio-metabolic consequence (Karlsson, Knutsson & Lindahl 2001; Morikawa et al. 2005; Tenkanen, Sjoblom & Harma 1998).

1.3 Autophagy

Autophagy is a cellular catabolic housekeeping process that clears the cellular milieu of toxic cellular components through lysosomal-dependent catabolic process and generates ATP generation including new organelles (Choi, Ryter & Levine 2013; Galluzzi et al. 2014; Levine, Packer & Codogno 2015). There are three major types of autophagy: microautophagy, macroautophagy and chaperon-mediated autophagy (CMA), and all of these promote proteolytic-degradation of cytosolic components in the lysosomal compartment (**Figure 1. 3**) (Kaur & Debnath 2015a; Thorburn 2014). Microautophagy is a direct invagination of the substrates into lysosome or endosomal membrane for the subsequent proteolytic degradation (Li, Li & Bao 2012). Macroautophagy involves formation of double-membrane bound intermediator vesicles, referred to as autophagosomes, to transfer cytoplasmic organelles to the lysosomes; where they are fused with the lysosomes for proteolytic degradation (Kaur & Debnath 2015a). In contrast, chaperon-mediated autophagy (CMA) is distinct from micro and macroautophagy, in that, membrane-bound sequestering compartment vesicles are not involved. Instead, target protein contains KFERQ-like pentapeptide motif, which is recognized by the cytosolic chaperon heat shock cognate 71-kDa protein (HSC70, also known as HSPA8). HSC70 promotes translocation of target protein through lysosomal membrane to lysosomal lumen via lysosomal-associated membrane protein 2A (LAMP2A) receptor, which is an isoform of LAMP-2 (Cuervo & Wong 2014; Kaur & Debnath 2015a; Mizushima & Komatsu 2011; Ueno & Komatsu 2017). Macroautophagy is the main route of incorporation of cellular components into the lysosomes, and also because microautophagy and CMA have limited roles in aging and aging-associated diseases, we will focus on macroautophagy, hereafter, will be referred as autophagy (Gelino & Hansen 2012). Autophagy is also distinct from the ubiquitin-proteasome system (UPS)-mediated protein degradation, as UPS targets

individual short-lived proteins while autophagy targets the bulk of long-lived proteins including other cellular organelles such as mitochondria, peroxisomes, endoplasmic reticulum, and nucleus (Kaur & Debnath 2015) and will not be discussed here. Also, the detailed mechanism of autophagy has been discussed elsewhere so here we will be focusing on the overview of this mechanism only (Codogno & Meijer 2010; Gelino & Hansen 2012; Glick, Barth & Macleod 2010; Kaur & Debnath 2015b; Yang, Z & Klionsky 2009).

Mechanism of autophagy

Biogenesis of autophagosome

Previous genetic studies in yeast have identified more than 30 autophagy-related proteins from highly conserved autophagy related genes (ATG) that are involved in the process (Kaur & Debnath 2015a; Nakatogawa et al. 2009; Vindis 2015; Yang, Z & Klionsky 2009). (Glick, Barth & Macleod 2010; Kaur & Debnath 2015a; Vindis 2015) autophagy can simply be categorized into four steps: 1) Initiation or phagophore assembly site (PAS), 2) Nucleation or formation of phagophore, 3) Expansion or formation of autosomes, and 4) Degradation (

Figure 1.2)

Initiation / Phagophore assembly site (PAS)

The initiation of autophagy begins at the phagophore assembly site (PAS) as depicted in the **Figure 1.2**. This is mediated by proteins of UNC51-like kinase (ULK) complex (which is composed of ULK-1 or ULK-2 and ATG-13, FAK family kinase interacting protein of 200 kDa (FIP200) and ATG101) assembly to form autophagosome (Kaur & Debnath 2015a; Lamb, Yoshimori & Tooze 2013).

Nucleation / Formation of phagophore

During nucleation, activated UKL complex targets class III PI3K complex consisting of Beclin-1 (Atg6 in yeast), vacuolar protein sorting-15 (VPS-15), VPS-34 and ATG14 to promote the local production of a pool of phosphatidyl-inositol 3-phosphate that is specific to autophagosomes. Interestingly, a new study has confirmed the involvement of ATG14 in fusion of autophagosome with endosomal compartment (Diao et al. 2015; Kaur & Debnath 2015a).

Expansion / Formation of autophagosome

During expansion, the ATG12-ATG5-ATG16 complex is recruited to the autophagosome membrane where it promotes the lipidation of microtubule-associated protein 1 light chain 3 (MAP1LC3; also known as LC3) with phosphatidyl ethanolamine. LC3 is the chief mammalian orthologue of yeast Atg8, which is required for expansion of isolation membrane (Kaur & Debnath 2015a). Recent studies suggest that deacetylation and cytosolic translocation of LC3 pool is necessary for lipidation with phosphatidylethanolamine during starvation-induced autophagy (Huang et al. 2015).

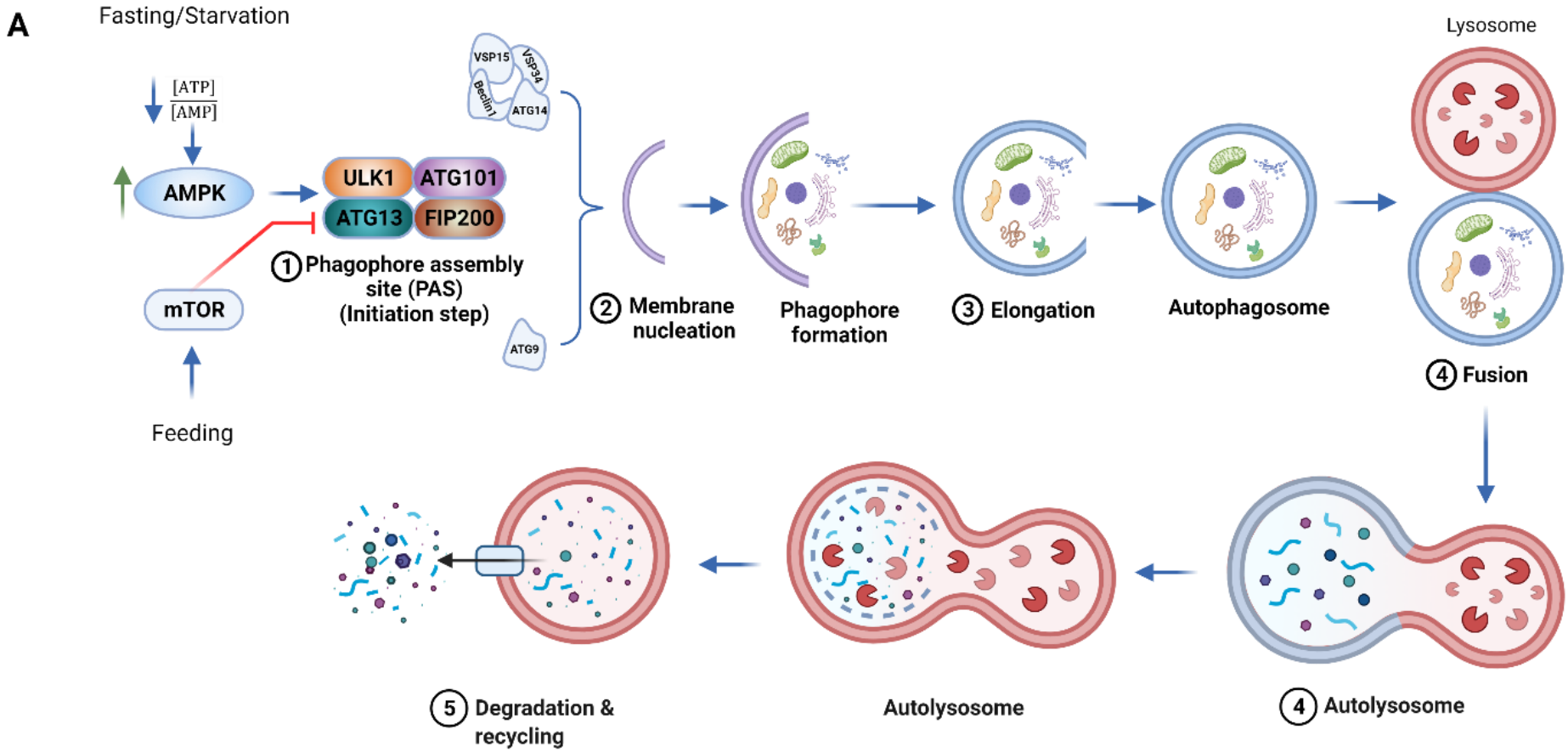

Figure 1.2 The basic steps in autophagy process (Kaur & Debnath 2015a).

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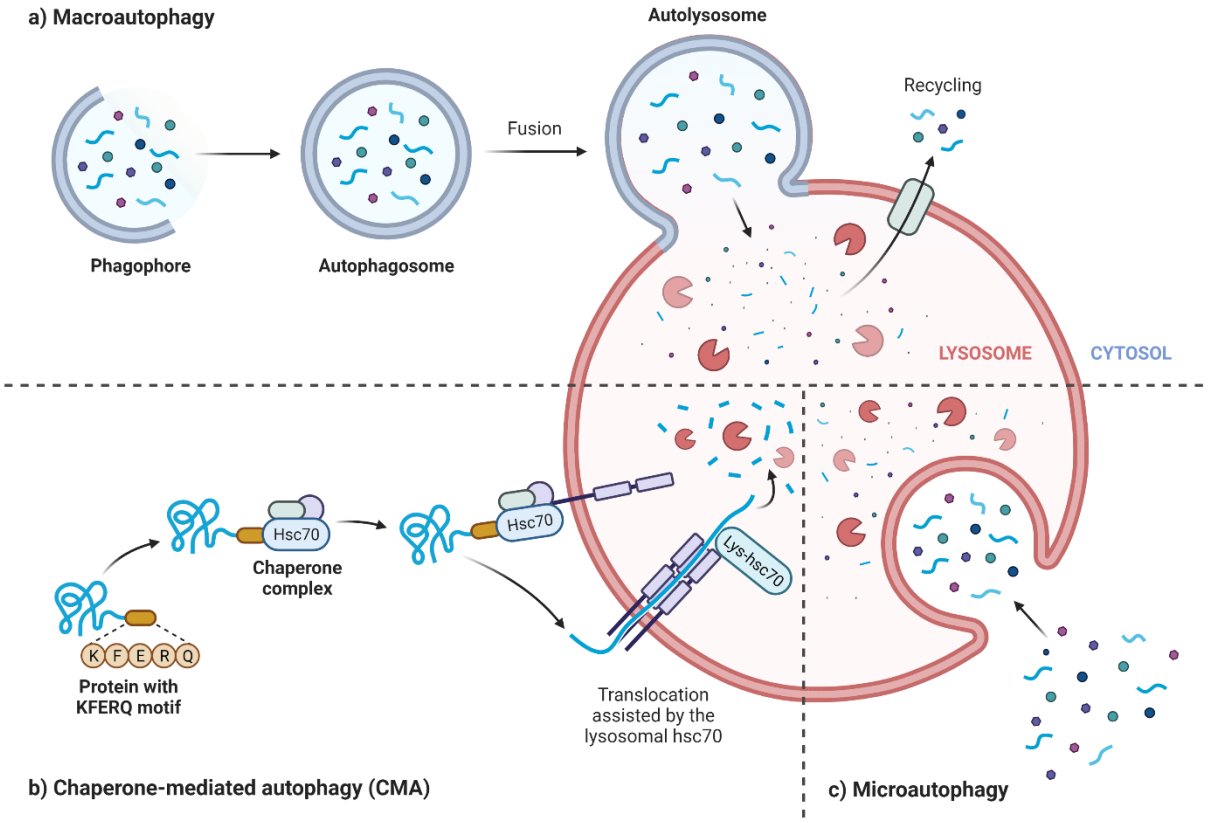

Figure 1. 3 The three major types of autophagy processes

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Role of autophagy in β-cell dysfunction and insulin resistance

In mice, β-cell specific Atg7 knockout (KO) displayed defective basal autophagy, morphological abnormalities of islets, reduced β-cell mass and decrease in basal and glucose-stimulated insulin secretion (Ebato et al. 2008; Jung et al. 2008). While in the same study, enhanced autophagy was also observed in control mice under HFD for 12-weeks. This may indicate an adaptive response of β-cell to combat diet-induced insulin resistance and insulin insufficiency. This was associated with mitochondrial dysfunction including reduced ATP production (Ebato et al. 2008). Further, electron microscopic examination revealed swollen mitochondria and cisternal distention of Golgi complex and rough endoplasmic reticulum (ER) (Jung et al. 2008). In another similar study, when mice with mild hyperglycaemia and β-cell specific Atg7 knock out were bred with *ob/ob* mice to induce ER stress *in vivo*, severe hyperglycemia was

manifested (Quan et al. 2012). The mice also revealed higher susceptibility to ER stress and had increased number of apoptotic β-cells in islet (Quan et al. 2012). Several studies also support the notion that autophagy may improve insulin sensitivity (Codogno & Meijer 2010; Liu, Y et al. 2015; Wang, Y et al. 2016). For example, a study on a transgenic C57BL/6 mouse has revealed that higher expression of *Risa* (a long non-coding RNA that is highly expressed in heat, kidney, liver, lung, muscle, spleen, testis, thyroid and white adipose tissue) results in down regulation of insulin-induced phosphorylation of insulin receptor *Akt* and *Gsk3*. This was associated with a reduction in LC3B-II protein level and LC3B-II/ I ratio and increases in phosphorylation of ULK1(Ser^{757}), contributing to inhibition of autophagy. This suggests that *Risa* induced insulin resistance by down regulating the autophagy process (Wang, Y et al. 2016). Together these findings highlight a defensive role of autophagy to maintain pancreatic β-cell function through regular cleaning of toxic ubiquitinated proteins and damaged organelles (Barlow & Thomas 2015). Human islets obtained from organ donors also showed increased autophagy as demonstrated by accumulation of autophagic vacuoles and autophagosomes in individuals with type 2 diabetes compared to donors without type 2 diabetes (Masini et al. 2009). However, muscle-specific autophagy-related genes collected from five males and two females with severe insulin resistance, namely *ATG14, GABARAPL1, SQSTM1/P62, LC3BII*, and *ATG5* were repressed (Moller et al. 2017).

Autophagy in obesity

Autophagy is altered (enhanced or suppressed) in both animals and humans models of obesity either diet or genetically predisposed (Ignacio-Souza et al. 2014; Jansen et al. 2012; Kosacka et al. 2015; Kovsan et al. 2011; Meng & Cai 2011; Mu et al. 2017; Yamahara et al. 2013). Autophagy deficiency resulting from knockdown of *Atg*-specific

genes have consistently shown to suppress autophagy either in whole body (He et al. 2013), liver (Settembre, De Cegli, et al. 2013) or in pancreas (Ebato et al. 2008) resulting in impaired glucose tolerance with reduced insulin secretion in HFD-fed mice including increased lipid content and insulin resistance (Settembre, De Cegli, et al. 2013), and impaired glucose tolerance and insulin secretion (Ebato et al. 2008; Jung et al. 2008). Autophagy is impaired (either increased or decreased) in HFD-diet-induced obese mice. For example, suppressed autophagy was observed in pancreatic islet of mice that were fed a high-fat, high-calorie for 12-36 weeks(Chang et al. 2017; Liu, H et al. 2017), in liver of mice fed HFD for 16-weeks(Lopez-Vicario et al. 2015), and in liver of rats fed HFD for 8-weeks (Sciarretta et al. 2012; Wang, S et al. 2018). While, enhanced hepatic autophagy was reported in rats that were fed with high fructose diet (60%) for 5 months (Aijala et al. 2013), in NASH and NAFLD rats models (Xiao et al. 2014), and in mice fed HFD from 2 to 8 weeks (Hsu et al. 2016).

Fasting-induced activation of autophagy

In mice, starvation resulted in 25-40% of total liver protein loss along with sub-cellular constituents (Yin, Ding & Gao 2008). Those losses were mostly confined in the cytosol resulting into 25% total reduction in cell volume within 24h of starvation indicating autophagy-mediated process (Mortimore & Poso 1987; Yin, Ding & Gao 2008). Considering fasting-induced activation of autophagy, a 24h fasting resulted in higher abundance of autophagosome in mice hepatocytes including Purkinje cells in the cerebella (Alirezaei et al. 2010). Similar findings were observed in mice skeletal muscle where prolonged fasting increased LC3 puncta via activation of AMPK pathway and prevented fasting-induced hypoglycaemia (Bujak et al. 2015). Moreover, hepatic autophagy was downregulated in both the genetic and dietary model of obesity as evident from reduced microtubule associated protein 1 light chain 3 Beta (MAP1LC3B),

mammalian orthologue for yeast autophagy related gene 6 (ATG6)/ (BECLIN1), autophagy related 5 (ATG5) and autophagy related (ATG7) with concomitant accumulation of sequestosome (SQSTM1) protein (Yang, L et al. 2010). Similarly, in a primary culture of muscle precursor cells, from control group showed accumulation of LC3II protein in response to treatment with Bafilomycin when subjected to starvation using HBSS medium for 4h compared to differentiated cells from T2DM patients, suggesting dampened autophagic response in T2DM (Henriksen et al. 2019). Together these findings underscores that autophagy is necessary for optimal cellular functionality irrespective of types of cells, such as hepatocytes (Komatsu et al. 2005), skeletal muscle (Bujak et al. 2015; Ehrlicher et al. 2018) and pancreatic β-cells (Bartolome et al. 2014; Sheng et al. 2017). And its suppression may result in adverse metabolic consequences.

Impacts of IF on autophagy

At least some of the fasting based health benefits are proposed to occur through activation of autophagy (DiNicolantonio & McCarty 2019; Fernandez et al. 2017; Liu, H et al. 2017). A study conducted by Liu et al (Liu, H et al. 2017) has found that in obesity-induced diabetic mice, despite continued high-fat intake, IF restored autophagy flux in islets and improved glucose tolerance by enhancing glucose-stimulated insulin secretion, β-cell survival, and nuclear expression of NEUROG3 (a marker of pancreatic regeneration). It also proved that intact autophagy functionality is necessary for IF to induced desired positive metabolic outcomes because IF failed to rescue β-cell death or induce NEUROG3 expression in obese mice that lack proper lysosomal function resulting from LAMP2 or haplo-insufficiency of BECN1/Beclin-1 deficient mice.

Similarly, metabolic benefits have been found in liver of C57BL/6J mice subjected to IF for 8-weeks irrespective of type of diet consumed (either high-fat or high-fructose) (Marinho et al. 2019). IF improved oral glucose tolerance test (OGTT), improved insulin level, enhanced markers of β-oxidation, and reduced hepatic steatosis and inflammation A previous study (Rickenbacher et al. 2014), showed that 1-day of acute fasting protected mice from hepatic ischemia-reperfusion (IR) injury. Fasted mice had dampened inflammatory response and reduction in circulating HMGB1 associated with cytoplasmic aggregate formation and autophagy. They also showed decreased level of serum AST/ALT, the necrotic area and the number of TUNEL-positive hepatocytes. Fasted mice had upregulated autophagy response as assessed by the autophagy markers at protein level LC3 (both I and II) and BECLIN-1. However, when the mice were subject to inhibition of autophagy, the protective effects of fasting were lost. Thus, basal levels of autophagy are necessary to elicit beneficial metabolic triggered by fasting. Here we used 8-weeks of IF to trigger the basal level of autophagy and study its impact in metabolic outcomes in mice.

Impact of CR on autophagy

CR upregulated autophagy response in peripheral tissues such as liver (Wohlgemuth et al. 2007), pancreas (Gao et al. 2015), skeletal muscle (Gutierrez-Casado et al. 2019) and heart (Wohlgemuth et al. 2007). Wohlgemuth et al., (Wohlgemuth et al. 2007) reported an increase in autophagy vacuoles in heart of Fisher rats by CR. CR increased the markers of autophagy, *Atg7* and *Atg9* and increased the lipidation of LC3I to LC3II in heart in aged rats. However, no such effect was observed in liver. Similarly, in another study (Gao et al. 2015), CR increased the β-cell autophagy in C57BL/6 mice when switched from HFD to normal chow with 40% of CR. The increased autophagy structures were also observed in muscle of CR mice compared

to control at six months of age (Gutierrez-Casado et al. 2019). Gutierrez-Casado et al., also reported that CR helps in maintaining the optimal level of autophagy during aging. As such, the sustained increased levels of BECLIN-1 and LC3II/I ratio was observed with CR during aging. Moreover, long-term CR increased SIRT1 in aged kidney and attenuated hypoxia-associated mitochondrial and renal damage by enhancing BCL2/adenovirus E1B 19-kDa interacting protein 3-dependent (*Bnip3*-dependent) autophagy (Kume et al. 2010). This was conducted by culturing primary renal proximal tubular cells (PTCs) in serum from CR mice promoted SIRT1-mediated forkhead box O3 (FOXO3) deacetylation – the activity that is essential for expression of *Bnip3* and *p27AKip1* and subsequent autophagy and cell survival during hypoxic conditions. While, Dogan et al., (Dogan et al. 2011) has found that 8-months of CR decreased mTOR and p70S6K while 24-months increased the activity of mTOR and p70S6K in mammary tumour, indicating suppression of autophagy with advancing age (Barbosa, Grosso & Fader 2018).

Autophagy is under circadian regulation: can it be modified by time-restricted feeding/eating (TRF/TRE)

As with most of the physiological process, autophagy is also under circadian regulation in several animal tissues (Ma, Panda & Lin 2011; Pfeifer & Scheller 1975; Pfeifer & Strauss 1981). The lipidation of autophagy marker, microtubule-associated protein 1 light chain 3 (MAP1LC3B or LC3B), from LC3I to LC3II peaks at Zeitgeber time (ZT), ZT6-9, and nadirs during the dark phase (Ma, Panda & Lin 2011). Similarly, temporal rhythmicity of autophagosome abundance also peaks at the end of the light phase (ZT11) before rapidly decreasing during the mid-dark phase (ZT17) (Ma, Panda & Lin 2011). A disruption in this rhythmicity could result in altered metabolic outcomes, such

as hepatic lipid metabolism and hepatic gluconeogenesis, as observed in obesity (Ma, Panda & Lin 2011; Toledo et al. 2018).

A study published in 2017 (Martinez-Lopez et al. 2017) in tissue-specific *Atg7* knockout mice has shown that feeding mice twice a day at ZT1-ZT3 and ZT10-12 for 16 months did not alter body weight, but reduced body fat and increased lean mass as early as 3 months. Autophagy activity analysed via LC3II flux across 24h revealed a gradual increase in LC3II flux from ZT12 and reaching zenith at ZT20 in isocaloric twice a day fed (ITAD) mice, an opposite trend to a decrease in autophagy in fed state. Similar increasing pattern was observed in this group of mice between ZT0-4 and were able to maintain the same level of autophagy flux till ZT7 before gradually declining to a level even lower than ad libitum-fed mice until ZT12 (Martinez-Lopez et al. 2017). The autophagy response varied in tissue-specific manner, such as: ~3-fold increase in LC3II-flux in brown adipose tissue, ~2-fold increase in LC3II in gastrocnemius at ZT4 in mice subjected to ITAD-feeding for 8 months. However, it is not well understood if TRF could mitigate the deleterious effect of high-fat-diet-induced obesity by enhancing autophagy at cellular level.

1.4 Research Questions

This research aims to answer the following questions:

1. Does calorie restriction (CR) and intermittent fasting (IF) alter the anti-inflammatory and cholesterol efflux capacity of HDL in human? And, if so, which one is better?

2. Does IF activate autophagy in mice and humans? And, if so, does the change in autophagy underpin the metabolic health benefit?

3. Does an alternate form of IF, time restricted feeding (TRF) improve metabolic phenotypes in chow or high fat diet fed mice? And if so, are similar benefits observed when TRF is initiated early at ZT12 (TRFe) or delayed by 4-hours at ZT16 (TRFd)?

4. Does TRFd shift the phase of key circadian genes and markers of NAD metabolism in liver? If yes, does this shift impact the improvement in metabolic phenotypes in TRF?

5. Does TRFd increases the amplitude of key autophagy genes in mouse liver, and is there a phase shift in the expression of key autophagy genes?

1.5 Aims and Hypothesis

Nutritional interventions such as CR, IF and TRF are of particular interest as a non-invasive intervention method in a fight against obesity and related pathophysiology. These interventions are effective to lose weight, fat mass and lower the atherogenic lipid levels such as low-density lipoprotein, very-low-density lipoprotein, and triglycerides. Moreover, they help in lowering the fasting blood glucose level and fasting insulin level. However, their impact at cellular level particularly in activating markers of autophagy and increasing their amplitudes remain to be explored. We hypothesised that nutritional intervention imparted as IF would increase the markers of autophagy in both human and mice and will increase the amplitudes of the marker genes in mice liver and help maintain the robustness of circadian rhythmicity in 24h cycle. We also hypothesise that nutritional intervention provided as early TRF (initiated during the early hours of active phase) would have a greater metabolic impact compared to the one initiated 4h later during the active phase (one akin to breakfast skipping). The individual hypothesis for individual chapter is mentioned below as such.

Study 1: Intermittent fasting and caloric restriction did not affect the cholesterol efflux and anti-inflammatory property of HDL in women with obesity.

Aim of the study

The aim of the study was to assess and compare the beneficial effects of IF and CR in modulating the anti-inflammatory and cholesterol efflux capacity of HDL in human.

Hypothesis

We hypothesised that IF would increase the anti-inflammatory and cholesterol efflux capacity of HDL as compared to CR.

Study 2: Intermittent fasting activates markers of autophagy in mouse liver, but not muscle from mouse or humans.

Aim of the study

The aim of this study was to examine the effect of intermittent fasting on markers of autophagy in human muscle and in mouse muscle and liver.

Hypothesis

We hypothesised that IF would increase markers of autophagy in both tissues from mice and in muscle in humans.

Study 3: Early or delayed time-restricted feeding prevents metabolic impact of obesity in mice.

Aim of the study

The aim of the study was to test the effects of 10h TRF initiated at ZT12 or ZT16 on metabolic phenotypes and circadian parameters in chow and HFD fed mice, and compare with *ad libitum* fed mice. We also aimed to test whether delaying the initiation time of TRF mitigated the adverse metabolic consequences of HFD.

Hypothesis

We hypothesised that TRFd would be equally beneficial to TRFe in the prevention of metabolic consequences of HFD, despite inducing a delay in the phase of key hepatic circadian genes and markers of NAD metabolism.

Study 4: Time-restricted feeding increases the amplitude of markers of hepatic autophagy in C57BL/6 mice.

Aim of the study

The aim of this study was to compare the effect of TRF on the mean, amplitude and phase of key autophagy markers at mRNA and protein level.

