Sleep, dietary patterns and metabolic health in UK adults

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Declaration

The candidate confirms that the work submitted is his own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

The literature review in Chapter 1 incorporates much of the work of two jointly authored publications:

Potter GDM, Cade JE, Grant PJ, Hardie LJ. Nutrition and the circadian system. Br J Nutr. 2016;116(3):434-42.

Potter GDM, Skene DJ, Arendt J, Cade JE, Grant PJ, Hardie LJ. Circadian Rhythm and Sleep Disruption: Causes, Metabolic Consequences, and Countermeasures. Endocr Rev. 2016;37(6):584-608.

The candidate was responsible for reviewing the literature and writing all drafts of both of these manuscripts. The contribution of the other authors was providing comprehensive feedback throughout preparation of the publications.

Chapter 2 incorporates much of the work of another jointly authored publication:

Wark PA, Hardie LJ, Frost GS, Alwan NA, Carter M, Elliott P, et al. Validity of an online 24-hour recall tool (myfood24) for dietary assessment in population studies: comparison with biomarkers and standard interviews. BMC Med (In press) 2018. ISSN 1741-7015.

The candidate was responsible for completing the urinary nitrogen and sugars laboratory analyses. The contribution of the other authors was as follows: Nisreen helped design the study. Janet conceived the project, oversaw the project, contributed to study design, and helped develop myfood24. Michelle Carter managed the project in Leeds for two years and helped develop myfood24. Paul helped design the study. Heather led participant recruitment and collection of biological samples. Gary helped design the study and oversaw the participant recruitment process. Darren helped design the study and completed the statistical analyses. Neil contributed to the study design, helped develop myfood24, and continues to manage the myfood24 database. Laura helped design the study and developed the biomarker methods. Michelle Morris contributed to management of the project in Leeds for one year and helped develop myfood24. Umme and Katerina managed the project in London, also helping with participant recruitment and biological sample collection. David did the plasma β-carotene, α-tocopherol, and vitamin C biomarker laboratory analyses. Essra also contributed to the vitamin C

biomarker laboratory analysis. Elio helped design the study. Petra jointly conceived the project, oversaw the project in London, and helped design the study.

Chapter 4 incorporates much of the work of another jointly authored publication:

Potter GDM, Cade JE, Hardie LJ. Longer sleep is associated with lower BMI and favorable metabolic profiles in UK adults: Findings from the National Diet and Nutrition Survey. PLoS One. 2017;12(7):e0182195.

Chapter 4 is based on a secondary analysis of a publicly available dataset (the National Diet and Nutrition Survey). The candidate was not involved in the design, data collection, or primary data processing of the National Diet and Nutrition Survey. Credit for these data is detailed in the Acknowledgements. The candidate was responsible for designing and completing the data analysis in Chapter 4. The candidate was also responsible for data cleaning and formatting, data interpretation, and writing all drafts of the manuscript. The contribution of the other authors was helping design the analysis and providing comprehensive feedback throughout preparation of the publication.

Acknowledgements

Abstract

The causes of the ongoing metabolic disease pandemic are complex. Changes in human population genetics proceed slowly, so the recent increase in metabolic disease prevalence likely reflects environmental and behavioural changes. Our circadian (~ 24-hour) systems optimise our biology according to time of day. Artificial stimuli like electric lighting and around-the-clock food access enable waking behaviours, such as eating, at times at which our circadian systems prime us to sleep. Such mistimed behaviours may contribute to metabolic disease, as exemplified by increased risk of diabetes in shift workers. However, few researchers have concurrently explored associations between sleep, diet composition and timing, and metabolic health. Furthermore, many dietary analysis methods used have not been validated, precluding accurate inferences about diet-disease relationships. And few studies have assessed the metabolic effects of interventions to resynchronise the circadian system each day.

I first helped validate myfood24, an online dietary recall tool, by completing all of the lab work for dietary protein and sugar biomarkers. myfood24 has comparable validity to the gold-standard recall method. Subsequent analysis of the myfood24 data showed that consuming calories later relative to sleep is associated with overweight and obesity. Next, analysis of a public database indicated that longer sleepers had lower body mass indices, smaller waists, and favourable blood lipid profiles. Finally, we assessed whether long-term supplementation of melatonin (a hormone that synchronises the circadian system) influences metabolic health, sleep, and diet in adults predisposed to diabetes. Contrary to our hypotheses, melatonin had few effects.

This project helped validate myfood24, a tool that could unveil diet-disease relationships in future studies. These studies might have key public health implications. Our findings also strengthen the notions that mistimed eating and insufficient sleep contribute to obesity. Finally, this project shows that melatonin may not substantially influence metabolic health in relatively healthy adults.

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List of abbreviations

ACTH   Adrenocorticotropic hormone

ADP   Adenosine diphosphate

AMP   Adenosine monophosphate

AMPK   5' AMP-activated protein kinase

ANS   Autonomic nervous system

ATP   Adenosine triphosphate

ANOVA   Analysis of variance

ASA24®   Automated Self-Administered 24-hour recall

BMAL1   Brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1

BMI   Body mass index

CAPI   Computer-assisted personal interview

CCG   Clock-controlled gene

CI   Confidence interval

CK   Casein kinase

CLOCK   Circadian locomotor output cycles kaput

CRF   Clinical Research Facility

CRH   Corticotropin-releasing hormone

CRP   C-reactive protein

CRY   Cryptochrome

FAA   Food anticipatory activity

FFQ   Food frequency questionnaire

FBXL3   F-box/LRR-repeat protein 3

GWA   Genome-wide association

HbA1c   Glycated haemoglobin

HDL	high-density lipoprotein
HPLC	High performance liquid chromatography
IL	Interleukin
LD	Light/dark
LDL	Low-density lipoprotein
MCTQ	Munich ChronoType Questionnaire
ME	Median eminence
MEQ	Morningness-Eveningness Questionnaire
MT	Melatonin receptor
MTNR	Melatonin receptor (gene and associated protein)
MPR	(Interviewer-administered) multiple-pass 24-hour recall method
myfood24	measure your food on one day 24-hour recall
NAD	Nicotinamide adenine dinucleotide
NDNS-RP	National Diet and Nutrition Survey Rolling Programme
NMES	Non-milk extrinsic sugar
NPAS2	Neuronal period-aryl hydrocarbon receptor nuclear translocator single-minded protein 2
OR	Odds ratio
p value	Probability value
PER	Period
Process C	Circadian process
Process S	Sleep process
PSQI	Pittsburgh Sleep Quality Index
QC	Quality control
REE	Resting energy expenditure

SCN	Suprachiasmatic nuclei
SD	Standard deviation
SIRT	Silent mating type information regulation 2 homolog (SIRTUIN)
SNP	Single-nucleotide polymorphism
T	Zeitgeber period
τ	Circadian system period
TEE	Total energy expenditure
TOR	Target of rapamycin
TRF	Time-restricted feeding
TSH	Thyroid-stimulating hormone
T3	(Free) triiodothyronine
T4	(Free) thyroxine
UK	United Kingdom
US	United States
VCO_2	Carbon dioxide production
VO_2	Oxygen consumption
WeLReN	North West London Primary Care Research Network

Chapter 1: Introduction

1.1 Obesity and diabetes are increasingly common and burdensome

Ongoing trends in non-communicable diseases are troubling. Perhaps for the first time in human history, the majority of people now live in countries where more people die prematurely from overweight and obesity than from being underweight (1). At present, efforts to curb changing disease trends are not working: The Lancet recently reported that obesity had not declined in a single nation in the previous 33 years (2). And if post-2000 trends continue, 18% of men and 21% of women will be obese worldwide by 2025 (3).

A recent systematic review of data from 195 countries found that obesity prevalence has at least doubled in 73 countries since 1980. In 2015, 5% of children and 12% of adults were obese, and high body mass index (BMI) contributed to 7.1% of deaths from any cause and 4.9% of disability-adjusted life years from any cause. More than two-thirds of these deaths and over half of these disability-adjusted life years resulted from cardiovascular disease. Diabetes, chronic kidney disease, musculoskeletal disorders, and several cancers were other causes of BMI-related deaths and disability-adjusted life years (4). Obesity is also a risk factor for other non-communicable diseases, increasing premature mortality from liver and respiratory diseases (5), for example.

Obesity-related disease is fuelled by widespread metabolic dysregulation. This is exemplified by metabolic syndrome, a cluster of metabolic risk factors comprising central obesity plus two or more of the following: elevated blood pressure, raised fasting glucose, elevated triglycerides, and low high-density lipoprotein (HDL) cholesterol. Metabolic syndrome is thought to currently affect about a quarter of adults worldwide (6) and is associated with diseases such as cancer, cardiovascular disease, and type two diabetes (7, 8).

It is therefore no surprise that diabetes is increasing at an alarming rate. From 1980 to 2014, the number of people with diabetes worldwide increased nearly four-fold, from 108 million to 422 million. Even with population growth, this is a rise in prevalence from 4.7% to 8.5%. Like obesity, diabetes is a major cause of numerous pathologies, from cardiovascular disease to kidney failure. Indeed, it was estimated that diabetes directly resulted in ~ 1.5 million premature deaths in 2012 alone, with raised blood glucose contributing to the premature mortality of an additional 2.2 million people (9).

It is clear that many metabolic diseases are inextricably related. With contemporary trends in obesity, metabolic syndrome, and diabetes prevalence in mind, identifying the

factors that influence risk of these disorders is a public health priority. We now live in a 24/7 society, and one of these factors appears to be discordance between the timing of our behaviours and that of our biology. The detrimental metabolic consequences of such discordance are increasingly evident.

1.2 Circadian system and sleep disruption are associated with metabolic diseases

Life evolved on Earth in the presence of environmental cycles that predictably emanate from the rotation of the planet around its axis, the rotation of the moon about Earth, and Earth's orbit around the sun. To thrive in ecosystems exposed to environmental cycles, organisms evolved internal timing systems to anticipate and adapt to reliable ecological changes, the most conspicuous of which is arguably the daily light/dark (LD) cycle. LD cycles result in ~ 24-hour changes in light and temperature, and these cycles tuned the development of organisms' circadian (~ 24-hour) systems (10) – endogenous 'clocks' that produce circadian rhythms in biology and behaviour (Table 1.1 is a glossary of relevant terms).

Table 1.1. Glossary.

Circadian rhythm	An endogenous rhythm with a period of ~ 24 hours that is entrainable, persists in the absence of external time cues (11), and is temperature compensated. Temperature compensation means that the rhythm is relatively unaffected by temperature changes, allowing stable circadian rhythms in different thermal environments (12). Like other biological rhythms, circadian rhythms have three important parameters: amplitude (the difference between a rhythm's acrophase (peak) or bathyphase (trough) and its mean), period (the time elapsed until the same phase of the rhythm oscillation recurs), and phase (the momentary state of an oscillation within its period). Circadian time is synonymous with internal time and spans one full circadian period. Circadian time zero is typically subjective dawn.
Circadian system disruption	Disruption of the endogenous timing system that regulates circadian patterns of behaviour and biology. This can occur from the level of the molecular clock that temporally regulates cellular activities, to misalignment between behavioural and environmental cycles. Circadian system disruption disturbs phase relationships in

	oscillatory subsystems, resulting in circadian system misalignment. For example, the timing of metabolic processes in the liver may be shifted by changes in meal timing, but the timing of activity in neural networks in the brain may be relatively unaffected. Our understanding of the range of healthy phase relationships between subsystems is currently limited (13).
Constant routine	An experimental protocol that attempts to enforce unchanging behavioural and environmental conditions to unmask true circadian rhythms from diurnal rhythms arising from exogenous factors such as eating. Constant routine typically entails unchanging dim lighting, evenly-spaced isocaloric snack consumption, semi-recumbent posture, and wakefulness, although modified constant routine protocols permit sleep.
Entrainment	Coupling of an endogenous rhythm to a zeitgeber, such that the oscillations have the same frequency (synchronisation) or frequencies that are whole multiples (frequency demultiplication) (14). When entrained, circadian system period (τ) matches the period of the zeitgeber (T), typically the light/dark (LD) cycle. The mean free-running human τ is ~ 24.2 hours (15), so circadian rhythms must be entrained to the 24-hour LD cycle daily. A short or long free-running τ typically entrains earlier or later, respectively. The result is variation in activity/rest cycle timing (chronotype) between members of a species.
Forced desynchrony	An experimental protocol that enforces LD cycles outside the range of entrainment. When repeated for a sufficient number of cycles, these protocols distribute sleep and wakefulness across the circadian cycle and hence uncouple effects of behavioural cycles from effects of the circadian system. An example protocol might involve 28-hour sleep/wake cycles with sleep permitted for a third of each 'day' (so for 9.33 hours). Three of these cycles would produce ~ 180° misalignment between the circadian clock and LD cycle, such that the person would now be engaging in waking behaviours like eating during his/her biological night-time.
Peripheral clocks	All endogenous oscillator systems outside the master clock in the suprachiasmatic nuclei. Peripheral clocks therefore include other clocks in the brain as well as those in organs such as the liver, and

	all serve to produce circadian rhythms to optimise the timing of local tissue processes.
Sleep disruption	Disruption to sleep continuity, timing, or duration. Sleep restriction entails reduced sleep duration, whereas sleep deprivation is the absence of sleep.
Zeitgeber	The stimulus that entrains a biological rhythm (14). The 24-hour LD cycle is the primary zeitgeber for the human circadian system. High amplitude, relatively consistently timed zeitgebers help ensure stable entrainment (for example, regularly timed high intensity light exposure during the day and minimal light exposure at night). Zeitgeber time is the duration of one zeitgeber cycle (one LD cycle, for example). Zeitgeber time zero is commonly dawn (the beginning of the warm phase).

It is likely that the circadian systems of our preindustrial ancestors were closely aligned with LD cycles. As humans are diurnal, our forebears' daytime activities would likely have included foraging, hunting, and eating. Night-time would perhaps mostly have entailed resting and fasting. This hypothesis is supported by studies in which people living in industrialised areas are exposed to only natural light sources (sunlight, moonlight, starlight, and firelight) while camping. While camping, people's circadian systems swiftly synchronise tightly with the LD cycle, and pre-camping inter-individual variation in circadian system and sleep timing decreases markedly (16, 17).

Industrialisation resulted in rapid modifications to the environment, a seminal example of which was Thomas Edison's invention of the incandescent lightbulb in 1879. This pivotal creation enabled workers to complete important jobs during the night, fuelling economic globalisation by extending working hours around the clock. Novel transportation modes such as trains, cars, and planes perhaps also contributed to the distortion of natural human activity/rest cycles. Furthermore, developments in indoor heating may have altered exposure to ambient temperature cycles, and the thermal environment is known to influence sleep and wakefulness (18). Last, the confluence of changing food production and distribution with innovative refrigeration methods may have altered food availability. So, with industrialisation humans now had more means to distort the daily LD, activity/rest, thermal, and eating/fasting cycles that shaped the lives of previous generations. It is plausible that these disruptions to human lifestyle patterns has contributed to the burgeoning prevalence of diseases of modernity (19), and an accumulating mass of evidence from cross-sectional studies supports this notion.

Some shift workers often work at times at which they would otherwise sleep, and these people are therefore at particular risk of circadian rhythm and sleep disruption (20, 21). Shift workers are predisposed to various health disorders, including certain cancers, myocardial infarction, stroke, and type two diabetes (22). As shift work exposure is related to risk of metabolic syndrome in a dose-response fashion (23), it seems that some deleterious health consequences of shift work may be cumulative. It is likely that disruption to both the circadian system and sleep influence disease risk in shift work (22). Compared to day shift workers matched for BMI, for example, some of the adverse metabolic consequences experienced by night shift workers are associated with sleep disturbances (24). As ~ 19% of the European workforce work nights at least once a month (25), the societal implications of harm resulting from shift work are substantial.

Flying across time-zones produces rapid changes in zeitgebers. The circadian system must synchronise to the new environment, and the result is jetlag, a syndrome that includes general malaise and sleep disturbances. A phenomenon similar to mild jetlag affects many individuals on a weekly basis. This is particularly prevalent among people with later sleep timing who must use alarms on work days to produce wakefulness when sleep would otherwise occur. Bed times therefore differ between work days and non-work days, and a discrepancy of at least one hour between mid-sleep time on work days and non-work days affects ~ 69% of Northern Europeans. These transitions have been termed 'social jetlag' and are associated with metabolic diseases like obesity and unhealthy behaviours such as high alcohol and cigarette consumption (26, 27). Similarly, greater social jetlag is related to cardiometabolic disease risk factors like insulin resistance (28, 29), and more variable intra-individual sleep timing is associated with higher fat mass and lower lean mass (30).

Alarm use contributes to insufficient sleep among adults, and ~ 80% of adults rely on alarms to get up on time for workdays (26). The significance of this is that sleep duration is associated with numerous chronic diseases. As an example, sleep duration generally has a U-shaped association with type two diabetes risk. The mechanisms underlying the association between short sleep and increased type two diabetes risk are discussed at length in section 1.5. Why long sleep is associated with increased type two diabetes risk is more contentious, but low socioeconomic status, depression, and other comorbidities are thought to underlie this relationship (31).

Short sleep has often been associated with obesity and increased waist circumference in cross-sectional studies (32, 33). The association between sleep duration and waist circumference is particularly noteworthy. Waist circumference is generally reflective of visceral adiposity, and visceral obesity is associated with many metabolic pathologies,

including cardiovascular diseases (34). The relationship between sleep duration and adiposity is not limited to adults. As sleep timing delays during growth and is latest on reaching physical maturity, enforcing early school starts disrupts sleep timing and duration during adolescence (35). Short sleep during this period is prospectively associated with obesity development (36). Relationships between sleep and adiposity may relate to eating behaviours. An extreme example is night eating syndrome, a disorder characterised by circadian system and sleep disruption, less healthy dietary habits, and overweight and obesity (37).

Finally, some individuals are at increased risk of circadian system and sleep disruption regardless of industrialisation. Sometimes this is related to the photoperiod. All 24 time zones converge at the Poles, where long summer days and protracted winter nights may increase the probability of disruption to the circadian system and sleep (38). In other instances, underlying pathologies are at fault. This is true in instances of circadian system misalignment in blind individuals without light perception (39), most of whom experience non-24-hour sleep-wake rhythm disorder in which sleep quality is highly variable. Sleep quality also deteriorates with advancing age, as do many circadian rhythms (40). It is therefore no surprise that many diseases associated with senescence, such as Alzheimer's, are also characterised by circadian system disorganisation (41). So, as life expectancy continues to rise in many parts of the world, more people are likely to experience sleep problems and circadian system dysfunction.

1.3 Regulation of the circadian system and sleep

1.3.1 The suprachiasmatic nuclei

The circadian system coordinates daily cycles of biology and behaviour. It does so using networks of molecular clocks throughout the body's cells that control the timing of local cellular processes. Only some cells are directly exposed to key zeitgebers such as the LD cycle, and the circadian system must therefore be regulated in a way that both senses environmental time cues and relays time of day information throughout the body to coordinate the timing of activities in a vast number of cells. To fulfil this complicated task, a hierarchical circadian system has evolved in mammals, at the helm of which are two suprachiasmatic nuclei (SCN) in the anterior hypothalamus.

The preeminent roles of the SCN in locomotor, hormonal and feeding circadian rhythms were demonstrated by early studies of rats in which SCN ablation abrogated circadian rhythms in physiology and behaviour (42-44). Having established the importance of the

SCN, researchers' attention turned to the mechanisms by which the SCN time biological processes. The primary zeitgeber that synchronises the SCN with the 24-hour day is the LD cycle. Together with rod and cone photoreceptor cells, entrainment occurs by way of melanopsin-containing intrinsically photosensitive retinal ganglion cells in the inner retinae that relay photic information to the SCN via a monosynaptic pathway (45). A multisynaptic pathway then links the SCN with the pineal gland. On light exposure, norepinephrine release from the fibres that make up the multisynaptic pathway inhibits pineal gland melatonin synthesis (46, 47). It should be noted that although multiple tissues synthesise melatonin, including the retinae (48), the pineal gland is the most important in circadian biology, and pinealectomy results in almost undetectable circulating melatonin levels (49).

The duration of pineal melatonin synthesis is therefore the primary endogenous signal of scotoperiod (darkness), as supported by experiments of timed melatonin infusion in various pinealectomised animals. These experiments have shown that melatonin synthesis duration is likely the key parameter of the melatonin rhythm in instigating photoperiodic changes in biology in seasonal animals (50). As the human melatonin rhythm is also sensitive to photoperiod changes, artificial lighting perhaps suppresses seasonal changes in human biology that might otherwise be evident (51). Indeed, melatonin rhythms are similar during summer and winter when people are exposed to modern electrical lighting, but when people go camping seasonal differences become apparent such that the duration of melatonin synthesis is markedly longer in the winter (17).

Mammals have two melatonin receptors, MT_1 and MT_2 (52, 53). (hMT_1 and hMT_2 in humans, encoded by *MTNR1A* and *MTNR1B*, respectively.) Melatonin receptors are G-protein-coupled receptors and have been found in many mammalian tissues, including the adrenals, kidneys, T and B lymphocytes (all MT_1), the small intestine (MT_2), and arteries, heart, lungs, and skin (all both MT_1 and MT_2) (54). MT_1 and MT_2 are also found in multiple brain regions, including the cerebellum, hippocampus, and thalamus (55). As the SCN have both MT_1 and MT_2 (56, 57), pineal gland melatonin synthesis feeds back to the SCN to help entrain the SCN to the LD cycle (58), ensuring that sleep occurs at appropriate times.

The circadian system has key roles in sleep/wake cycle regulation, as shown by the gating of sleep at specific circadian phases. Moreover, the circadian phase at which sleep occurs influences sleep duration and architecture (59). Perhaps the best-supported model of sleep regulation is Borbély's (60). This model comprises the interaction of a circadian process that changes the drive to stay awake and a sleep

homeostasis process that accumulates with increasing wakefulness duration, promoting sleep. The circadian wakefulness drive is at its strongest shortly before habitual sleep onset to counteract the increased sleep homeostasis that has accumulated during the day. Around sleep onset, a sudden drop in the circadian wakefulness drive no longer opposes the sleep pressure that has built up, the result of which is sleep initiation. Borbély's model has proven effective in simulating sleep in numerous experimental conditions (61), yet many mechanisms by which the circadian and homeostatic processes interact remain elusive.

1.3.2 Molecular clocks

Light exposure rapidly and transiently influences transcription of many genes in the SCN. Among these genes are 'clock' genes (62). Clock genes exist in almost all cells in humans. Simplistically, clock genes form delayed, interlocking gene transcription/translation negative feedback loops that result in ~ 24-hour changes in levels of clock gene proteins. These proteins are transcription factors that bind to promoter regions of 'clock-controlled genes' (CCGs) to activate their expression. In this way, clock genes regulate the timing of cellular processes. A logical question to ask is what makes a gene a clock gene as opposed to a CCG. In contrast to clock genes, mutations in CCGs do not markedly affect circadian rhythms in biology and behaviour.

The positive arm of the core molecular clock loop comprises the basic helix-loop-helix transcription factors circadian locomotor output cycles kaput (CLOCK) and brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1 (BMAL1). (In tissues such as the vasculature, CLOCK's functions are replaced by its paralogue neuronal period-aryl hydrocarbon receptor nuclear translocator single-minded protein 2 (NPAS2)). CLOCK and BMAL1 heterodimerise to activate transcription of CCGs. CCGs include the genes of the negative limb of the clock, *cryptochrome* (*CRY*) *1-2* and *period* (*PER*) *1-3*. CRY and PER proteins then accumulate in the cytosol, multimerise, translocate into the nucleus, repress CLOCK-BMAL1 transactivating function, and therefore ultimately end their own transcription. PER-CRY complexes are then degraded by casein kinase 1 (CK1) ε, CK1δ, and F-box/LRR-repeat protein 3 (FBXL3). CLOCK-BMAL1 inhibition ends, thus closing the negative feedback loop. At least five auxiliary feedback loops add robustness and couple the molecular clock to metabolic status (63).

Within a species, findings that similar proportions of the components of the transcriptome, proteome, and metabolome have oscillating 24-hour profiles is suggestive of their integration and pivotal roles in metabolic regulation (64-66). Nevertheless, there is not perfect concordance in these oscillations. Delays between gene transcription and

translation vary across the day (82,83), and although there are mostly minimal delays between gene transcription and translation, some constitutively expressed genes produce rhythmic products via 24-hour changes in translation (67). Similarly, 24-hour changes in gene transcript levels can result in unchanging levels of their protein products. Such discordance partly results from widespread post-translational clock protein modifications (68). Interestingly, compared to protein-coding gene transcripts, a similar proportion of non-coding RNAs have 24-hour changes in abundance, conferring another level of post-transcriptional regulation and likely influencing molecular clock regulation (64). Collectively, post-transcriptional processes contribute to appropriate, tissue-specific responses of peripheral clocks.

Finally, there exist non-transcriptional clocks in cells such as erythrocytes. These clocks regulate redox cycles in peroxiredoxins, antioxidant proteins involved in electron transfer (69). Interestingly, a recent study also showed antiphasic mitochondrial redox cycles of inactivated peroxiredoxin and sulfiredoxin, likely resulting in oscillating release of hydrogen peroxide, a key regulator of several cell signalling pathways (70). Peroxiredoxin cycles are sustained in the absence of clock gene expression feedback loops and are the most highly conserved clocks known (71). Their integration with the circadian system, sleep homeostasis, and metabolic networks is little understood, however.

1.3.3 Molecular clocks and metabolism

The many metabolic roles of the molecular clock are made evident in studies of clock gene disruption in non-human animals. Whole-body and tissue-specific mutations and knockouts of clock genes produce various metabolic aberrations in rodents (72, 73). Perhaps the most severe example of this is premature mortality and the abolition of all measured molecular and behavioural circadian rhythms in *Bmal1* double knockout mice (74). In humans, genome-wide association (GWA) studies also provide strong evidence for roles of clock gene variants in metabolism. GWA studies have associated *PER3* variants with type two diabetes (75), as well as *CRY2* variants with fasting glycaemia and insulin concentrations (76), for example.

Candidate gene studies provide less robust evidence roles of clock gene variation in metabolic phenotypes. In adults, two *BMAL1* haplotypes have been associated with hypertension and type two diabetes (77, 78). *CLOCK* single-nucleotide polymorphisms (SNPs) may be associated with non-alcoholic steatohepatitis, metabolic syndrome, small dense low-density lipoprotein levels, obesity, and type two diabetes (79-83). Perhaps the most studied of these outcomes is obesity: At least eight common *CLOCK* SNPs have

been associated with obesity, and three may be related to energy intakes (84). However, these candidate gene studies are limited by their sample sizes, their exclusion of all causative genes and gene variants, and their limited replicability.

The molecular clock helps temporally separate incompatible metabolic processes, such as anabolism and catabolism of glucose and lipids. By partitioning metabolic pathways, the molecular clock may offset accumulation of toxic intermediates. Furthermore, as the synthesis of proteins is energetically expensive, the molecular clock perhaps improves energy economy by optimising the timing of protein production. To fulfil these roles, the molecular clock must exert tissue-specific control. Hundreds of CCGs control the timing of tissue-specific functions (85), and while some genes may have 24-hour expression profiles in multiple tissues, their phases often differ between and even within tissues (86, 87). CCGs are enriched for metabolic pathways, and some metabolic genes that are direct targets of CLOCK-BMAL1 heterodimer also feedback to molecular clock components (63).

As metabolic state and the molecular clock are inextricably coupled, rhythmic cellular activities are modified by factors such as diet. The circadian system is reciprocally linked with eating/fasting cycles via interactions between the molecular clock and metabolic regulators. In the post-absorptive state, decreased energy availability increases 5' AMP-activated protein kinase (AMPK) phosphorylation, stimulating ATP formation by increasing processes such as fatty acid oxidation. AMPK impinges on the molecular clock by interacting with the NAD(+)-dependent protein deacetylase SIRTUIN (SIRT)1, which subsequently deacetylates PER2, leading to PER2 degradation and facilitating high amplitude daily transcription profiles of several clock genes (88). As deacetylases, SIRT1 and SIRT6 have particularly pivotal roles in the temporal control of metabolism by regulating chromatin modifications. Deacetylation physically modifies chromatin structure and thus alters access to promoter regions of genes. Deacetylases thereby alter gene transcription. In this way SIRT1 and SIRT6 modify the transcription timing of distinct sets of genes in the liver, with SIRT1 primarily acting on genes involved in peptide and cofactor metabolism, and SIRT6 targeting genes integral to carbohydrate and lipid metabolism (89).

In contrast to the pathways activated in the post-absorptive state, increased energy availability in the postprandial state stimulates anabolic processes such as protein synthesis via target of rapamycin (TOR) signalling. The TOR pathway is coupled to the molecular clock by influencing phosphorylation of glycogen synthase kinase 3β, which in turn regulates PER stability and hence period length (90). Thus, whereas fasting-induced AMPK activation may be important to high amplitude clock gene transcription

rhythms, feeding-induced TOR activation can modify period length. Such interactions between energy sensors and molecular clock components highlight the close coupling between diet and the circadian system.

During time-restricted feeding (TRF), food availability is typically restricted to a period of 8 to 12 hours. Whereas the SCN are primarily entrained by LD cycles, TRF studies have shown that peripheral clock phase is predominantly responsive to feeding. Indeed, when TRF occurs during an organism's rest phase, gene expression profiles are inverted in many peripheral tissues, including the heart, kidney, liver, pancreas, adipose tissue, and the gastrointestinal tract (91-94). The time course of this feeding entrainment is organ-specific in mice, with the liver clock responding to feeding particularly rapidly. As a result, peripheral tissue rhythm phases can be uncoupled from SCN phase (91). It is increasingly clear that endocrine responses to feeding contribute to feeding entrainment of many tissues, with important contributions made by hormones such as insulin and oxyntomodulin (95, 96).

Perhaps the first study to show that diet timing influences phases of peripheral clocks in humans was published very recently. Ten healthy young men self-selected their sleep patterns for 10 days before continuing these in the laboratory (97). Participants ate three standardised meals each day for five days, with five hours between meals and LD cycles. In the first part of the study, participants began the first meal 30 minutes after waking. In the second part, participants began the first meal 5.5 hours after waking. A constant routine protocol was used after each pattern. Meal timing did not alter hunger, triglycerides, sleepiness, or blood clock gene expression profiles. As cortisol and melatonin rhythms were unchanged, it seems that the phase of the SCN was also unaffected. Interestingly, however, *PER2* mRNA rhythms in gluteal adipocytes were delayed by about an hour after the later meal pattern, indicating that meal timing may shift some peripheral clocks without markedly affecting the SCN. Furthermore, the later pattern delayed plasma glucose rhythms by ~ 5.7 hours relative to melatonin phase, shifting the acrophase (peak) to ~ 4.4 hours after dim-light melatonin onset. Although not significant after correction for multiple testing (p = 0.029), the mean insulin acrophase was ~ 3.4 hours later after the later eating pattern too. It is plausible that changes in peripheral clocks contributed to altered glucose rhythms, and it is noteworthy that glucose but not triglyceride profiles were shifted, suggesting that meal timing can somewhat uncouple rhythms in glucose and lipid metabolism. As melatonin acutely worsens glucose tolerance (98), it is perhaps contrary to expectations that mean glucose levels were lower in the later pattern, as were both peak and trough levels. Interestingly, there were no changes in sleep between the patterns, so differences between groups were not confounded by altered sleep (97). So, meal timing indeed alters the timing of

some human circadian clocks, but the health consequences of the resulting changes in phase relationships between clocks are unclear.

In some mammals, effects of feeding/fasting cycles on the circadian system are so great that they can even entrain behavioural cycles with the 24-hour day. TRF in animals such as rats produces food-seeking behaviour coordinated with when food procurement is most likely. This food anticipatory activity (FAA) is directed towards places where food is available and may therefore be an adaptive strategy to enhance foraging success (99), as supported by findings that FAA is accentuated during energy restriction. As FAA is entrainable and persists during several days of food deprivation, FAA appears to be a true circadian rhythm. Interestingly, FAA persists following SCN ablation (100) as well as after genetic disruption of the molecular clock (101). The location of the food-entrainable oscillators thought to underlie FAA remains obscure.

1.3.4 The suprachiasmatic nuclei coordinate the timing of biology and behaviour

As we are diurnal organisms, our circadian systems optimise our bodies for physical activity and energy harvesting during the day, and inactivity and energy mobilisation during the night. The SCN principally coordinate these daily cycles. In isolation, individual cells have divergent periods of clock gene expression (102), and if all cells had functional clocks with different periods then the net result would be systemic arrhythmia. The timing of cellular clocks must therefore be coordinated. Functional SCN are integral to coordinating peripheral clock timing, as disruption of the SCN dampens and desynchronises peripheral tissue circadian rhythms (103). The SCN synchronise circadian rhythms by autonomic, temperature, and humoral mechanisms. The ensuing biological changes alter behaviours (such as eating/fasting and activity/rest cycles) that then also influence the circadian system.

Regarding autonomic pathways, the SCN relay temporal information via efferents to other brain regions (104), and the paraventricular nucleus is particularly important in the regulation of circadian rhythms in activity, autonomic processes, and the endocrine system (105). Furthermore, multisynaptic efferents from the SCN to the periphery help regulate the availability of nutrients like glucose in the blood (106), as well as hormone secretion rhythms by organs including the adrenal glands, adipose tissues, pancreas, and thyroid gland (107-110). In turn, humoral factors from the periphery relay information back to the hypothalamus through its median eminence (111).

With respect to temperature, humans are homeotherms and are hence resistant to temperature entrainment by the environment. The SCN confer this resistance. Although

thermoregulation is enacted by the interaction of structures located primarily in the hypothalamus, brainstem and spinal cord, the SCN are important to the core body temperature rhythm, a rhythm helps synchronise oscillators throughout the body (112). Interestingly, deletion of *reverse-erythroblastosis α* in mice abolishes the core body temperature rhythm by changing brown adipose tissue activity, implicating a specific component of the molecular clock in thermoregulation (113).

Finally, the SCN produce their own secretions to synchronise peripheral clocks (114-117), which they also achieve by controlling circadian rhythms in the endocrine system. As is true of melatonin, cortisol is a particularly pivotal hormone in the circadian system. Some clock genes are directly regulated by glucocorticoids via glucocorticoid response elements, so cortisol helps coordinate activities in diffuse networks of molecular clocks (118). To exemplify this, glucocorticoid receptor activation restores ~ 60% of liver gene transcription rhythms after SCN ablation, demonstrating important synchronising roles of cortisol in some peripheral clocks (119). How much glucocorticoids influence peripheral clock rhythms differs between tissues: The kidneys and lungs are more responsive to glucocorticoids than the liver, for example, which is mostly entrained by feeding signals (120). Figure 1.1 overviews how the circadian system and sleep are regulated, and also how each is often disrupted.
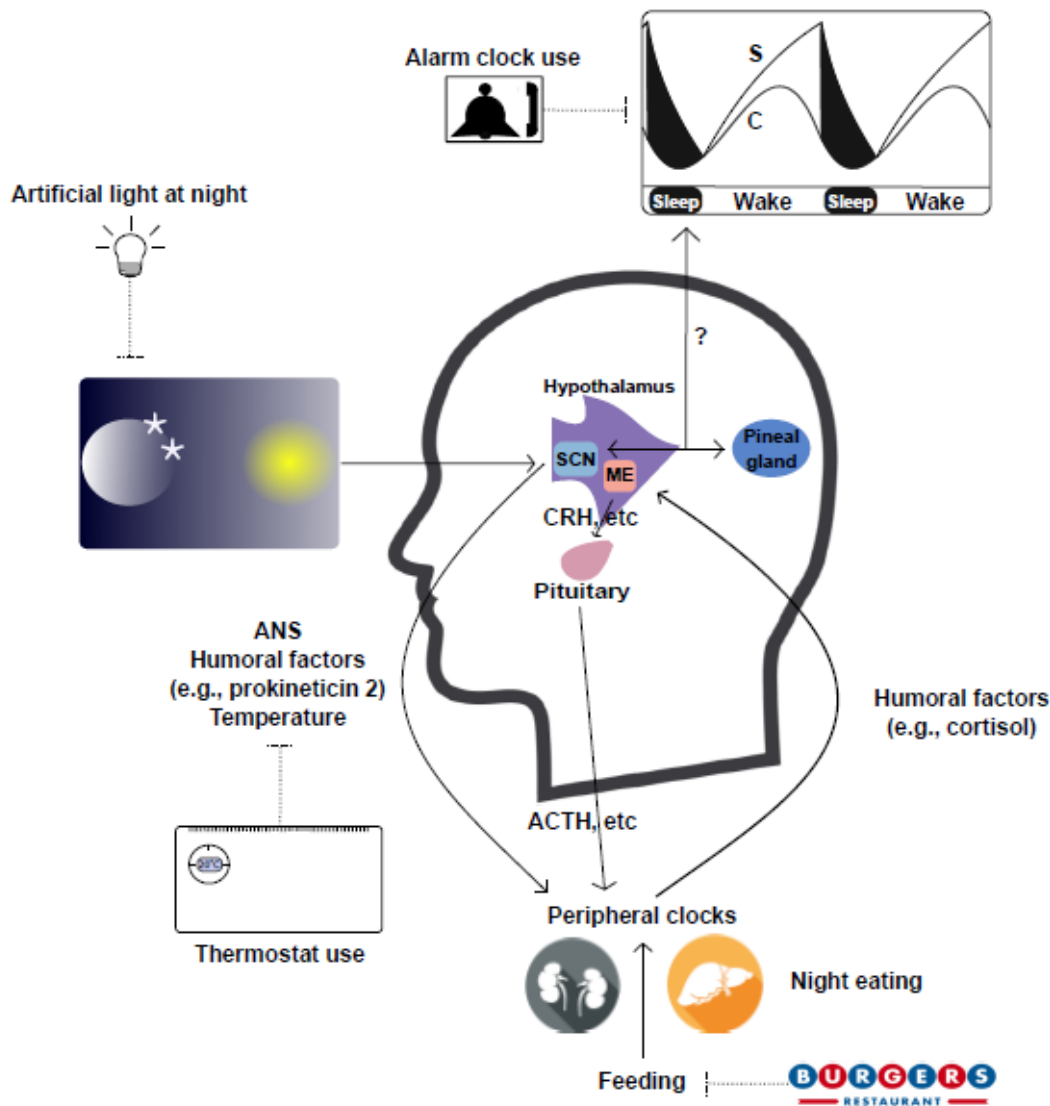
Figure 1.1 Temporal control of physiology.

Retinal light exposure is the primary zeitgeber for the central clock in the suprachiasmatic nuclei (SCN). Melatonin signals darkness, light suppresses melatonin synthesis by the pineal gland, and night-time light exposure can therefore disrupt SCN and melatonin rhythm timing. As a diurnal species, exogenous melatonin increases sleep propensity in humans. However, a prospective study of adults undergoing pinealectomy demonstrated that endogenous melatonin may not have a strong regulatory role in human sleep (121). The sleep/wake cycle can be effectively simulated by a two-process model in which a circadian process (C) influences wakefulness and interacts with a sleep-promoting process (S) that accumulates during wakefulness. Process C is highest late in the waking day to counter the soporific effects of process S at this time. A pronounced drop in process C around sleep onset no longer opposes process C, and so sleep begins. (Continued on next page.)

Within the hypothalamus the SCN influence the circadian rhythm of body temperature, a key synchroniser of the peripheral clocks that coordinate timing of local cellular processes outside the SCN. The use of thermostats can obviate daily oscillations in temperature, which may influence sleep propensity (18). The SCN also relay time of day information to peripheral clocks through neural pathways of the autonomic nervous system (ANS), as well as by their own secretion of signalling factors such as prokineticin 2. Hypothalamic-pituitary-peripheral organ axes are critical to endocrine regulation of the circadian system. For example, corticotropin-releasing hormone (CRH) enters the portal system through the median eminence (ME) of the hypothalamus and stimulates the secretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary gland. ACTH then increases adrenal cortex production of cortisol, a hormone with a high-amplitude circadian oscillation and synchronising effects in many peripheral clocks. The timing of metabolic processes in peripheral clocks is also influenced by nutritional status, and peripheral clocks convey metabolic signals back to the hypothalamus through the ME. Nowadays, around-the-clock access to food often distorts the clear eating/fasting cycles that probably typified much of human history, plausibly dampening peripheral clock oscillations.

1.3.5 Active phase biology and behaviour

The circadian system primes the human body for waking activities during daylight, beginning just before awakening. The cortisol awakening response increases alertness and blood pressure and mobilises energy reserves at the end of the sleep period in expectation of upcoming wakefulness. In this way the cortisol rhythm exemplifies anticipatory physiology. During the active phase, the circadian system modifies blood pressure, heart rate, and skeletal and heart muscle contractile efficiency and substrate oxidation to ready the body for physical activity by improving physical capacities (122-124). As both the organ that propels locomotion and as the body's largest glucose depot, skeletal muscle is a key determinant of substrate oxidation. Comprehensive lipidomics of human skeletal muscle has shown diurnal oscillations in levels of numerous lipids, and the timing of changing lipid levels coheres with the timing of clock gene expression in this tissue (125). The skeletal muscle clock therefore times fuel partitioning to meet the demands of physical activity. Mitochondria are also key determinants of substrate oxidation. Molecular clock-dependent daily oscillations in mitochondrial proteins include rate-limiting enzymes in carbohydrate and lipid metabolism, thereby also helping coordinate nutrient supply with demand according to time of day (126).

Over the course of evolution, physical activity has been necessary to procure food, and timely changes in the gastrointestinal system support efficient daytime digestion and energy storage. Gastric emptying, colonic motility and the speed of the migrating motor complex are all faster during the day (127-129). Early-phase postprandial insulin secretion is higher earlier in the biological day to promote efficient energy storage (130), and an acrophase in adiponectin in the middle of the active phase supports this change in insulin sensitivity (131). With respect to lipids, bile acid production is regulated by the circadian system to ensure timely cholesterol metabolism and absorption of nutrients including fat-soluble vitamins (132). Time of day changes in the activity of key intestinal nutrient transport proteins and enzymes is also important in optimising responses to feeding (133). Reduced postprandial lipoprotein lipase activity apparently contributes to impaired lipid tolerance in the evening, for example (134).

The composition of the gut microbiota and their bacterial fermentative end-products change with feeding and time of day in humans (135), although the significance of these dynamics is currently unclear. Microbiota rhythms in mice partly result from feeding but are also dependent on a functional circadian system, as genetic disruption of the molecular clock results in dysbiosis (136). One reason that the circadian system coordinates time-of-day appropriate changes in the gut microbiota may be to defend against various microorganisms present in foods and drinks. Immune system resilience differs according to circadian phase, and studies of mice have shown greater pathogen colonisation and lower survival when exposure to foodborne and airborne pathogens occurs at the beginning of the rest phase versus the active phase. Interestingly, many components of the inflammatory response differ in their acrophase timing, and this perhaps reduces the likelihood of damage incited by an inappropriately strong immune response (137). It is also noteworthy that immune responses are energetically costly, so circadian system regulation of immunity perhaps also enhances energy economy (138).

Although the extent to which some of these rhythmic changes are regulated by the circadian system is unclear, collectively these findings indicate that organisms are best prepared for physical activity and food consumption during the biological day.

1.3.6 Rest phase biology and behaviour

Of all hormones, melatonin has the most robust circadian rhythm and is therefore commonly used as a marker of circadian phase. Melatonin circulates a time of day signal throughout many body tissues by interacting with its receptors. In most (non-Polar) natural LD cycles, pineal gland melatonin synthesis increases substantially around dusk.

At this time melatonin may increase sleep propensity somewhat, perhaps in part via its hypothermic effects (139).

Nocturnal hunger would not be conducive to sleep in humans. Once sleep is underway, peak leptin secretion typically occurs shortly after midnight and may contribute to reduced appetite for most foodstuffs in the biological morning, allowing efficient sleep during declining energy availability (140). As energy availability falls during sleep, growth hormone stimulates lipolysis and ketogenesis in the liver, inducing insulin resistance and thereby sparing glucose and protein oxidation (141). Although a minor influence of the circadian system on growth hormone secretion profiles has sometimes been detected, clear pulses of growth hormone secretion are mostly attributable to sleep (142). Growth hormone has an acrophase near the onset of slow wave sleep, and growth hormone profiles are also characterised by episodic surges a few hours after meals (143, 144). Together, the interplay of nocturnal endocrine activity apparently prevents undue arousal from sleep and facilitates the retention of lean body mass overnight.

Our understanding of the circadian regulation of biology and behaviour has progressed remarkably in recent decades. A well-functioning circadian system confers many metabolic benefits, as shown by the adverse consequences instigated by disruption of the circadian system.

1.4 Circadian system disruption

1.4.1. Shift work

1.4.1.1 Field studies

Shift workers make up a large proportion of people regularly subject to circadian system disruption, and field studies have documented impaired metabolic health of night shift workers, even when diet is controlled. Night shift workers have higher plasma triglycerides (145), and as postprandial glucose and lipid tolerance to standard test meals are worsened on switching to night shifts, some metabolic impairments are perhaps direct consequences of shift work-associated circadian system misalignment (146).

An important question is whether it is possible for the circadian system to permanently adapt to night shift work. Isolated environments such as oil rigs can be more conducive to adaptation to shift work adaptation than more common shift working scenarios, and most workers can synchronise their circadian systems to night shifts within a week in

such circumstances (147). Even in isolated conditions, however, it can then take weeks for workers to re-entrain to day shifts (148-150).

Less isolated working environments are not so facilitative to entrainment to night shifts. Still, people who work night shifts exclusively might be expected to completely entrain their circadian systems to their work schedules. Yet < 3% of workers are reported to show complete entrainment in such circumstances (20). Indeed, abnormal circadian endocrine rhythms persist even among adults who have worked night shifts for over two years. Disrupted circadian rhythms in these workers include thyroid-stimulating hormone profiles, and the combination of reduced cortisol and increased prolactin during waking hours in such people may be detrimental to efficient work by impairing vigilance (151).

A recent review collated the findings of systematic reviews and meta-analyses of the health consequences of shift work and insufficient sleep. The analyses showed that night shift work is associated with weight gain as well as increased risk of breast, colorectal, and prostate cancers, as well as myocardial infarction, ischaemic stroke, and type two diabetes. The results generally indicated that shift schedules involving night shifts confer the largest increase in disease risk (22).

Interestingly, the magnitude of the increased risk of cardiometabolic diseases (not cancers) due to shift work was comparable to that of insufficient sleep alone, raising the question of whether associations between shift work and cardiometabolic diseases are simply the result of sleep disturbances (22). Indeed, a meta-analysis showed that self-reported sleep duration is shortest after night shifts (mean 5 hours 51 minutes) and longest after evening shifts (mean 8 hours 2 minutes) (152). Conversely, a narrative review concluded that mean sleep duration over the course of a shift cycle is very similar (14 minutes shorter) to permanent day workers (153).

It seems unlikely that insufficient sleep is the sole contributor to increased cardiometabolic disease risk in shift workers, and it would be facile to overlook the complexity of exposures that shift work entails. To mention but a few, shift workers may experience more psychosocial stress, be less physically active, consume more food at night, and be more likely to smoke (22). Laboratory experiments of simulated shift work are particularly suited to removing confounding exposures and thereby unveiling the relative importance of individual exposures.

1.4.1.2 Simulated shift work
Circadian misalignment protocols have provided important insights into the short-term health consequences likely to be experienced by shift workers. Within three days,

circadian misalignment was found to increase blood pressure (particularly during sleep) and inflammatory markers, reverse cortisol rhythms, and reduce heart rate variability and insulin sensitivity in healthy adults (154, 155). The magnitude of the effect on glucose metabolism was particularly remarkable: After just 72 hours of circadian misalignment nearly half of the previously healthy participants had pre-diabetic postprandial glucose responses. During circadian misalignment sleep efficiency and leptin levels were lower. Disturbed sleep may reduce circulating leptin, but the analysis showed a likely greater contribution of circadian misalignment (154). Furthermore, subsequent work from the same researchers showed that circadian misalignment that contributes more to increased postprandial glycaemia and insulin resistance than the behavioural cycle (130). Whereas these studies focused on circadian misalignment alone, shift workers often experience sleep loss too. It is therefore concerning that when compared to sleep restriction alone, the combination of circadian misalignment and sleep loss nearly doubles impairments in insulin sensitivity in men (156).

