Greased lighting : implications of circadian lipid metabolism for cardiometabolic health

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Chapter 8

General Discussion and Future Perspectives

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

For centuries, the use of artificial light permitted man to stay awake after dark. From campfires in prehistoric times, to the use of an oil lamp ancient Greece, humans have invented ways to bring light. The invention of the light bulb in 1879 by Thomas Edison is heralded as the birth of 'artificial light'. This coincided with the advent of the industrial revolution, and the increased need to working at night commenced to have a large impact on society. In the 19th century, factory labor was among the hardest shift work circumstances with shift work rotations of 12 hours for two weeks straight, without a day rest [1]. Today, it is estimated that in Europe, approximately 20% of the working population is involved in some form of shift work [2]. The 24 hour economy is not just exemplified by the common use of shift work but also by shifting of social activities to nightly hours. This habit has only further encouraged shift work, as nowadays leisure businesses are also open 24/7, including supermarkets, bars, gas stations and restaurants. In terms of our biological clock, these habits are severely disturbing via at least three different pathways. Firstly, humans are widely exposed to bright light during the natural occurring night. Since light exposure directly acts on the central biological clock, the mistimed light exposure may disrupt synchronization of the clock. Secondly, in our modern society physical activity, wakefulness and food intake take place throughout the light-dark cycle. This induces a misalignment between the metabolic demand, *i.e.* energy intake, storage and expenditure, and the endogenous circadian rhythms of metabolic organs, which are entrained to the light-dark cycle. Thirdly, staying up late in the evening and night for work or leisure has led to shortened sleep duration [3]. All of these human circadian disruptions have been associated with metabolic disease.

In this thesis we have investigated which metabolic pathways may mediate the interaction between the biological clock and energy metabolism. To gain more insight in how circadian disruption causes metabolic disease, we studied the effect of light exposure on metabolic phenotypes in mice (**Part I**). We demonstrated that prolonged light exposure attenuates brown adipose tissue (BAT) activity, thereby increasing adiposity (**Chapter 2**). Subsequently, we demonstrated that BAT activity displays a marked 24h rhythm, which determines the 24h rhythm in plasma lipid levels (**Chapter 3**), and which is attenuated upon dampening of the endogenous glucocorticoid rhythm (**Chapter 4**). Finally, we show that mistimed light exposure can aggravate atherosclerosis development in dyslipidemic mice (**Chapter 5**). In **Part** II, we evaluated the relationship between circadian rhythms and metabolism in humans. We showed that shortened sleep duration increases acylcarnitines in plasma, suggesting a defect in mitochondrial fatty acid oxidation (**Chapter 6**). Finally, we demonstrate 24h rhythms in plasma cholesterol and observed a higher rhythmicity in plasma cholesterol in the context of longevity (**Chapter 7**). In this chapter, I will discuss these novel findings in the context of the three pathways – mistimed light exposure, behavioral misalignment and short sleep – that mediate the relationship between light exposure and metabolic disease (see graphical summary). I will conclude by discussing the implications for future research and possible therapeutic strategies.

The rhythm in brown adipose tissue in relation to cardiometabolic disease

A very recent study on light pollution reports that an astonishing 83% of the world population lives under light-polluted skies [4]. In the US and Europe, this proportion even reaches 99%. The human retina has three photoreceptors sensitive to light: rods and cones, which are crucial for visual perception, and the melanopsin-containing intrinsically photoreceptive retinal ganglion cells (ipRGCs) [5]. The ipRGCs transmit light-dark information directly to the SCN, although mice lacking melanopsin can still entrain to light-dark cycle [6, 7]. Conversely, ipRGCs are also sufficient to entrain to light-dark cycle [8]. The illumination levels that constitute light pollution [4] not only reach the human retina, but are also sufficient to trigger the photoreceptors [9]. Evidence is accumulating that in humans, light exposure at night associates with increased body weight [10-13]. Although constant light exposure has previously been shown to induce a fast increase in weight gain due to a decrease in 24h energy expenditure in mice [14], it remained unknown which tissues contributed to this difference. In chapter 2, we provide evidence that BAT plays a crucial role. BAT is a highly metabolically active tissue that burns glucose and fatty acids (FA) to produce heat. Activation of BAT increases energy expenditure, decreases and prevents diet-induced obesity and decreases dyslipidemia [15-18]. We showed that prolonged light exposure duration decreases SNS output towards BAT and reduces the capacity of BAT to take up TG-derived FA from the circulation [19]. Without compensation in food intake, this led to increased storage of energy in WAT. Specific sympathetic denervation of BAT abolished the rhythm-attenuating effect of prolonged light exposure.

