IMPACT OF TIME RESTRICTED FEEDING ON GLUCOSE METABOLISM AND METABOLIC HEALTH

THE UNIVERSITY of ADELAIDE

A thesis submitted by

Prashant Regmi

BSc MLT, MSc clinical biochemistry

For the degree of

Doctor of Philosophy

Discipline of Medicine

Adelaide Medical School

The University of Adelaide

January 2021

Table of Contents

Summary

Lifestyle induced metabolic diseases such as obesity, type 2 diabetes and cardiovascular diseases are often associated with increased energy intake, and reduced levels of physical activity. However, accumulating evidence suggests that *when* we eat may be a contributing factor to chronic disease progression. Eating out of phase with daily circadian rhythms induces metabolic desynchrony in peripheral metabolic organs in mice, and may increase chronic disease risk. Time restricted feeding (TRF, also known as time restricted eating) is a novel dietary tool that limits the duration of the daily food intake window to 6-12 hours, without altering calorie intakes or diet quality. In preclinical models, TRF reduced diet-induced weight gain and hepatosteatosis, improved glucose tolerance, mitigated age-induced decline in cardiac function and improved muscle function, and protected from the metabolic milieu of diverse nutritional challenges, including high-fat-diet (HFD) and high-fat-high-sucrose diet. In human trials, that are currently limited in size and number, TRF also reduced body weight and fasting glucose, improved glucose tolerance, reduced blood pressure, and reduced atherogenic lipids in people with overweight and obesity.

However, the majority of TRF interventions in animal models and in humans have been initiated early in the active phase. Implementing TRF initiated early in the morning may be challenging in the general population both biologically and socially. Hunger is lowest in the morning due to a circadian nadir in ghrelin. Furthermore, family and communal get togethers are essential factors to increase social bonding capable of providing social and emotional support, however many social events are typically geared towards evening. Delaying the initiation time of TRF (i.e. allowing food consumption for identical time lengths later in the day) may overcome both of these issues, but the metabolic consequences of delayed TRF are unclear. This thesis examined the impact of early and delayed TRF in humans and in mice.

We carried out the first human trial (randomized cross-over) examining the acute effects of early versus delayed TRF in overweight men at the risk of type 2 diabetes. After baseline assessment for one week, participants were randomised to eat their habitual diets within a 9-hour period starting early (8am-5pm, TRFe) or delayed (12pm-9pm, TRFd), separated by a 2-week wash out period. One-week of TRF improved glucose tolerance, modestly reduced body weight, reduced fasting triglycerides, and reduced fasting glucose as measured by continuous glucose monitoring. These results were independent of gastric emptying or physical activity. Importantly, there was no statistically significant difference in any of above results between TRFe and TRFd. This study suggested that TRF improved markers of health, irrespective of whether it was initiated at 8am or 12pm.

To further examine the long-term effects of early versus delayed TRF on metabolic phenotypes and circadian rhythms in peripheral organs, we carried out an 8-week early versus delayed TRF study in chow or high-fat diet fed mice. After four weeks of *ad libitum* feeding with chow or high-fat diet, mice on each diet were randomized to one of three interventions: i) continue *ad libitum*, ii) 10-hour TRF initiated at ZT12 (TRFe), and iii) 10-hour TRF initiated at ZT16 (TRFd) for a further 8-weeks. This study showed that both forms of TRF reduced weight and fat gain, improved glucose tolerance, reduced hepatosteatosis, and increased metabolic flexibility. We measured the mRNA levels of key genes involved in circadian regulation in the liver as an index of peripheral circadian rhythm. We observed that both forms of TRF increased the amplitude of genes involved in circadian regulation and markers of nicotinamide adenine dinucleotide (NAD) metabolism in liver compared to *ad libitum*. TRFd marginally limited the benefits in weight and fat gain compared to TRFe, and induced a phase delay in body temperature, and clock genes and markers of NAD metabolism in liver. However, a phase delay in key circadian genes in liver did not adversely impact the improvement in metabolic phenotypes in TRFd, as well as there was no

statistically significant difference in measured metabolic phenotypes and amplitudes of circadian genes in liver between TRFe and TRFd.

Additionally, we explored whether intermittent fasting (IF) impacted markers of NAD metabolism in skeletal muscle, and whether this was associated with metabolic switching from fed to fasting day in overweight women and chow or high-fat diet fed mice. We demonstrated that insulin sensitivity was transiently reduced in overweight women on the fasting day during IF, which may help to spare glucose. At the molecular level, the rise in *NAMPT* expression on fasting day may facilitate the lipid oxidation pathways in skeletal muscle.

In conclusion, this research showed that TRF improves metabolic health whether initiated early or delayed (akin to skipping breakfast), when there are equidistant transitions between fasting-feeding cycles. Uniquely, we demonstrate the metabolic benefits of TRFd occur alongside a phase delay in hepatic clocks and metabolic markers, but with increases in the amplitude and/or mean of genes involved in nutrient signalling and circadian regulation. Flexibility to initiate TRF a few hours later in the day could increase the translational potential of this promising dietary tool in the general population. Further, a transient reduction in insulin sensitivity and rise in skeletal muscle *NAMPT* expression in response to the fasting day may facilitate metabolic switching from glucose to fat oxidation in intermittent fasting.

Declaration

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

I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Name: Prashant Regmi

Signature: Date: 12 January 2021

Acknowledgement

Foremost, I express my special thanks to my principle supervisor Associate Professor Leonie Heilbronn. It was a great privilege and honour to study under her supervision. I have been deeply inspired and influenced by her dynamism, vision, critical thinking and motivation in research. This research would not have been possible without her patient guidance, continuous encouragement, and crucial advice throughout my candidature. I would also like to thank my co-supervisors Professor Amanda Page and Dr. Amy Hutchison for their kind cooperation and precious time throughout the study.

My special thanks to Mr. Rajesh Chaudhary and Dr. Bo Liu for their support during animal study. Thanks to Dr. Andrew Vincent and Kylie Lange for their statistical support. I would also like to thank staffs of SAHMRI bioresources for their continuous support during mice study. Also, I would like to thank my group members Lijun Zhao, Xiao Tong Teong and Kai Liu for their continuous encouragement.

I would like to express my eternal gratitude to my parents Mr. Krishna Prasad Regmi & Mrs. Pabitra Regmi, wife Mrs. Manson Pandey, and brother Mr. Prachand Regmi for their constant support, attention and encouragement to build up my career. I feel privileged to acknowledge and thank everyone who contributed to the completion of this study.

Finally, I also wish to thank those critics who have helped me with their constructive suggestions and ideas, without which my study would be poorer.

Prashant Regmi

January 2021

Conference proceedings

Prashant Regmi, Amy T Hutchison, Gary A Wittert, Leonie K Heilbronn. Time restricted feeding improves glucose tolerance in men at risk of type 2 diabetes. Joint scientific meeting of the Australia and New Zealand obesity society and the obesity surgery society of Australia and New Zealand, Adelaide, Australia 2017 [oral presentation].

Prashant Regmi, Bo Liu, Gary A Wittert, Amanda J Page, Amy T Hutchison, Leonie K Heilbronn. Effect of intermittent fasting on whole body insulin sensitivity and skeletal muscle NAD and NAMPT mRNA levels in humans and mice. Florey postgraduate research conference, Adelaide, Australia 2018 [poster presentation].

Prashant Regmi, Bo Liu, Gary A Wittert, Amanda J Page, Amy T Hutchison, Leonie K Heilbronn. Effect of intermittent fasting on whole body insulin sensitivity and skeletal muscle NAD and NAMPT mRNA levels in humans and mice. SAHMRI annual meeting, Adelaide, Australia 2018 [poster presentation].

Prashant Regmi, Rajesh Chaudhary, Amanda J Page, Bo Liu, Leonie K Heilbronn. Time restricted feeding improves metabolic outcomes in mice with and without a phase delay in initiation. Australia and New Zealand obesity society conference 2019, Sydney, Australia [oral presentation].

Prashant Regmi, Rajesh Chaudhary, Amanda J Page, Bo Liu, Leonie K Heilbronn. Time restricted feeding improves metabolic outcomes in mice with and without a phase delay in initiation. Obesity Week, Las Vegas, USA 2019. This abstract was selected as one of the twelve top scoring abstracts in basic science and was invited to present in lightning talk [oral presentation].

List of Tables

List of Figures

List of Appendices

List of Abbreviations

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

ACTB: β-actin

ADH: Alcohol dehydrogenase

AICAR: 5-Aminoimidazole-4-carboxamide ribonucleotide

AL: Ad libitum

AMP: Adenosine monophosphate

AMPK: 5' AMP-activated protein kinase

ANOVA: Analysis of variance

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

Bmal1: Brain and muscle ARNT-like 1

BMI: Body mass index

cDNA: Complementary deoxy-ribonucleic acid

CGM: Continuous glucose monitoring

CKϵ: Casein kinase epsilon

Clock: Circadian locomotor output cycles kaput

CONGA: Continuous overall net glycaemic action

CRP: C-reactive protein

Cry1: Cryptochrome 1

CT: Cycle threshold

DBP: Diastolic blood pressure

DPP-IV inhibitor: Dipeptidyl peptidase-IV inhibitor

EDTA: Ethylenediaminetetraacetic 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

ϒ-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

HbA1c: Glycosylated haemoglobin

HFD: High fat diet

HOMA-IR: Homeostatic model assessment of insulin resistance

HPRT: Hypoxanthine phosphoribosyltransferase

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

LDL: Low-density lipoprotein

MAGE: Mean amplitude of glycaemic excursions

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 phosphoribosyltransferase

oxLDL: Oxidized-LDL

PCR: Polymerase chain reaction

Per2: Period 2

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

PPIA: Peptidylprolyl isomerase A

pS6/S6: Phospho/ ribosomal protein S6

PYY: Peptide tyrosine tyrosine

RCT: Randomized controlled trial

Rev-erb*a*: Nuclear receptor subfamily 1, group D, member 1

RMR: Resting metabolic rate

ROR*a*: Retinoic 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 sulfate polyacrylamide gel electrophoresis

SEM: Standard error of mean

SIRT1: Sirtuin 1

SREBP: Sterol regulatory element-binding protein

T2D: Type 2 diabetes

TCA cycle: Tricarboxylic acid cycle

TG: Triglycerides

TRF/TRE: Time restricted feeding/eating

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

TRFd/ dTRF/ dTRE: Delayed time restricted feeding/eating

TSH: Thyroid stimulating hormone

UCP1: Uncoupling protein 1

VCO2: Volume of carbon dioxide

VO2: Volume of oxygen

WC: Waist circumference

ZT: Zeitgeber time

Research questions, aims and hypothesis

Chapter 1: Research questions, aims and hypothesis

1.1 Background

Lifestyle induced metabolic disorders such as obesity, type 2 diabetes and cardiovascular diseases are major public health challenges, and their prevalence continues to rise worldwide, reaching the level of a global pandemic (Saklayen 2018). Lifestyle modification, such as nutrition interventions, are the first-line defence in the prevention and treatment of those metabolic disorders (Ricanati et al. 2011). However, current dietary practice guidelines have mainly focused on modification of quantity and quality of foods (NHMRC 2013).

As extensively reviewed in chapter 2 and 3, in addition to the effects of energy imbalance and suboptimal nutrient consumption, emerging evidences show that when we eat may be a contributing and modifiable risk factor in chronic disease progression (Jiang & Turek 2017). Time-restricted feeding (TRF, also known as time-restricted eating, TRE) is a dietary tool that restricts the food consumption for 6-12 hours during the active phase of the day without altering calories or diet quality. TRF shows pleiotropic metabolic health benefits in both preclinical models and humans (Chaix et al. 2014; Gabel et al. 2018; Gill & Panda 2015; Hatori et al. 2012; Sutton et al. 2018; Villanueva et al. 2019; Wilkinson et al. 2019), which has raised hope in TRF as a dietary tool for the prevention and therapy of metabolic disorders. However, most TRF studies have initiated their protocol early in the morning. Implementing early TRF in the general population may be challenging both biologically and socially (Dunbar 2017; Espelund et al. 2005; Qian et al. 2018). Delaying the initiation time of TRF can circumvent both issues, but the metabolic consequences of delayed TRF are unclear.

1.2 Research questions

This research aims to answer the following questions:

1. Does an equidistant TRF initiated early in the morning (TRFe) or delayed by 4-hour (TRFd) show similar improvements in glycaemic profile in overweight men at risk of type 2 diabetes?

2. Does an equidistant TRF initiated at ZT12 (TRFe) or ZT16 (delayed by 4-hour, TRFd) show similar improvements in metabolic phenotypes in chow or high fat diet fed mice?

3. Does an equidistant TRFe or TRFd show similar increase in the amplitudes of key circadian genes and markers of NAD metabolism in mouse liver?

4. Does 4-hour delay in TRF initiation 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 intermittent fasting impact markers of NAD metabolism in skeletal muscle? If yes, is this associated with metabolic switching from glucose to fat oxidation in skeletal muscle from fed to fast day?

1.3 Aims and hypothesis

a. TRF study in humans

The aim of this study was to examine the effects of 9-hour TRF initiated at 8am or 12pm for one week on glucose tolerance in overweight men at risk of type 2 diabetes, compared to baseline condition.

Hypothesis: We hypothesised that 9-hour TRF initiated at 8am or 12pm would improve glucose tolerance in overweight men at risk of type 2 diabetes.

b. TRF study in mice

The aim of this study was to test the effects of 10-hour 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 equidistant TRF initiated at ZT12 or ZT16 were capable to mitigate the adverse metabolic consequences of HFD.

Hypothesis: We hypothesised that TRFe or TRFd would be beneficial in the prevention of the metabolic consequences of HFD in mice, and there would be no statistical difference between the magnitudes of improvements in metabolic phenotypes between TRFe and TRFd. TRFd will induce a delay in the phase of body temperature, and key hepatic circadian genes and markers of NAD metabolism.

c. Intermittent fasting study in humans and mice

The aim of this study was to examine whether IF impacted markers of NAD metabolism in skeletal muscle, and to explore whether this was associated with metabolic switching from fed to fasting day in humans and mice.

Hypothesis: We hypothesized that IF would increase *NAMPT* expression in skeletal muscle along with plasma and physiological markers of lipid oxidation during the fed to fast transition.

1.4 Outline of thesis

In addition to the research questions, aims and hypothesis, this thesis is organized into a series of published and unpublished articles. This includes a review of the current literature, a perspective, and three original research articles (3 published, 1 submitted, and 1 written in manuscript format). The terms time restricted feeding (TRF) and time restricted eating (TRE) are used interchangeably in this thesis.

Chapter 2 is entitled "Time restricted eating: benefits, mechanisms and challenges in translations" and was published in *iScience* in 2020. This review discusses the importance of meal timing on the circadian system, the metabolic health benefits of TRE in preclinical models and humans, the possible mechanisms of action, the challenges we face in implementing TRE in humans, and the possible consequences of delaying initiation of TRE. We proposed that delaying TRE will address both biological and social issues of early TRE, and increase the translational potential of TRE in general population.

Chapter 3 is entitled "Will delaying breakfast mitigate the metabolic health benefits of time restricted eating?" and was published in *Obesity* in 2020. This perspective discusses the potential challenges in translating early TRE to the community and considers the potential metabolic consequences of delaying TRE. We proposed that delaying TRE will address both biological and social issues of early TRE, and increase translational potential of TRE in general population.

Chapter 4 is entitled "Time restricted feeding improves glucose tolerance in men at risk of type 2 diabetes: a randomized crossover trial" and was published in *Obesity* in 2019. This paper examined the effects of 9-hour TRF initiated at 8am or 12pm for one-week on body weight, glucose tolerance, gastro-intestinal hormones, continuous glucose monitoring, gastric emptying, and activity. We showed that 9-hour TRF initiated at 8am or 12pm

improved glucose tolerance to a mixed nutrient liquid meal. This paper highlighted that TRF shows comparable metabolic improvements whether initiated early or a few hours later in the morning, unless there is equidistant transition between fasting and feeding cycles.

Chapter 5 is entitled "Early or delayed time restricted feeding prevents the metabolic impacts of obesity in mice" and is accepted for publication in *Journal of Endocrinology*. This study examined the effects of 10-hour TRF initiated at lights off (ZT12, TRFe) or 4 hours after lights off (ZT16, TRFd) on body weight, fat mass, glucose tolerance, nutrient homeostasis, hepatosteatosis, and clock genes and markers of NAD metabolism in liver. This study showed that TRFd marginally limited the benefits in weight and fat gain compared to TRFe, but improved metabolic phenotypes and increased the amplitudes 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.

Chapter 6 is entitled "Intermittent fasting increases *NAMPT* expression in human skeletal muscle on fasting day" and will be submitted for publication. This study examined the effects of 8-weeks of intermittent fasting on skeletal muscle markers of NAD metabolism in women with obesity and a chow or HFD fed mice. This study showed that a reduction in peripheral insulin sensitivity and a rise in skeletal muscle *NAMPT* expression may underlie the mechanisms behind metabolic switching to fat oxidation in intermittent fasting in humans. However, intermittent fasting did not alter markers of NAD metabolism in mouse skeletal muscle, possibly due to difference in circadian time of tissue collection.

Literature Review

Chapter 2: Time restricted eating: benefits, mechanisms, and challenges in translation.

Prashant Regmi[1,2,3], Leonie K Heilbronn[1,2,3,4]

[1]Adelaide Medical School, The University of Adelaide, Adelaide 5000, South Australia, Australia

[2]Life-long health research theme, South Australian Health and Medical Research Institute, Adelaide 5000, South Australia, Australia

[3]NHMRC Centre of Research Excellence in Translating Nutritional Science to Good Health, University of Adelaide, Adelaide 5000, South Australia, Australia.

[4]Corresponding author and lead contact.

Statement of Authorship

Title of Paper	Time restricted feeding: Benefits, Mechanisms, and Challenges in Translation.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Regmi, P., Heilbronn, L.K, Time Restricted eating: Benefits, Mechanisms, and Challenges in Translation. iScience. 2020, 23(6): 101161. doi: https://doi.org/10.1016/j.isci.2020.101161.

Principal Author

Name of Principal Author (Candidate)	Prashant Regmi		
Contribution to the Paper	Searched and reviewed literature, interpreted data, wrote the manuscript, and approved 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	16 August 2020

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	Leonie Heilbronn		
Contribution to the Paper	Interpreted data, wrote the manuscript and approved final manuscript.		
Signature		Date	21/8/2020

2.1 Highlights

- Time restricted eating protects from metabolic consequences of obesity in animals.
- Most studies initiate TRE early during the active phase.
- The metabolic consequences of a short delay in initiation of TRE are unclear.

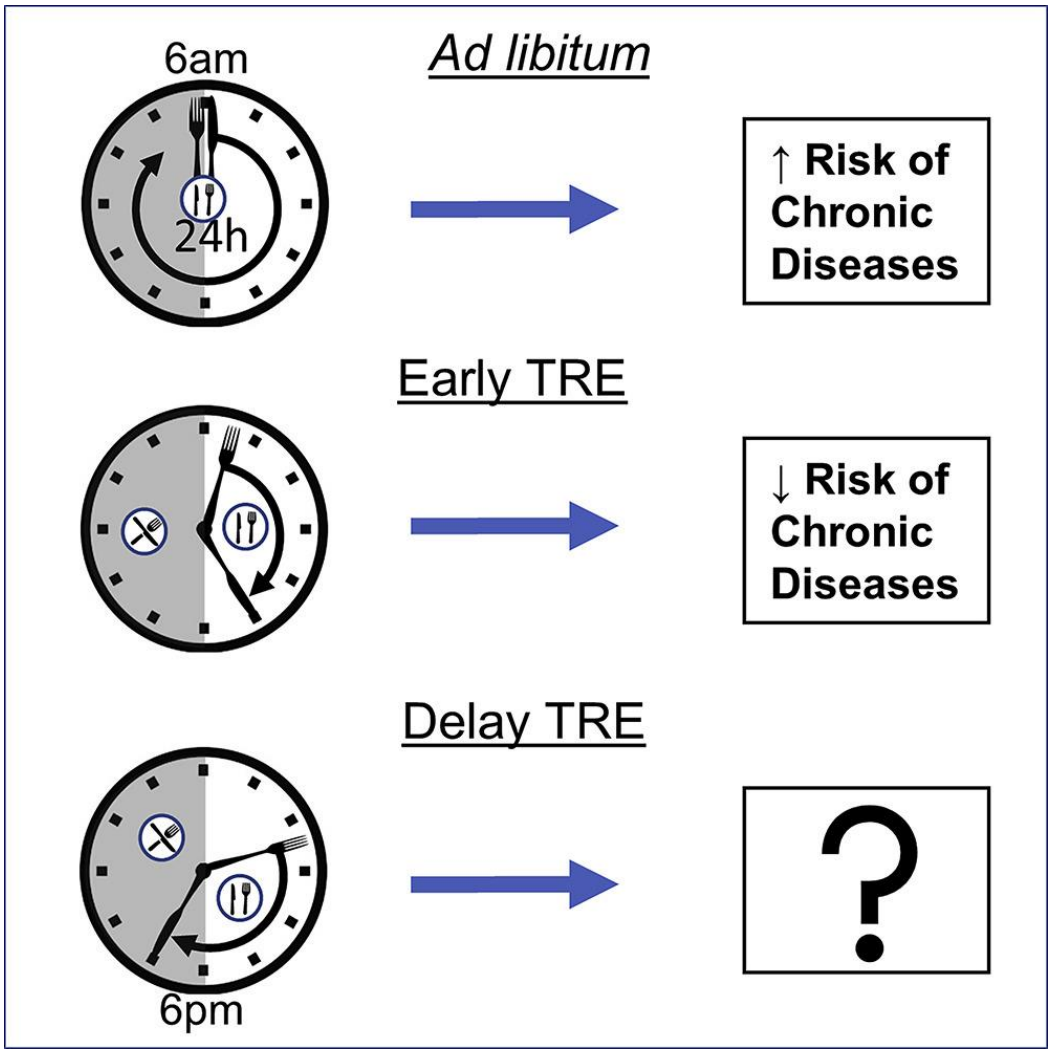


Figure 2. 1 Graphical abstract

2.2 Summary

Eating out of phase with daily circadian rhythms induces metabolic desynchrony in peripheral metabolic organs, and may increase chronic disease risk. Time restricted eating (TRE) is a dietary approach that consolidates all calorie intake to 6 to 10-hour periods during the active phase of the day, without necessarily altering diet quality and quantity. TRE reduces body weight, improves glucose tolerance, protects from hepatosteatosis, increases metabolic flexibility, reduces atherogenic lipids and blood pressure, and improves gut function and cardiometabolic health in preclinical studies. This review discusses the importance of meal timing on the circadian system, the metabolic health benefits of TRE in preclinical models and humans, the possible mechanisms of action, the challenges we face in implementing TRE in humans, and the possible consequences of delaying initiation of TRE.

Keywords: Biological sciences, chronobiology, nutrition

2.3 Introduction

Lifestyle induced metabolic diseases, such as type 2 diabetes (T2D) and cardiovascular disease, are often associated with obesity, and reductions in physical activity and increased consumption of energy dense foods. Accumulating evidence suggests that *when* we eat may be another contributing factor to chronic disease progression (Andrzejczak, Kapala-Kempa & Zawilska 2011). Lengthened daily eating patterns, in excess of 14 hours/day, were evident in studies conducted in the USA and India, with less than 25% of caloric intake occurring prior to 1 pm (Gill & Panda 2015; Gupta, NJ, Kumar & Panda 2017). Time restricted eating (TRE) is a novel dietary tool that recommends individuals shorten the duration of the daily eating window, without altering calorie intakes or diet quality. TRE restores circadian rhythms and imparts pleiotropic metabolic benefits in animal models (Chaix et al. 2014; Delahaye et al. 2018; Gill et al. 2015; Hatori et al. 2012; Olsen et al. 2017; Villanueva et al. 2019; Wang, HB et al. 2018; Woodie et al. 2018). TRE also reduces body weight and fat mass, improves glucose tolerance and reduces blood pressure in humans, particularly in those with overweight or obesity (Figure 2. 2) (Gabel et al. 2018; Gill & Panda 2015; Hutchison, Regmi, et al. 2019; Sutton et al. 2018; Wilkinson et al. 2019). The studies to date in humans are limited in size and duration, and the effectiveness and acceptability of TRE in the general population remains unclear. The majority of TRE studies have also initiated the eating window early in the active phase, presumably to maximize the metabolic benefits. This review will discuss the metabolic benefits of TRE in preclinical models and the possible mechanisms of action. We also discuss the likely challenges of implementing TRE in humans and the possible consequences of delaying initiation of TRE.

2.4 Regulation of central and peripheral clock machinery

Circadian rhythms are ubiquitous periodic oscillations in internal biological process that direct behaviour and metabolism such as hormonal signalling, body temperature, nutrient absorption and metabolism (Dongen 2017; Espelund et al. 2005; Panda et al. 2002; Reppert & Weaver 2002). The suprachiasmatic nucleus (SCN) is considered the master regulator of circadian rhythms and is primarily entrained by the light-dark cycle. At the molecular level, circadian rhythms arise from tightly controlled autonomous interlocked genetic transcriptional feedback loop that involves *circadian locomotor output cycles kaput* (*clock*) and *brain and muscle ARNT like protein 1* (*bmal1*) as positive transcriptional factors for *period* (*per1, per2, per3*) and *cryptochrome* (*cry1, cry2*) genes (extensively reviewed in (Hastings, Maywood & Brancaccio 2018)). The translation products of *per* and *cry* dimerize and act as negative regulators by inhibiting *clock* and *bmal1*. An additional feedback loop involves the transcriptional regulation of *bmal1* by *retinoic acid related orphan receptor* (*rorα*) and *nuclear receptor subfamily 1, group D, member 1*(*rev-erbα*). One cycle of this feedback loop takes ~24 hours and is the basis of circadian rhythms in many organisms. This feedback loop also operates in peripheral tissues, including the liver, skeletal muscle, adipose tissue, pancreas, and intestine, which are not directly entrained by light (Reppert & Weaver 2002; Yoo et al. 2004).

Peripheral clocks are exquisitely sensitive to the fasting - feeding cycle and as discussed in the next section, can be uncoupled from the central clock through modifications in meal delivery (Damiola et al. 2000). At the molecular level, fasting increases the AMP/ATP ratio, activating 5' AMP-activated protein kinase (AMPK). This in turn phosphorylates serine71 of cry1, reducing its stability (Lamia et al. 2009). AMPK also regulates the activity of casein kinase I epsilon via its phosphorylation at serine389, which is a critical regulator of *per*

phosphorylation and stability (Meng et al. 2008). Nicotinamide adenine dinucleotide (NAD^+) is a cofactor of several key pathophysiological enzymes and an absolute requirement of sirtuin 1 (SIRT1, a NAD^+ dependent histone deacetylase). The majority of cellular NAD^+ comes from its salvage pathway where nicotinamide phosphoribosyltransferase (NAMPT) is the rate-limiting enzyme (Poljsak 2018; Zhang et al. 2017). Fasting activates NAMPT, thus increasing the cellular availability of NAD^+, activating SIRT1. Activated SIRT1 has been shown to directly bind with *clock:bmal1* and represses the transcription of *per2* (Ramsey et al. 2009). Thus, fasting also reduces both transcription and stability of *per* and *cry*, which de-represses *clock:bmal1* targets and increases their amplitude (Lamia et al. 2009; Um et al. 2007). SIRT1 has also been described to regulate the acetyltransferase activity of *clock* (Figure 2. 3) (Doi, Hirayama & Sassone-Corsi 2006; Nakahata et al. 2008). In contrast, the mechanistic target of rapamycin (mTOR), a nutrient-activated serine/threonine protein kinase, is activated during the fed state. This post-transcriptionally induces *cry1* through an unknown mechanism (Ramanathan et al. 2018). Feeding also suppressed NAMPT function, reduced cellular NAD^+, inactivating SIRT1. This abrogated SIRT1 mediated suppression of *clock:bmal1* and increased *per2* transcription (Ramsey et al. 2009). Hence, fasting increases the positive limb of circadian clock (*clock* and *bmal1*), whereas feeding increases the negative limb of circadian clock (*cry* and *per*).

2.5 Mealtime is a strong entraining cue of peripheral clocks and subsequently impacts metabolism and risk factors for chronic disease:

Disrupted feeding therefore has a marked effect on the expression of the molecular clock in peripheral tissues (Jiang & Turek 2017) and uncouples this from the SCN (Damiola et al. 2000). For example, restricting food access solely to the light phase in mice, when this nocturnal animal normally sleeps, completely reversed the phase of the circadian clock in

liver, stomach, intestine, heart, pancreas and kidney, without affecting the phase in the SCN (Damiola et al. 2000; Davidson et al. 2003). Simply delaying meals by 4 hours also resulted in a phase shift in the circadian clock in mouse liver of a similar length (Shimizu et al. 2018). Delaying a single breakfast meal by 5 hours also delayed expression of genes under *per* control in human adipose tissue (Wehrens et al. 2017). Conversely, studies have shown that rhythmic feeding was sufficient to maintain circadian rhythms of clock genes in peripheral tissues during constant light or darkness or following lesion of SCN (Hamaguchi et al. 2015; Kolbe et al. 2019; Novakova et al. 2011). These findings show that cues from the fasting-feeding cycle are more powerful entraining cues for peripheral clocks than the light - dark cycle (Figure 2. 3) (Damiola et al. 2000; Wang, H et al. 2017).

Disrupting molecular clocks by altering feeding behaviours has subsequent impacts on metabolism in animal models. Reversing the phase of clock genes in peripheral organs by daytime restricted feeding was associated with weight gain, dyslipidaemia and fatty liver as compared with animals that were pair-fed to an equivalent calorie level solely during the active phase (Bray et al. 2013; Yasumoto et al. 2016). This also reversed the phase of several genes involved in glucose homeostasis such as *glut2*, *pyruvate kinase*, *glucokinase* and *glycogen synthase* in liver and several genes involved in lipid homeostasis such as *acetyl CoA carboxylase*, *diacylglycerol-O-acyltransferase*, *medium chain acylCoA dehydrogenase* in liver, muscle and epididymal fat (Bray et al. 2013; Yasumoto et al. 2016). Daytime restricted feeding also reversed the phase of insulin, leptin and ghrelin in plasma compared to mice fed only during nighttime (Yasumoto et al. 2016).

Similarly, a rotating light cycle (a mimic of shift-work) altered the phase and reduced oscillation of clock genes in liver, and caused higher weight gain, increased hepatosteatosis, and reduced β-cell function and glucose stimulated insulin secretion (Christie et al. 2018;

Gale et al. 2011; Zhong et al. 2019). Chow fed mice under rotating light cycle also had altered phase of insulin and corticosterone in plasma, and transcription factors *FOXO1*, *PPARa* and *PPARϒ* in liver (Zhong et al. 2019). Whereas, high-fat diet (HFD) fed mice under rotating light cycle completely lost the rhythmic expression of lipogenic gene *acylCoA carboxylase* in liver (Christie et al. 2018).

Further, delaying the feeding phase by just 4 hours shifted peripheral clocks and increased weight gain in rats exposed to HFD (Shimizu et al. 2018). Meal delay also delayed the peak of several genes involved in glucose homeostasis such as *glucokinase*, *glucose 6 phosphatase*, *phosphoenol pyruvate carboxykinase*, and several genes and transcription factors involved in lipid homoeostasis such as *SREBP, PPARa, fatty acid synthase, carnitine palmitoyl transferase, malic enzyme* in liver. Likewise, meal delay also delayed the peak time of insulin, free fatty acids and bile acids in plasma, and circadian rise in body temperature (Shimizu et al. 2018).

2.6 Health effects of TRE in animal models, and humans

2.6.1 TRE induces pleiotropic metabolic health benefits in animal models of obesity and ageing

TRE, defined here as the provision of food for up to 12 hours during the active phase, is commonly known as TRF in animal studies to depict the eating window or food availability is externally controlled. TRE limited weight and fat gain and protected nocturnal mice and diurnal flies from the metabolic consequences of HFD (Chaix et al. 2014; Delahaye et al. 2018; Gill et al. 2015; Hatori et al. 2012; Olsen et al. 2017; Sundaram & Yan 2016; Villanueva et al. 2019; Woodie et al. 2018). This included protection from inflammation (Sherman et al. 2011) and immune responses (Cisse et al. 2018), enhanced bile acid synthesis facilitating cholesterol excretion (Chaix et al. 2014) and reduced cholesterol levels

(Delahaye et al. 2018). TRE also prevented age and HFD induced reductions in cardiac contractile function (Gill et al. 2015; Tsai et al. 2013) in mice and flies, and restored HFD induced loss of gastric vagal afferent mechanosensitivity (Kentish et al. 2018). TRE restored HFD induced dampening of the circadian rhythms in the gut microbiome (Hu et al. 2019; Zarrinpar et al. 2014) and circadian rhythms in fatty acid oxidation (Chaix et al. 2019; Hatori et al. 2012). Thus, TRE has pleiotropic metabolic benefits to protect against chronic disease in mice and flies, and importantly was able to reverse the consequences of obesity (Chaix et al. 2014) and ageing (Duncan et al. 2016). The beneficial effects were also evident when TRE was implemented 5 days per week and food access was allowed *ad libitum* during weekends (Chaix et al. 2014; Olsen et al. 2017) in HFD fed mice.

At the molecular level, TRE increased the amplitude of expression of AMPK and mTOR (Hatori et al. 2012; Sherman et al. 2012) (Figure 2. 3) and *NAMPT* in the liver of mice that were fed HFD (Chaix et al. 2019). TRE also increased the amplitude of ribosomal protein phospho-S6 in skeletal muscle during the active phase (Chaix et al. 2014), suggesting increased mTOR activation during feeding. TRE increased the amplitude of *cry1* and *per1* in the liver of mice fed chow (Greenwell et al. 2019), and restored the amplitude of *bmal1*, *cry1*, *per2* and *rev-erbα* in mice that were fed HFD (Hatori et al. 2012). In liver, TRE reduced the amplitude of *pyruvate carboxylase* and *glucose 6-phosphatase*, and increased *glucokinase* during the active phase (Chaix et al. 2014; Hatori et al. 2012), potentially underpinning reductions in hepatic glucose production and increased glucose utilization. TRE also reduced the amplitude of genes *fatty acid synthase*, *stearoyl CoA desaturase* and *fatty acid elongase* during the active phase, and increased the amplitude of *hepatic triglyceride lipase* during the inactive phase, which was associated with reduced lipid storage and increased triglyceride hydrolysis.

Importantly, Chaix *et al* examined the effects of TRE in mice that were deficient in *cry1* and *cry2* at the whole-body level, or deficient in *bmal1* and *reverbα* & *β* only in liver. In this study, TRE was effective to restore robust rhythms in genes involved in energy metabolism and nutrient utilisation in the liver from all knockouts, as well as nutrient signalling pathways with higher AMPK and mTOR function (higher pS6 levels during feeding) in fasting and fed state respectively. TRE also protected knockouts from HFD induced weight gain, glucose intolerance, hepatic steatosis and dyslipidaemia (Chaix et al. 2019). This study proves that sustaining daily rhythms in the fasting and feeding cycle is sufficient to maintain metabolic homeostasis, independently of circadian clocks (Chaix et al. 2019).

From studies to date, it is difficult to separate whether TRE improves health, independently of changes in calorie intake. Certainly, some studies have suggested this (Chaix et al. 2019; Chaix et al. 2014; Hatori et al. 2012). However, food intake is difficult to measure accurately, and other studies have shown lower calorie consumption in TRE mice that are fed a HFD (Delahaye et al. 2018; Sundaram & Yan 2016) and marked weight loss initially in response to TRE (Kentish et al. 2018; Sundaram & Yan 2016). Thus, some of the metabolic benefits of TRE may well be mediated by calorie restriction and weight loss. However, a recent study in mice showed that TRE improved glucose tolerance and reduced HOMA-IR in rats following high fat-high-sugar diet, without any weight loss (Woodie et al. 2018). A recent human study also supported the notion that TRE imparts metabolic benefits independently of changes in body weight (Sutton et al. 2018).