Although difficult to study under controlled conditions in humans, a key question is whether the effects of circadian system disruption are cumulative. Whereas acute sleep deprivation increases cortisol secretion, long term circadian misalignment reduces cortisol secretion (157). Similarly, while acute circadian misalignment increases insulin secretion (154), three weeks of combined sleep restriction and circadian misalignment decreases insulin secretion (158). These findings imply that chronic circadian system disruption results in dysfunction of organs such as the adrenals and pancreas. A related question is whether chronic circadian system disruption eventually produces adaptations that mitigate the adverse effects of subsequent disruptions. It appears that this is not the case, however. Healthy long-term shift workers still experience the deleterious effects of circadian system misalignment on postprandial glucose tolerance and insulin action (159).

It should be noted that some studies have documented metabolic effects of acute circadian system misalignment that differ from those reported in other studies. During six days of simulated night shift work in healthy adults, there was an initial increase in fat oxidation, and carbohydrate and protein oxidation declined. Energy expenditure fell on the second and third days, particularly during sleep. Paradoxically, appetite fell despite reduced levels of the anorexigenic hormones leptin and peptide tyrosine (160). In contrast to these findings, another study reported that neither three consecutive three-hour LD cycle advances nor three consecutive three-hour LD cycle delays influenced appetite or energy expenditure in adults, and both shifts increased carbohydrate oxidation and reduced protein oxidation. This study also documented metabolic effects specific to the LD cycle shifts: LD cycle advances acutely reduced cortisol rhythm

amplitudes and increased insulinaemia, whereas LD cycle delays increased glycaemia, and decreased glucagon-like peptide-1 concentration and sleeping energy expenditure (161). Discrepancies between findings of different studies likely reflect differences in experimental design and participants, emphasising the need to compare a variety of circadian misalignment protocols in multiple populations to better understand the nuances of different shift work schedules.

Together, these studies indicate possible mechanisms linking shift work to increased metabolic disease risk. Note that as both shift work and jetlag initially entail abrupt changes in several zeitgebers, many reported metabolic aberrations seen in these studies may also occur in people experiencing jetlag.

1.4.2 Electric lighting

Shift work was facilitated by the invention of electric lighting, and many of the health ramifications of exposure to this evolutionarily novel stimulus are now being illuminated. Compared to life without artificial lighting, we tend to experience less daylight exposure and more night-time light exposure (16, 17), each of which may have detrimental effects on the circadian system and sleep.

1.4.2.1 Shelter from daylight

People in industrialised societies reportedly spend ~ 88% of their time in enclosed buildings (162). Differences in light exposure between enclosed environments and unsheltered environments are stark. Whereas light intensity in many rooms is 400 lux or less, midday outdoor light intensities generally range from 10,000 lux to well over 100,000 lux on a sunny, cloudless day at around midday. While living their normal lives (with access to electric lighting) in North America, people are exposed to ~ 4 to 13 times less light during the day compared to exposure to only natural light when they go camping (16, 17). Consequently, many individuals are sheltered from the multitude of beneficial effects of natural daytime light on biology and behaviour (163).

Some of the beneficial effects of daytime natural light exposure may result from vitamin D synthesis. Vitamin D is synthesised in response to ultraviolet-B irradiation, and being indoors during daylight contributes to low vitamin D status. Positive associations between vitamin D status and sleep duration (164-166) and sleep efficiency (164, 166) may relate to direct influences of vitamin D on clock gene transcription in vitro (167). Alternatively, associations might simply reflect beneficial effects of greater daytime light exposure on sleep. It is clearly advantageous to be exposed to outdoor light during daylight, but at

times this is not feasible. In such circumstances, greater daytime indoor light exposure from windows is still preferable and has been associated with better sleep in office workers (168).

1.4.2.2 Light at night

Light at night is particularly problematic for two reasons. First, it directly affects circadian biology. Second, light at night enables other activities that can contribute to circadian system disruption, such as night-time eating and physical activity.

About 80% of the world's population is exposed to artificial light at night (169), and nocturnal light pollution continues to sprawl and intensify (170). As a result, it has been estimated that people in environments with access to artificial light commonly experience light intensity levels over twice as high between sunset and sleep compared to when they are only exposed to natural light while camping (16). The introduction of electric lighting appears to have significantly influenced sleep. Among individuals of the same sociocultural background, electric lighting has been associated with increased light exposure shortly after dusk, delayed sleep onset, and shortened sleep duration. These effects may be particularly prominent on workdays (171, 172). Similarly, adults living in areas with the greatest exposure to light at night report delayed sleep times, shorter sleep, poorer sleep quality, and increased daytime sleepiness (173). Ecological studies of light at night such as this are susceptible to confounding from co-exposures such as late-night food and drink consumption, do not necessarily reflect retinal light exposure for individuals, and often use data from satellites that better detect light wavelengths responsible for visual acuity rather than wavelengths most important to circadian system entrainment. Nevertheless, such studies are still informative.

Laboratory studies have clarified how electric lighting affects the circadian system. Timing of light exposure can influence circadian phase. In contrast to the swift circadian phase delay evident in response to light exposure late in the biological day, phase advancing (early morning) light exposure causes only some components of the circadian system to advance rapidly while other elements remain in transition (174). This has implications for shift work schedules and helps explain why most people find it easier to adapt to forward shift work rotations (days then afternoons then nights, for example) than backwards schedules.

Intensity of light exposure is another important determinant of how the circadian system responds to light. Light intensity at full moon is typically barely one lux and twilight is around 10 lux. But nowadays it is common to exceed these levels by at least an order of

magnitude. Night-time light exposure suppresses pineal gland melatonin synthesis, and this is the predominant mechanism by which artificial light disrupts the circadian system. Several parameters of light determine melatonin rhythm amplitude, period, and phase, including light exposure duration, intensity, spectrum, and timing (175). By how much night-time melatonin synthesis is suppressed is influenced by factors including prior light exposure (176).

The built environment is a relatively ubiquitous source of nocturnal light pollution, but many electronic devices now increase night-time light exposure too. Some of these devices emit monochromatic blue light (λ_{max} 460-480 nm), to which intrinsically photosensitive retinal ganglion cells are especially sensitive (177, 178). As a result, exposure to even low levels of light at night from e-Book devices reduces melatonin synthesis, delays sleep, and impairs next-morning alertness. As ~ 90% of Americans use electronic devices within an hour of bed time on multiple nights each week, these devices are likely contributing to circadian phase delays and sleep disruption (179). It should also be noted that use of electronic devices is generally higher in young people, and this is concerning as adolescents appear to be the group most sensitive to the circadian phase delaying effects of night-time light exposure (180).

As a result of widespread concerns about detrimental effects of artificial light at night on health, light at night was recently selected by the National Toxicology Program as the subject of a health hazard assessment regarding cancer and non-cancer (including metabolic) outcomes (175). The metabolic consequences of light at night in humans are not yet well understood and are a worthy topic for future research. Nevertheless, cross-sectional studies provide tentative support for adverse metabolic effects of light at night. There are positive associations between nocturnal light pollution and obesity prevalence in > 80 countries (181), as well as associations between late light exposure above 500 lux and BMI in free-living adults (182). Given the aforementioned discussion of the sleep-disrupting effects of light exposure, it seems likely that increased light exposure at night is obesogenic, especially in the light of the multitude of factors that conspire to increase energy balance after sleep disruption.

1.5 Sleep disruption

1.5.1 Metabolic consequences

1.5.1.1 Disrupted glucose and lipid metabolism

Sleep restriction is widespread and its metabolic consequences are many. One of the most consistently reported effects of sleep restriction is disrupted glucose metabolism (183). This was first shown in 1999 in a study in which five nights of sleep restriction to four hours per night caused many otherwise healthy adults to temporarily become pre-diabetic. Altered 24-hour endocrine rhythms including increased nocturnal cortisol secretion were evident, perhaps contributing to impaired insulin action (184). Recent studies have added to this preliminary research by better simulating how much sleep restriction people often experience.

Many individuals use alarms to curtail sleep before work days, and simulating this behaviour by enforcing early waking during five nights of five hours of sleep reduced intravenous and oral insulin sensitivity by ~ 20% in healthy adults (185). Importantly, intravenous insulin sensitivity was not restored by three days of recovery sleep - longer than most working adults have to catch up on sleep each weekend. A limitation of most experimental sleep restriction studies is that they enforce unrealistic changes in sleep duration, but restriction by 90 minutes nightly - an amount similar to that experienced by many (26) - has also been shown to reduce insulin sensitivity after a week in young men (186). In this otherwise healthy population, impaired insulin sensitivity subsided with continued exposure to sleep restriction, however, implying that certain metabolic processes in some people may at least temporarily adapt to sleep restriction.

Much progress has been made in understanding the mechanisms by which sleep disruption predisposes to obesity, hyperglycaemia, and insulin resistance (Figure 1.2). One mechanism involves brain energetics. After eating, the brain accounts for roughly half of whole body glucose disposal, and sleep deprivation reduces this disposal (187). Reduced glucose disposal contributes to increased plasma glucose. This hyperglycaemia also reflects systemic insulin resistance following sleep restriction, which appears to result primarily from insulin resistance outside the liver (188). Changes in adipose tissue insulin signalling may contribute strongly to this insulin resistance (189). In accordance with the glucose fatty-acid cycle (190), increased release of non-esterified fatty acids from adipocytes after sleep restriction perhaps also contributes to insulin resistance (191), as does gluconeogenesis stimulation by heightened sympathetic activity of the autonomic nervous system (192).
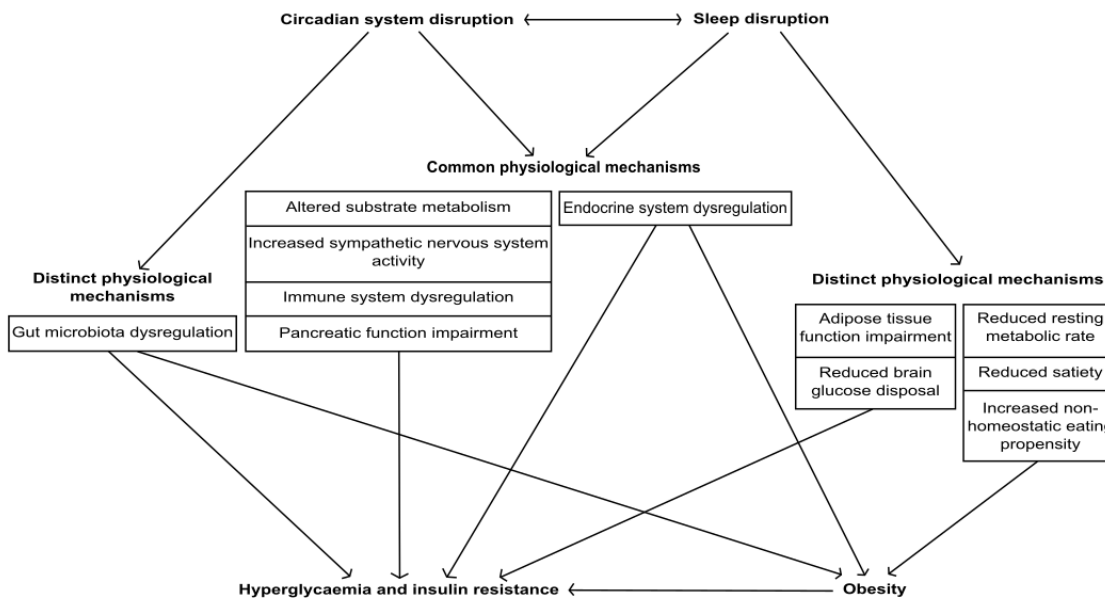
Figure 1.2. Mechanisms linking circadian system and sleep disruption to hyperglycaemia, insulin resistance, and obesity.

With further research, mechanisms that are currently listed as distinct may prove to be common.

As the molecular clock has roles in glucose metabolism, metabolic dysregulation after sleep disruption may also be related to epigenetic and transcriptional changes in the molecular clock in peripheral tissues important to glucose disposal, such as adipose tissue and skeletal muscle. Indeed, there is increased DNA methylation of the promoter region of *CRY1* and 2 regions near *PER1* in adipocytes, as well as reduced *BMAL1* and *CRY1* transcription in myocytes after sleep deprivation (193).

Another factor contributing to reduced insulin sensitivity after sleep disruption may be a shift in cytokine balance towards a more inflammatory state (194). Sleep curtailment affects numerous aspects of immune function. One week of sleep restriction in men increased circulating white blood cells and changed their daily rhythm. Notably, altered cell counts had not returned to baseline after nine days of recovery sleep (195). Such changes in immune function could conceivably influence the development of metabolic diseases associated with immune system changes, such as type two diabetes (196).

It is also known that different sleep stages have unique roles in metabolism. Restricting sleep to the first half of the night produces different endocrine effects to restriction to the second half (197), and each sleep stage is associated with distinct physiological changes. Changes in sleep architecture during sleep disruption may contribute to altered glucose metabolism. For example, independent of sleep duration, selective slow wave

sleep restriction dose-dependently reduces insulin sensitivity in adults, although adolescents may be more resistant to this effect (198, 199).

A detailed review of sleep disorders such as obstructive sleep apnoea is beyond the scope of this chapter. However, as studies have consistently shown that obstructive sleep apnoea is associated with impaired insulin and glucose metabolism, I will briefly outline its features. Individuals with obstructive sleep apnoea experience episodic upper airway closure and hence intermittent hypoxia during sleep. Obstructive sleep apnoea also entails reduced sleep duration, sleep fragmentation, reduced slow wave sleep, and increased oxidative stress and sympathetic nervous system activity, all of which summate to induce insulin resistance (183).

Obese individuals are at increased risk of obstructive sleep apnoea, and obstructive sleep apnoea prevalence has risen in recent years in some countries (200). However, although obesity increases the risk of obstructive sleep apnoea and is itself associated with insulin resistance, insulin resistance in obstructive sleep apnoea can occur independently of adiposity (201). Moreover, obstructive sleep apnoea is highly prevalent in people with type two diabetes (202), and a meta-analysis found that obstructive sleep apnoea is a strong risk factor for type two diabetes development (203). Pregnancy also predisposes women to obstructive sleep apnoea (204), which may thereby contribute to gestational diabetes (205).

1.5.1.2 Energy balance

Obesity is an important risk factor for type two diabetes. Therefore, if sleep disruption has obesogenic effects then these could compound direct effects of sleep disruption on glucose and insulin metabolism. A meta-analysis of sleep restriction studies found that sleep restriction increases energy intake, which may increase fat mass over time (206). One reason that people eat more after sleep loss is the extended period in which food can be consumed to compensate for the additional energetic cost of wakefulness. However, sleep restriction also increases the appeal and consumption of desserts among adolescents (207). As sleep deprivation has even been shown to increase the energy content of food purchased per unit of money in a mock supermarket (208), it seems that people not only eat more but actively seek energy-dense, rewarding foods.

Given the effects of sleep loss on appetite, it might be expected that sleep restriction influences satiety hormones. The best studied of these are the satiety hormones ghrelin and leptin. During ad libitum food availability, however, reported effects of sleep restriction on ghrelin and leptin have been contradictory (184, 209, 210). It is important

to understand that a multitude of hormones influence feeding behaviour, however, and sleep restriction has recently been found to increase plasma concentrations of the orexigenic endocannabinoids 2-arachidonoylglycerol and 2-oleoylglycerol (211).

Changes in weight fundamentally result from changes in energy balance, and some studies have found that the energy expenditure component of energy balance may be affected by sleep restriction. A working week simulation study found that sleep restriction reduced resting metabolic rate. This was particularly true of African Americans, a population that appears to be highly prone to the obesogenic effects of sleep restriction. Interestingly, this effect was seen *despite participants being in positive energy balances*, which might be expected to instead have raised participants' resting metabolic rates (212).

A recent meta-analysis did not find that sleep restriction influenced energy expenditure, however. It did corroborate previous findings that sleep restriction increases energy intake, and the net effect of these changes is, of course, a positive energy balance (213). Prospective field studies have also documented the importance of sleep in weight regulation, as longer sleep was recently found to predict greater reductions in BMI in overweight and obese adults consuming hypoenergetic diets (214). Furthermore, considering energy balance and weight alone may be misleading. Specifically, sleep restriction may affect body composition when diet is controlled for, as sleep restriction during hypoenergetic diet consumption accelerates lean body mass loss and impedes reductions in adiposity (215).

Interestingly, people may have trait-like responses to sleep disruption, as within-participant effects of one night of sleep restriction on energy intake and weight changes were stable when repeated exposures were separated by long periods, particularly among men. As there was large variation between individuals in changes in weight (- 2.3 to + 6.5 kg) and energy intakes (− 501 to + 1178 calories) after one night of sleep restriction, there is a need to identify biomarkers that highlight those vulnerable to obesogenic effects of sleep disruption (216).

Finally, it remains unclear whether men and women differ in their energy balance responses to sleep disruption. A relatively large study of five nights of sleep restriction to four hours of time in bed reported that men are predisposed to positive energy balances after sleep restriction (217), but a smaller, crossover study of five nights of sleep restriction to five hours of time in bed in a more homogeneous group of younger adults reported that women are more susceptible (218). Sex certainly influences some responses to sleep restriction. There are menstrual cycle phase-dependent endocrine responses to sleep restriction (219), indicating the need to carefully consider cycle phase

in future studies. Clearly additional research is required to clarify sex differences in effects of sleep loss, however.

1.5.2 Effects on diet quality

It has been estimated that US adults make ~ 230 food-related decisions daily (220), and if sleep disruption predisposes people to making less nutritious food choices then deleterious metabolic effects of sleep disruption could be compounded. Sleep restriction has sometimes been found to influence dietary macronutrient proportions, but conflicting evidence exists (217). Macronutrient intakes depend on available foods, and snack options are limited in experimental settings. Instead of altering macronutrient preferences, recent brain imaging studies support the hypothesis that sleep disruption increases non-homeostatic eating propensity.

Regional changes in brain activity suggest that sleep restriction increases sensitivity to rewarding properties of food (221). Brain activity changes after sleep deprivation are commensurate with increased appetite (222). In response to images of food perceived as 'unhealthy', sleep restriction strongly influences insula activation, a region involved in pleasure-seeking, even following a day in which sleep-restricted participants consumed more food than control participants (223). Sleep deprivation has also been shown to reduce activity in appetitive evaluation regions in the frontal and insular cortices as participants rated the desirability of foods. Activity in the amygdala was increased, however, and the combination of these brain activity changes was associated with cravings for energy-dense foods (224).

Collectively, these preliminary experiments demonstrate that sleep loss induces changes in brain circuits that predispose people to selection of energy-dense, rewarding foods, compounding the adverse effects of sleep loss on metabolic regulation that are independent of diet. Recent cross-sectional studies have highlighted that sleep timing is a key determinant of people's susceptibility to circadian system and sleep loss. It is therefore no surprise that sleep timing has also been associated with both metabolic health and dietary choices.

1.6 Sleep timing, diet, and metabolism

1.6.1 Chronotype

It has long been known that there are marked differences between organisms of a given species in chronotype. Chronotype is arguably best defined as an organism's phase angle of entrainment (for example, the timing of core body temperature nadir relative to dawn) and is related to an individual's circadian period such that a shorter period typically results in an earlier chronotype (225). The overt manifestation of chronotype is preferences in timing of activity and sleep (whether somebody is more of a 'morning lark' or 'night owl').

It is not always possible to objectively measure markers of circadian phase, so various methods to assess chronotype in cross-sectional studies have been proposed. An early method was Horne and Östberg's Morningness-Eveningness Questionnaire (MEQ) (226). Mostly comprising questions on when people subjectively feel best, the MEQ is still popular. Nevertheless, the MEQ is clearly limited by its lack of quantitative estimates of actual sleep timing. Researchers have since developed questionnaires to address limitations of the MEQ. Perhaps the most popular of the new alternatives is the Munich ChronoType Questionnaire (MCTQ) (227). After correction for sleep debt accumulated on work days, the MCTQ uses mid-sleep time on non-work days as an approximation of chronotype. The MCTQ has been validated against actimetry and markers of circadian phase (228), although it should be noted that the MCTQ may not produce significantly more accurate estimates of circadian phase than the MEQ (229). To avoid confusion in this section, I will refer to chronotype in discussing studies that have estimated sleep timing and morningness/eveningness to discuss other studies.

There exists such a range in chronotypes that some people in a given time-zone awake as others go to bed. The evolutionary basis of variation in chronotype is consistent with the sentinel hypothesis (230). This posits that in environments with inevitable dangers (such as predation), it is only safe to sleep if there are sentinels that remain vigilant to threats. A recent study of 33 adult Hadza hunter-gatherers supports this hypothesis. Sleep was monitored using actimetry for 20 days. Of all night-time actimetry epochs during this period, all participants were scored as being asleep only 0.002% of the time (less than one minute a night), and this was related to variation in chronotype. Interestingly, however, only age was associated with chronotype in this group: sex, nursing status, and co-sleeping did not predict chronotype. The authors therefore proposed that variability in age in bands of people in traditional societies leads to a range of chronotypes, resulting in adaptive sentinel-like behaviour (231).

Although sleep timing varies across one's lifespan, a person's sleep timing relative to others of the same age appears to be quite stable, and twin studies have shown that

morningness/eveningness is quite heritable (232). A striking example of this is a rare genetic mutation that results in familial advanced sleep phase syndrome (233). Recent GWA studies have sought specific gene variants involved in sleep timing, finding that genetic loci near established components of the molecular clock (*PER2*, *PER3*, and *FBXL3*) are associated with morningness/eveningness (234, 235).

Genetic variation influences sleep timing, but it is also clear that lifestyle is an important influence on chronotype, as shown by findings that the chronotypes of people in German cities are later and less closely aligned with the LD cycle than chronotypes of people in smaller settlements (236). Experiments in which young adults with a range of chronotypes went camping showed that exposure to only natural light for a week during both the winter and the summer advanced sleep times and dramatically reduced differences in sleep timing and melatonin phase between participants, such that the adults' circadian systems were more tightly synchronised with the LD cycle (16, 17).

With respect to public health, arguably the most important findings to emerge from chronotype research is that late chronotype/eveningness has been repeatedly associated with less healthy behaviours and a greater risk of some diseases. It is therefore particularly worrisome that chronotype in the general population has progressively delayed in recent years, perhaps because of less time spent outdoors. This may explain findings that ongoing declines in sleep duration worldwide appear to be from sleep loss on work days only (26), although changes in work schedules may have contributed to this in some instances.

Later chronotype is associated with unhealthy behaviours such as smoking (27). Consumption of more alcohol and processed foods (237-239) may result from less dietary restraint in evening types (240). Late chronotype is also associated with a range of metabolic health impairments, such as diabetes and sarcopaenia in men, metabolic syndrome in women (241), and development of gestational diabetes during pregnancy (242). As might be expected, it appears that evening types are more likely to skip breakfast and consume less food in the morning and more in the evening (243-245). Consuming more food in the evening may be associated with higher BMI, independent of chronotype (246), and higher energy intake at dinner may somewhat contribute to worse glycaemic control among late chronotypes with type two diabetes (247).

As shift workers make up nearly a fifth of the workforce in Europe (25), an important question is whether chronotype modifies susceptibility to the deleterious health effects of shift work. In research on 65,000 women, morning types working day shifts had 19% lower odds of developing type two diabetes over a 20 year period than intermediate types, whereas evening types had 51% increased odds. Interestingly, however, risk of

type two diabetes was not different among evening types who also worked night shifts. As this study adjusted for a range of confounders including diet and physical activity, it appears that work schedule is an independent determinant of associations between morningness/eveningness and metabolic disease risk (248). This work supports prior research on other non-communicable diseases, such as a nested case-control study that showed that breast cancer risk is far higher among morning types working night shifts (249).

Promisingly, personalising shift schedules may improve shift work tolerance. By removing the most taxing shifts (night shifts for early chronotypes, morning shifts for late chronotypes), self-reported sleep duration, sleep quality, and wellbeing improved, and sleep timing variability also decreased during the five-month intervention (250). The study sample size was small, however, and the intervention also changed other variables that could have influenced the results, such as shift rotation speed. It remains to be seen whether customising work schedules can abate the deleterious effects of shift work on metabolic health.

Why late chronotype is associated with poorer health is not yet clear, but multiple factors probably contribute. Whereas unhealthy behaviours such as excessive stimulant use (251) little time spent outdoors (227) and high screen-time (179, 252) may delay the phase of the circadian system, an important factor could be differences in sleep between chronotypes. Some researchers have found worse sleep quality and more insomnia symptoms in late chronotypes (241), as well as more prevalent sleep apnoea in evening types (244). During Daylight Savings Time (the one-hour advance in clock time from late March until late October), late chronotypes may also experience curtailed sleep (253). For shift workers, associations between chronotype and sleep are dependent on work schedules. Whereas early chronotypes sleep less, have more variable sleep timing, and have more disturbed sleep during night shifts, late chronotypes experience similar problems while working early shifts (254).

Chronotype is interconnected with social jetlag, and late chronotypes generally experience more social jetlag. Whereas chronotype is a proxy of phase angle of entrainment, social jetlag is thought to reflect circadian system misalignment. It is therefore possible that circadian system misalignment is another contributor to associations between chronotype and metabolic health.

1.6.2 Social Jetlag

Around 80% of people with regular work schedules use alarms on work days (26), and late chronotypes may be more likely to use alarms. It was recently reported that whereas 89% of people begin work before or at 09:00 and 83% awake on these days at 07:00 or earlier, 77% of people would naturally sleep until 08:00 or later (255). Indeed, late chronotypes typically fall asleep about two hours later than early chronotypes before work days but only wake up half an hour later (227). One result of this is sleep debt, which late chronotypes then try to pay off on non-work days. It seems that how well people accomplish this may be location-dependent. On work days, young adults in Singapore have later bed times but similar wake times to young adults in the UK, resulting in shorter sleep. On non-work days, however, their sleep duration is similar, and people in Singapore may therefore experience more sleep debt (256).

Later chronotypes may not only have larger sleep debts to rectify on non-work days, they may also have more discrepant sleep timing as sleep shifts later these days, partly to pay off the sleep debt (227). These people are therefore in a situation analogous to flying at least one time-zone further east as they shift their sleep timing an hour or more earlier on work days, and it is as if about a third of people fly across multiple time-zones (26). Unlike jetlag, however, social jetlag is chronic, and it is plausible that adverse effects of repeated minor circadian misalignment and sleep disturbances resulting from social jetlag could summate to substantial health consequences. This is consistent with previously discussed findings that shift workers do not seem to develop tolerance to repeated circadian misalignment (159).

Like chronotype, social jetlag has been associated with many ailments. In an analysis of > 65,000 people, social jetlag was positively associated with BMI among overweight and obese people, with an effect size more than half that of sleep duration (26). Social jetlag has been associated with other metabolic abnormalities, including dysglycaemia, dyslipidaemia, excessive inflammation, hypercortisolaemia, insulin resistance, and metabolic syndrome (28, 257). Social jetlag may also be associated with diminished amplitudes of some circadian rhythms, such as body temperature (258). As is true of evening types (244, 259), higher social jetlag is associated with lower physical activity and higher resting heart rate (260). And because late chronotypes tend to have more variable sleep times, they may also have more variable meal timing. Although few researchers have tested this idea, there is evidence that adolescents with late chronotypes shift their breakfast later on weekends (261).

It is plausible that associations between social jetlag and metabolic problems reflect reverse causality: poor health might influence occupation, which may in turn affect social

jetlag. However, as social jetlag has been associated with various health impairments after adjustment for many demographic variables, it seems more likely that circadian system and sleep loss are at fault. Although the preponderance of evidence associates late chronotype and social jetlag with worse metabolic health, associations with some outcomes have not always been consistent. This likely reflects many factors, including differences in participant demographics, methods used to assess chronotype, choices of statistical adjustments used, and statistical power.

As late chronotype and social jetlag have been associated with many deleterious health consequences, there is a need for studies exploring the effects of interventions to advance chronotype and sleep timing variability. A recent experiment compared the effects of spending the weekend in typical electric lighting environments to camping in Colorado for a weekend in July. Whereas melatonin onset delayed in the artificial lighting environment, melatonin onset advanced while camping. Weekend camping also prevented shifts in sleep timing (17). These findings suggest that large changes in the LD cycle can rapidly advance circadian phase and offset social jetlag.

A limitation of previous chronotype and social jetlag studies is that objective measures of sleep such as actimetry have rarely been used. Prior studies have not assessed diet with validated tools that also document dietary timing. Furthermore, there has been little research on the importance of diet timing relative to circadian phase, despite evidence from preliminary chrononutrition research showing that diet timing is an important determinant of postprandial metabolic responses.

1.7 Chrononutrition

Studies of chrononutrition explore the reciprocal relationship between the circadian system and nutritional status. Chrononutrition research has focused particularly on how both diet composition and diet timing influence the circadian system. For the purpose of this thesis I therefore use the term 'dietary patterns' to include both diet composition and timing, but I acknowledge that 'dietary patterns' has mostly been used in reference to the interaction of bioactive compounds and nutrients within the whole diet, and that various diet quality indices have been developed to assess how adherence to a given dietary pattern influences health (262). Much nutrition research has explored how diet composition influences health, and changes in diet quality are strongly related to mortality (263). Only recently have people begun studying roles of diet timing in health, however.

There are benefits and drawbacks to different ways of studying meal patterns (264). Components of meal patterns can include context (environment), format (composition, item combinations, item sequencing), and patterning (consistency, frequency, spacing, timing). In this thesis I focus on what seems most germane to studying chrononutrition: a 'time-of-day', patterning approach. A traditional time-of-day approach categorises events into arbitrary time bins (breakfast, for example, might be the event with the most calories between 06:00 and 10:00), but defining meals and snacks in this way is based on cultural preconceptions and may misclassify people with unusual sleep/wake patterns. I therefore do not define events into meals or snacks but am instead interested in the timing of caloric events, particularly in relation to the sleep/wake cycle. However, I recognise that a limitation of excluding non-caloric items is that some non-nutritive compounds such as caffeine (251) may influence the circadian system and health. Figure 1.3 outlines different time structures particularly relevant to chrononutrition.
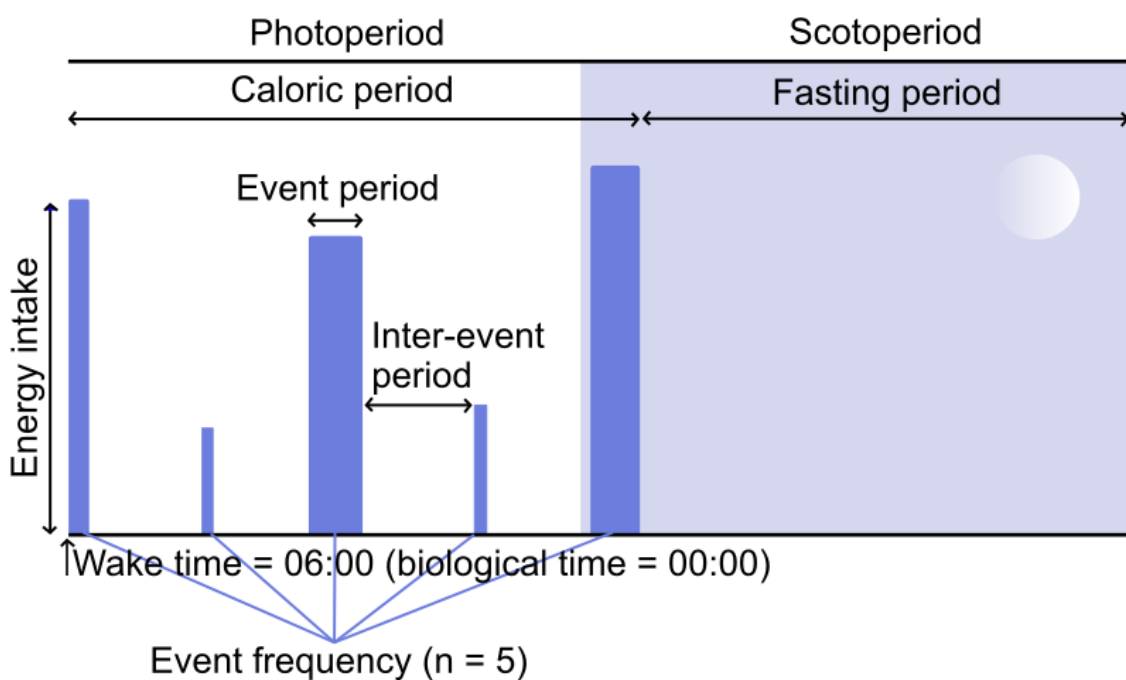
Figure 1.3. Diet timing variables.

This figure details an example dietary pattern in which the first caloric event (vertical bar) is on waking at 06:00 (the start of the biological day). On this day, the individual has five caloric events, the last of which begins shortly after dusk. The time elapsed from the beginning to the end of an individual event is the event period, the time between events is the inter-event period, and the time from the beginning of the first caloric event to the end of the last caloric event is the caloric period. The time from the end of the last caloric event until the first caloric event of the next day is the overnight fasting period. Most

studies to date have only considered dietary event timing relative to clock time, but it is noteworthy that each of these time structures can also be considered relative biological time.

Chrononutrition studies to date have mostly addressed how to modify diet timing to encourage consistent, high-amplitude circadian rhythms in metabolic processes. To this end, many researchers have focused on the duration of the caloric period. In discussing studies of humans in which it is unclear if consumption of all calorie-containing items (including drinks) was documented, I will refer to eating period, however.

1.7.1 Caloric period duration

1.7.1.1 Cross-sectional studies of humans

Cross-sectional research methods are arguably the best way of understanding human eating patterns in daily life. Epidemiological findings can be used to identify population trends and hence groups of people that may benefit most from dietary interventions. Such studies often also highlight valuable research questions to pursue in more controlled settings. The limitation of cross-sectional studies is that causal relationships cannot be inferred, and the use of a range of assessment tools and analytic methods in different populations often results in conflicting results between nutritional epidemiology studies. Cross-sectional studies have therefore produced insights into the timing of dietary patterns in many locations worldwide, but there have sometimes been contradictory findings regarding how eating period relates to metabolic health.

Most observational research of eating period duration has focused on associations with bodyweight. In a longitudinal study of > 50,000 ≥ 30-year-old adult members of Seventh-day Adventists churches in the US and Canada, eating period and number of meals consumed were associated with changes in BMI relative to their peers such that people with eating periods of less than seven hours lost weight and people with eating periods of > 12 hours gained weight over about seven years. Associations between eating patterns and BMI were similar when participants were stratified by age (265). The study had clear limitations, however, as diet was assessed only at baseline using food frequency questionnaires (FFQs), participants were not alcohol drinkers or smokers and so perhaps aren't representative of less healthy people, and results may have been confounded by response bias (~ 55% of the baseline cohort responded to the eight-year follow-up forms).

Seventh-day Adventists are in many ways unusual in their eating patterns. Whereas participants in the Seventh-day Adventist study only consumed four meals/snacks on average and had a mean eating period of only ~ 10 hours, studies of other US adults have found very different patterns. North American adults were found to eat in erratic patterns, with a median caloric period of nearly 15 hours, for example (266). Interestingly, these adults also consumed breakfast on weekend days over an hour later than weekdays, a phenomenon the researchers named 'metabolic jetlag'. Caloric period was not correlated with BMI in these adults (27).

This work was followed by a pilot study of eight obese adults from the sample who had habitual eating periods > 14 hours. Participants lost > 3 kg during a 16-week period in which they were told to restrict their consumption of everything but water to an 11-hour period each day, and participants reduced their energy intakes ~ 20% during the intervention. All participants expressed desire to continue beyond 16 weeks, so the researchers followed them up a year later, at which time participants had generally sustained their weight loss (266). The pilot study was clearly limited by its sample size as well as the lack of objective measures of sleep and body composition. Furthermore, the participants probably expected the restricted caloric period to benefit them, and they were not crossed over to a non-restricted condition.

Similar smartphone diet recording methods have been used to assess temporal profiles of adults' diets in India. Indian adults have similar caloric periods to the American adults and also eat in an erratic way. In contrast to the American adults, metabolic jetlag was largely absent among Indian participants. Similar to American adults, however, eating period was unrelated to BMI (267). A shortcoming of both studies was the use of a diet recording method that has not been validated, and this resulted in substantial under-reporting in Indian men. A more recent study that examined whether caloric period is related to BMI in free-living adults in America also did not find any associations (268), as was true of a large study of middle-aged adults from Belgium, the Czech Republic, France, the Netherlands, and Norway (269).

Breakfast-skipping is a way to shorten eating period and might therefore be expected to be associated with lower BMI if eating period is positively associated with bodyweight. But in the previously mentioned study of Seventh-day Adventist church members, breakfast eaters *lost* weight relative to breakfast-skippers, supporting findings of many prior cross-sectional studies (265). Indeed, breakfast-skipping has generally been associated with worse health, perhaps reflecting poorer dietary quality and lower micronutrient intakes (264).

Although studies of eating frequency have not always documented eating periods, daily eating period is generally related to the frequency of dietary events. Many cross-sectional studies have reported an inverse association between eating frequency and bodyweight. It appears, however, that this may reflect concurrent under-reporting of eating frequency and energy intake. Associations are often no longer evident when under-reporting is accounted for (270).

Finally, few cross-sectional studies have assessed associations between eating period and metabolic outcomes other than bodyweight. Some preliminary findings have associated shorter eating periods with superior glucose metabolism. In a large-scale study of US women, shorter self-reported eating periods were associated with lower postprandial glucose responses and lower odds of having elevated glycated haemoglobin (271). Additional studies are needed that explore relationships between caloric period and other metabolic health outcomes.

Inconsistencies between these cross-sectional studies highlights the fact that selecting appropriate dietary assessment methods to study eating behaviours in large numbers of people outside the laboratory is difficult. To accurately estimate nutrient intakes, the diet analysis method should have comprehensive and current food lists that contain the items commonly consumed by the participants, a criterion not met by some studies (265, 267). Furthermore, the validity of the method should have been proven against biomarkers of nutrient intakes, a criterion not met by several studies (265-267). Of particular interest to the study of temporal aspects of eating and drinking, the method should also document the time of day of all food and drink consumption. And to attempt to record every food and drink consumed, the method must also prompt participants regarding whether they forgot to record commonly forgotten foods.

Of available methods, interviewer-administered 24-hour dietary recalls as used by Park and colleagues (269) gather particularly accurate data but are time- and resource-expensive, requiring trained personnel to collect and code the data. More convenient methods such as FFQs have limited selections of foods included in their lists. Current usage of digital devices is unprecedented, so some researchers have begun using online recall methods to make data collection more efficient. Few such methods have been validated, however. For these reasons, validated, online dietary recall methods that document the time of food and drink consumption and have comprehensive food databases tailored to the relevant population may provide valuable, accurate insights into how diet timing and composition influence health.

1.7.1.2 Manipulation of caloric period in rodents

Associations between eating period and metabolic outcomes reported in cross-sectional studies of humans have been inconsistent. I have generally refrained from reviewing studies of non-human animals, but TRF experiments using rodents have comprehensively detailed how manipulation of feeding timing alters metabolism. I will now focus first on studies of rodents because 1) findings of these studies are consistent and encouraging, and 2) there have been few studies of humans.

Rodents are widely studied in pre-clinical research, and such studies have provided many insights into metabolic diseases like obesity. Perhaps the most common way of rapidly inducing obesity in mice is giving them ad libitum access to 'high-fat' diets (HFDs). HFD is really a misnomer since HFD chow is typically high in sugar too. Nevertheless, researchers soon realised that whereas mice normally consume 70 to 80% of daily energy intake during the scotoperiod (their active phase), feeding mice HFDs caused the animals to change their behavioural rhythms such that mice now consumed about twice as high a proportion of their energy intakes during the photoperiod (their rest phase). Researchers subsequently found that these flattened feeding/fasting rhythms were associated with altered 24-hour profiles of clock gene transcription (272, 273). Whether obesity precedes dampened circadian rhythms or vice-versa has since been contentious, but recent evidence indicates that HFDs induce rapid reorganisation of activity of the molecular clock and its targets before overt increases in adiposity in mice (274).

To offset the blunted feeding rhythms of mice with ad libitum HFD access, researchers have studied what happens when access to HFDs is restricted to the active phase. The results have included superior metabolic health - including reduced adiposity - despite similar energy intakes (273). Even without marked differences in energy intakes and locomotor activity, several TRF schedules have been found to be beneficial during various nutritional 'challenges', such as HFDs and high-fructose diets, and favourable metabolic effects of TRF are reportedly proportional to fasting duration in rodents (275). A recent review of the effects of TRF in rodents concluded that despite significant heterogeneity between studies in fasting duration, fasting timing relative to the LD cycle, and chow composition, TRF has consistently been shown to benefit bodyweight, blood lipids, glucose metabolism, insulin action, and inflammation (276).

The mechanisms underlying the beneficial effects of TRF are many. TRF mitigates the effects of HFD consumption such that nutrient sensor profiles (including AMPK and mechanistic TOR) are more similar to mice fed normal chow (273). TRF also counters reductions in cyclical changes in the gut microbiota that occur during HFD feeding, and

stool metabolite analyses have shown that TRF results in lower sugar uptake, perhaps contributing to protective effects of TRF against obesity (277).

Interestingly, many of the nutrient-sensing pathways by which energy restriction improves health and increases lifespan in model organisms are also affected by TRF (278). Such energy restriction studies may have been confounded by the fact that some animals eat all of their food as soon as it becomes available, raising the possibility that time restriction may also contribute to the beneficial metabolic effects of energy restriction (279). It is therefore essential that careful measures are made of the timing and duration of food consumption in such studies, and a recent study used a new feeding system to provide higher resolution timing measures of chow consumption in mice fed in different schedules (280).

After a week of ad libitum chow access, mice were fed in one of the following ways: 1) ad libitum for 24 hours each day, 2) 30% energy restriction, with 24-hour chow access beginning either at the start of the scotoperiod or the start of the photoperiod, 3) 12 hours of TRF during the scotoperiod, or 4) 12 hours of TRF during the photoperiod. Interestingly, both TRF groups consumed all of their food within 10 hours. Furthermore, the energy-restricted mice compressed their feeding period such that they consumed all chow within two hours of it becoming available. The mice had free access to running wheels, and the mice in these groups temporarily advanced their locomotor activity into the photoperiod in both groups, the period when these animals would normally be sleeping (280). This concurs with previous reports that a negative energy balance can shift previously nocturnal mice into a diurnal temporal niche (281). Whereas scotoperiod TRF mice consumed a similar amount of food to the ad libitum group, the photoperiod TRF mice ate ~ 15% fewer calories, so different TRF timing may also inadvertently affect energy intakes. A further experiment found that mice that only had chow access every other day ate twice their normal daily intakes, fully compensating for the fasting periods (280).

This comprehensive series of experiments showed that energy restriction affected feeding timing more than time restriction. The findings highlight the importance of meticulous data collection and indicate a need to re-evaluate many rodent energy restriction studies to determine if it is actually time restriction that underlies the many reported health benefits of energy restriction. Notably, these studies all used male C57/BL6 mice, animals highly susceptible to diet-induced obesity. As such, it may be premature to extrapolate these findings to humans.

1.7.1.3 Manipulation of caloric period in humans

Whereas TRF of HFDs in mice has marked beneficial effects on metabolism, TRF may not confer such striking metabolic advantages when mice are fed normal chow (273). Similarly, preliminary studies of humans fed standardised diets have not shown many benefits of a restricted caloric period on metabolic outcomes. A crossover trial of 15 healthy young adults found that consuming all food at a single evening meal increased fasting glycaemia and impaired glucose tolerance versus an isocaloric diet comprising three meals throughout the day (282), and a similar study reported that single meal consumption produced increased hunger, blood pressure, and blood cholesterol (283). Consumption of one daily meal is an extreme intervention that is unlikely to be sustainable, however, and findings may have been confounded by diurnal variations in these parameters, as outcomes were measured at different times of day.

Another way to shorten caloric period is to skip breakfast. In a study of overweight and obese adults, breakfast-skipping did not influence responses to weight loss diets (284). A comprehensive study of lean young adults found that one of the only metabolic effects of six weeks of breakfast omission was increased afternoon glycaemic variability (285). Interestingly, the breakfast-skippers consumed fewer calories than the breakfast eaters, but this was negated by lower physical activity thermogenesis. Subsequent research using the same protocol in obese adults also reported few differences between groups, other than higher insulin sensitivity in breakfast eaters (286). Most recently, a randomised crossover study assessed the acute effects of breakfast-skipping in adults with and without type two diabetes, none of whom was using insulin. Blood glucose responses to lunch were again higher when participants skipped breakfast. Interestingly, postprandial expression of several clock genes in white blood cells differed between the meal conditions, perhaps indicating an acute effect of nutritional status on peripheral clocks. Whether changes in the circadian system contributed to altered postprandial responses is unclear, however (287).

It is worth considering that skipping breakfast not only shortens caloric period but also delays it. Most recently, a crossover trial compared the acute effects of skipping breakfast to skipping dinner. In one condition three meals were consumed, in another breakfast was skipped, and in another dinner was skipped. Diet composition was the same in the three conditions (288). Contrary to findings of the longer-term Bath Breakfast Project studies (285, 286), energy expenditure was marginally higher in both of the meal-skipping conditions, although activity was constrained as participants had to remain in the laboratory. On breakfast-skipping days fat oxidation was slightly higher and carbohydrate oxidation slightly lower than when consuming three daily meals. Consistent with findings of the Bath Breakfast Project, however, blood glucose and insulin

responses were impaired after lunch in the breakfast-skipping condition when compared to the dinner-skipping condition, perhaps as a result of greater inflammatory responses to eating after skipping breakfast (288).

Together, findings from these studies do not support the hypothesis that a restricted caloric period improves metabolic health in humans. Similarly, experimentally manipulating meal frequency does not appear to markedly influence many metabolic outcomes. A recent systematic review included experiments in which higher meal frequencies were compared to more regular frequencies in adults without health conditions other than overweight or obesity. The authors did not search for restricted eating period studies. Most studies reported no effects of meal frequency on energy intake, adiposity measures, or diet-induced thermogenesis. Effects on blood lipids, blood glucose, and insulin were also inconsistent (289).

A limitation common to the majority of short- and longer-term restricted caloric period studies is that samples have typically comprised fewer than 50 participants (276). There is a glaring need for large-scale prospective studies assessing a restricted caloric period. Notably, meal-skipping not only alters the duration of the eating period but also its timing. And the timing of the eating period relative to the biological day may matter.

1.7.2 Timing of the eating period

Diet timing may be a key determinant of postprandial metabolic responses. Mice fed HFDs during the photoperiod (rest phase) tend to gain more fat mass than mice fed HFDs during the scotoperiod (290), and the same is true for mice fed normal chow. A recent study showed that although there were no differences in weight between mice with access to food only during the scotoperiod or photoperiod, the mice with chow access during the photoperiod consumed 15% fewer calories. Similarly, when researchers imposed 30% energy restriction beginning during either the scotoperiod or photoperiod, energy intakes were similar but only mice in the scotoperiod group lost weight, indicating that feeding timing was a key determinant of weight (280).

Within just nine days, TRF during the photoperiod in mice has been found to alter 24-hour clock and metabolic gene expression profiles in peripheral tissues, blunt corticosterone rhythm amplitudes, reduce energy expenditure despite comparable locomotor activity, and reduce lipid oxidation (291). It is possible that such deleterious metabolic effects of TRF during the photoperiod reflects misalignment between energy intake and energy expenditure, as a transgenic *hPER1* mutation in mice increases obesity risk by advancing peak feeding time relative to peak daily energy expenditure.

Subsequently restricting chow access to synchronise feeding with peak energy expenditure offsets obesity development in these animals (292).

Regarding humans, each year Ramadan provides a natural experiment to determine what happens when people eat during the scotoperiod, the rest phase for us diurnal beings. In contrast to the obesogenic effects of rest phase TRF reported in rodent studies, however, a meta-analysis of 35 studies found a mean *reduction* in weight of 1.24 kg during Ramadan, with differences between ethnicities and greater reductions in men. No effects on dietary macronutrient proportions were apparent, and fasting duration was not associated with weight changes. Of the 16 studies that followed participants after Ramadan, mean weight regain was 0.72 kg (293).