Our findings support recent human studies demonstrating that exposure to light can alter metabolism. Healthy individuals that were exposed to low levels of blue light in the evening showed a lower energy expenditure in the morning, without alterations in sleep duration or quality [20]. Other evidence for the effect of light exposure on metabolism in humans is mainly derived from epidemiological studies. Light duration inversely correlates to detectability of BAT throughout the year, as shown by uptake of [^{18}F]fluorodeoxyglucose. This association is stronger and more significant than the association between ambient temperature and BAT activity [21]. Take together with our findings in mice, it can be hypothesized that short light duration, presumably via the SCN, increases basal BAT activity in humans. Since light duration is a more stable predictor for seasonal changes than ambient temperature, this mechanism seems to have biological relevance: shortening of days predicts the approach of winter, a season in which higher BAT activity is needed for heat generation to maintain body temperature. Therefore, turning up the heat by activation of BAT as the days grow shorter may more likely be an adaptive mechanism than merely a reaction to the outside temperature. Whether light exposure duration regulates BAT activity in humans as well remains to be investigated.

Besides showing that disruption of circadian rhythm lowers BAT activity, we demonstrated a marked diurnal rhythm in TG-rich lipoprotein-derived FA uptake by BAT in mice (chapter 3). This diurnal rhythm aligns with the known rhythms of energy expenditure and core body temperature, which peak before the start of the wakeful period in both day-

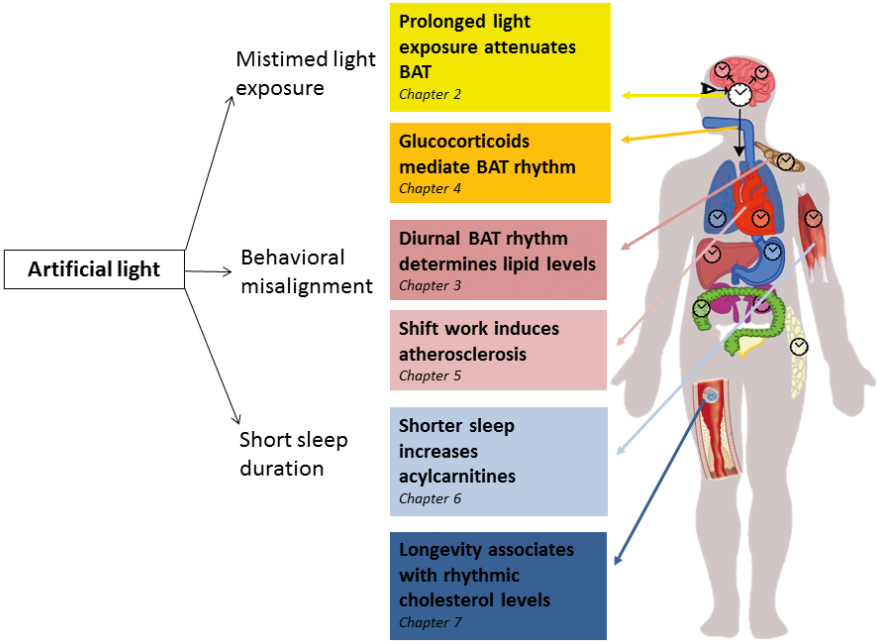

Figure 1. Graphical Summary representing the main conclusions based on the studies described in this thesis. *See text for explanation.*