Hypothesis

We hypothesised that HFD will dampen amplitude vs chow and both forms of TRF (both TRFe and TRFd) would increase amplitude of autophagy at mRNA levels compared to their ad libitum counterpart. Secondly, that early TRF (TRFe) would be better compared to delayed TRFd. Also delaying the TRF by 4h would result in a delay in the phase of these genes.

Chapter 2: Intermittent fasting and caloric restriction did not affect the cholesterol efflux and anti-inflammatory property of HDL in women with obesity.

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Short title: Intermittent fasting and HDL efflux

Keywords: Intermittent fasting, calorie restriction, cholesterol efflux, anti-inflammatory, obesity

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Principal Author

Name of Principal Author (Candidate)	Rajesh Chaudhary		
Contribution to the Paper	Performed the experiments, analysed and interpreted data, wrote the manuscript and approved the final manuscript.		
Overall percentage (%)	60%		
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. I am the primary author of this paper.		
Signature		Date	25 February 2021

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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2.1 Abstract

Background: The anti-inflammatory and cholesterol efflux function of high-density lipoprotein cholesterol (HDL) underlie its cardio-protective effect. This study compared the effects of energy restricted diets delivered as 70% daily caloric restriction (CR) or intermittent fasting (IF) on HDL function.

Methods: Women (N=88; 50±1y, BMI 32.3±0.5kg/m^2) were randomised to one of four groups (IF70, CR70 (provided 70% of calculated baseline energy requirements)) or IF100 and control (provided 100% of baseline energy requirements) for 8-weeks. This secondary analysis includes a subset of participants from CR70 (N=10) and IF70 (N=10) groups, who had lost >5% of body weight and were not taking lipid lowering medications. Cholesterol efflux of HDL was assessed from ^{3H}cholesterol-loaded macrophages and the anti-inflammatory properties of serum and HDL were assessed by exposure to endothelial cells (ECs) and mRNA levels of inflammatory genes, vascular adhesion molecule-1, intracellular adhesion molecule-1, *P65* and C-C Motif Chemokine Ligand 2 were assessed by qPCR.

Results: The IF70 group displayed greater weight loss (*P*=0.030) and greater reductions in total cholesterol (*P*=0.012) and low-density lipoprotein cholesterol (LDL) (*P*=0.021) versus CR70, but the reduction in HDL was not different between groups (*P*=0.253). There were no within or between group effects in the change in mRNA expression of inflammatory markers of ECs that were treated with either serum or isolated HDL. There were also no differences in the cholesterol efflux capacity of HDL within or between groups.

Conclusion: Clinically significant weight loss by CR or IF does not alter the biochemical properties of HDL.

2.2 Introduction

High-density lipoprotein cholesterol (HDL) inversely predicts cardiovascular disease (Castelli 1988; Goff et al. 2014). HDL is known to exert its cardioprotective effect through reverse cholesterol transport, whereby HDL accepts cholesterol from peripheral tissues, including from foam cell macrophages within atherosclerotic plaques (Di Bartolo et al. 2018), and delivers it to the liver for metabolism into bile acid/salts before excretion (Ouimet, Barrett & Fisher 2019). Preclinical studies show serum HDL is antiatherogenic and antithrombotic (Badimon, Badimon & Fuster 1990; Rubin et al. 1991) and possesses antioxidant properties (Kontush & Chapman 2010), but the cardiovascular benefits of high serum HDL remain contentious. In the Copenhagen General Population study (Mortensen et al. 2015), the addition of HDL to cardiovascular risk estimation reduced the sensitivity for detecting a fatal cardiovascular event. In a randomised controlled trial, effectively increasing HDL by administration of nicotinic acid and cholesterol ester transfer protein (CETP) inhibitors along with a statin, failed to alter cardiovascular outcomes (Barter et al. 2007; Investigators et al. 2011; Schwartz et al. 2012). The contention surrounding HDL may be because the cholesterol efflux and anti-inflammatory capacity of HDL, rather than its total concentration, determines its cardioprotective effect (Ebtehaj et al. 2019; Qiu et al. 2017).

The efflux capacity of HDL from macrophages is inversely associated with intima-media thickness and likelihood of angiographic coronary artery disease, independent of HDL concentration (Khera et al. 2011). HDL imparts anti-inflammatory action predominantly via its major protein components, apolipoprotein AI (apoA-I), as well as paraoxonase 1 (PON1) (Barter et al. 2004; Navab et al. 1991). ApoAI prevents the formation of low-density lipoprotein (LDL)-derived oxidised phospholipid by removing

the seeding molecule from LDL and/or from artery wall cells. ApoAI also prevents both LDL oxidation and LDL-induced monocyte chemotactic activity when incubated with LDL (Navab et al. 1997).

Lifestyle interventions, such as calorie restriction (CR), reduces atherosclerosis risk markers in humans (Fontana et al. 2004), and improves vascular function (Lefevre et al. 2009). However, cholesterol efflux capacity of HDL was not altered after 16-weeks of CR in individuals with type 2 diabetes, although plasma cholesterol ester transfer protein levels were decreased and apoAI levels were increased (Wang, Y et al. 2011). Similar results were observed in another diet-induced weight loss study where participants with obesity underwent 6 months of low-fat and reduced energy diet (Aicher et al. 2012).

Intermittent fasting (IF), a novel alternative to CR, is achieved by interspersing *ad libitum* eating with 24h of fasting or severe caloric deprivation for between 1-3 days/week. Studies have shown that IF provides similar metabolic benefits to CR such as reduced weight, fat mass, blood pressure and lipid profile (Gotthardt et al. 2016; Harvie, M & Howell 2017; Harvie, MN et al. 2011; Hutchison, Liu, et al. 2019; Liu, H et al. 2017; Patterson et al. 2015; Patterson & Sears 2017; Tinsley & La Bounty 2015; Varady et al. 2013). We recently showed that IF increased apolipoprotein A4 (APOA4) and decreased apolipoprotein C2 and C3 (Harney et al. 2019). To date, no study has reported the impact of IF on cholesterol efflux and anti-inflammatory capacity of HDL. Given the role of APOA4 in promotion of reverse cholesterol transport and APOC3 in suppressing the triglycerides lipolysis, we examined the effects of eight weeks of CR vs IF on the cholesterol efflux and anti-inflammatory properties of HDL and hypothesised that IF would be more effective.

2.3 Research Design and Methods

Study participants

Details of the study have been previously described (Harney et al. 2019; Hutchison, Liu, et al. 2019). Briefly, a total of 88 women, aged 35-70 years, with BMI of 25-42 kg/m^2 were enrolled in the study. Out of 79 completers (CR70: continuous restriction at 70% of baseline energy requirement, N=24; IF70: an IF diet at 70% of baseline energy requirement, N=22; IF100: an IF diet at 100% of baseline energy requirements, N=22; control, N=11), a subset of participants who had lost more than 5% of body weight was included in this secondary analysis study (CR70, N=10 and IF70, N=10). CR70 participants consumed only ~70% of their baseline energy requirement every day throughout the intervention period (8-weeks), while IF70 participants were provided with ~100% of baseline energy requirement on fed days, and consumed breakfast (~32% of baseline energy requirement) before 8 AM on fasting days. The estimated daily energy requirement for each participant was calculated by averaging predicted daily energy expenditure using a published equation, which use age, gender, height, and weight variables (Redman et al. 2009). Participants followed food dietary pattern regime throughout the study as previously described (Hutchison, Liu, et al. 2019). The inclusion and exclusion criteria have been previously described (Hutchison, Liu, et al. 2019). A CONSORT flowchart indicating the total number of participants enrolled, screened, completed intervention and total number of final serum samples processed is indicated in (**Figure 2. 1)**. This study was approved by the Royal Adelaide Hospital Research Ethics committee and registered as a clinical trial with Clinicaltrials.gov (NCT01769976).

Blood sampling, serum HDL, and reconstituted HDL (rHDL) preparation

Blood samples were collected at baseline (12h of overnight fast), on week 8 of IF intervention (12h overnight fast), and week 8 (24h of fast). Blood samples were centrifuged to prepare serum and quickly frozen at -80^{o}C until further processing. For HDL extraction, 1 ml of each serum sample was thawed on ice and centrifuged at 2500 rpm for 5 min at 4^{o}C to pellet any cell debris. Serum samples were separated for cell treatment and for HDL extraction. Equal amount of serum and polyethylene glycol (200 mg/ml, PEG-6000, P3015, Sigma-Aldrich, MO, USA) prepared in MiliQ water was mixed to precipitate apoB-containing lipoproteins. The samples were mixed by pipetting and were left for 5 min at room temperature to precipitate apoB-containing lipoproteins before centrifuging at 13000 rpm for 5 min at 4^{o}C. Supernatant containing HDL fraction was pipetted in a separate tube and filtered using 0.22 µm low protein binding durapore-PVDF membrane filter (SLGV033RC, Merck Milipore, Ireland). Purified APOA1 was complexed with 1-palmitoyl-2-linoleoyl-phosphatidycholine to form discoidal reconstituted HDL (rHDL) as previously described (Weisweiler 1987). Serum and reconstituted HDLs were equalised to 0.6 mg/ml and 0.025 mg/ml concentrations to treat ECs and immortalised bone marrow-derived macrophage (iBMDM) cells, respectively.

Epithelial cells culture and treatment

Human Coronary Artery Epithelial cell at passage 4 (HCAECs or ECs, p4) were grown in MesoEndo media (#212, Cell Applications Inc.) at 37^{o}C in a humidified environment at 5% CO_2. After 90 to 95% of confluency, cells were plated in 6-well flat bottom plates with lids (#3516, Corstar, China) at a cell density of 1.5×10^5 cells/well and returned to incubator at 37^{o}C with 5% CO_2 for 24 hours. The cells were plated in duplicate for each individual treatment group. After 24h cells were treated with 650 µl of individual

treatment samples: Phosphate-buffered saline (PBS, volume equivalent to largest corresponding volume), 15% serum, 15% fetal bovine serum (FBS, A1933, Sigma-Aldrich, USA), and 0.6 mg/ml of both reconstituted HDL (rHDL) and patients' HDL. After an overnight incubation, cells in all treatment groups were activated with TNFα (T0157, Sigma-Aldrich, Germany) for 4.5 hours.

Cell harvesting and gene expression analysis

Cells were washed once with 1X cold PBS, and were harvested using 500 µl of Tri Reagent (T9424, Sigma-Aldrich, USA). Total mRNA was extracted following manufacturer's protocol, and concentration and purity were assessed using NanoDrop Lite Spectrophotometer (Thermofisher Scientific, CA, USA). cDNA was synthesised from 800 ng of total mRNA using iScript Reverse Transcription Supermix (#1708841, Bio-Rad, USA) and qPCR was performed for inflammatory markers, namely vascular cell adhesion molecule (*VCAM-1)*, intracellular adhesion molecule 1 (*ICAM-1)*, rel-like domain-containing proteins (*RELA*/*P65)*, and c-c motif chemokine ligand 2 (*CCL2)*. These genes are predominantly expressed in the endothelial cells and have direct role in the formation of atherosclerotic lesion when triggered by tumour necrosis factor α. Beta-2-microglobuin was used as a housekeeper. Reactions were performed in 10 ul of final reaction volume using SsoAdvance Universal SYBR Green Supermix (#1725271, BioRad, USA) with internal negative control on QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, USA). Relative gene expression analysis was performed using $2^{-\Delta\Delta Ct}$ method, where ΔCt is ($Ct_{target} - Ct_{housekeeper}$) and $\Delta\Delta Ct$ is ($\Delta Ct_{target\ of\ unknown} - \Delta Ct_{target\ of\ internal\ control}$).

Cholesterol efflux assay

HDL-mediated cholesterol efflux was measured using immortalised bone marrow derived macrophages (iBMDM, J771). For the assay, cells were plated at a density of 0.8×10^5 cells/well in DMEM supplemented with 10% FBS (v/v), in 24-well plate (Corning Cell bind surface, 3337, Corstar, USA). The cells were labelled with 2 uCi/ml of ^{3}H-Cholesterol in DMEM with 1% FBS for 24 hours in 37^{o}C with 5% of CO_2. Cells were then equilibrated in serum free media (0.2% BSA in DMEM) for 18 hours after washing the cells twice with 1% BSA prepared in 1X PBS. Following equilibrium, the cells were washed with cold 1% BSA prepared in 1X PBS and treated with 25 ug/ml of apoB-depleted HDL from serum and/or reconstituted rHDL prepared in 0.2% BSA in DMEM media. The cells were incubated for 4 hours at 37^{o}C to induce cholesterol efflux. Following 4h of incubation, the media and the cell lysate was transferred in the scintillation vials to quantify 3H-cholesterol present therein by scintillation counting. The media was transferred in 1.5 ml Eppendorf tubes and centrifuged for 5 min at 2500 rpm to pellet cell debris and were transferred in the scintillation vials containing 4 ml of scintillation liquid. The cells were washed twice with 1X PBS before shaking on a table shaker for 5 min with 500 ul of 0.2M NaOH+0.15m NaCl in each well. The cell lysates were transferred to the separate scintillation vials. Both the supernatant and cell lysate containing scintillation vials were then transferred to the scintillation machine and the CPM were counted on liquid scintillation analyser (TriCarb 2810, Perkin Elmer). Samples were run in triplicate with serum free media (0.2% BSA in DMEM) as control. The inter assay variability was controlled by using inter assay control.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 25.0 (Armonk, NY: IBM Corp.) and data are presented as mean ± SEM. Analysis was

performed on log-transformed data if the skewness were observed in the residuals. For clinical data, a restricted maximum likelihood mixed-effect model was used to examine the group effect of 8-weeks of following intervention in 12h fast and 24h fast as well as time effect within each group (CR70 and IF70). The model included fixed factors for groups (CR70 and IF70), time (V0: baseline, V8A: 8-weeks of CR70 or IF70 under 12h fast, V8B: 8-weeks of IF70 (24h fast)), and group-by-time interaction and a random factor for participants with an unstructured covariance matrix to account for the repeated visits. For anti-inflammatory maker assessment, paired t-test was used for the within group comparison of visits in CR70 group to assess the impact of 8 weeks of CR70 intervention on anti-inflammatory capacity of HDL. ANCOVA was used for between CR70 and IF70 group comparison with baseline visit as a cofactor to assess the impact of baseline expression on the expression profile at week 8, and one-way repeated measure ANOVA was used to examine visits within IF70 group. The differences in the statistical tests performed was because of unequal subgroups between the CR70 and IF70 group, where CR70 had just two visits while IF70 group had three visits including 24h fast state on week 8 of intervention to assess the impact of fasting. For the effect of time within-group analysis was performed using Bonferroni-adjusted pairwise comparison of estimated marginal means. Percentage of cholesterol efflux capacity was assessed by paired t-test. The analysis was repeated after removal of an outlier that were >2SD (standard deviation) from the mean (denoted as black dots in **Figure 2.2**). *P*-value <0.05 was considered statistically significant.

2.4 Results

As previously reported, greater weight loss was observed in IF70 vs CR70 (*P*=0.030, **Table 2.1**), including greater reductions in total cholesterol (*P*=0.012) and LDL (*P*=0.021) levels (**Table 2.1**) (Hutchison, Liu, et al. 2019). There was no difference in

the reduction in HDL cholesterol between groups (*P*=0.253). In a subset of participants (N=10/group), there was no within or between group effects in the change in mRNA expression of inflammatory markers, namely *VCAM-1*, *ICAM-1*, *P65*, and *CCL2* in ECs that were treated with either serum or isolated HDL (**Figure 2.2A–H**). The analysis was performed after removal of outliers, but this did not alter the results. There were also no differences in the cholesterol efflux capacity of HDL within or between CR70 and IF70 groups (**Figure 2.3).**

2.5 Discussion

Nutritional interventions such as CR and IF have profound metabolic benefits and reduce cardiovascular risk markers (Bhutani et al. 2013; Hutchison, Liu, et al. 2019; Nematy et al. 2012; Varady et al. 2011). In this study, we compared the cholesterol efflux and anti-inflammatory properties of HDL utilising a novel *in vitro* system following eight weeks of CR or IF in women with overweight or obesity. Our data suggest that neither of the interventions altered the anti-inflammatory or cholesterol efflux capacity of HDL. To our knowledge, this is the first study to compare CR vs IF on the key functional properties of HDL, namely cholesterol efflux capacity and its anti-inflammatory properties.

In the present study we observed a greater reduction in body weight, total cholesterol and LDL in participants who were randomised to IF as compared to CR. As is often reported in response to active weight loss (Ditschuneit, Frier & Flechtner-Mors 2002; Eckel 1999), HDL concentrations were modestly but significantly reduced in both groups, with no between group effect detected. However, neither the cholesterol efflux nor the anti-inflammatory capacity of HDL was affected by the interventions. This finding is similar to a randomised controlled trial where 6 weeks of low calorie (500 kcal) diet induced weight loss in 52 participants with abdominal obesity, but did not

improve ABCA1-mediated cholesterol efflux (Talbot et al. 2018). Moreover, these findings were consistent in insulin-dependent individuals with type 2 diabetes (N=27; men=14 and women=13, age 55y) with obesity who underwent 16 weeks of a very low-calorie diet (VLCD) (~450 kCal/day) (Wang, Y et al. 2011). Interestingly, cholesterol efflux capacity of HDL was improved in 113 middle-age men with abdominal obesity and dyslipidaemia after 48-weeks of moderate calorie restriction plus exercise (Boyer et al. 2018). Exercise also increased the cholesterol efflux capacity in mice compared to their sedentary counterparts, even though phospholipids, total, non-HDL and HDL cholesterol were unaffected (Meissner et al. 2010). Thus, the differences observed could be attributed to the factors such as, longer duration of CR, larger numbers of participants or that exercise is required to alter HDL function (Boyer et al. 2018).

Long-term CR was also associated with lower inflammatory markers, C-reactive protein, interleukin 6 and TNF-α level (Fontana et al. 2004). Moreover, lifestyle intervention studies that have implemented a calorie-restricted Mediterranean diet and exercise also inhibited myeloperoxidase-mediated HDL oxidation in patients with metabolic syndrome (Mathew et al. 2018). In the present study, daily CR or IF, both at 70% of baseline energy requirements, did not significantly alter the mRNA levels of inflammatory markers in ECs that were treated with either serum or HDL, isolated through apoB-depletion. We have previously reported there were no between group differences in serum *MCP1*, *TNF-*α or *IL-6*, but we observed a modest but significant increase in *MCP-1* following 24h fast in IF70 group (Liu, B, Hutchison, et al. 2019). Weight loss of less than 10% also did not alter serum inflammatory biomarkers (Esposito et al. 2003; Tam et al. 2012), but the genes involved in oxidative stress, namely NAD(P)H dehydrogenase, 24-dehydrocholesterol reductase, and ubiquitin carboxyl-terminal esterase L1 were decreased in subcutaneous adipose tissue when

weight loss exceeded 10% (Magkos et al. 2016). These differences could be attributed to number of factors, ranging from the amount of weight loss, the length of intervention, age and the sex of participants and sample size.

Of note, HDL is found to harbour both anti as well as pro-inflammatory properties in an *in vitro* system (Fotakis et al. 2019) and thus its anti-inflammatory property may change to pro-inflammatory depending on the context, such as during acute phase response (Van Lenten et al. 1995) or under extreme cholesterol depletion (Fotakis et al. 2019). Moreover, the anti-inflammatory properties of HDL are influenced by its structural components such as apoAI, apoAII and including the paraoxonase 1 protein that is co-transported with it (Barter et al. 2004). However, we did not observe any evidence of change in pro-inflammatory or anti-inflammatory properties in response to either of the interventions. HDL function is also modulated by number of factors that includes HDL particle size, number and density, phospholipid and apoAI content, including cholesterol transporters such as ABCA1, ABCG1 and SR-B1 (Anastasius et al. 2016; Fournier et al. 1997; Kontush & Chapman 2006; Nissen et al. 2003), which were not measured in this study. Other limitations of this study include a small sample size. Hence, future studies should consider an extensive study comprising of a larger sample size with various levels of assessment from structural to biochemical. The findings of this study suggest that short term CR70 and IF70, with clinically significant weight losses do not alter the biochemical properties of HDL.

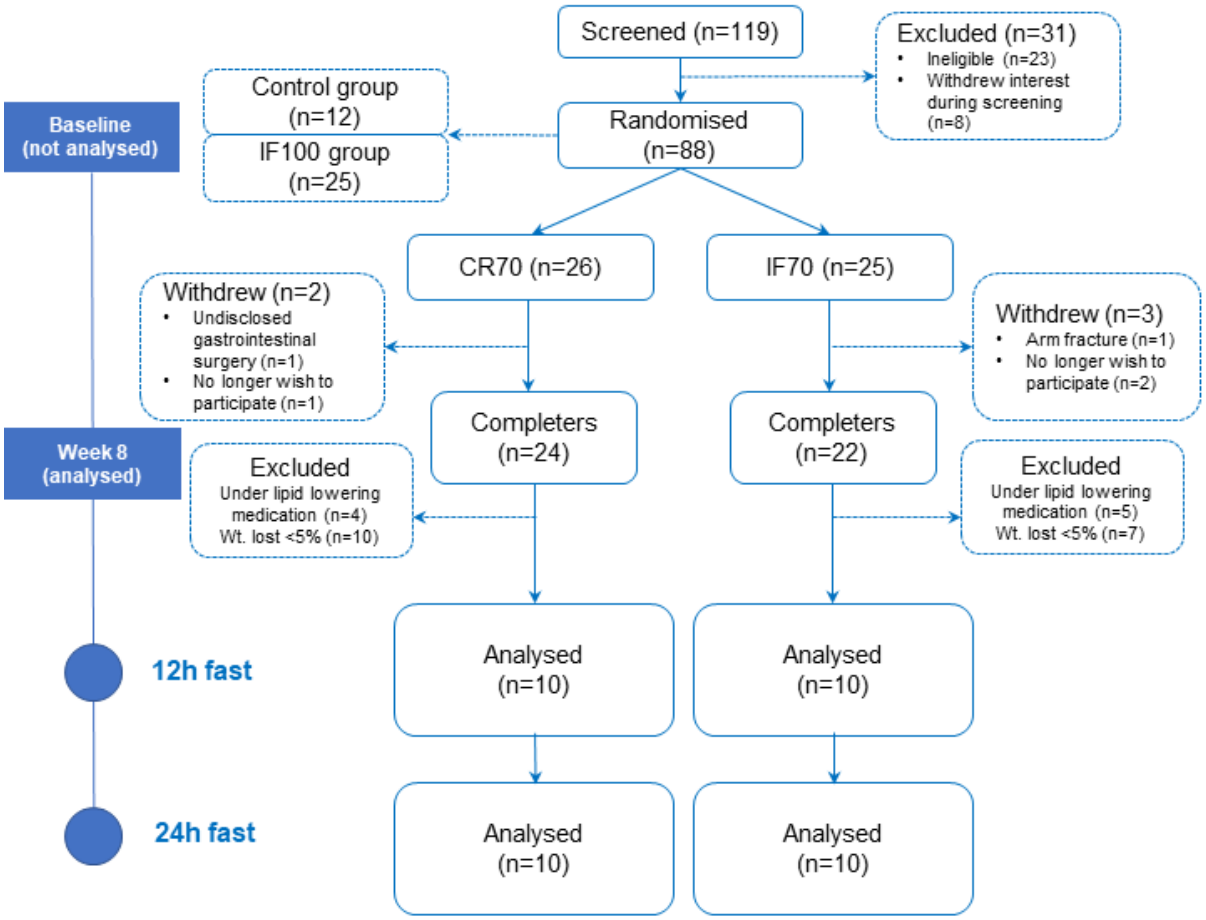

Figure 2. 1 CONSORT flowchart of the study. CR: Calorie restriction, IF: Intermittent fasting

Table 2.1 Clinical characteristics of the participants (Hutchison, Liu, et al. 2019)

	CR70 (N=25)		IF70 (N=25)		Between group comparison (P-value)
	V0 (baseline, 12h fast)	V8A (12h fast)	V0 (12h fast)	V8A (12h fast)	∆(V8A-V0)
Weight (kg)	89.14 ± 2.69	85.20 ± 2.73*	89.40 ± 2.69	84.06 ± 2.73*	0.030
Fat mass (kg)	44.33 ± 2.27	40.73± 2.30*	43.52± 2.27	39.23 ± 2.30*	0.224
Total cholesterol (mmol/L)	4.95 ± 0.14	4.70 ± 0.14*	4.81 ± 0.14	4.21 ± 0.14*	0.012
HDL (mmol/L)	1.37 ± 0.05	1.31 ± 0.05*	1.34 ± 0.05	1.24 ± 0.05*	0.253
LDL (mmol/L)	2.98 ± 0.12	2.86 ± 0.12	2.92 ± 0.12	2.54 ± 0.12*	0.021
Triglycerides (mmol/L)	1.28 ± 0.08	1.13 ± 0.07*	1.17 ± 0.08	0.93 ± 0.07*	0.299

Data are presented as mean ± SEM. Mixed-effects model performed to test the effects of time and group within and between the group following intervention in 12h fast. **CR70:** Calorie restriction at 70% of baseline energy requirements, **IF70:** Intermittent fasting at 70% of baseline energy requirements. **V0:** baseline visit in 12h of fast, **V8A:** visit at week 8 (12h fast state). **HDL:** High-density lipoprotein cholesterol, **LDL:** Low-density lipoprotein cholesterol. *P<0.05 vs baseline.