A more recent meta-analysis of 30 Ramadan studies also considered whether Ramadan fasting influenced other markers of metabolic health. The analysis showed that both fasting blood glucose and low-density lipoprotein cholesterol declined in both sexes during Ramadan. Bodyweight, total cholesterol, and triglycerides also decreased in men, and HDL cholesterol increased in women (294). Most Ramadan studies have not measured changes in body composition, and changes in a variety of other health behaviours during Ramadan make it difficult to identify the effects of dietary changes. Nevertheless, these meta-analyses provide unambiguous evidence that consuming all calories during darkness can be healthy in humans, at least in the short-term. As Ramadan has been shown to delay the circadian phase of cortisol and melatonin rhythms (295), it would be interesting to better understand when Ramadan adherents are eating and drinking relative to circadian phase, for it appears that diet timing relative to circadian phase may have salient metabolic consequences.

1.7.3 Energy intake distribution within the eating period

Before considering effects on metabolic health, it is useful to first consider findings of cross-sectional studies that have recorded how people commonly distribute energy intake. In previously discussed studies (266, 267), US adults had consumed ~ 23% of daily energy intakes by 12:00 and Indian adults ~ 29%. By 18:00, US adults had only consumed ~ 63% of daily energy intakes and Indian adults had consumed ~ 60%. Energy intakes were therefore distributed relatively late in the day. In a study of 24-hour dietary recalls from 10 European countries, distinct regional patterns were evident. Adults in Mediterranean countries ate larger lunches than adults in central and northern Europe (38 to 45% versus 16 to 27% of daily energy intakes, respectively) and consumed less energy from snacks (10 to 20% versus 23 to 35%, respectively). However, data were collected between 1995 and 2000 and may therefore not be representative of patterns

nowadays (296). This said, more recent work from these researchers showed that meal patterns of adults from the three countries included in both of their studies (France, the Netherlands, and Norway) have not changed markedly since. In their more recent study of middle-aged adults from Belgium, the Czech Republic, France, the Netherlands, and Norway, breakfast and lunch were consumed most often by Czech adults, dinner by French adults, and after-dinner snacks by Belgian and Dutch adults (269).

A recent review summarised the distributions of daily energy intake across meals and snacks reported in cross-sectional studies conducted in Europe and North and South America. Four patterns of energy distribution were reported. Again, patterns varied by geographical location (297). In a pattern common to North America and Northern Europe, later meals comprised a greater proportion of daily energy intake. This was true of the UK, where dinner contributed substantially more of daily energy intake than other countries. Furthermore, the distribution of energy intake in the UK appears to have shifted later in the day in recent years (298). In Eastern Europe, breakfast and lunch contributed the most energy. In South America, lunch contributed slightly more energy than breakfast and dinner. And a small breakfast, large lunch, and moderate-sized dinner were typical in Southern and Western Europe (297).

The significance of these findings is that energy intake distribution may also affect metabolism, even when variables such as nutrients consumed are unchanged. Studies of mice show this plainly. Mice fed a high-fat meal at the end of the scotoperiod develop higher adiposity, insulin, leptin, and triglyceridaemia versus mice that consume a high-fat meal at the beginning of the scotoperiod (299). Similarly, restricting fructose access to the photoperiod increases adiposity and insulin resistance in mice in comparison to restricting access to the scotoperiod (300).

Human studies generally give tentatively support for roles of energy intake distribution in metabolic health. In the aforementioned review of cross-sectional studies, the authors concluded that the research provided some evidence that greater evening energy intake may be associated with obesity. The authors decided that heterogeneity in the studies precluded a meta-analysis, however, and there is difficulty in interpreting many of the studies that collated all snacks and hence masked the timing of snack energy intake (297). Other researchers have since completed a meta-analysis of cross-sectional studies of dinner energy intake and adiposity, reporting a trend to higher BMIs in participants consuming larger dinners. In a separate analysis of intervention trials, no association between dinner energy intake and change in weight was found, however (301). A limitation of all of these studies is that they did not assess meal timing relative to circadian phase.

The first cross-sectional study to consider caloric timing relative to circadian phase was published only very recently. In this study, young adults used a time-stamped mobile phone application to record all dietary events for a week in the middle of a period of 30 days of wrist actimetry to monitor sleep. Body composition was assessed using bioelectrical impedance analysis, and the results were used to separate participants into lean (n = 70) and non-lean (n = 40) groups. Salivary melatonin profiles were measured hourly in constant dim lighting during an overnight laboratory visit from 16:00 to 07:00. Non-lean participants consumed most of their calories ~ 1.1 hours closer to dim-light melatonin onset, but there were no differences between groups in the clock time of caloric events. Non-lean participants also consumed their latest calories 0.9 hours later relative to dim-light melatonin onset. Furthermore, participants who consumed their last calories later relative to circadian phase also slept less. Interestingly, only caloric timing relative to melatonin phase was predictive of adiposity in a multiple regression analysis that included clock time of caloric events, diet composition, and sleep duration as predictor variables (268).

Prospective studies have also found that the timing of an individual meal may be important, as earlier lunch consumption has been associated with greater weight loss after a 20-week weight loss programme (302). Most recently, the Seventh-day Adventist church member researchers reported that participants who had their largest meal at breakfast (between 05:00 and 11:00) lost weight relative to participants who had their largest meal at dinner (between 17:00 and 23:00). Similarly, participants who had their largest meals at lunch lost weight relative to those who had their largest meals at dinner (265).

Findings from human studies of meal timing interventions give additional support for these observations. Among overweight and obese women matched for energy intakes, those who consumed a larger proportion of daily energy intake at breakfast lost more weight than those consuming a larger proportion at dinner (303), and like findings have since been reported in severely obese adults following bariatric surgery (304). As diet-induced thermogenesis is higher in the morning, and breakfast consumption is associated with more subsequent non-exercise activity thermogenesis and hence energy expenditure (285), perhaps assigning more of daily energy intake to earlier meals may encourage a negative energy balance during hypoenergetic diets.

1.7.4 Consistency of the eating period

Finally, the timing of dietary events from one day to the next is very inconsistent in some adults (266, 267), which may be pertinent to metabolic health. In mice, fixing feeding to

the same 12-hour period during twice weekly six-hour LD cycle advances might be expected to uncouple LD cycle-entrained SCN phase from feeding-entrained peripheral clock phase and thereby produce corresponding metabolic disorder. In these conditions, however, TRF actually offset the obesogenic effects of LD cycle shifts observed in ad libitum-fed mice, despite similar energy intakes. Meal regularity and not just its timing relative to activity may therefore also be important to benefits of TRF (305).

Studies of humans also indicate the importance of consistent meal patterns. A recent review summarised the results of the few studies that have considered the relationship between dietary regularity and metabolic health. Several cross-sectional and prospective cohort studies have associated irregularity with increased with blood pressure, BMI, and metabolic syndrome (306), including a study of UK adults (307). Furthermore, intervention studies to date have found adverse effects of irregular meal patterns. In a crossover study in which healthy young women consumed a variable number (three to nine) of 'meals' daily for 14 days and a fixed number (six) for 14 days with a 14 day washout period in the interim, diet-induced thermogenesis was higher and blood glucose regulation was better after the regular meal condition (308). This study built on previous work from the same group that used similar methods to show that irregular meal patterns may contribute to insulin resistance and raise low-density lipoprotein and total cholesterol (309, 310).

Together, it appears that consistent meal patterns and consuming meals in close proximity to physical activity may help optimise metabolic health. Furthermore, allocating a higher proportion of energy intake to earlier meals may promote a lower energy balance when diets are matched for energy intake. Preliminary research has also shown that diet timing relative to circadian phase may influence adiposity, but more research is needed to support this hypothesis.

1.7.5 Dietary compounds influence the circadian system and sleep

In addition to diet timing, diet composition may influence the circadian system and sleep, and an array of chronobiotic compounds in foods has been found. Chronobiotics are agents capable of modifying a biological rhythm's amplitude, period, or phase. Recent evidence has shown that macronutrient composition can modify numerous aspects of the circadian system.

Switching adults from higher carbohydrate (55%), lower fat (30%) diets to isocaloric lower carbohydrate (40%) and higher fat (45%) diets delayed and increased the amplitude of cortisol rhythms, altered inflammatory and metabolic gene expression

profiles, and modified *PER* gene expression profiles in monocytes (311). So, while macronutrient composition clearly appears to alter peripheral clocks, it does so in a tissue-specific way with long-term health consequences that are not yet clear.

Of all commonly consumed dietary compounds, alcohol appears to be particularly disruptive to molecular, endocrine, and behavioural circadian rhythms in humans and other animals (312-316). Caffeine, the most-used psychoactive compound worldwide, is present in many foods and drinks, and evening caffeine consumption delays the human circadian system in vivo and lengthens clock gene expression periods in vitro (251). Because of these chronobiotic effects, caffeine has been studied for whether it can aid entrainment in blind individuals with non-24-hour sleep/wake rhythm disorder, a disorder in which the LD cycle fails to synchronise the circadian system with the 24-hour day. In one study, 150 mg of morning caffeine was insufficient to entrain circadian rhythms in people with this disorder (317). Nevertheless, careful use of caffeine may expedite circadian rhythm entrainment in some circumstances, such as after jetlag (318). However, even if subjective sleepiness is unaffected by its ingestion, caffeine may impair sleep following jetlag (319). Of all oral chronobiotics commonly used to facilitate entrainment, melatonin has perhaps most consistently been proven efficacious.

1.8 Melatonin as a countermeasure against metabolic disease

Melatonin (5-methoxy-*N*-acetyltryptamine) is a particularly potent chronobiotic. Melatonin is a relatively ubiquitous compound in nature that is therefore also present in many foods and drinks, albeit perhaps in insufficient quantities to significantly influence the circadian system and sleep after ingestion. Melatonin was discovered in 1958 as a factor that inhibits the darkening effects of melanocyte-stimulating hormone on frog skin (320), and the structure ($C_{13}H_{16}N_2O_2$) of this indoleamine hormone was reported the following year (321). Now, however, melatonin is best known as an endogenous signal of scotoperiod, with critical roles in circadian system regulation. It has also been well documented that pharmacological doses of melatonin and its agonists improve sleep in various populations (54), and melatonin can scavenge free radicals within cells, independent of its receptors (322). This is particularly noteworthy as it is increasingly clear that oxidative stress is a key contributor to metabolic dysregulation in pathologies such as metabolic syndrome (323). Given the many roles of circadian system function and sleep in metabolic regulation, as well as the presence of melatonin receptors in many key metabolic tissues, it is intuitive that melatonin has roles in metabolism. Only recently, however, have researchers begun exploring some of these roles.

Lower circulating melatonin levels have been found in people with some metabolic diseases. This is true of women with hypertension (324), myocardial infarction patients (325), and type two diabetes (326), particularly among individuals with autonomic neuropathy (327, 328) and proliferative retinopathy (329). Not all studies have reported such results, however. Indeed, one study reported that adults with obesity and metabolic syndrome had comparable melatonin levels to healthy controls (330). Cross-sectional studies like these preclude inferences about causality, but their findings are supported by some prospective studies. Lower excretion of 6-sulphatoxymelatonin (the primary melatonin metabolite) has been associated with increased subsequent type two diabetes risk in a prospective study, for example (331). These studies generally suggest that there may be a connection between lower melatonin synthesis and some cardiometabolic diseases, and studies have therefore explored whether exogenous melatonin supplementation may benefit various metabolic processes.

1.8.1 Melatonin supplementation in rodents

Preclinical studies using rodent models of metabolic diseases have consistently shown that long-term melatonin supplementation has widespread benefits on physiology. In rat models of diabetes, for example, melatonin has been found to reduce hyperglycaemia, hyperinsulinaemia, hyperleptinaemia, and hypertriglyceridaemia (332, 333), also offsetting hepatic steatosis and improving liver mitochondrial function (334). Similar findings have been reported in studies of rat models of obesity (335, 336), and melatonin improved insulin sensitivity, glucose tolerance, and vascular function in diet-induced obese mice (337). It appears that exogenous melatonin may also help counter many of the adverse metabolic consequences associated with senescence in mice and rats, such as insulin resistance, increased weight, and reduced locomotor activity (338, 339). Finally, as an antioxidant, melatonin reduces increased oxidative stress in response to injuries in rats (340).

Studies of rodents have demonstrated numerous promising effects of long-term melatonin administration, but many of these studies used doses > 5 mg/kg. Relative to bodyweight, this is well above the 0.3 to 5 mg commonly taken by humans. Furthermore, there are considerable differences in melatonin receptor ligand pharmacology between species (55), and most of these studies used nocturnal rodents: In nocturnal animals, melatonin in many ways has opposite functions to those in humans, readying the animals for activity and feeding. Last, most mouse studies have used C57BL6/J mice. This congenic strain has melatonin receptors but synthesises little melatonin because of a

genetic deficit that results in no activity of N-acetyltransferase or hydroxyindole-O-methyltransferase, enzymes involved in melatonin synthesis (341).

1.8.2 Melatonin supplementation in humans

Like rodent studies, human studies of long-term melatonin supplementation have documented a variety of beneficial effects. Long-term, open-label use of a slow-release melatonin formulation reduced glycated haemoglobin in type two diabetes patients with insomnia (342). In metabolic syndrome patients whose health had not responded favourably to lifestyle modifications, melatonin use reduced blood pressure and weight after one month. After two months differences in weight were no longer evident, but patients retained blood pressure improvements and also had lower low-density lipoprotein cholesterol levels and measures of oxidative stress (343). Similarly, a crossover trial of metabolic syndrome patients reported that long-term melatonin supplementation tended to reduce weight, decreased blood pressure, and led to higher metabolic syndrome remission than placebo (344). Changes in weight are not always indicative of changes in fat mass, but a study of post-menopausal women demonstrated that one year of melatonin supplementation increased lean body mass without affecting weight (345). Other reports indicate that melatonin may increase HDL cholesterol levels in peri- and post-menopausal women (346).

Not all studies have reported beneficial effects of long-term melatonin supplementation, however. Using blood lipids to exemplify this, some have reported deleterious effects, such as raised triglycerides in normolipidaemic post-menopausal women (347) and increased low-density lipoprotein and very low-density lipoprotein cholesterol in post-menopausal women (348). Others found no effects on lipids or glucose in elderly women (349) or lipids in hypercholesterolaemic patients (350). There are likely many for reasons for discrepancies in findings between studies, including participant characteristics, differences in melatonin dosing, timing, pharmacokinetics, and concurrent medication use. Another reason may be differences in melatonin receptor genetics.

1.8.3 Melatonin receptor variants

A series of GWA studies published concurrently showed that a *MTNR1B* variant (rs10830963) present in ~ 30% of the population is associated with higher plasma glucose, lower early insulin responses, faster deterioration of insulin secretion, and increased type two diabetes risk (351-353). The mechanisms underlying these findings are unclear. A recent study of RNA sequencing of pancreatic islets from 204 donors

reported that the risk variant is an expression quantitative trait locus: more copies of the risk (G) allele result in greater *MTNR1B* transcription in human islets (354). The same researchers found that melatonin signalling inhibits insulin release in INS-1 832/13 β cells. They also reported insulin resistance in MT2 knockout (*MT2^-/-*) mice (354). Conversely, others have shown that melatonin receptor activation restored insulin secretion in human islets exposed to glucotoxicity and enhanced survival of rodent INS-1 832/13 β cells (355). Additional studies that compare *MTNR1B* mRNA expression between people with and without type two diabetes, quantify MTNR1B protein levels, and consider multiple signalling pathways in various human tissues will help clarify these contradictions (356).

Regardless of mechanisms, *MTNR1B* variants may influence how much melatonin ingestion acutely worsens oral glucose tolerance. Morning melatonin administration to healthy lean young women increased glucose AUC responses to a subsequent oral glucose tolerance test about six times more in carriers of the risk allele (CG) than in women with no copies (CC) (357). In a study of 23 adults with two copies of the risk allele (GG) and 22 with two of the non-risk allele (CC), three months of melatonin supplementation reduced oral glucose tolerance test first-phase insulin responses in all, but melatonin inhibited insulin secretion more in those with the risk allele, particularly in the first 30 minutes (354). Interestingly, this *MTNR1B* type two diabetes risk variant has been associated with prolonged melatonin synthesis duration and delayed melatonin offset phase in humans, without affecting objectively measured sleep. If melatonin indeed inhibits glucose-stimulated insulin secretion, it is plausible that extended melatonin synthesis into waking could contribute to type two diabetes risk, particularly among carriers with early sleep times (358).

Collectively, studies on the metabolic roles of melatonin suggest that 1) melatonin may be lower in type two diabetes; 2) melatonin has myriad beneficial effects on rodent physiology, but a shortcoming of studies is the use of nocturnal mammals and the large doses used; 3) long-term melatonin may benefit some metabolic processes and sleep in humans, but studies have had design issues including small sample sizes; and 4) effects of melatonin treatment may be *MTNR1B* genotype-dependent. Many questions remain unanswered, however. Thus far, longer-term studies have mostly tested use of melatonin in small groups of people with metabolic diseases, but no well-powered, controlled trial has determined whether melatonin is an effective countermeasure against metabolic disease development. Diet is an important determinant of disease risk, yet no study of humans has assessed whether melatonin supplementation affects food intake, despite clear indications of its interrelationships with key appetite-regulating hormones such as leptin (359). Last, there is little evidence regarding how different participant

characteristics (including basal melatonin levels, *MTNR1B* genotype, and sex) influence responses to melatonin use.

1.9 Aims

The literature review showed that the circadian system, sleep, and diet have pivotal and interrelated influences on metabolic health. But the review also underscored numerous research questions that have not been well answered. This project is designed to address some of these questions, within the resource and time constraints imposed. Specifically:

1) A limitation of many chrononutrition studies has been the use of dietary assessment methods that have not been validated. Some methods used have additional shortcomings, such as inability to record the timing of all dietary events and use of restricted food and drink databases. It is therefore my initial aim to help validate a tool that overcomes these limitations.

2) Only one small study of a homogeneous group of young US adults has assessed whether diet timing relative to circadian phase (dietary phase angle) is associated with adiposity. Furthermore, relatively little is known about diet and sleep timing in UK adults. I intend to use the validated dietary assessment tool to 1) explore if dietary phase angle is associated with adiposity in a relatively representative group of UK adults, and 2) more accurately describe the timing of dietary events in relation to sleep in these people.

3) No studies have concurrently reported on associations between sleep duration and nutrient intakes, as well as sleep duration and objective measures of metabolic health in UK adults. This knowledge gap will be addressed using a large, publicly available dataset.

4) If the circadian system and sleep influence diet and metabolic health, what will be the effects of an intervention to align the circadian system and improve sleep in people at above average risk of type two diabetes? As yet, no well-powered, controlled trial has determined whether 1) melatonin supplementation is an effective countermeasure against metabolic disease development, or 2) melatonin affects food intake.

Chapter 2: Validation of myfood24, an online dietary recall tool suited to chrononutrition studies

2.1 Chapter overview

Changes in eating behaviours contribute to the development of many non-communicable chronic diseases. Numerous studies have reported that sleep patterns influence dietary choices. However, many of these studies have used dietary assessment methods that have not been validated against biomarkers of dietary intakes, and accurate dietary measurement is essential to understanding how diet influences health. Monitoring trends in diet is rife with issues, such as use of onerous diet recording methods, inaccurate participant diet recollections, and staying current with the wealth of food and drink choices available nowadays. Several commonly used dietary recall methods have not kept pace with the digital revolution we are experiencing, so novel methods using widely available technologies may improve usability and hence compliance. Furthermore, as it is increasingly clear that when we eat influences metabolic responses to consumption of a given food, another limitation of numerous dietary recall methods is their failure to document diet timing. As the primary aim of this project is to better understand interactions between sleep, diet, and metabolic health in UK adults, a user-friendly diet recall method designed specifically for this population that also documents diet timing would be very useful. Especially if the method is known to provide valid estimates of dietary intakes.

The purpose of the work in this chapter was therefore to test the validity of measure your food on one day 24-hour recall (myfood24), an online dietary recall tool developed specifically for use with UK adults. myfood24 was compared with the interviewer-administered 24-hour recall method, the gold standard but burdensome approach often used in nutritional epidemiology. To this end, 212 UK adults completed up to three series of one myfood24 recall and one interviewer-administered recall (recalls took place within four days of each other). There was approximately two weeks between each series. Biomarkers of nutrient intakes were compared with estimated dietary intakes: urinary nitrogen with dietary protein, urinary sugar with total dietary sugar, urinary potassium with dietary potassium, urinary sodium with dietary sodium, and energy expenditure (estimated by combining indirect calorimetry with accelerometry) with energy intake (assuming energy balance). I was responsible for the laboratory analyses of urinary nitrogen and sugars. Results of the two dietary recall methods were largely similar and better than findings reported for frequently used food frequency questionnaires. This chapter therefore supports the rationale to use myfood24 to explore interactions between sleep, diet, and metabolic health in subsequent chapters of this project.

2.2 Background

Longstanding interest in dietary measurement is evidenced by dietary assessment reports published as far back as the 1930s (360). Even then, people understood that accurate dietary assessment is essential to understanding how diet influences disease risk (361). Much progress has since been made, but certain limitations of dietary recall methods may be inevitable. Some participants, for example, are self-conscious about their diets and hence misreport. In other instances, people forget to record some foods and drinks they consumed, and not all study participants can always record their intakes, even if they intend to.

To this day, however, many widely used dietary assessment methods share important limitations, one of which is use of methods that are particularly burdensome for participants, hence reducing compliance with dietary recording. Many frequently used recall methods are also time-consuming for researchers and are therefore expensive. Another issue with some methods is limited food and drink databases that often do not have items commonly consumed by a study's participants. Indeed, the Food Marketing Institute estimated that there were on average 42,000 items stocked in UK supermarkets in 2014. Yet the most widely used food composition tables in the UK (McCance and Widdowson's) contain less than a tenth of this number of items (362). Moreover, few dietary assessment methods are updated frequently to include new items.

Arguably a primary problem with popular dietary assessment methods is their validity. Food frequency questionnaires (FFQs) have become widely used as a convenient dietary assessment method in nutritional epidemiology since first appearing in the 1960s (363). Despite their popularity, however, FFQs are based on many assumptions, such as the types and consumption frequencies of food choices. As a result, comparison with biomarkers of energy expenditure (doubly-labelled water) and dietary protein (urinary nitrogen) has shown that FFQs lack precision and often result in under-reporting (364). Numerous cross-sectional studies that have explored associations between sleep and diet have assessed diet using FFQs and rudimentary lifestyle questionnaires that have not been validated against biomarkers of dietary intakes (365). It is perhaps unsurprising that these studies have produced some conflicting findings in recent decades.

A primary use of dietary biomarkers is as reference measures in validation studies of dietary assessment methods. There are currently four classes of dietary biomarkers: recovery, predictive, concentration, and replacement. (I will not discuss the latter as they were not used in this study.) Recovery biomarkers are the gold-standard and include urinary nitrogen (for dietary protein), urinary potassium (for dietary potassium), and

urinary sodium (for dietary sodium). Assuming metabolic balance between intake and excretion over a given period, recovery biomarkers can be used to estimate true dietary intakes with impressive accuracy, as exemplified by an early study of eight adults that reported a correlation coefficient between urinary nitrogen and dietary protein of 0.99 over a 28 day period (366). Shorter urine collection periods of course result in lower correlations, but accuracy remains high, especially when the completeness of the urine sample is verified (367). Para-amino benzoic acid (PABA) is commonly used to verify urine sample completeness, as PABA is actively absorbed, > 85% of PABA is typically excreted in urine within 24 hours, and urinary PABA is proportional to the dose ingested (368). Like nitrogen, urinary potassium and sodium biomarkers continue to be widely used to validate various dietary assessment methods (369, 370).

Dietary sugars include monosaccharides like fructose and disaccharides like sucrose, the sum of which is total sugars. Extrinsic sugars (those added during the processing or preparation of foods) are common ingredients in less healthy foods and drinks, and excessive sugar intake has sometimes been associated with various metabolic diseases (371). Importantly, however, sugar intakes are often misreported (372). Biomarkers of sugar intakes are therefore useful both as proxies of sugar intakes and in validating dietary assessment methods, and urinary fructose and sucrose are predictive biomarkers of total sugar intake.

Predictive biomarkers are sensitive to changes in intake over time, but unlike recovery biomarkers predictive biomarkers also have some intake-related, person-specific bias that is accounted for using calibration equations from controlled feeding studies (372). Furthermore, only a very small proportion of intake is present in urine (urinary fructose and sucrose comprise ~ 0.05% of total sugar intake) (373), which may particularly strongly reflect extrinsic sugar intake (374). The small amount of urinary fructose includes dietary fructose and fructose from cleavage of sucrose by the liver. A small proportion of sucrose that is not hydrolysed in the small intestine enters the circulation and is then excreted in urine. Urinary glucose, however, is mostly reabsorbed in the kidneys and is not reflective of dietary intakes. For this reason, urinary sugar concentration is the sum of urinary fructose and urinary sucrose alone, and when I refer to urinary sugars henceforth I am specifically referring only to total fructose and sucrose.

It should be noted that people with particular characteristics may be less likely to accurately report their dietary intakes. This is true of people with higher body mass indices (BMIs), for example (375). Importantly, a controlled feeding study found that BMI does not influence the accuracy of urinary fructose and sucrose as predictors of total

sugar intake, showing that these biomarkers appear to be quite robust and are hence useful for various applications (376).

The concentrations of nitrogen and sugars in urine reflect short-term intakes of protein and sugars, respectively. Other dietary nutrients are turned over more slowly, however, so biomarkers of these nutrients reflect intakes over longer timeframes. This is true of concentration biomarkers of vitamin intakes, such as β-carotene, vitamin C, and α-tocopherol in plasma. Concentration biomarkers cannot be used to estimate true intakes, but biomarker concentrations do correlate with dietary intakes (377). (Note that I refer to reference measure estimates of intakes as 'true' intakes for brevity, but I recognise that the reference measure estimates do not perfectly reflect true intakes.) At present, few dietary biomarkers are available, so combining different classes of dietary biomarkers is a useful way to comprehensively assess the validity of a dietary assessment method in measuring several nutrients. Few studies have used this approach yet, however (378), in part because of the costs and practicalities of doing so.

Countries such as the UK are undergoing a digital revolution in which technology is advancing at an unprecedented rate. In 2016, 86% of UK adults had home internet access (379). To attempt to stay current with societal trends, various digital dietary recall methods have become available recently. Many novel methods are internet-based (DietDay (380), for instance), downloadable app-based (such as e-DIA (381)), camera-based (The Remote Food Photography Method (382), for example), wearable (383), or some combination thereof (384). Most online 24-hour recall methods are based on the multiple-pass method of Moshfegh and colleagues (385), including the Automated Self-Administered 24-hour recall (ASA24®) (386). As of March 2017, the ASA24® had been used in over 3,000 studies and has been adapted for use in the US, Canada, and Australia (387). INTAKE24 is a similar tool recently developed for use in the UK (388). Other online recall methods designed for use in the UK are FFQs, such as the Oxford WebQ questionnaire that has been used by more than 200,000 participants in the UK Biobank project.

Online recall methods are either self-administered or interviewer-administered, with a participant recalling items to an interviewer who then uses the tool to collect and analyse the data. Although dependent on technology readiness, the majority of participants appear to prefer new digital recall methods to more traditional interviewer- and paper-based methods (389, 390). And new methods may not only be preferred by participants, as online methods make it far easier to collect dietary data from many geographic locations and may be less administratively burdensome for researchers. Before new digital recall methods become widely used, however, it is first necessary to compare their

validity with more established recall tools. One way to ensure that the new tool accurately represents true dietary intakes is by concurrently testing the validity of both the established and novel methods against biomarkers of dietary intakes.

measure your food on one day 24-hour recall (myfood24) is an online recall tool that was designed in response to the many difficulties that complicate accurate dietary assessment. myfood24 development has been described in detail (391). myfood24 is well suited to large-scale epidemiological studies. As it is self-administered, myfood24 removes the need for interviews, which are time-consuming and hence costly for researchers. Some recall methods still require manual food coding, but this is automated in myfood24. With a similar structure to the US Automated Multiple-Pass Method, myfood24 reduces the likelihood of omitting commonly forgotten items by allowing users to make initial lists of items consumed and by prompting for commonly forgotten foods. myfood24 users are both guided by portion size photographs in ~ 6,000 instances but also free to select atypical portion sizes if they wish. Notably, digital portion size images may improve portion size estimation accuracy (392).

Most digital dietary recall methods currently used were not developed specifically for UK participants (INTAKE24 is an exception). To address this, myfood24 development was an iterative process that was refined by feedback from focus groups comprising UK citizens of a broad age range (391). And not only is the myfood24 user interface designed to be intuitive for its target users, the myfood24 database also has back of pack nutrient information for > 50,000 branded UK food products, far more than any other online tool to date (378). Detailed search and recipe builder functions let myfood24 users seamlessly combine items into meals they consume. As participants enter times of consumption for each item, myfood24 is particularly useful in temporal profiling of dietary patterns. myfood24 provides detailed nutrient profiles for items consumed, including up to 120 nutrients. Finally, myfood24 was designed to have as few separate web pages, pop-ups, and prompts as possible to enhance simplicity and recall speed.

Given the numerous advantages of myfood24, we sought to validate the tool against biomarkers of dietary intakes and compare its performance with the gold-standard dietary assessment method, the interviewer-administered multiple-pass 24-hour recall method (MPR) (385).

2.3 Methods

2.3.1 Participants

The study was approved by the West London Research Ethics Committee (number 14/SC/1267) and was conducted in accordance with the Declaration of Helsinki. Participants gave written informed consent. Participants were intended to be representative of the UK adult population, and inclusion criteria were English speaking, non-pregnant, weight-stable, literate 18 to 68-year-old adults with internet and telephone access.

Participants were recruited through the North-West London Primary Care Research Network, a group of primary care professionals and practices that previously showed interest in participating in research projects. In addition, posters advertising the study were put up in the National Institute of Health Research/Wellcome Trust Clinical Research Facility at Hammersmith Hospital (Imperial College Healthcare NHS Trust, London, UK), and the Clinical Research Facility contact list was used to contact potential participants. A list of local addresses was also obtained from the post office, with prospective participants receiving postal invitations to take part. Participants received a financial reward on completion of the study (£100) as compensation for their time.

2.3.2 Study Design

Participants first completed a health screening visit at the Clinical Research Facility. On arrival at the facility participants were weighed using scales after voiding. Height was measured using a stadiometer (Seca, Hamburg, Germany), adiposity was estimated using bioelectrical impedance analysis (Tanita Corporation, Tokyo, Japan). Waist (level with the naval) and hip (at the largest diameter of the hips) circumferences were measured using tape measures, and cardiac function was assessed by electrocardiography. Participants rested supine for ~ 30 minutes, during which blood pressure was measured using a digital monitor. At this visit participants filled in a general health and lifestyle questionnaire, the SCOFF questionnaire (to exclude individuals with eating disorders) and a technology readiness questionnaire. A 22 ml blood sample was taken for analysis of immune function, kidney function, liver function, and blood lipids.

After screening, participants completed a series of dietary recalls using both myfood24 and the MPR three times, each of which occurred shortly after collecting reference measures (including energy expenditure estimates and biomarkers of nutrient intakes). Recalls were completed in a randomly allocated order, as determined by an orthogonal Latin square design. There were at least two weeks between series of recalls. In each series, biomarker measures were first made, one of the dietary recall methods followed the day after, and the other dietary recall method came about two to four days thereafter.

In this way, roughly half of dietary recalls were for biomarker measurement days. To avoid learning effects that often happen when the test and reference method are used for the same day, recalls were separated by two to four days, and the three cycles of recalls were separated by roughly two weeks (Figure 2.1).
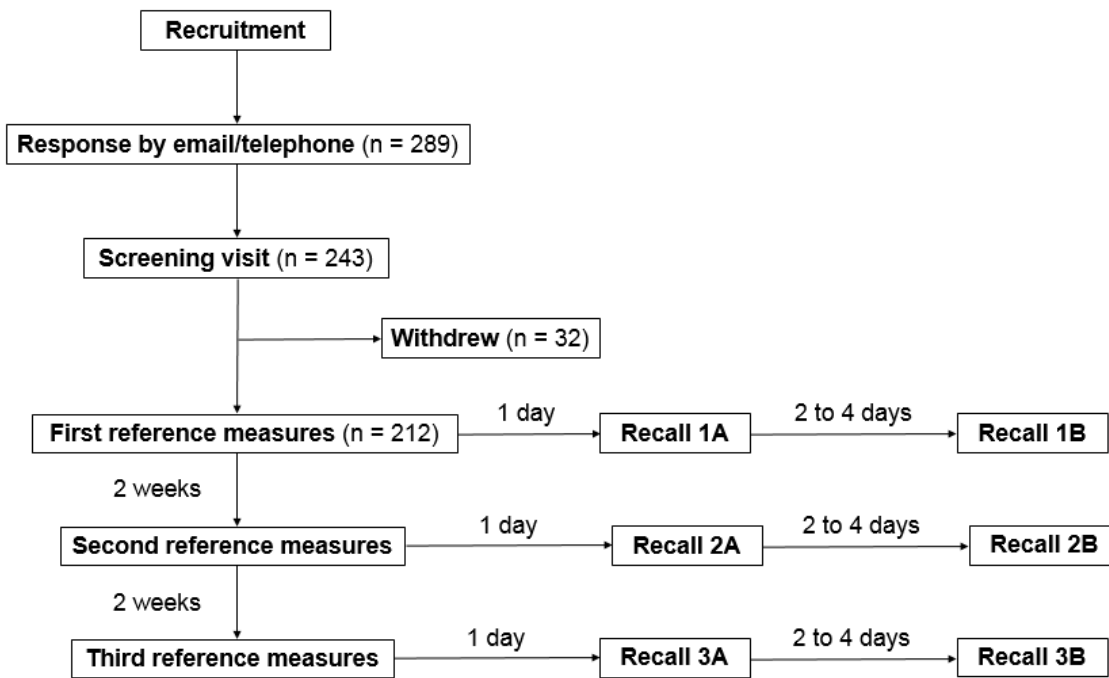
Figure 2.1. Study design overview.

After screening for eligibility, reference measures (energy expenditure estimates and dietary biomarkers) were collected for each participant up to three times. The day after each set of reference measures, participants completed a dietary recall using either the interviewer-administered multiple-pass 24-hour recall method or myfood24 ('A' recall days). Participants were randomised to determine whether they completed an interviewer-administered multiple-pass 24-hour recall or a myfood24 recall at 'A' recall days. Participants then completed another recall two to four days later ('B' recall days) using the method not used at the previous ('A') recall.

2.3.3 Urinary biomarkers
Urinary biomarkers were measured the day before study centre visits. Participants collected urine in 4-L containers for 24 hours (after the first void of the day up to and including the first void of the following day). Participants were asked to take 3 x 80 mg PABA tablets with meals (at ~ 08:00, 13:00 and 18:00) on urine collection days, and to record missing voids, spillages, and supplement and medication use. Participants were

instructed to store the urine containers in a cool place. Participants returned samples on the same day, volume was measured, and ~ 500 ml of each urine sample was stored at –20 °C as 50-ml aliquots.

Samples were then transported to the Molecular Epidemiology Unit at the University of Leeds. Here samples were verified for completeness by a coinvestigator who measured PABA by high performance liquid chromatography (HPLC) (368). An ADVIA 2400 Clinical Chemistry System (Siemens AG, Munich, Germany) with ion-selective electrode detection was used to measure urinary potassium and sodium concentrations. This was completed at the Clinical Biochemistry Department in the Leeds Teaching Hospitals NHS Trust.

2.3.3.1 Urinary nitrogen

To assess the accuracy of reported dietary protein intakes, I analysed urine samples for nitrogen using the Kjeldahl method. The method comprises three primary steps: digestion, distillation, and titration (Figure 2.2).

Preparation
Tubes prepared for run: 6 x urine samples, 1 x urine QC, and 1 x urea sample per run. Catalyst, salt, and acid added to each tube.

Digestion
Tube contents digested at 420 °C for 70 minutes.
Tubes allowed to cool for 35 minutes.

Distillation
Ammonia in sample distilled into a vessel where it combines with boric acid to produce ammonium borate.

Titration
Ammonium borate titrated with acid until colour change. Sample and QC concentrations quantified.

Figure 2.2. The Kjeldahl method.

QC (quality control).

First, polypeptides in the sample were digested into more simple chemicals (including ammonia), using heat, acid, and a catalyst. Specifically, 1 ml urine was digested using a digestion block (FOSS Tecator^{TM}, DK-3400 Hilleroed, Denmark) with 12 ml of 95 to 97% sulphuric acid at 420 °C for 70 minutes, using 7 g K_2SO_4 and 0.8 g $CuSO_4$ x $5H_2O$ as a catalyst to oxidise urea to ammonium sulphate. Next, the ammonia was separated from the digestion mixture by distillation. In this step, raising the pH by adding an alkali solution (40% NaOH) changes ammonium ions from a liquid to a gas that is captured in a boric acid receiving solution, forming ammonium borate. Ammonia was then distilled with a 2% boric acid indicator solution (Reagecon Tecator Mixed Indicator TECMXI01) using a Kjeltech^{TM} 8400 Analyzer Unit (FOSS, DK-3400 Hilleroed, Denmark). Finally, ammonium borate was titrated with an acid (0.1 M HCl) until a colour change.

Sample nitrogen concentration was then quantified: Approximately 81% of nitrogen is excreted through urine, and nitrogen comprises ~ 16% of protein (366). So, after adjusting for the volumes of the participants' 24-hour urine samples, results were divided by 0.81 and then multiplied by 6.25 to estimate 24-hour dietary protein intakes.

Before beginning sample analysis, I determined the sensitivity of the method using 4-fold serial dilutions of a stock urea solution containing 80 mg nitrogen/ml water, such that the most dilute solution contained 0.0048 mg/ml water. I aliquoted a urine quality control sample, 1 ml of which was also included with every run to confirm consistent nitrogen recoveries within the urine matrix. I also aliquoted three other urine quality controls: 1 ml of each of these other quality controls was included every 10 runs to ensure consistent nitrogen recoveries in a range of urine matrices. I reanalysed samples in instances where controls fell outside 2 SDs from the mean. During analysis of myfood24 samples, I also used 2 x 100 mg ammonium sulphate samples to check nitrogen recoveries each day before analysing participant samples, as well as a 100 mg urea sample alongside each run of participant samples. Again, I reanalysed samples in instances where these fell outside 2 SDs from the mean.

2.3.3.2 Urinary sugars

To assess the accuracy of reported dietary sugar intakes, I quantified urinary fructose, glucose, and sucrose using a colourimetric enzymatic assay (R-Biopharm sucrose/D-glucose/D-Fructose, An der neuen Bergstraße 17 64297 Darmstadt, Germany). To increase assay throughput and reduce costs, I modified this method from using cuvettes to a 96-well plates and a microplate reader. To accommodate the smaller wells in comparison to cuvettes, I simply used 10-fold smaller volumes of solutions used in the

cuvette assay (final volume reduced from 3.02 ml per cuvette to 302 µl per well). The method was otherwise as per the manufacturer's instructions.

In this method, I first made a series of dilutions to produce standards from 1 to 150 mg/L for each sugar. Dilutions were made at the start of each day to avoid potential issues with deterioration of standards. Standards (Sigma-Aldrich, St. Louis, Missouri, US) for each sugar were included in duplicate on each plate. Standards comprised 2-fold serial dilutions of fructose, glucose and sucrose stock solutions (each containing 0.8 mg sugar/L water), such that the most dilute solution contained 1 mg sugar/L water. I included these standards in duplicate with each run. Changes in optical densities for standards were plotted against their known concentrations, and the resultant linear regression equations were used to estimate urine sample sugar concentrations.

Regarding urine, each sample required two wells per measurement. In one of these wells (sucrose wells), sucrose was hydrolysed and D-glucose concentration was then determined. In the other (glucose and fructose wells), D-glucose was first measured before D-fructose concentration was determined. The concentrations of D-glucose and sucrose were then determined by comparing D-glucose concentrations in the sucrose wells to D-glucose concentrations in the glucose and fructose wells. All solutions and incubations were stored at 20 to 22 °C, unless otherwise indicated. In more detail:

1) In the sucrose wells only, sucrose was first hydrolysed by a solution (raised to 37 °C) containing β-fructosidase in a citrate buffer (pH 4.6):

   Sucrose + H2O ─ β-fructosidase→ D-glucose + D-fructose

2) Next, D-glucose concentration was measured in both the sucrose wells as well as the glucose and fructose wells. Optical density was measured using a microplate reader (wavelength 340 nm) before addition of the catalysts. In the first part of this step, hexokinase (pH 7.6) catalysed the phosphorylation of D-glucose to D–glucose-6-phosphate (G-6-P):

   D-Glucose + ATP ─hexokinase→ G-6-P + ADP

   Next, glucose-6-phosphate dehydrogenase (G6P-DH) catalysed the oxidation of G-6-P by nicotinamide adenine dinucleotide phosphate (NADP, in a triethanolamine buffer (pH 7.6)), forming D-gluconate-6-phosphate:

   G-6-P + NADP$^+$ ─G6P-DH→ D-gluconate-6-phosphate + NADPH + H$^+$

   The NADPH formed in this reaction is stoichiometric to the concentration of D–glucose, producing a colour change that was measured using a microplate reader (wavelength 340 nm).

3) Finally, D-fructose concentration was determined in the glucose and fructose wells only. In this step, D-fructose-6-phosphate was converted to G-6-P by phosphoglucose isomerase:

F-6-P ─phosphoglucose isomerase→ G-6-P
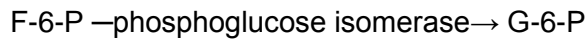
The G-6-P now reacted with NADP once more, as before:

G-6-P + NADP$^+$ ─G6P-DH→ D-gluconate-6-phosphate + NADPH + H$^+$
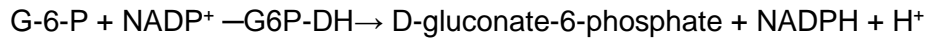
And this time the NADPH formed was stoichiometric to the concentration of D–fructose, producing a colour change that was measured using a microplate reader (wavelength 340 nm).

Dilutions were made if a sample's concentrations exceeded the range of the standard curve. Urinary sugars (the sum of fructose and sucrose) were then used to estimate total dietary sugar intakes by multiplying concentrations by sample volumes and then using the calibration equation from a controlled feeding study that accounts for age and sex of each participant (393). I aliquoted a urine quality control sample that was included with each run to assess the consistency of sugar recoveries within the urine matrix. I reanalysed samples in instances where controls were beyond 2 SDs from the mean.

Before sample analysis, I contacted other scientists who had used the assay in 96-well plates. Dr Johanna Lampe did the urinary sugars laboratory analyses for Dr Natasha Tasevska and kindly sent me the protocol they used. The protocol was the same as the manufacturer's methods, other than the following modifications: 1) samples were vortexed and adjusted to pH 8, 2) samples were mixed for five minutes (not three) before measuring optical density the first time, and 3) 4.5 µl (not 2 µl) of Suspension 3 (contains hexokinase and G6P-DH, used in the final step of D-glucose determination) and Suspension 4 (contains phosphoglucose isomerase, used in the final step of D-fructose determination) was used. I compared results for a series of standards (from 1 to 80 mg/L) using this method to the manufacturer's method. Once I had chosen which method to use, I spiked quality control urine samples, replacing half the normal quality control volume with a 0.4 g/L solution of each sugar to ensure that the assay detected known quantities of each sugar within urine matrices.

2.3.3.3 Urinary potassium and sodium

An ADVIA 2400 Clinical Chemistry System (Siemens AG, Munich, Germany) with ion-selective electrode detection was used to measure urinary potassium and sodium concentrations. This was completed at the Clinical Biochemistry Department in the Leeds Teaching Hospitals NHS Trust. To estimate dietary potassium and sodium intakes, it was assumed that 80% of potassium and 86% of sodium is excreted. Therefore, urinary

potassium was divided by 0.8 and urinary sodium by 0.86, as per previous studies (394, 395).

2.3.4 Energy expenditure

Energy expenditure data were collected and processed by colleagues at Imperial College London. On urinary biomarker collection days each participant also wore an accelerometer (SenseWear; BodyMedia Inc., Pittsburgh, US) for ~ 24 hours on the midline of the left triceps brachii to measure physical activity. Participants were instructed to only remove the armbands when bathing. The accelerometers measure triaxial acceleration, galvanic skin response and skin temperature to estimate physical activity level and metabolic equivalents using the manufacturer's proprietary algorithms.

The day after physical activity measurement, participants rested semi-supine for ~ 30 minutes, during which open-loop indirect calorimetry (GEM Nutrition Ltd., Daresbury, Cheshire, England) was used to measure resting energy expenditure (REE). After calorimeter calibration, VO_2 and VCO_2 were recorded every minute for 15 minutes. The first five minutes of data were discarded to allow stabilisation of measures, and the mean values of the last 10 minutes were used to calculate REE using a previously described equation (396). Ambient temperature was maintained at ~ 21 °C during all measurements. Activity energy expenditure (estimated from the accelerometry data) was multiplied by REE. An assumed diet-induced thermogenesis of 10% of total energy expenditure (TEE) (397) was added to estimate TEE. This method of estimating TEE has close agreement with TEE measurement by doubly-labelled water (398), the gold-standard TEE biomarker. Participants whose bodyweights changed by > 5% during the study were excluded, and it was otherwise assumed that participants were in energy balance. TEE was therefore used as a proxy of energy intake.

2.3.5 Plasma biomarkers

On REE measurement days coinvestigators also collected a 40 ml blood sample into lithium heparin tubes from each participant for biomarker analysis. Samples were centrifuged at 2,000 x g for 10 minutes, and plasma aliquots were collected and stored at -80 °C. Aliquots were then transported to the University of Leeds on dry ice, where HPLC was used to measure plasma concentrations of β-carotene, total vitamin C (ascorbic acid and dehydroascorbic acid), and vitamin E (α-tocopherol) in the Molecular Epidemiology Unit. Detection wavelengths were 452nm for β-carotene, 270nm for

ascorbic acid, and 292nm for α-tocopherol. A detailed description of these methods has been published previously (399).

2.3.6 Dietary recalls

Participants were instructed to recall all foods and drinks consumed the previous day for both recall methods. Participants completed up to three MPRs. These recalls were by telephone and were led by trained personnel. The interviewer used a standardised script with a prompt sheet based on the 5-step multiple-pass method of the US Automated Multiple-Pass Method (385). Dietary intakes were then estimated using Dietplan 6.7 software (Forestfield Software, Horsham, UK) in conjunction with McCance and Widdowson's Composition of Foods tables (sixth edition) (400). Trained coders mapped the foods and drinks recorded to McCance and Widdowson's The Composition of Foods integrated dataset using a standardised protocol described previously (401).

Participants were sent e-mail invitations with links to complete myfood24 recalls on three occasions. Participants had access to frequently asked questions and online videos while using myfood24 (https://www.youtube.com/channel/UCpbMxRnEK0lK8AcSJbg-bPA).

2.3.7 Statistical analyses

For this chapter I tested whether urinary nitrogen and total sugars and their corresponding myfood24 dietary measures (protein and sugar intakes, respectively) differed by participant characteristics. I used independent t-tests to determine if outcomes differed by sex and one-way ANOVAs to test if outcomes differed by age and BMI. For independent t-tests and one-way ANOVAs I used dot plots to identify any unfeasible values, QQ plots to assess normality of distribution in each category, and SDs to check homogeneity of variances. (Variances were considered homogeneous if SDs differed by a factor of < 2.) Bonferroni correction was used for post-hoc testing of one-way ANOVAs. Data that remained positively skewed after log-transformation were tested using the non-parametric Mann-Whitney and Kruskal-Wallis tests, as appropriate. p values ≤ 0.05 were considered significant. My statistical analyses were completed in Stata version 13 (Texas, US).

Given the complexity of the study, all of the main statistical analyses for the validation study were completed by a specialist biostatistician. I did not partake at all in the following analyses. Assuming similar parameters to the EPIC Norfolk and OPEN studies (402, 403), power calculations indicated that a sample size of 200 would allow the attenuation

factor for protein intake to be estimated to about ± 0.08. (The attenuation factor is usually a value between zero and one that indicates how well each dietary recall method detects diet–disease relationships. Lower attenuation factors indicate greater attenuation of diet-disease associations, biasing estimates of disease closer to one and therefore requiring larger sample sizes to compensate for lower statistical power.) Furthermore, a sample size of 200 would allow the correlation between myfood24 and estimates of true long-term intakes to be estimated to about ± 0.1. (Correlation is a measure of the loss of statistical power to identify diet-disease associations when using reported rather than true intakes.) This would also allow the mean difference between the MPR and myfood24 to be estimated to about ± 0.4 g nitrogen.