active and night-active mammals [22]. In our study, prolonged and shortened light exposure shifted the peak time in BAT activity to the respective onsets of the dark (active) period. This was accompanied by a fast postprandial lipid clearance at the onset of the dark period irrespective of the light exposure period. These data suggest that BAT should be influenced by circadian misalignment. Indeed, an experimental jet-lag protocol of 6 days in healthy individuals led to decreased energy expenditure and a decreased thermic effect of food [23]. These results may be explained by misalignment between food intake and the endogenous BAT rhythm. In another misalignment study, 10 healthy individuals were subjected to 7 repeated cycles of a 28h 'day'. Metabolic hormones changed, including decreased total leptin levels, increased insulin levels, but interestingly also decreased plasma epinephrine [24]. This suggests that reduced sympathetic output towards BAT may play a role in lowering BAT activity upon circadian disruption.

Although humans are day-active and mice are night-active, it is likely that the circadian BAT activity found in mice is analogous to humans, *i.e.* highest activity at wakening. A recent human study found a circadian rhythm in supraclavicular temperature, a proxy for human BAT activity, which was low during the night and higher during daytime. Furthermore, human brown adipose tissue explants sustain a circadian rhythm in gene expression [25]. Circumstantial evidence also points towards a circadian human BAT activity with a trough at night and peak at wakening. In humans, BAT activation also contributes to energy

expenditure [15] and decreases free FA [18]. Energy expenditure displays a robust circadian rhythm in humans [26]. Our data demonstrate that plasma free FA display a circadian rhythm, with low FA in the morning, which is not explained by food intake (chapter 3). From an evolutionary point of view, a circadian rhythm in BAT constitutes an energy saving ability. BAT developed to maintain body temperature in homeotherms, however keeping BAT constitutively active would be a 'waste' of energy. Moreover, circadian body temperature may serve as a synchronization signal for peripheral clocks. *In vitro*, temperature cycles specifically regulate the cyclic expression of cold-inducible RNA-binding protein (CIRP) and mice lacking CIRP have a decreased circadian amplitude in rhythmic gene expression [27]. Since BAT contributes to body temperature, the diurnal BAT rhythm may be important in the synchronization circadian rhythms throughout the body and contribute to wakening. Together, human data support the hypothesis that circadian BAT activity is high at wakening and low during sleep.

A key question remains to be elucidated: how does the SCN regulate the rhythm in BAT activity? Possible pathways can be divided in indirect *vs*. direct regulation of BAT by the SCN. Meal timing has been shown to entrain peripheral circadian clocks in metabolic tissues, and is therefore a likely indirect regulator of rhythmic BAT activity. Restricting food intake to the usual sleep period of rodents induces a shift in the rhythm of metabolic tissues, both in clock gene expression and metabolic function [28]. The SCN itself however is not affected by the restricted feeding regimens [29]. Therefore, food intake rhythm has an independent effect on synchronizing metabolic tissues. The SCN does align food intake behavior to the light-dark cycle. SCN lesioned rats display arrhythmic locomotor activity and food intake behavior [30, 31]. Light exposure duration induces adaptations in food intake rhythms in wild type animals [32]. These adaptations are dependent on intact SCN functioning, as VIP-knockout mice (a genetic model for SCN dysfunction) cannot entrain to light exposure duration [33]. The effect of food intake rhythm on BAT activity has not extensively been characterized. Under normal chow feeding, there is no effect of night-restricted feeding on gene expression rhythms of BAT. However, after 20 weeks of high fat diet feeding, night time restriction of food intake increases overall BAT activity and improves the rhythm in gene expression of several thermogenic genes [34]. The authors attribute this effect to increased bile acid synthesis during feeding within the liver. Due to increased production by liver after a meal, the spill-over into the circulation would ultimately result in higher bile acid plasma levels that can directly increase BAT activity [34]. Hormones released upon feeding such as insulin and GLP-1 are also known to increase BAT activity [35, 36]. Changes in hormone release due to changes in food intake behavior may provide additional indirect pathways by which the SCN regulates rhythmic BAT.