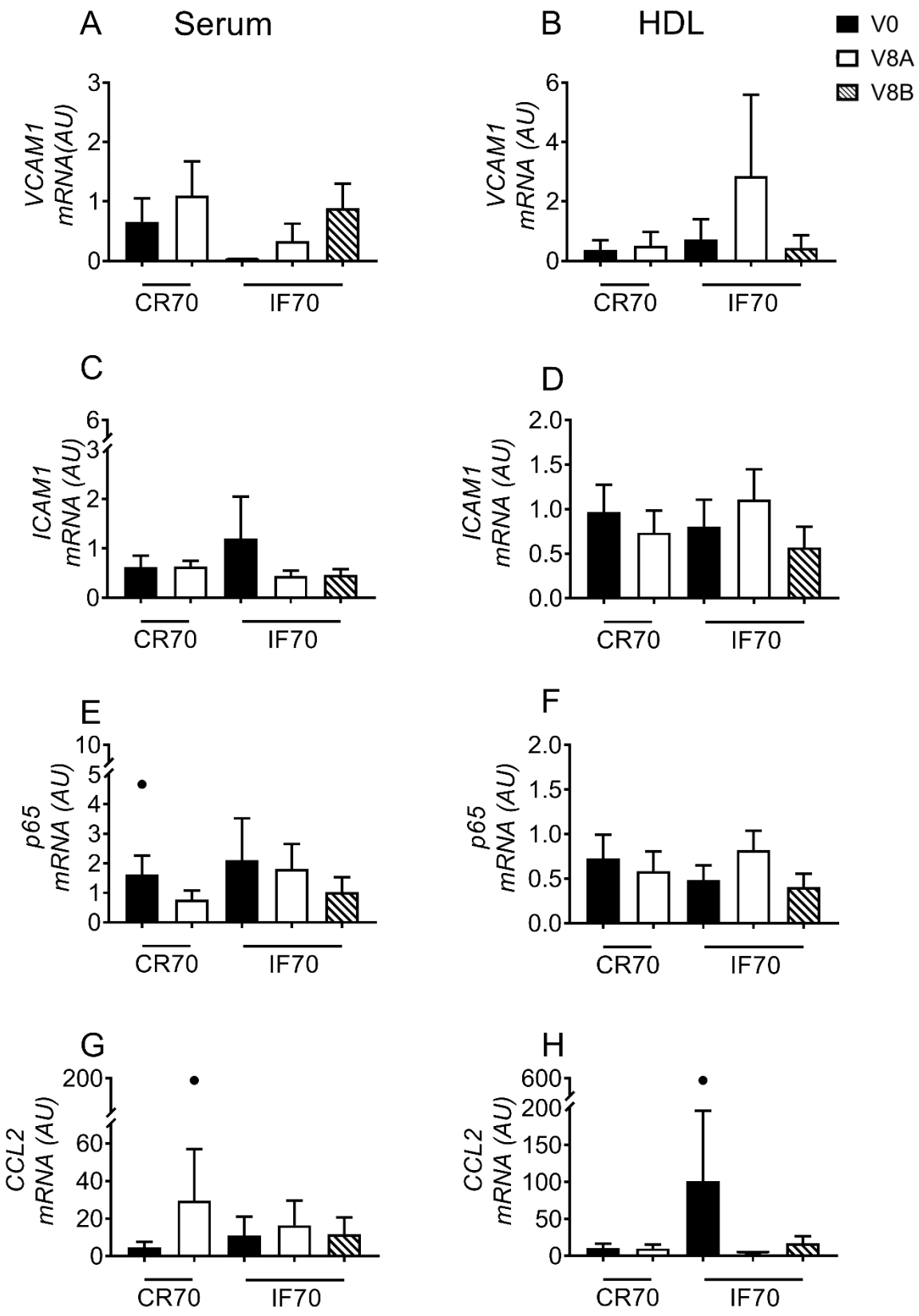

Figure 2.2 IF70 and CR70 did not significantly alter the anti-inflammatory property of HDL.

(A-H) Changes in the mRNA markers of inflammation relative to beta-2-microglobulin as housekeeper in ECs cells treated with HDL from participants in calorie restriction at 70% of the baseline energy requirements (CR70, N=6-8/treatment group) and intermittent fasting at 70% of the baseline energy requirements groups (IF70, N=6-8/treatment group) at baseline (V0, 12h fast) and following 8 weeks of intervention in fed state (V8A, 12h fast) and fast state (V8B, 24h fast). Values are presented as mean±SEM. Paired t-test used for between visits comparison in CR70 group, ANCOVA between CR70 and IF70 group, and repeated-measure ANOVA with Bonferroni post-hoc correction between visits in IF70 group. AU: Arbitrary unit. Black dots in the graphs are the outliers.

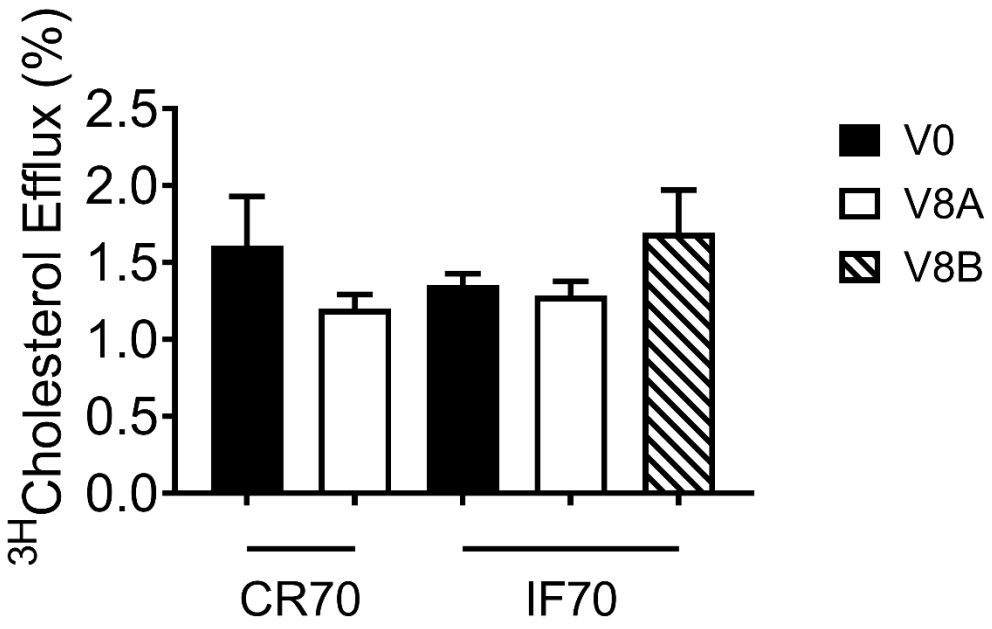

Figure 2.3 IF70 and CR70 did not significantly alter the cholesterol efflux capacity of HDL.

Changes in ^{3H}Cholesterol efflux capacity of participants HDL from calorie restriction at 70% of the baseline energy requirements (CR70, N=10) and intermittent fasting at 70% of the baseline energy requirements group (IF70, N=10) at baseline (V0, 12h fast) and following 8 weeks of intervention in fed state (V8A, 12h fast) and fast state (V8B, 24h

fast) in iBMDM cells loaded with [3H]-Cholesterol (2μCi/mL). Samples were run in triplicate. Paired t-test was used between visits within both CR and IF groups. Values are presented as mean±SEM.

Research Question and Future direction

Our current findings indicate that IF is an effective nutritional intervention to lose weight and lowering of atherogenic lipid LDL compared to CR at physiological level. However, these interventions do not differ from each other in terms of either enhancing cholesterol efflux capacity or imparting anti-inflammatory response via HDL at cellular level. However, considering a greater effect of IF on metabolic outcome and a potential to activate autophagy, we hypothesised that IF may impart metabolic outcome by activating autophagy at cellular level at least in metabolically active tissues such as liver and muscle. Hence, we move on to examining the effect of IF at cellular level in liver from mice and muscle from both mice and human.

Chapter 3: Intermittent fasting activates markers of autophagy in mouse liver, but not muscle from mouse or humans.

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Principal Author

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Contribution to the Paper	Performed the experiments, analysed and interpreted data, wrote the manuscript and approved the final 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. I am the primary author of this paper.		
Signature		Date	25 February 2021

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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3.1 Abstract

Background: Intermittent fasting (IF) activates autophagy in mouse cardiac muscle and pancreatic islets, which may underpin its beneficial health effects. However, the autophagy response to IF in fed and fasted state is less understood. Here, we examined the effect of 8-weeks of IF on markers of autophagy in liver from mice and skeletal muscle from both mice and human with obesity or overweight.

Methods: Ten-week-old C57BL/6J male mice were fed a high-fat diet (HFD) (43% energy from fat) or chow diet ad libitum (AL) for 8 weeks, before randomisation to continue AL or to IF (24h fast initiated at Zeitgeber time 11 (ZT11) on 3 non-consecutive days/week) for a further 8 weeks (8-16/group). Tissue was collected in the fed-state or after a 22h fast. Fifty women (age 51±2y, BMI 31.8±4.3 kg/m^2) were randomised to one of two IF protocols and provided with all foods either at 70% (IF70) or 100% (IF100) of baseline energy requirements for 8 weeks. Subjects underwent a 24h fast from 0800h on 3 non-consecutive days/week. *Vastus lateralis* muscle was collected following a 12h and 24h fast. Microtubule associated protein light chain 1 *(Map1lc3b)*, Beclin1 (*Becn1),* Sequestosome 1 *(Sqstm1/p62),* Transcription factor EB *(Tfeb), and* Lysosomal associated membrane protein 2 *(Lamp2)* were assessed by qPCR, and LC3 (LC3I and LC3II), BECLIN1 and LAMP1 by immunoblotting.

Results: Fasting for 22h increased hepatic LC3I protein and *Map1lc3b* mRNA levels, but did not alter LC3II in IF mice that were fed chow or HFD. LAMP1 protein and *Beclin1* mRNA levels were also increased by fasting but only in IF mice that were fed a chow diet. IF did not activate markers of autophagy in mouse muscle. In humans, a 24h fast only increased *SQSTM1*. However, *BECLIN1*, *SQSTM1* and *LAMP2* mRNA

levels were decreased after 8 weeks of intervention following a 12h overnight fast, in the IF70 group only, who also lost significantly more weight than the IF100 group.

Conclusion: Markers of autophagy in liver, but not in skeletal muscle, were elevated in response to intermittent fasting in mice. In humans, autophagy markers in muscle were reduced from baseline following IF, likely in response to weight loss.

3.2 Introduction

Autophagy is a cellular catabolic housekeeping process that recycles unwanted or damaged material to maintain cellular health and energy homeostasis during times of stress or nutritional deficiency (e.g. fasting or amino acid deficiency) (Jackson & Hewitt 2016; Kanki & Klionsky 2010; McWilliams & Muqit 2017; Pickles, Vigie & Youle 2018; Singh & Cuervo 2011; Webster et al. 2014). Autophagosomes engulf cellular organelles in a double-membraned vesicle and digest them with the help of a lysosome upon forming an autolysosome.

Impairments in autophagy are linked to diseases including diabetes (Marasco & Linnemann 2018), neurodegenerative diseases (Menzies et al. 2017) and Crohn's disease (Lassen & Xavier 2018). Reduced levels of Microtubule associated protein 1 light chain 3 Beta (*Map1lc3b*), mammalian orthologue for yeast autophagy related gene 6 (*Atg6*), *Atg5, Atg7, Beclin1*, and Sequestosome 1 (*Sqstm1/p62*) have also been observed in the liver in genetic and diet-induced models of obesity, suggesting that obesity impairs hepatic autophagy (Yang, L et al. 2010). However, this is not consistently observed and elevated markers of hepatic autophagy have also been reported in diet-induced obese rodents (Aijala et al. 2013; Hsu et al. 2016).

Intermittent fasting (IF) involves fasting for one to three days per week, interspersed with *ad libitum* (AL) food intake on non-fasting days (Barnosky et al. 2014). IF has physiological and metabolic health benefits in animals and humans alike (Fontana & Partridge 2015), including reduced body weight (Harvie, MN et al. 2011; Klempel et al. 2012; Klempel, Kroeger & Varady 2013), reduced fasting insulin and improved glucose tolerance (Liu, B, Page, Hatzinikolas, et al. 2019). One possible mechanism that underpins the metabolic benefit of fasting is via the activation of autophagy (Liu, H et

al. 2017). To date, one study has shown that six weeks of IF restored autophagic flux in pancreatic islets, increased beta-cell mass, and enhanced glucose-stimulated insulin secretion in mice that were fed a HFD (Liu, H et al. 2017). Six weeks of IF also protected chow-fed mice from myocardial ischemia-reperfusion injury with marked reductions in infarct size in both sexes compared to control via repetitive stimulation of the autophagy-lysosomal machinery (Godar et al. 2015)

The purpose of this study was to investigate the effects of an eight week IF regime on markers of autophagy in mouse liver and skeletal muscle. Additionally, we examined the effect of IF for 8 weeks on markers of skeletal muscle autophagy in women with overweight or obesity. We hypothesised that IF would increase markers of autophagy in both tissues from mice and in muscle in humans.

3.3 Research Design and Methods

Ethical approval

All experimental protocols involving animals were approved by the South Australian Health and Medical Research Institute (SAHMRI) and The University of Adelaide Animal Ethics Committee (SAM157). Animal experiments were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. For the human study, participants provided their written informed consent prior to the commencement of the study (Hutchison, Liu, et al. 2019). The human study was approved by the Royal Adelaide Hospital Research Ethics Committee (RAH121013) and registered as a clinical trial with Clinicaltrials.gov (NCT01769976).

Animal and study design

Forty-eight C57BL/6J male mice, aged 10 weeks, were housed four per cage under a 12:12 hour of light/dark cycle, with lights on at 0700h (Zeitgeber time 0, ZT0) and were fed either a lard based high-fat diet (HFD, SF04-001, Speciality Feeds, Glen Forrest, Australia) comprising 43%, 21%, and 36% of energy from fat, protein, and carbohydrate respectively or standard chow diet (Chow, 2018SX, Envigo, Madison Wisconsin, USA) with 18%, 24%, and 58% of energy from fats, protein, and carbohydrate respectively for eight weeks. Mice on each diet were then randomised to either ad libitum (AL, N = 8) or intermittent fasting (IF, N = 16) for another eight weeks. IF was initiated at ZT11 (one hour prior to lights off) for 24h for 3 non-consecutive days per week, as previously described (Liu, B, Page, Hatzinikolas, et al. 2019). Daily food access was controlled by transferring the mice between cages with or without food. AL-fed mice were also transferred at the same time to standardize the handling. Weekly and daily body weight and food intake data were collected before and after introducing IF, respectively. An oral glucose tolerance test (OGTT) was performed (with 2 grams glucose/kg body weight) at ZT7 after 6 weeks of IF intervention with mice fasted for 6 hours. Glucose was assessed at 0, 15, 30, 60, 90, and 120 minutes via the tail-prick method using a Glucometer (AccuChek Performa Monitor, Roche, Basel , Switzerland) as previously described (Liu, B, Page, Hatzinikolas, et al. 2019). Mice were euthanised at ZT7-9 in fed (IF-FED) or after a 22h fast (IF-FAST). Liver, quadriceps muscle, inguinal and gonadal adipose tissues were collected, weighed and snap frozen in liquid nitrogen before storing them at -80^{O}C until further processing. Adipose tissue mass (inguinal and gonadal) was adjusted to terminal body weight.

Human study

Details of this study are reported elsewhere (Hutchison, Liu, et al. 2019). Briefly, women aged 35-70 years, with BMI of 25-42 kg/m^2 were randomised to one of four groups for eight weeks: 1) IF70 (IF diet at 70% of baseline energy requirement per week), 2) IF100 (IF diet at 100% of baseline energy requirement per week), 3) DR70 (continuous dietary restriction; continuous restriction at 70% of baseline energy requirement daily), and 4) control (100% of calculated baseline energy requirement daily). However, in this study only the IF70 and IF100 groups were used, as we were interested in the across-species effect of similar IF diets on autophagy. Both groups were instructed to consume breakfast (30-35% of baseline energy requirements) before 0800h and then fast for ~24h on 3 non-consecutive days per week for 8 weeks. On feeding days, IF70 group consumed ~100% of their baseline energy requirements per day, while IF100 group consumed ~145% of their baseline energy requirements per day to meet the prescribed overall intake of 70% and 100% energy requirements per week. The inclusion and exclusion criteria have been previously defined and described, and the assessment of insulin sensitivity, body weight and body composition have been previously described (Hutchison, Liu, et al. 2019). Percutaneous muscle (*vastus lateralis*) biopsies for autophagy markers were obtained from a subset of participants: 1) IF70 (N = 17) and 2) IF100 (N = 14), at baseline (V0:12h fast), and after 8 weeks of IF (V8A: 12h fast or V8B: 24h fast), and then snap-frozen in liquid nitrogen before storing at -80^{O}C until further processing.

Gene Expression Analysis

Tissues were lysed and total RNA was extracted using 20-30 mg of *vastus lateralis* muscle tissue from humans and quadriceps muscle and liver from mice in TRI Reagent (T9424, Sigma-Aldrich, Missouri, USA) using TissueLyser LT (Qiagen, Hilden, Germany) at 50 Hz. The concentration and purity of RNA was assessed using a

NanoDrop Lite Spectrophotometer (ThermoFisher Scientific, Massachusetts, USA). cDNA was synthesised from 1000 ng of total RNA from each sample using the QuantiTect reverse transcription kit (Catalogue no. 205313; Qiagen, Venlo, Netherlands) on a T100 Thermal Cycler (Bio-Rad, California, USA) as per kit instruction. Quantitative real-time PCR (qPCR) was performed for mouse autophagy markers, namely: *Beclin-1*, a mammalian orthologue for yeast autophagy related gene 6 (*Atg6*) [(*Becn1*], Microtubule associated protein 1 light chain 3 beta [*Map1lc3b*], Sequestosome-1, a selective autophagy receptor [*Sqstm1/p62*], Transcription factor EB [*Tfeb*]. Similarly for human, [*BECN1*], [*MAP1LC3B*], [*SQSTM1*], and Lysosome-associated membrane glycoprotein 2, a lysosomal membrane protein receptor required for fusion of autophagosome to lysosome [*LAMP2*] genes were amplified. All genes were amplified using TaqMan primers (Error! Reference source not found.) and Fast Universal PCR Master mix (Applied Biosystems, USA) on 7500 Fast Real-time PCR system (Applied Biosystems).. Internal negative controls were used in all the reactions performed. Relative gene expression analysis was performed using $2^{-\Delta Ct}$, where $\Delta Ct = CT_{(target\ gene)} - CT_{(reference\ gene)}$ as previously described (Liu, B, Page, Hatzinikolas, et al. 2019). For the mouse study, four housekeepers including beta-2 microglobulin [*B2m*], peptidylprolyl isomerase A [*Ppia*], 18S ribosomal RNA [*Rn18s*], Beta-actin [*Actb*] were examined (Error! Reference source not found.). For the human study, three housekeepers including beta-actin [*ACTB*], LDL receptor related protein 10 [*LPR10*], and hypoxanthin phosphoribosyltransferase [*HPRT1*] were analysed (Error! Reference source not found.). A combination of *B2m* and *Ppia* for mouse liver (stability value: 0.002), *Actb* and *B2m* for mouse muscle (stability value: 0.118), and *ACTB* and *HPRT1* (stability value: 0.052) for human muscle were determined as the best pair of genes for normalisation by NormFinder (Aarhus N, Denmark) as previously reported (Hildyard & Wells 2014).

Immunoblotting

Frozen quadriceps muscle and liver (~20-30 mg) from mice were lysed in Cell Lytic MT Cell lysis Reagent (C3228, Sigma, Rehovot, Israel) with Halt Protease and Phosphatase Inhibitor cocktail (#1861281, ThermoFisher Scientific) in Tissue Lyser LT (Qiagen). Protein concentrations were determined using Pierce BCA Protein Assay Kit as per manufacturer protocol (#23225, Pierce Biotechnology, ThermoFisher Scientific). Ten micrograms of proteins were separated in either Criterion TM Precast Gel (4-15% Tris-HCl) (#3450029, Bio-Rad) or Bolt 4-12% Bis-Tris Plus gel (#NW04122BOX, ThermoFisher Scientific) and transferred to PVDF membrane (#162-0177 Bio-Rad). The membranes were blocked with 2% BSA prepared in TBS tween 0.1% or 1% blocking solution (#11921673001, Roche, Germany). The membranes were then probed with anti-MAP1LC3B (1:250 dilution in 0.5% blocking solution from Roche, Rabbit polyclonal, NB100-2220, Novus Biologicals, Colorado, USA), anti-BECLIN1, (1:1000 dilution in 2% BSA, Rabbit polyclonal, NB110-87318, Novus Biologicals), anti-LAMP1 (1:1000 dilution in 2% BSA, Rabbit polyclonal, ab24170, Abcam, Cambridge, USA), and anti-Beta Tubulin (1:500 dilution in 0.5% blocking solution from Roche, Rabbit polyclonal, ab6046, Abcam) by incubating overnight at 4^0C. Secondary antibody, Goat anti-rabbit IgG (H+L) HRP conjugate (1:10,000 dilution in 1X TBS, #AP307P, Millipore, Danvers, USA), was used against the primaries. Membranes were developed using SuperSignalTM West Femto Maximum Sensitivity Substrate (#34095, ThermoFisher Scientific), scanned on ImageQuant LAS-4000 Chemiluminescence and Fluorescence Imaging System (GE Healthcare, Uppsala, Sweden), and bands were quantified using ImageJ software (National Institute of Health, Maryland, USA).

Statistical analysis

Data are presented as mean ± SEM. The normality of the data was checked using Shapiro-Wilk test and all statistical analysis was performed using IBM SPSS Statistics for Windows, Version 25.0 (Armonk, NY: IBM Corp.) on log-transformed data if not normally distributed. For mouse data, single comparison analysis was performed using a two-way ANOVA with diets (chow and HFD) and interventions (AL and IF) as between-group factors. For human data, a linear mixed-effects model was used with visits (baseline, 12h and 24h fast at week 8) and groups (IF70 and IF100) as fixed factors. Bonferroni *post hoc* test was applied when a diet by intervention effects was observed and Bonferroni *post hoc* test with pair-wise comparison when visit effect was observed. *P*-value <0.05 was considered statistically significant.

3.4 Results

IF decreased fat mass, improved glucose tolerance and increased markers of autophagy in mouse liver but not in muscle

As previously reported (Liu, B, Page, Hatzinikolas, et al. 2019), a diet by intervention effect was observed in food intake (**Figure 3.1A**). Intermittent fasting did not alter food intake in chow-fed mice. However, intermittent fasting reduced cumulative food intake by 28% in mice that were fed HFD ($P = 0.006$). As previously reported, there was no difference in body weight between chow-IF and chow-AL. However, HFD-IF mice had significant weight loss vs HFD-AL ($P < 0.001$). A diet by intervention effect was also observed in the fat mass: body weight ratio (**Figure 3.1B**), which was increased by HFD-AL feeding and decreased by IF in both diet groups (all $P < 0.001$). Diet and intervention effects were also observed in oral glucose tolerance (**Figure 3.1C** (**i and ii**)), which was impaired by HFD feeding and improved by IF in both diet groups (both $P < 0.001$).

In liver, a diet by intervention effect was observed in LC3I protein levels (**Figure 3.2A**). LC3I was increased by fasting in chow-fed IF mice (P = 0.006) and by fasting in HFD-fed IF mice vs AL (P <0.001). There was no diet or intervention effects on LC3II (**Figure 3.2B**). Thus, a diet by intervention effect was observed in LC3II/I ratio (**Figure 3.2C**). The LC3II/I ratio was increased by HFD (P = 0.003) and decreased by fasting as compared to the HFD-AL group (P <0.001). A diet by intervention effect was observed in LAMP1 protein (**Figure 3.2D**). LAMP1 protein was increased by fasting in chow-fed IF mice (P = 0.034) and as compared to chow-AL (P = 0.024). A diet by intervention effect was also observed in *Beclin1* mRNA (**Figure 3.2E**), which was increased by fasting in chow-fed IF mice (P = 0.020). An intervention effect was also observed in *Map1lc3b* mRNA (**Figure 3.2F**), which was increased by fasting vs AL (P = 0.043). In muscle, a diet by intervention effect was observed in BECLIN1 protein levels only (**Figure 3.2H**). BECLIN1 protein level was lower in HFD-AL vs chow-AL (P = 0.018) and lowered by fasting in chow-fed IF mice versus fed (P <0.001) and AL (P = 0.003). A diet effect was observed in LC3II/I ratio which was increased by HFD (**Figure 3.2K**). A diet by intervention effect was also observed in *Sqstm1* mRNA levels (**Figure 3.2N**), which were decreased by HFD vs chow AL feeding (P <0.001) and by feeding in IF chow-fed mice vs AL (P = 0.036). The mRNA levels of *Tfeb* was not altered by HFD feeding or IF (Error! Reference source not found.).

IF reduced body weight, fat mass and mRNA levels of autophagy markers in human muscle

As previously reported (Hutchison, Liu, et al. 2019), there were greater reductions in body weight in IF70 as compared to IF100 (5.12 ± 0.60 vs 3.15 ± 0.60kg; P = 0.029, **Table 3.1**). A group by visit effect was observed in *BECLIN1* mRNA level (**Figure 3.3A**), which was significantly reduced from baseline following a fed day (V8A) in the IF70 group only (P = 0.009). There was no change in *MAP1LC3B* mRNA levels (**Figure**

3.3B). However, a visit effect was observed in both *SQSTM1* and *LAMP2* mRNA levels (**Figure 3.3C** and **D**). *SQSTM1* was decreased from baseline when measured following a fed day and increased by a 24h fast, whereas *LAMP2* was decreased from baseline when measured following a fed day only.

3.5 Discussion

Intermittent fasting has previously been shown to preserve beta-cell mass (Liu, H et al. 2017) and protect mice from ischemia reperfusion injury (Godar et al. 2015) via activation of autophagy. However, the effects of IF on markers of autophagy in other tissues, such as liver and muscle, are unknown. In this study, the 24h fasting period that was imposed during an IF protocol elevated the markers of autophagy in mouse liver. Activation of autophagy was particularly evident in mice that were fed a chow diet. There was very little evidence that fasting increased the markers of autophagy in mouse muscle, suggesting there is tissue-specific regulation of autophagy in response to fasting. In humans, there was no evidence that fasting activated markers of autophagy in human *vastus lateralis* muscle. However, some markers of autophagy, namely *BECLIN1*, *SQSTM1* and *LAMP2* were reduced from baseline on non-fasting days, suggesting that weight loss may have dampened autophagy overall.

In the present study, we observed that a 24h fast markedly increased LC3I and *Map1lc3b* in mouse liver in both diet groups, suggesting that prolonged fasting transiently increases abundance of autophagy proteins. However, there was no change in LC3II, which resulted in a reduced LC3II/I ratio. The reduced LC3II/I ratio indicates that autophagy is responding to fasting, however without a flux measurement, the results remains difficult to interpret (Mizushima, Yoshimori & Levine 2010). We speculate that fasting caused increasing amount of LC3I to be lapidated to form LC3II, and that LC3II was cleared in the autolysosome at an increased rate. The observed

increase in LAMP1 protein along with *Beclin1* mRNA transcript by fasting in chow-IF mice indicates an increase in autophagic activity along with lysosomal function. Overall, this data suggests a transient increase in hepatic autophagy in response to fasting, particularly in the chow-fed animals along with an increase in glucose tolerance and lowering of insulin in both diet groups indicating convergence of health benefits provided by IF from physiological to cellular level (Moruno, Perez-Jimenez & Knecht 2012). Unfortunately, measures of autophagy flux in animals are incredibly challenging. However, a true autophagy flux assessment can be achieved via measurement of LC3II in conjugation with a lysosomal inhibitor such as leupeptin or chloroquine (du Toit et al. 2018). Flux can also be measured using tf-LC3 mice (Bensalem et al. 2021; Lee, JH et al. 2019).