Other than participants with two or more missing urine voids during a 24-hour collection period (404), all participants were included in analyses. The main analysis was of long-term intakes. Attenuation factors and partial correlation coefficients between each dietary assessment tool and estimated long-term intakes (measuring the loss of power and attenuation of log relative risks between intake categories) were estimated from structural equation models. The models used the method of maximum likelihood and assumed that 1) multivariate data distributions were normal after log-transformation, and 2) any missing observations were missing at random. The models estimate the bias in each dietary assessment tool compared to reference measures based on mean self-reported intakes over the replicates for each participant minus the means over the replicates for the biomarkers. Another assumption the project statistician made was that of two systematic components of dietary measurement error: 1) positive correlation between true intake and error and 2) person-specific bias that is independent of intake (405). The data were back-transformed and are expressed as a percentages. This is the equivalent to the mean difference in the Bland-Altman approach (406).

Intraclass correlation coefficients (ICCs) for absolute agreement between estimated intakes and the concentration biomarkers (β-carotene, total vitamin C, and vitamin E) were derived from two-way mixed effects models (dietary assessment method as the fixed effect). To allow for different responses for the two dietary assessment tools, a subject-by-method interaction test was included. The variance of random coefficients was allowed to vary. This was also true of measurement error variances between methods. The analysis focussed on individual 24-hour periods rather than averages of the three time periods (407-409).

The mean differences in estimates between the dietary assessment methods (an estimate of relative bias) are also presented for nutrients without biomarkers, as well as estimated limits of agreement (a proxy of precision) (406).

Sensitivity analyses were completed including only participants with complete PABA recovery (85-110%) and also adjusting urinary nitrogen, potassium, and sodium to 93% PABA recovery if recovery was 50-85% (410). To determine whether participant characteristics influenced how robust results were, analyses were also repeated after stratification by age, BMI, and sex. Finally, to determine if repeated recalls improve estimates of long-term intakes, attenuation factors and correlations were estimated for a series of two, four, or seven administrations of myfood24, using a previously published approach (411).

Data were log-transformed for analyses to normalise their distribution. The main statistical analyses were completed in Stata version 14.2 (Texas, US).

2.4 Results

In total, 19% of people contacted through Clinical Research Facility lists responded and 4% of people contacted by post responded. Of the 289 respondents invited, 243 attended and were deemed eligible during the screening visit. During the study, 31 participants then withdrew. At least one dietary recall for each of the recall methods was completed by 212 participants (Table 2.1 shows participant characteristics).

Table 2.1. Participant characteristics.

Characteristic	Men (n = 85)	Women (n = 127)
Age	43 ± 15	44 ± 16
Body mass index (kg/m^2)	26 ± 4	25 ± 5
Race (% white)	74%	72%
Smoking (% current smoker)	14%	10%

Data are means ± SDs.

There were 12 x 24-hour collection periods (accounted for by 11 of the participants) at which more than one urine sample was missed. Urine biomarker results from these collection periods were excluded from the main analysis, but no participants were excluded entirely from the main analysis because samples were collected up to three times. Weight changed substantially (> 5% weight change from first clinic appointment) in six participants, and energy expenditure results for these participants were excluded from the main analysis.

2.4.1 My laboratory analyses and the corresponding dietary outcomes

2.4.1.1 Urinary nitrogen and dietary protein

The most important preliminary check I made was of the sensitivity of the urinary nitrogen method. This confirmed consistently accurate detection in concentrations ≥ 0.0195 mg/ml (Figure 2.3), well below the lowest concentration of any sample analysed (0.91 mg/ml).
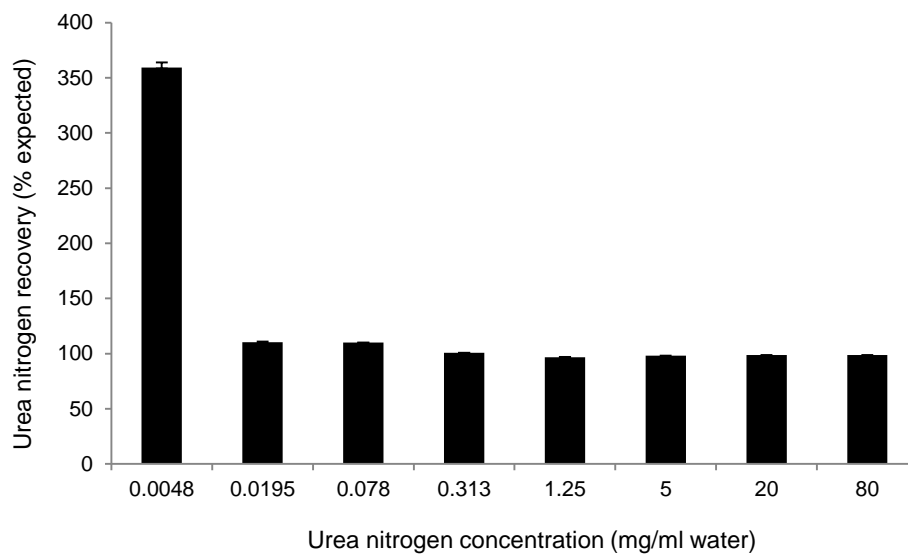
Figure 2.3. Kjeldahl nitrogen detection sensitivity analysis.

Nitrogen was accurately detected in concentrations ≥ 0.0195 mg/ml. Data are means of four runs, error bars are SDs.

During analysis of myfood24 samples, the inter-assay CV of the pre-run nitrogen recovery check using ammonium sulphate was 0.6%, and the inter-assay CV of the urea sample included alongside samples each run was 1.0%. Urea sample nitrogen recovery was slightly below 100% (Figure 2.4).
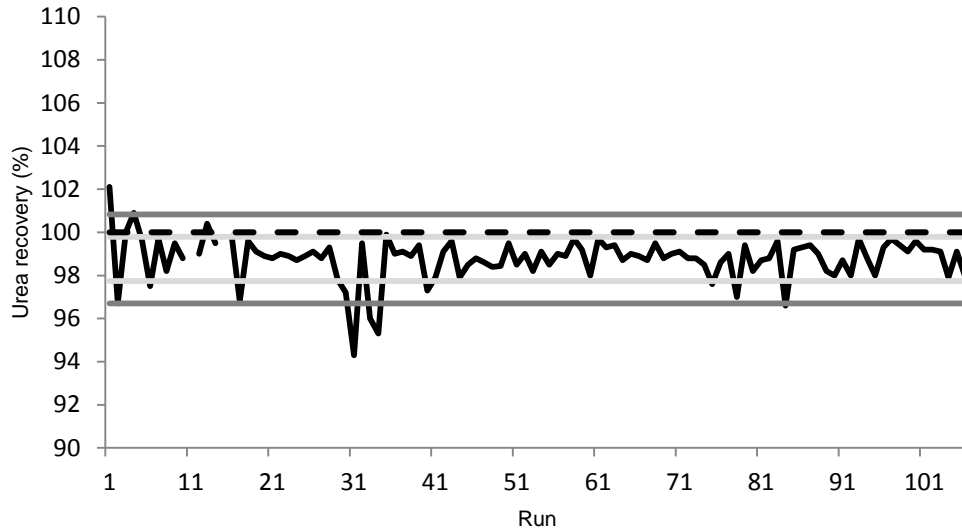
Figure 2.4. Kjeldahl Levey-Jennings chart displaying inter-assay nitrogen recovery from a urea control.

Light grey lines 1 SD from mean, dark grey lines 2 SDs from mean, dashed black line theoretical 100% recovery.

The inter-assay CV of the primary urinary nitrogen quality control sample was 1.4% (Figure 2.5), and the inter-assay CVs of the three other urinary nitrogen quality controls that were included every 10 runs were 1.0% for the least concentrated, 2.7% for the intermediate concentration, and 0.6% for the most concentrated.
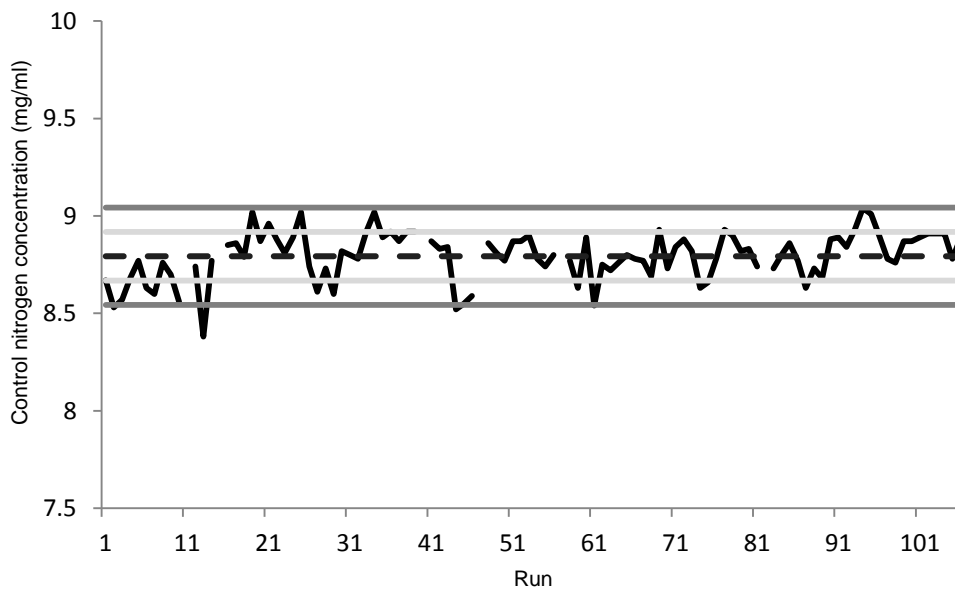
Figure 2.5. Kjeldahl Levey-Jennings chart displaying inter-assay reproducibility of nitrogen measurement in a control urine sample.

Light grey lines 1 SD from mean, dark grey lines 2 SDs from mean, dashed black line mean recovery.

Excluding samples that were reanalysed, I analysed 575 samples for nitrogen. Urinary nitrogen concentration data were positively skewed and so were log-transformed (Figure 2.6). An independent t-test showed that urinary nitrogen concentration was lower in women than men ($t(572) = -6.44$, $p < 0.001$, urinary nitrogen and dietary protein intake descriptive data are summarised in Table 2.2). One-way ANOVA testing showed that urinary nitrogen concentrations differed between age groups ($F(4,569) = 10.76$, $p < 0.001$). Post-hoc pairwise tests with Bonferroni correction showed that 18- to 27-year-olds had higher urine nitrogen concentrations than 48- to 57-year-olds ($p < 0.001$) and 58- to 68-year-olds ($p < 0.001$). Twenty-eight- to 37-year-olds tended to have higher urinary nitrogen concentrations than 58- to 68-year-olds ($p = 0.02$). And 38- to 47-year-olds tended to have higher urine nitrogen concentrations than 58- to 68-year-olds ($p = 0.04$). One-way ANOVA testing also showed that urinary nitrogen concentrations differed between BMI categories ($F(2,552) = 4.65$, $p = 0.001$). Post-hoc pairwise tests with Bonferroni correction showed that participants with BMIs ≥ 30 kg/m^2 tended to have higher urinary nitrogen concentrations than both those with BMIs of 25 to 29.99 kg/m^2 ($p = 0.014$) and those with BMIs < 25 kg/m^2 ($p = 0.013$).
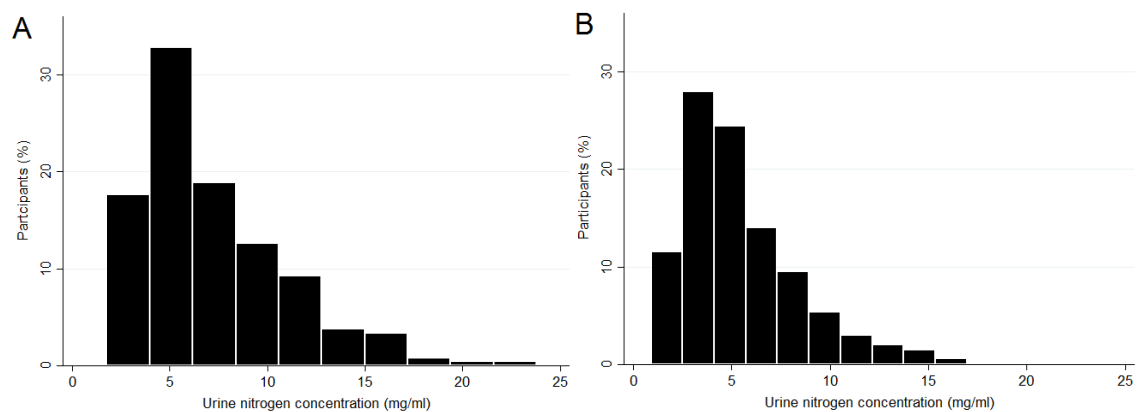
Figure 2.6. Urinary nitrogen concentration data distribution in men (A) and women (B).

Table 2.2. Urinary nitrogen and dietary protein data according to participant characteristics.

Characteristic	Group	Urinary nitrogen concentration (mg/L)[a]	n[b]	p value	myfood24 protein intake (g)[a]	n[b]	p value	myfood24 protein intake (% total energy)[a]	n[b]	p value
Sex	Females	4.83 (3.33, 6.85)	336	< 0.001	65 (50, 84)	320	< 0.001	15.7 (12.6, 19.8)	320	0.24
	Males	6.16 (4.45, 9.54)	238		87 (62, 113)	218		16.2 (13.5, 19.2)	218	
Age category	18 to 27 years	6.72 (4.60, 9.55)	127	< 0.001	73 (50, 107)	121	0.64	16.6 (13.5, 21.1)	121	0.41
	28 to 37 years	5.91 (3.87, 8.70)	97		63 (54, 97)	94		14.8 (12.4, 18.4)	94	
	38 to 47 years	5.46 (4.09, 8.54)	76		78 (57, 102)	73		16.3 (12.2, 19.4)	73	
	48 to 57 years	4.65 (3.22, 6.75)	139		73 (53, 92)	125		15.9 (12.9, 19.6)	125	
	58 to 68 years	4.64 (3.31, 6.35)	135		71 (57, 92)	125		15.7 (12.9, 19.4)	125	
BMI category	18 to 24.99 kg/m^{2}	5.26 (3.57, 7.66)	281	0.001	67 (51, 89)	261	0.002	15.4 (12.4, 18.5)	261	0.001
	25 to 29.99 kg/m^{2}	5.07 (3.75, 7.46)	195		79 (60, 106)	189		17.1 (13.3, 20.6)	189	
	≥ 30 kg/m^{2}	6.21 (4.54, 10.03)	79		77 (59, 98)	70		14.7 (12.6, 19.2)	70	

Legend: BMI (body mass index).

[a]Values are medians (quartile 1, quartile 3) because data were positively skewed.

[b]Numbers differ because not all participants with nitrogen data also had all dietary and descriptive data recorded by coinvestigators.

Protein intake data (g per day) were positively skewed and so were log-transformed (Figure 2.7). An independent t-test showed that protein intake was higher in men than women ($t(536)$ = -6.75, $p < 0.001$, data are summarised in Table 2.2). A one-way ANOVA test showed that dietary protein intake did not differ between age groups ($F(4,533)$ = 0.64, $p = 0.64$). Another one-way ANOVA test showed that dietary protein intake differed between BMI categories ($F(2,517)$ = 6.48, $p = 0.002$). Post-hoc pairwise tests with Bonferroni correction showed that participants with BMIs of 25 to 29.99 kg/m^2 had higher dietary protein intake than those with BMIs < 25 kg/m^2 ($p = 0.001$).
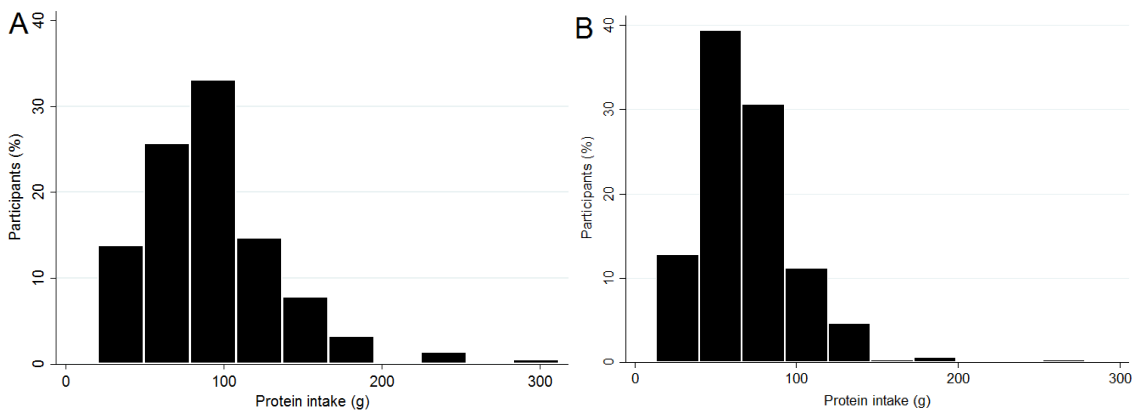
Figure 2.7. Dietary protein intake data distribution in men (A) and women (B).

Protein intake data (% daily energy intake) were positively skewed and so were log-transformed (Figure 2.8). An independent t-test showed that protein intakes (% daily energy intake) did not differ between sexes ($t(536)$ = -1.17, $p = 0.24$, data are summarised in Table 2.2). A one-way ANOVA test showed that dietary protein intake (% daily energy intake) did not differ between age groups ($F(4,533)$ = 0.99, $p = 0.41$). Another one-way ANOVA test showed that dietary protein intake (% daily energy intake) differed between BMI categories ($F(2,517)$ = 4.66, $p = 0.001$). Post-hoc pairwise tests with Bonferroni correction showed that participants with BMIs of 25 to 29.99 kg/m^2 had higher dietary protein intake (% daily energy intake) than those with BMIs < 25 kg/m^2 ($p = 0.01$).
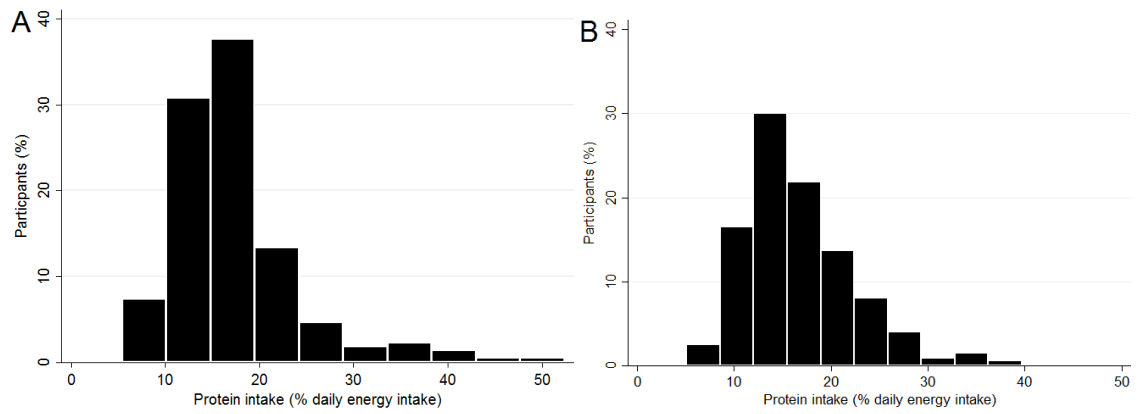
Figure 2.8. Dietary protein intake (% daily energy intake) data distribution in men (A) and women (B).

Analysis of the contributions of each of the myfood24 food and drink categories to protein intake of all participants showed that, together, three categories (1) ready meals, 2) meat and poultry, 3) dairy and eggs) comprised more than half of dietary protein intake (Figure 2.9).
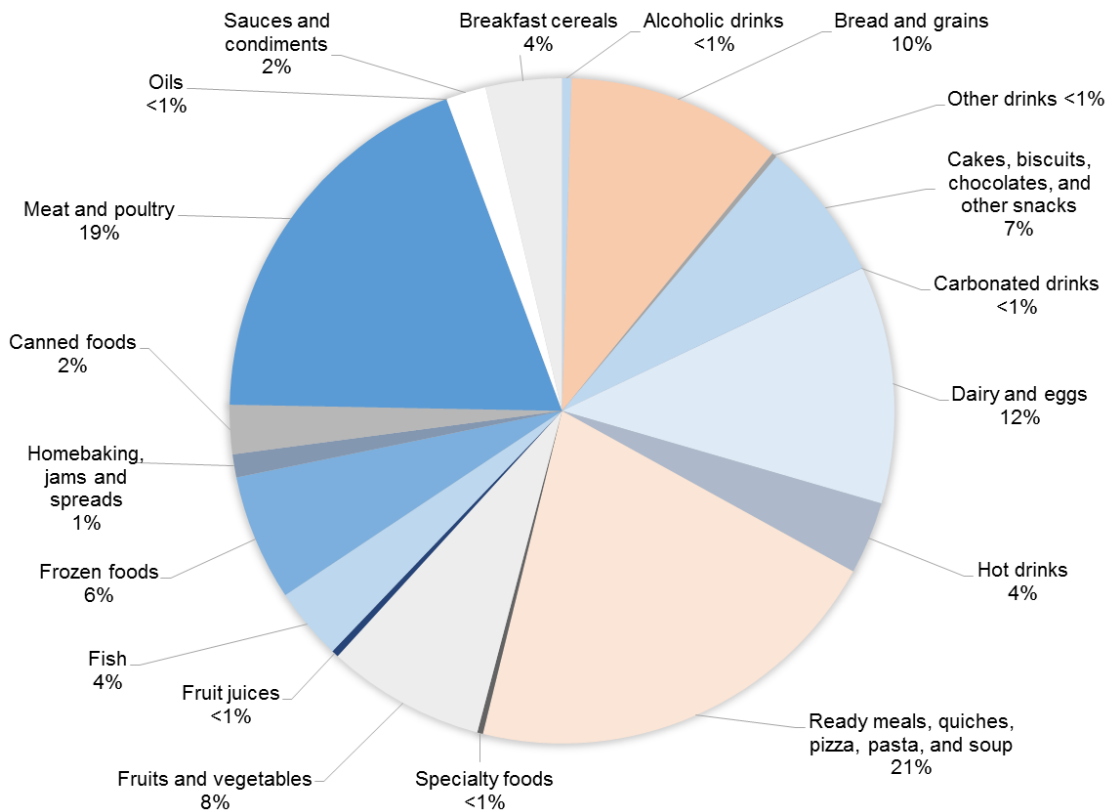
Figure 2.9. Contributions of food and drink categories to protein intake (n = 550 recalls, men and women combined).

2.4.1.2 Urinary and dietary sugars

For the sugars analysis, I first compared the methods of Dr Lampe to those of the manufacturer. The results were similar for fructose and glucose, but results for sucrose were superior using the manufacturer's method (Figure 2.10). I therefore used the manufacturer's method for sample analysis.
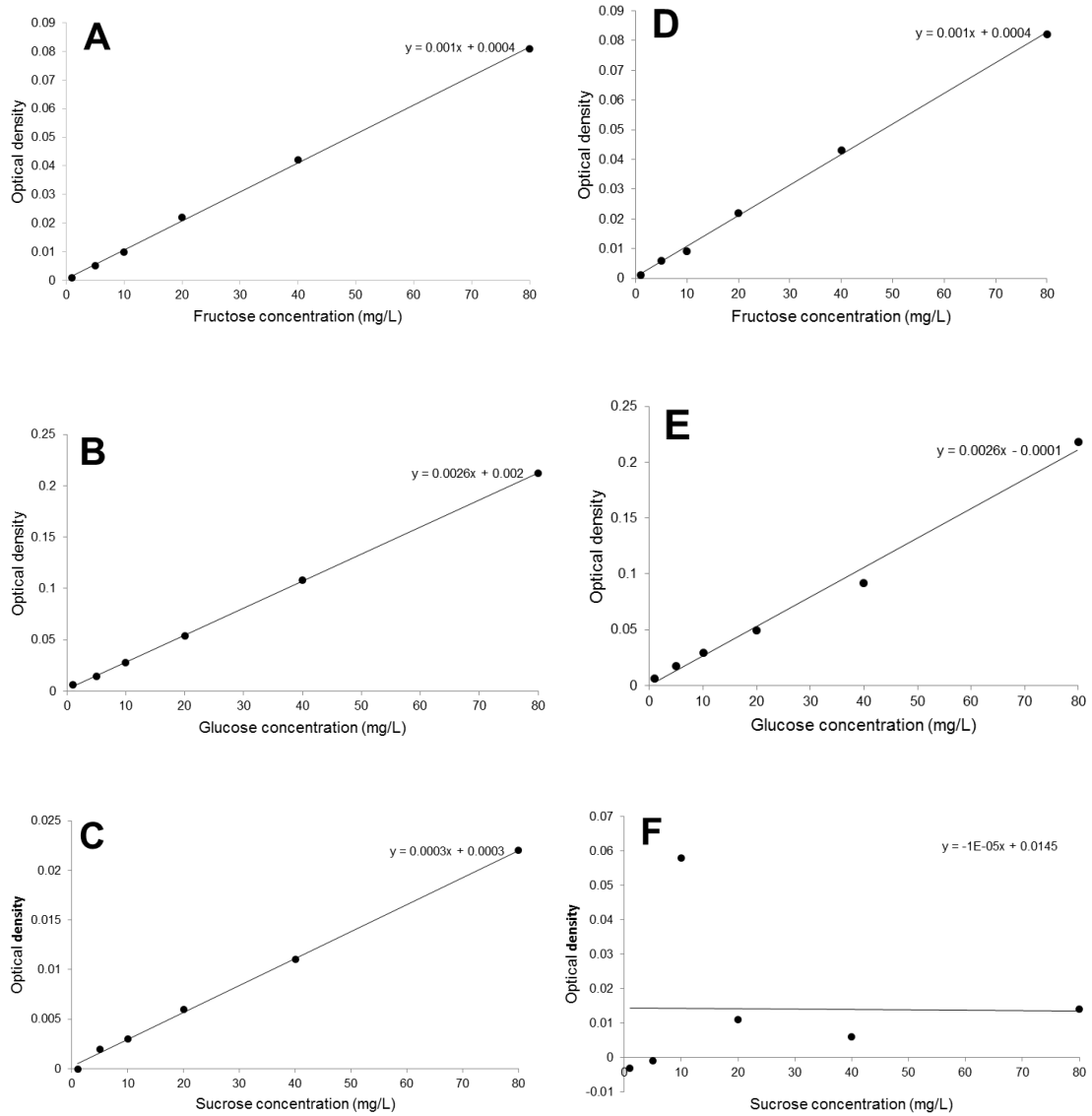
Figure 2.10. Urinary sugars method comparison.

Results for standards of each sugar were compared using the manufacturer's method (A to C) and a modified method (D to F). The modified method did not produce the expected curve for sucrose.

The urine spiking experiments showed that known sugar quantities added to urine could be detected: spike recovery was 103 ± 10%. The fructose inter-assay CV was 31.6%, the glucose inter-assay CV 13.2%, and the sucrose inter-assay CV 66.1%.

Excluding samples that were reanalysed, I analysed 574 samples for urinary sugars. The limit of detection was 1 mg/L for fructose and 25 mg/L for sucrose. Samples below these limits were assigned values of half of the limits of detection. Fifteen samples exceeded a concentration of 200 mg/L. Of these, six samples were above 1,000 mg/L, and such large outliers meant that urinary sugar concentration data remained positively skewed after log-transformation (Figure 2.11). A Mann-Whitney test showed that urinary sugar concentration did not differ between the sexes (z = -1.11, p = 0.27, urinary and dietary sugar descriptive data are summarised in Table 2.3). A Kruskal-Wallis test showed that urinary sugar concentrations differed between the five age groups ($\chi^2(4)$ = 46.18, p < 0.001), with a mean rank of 346 in the 18- to 27-year-olds, 307 in the 28- to 37-year-olds, 331 in the 38- to 47-year-olds, 252 in the 48- to 57-year-olds, and 228 in the 58- to 68-year-olds. (Mean ranks are detailed because variances were heterogeneous between groups: higher ranks indicate higher urinary sugar concentrations.) Another Kruskal-Wallis test showed that urinary sugar concentrations differed between the three BMI categories ($\chi^2(2)$ = 13.66, p = 0.001), with a mean rank of 294 in the 18 to 24.99 kg/m^2 group, 244 in the 25 to 29.99 kg/m^2 group, and 304 in the ≥ 30 kg/m^2 group.
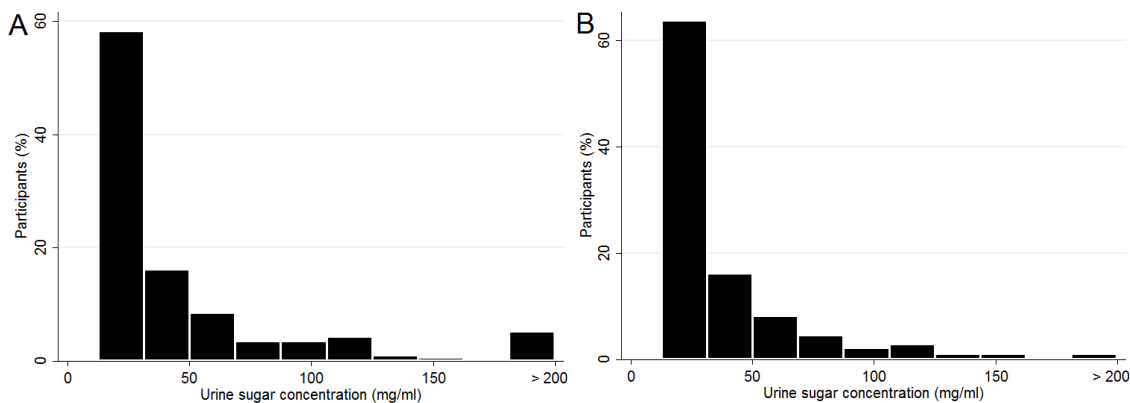
Figure 2.11. Total (fructose and sucrose) urinary sugar concentration data distribution in men (A) and women (B).

Table 2.3. Total urinary and dietary sugar data according to participant characteristics.

(Note that total urinary sugar comprises urinary fructose and urinary sucrose alone.)

Characteristic	Group	Urinary sugar concentration (mg/L)[a]	n[b]	p value	myfood24 total sugar intake (g)[a]	n[b]	p value	myfood24 total sugar intake (% total energy)[a]	n[b]	p value
Sex	Females	24 (17, 45)	336	0.27	71 (46, 99)	320	0.03	17.4 (12.5, 23.2)	320	0.03
	Males	25 (17, 51)	237		79 (51, 122)	217		15.3 (10.9, 21.5)	217	
Age category	18 to 27 years	33 (20, 64)	127	< 0.001	69 (49, 98)	121	0.28	15.0 (10.8, 22.9)	121	0.52
	28 to 37 years	27 (17, 51)	97		75 (46, 108)	94		16.8 (10.8, 21.4)	94	
	38 to 47 years	29 (20, 56)	76		88 (53, 136)	73		17.1 (12.1, 23.9)	73	
	48 to 57 years	21 (16, 36)	137		71 (47, 96)	123		16.1 (12.6, 21.3)	123	
	58 to 68 years	20 (15, 34)	136		81 (53, 110)	126		18.3 (12.7, 23.2)	126	
BMI category	18 to 24.99 kg/m^2	27 (18, 50)	281	0.001	77 (53, 112)	261	0.47	17.8 (13.0, 23.3)	261	0.03
	25 to 29.99 kg/m^2	21 (16, 38)	196		75 (46 to 103)	190		15.3 (10.8, 22.1)	190	
	≥ 30 kg/m^2	27 (17, 73)	77		70 (46, 106)	68		14.7 (11.6, 20.5)	68	

Legend: BMI (body mass index).

[a]Values are medians (quartile 1, quartile 3) because data were positively skewed.

[b]Numbers differ because not all participants with biomarker data also had all dietary and descriptive data recorded by coinvestigators.

myfood24 dietary sugar intake data were positively skewed and so were log-transformed (Figure 2.12). An independent t-test showed that myfood24 dietary sugar intake tended to be lower in women than men ($t(535) = -2.15$, p = 0.03, data are summarised in Table 2.3). One-way ANOVA testing showed that myfood24 dietary sugar intake did not differ between age groups ($F(4,532) = 1.28$, p = 0.28) or BMI categories ($F(2,516) = 0.76$, p = 0.47).
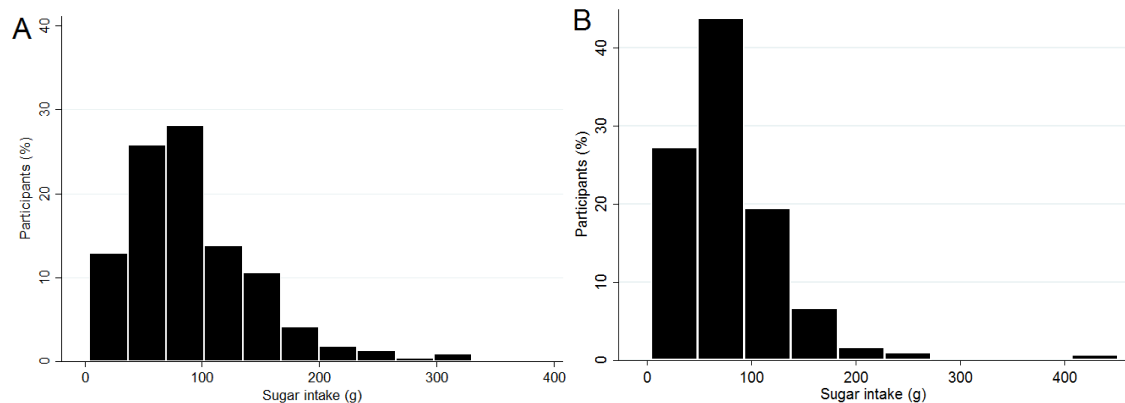
Figure 2.12. Total dietary sugar intake data distribution in men (A) and women (B).

myfood24 dietary sugar intake (% daily energy intake) data were normally distributed (Figure 2.13). An independent t-test showed that myfood24 dietary sugar intake (% daily energy intake) tended to be higher in women than men ($t(535) = 2.21$, p = 0.03, data are summarised in Table 2.3). One-way ANOVA testing showed that myfood24 dietary sugar intakes (% daily energy intake) were not different between age groups ($F(4,532) = 0.80$, p = 0.52) but tended to differ between BMI categories ($F(2,516) = 3.62$, p = 0.03). However, post-hoc pairwise tests with Bonferroni correction showed that the difference between the most dissimilar groups (the lowest and highest BMI categories) was not quite significant (p > 0.06), perhaps because the Bonferroni correction is conservative.
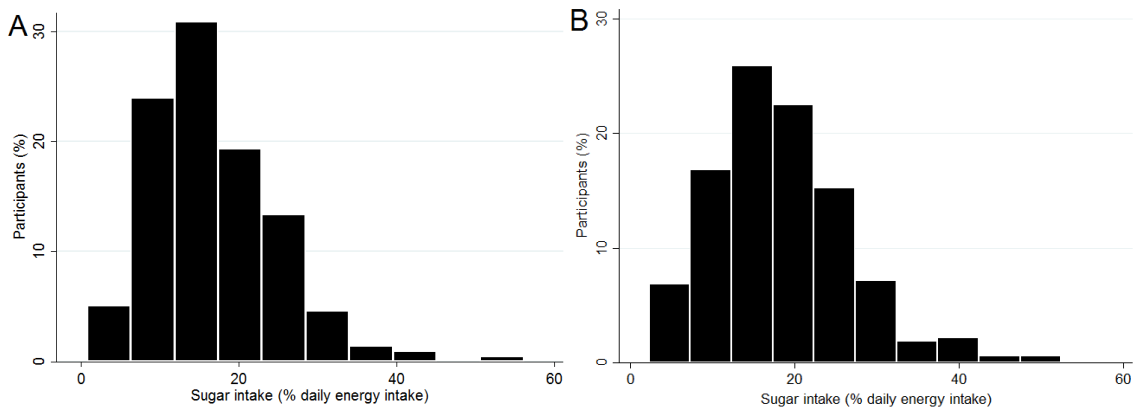
Figure 2.13. Total dietary sugar intake (% daily energy intake) data distribution in men (A) and women (B).

Analysis of the contributions of each of the myfood24 food and drink categories to sugar intake of all participants showed that, together, two categories (1) fruits and vegetables, 2) cakes, biscuits, chocolates, and other snacks) comprised nearly half of dietary sugar intake (Figure 2.14).
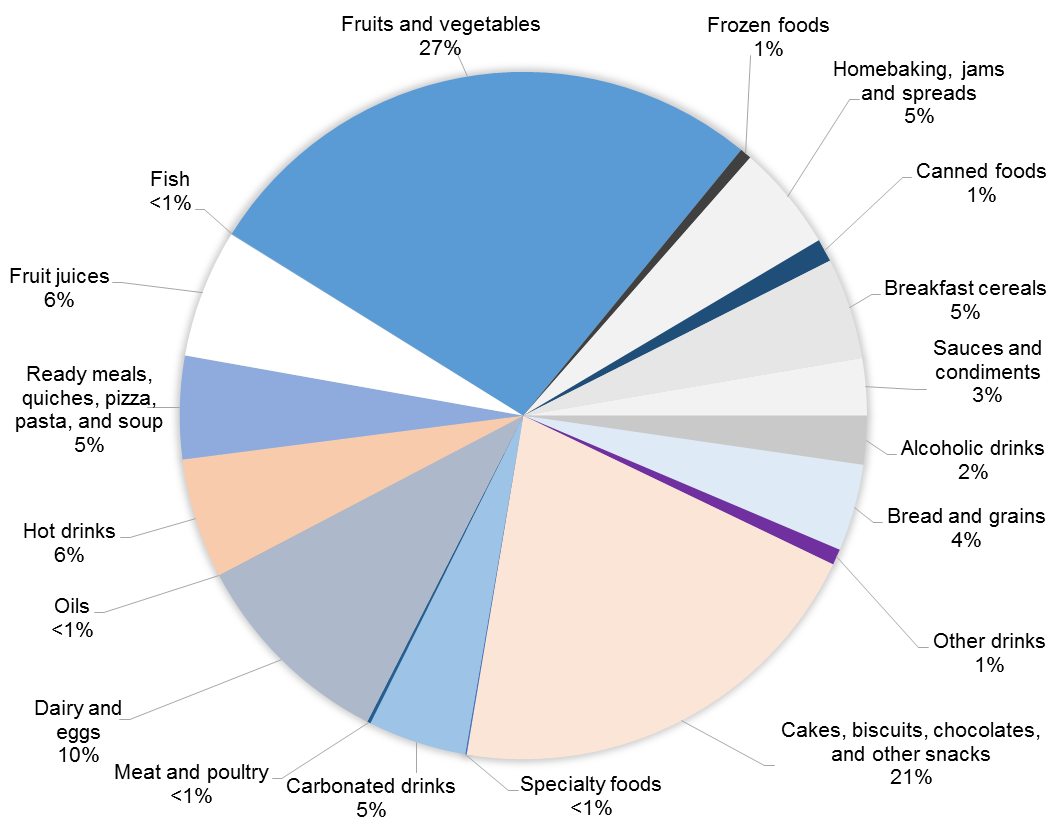
Figure 2.14. Contributions of food and drink categories to sugar intake (n = 550 recalls, men and women combined).

2.4.2 Main analyses

Table 2.4 shows the geometric means and 95% confidence intervals for protein, potassium, sodium, and total sugar intakes at the first clinic visit as assessed by the MPR, myfood24, and the reference measures. myfood24 estimates at the first clinic visit were similar to reference measures for protein, higher for potassium and sodium, and lower for total sugars and energy intake (compared to estimated TEE). Whereas the MPR overestimated protein intake, myfood24 protein intake was similar to estimated true protein intake, however. The MPR and myfood24 resulted in largely similar results. myfood24 resulted in slightly lower estimates of nutrient intakes, as well as lower estimates of nutrient densities (the ratio (%) of energy from each nutrient relative to total energy), with the exception of sodium. Differences between the recall methods and the reference measures indicated that the recall methods overestimated potassium and sodium intakes and underestimated total sugar and energy intakes. Recall method results for nutrient intakes were slightly better than those for energy intake and nutrient densities. Other than sugars, myfood24 nutrient densities were higher than the reference measures because myfood24 energy intakes were lower than reference measure estimates.

Table 2.4. Estimated intakes (geometric means) for protein, potassium, sodium, and total sugar intakes and densities as assessed by myfood24, the interviewer-administered multiple-pass 24-hour recall method, and reference measures for the first clinic visit.

Characteristic		myfood24		MPR		Reference measures
	n	Geometric mean (95% CI)	n	Geometric mean (95% CI)	n	Geometric mean (95% CI)
Nutrient intakes						
Protein (g)	208	70.5 (66.1 to 75.2)	197	81.7 (77.3 to 86.4)	192	68.4 (64.1 to 72.8)
Total sugar (g)[a]	208	72.8 (66.4 to 79.8)	197	91.0 (85.0 to 97.5)	191	128.3 (115.9 to 142.0)
Potassium (g)	208	2.7 (2.5 to 2.9)	197	3.1 (3.0 to 3.3)	192	2.1 (1.9 to 2.3)
Sodium (g)	208	2.3 (2.1 to 2.5)	197	2.4 (2.2 to 2.6)	192	1.8 (1.7 to 2.0)
Protein intake density (g/MJ energy intake)	208	9.5 (9.0 to 9.9)	197	9.6 (9.2 to 10.0)	180	6.2 (5.8 to 6.7)
Total sugar intake density (g/MJ energy intake)[a]	208	9.8 (9.1 to 10.5)	197	10.7 (10.1 to 11.4)	179	11.6 (10.4 to 12.9)
Potassium intake density (g/MJ energy intake)	208	0.36 (0.35 to 0.38)	197	0.37 (0.35 to 0.39)	180	0.19 (0.18 to 0.21)
Sodium intake density (g/MJ energy intake)	208	0.31 (0.29 to 0.33)	197	0.28 (0.27 to 0.30)	186	0.16 (0.15 to 0.18)
Energy expenditure						
Total energy expenditure (MJ)	208	7.5 (7.1 to 7.9)	197	8.5 (8.1 to 8.9)	185	11.0 (10.5 to 11.6)

Legend: CI (confidence interval), MPR (interviewer-administered multiple-pass 24-hour recall method).

[a]Based on recall intakes and predicted intakes for the reference measure, according to feeding studies (393).

The attenuation factors (the degree to which diet–disease relationships are attenuated) used to estimate long-term nutrient intakes and densities were relatively poor for both dietary recall methods, and myfood24 attenuation factors were slightly lower than the MPR (Table 2.6). Partial correlation coefficients comparing recall method values with estimated true long-term intakes were similar for myfood24 and the MPR. Analysis of how repeated use of myfood24 influences estimates of true long-term intakes showed that estimates improved with more recalls (protein and sugar results are in Table 2.5).

Table 2.5. Attenuation factors and correlations between myfood24 and estimated true protein and total sugar intakes for different numbers of administrations of myfood24.

Dietary variable	Number of administrations	Attenuation factor (95% CI)	Correlation with estimated true intake (95% CI)
Protein intake (g)	1	0.30 (0.21 to 0.38)	0.43 (0.32 to 0.53)
	2	0.42 (0.30 to 0.53)	0.51 (0.39 to 0.63)
	4	0.52 (0.38 to 0.67)	0.57 (0.44 to 0.70)
	7	0.59 (0.42 to 0.75)	0.60 (0.46 to 0.74)
Total sugar intake (g)	1	0.15 (0.06 to 0.24)	0.24 (0.09 to 0.38)
	2	0.20 (0.08 to 0.33)	0.28 (0.11 to 0.45)
	4	0.25 (0.09 to 0.40)	0.31 (0.12 to 0.49)
	7	0.27 (0.10 to 0.44)	0.32 (0.13 to 0.52)

Legend: CI (confidence interval).

All data were positively skewed and so were log-transformed.

After excluding urine collections in which PABA recovery was below 50% or above 110%, and after adjusting urinary nitrogen, potassium, and sodium to 93% PABA recovery if sample PABA recovery was 50 to 85%, derived protein (77g versus 68g), potassium (2.4g versus 2.1g), and sodium (2.1g versus 1.8g) intakes all increased, and the results were closer to self-reported intakes. Exclusion and adjustment did not markedly influence attenuation factor estimates, however (protein 0.27 versus 0.30, potassium 0.29 versus 0.31, sodium 0.19 versus 0.21). Furthermore, correlations between self-reported and estimated true intakes were not substantially different (protein 0.50 versus 0.43, potassium 0.48 versus 0.40, sodium 0.37 versus 0.30). After stratification by age, BMI, and sex, attenuation factors were generally similar, but with less attenuation for energy and nutrient intakes among younger participants (protein and sugar results are in

Appendix A, Table 1), leaner participants (protein and sugar results are in Appendix A, Table 2), and male participants (protein and sugar results are in Appendix A, Table 3). Intraclass correlations between plasma antioxidant concentrations and estimated intakes from myfood24 and the MPR were similar, although myfood24 estimates were lower than MPR estimates (Table 2.7).

Finally, myfood24 estimates of dietary intakes were compared to MPR estimates for nutrients, including those without reference measures. myfood24 estimates were generally 10 to 20% lower than MPR estimates. Limits of agreement were wide, reflecting substantial day-to-day variation in diets. Intraclass correlation coefficients comparing myfood24 and MPR estimates were generally 0.4 to 0.5, indicating moderate agreement. Table 2.8 includes results for energy, macronutrient, and fibre intakes.

Table 2.6. Attenuation factors, correlations between dietary recall methods and estimated true intakes, and mean differences between dietary recall methods and reference measures for protein, potassium, sodium, and total sugar intakes and densities for myfood24 and the interviewer-administered multiple-pass 24-hour recall method.

	Dietary recall method	Attenuation factor (95% CI)	Correlation with estimated true intake (95% CI)	Mean % difference compared to reference measures (95% CI)
Nutrient intakes				
Protein (g)	myfood24	0.30 (0.21 to 0.38)	0.43 (0.32 to 0.53)	1% (-4% to 7%)
	MPR	0.38 (0.29 to 0.47)	0.48 (0.39 to 0.58)	11% (6% to 17%)
Total sugars (g)[b]	myfood24	0.15 (0.06 to 0.24)	0.24 (0.09 to 0.38)	-45% (-39% to -50%)
	MPR	0.25 (0.14 to 0.36)	0.31 (0.18 to 0.44)	-30% (-24% to -35%)
Potassium (g)	myfood24	0.31 (0.21 to 0.41)	0.40 (0.28 to 0.52)	26% (19% to 35%)
	MPR	0.35 (0.23 to 0.46)	0.38 (0.27 to 0.49)	48% (39% to 57%)
Sodium (g)	myfood24	0.21 (0.12 to 0.30)	0.30 (0.18 to 0.41)	22% (13% to 32%)
	MPR	0.22 (0.11 to 0.32)	0.28 (0.15 to 0.40)	28% (18% to 38%)
Protein intake density (g/MJ energy intake)	myfood24	0.16 (0.03 to 0.29)	0.17 (0.03 to 0.32)	48% (39% to 58%)
	MPR	0.26 (0.11 to 0.40)	0.24 (0.11 to 0.37)	46% (38% to 55%)
Total sugar intake density (g/MJ energy intake)[a]	myfood24	0.16 (0.04 to 0.28)	0.21 (0.06 to 0.36)	-19% (-11% to -26%)
	MPR	0.23 (0.09 to 0.37)	0.25 (0.10 to 0.39)	-8% (-15% to 0%)
Potassium intake density (g/MJ energy intake)	myfood24	0.25 (0.09 to 0.41)	0.23 (0.09 to 0.37)	85% (72% to 99%)
	MPR	0.38 (0.23 to 0.53)	0.34 (0.22 to 0.47)	93% (81% to 107%)
Sodium intake density (g/MJ energy intake)	myfood24	0.08 (-0.03 to 0.19)	0.09 (-0.04 to 0.21)	78% (63% to 94%)
	MPR	0.11 (-0.02 to 0.24)	0.11 (-0.02 to 0.24)	66% (52% to 80%)

Energy expenditure				
Total energy expenditure (MJ)	myfood24	0.19 (0.10 to 0.29)	0.29 (0.15 to 0.42)	-31% (-27% to -35%)
	MPR	0.32 (0.21 to 0.43)	0.37 (0.25 to 0.49)	-23% (-19% to -27%)

Legend: CI (confidence interval), MPR (interviewer-administered multiple-pass 24-hour recall method).