The SCN may also directly signal towards BAT. I will start by evaluating how the SCN in general synchronizes peripheral tissues. Presumably, the SCN synchronizes peripheral organs through the sympathetic nervous system (SNS) and through endocrine output [37]. In case of metabolic tissues, it is demonstrated in rats that specific ablation of the SCN abolishes circadian both at both gene expression level and the functional level, *e.g.* production of insulin and leptin. Furthermore, liver denervation abolishes the circadian

rhythm in glucose plasma levels [38], demonstrating that the SNS is necessary to sustain a circadian rhythm in liver function. BAT is densely innervated by the SNS. The best known trigger for SNS activation towards BAT is cold sensing [39]. Tracing studies in Siberian hamsters demonstrated a direct neural connection between the SCN and BAT [40]. In rats, administration of the excitatory neurotransmitter glutamate to the retinohypothalamic tract or directly to the SCN induced a thermogenic response of BAT [41, 42]. Furthermore, we found that light exposure duration dose dependently decreased noradrenergic outflow as demonstrated by reduced tyrosine hydroxylase staining within BAT. In our study, prolonged light exposure duration was associated with decreased intracellular β-adrenergic signaling, such as CREB phosphorylation. Specific denervation of the BAT depots abolished the attenuating effects of prolonged light exposure on BAT activity, suggesting mediation via SNS [19] (chapter 2). The SCN likely accomplishes the SNS output towards BAT by signaling through the ventromedial hypothalamus (VMH). Specific knock out of clock gene *Bmal1* in the VMH dampened energy expenditure and body temperature rhythms, and dampened the naturally occurring increase in nocturnal expression of *Ucp1* and mitochondrial genes *Nrf1* and *Cpt1b* within BAT. From these data, it seems that the VMH produces an intrinsic rhythm in SNS output towards BAT. Light exposure modulates SCN output towards brain areas including the VMH, thereby changing the amplitude and timing of SNS output [43]. For the SCN to be able to signal towards BAT, an intact signaling via VMH and SNS is thus at least necessary.

Besides SNS input, endocrine systems have been implicated in synchronizing peripheral tissue activity. Glucocorticoid rhythms are a likely candidate to transmit the timing signal. *In vitro*, non-SCN cells do not retain rhythmicity in gene expression [44, 45]. Synchronization of these *in vitro* rhythms can be accomplished by at least three methods: heat shock, serum shock or administration of the glucocorticoid receptor (GR) agonist dexamethasone [46-48]. Interestingly, the intracellular mechanisms by which heat shock and serum shock achieve synchronization are not fully understood. Moreover, attempts to identify which factors within serum synchronize the cells have failed. In this respect, it is intriguing that the dexamethasone is the only single molecule known to synchronize cells *in vitro*. There is some evidence that glucocorticoids can synchronize the rhythm in BAT activity. In chapter 4, we provide evidence that dampening of glucocorticoid rhythms flatten the TG-derived FA uptake by BAT. The finding that the peak corticosterone aligns with peak FA uptake by BAT may seem surprising. Previous studies showed that high concentrations of dexamethasone strongly inhibit BAT activity *in vivo* and *in vitro* [49, 50]. The peak concentration of corticosterone during circadian peak is two-fold lower than the corticosterone peak in response to stress [51]. Therefore, we hypothesize that the concentration of corticosterone is crucial in determining whether BAT is activated or inhibited. In our laboratory, we have investigated the effect of a high versus a low dose of dexamethasone on brown adipocytes. Indeed, our preliminary data show that only high concentrations of dexamethasone downregulate thermogenic gene expression, but that both a high and a low dose of dexamethasone are able to synchronize clock gene expression of brown adipocytes.