The present study tested mice at 16 weeks of HFD-AL feeding (43% kcal as fat). There was an increase in the LC3II/I ratio as a result of HFD, suggesting that HFD stimulated hepatic autophagy, which may have resulted due to higher lipidation of LC3I to LC3II considering lower LC3I relative to LC3II. The effects of HFD on hepatic autophagy are controversial (Hsu et al. 2016; Klionsky et al. 2016; Lopez-Vicario et al. 2015; Xiao et al. 2014; Yan, Gao & Zhang 2017). Enhanced hepatic autophagy was reported in rats that were fed with high fructose diet (60%) for 5 months (Aijala et al. 2013), in NASH and NAFLD rat models (Xiao et al. 2014; Yan, Gao & Zhang 2017), and in mice fed HFD from 2 to 8 weeks (Hsu et al. 2016). In the latter study, a time course was conducted whereby autophagy was supressed at 16 weeks (Hsu et al. 2016). Reductions in hepatic autophagy were also observed in mice that were fed HFD (60% kCal in fat) for 16 weeks (Rodriguez-Navarro et al. 2012), and in mice that were fed high fat - high fructose for 8-weeks (Sharma, S et al. 2011). The discrepancy in the effects of HFD on autophagy may be the result of differences in duration of feeding or

macronutrient composition and require further investigation and should also include a time course and functional assessments of autophagy flux.

In the present study, there was limited evidence that fasting increased markers of autophagy in mouse muscle. To our knowledge, there is no study that has previously examined the effects of IF in fed and fasted state on autophagy markers in a wildtype C57BL/6 mouse muscle. The discrepancy in findings between the liver and muscle may be attributed to the primary role of liver in starvation-induced ketone body generation, where autophagy has been shown to be required for ketone generation (Takagi et al. 2016). Mechanistically, hepatic autophagy ablation achieved by Atg7 knockdown resulted in accumulation of nuclear receptor co-repressor 1 (NCoR1), a transcription factor and a negative regulator of nuclear receptor peroxisome proliferator-activated receptor-alpha (PPAR-α), suppressing PPAR-α, and hence enzymes responsible for β-oxidation, leading to reduced production of ketone bodies (Saito et al. 2019).

In human *vastus lateralis* muscle, mRNA levels of *BECLIN1, SQSTM1,* and *LAMP2* were decreased in response to IF, but solely when assessed following a fed day. This data suggests skeletal muscle autophagy may be dampened in women with obesity in response to weight loss, although we did not observe a correlation between the change in these markers and the degree of weight loss in this study. We did not find literature reporting changes in autophagy markers in response to weight loss in humans, however, methionine restriction reduced total body weight and activated chaperone-mediate autophagy in inguinal white adipose tissue in mice (Cooke et al. 2020). In the present study, there was no evidence of an increase in markers of autophagy in response to the 24h fast, except an increase in *SQSTM1* mRNA transcript that was

imposed by the IF protocol. To our knowledge, only one previous study has reported the effects of an acute fast in humans. In that study, 24h of fasting decreased the levels of LC3I and LC3II proteins in muscle of healthy lean males, without altering the LC3II/I ratio (Dethlefsen et al. 2018) and decreased BECLIN1 protein levels. However, when exercise was added with fasting, BECLIN1 protein level was increased (Dethlefsen et al. 2018). Based on the current literature, exercise induces autophagy by activation of AMP-activated protein kinase (AMPK), leading to inhibition of mechanistic target of rapamycin, activation of the Unc-51 like autophagy activating kinase 1 complex and the Phosphatidylinositol 3-kinase complex (Andreotti et al. 2020). However, fasting also activates AMPK in mouse muscle (Bujak et al. 2015), although this was not tested in the present study due to limited tissue sample. In one recent study, 6h of time-restricted eating, a form of IF in human, increased the marker of autophagy, *MAP1LC3A,* in white blood cells in morning, in 18h of fasting state in addition to clock genes Brain and Muscle ARNT-like 1, Cryptochrome Circadian Regulator 1, Cryptochrome Circadian Regulator 2 and Retinoid-related Orphan Receptor A, in a short 4-day crossover trial (Jamshed et al. 2019). It may also be worth noting that autophagy possesses robust circadian rhythm in mouse organs, namely liver and heart and to a lesser extent in muscle which peaks at ZT11 before rapidly decreasing at night (ZT17), and again rising throughout the light phase (ZT5) (Ma, Panda & Lin 2011). Thus, it may be possible that circadian rhythm may have influenced the regulation of autophagy genes at different time points. The study in humans was limited to the mRNA level due to sample availability. Additionally, it is worth noting that male mice were used while the human participants were females. Finally, although the fasting length was equalised across species, a 24h fast is a greater metabolic stressor in mice which have approximately 7-times higher metabolic rate as compared to humans (Demetrius 2005).

The findings from this study suggest that hepatic autophagy was activated by IF in mice, but this was mitigated in mice that were exposed to HFD. Considering the anti-aging effect of autophagy (Barbosa, Grosso & Fader 2018; Stead et al. 2019), the findings of mouse liver suggest that a chow diet with IF intervention is more beneficial at cellular level over HFD. There was little evidence of activation of autophagy in muscle in response to IF in either mouse or humans.

Table 3.1 Anthropometric measurements at baseline and following IF intervention in women

	IF70 (n=25)			IF100 (n=25)			Between-group comparison (P-value)		
	V0 (baseline)	V8A (12h Fast)	V8B (24h Fast)	V0 (baseline)	V8A (12h Fast)	V8B (24h Fast)	Δ (V8A-V0)	Δ (V8B-V0)	Δ (V8A-V8B)
Age (years)	49±2			51±2			-	-	-
Weight (kg)	89.4±2.6	84.0±2.6*	83.3±2.6$^{\#}$	84.1±2.6	81.3±2.6*	80.5±2.6$^{\#}$	**<0.001**	**0.001**	0.890
Total %FM	48.3±1.2	46.0±1.3*	-	47.0±1.2	45.2±1.3*	-	0.332	-	-
FPG (mmol/L)	4.8±0.0	4.6±0.0	4.6±0.0	4.9±0.0	4.9±0.0	4.7±0.0	0.052	0.389	0.230
FPI (mU/L)	19.5±1.4	15.9±1.7*	13.5±1.2	18.5±1.4	23.0±1.7*	14.4±1.2$^{\#}$	**<0.001**	0.247	**0.009**
Fasting NEFA (mmol/L)	0.6±0.0	0.4±0.04*	0.8±0.0$^{\#}$	0.6±0.0	0.5±0.0*	0.8±0.0$^{\#}$	0.242	0.449	0.059
HOMA-IR	4.2±0.3	3.3±0.3	2.7±0.3	4.1±0.3	4.8±0.3	3.3±0.3	**<0.001**	0.108	0.085

Data are presented as mean ± SEM.

A mixed effect model performed with Bonferroni *post hoc* correction to test group differences of an 8 week of IF intervention following a 12h overnight fast (Fed, V8A) and 24h fast (fast, V8B), including Bonferroni *post hoc* with pair-wise comparison for time effects within each group.

IF70: intermittent fasting diet at 70% of the baseline energy requirements; IF100: intermittent fasting diet at 100% of the baseline energy requirements; %FM: Percentage fat mass; FPG: Fasting plasma glucose; FSI: Fasting serum insulin; NEFA: Non-esterified fatty acids; HOMA-IR: Homeostatic model assessment of insulin resistance.

P-value <0.05 was considered statistically significant. $^{*}P<0.05$: differences within group versus baseline, and $^{\#}P<0.05$: differences between V8A versus V8B within group.

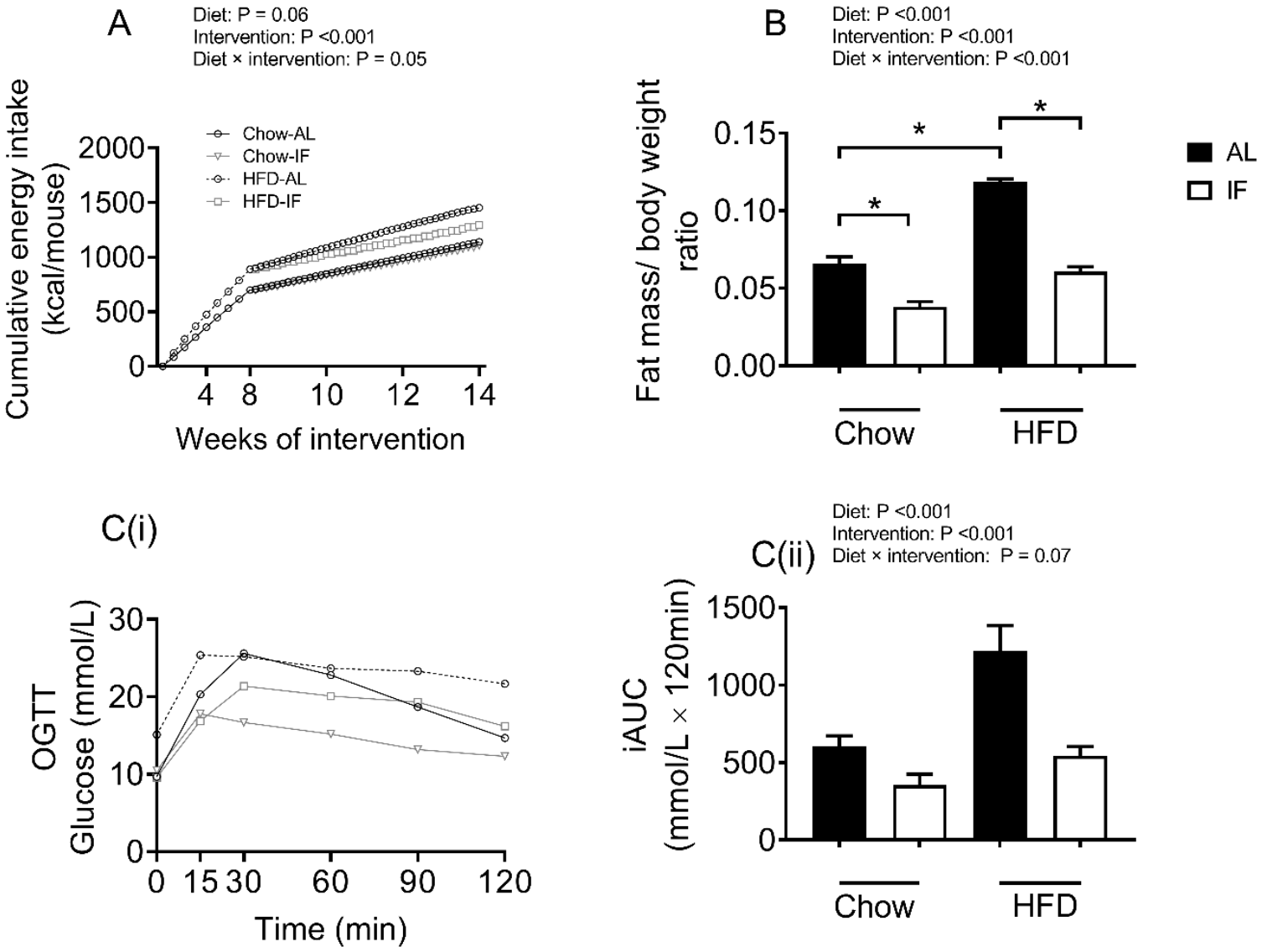
A
Diet: P = 0.06
Intervention: P <0.001
Diet × intervention: P = 0.05
Cumulative energy intake (kcal/mouse)
2000
1500
1000
500
0
Chow-AL
Chow-IF
HFD-AL
HFD-IF
4 8 10 12 14
Weeks of intervention
B
Diet: P <0.001
Intervention: P <0.001
Diet × intervention: P <0.001
Fat mass/ body weight ratio
0.15
0.10
0.05
0.00
*
*
*
AL
IF
Chow
HFD
C(i)
OGTT
Glucose (mmol/L)
30
20
10
0
0 15 30 60 90 120
Time (min)
C(ii)
Diet: P <0.001
Intervention: P <0.001
Diet × intervention: P = 0.07
iAUC
(mmol/L × 120min)
1500
1000
500
0
Chow
HFD

Figure 3.1 IF decreased fat mass and increased glucose tolerance in chow and HFD-fed mice

A) Cumulative calorie intake in lean chow-fed and high fat diet (HFD)-induced obese mice fed ad libitum (AL) or on an intermittent fasting (IF) protocol (N = 8/group). **B)** Fat mass (gonadal and inguinal) normalised to body weight (N = 22-23/group). **C(i)** Glucose response to an oral glucose tolerance test (OGTT; N=7-8/group). **C(ii)** The incremental glucose area under the curve (iAUC) during OGTT. Two-way ANOVA with Bonferroni *post hoc* test. Diet by intervention effect: **P*<0.05.

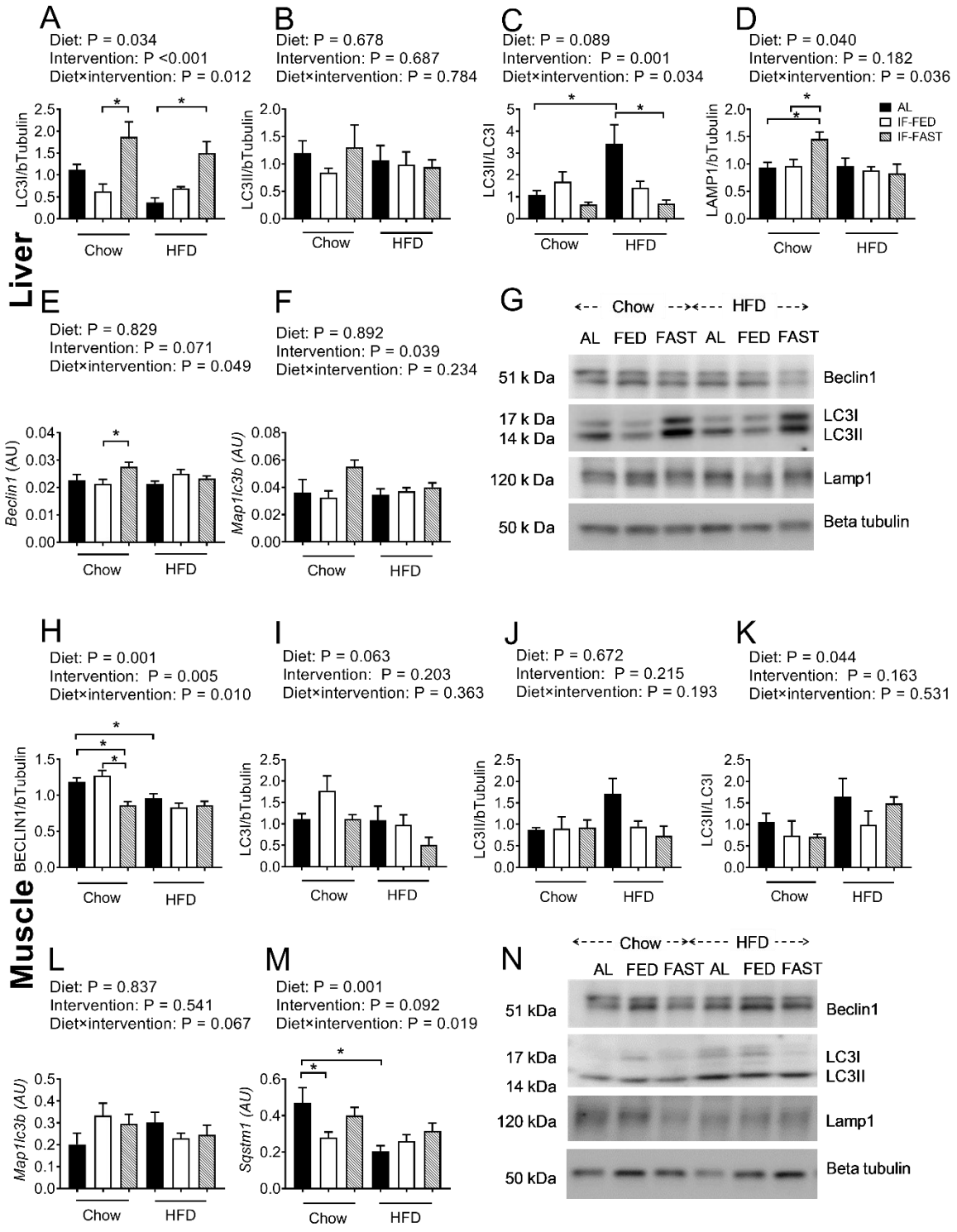

Figure 3.2 IF activated mRNA markers of autophagy in mouse liver only

(**A, B, C, D**) Changes in protein markers of autophagy in liver of mice fed ad libitum (AL), high-fat diet (HFD) on an 8-week intermittent fasting (IF) protocol with tissue collected in the fed (IF-FED) or fasted (24h) state (IF-FAST; N=4/group). (**E** and **F)** Changes in mRNA markers of mouse liver autophagy in AL, IF-FED and IF-FAST groups (N = 7-8/group). (**G**) Representative western blot for mouse liver protein (**H, I,**

J, and **K**) Changes in protein markers of mouse muscle autophagy in AL, IF-FED and IF-FAST groups (N = 4/group). (**L** and **M**) Changes in mRNA markers of mouse muscle autophagy in AL, IF-FED and IF-FAST groups (N = 7-8/group). (**N**) Representative western blot for mouse muscle protein. Beta-tubulin used as loading control. Two-way ANOVA with Bonferroni *post hoc* test was applied for intervention and diet by intervention effect. Diet by intervention effect: **P*<0.05. AU: Arbitrary unit

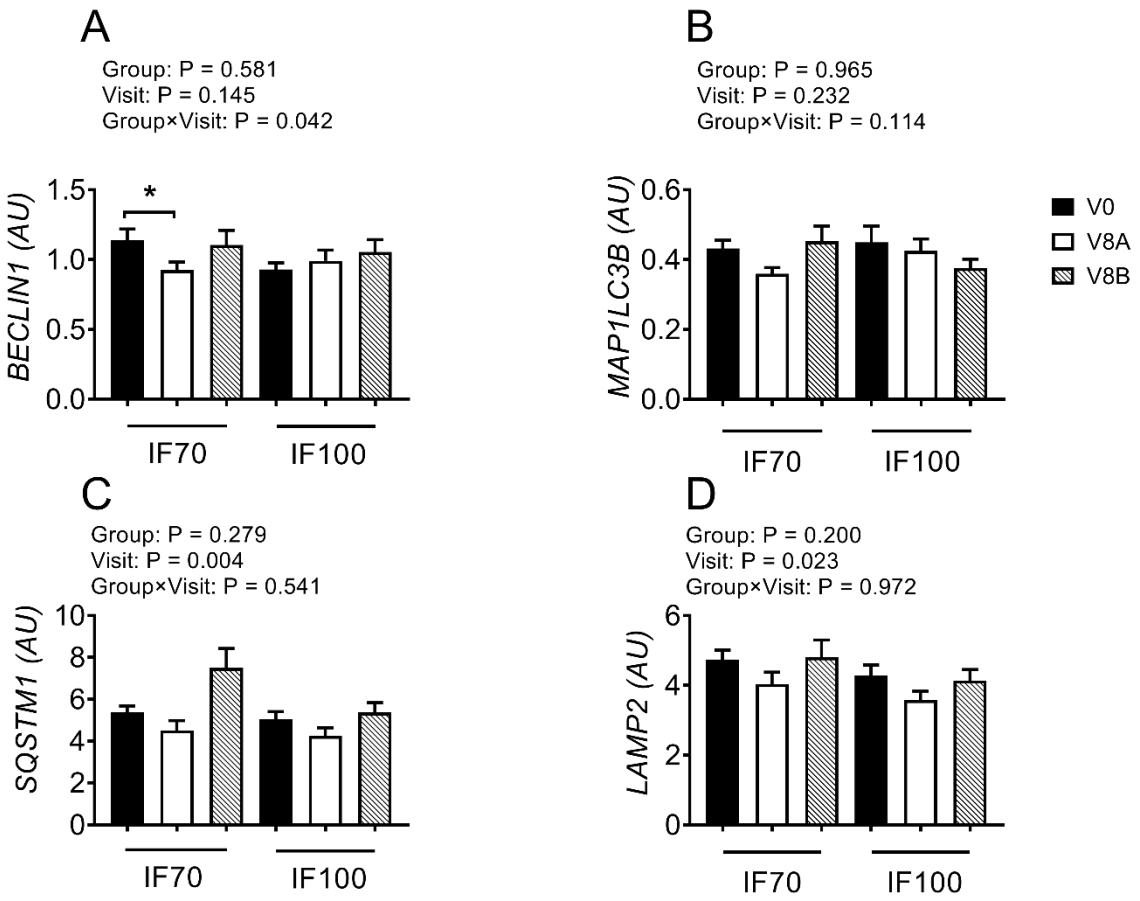

Figure 3.3 IF reduced body weight but did not increase the mRNA levels of autophagy in human muscle

(**A, B, C and D**) Changes in mRNA markers of autophagy in human *vastus lateralis* muscle following 8 weeks of IF intervention (IF70, N = 17; IF100, N = 14). **V0:** Baseline

(12h fast), **V8A:** 8 weeks of IF (12h fast), **V8B:** 8 weeks of IF (24h fast). Values are presented as mean ± SEM. Linear mixed-effect model with Bonferroni *post hoc* correction. Group by visit effect: **P*<0.05 as indicated. AU: Arbitrary unit.

Chapter 4: Early or delayed time-restricted feeding prevents metabolic impact of obesity in mice.

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Short running title: Early or delayed TRF improves metabolic outcome

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Statement of Authorship

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Signature		Date	25 February 2021

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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Signature		Date	25/2/2021

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4.1 Abstract

Time-restricted feeding (TRF) initiated early during the dark phase prevents the metabolic consequences of high fat diet in rodent models. However, the metabolic consequences of delaying the initiation of TRF, akin to breakfast skipping in humans, is unclear. We assigned 8-week-old male C57BL6J mice (n=192) to chow or high-fat-diet *ad libitum* (AL) for 4-weeks, before randomization to continue AL or 10-hours of TRF, initiated at lights off (TRFe) or 4-hour after lights off (TRFd) for a further 8-weeks. Oral glucose tolerance tests (1g/kg), metabolic monitoring and body composition by echoMRI was performed, and tissues were collected at six time points. TRF reduced weight gain and fat mass versus AL, with a greater reduction in TRFe versus TRFd. TRF improved glucose tolerance and protected mice from high fat diet induced hepatosteatosis versus AL, with no difference between TRFe and TRFd. TRF increased the amplitude of *Bmal1*, *Cry1*, *Per2, Nampt*, and *Nocturnin* in liver. A phase delay in *Bmal1*, *Cry1*, *Per2, Reverbα, Nampt*, NAD, *Sirt1*, and *Nocturnin* was observed in TRFd. Thus, delaying TRF limited the weight benefit and induced a phase delay in the hepatic clock, but improved metabolic health. Allowing more flexibility in when TRF is initiated may increase the translational potential of this dietary approach in humans.

4.2 Introduction

Time-restricted feeding (TRF) is a dietary tool that limits the duration of food intake for 6-12 hours during the active phase of the day, without altering either the amount or quality of food provided (Regmi & Heilbronn 2020). In rodents, TRF limited diet-induced weight gain and protected mice from the metabolic consequences of diverse nutritional challenges, including high-fat-diet (HFD) and high-fat-high-sucrose diet (Chaix et al. 2014; Duncan et al. 2016; Hatori et al. 2012; Sundaram & Yan 2016; Woodie et al. 2018). TRF also reduced body weight and fasting glucose, improved glucose tolerance, reduced blood pressure and reduced atherogenic lipids in people with overweight and obesity (Gill & Panda 2015; Sutton et al. 2018; Wilkinson et al. 2019).

Most TRF studies have initiated TRF early (TRFe), at the onset of the dark phase (Chaix et al. 2014; Gill & Panda 2015; Hatori et al. 2012; Sundaram & Yan 2016; Sutton et al. 2018; Wilkinson et al. 2019; Woodie et al. 2018). This is likely the optimal time to initiate TRF since glucose tolerance and insulin sensitivity are highest during the dark phase (Rudic et al. 2004). Skipping breakfast in humans (Bi et al. 2015; Jakubowicz et al. 2019), or eating late during the dark phase in mice (Bray et al. 2010) are also linked to weight gain and poorer glucose control. However, implementing TRFe in the general population may be challenging both biologically and socially (Regmi & Heilbronn 2020). Delaying the initiation time of TRF (TRFd) may increase the acceptability of this as a dietary tool in the community.

However, the metabolic consequences are not yet clear. In the only human trial to date, TRF initiated at 8am or 12pm for one week equally improved glucose tolerance in participants with obesity (Hutchison, Regmi, et al. 2019). However, there is some evidence that TRFd could limit weight benefit (Delahaye et al. 2018; Shimizu et al.

2018) and induced a phase delay in hepatic clocks after two weeks in rodents (Shimizu et al. 2018).

TRF acts partially by facilitating the robust oscillation of clock genes in peripheral organs (Chaix et al. 2014; Greenwell et al. 2019; Hatori et al. 2012; Velingkaar et al. 2020). Interestingly, robust physiological rhythms were restored in clock deficient mice when fed under TRF (Chaix et al. 2018; Vollmers et al. 2009). This suggests that TRF impacts other regulatory factors that drive rhythmic transcriptomes, independently of clock. Nicotinamide adenine dinucleotide (NAD) is a cofactor that plays a pivotal role in energy metabolism, sirtuin (SIRT) function, and biological ageing (Poljsak 2018). The majority of cellular NAD was thought to come from the nicotinamide phosphoribosyltransferase (NAMPT) mediated salvage pathway, whose amplitude in liver was reduced by HFD (Eckel-Mahan et al. 2013). However, another novel source of NAD is Nocturnin, a member of the exonuclease-endonuclease family of proteins, initially considered a deadenylase (Stubblefield et al. 2018), but recently shown to be a NADPH phosphatase (Estrella et al. 2019).

This study examined whether delaying the initiation of TRF improves glucose tolerance and mitigates the adverse health consequences of HFD, and the effects on genes involved in circadian regulation and markers of NAD metabolism in mouse liver. We hypothesized that TRFd would be equally beneficial to TRFe in the prevention of metabolic consequences of HFD, despite inducing a delay in the phase of key hepatic circadian genes and markers of NAD metabolism.