2.6.2 TRE improves metabolic health outcomes in humans:

Several TRE protocols with daily meal intakes prescribed from 4 to 13 hours have been trialled in people of normal weight and overweight (summarised in Table 2. 1), although all of these trials are short-term (4 days to 16 weeks) and conducted in a small number of

participants. The majority of studies report modest reductions in body weight and fat mass (Anton et al. 2019; Chow et al. 2020; Gabel et al. 2018; Gill & Panda 2015; LeCheminant et al. 2013; Moro et al. 2016; Stote et al. 2007; Wilkinson et al. 2019), and waist circumference (Kesztyus et al. 2019). TRE also reduced plasma triglycerides and inflammatory markers (LeCheminant et al. 2013; Moro et al. 2016), blood pressure and atherogenic lipids (Gabel et al. 2020; Wilkinson et al. 2019). Some studies have also reported significant improvement in glucose control (Hutchison, Regmi, et al. 2019; Jamshed et al. 2019), although this was not universally observed (Wilkinson et al. 2019). TRE for 12-weeks did not alter gut microbiome in humans (Gabel et al. 2020).

Most of the studies performed in humans have observed a reduction in self-reported energy intake (Gabel et al. 2018; Gill & Panda 2015; Wilkinson et al. 2019), which may account for some of the beneficial weight and health effects. However, in a highly controlled, cross-over feeding trial, 5 weeks of early (e) TRE (dinner before 3pm) increased insulin sensitivity and β-cell responsiveness, reduced oxidative stress as compared to the control condition in the absence of energy restriction and weight loss (Sutton et al. 2018). However, eTRE increased fasting triglyceride, as a physiological response to the increased fasting duration. Four days of eTRE (8am-2pm) also reduced fasting and postprandial glucose and increased daytime energy expenditure and the expression of *SIRT1*, clock genes and genes involved in autophagy in blood (Jamshed et al. 2019; Ravussin 2019). Five days of TRE (10am-5pm) also reduced night-time glucose in participants who were overweight (Parr et al. 2020). These studies suggest that TRE could be a promising tool for the improvement of metabolic outcomes in general population.

2.7 Challenges in translating TRE to humans

TRE is a simple approach that could be highly beneficial in primary practice, since it does not require extensive nutrition knowledge or significant time commitment to convey to the patient in need, unlike current dietary practice guidelines (NHMRC 2013). However, the majority of TRE interventions in animal models and in humans have been initiated early in the active phase (Chaix et al. 2014; Gabel et al. 2018; Gill & Panda 2015; Hatori et al. 2012; Jamshed et al. 2019; Olsen et al. 2017; Ravussin 2019; Sutton et al. 2018). Here, we discuss possible challenges with the translation of eTRE in general population and the possible outcomes of delayed TRE (dTRE, i.e. allowing food consumption for identical time lengths late in the day).

Early morning is likely to be the optimal time to initiate TRE to maximise the metabolic benefits. For example, insulin sensitivity and glucose uptake are higher at the beginning of the active phase in nocturnal mice (Basse et al. 2018; Rudic et al. 2004) and diurnal humans (Sonnier et al. 2014). Similarly, lipid absorption in the intestine (Douris et al. 2011) and *de novo* lipogenesis in the liver are higher during the active phase in mice (Gilardi et al. 2014). Cholesterol and bile acid synthesis are also elevated early during the active phase (Chaix et al. 2014). Furthermore, findings from observational and epidemiological studies suggest that breakfast skippers are also more likely to be overweight, have poorer glucose control and develop T2D as compared to people who identify as breakfast consumers (Bi et al. 2015). Although other observational studies have reported that skipping breakfast, without eating late, does not link to obesity, suboptimal glycaemic control, or poorer metabolic health (Azami et al. 2019; Nakajima & Suwa 2015; Okada et al. 2019). Women who were overweight were randomized to high calorie breakfast vs high calorie dinners, where the high calorie breakfast group lost more body weight, and had greater reductions in waist

circumference, fasting glucose and fasting insulin (Jakubowicz et al. 2013). In another study, individuals with T2D were provided with a three-meal per day diet (light dinner before 8pm) or an isocaloric six-meal diet (heavy dinner and snacks continued until 11pm) for 12 weeks. The three-meal diet reduced body weight, glycosylated haemoglobin and therapeutic insulin dose and significantly lowered hyperglycaemic episodes by continuous glucose monitoring. Clock gene expression in blood samples also showed higher oscillation in three-meal diet (Jakubowicz et al. 2019). Eating breakfast and lunch only also reduced body weight, plasma glucose and hepatic fat more than eating six meals spread throughout the day (Kahleova et al. 2014). Together, these studies show that consuming meals earlier in the day are optimal for weight control and improvements in glycaemic profile under isocaloric conditions.

Implementing TRE early in the morning may be challenging to implement in the general population both biologically and socially. There is large endogenous circadian variation in hunger, with peak in the evening and nadir in the morning (Qian et al. 2018; Scheer, Morris & Shea 2013). This is because ghrelin, a hormone secreted by stomach that increases feelings of hunger, is under circadian regulation, and is at biological nadir in the morning (Espelund et al. 2005) and peaks in the afternoon. Furthermore, family and communal get togethers are essential factors to increase social bonding, feeling of physical and mental wellbeing, and overall happiness in humans. Group eating and food sharing is considered the easiest way to strengthen family and community bonds capable of providing social and emotional support (Dunbar 2017). However, many social events are typically geared towards evening. Delaying the start time of TRE may overcome both of these issues, but the metabolic consequences of dTRE are not clear. As described earlier, fasting and feeding are known regulators of peripheral molecular clocks. Thus, it is likely that delaying TRE will delay peripheral clocks in metabolic organs. This was seen in recent human studies where skipping breakfast delayed *per* rhythms in adipose tissue (Loboda et al. 2009; Wehrens et al. 2017).

However, whether there is a net consequence of a short phase delay in clocks on metabolic health in humans is currently unknown.

In athletes undertaking resistance training, dTRE (12-8pm) reduced fat mass without altering fat free mass and improved muscle performance (Tinsley et al. 2019). Blood glucose, insulin, total testosterone and IGF-1 were also reduced in this study. However, severe time restriction, whereby all food intake was limited to one large daily meal eaten between 5 – 9 pm, impaired glucose tolerance in the following morning (Carlson et al. 2007). dTRE also failed to show any benefits in glycaemic profile and body weight reduction when meal intake was limited to 4 hours in the evening (anytime between 4pm-midnight) for 4 days per week (Tinsley et al. 2017). However, the irregular patterning of meals in that study could also contributed to this result (Farshchi, Taylor & Macdonald 2004).

Only two animal studies have directly compared eTRE with dTRE. In one study, mice underwent 6-hours of TRE with HFD given either during first half (ZT12-18) or second half (ZT18-24) of the night for 8 weeks. Body weight gain and insulin resistance as measured by HOMA-IR were higher in dTRE versus eTRE. However, both TREs equally improved glucose tolerance compared to *ad libitum* (Delahaye et al. 2018). The fasting length prior to the glucose assessment was not standardized in that study (7-16 hours depending on intervention), which may have contributed to the results as glucose tolerance is higher after 18-hours of fasting versus 6-hours of fasting (Andrikopoulos et al. 2008). In another study, rats were fed for 12-hours during night whether at ZT12-24 (eTRE) or ZT16-4 (dTRE) for 2 weeks. Despite similar calorie consumption, body weight gain was higher in dTRE group and dTRE also delayed the phase of *clock*, *bmal1*, *per1*, *cry2* and *rev-erbα* by 2 hours, and that of *cry1* by 4 hours in liver (Shimizu et al. 2018). The amplitudes of those genes were also lower in dTRE. However, the study was of short duration and did not include an *ad libitum* fed group. We conducted a preliminary study comparing dTRE with eTRE in men

with obesity. This study showed that dTRE produced similar improvements in glucose tolerance as eTRE (Hutchison, Regmi, et al. 2019). However, when glycaemic measurements were measured by continuous glucose monitoring, only eTRE significantly reduced fasting glucose vs baseline. This reduction was at trend level for dTRE vs baseline and there was no statistical difference in this improvement between TRE groups (Hutchison, Regmi, et al. 2019). The impact on clock genes were not examined. Larger trials comparing effects of eTRE vs dTRE are warranted.

Studies in mice have shown the beneficial effects of TRE are dose dependent, with greater reductions in body weight, fat mass and improvement in glucose tolerance when a 9-hour protocol was implemented vs 12 and 15 hours (Chaix et al. 2014; Sundaram & Yan 2016). The optimal TRE time frame to recommend for people has not been tested. Clear improvements have been noted after 6, 8, 9 and 10 hour protocols (Gabel et al. 2018; Hutchison, Regmi, et al. 2019; Sutton et al. 2018; Wilkinson et al. 2019). It is likely that the greater time restriction would result in greater weight losses, which may maximise the metabolic benefits. However, very short feeding windows could also reduce adherence or result in poorer food choices, if the individual feels under too great a time pressure. Extending the eating window beyond 12-hours is unlikely to have major beneficial metabolic effects (LeCheminant et al. 2013).

2.8 Conclusion & future directions

TRE initiated early in the active phase shows pleiotropic metabolic benefits in animal models of diet-induced obesity and ageing. Short-term TRE trials in humans have shown modest reductions in body weight and improved cardio-metabolic health in people who are overweight or obese, suggesting that TRE may be a promising therapeutic tool. However, these studies are limited in number, sample size, and study duration. The feasibility of

implementing early TRE in the general population on a daily basis is unclear, and the effects of delaying TRE to increase the potentially translatability and acceptability of this dietary approach are unknown. Large scale, long-term trials are warranted to determine if TRE is a viable alternative to current practice dietary guidelines.

Author contributions: PR searched the literature and both authors contributed to data interpretation and preparation of the manuscript. LKH had primary responsibility for the final publication.

Declaration of interest: The authors declare no competing interests.

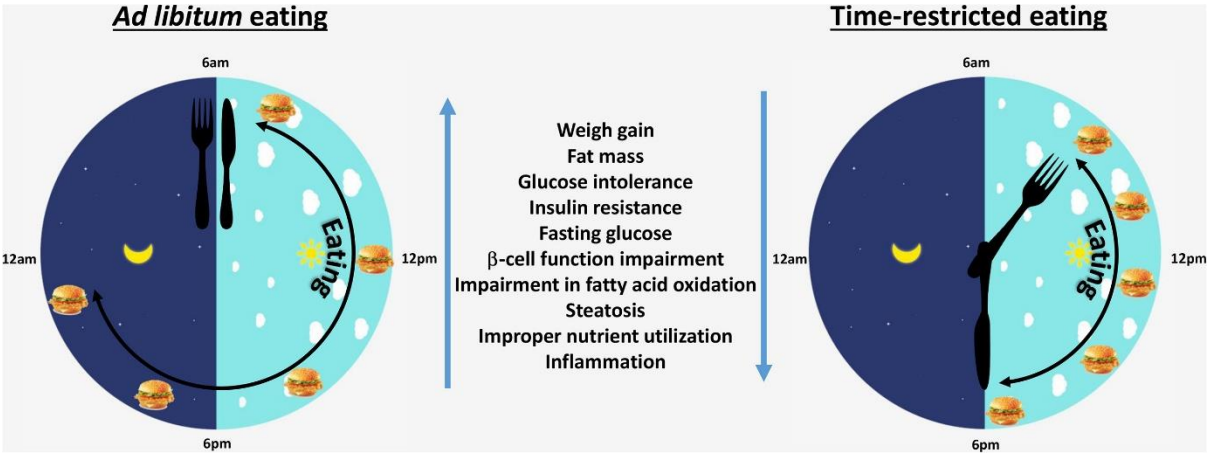


Figure 2. 2 TRE metabolic benefits

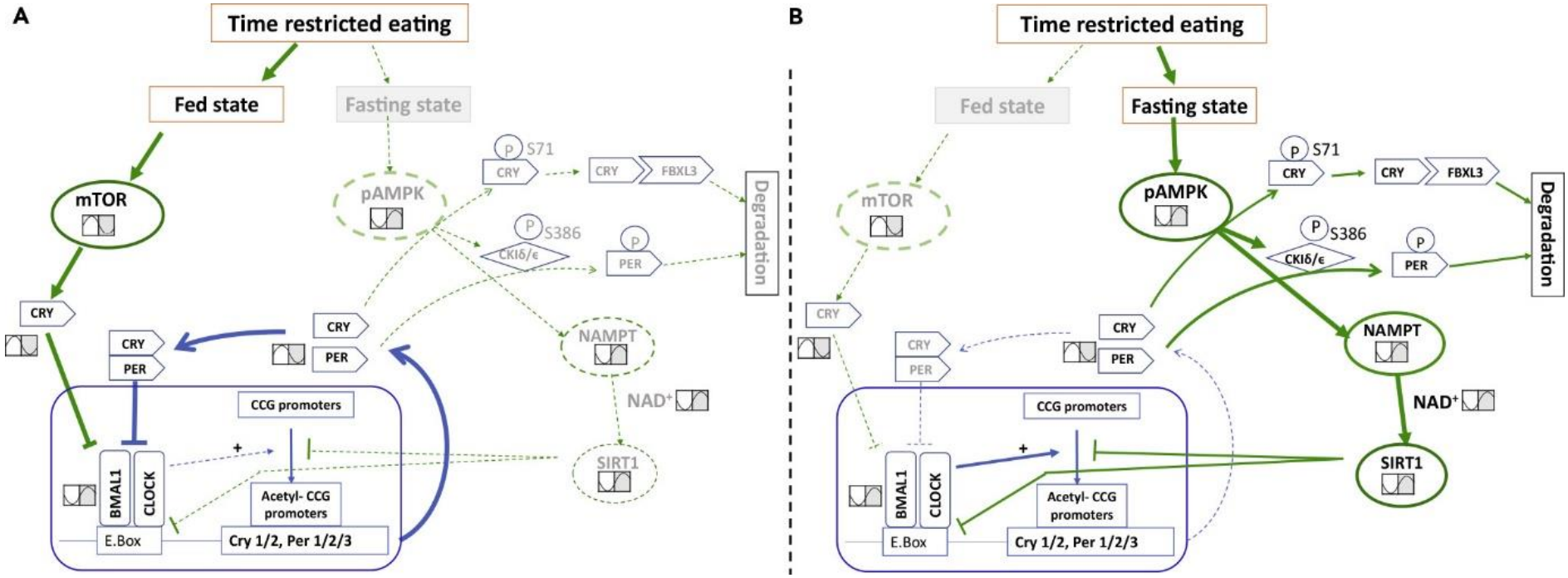


Figure 2. 3 TRE regulation of peripheral circadian clocks.

TRE can reprogram circadian clock in fasting state via AMPK and in fed state via mTOR. a) In fed state, nutrient availability activates mTOR. Activated mTOR induces cry, which repress *Clock:Bmal1*, b**)** In fasting state, nutrient depletion activates AMPK that directly and indirectly enhances phosphorylation on cry, and per. Phosphorylation is key for degradation of these proteins. Next, AMPK can activate SIRT1 activity via NAMPT. SIRT1 binds with clock:bmal1 and represses the transcription of *per2*. The acetyltransferase activity of *clock* is counteracted by SIRT1. Blue arrows represent core clock machinery, green arrows represent effect of TRE. AMPK: AMP-activated protein kinase, bmal1: brain and muscle arnt like 1, CCG: clock-controlled genes, CK: casein kinase, clock: circadian locomotor output cycle kaput, cry: cryptochrome, mTOR: mechanistic target of rapamycin, NAD: nicotinamide adenine dinucleotide, NAMPT: nicotinamide phosphoribosyl transferase, per: period, SIRT1: sirtuin1

Table 2. 1 List of time restricted eating trials in humans and their major findings.

Study	**Participants**	**Trial length**	**Design**	**Intervention, Meal time**	**Major Findings**
(Carlson et al. 2007; Stote et al. 2007)	n = 15 (10 females, 5 males), normal weight	8 weeks	Cross-over	TRE: one isocaloric meal (5pm-9pm) Control: three meal/day	↓ body weight, fat mass, blood pressure, glucose tolerance ↑ fasting glucose
(LeCheminant et al. 2013)	n=27 males, normal weight	2 weeks	Cross-over	TRE: 13-hour TRE (6am-7pm) Control: *AL*	↓ 0.4kg body weight (vs ↑ 0.6kg control condition)
(Gill & Panda 2015)	n=8 (3 females, 5 males), overweight	16 weeks	Within participant	TRE: 10-11 hour (self-selected) Baseline: >14 hours	↓ body weight
(Moro et al. 2016)	n=34 males, normal weight	8 weeks	Randomized controlled	TRE: 8-hour (1pm-8pm) Control: 12-hour (8am-8pm)	↓ fat mass, fasting glucose, fasting insulin, total testosterone, IGF-1
(Tinsley et al. 2017)	n=18 resistance trained males (10: RT-TRE; 8:RT-AL)	8 weeks	Randomized controlled	TRE: 4-hour (anytime 4pm-midnight) for 4 days a week Control: *AL*	↔ body weight, fat mass
(Gabel et al. 2018)	n=23 (20 females, 3 males), obese	12 weeks	Historical control	TRE: 8-hour (10am-6pm) Control: *AL*	↓ body weight and blood pressure ↔ fat mass, fasting glucose, LDL cholesterol, TG.
(Sutton et al. 2018)	n=8 males, overweight	5 weeks	Cross-over	eTRE: 6-hour (8am-2pm, dinner before 3pm) Control: 12-hour	↓ fasting TG, desire to eat in the evening ↑ insulin sensitivity, β-cell responsiveness ↔ body weight
(Jamshed et al. 2019; Ravussin 2019)	n=11 (4 females and 7 males), overweight	4 days	Cross-over	TRE: 6-hour (8am-2pm) Control: 12-hour (8am-8pm)	↓ mean 24-hour glucose, glycaemic excursions, morning ghrelin, desire to eat ↑ daytime EE, metabolic flexibility, fullness, plasma ketones
(Hutchison, Regmi, et al. 2019)	n=15 males, overweight	1 week	Cross-over	eTRE: 9-hour (8am-5pm) dTRE: 9-hour (12pm-9pm) Baseline: *AL*	↓ body weight, fasting TG and hunger ↓ mean fasting glucose by CGM in eTRE ↑ glucose tolerance
(Tinsley et al. 2019)	n=40 females, resistance trained	8 weeks	Randomized controlled	TRE: 8-hour (12pm-8pm) TRE plus β-hydroxy β-methyl butyrate.	↓ fat mass ↑ muscle performance
(Anton et al. 2019)	n=10 (6 females, 4 males), overweight, ≥65 years	4 weeks	Within participant	TRE: 8-hour Baseline: *AL*	↓ body weight
(Wilkinson et al. 2019)	n=19 (6 females, 13 males), overweight	12 weeks	Within participant	TRE: 10-hours (self-selected, dinner before 8pm) Baseline: ≥ 14-hours	↓ body weight, fat mass, waist circumference, blood pressure, plasma cholesterol. ↔ fasting glucose, HbA1c, HOMA-IR, fasting insulin.
(Kesztyus et al. 2019)	N=40 (31 females, 9 males), with abdominal obesity	3 months	Within participant	TRE: 8-9 hour Baseline: *AL*	↓waist circumference, HbA1c
(Parr et al. 2020)	n=11 males, overweight	5 days	Cross-over	TRE: 8-hour (10am-6pm) Extended eating: 15-hour (7am-10pm)	↓ night-time glucose, glucose and insulin iAUC after lunch ↔ daytime glucose ↑ TG after lunch
(Chow et al. 2020)	N=20 (17 females, 3 males), overweight	12 weeks	Randomized controlled	TRE: 8-hours Non-TRE: *AL*	↓body weight, lean mass and visceral fat mass.
(Gabel et al. 2020)	N=14, overweight	12 weeks	Within participant	TRE: 8-hours (10am-6pm) Baseline: *AL*	↓body weight, fat mass, systolic blood pressure ↔ Gut microbiome

n: number, TRE: time restricted eating, RT: resistance trained, AL: *ad libitum*, RCT: randomized controlled trial, IGF: insulin like growth factor, TG: triglycerides, LDL: low-density lipoprotein, EE: energy expenditure, SIRT1: sirtuin1, mTOR: mechanistic target of rapamycin, CGM: continuous glucose monitoring, eTRE: early TRE, dTRE: delayed TRE, HbA1c: glycosylated haemoglobin, HOMA-IR: homesostatic model assessment of insulin resistance, iAUC: incremental area under the curve, ↓: reduced, ↑: increased, ↔: no change.

2.9 Update of literature review

After publication of review paper presented earlier in this chapter, several TRE studies in mice and humans were published. The summary of updates in the literature is presented here. Major findings of human TRE studies published during the preparation of this thesis are also summarized in Table 2. 2.

2.9.1 Mice studies

Cafeteria diet is a model diet in animals mimicking western-style foods in humans, and adverse metabolic effects such as induction of metabolic syndrome, and liver and adipose tissue inflammation have been demonstrated with this diet (Sampey et al. 2011). In male Wister rats fed cafeteria diet, TRE (8 hour, ZT13-21) reduced weight gain, adiposity, plasma total cholesterol, LDL-cholesterol, and triglycerides compared to *ad libitum* fed group. TRE also reduced the adipocyte size in both diets, and increased the protein levels of UCP1 in inguinal fat suggesting adipose tissue browning.

People with obesity and type 2 diabetes are more likely to experience sleep disturbance than those without it (Lecube et al. 2016). *Db/db* mice, an animal model of type 2 diabetes, exhibited an abnormal sleep pattern with shorter sleep time during the light-phase and longer sleep time during the dark-phase compared to control mice. Whereas, TRE effectively restored the circadian patterns in the sleep-wake cycle in those mice (Hou et al. 2019). Further, constant light exposure completely disrupted the circadian rhythm in the expression of clock genes and several clock-controlled genes in liver and white adipose tissue. Whereas, 12-hour TRE (19:00-7:00) restored the circadian rhythm in clock genes, genes involved in lipid metabolism, and transcription factors such as PPAR α and ϒ, SREBP in liver and white adipose tissue (Yamamuro et al. 2020).

2.9.2 Human studies

In adults with obesity, both 4-hour (n=16) or 6-hour (n=19) TRE for 8-weeks reduced body weight, fat mass, fasting insulin, insulin resistance, and oxidative stress to lipid compared to control group (n=14). However, there was no change in fasting glucose, glycosylated haemoglobin, blood pressure, heart rate, HDL-cholesterol, LDL-cholesterol, triglyceride, TNFα, and IL-6 in both TRE protocols compared to controls. Daily energy intake was reduced by ~550 kcal/day in both TRE groups. Contrary to the authors' hypothesis that a shorter eating window would produce better outcomes, 4h TRE did not produce superior changes in any measured parameters compared to 6hTRE (Cienfuegos et al. 2020).

In another study, 8-hour TRE for 6-weeks in normal weight adults (n=22) increased glucose tolerance, reduced feeling of hunger, but did not affect body mass, bone density, respiratory quotient, fasting glucose, insulin AUC, ketones, oxLDL, CRP, IL-6, and markers of cardiovascular function. This study showed that 8-hour TRE had 84% adherence. The adherence rose to 95% when eating window was extended to 8.5 hours. 8-hour TRE was well tolerated in old age normal weight participants, and no adverse events were reported (Martens et al. 2020). Further, Two other studies examined the effects of 8-hour TRE for 4-weeks in normal weight or recreationally active young males (n=22 and 26) under unrestricted calories or 25% calorie deficit condition. TRE, whether under calorie unrestricted or calorie deficit condition, did not differentially impact body weight, fat mass, and the markers of cardiometabolic health compared to controls (McAllister et al. 2020; Stratton et al. 2020).

In another controlled trial, 58 women with obesity were provided with hypo-energetic diet (reduction of 500 to 1000 kcal/day based on total energy expenditure of each participant) whether under TRE (12-hour, n=31) or non-TRE (n=27). Outcomes were measured after 21 days and after 12 months. After 21 days, TRE raised axillary temperature and reduced

percentage fat mass, but did not alter body weight, respiratory quotient, plasma glucose, insulin, and thyroid hormones. After 12 months of TRE, percentage fat mass and waist circumference reduced, but body weight was unchanged (de Oliveira Maranhao Pureza et al. 2020; Pureza et al. 2020).

Recently, Moon et al (Moon et al. 2020) carried out the first systematic review and meta-analysis of TRE studies. In total 19 studies were included in the meta-analysis and showed that TRE reduced body weight and fat mass, while preserving fat-free mass. TRE also improved the markers of cardiometabolic health such as blood pressure and cholesterol levels, and reduced fasting glucose. Altogether, these studies show that TRE is a well-tolerated nutritional intervention, but shortening the eating window to less than 6-hours does not produce superior results.

Table 2. 2 Human TRE studies published recently, and their major findings.

Study	Participants	Trial length	Design	Intervention, Meal time	Major Findings
(Cienfuegos et al. 2020)	n=58 (females and males with obesity) 4-hour TRE (n=16) 6-hour TRE (n=19) Control (n=14)	8-weeks	Randomised controlled trial	TRE: 4-hour (3-7pm) TRE: 6-hour (1-7pm) Control: *ad libitum*	↓ BW, FM, fasting insulin, IR, oxidative stress, ↔ fasting glucose, HbA1c, SBP, DBP, HR, LDL, HDL, TG, TNFα, IL-6 Energy intake reduced by ~550 kcal/day in both TRE groups. 4h TRE did not produce superior changes in any measured parameters compared to 6hTRE.
(Jones et al. 2020)	n=16 (young normal weight males)	2-weeks	Randomised controlled trial	eTRE: 8-hour (8am-4pm) Control/CR: *ad libitum* (food provided to match eTRE group)	↑glucose tolerance, Matsuda insulin sensitivity index, forearm glucose uptake ↓ glucose variability in CGM, ↔ BW, FM, lean mass, glucose, TG, FFA, BCAA
(McAllister et al. 2020)	n = 22, college aged males, normal weight	4-weeks	Randomised controlled trial	TRE: 8-hour Control: *ad libitum*	TRE did not differentially impact the markers of cardiometabolic health in normal weight young males.
(Martens et al. 2020)	n=22 (females and males), midlife to old age (age 55-79 years), normal weight	6-weeks	Within participant	TRE: 8-hour Control *ad libitum*	↑glucose tolerance. ↓feeling of hunger. ↔body mass, bone density, RER, markers of cardiovascular function, fasting glucose, insulin AUC, oxLDL, CRP, IL6, acetoacetate, βHBD 8-hour TRE was well tolerated in old age normal weight participants, and no adverse events were reported.
(Stratton et al. 2020)	n=26 (recreationally active males), young	4-weeks	Randomised controlled trial	TRE: 8-hour (25% calorie deficit) Control: *ad libitum* (25% calorie deficit)	At 25% calorie deficit diet, TRE did not differentially impact body weight or fat mass in recreationally active young males.
(Zeb et al. 2020)	N=80 (TRE:n=56, non-TRE: n=24), young healthy adults	25-days	Randomised controlled trial	TRE: 8-hour (19:30-3:30) Non-TRE: *ad libitum*	↑HDL-c, microbiome diversity in gut ↓TC, TG, ALT, AST, ALP, ϒ-GT, serum albumin ↔LDL-c, IL-1β, TNFα
(de Oliveira Maranhao Pureza et al. 2020; Pureza et al. 2020)	N=58 (TRE:n=31, non-TRE:n=27) young women with obesity.	21-days & 12-months	Randomised controlled trial	TRE: 12-hour (hypo-energetic diet) Non-TRE: *ad libitum* (hypo-energetic diet)	**21 days** ↑axillary temperature ↓ %FM, ↔BW, RMR, glucose, insulin, fT3, fT4, TSH **12-months** ↓ %FM, WC ↔BW

n: number, TRE: time restricted eating, eTRE: early time restricted eating, BW: body weight, FM: fat mass, IR: insulin resistance, HbA1c: glycosylated haemoglobin, SBP: systolic blood pressure, DBP: diastolic blood pressure, HR: heart rate, TC: total cholesterol, LDL-c: low density lipoprotein cholesterol, HDL-c: high-density lipoprotein cholesterol, TG: triglycerides, TNFα: tissue necrosis factor alpha, IL-6: interleukin 6, CGM: continuous glucose monitoring, FFA: free fatty acids, BCAA: branched chain amino acids, RER: respiratory exchange ratio, AUC: incremental area under the curve, oxLDL: oxidized low-density lipoprotein, CRP: C-reactive protein, β-HBD: beta hydroxybutyrate, ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, ϒ-GT: gamma glutamyl transferase, WC: waist circumference, fT3: free tri-iodothyronine, fT4: free thyroxine, TSH: thyroid stimulating hormone.

Chapter 3: Will delaying breakfast mitigate the metabolic health benefits of time restricted eating?

Leonie K Heilbronn[1,2], Prashant Regmi[1,2].

[1]Faculty of Health Sciences, The University of Adelaide

[2]Lifelong Health Theme, South Australian Health and Medical Research Institute

Corresponding author: Leonie.heilbronn@adelaide.edu.au

Statement of Authorship

Title of Paper	Will delaying breakfast mitigate the metabolic health benefits of time restricted eating?
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Leonie K Heilbronn, Prashant Regmi. Will delaying breakfast mitigate the metabolic health benefits of time restricted eating? *Obesity* (2020) 28, S6-S7. https://doi.org/10.1002/oby.22776

Principal Author

Name of Principal Author (Candidate)	Prashant Regmi		
Contribution to the Paper	Prepared the first draft.		
Overall percentage (%)	40%		
Certification:	This paper reports on 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 co-primary author of this paper.		
Signature		Date	16 August 2020

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	Leonie Heilbronn		
Contribution to the Paper	Prepared final manuscript and had primary responsibility of the publication.		
Signature		Date	20/8/20

3.1 Abstract

Eating out of phase with the biological clock induces circadian misalignment in peripheral organs and impairs glucose tolerance in preclinical models. TRE is a dietary approach that consolidates energy intake to 6 to 10 hours during the biologically active phase of the day, without necessarily altering diet quality and quantity. TRE induces pleiotropic metabolic benefits in mice, flies, and humans. Most studies have initiated TRE early in the biological morning. This perspective discusses the potential challenges in translating early TRE to the community and considers the potential metabolic consequences of delaying TRE.

Key Words: Time restricted eating, intermittent fasting, glucose tolerance, obesity, circadian rhythm.

3.2 Perspective

TRE is a novel dietary tool that limits energy intake to a shortened daily window (e.g., 6-10 hours), without necessarily altering diet quality or quantity. TRE may be particularly effective in modern society, which has shifted to a grazing and prolonged daily pattern in eating (Gill & Panda 2015). TRE restores circadian rhythmicity in peripheral metabolic organs, improves glucose tolerance, reduces hepatosteatosis, and prevents metabolic dysfunction in animal models of diet-induced obesity and aging (Chaix et al. 2014). One study in humans indicated there are improvements in metabolism that occur independently of weight loss (Sutton et al. 2018). These studies have led to marked scientific and community interest in TRE as a tool to combat chronic metabolic disease.

In nocturnal rodent models, TRE is typically initiated at the onset of the dark phase for 6 to 12 hours (Chaix et al. 2014; Delahaye et al. 2018). In diurnal humans, most studies have also

initiated TRE early (eTRE) in the morning. eTRE reduces body weight, improves glucose tolerance, reduces oxidative stress, and improves markers of cardiovascular health in individuals with obesity (Jamshed et al. 2019; Sutton et al. 2018). Physiologically, eTRE is most likely to maximize the metabolic health benefits. Glucose tolerance exhibits clear circadian variation, with insulin sensitivity and secretion being much higher in the morning than the evening, even when there are 12-hour equidistant fasts between meals (Sonnier et al. 2014). The reverse is true for nocturnal rodents (Rudic et al. 2004). Epidemiological studies have also linked breakfast skipping with poorer glycaemic control and cardiovascular health, although a combination of breakfast skipping with late-night eating may be necessary to significantly elevate risk (Kutsuma, Nakajima & Suwa 2014). Individuals who choose to eat earlier in the day also lose more body weight on an energy-restricted diet versus individuals who chose to eat later in the day (Garaulet et al. 2013). A similar result was found in a group that was randomized to receive more calories at breakfast versus dinner, along with greater reductions in fasting glucose after 12 weeks (Jakubowicz et al. 2013). These studies all suggest that eTRE will provide optimal body weight and health benefits.

However, the acceptability and long-term feasibility of implementing eTRE in the community is unclear. eTRE may be less feasible because the stereotypical working day is 9 AM to 5 PM, and thus many individuals will find it difficult to consume a home-cooked dinner before 6 or 7 PM. There are also other social limitations in following a strict eTRE approach, as many get-togethers with friends and the wider community are often geared toward the evening. Furthermore, the biological drive to eat is also lowest in the morning, when ghrelin is at its circadian nadir (Qian et al. 2018). This means that people are generally less hungry in the morning versus the evening. Although eTRE was recently shown to reduce mean fasting ghrelin and reduce perceived appetite after 4 days (Jamshed et al. 2019), in our

experience, approximately three-quarters of participants expressed a preference for eating later rather than earlier in the day (unpublished data).

The alternative is to advocate for a short phase delay in initiation of TRE. However, the metabolic consequences of delaying TRE (dTRE) are unclear (Figure 3. 1). With this approach, breakfast would be delayed or skipped entirely, which is known to acutely impair daylong glucose control in people with type 2 diabetes (Jakubowicz et al. 2015). Severe restriction of the daily eating window, late in the day (between 4 and 8 PM), has also been shown to impair glucose tolerance as compared with the control condition of eating the same energy over breakfast, lunch, and dinner in normal weight individuals after 8 weeks (Carlson et al. 2007). Tinsley et al. (Tinsley et al. 2017) conducted dTRE during any 4-hour window between 4 PM and 12 AM, which did not alter body weight or fat mass in resistance-trained athletes. However, when dTRE was performed over a longer time period (scheduled from 1 PM to 8 PM), versus the control condition (eating three meals over a 12-hour period), dTRE significantly reduced body weight, fat mass, leptin, triglyceride, and triiodothyronine and increased adiponectin (Moro et al. 2016). There was no differential effect on fasting glucose or blood lipids.

To our knowledge, only two studies have directly compared the effects of eTRE and dTRE. Delahaye et al. (Delahaye et al. 2018) compared the effects of 6 hours of TRE initiated at lights off (eTRE) or after a 6-hour delay (dTRE) in mice that were fed a high-fat diet for 8 weeks. Both were effective to limit body weight gain, fat gain, and hepatic lipid accumulation versus animals fed *ad libitum*. However, dTRE animals weighed more and had higher fasting glucose and insulin versus eTRE. Blood samples were collected after a 10-hour fast in the dTRE group and a 16-hour fast in the eTRE group, which could account for this difference. Improvements in glucose tolerance were not different between TRE groups.

This result is similar to the only human trial comparing the eTRE and dTRE to date. In that study, eTRE and dTRE were equally effective in improving glucose tolerance as assessed in response to a mixed liquid meal in men with obesity (Hutchison, Regmi, et al. 2019). Importantly, meal tests were conducted after equidistant fasting lengths in both groups. However, this study was only 1-week in duration. While there was no statistical difference between TRE groups in the improvement in fasting glucose as measured by continuous glucose monitoring, only eTRE significantly reduced overnight fasting glucose levels from baseline. These studies suggest that delaying TRE may mitigate some of the weight benefits, but dTRE may be equally as effective for glucose tolerance.

In summary, TRE is a promising dietary approach that modestly reduces body weight and improves several markers of cardiometabolic health. However, studies in humans have been limited, and large-scale studies examining the long-term outcomes are warranted. While TRE is a practical approach, the majority of studies have implemented TRE early in the day. Future studies should compare eTRE and dTRE to assess social acceptability and long-term compliance as well as the potential metabolic impacts of delaying initiation of TRE.

Disclosure

The authors declare no conflict of interest.