All data were positively skewed and so were log-transformed.

[b]Based on recall intakes and predicted intakes for the reference measure, according to feeding studies (393).

Table 2.7. Estimated intakes at first dietary recalls (geometric means) and intraclass correlation coefficients between plasma antioxidant biomarkers and myfood24 and interviewer-administered multiple-pass 24-hour recall method across the three clinic visits.

Antioxidant	Biomarker	myfood24		MPR	
	Geometric mean (95% CI)	Geometric mean (95% CI)	Intraclass correlation coefficient with biomarker	Geometric mean (95% CI)	Intraclass correlation coefficient with biomarker
β-carotene	0.59 (0.52 to 0.67) µM	0.65 (0.51 to 0.83) mg	0.56 (0.52 to 0.60)	1.53 (1.20 to 1.95) mg	0.52 (0.48 to 0.56)
Vitamin C	60 (57 to 64) µM	59 (51 to 69) mg	0.53 (0.50 to 0.57)	75 (66 to 85) mg	0.53 (0.49 to 0.56)
Vitamin E	37 (35, 40) µM	1.6 (1.3 to 1.9) mg	0.55 (0.50 to 0.59)	2.3 (2.0 to 2.8) mg	0.53 (0.49 to 0.57)

Legend: CI (confidence interval), MPR (interviewer-administered multiple-pass 24-hour recall method).

[a]Data for differences in means and limits of agreement were log-transformed. They are presented as % differences.

Table 2.8. Geometric mean intakes at first dietary recalls only, percent differences in means for all recalls, limits of agreement, and intraclass correlation coefficients for nutrient intakes estimated using myfood24 and interviewer-administered multiple-pass 24-hour recall method across the three clinic visits.

Diet variable	myfood24	MPR			
	Geometric mean (95% CI)	Geometric mean (95% CI)	Difference in means (%, MPR as reference)[a]	Limits of agreement (%, MPR as reference)[a]	Intraclass correlation coefficient
Energy (MJ)	7.5 (7.1 to 7.9)	8.5 (8.1 to 8.9)	-10% (-14% to -6%)	-63% to 118%	0.51 (0.48 to 0.54)
Carbohydrate (g)	198 (186 to 211)	224 (211 to 236)	-12% (-17% to -7%)	-70% to 162%	0.54 (0.51 to 0.56)
Fat (g)	68 (64 to 73)	82 (77 to 88)	-14% (-18% to -9%)	-74% to 182%	0.42 (0.40 to 0.45)
Protein (g)	72 (68 to 78)	78 (73 to 82)	-9% (-14% to -5%)	-68% to 158%	0.45 (0.42 to 0.48)
Englyst fibre (g)	14 (13 to 15)	15 (14 to 16)	-14% (-19% to -9%)	-75% to 199%	0.43 (0.41 to 0.46)

Legend: CI (confidence interval), MPR (interviewer-administered multiple-pass 24-hour recall method).

[a]Data for differences in means and limits of agreement were log-transformed. They are presented as % differences.

2.5 Discussion

Comparison between myfood24 and the MPR shows that the methods produce relatively similar results, although myfood24 systematically resulted in slightly lower estimates of dietary intakes. Our findings are mostly consistent with results of similar studies of online dietary assessment methods, which have generally found good agreement between online methods and reference measures (378). Comparison between multiple 24-hour recall methods and multiple FFQs suggests that 24-hour recall methods have stronger agreement with reference measures of dietary intakes (369, 370). When myfood24 was used to estimate nutrient intakes, attenuation values were generally ~ 0.2 to 0.3. Attenuation factors are useful for de-attenuating observed relative risks during data analysis. Attenuation and correlation values below ~ 0.4 are generally considered poor. For example, an attenuation factor of 0.4 would mean a true relative risk of 2.0 would be attenuated to 1.32 ($2.0^{0.4}$) (369). Although less than 0.4, the attenuation factors for myfood24 are comparable to values for other 24-hour dietary recall methods and are mostly superior to attenuation factors for FFQs reported in previous studies, although there is substantial heterogeneity between study findings using both recalls and FFQs (369, 370). myfood24 is therefore better able to detect diet-disease relationships than FFQs and is comparable to the MPR, the gold-standard dietary recall method. myfood24 will be a valuable tool for large-scale studies exploring diet-health associations in the UK.

Comparing the geometric means for myfood24 and the reference measures, it appears that myfood24 underestimated energy intakes, and this is commensurate with previous results using 24-hour recall methods (369). Few contemporary dietary assessment methods seem to estimate energy intake well as yet. Both myfood24 and the MPR produced slightly higher protein, potassium, and sodium intake estimates, and substantially lower sugar intake estimates than the reference measures. These differences between reported intakes and estimated true intakes (reporting bias) are a crucial consideration when estimating and comparing mean intakes in different groups of people. Direct comparison between myfood24 and the MPR showed moderate agreement, with intraclass correlation coefficients of ~ 0.4 to 0.5. Why myfood24 typically resulted in estimates that were 10 to 20% lower than the MPR is unclear, however. As only a few reference measures were used, for many nutrients it is unknown if myfood24 underestimated intakes, the MPR overestimated intakes, or if neither method was accurate. This is also true for the plasma antioxidant biomarkers, as their quantitative relationships to dietary intakes are currently unknown (412).

It is important to note that participants were assessed in free-living conditions, which compromises the precision of measures but enhances external validity. It is therefore expected that measures of agreement are lower in such circumstances. Furthermore, using a study design in which different tools were used to estimate intakes for a single day would likely have produced closer estimates of agreement. This would have been appropriate for assessment of short-term intakes, but we sought to validate myfood24 for use in studies of longer-term dietary intakes and therefore distributed dietary recalls over several weeks. Collection of reference measures sometimes coincided with dietary recalls. If more recalls using either myfood24 or the MPR coincided with reference measures, measures of agreement might have been biased. We addressed this by randomising the order of which recall method was used first.

Online dietary recall methods similar to myfood24 have been developed and used elsewhere in the world. Although not yet validated against biomarkers of dietary intakes, the ASA24® has been compared to standard interviewer-administered recalls and been found to produce similar estimates (390). Importantly, participant attrition was lower when using the ASA24®, suggesting that online methods such as the ASA24® and myfood24 might improve participant response rates in larger studies involving multiple recalls. We did not explore attrition in this study, but previous assessment of usability has shown that myfood24 appears to be appropriate for use among UK participants. It should be noted, however, that even if online methods are less costly and time-consuming for researchers, familiarisation with digital methods is arguably more burdensome for participants than simply answering a series of questions by telephone.

All dietary recall methods have limitations. All are of course prone to correlated person-specific biases and measurement error (413), and a limitation of 24-hour recall methods like myfood24 is that FFQs are perhaps better at documenting intakes of rarely consumed foods (414). This said, an advantage of online methods like myfood24 is that collection of multiple recalls across time is scarcely more expensive than collection of single recalls, allowing for better monitoring of trends across time (and therefore seasons), as well as new insights into intra-individual dietary variability. And myfood24 is unique in having a database comprehensive enough to include items consumed by only a small minority of people (there are > 50,000 items in the myfood24 database).

Doubly-labelled water is the gold-standard measure of energy expenditure and was not used in this study because of its cost. We did, however, use the gold-standard measure of protein intake, urinary nitrogen. The method was highly consistent, as shown by the consistency of the quality control CV values. Interestingly, urinary nitrogen values were lower among overweight participants than among people with healthy BMI values or

obese BMI values. The same was true of urinary sugars. Yet overweight participants had the highest protein intakes. The reasons for these observations are not clear, but the findings highlight the need to better determine the participant characteristics associated with dietary misreporting.

The urinary sugars quality controls were more variable than the nitrogen controls, and this may be related to the method used. The method was designed for use with cuvettes, but we modified it for use with 96-well plates. The volumes of reagents used were too small for use with multi-channel pipettes, however, so I pipetted all wells individually rather than using a multi-channel pipette. A potential limitation of urinary fructose and sucrose as biomarkers is that the calibration equations used to estimate dietary intakes are from only a few, small feeding studies, perhaps limiting their applicability to people elsewhere with very dissimilar dietary intakes (372). It is noteworthy, however, that the feeding studies were done using UK participants, as was the study of this chapter.

Although not the purpose of this study, the question of how accurately online dietary recall methods document the timing of items consumed is not well understood and will be a valuable point of inquiry to address in future studies that profile the timing of dietary intakes in large-scale studies. Prospective digital dietary records may be particularly advantageous in chrononutrition studies by providing time stamps of each dietary event, and preliminary studies using smartphone cameras show the promise of such methods (266-268).

It is important to note that validation results are dependent on the reference measures used: Validation studies that have reported greater tool accuracy have often used direct observation as the reference, for example (378). An advantage of direct observation is the ability to document the incidence of matches, intrusions, and omissions, and this was not possible in this study. Nevertheless, perhaps this study's greatest strength is validation of myfood24 against objective biomarkers of dietary intakes. Biomarkers are not prone to person-specific bias that might be seen in comparisons between the recall method and another recall method only. Such studies are *comparison* studies and may overestimate the performance of the tool (403). In doing so, researchers may then underestimate sample sizes needed for studies using the new tool to assess diet-disease relationships. True validation studies (like this one) compare the new method with objective measures of intakes such as biomarkers or direct observations of intakes.

About half of the other online 24-hour recall methods have been validated, about half have only been compared to other recall methods (378). Most of these studies showed strong correspondence between nutrient intake estimates using the online tool and the comparison recall method. Studies comparing online tools to urinary nitrogen,

potassium, and sodium generally reported that the tools provide relatively accurate estimates of intakes of the corresponding nutrients. Online tool estimates of energy intakes have generally been weaker, however. Although my own lab work was on urinary biomarkers, this is the first validation study of an online 24-hour recall method that has included blood biomarkers of nutrient intakes, to my knowledge (378).

Another strength of our work is the use of measures of agreement to estimate how much the diet-disease association in a large-scale study would be attenuated if myfood24 was being used. Correlation analysis has been the most common analysis method in other validation studies (378) but is not a true measure of agreement (406). Using measures of agreement both shows the utility of myfood24 and allows comparison with other dietary assessment methods that have been assessed similarly.

Last, it is noteworthy that response rates were low during recruitment, so selection bias is a possibility. It is also possible that participants were not representative of the UK population. Furthermore, the study included participants with a wide range of ages. Participants differ in how technology-savvy they are, raising the possibility that online methods may be unsuited to populations such as the elderly. It has already been shown that myfood24 has acceptable usability in a range of populations, however, including young people (415) and older people (391). Furthermore, stratification by age, BMI, and sex did not markedly affect results in this study, although there was less attenuation among younger, leaner participants.

In conclusion, myfood24 produced largely similar results to the MPR, the gold-standard dietary recall method. Furthermore, attenuation using myfood24 was generally lower than previously reported in studies of FFQs. Coupled with the many advantages afforded by being digital and designed for use in UK adults, myfood24 will be a useful method in future research exploring diet-disease associations in the UK, including studies of sleep. This is in part because myfood24 is well-suited to recording diet timing data. Development, validation, and refinement of similar methods elsewhere will be valuable. Direct comparison of similar tools (such as INTAKE24) will also be important to ensure that the most accurate methods are being used in important large-scale studies. This chapter justifies the subsequent use of myfood24 in this project.

Chapter 3: Sleep timing and diet timing in UK adults: Late eating relative to sleep is associated with overweight and obesity

3.1 Chapter overview

The global prevalence of obesity is rising. Recent findings show that it is not only what we eat but also *when* we eat relative to circadian phase that influences bodyweight. Only a single study has shown this in humans, however. I therefore used the measure your food on one day 24-hour recall (myfood24) dataset of the previous chapter to explore whether diet timing relative to sleep timing is associated with overweight and obesity.

Sleep was measured one night at a time, up to three times, with at least two weeks between measurements in 176 UK adults (age 18 to 68 years, 59% female) using SenseWear® armbands. I scored all sleep records. To check agreement with the armband sleep measures, a subset of participants completed the Munich ChronoType Questionnaire, of whom 68 provided usable data. I distributed these questionnaires online and then scored participants' responses. Height and weight were measured the day following each sleep bout, and participants completed an online diet recall using myfood24 one to five days after each bout to assess diet timing and composition. I scored the timing variables for all diet recalls. Dietary phase angle was defined as the time elapsed between the time at which a participant consumed 50% of daily calorie intake and their subsequent mid-sleep time. Participants were divided into two groups: those with larger and those with smaller dietary phase angles. I then used regression models to test whether dietary phase angle was associated with overweight and obesity, and if timing of the final caloric event of the day before sleep was associated with sleep duration. I also used linear regression to determine relationships between sleep timing and diet timing, as well as paired t-tests to assess whether diet differed between weekdays and weekends.

The group with smaller dietary phase angles were more likely to be overweight or obese, and later consumption of the last caloric event of the day relative to sleep onset was associated with longer sleep. Sleep timing was generally related to diet timing, but sleep timing variability was not associated with diet timing variability. Finally, several diet timing measures differed between weekdays and weekends, with participants generally eating later on weekends. Our findings build on evidence implicating late eating in the pathogenesis of obesity and provide novel insights into the temporal profiles of sleep and diet in UK adults.

3.2 Background

The number of people with obesity worldwide has more than doubled in > 70 countries since 1980. Overweight and obesity now account for about four million premature deaths each year, primarily as a result of cardiovascular comorbidities (4). Many interacting factors contribute to overweight and obesity, including both sleep disruption and misalignment of the circadian (~ 24-hour) system that regulates the daily phase (timing) of biology and behaviour (416).

The primary time cue that entrains (synchronises) the circadian system with the 24-hour day is the light/dark cycle. Our environments now enable waking behaviours like eating at times at which our biology would otherwise programme us to sleep, and shift workers exemplify the increased risk of metabolic sequelae that results from misalignment between behaviour and biology (22). Weight gain is among these consequences (417, 418). Night shift work overtly disrupts the circadian system and sleep, but more insidious disturbances may be common. An example of this is the positive association between 'social jetlag' (the discrepancy between weekday and weekend sleep timing that results largely from social factors such as enforced work schedules) and body mass index (BMI) among overweight adults (26). In addition to these social factors, aspects of the built environment may also contribute to circadian system and sleep disruption, including the ongoing sprawl and intensification of nocturnal light pollution (170).

The light/dark cycle is the primary time cue for the suprachiasmatic nuclei (SCN), the central clock in relaying time of day information to peripheral clocks elsewhere in the circadian system (419). Peripheral clocks coordinate the timing of local cellular processes to meet tissue-specific needs. Whereas the phase of the SCN is relatively impervious to nutritional status, peripheral clock timing is strongly influenced by diet timing. Altered diet timing can therefore uncouple peripheral clock timing from SCN phase (420), and loss of optimal phase relationships between central and peripheral clocks is thought to influence risk of various diseases (13).

Mice allowed ad libitum access to 'high-fat' diets have attenuated circadian rhythms and hence consume more chow at times when mice fed regular chow would sleep. Mice fed high-fat diets also rapidly become obese (273). Time-restricted feeding limits an animal's access to food to 8 to 12 hours each day, and time-restricted feeding offsets the obesogenic effects of high-fat diets in mice (421). Interestingly, however, restricting food availability to times at which mice would normally rest and fast predisposes these rodents to weight gain (290, 291).

Studies of humans have also shown that diet timing may influence body composition. Among overweight and obese women consuming isocaloric weight loss diets for 12 weeks, women who ingested half of daily caloric intake at breakfast lost more weight than those who ingested half of daily caloric intake at dinner (303). The latter study only assessed diet timing relative to clock time, but a more recent study measured diet timing relative to circadian phase (268). Specifically, participants recorded their diets for seven days, and researchers assessed circadian phase using a constant routine protocol. Adiposity was estimated using bioelectrical impedance analysis, and researchers divided participants into a lean group or a non-lean group according to sex-specific cut-offs. Participants in the non-lean group consumed most of their calories 1.1 hours closer to dim-light melatonin onset. Consistent with these findings that eating at a late circadian phase is obesogenic, people with Night Eating Syndrome have nocturnal hyperphagia and are generally predisposed to weight gain (422). Why later caloric intake is obesogenic is not well understood, but substantially lower diet-induced thermogenesis in the biological evening compared to the biological morning may contribute (423).

Interestingly, more variable eating patterns are also associated with lower diet-induced thermogenesis (308), but little is known about what leads to more variable eating patterns in free-living individuals. Given that people only eat when they are awake (except in rare sleep disorders, perhaps), it follows that more variable sleep patterns may lead to more variable eating patterns. I am not aware of any studies that have explored this, however.

No study has yet tested whether when people consume calories relative to sleep is associated with adiposity in a relatively representative group of UK adults (primary analysis). There has also been little research on whether timing of the final caloric event before sleep is related to the duration of the subsequent sleep bout (secondary analysis). Finally, it is unclear whether mid-sleep time is associated with the timing of the caloric period, whether sleep period is inversely related to caloric period, and whether diet timing variability tracks sleep timing variability (tertiary analyses).

3.3 Methods

The data analysed in this chapter are from a study designed to assess the validity of the online dietary recall tool used, as described in the previous chapter. In this study I used data from the validation study for a different purpose.

3.3.1 Participants

The study was approved by the West London Research Ethics Committee (number 14/SC/1267) and was conducted in accordance with the Declaration of Helsinki. Participants provided written informed consent. Participants were intended to be representative of the UK adult population and were English speaking, non-pregnant, weight-stable, literate 18- to 68-year-old adults with internet and telephone access. Participants were recruited through the North-West London Primary Care Research Network, a group of primary care professionals and practices that previously showed interest in participating in research projects. In addition, posters advertising the study were put up in the National Institute of Health Research / Wellcome Trust Clinical Research Facility at Hammersmith hospital (Imperial College Healthcare NHS Trust, London, UK), and the Clinical Research Facility contact list was used to contact potential participants. A list of local addresses was also obtained from the post office, with prospective participants receiving postal invitations to take part. Participants received a financial reward on completion of the study (£100).

3.3.2 Study design

Participants first completed a health screening visit at the Clinical Research Facility. Participants were instructed to not eat for at least four hours before each visit. On arrival, participants were weighed using scales (Tanita Corporation, Tokyo, Japan) after voiding, and height was measured using a stadiometer (Seca, Hamburg, Germany). BMI was then calculated. Cardiac function was assessed by electrocardiography, and blood pressure was measured using a digital monitor (Omron, Kyoto, Japan) after participants had rested supine for ~ 30 minutes. A 22 ml blood sample was taken for analysis of immune function, kidney function, liver function, and blood lipids. Participants filled in a general health and lifestyle questionnaire, the SCOFF questionnaire (to exclude individuals with eating disorders), and a technology readiness questionnaire, as participants would be completing dietary recalls online.

After the screening visit, participants returned to the Clinical Research Facility for study visits up to three times, separated by at least two weeks. Study visits took place between 12/06/2014 and 07/08/2015. The day before each study visit, each participant was instructed to wear a SenseWear® armband (BodyMedia Inc., Pittsburgh, Pennsylvania, US) for ~ 24 hours to monitor sleep. To check agreement with the armband data, a subset of participants completed the Munich ChronoType Questionnaire (MCTQ (227)) at one visit. Up to five days after each study visit, participants completed an online 24-hour dietary recall. Figure 3.1 outlines the study design.

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┌─────────────────────────────────────────────────┐
│                  Recruitment                     │
│   WeLReN, posters, CRF contact list, postal adverts. │
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          ┌─────────────────────────────┐
          │   Response by email/telephone │
          └─────────────────────────────┘
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│                 Screening visit                  │
│   Study explanation, anthropometry, electrocardiogram, │
│   blood pressure measures, 22 ml blood sample (for liver │
│   function, kidney function, and blood lipids), questionnaires. │
└─────────────────────────────────────────────────┘
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┌─────────────────────────────────────────────────┐
│     Study visits 1 to 3 (separated by ≥ 2 weeks)  │
│      Day before: SenseWear® sleep measures.       │
│   Study visit days: anthropometry, MCTQ (at one visit). │
│        1 to 5 days later: online dietary recall.   │
└─────────────────────────────────────────────────┘
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                        ▼
             ┌──────────────────┐
             │   Compensation   │
             └──────────────────┘
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Figure 3.1. Study design overview.

CRF (Clinical Research Facility), MCTQ (Munich ChronoType Questionnaire), WeLReN (North West London Primary Care Research Network).

3.3.3 Sleep

The day before each study visit, participants wore SenseWear® armbands on the midline of the left triceps brachii. To avoid feedback effects, the accelerometers did not give participants information regarding activity and sleep. Participants were instructed to only remove the armbands when bathing. The armbands have triaxial accelerometers and also measure galvanic skin response and skin temperature to estimate sleep using the manufacturer's proprietary algorithms. SenseWear® armband sleep estimates have been shown to generally agree well with polysomnographic sleep measures in several populations (424-426). Unlike polysomnography, however, the armbands are well suited to use in studies of free-living participants. As is true of widely used actimetric watches, these armbands are sensitive in detecting sleep but have lower wake detection rates.

I scored all of the sleep records (n = 564 before exclusions). Only nocturnal weekday sleep was scored because 1) all participant sleep bouts other than one were during weekday nights, and 2) sleep tends to differ between weekdays and weekends, as during the weekend people typically attempt to catch up on sleep lost during the working week (27). What appeared to be daytime naps were not scored. I used the mean values for our analysis if participants had multiple nights of sleep recorded. I defined sleep onset as the first minute of registered sleep in a 20-minute period in which there were ≥ 19 minutes of sleep recorded, because doing so has been shown to improve agreement between actimetric estimates of sleep and polysomnographic measures (427). I defined sleep offset as the first minute of registered wakefulness in a 20-minute period in which there were ≥ 19 minutes of wakefulness recorded. Sleep period was calculated as sleep offset minus sleep onset. Given its strong correlation with dim-light melatonin onset (428), mid-sleep time was used as a proxy of circadian phase. I calculated mid-sleep time as the halfway time in the sleep period, sleep duration as the sum of sleeping minutes recorded during the sleep period, and sleep efficiency as the percentage of the sleep period spent asleep. To assess sleep variability, I used the SDs of sleep period and mid-sleep time for participants with multiple nights of sleep recorded.

To check agreement with the armband data, a subset of participants completed the MCTQ to estimate mid-sleep time on weekdays at one visit. After initiation of recruitment in the myfood24 study, I persuaded the myfood24 consortium to include the MCTQ. Coinvestigators from Imperial College London gave paper MCTQs to participants still coming to the clinic for study visits (Appendix B), and I sent an online MCTQ survey (via Bristol Online Survey (now called Online Surveys)) to participants who had already completed their study visits. I transcribed paper questionnaires and scored all questionnaires. I calculated weekday mid-sleep time from the questionnaires as described for the armband data.

3.3.4 Diet

One to five days after each study visit participants used an online dietary recall tool named measure your food on one day 24-hour recall (myfood24) to record the quantities and times of ingestion of all foods and drinks consumed the previous day. myfood24 has comprehensive and current UK food lists and proven usability in adults (391). As described in the previous chapter, myfood24 was recently validated against biomarkers of dietary intakes and was found to give largely similar results to the gold-standard dietary recall method.

I scored all of the diet timing records (n = 550 before exclusions). As I exclusively used weekday sleep measures, only weekday diet recalls were included in the main analysis. I used the mean values for our analysis where participants had multiple days of diet recalls. Caloric onset was defined as the first caloric event (> 0 calories from either food or drink) after mean mid-sleep time, caloric offset as the last caloric event before mean mid-sleep time, and caloric period as caloric offset minus caloric onset. Caloric period midpoint was calculated as the halfway time between caloric onset and caloric offset. I also calculated the time at which each participant had accumulated 50% of daily caloric intake as a dietary phase marker (268). I therefore used the difference between time at 50% of daily caloric intake and mid-sleep time as an approximation of dietary phase angle. (Note that sleep and diet records were generally not for the same days.) Figure 3.2 depicts key diet and sleep timing measures. To assess diet timing variability, I used the SDs of caloric period and caloric period midpoint for participants with multiple weekday diet recalls.
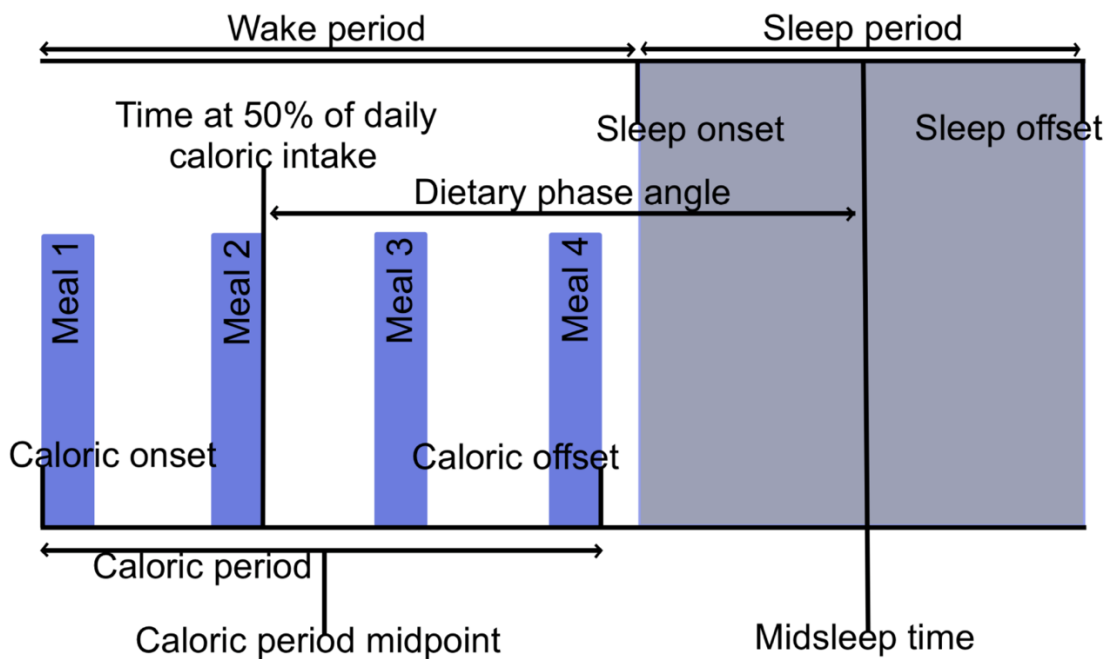
Figure 3.2. Diet and sleep timing measures.

Caloric period is the time elapsed from ingestion of the first calorie (caloric onset) to the last (caloric offset). Caloric period midpoint occurs halfway through the caloric period. For simplicity, this hypothetical daily dietary pattern includes four isocaloric meals (each containing 25% of daily caloric intake) that are evenly spaced. The time at 50% of daily caloric intake is therefore the end of the second meal. Sleep period is the time elapsed from sleep onset to sleep offset. Mid-sleep time is halfway through the sleep period.

Dietary phase angle is the difference between time at 50% of daily caloric intake and mid-sleep time.

Many participants (n = 71) completed weekend diet recalls as well as weekday recalls. For descriptive purposes, I calculated the above timing measures for weekdays and weekends separately for these people. I also recorded the onset and caloric content of self-defined breakfast, lunch, and dinner for weekday and weekend meals for these participants. Similar to previous work (266), events registered within 15 minutes of meal onset were combined into the same meal. Where participants reported consuming the same meal multiple times in a single recall, I only recorded the event with the most calories for the meal in question. Only events of ≥ 50 calories were scored as meals (264).

3.3.5 Statistical analyses

A directed acyclic graph was used to select variables for adjustment (Appendix C). For the primary analysis, I divided participants into those with smaller dietary phase angles and those with larger dietary phase angles, as well as those with healthy BMIs (healthy range 18.5 kg/m^2 ≥ BMI < 25 kg/m^2) or overweight and obese BMI values (BMI ≥ 25 kg/m^2). I then used logistic regression to determine if dietary phase angle was associated with overweight and obesity, adjusting for age, race, sex, and sleep duration. For regression analyses, I used dot plots to identify any unfeasible values and checked the distribution of residuals using QQ plots. Positively skewed outcomes were log-transformed.

Linear regression was used for secondary and tertiary analyses. Secondary analyses included 1) whether time from caloric offset to sleep onset was associated with sleep duration (adjusting for age, race, and sex), and 2) if caloric period was associated with daily caloric intake (adjusting for age, race, sex, and sleep duration). Tertiary analyses included whether mid-sleep time was associated with sleep duration, if sleep period was associated with caloric period, and whether mid-sleep time was associated with time at 50% of daily caloric intake (all adjusting for age, race, and sex). I also determined whether mid-sleep time variability was associated with caloric period midpoint variability, and if sleep period variability was associated with caloric period variability (both adjusting for age and race).

Paired t-tests were used to compare diet timing on weekdays and weekends for participants with both weekday and weekend diet recalls. For these, I used dot plots to

identify any unfeasible values, QQ plots to assess normality of distribution in each category, and SDs to check homogeneity of variances. (Variances were considered homogeneous if SDs differed by a factor of < 2.) A Bland-Altman plot was also used to assess agreement between SenseWear® and MCTQ measures of weekday mid-sleep time. Data are means ± SDs. p values ≤ 0.05 were considered significant. Statistical analyses were completed in Stata version 13 (Texas, US).

3.4 Results

Two hundred and eighty-nine respondents were invited to the first clinic. Of these participants, 240 attended, consented to the study, and passed the health screen. Twenty-seven participants then withdrew before beginning the study, 29 participants completed diet recalls but did not provide useable sleep data, four participants had descriptive data missing, and I could not calculate valid diet timing measures for four participants (three had weekend diet recalls only, one had breakfast listed as occurring before mid-sleep time). I therefore included 176 participants in the main analysis. Participant characteristics are summarised in Table 3.1.

Table 3.1. Participant characteristics, stratified by sex.

Characteristic	Women (n = 104)	Men (n = 72)
Age (years)	44.5 ± 15.2	43.4 ± 15.5
Race (% white)	75	72
Smoking (% current smokers)	9	8
Occupation (% managerial and professional)	36	53
Body mass index (kg/m^2)	25.1 ± 4.2	26.2 ± 4.1
Weekday sleep		
Sleep onset time	23:59 ± 1:20	00:25 ± 1:27
Mid-sleep time	03:37 ± 1:01	03:51 ± 1:18
Sleep offset time	07:14 ± 1:00	07:18 ± 1:24
Sleep duration (hours)	6:21 ± 1:16	5:49 ± 1:11

Sleep efficiency (%)	88 ± 9	85 ± 10

Weekday diet

Daily caloric intake	1,682 ± 573	2,118 ± 690
Caloric onset	08:25 ± 1:09	08:29 ± 1:37
Time at 50% daily caloric intake	15:40 ± 2:23	15:45 ± 2:51
Caloric offset	20:38 ± 1:55	21:08 ± 1:20
Caloric period	12:12 ± 2:09	12:39 ± 1:53

Data are means ± SDs.

Ninety-seven (55%) participants had three days of sleep recorded, 58 (33%) had two days, and 21 (12%) had one day. Of the 110 participants who completed three diet recalls, 57 participants completed three weekday diet recalls, 47 participants completed two weekday diet recalls and one weekend diet recall, and six participants completed one weekday and two weekend diet recalls. Of the 55 participants who completed two diet recalls, 37 participants completed two weekday diet recalls, and 18 participants completed one weekday diet recall and one weekend day diet recall. The remaining 11 participants completed one weekday diet recall only.

A Bland-Altman plot to assess agreement between SenseWear® and MCTQ measures of weekday mid-sleep time showed moderate agreement between the methods (Figure 3.3). The mean difference showed that SenseWear® estimates were 12 minutes later (95% limits of agreement -85 minutes to 109 minutes), on average, and only 2/68 (3%) observations were outside the limits of agreement.
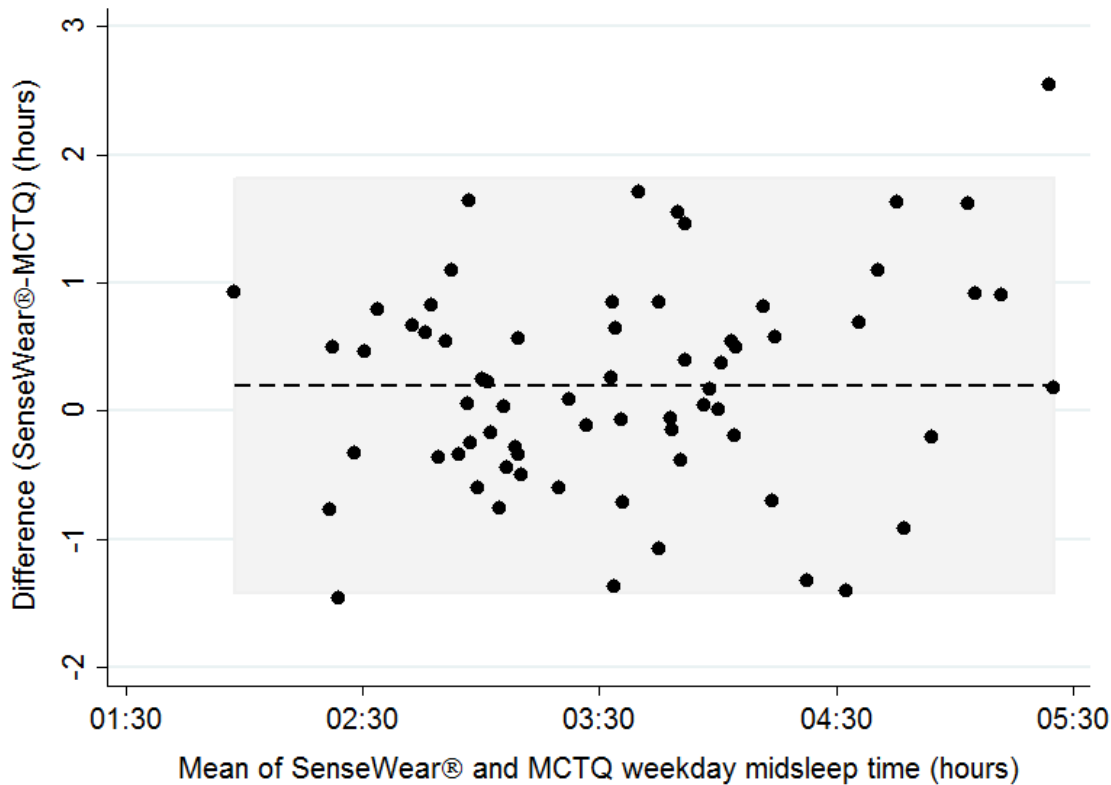
Figure 3.3. Bland-Altman plot comparing SenseWear® and Munich ChronoType Questionnaire estimates of weekday mid-sleep time.

3.4.1 Dietary phase angle and body mass index

In the unadjusted logistic regression analysis, participants with smaller dietary phase angles (9:52 ± 1:43 hours, n = 88) did not have higher odds of overweight or obesity than those with larger phase angles (14:08 ± 1:26 hours, n = 88) (OR 1.73, 95% CI 0.95 to 3.14, p = 0.07). After adjustment, however, participants with smaller dietary phase angles had higher odds of overweight or obesity (OR 2.13, 95% CI 1.11 to 4.10, p = 0.02). This association persisted after inclusion of daily caloric intake as an adjustment (OR 2.09, 95% CI 1.08 to 4.05, p = 0.03).

3.4.2 Caloric offset and sleep duration

Time between the last caloric event and sleep onset was negatively associated with sleep duration in the unadjusted analysis (-8.9 minutes of sleep per hour, 95% CI -14.7 to -3.0 minutes, p = 0.003). This association remained after adjustment (-9.3 minutes of sleep per hour, 95% CI -15.3 to -3.2 minutes, p = 0.003).

3.4.3 Sleep timing and diet timing

Mid-sleep time was not associated with sleep duration in the unadjusted analysis (-8.8 minutes of sleep per hour later mid-sleep, 95% CI -18.6 minutes to 1.0 minute, p = 0.08) or after adjustment (-7.3 minutes of sleep per hour later mid-sleep, 95% CI -17.3 to 2.8 minutes, p = 0.16). Later mid-sleep was associated with later caloric period midpoint in both the unadjusted analysis (33.1 minutes later caloric period midpoint per hour later mid-sleep, 95% CI 25.4 to 40.8 minutes, p < 0.001) and after adjustment (31.4 minutes later caloric period midpoint per hour later mid-sleep, 95% CI 23.5 to 39.2 minutes, p < 0.001).

Sleep period was not associated with caloric period in the unadjusted analysis (14.5 minutes shorter caloric period per hour longer sleep period, 95% CI -29.5 to 0.5 minutes, p = 0.06). This association was significant after adjustment, however (16.1 minutes shorter caloric period per hour longer sleep period, 95% CI -30.8 to -1.5 minutes, p = 0.03). Caloric period was not associated with caloric intake in the unadjusted analysis (31 calories per hour longer caloric period, 95% CI -17 to 78 calories, p = 0.20) or after adjustment (29 calories per hour longer caloric period, 95% CI -20 to 78 calories, p = 0.24).

As 126 participants had both multiple nights of sleep bouts and multiple diet recalls, 126 people were included in the timing variability analysis. Weekday sleep period variability was not associated with weekday caloric period variability in the unadjusted analysis (logged coefficient 0.09, 95% CI -0.13 to 0.30, p = 0.43) or after adjustment (logged coefficient 0.05, 95% CI -0.18 to 0.28, p = 0.65). Weekday mid-sleep time variability was not associated with weekday caloric period midpoint variability in the unadjusted analysis (logged coefficient 0.05, 95% CI -0.34 to 0.44, p = 0.81) or after adjustment (logged coefficient -0.09, 95% CI -0.49 to 0.31, p = 0.66).

3.4.4 Weekday and weekend diet timing

On average, caloric onset was 31 minutes later on weekends than weekdays (95% CI 9 to 53 minutes, $t(70) = 2.8542$, p = 0.006), and breakfast began 32 minutes later on weekends than weekdays (95% CI 8 to 54 minutes, $t(63) = 2.7630$, p = 0.008). Lunch was 22 minutes later on weekends than weekdays (95% CI 0 to 44 minutes, $t(62) = 1.9660$, p = 0.05). Caloric offset, caloric period midpoint, caloric period, time at 50% daily caloric intake, and dinner onset did not differ between weekdays and weekends. These results are summarised in Table 3.2.

Table 3.2. Comparisons between participant weekday and weekend diet patterns.

Characteristic	Weekday	Weekend	p value	n
Timing variables				
Caloric onset	08:24 ± 1:16	08:55 ± 1:36	0.006	71
Breakfast onset	08:34 ± 1:07	09:06 ± 1:10	0.008	64
Lunch onset	13:27 ± 1:03	13:49 ± 1:16	0.05	63
Caloric period midpoint	14:36 ± 1:22	14:54 ± 1:26	0.10	71
Time at 50% daily caloric intake	16:06 ± 2:49	15:41 ± 2:57	0.44	71
Dinner onset	19:29 ± 1:11	19:33 ± 1:29	0.66	69
Caloric offset	20:51 ± 2:05	20:53 ± 1:58	0.90	71
Caloric period	12:26 ± 2:16	11:58 ± 2:10	0.12	71
Caloric intakes				
Daily caloric intake	1,927 ± 750	2,105 ± 750	0.09	71
Breakfast caloric intake (% daily caloric intake)	19.0 ± 12.3	18.7 ± 9.8	0.86	64
Lunch caloric intake (% daily caloric intake)	31.2 ± 31.54	30.4 ± 13.4	0.83	63
Dinner caloric intake (% daily caloric intake)	40.0 ± 32.4	37.2 ± 15.3	0.53	69

Data are means ± SDs.

For participants with both weekday and weekend diet recalls, I also plotted the proportion of daily calories, carbohydrates, fats, and proteins consumed across the 24-hour day,

using 04:00 as the beginning of the day because 04:00 was the closest hour to mean mid-sleep time (Figure 3.4).
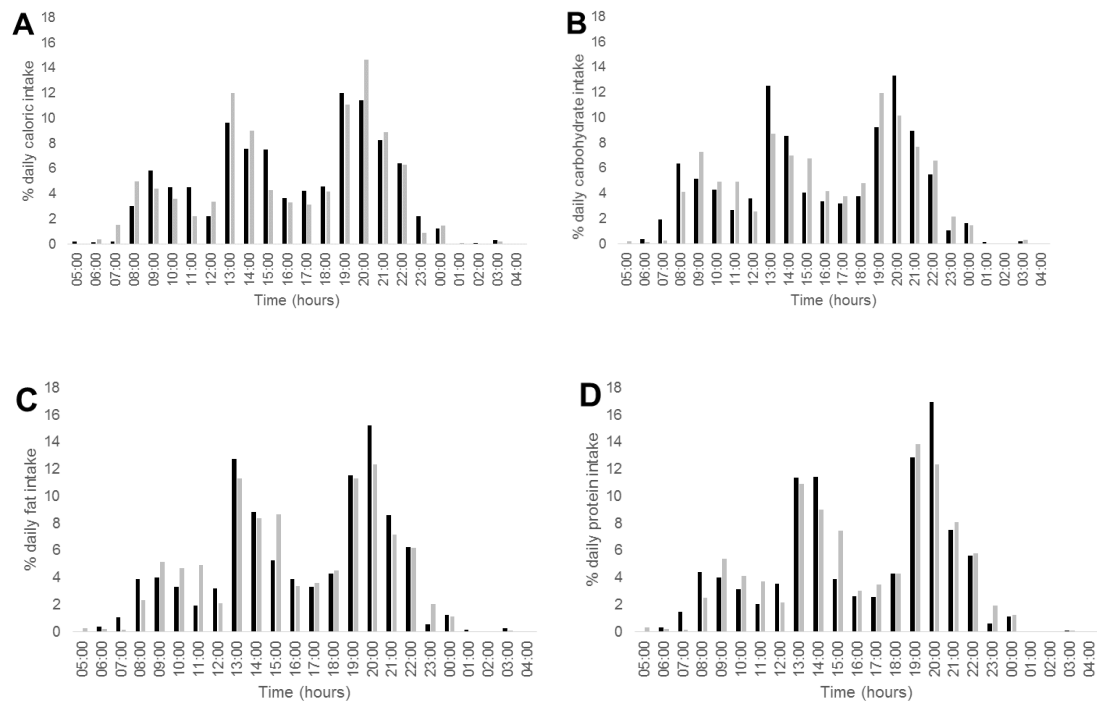
Figure 3.4. Comparisons between participant weekday and weekend diet timing (n = 71).

Percentages of daily calorie (A), carbohydrate (B), fat (C), and protein (D) intakes are plotted in 1-hour bins. As the closest hour to mean mid-sleep time was 04:00, the first bin is for events from 04:01 to 05:00, the second is from 05:01 to 06:00, and so on.

3.5 Discussion

The prevalence of obesity has increased unremittingly in most countries in recent decades (4). Numerous factors have fuelled this trend, but diet timing has garnered little research attention. In this study we show that adults who consume the majority of daily caloric intake closer to mid-sleep time are more likely to be overweight or obese, even after inclusion of daily caloric intake as an adjustment. Preclinical studies have shown obesogenic effects of rest phase feeding in rodents (290, 291), but a clear limitation of these studies is the use of other animals: it would be premature to extrapolate that the same would be seen in humans. A small initial study of 52 adults with intermediate and late chronotypes reported that calorie consumption after 20:00 was correlated with BMI (246), but the first study to measure diet timing relative to circadian phase reported that

dietary phase angle but not clock time of caloric intake independently associates with adiposity in a group of 110 young adults (268). Our study supports the findings of the latter study and builds on it by including a larger group of adults.

In contrast to some prior research (268), we found that calorie consumption closer to sleep onset was associated with longer sleep. The reason for this discrepancy between studies is unclear. We used different statistical methods to the previous work, and another plausible explanation for our divergent results is that we only considered the nocturnal sleep bout, whereas McHill and colleagues also considered naps (268). On one hand, diet-induced thermogenesis might be expected to offset the decline in body temperature that accompanies sleep onset, and pre-sleep caloric intake might thereby disrupt sleep. On the other, sufficient energy availability may be necessary to sustain sleep and prevent premature awakening to acquire food. Both studies only considered caloric intake *timing*, but the energy content and nutrient profile of the final dietary event are also likely to influence sleep. Experimental manipulation of diet composition and timing relative to sleep onset will rectify contradictory results of these studies.

Digital means of recording diet have produced higher resolution insights into diet timing of late (266-268). Like the two of these studies carried out in the West (266, 268), we report that participants began consuming calories later on the weekends, and participants also consumed breakfast and lunch later. We also plotted macronutrient intakes according to time of day, showing subtle differences between weekdays and weekends. Unlike previous studies, a clear strength of our work is the use of a diet recall tool that has both been validated against biomarkers of dietary intakes and was developed explicitly for use with the population from which participants came.

As later sleep timing has been associated with later meal timing (246), we anticipated that later mid-sleep time would be associated with a later caloric midpoint. We also hypothesised that sleep period would be inversely associated with caloric period. The data supported our contentions. Interestingly, however, we did not find that more variable sleep timing was related to more variable diet timing. Perhaps eating schedules are more dependent on cultural norms than individual variation in sleep timing. Alternatively, our study may have been underpowered to detect subtle associations between sleep timing variability and fluctuating diet timing.

Our study has notable strengths, including use of relatively accurate sleep measurement devices, use of a validated diet recall tool, and a larger participant sample than similar previous research (246, 268). Nevertheless, we acknowledge that our work has limitations. Few nights of sleep were measured for each participant, and no weekend nights were included. We also did not collect information about recent trans-meridian

travel or use of alarms, and participants did not complete sleep logs to verify time in bed. Furthermore, we did not directly measure circadian phase.

More experiments are necessary to clarify the metabolic and behavioural consequences of manipulating diet timing relative to circadian phase, and we can only speculate about the best measures of diet timing at present. It is plausible that foods consumed later in the waking day are less nutritious, and this could contribute to associations between dietary phase angle and body composition. Additional studies will benefit from precise methods of body composition measurement, like dual-energy X-ray absorptiometry. Furthermore, circadian phase is not fixed. If sleep timing and circadian phase shift over the course of the working week, should diet timing shift in lockstep? This is a particularly pertinent question for rotating shift workers and frequent flyers. It has been shown that diet-induced thermogenesis is lower late than early in the biological day (423), but effects of diet timing on key determinants of body composition such as substrate oxidation and skeletal muscle protein synthesis are not well characterised. Exactly how diet influences components of physical activity such as non-exercise activity thermogenesis is also unclear. Furthermore, experiments using polysomnography are required to show how diet composition and timing interact to influence sleep parameters.

Our study adds credence to the notion that when we eat may be a critical determinant of our metabolic health. Should other studies continue to support this idea, it would make sense to include advice on diet timing in dietary guidelines. Perhaps diet timing is another piece in the complex puzzle we face in preventing and reversing obesity.

Chapter 4: Sleep duration, nutrient intakes, and metabolic health in UK adults: findings from the National Diet and Nutrition Survey

4.1 Chapter overview

In the previous chapter we identified diet timing relative to sleep as a possible contributor to overweight and obesity. We did not directly explore associations between sleep duration and diet or sleep duration and metabolic health, however. We therefore sought a publicly available dataset with sleep, diet, and metabolic health data. We chose the National Diet and Nutrition survey, a programme that aims to monitor trends in diet and nutritional status in 500 UK adults each year.

A growing body of evidence associates short sleep with increased risk of metabolic diseases such as obesity, which may be related to diet. Yet few studies have concurrently determined associations between sleep duration and objective measures of metabolic health, as well as how sleep duration relates to diet. We therefore used National Diet and Nutrition Survey data to address this.

In total, 1,615 UK adults completed questions about sleep duration, as well as three to four days of food diaries. Body mass index (BMI), waist circumference, and blood pressure were recorded. Fasting blood lipids, glucose, glycated haemoglobin (HbA1c), thyroid hormones, and C-reactive protein (CRP) were measured in a subset of participants. I used regression analyses and restricted cubic spline modelling to assess associations between sleep duration and outcomes.