Taken together, the link between artificial light and cardiometabolic disease in humans may be mediated via decreased BAT activity. What are the implications to prevent or treat cardiometabolic diseases? Firstly, exposure to artificial light at otherwise dark periods could be reduced or altered. Blue light is the most potent signal for the SCN [52]. Indeed, exposure to light-emitting screens in the evening disturbs sleep in healthy individuals [53]. One study investigated whether selectively removing blue light wavelength prevents adverse health effects. Although sleepiness was indeed improved without blue light exposure, effects on physical parameters, such as cortisol and melatonin levels, were not significant [54]. More studies are needed to further investigate whether blue light specifically mediates the effects of artificial light on metabolism, which would support the use of blue light filters of screens to maintain cardiometabolic health. Although there are no systematic data available, the amount of smartphone software-applications to filter out blue light from smartphone and tablet screens is increasing; the newest Windows operating system has a blue light filter incorporated aimed at "reducing blue light at night to help you sleep" [55]. Secondly, pharmacological activation of BAT may be tailored to the circadian rhythm of its activity. This concept of 'chronotherapy' is gaining ground in various research areas [56]. For example, cancer cells are not synchronized in their circadian rhythm of cell proliferation, making them relatively more susceptible to chemotherapy during the night compared to healthy cells, as these are in their resting phase during night time [57]. In cardiovascular disease, it has been known that myocardial infarctions occur mostly in the morning [58], coinciding with the circadian rise in blood pressure [59] and increased platelet aggregation [60]. In fact, aspirin therapy in the evening more effectively lowers the morning peak in platelet aggregation function than aspirin in the morning [61]. Interestingly, preliminary data have shown that cooling of individuals in the morning increases their energy expenditure more than cooling in the evening (Kooijman, unpublished data), suggesting that activation of BAT may be optimal in the morning, when it is presumably peaking in humans.

Of note, impaired BAT activity may also explain the relationship between disturbed glucocorticoid rhythms and obesity in humans. Individuals who depend on oral supplementation of glucocorticoids, *e.g.* with adrenal insufficiency, have increased risk of metabolic syndrome even though they are adequately treated [62]. Current standard replacement therapy of glucocorticoids attempts to mimic the circadian rhythm with 2-3 doses divided over the day; one high dose in the morning and two lower doses in the afternoon and evening. Even so, the resulting glucocorticoid levels poorly resemble the endogenous circadian – not the mention ultradian – rhythm [63]. If glucocorticoid rhythms are indeed an important synchronizer for BAT activity in humans, individuals on exogenous glucocorticoids may have a disturbed rhythm of BAT, which may lead to decreased thermogenesis and subsequent increased lipid levels and adiposity.

The role of light exposure in the relationship between shift work and atherosclerosis

While studies have established a clear association between shift work and cardiovascular events in humans [64], proving a direct causal relationship is challenging. Shift work is accompanied by many confounding factors. Shift workers may adopt a different life style due to their irregular working hours, as their sleep pattern, physical activity and food intake habits may be different compared to non-shift workers [65, 66]. Furthermore, there is a difference among individuals in shift work tolerance, which is commonly defined as the ability to carry out shift work without adverse consequences [67]. On the other hand, the causes of cardiovascular diseases (CVD) are multifactorial. Contributing factors include dyslipidemia, a pro-inflammatory state, hypertension, pro-thrombotic state and obesity. Shift work and/or circadian rhythm disturbances have been linked independently to all of these individual contributing factors. The work described in this thesis aimed to dissect the contributing pathways, in particular whether mistimed light exposure can induce CVD.