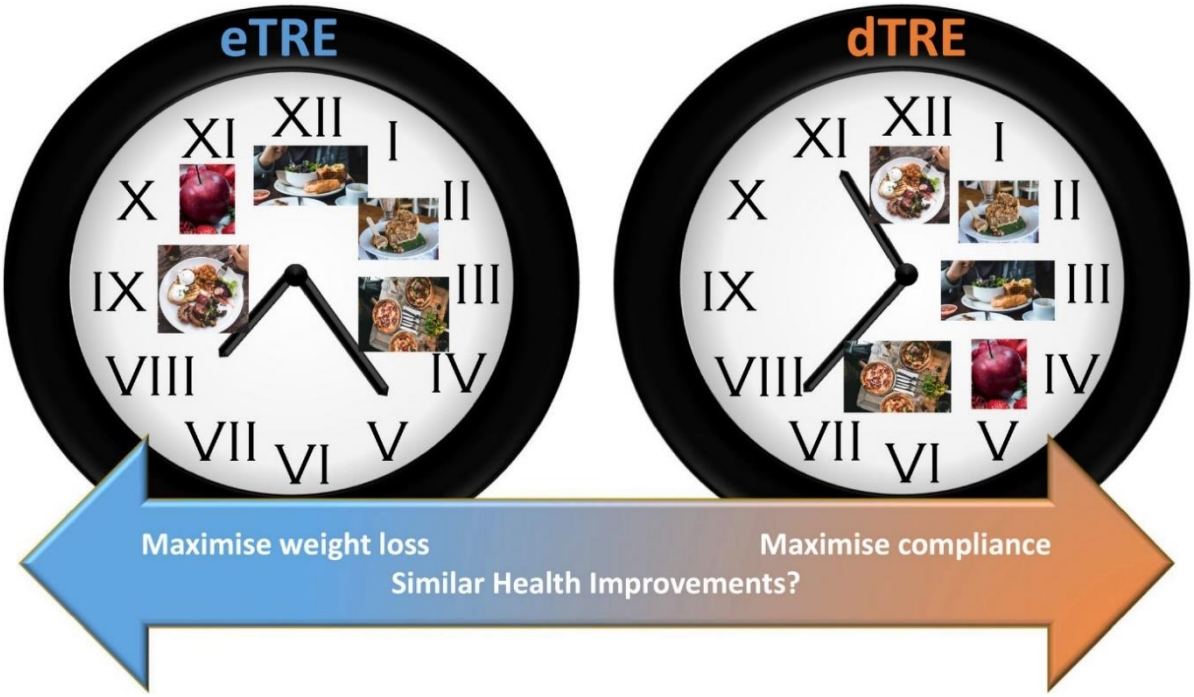


Figure 3. 1 Representative eating times in a 9-hour eTRE and dTRE.

eTRE, early time restricted eating; dTRE, delayed time restricted eating.

Chapter 4: Time restricted feeding improves glucose tolerance in men at the risk of type 2 diabetes: a randomized cross-over trial.

Amy T. Hutchison [1,2,3], Prashant Regmi[1,2,3], Emily N.C. Manoogian[4], Jason G. Fleischer[5], Gary A. Wittert[1,2,3], Satchidananda Panda[4], and Leonie K. Heilbronn[1,2,3]

Amy T. Hutchison and Prashant Regmi contributed equally to this work.

1 Adelaide Medical School, University of Adelaide, Adelaide, South Australia, Australia. Correspondence: Leonie K. Heilbronn (leonie.heilbronn@adelaide.edu.au)

2 NHMRC Centre of Research Excellence in Translating Nutritional Science to Good Health, University of Adelaide, Adelaide, South Australia, Australia

3 Nutrition and Metabolism Theme, South Australian Health and Medical Research Institute, Adelaide, South Australia, Australia

4 Regulatory Biology Laboratory, Salk Institute for Biological Studies, La Jolla, California, USA

5 Integrative Biology Laboratory, Salk Institute for Biological Studies, La Jolla, California, USA.

Clinical trial registration: ClinicalTrials.gov identifier NCT02633722.

Statement of Authorship

Title of Paper	Time restricted feeding improves glucose tolerance in men at the risk of type 2 diabetes: a randomized cross-over trial.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	**Hutchison AT, Regmi P**, Manoogian ENC, Fleischer JG, Wittert GA, Panda S, Heilbronn LK. Time-Restricted Feeding Improves Glucose Tolerance in Men at Risk for Type 2 Diabetes: A Randomized Crossover Trial. *Obesity* (2019) 27, 724-732. doi:10.1002/oby.22449

Principal Author

Name of Principal Author (Candidate)	Prashant Regmi		
Contribution to the Paper	Analysed data, drafted manuscript and approved final manuscript.		
Overall percentage (%)	40%		
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 co-primary author of this paper.		
Signature		Date	9 July 2020

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	Amy T Hutchison		
Contribution to the Paper	Designed and conducted study, analysed data, drafted manuscript and approved final manuscript.		
Signature		Date	16 July 2020

Name of Co-Author	Emily NC Manoogian		
Contribution to the Paper	Analysed data and approved final manuscript.		
Signature		Date	9 July 2020

Name of Co-Author	Jason G Fleischer		
Contribution to the Paper	Analysed data and approved final manuscript.		
Signature		Date	9 July 2020

Name of Co-Author	Gary A Wittert		
Contribution to the Paper	Contributed to data interpretation and approved final manuscript.		
Signature		Date	17 July 2020

Name of Co-Author	Satchidananda Panda		
Contribution to the Paper	Contributed to data interpretation and approved final manuscript.		
Signature		Date	22 July 2020

Name of Co-Author	Leonie K Heilbronn		
Contribution to the Paper	Designed and conducted study, supervised the study, had primary responsibility for the study and publication.		
Signature		Date	20/8/20

4.1 Abstract

Objective: This study aimed to assess the effects of 9-hour time-restricted feeding (TRF), early (TRFe) or delayed (TRFd), on glucose tolerance in men at risk for type 2 diabetes.

Methods: Fifteen men (age 55 ± 3 years, BMI 33.9 ± 0.8 kg/m2) wore a continuous glucose monitor for 7 days of baseline assessment and during two 7-day TRF conditions. Participants were randomized to TRFe (8 am to 5 pm) or TRFd (12 pm to 9 pm), separated by a 2-week washout phase. Glucose, insulin, triglycerides, non-esterified fatty acids, and gastrointestinal hormone incremental areas under the curve were calculated following a standard meal on days 0 and 7 at 8 am (TRFe) or 12 pm (TRFd).

Results: TRF improved glucose tolerance as assessed by a reduction in glucose incremental area under the curve (P = 0.001) and fasting triglycerides (P = 0.003) on day 7 versus day 0. However, there were no mealtime by TRF interactions in any of the variables examined. There was also no effect of TRF on fasting and postprandial insulin, non-esterified fatty acids, or gastrointestinal hormones. Mean fasting glucose by continuous glucose monitor was lower in TRFe (P = 0.02) but not TRFd (P = 0.17) versus baseline, but there was no difference between TRF conditions.

Conclusions: While only TRFe lowered mean fasting glucose, TRF improved glycemic responses to a test meal in men at risk for type 2 diabetes regardless of the clock time that TRF was initiated.

4.2 Introduction

TRF is a novel dietary tool that time-limits energy intake for up to 12 h/d without necessarily altering diet quality or quantity. TRF reduces body weight, improves glucose and insulin profiles, and reduces insulin resistance in mouse models (Belkacemi et al. 2010; Hatori et al. 2012; Tsai et al. 2013), even in the presence of a high-fat diet (Chaix et al. 2014; Hatori et al. 2012; Sundaram & Yan 2016).

Few studies have explored the metabolic effects of TRF in humans. TRF reduced body weight by ~3% in individuals with obesity over 12 to 16 weeks (Gabel et al. 2018; Gill & Panda 2015). However, these studies were uncontrolled. Lean males also lost 0.4 kg when they followed a 13-hour TRF protocol (6 am to 7 pm) for 2 weeks versus a control condition in which they gained +0.6 kg (LeCheminant et al. 2013). When healthy men and women restricted eating to one meal per day during a 4-hour window (5 pm to 9 pm), modest improvements in body weight and fat mass were noted compared with the same diet that was given as three meals per day (Stote et al. 2007). In lean young men, TRF for 8 h/d (between 1 pm and 9 pm) combined with 8 weeks of resistance training reduced fat mass, fasting plasma glucose, and insulin versus a 13-h/d protocol (8 am to 9 pm) (Moro et al. 2016). In contrast, self-selection of a 4-hour TRF window (between 4 pm and midnight) on 4 d/wk did not alter body composition in lean young men performing resistance training despite an overall reduction in energy intake (Tinsley et al. 2017).

Recently, a controlled feeding trial compared a 6-h/d eating period (dinner before 3 pm) with a 12-h/d eating period in men with prediabetes (Sutton et al. 2018). This study carefully matched and supervised all energy intakes in both conditions. There was no change in body weight in either condition, but TRF improved postprandial insulin, insulin sensitivity, and β-cell function after 5 weeks (Sutton et al. 2018). This study is the first to show that limiting

food intake prior to 3 pm is beneficial for glycaemic control in humans, independently of weight change. However, the feasibility of implementing a strict TRF approach daily in the general population is unclear.

There is also a known circadian impact of meal timing, with poorer glucose tolerance at night despite identical meals and equidistant fasting lengths (Gupta, CC et al. 2017; L M Morgan 1999). Whether changes in gastric emptying, which modulates glycemia, underlie this observation is unclear. The aim of this study was to examine the effects of 9-hour TRF commenced at 8 am or 12 pm (i.e., with a phase delay) on glucose response to a mixed-nutrient meal in men at risk for type 2 diabetes. Secondary outcomes were gastric emptying, insulin and gastrointestinal hormone release, markers of appetite, weight, and glucose profiles by continuous glucose monitoring (CGM). We hypothesized that TRF would improve glycemic control and that TRF-early (TRFe) would produce greater improvements than TRF-delayed (TRFd).

4.3 Methods

4.3.1 Participants

Fifteen men were recruited into this randomized crossover trial (Figure 4.2). One participant withdrew from the study after completing the TRFe condition only because of scheduling conflicts. All participants habitually ate breakfast and did not perform shift work. Inclusion criteria were age 30 to 70 years, waist circumference ≥ 102 cm, weight stability (within 5% of screening weight) for > 6 months prior to study entry, non-smoker, being sedentary or lightly active (i.e., took part in < 2 moderate- to high-intensity exercise sessions per week), consuming < 140 g/wk of alcohol, no prior diagnosis of type 2 diabetes, not taking antihyperglycemic medication, and no personal history of cardiovascular disease, eating

disorders, or major psychiatric disorders. All participants were assessed for their risk of developing diabetes by the Australian Type 2 Diabetes Risk Assessment Tool (AUSDRISK) score (Chen, L et al. 2010). A score of 9 to 11 indicates intermediate risk (1 in 30 will develop diabetes within 5 years, n = 1), 12 to 15 indicates high risk (1 in 14, n = 2), 16 to 19 indicates very high risk (1 in 7, n = 6), and ≥ 20 indicates 1 in 3 will develop diabetes within 5 years (n = 6). The Royal Adelaide Hospital Research Ethics Committee approved the study protocol, and all participants provided written informed consent prior to their inclusion. This study was registered as a clinical trial.

4.3.2 Study design

After an initial screening visit, participants underwent a baseline assessment for 1 week (Figure 4.1). Whole body composition was measured by dual-energy x-ray absorptiometry (Lunar Prodigy; GE Healthcare, Madison, Wisconsin). Participants were fitted with a CGM device to measure blood glucose (Dexcom G4 Platinum, San Diego, California) and an accelerometer (MF-SW/DD100; Body Media Sense Wear, Sydney, New South Wales, Australia) to measure activity and sleep patterns for the 7 days. Participants also kept a diary briefly describing the meal, snack, or beverage that was eaten and the time that this food was consumed.

Following the baseline monitoring period, participants were randomized to one of two TRF protocols for 7 days in a crossover design, separated by a 2-week washout period (Figure 4.1). These were TRFe (eating window between 8 am and 5 pm) and TRFd (eating window between 12 pm and 9 pm). Participants were asked to maintain their usual lifestyles, including sleep patterns, to consume their habitual diet within the specified TRF times in each condition, and to record the times they started and finished eating each day. Outside the eating window, participants were allowed to consume water and limited amounts (one to two

servings) of very low-calorie drinks and foods (e.g., herbal tea, diet drinks, mints, gum containing < 4 kcal/serving) as a tool that may increase compliance.

4.3.3 Testing days

Metabolic testing was conducted prior to (day 0) and on day 7 of each intervention. Participants arrived at our research facility at either 7:30 am (TRFe) or 11:30 am (TRFd) after fasting from 5 pm or 9 pm, respectively, to standardize the length of the fast. Body weight and waist and hip circumference were measured in a gown after voiding, and blood pressure was measured after a 10-minute seated rest. A 20-gauge cannula was inserted into an antecubital vein of the nondominant arm, and a fasting blood sample (20 mL) was collected, followed by a baseline breath sample. Participants were given a mixed-nutrient liquid test meal (474 mL, 700 kcal; 57% carbohydrate, 28% fat, 15% protein) (Ensure Plus; Abbott Laboratories, Abbott Park, Illinois) labeled with 100 mg of ^{13}C sodium acetate (Cambridge Isotopes Inc., Tewksbury, Massachusetts) and asked to consume the entire drink within 2 minutes. Test drinks were given at 8 am (TRFe) or at 12 pm (TRFd), according to study protocol. Breath samples were collected every 5 minutes for the first 30 minutes of the meal test (0, 5, 10, 15, 20, 25, and 30 minutes) and at 45, 60, 75, 90, 120, 150, and 180 minutes. Venous blood was sampled immediately prior to the test meal (0 minutes) and at 15, 30, 60, 90, 120, and 180 minutes. Blood was collected in ice-chilled EDTA vacuum tubes, and 40 µL of 200mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride and 20 µL of dipeptidyl peptidase-IV inhibitor were added to 2 mL of whole blood for the measurement of gastrointestinal hormones (ghrelin, peptide YY [PYY], glucagon-like peptide-1 [GLP-1], glucose-dependent insulinotropic peptide [GIP], and amylin). Plasma was separated immediately by centrifugation at 3,000 rpm for 15 minutes at 4°C and immediately snap frozen to −80°C for later analysis.

4.3.4 Gastric emptying and appetite responses

$^{13}CO_2$ concentrations in exhaled air from end-expiratory breath samples were analyzed with the use of infrared spectroscopy (FANci2 breath test analyzer; Fischer Analysen Instrumente GmbH, Leipzig, Germany). The half-emptying time was calculated after curve fitting of the 13C exhalation to a modified power exponential function (Braden et al. 1995) in GraphPad Prism 7.02 (GraphPad Software, La Jolla, California). Measures of appetite (hunger, fullness, and desire to eat during the meal test) were quantified using visual analog scales administered at 0, 30, 60, 120, and 180 minutes.

4.3.5 Continuous glucose monitoring

All participants were fitted with a CGM during baseline and both intervention periods. A glucose-oxidase-based electrochemical sensor was inserted under the skin on the abdomen and remained in situ for each 7-day period. The electrode transmitted interstitial glucose measurements every 5 minutes wirelessly to a receiver where they were stored until the participant returned to the department. This produced 12 readings per hour. Participants calibrated their CGM twice daily by finger prick (OneTouch Verio IQ; Lifescan, Inc., Milpitas, California).

4.3.6 Activity

Energy expenditure, number of steps, and time spent sleeping were measured by the SenseWear armband, a multisensor monitor worn on the back of the right upper arm (over the triceps muscle) per manufacturer instructions. This device continuously records physiological data during daily activities, and the information is integrated and processed by software using algorithms that utilize an individual's physical characteristics (sex, age, height, and weight). Energy expenditure estimated by the armband correlated strongly with estimates from doubly labeled water (Johannsen et al. 2010).

4.3.7 Biochemical measures

Blood glucose was measured using a commercially available enzymatic kit on an AU480 clinical analyzer (Beckman Coulter, Inc., Brea, California). Nonesterified fatty acid (NEFA) concentrations were measured using an enzymatic spectrophotometric assay (NEFA-HR; Wako Diagnostics, Mountain View, California). Insulin, ghrelin, GLP-1, GIP, amylin, and PYY were measured from the same sample in duplicate using a MILLIPLEX magnetic bead-based quantitative immunoassay (MilliporeSigma, Burlington, Massachusetts), with MAGPIX instrumentation. Samples from each participant were analysed within the same run to minimize inter-assay variation.

4.3.8 Statistical analysis

For all biochemical outcomes, incremental area under the curve (iAUC) was calculated using the trapezoidal rule. Statistical analysis was performed using linear mixed modeling, with effects of mealtime (meal commenced at 8 am or 12 pm), TRF (day 0 vs. day 7), and sequence (TRFe followed by TRFd, or vice versa) as fixed variables. A differential effect of mealtime in each condition was tested via the interaction between mealtime and TRF. Student t tests for carryover effects were conducted on the day 0 to day 7 differences, and no significant effects were found for any of the outcomes. For CGM data, accelerometry, and weight, a separate model was constructed with measures from the 7-day baseline monitoring period included and treatment (baseline, TRFe, or TRFd) as a fixed factor.

For the CGM analysis, the fed and fasting windows were determined from meal log data. The “fasting” window lasted from 4 hours after the latest occasion a participant ate in a given condition until the earliest occasion a participant ate in that condition. Mean fasting glucose for a participant in a given condition (baseline, TRFe, and TRFd) was calculated over this time window and across all days the participant spent in that condition. The “fed” window

was calculated from the earliest eating occasion until the latest meal occasion, plus 4 hours to allow for postprandial fluctuations in blood glucose. We also calculated 3-hour fasting glucose concentrations as the mean of glucose measurements obtained from CGM for the 3 hours preceding the first meal of each day in all three conditions. Mean amplitude of glycaemic excursions (MAGE) (Service et al. 1970), mean of daily differences (MODD), and continuous overall net glycaemic action (CONGA) (McDonnell et al. 2005) were calculated from CGM data as described by others. Because of technical issues, CGM data were lost from one participant during baseline (n = 14) and from one during TRFd (n = 13). Body weight change during baseline was calculated as weight on day 0 (phase 1) minus weight at start of baseline.

Sample size requirements were calculated on statistical power functions using within-subject contrasts for the primary end point Δglucose iAUC. The study was powered to detect a 1.4-mmol/L/min difference in glucose iAUC between groups, assuming an SD of 1.25 mmol/L/min (unpublished data), with $\alpha < 0.05$ and statistical power of 80%. Statistical analysis was performed in SPSS Statistics (version 24; IBM Corp., Armonk, New York). All data are presented as mean ± SEM, with $P < 0.05$ considered significant.

4.4 Results

Fifteen men (mean age 55 [SEM 3] years, BMI 33.9 [SEM 0.8] kg/m^2) were recruited into the study. Baseline characteristics are presented in Figure 4.1. Body weight was lower on day 7 compared with day 0 ($P < 0.001$) for each treatment, but there were no significant differences between treatments (all $P > 0.66$; baseline: −0.8 ± 0.3 kg; TRFe: −1.3 ± 0.2 kg; TRFd: −0.8 ± 0.2 kg). Participants' mean self-reported eating windows are shown in Figure 4. 7. The mean first eating occasion (SEM) occurred at 7:45 am (16 minutes) during baseline, 8:22 am (10 minutes) in TRFe, and at 12:15 pm (14 minutes) in TRFd. The mean last eating

occasions were 7:18 pm (37 minutes), 4:37 pm (21 minutes), and 7:56 pm (16 minutes) in baseline, TRFe, and TRFd, respectively. All effect sizes and P values for each variable are given in Table 4. 2. There were no significant mealtime by TRF interactions for any of the variables reported.

4.4.1 Insulin and glucose responses to meal test

There was a significant effect of TRF (P = 0.001) and mealtime (P = 0.002) on glucose iAUC. TRF reduced glucose iAUC by ~36% (−1.6 ± 0.4 mmol/L/h), whereas consuming a meal at 12 pm resulted in a ~21% (0.87 ± 0.5 mmol/L/h) higher glucose iAUC (Figure 4.3A). There was no effect of mealtime or TRF on fasting glucose or fasting insulin, although a trend toward a reduction in insulin iAUC was observed as a result of TRF (P = 0.09; Figure 4.3B).

4.4.2 Gastrointestinal hormone, triglyceride, and NEFA responses to meal test

There was an effect of mealtime, but not TRF, on fasting ghrelin and ghrelin iAUC. Fasting ghrelin was lower at 8 am versus 12 pm (P = 0.01), while ghrelin iAUC was lower at 12 pm versus 8 am (P = 0.038; Figure 4.4A). TRF, but not mealtime, reduced fasting GLP-1 (P = 0.002; Figure 4.4B), while there was no effect of TRF or mealtime on postprandial GLP-1. There was no effect of mealtime or TRF on fasting or postprandial GIP (Figure 4.4C), amylin (Figure 4.4D), or PYY (Figure 4.4E). TRF reduced fasting triglycerides (P = 0.003) but did not alter triglyceride iAUC (P = 0.57; Figure 4.4F). There was no effect of mealtime on fasting or postprandial triglycerides. There was an effect of mealtime, but not TRF, on fasting and NEFA iAUC (Figure 4.4G). Fasting NEFA was higher at 12 pm versus 8 am (P < 0.001), while NEFA iAUC was lower (i.e., meal-induced suppression was greater) at 12 pm (P = 0.001).

4.4.3 Gastric emptying and perceived appetite responses to meal test

There was no effect of mealtime or TRF on the rate of gastric emptying. There was a significant effect of mealtime only on perceived fullness iAUC, which was increased when the meal was initiated at 8 am versus 12 pm (P = 0.038). There was no effect of mealtime or TRF on perceived hunger, fullness, or desire to eat.

4.4.4 Mean glucose responses from CGM

Mean hourly glucose concentrations are shown in Figure 4. 5A. There was no statistical difference in the mean 24-hour blood glucose concentration (P = 0.37; Figure 4. 5B), CONGA (P = 0.37), MAGE (P = 0.27), or MODD (P = 0.22) between treatments (data not shown). There was an effect of treatment on mean fasting glucose by CGM (P = 0.023; Figure 4. 5C, Figure 4. 6). This was significantly lower during TRFe compared with baseline (P = 0.02) but not in TRFd compared with baseline (P = 0.17), and there were no differences between TRF treatments (P = 0.47). The mean blood glucose concentration for the 3 hours preceding the first meal of the day was reduced in TRFe versus baseline (P = 0.03), with a tendency to be reduced in TRFd versus baseline (P = 0.07). There was no difference between treatments (P = 0.92), and there was no effect of treatment on mean fed blood glucose assessed by CGM (P = 0.77; Figure 4. 5D).

4.4.5 Activity

There was no effect of treatment on total energy expenditure, number of steps, or duration of sleep assessed by accelerometry (Table 4. 3).

4.5 Discussion

This study examined the effects of 1 week of TRF, with and without a short phase delay, on glucoregulatory responses to a standard test meal and 24-hour glucose profiles in men at risk for type 2 diabetes. We observed that 9-hour TRF produced a ~36% reduction in the glycaemic responses to a test meal, with no differences between TRF treatments. The improvement in glycaemic control was not explained by changes in gastric emptying or gastrointestinal hormone release, but there was a tendency to reduce postprandial insulin. Together, these data show that TRF improves glycaemic responses to a test meal in men who are at risk for type 2 diabetes, regardless of the clock time TRF was initiated. TRF improves glucose tolerance and insulin sensitivity in mice (Belkacemi et al. 2012; Hatori et al. 2012). In humans, markers of β-cell function and insulin sensitivity were also improved by TRF (6-hour feeding period, with dinner before 3 pm) (Sutton et al. 2018). In that study, the increased fasting length (18 hours versus 12 hours in the control condition) may have contributed to the result (Sutton et al. 2018). To achieve equal fasting lengths in this study, meal tests were conducted at 8 am (TRFe) or 12 pm (TRFd). As expected, we observed a reduced glucose iAUC response to the test meal given at 8 am versus 12 pm, which reflects known variations in circadian rhythm (Carroll & Nestel 1973; Zimmet et al. 1974). However, TRF improved the glucose response to the standardized test meal, regardless of whether it was initiated at breakfast or lunch. This study suggests that there may be some flexibility in the clock time that TRF is initiated. We highlight that modest weight loss occurred during both TRF conditions, which may have contributed to the observed improvement in glycemia, rather than TRF *per se*. However, Sutton et al. (Sutton et al. 2018) recently established that the insulin-sensitizing effects of TRF occurred independently of weight loss.

Several uncontrolled studies in humans have shown that TRF reduces body weight by 1% to 3% over a period of 2 to 16 weeks (Fakhrzadeh et al. 2003; Gill & Panda 2015; LeCheminant et al. 2013; Nematy et al. 2012). In our study, participants lost a modest amount of weight (~1%) during TRF. However, an equivalent weight loss occurred during the baseline monitoring period, despite participants being instructed to consume their habitual diets, and was not different between conditions. This finding suggests that diet monitoring by the investigators, rather than TRF, may underpin the modest weight loss reported in past uncontrolled trials of TRF. Clearly, long-term randomized controlled trials are required to test the efficacy of TRF to reduce body weight. Despite similar weight loss across the three treatment periods, mean fasting glucose by CGM was lower only during the TRFe treatment versus baseline. Firstly, this finding supports past evidence that TRF improves glycaemic control independently of weight loss (Stote et al. 2007). Second, there was no statistical difference between TRFd and baseline. Thus, we speculate that restricting calories to an earlier time frame may be optimal for 24-hour glucose management, although there was not a statistical difference in this response between TRF conditions. However, it is well described that glucose tolerance diminishes over the course of the day under natural circadian variation (Carroll & Nestel 1973; Lee et al. 1992; Zimmet et al. 1974).

While gastric emptying rate is a known modulator of the glycaemic response to a meal, there was no change in gastric emptying in response to TRF. There was also no effect of TRF on gastrointestinal hormone release. However, fasting ghrelin and the postprandial suppression of ghrelin were higher at 12 pm versus 8 am. The natural ghrelin nadir occurs in the biological morning (~8 am) in humans, irrespective of the sleep-wake cycle (Espelund et al. 2005), and correlates with circadian variations in hunger, which peak later in the day (Scheer, Morris & Shea 2013). This finding suggests that prescription of TRF later in the day may have greater effects on reducing appetite and may aid compliance. Studies of extended

morning fasting have shown greater suppression of ghrelin when a meal is consumed around midday (i.e., breakfast skipping) compared with consuming the same meal at breakfast and lunch (Chowdhury et al. 2016; Chowdhury et al. 2015). Some authors have linked this to reduced insulin responses, occurring as a result of the second-meal effect, because insulin was proposed to play in role in ghrelin suppression (Flanagan et al. 2003). In a study by Carlson et al., consuming all calories within a single 4-hour window (4 pm to 8 pm) had no effect on morning fasting and postprandial ghrelin when compared with three meals per day after 8 weeks (Carlson et al. 2007). Further exploration of how meal timing and TRF may manipulate appetite signalling is warranted.

We observed a reduction in triglycerides in response to TRF, with no difference between TRFe and TRFd. In contrast, previous observations of 8 weeks of TRF in lean males reported no change in fasting triglycerides (Moro et al. 2016) and whilst others reported increased total cholesterol and LDL and HDL cholesterol after consuming all calories as one meal per day for 6 weeks between 5 pm and 9 pm (Stote et al. 2007). It is unclear whether consumption of the meal during the morning may have altered this outcome. We also observed mealtime-related changes in fasting and postprandial NEFA, which are congruent with previous reports of diurnal rhythms of circulating NEFA and lipid metabolism (Dallmann et al. 2012).

This was a 1-week controlled crossover intervention that was conducted solely in overweight men. As such, our findings cannot be directly extended to women, individuals with normal weight, or those with established metabolic disturbances, such as type 2 diabetes mellitus. The study was conducted in a free-living population, and we made no attempt to standardize food intake. During the free-living periods (1-week baseline assessment prior to randomization to TRFd or TRFe and during the washout before crossing over to the alternate TRF condition), participants were instructed to continue to follow their regular dietary habits. This lack of standardization could have influenced the baseline result and may have

lessened our ability to detect differences between TRFe and TRFd. The long-term feasibility of this eating pattern compared with *ad libitum* intake and its effectiveness in improving metabolic outcomes over longer periods require further investigation in larger populations. Finally, this study may have been underpowered to detect changes in secondary outcomes. Future studies should also consider how an individual's own chronotype may impact the magnitude of responses when TRF is commenced early or late. In conclusion, this study has demonstrated that 1 week of TRF improves glucose responses to a meal in men at risk for type 2 diabetes, irrespective of when TRF is commenced. This trial should be repeated in larger cohorts with more tightly controlled free-living periods to confirm this result. Overall, the simplicity of TRF and the efficacy in improving glycaemic outcomes indicate that large-scale, long-term randomized controlled trials are warranted.

Acknowledgments: Individual deidentified participant data will not be available for this clinical trial.

Author contributions: ATH and LKH designed and conducted the study. ATH, PR, ENCM, and JGF analyzed data. All authors contributed to data interpretation and preparation of the manuscript. LKH had full access to the data and had primary responsibility for the final publication.

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

Table 4. 1 Baseline characteristics of participants

Age (years)	55±3
Body weight (kg)	106.0±2.5
BMI (kg/m^2)	33.9±0.8
Waist circumference (cm)	115±2
Waist: hip ratio	0.99±0.01
SBP (mmHg)	141±3
DBP (mmHg)	87±2
Fasting glucose (mmol/L)	6.1±0.5
Total fat mass (kg)	32.5±1.7
Total lean mass (kg)	62.5±2.3
Fat mass (%)	35.1±1.2
AUSDRISK score	20±1

AUSDRISK: Australian Type 2 Diabetes Risk Assessment Tool; BMI: Body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure (Mean±SEM)

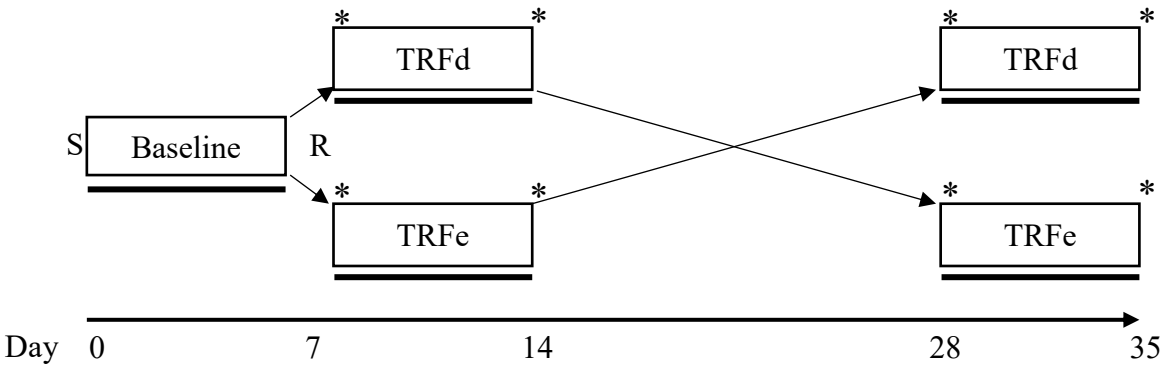


Figure 4.1: Study design

S: screening; R: randomization; *: meal test, TRFe: meal time between 8am to 5pm, TRFd: meal time between 12pm to 9pm. ——— Denotes continuous glucose monitoring periods.

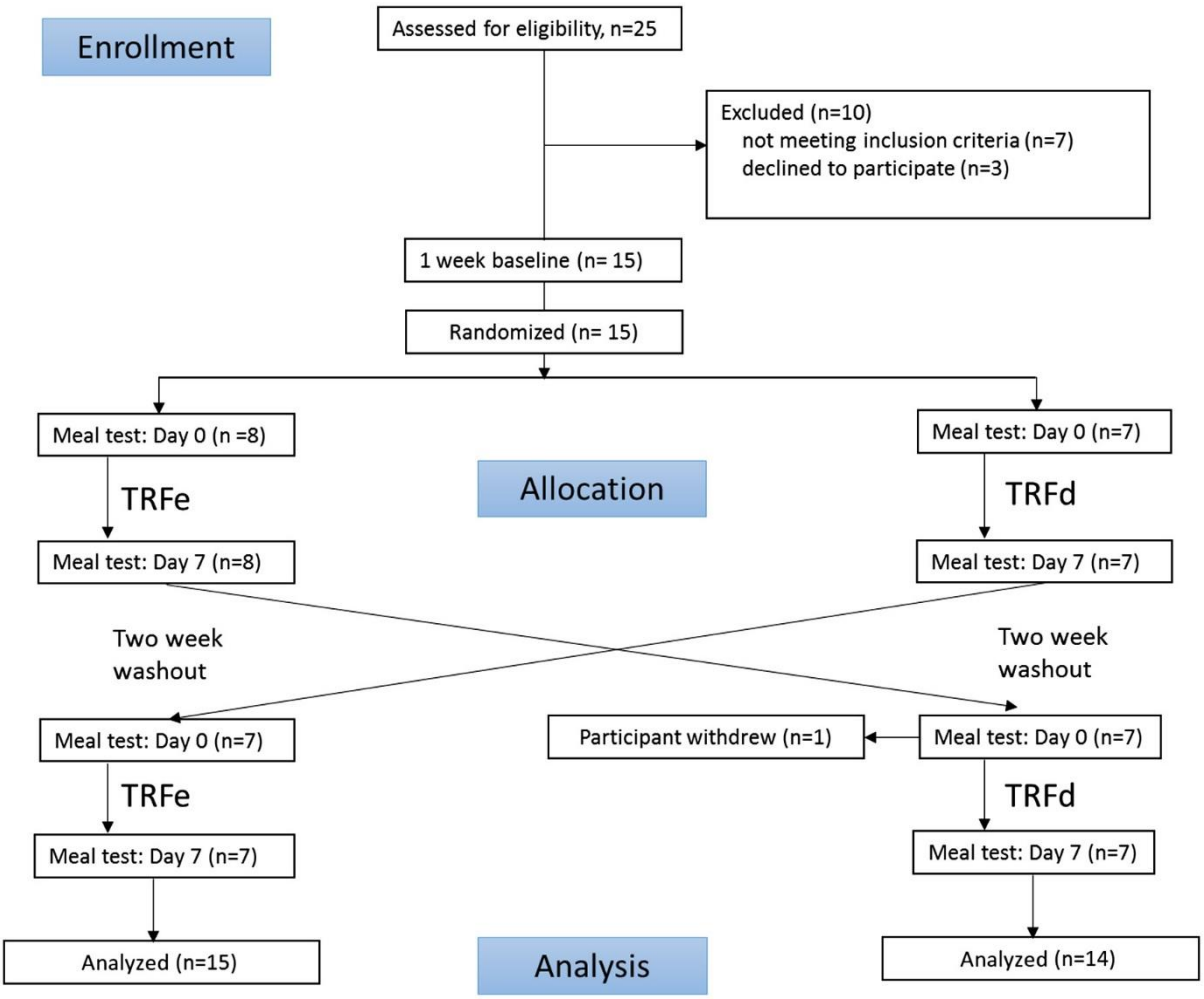


Figure 4.2 Consort diagram

TRFe: Time restricted feeding early, TRFd: time restricted feeding delayed

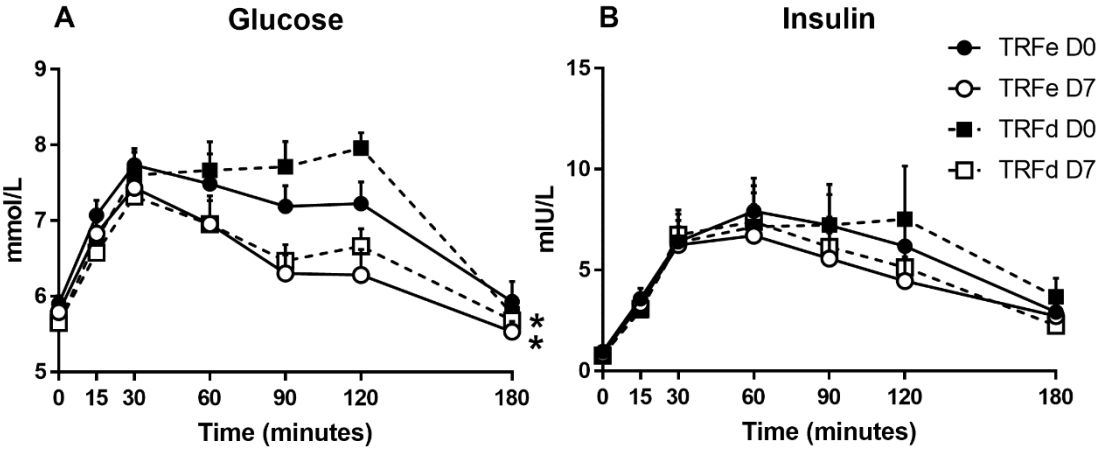


Figure 4.3 Glucose and insulin iAUC responses to a standardized meal test

Mean glucose (A) and insulin (B) iAUC responses to a 3 hour mixed-nutrient meal test at baseline (D0) and after 7 days (D7) of time-restricted feeding commenced early (TRFe, 8 am -5 pm) or with a phase delay (TRFd, 12 pm-9 pm). Data are presented as mean ± SEM. Closed circles: TRFe D0; open circles: TRFe D7; closed squares: TRFd D0; open squares: TRFd D7. Statistical analysis was performed using linear mixed modelling, with TRF condition (TRFe or TRFd) and time (day 0 vs day 7) as fixed variables. (*: $P<0.05$ vs. D0). iAUC: incremental area under curve; TRFe: time-restricted feeding commenced early, 8 am -5 pm; TRFd: time-restricted feeding with a phase delay, 12 pm-9 pm.