Consistent with our hypotheses, sleep duration was negatively associated with BMI and waist circumference. Sleep duration also tended to be positively associated with high-density lipoprotein cholesterol. Sleep duration was not associated with any dietary measures.

We found that longer sleep was associated with lower BMI and waist circumference, and favourable metabolic health profiles in general. We found little evidence of strong associations between sleep and diet, however. Findings from this chapter highlight the importance of sufficient sleep in curbing current trends in metabolic disease prevalence.

4.2 Background

Including undiagnosed cases, ~ 4.5 million people in the UK have diabetes, and it was estimated that in 2015 ~ 415 million 20 to 70 year old adults had diabetes worldwide (429). Roughly 24,000 individuals die prematurely each year in the UK as result of diabetes (430). Diabetes is therefore a large economic burden, costing the National Health Service in the UK ~ £10 billion in direct costs each year, 10% of its budget (431).

Type two diabetes accounts for the majority of diabetes cases and costs, and obesity is the most potent risk factor for type two diabetes. Although not all people with obesity develop the disease, obesity accounts for much of type two diabetes risk (432). About 59% of women and 68% of men in the UK are now overweight or have obesity (2). Obesity predisposes the affected to other metabolic dysfunction, and central obesity appears to explain much of this (433). Metabolic syndrome (central obesity, dyslipidaemia, hyperglycaemia, and hypertension) is a cluster of risk factors that also increases risk of type two diabetes (7) and is thought to affect about a quarter of adults worldwide (6). Identifying the lifestyle factors that influence risk of obesity, metabolic syndrome, and type two diabetes is therefore a public health priority.

Short sleep is increasingly common in many countries, and findings from an analysis of ~ 250,000 sleep questionnaires worldwide suggest that ~ 80% of adults use alarms to shorten their sleep on work days (26). Large-scale epidemiologic studies have consistently linked short sleep to type two diabetes, obesity (and central obesity), and metabolic syndrome (31-33, 434), and some of the mechanisms contributing to associations between short sleep and metabolic diseases are increasingly well understood (416). Among these mechanisms, short sleep may affect dietary choices, predisposing individuals to selection of energy-dense, rewarding foods, and non-homeostatic eating (435). The increases in type two diabetes, obesity and metabolic syndrome prevalence that have occurred concurrently with declines in sleep duration are hence unlikely to be mere coincidences.

Given the small sample size of the previous chapter, we sought a larger dataset to better determine associations between sleep duration and diet, and sleep duration and metabolic health in UK adults. We therefore used data from years 1 to 4 of the National Diet and Nutrition Survey Rolling Programme (NDNS-RP) to determine whether sleep duration was associated with diet, adiposity, glucose and lipid metabolism, metabolic syndrome criteria, thyroid function, and inflammation in UK adults. In doing so, we are the first to concurrently report on associations between sleep duration and nutrient intakes, as well as sleep duration and objective measures of metabolic health in UK adults, to our knowledge. We hypothesised that short sleep would be associated with 1)

less healthy dietary habits, 2) obesity, 3) dysglycaemia, 4) dyslipidaemia, 5) metabolic syndrome, 6) impaired thyroid function, and 7) higher systemic inflammation.

4.3 Methods

The NDNS-RP aims to track diet and nutritional status in 1,000 individuals per year (500 children aged 1.5 to 18 years, and 500 adults aged 19 years and over) living in private households in England, Northern Ireland, Scotland, and Wales. NDNS-RP results are used to monitor diet trends in the UK to develop policies to improve health, and data are available online at the UK Data Service website (436). A detailed overview of the NDNS-RP methods has been described previously (437).

Briefly, households were randomly selected from the Postcode Address File (all addresses in the UK) and grouped into units by location. Information about the purpose of the NDNS-RP was then sent to addresses randomly selected from these units, after which interviewers contacted the households to arrange visits to recruit participants and distribute diet diaries for four consecutive days of diet recording. With help from an interviewer, participants completed a computer-assisted personal interview to collect data on background and lifestyle. Height and weight were measured at these visits also. Individuals who completed diet diaries for at least three of the four days were eligible for visits by nurses for additional anthropometry and physiological measures. The Oxfordshire A Research Ethics Committee approved the study, which was conducted in accordance with the Declaration of Helsinki. We used the data for 19 to 65 year old, non-pregnant adults. Written informed consent was obtained from all participants. Participants who provided blood samples were compensated with £15 in high street shop vouchers for their contributions.

4.3.1 Sleep

Participants were asked the following two computer-assisted personal interview questions about habitual sleep duration at interviews: "How long (do you) usually sleep on week nights?" and "How long (do you) usually sleep on weekend nights?" We therefore estimated participants' mean daily sleep duration using the formula ((5 x self-reported usual weekday sleep duration) + (2 x self-reported usual weekend sleep duration) / 7)). Participants were not asked about napping or shift work schedules.

4.3.2 Blood pressure

After resting for five minutes in a seated position, blood pressure was measured three times with one minute between readings, using an automated sphygmomanometer (Omron HEM907, Kyoto, Japan). To avoid behaviours that can acutely influence blood pressure, participants had not eaten, exercised, drunk alcohol, or smoked in the preceding 30 minutes. For consistency, we used the mean of the second and third readings because the first reading is often the highest (438).

4.3.3 Anthropometry

Participants were measured for height and weight using portable stadiometers and weighing scales, respectively. Nurses measured waist circumference using tape measures at follow-up household visits. Height, weight, and waist circumference were measured twice. If there were unacceptable discrepancies (height ± 0.5 cm, weight ± 0.2 kg, waist circumference, ± 3 cm) then a third measurement was completed, and the mean value of the two most similar measurements was used.

4.3.4 Blood Measures

After anthropometry and blood pressure measures were taken, eligible participants provided up to ~ 35 ml of fasted blood via venepuncture. Venepuncture exclusion criteria included bleeding and clotting disorders, use of anticoagulant medications, an epileptic fit within the previous five years, and self-disclosed infection with hepatitis B or human immunodeficiency virus. To stabilise samples, blood was collected into tubes containing appropriate anticoagulants/stabilising agents. Samples were then processed at suitably equipped field laboratories located within two hours and stored at -20 to -80°C before subsequent analyses after transportation to the National Health Service laboratory at Addenbrooke's Hospital or the Human Nutrition Research centre in Cambridge, UK. Detailed information about sample processing (439) and the range of analytes measured (440) is available online.

As we were interested in the associations between sleep duration and metabolic health, we analysed data for analytes of particular clinical relevance to metabolic diseases such as type two diabetes and obesity. Specifically, we used data for the following analytes: 1) fasting glucose and glycated haemoglobin (HbA1c) (measures of glucose metabolism), 2) high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides (measures of lipid metabolism), 3) free triiodothyronine

(T3), free thyroxine (T4), and thyroid-stimulating hormone (TSH) (measures of thyroid function), and 4) C-reactive protein (CRP) (a measure of systemic inflammation).

4.3.5 Metabolic syndrome

I determined whether participants had the metabolic syndrome using the International Diabetes Federation (2006) criteria. According to these criteria, metabolic syndrome is defined as central obesity (using waist circumference values that are race- and sex-specific) plus at least two of the following: raised blood pressure (systolic blood pressure ≥ 130 mmHg, diastolic blood pressure ≥ 85 mmHg, or treatment for hypertension), raised fasting plasma glucose (≥ 5.6 mmol/L or treatment for diabetes), raised triglycerides (≥ 1.7 mmol/L or treatment for hypertriglyceridaemia), and low HDL cholesterol (< 40 mg/dL for males, < 50 mg/dL for females, or treatment for low HDL cholesterol levels). In accordance with the criteria, central obesity was assumed if BMI was > 30 kg/m^2 (6).

4.3.6 Diet

Participants completed three to four food diaries on consecutive days. These were collected no later than three days after the final diet day. Participants were asked to provide detailed descriptions of all items consumed, including time and estimated (not weighed) quantity of consumption. Weekend days were over-represented in year 1 of the NDNS-RP and were subsequently under-sampled in year 2 to address this.

Food diary data were processed by trained coders and editors who entered diaries into the Medical Research Council Human Nutrition Research dietary assessment system, Diet In Nutrients Out, using food composition data for > 6,000 foods (441). Component parts of composite items (such as sandwiches) were assigned individual food codes. Detailed information on data coding and editing is provided in Appendix A of the NDNS-RP official report (440).

We decided to assess energy intake; macronutrient intakes (including alcohol); and fibre, fruit (excluding fruit juice) and vegetable intakes as markers of healthy dietary habits. We also assessed non-milk extrinsic sugar, saturated fatty acid, trans-fatty acid, and sodium intakes as indices of unhealthy dietary habits. Dietary fibre comprised non-starch polysaccharides, as defined by the Englyst method (442). Non-milk extrinsic sugars were defined as sugars added during processing or by participants, sugars in fruit juices, and 50% of sugars in canned, dried and stewed fruits. Our choices of which foods and nutrients to analyse were partly informed by various diet quality indices (443).

4.3.7 Statistical analyses

I used linear regression analyses to test associations between sleep duration and 1) energy intake and macronutrient intakes (including macronutrient intakes as percentages of total energy intake); 2) indices of diet quality, including fibre, saturated fatty acids, trans-fatty acids, total sugar, non-milk extrinsic sugar, sodium, total fruit, and total vegetable intakes; 3) body mass index (BMI); 4) waist circumference; 5) blood measures (fasting glucose, HbA1c, HDL cholesterol, LDL cholesterol, triglycerides, free T3, free T4, TSH, and CRP); and 6) metabolic syndrome score (out of the five criteria). I used dot plots to identify any unfeasible values and checked the distribution of residuals using QQ plots. Positively skewed outcomes were log-transformed. I also divided participants by tertiles of sleep duration to produce short, middle and long sleep categories and then used binary logistic regression analysis to determine whether metabolic syndrome prevalence differed between sleep duration categories. I used a directed acyclic graph to select variables to adjust for (Appendix D), and models were adjusted for age, race, sex, smoking, and socioeconomic status. Additional models included BMI as an adjustment for all outcomes other than indices of diet quality.

As meta-analysis has shown there may not be simple linear relationships between sleep duration and diabetes risk (31), I also used restricted cubic splines to model relationships between sleep duration and metabolic outcomes. The splines comprised 4 polynomial segments separated by five knots (at the following percentiles of sleep duration: 5, 27.5, 50, 72.5, and 95, as recommended by Harrell (444)), with linear regions before the first knot and after the last.

Data are means ± SDs. Many people correct alpha levels using Bonferroni adjustments to reduce risk of Type I errors (incorrect rejection of null hypotheses). In doing so, however, they increase risk of Type-two errors (incorrect acceptance of null hypotheses). We therefore chose a conservative p value of ≤ 0.01 to account for multiple testing. Statistical analyses were completed in Stata version 13 (Texas, US).

4.4 Results

NDNS-RP data from years 1 to 4 are available for 1,692 19 to 65 year old adults. As not all participants were willing to give blood or met the blood sample eligibility criteria, blood data are available for only 51% of all participants, and some participants do not have data for various measures because of missing or invalid measurements. After excluding 75 participants without sleep data and two participants because of

pregnancy/breastfeeding, we analysed data for the remaining 1,615 non-pregnant adults (Table 4.1), of whom 448 were aged 19 to 34 years, 655 were aged 35 to 50 years, and 512 were aged 51 to 65 years. Two participants with TSH levels more than four times higher than the next highest value were excluded from thyroid hormone analyses. In total, 24.8% of participants reported being current smokers (sleep duration 7.15 ± 1.38 hours), 20.5% ex-smokers (sleep duration 7.14 ± 1.18 hours) and 54.7% reported never having smoked regularly (sleep duration 7.24 ± 1.18 hours). Men reported sleeping 7.17 ± 1.15 hours, women 7.22 ± 1.29 hours.

Table 4.1. National Diet and Nutrition Survey Rolling Programme participant characteristics, stratified by tertiles of mean sleep duration.

Characteristic	Shortest third of sleep duration (5.88 ± 0.86 hours, n = 538)	Middle third of sleep duration (7.26 ± 0.26 hours, n = 538)	Longest third of sleep duration (8.44 ± 0.66 hours, n = 539)
Age (years)	44.7 ± 12.2	43.8 ± 12.5	41.2 ± 13.2
Race (% white)	92.8	89.4	88.5
Sex (% female)	55.4	53.5	62.3
Smoking (% current smokers)	27.3	23.2	23.8
Occupation (% managerial and professional)	50	57.6	47.1
Diet			
Energy (calories)	1,822 ± 606	1,885 ± 578	1,783 ± 583
Carbohydrate (g)	218 ± 73	225 ± 73	215 ± 71
Carbohydrate (% total energy)	45.6 ± 8.0	45.2 ± 7.2	45.7 ± 7.6
Fat (g)	67 ± 27	71 ± 26	66 ± 26
Fat (% total energy)	32.7 ± 6.6	33.3 ± 6.1	33.1 ± 6.5

Protein (g)	73 ± 31	76 ± 23	72 ± 24
Protein (% total energy)	16.3 ± 3.9	16.5 ± 3.5	16.6 ± 4.3
Alcohol (g)	15.5 ± 25.6	14.7 ± 21.6	13.2 ± 23.7
Fibre (g)	13.5 ± 5.1	14.3 ± 5.2	13.4 ± 5.0

Metabolism

BMI (kg/m^2)	28.6 ± 5.5 (n = 499)	27.3 ± 5.3 (n = 506)	27.1 ± 5.4 (n = 505)
Waist circumference (cm)	95 ± 15 (n = 405)	92 ± 15 (n = 403)	91 ± 15 (n = 395)
Fasting glucose (mmol/L)	5.32 ± 1.35 (n = 252)	5.26 ± 1.12 (n = 246)	5.09 ± 1.14 (n = 247)
HbA1c (%)	5.64 ± 0.70 (n = 271)	5.55 ± 0.57 (n = 260)	5.46 ± 0.55 (n = 254)
HDL cholesterol (mmol/L)	1.45 ± 0.43 (n = 272)	1.53 ± 0.41 (n = 267)	1.54 ± 0.47 (n = 257)
LDL cholesterol (mmol/L)	3.24 ± 0.95 (n = 266)	3.27 ± 1.00 (n = 262)	3.15 ± 0.96 (n = 250)
Triglycerides (mmol/L)	1.50 ± 1.14 (n = 270)	1.25 ± 0.83 (n = 267)	1.32 ± 1.05 (n = 257)
Systolic blood pressure (mmHg)	125 ± 14 (n = 309)	125 ± 16 (n = 319)	124 ± 16 (n = 298)
Diastolic blood pressure (mmHg)	75 ± 10 (n = 309)	75 ± 11 (n = 319)	75 ± 11 (n = 298)
CRP (mg/L)	3.40 ± 4.15 (n = 273)	2.79 ± 3.11 (n = 267)	3.35 ± 5.25 (n = 257)
Free T3 (pmol/L)	5.06 ± 0.58 (n = 102)	5.06 ± 0.52 (n = 100)	5.04 ± 0.51 (n = 79)
Free T4 (pmol/L)	13.07 ± 1.91 (n = 102)	13.23 ± 2.04 (n = 100)	13.39 ± 1.74 (n = 79)
TSH (mIU/L)	2.57 ± 2.18 (n = 102)	2.59 ± 1.76 (n = 100)	2.63 ± 1.68 (n = 79)

Legend: BMI (body mass index), CRP (C-reactive protein), HbA1c (glycated haemoglobin), HDL (high-density lipoprotein), LDL (low-density lipoprotein), TSH (thyroid-stimulating hormone), T3 (triiodothyronine), T4 (thyroxine).

Data are means ± SDs.

4.4.1 Sleep and diet

Sleep duration was not associated with any dietary measure in the unadjusted and adjusted linear regression analyses (Table 4.2).

Table 4.2. Sleep duration and dietary intakes.

Characteristic	Unadjusted model		Adjusted model[a]	
	Coefficient per additional hour of sleep (95% CI)	p value	Coefficient per additional hour of sleep (95% CI)	p value
Energy (calories)	3 (-20 to 27)	0.78	3 (-17 to 24)	0.75
Carbohydrate (g)	1 (-2 to 3)	0.72	0 (-3 to 2)	0.83
Carbohydrate (% total energy)	0.0 (0.0 to 0.0)	0.74	-0.2 (-0.5 to 0.1)	0.13
Fat (g)	0 (-1 to 1)	0.55	0 (-1 to 1)	0.52
Fat (% total energy)	0.1 (-0.1 to 0.4)	0.27	0.2 (-0.1 to 0.4)	0.25
Protein (g)	0 (-1 to 1)	1.0	0 (-1 to 1)	0.79
Protein (% total energy)	0.0 (-0.1 to 0.2)	0.77	0.1 (-0.1 to 0.2)	0.47
Alcohol (g)[b]	0.00 (-0.07 to 0.06)	0.90	0.01 (-0.06 to 0.07)	0.83
Fibre (g)	0.0 (-0.2 to 0.2)	0.71	0.1 (-0.1 to 0.3)	0.33
Sugar (g)	-1 (-2 to 1)	0.42	-1 (-2 to 1)	0.38
NMES (g)	0 (-1 to 1)	0.95	-1 (-2 to 1)	0.45
Trans-fatty acids (g)	0.00 (-0.03 to 0.02)	0.75	0.00 (-0.03 to 0.03)	0.94
Saturated fatty acids (g)	0.0 (-0.5 to 0.4)	0.88	0.0 (-0.4 to 0.5)	0.85

Sodium (g)	0.02 (0.00 to 0.06)	0.10	0.02 (0.00 to 0.05)	0.14
Fruits (not juices) (g)	-2 (-6 to 2)	0.32	0 (-4 to 4)	0.99
Vegetables (g)	1 (-3 to 5)	0.62	2 (-2 to 7)	0.28

Legend: CI (confidence interval), NMES (non-milk extrinsic sugars).

[a]Adjusted for age, race, smoking, socioeconomic status, and sex.

[b]Alcohol data were log-transformed.

In the models that also adjusted for BMI (n = 1,510 participants), sleep duration was again not associated with energy (-2 calories per additional hour of sleep, 95% CI -24 to 20 calories, p = 0.84), carbohydrate (-1 g per additional hour of sleep, 95% CI -3 to 2 g, p = 0.66), fat (0 g per additional hour of sleep, 95% CI -1 to 1 g, p = 0.86), or protein (0 g per additional hour of sleep, 95% CI -1 to 1 g, p = 0.77) intakes.

4.4.2 Sleep and metabolic health

After adjustment for age, race, sex, smoking, and socioeconomic status, HDL cholesterol tended to be higher in longer sleepers (0.03 mmol/L higher per additional hour of sleep, 95% CI 0.00 to 0.05 mmol/L, p = 0.03, metabolic data are summarised in Table 4.3). For each additional hour of sleep, participants had lower HbA1c levels in the unadjusted model (0.05% lower, 95% CI -0.09 to -0.01, p = 0.006), but this association did not approach significance after adjustment (p = 0.09).Similarly, for each additional hour of sleep, participants tended to have lower triglyceride levels in the unadjusted model (0.07 mmol/L lower, 95% CI -0.13 to -0.01, p = 0.02), but this association was not significant after adjustment (p = 0.11). Of the five criteria used to diagnose an individual with metabolic syndrome, for each additional hour of sleep, participants tended to have fewer of these criteria in the unadjusted model (0.10 fewer criteria, 95% CI -0.20 to -0.02, p = 0.02), but again this association was not evident after adjustment (p = 0.36). In linear regression analyses, sleep duration was not associated with CRP, fasting glucose, LDL cholesterol, free T3, free T4, or TSH. In the logistic regression analysis, sleep duration was not associated with the presence of metabolic syndrome.

Table 4.3. Sleep duration and measures of metabolic health.

Characteristic	Unadjusted model		Adjusted model[a]		
	Coefficient per additional hour of sleep (95% CI)	p value	Coefficient per additional hour of sleep (95% CI)	p value	n
BMI (kg/m^2)	-0.58 (-0.81 to -0.36)	< 0.001	-0.46 (-0.69 to -0.24)	< 0.001	1,510
Waist circumference (cm)	-1.4 (-.2.1 to -0.8)	< 0.001	-0.9 (-1.5 to -0.3)	0.004	1,203
HbA1c (%)	-0.05 (-0.09 to -0.01)	0.006	-0.03 (-0.07 to 0.00)	0.09	785
Fasting glucose (mmol/L)	-0.05 (-0.12 to 0.02)	0.18	-0.03 (-0.10 to 0.04)	0.44	745
HDL cholesterol (mmol/L)	0.02 (0.00 to 0.05)	0.08	0.03 (0.00 to 0.05)	0.03	795
Triglycerides (mmol/L)	-0.07 (-0.13 to -0.01)	0.02	-0.05 (-0.10 to 0.01)	0.11	794
LDL cholesterol (mmol/L)	-0.02 (-0.08 to 0.04)	0.48	0.01 (-0.05 to 0.07)	0.73	778
Free T3 (pmol/L)	0.00 (-0.06 to 0.06)	0.97	-0.01 (-0.06 to 0.04)	0.72	281
Free T4 (pmol/L)	0.18 (-0.02 to 0.38)	0.08	0.17 (-0.04 to 0.38)	0.10	281
TSH (mIU/L)	0.03 (-0.17 to 0.24)	0.74	0.03 (-0.18 to 0.23)	0.79	281
CRP (mg/L)[b]	-0.05 (-0.09 to 0.00)	0.05	-0.04 (-0.09 to 0.01)	0.09	797
Number of metabolic syndrome factors (out of 5[c])	-0.10 (-0.20 to -0.02)	0.02	-0.04 (-0.13 to 0.05)	0.36	554
	Metabolic syndrome OR (95% CI)	p value	Metabolic syndrome OR (95% CI)	p value	n
Shortest third of sleep duration (5.88 ± 0.79 hours)[d]	1.33 (0.84 to 2.13)	0.23	1.22 (0.73 to 2.06)	0.44	175
Longest third of sleep duration (8.38 ± 0.65 hours)[c]	1.05 (0.65 to 1.68)	0.85	1.18 (0.69 to 2.01)	0.55	184

Legend: BMI (body mass index), CI (confidence interval), CRP (C-reactive protein), HbA1c (glycated haemoglobin), HDL (high-density lipoprotein), LDL (low-density lipoprotein), OR (odds ratio), T3 (triiodothyronine), T4 (thyroxine).

[a]Adjusted for age, race, smoking, socioeconomic status, and sex.

[b]Data were positively skewed and so were log-transformed.

[c]Using the five International Diabetes Federation (2006) criteria: central obesity, raised blood pressure, raised fasting plasma glucose, raised triglycerides, and reduced HDL cholesterol.

[d]Reference group sleep duration, 7.26 ± 0.26 hours (n = 195).

Sleep duration was negatively associated with BMI and waist circumference, such that participants had 0.46 kg/m^2 lower BMI values (95% CI -0.69 to -0.24 kg/m^2, p < 0.001, Figure 4.1, panel A) and 0.9 cm lower waist circumferences (95% CI -1.5 to -0.3 cm, p = 0.004, Figure 4.1, panel B) per additional hour of sleep. Restricted cubic spline modelling showed that the negative association between sleep duration and these outcomes was linear.
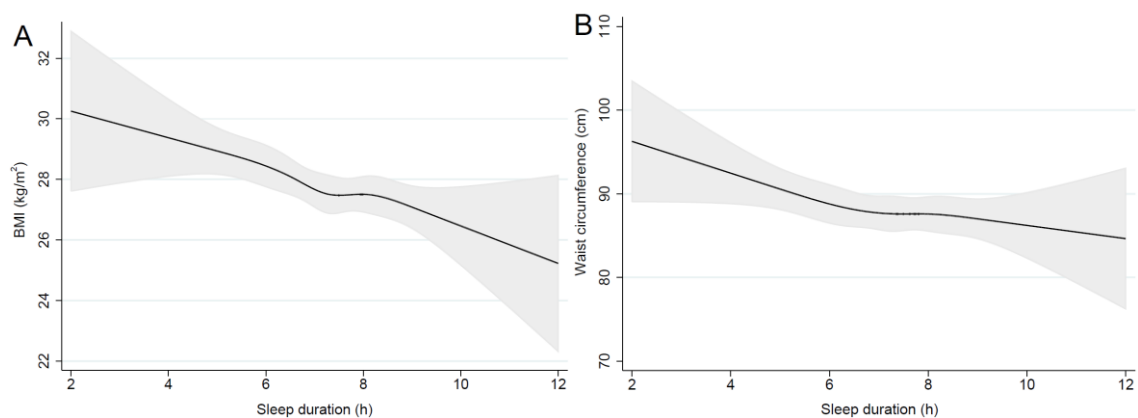
Figure 4.1. Sleep duration, BMI and waist circumference.

Black lines plot the predicted BMI (A) and waist circumference (B) values with 95% confidence intervals (grey fill) for typical females from the sample (white, never smokers, lower managerial and professional occupation, using the mean age). Very similar associations were apparent in males.

After inclusion of BMI as an additional adjustment, sleep duration was not associated with any other metabolic outcomes (Table 4.4).

Table 4.4. Sleep duration and measures of metabolic health, including body mass index as an adjustment.

Characteristic	Coefficient per additional hour of sleep (95% CI)	p value	n
Waist circumference (cm)	0.1 (-0.2 to 0.4)	0.56	1,157
HbA1c (%)	-0.02 (-0.06 to 0.01)	0.18	762
Fasting glucose (mmol/L)	-0.01 (-0.08 to 0.06)	0.77	726
CRP (mg/L)[a]	0.00 (-0.05 to 0.04)	0.85	775
Triglycerides	-0.03 (-0.08 to 0.03)	0.38	772
LDL cholesterol	0.02 (-0.04 to 0.07)	0.54	758
HDL cholesterol	0.01 (-0.01 to 0.04)	0.22	774
Free T3 (pmol/L)	-0.01 (-0.07 to 0.04)	0.66	272
Free T4 (pmol/L)	0.16 (-0.06 to 0.37)	0.16	272
TSH (mIU/L)	0.02 (-0.19 to 0.23)	0.82	272

Legend: CI (confidence interval), CRP (C-reactive protein), HbA1c (glycated haemoglobin), HDL (high-density lipoprotein), LDL (low-density lipoprotein), OR (odds ratio), T3 (triiodothyronine), T4 (thyroxine).

Adjusted for age, body mass index, race, smoking, socioeconomic status, and sex.

[a]Data were positively skewed and so were log-transformed.

4.5 Discussion

Consistent with our predictions, sleep duration was negatively associated with BMI and waist circumference and tended to be positively associated with HDL cholesterol levels. In unadjusted models, sleep duration was negatively associated with HbA1c and also tended to be negatively associated with triglycerides and metabolic syndrome criteria, but these associations were no longer apparent after adjustment. Associations were strongly attenuated after inclusion of BMI as an adjustment, suggesting that higher BMI values contribute to metabolic dysfunction in shorter sleepers. Interestingly-and contrary to our expectations-sleep duration was not associated with diet. Collectively these findings suggest that among UK adults longer sleepers have favourable metabolic profiles in comparison to shorter sleepers, but not substantially different dietary habits.

Our observation that sleep duration was negatively associated with BMI and waist circumference is consistent with meta-analyses showing that short sleep is associated with obesity and central obesity (32, 33). The magnitudes of the associations that we found are within the ranges of those documented previously. Interestingly, a recent study of a large group of UK adults showed that gene/environment interactions may be key determinants of obesity risk, as the adverse effects of various sleep behaviours (including short sleep) on adiposity were more pronounced among those with genetic predisposition to obesity (445). These findings suggest a particular need to improve sleep patterns among people who are genetically susceptible to obesity.

Blood lipids are important determinants of risk of various metabolic diseases. HDL cholesterol helps offset excessive inflammation and regulates reverse cholesterol transport, thereby influencing risk of health problems such as atherosclerotic cardiovascular disease (446). Whereas some studies have found that both short and long sleep may contribute to lower HDL cholesterol levels (447), we observed a more linear relationship. A consistency among studies, however, seems to be that short sleep is associated with adverse effects on lipid metabolism, including lower HDL cholesterol levels (448).

As is true of lipid metabolism, sleep restriction has detrimental effects on glucose metabolism (416), so we tested whether sleep duration was associated with fasting glucose and HbA1c, markers used to diagnose type two diabetes. Sleep duration was associated with neither but was closer to being associated with HbA1c. This discrepancy may be partly explained by the different factors that fasting glucose and HbA1c reflect (449): HbA1c is a more stable marker of longer term glucose homeostasis, but fasting glucose is more susceptible to acute fluctuations resulting from variables such as diet and physical activity. A meta-analysis of prospective studies reported a U-shaped association between sleep duration and type two diabetes risk, but our restricted cubic spline models did not show nonlinear relationships with fasting glucose or HbA1c. This could reflect the relative scarcity of people whose sleep might be considered pathologically long. Whereas short sleepers comprised a significant proportion of the study population (9.9% reported sleeping less than six hours, for example), perhaps there were not enough long sleepers to see if long sleep might be pathological: Only 1.1% of participants reported sleeping longer than 10 hours.

A recent prospective study of > 160,000 adults found that compared to participants who self-reported sleeping six to eight hours a night, those sleeping less than six hours were at greater risk of developing each of the metabolic syndrome criteria and therefore had a 9% higher risk of metabolic syndrome. Furthermore, people who slept more than eight

hours had a 7% lower risk of metabolic syndrome development (450). Given our findings of greater adiposity, lower HDL cholesterol, and a tendency to impaired glucose metabolism among shorter sleepers, we expected sleep duration to be associated with metabolic syndrome. Whereas sleep duration was negatively associated with number of metabolic syndrome criteria in the unadjusted linear regression model, this relationship was not apparent after adjustment. As the full complement of metabolic syndrome components was only available for 554 participants, the study may have had insufficient statistical power to document an association.

Thyroid hormones have myriad roles in metabolic regulation (451), so we determined if sleep duration was associated with thyroid function. We found that sleep duration was not associated with measures of thyroid function. Experimental sleep restriction has been shown to acutely increase thyroid hormone secretion, but chronic sleep restriction may lower free T4 levels (452). As hypothyroidism predisposes people to weight gain, impaired thyroid function could be a mechanism by which long-term sleep loss increases susceptibility to obesity. However, effects of sleep on thyroid function are somewhat unclear at present.

Higher CRP levels increase susceptibility to metabolic diseases such as type two diabetes (453). Consequently, we also tested whether sleep duration was associated with blood CRP concentrations. We did not find that that sleep duration was associated with CRP, in contrast to evidence that sleep restriction generally induces a proinflammatory state, including elevated CRP levels (454). It is possible that our sample size was insufficient to determine any associations between sleep and CRP, and additional studies will help clarify whether dysregulated systemic inflammatory responses are another means by which short sleep adversely affects metabolic regulation.

Epidemiologic studies have often associated short sleep with higher energy intake and have sometimes found that short sleep coincides with reduced dietary quality (365). Some of the underlying mechanisms are well characterised (416), and meta-analysis of experimental sleep restriction studies has shown that sleep restriction increases energy intake (213). It is unclear why we did not find that short sleep was associated with increased energy intake and indices of processed food intake, especially when we found that sleep duration was inversely associated with BMI. Perhaps other components of energy balance were affected by sleep duration. It has been found, for example, that sleep restriction may acutely reduce resting metabolic rate (455) and could hence lead to a positive energy balance if energy intake remained constant. The extra energy cost of wakefulness could compensate for this, however, and a recent meta-analysis of sleep

restriction intervention studies reported that curtailed sleep does not alter daily energy expenditure (213). It could be that sleep duration has a reciprocal relationship with free-living physical activity, but we did not feel that there was sufficient experimental evidence that physical activity independently influences sleep duration to include physical activity as an adjustment.

Another possibility is that dietary underreporting is more pervasive among individuals with higher BMI and those attempting to lose fat mass (456), hindering our ability to observe a relationship between sleep duration and energy intake using such self-reported measures of diet. It is also plausible that sleep duration may have been related to diet in a nonlinear manner. Based on our interpretation of the literature, however, we decided a priori to use linear regression analyses to model dietary outcomes. Finally, other sleep parameters such as sleep efficiency, sleep timing (a simple estimate of chronotype), and sleep timing variability may influence diet and metabolic health (28, 237, 457), but questions related to these variables are not included in the NDNS-RP.

This study had several strengths, including comprehensive dietary assessment by way of four-day estimated food diaries, thorough metabolic profiling, concurrent measures of diet and metabolic health, and the study of a representative adult population. Nevertheless, this work also had limitations. The study used self-reported sleep duration instead of a more objective measure such as actimetry or polysomnography, and participants were not asked about napping or effects of work schedules on sleep. Correlations between self-reported sleep duration and actimetry-estimated sleep duration of 0.43 to 0.45 have been reported in adults, and self-reports tend to underestimate sleep duration, a discrepancy that may increase with longer self-reported sleep (458). Other limitations include the absence of information regarding behaviours that influence sleep (such as alarm use), missing data for some variables, and the possibility that participants' behaviours were not rigorously standardised before collection of fasted samples.

As this was a cross-sectional study, it is of course not possible to infer that insufficient sleep results in adverse metabolic consequences. Prospective studies using more objective measures of sleep duration and quality are needed to better clarify the relationships between sleep, dietary habits and metabolic health. As recent studies have documented an array of beneficial effects of sleep extension on dietary choices and metabolic health in habitual short sleepers (459-461), the optimisation of such interventions should be further studied.

In conclusion, longer sleepers generally had more favourable metabolic profiles, but sleep duration was scarcely associated with dietary intakes. Our findings support the

accumulating evidence showing an important contribution of short sleep to metabolic diseases such as obesity. This evidence raises the question of whether interventions to enhance sleep benefit metabolic health, and the next chapter focuses on this question.

Chapter 5: A randomised controlled trial to determine if melatonin improves metabolic health and alters diet in adults at increased risk of type two diabetes

5.1 Chapter overview

In Chapter 4 we found that longer sleep was associated with favourable metabolic health profiles in UK adults, supporting the substantial body of evidence showing that sleep duration influences risk of cardiometabolic diseases. Findings from many studies also indicate important roles of the circadian system in metabolism. This background provides the rationale to explore whether a compound that influences sleep and circadian system function might also effect metabolic changes.

Of the compounds that meet these criteria, exogenous melatonin is widely available in many countries and is established to be safe. Recent studies of humans have reported that melatonin signalling influences key metabolic processes, including blood glucose homeostasis and insulin action. Yet few studies have comprehensively assessed effects of melatonin supplementation on metabolic health in humans, and none has explored whether melatonin influences dietary choices. We aimed to address this by exploring the effects of long-term melatonin supplementation on metabolic health, sleep, and diet in UK adults at elevated risk of type two diabetes. The study was first conceived to exclusively determine whether melatonin affects metabolic health. To also explore if melatonin influences sleep and diet, we amended the study protocol to also assess these outcomes among participants in the second half of participant recruitment.

Adults with first-degree relatives with type two diabetes were randomised to take either 2 mg prolonged-release melatonin or placebo two hours before sleep each day for 24 weeks. Participants visited the study centre at baseline, 12 weeks, 24 weeks, and 36 weeks (after a 12 week washout). Bodyweight, waist circumference, and blood pressure were measured at each visit. Fasted blood samples were also taken to assess measures related to glucose metabolism (fasting glucose, glycated haemoglobin, glucagon, and adiponectin), lipids (high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, total cholesterol, total: HDL cholesterol ratio, and oxidised LDL cholesterol), energy availability (leptin), and inflammation (CRP and IL-6). Of these markers, I completed the glucagon, adiponectin, leptin, interleukin-6, and oxidised LDL cholesterol laboratory analyses. Sleep quality was assessed at each visit using the Pittsburgh Sleep Quality Index. A subset of participants also completed the Munich ChronoType Questionnaire at each visit to estimate sleep duration, chronotype, and social jetlag, as well as a myfood24 recall under my supervision to record their dietary

intakes (energy, carbohydrate, protein, fat, fibre, fruit, and vegetable intakes). I analysed the data using multilevel mixed models according to the intention-to-treat principle.

Contrary to our hypotheses, long-term melatonin supplementation did not significantly affect any of the outcomes measured, although participants in the placebo group tended to sleep longer at 36 weeks than at previous time-points. I discuss these null findings in the context of other studies and suggest related research questions that may be fruitful to pursue.

5.2 Background

Cardiometabolic diseases result in widespread suffering, the burden of which continues to escalate. From 1990 to 2015 there was a 28.3% increase in global death rate related to overweight and obesity, and a 35.8% increase in disability-adjusted life years because of excessive adiposity. Worryingly, the rate of overweight- and obesity-related deaths increased in countries from across the spectrum of income per capita in this period (4). Diabetes contributes considerably to overweight and obesity-related deaths, and diabetes climbed from the 18th to 15th highest cause of years of life lost from 2005 to 2015 alone, an increase of over 25% (462). A recent forecast projected that diabetes will be the 7th leading cause of death by 2030 (463). It is clear that efforts to address current trajectories in cardiometabolic diseases are ineffective, and there is much interest in measures to reverse these trends. Findings from preliminary studies indicate that melatonin and other melatonin receptor agonists might be promising agents in such reversal.

A multisynaptic pathway relays photoperiodic information from specialised retinal photoreceptors to the pineal gland, where melatonin is primarily synthesised. As light exposure reduces melatonin synthesis, this photoneuroendocrine system ensures that circulating melatonin levels are highest during darkness. Melatonin therefore provides a biological signal of scotoperiod duration that is relayed systemically through the interaction of melatonin with two receptors in humans (hMT$_1$ and hMT$_2$, encoded by *MTNR1A* and *MTNR1B*, respectively). These receptors are present in several brain regions (55) and in many peripheral tissues. The suprachiasmatic nuclei of the hypothalamus also have melatonin receptors. As these nuclei are at the helm of regulation of the circadian system that synchronises timing of physiology and behaviour with the 24-hour day, melatonin is a critical hormone in coordinating biological rhythms (54).

Although many consider melatonin as an important hormone in sleep regulation, pinealectomised patients have relatively normal sleep, so whether endogenous melatonin has key roles in human sleep is contentious (121). Rather, the presence of melatonin receptors in key metabolic tissues suggests roles of melatonin in metabolic regulation. As genome-wide association studies have associated *MTNR1B* variants with glycaemia, insulin responses, and type two diabetes risk (351-353), roles of melatonin in insulin and glucose metabolism have received much attention. Recent work has shown that pancreatic melatonin signalling may reduce glucose-stimulated insulin secretion. This same study reported that the common *MTNR1B* variant associated with increased diabetes risk increases pancreatic islet melatonin signalling. Melatonin synthesis may therefore reduce the likelihood of nocturnal hypoglycaemia (354), and so it is little

surprise that ingestion of exogenous melatonin acutely worsens oral glucose tolerance in humans (98).

The significance of roles of melatonin in metabolic regulation is that artificial lighting now hampers melatonin synthesis in many people. About 99% of people in Europe live in settlements lit by artificial light at night (169). The introduction of electric lighting is associated with delayed melatonin synthesis and sleep onset, as well as shortened sleep duration (171, 172). Most people in Europe probably now experience disrupted melatonin rhythms and sleep, as supported by findings that self-reported sleep duration is declining during the working week (26). Interestingly, lower circulating melatonin levels have been found in people with metabolic diseases such as type two diabetes (326-329), and lower excretion of the primary melatonin metabolite has been associated with increased type two diabetes risk in a prospective study (331), raising the question of what effects increasing circulating melatonin may have.

Preclinical studies using rodent models of metabolic diseases have consistently shown that long-term melatonin supplementation has numerous benefits on physiology, such as reducing hyperglycaemia, hyperinsulinaemia, hyperleptinaemia, and hypertriglyceridaemia (332, 333). Melatonin may also offset hepatic steatosis, improve liver mitochondrial function (334), and enhance vascular function in diet-induced obesity (337). Melatonin appears to counter adverse metabolic consequences associated with senescence in mice and rats (338, 339).

Exogenous melatonin rarely results in side-effects in humans, even after single doses as high as 300 mg (464), and some preliminary studies of long-term melatonin supplementation in small groups of people have reported beneficial effects. Long-term use of a slow-release melatonin formulation reduced glycated haemoglobin (HbA1c) in type two diabetes patients with insomnia (342). Melatonin reduced blood pressure, low-density lipoprotein cholesterol levels and measures of oxidative stress in metabolic syndrome patients (343). Similarly, long-term melatonin supplementation tended to reduce weight and blood pressure and led to greater metabolic syndrome remission after treatment than placebo (344). Other studies have reported that melatonin supplementation increased lean body mass in post-menopausal women without affecting weight (345), and that melatonin improves blood lipids in peri- and post-menopausal women (346). Not all studies have documented beneficial effects of long-term melatonin supplementation (347-350), although there is little evidence of any adverse effects of exogenous melatonin consumption.

There are likely several reasons for discrepancies in findings between studies, including differences in participant characteristics, melatonin formulations, and ingestion timing.

No controlled trial has yet determined whether melatonin is an effective countermeasure against metabolic disease development. Furthermore, diet is an important determinant of disease risk, but no study of humans has assessed whether melatonin supplementation affects food intake, despite clear indications that supraphysiological doses of melatonin may improve some sleep parameters (465), inter-relationships between melatonin and key appetite hormones, and possible effects of melatonin supplementation on body composition (359). The purpose of this study was therefore to assess whether long-term melatonin improves metabolic health measures and alters dietary intakes in adults at increased risk of type two diabetes.

5.3 Methods

5.3.1 Participants

Non-pregnant, 18 to 75 year old first-degree relatives of individuals with type two diabetes were recruited through a diabetes clinic in Leeds, UK, as well as through local general practice surgeries, research databases, and poster adverts targeting University of Leeds staff. All study visits took place at the Leeds Institute of Genetics and Health Therapeutics (LIGHT) laboratories at the University of Leeds. Inclusion criteria included at least one first-degree relative with a confirmed diagnosis of type two diabetes, absence of infection or systemic disease, use of stable doses medications for three months before the study, no use of melatonin in the four weeks preceding the study, and no use of medication contra-indicated for use in conjunction with melatonin.

Recruited participants first attended a screening visit to assess their eligibility. A study nurse took a fasted blood sample (after ≥ 12 hours of fasting) via venepuncture for routine pathology testing and exclusion of diabetes. Anthropometrics, demographic information, and clinical histories were recorded for each participant. Participants also completed questionnaires to estimate morningness/eveningness (Morningness/Eveningness Questionnaire (226)), physical activity (International Physical Activity Questionnaire), and diabetes risk (Finnish Diabetes Risk Score).

Participants gave written informed consent before participation. The local National Health Service Research Ethics Committee approved the study (number 14/YH/0172), which was conducted according to the guidelines outlined in the Declaration of Helsinki. Participants were not financially compensated for their participation.

5.3.2 Study design

Eligible participants were randomised into either a group receiving a daily placebo tablet or a group receiving a daily 2 mg prolonged-release melatonin tablet (Circadin®, Neurim Pharmaceuticals, Zug, Switzerland) that was designed to mimic endogenous melatonin rhythms. This formulation is typically used to treat primary insomnia. Circadin® is a non-selective melatonin receptor agonist, and its use results in no significant withdrawal symptoms (466). Access to the randomisation code was exclusively given to the pharmacist who prepared the tablet containers before tablets were shipped to the LIGHT laboratories from the University Medical Centre Hamburg-Eppendorf in Germany. The trial was therefore double-blind, with participants and investigators not knowing which group participants had been assigned to during data collection.

After screening, the first study visits occurred between October 10th 2014 and February 22nd 2016. Participants were instructed to take their tablets once daily in the evening, two hours before habitual bedtime. To check compliance, research nurses documented the number of tablets returned by participants at every visit. Participants took tablets from the first study visit at week 0 (randomisation) until the third visit at week 24, between which the second visit took place at week 12. To determine if any effects of the treatment persisted, participants returned at 36 weeks, 12 weeks after discontinuing treatment. All visits were scheduled in the morning (between 07.30 and 11:00), and visits occurred at approximately the same time for each participant to minimise noise in measurements from diurnal variations in anthropometric and physiological measures. Based on previous blood test results, study nurses gave participants advice at each visit regarding health behaviours, including dietary choices, physical activity, and smoking cessation. Where relevant, nurses gave participants British Heart Foundation advice booklets.

After collection of a fasted blood sample for determination of multiple cardiometabolic measures, participants had an oral glucose tolerance test at each study visit, with blood sampling at 30, 60, 90, and 120 minutes after ingestion of 75 g glucose drink. Bodyweight, height, abdominal circumference, and blood pressure were measured, and sleep quality was assessed in all participants using the Pittsburgh Sleep Quality Index (PSQI).

I applied to the local National Research Ethics Service Committee for a minor protocol amendment to also record sleep duration, sleep timing, and dietary intakes. The amendment was approved, so the Munich ChronoType Questionnaire (MCTQ (227), Appendix B) was completed by participants recruited in the second year of the study, and second-year participants also completed online 24-hour dietary recalls using measure your food on one day 24-hour recall (myfood24).

5.3.3 Metabolic health, anthropometry, and blood pressure

At each study visit, a cannula was first inserted into the antecubital vein, where possible. From this, a 75 ml fasted blood sample was collected. This blood sample was distributed into multiple tubes according to the measures to be made. Of these, a 4 ml serum tube sample was used to analyse blood lipids (low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides) and C-reactive protein (CRP). A 4 ml ethylenediaminetetraacetic acid tube sample was used to analyse HbA1c. A 4 ml fluoride oxalate tube sample was used to analyse fasting glucose. Blood samples for these routine clinical blood measures were transported on the same day to the Leeds Teaching Hospitals Trust Laboratories for processing and analysis.

The ethylenediaminetetraacetic acid tube sample was also used to measure plasma melatonin by coinvestigators at the University of Surrey. These samples were immediately spun at 3,000 x g at 4°C for 30 minutes. The plasma was then aliquoted and stored at -80°C before transport to the Chronobiology Section at the University of Surrey where samples were analysed using a previously described radioimmunoassay (467).

Other blood samples were immediately processed in the LIGHT laboratories by the study technician, who then stored samples at -80°C for future analysis of other cardiometabolic function measures not detailed in this chapter. Fasted blood samples were also used to measure multiple other measures of cardiometabolic health. I only analysed data for the measures mentioned above (fasting glucose, HbA1c, blood lipids, and CRP). Participants then had an oral glucose tolerance test, with 6 to 8 ml blood samples taken at 30, 60, 90, and 120 minutes after ingestion of the 75 g glucose drink. The samples collected after ingestion of the glucose drink were used to assess multiple measures of glucose metabolism. Because of data unavailability, I did not analyse these data for this project.

In between blood sample collections, participant height was measured using a stadiometer (Seca, Hamburg, Germany), weight was measured while wearing minimal clothing using calibrated scales (Seca, Hamburg, Germany), and waist circumference (level with the naval) was measured by tape measure. Blood pressure was measured in the non-cannulated arm while seated, using an automated blood pressure cuff (Omron M7, Kyoto, Japan). Blood pressure was measured after the participants had sat for at least five minutes. All measurements were completed by trained nurses.

5.3.4 My laboratory analyses

The Principle Investigator on the project asked me to suggest other outcomes to measure that would complement the original suite of measures. I suggested adiponectin, glucagon, interleukin-6 (IL-6), leptin, and oxidised LDL, for reasons that will be discussed subsequently. I completed the laboratory assays for these measures. I prepared plasma samples according to the manufacturer's instructions and aliquoted a quality control blood sample to include in duplicate each run. I used the same blood sample as a quality control for all of the assays.

I processed the quality control blood sample in the same way that the participant samples had been processed. The sample was first collected into a tube (kept on ice) containing the anticoagulant ethylenediaminetetraacetic acid. The sample was immediately spun at 3,000 x g at 4°C for 30 minutes. The plasma was then aliquoted and stored at -80°C. Individual aliquots were taken out to thaw beginning about two hours before using them. This quality control sample was included in all assays.

First, I used commercial ELISA kits to measure adiponectin (Human Total Adiponectin/Acrp30 Quantikine ELISA Kit, Bio-Techne Ltd, Abingdon, UK). The kits use the quantitative sandwich enzyme immunoassay technique in which a monoclonal antibody specific to human adiponectin is pre-coated on 96-well microplates. A series of standards ranging from 3.9 to 250 ng/ml was included with samples and the quality control on each microplate, and everything was run in duplicate for all assays that I did in the EuRhythDia project. Samples were diluted 100-fold, as per the manufacturer's instructions.