4.3 Research Design and Methods

Animals and diets

All experiments were approved by the SAHMRI and University of Adelaide Animal Ethics Committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Eight-week-old C57BL/6J male mice (N=192, SAHMRI bioresources, Adelaide, Australia) were housed 4 mice/cage under 12:12h light:dark cycle with lights on at 7 am (Zeitgeber time (ZT) 0) at 18-24°C. Mice were fed either a standard chow (18% calorie from fat, Teklad global 2018SX, Envigo, Madison, USA) or a lard-based HFD (43% calories from fat, SF16-001, Specialty Feeds, WA, Australia) for 4-weeks. Mice on each diet were then randomized to one of three interventions: i) continue AL, ii) 10-hour TRF initiated at ZT12 (TRFe), and iii) 10-hour TRF initiated at ZT16 (TRFd) for a further 8-weeks **(Figure 4.1)**. Food consumption was recorded on a weekly basis throughout the study. During TRF, food access was controlled by transferring mice between cages with and without food. AL 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. Feeding efficiency was calculated as the ratio of body weight gain to calories consumed (Yasumoto et al. 2016). After 8-weeks, mice were sedated with isoflurane at 4 hourly intervals (ZT 0, 4, 8, 12, 16, 20), to collect blood by cardiac puncture, and were euthanised by cervical dislocation prior to collection of liver, inguinal and gonadal fat pads.

Body weight and composition

Body weight was recorded weekly at the end of fasting period during cage transfer (AL: ZT11-12, TRFe: ZT12 and TRFd: ZT16). At the 20 weeks of age, body composition

was examined at ZT4-5 using an EchoMRI™-500 Body Composition Analyzer (N=6/group).

Oral glucose tolerance test (GTT) and Insulin measurement

At the 19 weeks of age, mice were fasted for 6 hours and an oral GTT (1g glucose/kg body weight) was performed at ZT4 (light phase, N=8/group) or ZT16 (dark phase, N=7-8/group). Blood glucose was measured at 0, 15, 30, 60, 90 and 120 minutes via tail vein bleeding using a glucometer (Accu-Chek® Performa II, Roche). Plasma samples at 0, 15, 30 and 60 minutes were stored at -80°C and later insulin was measured using Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem, USA). Glucose and insulin area under the curve (AUC) were calculated by trapezoidal rule (Allison et al. 1995).

Metabolic cage

At the 20 weeks of age, a subset of mice (N=4-6/group) were individually housed in Promethion® metabolic cages (Sable Systems, Las Vegas, USA) for indirect calorimetry. Mice were acclimatized for 22-24 hours and metabolic data acquired for 24 hours. Food and water consumption, x, y and z beam breaks, VCO2 and VO2 were measured at 5-minute intervals. Respiratory quotient (RQ) and energy expenditure (EE) were calculated as described by Weir equation (Weir 1949). EE was adjusted as raw EE/(body weight)3/4 as previously described (Tschop et al. 2011).

Liver triglycerides and enzyme activity measurement

For triglyceride measurement, liver tissue samples (~50 mg) were first homogenized in 5% NP-40 solution (in ddH2O). The supernatant was separated, and triglyceride was measured using a Triglycerides Assay Kit (Abcam) and was adjusted for tissue

weight. For enzyme activity, citrate synthase and β-hydroxyacyl CoA dehydrogenase activity were measured in 6-10mg liver tissue homogenates by kinetic assay as previously described (Bergmeyer 1974), and were adjusted for tissue weight.

Gene expression analysis

Total RNA was extracted from liver using Trizol (Invitrogen) and cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen). Quantitative real-time PCR was performed as described previously (Liu, B, Page, Hutchison, et al. 2019) using the TaqMan primers and master mix listed in (**Table 4.1**). *Hypoxanthine phosphoribosyl transferase (Hprt)* was used as reference gene and relative gene expression was calculated using $2^{-\Delta CT}$, where ΔCT = (CTtarget gene - CTreference gene).

Histology

Fresh liver tissue was fixed in 4% buffered paraformaldehyde for ~8 hours, dehydrated in 30% sucrose, mounted in Tissue-Tek OCT Compound, and frozen at -80°C. Cryosections (10μm) were air-dried on gelatine-coated slides, stained using oil red O as previously described (Christie et al. 2018), and scanned under brightfield microscopy.

NAD measurement

NAD was extracted from liver tissue (~20 mg) and NAD-NADH cycling assay was performed using ADH cycling mix at 37°C in dark for 15 minutes as previously described (Bertoldo et al. 2020). Fluorescence was measured (excitation 340 nm and emission 445 nm) (Chance et al. 1979), and the NAD concentration was determined using a standard curve, and corrected for amount of tissue used.

Western Blot

Liver tissue lysates (10μg of protein) were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Membranes were probed for NAMPT (E-3, sc-393444, Santa-Cruz) and β-tubulin (Ab-6046, Abcam). Bands were visualized by chemiluminescence, the intensity was measured using ImageJ software, and presented as relative protein levels.

Calculation and Statistical analysis

Statistical analysis was performed by two-way ANOVA with diet (chow and HFD) and intervention (AL, TRFe and TRFd) as fixed factors. A differential effect of intervention in each diet was tested via the interaction between diet and intervention, and Bonferroni's *post hoc* was applied. For glucose AUC an additional two-way ANOVA analysis was performed with body weight as co-variate in the model. Comparison of glucose AUCs between chow-AL and HFD- TRFe or TRFd, 10-hour fasting triglyceride between TRFe and TRFd, and western blot results were performed using t-tests (SPSS, IBM). This study was powered to detect observed changes in weight gain, feeding efficiency, glucose AUC, liver triglycerides, and circadian genes. The point estimates of metabolic phenotypes by TRFe and TREd were very similar; hence would require sample size of several hundred in every group to achieve statistical power. All data were included in the analysis. All data are presented as mean±SEM and P<0.05 was considered statistically significant. Circadian data was analysed by Cosinor regression using R package *cosinor* of the log transformed gene expression, y_i.

$$\log y_i = A + B\cos\left(\frac{2\pi t}{24} + C\right) + \epsilon_i, \epsilon_i \sim N(0, \sigma^2)$$

Where $\boldsymbol{A}$ is the mean, $\boldsymbol{B}$ is the amplitude, and $\boldsymbol{C}$ is the phase shift.

4.4 Results

TRF mitigates weight gain, but a 4-hour phase delay lessens the effect

Body weight gain was lower in both TRF groups versus AL and in TRFe vs TRFd on both diets (all $P \leq 0.04$, **Figure 4.1B**). Body composition by MRI was not different between groups in the chow-fed mice (**Figure 4.1C**). In mice that were fed a HFD, percent fat mass was lower and percent lean mass was higher in both TRF groups vs AL (both $P<0.001$) and in TRFe vs TRFd (both $P=0.001$, **Figure 4.1C**). TRF also reduced gonadal and inguinal fat vs AL in HFD fed mice (both $P<0.001$), and in TRFe vs TRFd ($P \leq 0.037$, **Figure 4.1D & E**). TRF did not alter cumulative calorie consumption in chow fed mice (**Figure 4.1F**). However, a trend towards lower calorie consumption was observed in both TRF groups vs AL in HFD fed mice ($P=0.07$). TRF mice on both diets consumed fewer calories in the first week of TRF, and this was partially sustained for 8 weeks in HFD fed mice (**Figure 4.6A**). Feeding efficiency was reduced in TRFe vs AL in chow fed mice ($P<0.001$, **Figure 4.1G**). In HFD mice, feeding efficiency was reduced in both TRF groups vs AL (both $P<0.001$) and in TRFe vs TRFd ($P=0.025$). Liver triglyceride, assessed at ZT8, 12 & 20, was reduced in TRF vs AL (both $P<0.001$) in HFD fed mice only (**Figure 4.1H**, **Figure 4.7A-C** & **Figure 4.8**), with no difference between TRFe and TRFd. Assessing liver triglyceride after identical fasting length in TRF groups [i.e., ZT8 (TRFe) and ZT12 (TRFd)] did not alter these results (**Figure 4.1I**).

TRF improved the 24-hour rhythm in nutrient utilization, irrespective of a 4-hour delay

Food intake patterns over 24 hours were examined in the metabolic chamber and presented as average hourly Kcal consumption (**Figure 4.2A-C**). Chow-fed AL mice appeared to increase food intake at ZT11, approximately 1-hours before the initiation

of a dark phase, with two peaks observed at ZT14 and ZT22. HFD-AL mice consumed ~45% of their total calories during the light phase (vs ~30% in chow-AL) and did not exhibit a discernible peak in calorie consumption during the dark phase. The TRF groups exhibited two distinct peaks in food consumption, with both peaks delayed in TRFd mice. TRF did not alter average RQ during the light phase in chow or HFD mice. However, carbohydrate oxidation exceeded 1.0 during the dark phase in chow fed TRFe mice and was significantly higher vs AL and TRFd (both $P<0.001$, **Figure 4.2D-F**). Activity was higher during the dark phase in both TRF groups vs AL ($P\leq0.05$) in both diet groups, with no difference between TRFe and TRFd (**Figure 4.2G-I**). Active phase EE and total 24-hour EE was not significantly different between groups (all $P\geq0.059$, **Figure 4.2J-L**). TRF did not alter β-hydroxyacyl CoA dehydrogenase or citrate synthase activity, key enzymes of β-oxidation and TCA cycle respectively, in liver (**Figure 4.7D-I**).

TRF improved glycaemic profile

Glucose tolerance, as assessed by glucose AUC, was improved in both TRF groups vs AL in both diets, when measured during the light and dark phase (all $P\leq0.007$). This significance was maintained after adjusting for body weight, in the dark phase, but not in the light phase. There were similar point estimates and no significant difference in the improvement in glucose tolerance between TRFe and TRFd groups (**Figure 4.3A, B, E & F**). Furthermore, glucose AUC in TRF groups that were fed HFD was not different to chow-fed AL mice, suggesting that TRF completely protected mice from HFD induced glucose intolerance. Insulin AUC was also lower in both TRF vs AL in mice fed HFD (all $P<0.045$, **Figure 4.3C, D, G & H**). Fasting glucose and insulin at ZT4 were also lower in both TRF vs AL mice that were fed HFD (all $\underline{P}\leq0.003$), but fasting glucose was higher at ZT16 in TRFd vs AL and TRFe in mice fed chow or HFD.

Both forms of TRF increased amplitude of genes involved in circadian rhythm in liver, but with a phase delay in TRFd

In *ad libitum* fed mice, HFD did not alter the amplitude (all *P*≥0.11), mean (all P≥0.38) or phase (all P≥0.06) of any of the circadian regulators versus chow (**Figure 4.4A-F**), except for a phase advance in *Reverbα* (*P*=0.01). In chow fed mice, TRF increased the amplitude of *Bmal1*, *Cry1* and *Per2* versus AL (all *P*≤0.04). In HFD mice, the amplitude of *Per2* was increased in both TRF groups vs AL and the amplitude of *Reverba* was increased in TRFe vs AL (*P*≤0.04). There was no difference in mean or amplitude of any genes between TRFe and TRFd in either diet. The phase of *Bmal1*, *Cry1*, *Per2* and *Reverbα* was delayed and *Rora* was advanced in TRFd vs AL and TRFe (all *P*<0.03) in both diet groups. Additionally, the phase of *Per2* was delayed and *Rora* advanced in TRFe vs AL on both diets.

5.4.5 The circadian rhythms in hepatic levels of markers of NAD metabolism were restored by TRF in mice that were fed a HFD.

In *ad libitum* fed mice, HFD reduced the mean mRNA and protein level of NAMPT (**Figure 4.5A and Figure** 4.9**A** and **B**), and delayed the phase of NAD and *Sirt1* (all *P*<0.05). TRF increased the amplitude of *Nampt* in both diets (all *P*≤0.006), but did not alter NAMPT protein levels. The amplitude of *Nocturnin* was also increased by TRFe in mice that were fed a HFD (*P*=0.03), and by TRFd in mice that were fed a chow diet (*P*=0.04). The mean of NAD and *Sirt1* was increased by TRF in chow fed mice, but this was significant only in TRFd vs AL in HFD mice (all *P*≤0.02). TRFe restored the HFD induced phase shift in NAD and *Sirt1* (*P*<0.03), whereas the phase of NAD and *Sirt1* was delayed in TRFd vs AL in chow fed mice. The phase of *Nampt* and *Nocturnin* was also delayed in TRFd vs AL and TRFe in both diets.

4.5 Discussion

TRF is a dietary approach that protects mice from the metabolic consequences of obesity and aging (Chaix et al. 2014; Duncan et al. 2016; Hatori et al. 2012). To date, most protocols have initiated TRF at the onset of the active phase. Humans are geared both biologically (Espelund et al. 2005) and socially (Dunbar 2017) to eat more food later in the day. If there is no allowance for food consumption in the early evening, many individuals could struggle with long term adherence to TRF. The present study examined the effects of delaying the initiation of TRF by four hours, akin to breakfast skipping, on metabolic parameters in mice that were fed chow or HFD. We showed that TRFd was less effective to reduce body weight and fat mass as compared to TRFe, and induced a phase delay in the hepatic expression of clock genes and markers of NAD metabolism. However, TRFd was effective to increase the amplitude of *Per2, Nampt*, and *Nocturnin*, and the mean levels of *Cry1*, NAD and *Sirt1,* and protected the mice against the metabolic consequences of HFD.

The present study showed that both forms of TRF improved glucose tolerance in mice that were fed chow or HFD and rescued hepatic steatosis in mice that were fed HFD. The magnitude of improvements in glucose tolerance in TRFe and TRFd were 17-23% in chow fed mice and 20-26% in HFD fed mice. Improvements in glucose metabolism were previously reported in TRF mice that were fed a HFD, either the first 6 hours or last 6 hours of the dark phase (Delahaye et al. 2018). Unlike that study, we allowed 10 hours of food access, and standardized the fasting length prior to the assessment of glucose tolerance, which is a known factor in glucose responsiveness (Andrikopoulos et al. 2008; Rudic et al. 2004). We have also previously shown that one week of TRF, initiated from 8am-5pm or from 12-9pm, was equally effective at improving glucose

tolerance in response to a mixed nutrient meal test, after standardised fasting lengths, in men with obesity (Hutchison, Regmi, et al. 2019).

Two studies to date have shown that TRF improves metabolic health, independently of body weight and food intake, in mice and humans (Sutton et al. 2018; Woodie et al. 2018). In the present study, lower body weight and fat mass was observed in TRF vs AL mice on both diets, although the improvement in glucose tolerance during the dark phase held after adjusting for body weight. In mice that were fed a HFD, there was a marked reduction in food intake at the start of the TRF protocol, which was partially sustained for 8 weeks. The effects of TRF on food intake is controversial. In mice that were fed HFD, previous studies have reported no differences in food intake (Chaix et al. 2019; Chaix et al. 2014; Hatori et al. 2012), initial reductions in food intake (Velingkaar et al. 2020), or reduced cumulative food intake (Delahaye et al. 2018; Serra et al. 2019; Sundaram & Yan 2016). In the present study, food intake was not different between groups in mice that were fed a chow diet, but the TRF mice were more active throughout the dark phase, potentially accounting for the weight difference. An increase in locomotor activity is commonly observed in mice that are fed under restricted feeding schedules (Duncan et al. 2016; Sundaram & Yan 2016; Woodie et al. 2018), and has been coined ‘food anticipatory activity’ (Mistlberger 1994). Furthermore, the increased activity could have partially contributed to the improved metabolic phenotype (Sato et al. 2019) that we observed in TRF mice fed a chow diet in this study. As this study and previous TRF studies have observed reduced body weight (Chaix et al. 2014; Hatori et al. 2012)s and arguably reduced food intake (Delahaye et al. 2018; Sundaram & Yan 2016) and increased activity (Duncan et al. 2016; Sundaram & Yan 2016), future studies should include pair-fed groups to unequivocally determine whether the TRF or the reduction in body weight/food intake

that occur as a result of the TRF drive the metabolic phenotype observed in these animals. This undertaking would need to be carefully controlled as pair-fed animals tend to consume their allocated food more quickly than ad libitum fed animals (Ellacott et al. 2010), undergoing a form of TRF. This could be overcome by allocating food as discrete meals over 24-hours, in a pattern that mimics their *ad libitum* feeding behaviour (Greenwell et al. 2019).

Some previous studies have observed that TRF increases energy expenditure, independently of activity and body weight (Chaix et al. 2019; Hatori et al. 2012), which could indicate adipose tissue browning, as we have shown previously occurs in response to intermittent fasting (Liu, B, Page, Hutchison, et al. 2019). However, those studies have calculated energy expenditure per kilogram of body weight (Chaix et al. 2019; Hatori et al. 2012). Adjusting for total body weight leads to artificial reductions in energy expenditure as adipose tissue is less metabolically active and represents a larger proportion of body weight in obese mice (Tschop et al. 2011). There was no evidence of unexplained differences in energy expenditure in TRF mice fed chow or HFD in the present study. However, delaying TRF was less effective to reduce body weight and fat mass versus TRFe despite eqivalent food intake. This difference in feeding efficiency between TRF subgroups could either be the result of a lower than detectable difference in energy expenditure, or increased nutrient absorption, but the latter was not assessed.

Both forms of TRF increased the amplitude of key genes that are involved in circadian regulation in liver of mice that were fed a chow diet, but this was significant only for *Per2* in mice that were fed a HFD. This contrasts previous studies that have reported increased *Bmal1*, *Cry1*, *Per2* and *Reverb*α in TRF mice fed HFD (Chaix et al. 2019; Cho et al. 2012). However, those studies relied on a visual inspection of the data (Chaix

et al. 2019; Greenwell et al. 2019; Hatori et al. 2012), whereas this study applied a more rigorous statistical analysis. The discrepancy between studies could also be due to the lower percentage of dietary fat utilised in this study (43%) versus past studies (60%). As there was not a universal increase in the amplitude and/or mean of genes controlling clocks, particularly in mice fed a HFD, this suggests there is an alternative driving force underpinning improvements in glucose metabolism. This is supported by a recent study that showed that TRF restored glucose metabolism in clock deficient mice (Chaix et al. 2019). In the present study, we observed that both forms of TRF increased the amplitude of *Nampt* and *Nocturnin* and increased the mean levels of NAD and *Sirt1* on both diets. To our knowledge, this has not previously been examined. A rise in cellular NAD and gain of SIRT1 function delays ageing and improves the metabolic phenotype in animal models (Campbell et al. 2015; Poljsak 2018; Ramsey et al. 2008; Stromsdorfer et al. 2016) and thus could underpin the anti-aging benefits of TRF. Increased NAD availability also drives β-oxidation, including β-hydroxyacyl CoA dehydrogenase activity (Canto, Menzies & Auwerx 2015), enabling increased metabolic flexibility during TRF, which is the capacity of an organism to adapt fuel oxidation according to fuel availability (Galgani, Moro & Ravussin 2008).

Delaying the initiation of food intake induced a clear phase delay in multiple genes that are under circadian regulation. In particular, TRFd induced a linear phase delay in *Bmal1*, *Cry1*, *Per2*, *Reverbα*, *Nampt*, NAD, *Sirt1* and *Nocturnin.* The phase delay in NAD and *Sirt1* could drive the delay in *Per2*, given the known function of SIRT1 in regulation of *Per2* transcription by binding with clock: bmal1 {Ramsey, 2009 #214}. Interestingly, we observed the phase of NAD coincided with that of *Nampt* and *Nocturnin.* Whilst *Nampt* is a known source of NAD, the latter finding supports the recent notion the NADPH phosphatase activity of nocturnin provides an alternative

source of NAD (Estrella et al. 2019). Future studies should examine whether metabolic improvements in response to TRF are abrogated in *Nampt, nocturnin* and S*irt1* deficient animal models. This study extends previous findings (Shimizu et al. 2018) which analysed the effects after just two weeks, when animals are still adapting to the new diet schedule (Kentish et al. 2018), and did not examine nutrient signalling pathways.

Finally, we observed that fasting glucose at ZT16 was higher in TRFd mice as compared to TRFe and AL in both diets. This could be the result of a delay in the 'dawn phenomenon', whereby the early morning rise in cortisol/corticosterone increases hepatic glucose production and blood glucose occurred in TRFd, the equivalent effect takes place in the early dark phase in mice (Ando et al. 2016; Bolli et al. 1984). Daytime restricted feeding also shifts the rise in blood glucose from the pre-dark phase to the pre-light phase (Ando et al. 2016). Together, this study highlights the clear entraining effect of food intake on metabolism. However, the short delay imposed by TRFd did not adversely impact the TRF induced improvements in glucose metabolism and metabolic phenotype. This study provides strong support for allowance to delay the initiation of TRF, so long as there is a stable daily timing of food intake.

This study shows that delaying the initiation of feeding by four hours does not adversely impact the known beneficial effects of TRF, with comparable increases in glucose tolerance. Uniquely, we demonstrate the metabolic benefits of TRFd occur alongside a phase delay in hepatic clocks and metabolic markers, but with a comparable increase in the amplitude and/or mean of genes involved in nutrient signalling and circadian regulation. There are many physiological and metabolic differences between small animal model organisms and humans, but if this finding translates to humans, the

delayed form of TRF is likely to be more acceptable, long-term, in the general population.

Author contribution: PR and LKH designed the study. PR, RC, LKH, BL, AJP conducted study. PR & RC performed the experiments. PR, RC & AV analysed data. PR, RC, AJP, ATH, AV, BL and LKH contributed to data interpretation and preparation of the manuscript. LKH had full access to the data and had primary responsibility for the final publication.

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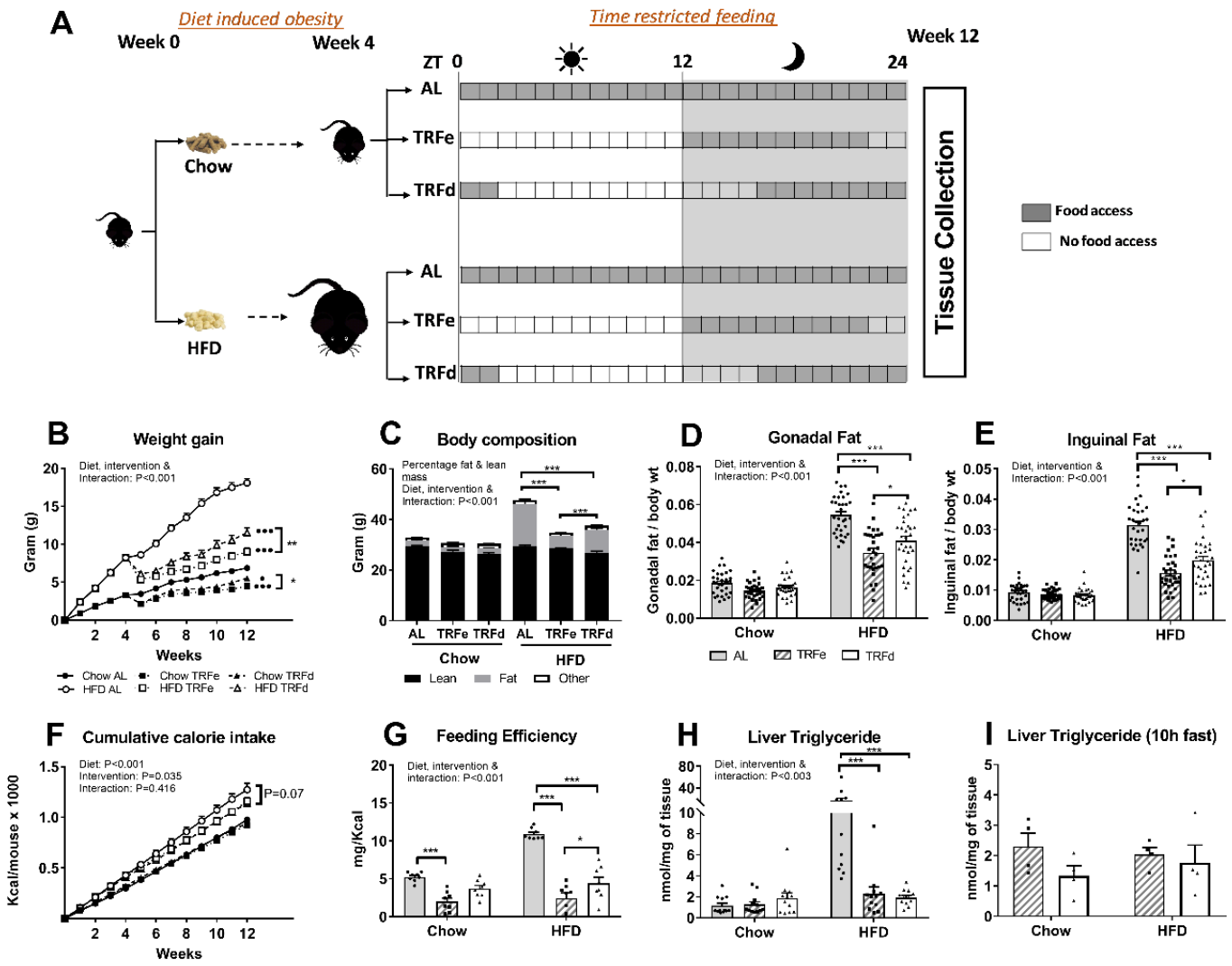
A
Diet induced obesity
Time restricted feeding
Week 0
Week 4
Week 12
ZT 0
12
24
Chow
HFD
AL
TRFe
TRFd
Tissue Collection
Food access
No food access
B
Weight gain
Diet, intervention & Interaction: P<0.001
Gram (g)
Weeks
Chow AL
HFD AL
Chow TRFe
HFD TRFe
Chow TRFd
HFD TRFd
C
Body composition
Percentage fat & lean mass
Diet, intervention & Interaction: P<0.001
Gram (g)
AL TRFe TRFd AL TRFe TRFd
Chow
HFD
Lean
Fat
Other
D
Gonadal Fat
Diet, intervention & Interaction: P<0.001
Gonadal fat / body wt
Chow
HFD
AL
TRFe
TRFd
E
Inguinal Fat
Diet, intervention & Interaction: P<0.001
Inguinal fat / body wt
Chow
HFD
F
Cumulative calorie intake
Diet: P<0.001
Intervention: P=0.035
Interaction: P=0.416
P=0.07
Kcal/mouse x 1000
Weeks
G
Feeding Efficiency
Diet, intervention & interaction: P<0.001
mg/Kcal
Chow
HFD
H
Liver Triglyceride
Diet, intervention & interaction: P<0.003
nmol/mg of tissue
Chow
HFD
I
Liver Triglyceride (10h fast)
nmol/mg of tissue
Chow
HFD

Figure 4.1 TRF protects mice from weight gain, adiposity and hepatic fat accumulation.

A) study design, B) body weight gain throughout the study (n=31-33/group), C) body composition by echoMRI (n=6/group), D) gonadal fat to body weight ratio(n=31-33/group), E) inguinal fat to body weight ratio(n=31-33/group), F) average total calorie consumption per mouse (n=8/group), G) feeding efficiency (n=8/group), H) liver triglyceride (n=12/group), I) liver triglyceride after equal 10 hours of fasting in TRFe and TRFd (n=4/group, statistics for this sub-group was done by t-test). Statistics were performed by two-way ANOVA with diet (chow vs HFD) and intervention (AL, TRFe and TRFd) as fixed variables. Bonferroni's correction was applied *post hoc*. Filled bars: AL, hatched bars: TRFe, and open bars: TRFd. (•:P<0.05 vs AL, •••: P<0.01 vs AL, *: P<0.05, **:P<0.01, ***P<0.001).