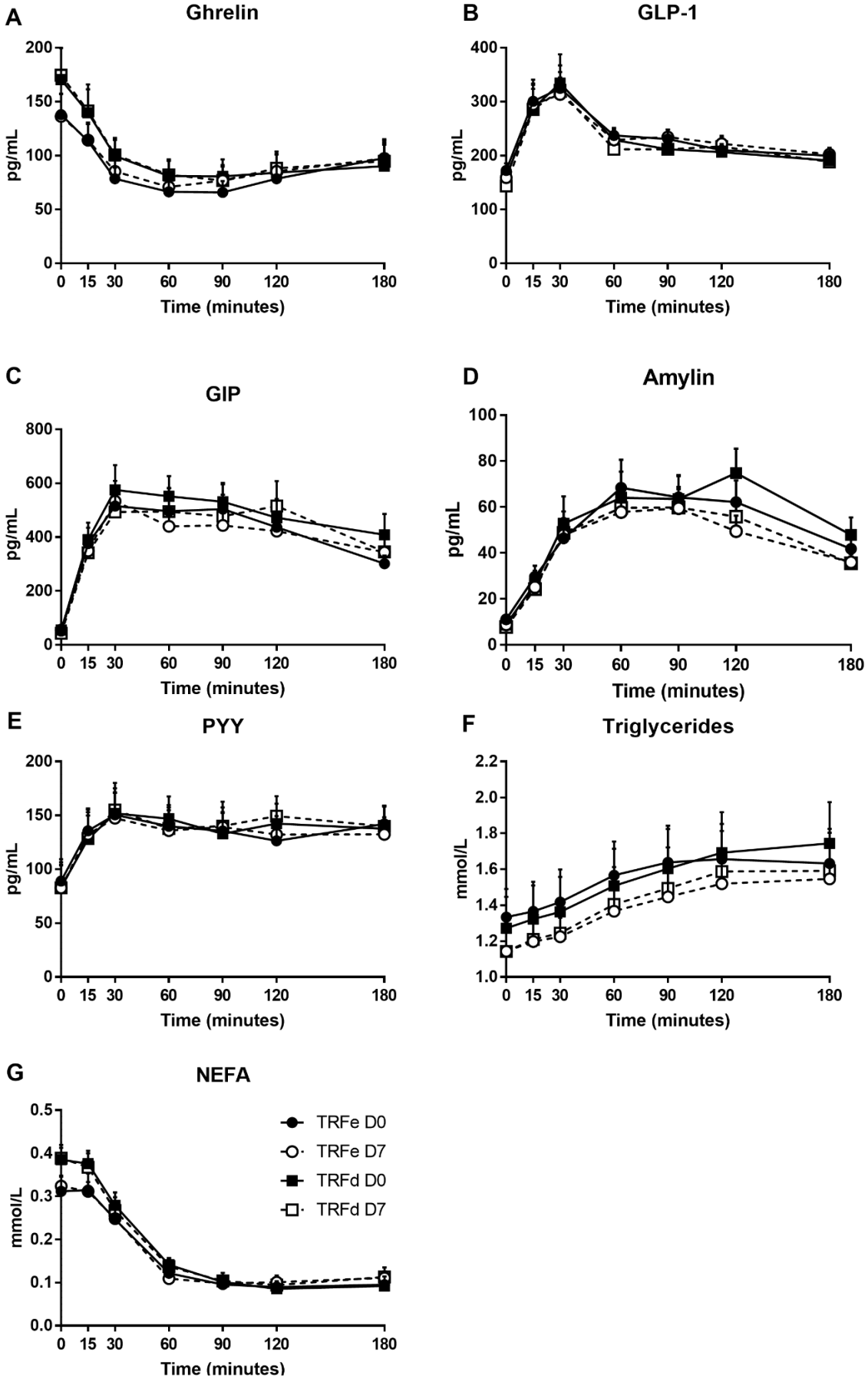

A
Ghrelin
pg/mL
Time (minutes)
B
GLP-1
pg/mL
Time (minutes)
C
GIP
pg/mL
Time (minutes)
D
Amylin
pg/mL
Time (minutes)
E
PYY
pg/mL
Time (minutes)
F
Triglycerides
mmol/L
Time (minutes)
G
NEFA
mmol/L
Time (minutes)
TRFe D0
TRFe D7
TRFd D0
TRFd D7

Figure 4.4 Gastrointestinal hormone, triglyceride and non-esterified fatty acid responses to a standardized meal test

Mean ghrelin (A), GLP-1 (B), GIP (C), amylin (D), PYY (E), triglyceride (F) and NEFA (G) responses to a 3 hour mixed-nutrient meal test at baseline (D0) and after 7 days (D7) of time restricted feeding commenced early (TRFe, 8 am -5 pm) or with a phase delay (TRFd, 12 pm-9 pm). Data are presented as mean ± SEM. Statistical analysis was performed using linear mixed modelling, with TRF condition (TRFe or TRFd) and time (day 0 vs day 7) as fixed variables. GIP: gastric inhibitory peptide; GLP1: glucagon like peptide 1; NEFA: non-esterified fatty acids; PYY: peptide YY; TRFe: time-restricted feeding commenced early, 8 am -5 pm; TRFd: time-restricted feeding with a phase delay, 12 pm-9 pm.

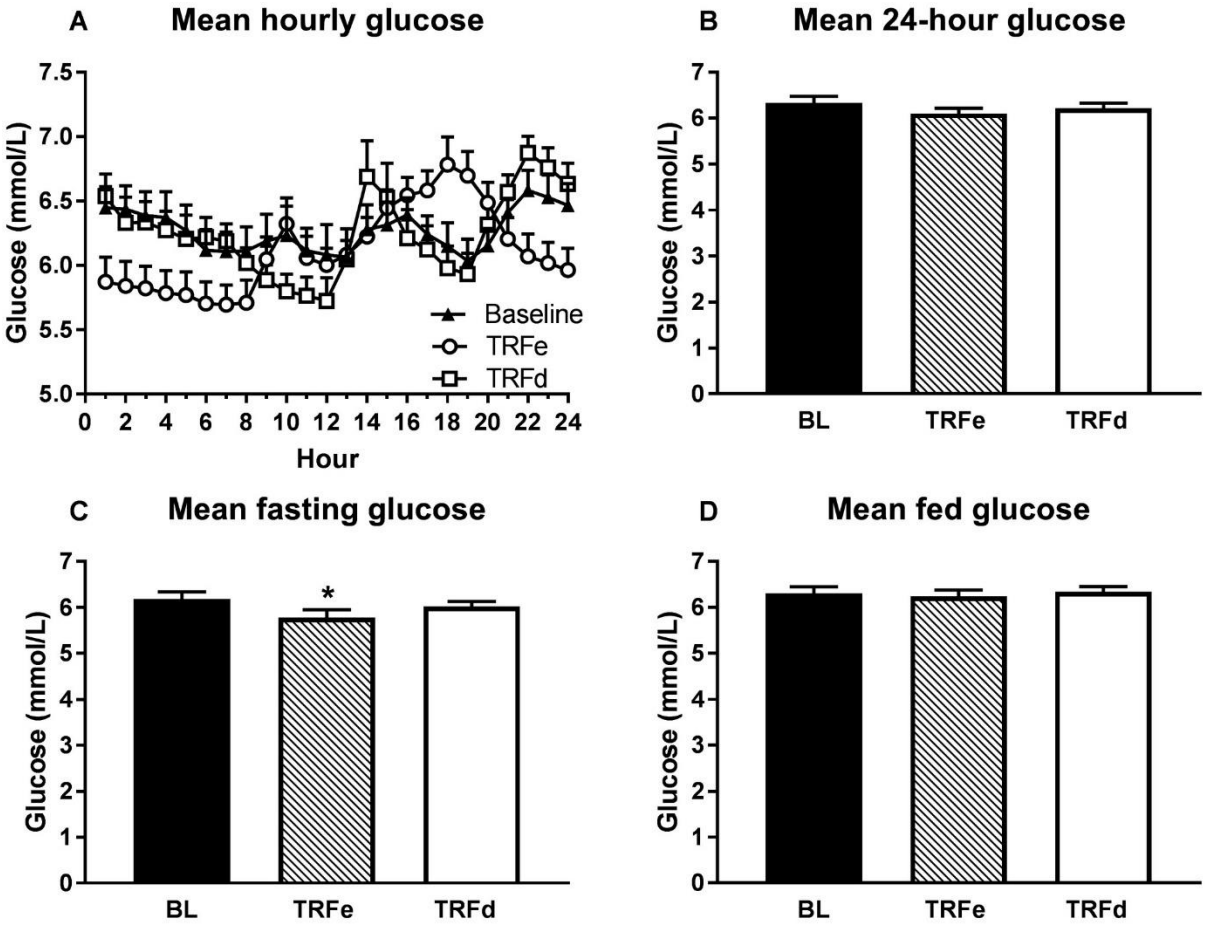


Figure 4. 5 Mean hourly glucose and mean 24-hour glucose during 7 days of time-restricted feeding

(A) Mean hourly glucose from continuous glucose monitoring over 7 days during baseline monitoring period (closed triangles), time-restricted feeding commenced early (TRFe, 8 AM-5 PM; open circles), or time-restricted feeding with a phase delay (TRFd, 12 PM-9 PM; open squares). Glucose readings were averaged every hour and each time period over 7 days, plotted as mean ± SEM. (B) Mean 24-hour blood glucose concentrations from CGM over 7 days during baseline monitoring period (black bar), TRFe (8 AM-5 PM; hatched bar), or TRFd (12 PM-9 PM; open bar). (C) Mean fasting blood glucose concentrations from CGM over 7 days during baseline monitoring period (black bar), TRFe (8 AM-5 PM; hatched bar), or TRFd,(12 PM-9 PM; open bar). Mean fasting glucose for a participant in a given condition

(baseline, TRFe, and TRFd) was calculated between 4 hours after a participant consumed first meal until the time participant consumed last meal in that condition. (D) Mean fed blood glucose concentrations from CGM over 7 days during baseline monitoring period (black bar), TRFe (8 AM-5 PM; hatched bar), or TRFd (12 PM-9 PM; open bar). Mean fed glucose for a participant in a given condition (baseline, TRFe, and TRFd) was calculated from the time a participant consumed first meal until the time participant consumed last meal, plus 4 hours in a given condition. Data are mean ± SEM ($n = 14$). Statistical analysis was performed using linear mixed modeling with effects of treatment (baseline, TRFe, and TRFd) as a fixed variable. *$P < 0.05$ vs. baseline.

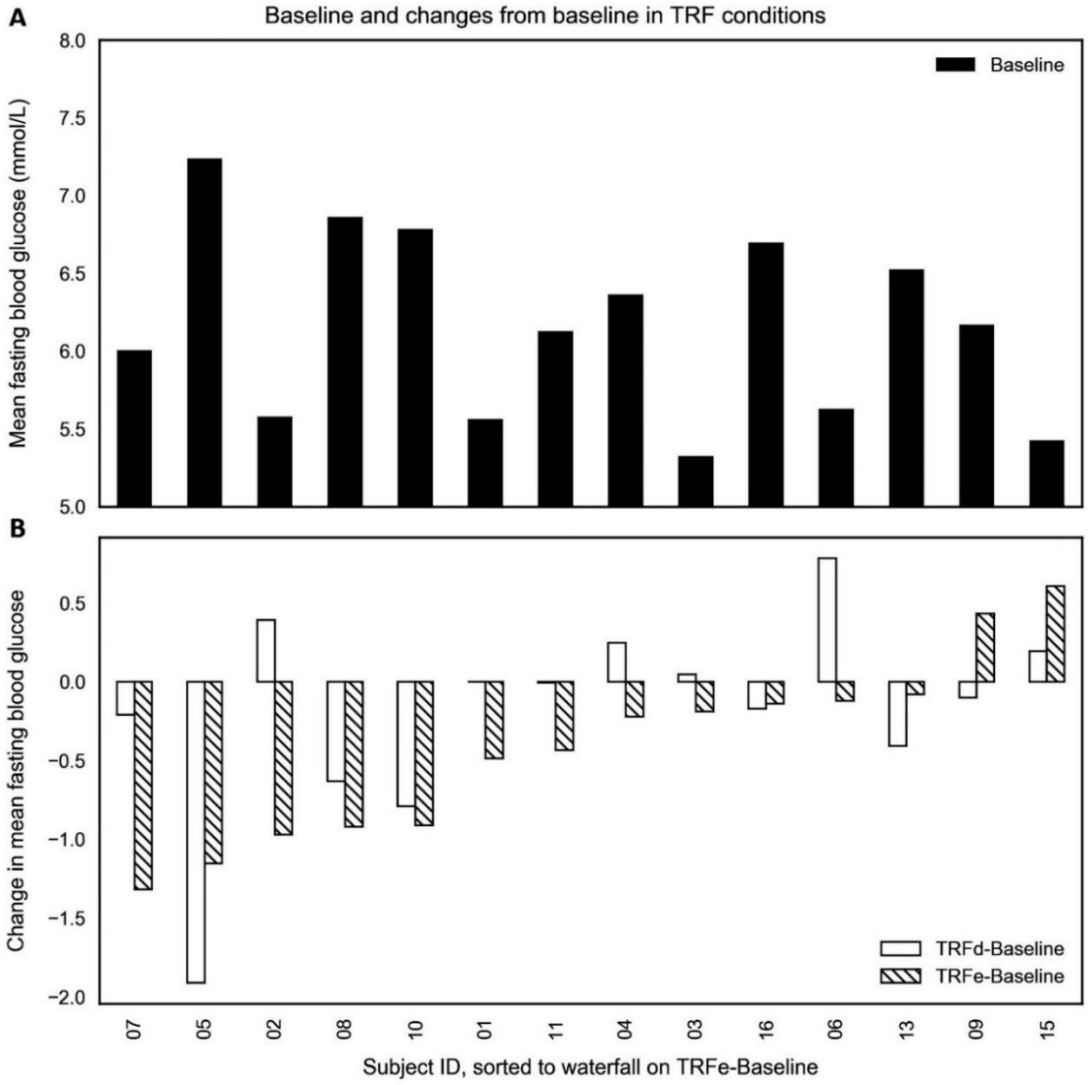


Figure 4. 6 Waterfall graph of mean fasting glucose measured by CGM during baseline and 7 days of time-restricted feeding.

(A) Baseline mean fasting glucose and (B) change in mean fasting glucose during time-restricted feeding commenced early (TRFe, 8 AM-5 PM) or with a phase delay (TRFd, 12 PM-9 PM) from baseline (mmol/L). Mean fasting glucose was determined by CGM data between 4 hours after a participant consumed first meal until the time participant consumed last meal based on food logs. Data sorted from the largest decrease in mean glucose to increased glucose based on TRFe condition-baseline. Data from both conditions shown for each participant, stacked below participant's own baseline fasting glucose. Baseline CGM data was collected from 14 participants.

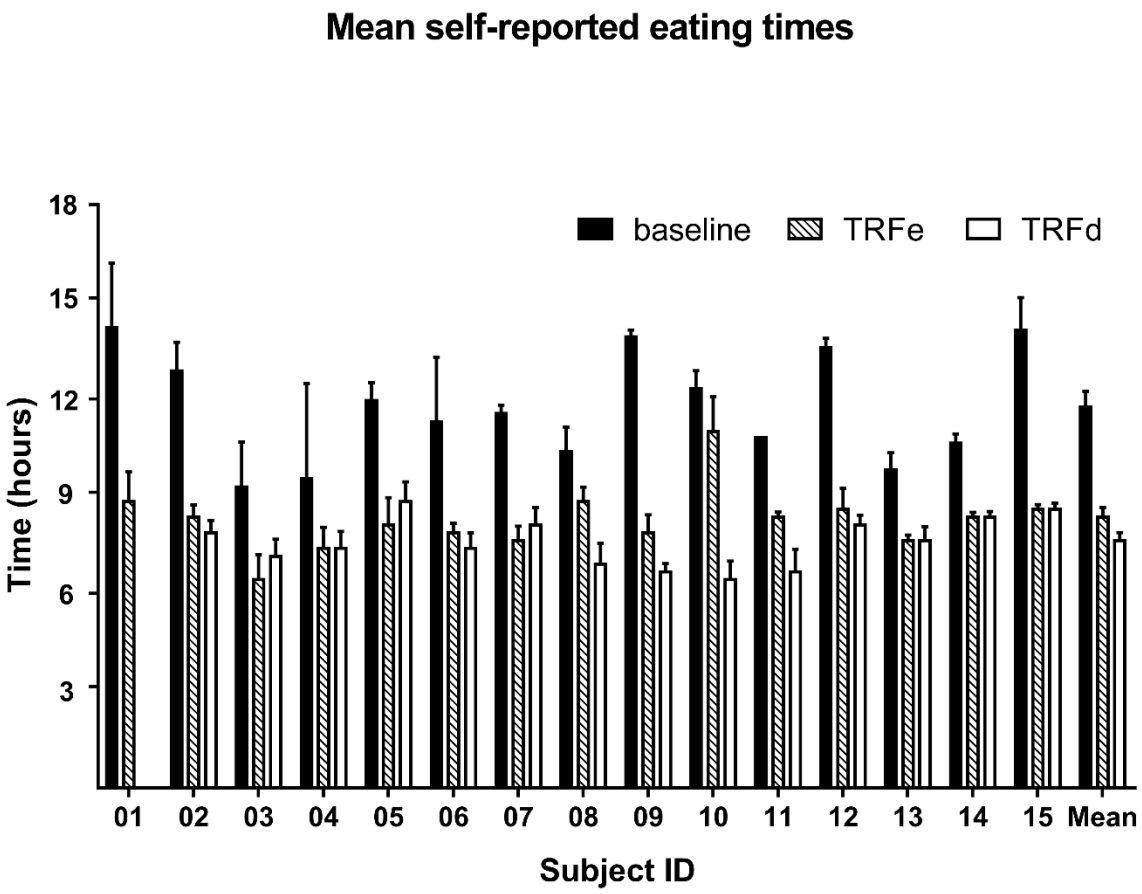


Figure 4. 7 Mean self-reported eating windows by individual, and overall group mean during baseline, and time-restricted feeding commenced early, or with a phase delay

Self-reported eating windows for individual participants, and group mean, determined from food logs during baseline (black bar), or time restricted feeding commenced early (TRFe, 8 am -5 pm; hatched bar) or with a phase delay (TRFd, 12 pm-9 pm; open bar). Participants were asked to keep a food diary briefly describing the meal, snack or beverage that was eaten, and the time that this food was consumed. Data are means ± SEM.

Table 4. 2 Effects of meal time and TRF on fasting values and postprandial iAUCs of measured parameters in response to a standardized mixed nutrient meal test

Outcome measure	TRFe		TRFd		Meal time effect	TRF effect	Mealtime x TRF effect
	Day 0	Day7	Day 0	Day 7	P-value	P-value	P-value
Fasting Glucose (mmol/L)	5.8±0.1	5.7±0.1	5.6±0.1	5.7±0.1	0.13	0.58	0.24
Fasting Insulin (pg/ml)	952±108	840±105	797±81	755±72	0.10	0.19	0.51
Fasting Ghrelin (pg/mL)	138±19	136±21	175±29	178±24	**0.01**	0.93	0.80
Fasting GLP-1 (pg/mL)	172±13	159±12	165±11	148±10	0.20	**0.002**	0.69
Fasting GIP (pg/mL)	52±7	55±15	55±8	43±5	0.57	0.51	0.55
Fasting Amylin (pg/mL)	11±2	9±2	9±1	8±1	0.18	0.18	0.68
Fasting PYY (pg/mL)	89±20	83±21	82±22	82±19	0.24	0.42	0.54
Fasting TG (mmol/L)	1.3±0.1	1.1±0.1	1.2±0.2	1.1±0.1	0.32	**0.003**	0.57
Fasting NEFA (mmol/L)	0.31±0.03	0.32±0.02	0.39±0.02	0.39±0.03	**0.000**	0.82	0.73
Feelings of hunger (AU)	37±8	44±7	37±8	49±8	0.71	**0.01**	0.67
Feelings of fullness (AU)	18±5	27±7	14±4	14±5	**0.04**	0.36	0.31
Desire to eat (AU)	50±7	51±8	44±8	60±7	0.80	**0.04**	0.35
Glucose iAUC (mmol/L.hour)	3.7±0.4	2.5±0.5	5.0±0.5	3.0±0.5	**0.002**	**0.001**	0.18
Insulin iAUC (mIU/L.hour)	404±86	328±79	438±123	361±73	0.30	0.09	0.99
Ghrelin iAUC(pg/mL.hour)	-165±25	-152±32	-242±48	-239±38	**0.04**	0.73	0.84
GLP-1 iAUC (pg/mL.hour)	224±34	250±39	218±41	258±45	0.98	0.12	0.76
GIP iAUC (pg/mL.hour)	-143±22	-152±48	-153±24	-117±15	0.57	0.51	0.55
Amylin iAUC (pg/mL.hour)	127±17	109±18	144±21	122±13	0.07	0.07	0.85
PYY iAUC (pg/mL.hour)	141±22	170±26	167±38	173±37	0.51	0.34	0.35
Triglyceride iAUC (mmol/L.hour)	0.7±0.1	0.8±0.1	0.8±0.1	0.9±0.1	0.14	0.57	0.96
NEFA iAUC (mmol/L.hour)	-0.85±0.10	-0.90±0.08	-1.09±0.07	-1.08±0.08	**0.001**	0.82	0.43
Hunger iAUC (AU.hour)	-13±15	-27±17	-6±13	-22±15	0.62	0.22	0.94
Fullness iAUC (AU.hour)	23±13	7±23	41±16	19±10	0.24	0.13	0.77
Desire to eat iAUC (AU.hour)	-29±17	-27±17	-8±16	-36±13	0.65	0.38	0.36
Half emptying time, T_{50} (min)	96±3	100±4	97±4	107±5	0.30	0.08	0.47

Responses to a 3-hour mixed-nutrient meal test at baseline (D0) and after 7 days (D7) of time restricted feeding commenced early (TRFe, 8 am -5 pm) or with a phase delay (TRFd, 12 pm-9 pm). Data are presented as mean ± SEM. Statistical analysis was performed using linear mixed modelling, with effects of meal time (meal commenced at 8 am or 12 pm) and TRF (day 0 vs day 7) as fixed variables. AU: arbitrary unit, iAUC: incremental area under the curve, GIP: gastric inhibitory peptide, GLP-1: glucagon like peptide-1, NEFA: non-esterified fatty acids, PYY: peptide YY, TG: triglyceride, TRF: time restricted feeding.

Table 4. 3 Measures of activity assessed by accelerometry during baseline, and time-restricted feeding commenced early, or with a phase delay

Parameters	**Baseline**	**TRFe**	**TRFd**	**P-value**
Hours of Armband Data/day (hh:mm)	23:25±00:10	23:40±00:05	23:41±00:03	0.28
Hours Offbody/day (hh:mm)	00:34±00:10	00:18±00:05	00:17±00:03	0.19
Percent Onbody/day (%)	97.6±0.7	98.7±0.4	98.8±0.2	0.19
Total EE (kJ)	14599±715	13683±485	14361±661	0.60
Measured EE (kJ)	14396±755	13581±490	14266±663	0.67
Offbody EE (kJ)	203±67	102±29.8	95.3±19.9	0.20
Measured Active EE (kJ)	3290±666	2351±543	3475±567	0.39
Physical Activity Duration (hh:mm)	01:56±00:23	01:22±00:16	02:04±00:20	0.37
Steps (n)	8745±1740	7197±1640	8977±1222	0.70
Lying Down (hh:mm)	08:56±00:23	08:17±00:25	09:00±00:17	0.35
Measured Sleep (hh:mm)	07:12±00:15	06:45±00:19	07:20±00:08	0.26
Average MET	1.4±0.1	1.3±0.1	1.4±0.1	0.56
Sedentary (hh:mm)	17:44±00:41	18:37±00:47	17:50±00:44	0.67
Light (hh:mm)	03:44±00:27	03:40±00:36	03:46±00:28	0.99
Moderate (hh:mm)	01:54±00:23	01:20±00:15	02:01±00:19	0.36
Vigorous (hh:mm)	00:02±00:01	00:02±00:01	00:03±00:02	0.92

Measures of activity assessed by accelerometry during 7 days of baseline, time restricted feeding commenced early or with a phase delay. Data are presented as mean ± SEM. Statistical analysis was performed using linear mixed modelling with intervention (baseline, TRFe and TRFd) included as an independent variable. EE: energy expenditure, MET: metabolic equivalent, TRFe: time-restricted feeding between 8 am and 5 pm, TRFd: time restricted feeding between 12 pm and 9pm.

Chapter 5: Early or delayed time-restricted feeding prevents metabolic impact of obesity in mice.

Prashant Regmi[1,2*], Rajesh Chaudhary[1,2*], Amanda J Page[1,2], Amy T Hutchison[1,2], Andrew Vincent[1], Bo Liu[1,2], Leonie K Heilbronn[1,2]

* Prashant Regmi and Rajesh Chaudhary equally contributed to this work.

[1]Adelaide Medical School, University of Adelaide, Adelaide, South Australia, Australia 5000

[2]Lifelong Health Theme, South Australian Health and Medical Research Institute (SAHMRI), Adelaide, South Australia, Australia 5000

Short running title: Early or delayed TRF improves metabolic outcome

Corresponding author:

A/Prof. Leonie Heilbronn, Ph.D.

Adelaide Medical School, The University of Adelaide,

North Terrace, Adelaide, SA 5000, Australia

Email: leonie.heilbronn@adelaide.edu.au

Phone: +61 8 8128 4838

Statement of Authorship

Title of Paper	Early or delayed time restricted feeding prevents the metabolic impacts of obesity in mice.
Publication Status	☐ Published ☑ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Prashant Regmi, Rajesh Chaudhary, Amanda Page, Amy Hutchison, Andrew Vincent, Bo Liu, Leonie Heilbronn. Early or delayed time restricted feeding prevents the metabolic impacts of obesity in mice.

Principal Author

Name of Principal Author (Candidate)	Prashant Regmi		
Contribution to the Paper	Designed and carried out study, collected data, performed experiments, analysed the data, wrote the manuscript.		
Overall percentage (%)	50%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the co-primary author of this paper.		
Signature		Date	20 August 2020

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	Rajesh Chaudhary		
Contribution to the Paper	Carried out study, collected data, performed experiments, analysed the data, and approved final manuscript.		
Signature		Date	20 August 2020

Name of Co-Author	Amanda J Page		
Contribution to the Paper	Supervised the study, collected metabolic data. Approved final manuscript.		
Signature		Date	31/8/20

Name of Co-Author	Amy T Hutchison		
Contribution to the Paper	Contributed to interpretation of data and approved final manuscript.		
Signature		Date	21/08/2020

Name of Co-Author	Andrew Vincent		
Contribution to the Paper	Analysed the data.		
Signature		Date	21/08/2020

Name of Co-Author	Bo Liu		
Contribution to the Paper	Supervised the study, collected metabolic data. Approved final manuscript.		
Signature		Date	20 August 2020

Name of Co-Author	Leonie Heilbronn		
Contribution to the Paper	Designed and carried out study, supervised the study, had full access to the data and had primary responsibility of the final publication.		
Signature		Date	20/8/20

5.1 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.

5.2 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. 2019; 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.

5.3 Materials and Methods

5.3.1 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 5.1A). 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.

5.3.2 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^{TM}-500 Body Composition Analyzer (n=6/group).

5.3.3 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).

5.3.4 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, VCO_2 and VO_2 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).

5.3.5 Liver triglycerides and enzyme activity measurement

For triglyceride measurement, liver tissue samples (~50 mg) were first homogenized in 5% NP-40 solution (in ddH_2O). 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; Srere 1969), and were adjusted for tissue weight.

5.3.6 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, Page, Hutchison, et al. 2019) using the TaqMan primers and master mix listed in Table 5. 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).

5.3.7 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.

5.3.8 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.

5.3.9 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.

5.3.10 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.

5.4 Results

5.4.1 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≤0.04, Figure 5.1B). Body composition by MRI was not different between groups in the chow-fed mice (Figure 5.1C). 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 5.1C). TRF also reduced gonadal and inguinal fat vs AL in HFD fed mice (both P<0.001), and in TRFe vs TRFd (P≤0.037, Figure 5.1D & E). TRF did not alter cumulative calorie consumption in chow fed mice (Figure 5.1F). 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 5. 6A). Feeding efficiency was reduced in TRFe vs AL in chow fed mice (P<0.001, Figure 5.1G). 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 5.1H, Figure 5. 7A-C & Figure 5. 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 5.1I).

5.4.2 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 5. 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 5. 2D-F). Activity was higher during the dark phase in both TRF groups vs AL (P≤0.05) in both diet groups, with no difference between TRFe and TRFd (Figure 5. 2G-I). Active phase EE and total 24-hour EE was not significantly different between groups (all P≥0.059, Figure 5. 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 5. 7D-I).

5.4.3 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≤0.007). 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 5.3A, 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 5.3C, D, G & H). Fasting glucose and insulin at ZT4 were also lower in both TRF vs AL mice that were fed HFD (all P≤0.003), but fasting glucose was higher at ZT16 in TRFd vs AL and TRFe in mice fed chow or HFD.

5.4.4 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 5. 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 5. 5A-D, Figure 5. 9A-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.

5.5 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) 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, 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 *Reverbα* in TRF mice fed HFD (Chaix et al. 2019; Hatori 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 (Mitchell et al. 2014; 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 et al. 2009). 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.

Acknowledgement: PR and RC were supported by Adelaide scholarship international from The University of Adelaide.

Conflict of Interest: Authors declare no conflict of interest.

Funding: This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Prior Presentation: Parts of this study were presented as an oral presentation at the Australia and New Zealand obesity society annual conference 2019, 16-18 October, Sydney, Australia, and Obesity week 2019, 3-7 November, Las Vegas, USA.

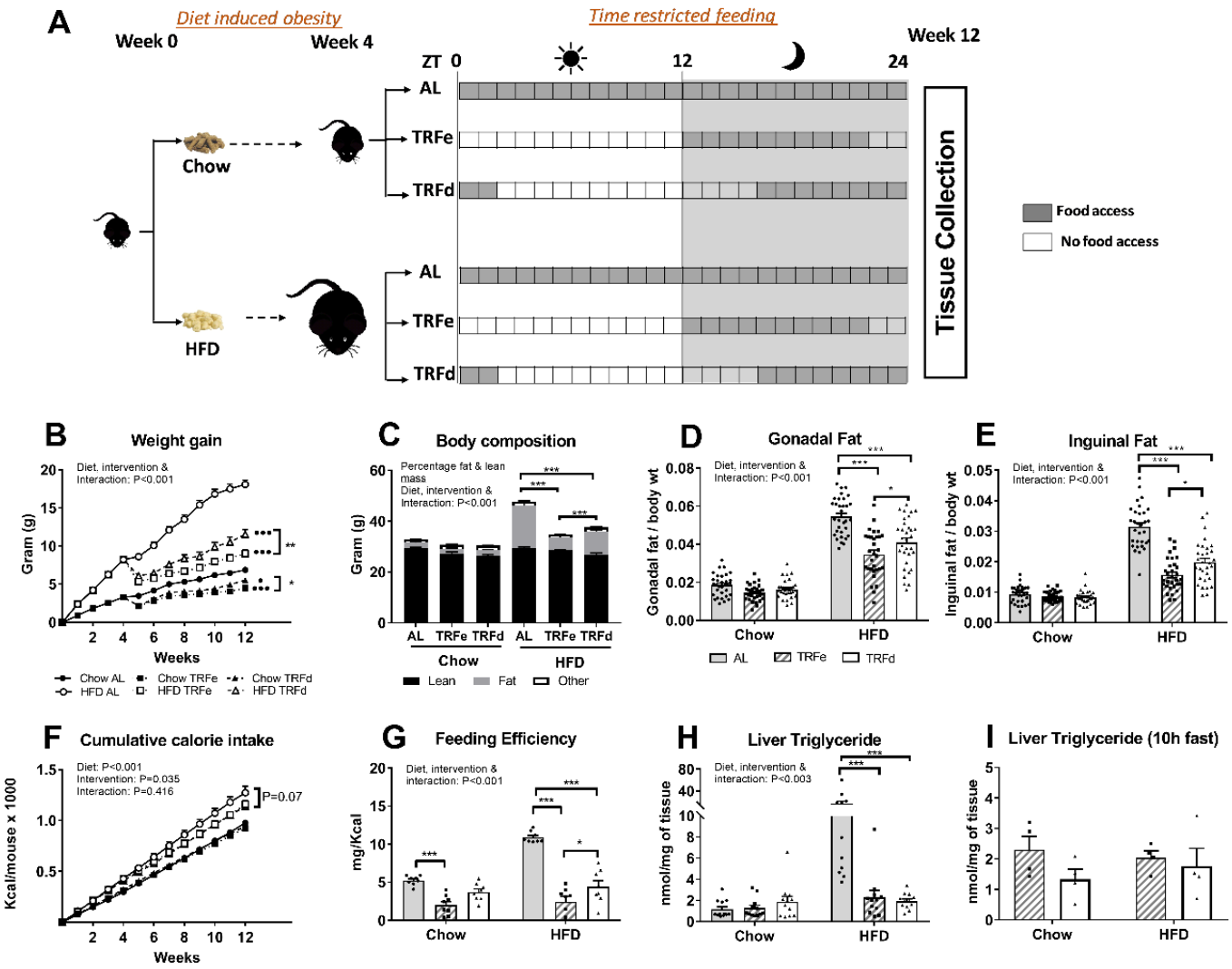

A
Diet induced obesity
Time restricted feeding
Week 0
Week 4
Week 12
ZT 0
12
24
Chow
HFD
AL
TRFe
TRFd
Tissue Collection
Food access
No food access
B
Weight gain
Diet, intervention & Interaction: P<0.001
Gram (g)
Weeks
Chow AL
Chow TRFe
Chow TRFd
HFD AL
HFD TRFe
HFD TRFd
C
Body composition
Percentage 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 5.1 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).