The data described in chapter 5 are the first to demonstrate that mistimed light exposure is a causal factor in atherosclerosis formation. We previously showed that continuous light (LL) exposure in mice decreases SCN amplitude, decreases energy expenditure [14] and decreases BAT activity [19]. Since pharmacological activation of BAT lowers the levels pro-atherogenic VLDL and chylomicron remnants in dyslipidemic mice by accelerating their clearance from plasma [16], it stands to reason that LL would be pro-atherogenic by reducing BAT activity. Although we did observe increased total cholesterol levels, gene expression of BAT activation markers and lipid content within BAT was not affected by chronic LL exposure. Possibly, LL transiently disturbs the rhythm in mice and consequently, transiently lowers BAT activity. A five week LL exposure in wild type male mice induced an arrhythmic phenotype, evidenced by arrhythmic locomotor behavior and flattened plasma corticosterone levels [14]. Exposing dyslipidemic mice to 15 weeks of LL induced arrhythmic locomotor behavior in a small subset of mice (chapter 5). In contrast to LL exposure, 15 weeks of mistimed light exposure did aggravate atherosclerosis development. The lesion size and severity depended on the severity of SCN disturbance. The SCN is disturbed more by a 12 h shift than by a 6 h shift. Moreover, the SCN adapts faster to phase delays than to phase advances [68]. In mice, phase advance but not delay was shown to increase mortality [69]. Atherosclerosis development was not affected by 6 h delay, modestly affected by 6 h advance and severely affected by 12 h reversal of the light-dark cycle. Therefore, the extent (i.e. 6 h vs 12 h) and direction (advance vs delay) of circadian disruption possibly correlates to the extent of atherosclerosis formation.

A crucial question is how mistimed light exposure induces a pro-atherogenic phenotype. Although plasma cholesterol concentrations play a dominant role in atherosclerosis development we did not find an increase in total cholesterol exposure after exposure to 12h reversal and 6h phase advance for 15 weeks compared to a regular LD light regime. However, absence of increased plasma cholesterol levels do not exclude the possibility that mistimed light exposure may affect cholesterol metabolism in various other ways and thereby contributes to atherosclerosis development. LDL particles may be more prone to

oxidation, which makes them prone to be taken up by macrophages, resulting in foam cell formation. Furthermore, the cholesterol efflux capacity of macrophages may play a role. Efflux capacity depends on expression of cholesterol transporters on the macrophages, which is at least partly regulated by circadian genes. For instance, C*lock* regulates the expression of *Abca1*. Macrophages from *Clock*$^{\Delta 19/\Delta 19}$*Apoe-/-* mice have an impaired cholesterol efflux capacity [70].

Another explanation that we did not observe increased total cholesterol levels after mistimed light may be the timing of sampling. Plasma cholesterol levels display a modest diurnal rhythm in humans [71]; we confirmed an oscillation of approximately 2 mM during the day in APOE*3-Leiden.CETP dyslipidemic mice (van den Berg, unpublished data). Since the biological clock adapts to light shifts during the first days after the light regime changes, the 24h rhythms in plasma cholesterol are possibly in phase between the groups. The cholesterol levels of different groups could be similar at a specific time point, even though the groups exhibit different 24h rhythms in plasma cholesterol levels. To determine whether total cholesterol exposure is affected by mistimed light exposure, repeated 24h plasma curves of cholesterol levels should therefore be determined in future studies.

Alternatively, mistimed light exposure may induce atherosclerosis without modulating cholesterol metabolism but via inducing a proinflammatory phenotype, since the biological clock also influences the immune system. Circulating leukocytes and monocytes display a circadian rhythm [72]. Macrophages from mice with *Clock*$^{\Delta 19/\Delta 19}$,an arrhythmic mouse model, were previously shown to be more proinflammatory and to exacerbate metabolic syndrome upon high fat feeding [73]. Macrophages from *Rev-erbα-/-* mice were also shown to be more proinflammatory [74]. We infer that inflammatory cells are regulated by the biological clock and that its disturbance induces a proinflammatory phenotype. Therefore, disturbance of the biological clock via other pathways, such as mistimed light exposure, likely also induce a proinflammatory state.