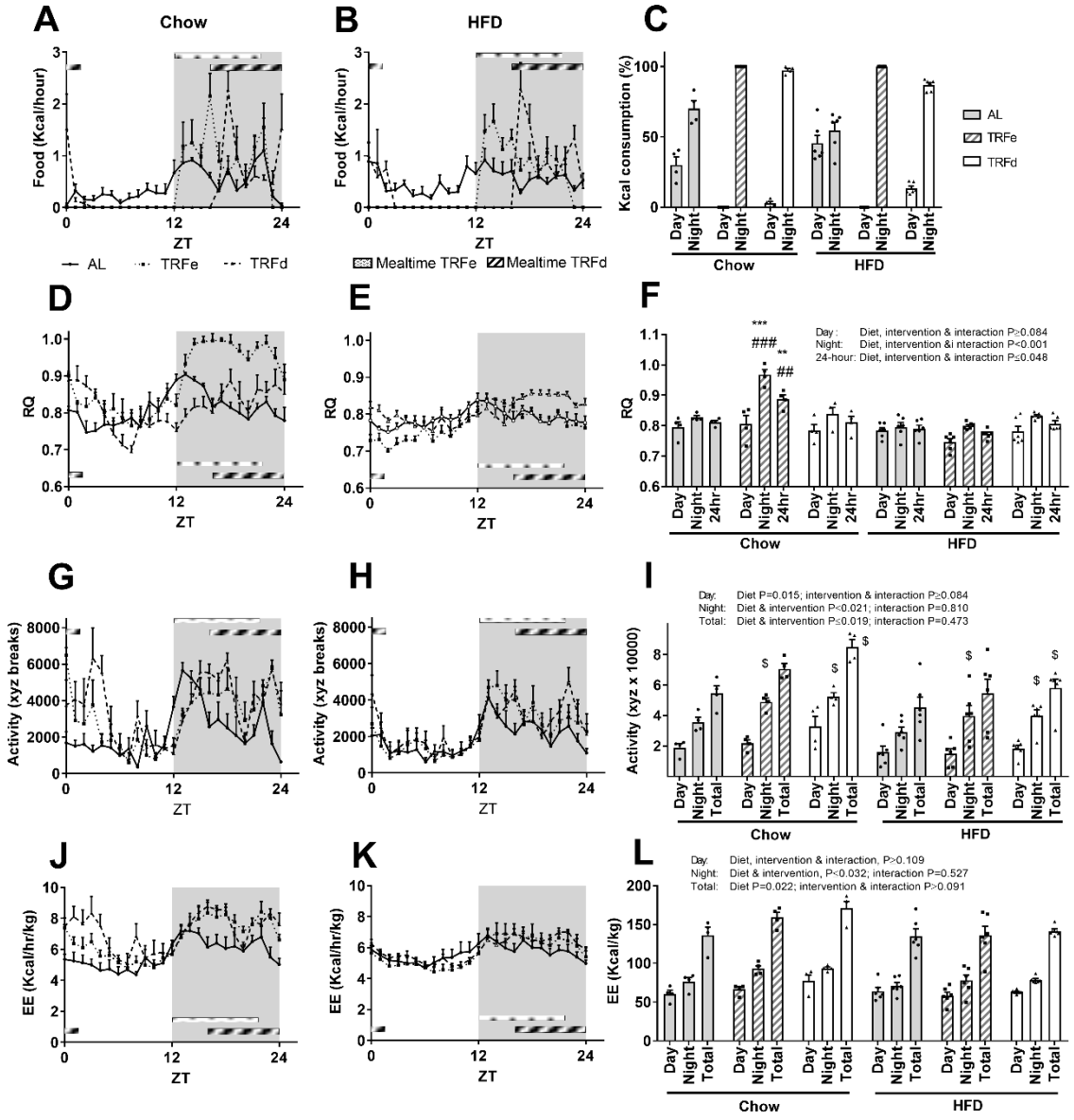

Figure 4.2 TRF drives rhythm in nutrient utilization.

A&B) hourly calorie consumption in chow and HFD (n=4-6/group), C) total day and night percentage calorie consumption (n=4-6/group), D&E) 24-hour hourly RQ (CO_2 exhaled/ O_2 inhaled) in chow and HFD (n=4-6/group), F) average day and night RQ (n=4-6/group), G&H) total hourly activity in chow and HFD (n=4-6/group), I) total day and night activity (n=4-6/group), J&K) 24-hour hourly energy expenditure in chow and HFD (n=4-6/group), L) total day and night energy expenditure (n=4-6/group). Statistics were performed by two-way ANOVA with diet (chow and HFD) and intervention (AL, TRFe and TRFd) as fixed variables. Bonferroni's correction was applied *post hoc*. Grey

area represent dark phase and food availability is indicated by dotted (TRFe) and hatched (TRFd) boxes. Filled bars: AL, hatched bars: TRFe, and open bars: TRFd. *: $P<0.05$, **:$P<0.01$, ***$P<0.001$, $^{\$}P<0.05$ overall intervention effect in both diets.

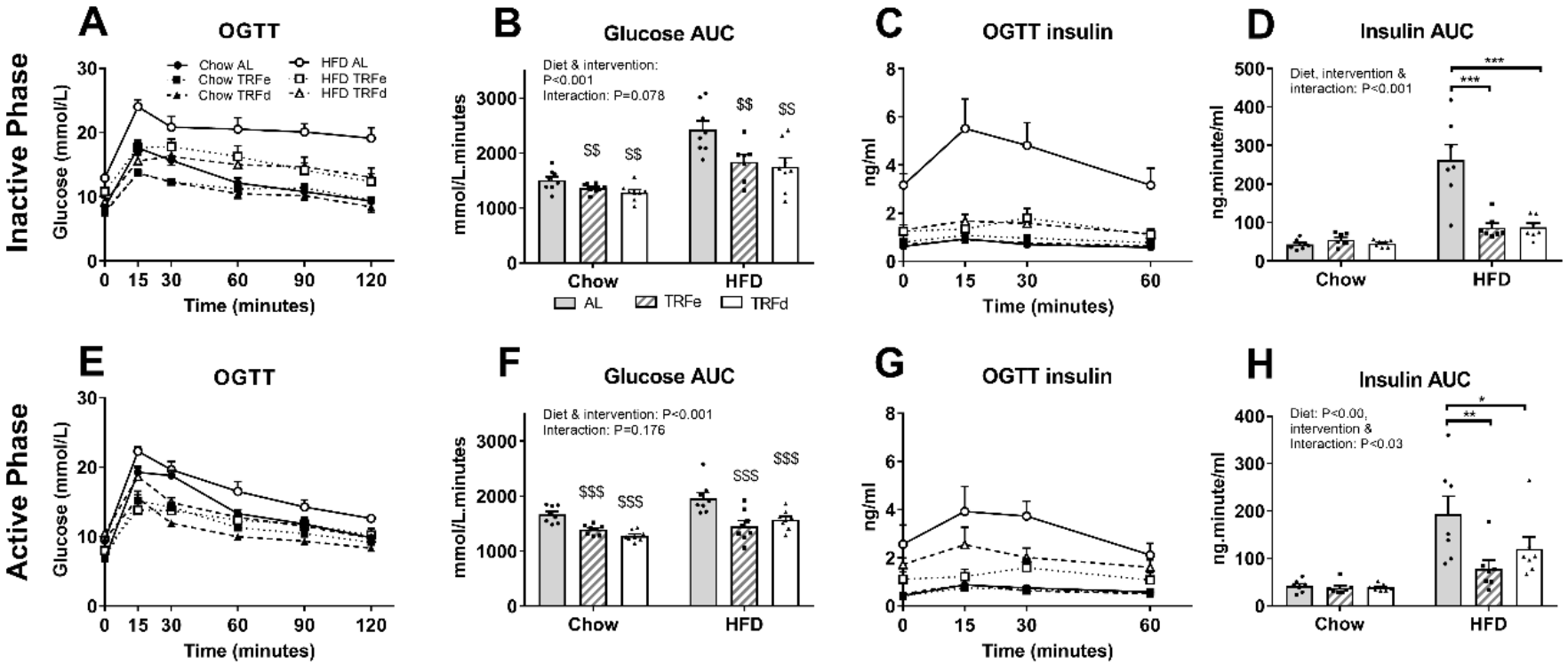

Figure 4.3 TRF improves glycaemic profile.

A) blood glucose after 1g/kg body weight of oral glucose load at ZT4 (N=8/group) , B) glucose area under the curve at ZT4 (N=8/group), C) blood insulin after 1g/kg of body weight of oral glucose load at ZT4 (N=7/group), D) insulin area under the curve at ZT4 (N=7/group), E) blood glucose after 1g/kg body weight of oral glucose load at ZT16 (N=7-8/group), F) glucose area under the curve at ZT16 (N=7-8/group), G) blood insulin after 1g/kg of body weight of oral glucose load at ZT16 (N=7/group), H) insulin area under the curve at ZT16 (N=7/group). Statistics were performed by two-way ANOVA with diet (chow and HFD) and intervention (AL,

TRFe and TRFd) as fixed variables. Bonferroni's correction was applied *post hoc*. Filled bars: AL, hatched bars: TRFe, and open bars: TRFd. *: P<0.05, **:P<0.01, ***P<0.001; $$ P<0.01, $$$ P<0.001: overall intervention effect in both diets.

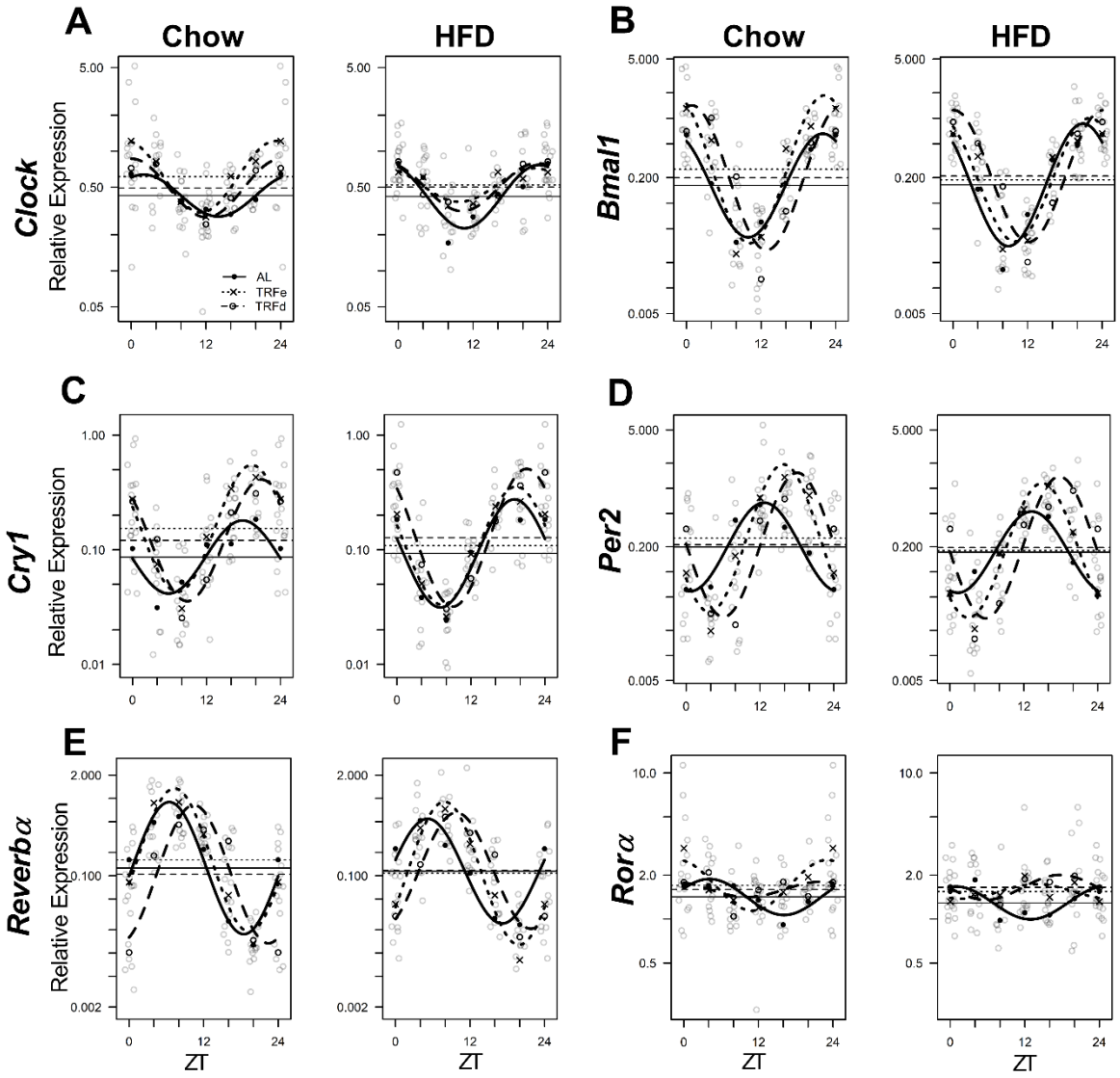

Figure 4.4 TRF facilitates robust oscillation of genes involved in circadian rhythm, despite inducing phase delay in TRFd.

A-F) Cosinor plots of clock gene expression based on relative mRNA expression at six time points of the day (ZT0, 4, 8, 12, 16 & 20; N=5-6/time point/group).

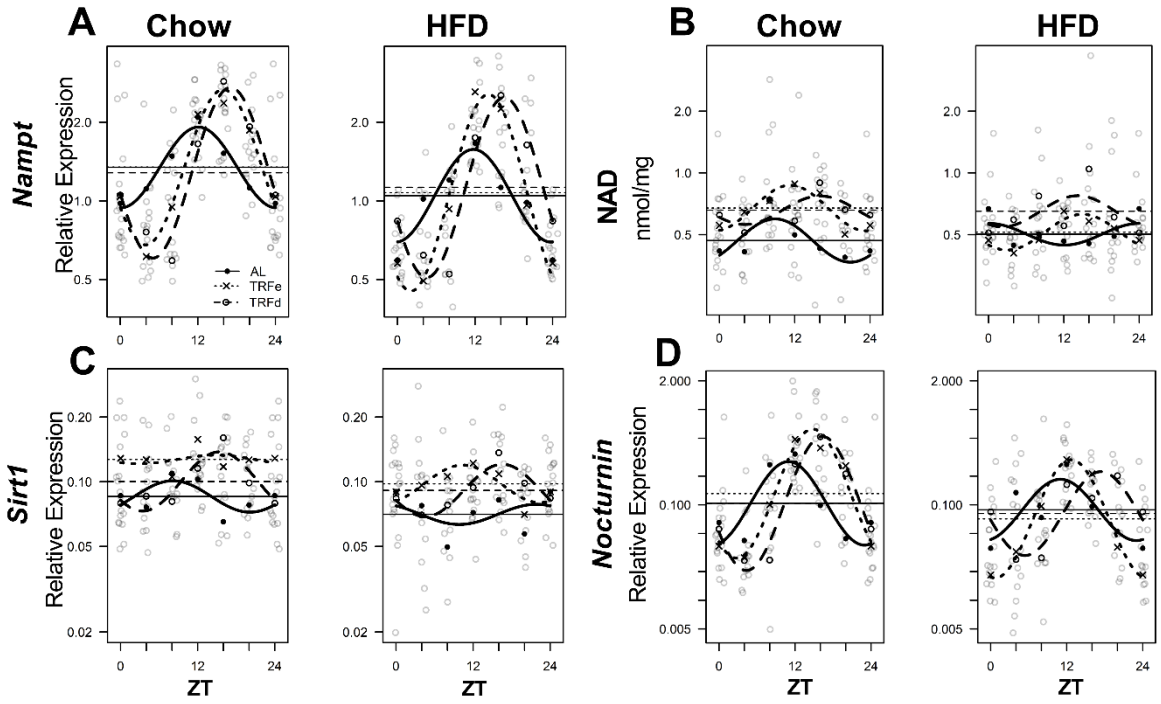

Figure 4.5 TRF facilitates robust oscillation and restores HFD induced phase shift in markers of NAD metabolism in liver.

A-D) Cosinor plots of *Nampt*, NAD, *Sirt1* and *Nocturnin* based on relative mRNA expression or tissue levels at six time points of the day (ZT0, 4, 8, 12, 16 & 20; N=5-6/time point/group).

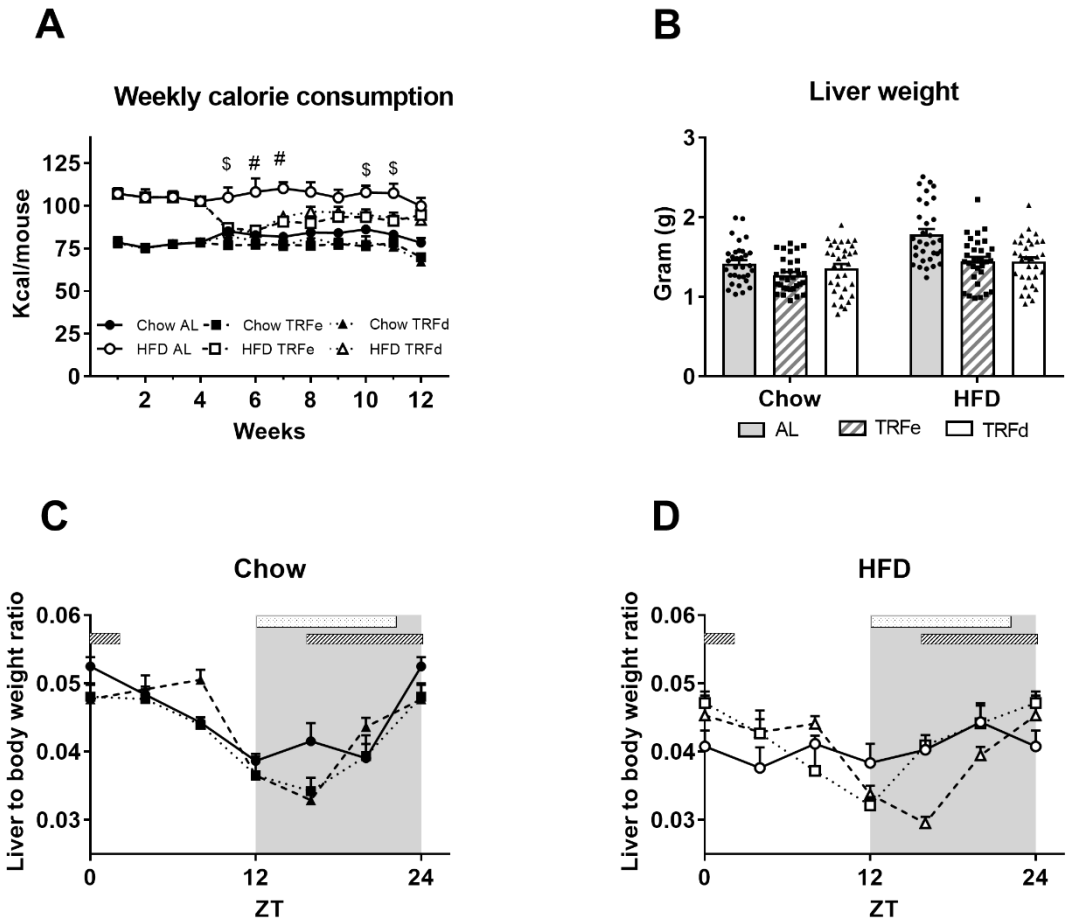

Figure 4.6 Weekly calorie consumption and liver to body weight ratio.

A) weekly calorie consumption (N=8/group), B) liver weight in grams (N=31-33/group), C&D) liver to body weight ratio at different circadian time (N=5-6/group/time point). Filled bars: AL, hatched bars: TRFe, and open bars: TRFd. Grey area represent dark phase and food availability is indicated by dotted and hatched boxes for TRFe and TRFd respectively. $^{\$}P<0.05$ overall TRFe or TRFd intervention effect vs AL, $^{\#}P<0.05$ TRFe or TRFd vs AL in HFD mice only.

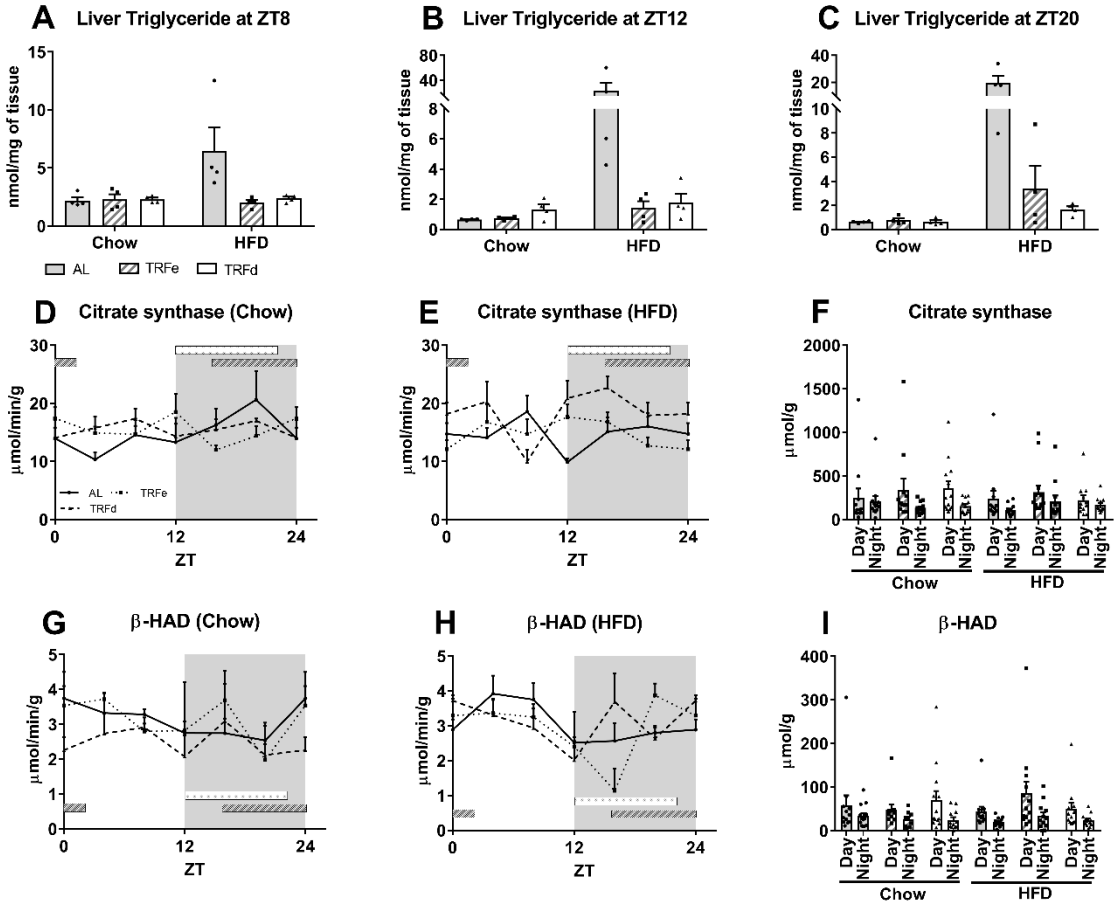

Figure 4.7 Triglycerides levels, and citrate synthase and β-HBD activity in liver. A) liver triglyceride at ZT8 (N=4/group), B) liver triglyceride at ZT12 (N=4/group), C) liver triglyceride at ZT20 (N=4/group), D-F) citrate synthase activity (N=4-5group/time point), G-I) β-hydroxyacyl CoA dehydrogenase activity (N=4/group/time point). Filled bars: AL, hatched bars: TRFe, and open bars: TRFd. Solid lines: AL, dotted line: TRFe, and dashed line: TRFd. Grey area represent dark phase and food availability is indicated by dotted and hatched boxes for TRFe and TRFd respectively. β-HAD: β-hydroxyacyl CoA dehydrogenase.

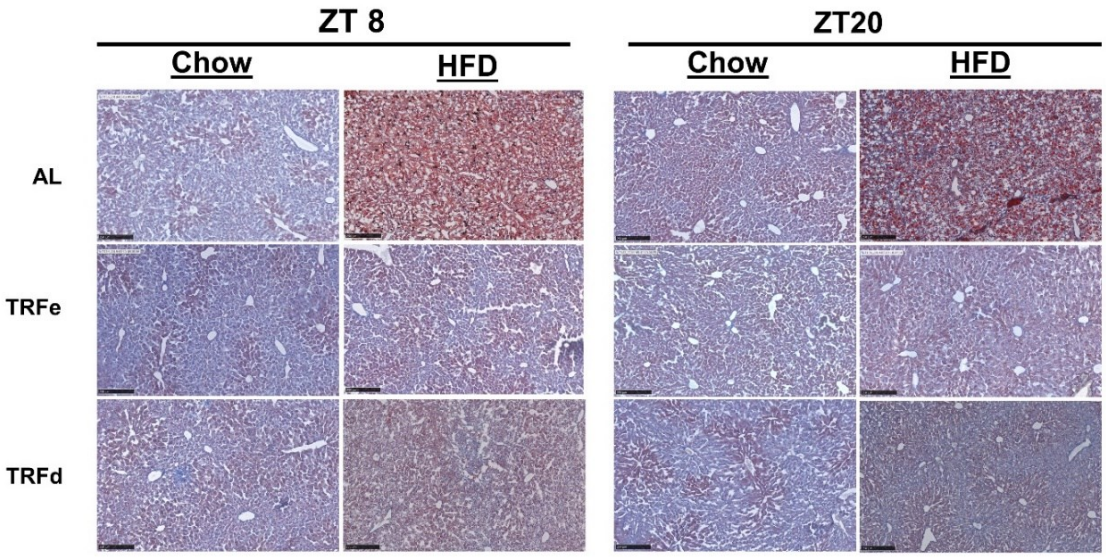

Figure 4.8 Representative images of oil red O staining in liver sections.