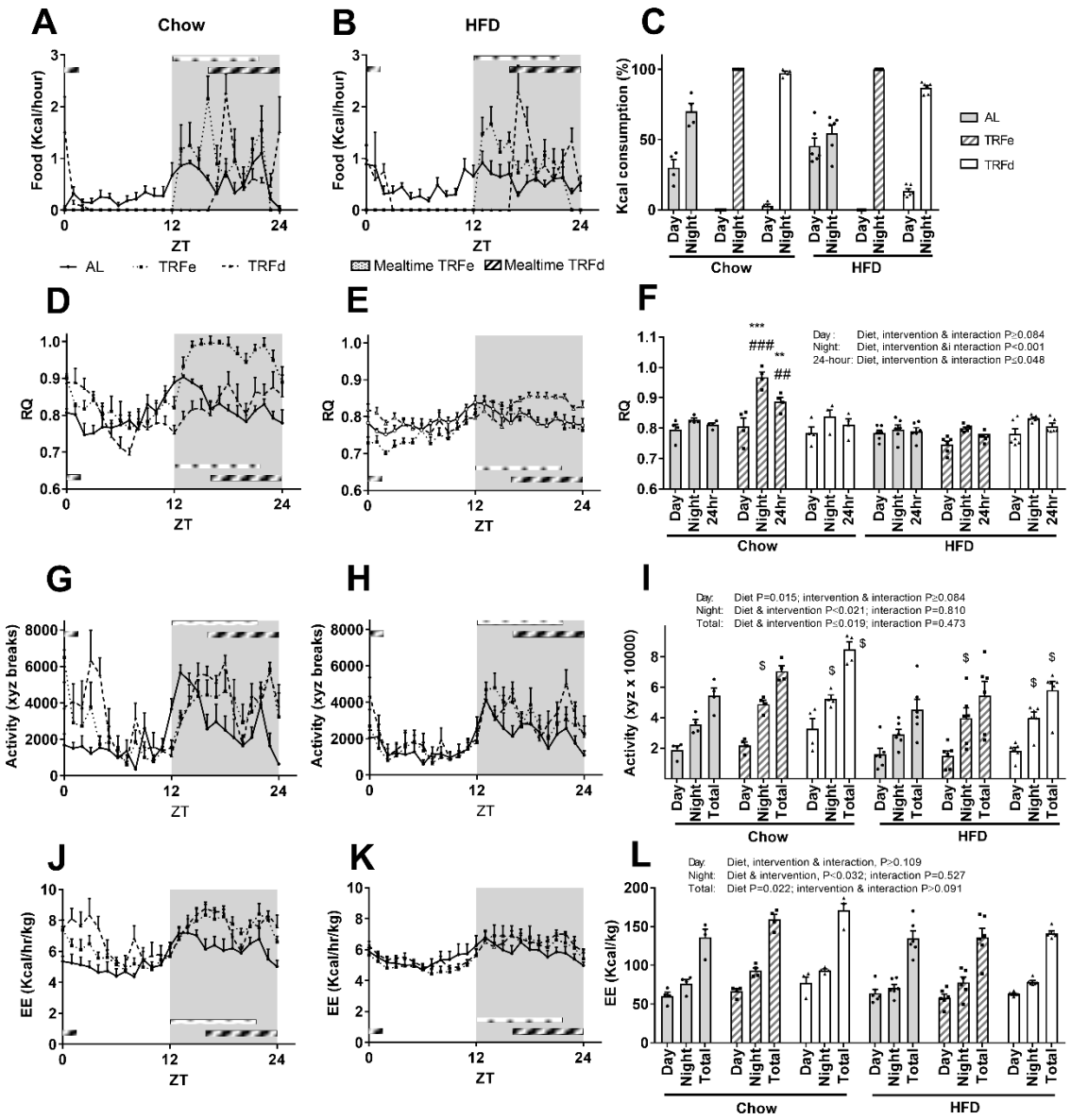


Figure 5. 2 TRF drives rhythm in nutrient utilization.

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.

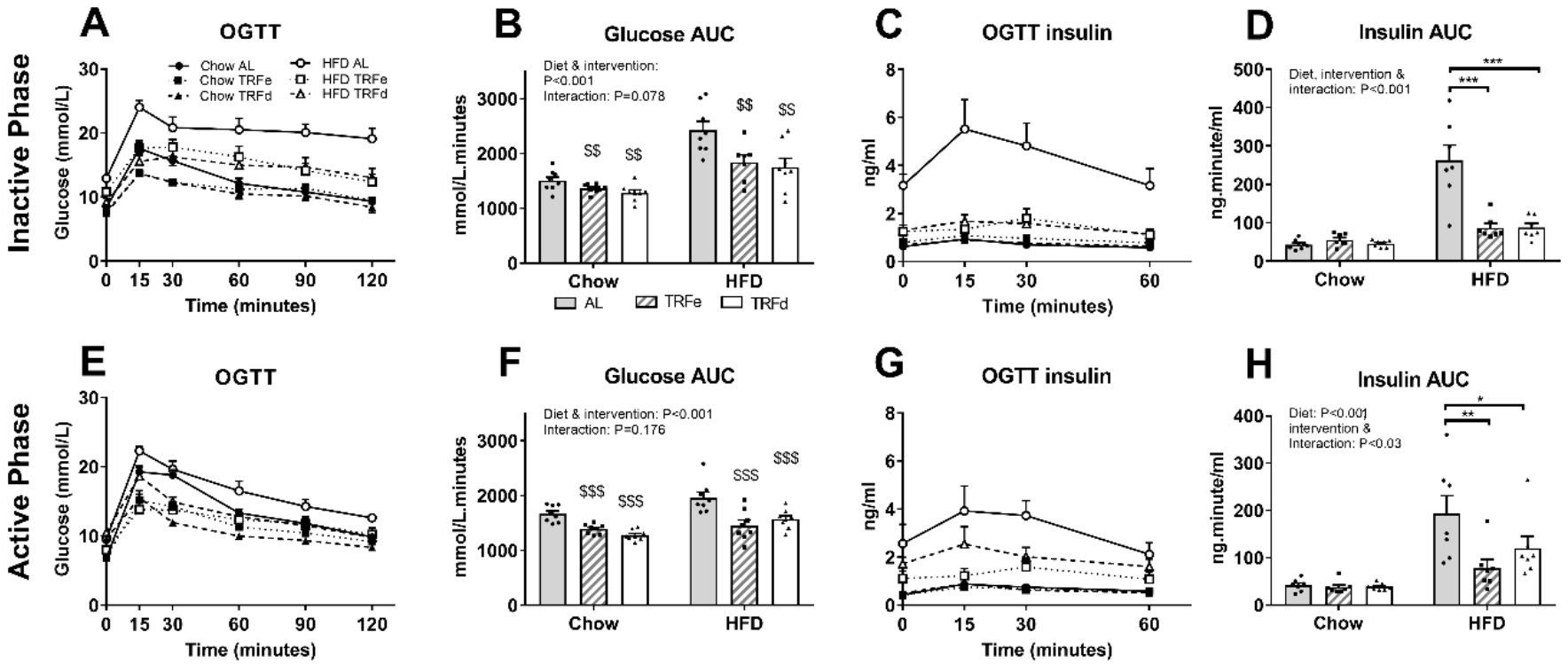

A
OGTT
Inactive Phase
Chow AL
Chow TRFe
Chow TRFd
HFD AL
HFD TRFe
HFD TRFd
Glucose (mmol/L)
Time (minutes)
B
Glucose AUC
Diet & intervention: P<0.001
Interaction: P=0.078
mmol/L.minutes
Chow
HFD
AL
TRFe
TRFd
C
OGTT insulin
ng/ml
Time (minutes)
D
Insulin AUC
Diet, intervention & interaction: P<0.001
ng.minute/ml
Chow
HFD
E
OGTT
Active Phase
Glucose (mmol/L)
Time (minutes)
F
Glucose AUC
Diet & intervention: P<0.001
Interaction: P=0.176
mmol/L.minutes
Chow
HFD
G
OGTT insulin
ng/ml
Time (minutes)
H
Insulin AUC
Diet: P<0.001
intervention &
Interaction: P<0.03
ng.minute/ml
Chow
HFD

Figure 5.3 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.

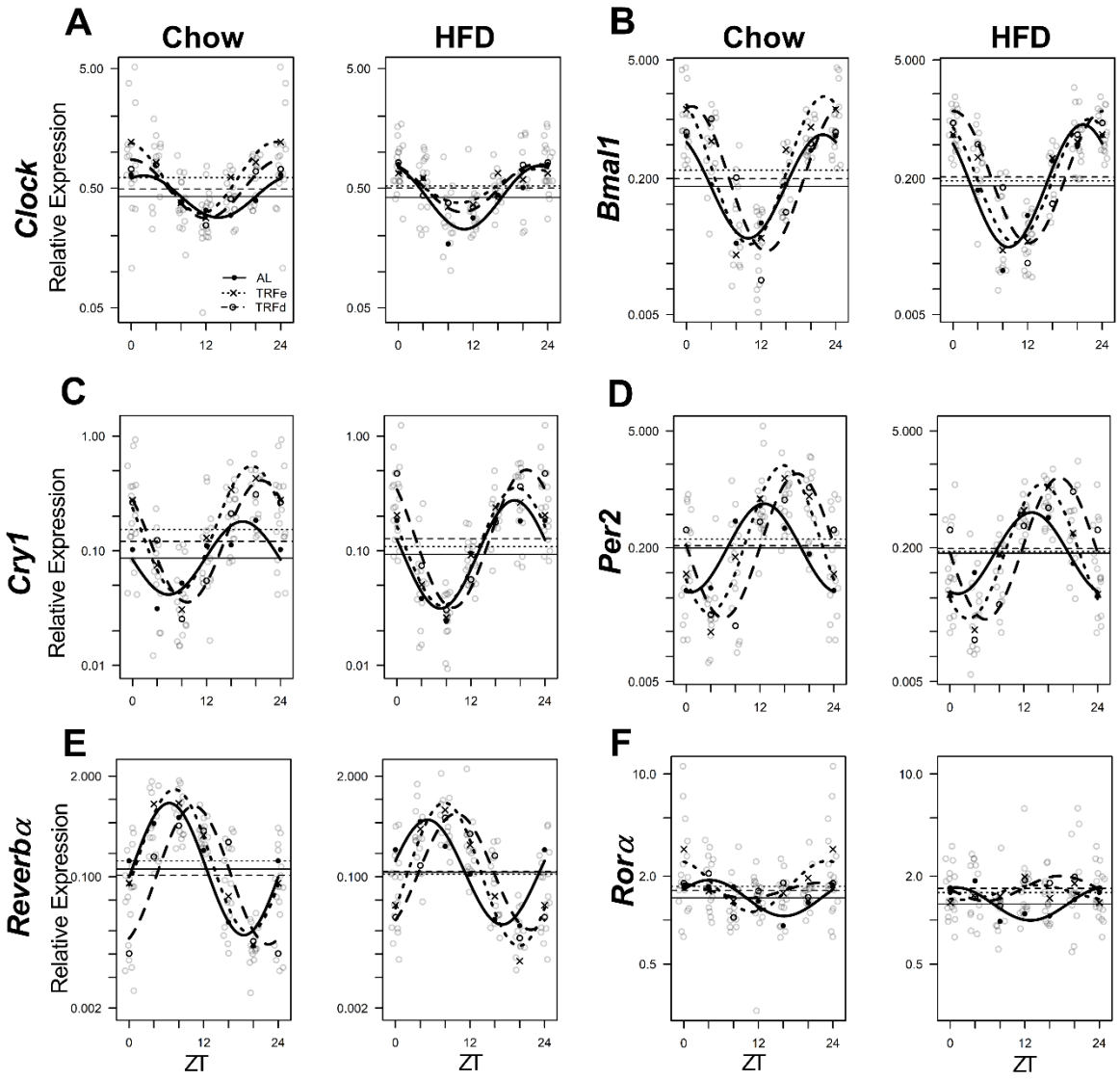


Figure 5. 4 TRF facilitates robust oscillation of genes involved in circadian rhythm, despite inducing phase delay in TRFd.

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).

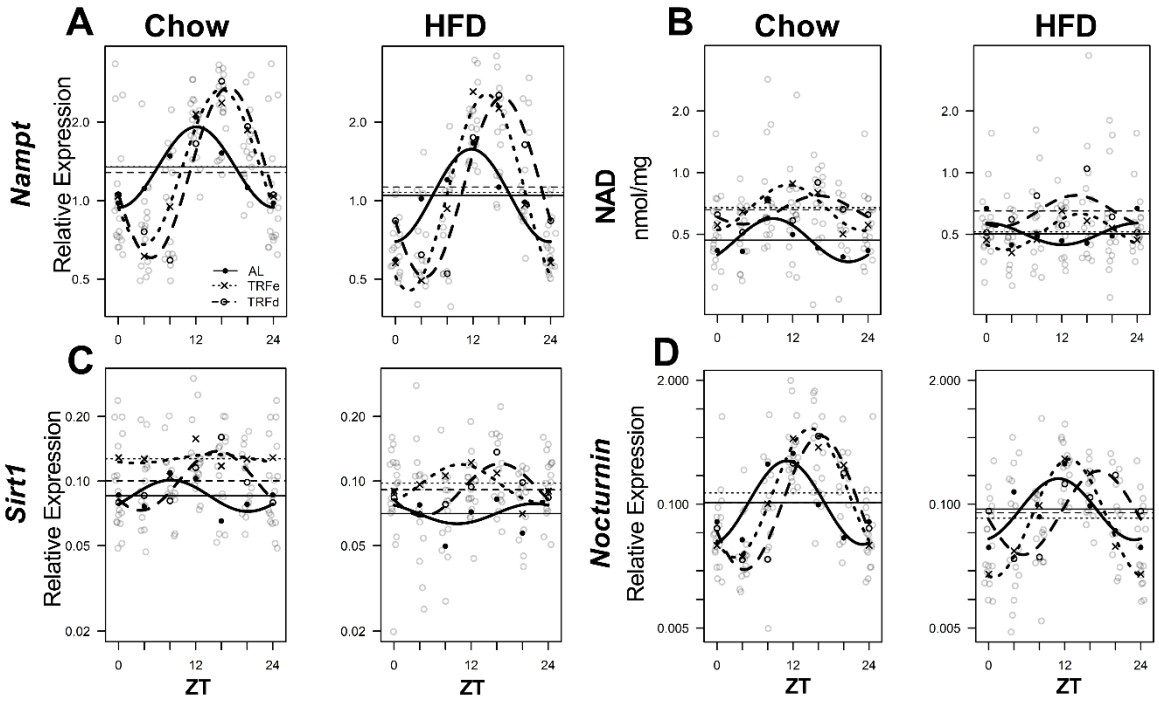


Figure 5. 5 TRF facilitates robust oscillation and restores HFD induced phase shift in markers of NAD metabolism in liver.

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).

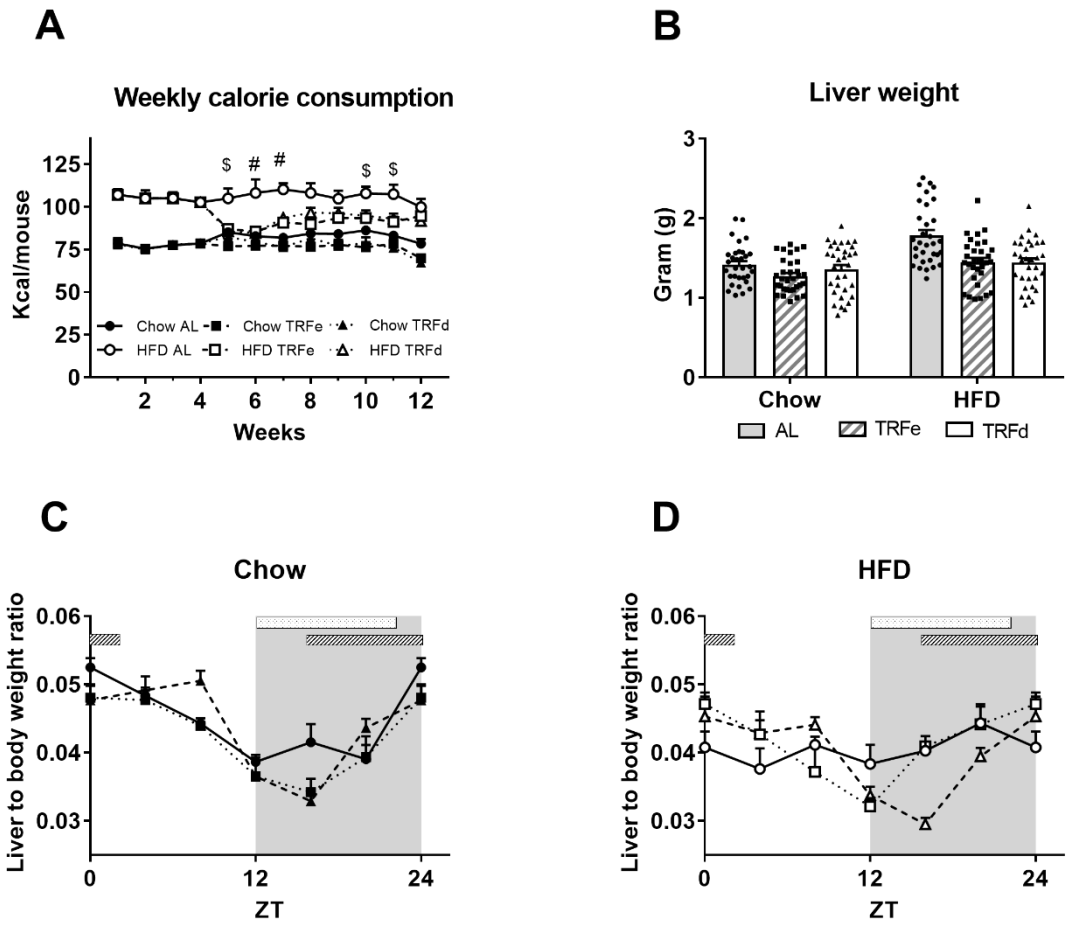


Figure 5. 6 Weekly calorie consumption and liver to body weight ratio.

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.

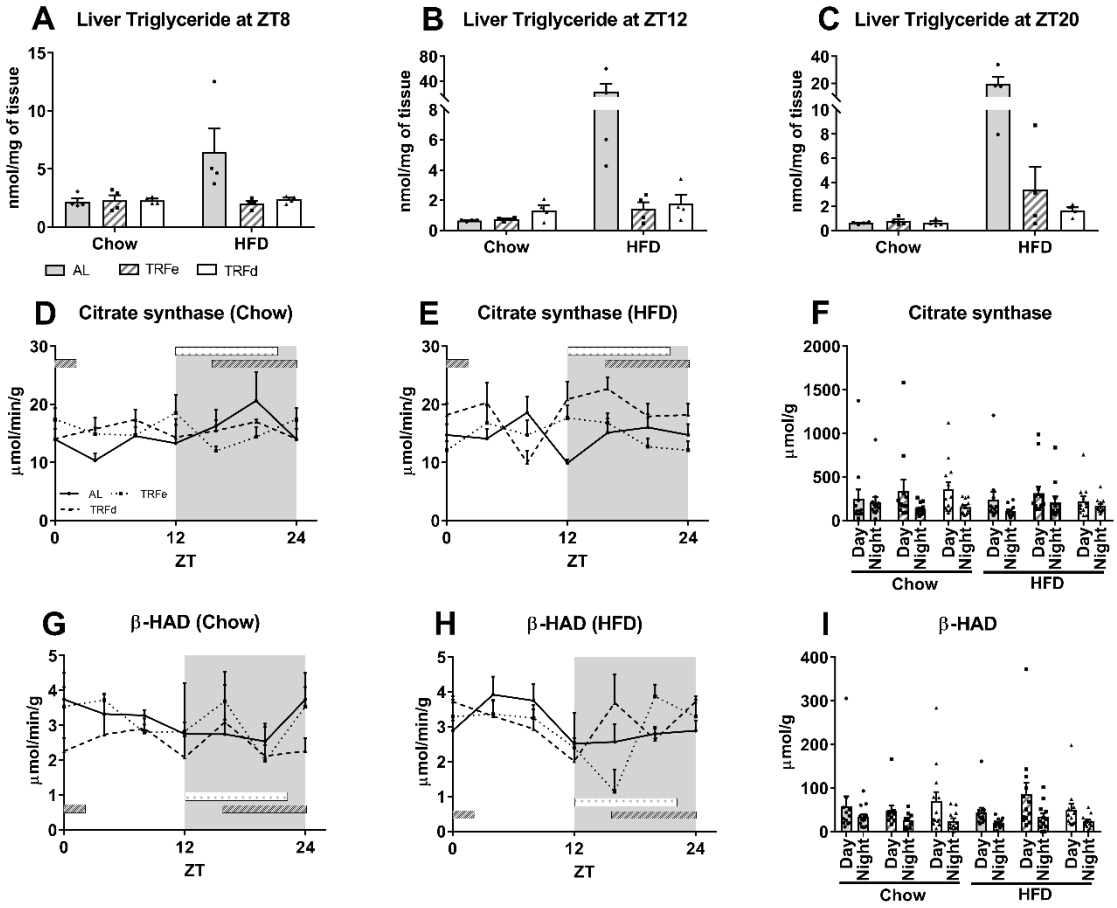


Figure 5. 7 Triglycerides levels, and citrate synthase and β-HBD activity in liver.

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.

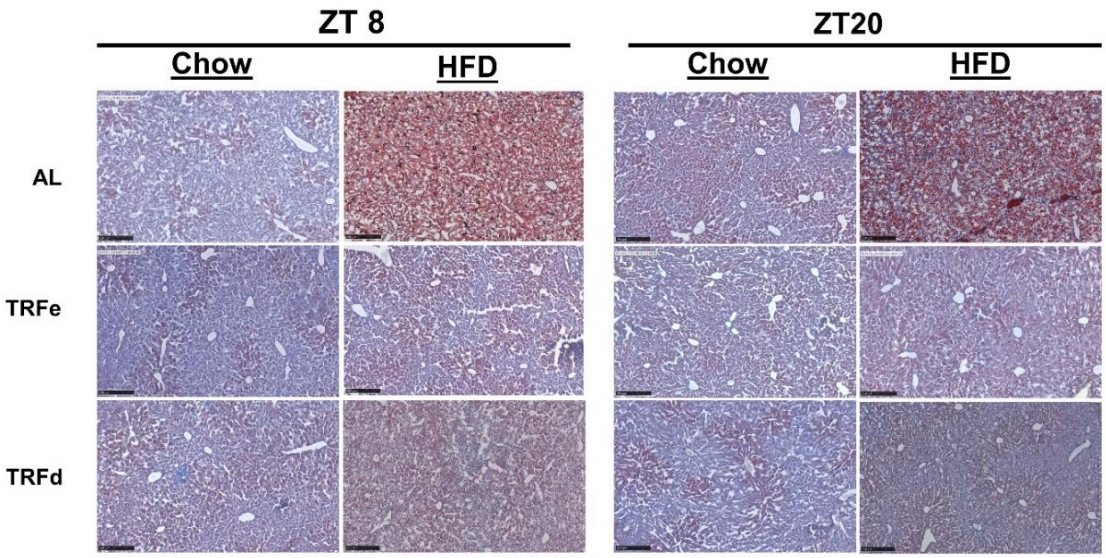


Figure 5. 8 Representative images of oil red O staining in liver sections.

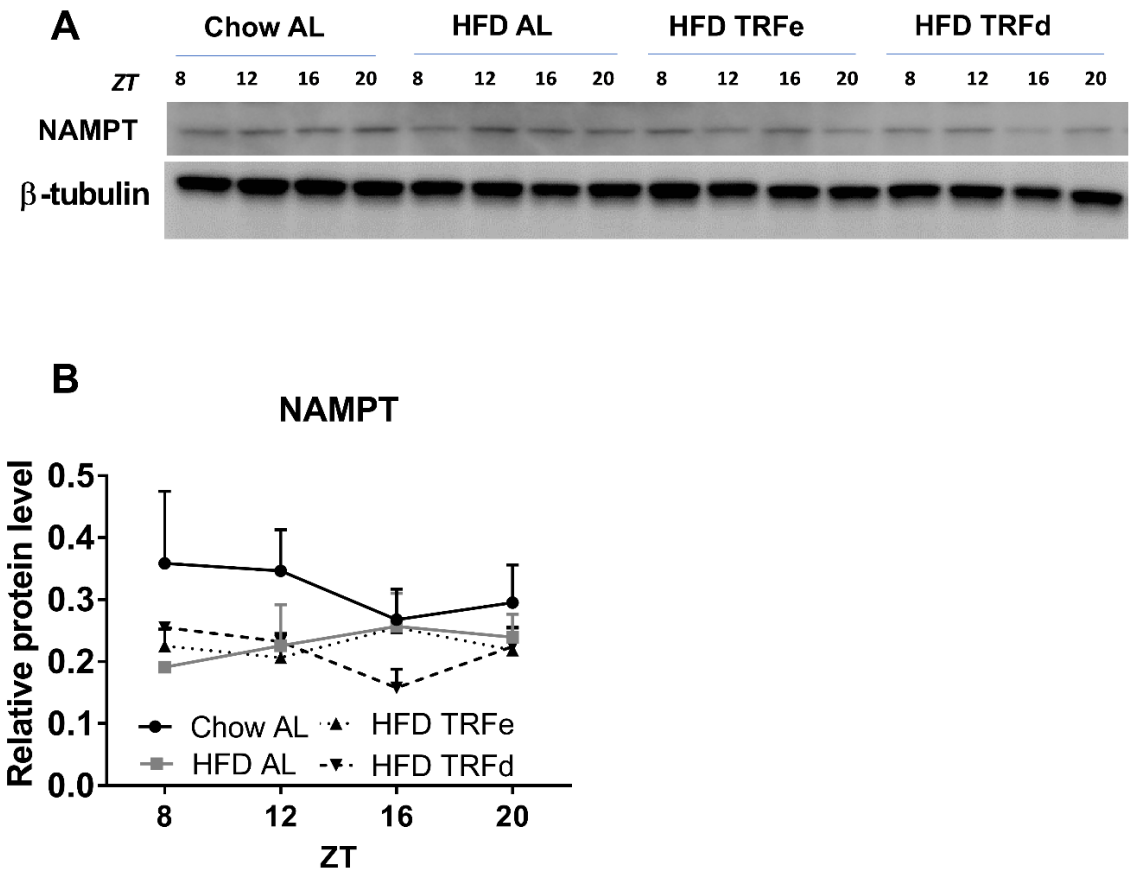


Figure 5. 9 Western blots of NAMPT

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

Table 5. 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 5. 2 Diets, instruments, kits, chemicals and software used in this 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	EchoMRI™-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 ™ Fast Universal PCR Master Mix (2X) (#4352042, Applied Biosystems, Lithuania)
BCA assay	Thermofisher (23228 & 1859078)
Chemiluminescence substrate	Thermofisher (Super signal, 34095)
Chemicals	
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 6: Intermittent fasting increases *NAMPT* expression in human skeletal muscle on fasting day.

Prashant Regmi[1,2], Bo Liu[1,2], Gary A Wittert[1,2], Amanda J Page[1,2], Amy T Hutchison[1,2], Leonie K Heilbronn[1,2]

[1]Adelaide Medical School, The University of Adelaide, Adelaide, SA

[2]Nutrition and Metabolism Theme, South Australian Health and Medical Research Institute, Adelaide, SA

Correspondence to:

A/Prof. Leonie Heilbronn, Ph.D.

Adelaide Medical School, The University of Adelaide,

North Terrace, Adelaide, SA 5000, Australia

Email: leonie.heilbronn@adelaide.edu.au

Phone: +61 8 8128 4838

Statement of Authorship

Title of Paper	Intermittent fasting increases *NAMPT* expression in human skeletal muscle on fasting day.
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Prashant Regmi, Bo Liu, Gary A Wittert, Amanda J Page, Amy T Hutchison, Leonie K Heilbronn. Intermittent fasting increases *NAMPT* expression in human skeletal muscle on fasting day.

Principal Author

Name of Principal Author (Candidate)	Prashant Regmi		
Contribution to the Paper	Performed experiments, analysed the data, interpreted the 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	31 August 2020

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	Bo Liu		
Contribution to the Paper	Commenced the study and collected samples, interpreted data and approved final manuscript.		
Signature		Date	31 August 2020

Name of Co-Author	Gary A Wittert		
Contribution to the Paper	Designed the study, collected biopsies and supervised clamps, interpreted data, and approved final manuscript.		
Signature		Date	07/09/20

Name of Co-Author	Amanda J Page		
Contribution to the Paper	Supervised study, interpreted data, and approved final manuscript.		
Signature		Date	31/8/20

Name of Co-Author	Amy T Hutchison		
Contribution to the Paper	Commenced study and collected samples, analysed and interpreted data, and approved final manuscript.		
Signature		Date	2/09/2020

Name of Co-Author	Leonie K Heilbronn		
Contribution to the Paper	Designed and supervised the study, interpreted data, approved final manuscript, and had primary responsibility for the study and publication.		
Signature		Date	14/9/2020

6.1 Abstract

Intermittent fasting (IF) reduces adiposity, potentially by switching from anabolic to catabolic states. We examined the effect of IF on metabolic switching, and skeletal muscle nicotinamide phosphoriboslytransferase (NAMPT) and sirtuin1 (SIRT1) from fed to fast transition.

Women with obesity underwent 24h-fasts initiated at 8am on three non-consecutive days/week, with foods provided at 70%, or 100% of weekly energy requirements on non-fast days. Insulin sensitivity, body weight and composition were measured, and *vastus lateralis* biopsies were collected at baseline (12h-fast), and twice at 8-weeks (12 & 24h-fast). We additionally fed 10-week-old C57BL/6 male mice with a chow or high-fat-diet for 8-weeks before randomized to continue *ad libitum* or IF (24h-fast initiated at ZT11, three alternate-days/week) for a further 8-weeks. Mice underwent an oral glucose tolerance test, and were sacrificed in a fed or 24-h fasted state.

IF reduced body weight and fat mass in women with obesity (both $P<0.001$). IF reduced mRNA levels of *NAMPT* and *SIRT1* following a fed day (both $P\leq0.005$), whereas *NAMPT* mRNA levels were increased after a 24h-fast ($P=0.005$) along with a transient reduction in insulin sensitivity ($P<0.001$). In mice, IF completely protected from high-fat-diet induced weight gain, adiposity, glucose intolerance, and fasting hyperglycaemia, but did not alter markers of NAD metabolism in skeletal muscle.

The reduction in peripheral insulin sensitivity and a rise in skeletal muscle NAMPT may underlie the metabolic switch to fat oxidation in IF. Circadian timing of tissue collection might have contributed to the differential effects of IF on markers of NAD metabolism in humans and mice.

Key words: intermittent fasting, metabolic switching, insulin sensitivity, skeletal muscle, NAD

6.2 Introduction

'Metabolic switching' is the process of shifting the source of energy from utilization of glucose to fatty acids and fatty acid-derived ketones (Cahill 2006). Intermittent metabolic switching enhances neurogenesis and cognition in brain, reduces hepatic steatosis, mitigates age-induced decline in muscle mass, reduces blood pressure, and activates cellular recycling and repair pathways (Anton et al. 2018). Weight loss (particularly reduction in body fat) has several health benefits including improvement in glycaemic profile and reduction in cardiovascular disease risk, and also typically involves the shift from lipogenesis and fat storage to mobilization of fat in the form of non-esterified fatty acids (NEFA) and ketones (Cahill 2006; Look et al. 2007; Wing et al. 2011).

Nicotinamide adenine dinucleotide (NAD) is a cofactor that plays a pivotal role in cellular metabolism and sirtuin (SIRT) function (Canto, Menzies & Auwerx 2015; Imai et al. 2000). The majority of NAD is recovered from the nicotinamide phosphoribosyltransferase (NAMPT) mediated salvage pathway (Zhang et al. 2017). Reduction in cellular NAD leads to accelerated ageing, and induces insulin resistance in mice (Poljsak 2018; Stromsdorfer et al. 2016). Whereas, dietary supplementation of NAD precursors and genetic overexpression or chemical activation of *Nampt* improves healthspan and lifespan in rodents (Mitchell et al. 2018; Mitchell et al. 2014; Peek et al. 2013; Stromsdorfer et al. 2016). Further, cellular NAD availability can also influence lipid oxidation because NAD is a cofactor of key enzymes of β-oxidation (Houten & Wanders 2010).

Intermittent fasting (IF) is a pattern of eating based on alternating periods of zero or minimal calories consumed 1 to 4 days per week, followed by periods of unrestricted eating (Heilbronn & Panda 2019). IF exhibits several metabolic health benefits in both preclinical models and humans (Halberg et al. 2005; Heilbronn et al. 2005; Hoddy et al. 2014; Hutchison, Liu, et al. 2019; Li et al. 2017; Liu, Page, Hatzinikolas, et al. 2019; Liu, Page,

Hutchison, et al. 2019; Varady et al. 2013). Although reduced calorie intake and weight loss has been observed in several IF studies in diet induced obese mice and humans (Halberg et al. 2005; Hutchison, Liu, et al. 2019; Liu, Page, Hatzinikolas, et al. 2019; Varady et al. 2013), some studies have suggested that metabolic switching between fed and fasting states, rather than weight loss, may underlie the metabolic health benefits of IF (Anton et al. 2018; Antoni et al. 2017).

The aim of this study was to examine whether IF impacts markers of NAD metabolism in skeletal muscle, and to explore whether this was associated with metabolic switching from fed to fasting day in humans and mice. We hypothesized that IF would increase *NAMPT* expression in skeletal muscle along with plasma and physiological markers of lipid oxidation during the fed to fast transition.

6.3 Methods

6.3.1 Human Study

Detailed study design has been described previously (Hutchison, Liu, et al. 2019). To distinguish whether the effects of IF are independent of calorie restriction, diets at two levels were prescribed in this study. Briefly, women with overweight or obesity (baseline characteristics presented in (Table 6. 1) were provided with foods at 70% (IF70, n=25), or 100% (IF100, n=25) of baseline energy requirements, with 24h fasts initiated at 8am on three non- consecutive days per week for 8 weeks. On four feeding days per week, IF70 group consumed ~100% of baseline energy requirement, whereas IF100 group consumed ~145% of baseline energy requirements to fulfil their weekly assigned energy targets. Participants underwent three metabolic visits, two after a 12-hour fast at baseline (V0) and week 8 (V8a), and one after 24-hour fast at week 8 (V8b). Body weight, body composition by DXA, and insulin sensitivity by hyperinsulinemic-euglycemic clamp [60mIU/m^2/min, presented as M-

value (GIR/kg FFM+17.7)] were measured. In addition, *vastus lateralis* biopsies (at ~8am) were collected on each visit and snap frozen in liquid nitrogen. As we were interested in markers of NAD metabolism in skeletal muscle, only those participants who donated skeletal muscle biopsy are included in this study (IF70, n=17, and IF100, n=14). The study was approved by Royal Adelaide Hospital Research Ethics Committee, and participants provided written informed consent. The study was registered with ClinicalTrials.gov (NCT01769976).

6.3.2 Mouse study

The details on study design have been published previously (Liu, Page, Hatzinikolas, et al. 2019). In brief, fourty-eight ten-week old C57BL/6 male mice were fed chow (18% energy from fat) or HFD (43% energy from fat) for 8-weeks before randomized to continue *ad libitum* or IF for a further 8-weeks on their respective diets. IF mice fasted for 24 hours starting from ZT11 (ZT0 when lights on) for three non-consecutive days per week. After 7-weeks on intervention (at the 25 week age), mice underwent an oral glucose tolerance test (OGTT, 2g/kg body weight) at ZT6, and incremental area under the curve (iAUC) was calculated by trapezoidal rule as the index of glucose tolerance (Allison et al. 1995). After one week of recovery, mice were anaesthetised by isoflurane and euthanized by cervical dislocation. AL mice were euthanized in fed state, and IF mice were euthanized in fed (n=7-8/diet) or 22-hour fast (n=7-8/diet) states at ZT7-9. A terminal blood sample and *quadriceps* muscle were collected and snap frozen in liquid nitrogen for further analysis. All experiments were approved by the SAHMRI and The 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.

6.3.3 NAD measurement

Mouse skeletal muscle tissue samples (~30-40 mg) were homogenized in NAD extraction buffer and NAD-NADH cycling assay was performed using alcohol dehydrogenase (ADH) cycling mix at 37°C in the dark for 15 minutes as previously described (Bertoldo et al. 2020). Absorbance was measured at 340 nm, NAD concentration was determined using a standard curve, and corrected for the amount of tissue used.