From clock disturbance to metabolic disease: scientific challenges and future perspectives

The relationship between the circadian clock and metabolism is steadily being unraveled. Animal studies, including those described in this thesis, have convincingly shown that a malfunctioning clock causes metabolic disease. Nevertheless, there are some challenges ahead. Future research has yet to answer the following questions: 1) What are the timing signals for peripheral tissues? 2) What are the intracellular pathways that cause metabolic derangements upon circadian rhythm disturbances? 3) Which data from animal experiments are translatable to humans and which are not? 4) How can we 'cure' our diseased clock, if at all? 5) Since timing determines virtually every tissue function, how should we integrate this fourth dimension into other research fields? These questions are challenging due to the several aspects in the current research on this topic, of which I will discuss the following three.

I. The use of knock out models. Many studies on circadian disturbances have been performed by studying the effect of knock out or mutant clock genes on metabolism using

genetically modified mice. Although very useful to uncover vital functions of genes, the use of genetic animal models has drawbacks. Any genetic model has the downside that animals develop without the gene of interest. Any phenotype may therefore be a developmental problem and compensatory mechanisms may have occurred. This issue may be solved by using conditional knock-out mice. To date, these have not been used in the context of circadian research. In addition, genetic manipulation of a clock gene always affects both the central and peripheral clocks, since they share the same molecular mechanism. This issue is slowly being solved by the use of tissue-specific knock-out mice, although the tissue specificity is often an issue. For example, in adipose tissue-specific *Bmal1* knock-out mice, both WAT and BAT are affected, while only WAT was studied [75]. The SCN-specific *Bmal1* knock-out mouse has been developed using a synaptotagmin10-Cre driven mouse strain, which is specifically expressed in the SCN [76, 77]. SCN-specific *Bmal1* knock-out leads to disturbed gene expression rhythm in WAT, showing that SCN specific rhythm is necessary for rhythmic gene expression in metabolic tissues [78]. Whether the gene expression disturbance translates to disturbed tissue function and whether that results in metabolic disease remains to be investigated. Another drawback of genetic manipulation is that the translatability of findings in mice to human settings of circadian disruptions is very limited. We have shown that, by using artificial light exposure in our studies to manipulate the clock, the human exposure to artificial light can be mimicked, making it possible to study metabolic consequences in an intact animal model. Therefore, findings obtained by varying light exposure regimes are likely more translatable to the human situation compared with genetic intervention. Future research in humans should prove whether indeed BAT activity is influenced by light exposure regimes.

II. Discovery of intracellular mechanisms. It is becoming increasingly clear which metabolic organs mediate the relationship between disturbed clock function and metabolic disease. Nevertheless, the intracellular mechanisms involved are still obscure. Gene expression analysis is the most widely used method to investigate rhythms. However, gene expression analysis alone may be insufficient to predict functional rhythms in organs. We demonstrated a clear adaptive rhythm in BAT with respect to its capacity to take up FA from TG-rich lipoproteins. Upon examination of gene expression, many canonical BAT 'activity' genes, such as *Ucp1,* did not display a clear rhythmicity (chapter 3). In general, regulation of gene expression is only one layer of rhythmic regulation within the cell. In an extensive paper by Koike *et al.* [79], immunochromatin precipitation-sequencing of seven core clock genes showed thousands of genes with bound clock proteins. They compared intronic and exonic RNA expression and concluded that only 22% of cycling transcripts are driven by transcriptional regulation, demonstrating that oscillating mRNA levels depend more on non-transcriptional regulation, *e.g.* post-transcriptional regulation. The highly rhythmic gene *Nocturnin* regulates poly-adenylation of mRNA transcripts [80]. This is likely the tip of the iceberg of non-transcriptional circadian regulation.