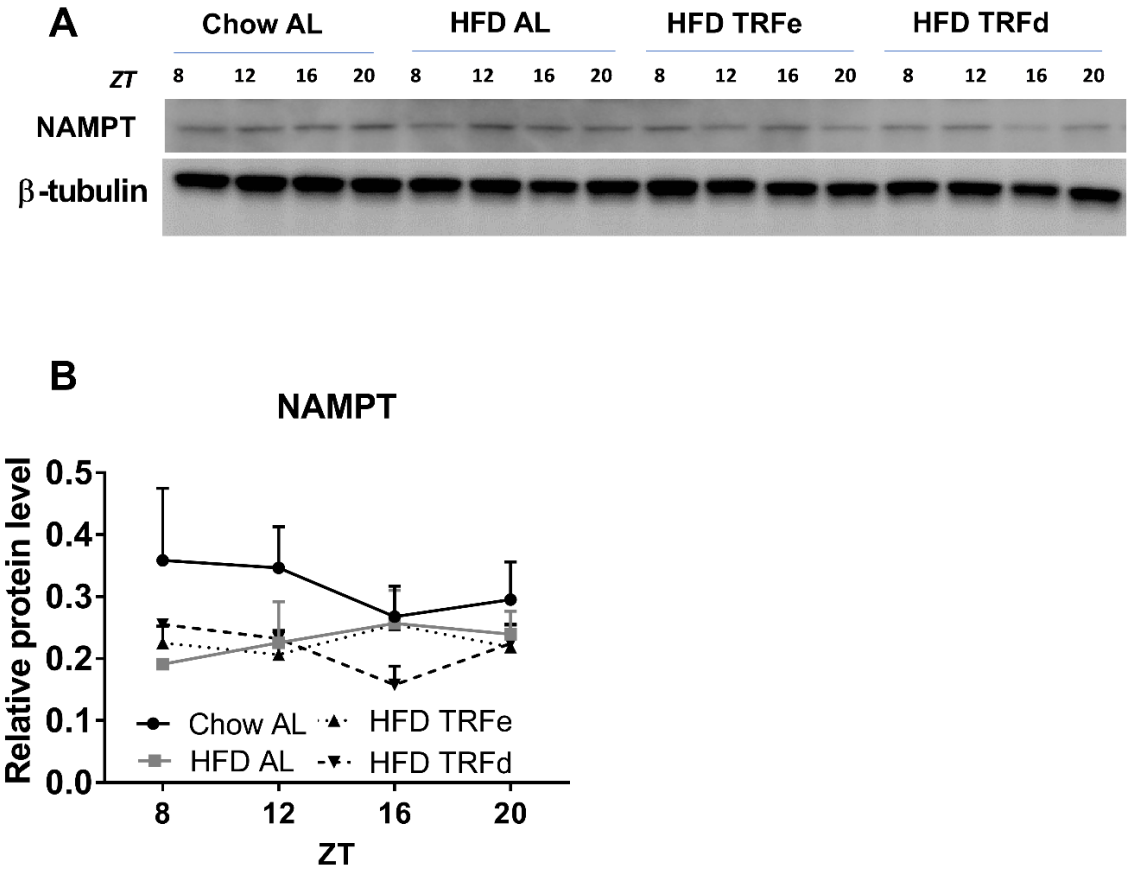

Figure 4.9 Western blots of NAMPT

A) representative western blot images, B) NAMPT protein levels (N=3/group/time point). NAMPT: nicotinamide phosphoribosyltransferase.

Table 4.1 Primers (Taqman) and antibodies

Primers		
	Gene name	Assay ID (catalogue number)
1	*Clock*	Mm00455950_m1
2	*Bmal1 (Arnt1)*	Mm00500223_m1
3	*Per2*	Mm00478099_m1
4	*Cry1*	Mm00514392_m1
5	*Rev-erba (nr1d1)*	Mm00520708_m1
6	*Rora*	Mm1173766_m1
7	*Hprt*	Mm1545399_m1
8	*Nampt*	Mm00451938_m1
9	*Sirt1*	Mm01168521_m1
10	*Nocturnin*	Mm00802276_m1
Antibodies		
11	NAMPT	E-3, sc-393444, Santa-Cruz
13	β-tubulin	Ab-6046, Abcam

Clock: circadian locomotor output cycle kaput, *Bmal1*: brain and muscle ARNT like protein 1, *Per2*: Period2, *Cry1*: cryptochrome1, *Reverba*: nuclear receptor subfamily 1, group D, member 1; *Rorα*: retinoic acid related orphan receptor alpha, *Nampt*: nicotinamide phosphoribosyltransferase, *Sirt1*: sirtuin1.

Table 4. 2 Diets, instruments, kits, chemicals and software used in the study

Items	Source and identifier
Diets	
Chow	Teklad Global 18% Protein Rodent Diet (2018SX) https://www.envigo.com/resources/data-sheets/2018sx-datasheet-0915.pdf
HFD	SF16-001, Specialty Feeds, Australia http://www.specialtyfeeds.com/diets/sf16-001/
Instruments	
echoMRI	EchoMRITM-500 Body Composition Analyzer (EchoMRI LLC, Texas, USA)
Glucometer	Accu-Chek® Performa II, Roche
Metabolic cages	Promethion® BX1 metabolic cages (Sable Systems International, Las Vegas, USA)
Plate reader (Spectrophotometric)	Versamax™ microplate reader, Molecular Devices
Plate reader (Fluorometry)	Glomax® Discover microplate reader, Promega
NanoDrop	NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, CA)
Cryostat	Leica CM 1950, Leica Biosystems
Nanozoomer	NanoZoomer S60 Digital Slide Scanner C13210-01, Hamamatsu, Japan
Thermal cycler (RT)	T100 thermal cycler (Bio-Rad, CA, USA)
Thermal cycler (real time PCR)	QuantStudio 7 (Thermofisher)
Chemiluminescence and fluorescence imaging system	ImageQuant LAS-4000, Fujifilm
Kits	
Insulin	Ultra-Sensitive Mouse Insulin ELISA Kit (#90080 Crystal Chem, USA)
Triglyceride	Triglycerides Assay Kit-Quantification (ab65336, Abcam, USA)
RT kit	QuantiTect Reverse Transcription kit (#205313, Qiagen, USA)
Master mix	Taqman TM Fast Universal PCR Master Mix (2X) (#4352042, Applied Biosystems, Lithuania)
BCA assay	Thermofisher (23228 & 1859078)
Chemiluminescence substrate	Thermofisher (Super signal, 34095)
Chemical	
Phosphate Buffer	Cat. No P4417, Sigma Life Science, USA
TRIZOL	TRI Reagent (T9424, Sigma, USA)
OCT compound	Tissue-Tek O.C.T Compound (IA018, Sakura, Finetek, USA)
Mounting media	glycerine jelly medium (108562; Aquatex, Merk Millipore, VIC, Australia)
Durex K-Y ® Jelly	Reckitt Benckister, NSW, Australia
NP-40	Sigma (MKCD6607)

Sucrose	Sigma (S9378)
Oil red O	Sigma-Aldrich (O-0625)
Tissue-Tek Cryomold	Sakura Finetek (4566), USA
Sodium Dihydrogen Orthophosphate	Chem Supply (SA061)
Sodium Phosphate dibasic	Sigma Aldrich (S0876), USA
Paraformaldehyde	Aldrich Chemistry (441244), USA
Triton X-100	Aldrich (234729), USA
Phosphate buffered saline	Sigma Life Science (P4417)
Potassium hydroxide	Sigma Aldrich (P5958), USA
Ethylenediaminetetraacetic acid disodium salt dihydrate	Sigma (E1644), USA
Oxaloacetic acid	Sigma Life Sciences (04126)
Acetoacetyl Coenzyme A (Sodium salt dihydrate)	Sapphire Bioscience (000-25365)
Acetyl-CoA	Roche Diagnostics GmbH (10101893001), Germany
NADH, disodium salt	Roche Diagnostic (10107735001), Germany
TRIS	Sigma (T6066)
Alcohol dehydrogenase	Sigma (A3263)
Tissue lysis buffer	Sigma (CellLytic MT cell lysis reagent (C3228)
Nicotinamide	Sigma (72340)
NAD	Roche Diagnostics (10127955001)
DTNB	Sigma 9D8130)
Oxaloacetic acid	Sigma (O-4126)
Software	
SPSS	Version 26; IBM Corp., Armonk, New York
GraphPad Prism	Prism for windows, version 7.02
ExpeData software	Version 1.8.2 Sable Systems, Las Vegas, USA
Universal Macro Collection	Version 10.1.3, Sable Systems, Las Vegas, USA
R	R for windows, version 4.0.1
Cosinar	Michael Sachs. Package cosinor. February 19, 2015 (https://cran.r-project.org/web/packages/cosinor/cosinor.pdf)
ImageJ	ImageJ 1.52a, National Institutes of Health, Bethesda, M)

Chapter 5: Time-restricted feeding increases the amplitude of markers of hepatic autophagy in C57BL/6 mice

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Statement of Authorship

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Publication Status	☐ ☐ ☐ ☑
Publication Details	Rajesh Chaudhary, Prashant Regmi, Bo Liu, Julien Bensalem, Timothy J Sargeant, Amanda J Page, Amy T Hutchison, Andrew Vincent, Leonie K Heilbronn. Time-restricted feeding increases the amplitude of markers of hepatic autophagy in C57BL/6 mice.

Principal Author

Name of Principal Author (Candidate)	Rajesh Chaudhary		
Contribution to the Paper	Designed and carried out the study, collected data, performed experiments, analysed data, wrote the manuscript, and approved the final 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. I am the primary co-author of this paper.		
Signature		Date	14 March 2021

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	Prashant Regmi		
Contribution to the Paper	Designed and carried out the primary study, collected data, performed experiments, analysed data, contributed to data interpretation, and approved the final manuscript.		
Signature		Date	15 March 2021

Name of Co-Author	Bo Liu
Contribution to the Paper	Supervised the study, collected metabolic data, contributed to data interpretation, and approved the final manuscript.

Signature		Date	15 March 2021

Name of Co-Author	Julien Bensalem
Contribution to the Paper	Contributed to data interpretation and approved the final manuscript.

Signature		Date	15 March 2021

Name of Co-Author	Timothy J Sargeant
Contribution to the Paper	Contributed to data interpretation and approved the final manuscript.

Signature		Date	15 March 2021

Name of Co-Author	Amanda J page
Contribution to the Paper	Supervised the study, collected metabolic data, contributed to data interpretation and approved final manuscript.

Signature		Date	15 March 2021

Name of Co-Author	Amy T Hutchison
Contribution to the Paper	Contributed to data interpretation and approved final manuscript.

Signature		Date	15 March 2021

Name of Co-Author	Andrew Vincent

Contribution to the Paper	Analysed the data.		
Signature		Date	15 March 2021

Name of Co-Author	Leonie Heilbronn		
Contribution to the Paper	Designed and carried out the study, supervised the study, had full access to the data and had primary responsibility of the final publication.		
Signature		Date	15 March 2021

5.1 Abstract

Background: Hepatic autophagy exhibits circadian rhythmicity and loss of the rhythm affects circadian periodicity of blood glucose level in mice. We recently showed that time-restricted feeding (TRF) increased the amplitude of core clocks in line with the timing of food intake. Here, we assessed the impact of TRF initiated at lights off, or four hours after lights off, on circadian rhythmicity of hepatic autophagy in mice fed chow or high-fat diet.

Methods: C57BL/6 mice (aged 8 weeks, N = 192) were acclimatised to either chow or high-fat diet *ad libitum* (AL) for 4 weeks, before randomisation to one of three regimes: AL, 10 h-TRF at lights off (TRFe), or 4 h after lights off (TRFd) for a further of 8 weeks. An oral glucose tolerance test (1 g/kg) was conducted at 19 weeks of age at ZT4 and ZT16 and body composition determined using an echoMRI. Tissue samples were collected at six timepoints and markers of autophagy were assessed by qPCR and immunoblotting.

Results: TRF reduced body weight and fat mass versus AL mice, with a greater reduction in TRFe vs TRFd. TRF also improved glucose tolerance, but with no significance between TRF groups. TRF increased both the mean and amplitude of *Map1lc3b mRNA levels,* with no significance between TRFe and TRFd. TRFe increased mean *Tfeb* mRNA levels vs TRFd and AL. TRFd delayed the phase in *Map1lc3b* and *Tfeb* mRNA levels vs TRFe and AL. We did not detect an effect of TRF at the protein level.

Conclusions: This study suggests that a structured feeding and fasting cycle drives hepatic autophagy at the mRNA level.

5.2 Introduction

Numerous biological processes at both the physiological and cellular levels exhibit circadian oscillation (Gachon et al. 2006; Hems, Rath & Verrinder 1975; Phillips & Berry 1970). Autophagy, a cellular catabolic housekeeping process, also exhibits circadian regulation in several animal tissues (Ma, Panda & Lin 2011; Pfeifer & Scheller 1975; Pfeifer & Strauss 1981). Microtubule associated protein 1 light chain 3 beta (MAP1LC3B or LC3) lipidation from LC3I to LC3II reaches a peak at Zeitgeber time (ZT), ZT6-9, with a nadir during the dark (feeding) phase (Ma, Panda & Lin 2011). In accordance with autophagy flux, the temporal rhythmicity of autophagosome abundance also peaks at the end of light phase (ZT11) before rapidly decreasing during the mid-dark phase (ZT17) (Ma, Panda & Lin 2011).

Time-restricted feeding (TRF) is a novel dietary intervention that limits daily food intake to 6-12h per day (Longo & Panda 2016). Studies show that TRF reduces body weight, fat mass, fasting glucose, and fasting insulin in both animal (Chaix et al. 2014; de Goede et al. 2019; Hatori et al. 2012; Sundaram & Yan 2016) and humans (Cienfuegos et al. 2020; Hutchison, Regmi, et al. 2019; Jamshed et al. 2019; Sutton et al. 2018) alike. The majority of these findings are based on early TRF (TRFe; TRF initiated during the early hours of biological active phase) versus control (Jamshed et al. 2019; Ravussin et al. 2019; Sutton et al. 2018). However, in our recent animal study, TRF reduced body weight and fat mass with a greater reduction in TRFe vs TRFd (delayed TRF 4h after lights off) and improved glucose tolerance with no difference between TRFe and TRFd (Regmi et al. 2020). This was also accompanied by an increase in amplitude of genes involved in the regulation of circadian rhythms, but with a phase delay in the TRFd group. These findings suggest that despite improving metabolic

health, delaying TRF limits body weight benefits and induces a phase delay in the hepatic clock in mice.

Autophagy is either suppressed or dysfunctional in a number of disease conditions (Alirezaei et al. 2010; Bharath et al. 2020; Henriksen et al. 2019), and is a hallmark of metabolic phenotypes, including obesity (Zhang, Y, Sowers & Ren 2018), insulin resistance (Yamamoto et al. 2018), and hepatic steatosis (Tong et al. 2019). These conditions can be improved by increasing the autophagy response via fasting, starvation or use of drugs such as metformin (Alirezaei et al. 2010; Bharath et al. 2020; Bjorkoy et al. 2005). Thus, we aimed to assess the impact of TRF on mouse hepatic autophagy markers and hypothesised that the metabolic benefits of TRF could be underpinned by increasing the amplitudes of markers of autophagy at the cellular level, but delaying the TRF by 4h would induce a phase delay.

5.3 Research Design and Methods

Animal, diet and metabolic assessment

Ethical clearance for experiments involving animals was acquired from the South Australian Health and Medical Research Institute (SAHMRI) and The University of Adelaide. Animal experiments were performed as per the Australian Code of Practice for The Care and Use of Animals for Scientific Purposes. The animal study design has been previously described (Regmi et al. 2020). In brief, 8-week-old C57BL/6J male mice (N = 192) were acquired from SAHMRI Bioresources and housed 4 mice/cage under a 12h of light:dark cycle with lights on at 19:00 h (ZT0) at 18-24˚C. Mice were fed either the standard laboratory chow (18% calorie from fat, Teklad global 2018SX, Envigo, Madison, USA) or lard-based high fat diet (HFD; 42% calorie from fat, SF16-001, Specialty Feeds, Western Australia, Australia) for 4 weeks. Mice from both diets were then randomised to one of the three following interventions for further 8 weeks:

1) *ad libitum* (AL), 2) 10 h of TRFe initiated at ZT12, and 3) 10 h of TRFd initiated at ZT16. During the study food access was controlled by transferring the mice between cages with and without food, with free access to water. AL mice were also transferred between cages at the same time to standardise the handling. Food consumption was recorded on a weekly basis. After 8 weeks of intervention, mice were sedated using isoflurane at four-hourly intervals (ZT 0, 4, 8, 12, 16, and 20) for blood collection via cardiac puncture. Mice were euthanised following blood collection via cervical dislocation prior to tissue collection (liver, inguinal and gonadal fat pads). Body weight was recorded weekly at the end of the fasting period during cage transfer at the following times: 1) AL, ZT11-12; 2) TRFe, ZT12, and 3) TRFd, ZT16. Body composition was measured at 20 weeks of age at ZT4-5 using an EchoMRI^{TM}-500 Body Composition Analyzer (EchoMRI LLC, Texas, USA) (N = 6/group). At week 19, an oral glucose tolerance test (OGTT) was performed (1 g glucose/kg body weight) during the light phase (ZT4; N = 8/group) and dark phase (ZT16; N = 7-8/group). Blood glucose was measured at 0, 15, 30, 60, 90, and 120 min via tail-bleeding using a glucometer (Accu-Chek ® Performa II, Roche). Plasma samples prepared at 0, 15, 30, and 60 min were stored at -80˚C until insulin assessment was performed using an Ultra-Sensitive Mouse Insulin ELISA Kit (#90080, Crystal Chem, USA). The glucose and insulin area under the curve (AUC) were calculated using the trapezoidal rule (Allison et al. 1995). Metabolic data are presented in Regmi *et al.,* 2020 (Regmi et al. 2020) and in Table 5.1.

Gene expression analysis

Approximately 20-30 mg of liver tissue samples were lysed using TissueLyser LT (Qiagen, Hilden, Germany) at 50 Hz, and total RNA was extracted using a Tri Reagent (T9424, Sigma, St. Louis, USA). The concentration and purity were assessed using a

NanoDrop Lite Spectrophotometer (Thermofisher Scientific, CA, USA) before synthesising cDNA from 1,000 ng of total RNA using a QuantiTect Reverse transcription Kit (#205313, Qiagen, Venlo, Netherlands) on T100 Thermal Cycler (Bio-Rad, Hercules, CA) following the kit instructions. Quantitative real-time PCR (qPCR) was performed for autophagy markers, namely Microtubule Associated Protein 1 Light Chain 3 Beta [*Map1lc3b*, (Mm00782868_sh)], Sequestosome-1, a selective autophagy receptor [*Sqstm1/p62,* (Mm00448091_m1)], and transcription factor EB [*Tfeb*, (Mm00448968_m1)] using TaqMan primers and Fast Universal PCR Master mix (Applied Biosystems, Foster City, CA, USA) with internal negative controls on QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, USA). Relative gene expression analysis was performed using $2^{-\Delta Ct}$ method, where ΔCt is ($Ct_{(target)}$ – $Ct_{(housekeeper)}$). Hypoxanthine phosphoribosyl transferase [*Hprt*, (Mm1545399_m1)] was used as the reference gene.

Immunoblotting

Frozen liver tissues (~20-30 mg) were lysed in Cell Lytic MT Cell Lysis Reagent (C3228, Sigma, Israel) with Halt™ Protease and Phosphatase Inhibitor cocktail (#1861281, Thermo Scientific) using TissueLyser LT (Qiagen, Germany). Protein concentrations were determined using Pierce BCA Protein Assay Kit as per the manufacturer's instructions (#23225, Pierce Biotechnology, Thermo Scientific, USA). Ten micrograms of protein samples were separated in Criterion TM Precast Gel (4-15% Tris-HCL) (#3450029, BioRad, USA) and transferred to polyvinylidene fluoride (PVDF) membrane (#162-0177, BioRad, USA). The membranes were blocked with 1% blocking solution (#11921673001, Roche, Germany) and then probed overnight at 4˚C with the primary antibodies: anti-MAP1LC3B (1:250 dilution, Rabbit polyclonal, NB100-2220, Novus Biologicals, USA), anti-BECLIN1, (1:1000 dilution, Rabbit polyclonal,

NB110-87318, Novus Biologicals, USA), anti-LAMP1 (1:1000 dilution, Rabbit polyclonal, ab24170, Abcam, USA), anti-phospho-S6 Ribosomal protein (Ser240/244) (1:500 dilution, Rabbit polyclonal, #2215, Cell Signaling Technology, USA), anti-Ribosomal protein S6 (C-8) (1:500 dilution, SC-74459, Santa Cruz Biotechnology Inc, CA, USA), anti-Beta Tubulin (1:500 dilution, Rabbit polyclonal, ab6046, Abcam, USA), and respective secondary antibodies (1:10,000 dilution) were used against the primaries for 1 h at room temperature. Membranes were developed using SuperSignalTM West Femto Maximum Sensitivity Substrate (#34095, Thermo Scientific, USA), and scanned on ImageQuant LAS-4000 Chemiluminescence and Fluorescence Imaging System (Fugi Films), and bands were quantified using ImageJ software (National Institute of Health, USA).

Statistical analysis

Non-normally distributed data was log-transformed, and statistics were performed using IBM SPSS Statistics, Version 25.0 (Armonk, NY: IBM Corp.). Statistical analysis was performed by two-way ANOVA with diet (Chow and HFD) and interventions (AL, TRFe, and TRFd) as fixed factors. A differential effect of intervention in each diet was tested via the interaction between diet and intervention, and Bonferroni's *post hoc* was applied. For protein data, comparison between Chow and HFD diets at every individual time-point was made by independent t-test, while the effects of intervention at each time-point within HFD group was assessed by one-way ANOVA. The circadian data on gene expression was analysed on log-transformed data using Cosinor regression using R package *cosinor*, yi:

$$\log y_i = A + B\cos\left(\frac{2\pi t}{24} + C\right) + \epsilon_i, \epsilon_i \sim N(0, \sigma^2)$$

Where A is the mean, B is the amplitude, and C is the phase shift.

5.4 Results

Both forms of TRF increased the mean and amplitude of autophagy genes in mouse liver, but with a phase delay in TRFd

As described previously, body weight gain was lower in both TRFe and TRFd vs AL (Chow: $P < 0.001$ and $P = 0.02$ respectively; HFD: both $P < 0.001$) and in TRFe vs TRFd (Chow: $P = 0.04$; HFD: $P = 0.002$) in chow-fed and HFD mice (**Table 5.1**) (Regmi et al. 2020). In AL-fed mice, HFD did not alter the mean and amplitude (all $P > 0.2$) of any autophagy markers versus chow. TRF increased both mean and amplitude of *Map1lc3b* vs AL (both $P < 0.03$) in both diet groups (**Figure 5.1A**) with no difference between TRFe and TRFd. No effect of HFD or TRF was detected on *Sqstm1* (**Figure 5.1B**). TRFe increased the mean of *Tfeb* vs AL ($P = 0.0003$) and TRFd ($P = 0.03$) (**Figure 5.1C**) in chow-fed mice. There was a phase delay in *Map1lc3b* in TRFd vs TRFe in both Chow and HFD-fed mice ($P = 0.001$ and $P = 0.0003$, respectively) and in *Tfeb* in both Chow and HFD-fed mice ($P = 0.04$ and $P = 0.02$, respectively) (**Figure 5.1A** and **C**). Also, *Map1lc3b* and *Tfeb* had phase delay by TRFd vs AL in Chow-fed mice ($P = 0.03$ and $P = 0.004$, respectively) (**Figure 5.1A** and **1C**).

HFD increased the lipidation of MAP1LC3B and decreased BECLIN1 protein at the end of light phase

TRF did not significantly alter protein levels of any of the autophagy markers that were assessed at any time point (**Figure 5.2A-G**). We limited assessment of protein markers to four time points, and thus cannot perform sinusoidal analysis to assess the circadian rhythms at the protein level. However, BECLIN1 protein levels, were reduced by HFD at ZT8 and ZT12 ($P = 0.035$ and $P = 0.004$, respectively), and HFD increased the

LC3II/I ratio at ZT8 and ZT12 (P = 0.003 and P = 0.018, respectively) versus Chow-AL (**Figure 5.2A** and **2B**).

5.5 Discussion

Autophagy is under circadian oscillation in various mouse tissues (Ma, Panda & Lin 2011) and dysregulation in this system could underpin dysregulated metabolic behaviour, such as altered hepatic lipid metabolism in obesity (Ma, Panda & Lin 2011) and hepatic gluconeogenesis (Toledo et al. 2018). Although TRFe is found to provide metabolic benefits in humans (Jamshed et al. 2019; Ravussin et al. 2019; Sutton et al. 2018) and mice (Delahaye et al. 2018) and increased *MAP1LC3B* in human blood, the effect of delaying initiation of TRF, akin to breakfast skipping, on hepatic autophagy is unclear. This study confirms that autophagy markers are under circadian regulation and showed that both forms of TRF increased the mean and amplitude of hepatic levels of autophagy marker *Map1lc3b,* although only TRFe increased the mean of *Tfeb*. Further, there was a clear phase delay in the expression of autophagy markers in TRFd, confirming that circadian regulation of autophagy is determined by food intake.

Abnormal (either reduced or defective) autophagy is a hallmark of a number of pathological conditions, including insulin resistance in diet-induced obesity (Yang, L et al. 2010), type 2 diabetes (Henriksen et al. 2019), neurodegenerative disease (Alirezaei et al. 2010), age-associated inflammation (Bharath et al. 2020), huntingtin-induced cell death (Bjorkoy et al. 2005). Autophagy is induced by fasting and metformin treatment and is vital for improved metabolic function and healthy aging (Bharath et al. 2020). Autophagy exhibits circadian rhythmicity, as indicated by the change in the relative abundance of LC3 proteins (both LC3I and LC3II) at different time points in mouse liver lysate (Ma, Panda & Lin 2011), lipidation of LC3 (LC3I to LC3II) peaking

between ZT6-9 (mid light phase) and at a nadir between ZT18-1 (mid dark phase). Similarly, abundance of autophagosome was highest at ZT11 (end of light phase) and lowest at ZT17 (beginning of dark phase) before rising again at ZT5 (beginning of light phase), although high-fat feeding appeared to flatten the rhythmicity of the curve, we did not observe any statistical significance in any of the autophagy markers.

In the current study, we have shown for the first time that both forms of TRF (TRFe and TRFd) increased the mean and amplitude of *Map1lc3b* mRNA transcript in both diet groups. Levels peaked by the end of biological inactive phase in both groups, suggesting the response is nutrient-driven. This result concurs with a recent finding in humans, where *MAP1LC3B* mRNA was increased by TRFe in the morning at the end of an 18h fast versus control (12 h fast)(Jamshed et al. 2019). Notably, in that study, the blood samples were collected at only two time points (8pm/ZT13 and 5am/ZT22) and comparisons were made at the single time point between case and control, and thus may be an effect of a phase shift rather than a net change (Jamshed et al. 2019). In the current study, TRFd shifted the phase of *Map1lc3b* by 4h in both diet groups, indicating fasting-driven response. Nutrient starvation activates the AMP-kinase (AMPK) pathway, which in turn inhibits the mechanistic target of rapamycin complex 1, to relieve Unc-51 like autophagy activating kinase 1 Ser757 phosphorylation, leading to ULK1-AMPK interaction (Kim, J et al. 2011). AMPK then phosphorylates ULK1 on Ser317 and Ser777, activating ULK1 kinase, and autophagy induction (Kim, J et al. 2011). Thus, separating feeding time between early and delayed by 4h shifts the two troughs in the rhythmicity by 4h leading to shift in surge of peak of the rhythmicity thereafter by same hours.