6.3.4 RNA extraction and real-time PCR

Total RNA was extracted from muscle (*vastus lateralis* in humans, and *quadriceps* in mice) using Trizol (Invitrogen) as per manufacturer's instruction. RNA quality was checked using NanoDrop (240/280 absorbance ratio, Thermo Fisher). After a gDNA wipe out reaction, cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen, 15 min at 42°C, 3 min at 95 °C, and then put into ice). Quantitative real-time PCR was performed as described previously (Liu, Page, Hutchison, et al. 2019) using the TaqMan primers and master mix. *NAMPT* (human: Hs00237184_m1, mouse: Mm00451938_m1) and *SIRT1* (human: Hs01009006_m1, mouse: Mm01168521_m1) genes were measured in muscle of human and mice. *ACTB* (β-actin, Hs01060665_g1) and *HPRT* (hypoxanthine-guanine phosphoribosyltransferase, Hs02800695_m1) were used as reference gene in humans (Zhao et al. 2020), and *B2M* (β-2-microglobulin, Mm00437762_m1) and *PPIA* (peptidylprolyl isomerase A, Mm02342430_g1) as reference gene in mice as recommended by NormFinder software. Relative gene expression was calculated using $2^{-\Delta CT}$, where ΔCT = (CTtarget gene - CTreference gene).

6.3.5 Western Blot

Mouse *quadriceps* muscle (~20 mg) was lysed in tissue lysis buffer (CellLytic MT cell lysis reagent, Sigma) with phosphatase inhibitors. The lysate was centrifuged at 12,000g for 10

minutes, and supernatant was separated. Protein concentration in the supernatant was measured using BCA assay, and diluted with distilled water and reducing agent to make final protein concentration of 2μg/μl. Final protein solution was heated to 95-100 °C for 5 minutes and immediately transferred on ice. 15ul of tissue lysates (30μg of protein) were resolved by SDS-PAGE (10-20% polyacrylamide gradient gel [Bio-Rad], 130 V, 1 hour 30 minutes) and transferred onto polyvinylidene fluoride membranes (35V, 1 hour). Membranes were blocked in 2% blocking solution (Bio-Rad,1 hour at room temperature with continuous agitation) and probed for NAMPT (1:500, E-3, sc-393444, Santa-Cruz) and β-tubulin (1:500, Ab-6046, Abcam) overnight at 4 °C with continuous agitation. After washing, membranes were incubated with secondary antibodies (anti-mouse or anti-rabbit, 1:10,000, 1 hour). Bands were visualized by fluorescence, the intensity was measured using ImageJ software as previously described (Chen, M et al. 2014), and protein concentration was presented as relative protein levels adjusted for β-tubulin.

6.3.6 Calculation and Statistical analysis

In the human study, data were analysed by linear mixed-model with group (IF70 and IF100) and visits (V0, V8, and V8b) as fixed factors. A differential effect of visits in each group was tested via the interaction between group and visits, and Bonferroni's *post hoc* was applied. Correlation was calculated by Pearson's correlation coefficient. In the mouse study, statistical analysis was performed by two-way ANOVA with diet (Chow and HFD) and intervention (AL, IF-fed, and IF-fast) 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. Comparison of final body weight, fat mass, fasting glucose, and glucose iAUC between chow-AL and HFD-IF, and NAMPT protein, *Nampt* and *Sirt1* mRNA between chow AL and HFD AL were performed using a t-test (SPSS, IBM). All data are presented as mean±SEM and $P<0.05$ was considered statistically significant.

6.4 Results

6.4.1 Human study results

As previously reported (Hutchison, Liu, et al. 2019), both forms of IF reduced body weight and fat mass (both P<0.001). IF did not change insulin sensitivity after a fed day, but insulin sensitivity was impaired in both IF groups after a 24-hour fast compared to fed day (both P<0.001, Figure 6. 1A). IF did not alter respiratory quotient (RQ) on the fed day, but reduced the RQ on fasting day compared to fed day (P<0.001, Figure 6. 1B). IF reduced skeletal muscle *NAMPT* mRNA expression after a fed day (P=0.005), but *NAMPT* mRNA level was increased after a 24-hour fast compared to fed day (P=0.005, Figure 6. 1C). IF reduced *SIRT1* expression after a fed day (P=0.001, Figure 6. 1D), whereas *SIRT1* expression was not altered after a 24-hour fast (P=0.175). Skeletal muscle *NAMPT* mRNA expression was positively correlated with insulin sensitivity at baseline (r=0.348, P=0.048, Figure 6. 1E), however, there was no longer an association with the change in this response after IF in the fed or fasted state.

6.4.2 Mice study results

As previously reported (Liu, Page, Hatzinikolas, et al. 2019), IF reduced weight gain, fat accumulation, glucose intolerance, and fasting glucose in mice fed HFD (all P<0.05). Final body weight, dissected total fat mass, glucose iAUC, and fasting glucose in HFD-IF mice were not statistically different than that of chow-AL (all P≥0.383, Figure 6. 2A-D). Hence, IF completely protected mice from HFD induced weight gain, adiposity, glucose intolerance, and hyperglycaemia. As previously reported (Liu, Page, Hatzinikolas, et al. 2019; Liu, Page, Hutchison, et al. 2019), IF did not alter RQ and plasma NEFA on the fed day, but RQ was decreased and plasma NEFAs were increased on fasting day (P<0.05). HFD AL reduced NAMPT protein (P=0.002) vs chow AL in skeletal muscle, but did not alter *Nampt* or *Sirt1*

mRNA levels (P≥0.722). IF did not alter NAMPT mRNA and protein, NAD levels and *Sirt1* mRNA expression in skeletal muscle on either diet (Figure 6. 3A-E).

6.5 Discussion

IF shows pleiotropic metabolic benefits in response to diverse nutritional challenges in both preclinical models and humans (Halberg et al. 2005; Heilbronn et al. 2005; Hoddy et al. 2014; Hutchison, Liu, et al. 2019; Joslin, Bell & Swoap 2017; Li et al. 2017; Liu, Page, Hatzinikolas, et al. 2019; Liu, Page, Hutchison, et al. 2019; Varady et al. 2013). Some studies have suggested that 'metabolic switching' between fed and fasting states may underlie the health benefits of IF (Anton et al. 2018; Antoni et al. 2017). Conversely, others have also suggested that improvement in metabolic phenotypes are related to cellular NAD levels and SIRT1 function in model animals (Mitchell et al. 2018; Mitchell et al. 2014; Poljsak 2018; Ramsey et al. 2008; Stromsdorfer et al. 2016). This study examined the effects of IF for 8-weeks, and the changes during fed to fasting transition in IF on markers of NAD metabolism in skeletal muscle of women with obesity and in chow or HFD fed mice. The results of this study show that IF reduced *NAMPT* and *SIRT1* expression in human skeletal muscle. From the fed to fast day, there was an increase in skeletal muscle *NAMPT* expression, and reduction in insulin sensitivity and RQ. However, IF did not alter markers of NAD metabolism in mouse skeletal muscle.

On the fed day, IF did not alter insulin sensitivity in women who were in energy deficit or balance, whereas the insulin sensitivity was transiently reduced in both group in response to the 24 h fast. Reduced insulin sensitivity has also been observed previously in humans following an intravenous glucose tolerance test after a 24-hour fast (Salgin et al. 2009). This is likely a protective mechanism to preserve glucose for the central nervous system and

facilitate skeletal muscle to utilise lipids and ketones for the source of energy (Cahill 2006). Mechanistically, fasting increases the AMP:ATP ratio and activates 5'AMP-activated protein kinase (AMPK), a fasting activated nutrient sensor in skeletal muscle (Hardie 2011). AMPK activation by 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) also increased fatty acid oxidation in rat muscle, but at the same time reduced glucose oxidation, glycogen synthesis and lactate release (Kaushik et al. 2001), indicating reduction in glucose utilization. *NAMPT* is a downstream target of AMPK (Brandauer et al. 2013), and the *NAMPT* mRNA expression was elevated after 24-hour fasting in women in this study. This elevation in NAMPT should increase production of NAD, facilitating lipid oxidation (Houten & Wanders 2010). Glucose restriction also increased NAMPT expression in skeletal muscle cell lines (Fulco et al. 2008), and the fasting-induced increase in *NAMPT* mRNA level in skeletal muscle was blunted in skeletal muscle from AMPKϒ3 knockout mice (Canto et al. 2010). In the present study, plasma NEFAs and β-hydroxy butyrate (a ketone body) were increased on fasting day in both IF70 or IF100 groups vs fed day (Hutchison, Liu, et al. 2019). Further, IF did not alter RQ on the fed day, but RQ was reduced after the 24-hour fast in both IF groups, suggesting an increased fat oxidation for the source of energy. Thus, the reduced peripheral insulin sensitivity, rise in plasma NEFAs and ketones, and reduction in RQ confirms that there was metabolic switching from fed to fast days in IF, and rise in *NAMPT* expression (suggesting increase in NAMPT function) on fasting day could facilitate lipid oxidation in skeletal muscle.

Interestingly, insulin sensitivity was positively correlated with *NAMPT* expression at baseline. This suggests that insulin sensitivity in skeletal muscle might be related to *NAMPT* expression. Transgenic loss of *NAMPT* in adipose tissue induced severe insulin resistance in mice even at the age of 8 weeks. Whereas, supplementation with nicotinamide mononucleotide, a product of NAMPT, improved insulin sensitivity (Stromsdorfer et al.

2016). Lower skeletal muscle NAMPT protein levels were also observed in sedentary and obese participants than lean and active participants (Costford et al. 2010).

Although both forms of IF reduced *SIRT1* expression on the fed day, *SIRT1* expression was not changed after a 24-hour fast. In a previous study, *SIRT1* gene expression was increased after 48-hour fasting in human skeletal muscle (Edgett et al. 2016), when the fasting was initiated at lunch. Conversely, alternate day fasting initiated from midnight for 3-weeks increased *SIRT1* expression in skeletal muscle samples taken after 32-hour fast at ~8am from lean individuals (Heilbronn et al. 2005). In contrast, a study in Ramadan population where people fasted for about ~15 hours did not alter *SIRT1* expression in blood (Madkour et al. 2019). This data suggests that a prolonging the fast for more than 24 hours is required to increase *SIRT1* expression. The time of fasting initiation in IF may also impact this response, as *SIRT1* expression displays a circadian rhythm in muscle (Wallace et al. 2018).

In mice, IF completely protected from HFD induced weight and fat gain, glucose intolerance and hyperglycaemia. However, there was no change in NAMPT mRNA and protein, NAD level and *SIRT1* mRNA in skeletal muscle. Apart from species and gender difference, this could be related to the circadian time of tissue collection. *NAMPT*, NAD and *SIRT1* show a circadian rhythm in mouse skeletal muscle and liver (Eckel-Mahan et al. 2013; Um et al. 2011; Wallace et al. 2018), with peak at the onset of active phase and nadir around mid-inactive phase. Skeletal muscle tissues were collected at ~8am in humans (early active phase), whereas tissues were collected at ZT7-9 in mice (mid-inactive phase). Hence, the circadian time of tissue collection might have led to the discrepancy in findings.

In conclusion, this study demonstrates that IF reduced body weight and fat mass in humans, and protected mice from HFD induced weight gain, adiposity, glucose intolerance and fasting hyperglycaemia. IF did not alter insulin sensitivity on the fed day, but insulin

sensitivity was transiently reduced on the fasting day, which may underlie the basis for metabolic switching from glucose to fat oxidation. At the molecular level, AMPK induced rise in *NAMPT* expression on fasting day may facilitate the lipid oxidation pathways in muscle. Circadian time of tissue collection should be considered when collecting tissues for the analysis of markers of NAD metabolism.

Funding agencies: The human study was funded by a National Health and Medical Research Council (NHMRC) Project Grant APP1023401. PR was supported by Adelaide scholarship international from The University of Adelaide.

Conflict of interest: Authors declare no conflict of interest.

Author contribution: LKH and GAW designed the study. ATH and BL commenced the study and collected data. PR performed experiments, statistical analysis and wrote the manuscript. GAW provided clinical support, supervised clamps and performed muscle biopsies. AJP supervised mouse study and metabolic monitoring. All authors contributed to data interpretation and preparation of manuscript. LKH had full access to the data and had primary responsibility for the final publication.

Prior Presentation: The parts of this study were presented as poster presentation in Florey postgraduate research conference, Adelaide, Australia 2018, and SAHMRI annual meeting, Adelaide, Australia 2018.

Table 6. 1 Baseline characteristics of participants in intermittent fasting study.

	IF70 (n=25)	**IF100 (n=25)**
Age at enrolment (y)	49±2	51±2
Weight (kg)	89.4±2.8	84.1±2.8
BMI (kg/m^2)	32.4±0.8	31.2±0.9
Waist circumference (cm)	101±2	99±3
Hip circumference (cm)	115±2	112±2
SBP (mmHg)	117±3	125±4
Body fat (%)	48.3±1.4	47.0±1.3
Fasting glucose (mmol/L)	4.9±0.1	4.9±0.1
Fasting insulin (mU/L)	19.5±1.5	18.6±1.5

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

IF70: intermittent fasting diet at 70% of baseline energy requirements; IF100: intermittent fasting diet at 100% of baseline energy requirement; BMI: body mass index; SBP: systolic blood pressure.

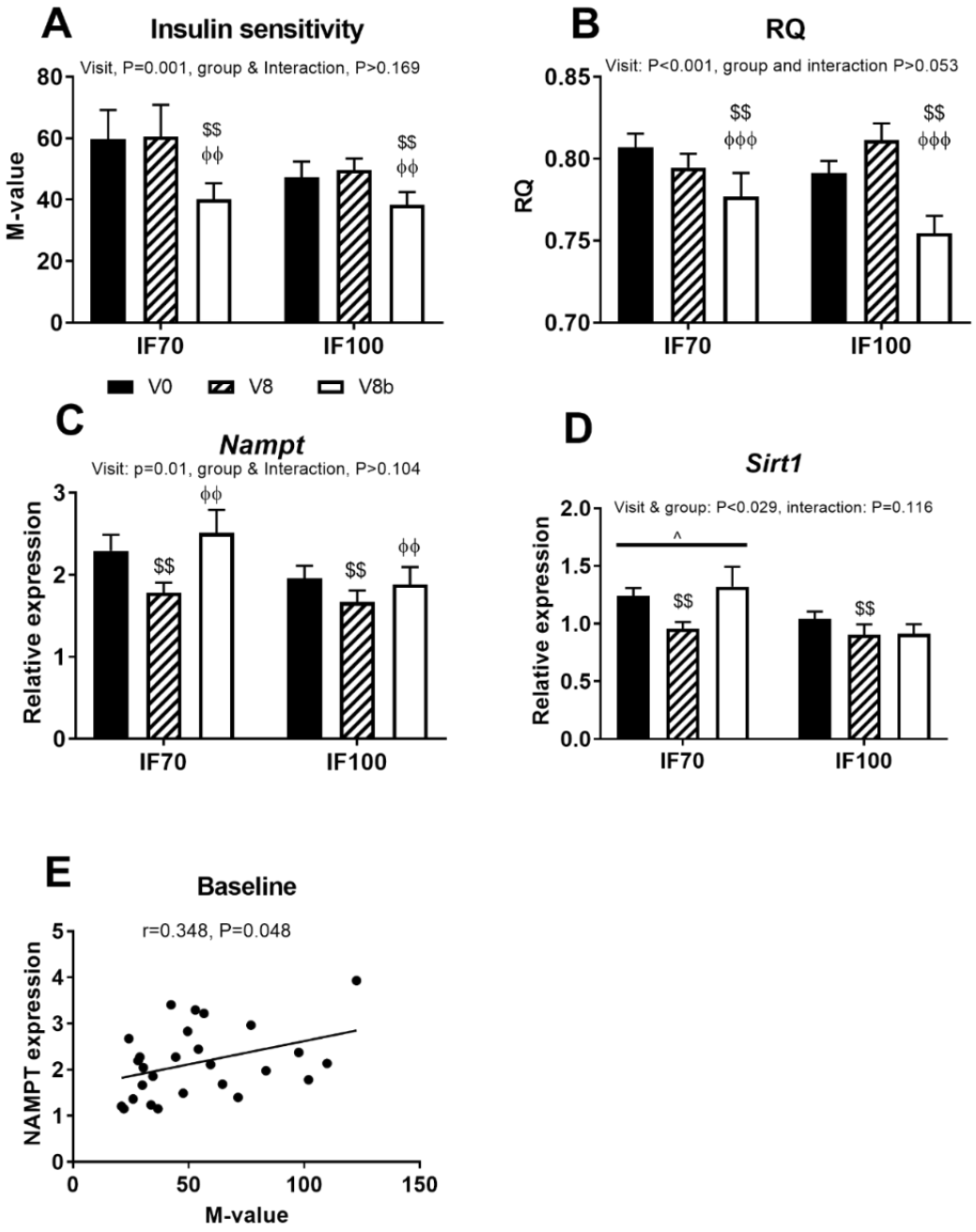


Figure 6. 1 Intermittent fasting reduced peripheral insulin sensitivity and RQ, and increased skeletal muscle *NAMPT* expression from fed to fast transition in humans.

A) insulin sensitivity expressed as M-value (GIR per kilogram FFM + 17.7) at V0, V8 and V8b, B) respiratory quotient at V0, V8 and V8b, C) skeletal muscle *NAMPT* expression at V0, V8 and V8b, D) skeletal muscle *SIRT1* expression at V0, V8 and V8b, E) correlation of skeletal muscle *NAMPT* expression with insulin sensitivity at baseline (V0). Statistics was

performed by linear mixed model with group (IF70 vs IF100) and visit (0, 8 and 8b) as fixed factors and Bonferroni's *post hoc* was applied. Correlation analysis was performed by Perason's correlation. Solid bars: baseline (12h fast), hatched bars: after 8-week IF (12h fast), open bars: after 8-week IF (24h fast), *: P<0.05, ** P<0.01, ***: P<0.001 vs respective visit 0, $: P<0.05, $$: P<0.01 overall visit effect vs visit 0; φφ: P<0.01 overall visit effect vs visit 8. ^ P<0.05 overall group effect (IF70 vs IF100).

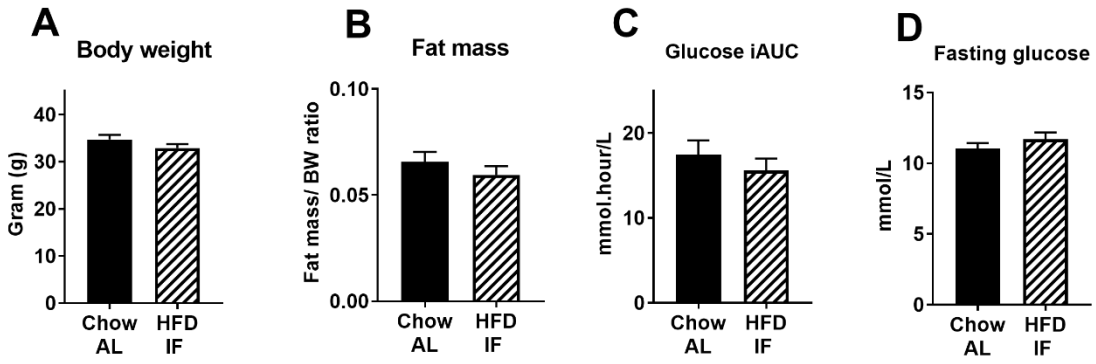


Figure 6. 2 Intermittent fasting protects mice from HFD induced metabolic disorders.

A) body weight comparison between chow AL and HFD IF (n=7-8/group), B) dissected fat mass comparison between chow AL and HFD IF (n=7-8/group), C) glucose iAUC comparison between chow AL and HFD IF (n=7-8/group), D) fasting glucose comparison between chow AL and HFD IF (n=7-8/group). Statistics was performed using t-test.

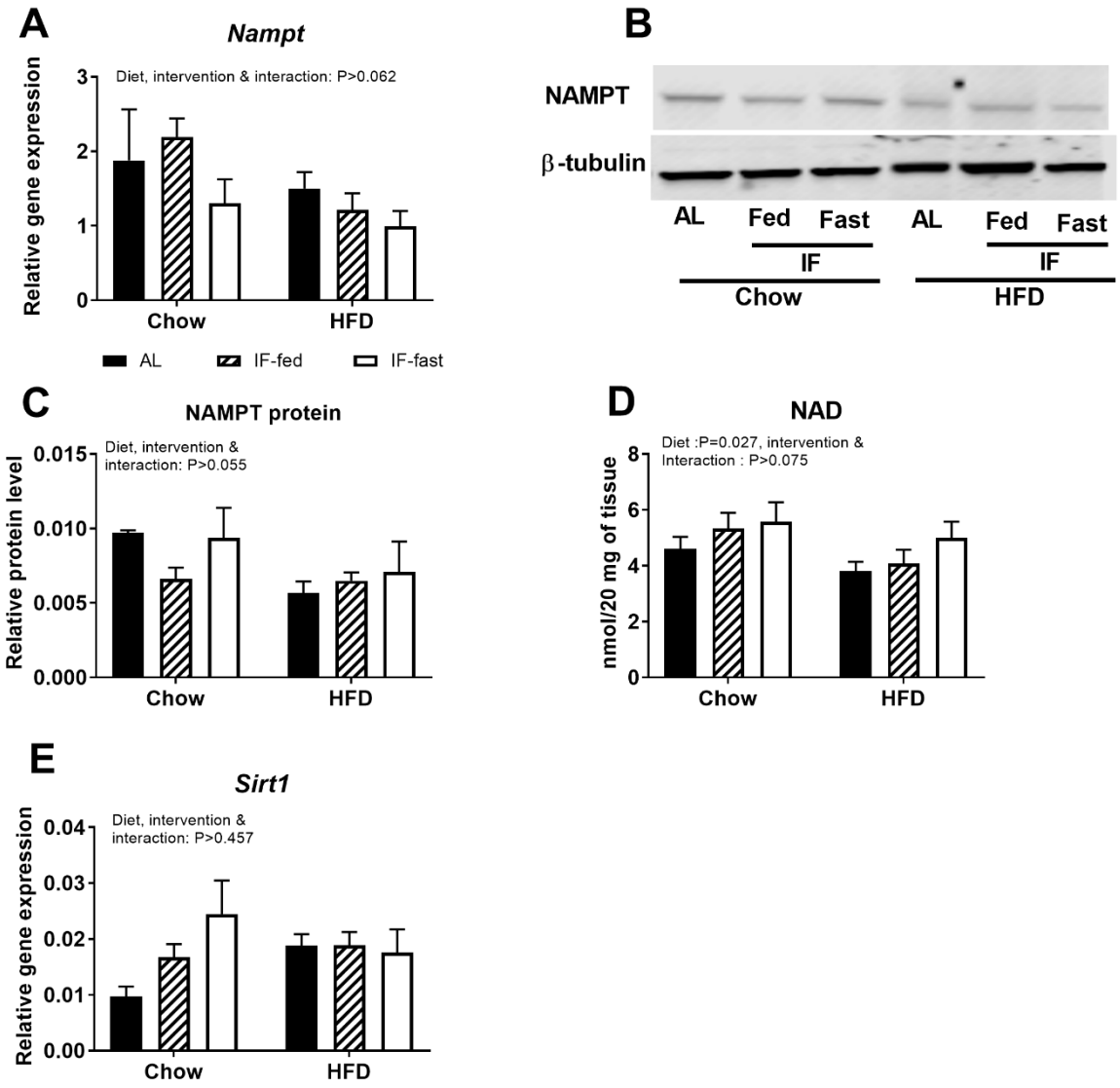


Figure 6. 3 Effect of intermittent fasting on skeletal muscle *Nampt*, NAD and *Sirt1* in mice.

A) skeletal muscle *Nampt* expression (n=7-8/group), B) representative Western blot images, C) skeletal muscle NAMPT protein (n=4/group), D) skeletal muscle NAD level (n=7-8/group), E) skeletal muscle *Sirt1* expression (n=7-8/group). Statistics was performed by two-way ANOVA with diet (chow and HFD) and intervention (AL, IF-fast, and IF-fed) as fixed factors and Bonferroni's *post hoc* was applied.

Chapter 7: Conclusions

The randomized-cross over trial demonstrated that TRF improved glucose tolerance, reduced fasting triglycerides, modestly reduced body weight, and reduced fasting glucose measured by continuous glucose monitoring, independent of gastric emptying or physical activity in overweight men at risk of type 2 diabetes. Importantly, there was no statistically significant difference in any of above results between TRFe and TRFd. This suggests that identical length TRF protocols exhibit similar improvement in metabolic health despite short delay in the time of initiation.

Our mouse study supported the findings in the human study, and further demonstrated that TRF reduced weight and fat gain, improved glucose tolerance, reduced hepatosteatosis, increased metabolic flexibility, and increased amplitudes of genes involved in circadian regulation and markers of NAD metabolism in liver compared to *ad libitum*. Cumulative calorie consumption was not different in chow fed mice, whereas both TRF groups tended to consume less calories than their *ad libitum* counterparts when exposed to HFD. As the magnitude of improvement was greater in mice that were fed a HFD versus chow, lower calorie consumption might have contributed to the metabolic benefits apart from TRF *per se* in these animals.

TRFd marginally limited the benefits in weight and fat gain compared to TRFe, but improved metabolic phenotypes and increased the amplitudes 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. This suggests that a short delay in peripheral circadian rhythm does not adversely impact metabolic improvements in TRF, unless there are similar increases in the amplitude of rhythm.

Thus, this research demonstrated that TRF improves metabolic health whether initiated early in the morning or a few hours later in the day, when there are equidistant transitions between fasting-feeding cycles. These findings confirm our hypothesis that TRFe or TRFd would be beneficial in the improvement of glucose tolerance in overweight men and in the prevention of the metabolic consequences of HFD in mice. Uniquely, we demonstrate that the metabolic benefits of TRFd occur alongside a phase delay in hepatic clocks and metabolic markers, but with a similar 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. However, the flexibility to initiate TRF a few hours later in the day will increase the translational potential of this promising dietary tool in the general population.

Further, a transient reduction in insulin sensitivity and rise in skeletal muscle *NAMPT* expression on the fasting day may underlie the basis of metabolic switching from glucose to fat oxidation in overweight women undergoing intermittent fasting.

Future directions

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

This research, for the first time, showed that TRFd was effective to improve glycaemic profile in overweight men at risk of type 2 diabetes. The flexibility in the time of TRF initiation could make it more widely accepted nutrition intervention. Yet, long-term effects of TRF/TRFd and associated compliance in humans are unclear. Future studies should establish whether the health benefits are observed in long term, and in larger populations.

Does circadian delay in TRFd impact lifespan?

This research demonstrated that the metabolic benefits of TRFd occur along with a phase delay in body temperature and hepatic circadian rhythms in mice. The phase of several circadian markers such as body temperature, melatonin and cortisol has been reported to shift with ageing (Duffy, Zitting & Chinoy 2015). Studies in hamsters found that activity onset is earlier even with the same light cycle in older animals (Scarbrough et al. 1997). Further, there is literature to support the notion that shift in circadian rhythm is related to premature ageing, and a reduction in healthy lifespan (Giebultowicz & Long 2015). Future studies should examine whether TRFd impacts lifespan in animal models.

Does TRF act by modulating cellular NAD levels?

Reduction in cellular NAD leads to deterioration in glycaemic profile and induces accelerated ageing in mice, whereas dietary supplementation or transgenic rise in cellular NAD improves healthspan and lifespan in rodents (Mitchell et al. 2018; Mitchell et al. 2014; Poljsak 2018; Stromsdorfer et al. 2016). The findings of this study showed that TRF increased mean and/or amplitudes of hepatic markers of NAD metabolism, facilitating their robust oscillation. This suggests a potential role of cellular NAD (and *Sirt1*) behind the

improvement in metabolic phenotypes, and change in hepatic circadian rhythm in TRF. Future studies should examine whether metabolic benefits, and change in hepatic circadian rhythm in response to TRF are abrogated in *Nampt* or *Sirt1* deficient animal models. Further, intermittent fasting increased skeletal muscle *Nampt* expression from the fed to fast transition in women with obesity, suggesting role of NAMPT in metabolic switching. Future studies should examine whether metabolic switch in response to alternate day fasting is hindered in *Nampt* deficient animal models.

Can change in meal window minimize the jetlag effect in people?

Jet lag, also known as time zone change syndrome, occurs when people travel rapidly across different time zones. Jet lag disturbs the normal physiology and can induce several disturbances including sleep disorders, fatigue, difficulty in focusing, loss of appetite and gastrointestinal disorders (Jankowski 2017). Many individuals suffer this syndrome for several days, before adjusting to the new time-zone. The current study showed that mealtime has strong influence on peripheral circadian rhythms, and suggest that adjusting meal window to match with the destination zone may help to minimize the jet lag. Future studies should examine whether jet lag can be minimized by shifting meal window in humans.

Is calorie restriction or weight loss essential for TRF metabolic health benefits?

Some TRF studies in animals have suggested that TRF improves health, independently of changes in calorie intake (Chaix et al. 2019; Chaix et al. 2014; Hatori et al. 2012). Whereas others have shown lower calorie consumption in TRF mice that are fed a HFD (Delahaye et al. 2018; Sundaram & Yan 2016), and we also observed reduced calorie consumption in TRF mice fed a HFD. In humans also, reported calorie consumption was found to be lower than control condition (Cienfuegos et al. 2020; Gabel et al. 2018; Gill & Panda 2015; Wilkinson

et al. 2019). Thus, some of the metabolic benefits of TRF may be mediated by calorie restriction and weight loss.

A recent study showed that TRF improved glucose tolerance in rats following high fat-high sugar diet, without any weight loss (Woodie et al. 2018). However, the TRF animals in that study had 24-hour access to sugary water. Additionally, a recent human study also supported the notion that TRF imparts metabolic benefits independently of changes in body weight (Sutton et al. 2018). However, this study was conducted only in 8 participants. 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/ health observed.

Does TRF have a dose dependent effect? If yes, what is the optimal eating window of TRF?

Several TRF protocols from 4-13 hours have been trialled in humans, but the optimal time frame of TRF to recommend for people has not been tested. Some studies in mice showed that there was greater reduction in body weight and fat mass, and improvement in glucose tolerance with 9-hour protocol vs 12- or 15-hour protocol (Chaix et al. 2014; Sundaram & Yan 2016). In humans, clear improvements have been observed with 6-,8-, 9- and 10-hour TRF protocols (Gabel et al. 2018; Hutchison, Regmi, et al. 2019; Sutton et al. 2018; Wilkinson et al. 2019). Shorter eating window can show better metabolic improvements as there is higher weight loss, but can also can lead to lower food choice and reduced long-term compliance. Further, Cienfuegos *et al* recently showed that the health benefits of 4-hour and 6-hour TRF were similar in humans, suggesting a 4 h eating window does not produce superior health benefits (Cienfuegos et al. 2020). Conversely, extending eating window beyond 12-hour is less likely to show beneficial metabolic effects in humans (LeCheminant

et al. 2013). Future studies should examine whether TRF has a dose dependent effects, and if yes, what is the optimal time of eating window to recommend.

Does sex differentially impact the metabolic response to TRF?

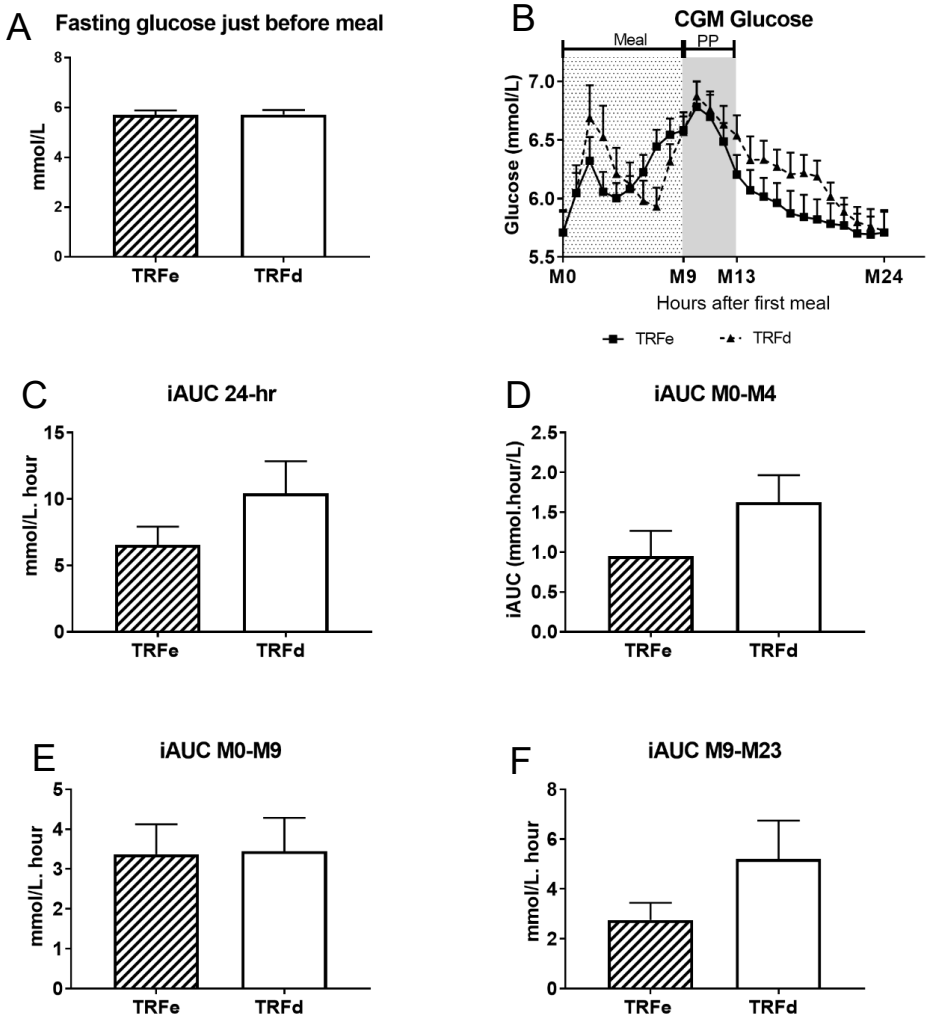
We examined the effects of TRF in overweight men and male mice. However, both during normal physiology or under therapeutic condition, there are sex differences in body composition, regional fat distribution, glucose homeostasis, hepatic steatosis, and lipid metabolism (Chella Krishnan, Mehrabian & Lusis 2018). As a result, sex differences are prevalent in both metabolic and cardiovascular traits, where TRF is a promising preventive and therapeutic tool. Therefore, the findings of current study cannot directly be extrapolated to women. Almost all TRF studies carried out yet have mainly focused in males. Future studies should examine the effects of TRF in women and female mice to examine whether sex differentially impact the metabolic response to TRF.

Does TRF improve metabolic health in individuals with established metabolic disorder?

In this research, we showed that TRFe or TRFd was effective to improve glycaemic profile in overweight human participants and metabolic phenotypes in chow or high fat diet fed mice. As such, this finding cannot directly be implemented/translated in those individuals with established metabolic disorder such as type 2 diabetes or cardiovascular diseases. Future studies should examine whether TRF is effective in those individuals with specific metabolic disorders.