In each run, adiponectin is first bound by the antibody during assay incubation. Unbound substances are then washed away and an enzyme-linked monoclonal antibody specific to human adiponectin is added. A last wash removes unbound antibody-enzyme reagents before a tetramethylbenzidine substrate solution is added. The substrate solution colour develops in proportion to the quantity of adiponectin bound in the first step. Finally, a sulphuric acid solution is added to stop colour development, and I measured colour within 30 minutes using a microplate reader (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer, Waltham, Massachusetts, US). I subtracted optical density readings at 540 nm from readings at 450 nm to correct for optical imperfections in the plate, as recommended by the manufacturer. I then used the linear regression equation from the standard curve to quantify sample concentrations. Samples were re-run in instances in which there was an apparent outlier. No samples were outside the range of the standard curve. The quality control inter-assay CV for adiponectin was 6.98%.

I measured glucagon using commercial ELISA kits (Glucagon Quantikine ELISA Kit, Bio-Techne Ltd, Abingdon, UK). The kits use a similar quantitative sandwich enzyme immunoassay technique to the adiponectin kits described above. A series of standards ranging from 15.6 to 1,000 pg/ml was included with samples and the quality control on each microplate. Samples were not diluted. Samples were re-run in instances in which 1) one of two sample duplicates was below the lowest standard or 2) there was an apparent outlier. The Principle Investigator insisted that results below the lowest standard were given the value of the lowest standard for all assays, where applicable. The quality control inter-assay CV for glucagon was 22.02%.

Next, I measured IL-6 using commercial ELISA kits (Human IL-6 Quantikine ELISA Kit, Bio-Techne Ltd, Abingdon, UK). The kits use a similar quantitative sandwich enzyme immunoassay technique to the kits described above. A series of standards ranging from 0.78 to 50 pg/ml was included with samples and the quality control on each microplate. Samples were not diluted. Samples were re-run in instances in which 1) one of two sample duplicates was below the lowest standard or 2) there was an apparent outlier. The quality control inter-assay CV for IL-6 was 4.20%.

I measured leptin using commercial ELISA kits (Human Leptin Quantikine ELISA Kit, Bio-Techne Ltd, Abingdon, UK). The kits use a similar quantitative sandwich enzyme immunoassay technique to the kits described above. A series of standards ranging from 15.6 to 1,000 pg/ml was included with samples and the quality control on each microplate. Samples were not diluted. Samples were re-run in instances in which there was an apparent outlier. No samples were outside the range of the standard curve. The quality control inter-assay CV for leptin was 9.87%.

I measured oxidised LDL cholesterol using commercial ELISA kits (Mercodia oxidized LDL ELISA, Mercodia, Uppsala, Sweden). The kits use a similar quantitative sandwich enzyme immunoassay technique to the kits described above, except that two monoclonal antibodies are directed against separate antigenic determinants on oxidised apolipoprotein B molecules. The samples were first diluted in 1/6,561 ratios (1/81 dilution of a 1/81 dilution). A series of five standards was included with samples and the quality control on each microplate. The manufacturer provides a high control and a low control with each kit, and these were also included on each microplate. Results were multiplied by 6,561 to account for the dilution. Samples were re-run in instances in which there was an apparent outlier. No samples were outside the range of the standard curve. The quality control inter-assay CV for oxidised LDL was 6.34%.

5.3.5 Sleep assessment

The PSQI is a series of questions about sleep quality in the preceding month, and questions are scored and tallied to give an overall score in which higher scores are indicative of lower sleep quality (468). The MCTQ uses separate questions for work days and non-work days to estimate mid-sleep time (the halfway time between sleep onset and offset) on non-work days as a proxy of chronotype (corrected for age, sex and sleep debt). Results are also used to estimate sleep duration (mean sleep duration on work days and non-work days, assuming five work days and two free days) and social jetlag (the difference between mid-sleep time on work days and non-work days) (227). Full calculations for chronotype and social jetlag are available on the Worldwide experimental Platform website (469). I scored the PSQI and MCTQ questionnaires.

5.3.6 Dietary assessment

At each study visit I sat with participants to record their dietary intakes for the previous day using myfood24. I then contacted participants by e-mail, asking them to complete two further dietary recalls per visit. Because I supervised each of the study visit recalls, I am confident that visit recalls were completed correctly. I therefore chose to only analyse the recalls completed in my presence. From these recalls I calculated 1) energy intake, 2) macronutrient intakes, 3) fibre intake, and 4) total fruit and total vegetable consumption. I also recorded response rates to e-mail invitations to complete dietary recalls to assess the feasibility of using myfood24 in a clinical trial.

5.3.7 Statistical analyses

The primary endpoint of the study was differences in HbA1c between groups, and a power calculation performed by a coinvestigator indicated that, allowing for 20% of participants to dropout, a minimum sample size of 80 individuals would be required in each group to detect 7% decrease in HbA1c after 12 months, with α at 0.01 (to allow for multiple comparisons) and β at 0.2. These calculations were based on 1) a study in which five months of melatonin treatment resulted in an absolute decrease in HbA1c of 0.66% (a 7% relative decrease) (342), and 2) the HbA1c levels found in a previous study of first-degree relatives of people with type two diabetes (470).

I completed the statistical analyses presented in this chapter. I tested whether melatonin treatment influenced the following outcomes: 1) measures related to glucose metabolism (fasting glucose, HbA1c, glucagon, and adiponectin), 2) lipids (HDL cholesterol, LDL cholesterol, total cholesterol, total: HDL cholesterol ratio, and oxidised LDL cholesterol),

3) adiposity (BMI and waist circumference), 4) energy availability (leptin), 5) inflammation (CRP and IL-6), 6) sleep (duration, quality, chronotype, and social jetlag), and 7) diet (energy, carbohydrate, protein, fat, fibre, fruit, and vegetable intakes).

To determine whether study visit one values needed to be included as model covariates, independent-samples t-tests were first used to check whether there were differences between groups at the first study visit. For t-tests I used dot plots to identify any unfeasible values, QQ plots to assess normality of distribution in each condition, and standard deviations to check homogeneity of variances. (Variances were considered homogeneous if they differed by a factor of less than two). One HDL datum was excluded from the analysis because the value was clearly unfeasible (206 mmol/L). All other data were included in the analyses. Positively skewed data were log-transformed. Study visit one data that remained positively skewed after log-transformation were tested using the non-parametric Mann-Whitney test.

Multilevel mixed-effects linear regression models were then used to determine whether outcomes differed between groups after 12 weeks of treatment (study visit two), 24 weeks of treatment (study visit three), and after a 12 week washout (study visit four). I chose these models because mixed models are relatively robust to 1) correlated errors due to measures repeated for each participant across time within a hierarchical data structure, and 2) missing data. The models used the restricted maximum likelihood approach (471), as restricted maximum likelihood variance components are less biased in small samples. Model predictors included fixed effects (treatment, time, and treatment * time interaction), and I included participant as a random effect to control for repeated measures. Random effects are presented as 1 SD of the residual variance (within-participant variance) and 1 SD of the variance of the intercepts (between-participants variance). Joint (multiple degrees of freedom) tests were used to test for treatment by time interactions. If time effects were significant, I changed the reference study visit to that with the biggest contrast for data presentation purposes. (Doing so has no bearing on model estimates and fit.) If interactions were significant, tests of simple effects and pairwise comparisons were used to determine their nature. For multilevel mixed-effects models I checked the distribution of residuals in each group using QQ plots. Positively skewed outcomes were log-transformed.

The Principle Investigator had reservations about treatment compliance and therefore wanted to do exploratory analyses using spot plasma melatonin concentration as the predictor variable and the same outcomes as the main analyses. For the exploratory analyses, model predictors included plasma melatonin concentration as the fixed effect and participant as a random effect to control for repeated measures. Two participants

had plasma melatonin concentrations that exceeded the melatonin assay limit of detection (500 pg/ml) at at least one study visit. Plasma melatonin concentration exceeded 500 pg/ml at three of the four visits in one of these participants. As this participant was in the control group, such high melatonin levels are remarkably unlikely (472). A participant in the treatment group had a plasma melatonin concentration that exceeded 500 pg/ml at one of the four visits. This concentration was more than 15 times higher than the participant's melatonin concentrations at other visits. These two participants were therefore from the exploratory analyses.

Data are reported as means ± SDs. p values of ≤ 0.01 were considered significant to account for multiple testing. All analyses were performed in Stata version 13 (Texas, US).

5.4 Results

5.4.1 Participant characteristics

Difficulties recruiting participants meant that the final sample size was unfortunately far smaller than the target sample size. Approximately 340 adults with first-degree relatives with type two diabetes were approached. Of these, 78 participants were recruited. Four participants failed screening visits because of past medical histories, six requested withdrawal from the study (five because of difficulties with blood sampling, one because of mental health difficulties), four were lost because of communication problems, three were withdrawn by the study staff (two because of limited blood vessel access during sample collection, one because of dumping syndrome), and three stopped taking their tablets and withdrew. Complete data were therefore collected for 58 participants. First study visit participant characteristics are summarised in Table 5.1.

Table 5.1. Participant characteristics at the first study visit.

Characteristic	Placebo	n[a]	Melatonin	n[a]	p value
Age (years)	47.1 ± 12.5	38	47.1 ± 15.6	37	1.0
Sex (% female)	73.7	38	83.8	37	0.29
Anthropometry and metabolic health					
BMI (kg/m^2)	27.9 ± 4.8	34	27.1 ± 4.9	32	0.50
Waist circumference (cm)	91 ± 13	35	88 ± 14	32	0.39
Systolic blood pressure (mmHg)	115 ± 16	35	114 ± 14	34	0.83

Diastolic blood pressure (mmHg)	77 ± 10	35	74 ± 9	34	0.14
Fasting glucose (mmol/L)	4.82 ± 0.56	34	4.66 ± 0.49	35	0.20
HbA1c (mmol/L)	35.9 ± 4.0	21	35.2 ± 4.0	21	0.59
Glucagon (pg/ml)	46.3 ± 24.4	35	36.4 ± 20.9	35	0.06
Adiponectin (µg/ml)	6.60 ± 2.98	35	6.46 ± 3.15	35	0.85
Leptin (pg/ml)	25.1 ± 20.5	35	20.5 ± 15.6	35	0.39
HDL cholesterol (mmol/L)	1.48 ± 0.43	19	1.77 ± 0.49	22	0.05
LDL cholesterol (mmol/L)	2.87 ± 0.79	20	3.12 ± 0.94	22	0.34
Total cholesterol (mmol/L)	4.88 ± 0.80	21	5.35 ± 0.87	22	0.07
Total: HDL cholesterol ratio	3.39 ± 1.00	20	3.25 ± 1.09	22	0.68
Triglycerides (mmol/L)	1.05 ± 0.45	21	1.04 ± 0.45	22	0.93
Oxidised LDL (U/L)	51.2 ± 14.8	35	54.4 ± 15.8	35	0.39
IL-6 (ng/ml)	1.70 ± 1.43	35	1.50 ± 1.22	35	0.58
CRP (mg/L)	2.83 ± 4.13	20	1.66 ± 2.57	22	0.11
Diet					
Energy (calories)	1,760 ± 764	16	1,760 ± 453	19	1.00
Carbohydrate (g)	202 ± 108	16	172 ± 57	19	0.29
Fat (g)	67 ± 36	16	76 ± 28	19	0.32
Protein (g)	75 ± 27	16	84 ± 27	19	0.35
Fibre	17.5 ± 16.00	16	21.1 ± 9.59	19	0.42
Fruits (g)	177 ± 251	16	190 ± 179	19	0.64
Vegetables (g)	204 ± 205	16	223 ± 213	19	0.75
Sleep					
Sleep duration	6.98 ± 1.69	9	7.69 ± 0.54	10	0.26
Chronotype	27.24 ± 1.15	9	27.59 ± 0.78	10	0.44
Social jetlag	0.55 ± 0.90	9	0.62 ± 0.57	10	0.85
PSQI score	6.73 ± 3.37	33	7.35 ± 3.50	31	0.44

Legend: BMI (body mass index), CRP (C-reactive protein), HbA1c (glycated haemoglobin), HDL (high-density lipoprotein), IL (interleukin), LDL (low-density lipoprotein), PSQI (Pittsburgh Sleep Quality Index).

Data are means ± SDs.

[a]Numbers vary because of missing data for some measures. Numbers for diet and sleep outcomes (other than PSQI score) are low because these data were only collected for participants recruited in the second year of the study.

5.4.2 Study compliance

Excluding participants who dropped out (n = 20) and participants with missing tablet consumption data (n = 24), participants (n = 34) took 85% of their tablets on average. Of

the 35 participants recruited in the second year of the study, 35 participants completed at least one dietary recall, including those who eventually dropped out of the study. Together, these participants completed 96 of 231 (41.6%) unsupervised dietary recall invitations sent by e-mail. Two participants did not have e-mail addresses and so could not be contacted. These people therefore only completed dietary recalls under my supervision.

5.4.3 Melatonin, anthropometry, and metabolic health

There were no main effects of either treatment or time on waist circumference, leptin, blood pressure, fasting glucose, HbA1c, Adiponectin, LDL cholesterol, total cholesterol, total: HDL cholesterol ratio, triglycerides, oxidised LDL cholesterol, CRP, or IL-6 (Table 5.2).

Table 5.2. Effects of melatonin supplementation on anthropometry and metabolic health.

Characteristic	Main effect of treatment X^2 (1 df)	p value	Main effect of time X^2 (3 df)	p value	Treatment * time interaction X^2 (3 df)	p value	Random effects Within-participant variance (1 SD (95% CI))	Between-participants variance (1 SD (95% CI))
BMI	0.42	0.52	13.77	0.003	0.99	0.80	0.59 (0.48 to 0.73)	24.5 (17.4 to 34.4)
Waist circumference	0.77	0.38	4.60	0.20	5.73	0.13	10.9 (8.8 to 13.4)	169 (119 to 239)
Systolic blood pressure	0.46	0.50	5.49	0.14	0.65	0.88	80 (65 to 99)	134 (89 to 202)
Diastolic blood pressure	1.99	0.16	5.25	0.15	0.86	0.83	39.7 (32.2 to 48.9)	45.4 (29.2 to 70.5)
Fasting glucose	2.98	0.08	2.29	0.51	2.19	0.53	0.14 (0.11 to 0.17)	0.16 (0.10 to 0.25)
HbA1c	1.80	0.18	7.21	0.07	3.59	0.31	1.69 (1.34 to 2.12)	13.5 (9.4 to 19.4)
Glucagon	4.92	0.03	3.21	0.36	0.86	0.84	239 (195 to 294)	252 (164 to 387)
Adiponectin	0.05	0.83	5.10	0.16	2.21	0.53	0.59 (0.48 to 0.72)	9.4 (6.6 to 13.2)
Leptin	0.38	0.54	0.67	0.88	4.50	0.21	24.1 (19.5 to 29.6)	290.5 (205.5 to 410.7)
HDL cholesterol	5.06	0.025	6.70	0.08	5.05	0.17	0.03 (0.02 to 0.03)	0.14 (0.10 to 0.21)
LDL cholesterol	0.32	0.57	4.74	0.19	1.62	0.66	0.11 (0.09 to 0.14)	0.57 (0.39 to 0.82)

Total cholesterol	1.97	0.16	6.14	0.11	2.25	0.52	0.14 (0.11 to 0.18)	0.59 (0.41 to 0.85)
Total: HDL cholesterol	0.68	0.41	7.43	0.06	1.93	0.59	0.09 (0.07 to 0.11)	0.88 (0.61 to 1.26)
Triglycerides	0.22	0.64	7.09	0.07	2.34	0.51	0.09 (0.07 to 0.11)	0.21 (0.14 to 0.31)
Oxidised LDL	0.76	0.38	1.64	0.65	1.50	0.68	51 (41 to 62)	168 (116 to 241)
IL-6[a]	0.93	0.33	5.22	0.16	5.29	0.15	0.23 (0.19 to 0.28)	0.23 (0.15 to 0.36)
CRP[a]	2.46	0.12	1.31	0.73	6.59	0.09	1.40 (1.12 to 1.76)	1.01 (0.61 to 1.66)

Legend: BMI (body mass index), CI (confidence interval), CRP (C-reactive protein), HbA1c (glycated haemoglobin), HDL (high-density lipoprotein), IL (interleukin), LDL (low-density lipoprotein).

[a]Data were positively skewed and so were log-transformed.

BMI differed across study visits ($X^2(3)$ =13.77, p = 0.003) such that participants tended to have higher BMI values at study visit three than study visit one (0.46 kg/m^2, 95% CI 0.08 to 0.84 kg/m^2, p = 0.02, Figure 5.1).
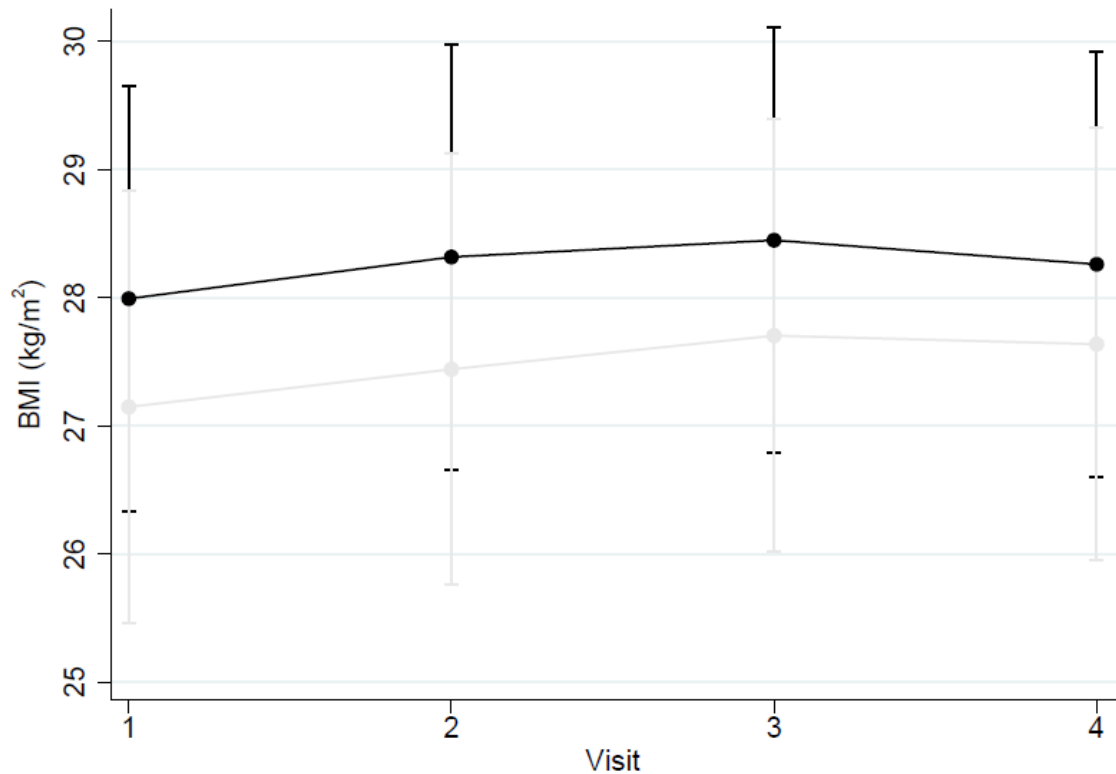
Figure 5.1. Effects of melatonin supplementation on body mass index (BMI).

There was a main effect of time such that participants had higher BMI values at study visit three than at study visit one. Black, placebo group. Grey, melatonin group. Error bars are 95% confidence intervals.

Plasma glucagon tended to differ between groups ($X^2(1)$=4.92, p = 0.03) such that participants in the melatonin group tended to have lower glucagon levels (-9.8 pg/ml, 95% CI -20.2 to 0.5 pg/ml, p = 0.06, Figure 5.2). (The reason that these values unavoidably differ slightly from those reported in Table 5.2 is that the χ^2 squared test separates the main effects (treatment and time), whereas the estimates in the whole model are not independent.)
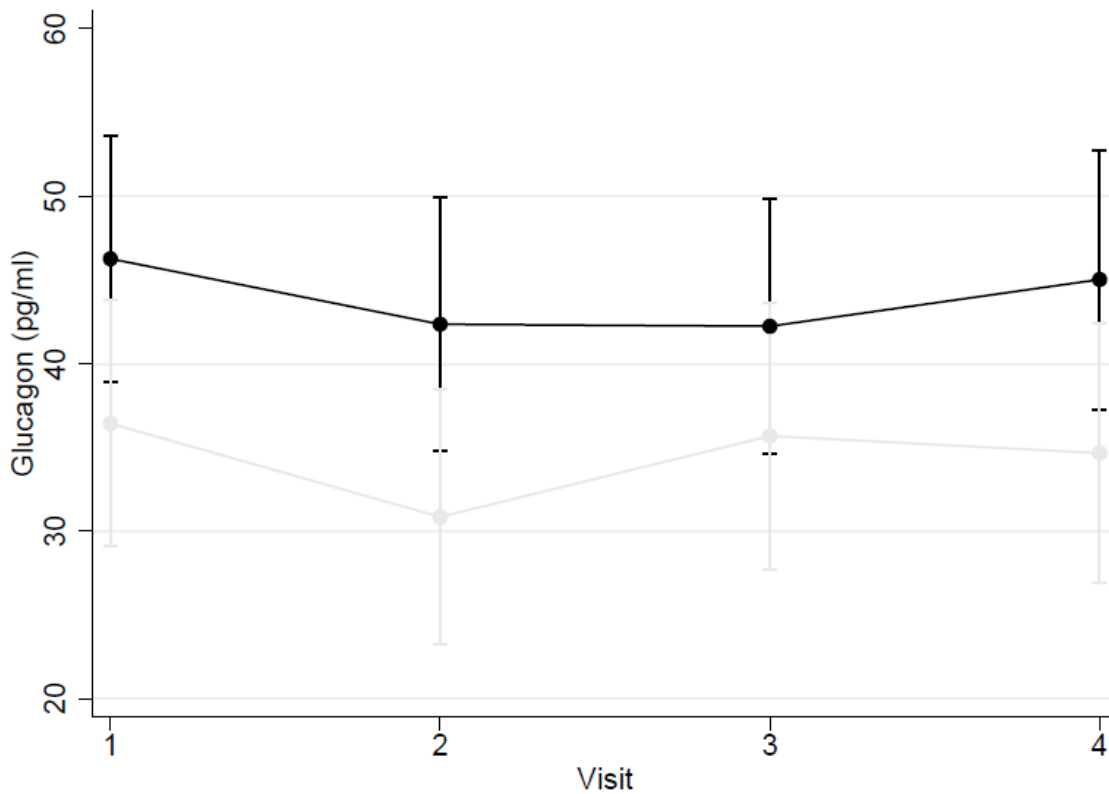
Figure 5.2. Effects of melatonin supplementation on plasma glucagon.

There was a main effect of treatment group such that participants had lower values in the melatonin group. Black, placebo group. Grey, melatonin group. Error bars are 95% confidence intervals.

Plasma HDL cholesterol also tended to differ between groups ($X^2(1)=5.06$, $p = 0.03$) such that participants in the melatonin group tended to have higher HDL cholesterol levels (0.31 pg/ml, 95% CI 0.10 to 0.52 mmol/L, $p = 0.004$, Figure 5.3).
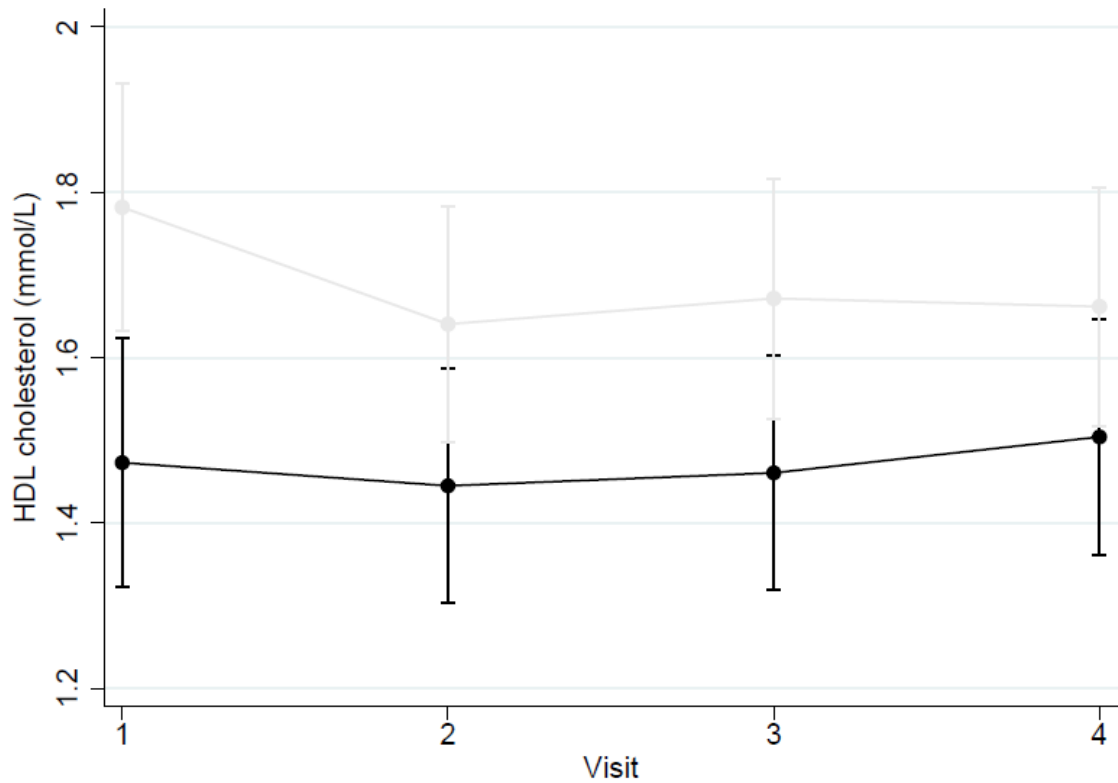
Figure 5.3. Effects of melatonin supplementation on plasma high-density lipoprotein (HDL) cholesterol.

There was a main effect of treatment group such that participants had higher values in the melatonin group. Black, placebo group. Grey, melatonin group. Error bars are 95% confidence intervals.

5.4.4 Melatonin and sleep

There were no main effects of either treatment or time on chronotype or social jetlag (Table 5.3).

Table 5.3. Effects of melatonin supplementation on sleep.

Characteristic	Main effect of treatment		Main effect of time		Treatment * time interaction		Random effects	
	X^2 (1 df)	p value	X^2 (3 df)	p value	X^2 (3 df)	p value	Within-participant variance (1 SD (95% CI))	Between-participants variance (1 SD (95% CI))
Sleep duration	0.59	0.44	8.06	0.045	11.56	0.009	0.23 (0.15 to 0.34)	0.98 (0.53 to 1.80)
Chronotype	0.71	0.40	0.28	0.96	0.28	0.96	0.09 (0.06 to 0.13)	0.62 (0.34 to 1.13)
Social jetlag	0.15	0.70	3.91	0.27	1.10	0.78	0.19 (0.12 to 0.29)	0.38 (0.18 to 0.77)
PSQI score	0.18	0.67	8.92	0.03	1.48	0.69	3.28 (2.63 to 4.08)	8.33 (5.64 to 12.29)

Legend: CI (confidence interval), PSQI (Pittsburgh Sleep Quality Index).

Sleep duration tended to differ significantly across study visits ($X^2(3)$=8.06, p = 0.05), and there was a significant treatment * time interaction ($X^2(3)$=11.56, p = 0.009, Figure 5.4). Pairwise comparisons showed that sleep duration differed across study visits in the placebo group only. In the placebo group, sleep duration at study visit four was longer than at study visit three (0.93 hours, 95% CI 0.38 to 1.48 hours, p = 0.001), sleep duration at study visit four was longer than at study visit two (0.81 hours, 95% CI 0.30 to 1.32 hours, p = 0.002), and sleep duration at study visit four was longer than at study visit one (0.65 hours, 95% CI 0.17 to 1.13 hours, p = 0.008). In the melatonin group, however, sleep duration at study visit four did not differ from study visit three (-0.05 hours, 95% CI -0.44 to 0.33, p = 0.78), study visit two (-0.11 hours, 95% CI -0.50 to 0.29, p = 0.60), or study visit one (-0.07 hours, 95% CI -0.48 to 0.35, p = 0.75).
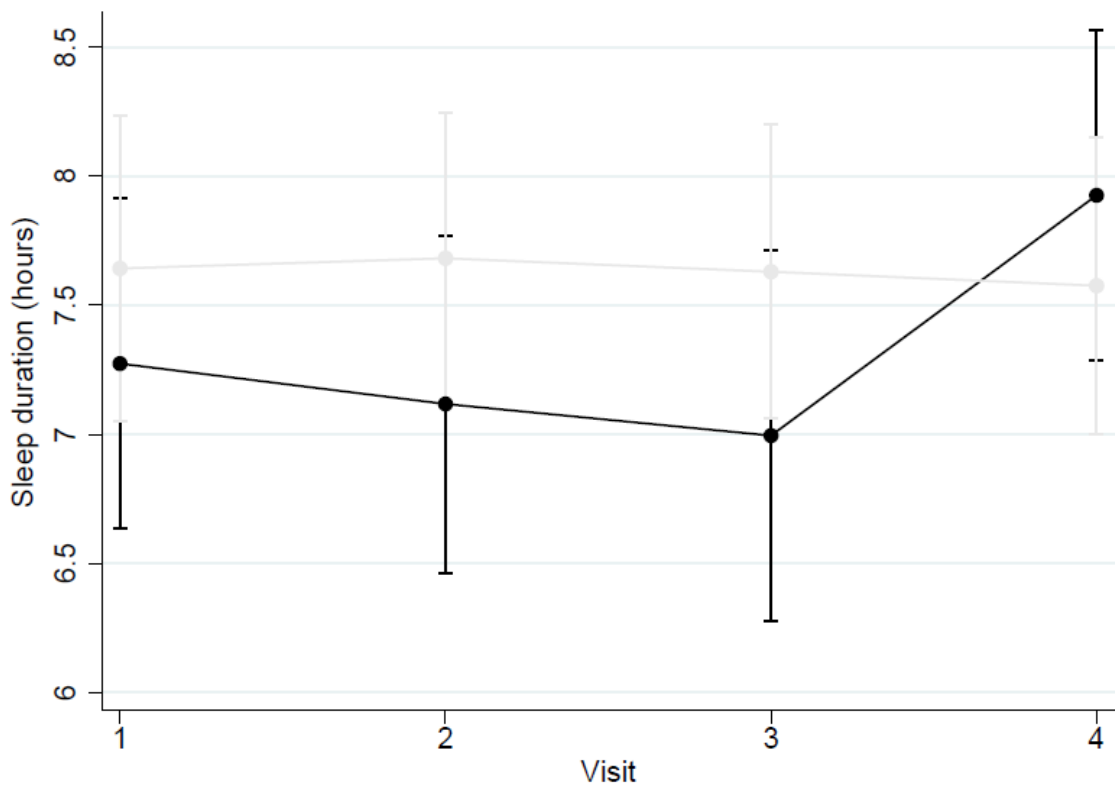
Figure 5.4. Effects of melatonin supplementation on sleep duration.

Sleep was longer at study visit four than visit one, two, and three in the placebo group only. Black, placebo group. Grey, melatonin group. Error bars are 95% confidence intervals.

Sleep quality (PSQI score) tended to differ across study visits ($X^2(3)$=8.92, p = 0.03) such that participants appeared to have the lowest PSQI scores at study visit three (-0.74

compared to study visit one, 95% CI -1.64 to 0.16, p = 0.11, Figure 5.5). (Lower PSQI scores reflect higher sleep quality.)
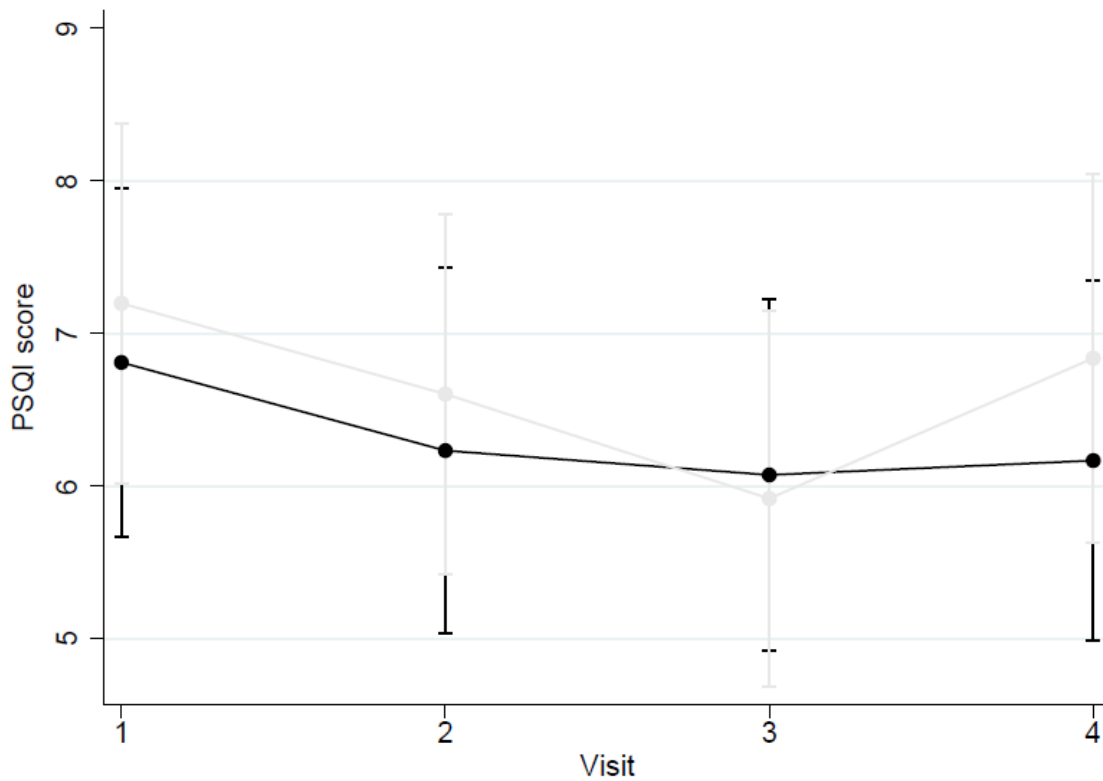
Figure 5.5. Effects of melatonin supplementation on Pittsburgh Sleep Quality Index (PSQI) scores.

There tended to be a main effect of time such that participants had the lowest PSQI scores at study visit three. Black, placebo group. Grey, melatonin group. Error bars are 95% confidence intervals.

5.4.5 Melatonin and diet

There were no main effects of either treatment or time on energy, carbohydrate, protein, fibre, fruit, or vegetable intakes (Table 5.4).

Table 5.4. Effects of melatonin supplementation on diet.

Characteristic	Main effect of treatment		Main effect of time		Treatment * time interaction		Random effects	
	X^2 (1 df)	p value	X^2 (3 df)	p value	X^2 (3 df)	p value	Within-participant variance (1 SD (95% CI))	Between-participants variance (1 SD (95% CI))
Energy	0.02	0.90	4.22	0.24	0.43	0.93	244,585 (175,255 to 341,342)	186,182 (81,526 to 425,186)
Carbohydrate	0.07	0.79	3.73	0.29	1.24	0.74	6,265 (4,524 to 8,676)	2,853 (1,107 to 7,356)
Fat	0.16	0.69	9.11	0.03	0.53	0.91	586 (419 to 819)	472 (208 to 1,071)
Protein	1.04	0.31	3.92	0.27	3.28	0.35	528 (380 to 733)	244 (92 to 643)
Fibre	0.55	0.46	1.59	0.66	1.24	0.74	91 (67 to 125)	63 (31 to 129)
Total vegetables	0.64	0.42	0.92	0.82	0.56	0.91	28,225 (20,658 to 38,563)	16,067 (7,465 to 34,580)
Total fruits	0.01	0.93	3.37	0.34	0.38	0.95	22,376 (16,354 to 30,616)	23,170 (12,155 to 44,169)

Legend: CI (confidence interval).

Fat intake tended to differ across study visits ($X^2(3)=9.11$, $p = 0.03$) such that participants tended to have higher fat intakes at study visit two than study visit three (16.5 g, 95% CI -1.8 to 34.9 g, $p = 0.08$, Figure 5.6).
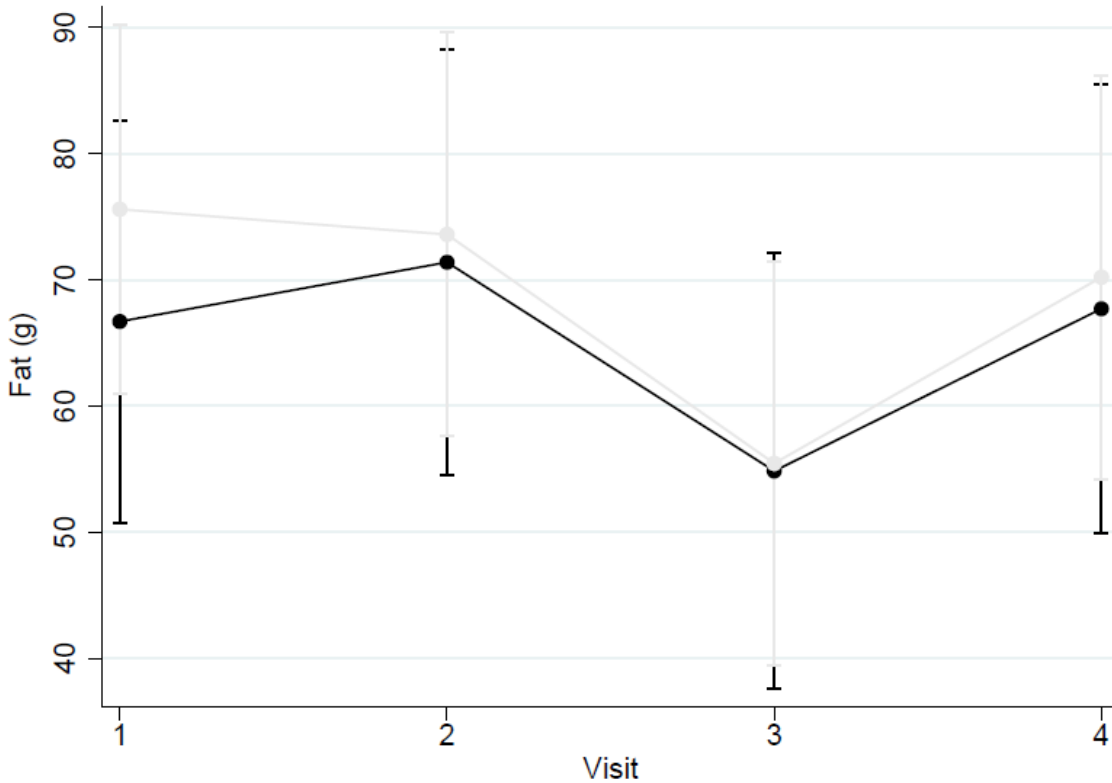
Figure 5.6. Effects of melatonin supplementation on fat intake.

Fat intake tended to be higher at study visit two than at study visit three. Black, placebo group. Grey, melatonin group. Error bars are 95% confidence intervals.

5.4.6 Exploratory analyses

None of the exploratory analyses that used spot plasma melatonin concentration as the predictor variable and the same outcomes as the main analyses was significant (Tables 5.5 to 5.7). Exploratory results therefore corroborate findings of the main analyses, which also showed little effect of melatonin treatment.

Table 5.5. Plasma melatonin, anthropometry and metabolic health.

Characteristic	Main effect of melatonin		Random effects		Number of participants (number of observations)
	X² (95% CI)	p value	Within-participant variance (1 SD (95% CI))	Between-participants variance (1 SD (95% CI))	
BMI	0.002 (-0.002 to 0.006)	0.30	0.61 (0.49 to 0.76)	25 (18 to 35)	66 (226)
Waist circumference	0.008 (-0.010 to 0.025)	0.40	10.8 (8.7 to 13.5)	177 (124 to 251)	66 (225)
Systolic blood pressure	-0.004 (-0.050 to 0.042)	0.87	84 (68 to 105)	133 (87 to 204)	67 (230)
Diastolic blood pressure	0.004 (-0.028 to 0.036)	0.79	41 (33 to 52)	45 (29 to 72)	67 (230)
Fasting glucose	0.000 (-0.002 to 0.002)	0.93	0.14 (0.12 to 0.18)	0.17 (0.11 to 0.27)	67 (227)
HbA1c	-0.002 (-0.009 to 0.006)	0.67	1.84 (1.45 to 2.35)	14 (10 to 20)	63 (196)
Glucagon	-0.021 (-0.010 to 0.053)	0.58	222 (179 to 276)	292 (191 to 448)	67 (230)
Adiponectin	0.000 (-0.004 to 0.004)	0.94	0.55 (0.44 to 0.69)	9.33 (6.58 to 13.22)	67 (230)
Leptin	0.018 (-0.009 to 0.044)	0.19	25 (20 to 32)	296 (208 to 421)	67 (230)
HDL cholesterol	0.000 (-0.001 to 0.001)	0.76	0.027 (0.021 to 0.034)	0.16 (0.11 to 0.23)	62 (204)
LDL cholesterol	0.001 (-0.001 to 0.003)	0.18	0.11 (0.09 to 0.14)	0.55 (0.38 to 0.81)	63 (205)
Total cholesterol	0.001 (-0.001 to 0.003)	0.22	0.14 (0.11 to 0.18)	0.59 (0.40 to 0.86)	63 (206)
Total: HDL cholesterol	0.001 (-0.001 to 0.002)	0.48	0.09 (0.07 to 0.12)	0.91 (0.63 to 1.31)	63 (205)
Triglycerides	0.001 (-0.001 to 0.002)	0.44	0.08 (0.06 to 0.10)	0.19 (0.12 to 0.28)	63 (206)

	Main effect of melatonin		Random effects		
	X² (95% CI)	p value	Within-participant variance (1 SD (95% CI))	Between-participants variance (1 SD (95% CI))	Number of participants (number of observations)
Oxidised LDL	0.014 (-0.021 to 0.049)	0.44	45 (37 to 57)	171 (118 to 248)	67 (230)
IL-6[a]	-0.001 (-0.003 to 0.002)	0.61	0.22 (0.18 to 0.27)	0.23 (0.14 to 0.35)	67 (230)
CRP[a]	-0.001 (-0.007 to 0.006)	0.87	1.35 (1.07 to 1.72)	0.99 (0.59 to 1.67)	63 (199)

Legend: BMI (body mass index), CI (confidence interval), CRP (C-reactive protein), HbA1c (glycated haemoglobin), HDL (high-density lipoprotein), IL (interleukin), LDL (low-density lipoprotein).

[a]Data were positively skewed and so were log-transformed.

Table 5.6. Plasma melatonin and sleep.

	Main effect of melatonin		Random effects		
Characteristic	X² (95% CI)	p value	Within-participant variance (1 SD (95% CI))	Between-participants variance (1 SD (95% CI))	Number of participants (number of observations)
Sleep duration	0.002 (-0.003 to 0.007)	0.37	0.23 (0.15 to 0.35)	1.13 (0.61 to 2.09)	28 (74)
Chronotype	-0.001 (-0.003 to 0.002)	0.67	0.08 (0.05 to 0.13)	0.62 (0.34 to 1.10)	28 (71)
Social jetlag	0.000 (-0.005 to 0.004)	0.83	0.20 (0.13 to 0.31)	0.37 (0.18 to 0.75)	27 (71)
PSQI score	-0.003 (-0.013 to 0.006)	0.49	3.3 (2.6 to 4.1)	8.8 (5.9 to 13.1)	65 (214)

Legend: CI (confidence interval), PSQI (Pittsburgh Sleep Quality Index).

Table 5.7. Plasma melatonin and diet.

| Characteristic | Main effect of melatonin | | Random effects | | Number of participants |
	X^2 (95% CI)	p value	Within-participant variance (1 SD (95% CI))	Between-participants variance (1 SD (95% CI))	(number of observations)
Energy	-2.1 (-6.3 to 2.1)	0.32	244,827 (173,812 to 344,857)	184,983 (80,801 to 423,490)	34 (107)
Carbohydrate	0.002 (-0.65 to 0.66)	1.00	6,516 (4,662 to 9,108)	2,410 (833 to 6,977)	34 (107)
Fat	-0.17 (-0.38 to 0.05)	0.13	638 (451 to 902)	478 (204 to 1,122)	34 (107)
Protein	-0.16 (-0.35 to 0.03)	0.10	534 (381 to 717)	319 (134 to 757)	34 (107)
Fibre	-0.04 (-0.12 to 0.04)	0.31	97 (70 to 133)	61 (29 to 129)	34 (107)
Total vegetables	0.19 (-1.23 to 1.60)	0.79	29,270 (21,207 to 40,397)	14,515 (6,336 to 33,252)	34 (107)
Total fruits	0.10 (-1.15 to 1.36)	0.87	21,038 (15,223 to 29,075)	22,293 (11,689 to 42,518)	34 (107)

Legend: CI (confidence interval).

5.5 Discussion

Contrary to our hypotheses, we found few effects of long-term melatonin supplementation on measures of metabolic health, sleep, and diet in adults at elevated risk of type two diabetes. Plasma glucagon was lower and plasma HDL was higher in the melatonin group, but this was true across all study visits. Subjective sleep quality was highest in both groups at study visit three, and dietary fat intake was higher in both groups at study visit two than at study visit three, but there was no effect of melatonin treatment on any of these outcomes. The only outcome for which the effects of treatment differed across time was sleep duration, which was longer at visit four in the placebo group only. Visit four was after a 12 week washout period, however. Together, these findings do not support the use of melatonin as a countermeasure against development of metabolic diseases such as type two diabetes, although this does not mean that melatonin might not influence cardiometabolic health in other circumstances.

The primary outcome of the study was change in HbA1c, a measure of long-term blood glucose levels. It was previously found that open-label use of the same slow-release melatonin formulation used in the study of this chapter reduced HbA1c after five months in type two diabetes patients with insomnia. Before the open-label period, however, participants were blinded to treatment and crossed over between melatonin and placebo conditions. During this initial period, treatment only improved sleep quality and did not influence HbA1c (342). A few factors may explain the discrepant findings between this previous study and that of this chapter. First, baseline HbA1c levels were roughly twice as high in the previous study. Second, participants in the prior study were also using either oral hypoglycaemic agents or insulin. Third, participants in the previous study complained of insomnia, although changes in sleep quality during treatment were not predictive of improvements in HbA1c.

HbA1c and fasting glucose are arguably the most clinically relevant measures of glucose metabolism, as both are routinely used to diagnose diabetes. Interestingly, the study discussed above also found no effects on fasting glucose after the crossover or open-label periods (342). This is commensurate with our findings, two previous studies of multiple weeks of melatonin supplementation in adults with metabolic syndrome (343, 344), and an open-label study of six months of 2 mg of melatonin treatment in elderly women (349). It therefore seems that long-term melatonin supplementation minimally influences fasting glucose.

Several hormones have critical roles in glucose metabolism. Of these, I analysed data for glucagon and adiponectin. Glucagon is a pancreatic peptide hormone that antagonises the effects of insulin by stimulating gluconeogenesis, glycogenolysis, and

lipolysis, also inhibiting glycogenesis and lipogenesis (473). In vitro experiments and pre-clinical studies of rodents have shown that melatonin tends to increase glucagon levels (474). To my knowledge, only one study has documented effects of long-term melatonin supplementation on glucagon in humans, reporting lower insulin/glucagon ratios after three months of 4 mg melatonin each night, an effect only present in carriers of the rs10830963 risk variant of *MTNR1B* (354). Such previous studies indicate that it might be useful to determine if *MTNR1B* genotypes mediate how metabolic outcomes respond to melatonin supplementation. Although we found that plasma glucagon was lower in the melatonin group, this was across all study visits, so melatonin did not seem to meaningfully influence glucagon levels.

Adiponectin is a protein produced by adipocytes and other cells. Adiponectin has anti-inflammatory and insulin-sensitising effects through interactions with its receptors at tissues such as the liver and skeletal muscle. Specifically, adiponectin enhances muscle glucose uptake, reduces hepatic gluconeogenesis, and increases lipid oxidation (475). Several studies of rodents reported that melatonin supplementation or injection raises adiponectin levels (359). Regarding humans, a four-week trial found that twice-daily (once in the morning, once in the evening) melatonin supplementation increased plasma adiponectin levels by 119% in adults with non-alcoholic steatohepatitis (476). Another study reported that one year of nightly treatment with melatonin tended to increase adiponectin in post-menopausal women with osteopaenia (345). Again, however, we found no effects of melatonin supplementation on plasma adiponectin. Collectively, our findings show that long-term melatonin supplementation has negligible effects on key hormones involved in glucose regulation in relatively healthy adults.