TFEB controls autophagy and lysosomal function (Napolitano & Ballabio 2016). TFEB overexpression reduced obesity and metabolic syndrome in mice (Settembre, De Cegli, et al. 2013), while liver-specific knockdown of *Tfeb* exacerbates autophagy-mediated cellular catabolism in liver and induces metabolic imbalance such as, impaired free fatty acid oxidation, reduced circulating level of ketone bodies and reduced peripheral fat mobilisation after 24h and 48h of fasting in obese mice (Napolitano & Ballabio 2016). Mechanistically, in nutrient rich conditions, TFEB is phosphorylated by mTOR and retained in the cytoplasm. During fasting, mTOR is inactivated with a concomitant activation of the phosphatase calcineurin that induces dephosphorylation of TFEB and is translocated to the nucleus (Medina et al. 2015; Settembre, Fraldi, et al. 2013), where it binds with the promoter region of a number of autophagy genes (Settembre et al. 2011). Interestingly, TFEB exhibits circadian rhythmicity and physically interacts with CLOCK/BMAL1 through its N-terminal region and enhances CLOCK/BMAL1-mediated transcription (Luo et al. 2016). In this study, *Tfeb* mRNA transcript exhibited circadian rhythmicity, but was delayed by TRFd, confirming a nutrient driven response in rhythmicity, as previously observed in the mouse liver (Luo et al. 2016). Interestingly, the amplitude of *Tfeb* mRNA transcript was not altered by either TRF group, although mean levels of *Tfeb* were elevated by TRFe. This result indicates that early TRF effectively increases overall *Tfeb* expression and may subsequently increase lysosomal activity.

For the first time we have shown that HFD appeared to flatten the rhythmicity of autophagy markers BECLIN1 and LC3II following visual inspection. This was not statistically significant between the chow and HFD mice possibly due to the following reasonings: 1) Western blot was run on 3-5 samples/time point, as it was not technically and logistically possible to run all 192 samples for 6 targets including

housekeeper from the study that would have resulted into 5-6 samples/ time point to achieve statistical significance, and 2) the protein data are usually difficult to achieve statistical significance compared to gene data and physiological data such as body weight, fat mass, activity and feeding rhythms, to name few. less number of samples (n=3/time point), but HFD has shown to attenuate the diurnal pattern of feeding, locomotor activity behaviour in mice including amplitudes of core clock genes, namely *Clock*, *Bmal1* and *Per2*, in mouse hypothalamus, fat and liver cells when conducted in higher number of mice (n=6-8/time point) (Kohsaka et al. 2007). A limitation of this study is that the protein markers were assessed only in 3 or 5 mice per time point, and thus it is possible that we were simply underpowered to detect the effect.

In conclusion, both forms of TRF increased the amplitude of markers of autophagy at the mRNA level, with a phase shift observed in TRFd mice. The increase in rhythmicity and amplitude of autophagy markers at the cellular level contribute to the metabolic benefits of TRF. Future studies should assess whether TRF increases autophagy or simply results in a phase shift in humans.

Declaration of interest

The authors have no conflict of interest to declare.

Authors' contribution

The primary study was designed by PR and LKH. RC and LKH designed this secondary analysis study. RC performed the experiments and collected data. RC, LKH and AV analysed the data. RC, JB, TJS, BL, ATH, AJP, and LKH contributed to data interpretation and preparation of manuscript. LKH had full access to the data and had primary responsibility for the final publication.

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Table 5.1 TRF-mediated changes in metabolic profile of diet-induced obese mice

Metabolic parameters	Chow (N = 18)			HFD (N = 18)		
	AL	TRFe	TRFd	AL	TRFe	TRFd
Cumulative calorie intake (kcal)	978.05±36.95	920.45±34.56	940.96±34.56	1273.22±36.95	1159.81±36.95	1138.87±36.95*
Fat mass (%)	6.62±3.29	6.15±2.60	7.80±1.19	35.17±2.08	14.04±5.97*	23.79±3.50*
Lean mass (g)	29.36±0.52	27.17±0.52	26.30±0.52	29.25±0.52	28.48±0.52	26.83±0.52
Body weight (g)	31.79±0.51	30.07±0.51	30.83±0.51	43.78±0.51	34.86±0.52*	36.51±0.51*
Gonadal fat (g)	0.018±0.00	0.014±0.00	0.016±0.00	0.055±0.00	0.035±0.00*	0.041±0.00*$^{\$}$
Inguinal fat (g)	0.009±0.001	0.009±0.001	0.008±0.001	0.031±0.001	0.016±0.001*	0.020±0.001$^{\$}$

Data are presented as mean±SEM. Two-way ANOVA performed with Bonferroni *post hoc* correction with diet (chow and HFD) and intervention (AL, TRFe and TRFd) as fixed factors. *P<0.05 vs AL and $^{\$}$P<0.05 vs TRFe. AL: *ad libitum*, TRFe: Time-restricted feeding early, TRFd: Time-restricted feeding delayed.

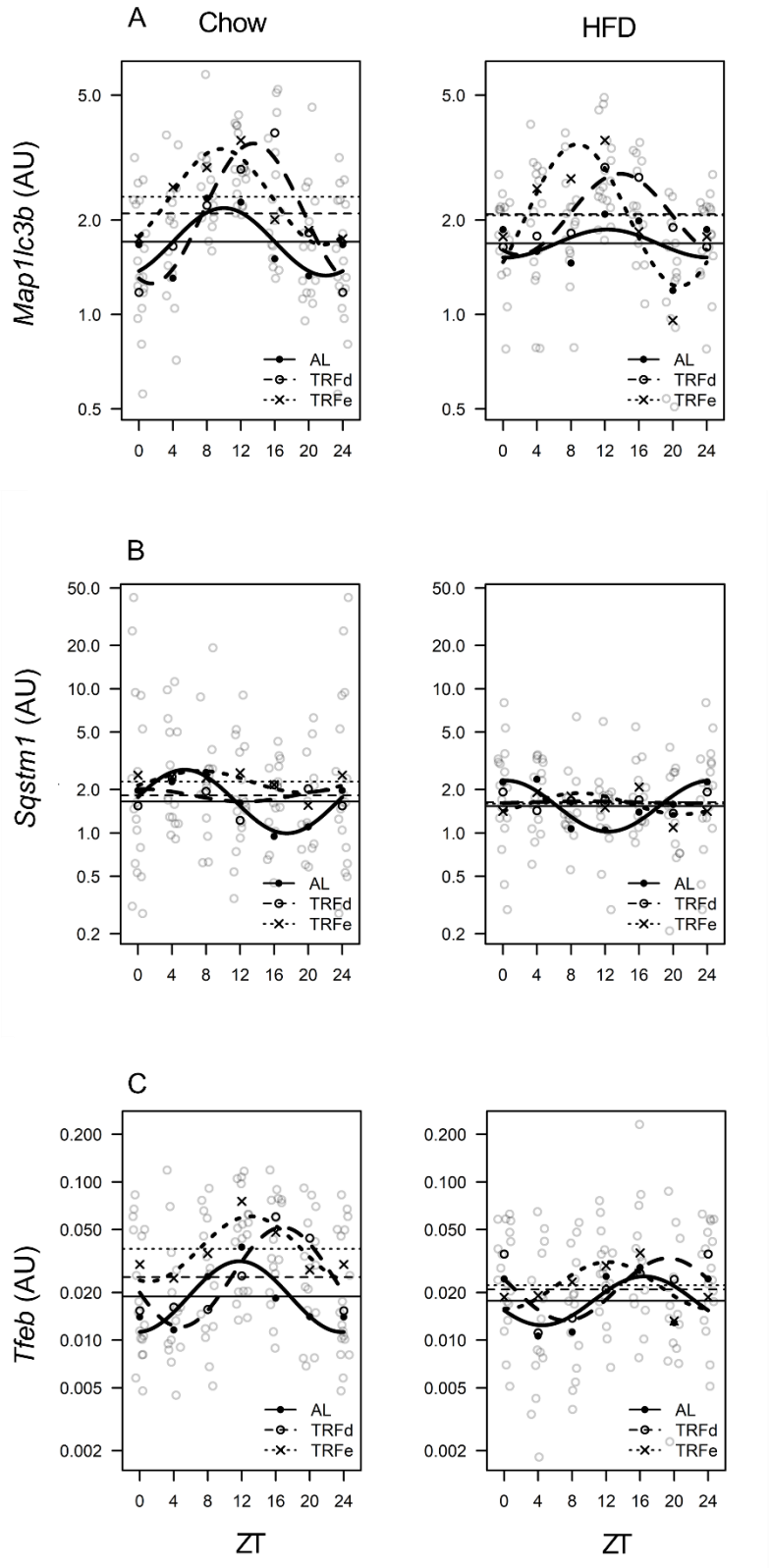

Figure 5.1 TRF increased mean and amplitude of autophagy genes in mouse liver, but with a phase delay in TRFd

(A-C) Cosinor plots of mRNA markers of autophagy based on their relative gene expression at six time points of the day (ZT0, 4, 8, 12, 16, and 20; N = 5-6/time

point/group). Straight horizontal lines represent the mean of all timepoints for each group. ZT0 = ZT24. AL: *ad libitum*, TRFe: Time-restricted feeding early, TRFd: Time-restricted feeding delayed, ZT: Zeitgeber time, AU: Arbitrary unit.

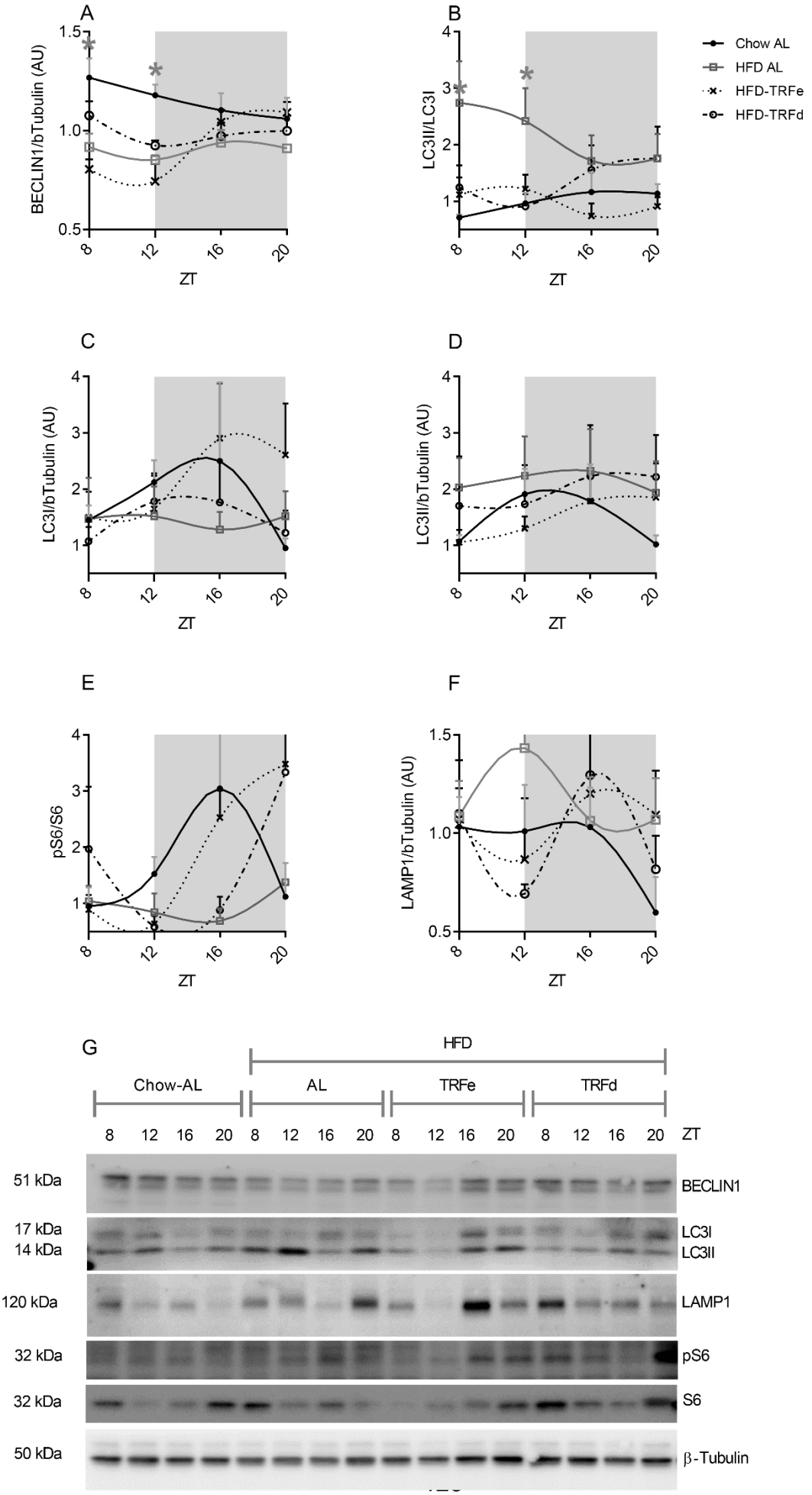
A
BECLIN1/bTubulin (AU)
1.5
1.0
0.5
8
12
16
20
ZT
B
LC3II/LC3I
4
3
2
1
Chow AL
HFD AL
HFD-TRFe
HFD-TRFd
C
LC3I/bTubulin (AU)
D
LC3II/bTubulin (AU)
E
pS6/S6
F
LAMP1/bTubulin (AU)
G
HFD
Chow-AL
AL
TRFe
TRFd
51 kDa
BECLIN1
17 kDa
14 kDa
LC3I
LC3II
120 kDa
LAMP1
32 kDa
pS6
32 kDa
S6
50 kDa
β-Tubulin

Figure 5.2 HFD increased the lipidation of MAP1LC3B and decreased the BECLIN1 protein at the end of light phase.

(A-F) Graphical representation of immunoblot band intensity quantitation of protein markers of autophagy in liver of mouse fed ad libitum (AL) and high-fat diet (HFD) in time-restricted feeding early (TRFe) and delayed (TRFd) protocol (N = 3-5/time point/group). **(G)** Representative immunoblot image of protein markers of autophagy in mouse liver normalised with β-tubulin as loading control. Grey area represents the dark/active phase. Independent t-test between Chow and HFD diets at individual time points, and one-way ANOVA was used between subgroups of HFD mice at each individual time point. **P*-value <0.05 was considered statistically significant. ZT: Zeitgeber time, AU: Arbitrary unit.

Chapter 6: Conclusions

In a randomised controlled trial, the IF intervention led to a greater weight loss including a greater reduction in total cholesterol and LDL vs CR, following 8 weeks of intervention. However, this clinically significant weight loss either by the IF or CR did not alter the anti-inflammatory capacity of isolated HDL or serum from the CR or IF participants who had lost more than 5% of their body weight and were not under lipid lowering medication, when tested in an *in vitro* model. Furthermore, the interventions did not alter the cholesterol efflux capacity of HDL. The findings of this study suggest that moderate weight losses, or fasting, does not alter the biochemical properties of HDL.

Moreover, IF and its sub-form, also known as TRF, is proposed to provide metabolic benefits in mice via activation of autophagy in peripheral organs such as liver and muscle triggered by IF schedule. However, this is not very well tested. Our results suggest that hepatic autophagy in mice is influenced by fasting-feeding cycles and is increased by IF and TRF schedule but are mitigated when exposed to HFD. These results also suggest that ad libitum feeding of HFD increases autophagy in mouse liver probably to protect from adverse metabolic consequences of HFD. Taken together, these data suggest that IF of more than 12 h is sufficient to activate hepatic markers of autophagy in mouse and may aid in beneficial metabolic phenotype. Moreover, muscle autophagy in mouse or human was, however, not affected by IF intervention. This is probably because fasting-induced production of hepatic ketone bodies are avidly oxidised and utilised as energy source by the peripheral organs such as muscle, heart and brain, which may mitigate the necessity to induce autophagy for maintaining cellular energy homeostasis during fasting.

The cumulative calorie consumption was not different between the groups of chow-fed mice, but they tend to consume less when subjected to HFD. Furthermore, both IF and TRF are effective interventions to reduce weight and fat gain and improves glucose tolerance in mice. The lower calorie consumption in HFD fed mice including TRF schedule may have partially contributed to the greater metabolic benefits in these mice compared to chow-fed. TRFd improved metabolic phenotypes, increased amplitudes of genes involved in circadian regulation despite a marginally limited benefits in weight and fat gain including a phase delay in clock genes and markers of NAD metabolism in liver compared to TRFe. The findings of this study suggest that a short delay in food access via TRF schedule, akin to breakfast skipping, do not adversely impacts the metabolic improvements as long as the length of TRF intervention is kept constant. Of note, metabolic difference exists between human and mouse, yet the findings are promising considering its greater potential on transability in human.

In conclusion, this research highlights that energy-restricted intermittent fasting may provide additional health benefits in human compared to continuous energy restriction although neither altered HDL cholesterol efflux and anti-inflammatory action at cellular level. This study also suggests that IF schedule of more than 12h is able to activate autophagy in mouse liver and may aid in metabolic health benefits. However, muscle in both human and mice are spared of IF effect on cellular autophagy level probably due to its ability to utilise ketone as alternative source of energy.

Future directions

The findings of our study suggest that nutritional intervention such as IF is one of the most effect strategies to lose weight and lowering the pro-atherogenic lipids such as LDL and TG. However, it did not show an influence on the cholesterol efflux capacity of HDL or inflammatory markers that have a direct role in the development and progression of atherogenesis. This may be possibly due to the following reasons: 1) lower number of sample process, 2) it may also require looking into chemical composition of the HDL particles and number of HDL subfractions, 3) may be due to variation in subjects under study from different ethnic backgrounds – termed as population-dependent phenomena.

Secondly, upon further examination on the impact of IF at cellular level, we examined its effect on cellular cleaning process known as autophagy. Our findings suggest that IF was able to increase the hepatic autophagy in mice though not in muscle from mouse or human. The observed divergent result between human and mice may be due to the following facts: 1) muscle autophagy from human and mice behave differently to a particular intervention as observed in one recent study where exercise increased the marker of autophagy in mouse while decreased in human.

Thirdly, nutritional intervention such as TRF imparted as either early or delayed settings would have the same metabolic benefits including increasing the amplitudes of CLOCK genes responsible for controlling the metabolism in 24h cycle or the autophagy genes responsible for maintaining the healthy cellular environment.

So, based on the findings of the studies in this thesis and shortcoming of the studies, future study designs should try to answer the following questions:

Is cholesterol efflux capacity of HDL a population-dependent phenomenon?

A large systemic review and meta-analysis suggests that cholesterol efflux capacity of HDL is inversely associated with the cardiovascular risk (Qiu et al. 2017), and is not influenced by either gender or estradiol concentration (Badeau et al. 2013). Lifestyle interventions, such as CR reduces atherosclerosis risk markers in human (Fontana et al. 2004) and improves vascular function (Lefevre et al. 2009). However, cholesterol efflux capacity of HDL was not altered after 16 weeks of CR in individuals with type 2 diabetes, although plasma cholesterol ester transfer protein levels were decreased and apoA1 levels were increased (Wang, Y et al. 2011). In our study also, we did not observe any significant effect of either of IF or CR interventions on the cholesterol efflux capacity of HDL. These discrepancies may be attributed to number of factors that includes type of intervention, degree of energy restriction, length of intervention and the type of population and subjects under study. Interestingly, a large study on Lithuanian population shows that more than half of the participants with normal weight and BMI had below average HDL cholesterol efflux capacity and was inversely associated with their HDL concentration (Kutkiene et al. 2019). The divergent results indicate that future studies should explore population-based approaches with comparative assessment between different populations to rule out if or not cholesterol efflux capacity of HDL is population dependent.

Is autophagy differentially impacted by gender in response to IF interventions?

Several autophagy-related genes are located on the X chromosome including: dynamin and VMA21 proteins involved in lysosomal acidification (Dowling et al. 2015), RAB39B involved in autophagy flux (Cheng et al. 2002; Sellier et al. 2016), and histone deacetylase 6 which promotes fusion of autophagosome and lysosome (Lee, JY et al. 2010; Mahlknecht et al. 2001). Despite this, females appear to have lower level of

autophagy. Human umbilical cord cells from male cells had higher level of BECLIN-1 while higher ratio of LC3II/I was observed in male cells (Addis et al. 2014; Campesi et al. 2016). Similar pattern was observed in animal model, where male rat pups had higher level of LC3II/I compared to female counterpart from same line (Demarest et al. 2016). In our study, although muscle autophagy was not affected by IF in either male mice or women, it is unclear if gender differences may also have played role in this outcome. Thus, future studies should explore the impact of IF in age and sex-matched autophagy in both human and animal models.

Does IF impacts circadian rhythmicity of hepatic autophagy in mice?

Autophagy is under circadian oscillation in various mouse tissues (Ma, Panda & Lin 2011) and dysregulation in this system could underpin dysregulated metabolic behaviour, such as altered hepatic lipid metabolism in obesity (Ma, Panda & Lin 2011) and hepatic gluconeogenesis (Toledo et al. 2018). Time-restricted feeding has found to provide metabolic benefits in humans (Jamshed et al. 2019; Ravussin et al. 2019; Sutton et al. 2018) and mice (Delahaye et al. 2018) and increased blood marker of autophagy, *MAP1LC3B* in human (Jamshed et al. 2019). In our study, for the first time we show that TRF increases the mean and amplitude of hepatic makers of autophagy in mice. However, the impact of IF on the rhythmicity of hepatic autophagy in mice is unknown although IF was found to cause arrhythmicity in *Clock* gene expression in mouse liver and advanced *Per2* and *Clock* expression (Froy, Chapnik & Miskin 2009). Future studies should explore the effect of 8 weeks of IF protocol on rhythmicity of autophagy markers in mice.

Could longer duration of IF regime activate markers of muscle autophagy in mouse and human?

In our study, 8 weeks of IF increased the hepatic markers of autophagy in mouse but not in muscle from either mouse or human. Autophagy was, however, increased in muscles from human (Dethlefsen et al. 2018) and mice (Jamart et al. 2013) in fasted state when exercise was combined, indicating nutritional intervention alone may be mild enough to activate autophagy in muscle or that the fasting length used may be shorter for muscle in comparison to liver. Inflammation on the other hand, can increase or decrease autophagy depending on the type of cytokines involved in a particular setting. Th1 cytokines, including IFN-γ, TNF-α, IL-1, IL-2, IL-6 and TGF-β activates autophagy, while the classical Th2 cytokines, including IL-4, IL-10 and IL-13 inhibits autophagy (Matsuzawa-Ishimoto, Hwang & Cadwell 2018). In our previous finding, IF participants in fasting state had increased M1-macrophage (CD40+) in adipose tissue and M2-macrophage (CD163+) in muscle including an increase in CD68 mRNA expression (Liu, B, Hutchison, et al. 2019). Thus, future studies should explore the impact of longer lengths of fasting on muscle autophagy from human and mice in relation to change in the markers of inflammation in the sample under study.

Can melatonin therapy ameliorate aging-related metabolic outcomes by activating autophagy?

Melatonin exhibits circadian regulation where secretion peaks at night and drops during light phase (Hu et al. 2017; Jauhari et al. 2020; Tordjman et al. 2017). Melatonin secretion in human declines with age including its circadian rhythmicity in majority of elderly people which is accompanied by poor sleep at night and cognitive decline during the daytime (Magri et al. 2004). Skene et al., (Skene 2003) reported that melatonin affects circadian rhythm including sleep quality, immunity, oxidative stress

and incidences of tumour even in visually-impaired person with non-2-h sleep/wake disorder. Melatonin can increase autophagy (Zhang, LF et al. 2015) and found to prevent tauopathy and cognitive decline in mice with Alzheimer's disease (Polito et al. 2014). However, it is unknown whether melatonin therapy protects from age-associated metabolic decline via induction of autophagy.

Does intermittent fasting or caloric restriction have superiority over another in activating autophagy in muscle?

IF and CR provides profound metabolic benefits in both human and rodents alike and partially exerts this effect by activating the autophagy in various peripheral tissues at cellular level (Chung & Chung 2019; Gao et al. 2015; Godar et al. 2015; Liu, H et al. 2017; Wohlgemuth et al. 2007). IF is found to protect mice from ischemia-reperfusion injury (Godar et al. 2015) and short-term food restriction of 24h in mice increased neural and hepatic autophagy (Alirezaei et al. 2010). Similarly, 0-40% of CR activated hepatic autophagy (Derous et al. 2017), mild CR of 8% for life-long attenuated impairment of autophagy in rodent muscle (Wohlgemuth et al. 2010) and 40% of CR for 2 months increased autophagy in rodent (Ning et al. 2013). Our findings show that 8 weeks of IF increases markers of autophagy in mouse liver but not in muscle from mouse or human. Of note, most of the studies are based on different lengths of fasting and/or caloric restriction with different degree of energy restriction in different species and in different interventional settings, and hence the superiority of CR or IF over another is still unanswered. Therefore, future study should explore the impact of energy-matched IF and CR on modulation of autophagy activity in liver and muscle in a sex and age-matched case-control study.

Can long-term energy matched IF and daily CR activate autophagy in human peripheral blood mononuclear cells, and if so whether they have equal impact on autophagy?

IF and CR are effective nutritional interventions that provided greater metabolic benefits by reducing weight, fat mass and increasing glucose tolerance and increasing insulin sensitivity in both human and animal alike (Furmli et al. 2018; Gotthardt et al. 2016; Halberg et al. 2005; Liu, B, Page, Hatzinikolas, et al. 2019). These metabolic benefits are partially imparted via activation of autophagy in peripheral tissues and more so in the metabolically active tissues such as muscle and liver (Godar et al. 2015; Martinez-Lopez et al. 2017). However, the effect on autophagy have been divergent depending on the type of tissue, mode and degree of intervention under study and thus remains controversial (Hsu et al. 2016; Klionsky et al. 2016; Lopez-Vicario et al. 2015; Xiao et al. 2014). Of note, majority of human studies have only been able to assess the markers of autophagy in muscle (Schwalm et al. 2015) and adipose tissue (Kovsan et al. 2011) due to limitation of autophagy flux assessment in, *in vivo* system in human. The assessment of autophagy flux in human, however, is now possible via use of chloroquine in human peripheral blood mononuclear cells (PBMC) (Bensalem et al. 2020). Thus, future study should consider using PBMC from both IF and daily CR participants and perform a comparative assessment of autophagy flux in long run.

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