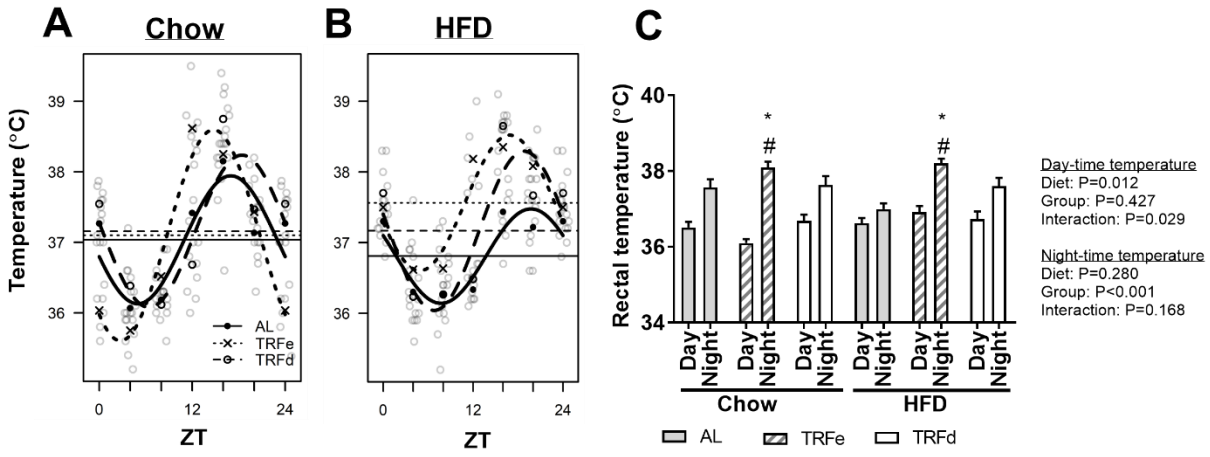
Appendices

Appendix 1: Hourly glucose measured by continuous glucose monitoring and glucose iAUC after first meal of the day in TRFe and TRFd in overweight men at risk of type 2 diabetes. A) average fasting glucose on immediate prior hour of first meal measured by continuous glucose monitoring, B) hourly average glucose measured by continuous glucose monitoring, C) 24-hour glucose iAUC based on fasting glucose at M0, D) first 4-hour glucose iAUC based on fasting glucose at M0, E) glucose iAUC of eating window based on fasting glucose at M0, F) glucose iAUC of non-eating window based on fasting glucose at M0. M: mealtime, M0: just before initiation of first meal of the day; M9, M13, M24: 9, 13, 24 -hours after first meal of the day; iAUC: incremental area under the curve; PP: post-prandial.



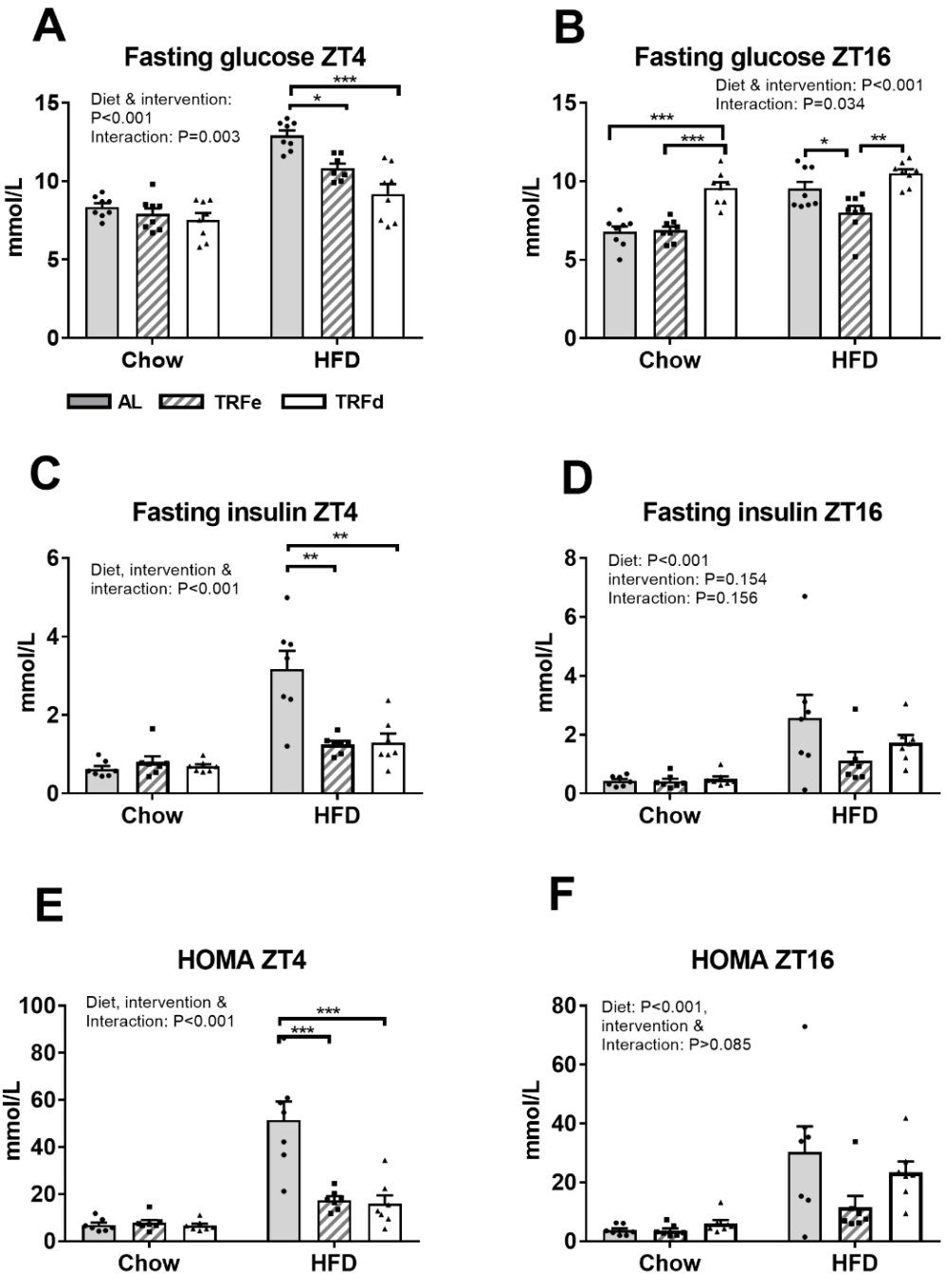
Appendix 2: TRF increased amplitude of rectal temperature of mice.

A & B) cosinor plots of mice rectal temperatures measured at six time points of the day (n=6/group/time point), C) average day and night rectal temperature in all intervention groups. Statistics were performed by cosinor regression for A & B, and by two-way ANOVA for C. AL: ad libitum, TRFe: time-restricted feeding early (ZT12-22); TRFd: time-restricted feeding delay (ZT16-2); ZT: zeitgeber time.



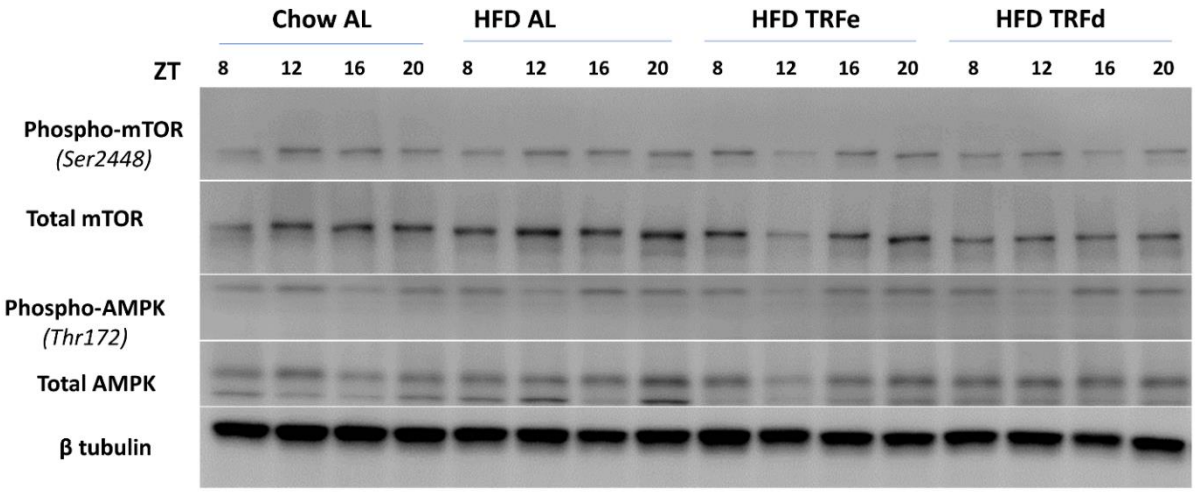
Appendix 3: TRF improved glycaemic profile in mice

A & B) fasting glucose at ZT4 and ZT16 (n=7-8/group), C & D) fasting insulin at ZT4 and ZT16 (n=7/group), E & F) HOMA-IR at ZT4 and 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; for overall intervention effect in both diets $$ P<0.01, $$$ P<0.001. AL: ad libitum, TRFe: time-restricted feeding early (ZT12-22); TRFd: time-restricted feeding delay (ZT16-2); ZT: zeitgeber time; HOMA-IR: homeostatic model assessment of insulin resistance.


A
Fasting glucose ZT4
Diet & intervention: P<0.001
Interaction: P=0.003
mmol/L
Chow
HFD
AL
TRFe
TRFd
B
Fasting glucose ZT16
Diet & intervention: P<0.001
Interaction: P=0.034
C
Fasting insulin ZT4
Diet, intervention & interaction: P<0.001
D
Fasting insulin ZT16
Diet: P<0.001
intervention: P=0.154
Interaction: P=0.156
E
HOMA ZT4
Diet, intervention & Interaction: P<0.001
F
HOMA ZT16
Diet: P<0.001,
intervention &
Interaction: P>0.085

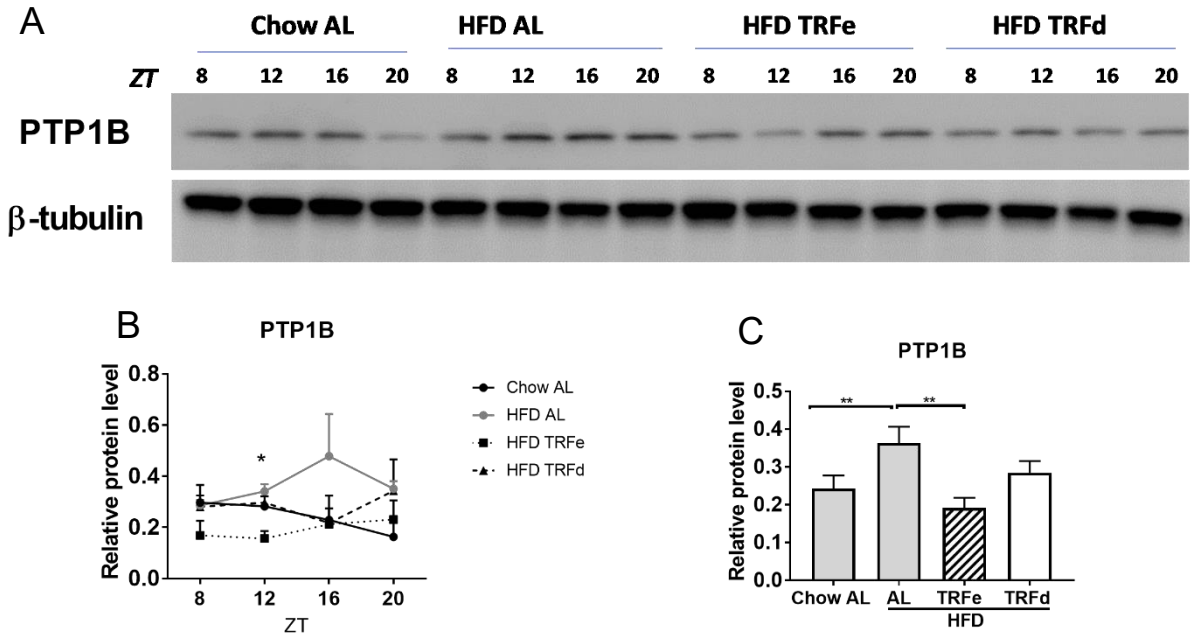
Appendix 4: Representative western blot images of AMPK and mTOR in liver of mice undergoing TRF.

AMPK: AMP-activated protein kinase; mTOR: mechanistic target of rapamycin; AL: ad libitum, TRFe: time-restricted feeding early (ZT12-22); TRFd: time-restricted feeding delay (ZT16-2); ZT: zeitgeber time.



Appendix 5: Representative western blot images of PTP1B in liver of mice undergoing TRF.

A) representative western blot images, B) relative PTP1B level at different time points (n=3/time point/group), C) total PTP1B level. Statistics were performed by t-test. *:P<0.05, **:P<0.01. PTP1B: protein tyrosine phosphatase 1B; AL: ad libitum, TRFe: time-restricted feeding early (ZT12-22); TRFd: time-restricted feeding delay (ZT16-2); ZT: zeitgeber time.



References

Allison, DB, Paultre, F, Maggio, C, Mezzitis, N & Pi-Sunyer, FX 1995, 'The use of areas under curves in diabetes research', *Diabetes Care*, vol. 18, no. 2, Feb, pp. 245-250.

Ando, H, Ushijima, K, Shimba, S & Fujimura, A 2016, 'Daily Fasting Blood Glucose Rhythm in Male Mice: A Role of the Circadian Clock in the Liver', *Endocrinology*, vol. 157, no. 2, Feb, pp. 463-469.

Andrikopoulos, S, Blair, AR, Deluca, N, Fam, BC & Proietto, J 2008, 'Evaluating the glucose tolerance test in mice', *Am J Physiol Endocrinol Metab*, vol. 295, no. 6, Dec, pp. E1323-1332.

Andrzejczak, D, Kapala-Kempa, M & Zawilska, JB 2011, '[Health consequences of shift work]', *Przegl Lek*, vol. 68, no. 7, pp. 383-387.

Anton, SD, Lee, SA, Donahoo, WT, McLaren, C, Manini, T, Leeuwenburgh, C & Pahor, M 2019, 'The Effects of Time Restricted Feeding on Overweight, Older Adults: A Pilot Study', *Nutrients*, vol. 11, no. 7, Jun 30.

Anton, SD, Moehl, K, Donahoo, WT, Marosi, K, Lee, SA, Mainous, AG, 3rd, Leeuwenburgh, C & Mattson, MP 2018, 'Flipping the Metabolic Switch: Understanding and Applying the Health Benefits of Fasting', *Obesity (Silver Spring)*, vol. 26, no. 2, Feb, pp. 254-268.

Antoni, R, Johnston, KL, Collins, AL & Robertson, MD 2017, 'Effects of intermittent fasting on glucose and lipid metabolism', *Proc Nutr Soc*, vol. 76, no. 3, Aug, pp. 361-368.

Azami, Y, Funakoshi, M, Matsumoto, H, Ikota, A, Ito, K, Okimoto, H, Shimizu, N, Tsujimura, F, Fukuda, H, Miyagi, C, Osawa, S, Osawa, R & Miura, J 2019, 'Long working hours and skipping breakfast concomitant with late evening meals are associated with suboptimal glycemic control among young male Japanese patients with type 2 diabetes', *J Diabetes Investig*, vol. 10, no. 1, Jan, pp. 73-83.

Basse, AL, Dalbram, E, Larsson, L, Gerhart-Hines, Z, Zierath, JR & Treebak, JT 2018, 'Skeletal Muscle Insulin Sensitivity Show Circadian Rhythmicity Which Is Independent of Exercise Training Status', *Front Physiol*, vol. 9, p. 1198.

Belkacemi, L, Selselet-Attou, G, Hupkens, E, Nguidjoe, E, Louchami, K, Sener, A & Malaisse, WJ 2012, 'Intermittent fasting modulation of the diabetic syndrome in streptozotocin-injected rats', *Int J Endocrinol*, vol. 2012, p. 962012.

Belkacemi, L, Selselet-Attou, G, Louchami, K, Sener, A & Malaisse, WJ 2010, 'Intermittent fasting modulation of the diabetic syndrome in sand rats. II. In vivo investigations', *Int J Mol Med*, vol. 26, no. 5, Nov, pp. 759-765.

Bergmeyer, HU 1974, 'Methods of Enzymatic Analysis', in *Academic*, vol. 1, New York, p. 1.

Bertoldo, MJ, Listijono, DR, Ho, WJ, Riepsamen, AH, Goss, DM, Richani, D, Jin, XL, Mahbub, S, Campbell, JM, Habibalahi, A, Loh, WN, Youngson, NA, Maniam, J, Wong, ASA, Selesniemi, K, Bustamante, S, Li, C, Zhao, Y, Marinova, MB, Kim, LJ, Lau, L, Wu, RM, Mikolaizak, AS, Araki, T, Le Couteur, DG, Turner, N, Morris, MJ, Walters, KA, Goldys, E, O'Neill, C, Gilchrist, RB, Sinclair, DA, Homer, HA & Wu, LE 2020, 'NAD(+) Repletion Rescues Female Fertility during Reproductive Aging', *Cell Rep*, vol. 30, no. 6, Feb 11, pp. 1670-1681 e1677.

Bi, H, Gan, Y, Yang, C, Chen, Y, Tong, X & Lu, Z 2015, 'Breakfast skipping and the risk of type 2 diabetes: a meta-analysis of observational studies', *Public Health Nutr*, vol. 18, no. 16, Nov, pp. 3013-3019.

Bolli, GB, De Feo, P, De Cosmo, S, Perriello, G, Ventura, MM, Calcinaro, F, Lolli, C, Campbell, P, Brunetti, P & Gerich, JE 1984, 'Demonstration of a dawn phenomenon in normal human volunteers', *Diabetes*, vol. 33, no. 12, Dec, pp. 1150-1153.

Braden, B, Adams, S, Duan, LP, Orth, KH, Maul, FD, Lembcke, B, Hor, G & Caspary, WF 1995, 'The [13C]acetate breath test accurately reflects gastric emptying of liquids in both liquid and semisolid test meals', *Gastroenterology*, vol. 108, no. 4, Apr, pp. 1048-1055.

Brandauer, J, Vienberg, SG, Andersen, MA, Ringholm, S, Risis, S, Larsen, PS, Kristensen, JM, Frosig, C, Leick, L, Fentz, J, Jorgensen, S, Kiens, B, Wojtaszewski, JF, Richter, EA, Zierath, JR, Goodyear, LJ, Pilegaard, H & Treebak, JT 2013, 'AMP-activated protein kinase regulates nicotinamide phosphoribosyl transferase expression in skeletal muscle', *J Physiol*, vol. 591, no. 20, Oct 15, pp. 5207-5220.

Bray, MS, Ratcliffe, WF, Grenett, MH, Brewer, RA, Gamble, KL & Young, ME 2013, 'Quantitative analysis of light-phase restricted feeding reveals metabolic dyssynchrony in mice', *Int J Obes (Lond)*, vol. 37, no. 6, Jun, pp. 843-852.

Bray, MS, Tsai, JY, Villegas-Montoya, C, Boland, BB, Blasier, Z, Egbejimi, O, Kueht, M & Young, ME 2010, 'Time-of-day-dependent dietary fat consumption influences multiple cardiometabolic syndrome parameters in mice', *Int J Obes (Lond)*, vol. 34, no. 11, Nov, pp. 1589-1598.

Cahill, GF, Jr. 2006, 'Fuel metabolism in starvation', *Annu Rev Nutr*, vol. 26, pp. 1-22.

Canto, C, Jiang, LQ, Deshmukh, AS, Mataki, C, Coste, A, Lagouge, M, Zierath, JR & Auwerx, J 2010, 'Interdependence of AMPK and SIRT1 for metabolic adaptation to fasting and exercise in skeletal muscle', *Cell Metab*, vol. 11, no. 3, Mar 03, pp. 213-219.

Canto, C, Menzies, KJ & Auwerx, J 2015, 'NAD(+) Metabolism and the Control of Energy Homeostasis: A Balancing Act between Mitochondria and the Nucleus', *Cell Metab*, vol. 22, no. 1, Jul 07, pp. 31-53.

Carlson, O, Martin, B, Stote, KS, Golden, E, Maudsley, S, Najjar, SS, Ferrucci, L, Ingram, DK, Longo, DL, Rumpler, WV, Baer, DJ, Egan, J & Mattson, MP 2007, 'Impact of reduced meal frequency

without caloric restriction on glucose regulation in healthy, normal-weight middle-aged men and women', *Metabolism*, vol. 56, no. 12, Dec, pp. 1729-1734.

Carroll, KF & Nestel, PJ 1973, 'Diurnal variation in glucose tolerance and in insulin secretion in man', *Diabetes*, vol. 22, no. 5, May, pp. 333-348.

Chaix, A, Lin, T, Le, HD, Chang, MW & Panda, S 2019, 'Time-Restricted Feeding Prevents Obesity and Metabolic Syndrome in Mice Lacking a Circadian Clock', *Cell Metab*, vol. 29, no. 2, Feb 5, pp. 303-319 e304.

Chaix, A, Zarrinpar, A, Miu, P & Panda, S 2014, 'Time-restricted feeding is a preventative and therapeutic intervention against diverse nutritional challenges', *Cell Metab*, vol. 20, no. 6, Dec 02, pp. 991-1005.

Chance, B, Schoener, B, Oshino, R, Itshak, F & Nakase, Y 1979, 'Oxidation-reduction ratio studies of mitochondria in freeze-trapped samples. NADH and flavoprotein fluorescence signals', *J Biol Chem*, vol. 254, no. 11, Jun 10, pp. 4764-4771.

Chella Krishnan, K, Mehrabian, M & Lusis, AJ 2018, 'Sex differences in metabolism and cardiometabolic disorders', *Curr Opin Lipidol*, vol. 29, no. 5, Oct, pp. 404-410.

Chen, L, Magliano, DJ, Balkau, B, Colagiuri, S, Zimmet, PZ, Tonkin, AM, Mitchell, P, Phillips, PJ & Shaw, JE 2010, 'AUSDRISK: an Australian Type 2 Diabetes Risk Assessment Tool based on demographic, lifestyle and simple anthropometric measures', *Med J Aust*, vol. 192, no. 4, Feb 15, pp. 197-202.

Chen, M, Wu, L, Zhao, J, Wu, F, Davies, MJ, Wittert, GA, Norman, RJ, Robker, RL & Heilbronn, LK 2014, 'Altered glucose metabolism in mouse and humans conceived by IVF', *Diabetes*, vol. 63, no. 10, Oct, pp. 3189-3198.

Chow, LS, Manoogian, ENC, Alvear, A, Fleischer, JG, Thor, H, Dietsche, K, Wang, Q, Hodges, JS, Esch, N, Malaeb, S, Harindhanavudhi, T, Nair, KS, Panda, S & Mashek, DG 2020, 'Time-Restricted Eating Effects on Body Composition and Metabolic Measures in Humans with Overweight: A Feasibility Study', *Obesity (Silver Spring)*, Apr 9.

Chowdhury, EA, Richardson, JD, Holman, GD, Tsintzas, K, Thompson, D & Betts, JA 2016, 'The causal role of breakfast in energy balance and health: a randomized controlled trial in obese adults', *Am J Clin Nutr*, vol. 103, no. 3, Mar, pp. 747-756.

Chowdhury, EA, Richardson, JD, Tsintzas, K, Thompson, D & Betts, JA 2015, 'Carbohydrate-rich breakfast attenuates glycaemic, insulinaemic and ghrelin response to ad libitum lunch relative to morning fasting in lean adults', *Br J Nutr*, vol. 114, no. 1, Jul 14, pp. 98-107.

Christie, S, Vincent, AD, Li, H, Frisby, CL, Kentish, SJ, O'Rielly, R, Wittert, GA & Page, AJ 2018, 'A rotating light cycle promotes weight gain and hepatic lipid storage in mice', *Am J Physiol Gastrointest Liver Physiol*, vol. 315, no. 6, Dec 1, pp. G932-G942.

Cienfuegos, S, Gabel, K, Kalam, F, Ezpeleta, M, Wiseman, E, Pavlou, V, Lin, S, Oliveira, ML & Varady, KA 2020, 'Effects of 4- and 6-h Time-Restricted Feeding on Weight and Cardiometabolic Health: A Randomized Controlled Trial in Adults with Obesity', *Cell Metab*, Jul 8.

Cisse, YM, Borniger, JC, Lemanski, E, Walker, WH, 2nd & Nelson, RJ 2018, 'Time-Restricted Feeding Alters the Innate Immune Response to Bacterial Endotoxin', *J Immunol*, vol. 200, no. 2, Jan 15, pp. 681-687.

Costford, SR, Bajpeyi, S, Pasarica, M, Albarado, DC, Thomas, SC, Xie, H, Church, TS, Jubrias, SA, Conley, KE & Smith, SR 2010, 'Skeletal muscle NAMPT is induced by exercise in humans', *Am J Physiol Endocrinol Metab*, vol. 298, no. 1, Jan, pp. E117-126.

Dallmann, R, Viola, AU, Tarokh, L, Cajochen, C & Brown, SA 2012, 'The human circadian metabolome', *Proc Natl Acad Sci U S A*, vol. 109, no. 7, Feb 14, pp. 2625-2629.

Damiola, F, Le Minh, N, Preitner, N, Kornmann, B, Fleury-Olela, F & Schibler, U 2000, 'Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus', *Genes Dev*, vol. 14, no. 23, Dec 01, pp. 2950-2961.

Davidson, AJ, Poole, AS, Yamazaki, S & Menaker, M 2003, 'Is the food-entrainable circadian oscillator in the digestive system?', *Genes Brain Behav*, vol. 2, no. 1, Feb, pp. 32-39.

de Oliveira Maranhao Pureza, IR, da Silva Junior, AE, Silva Praxedes, DR, Lessa Vasconcelos, LG, de Lima Macena, M, Vieira de Melo, IS, de Menezes Toledo Florencio, TM & Bueno, NB 2020, 'Effects of time-restricted feeding on body weight, body composition and vital signs in low-income women with obesity: A 12-month randomized clinical trial', *Clin Nutr*, Jul 14.

Delahaye, LB, Bloomer, RJ, Butawan, MB, Wyman, JM, Hill, JL, Lee, HW, Liu, AC, McAllan, L, Han, JC & van der Merwe, M 2018, 'Time-restricted feeding of a high-fat diet in male C57BL/6 mice reduces adiposity but does not protect against increased systemic inflammation', *Appl Physiol Nutr Metab*, vol. 43, no. 10, Oct, pp. 1033-1042.

Doi, M, Hirayama, J & Sassone-Corsi, P 2006, 'Circadian regulator CLOCK is a histone acetyltransferase', *Cell*, vol. 125, no. 3, May 5, pp. 497-508.

Dongen, SMJAHGPAV 2017, 'Shift Work: Disrupted Circadian Rhythms and Sleep—Implications for Health and Well-being', *Current Sleep Medicine Reports*, vol. 3, no. 2, p. 9.

Douris, N, Kojima, S, Pan, X, Lerch-Gaggl, AF, Duong, SQ, Hussain, MM & Green, CB 2011, 'Nocturnin regulates circadian trafficking of dietary lipid in intestinal enterocytes', *Curr Biol*, vol. 21, no. 16, Aug 23, pp. 1347-1355.

Duffy, JF, Zitting, KM & Chinoy, ED 2015, 'Aging and Circadian Rhythms', *Sleep Med Clin*, vol. 10, no. 4, Dec, pp. 423-434.

Dunbar, RIM 2017, 'Breaking Bread: the Functions of Social Eating', *Adapt Human Behav Physiol*, vol. 3, no. 3, pp. 198-211.

Duncan, MJ, Smith, JT, Narbaiza, J, Mueez, F, Bustle, LB, Qureshi, S, Fieseler, C & Legan, SJ 2016, 'Restricting feeding to the active phase in middle-aged mice attenuates adverse metabolic effects of a high-fat diet', *Physiol Behav*, vol. 167, Dec 1, pp. 1-9.

Eckel-Mahan, KL, Patel, VR, de Mateo, S, Orozco-Solis, R, Ceglia, NJ, Sahar, S, Dilag-Penilla, SA, Dyar, KA, Baldi, P & Sassone-Corsi, P 2013, 'Reprogramming of the circadian clock by nutritional challenge', *Cell*, vol. 155, no. 7, Dec 19, pp. 1464-1478.

Edgett, BA, Scribbans, TD, Raleigh, JP, Matusiak, JB, Boonstra, K, Simpson, CA, Perry, CG, Quadrilatero, J & Gurd, BJ 2016, 'The impact of a 48-h fast on SIRT1 and GCN5 in human skeletal muscle', *Appl Physiol Nutr Metab*, vol. 41, no. 9, Sep, pp. 953-962.

Ellacott, KL, Morton, GJ, Woods, SC, Tso, P & Schwartz, MW 2010, 'Assessment of feeding behavior in laboratory mice', *Cell Metab*, vol. 12, no. 1, Jul 7, pp. 10-17.

Espelund, U, Hansen, TK, Hojlund, K, Beck-Nielsen, H, Clausen, JT, Hansen, BS, Orskov, H, Jorgensen, JO & Frystyk, J 2005, 'Fasting unmasks a strong inverse association between ghrelin and cortisol in serum: studies in obese and normal-weight subjects', *J Clin Endocrinol Metab*, vol. 90, no. 2, Feb, pp. 741-746.

Estrella, MA, Du, J, Chen, L, Rath, S, Prangley, E, Chitrakar, A, Aoki, T, Schedl, P, Rabinowitz, J & Korennykh, A 2019, 'The metabolites NADP(+) and NADPH are the targets of the circadian protein Nocturnin (Curled)', *Nat Commun*, vol. 10, no. 1, May 30, p. 2367.

Fakhrzadeh, H, Larijani, B, Sanjari, M, Baradar-Jalili, R & Amini, MR 2003, 'Effect of Ramadan fasting on clinical and biochemical parameters in healthy adults', *Ann Saudi Med*, vol. 23, no. 3-4, May-Jul, pp. 223-226.

Farshchi, HR, Taylor, MA & Macdonald, IA 2004, 'Regular meal frequency creates more appropriate insulin sensitivity and lipid profiles compared with irregular meal frequency in healthy lean women', *Eur J Clin Nutr*, vol. 58, no. 7, Jul, pp. 1071-1077.

Flanagan, DE, Evans, ML, Monsod, TP, Rife, F, Heptulla, RA, Tamborlane, WV & Sherwin, RS 2003, 'The influence of insulin on circulating ghrelin', *Am J Physiol Endocrinol Metab*, vol. 284, no. 2, Feb, pp. E313-316.

Fulco, M, Cen, Y, Zhao, P, Hoffman, EP, McBurney, MW, Sauve, AA & Sartorelli, V 2008, 'Glucose restriction inhibits skeletal myoblast differentiation by activating SIRT1 through AMPK-mediated regulation of Nampt', *Dev Cell*, vol. 14, no. 5, May, pp. 661-673.

Gabel, K, Hoddy, KK, Haggerty, N, Song, J, Kroeger, CM, Trepanowski, JF, Panda, S & Varady, KA 2018, 'Effects of 8-hour time restricted feeding on body weight and metabolic disease risk factors in obese adults: A pilot study', *Nutr Healthy Aging*, vol. 4, no. 4, Jun 15, pp. 345-353.

Gabel, K, Marcell, J, Cares, K, Kalam, F, Cienfuegos, S, Ezpeleta, M & Varady, KA 2020, 'Effect of time restricted feeding on the gut microbiome in adults with obesity: A pilot study', *Nutr Health*, Mar 30, p. 260106020910907.

Gale, JE, Cox, HI, Qian, J, Block, GD, Colwell, CS & Matveyenko, AV 2011, 'Disruption of circadian rhythms accelerates development of diabetes through pancreatic beta-cell loss and dysfunction', *J Biol Rhythms*, vol. 26, no. 5, Oct, pp. 423-433.

Galgani, JE, Moro, C & Ravussin, E 2008, 'Metabolic flexibility and insulin resistance', *Am J Physiol Endocrinol Metab*, vol. 295, no. 5, Nov, pp. E1009-1017.

Garaulet, M, Gomez-Abellan, P, Alburquerque-Bejar, JJ, Lee, YC, Ordovas, JM & Scheer, FA 2013, 'Timing of food intake predicts weight loss effectiveness', *Int J Obes (Lond)*, vol. 37, no. 4, Apr, pp. 604-611.

Giebultowicz, JM & Long, DM 2015, 'Ageing and Circadian rhythms', *Curr Opin Insect Sci*, vol. 7, Feb 1, pp. 82-86.

Gilardi, F, Migliavacca, E, Naldi, A, Baruchet, M, Canella, D, Le Martelot, G, Guex, N, Desvergne, B & Cycli, XC 2014, 'Genome-wide analysis of SREBP1 activity around the clock reveals its combined dependency on nutrient and circadian signals', *PLoS Genet*, vol. 10, no. 3, Mar, p. e1004155.

Gill, S, Le, HD, Melkani, GC & Panda, S 2015, 'Time-restricted feeding attenuates age-related cardiac decline in Drosophila', *Science*, vol. 347, no. 6227, Mar 13, pp. 1265-1269.

Gill, S & Panda, S 2015, 'A Smartphone App Reveals Erratic Diurnal Eating Patterns in Humans that Can Be Modulated for Health Benefits', *Cell Metab*, vol. 22, no. 5, Nov 03, pp. 789-798.

Greenwell, BJ, Trott, AJ, Beytebiere, JR, Pao, S, Bosley, A, Beach, E, Finegan, P, Hernandez, C & Menet, JS 2019, 'Rhythmic Food Intake Drives Rhythmic Gene Expression More Potently than the Hepatic Circadian Clock in Mice', *Cell Rep*, vol. 27, no. 3, Apr 16, pp. 649-657 e645.

Gupta, CC, Dorrian, J, Grant, CL, Pajcin, M, Coates, AM, Kennaway, DJ, Wittert, GA, Heilbronn, LK, Della Vedova, CB & Banks, S 2017, 'It's not just what you eat but when: The impact of eating a meal during simulated shift work on driving performance', *Chronobiol Int*, vol. 34, no. 1, pp. 66-77.

Gupta, NJ, Kumar, V & Panda, S 2017, 'A camera-phone based study reveals erratic eating pattern and disrupted daily eating-fasting cycle among adults in India', *PLoS One*, vol. 12, no. 3, p. e0172852.

Halberg, N, Henriksen, M, Soderhamn, N, Stallknecht, B, Ploug, T, Schjerling, P & Dela, F 2005, 'Effect of intermittent fasting and refeeding on insulin action in healthy men', *J Appl Physiol (1985)*, vol. 99, no. 6, Dec, pp. 2128-2136.

Hamaguchi, Y, Tahara, Y, Hitosugi, M & Shibata, S 2015, 'Impairment of Circadian Rhythms in Peripheral Clocks by Constant Light Is Partially Reversed by Scheduled Feeding or Exercise', *J Biol Rhythms*, vol. 30, no. 6, Dec, pp. 533-542.

Hardie, DG 2011, 'AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function', *Genes Dev*, vol. 25, no. 18, Sep 15, pp. 1895-1908.

Hastings, MH, Maywood, ES & Brancaccio, M 2018, 'Generation of circadian rhythms in the suprachiasmatic nucleus', *Nat Rev Neurosci*, vol. 19, no. 8, Aug, pp. 453-469.

Hatori, M, Vollmers, C, Zarrinpar, A, DiTacchio, L, Bushong, EA, Gill, S, Leblanc, M, Chaix, A, Joens, M, Fitzpatrick, JA, Ellisman, MH & Panda, S 2012, 'Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet', *Cell Metab*, vol. 15, no. 6, Jun 06, pp. 848-860.

Heilbronn, LK, Civitarese, AE, Bogacka, I, Smith, SR, Hulver, M & Ravussin, E 2005, 'Glucose tolerance and skeletal muscle gene expression in response to alternate day fasting', *Obes Res*, vol. 13, no. 3, Mar, pp. 574-581.

Heilbronn, LK & Panda, S 2019, 'Alternate-Day Fasting Gets a Safe Bill of Health', *Cell Metab*, vol. 30, no. 3, Sep 3, pp. 411-413.

Hoddy, KK, Kroeger, CM, Trepanowski, JF, Barnosky, A, Bhutani, S & Varady, KA 2014, 'Meal timing during alternate day fasting: Impact on body weight and cardiovascular disease risk in obese adults', *Obesity (Silver Spring)*, vol. 22, no. 12, Dec, pp. 2524-2531.

Hou, T, Wang, C, Joshi, S, O'Hara, BF, Gong, MC & Guo, Z 2019, 'Active Time-Restricted Feeding Improved Sleep-Wake Cycle in db/db Mice', *Front Neurosci*, vol. 13, p. 969.

Houten, SM & Wanders, RJ 2010, 'A general introduction to the biochemistry of mitochondrial fatty acid beta-oxidation', *J Inherit Metab Dis*, vol. 33, no. 5, Oct, pp. 469-477.