Another challenge in elucidating intracellular mechanisms is to obtain human tissue. Gene expression rhythms in humans are often assessed in easily accessible peripheral blood mononuclear cells [81-83]. Subcutaneous white adipose tissue was also shown to

display rhythmic gene expression [84]. In a recent study, repeated muscle biopsies were taken from healthy men under regular feeding and sleep-wake conditions. Oxidative capacity of muscle displayed a rhythm with a peak in the afternoon. While mitochondrial genes did not show oscillations, protein involved in mitochondrial dynamics did oscillate [85]. We also obtained evidence for the involvement of mitochondria in circadian rhythm of metabolism in humans. After one night of short sleep, we found an increase in plasma of specifically the metabolite class of acylcarnitines, which may indicate a mitochondrial dysfunctional (chapter 6). To examine the hypothesis that acylcarnitines derive from muscle tissue and are a result of mitochondrial dysfunction, we are currently investigating the effect of short sleep energy metabolism, including measuring mitochondrial function directly in muscle and white adipose tissue. Although taking human biopsies is invasive, these studies will be necessary to advance the knowledge on intracellular mechanisms in the context of circadian disturbances of energy metabolism.

III. Microbiome involvement. We are not alone. Humans are a symbiosis of human cells and bacteria. The importance of the microbiome for metabolism in general is increasingly recognized. Interestingly, circadian rhythms are also present in the function and composition of gut microbiota [86]. Disturbance of the circadian clock either by genetic modification or by jet-lag resulted in a loss of circadian rhythms of microbiota in mice. Restricting the feeding time to the dark period – the natural feeding time – rescued this effect. Thus, timed feeding controls the circadian rhythm in microbiome [87]. Interestingly, the same group recently reported that the inverse relationship holds true as well. Rhythmicity in the microbiota influences the metabolic pathways in the intestine and in the liver, as loss of rhythmicity in the microbiota changed rhythmic expression in metabolic pathways in these organs [88]. In these studies, rhythms in adipose depots were not established. Since the gut microbiome has been shown to influence the function of both brown and white adipose tissue, it seems highly likely that these organs are also under control of the microbiome [89, 90]. Sleep fragmentation in mice was shown to both change the gut microbiome and lead to inflammation of visceral WAT [91]. Thus, disturbed circadian rhythms may indeed lead to metabolic diseases via changes in gut microbiota, although this relationship needs to be investigated in more detail.

Concluding remarks

The data presented in this thesis have added to the growing body of literature demonstrating that disturbances of the biological clock can lead to cardiometabolic disease. We show that BAT plays a crucial role in mediation of these effects and that this is regulated by both the sympathetic nervous system and glucocorticoid rhythms. The clinical implications of these observations are that prevention of disturbances to our day-night rhythms provides additional handles to improve cardiometabolic health of both the general population and CVD patients. These can be summarized as follows: 1) diminishing (blue) light exposure at dark (inactive) periods, 2) aligning behavior (*e.g.* food intake, physical activity and sleep) to the natural light-dark cycle, 3) aiming to sleep at least 7 hours per night, 4) use of chronotherapy in CVD, 5) BAT activating strategies, probably restricted to

the early wakeful phase, 6) administration of glucocorticoids aimed to mimic the circadian rhythm. Our data showing marked day-night rhythms in metabolism also have diagnostic implications. Measuring lipids at multiple daily time points may give better predictive value for development of CVD. For other CVD risk factors such as blood pressure, it is already common practice to measure multiple times. Blood pressure 'dips' during the night, and 'non-dippers' are known to have an increased risk of CVD in absence of hypertension [92]. Analogous to the 24h blood pressure monitoring, repeated determination of plasma lipid levels may refine risk assessments.

Future research should investigate whether humans display a diurnal rhythm in TG-derived FA uptake by BAT and whether specific flattening of BAT rhythm contributes to cardiometabolic disease. The timing signals and intracellular pathways that synchronize peripheral tissues need further elucidation, *e.g.* with respect to whether the SNS outflow towards BAT is rhythmic. If so, this would provide an additional target for pharmacological activation. In conclusion, the circadian clock crucially regulates energy metabolism. Increasing our understanding of the interplay between clock and energy metabolism will provide new avenues to improve cardiometabolic health.

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