Like adiponectin, leptin is a peptide hormone secreted primarily by adipocytes. Leptin is produced in proportion to adipocyte mass and hence signals energy availability in the brain. Leptin has widespread roles in determining energy balance, influencing autonomic nervous system activity, many neuroendocrine processes, and behaviours such as physical activity (477). Studies of rodents have generally found that melatonin supplementation or injection reduces weight and leptin levels, although there is some evidence to the contrary, likely as a result of differences between studies in variables such as dietary composition (359). Few human studies have yet assessed effects of melatonin supplementation on leptin. A single daytime melatonin dose administered at three different times did not acutely influence leptin levels in post-menopausal women (478). Conversely, a larger dose reduced leptin levels in male patients with liver cirrhosis and portal hypertension but increased levels in healthy male controls, a difference perhaps related to variation in liver metabolism of melatonin (479). The only longer-term trial was the aforementioned study of adults with non-alcoholic steatohepatitis, in whom

melatonin increased plasma leptin levels by 33% (476). In contrast to these studies, we found no effect of long-term melatonin supplementation on plasma leptin levels in adults. As leptin levels generally reflect energy availability, some might expect plasma leptin to have tracked BMI. It is interesting that there were no differences in leptin, despite BMI increasing from visit one to visit three. Effects of leptin are dependent on leptin sensitivity, so altered leptin sensitivity across study visits is a plausible but speculative explanation for this result. Regardless, there was no difference between groups.

Previous studies found that adults with metabolic syndrome tended to lose weight during long-term melatonin supplementation (343, 344), and numerous studies of rats have also reported that melatonin protects against development of obesity and central obesity (480). Beyond weight alone, melatonin may influence body composition in some circumstances, as one year of melatonin supplementation increased lean body mass in post-menopausal women, without affecting weight (345). We therefore expected to melatonin to influence body composition, but we found no effects of melatonin treatment on waist circumference or changes in BMI, which increased from study visit one to study visit three in both groups. It is noteworthy that not all previous studies have reported consistent effects of melatonin on bodyweight. In the study of metabolic syndrome patients whose health had not responded to a lifestyle intervention, BMI fell after one month of 5 mg melatonin each evening, but effects were not apparent after two months of melatonin treatment (343). Furthermore, the study in which metabolic syndrome patients were crossed over for 10 weeks found that weight fell 1.9 kg on average while taking melatonin, but this was not quite significant (p = 0.09). If melatonin influences bodyweight, it appears that effects are modest. Furthermore, our findings do not suggest that melatonin markedly influences diet. Diet is a key determinant of bodyweight, but only fat intake was found to differ across time. Notably, however, diet was only assessed in a subset of participants, limiting statistical power to detect differences.

Excess adiposity often coincides with dyslipidaemia, a core characteristic of many cardiometabolic diseases. Multiple studies of rats have reported that melatonin supplementation protects against dyslipidaemia (480). Results from studies of humans are equivocal, however. Whereas one month of 1 mg of melatonin each evening was found increase HDL cholesterol in peri- and post-menopausal women (346), others reported that melatonin increased triglycerides (347) and very-low-density lipoprotein cholesterol in normolipidaemic post-menopausal women (348). Conversely, pilot studies found negligible effects on lipids in elderly women (349) and hypercholesterolaemic patients (350). Although we found that HDL cholesterol levels were higher in the melatonin group, this was true across all study visits, and LDL cholesterol and

triglycerides were not influenced by treatment. Melatonin did not appreciably influence routinely-measured blood lipids in this study.

The atherogenic potential of blood lipids is increased by their oxidation (481), and systemic oxidative stress contributes to the pathogenesis of many disorders, including metabolic syndrome (323). In vitro work has shown that melatonin and its metabolites have many roles in protecting against mitochondrial dysfunction and hence excessive production of reactive oxygen species (482). Furthermore, melatonin is directly involved in defence against oxidative stress and scavenging free radicals (322), and in vitro work has shown that melatonin inhibits oxidation of LDL cholesterol (347, 483). We therefore hypothesised that melatonin would counter any excessive inflammation and hence reduce oxidised LDL, CRP, and IL-6. However, melatonin supplementation did not influence any of these outcomes. A previous study found that long-term melatonin supplementation did not influence CRP in adults with metabolic syndrome (343). There is otherwise little evidence regarding changes in CRP and IL-6 after melatonin supplementation in humans, other than small trials in which various doses of melatonin have been assessed for their effects on inflammatory responses to surgery. In none of these did melatonin influence CRP or IL-6 (484-486).

Inflammation influences blood pressure, and melatonin has been found to alter blood pressure through effects on the autonomic nervous system and nitric oxide signalling. Numerous studies have reported that melatonin reduces blood pressure, and a meta-analysis found that the same prolonged-release melatonin formulation used in this study reduced blood pressure in nocturnal hypertension (487). We did not find any effects of melatonin supplementation on blood pressure, however, perhaps because participants mostly had healthy blood pressure values. It is possible that we might have detected differences had 24-hour blood pressure monitoring been used rather than a single morning measure, and this is true of most outcomes in this study. It is also noteworthy that effects of melatonin on vascular function may depend on which vessels are measured: A study of adults reported that whereas melatonin reduced renal blood flow velocity and vascular conductance, melatonin increased forearm blood flow velocity and vascular conductance (488).

Many studies have found that use of melatonin and other melatonin receptor agonists improve multiple aspects of sleep, including sleep duration and quality in people with difficulty sleeping (465). We found sleep was longer at visit four in the placebo group only, a finding that is difficult to explain if the possibility of a false-positive finding is overlooked. We also found that sleep quality improved from study visit one to study visit three. However, melatonin supplementation did not influence this trend, and a placebo

effect is therefore perhaps the most plausible explanation. PSQI scores of five or more are generally considered indicative of poor sleep quality, and mean PSQI scores at the first study visit in each group exceeded five. I therefore expected melatonin to influence sleep quality. Because participants were instructed to take melatonin at a consistent time shortly before bed, I did not anticipate that melatonin would influence chronotype or social jetlag, and this was the case.

The melatonin dose used in this study may have been suboptimal, and it is tempting to speculate that a higher dose might have produced stronger effects. However, the dose used in this study was certainly supra-physiological. Peak daytime and night-time serum melatonin concentrations in healthy young adults are ~ 10 and 60 pg/ml blood, respectively (472). However, just 1 mg of exogenous melatonin can increase daytime serum melatonin concentration to over 400 pg/ml in healthy adults, and 10 mg can produce serum levels exceeding 6,000 pg/ml (489). Therefore, the 2 mg dose taken by participants was likely sufficient, regardless of whether it was a slow-release formulation. It is also possible that effects of melatonin supplementation may have differed according to differences between participants in pre-intervention melatonin signalling, but we did not measure overnight measure melatonin levels at this time or any other.

Participants in the previous human studies that have found beneficial effects of melatonin have generally had metabolic health problems such as metabolic syndrome. As participants in this study had at least one first-degree relative with type two diabetes, participants were at increased risk of this disease. However, most of participants' metabolic characteristics at the first study visit were within recommended ranges, even if they were typically overweight. This could explain why we found few effects of melatonin: perhaps melatonin supplementation is more beneficial in people with existing health problems.

This study had several strengths. First, the randomised, double-blind, placebo-controlled design was suited to making inferences about causality. Second, the intervention duration exceeded that of similar previous studies (342-344), and repeated measures after a washout period showed whether any treatment effects persisted. Third, this study more comprehensively profiled metabolic health than prior studies. Fourth, this is the first study to assess whether melatonin supplementation influences diet in humans. Fifth, this is the first study to assess the effects of long-term melatonin supplementation on metabolic health in relatively healthy adults.

This study also had limitations, however. First, the sample size was smaller than planned, limiting statistical power needed to detect differences between groups. Second, overnight measures of melatonin at the first study visit were not collected to determine

whether differences in endogenous melatonin mediate treatment responses. Third, *MTNR1B* genotype was not determined, despite previous work showing that *MTNR1B* genotype influences responses to treatment (354). Fourth, the study did not include objective measures of sleep. Fifth, as is true of other similar studies, the study had no objective biomarker of compliance with the intervention, instead using tablet counts. Sixth, the participants were given lifestyle advice at study visits that was not standardised.

In summary, long-term melatonin use had negligible effects on most anthropometric, metabolic, sleep, and dietary outcomes in first-degree relatives of people with type two diabetes. Future studies that build on these findings are needed. In particular, studies with larger participant samples that consider how different participant characteristics influence responses to melatonin use will be insightful. Studies should also consider different clinical populations and test various melatonin doses and formulations. Melatonin could still prove to be a useful prophylactic agent against cardiometabolic disease development, but findings presented in this chapter do not support its use in similar populations.

Chapter 6: Discussion, directions for future work, and conclusions

6.1 Summary of project contributions to existing knowledge

The vital roles of the circadian system, sleep, and diet in metabolic health were evident long before this project began. Nevertheless, my initial literature review identified novel questions that are relevant to public health within these research topics. Several of these questions were within the scope of this project, and the previous four chapters document most of our efforts to answer these questions. In this final chapter I will summarise project findings (Figure 6.1) and their implications, discuss project strengths and limitations, and suggest ideas for future studies to build on this work and respond to ongoing societal developments.

1. Literature review

2. Validation of my**food**24, an online dietary assessment tool suited to large-scale studies

3. Use of my**food**24 shows later circadian timing of food intake associates with overweight and obesity

4. Sleep duration is unrelated to diet but is inversely associated with body mass index in a national database

5. Small RCT shows long-term melatonin supplementation does not affect sleep, diet, or metabolism

Figure 6.1. Summary of project findings.

Numbers correspond to chapters. Legend: RCT (randomised controlled trial).

6.1.1 Validation of a new dietary recall tool that might help unveil diet-disease associations

Diet quality influences disease risk and human lifespan (263). Studies that have assessed the interplay between sleep, diet, and health have produced some conflicting findings, perhaps in part because of use of dietary assessment methods with unknown validity in documenting diet composition. As it appears that it is not only what we eat but *when* we eat that matters (490), another pitfall of many dietary assessment methods used is their failure to document diet timing.

We therefore assessed the validity of measure your food on one day 24-hour recall (myfood24), an online dietary recall tool developed to overcome some of the limitations inherent to other dietary assessment methods used with UK adults. Usability testing completed before the myfood24 validation study had shown that myfood24 is appropriately designed for use with UK adolescents and adults (391), and a subsequent study demonstrated that myfood24 has good agreement with the gold-standard interviewer-administered multiple-pass 24-hour recall method (MPR) in adolescents (415). But we still knew nothing of the validity of myfood24 in adults, as shown by its performance relative to reference measures.

In the myfood24 validation study, we compared agreement between myfood24 and a suite of reference measures to agreement between the MPR and the same reference measures. I completed all of the laboratory analyses for two of the reference measures. We found that the dietary recall methods produced similar results, validating myfood24 for use in Chapters 3 and 5 of this project and showing that use of myfood24 has great potential to document diet-disease relationships in future studies of UK adults.

6.1.2 Use of the myfood24 validation study dataset to show that the circadian timing of food intake is associated with body mass index

While reviewing the literature I realised that although there is some evidence that late eating is associated with body mass index (BMI) (246), nobody had directly studied whether diet timing *relative to circadian phase* was associated with metabolic outcomes, despite considerable evidence that the circadian system tunes many metabolic functions according to circadian time (491). My impression was that a salient question was how to best measure diet timing. Human sleep is relatively consolidated and is therefore quite evenly spread across the night, so using mid-sleep time is a reasonable way to assess sleep timing. People are likely to distribute their energy intakes unevenly across the

waking day, however, so using the halfway time between ingestion of the first and last caloric event of the day (the caloric midpoint) might be inappropriate. Mammals like mice do not have such consolidated sleep as humans, and time at 50% of daily locomotor activity is sometimes used to assess circadian phase in these animals (492). I therefore applied this same logic to assess diet timing, using the time of day at which people surpass 50% of daily caloric intake to approximate dietary phase.

I found no evidence regarding whether more variable sleep timing was associated with less consistent diet timing. This seemed significant, as 1) irregular sleep is associated with poorer metabolic health (26), and 2) inconsistent eating patterns adversely affect metabolic responses to feeding, including diet-induced thermogenesis and blood glucose regulation (308). I therefore used the myfood24 dataset of Chapter 2 to address these gaps in the literature.

After I analysed the data presented in Chapter 3, a paper was published addressing the same fundamental question that I sought to answer (268). The paper reported that later circadian timing of food intake was indeed associated with adiposity in a homogeneous group of young US adults. Chapter 3 adds to this published work by studying a larger group of adults from a different country, and also by assessing associations between sleep timing variability and diet timing variability.

In Chapter 3 I revisited the myfood24 data, scoring 564 sleep records, and cleaning and scoring 550 diet recalls. Results of the main analysis agree with the findings of McHill and colleagues (268): Participants with smaller dietary phase angles were more likely to be overweight or obese. Although sleep timing was generally related to diet timing, sleep timing variability was not associated with diet timing variability. As I assessed the exact timing of all caloric events, the chapter also gives a higher resolution analysis of temporal patterns of diet in UK adults than previously reported. The findings strengthen the evidence implicating eating at suboptimal circadian phases in the pathogenesis of obesity and provide new insights into how sleep relates to diet in UK adults.

6.1.3 Use of the National Diet and Nutrition Survey to explore associations between sleep duration, diet, and objective measures of metabolic health concurrently for the first time in UK adults

Cross-sectional studies have sometimes reported that short-sleeping adults consume less healthy diets (365), and many large-scale studies have identified short sleep as a risk factor for obesity (32). In my literature review, however, I found no studies that had concurrently assessed associations between sleep duration and diet as well as between

sleep duration and objective measures of metabolic health in UK adults. We therefore sought a larger dataset to explore these relationships, choosing to use data from years 1 to 4 of the National Diet and Nutrition Survey Rolling Programme (NDNS-RP) to do so. I designed and completed all of the analysis.

As anticipated, sleep duration was negatively associated with BMI and waist circumference. Sleep duration tended to be associated with more healthy blood lipids. Contrary to our expectations, however, sleep duration was not associated with dietary measures. Chapter 4 therefore reinforces findings of other studies showing that sleep duration influences metabolic health. Chapter 4 also adds to these studies by more comprehensively profiling metabolic health than similar studies of UK adults (445, 493).

6.1.4 A small randomised controlled trial showed that long-term melatonin supplementation does not substantially influence sleep, diet, and metabolic health in UK adults predisposed to type two diabetes

Against the background of ongoing environmental changes, many people are at increasing risk of the unhealthy metabolic correlates of circadian system and sleep disruption (416). Safe therapies to facilitate circadian system alignment and enhance sleep are therefore attractive candidates in the prevention of metabolic diseases. As a proven chronobiotic with somnogenic effects when supraphysiological doses are ingested, melatonin meets these criteria (54). Several preliminary studies reported that melatonin improves various health parameters in adults with metabolic disorders (342, 343). These studies have significant methodological issues, however. Furthermore, no randomised controlled trial had yet assessed whether 1) melatonin is an effective countermeasure against metabolic disease development, and 2) melatonin affects diet.

We therefore assessed the effects of long-term melatonin supplementation on metabolic function, sleep, and diet in first-degree relatives of people with type two diabetes. I supervised myfood24 diet recalls at study visits. I then completed the glucagon, adiponectin, leptin, interleukin-6, and oxidised LDL cholesterol laboratory analyses. I also scored the Pittsburgh Sleep Quality Index and Munich ChronoType Questionnaires to assess sleep. Finally, I analysed the data.

Contrary to our hypotheses, long-term melatonin supplementation did not significantly affect any of the metabolic or dietary outcomes measured. Although null findings can reflexively feel disheartening, the study results are important, as they indicate the need to study alternative interventions to enhance metabolic regulation in people with similar phenotypes (discussed in section 6.4).

6.2 Implications of our findings

Our findings have two main implications that may contribute to informing public health policies concerning healthy lifestyle patterns. First, dietary guidelines should perhaps include more definitive advice on when to eat. Current UK government dietary recommendations focus exclusively on what to eat and drink but contain no advice on *when* to eat and drink. This is true of dietary guidelines in most countries. Perhaps the closest advance towards advice on when to eat is incorporation by some governments of the recommendation to consume breakfast, as was true when changes were made to the US dietary guidelines in 2010 (494). If future studies continue to support previous findings that mistimed food and drink intake initiates a panoply of adverse metabolic consequences (495), governments should revise their guidelines accordingly. A large proportion of people engage in shift work, so dietary recommendations tailored to these people would ultimately be another worthwhile development. Such recommendations may not yet be feasible, however, as few studies have directly addressed this question in humans yet.

The second implication is that many people might experience improved health if the stakeholders who make decisions about changes in clock time and employee working hours modified their practices to allow people more complete sleep wherever possible. While one can hope that instructing people to go to bed earlier and sleep more leads to the desired outcomes, lasting behaviour change is often frustratingly elusive. Indeed, recidivism to prior behaviours and hence health status is frequently the norm, as exemplified by people who unsuccessfully try to lose weight and then retain the weight loss (496). Many strategies to facilitate the widespread, sustainable adoption of more healthy behaviours have been proposed. One such strategy is a libertarian paternalistic approach, in which governments implement changes to gently coax people into healthier lifestyle patterns (497).

An example of the deleterious effects of changes in clock time is what appears to be an increase in traffic accidents following Daylight Savings Time (498). This is of little surprise given the sleep loss that people may experience after this transition. Circadian misalignment during changes in clock time is also a plausible contributor to some health problems, such as the apparent rise in unipolar depressive episodes that certain individuals suffer on transition from Summer Time (499). China is perhaps the most severe instance of political enforcement of inappropriate clock times. China spans five geographical time-zones, but the entire country follows Beijing time. The health effects of this discordance between clock time and solar time in China is a topic ripe for research.

Regarding work schedules, flexible working hours and shift work schedules tailored to individual chronotypes might help reduce sleep loss and circadian misalignment (250). In some circumstances the initial logistical difficulties posed by implementing flexible working hours may be outweighed by the benefits of enhanced worker satisfaction and productivity. If flexible schedules are unfeasible, delaying work times makes sense for the majority of workers, as ~ 80% of adults curtail sleep by using alarms to wake before work (26). And if this is still not an option, permitting workers naps may enhance productivity if they have accumulated sleep debts (500).

School start times exemplify the benefits of delaying the start of the work day. Most studies of the effects of delaying school start times in adolescents have found that adolescents sleep longer as a result of waking later, have fewer motor vehicle crashes, are less likely to be late or skip school, fall asleep in lessons less, and achieve better grades (501). Influencing stakeholders to enforce change can seem an insurmountable challenge, but educating these people about anticipated economic benefits may help persuade them. Recent macroeconomic modelling of the effects of delayed school start times in the US forecast that delaying school start to 08:30 would increase high school graduation, reduce adolescent car crashes, and result in an $83 billion surplus after a decade (502).

6.3 Project limitations and strengths

Projects inevitably have shortcomings, and a limitation of this project is inconsistencies in methods between chapters. Whereas sleep was assessed using wearable devices in Chapter 3, for example, questionnaires were used in chapters 4 and 5. Similarly, diet was assessed using different methods in these chapters. This inconsistency is an unavoidable consequence of using data collected by other groups (like the NDNS-RP data).

Cross-sectional research such as that of chapters 2, 3, and 4 has inherent limitations, including the possibility of reverse causality, undetected bias, and unmeasured confounding. Related to this, nutrition research has been heavily criticised on many occasions, often for good reasons (503). A huge array of dietary compounds has been associated with increased risk of cancer or protection from cancer, for instance, yet the reported associations generally shrink substantially in meta-analyses (504). Indeed, results from randomised trials of specific nutrients indicate that the magnitude of probable effects on many diseases is practically negligible (505). For this reason, many nutritionists have transitioned to using randomised trials to study the interplay of various nutrients via dietary pattern approaches, and this shift may overcome limitations of a

more nutrient-centric approach. Furthermore, much attention has recently been given to irreproducible study results (506), and many scientists are vying for use of more stringent significance thresholds.

I acknowledge that as is true of the vast majority of similar published research, my project is not immune to these limitations. Research that fulfils these criteria would be remarkably costly, and my intention with this project was to maximise my contribution to the literature within the constraints of the resources available. This is not to dismiss the potential value of adopting the above ideas, however, and I consider the growing focus on experimental rigour to be a productive development.

A key strength of this project is its contribution to the validation of a new tool suited to nutrition research in the 21st century. There is growing interest in big data – datasets so vast and complex that mining the information with traditional analytic methods is inappropriate. Online tools like myfood24 can record an unprecedented amount of dietary information, and studies of larger scales than existing research will help overcome the aforementioned pitfalls of nutritional research.

I suggest that another strength of this project is its breadth. This breadth encompasses the methods used: from validation of a dietary assessment method, to nutritional epidemiology research, to a randomised controlled trial. But this breadth also encompasses the cross-disciplinary nature of the project, bringing together the unique research disciplines of chronobiology, sleep, and nutrition. In doing so, the project recognises that all are interdependent. Projects with narrow scopes are of course necessary to elucidating nuances in the biological mechanisms that underlie health and disease, but such projects also risk detachment from the greater context in which the work fits. The variety of this project has therefore fostered both my enthusiasm and my appreciation for multidisciplinary research.

6.4 Possible directions for future work

I acknowledge that some of the suggestions I discuss here present considerable logistical difficulties. My ideas are only intended to encourage reflection.

Beginning with chapter 2, it would be productive to reach consensus on the best ways to assess validity of dietary recall methods. Next, we need to determine which dietary assessment tools are best by pitting similar methods head-to-head in the least biased way possible. An example of this would be independent researchers with no conflicts of interest directly comparing myfood24 to INTAKE24, against biomarkers of dietary intakes. Next, the best tool could be optimised for the needs of different populations, as

has been done for some 24-hour recall methods (507, 508). Digital methods could also be tweaked at this stage to capture other components of dietary patterns, such as the context in which foods and drinks are ingested (for example, the place of consumption and presence of others). Another consideration is that some participant characteristics clearly influence dietary recall accuracy. This is overtly evident in the tendency for people with obesity to underestimate intakes, and recall accuracy raises a consideration relevant to much human research: How do we best design studies to maximise compliance? It seems that this is a hugely important yet neglected research question.

Recovery biomarkers of dietary intakes are currently only available for energy, protein, potassium, and sodium intakes, so there is a glaring need to develop novel biomarkers of nutrient intakes. New biomarkers of intakes have recently been identified, such as proline betaine as a biomarker of citrus intake (509), and developments in '-omics' methods might be particularly well suited to identifying new biomarkers. Metabonomics is similar to metabolomics but uses a systems biology approach to determine metabolic responses to changes in stimuli such as diet (510). By identifying changes in metabolic profiles that correspond to dietary changes, nutritional metabonomics may be a particularly promising approach. Furthermore, development of new statistical analysis techniques could also be important to unveiling new biomarkers (377).

We also need to better understand how gene-nutrient and other interactions influence the validity of biomarker measures. Myriad factors conceivably influence the kinetics of the absorption, distribution, metabolism, and excretion of nutrients, and such factors might lead to physiological confounding of how accurately biomarkers predict true intakes (511). It is also important to consider that many of the studies that first identified recovery biomarkers only included small groups of relatively homogeneous participants. The study that validated urinary nitrogen as a recovery biomarker of protein intake included only eight participants, for example (366).

Chronobiomic studies that identify time-dependent biological signals could be particularly useful means of enhancing the precision of biomarker measures. Spot (single time-point) biomarker measurements are far more practical than 24-hour measurements but are also less accurate. The intersection of studies to identify novel biomarkers of circadian phase with research to determine effects of biological rhythms on associations between dietary intakes and nutritional biomarkers could therefore enhance the accuracy of spot biomarker measurements and hence improve power to detect diet-disease relationships.

Relevant to both Chapter 2 and Chapter 3, many approaches to studying dietary patterns have been used, including participant-identified meal patterns and time of day patterns. The variables studied partly depend on features of different dietary assessment methods,

but variables have commonly included meal frequency, skipping, spacing, and timing (264). It might be fruitful to reach consensus on the best ways to characterise dietary patterns of both diet composition and timing.

Studies of the effects of manipulating each caloric event will be instructive. An example is comparing the effects of long-term breakfast-skipping to long-term dinner-skipping on diet, metabolism, activity, and sleep. Another example is comparing the effects of long-term consumption of a high-protein event at a late circadian phase to long-term consumption of an isocaloric event comprised of carbohydrate and/or fat at a similar circadian time. Indeed, whereas findings of Chapter 3 and other research (268) indicate that caloric intake at a late circadian phase might be obesogenic, sports nutrition research has shown that pre-sleep protein intake improves body composition responses to resistance training (512).

Additional studies of effects of nutrient intake distributions across the day in different populations would also be useful. Elderly people, for example, are relatively resistant to the anabolic effects of protein intake on skeletal muscle mass, and their habitual patterns of protein intake are rarely conducive to offsetting the debilitating effects of sarcopaenia (513). The elderly also exemplify the fact that it is misguided to only focus on obesity: Some people would actually benefit from gaining weight, provided that the composition of the mass accrued is appropriate. We need more studies of clinical populations, an issue relevant to so much human health research.

Chapter 3 considered the circadian phase of eating, a subject in its infancy and therefore a topic on which many related studies are needed. First, precise measures of circadian phase are currently laborious, and we need to identify biomarkers of circadian phase, circadian misalignment, and sleep debt for use in field studies. Another consideration is that circadian phase is not fixed, and dynamic modelling of circadian phase may ultimately enhance experimental precision.

Chapter 3 is particularly relevant to shift workers, who are likely to often eat during the biological night. As shift work is a complex exposure scenario, there is a clear need to develop methods for field studies that better document the suite of exposures that shift work entails. For example, little is known about light exposure patterns among shift workers. A recent review reported that light intensity levels of 50 to 100 lux at the eye are typical during shift work, with levels periodically exceeding 200 lux (514). Validating new tools that are suited to large-scale studies (such as questionnaires) against calibrated light exposure measurement devices will help clarify how shift work exposures interact to influence health. It is plausible, for example, that sedentary behaviour during shift work is particularly detrimental to metabolic health, but at present this is mere

speculation. A neglected group of people who may also be particularly susceptible to similar health consequences is the cohabitants of shift workers - 'second-hand shift workers'. Little is known about circadian alignment, sleep, diet, and metabolic health in these people, to my knowledge.

Artificial lighting enables shift work, and we must consider the effects of nocturnal light pollution on the entire biosphere. To exemplify this, ~ 30% of vertebrates and > 60% of invertebrates are nocturnal (515). Like mammals, a diversity of plants and microorganisms also have circadian clocks that are being disrupted by artificial light at night (516, 517). Disruption at any level of an ecosystem is likely to produce numerous and hard to predict consequences at other levels too.

In Chapter 4 we focused on sleep. Given the lack of objective data, we did not consider physical activity. However, we need to better understand the reciprocal relationships between physical activity and sleep. We also did not study sleep disorders in this project, but sleep disorders are becoming increasingly problematic. National surveys in countries like Canada, the US, and several parts of Africa and Asia have shown that perhaps 25 to 40% of people worldwide report sleep difficulties including insomnia (518-520). If trends continue then insomnia will affect more people still in the coming years, and the economic cost of sleep problems is substantial (521, 522). Recent studies have explored the genetic basis of sleep phenotypes and their behavioural and metabolic correlates, an example of which is a recent genome-wide association study using UK Biobank data (523). Nevertheless, little is currently known about associations between many sleep problems, metabolic health, diet, and physical activity.

The digital revolution is a great opportunity to use data collected through digital devices to gain new insights into sleep and health. A good example of this is a recent analysis of self-reported light exposure and sleep data generated worldwide through a mobile phone app designed to reduce jetlag by prescribing optimal light schedules (524). By analysing the data in relation to solar data for the date and location from which data were sent, the researchers found that home country was a stronger predictor of sleep onset than sunset, indicating that social cues are important determinants of sleep duration through their influence on sleep onset time. Perhaps unsurprisingly, sleep duration was therefore correlated with sleep onset time but not wake time in the 20 countries with the most respondents. The study highlights how simple digital tools can be used to test hypotheses worldwide with relative ease.

Using big data to better understand global health is not yet so feasible in many parts of the world, however, and very little research has been done in many countries rife with poverty. The significance of this cannot be downplayed, and the financial cost of

improving health and quality of life in 'less-developed' countries is typically so much lower than in more affluent countries. There has been comparatively little sleep research in countries in Africa and Asia, but a recent study found that 17% of adults aged ≥ 50 years reported sleep problems in eight countries across Africa and Asia. If representative of the United Nations definition of less-developed countries, by 2030 this would correspond to more than 260 million adults in this age range worldwide (520). And this could be an underestimate, as the researchers only included people reporting severe sleep difficulties. Within the countries studied, there was large variability in sleep problems prevalence (from 4 to 44%), and differences were not due to poverty alone. Future research in such parts of the world could be hugely valuable to public health worldwide. We must therefore also acknowledge and rectify the problems that accompany the substantial racial bias in research, a bias exemplified by the difficulties in applying personalised genomic medicine approaches in minority populations that result from the disproportionately large amount of data collected on Caucasian people (525).

Sometimes sleep loss is inevitable, and identification of strategies to counter the biological and behavioural consequences of sleep loss will be beneficial. Sleep extension appears to benefit many aspects of metabolic health. Among short-sleeping adults, for example, increased time in bed after a sleep extension intervention has been associated with improvements in glucose regulation and insulin sensitivity (460), and as few as three days of sleep extension may benefit insulin action and increase testosterone in habitually short-sleeping men (526). Sleep extension might also improve body composition. Increased sleep duration has been prospectively associated with attenuated increases in adiposity in short-sleepers (527), and findings from a study of overweight, habitually short-sleeping young adults show that this may be related to increased energy expenditure. Among these individuals, two weeks of two hours of increased time in bed in home environments increased sleep duration and daytime energy expenditure, also reducing appetite and desire for highly palatable foods (459).

One factor that may influence the effects of sleep extension interventions on diet is chronotype. Using a crossover design to change the time at which adolescents went to bed and thereby compare a 6.5- to a 10-hour sleep opportunity for five nights, longer sleep opportunity reduced evening eating *among individuals with earlier chronotypes only*, despite similar sleep timing and duration between chronotypes (528). Hence there is a need to further study the influence of chronotype on appropriate lifestyle recommendations. Improvements in sleep hygiene are a natural starting point in attempts to enhance sleep, and future research on optimising such variables as sound, bedding, mattresses, and temperature may benefit people with sleep problems.

Of all pharmaceutical means of offsetting the effects of sleep loss, caffeine has perhaps most commonly been studied. However caffeine use can be problematic in that it interferes with recovery sleep after sleep loss (529). A little-studied sleep loss countermeasure is creatine monohydrate. By enhancing ATP replenishment, creatine appears to offset sleep homeostasis and so reduce sleep need, at least in rats (530). Creatine has also been shown to mitigate the negative effects of sleep loss on mood (531) and athletic skills in adults (532). As discussed at length in Chapter 1, sleep loss often has detrimental effects on body composition. It is therefore especially interesting that creatine generally improves body composition and neuromuscular performance (533).

In Chapter 5 we studied the effects of melatonin on sleep and metabolic health. We found that this chronobiotic scarcely influenced any outcome, but more research on melatonin and melatonin receptor agonists is needed. There is currently a lack of selective MT_1 receptor ligands (534), and separate roles of *MTNR1A* and *MTNR1B* signalling in metabolic regulation should be explored (535). Other pharmaceutical chronobiotics may have potential in countering circadian system disruption. Pharmacological inhibition of casein kinase 1 helps synchronise misaligned oscillators and hence speeds adaptation to light/dark cycle shifts in mice (536, 537), as does therapeutic suprachiasmatic nuclei (SCN) neuropeptide modulation (538). An array of clock-enhancing small molecules may ultimately provide effective therapies for disorders of the circadian system (539). Of note, some of these pharmaceutical chronobiotics may also benefit metabolic health. *Reverse-erythroblastosis* agonists, for example, diminish adiposity, hyperglycaemia and hyperlipidaemia in diet-induced obese mice (540). However, none of these compounds has been tested for safety or efficacy in humans yet.

Intriguingly, a circadian clock was recently transplanted into a non-circadian organism for the first time, and such methods could have chronotherapeutic applications, such as regulating timely drug release (541). This is particularly pertinent given that most of the highest-selling drugs target the products of genes with 24-hour transcription profiles (64).

Non-pharmaceutical interventions may also enhance circadian system function and sleep. These include blue-blocking glasses and apps to filter short-wavelength emissions from electronic devices (542, 543). As the brightness, colour, duration, and timing of light exposure influence many physiological functions (544-546), it is feasible that these interventions could influence SCN phase and numerous other processes independent of the SCN, including activity in other brain regions and endocrine networks that help regulate appetite. Novel developments in sleep technology may be integral to enhancing people's sleep in the coming years. By enabling consumers to have greater control of

their light environments, developments in 'smart' lighting technology may also be particularly important to optimising circadian system alignment for individual chronotypes. In a similar vein, consideration should be given to light exposure when designing buildings and their windows.

Reciprocity between physical activity and the circadian system exists, as both experimental circadian rhythm disruption and diseases associated with SCN dysfunction disrupt physical activity patterns (547, 548). Furthermore, physical activity influences melatonin rhythms a little, as well as peripheral tissue gene expression timing (549, 550). The circadian system also regulates the autonomic control of cardiovascular responses to exercise, resulting in peak cardiac vagal tone withdrawal in the morning. A bimodal acrophase in adrenaline and noradrenaline reactivity to exercise in both the morning and evening perhaps helps explain the increased risk of cardiovascular events at these times (551).

Studies of rodents suggest that physical activity offsets some adverse metabolic effects of circadian system disruption that result from light exposure at night (552). Exercise may also offset some of the deleterious effects of sleep disruption, as resistance training attenuates the catabolic effects of sleep deprivation on lean body mass in rats, perhaps by nullifying changes in testosterone, insulin-like growth factor-1, and corticosterone (553). As there is a paucity of human studies on the subject, it is important to study how to optimise exercise protocols to mitigate metabolic dysfunction induced by circadian system and sleep disruption.

6.5 Conclusions

Metabolic diseases such as obesity and type two diabetes are increasing at an unprecedented rate in many parts of the world. These increases are coinciding with rapid overhaul of the built world, environmental exposures, and people's lifestyle patterns, which independently and collectively instigate circadian system and sleep disruption in a growing number of the population.

Precise recording of lifestyle patterns depends on use of effective research tools, and the first part of this project validated a new dietary recall method that we hope will be used to identify diet-disease relationships in future studies. As we then used the new method in a study that supported the hypothesis that timing of caloric intake associates with obesity, we also showed how the tool can be applied to unveil underappreciated dietary influences on health. We next focused on sleep, finding that short-sleeping UK adults are not only more likely to be overweight but also have poorer metabolic health in

general. Finally, we showed that long-term melatonin supplementation scarcely influences metabolic health, sleep, and diet in adults predisposed to diabetes, paving the way for studies of whether alternative chronobiotic agents are efficacious in improving metabolic health. As will always be true, more research is needed.

I once more express my sincere thanks to everyone who made this project possible. The project has truly been a pleasure to complete, in large part because of its participants and my colleagues. I just hope that my sleep loss in recent weeks has no enduring effects.

With that I thank you for reading this thesis and wish you a night of sublime sleep.

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Appendices

Appendix A: Chapter 2 supplementary tables

Appendix table 1. Attenuation factors and correlations between dietary assessment methods and true protein and total sugar intakes and densities, stratified by age.

Dietary variable	Dietary recall method	Age < 40 years		Age ≥ 40 years	
		Attenuation factor (95% CI)	**Correlation with true intake (95% CI)**	**Attenuation factor (95% CI)**	**Correlation with true intake (95% CI)**
Protein intake (g)	MPR	0.41 (0.28 to 0.54)	0.54 (0.41 to 0.68)	0.25 (0.15 to 0.36)	0.35 (0.23 to 0.47)
	myfood24	0.38 (0.25 to 0.51)	0.50 (0.36 to 0.65)	0.22 (0.11 to 0.33)	0.35 (0.20 to 0.51)
Total sugar intake (g)	MPR	0.13 (0.01 to 0.26)	0.22 (0.02 to 0.42)	0.17 (0.06 to 0.30)	0.24 (0.11 to 0.37)
	myfood24	0.07 (-0.06, 0.19)	0.13 (-0.12, 0.37)	0.14 (0.03, 0.24)	0.23 (0.07, 0.39)
Protein density (g/MJ energy intake)	MPR	0.33 (0.16 to 0.51)	0.38 (0.20 to 0.55)	0.07 (-0.07 to 0.21)	0.07 (-0.07 to 0.22)
	myfood24	0.28 (0.09 to 0.47)	0.32 (0.11 to 0.52)	0.04 (-0.13 to 0.21)	0.05 (-0.14 to 0.23)
Total sugar density (g/MJ energy intake)	MPR	0.02 (-0.13 to 0.17)	0.03 (-0.21 to 0.28)	0.25 (0.10 to 0.39)	0.28 (0.14 to 0.43)
	myfood24	0.06 (-0.10 to 0.22)	0.10 (-0.17 to 0.38)	0.18 (0.04 to 0.33)	0.23 (0.06 to 0.40)

Legend: CI (confidence interval), MPR (interviewer-administered multiple-pass 24-hour recall method).

All data were positively skewed and so were log-transformed.

Appendix table 2. Attenuation factors and correlations between dietary assessment methods and true protein and total sugar intakes and densities, stratified by body mass index.

Dietary variable	Dietary recall method	BMI < 25 kg/m^2		BMI ≥ 25 kg/m^2	
		Attenuation factor (95% CI)	Correlation with true intake (95% CI)	Attenuation factor (95% CI)	Correlation with true intake (95% CI)
Protein intake (g)	MPR	0.37 (0.24 to 0.50)	0.44 (0.32 to 0.57)	0.33 (0.21 to 0.45)	0.47 (0.33 to 0.61)
	myfood24	0.32 (0.20 to 0.45)	0.45 (0.30 to 0.60)	0.28 (0.16 to 0.40)	0.42 (0.26 to 0.57)
Total sugar intake (g)	MPR	0.14 (0.02 to 0.26)	0.22 (0.05 to 0.40)	0.24 (0.10 to 0.37)	0.30 (0.14 to 0.45)
	myfood24	0.14 (0.02 to 0.26)	0.25 (0.04 to 0.47)	0.14 (-0.01 to 0.28)	0.19 (-0.01 to 0.39)
Protein density (g/MJ energy intake)	MPR	0.34 (0.17 to 0.51)	0.34 (0.20 to 0.49)	0.04 (-0.13 to 0.20)	0.04 (-0.14 to 0.23)
	myfood24	0.29 (0.11 to 0.48)	0.32 (0.14 to 0.51)	-0.06 (-0.25 to 0.13)	-0.06 (-0.27 to 0.14)
Total sugar density (g/MJ energy intake)	MPR	0.08 (-0.06 to 0.22)	0.14 (-0.09 to 0.37)	0.27 (0.09 to 0.44)	0.28 (0.11 to 0.44)
	myfood24	0.07 (-0.08 to 0.22)	0.12 (-0.15 to 0.38)	0.24 (0.05 to 0.43)	0.27 (0.07 to 0.47)

Legend: CI (confidence interval), MPR (interviewer-administered multiple-pass 24-hour recall method).

All data were positively skewed and so were log-transformed.

Appendix table 3. Attenuation factors and correlations between dietary assessment methods and true protein and total sugar intakes and densities, stratified by sex.

Dietary variable	Dietary recall method	Men (n = 85)		Women (n = 127)	
		Attenuation factor (95% CI)	Correlation with true intake (95% CI)	Attenuation factor (95% CI)	Correlation with true intake (95% CI)
Protein intake (g)	MPR	0.30 (0.19 to 0.41)	0.45 (0.34 to 0.55)	0.21 (0.11 to 0.30)	0.35 (0.20 to 0.49)
	myfood24	0.23 (0.13 to 0.34)	0.38 (0.26 to 0.51)	0.20 (0.10 to 0.30)	0.36 (0.20 to 0.52)
Total sugar intake (g)	MPR	0.24 (0.12 to 0.36)	0.37 (0.23 to 0.52)	0.14 (0.03 to 0.25)	0.20 (0.04 to 0.36)
	myfood24	0.14 (0.02 to 0.26)	0.25 (0.05 to 0.44)	0.13 (0.01 to 0.25)	0.22 (0.02 to 0.41)
Protein intake density (g/MJ energy intake)	MPR	0.20 (0.02 to 0.38)	0.21 (0.03 to 0.39)	0.20 (0.05 to 0.35)	0.22 (0.06 to 0.37)
	myfood24	0.20 (0.00 to 0.41)	0.22 (0.00 to 0.43)	0.13 (-0.04 to 0.30)	0.15 (-0.04 to 0.34)
Total sugar intake density (g/MJ energy intake)	MPR	0.30 (0.12 to 0.47)	0.34 (0.17 to 0.52)	0.07 (-0.07 to 0.21)	0.09 (-0.09 to 0.27)
	myfood24	0.21 (0.04 to 0.39)	0.26 (0.05 to 0.47)	0.12 (-0.03, 0.27)	0.17 (-0.04 to 0.38)

Legend: CI (confidence interval), MPR (interviewer-administered multiple-pass 24-hour recall method).

All data were positively skewed and so were log-transformed.

Appendix B: Munich ChronoType Questionnaire

Morning/evening-type Questionnaire

Roenneberg T, Wirz-Justice A, Merrow M (2003) Life between clocks: daily temporal patterns of human chronotypes. J Biol Rhythms, 18, 80-90.

Purpose

The purpose of this questionnaire is to explore associations between your 'chronotype' – a measure of whether you are more of a morning 'lark' or a night 'owl' based on your sleeping habits - and the foods you eat. In order to accomplish this, the questionnaire is divided into separate sections for work and work-free days, respectively.

Thanks again for your time!

Instructions

- Please note that there are **separate sections** for work days and work-free days

- Some questions give the option of **either** using the 24-hour clock **or** using the 12-hour clock. Please use **one** of these throughout. If, for example, your answer was 18:00 (6 PM), you would either enter | 18:00 | or | 6:00 | | ~~AM~~/PM | If you use the 12-hour clock, please **cross-out** the option that does not apply (AM is crossed-out in this example)

- In multiple-choice questions, please **cross-out** the answer which does not apply to you. In these questions, choices are *italicised*

- Please enter information for **typical** days (not days involving late-night parties, for example)

- Please enter today's date and your participant number in the boxes provided at the top of **every** page

Work Schedule

1) I   *do*   /   *do not*   have a regular work schedule

2) I   *have*   /   have *not*   engaged in rotating shift work within the last three months

3) I   *have*   /   have *not*   engaged in night shift work within the last three months

Work days

1) On nights before work days, I typically go to bed at [:] (24-hour)   / [:] [AM/PM] (note that some people stay awake for some time when in bed)

2) On nights before work days, I actually get ready to fall asleep at [:] (24-hour) / [:] [AM/PM]

… and it then typically takes me [ ] minutes to fall asleep

3) I typically wake up at [:] (24-hour) / [:] [AM/PM] on   work days

4) After a further [ ] minutes, I typically get up

5) On average, I spend [ ] hours and [ ] minutes outdoors in daylight without a roof over my head on work days

6) I   *do*   /   *do not*   typically use a device like an alarm or radio to wake up on work days. Please leave a comment **if** you cannot otherwise freely choose your sleep times on work days (because of pets or children, for example):

[ ]

Work-free days

7) On nights before work-free days, I typically go to bed at [:] (24-hour) / [:] [AM/PM] (note that some people stay awake for some time when in bed)

8) On nights before work-free days, I actually get ready to fall asleep at [:] (24-hour) / [:] [AM/PM]

… and it then typically takes me [ ] minutes to fall asleep

9) I typically wake up at [:] (24-hour) / [:] [AM/PM] on work-free days

10) After a further [ ] minutes, I typically get up

11) On average, I spend [ ] hours and [ ] minutes outdoors in daylight without a roof over my head on work-free days

12) I *do* / *do not* typically use a device like an alarm or radio to wake up on work-free days. Please leave a comment **if** you cannot otherwise freely choose your sleep times on work days (because of pets or children, for example):

[   ]

Appendix C: Chapter 3 directed acyclic graph
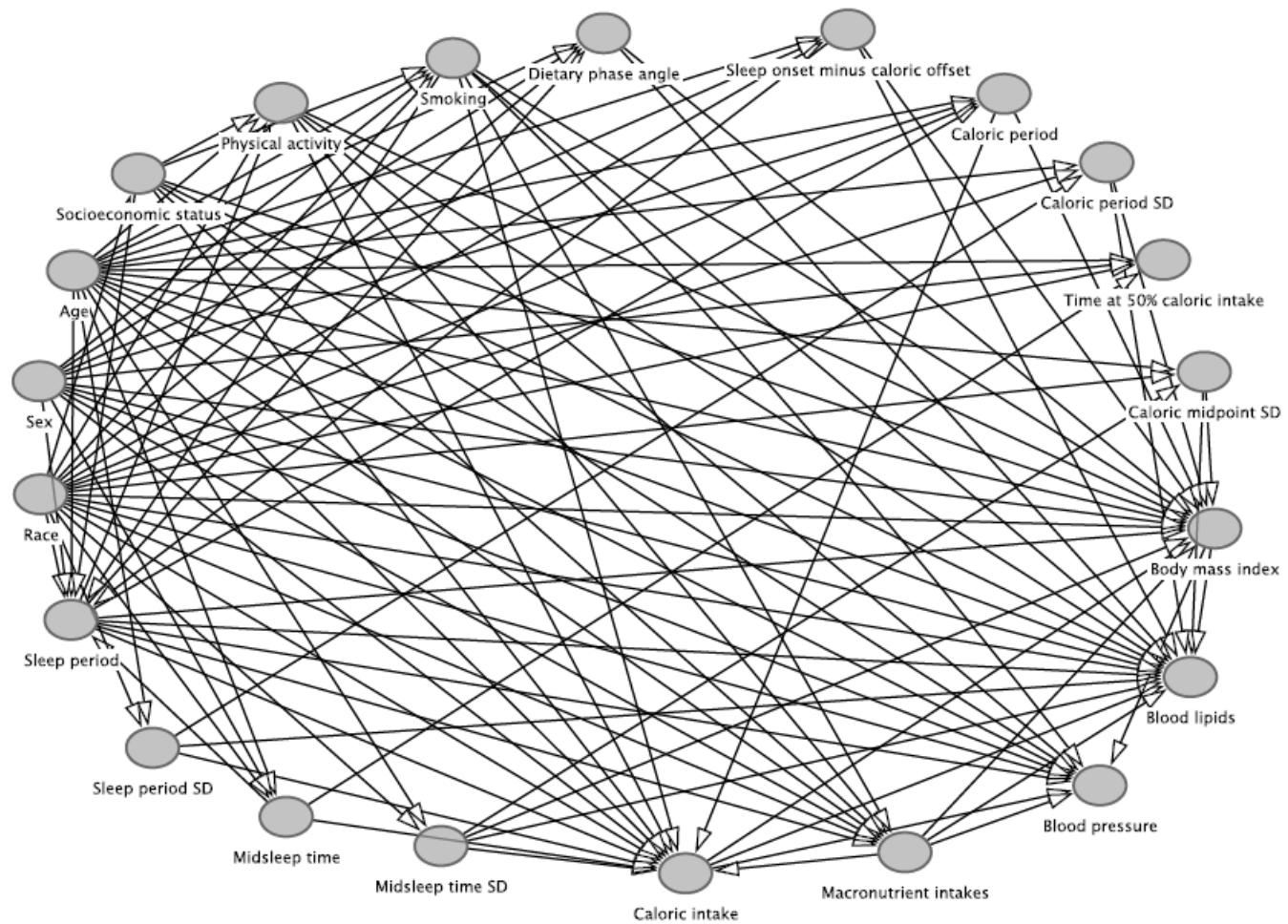
Appendix D: Chapter 4 directed acyclic graph