Hu, D, Mao, Y, Xu, G, Liao, W, Ren, J, Yang, H, Yang, J, Sun, L, Chen, H, Wang, W, Wang, Y, Sang, X, Lu, X, Zhang, H & Zhong, S 2019, 'Time-restricted feeding causes irreversible metabolic disorders and gut microbiota shift in pediatric mice', *Pediatr Res*, vol. 85, no. 4, Mar, pp. 518-526.

Hutchison, AT, Liu, B, Wood, RE, Vincent, AD, Thompson, CH, O'Callaghan, NJ, Wittert, GA & Heilbronn, LK 2019, 'Effects of Intermittent Versus Continuous Energy Intakes on Insulin Sensitivity and Metabolic Risk in Women with Overweight', *Obesity (Silver Spring)*, vol. 27, no. 1, Jan, pp. 50-58.

Hutchison, AT, Regmi, P, Manoogian, ENC, Fleischer, JG, Wittert, GA, Panda, S & Heilbronn, LK 2019, 'Time-Restricted Feeding Improves Glucose Tolerance in Men at Risk for Type 2 Diabetes: A Randomized Crossover Trial', *Obesity (Silver Spring)*, vol. 27, no. 5, May, pp. 724-732.

Imai, S, Armstrong, CM, Kaeberlein, M & Guarente, L 2000, 'Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase', *Nature*, vol. 403, no. 6771, Feb 17, pp. 795-800.

Jakubowicz, D, Barnea, M, Wainstein, J & Froy, O 2013, 'High caloric intake at breakfast vs. dinner differentially influences weight loss of overweight and obese women', *Obesity (Silver Spring)*, vol. 21, no. 12, Dec, pp. 2504-2512.

Jakubowicz, D, Landau, Z, Tsameret, S, Wainstein, J, Raz, I, Ahren, B, Chapnik, N, Barnea, M, Ganz, T, Menaged, M, Mor, N, Bar-Dayan, Y & Froy, O 2019, 'Reduction in Glycated Hemoglobin and Daily Insulin Dose Alongside Circadian Clock Upregulation in Patients With Type 2 Diabetes Consuming a Three-Meal Diet: A Randomized Clinical Trial', *Diabetes Care*, vol. 42, no. 12, Dec, pp. 2171-2180.

Jakubowicz, D, Wainstein, J, Ahren, B, Landau, Z, Bar-Dayan, Y & Froy, O 2015, 'Fasting until noon triggers increased postprandial hyperglycemia and impaired insulin response after lunch and dinner in individuals with type 2 diabetes: a randomized clinical trial', *Diabetes Care*, vol. 38, no. 10, Oct, pp. 1820-1826.

Jamshed, H, Beyl, RA, Della Manna, DL, Yang, ES, Ravussin, E & Peterson, CM 2019, 'Early Time-Restricted Feeding Improves 24-Hour Glucose Levels and Affects Markers of the Circadian Clock, Aging, and Autophagy in Humans', *Nutrients*, vol. 11, no. 6, May 30.

Jankowski, KS 2017, 'Social jet lag: Sleep-corrected formula', *Chronobiol Int*, vol. 34, no. 4, pp. 531-535.

Jiang, P & Turek, FW 2017, 'Timing of meals: when is as critical as what and how much', *Am J Physiol Endocrinol Metab*, vol. 312, no. 5, May 01, pp. E369-E380.

Johannsen, DL, Calabro, MA, Stewart, J, Franke, W, Rood, JC & Welk, GJ 2010, 'Accuracy of armband monitors for measuring daily energy expenditure in healthy adults', *Med Sci Sports Exerc*, vol. 42, no. 11, Nov, pp. 2134-2140.

Jones, R, Pabla, P, Mallinson, J, Nixon, A, Taylor, T, Bennett, A & Tsintzas, K 2020, 'Two weeks of early time-restricted feeding (eTRF) improves skeletal muscle insulin and anabolic sensitivity in healthy men', *Am J Clin Nutr*, Jul 30.

Joslin, PMN, Bell, RK & Swoap, SJ 2017, 'Obese mice on a high-fat alternate-day fasting regimen lose weight and improve glucose tolerance', *J Anim Physiol Anim Nutr (Berl)*, vol. 101, no. 5, Oct, pp. 1036-1045.

Kahleova, H, Belinova, L, Malinska, H, Oliyarnyk, O, Trnovska, J, Skop, V, Kazdova, L, Dezortova, M, Hajek, M, Tura, A, Hill, M & Pelikanova, T 2014, 'Eating two larger meals a day (breakfast and lunch) is more effective than six smaller meals in a reduced-energy regimen for patients with type 2 diabetes: a randomised crossover study', *Diabetologia*, vol. 57, no. 8, Aug, pp. 1552-1560.

Kaushik, VK, Young, ME, Dean, DJ, Kurowski, TG, Saha, AK & Ruderman, NB 2001, 'Regulation of fatty acid oxidation and glucose metabolism in rat soleus muscle: effects of AICAR', *Am J Physiol Endocrinol Metab*, vol. 281, no. 2, Aug, pp. E335-340.

Kentish, SJ, Hatzinikolas, G, Li, H, Frisby, CL, Wittert, GA & Page, AJ 2018, 'Time-Restricted Feeding Prevents Ablation of Diurnal Rhythms in Gastric Vagal Afferent Mechanosensitivity Observed in High-Fat Diet-Induced Obese Mice', *J Neurosci*, vol. 38, no. 22, May 30, pp. 5088-5095.

Kesztyus, D, Cermak, P, Gulich, M & Kesztyus, T 2019, 'Adherence to Time-Restricted Feeding and Impact on Abdominal Obesity in Primary Care Patients: Results of a Pilot Study in a Pre-Post Design', *Nutrients*, vol. 11, no. 12, Nov 21.

Kolbe, I, Leinweber, B, Brandenburger, M & Oster, H 2019, 'Circadian clock network desynchrony promotes weight gain and alters glucose homeostasis in mice', *Mol Metab*, vol. 30, Dec, pp. 140-151.

Kutsuma, A, Nakajima, K & Suwa, K 2014, 'Potential Association between Breakfast Skipping and Concomitant Late-Night-Dinner Eating with Metabolic Syndrome and Proteinuria in the Japanese Population', *Scientifica (Cairo)*, vol. 2014, p. 253581.

L M Morgan, FA, J Wright, R Gama 1999, 'Diurnal Variations in Peripheral Insulin Resistance and Plasma Non-Esterified Fatty Acid Concentrations: A Possible Link?', *Annals of Clinical Biochemistry: International Journal of Laboratory Medicine*, vol. 36, no. 4, p. 4.

Lamia, KA, Sachdeva, UM, DiTacchio, L, Williams, EC, Alvarez, JG, Egan, DF, Vasquez, DS, Juguilon, H, Panda, S, Shaw, RJ, Thompson, CB & Evans, RM 2009, 'AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation', *Science*, vol. 326, no. 5951, Oct 16, pp. 437-440.

LeCheminant, JD, Christenson, E, Bailey, BW & Tucker, LA 2013, 'Restricting night-time eating reduces daily energy intake in healthy young men: a short-term cross-over study', *Br J Nutr*, vol. 110, no. 11, Dec 14, pp. 2108-2113.

Lecube, A, Sanchez, E, Gomez-Peralta, F, Abreu, C, Valls, J, Mestre, O, Romero, O, Martinez, MD, Sampol, G, Ciudin, A, Hernandez, C & Simo, R 2016, 'Global Assessment of the Impact of Type 2 Diabetes on Sleep through Specific Questionnaires. A Case-Control Study', *PLoS One*, vol. 11, no. 6, p. e0157579.

Lee, A, Ader, M, Bray, GA & Bergman, RN 1992, 'Diurnal variation in glucose tolerance. Cyclic suppression of insulin action and insulin secretion in normal-weight, but not obese, subjects', *Diabetes*, vol. 41, no. 6, Jun, pp. 750-759.

Li, G, Xie, C, Lu, S, Nichols, RG, Tian, Y, Li, L, Patel, D, Ma, Y, Brocker, CN, Yan, T, Krausz, KW, Xiang, R, Gavrilova, O, Patterson, AD & Gonzalez, FJ 2017, 'Intermittent Fasting Promotes White Adipose Browning and Decreases Obesity by Shaping the Gut Microbiota', *Cell Metab*, vol. 26, no. 4, Oct 3, pp. 672-685 e674.

Liu, B, Page, AJ, Hatzinikolas, G, Chen, M, Wittert, GA & Heilbronn, LK 2019, 'Intermittent Fasting Improves Glucose Tolerance and Promotes Adipose Tissue Remodeling in Male Mice Fed a High-Fat Diet', *Endocrinology*, vol. 160, no. 1, Jan 1, pp. 169-180.

Liu, B, Page, AJ, Hutchison, AT, Wittert, GA & Heilbronn, LK 2019, 'Intermittent fasting increases energy expenditure and promotes adipose tissue browning in mice', *Nutrition*, vol. 66, Oct, pp. 38-43.

Loboda, A, Kraft, WK, Fine, B, Joseph, J, Nebozhyn, M, Zhang, C, He, Y, Yang, X, Wright, C, Morris, M, Chalikonda, I, Ferguson, M, Emilsson, V, Leonardson, A, Lamb, J, Dai, H, Schadt, E, Greenberg, HE & Lum, PY 2009, 'Diurnal variation of the human adipose transcriptome and the link to metabolic disease', *BMC Med Genomics*, vol. 2, Feb 9, p. 7.

Look, ARG, Pi-Sunyer, X, Blackburn, G, Brancati, FL, Bray, GA, Bright, R, Clark, JM, Curtis, JM, Espeland, MA, Foreyt, JP, Graves, K, Haffner, SM, Harrison, B, Hill, JO, Horton, ES, Jakicic, J, Jeffery, RW, Johnson, KC, Kahn, S, Kelley, DE, Kitabchi, AE, Knowler, WC, Lewis, CE, Maschak-Carey, BJ, Montgomery, B, Nathan, DM, Patricio, J, Peters, A, Redmon, JB, Reeves, RS, Ryan, DH, Safford, M, Van Dorsten, B, Wadden, TA, Wagenknecht, L, Wesche-Thobaben, J, Wing, RR & Yanovski, SZ 2007, 'Reduction in weight and cardiovascular disease risk factors in individuals with type 2 diabetes: one-year results of the look AHEAD trial', *Diabetes Care*, vol. 30, no. 6, Jun, pp. 1374-1383.

Madkour, MI, A, TE-S, Jahrami, HA, Sherif, NM, Hassan, RE, Awadallah, S & Faris, MAE 2019, 'Ramadan diurnal intermittent fasting modulates SOD2, TFAM, Nrf2, and sirtuins (SIRT1, SIRT3) gene expressions in subjects with overweight and obesity', *Diabetes Res Clin Pract*, vol. 155, Sep, p. 107801.

Martens, CR, Rossman, MJ, Mazzo, MR, Jankowski, LR, Nagy, EE, Denman, BA, Richey, JJ, Johnson, SA, Ziemba, BP, Wang, Y, Peterson, CM, Chonchol, M & Seals, DR 2020, 'Short-term time-restricted feeding is safe and feasible in non-obese healthy midlife and older adults', *Geroscience*, vol. 42, no. 2, Apr, pp. 667-686.

McAllister, MJ, Pigg, BL, Renteria, LI & Waldman, HS 2020, 'Time-restricted feeding improves markers of cardiometabolic health in physically active college-age men: a 4-week randomized pre-post pilot study', *Nutr Res*, vol. 75, Mar, pp. 32-43.

McDonnell, CM, Donath, SM, Vidmar, SI, Werther, GA & Cameron, FJ 2005, 'A novel approach to continuous glucose analysis utilizing glycemic variation', *Diabetes Technol Ther*, vol. 7, no. 2, Apr, pp. 253-263.

Meng, QJ, Logunova, L, Maywood, ES, Gallego, M, Lebiecki, J, Brown, TM, Sladek, M, Semikhodskii, AS, Glossop, NRJ, Piggins, HD, Chesham, JE, Bechtold, DA, Yoo, SH, Takahashi, JS, Virshup, DM, Boot-Handford, RP, Hastings, MH & Loudon, ASI 2008, 'Setting clock speed in mammals: the CK1 epsilon tau mutation in mice accelerates circadian pacemakers by selectively destabilizing PERIOD proteins', *Neuron*, vol. 58, no. 1, Apr 10, pp. 78-88.

Mistlberger, RE 1994, 'Circadian food-anticipatory activity: formal models and physiological mechanisms', *Neurosci Biobehav Rev*, vol. 18, no. 2, Summer, pp. 171-195.

Mitchell, SJ, Bernier, M, Aon, MA, Cortassa, S, Kim, EY, Fang, EF, Palacios, HH, Ali, A, Navas-Enamorado, I, Di Francesco, A, Kaiser, TA, Waltz, TB, Zhang, N, Ellis, JL, Elliott, PJ, Frederick, DW, Bohr, VA, Schmidt, MS, Brenner, C, Sinclair, DA, Sauve, AA, Baur, JA & de Cabo, R 2018, 'Nicotinamide Improves Aspects of Healthspan, but Not Lifespan, in Mice', *Cell Metab*, vol. 27, no. 3, Mar 6, pp. 667-676 e664.

Mitchell, SJ, Martin-Montalvo, A, Mercken, EM, Palacios, HH, Ward, TM, Abulwerdi, G, Minor, RK, Vlasuk, GP, Ellis, JL, Sinclair, DA, Dawson, J, Allison, DB, Zhang, Y, Becker, KG, Bernier, M & de Cabo, R 2014, 'The SIRT1 activator SRT1720 extends lifespan and improves health of mice fed a standard diet', *Cell Rep*, vol. 6, no. 5, Mar 13, pp. 836-843.

Moon, S, Kang, J, Kim, SH, Chung, HS, Kim, YJ, Yu, JM, Cho, ST, Oh, CM & Kim, T 2020, 'Beneficial Effects of Time-Restricted Eating on Metabolic Diseases: A Systemic Review and Meta-Analysis', *Nutrients*, vol. 12, no. 5, Apr 29.

Moro, T, Tinsley, G, Bianco, A, Marcolin, G, Pacelli, QF, Battaglia, G, Palma, A, Gentil, P, Neri, M & Paoli, A 2016, 'Effects of eight weeks of time-restricted feeding (16/8) on basal metabolism, maximal strength, body composition, inflammation, and cardiovascular risk factors in resistance-trained males', *J Transl Med*, vol. 14, no. 1, Oct 13, p. 290.

Nakahata, Y, Kaluzova, M, Grimaldi, B, Sahar, S, Hirayama, J, Chen, D, Guarente, LP & Sassone-Corsi, P 2008, 'The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control', *Cell*, vol. 134, no. 2, Jul 25, pp. 329-340.

Nakajima, K & Suwa, K 2015, 'Association of hyperglycemia in a general Japanese population with late-night-dinner eating alone, but not breakfast skipping alone', *J Diabetes Metab Disord*, vol. 14, p. 16.

Nematy, M, Alinezhad-Namaghi, M, Rashed, MM, Mozhdehifard, M, Sajjadi, SS, Akhlaghi, S, Sabery, M, Mohajeri, SA, Shalaey, N, Moohebati, M & Norouzy, A 2012, 'Effects of Ramadan fasting on cardiovascular risk factors: a prospective observational study', *Nutr J*, vol. 11, Sep 10, p. 69.

NHMRC 2013, *Australian dietary guideline*, viewed 22 January 2020, <https://www.nhmrc.gov.au/about-us/publications/australian-dietary-guidelines#block-views-block-file-attachments-content-block-1>.

Novakova, M, Polidarova, L, Sladek, M & Sumova, A 2011, 'Restricted feeding regime affects clock gene expression profiles in the suprachiasmatic nucleus of rats exposed to constant light', *Neuroscience*, vol. 197, Dec 1, pp. 65-71.

Okada, C, Imano, H, Muraki, I, Yamada, K & Iso, H 2019, 'The Association of Having a Late Dinner or Bedtime Snack and Skipping Breakfast with Overweight in Japanese Women', *J Obes*, vol. 2019, p. 2439571.

Olsen, MK, Choi, MH, Kulseng, B, Zhao, CM & Chen, D 2017, 'Time-restricted feeding on weekdays restricts weight gain: A study using rat models of high-fat diet-induced obesity', *Physiol Behav*, vol. 173, May 01, pp. 298-304.

Panda, S, Antoch, MP, Miller, BH, Su, AI, Schook, AB, Straume, M, Schultz, PG, Kay, SA, Takahashi, JS & Hogenesch, JB 2002, 'Coordinated transcription of key pathways in the mouse by the circadian clock', *Cell*, vol. 109, no. 3, May 03, pp. 307-320.

Parr, EB, Devlin, BL, Radford, BE & Hawley, JA 2020, 'Delaying breakfast as a modified time-restricted 2 feeding protocol for improving glycemic control and 3 encouraging dietary adherence for men with 4 overweight/obesity: a randomized controlled trial', *Nutrients*, vol. 12.

Peek, CB, Affinati, AH, Ramsey, KM, Kuo, HY, Yu, W, Sena, LA, Ilkayeva, O, Marcheva, B, Kobayashi, Y, Omura, C, Levine, DC, Bacsik, DJ, Gius, D, Newgard, CB, Goetzman, E, Chandel, NS, Denu, JM, Mrksich, M & Bass, J 2013, 'Circadian clock NAD+ cycle drives mitochondrial oxidative metabolism in mice', *Science*, vol. 342, no. 6158, Nov 01, p. 1243417.

Poljsak, B 2018, 'NAMPT-Mediated NAD Biosynthesis as the Internal Timing Mechanism: In NAD+ World, Time Is Running in Its Own Way', *Rejuvenation Res*, vol. 21, no. 3, Jun, pp. 210-224.

Pureza, I, Melo, ISV, Macena, ML, Praxedes, DRS, Vasconcelos, LGL, Silva-Junior, AE, Florencio, T & Bueno, NB 2020, 'Acute effects of time-restricted feeding in low-income women with obesity placed on hypoenergetic diets: Randomized trial', *Nutrition*, vol. 77, Mar 6, p. 110796.

Qian, J, Morris, CJ, Caputo, R, Garaulet, M & Scheer, F 2018, 'Ghrelin is impacted by the endogenous circadian system and by circadian misalignment in humans', *Int J Obes (Lond)*, Sep 19.

Ramanathan, C, Kathale, ND, Liu, D, Lee, C, Freeman, DA, Hogenesch, JB, Cao, R & Liu, AC 2018, 'mTOR signaling regulates central and peripheral circadian clock function', *PLoS Genet*, vol. 14, no. 5, May, p. e1007369.

Ramsey, KM, Mills, KF, Satoh, A & Imai, S 2008, 'Age-associated loss of Sirt1-mediated enhancement of glucose-stimulated insulin secretion in beta cell-specific Sirt1-overexpressing (BESTO) mice', *Aging Cell*, vol. 7, no. 1, Jan, pp. 78-88.

Ramsey, KM, Yoshino, J, Brace, CS, Abrassart, D, Kobayashi, Y, Marcheva, B, Hong, HK, Chong, JL, Buhr, ED, Lee, C, Takahashi, JS, Imai, S & Bass, J 2009, 'Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis', *Science*, vol. 324, no. 5927, May 01, pp. 651-654.

Ravussin, E 2019, 'Early Time-Restricted Feeding Reduces Appetite and Increases Fat Oxidation But Does Not Affect Energy Expenditure in Humans', *Obesity (Silver Spring)*.

Regmi, P & Heilbronn, LK 2020, 'Time-Restricted Eating: Benefits, Mechanisms, and Challenges in Translation', *iScience*, vol. 23, no. 6, May 15, p. 101161.

Reppert, SM & Weaver, DR 2002, 'Coordination of circadian timing in mammals', *Nature*, vol. 418, no. 6901, Aug 29, pp. 935-941.

Ricanati, EH, Golubic, M, Yang, D, Saager, L, Mascha, EJ & Roizen, MF 2011, 'Mitigating preventable chronic disease: Progress report of the Cleveland Clinic's Lifestyle 180 program', *Nutr Metab (Lond)*, vol. 8, Nov 23, p. 83.

Rudic, RD, McNamara, P, Curtis, AM, Boston, RC, Panda, S, Hogenesch, JB & Fitzgerald, GA 2004, 'BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis', *PLoS Biol*, vol. 2, no. 11, Nov, p. e377.

Saklayen, MG 2018, 'The Global Epidemic of the Metabolic Syndrome', *Curr Hypertens Rep*, vol. 20, no. 2, Feb 26, p. 12.

Salgin, B, Marcovecchio, ML, Humphreys, SM, Hill, N, Chassin, LJ, Lunn, DJ, Hovorka, R & Dunger, DB 2009, 'Effects of prolonged fasting and sustained lipolysis on insulin secretion and insulin sensitivity in normal subjects', *Am J Physiol Endocrinol Metab*, vol. 296, no. 3, Mar, pp. E454-461.

Sampey, BP, Vanhoose, AM, Winfield, HM, Freemerman, AJ, Muehlbauer, MJ, Fueger, PT, Newgard, CB & Makowski, L 2011, 'Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: comparison to high-fat diet', *Obesity (Silver Spring)*, vol. 19, no. 6, Jun, pp. 1109-1117.

Sato, S, Basse, AL, Schonke, M, Chen, S, Samad, M, Altintas, A, Laker, RC, Dalbram, E, Barres, R, Baldi, P, Treebak, JT, Zierath, JR & Sassone-Corsi, P 2019, 'Time of Exercise Specifies the Impact on Muscle Metabolic Pathways and Systemic Energy Homeostasis', *Cell Metab*, vol. 30, no. 1, Jul 2, pp. 92-110 e114.

Scarbrough, K, Losee-Olson, S, Wallen, EP & Turek, FW 1997, 'Aging and photoperiod affect entrainment and quantitative aspects of locomotor behavior in Syrian hamsters', *Am J Physiol*, vol. 272, no. 4 Pt 2, Apr, pp. R1219-1225.

Scheer, FA, Morris, CJ & Shea, SA 2013, 'The internal circadian clock increases hunger and appetite in the evening independent of food intake and other behaviors', *Obesity (Silver Spring)*, vol. 21, no. 3, Mar, pp. 421-423.

Serra, M, Marongiu, F, Pisu, MG, Serra, M & Laconi, E 2019, 'Time-restricted feeding delays the emergence of the age-associated, neoplastic-prone tissue landscape', *Aging (Albany NY)*, vol. 11, no. 11, Jun 12, pp. 3851-3863.

Service, FJ, Molnar, GD, Rosevear, JW, Ackerman, E, Gatewood, LC & Taylor, WF 1970, 'Mean amplitude of glycemic excursions, a measure of diabetic instability', *Diabetes*, vol. 19, no. 9, Sep, pp. 644-655.

Sherman, H, Frumin, I, Gutman, R, Chapnik, N, Lorentz, A, Meylan, J, le Coutre, J & Froy, O 2011, 'Long-term restricted feeding alters circadian expression and reduces the level of inflammatory and disease markers', *J Cell Mol Med*, vol. 15, no. 12, Dec, pp. 2745-2759.

Sherman, H, Genzer, Y, Cohen, R, Chapnik, N, Madar, Z & Froy, O 2012, 'Timed high-fat diet resets circadian metabolism and prevents obesity', *FASEB J*, vol. 26, no. 8, Aug, pp. 3493-3502.

Shimizu, H, Hanzawa, F, Kim, D, Sun, S, Laurent, T, Umeki, M, Ikeda, S, Mochizuki, S & Oda, H 2018, 'Delayed first active-phase meal, a breakfast-skipping model, led to increased body weight and shifted the circadian oscillation of the hepatic clock and lipid metabolism-related genes in rats fed a high-fat diet', *PLoS One*, vol. 13, no. 10, p. e0206669.

Sonnier, T, Rood, J, Gimble, JM & Peterson, CM 2014, 'Glycemic control is impaired in the evening in prediabetes through multiple diurnal rhythms', *J Diabetes Complications*, vol. 28, no. 6, Nov-Dec, pp. 836-843.

Srere, PA 1969, 'Citrate Synthase', in CaNO Kaplan (ed.), *Methods in Enzymology*, vol. 13, Academic Press, New York, p. 5.

Stote, KS, Baer, DJ, Spears, K, Paul, DR, Harris, GK, Rumpler, WV, Strycula, P, Najjar, SS, Ferrucci, L, Ingram, DK, Longo, DL & Mattson, MP 2007, 'A controlled trial of reduced meal frequency without caloric restriction in healthy, normal-weight, middle-aged adults', *Am J Clin Nutr*, vol. 85, no. 4, Apr, pp. 981-988.

Stratton, MT, Tinsley, GM, Alesi, MG, Hester, GM, Olmos, AA, Serafini, PR, Modjeski, AS, Mangine, GT, King, K, Savage, SN, Webb, AT & VanDusseldorp, TA 2020, 'Four Weeks of Time-Restricted Feeding Combined with Resistance Training Does Not Differentially Influence Measures of Body Composition, Muscle Performance, Resting Energy Expenditure, and Blood Biomarkers', *Nutrients*, vol. 12, no. 4, Apr 17.

Stromsdorfer, KL, Yamaguchi, S, Yoon, MJ, Moseley, AC, Franczyk, MP, Kelly, SC, Qi, N, Imai, S & Yoshino, J 2016, 'NAMPT-Mediated NAD(+) Biosynthesis in Adipocytes Regulates Adipose Tissue

Function and Multi-organ Insulin Sensitivity in Mice', *Cell Rep*, vol. 16, no. 7, Aug 16, pp. 1851-1860.

Stubblefield, JJ, Gao, P, Kilaru, G, Mukadam, B, Terrien, J & Green, CB 2018, 'Temporal Control of Metabolic Amplitude by Nocturnin', *Cell Rep*, vol. 22, no. 5, Jan 30, pp. 1225-1235.

Sundaram, S & Yan, L 2016, 'Time-restricted feeding reduces adiposity in mice fed a high-fat diet', *Nutr Res*, vol. 36, no. 6, Jun, pp. 603-611.

Sutton, EF, Beyl, R, Early, KS, Cefalu, WT, Ravussin, E & Peterson, CM 2018, 'Early Time-Restricted Feeding Improves Insulin Sensitivity, Blood Pressure, and Oxidative Stress Even without Weight Loss in Men with Prediabetes', *Cell Metab*, vol. 27, no. 6, Jun 5, pp. 1212-1221 e1213.

Tinsley, GM, Forsse, JS, Butler, NK, Paoli, A, Bane, AA, La Bounty, PM, Morgan, GB & Grandjean, PW 2017, 'Time-restricted feeding in young men performing resistance training: A randomized controlled trial', *Eur J Sport Sci*, vol. 17, no. 2, Mar, pp. 200-207.

Tinsley, GM, Moore, ML, Graybeal, AJ, Paoli, A, Kim, Y, Gonzales, JU, Harry, JR, VanDusseldorp, TA, Kennedy, DN & Cruz, MR 2019, 'Time-restricted feeding plus resistance training in active females: a randomized trial', *Am J Clin Nutr*, Jul 3.

Tsai, JY, Villegas-Montoya, C, Boland, BB, Blasier, Z, Egbejimi, O, Gonzalez, R, Kueht, M, McElfresh, TA, Brewer, RA, Chandler, MP, Bray, MS & Young, ME 2013, 'Influence of dark phase restricted high fat feeding on myocardial adaptation in mice', *J Mol Cell Cardiol*, vol. 55, Feb, pp. 147-155.

Tschop, MH, Speakman, JR, Arch, JR, Auwerx, J, Bruning, JC, Chan, L, Eckel, RH, Farese, RV, Jr., Galgani, JE, Hambly, C, Herman, MA, Horvath, TL, Kahn, BB, Kozma, SC, Maratos-Flier, E, Muller, TD, Munzberg, H, Pfluger, PT, Plum, L, Reitman, ML, Rahmouni, K, Shulman, GI, Thomas, G, Kahn, CR & Ravussin, E 2011, 'A guide to analysis of mouse energy metabolism', *Nat Methods*, vol. 9, no. 1, Dec 28, pp. 57-63.

Um, JH, Pendergast, JS, Springer, DA, Foretz, M, Viollet, B, Brown, A, Kim, MK, Yamazaki, S & Chung, JH 2011, 'AMPK regulates circadian rhythms in a tissue- and isoform-specific manner', *PLoS One*, vol. 6, no. 3, Mar 31, p. e18450.

Um, JH, Yang, S, Yamazaki, S, Kang, H, Viollet, B, Foretz, M & Chung, JH 2007, 'Activation of 5'-AMP-activated kinase with diabetes drug metformin induces casein kinase Iepsilon (CKIepsilon)-dependent degradation of clock protein mPer2', *J Biol Chem*, vol. 282, no. 29, Jul 20, pp. 20794-20798.

Varady, KA, Bhutani, S, Klempel, MC, Kroeger, CM, Trepanowski, JF, Haus, JM, Hoddy, KK & Calvo, Y 2013, 'Alternate day fasting for weight loss in normal weight and overweight subjects: a randomized controlled trial', *Nutr J*, vol. 12, no. 1, Nov 12, p. 146.

Velingkaar, N, Mezhnina, V, Poe, A, Makwana, K, Tulsian, R & Kondratov, RV 2020, 'Reduced caloric intake and periodic fasting independently contribute to metabolic effects of caloric restriction', *Aging Cell*, Mar 11, p. e13138.

Villanueva, JE, Livelo, C, Trujillo, AS, Chandran, S, Woodworth, B, Andrade, L, Le, HD, Manor, U, Panda, S & Melkani, GC 2019, 'Time-restricted feeding restores muscle function in Drosophila models of obesity and circadian-rhythm disruption', *Nat Commun*, vol. 10, no. 1, Jun 20, p. 2700.

Vollmers, C, Gill, S, DiTacchio, L, Pulivarthy, SR, Le, HD & Panda, S 2009, 'Time of feeding and the intrinsic circadian clock drive rhythms in hepatic gene expression', *Proc Natl Acad Sci U S A*, vol. 106, no. 50, Dec 15, pp. 21453-21458.

Wallace, E, Wright, S, Schoenike, B, Roopra, A, Rho, JM & Maganti, RK 2018, 'Altered circadian rhythms and oscillation of clock genes and sirtuin 1 in a model of sudden unexpected death in epilepsy', *Epilepsia*, vol. 59, no. 8, Aug, pp. 1527-1539.

Wang, H, van Spyk, E, Liu, Q, Geyfman, M, Salmans, ML, Kumar, V, Ihler, A, Li, N, Takahashi, JS & Andersen, B 2017, 'Time-Restricted Feeding Shifts the Skin Circadian Clock and Alters UVB-Induced DNA Damage', *Cell Rep*, vol. 20, no. 5, Aug 1, pp. 1061-1072.

Wang, HB, Loh, DH, Whittaker, DS, Cutler, T, Howland, D & Colwell, CS 2018, 'Time-Restricted Feeding Improves Circadian Dysfunction as well as Motor Symptoms in the Q175 Mouse Model of Huntington's Disease', *eNeuro*, vol. 5, no. 1, Jan-Feb.

Wehrens, SMT, Christou, S, Isherwood, C, Middleton, B, Gibbs, MA, Archer, SN, Skene, DJ & Johnston, JD 2017, 'Meal Timing Regulates the Human Circadian System', *Curr Biol*, vol. 27, no. 12, Jun 19, pp. 1768-1775 e1763.

Weir, JB 1949, 'New methods for calculating metabolic rate with special reference to protein metabolism', *J Physiol*, vol. 109, no. 1-2, Aug, pp. 1-9.

Wilkinson, MJ, Manoogian, ENC, Zadourian, A, Lo, H, Fakhouri, S, Shoghi, A, Wang, X, Fleischer, JG, Navlakha, S, Panda, S & Taub, PR 2019, 'Ten-Hour Time-Restricted Eating Reduces Weight, Blood Pressure, and Atherogenic Lipids in Patients with Metabolic Syndrome', *Cell Metab*, Dec 2.

Wing, RR, Lang, W, Wadden, TA, Safford, M, Knowler, WC, Bertoni, AG, Hill, JO, Brancati, FL, Peters, A, Wagenknecht, L & Look, ARG 2011, 'Benefits of modest weight loss in improving cardiovascular risk factors in overweight and obese individuals with type 2 diabetes', *Diabetes Care*, vol. 34, no. 7, Jul, pp. 1481-1486.

Woodie, LN, Luo, Y, Wayne, MJ, Graff, EC, Ahmed, B, O'Neill, AM & Greene, MW 2018, 'Restricted feeding for 9h in the active period partially abrogates the detrimental metabolic effects of a Western diet with liquid sugar consumption in mice', *Metabolism*, vol. 82, May, pp. 1-13.

Yamamuro, D, Takahashi, M, Nagashima, S, Wakabayashi, T, Yamazaki, H, Takei, A, Takei, S, Sakai, K, Ebihara, K, Iwasaki, Y, Yada, T & Ishibashi, S 2020, 'Peripheral circadian rhythms in the liver and white adipose tissue of mice are attenuated by constant light and restored by time-restricted feeding', *PLoS One*, vol. 15, no. 6, p. e0234439.

Yasumoto, Y, Hashimoto, C, Nakao, R, Yamazaki, H, Hiroyama, H, Nemoto, T, Yamamoto, S, Sakurai, M, Oike, H, Wada, N, Yoshida-Noro, C & Oishi, K 2016, 'Short-term feeding at the wrong time is sufficient to desynchronize peripheral clocks and induce obesity with hyperphagia, physical inactivity and metabolic disorders in mice', *Metabolism*, vol. 65, no. 5, May, pp. 714-727.

Yoo, SH, Yamazaki, S, Lowrey, PL, Shimomura, K, Ko, CH, Buhr, ED, Siepka, SM, Hong, HK, Oh, WJ, Yoo, OJ, Menaker, M & Takahashi, JS 2004, 'PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues', *Proc Natl Acad Sci U S A*, vol. 101, no. 15, Apr 13, pp. 5339-5346.

Zarrinpar, A, Chaix, A, Yooseph, S & Panda, S 2014, 'Diet and feeding pattern affect the diurnal dynamics of the gut microbiome', *Cell Metab*, vol. 20, no. 6, Dec 2, pp. 1006-1017.

Zeb, F, Wu, X, Chen, L, Fatima, S, Haq, IU, Chen, A, Majeed, F, Feng, Q & Li, M 2020, 'Effect of time-restricted feeding on metabolic risk and circadian rhythm associated with gut microbiome in healthy males', *Br J Nutr*, vol. 123, no. 11, Jun 14, pp. 1216-1226.

Zhang, LQ, Van Haandel, L, Xiong, M, Huang, P, Heruth, DP, Bi, C, Gaedigk, R, Jiang, X, Li, DY, Wyckoff, G, Grigoryev, DN, Gao, L, Li, L, Wu, M, Leeder, JS & Ye, SQ 2017, 'Metabolic and molecular insights into an essential role of nicotinamide phosphoribosyltransferase', *Cell Death Dis*, vol. 8, no. 3, Mar 23, p. e2705.

Zhao, L, Hutchison, AT, Wittert, GA, Thompson, CH, Lange, K, Liu, B & Heilbronn, LK 2020, 'Intermittent Fasting Does Not Uniformly Impact Genes Involved in Circadian Regulation in Women with Obesity', *Obesity (Silver Spring)*, May 21.

Zhong, LX, Li, XN, Yang, GY, Zhang, X, Li, WX, Zhang, QQ, Pan, HX, Zhang, HH, Zhou, MY, Wang, YD, Zhang, WW, Hu, QS, Zhu, W & Zhang, B 2019, 'Circadian misalignment alters insulin sensitivity during the light phase and shifts glucose tolerance rhythms in female mice', *PLoS One*, vol. 14, no. 12, p. e0225813.

Zimmet, PZ, Wall, JR, Rome, R, Stimmler, L & Jarrett, RJ 1974, 'Diurnal variation in glucose tolerance: associated changes in plasma insulin, growth hormone, and non-esterified fatty acids', *Br Med J*, vol. 1, no. 5906, Mar 16, pp. 485-488.