THE UNIVERSITY OF CHICAGO

# ROLE OF CHRONOTYPE IN TYPE 2 DIABETES RISK

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## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

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# CHAPTER 1

## UNDERSTANDING THE ROLE OF CIRCADIAN RHYTHMS IN TYPE 2 DIABETES

Circadian rhythms are physiological and behavioral rhythms governed by the 24-hour internal clocks in humans. The circadian clocks regulate the timing of sleep, activity and meal consumption. About 10% of genes in mammals are found to exhibit circadian oscillations that impact several key endocrine, metabolic and neurological pathways (Panda et al., 2002). However, the rise of modern conveniences such as artificial lighting, regulated-indoor temperature, and round-the-clock accessibility to food and snacks disturbs these rhythms thereby placing an increased stress on our body ultimately impacting health. The circadian clock communicates with the environment through various signals, with light being the strongest modulator. The presence of artificial lights during the night causes an unnatural exposure to light, thus perturbing the circadian clocks. These perturbations also arise due to irregular eating periods that may partly be due to irregular sleep/wake cycles (such as nighttime eating).

Now, the big question is whether irregular sleeping and eating behaviors impact metabolic health? With the advent of technology and the availability of resources, a steady rise in chronic metabolic diseases such as diabetes and obesity has been observed in parallel to changes in behaviors such as sleep (timing and duration of sleep) and activity (frequency of exercise). This has led to the exploration of interactions between behavioral rhythms and the circadian clocks in the onset and prevalence of metabolic disorders. The majority of research has been studies that elucidated the negative effects of chronic shiftwork or laboratory-stimulated acute shiftwork on metabolic homeostasis. The impact of chronic extreme circadian desynchronization, as manifested during shiftwork on diabetes onset, prevalence and progression is well established. However, the impact of milder circadian desynchronization that occurs in daytime workers who

have irregular sleeping and eating behaviors on diabetes risk is not entirely understood. Whether these perturbations due to mistiming of circadian and social behavior in otherwise healthy people increases disease risk remains unclear. It is particularly interesting to analyze the timing of sleep and dietary behaviors on diabetes risk under natural environmental conditions, to better understand the effect of our daily behavior on health. Additionally, this will inform of possible mechanisms of disease risk and contribute to the development of future, more effective lifestyle interventions for high-risk individuals. Given the co-prevalence of behaviors such as irregular sleep schedules, excessive use of light-emitting electronic devices before sleep, sporadic calories consumption, understanding the impact of these habitual behaviors on health by evaluating specific markers of sleep and dietary timing, together with the determination of the degree of the circadian perturbations and its impact on diabetes risk is critical.

This chapter will review the relationship between type 2 diabetes and circadian rhythms. I will 1. Summarize diabetes pathophysiology and diabetes risk factors 2. Review the circadian system components and properties and 3. Discuss the role of circadian disruption in diabetes.

## a) TYPE 2 DIABETES

Diabetes is a chronic metabolic disease and the seventh leading cause of mortality in the US primarily because it can lead to a variety of health complications, including heart disease, stroke, kidney failure, blindness and limb amputation. Although endemic to the western world, it is a growing problem in poorer countries as well. The number of people diagnosed with diabetes in the United States has quadrupled in the last 40 years to 29.1 million or 9.3% of the population ( National Diabetes Statistics Report: Estimates of Diabetes and Its Burden in the United States, 2014). About 90-95% of that represents adults with type 2 diabetes formerly called adult-onset or

non-insulin-dependent diabetes. Moreover, it is estimated that 28% of the people with diabetes remain undiagnosed in the United States. The number of individuals with diabetes is estimated to observe a 54% increase by 2030 (Rowley, Bezold, Arikan, Byrne, & Krohe, 2016). Besides the debilitating effect on life expectancy and life quality, it imposes a huge economic burden on the individual and the society at large. It is estimated that the national cost of diabetes is about 245 billion dollars (American Diabetes Association, 2013). These startling numbers increase the need to understand the pathophysiology of the disease to develop improved prevention and treatment strategies.

In the context of diabetes pathology, insulin resistance has been shown to increase the risk for type 2 diabetes and prediabetes (the transition state between normal glycemic control and type 2 diabetes). Insulin resistance is a state where more insulin than normal is needed to clear a said amount of glucose. This state induces a burden on the β-cells of the pancreas for insulin production. When the β-cells cannot produce sufficient insulin, it increases blood glucose to levels above normal but below the diabetic range and this state is called prediabetes. Eventually the loss of function of the pancreatic β-cells leads to overt diabetes or high blood glucose levels. For clinical purposes, the stages of diabetes progression are classified based on the levels of glycosylated hemoglobin (HbA1C) or fasting plasma glucose (FPG) and 2-hour postprandial glucose (2hPG) derived from an oral glucose tolerance test (OGTT) (Table 1).

### a. (i) REGULATION OF GLUCOSE PRODUCTION

Blood glucose is under tight homeostatic regulation with a net change of less than 3 mmol/L in metabolically healthy individuals. This controlled process is orchestrated primarily through the interplay of two counter-regulatory hormones: insulin and glucagon (Tonelli,

Kishore, Lee, & Hawkins, 2005). Besides insulin and glucagon, other hormones with glucoregulatory functions include amylin, incretins: GLP-1 (Glucagon-Like Peptide-1) and GIP (Glucose-dependent Insulinotropic Peptide), epinephrine, cortisol, and growth hormone. Of these, insulin and amylin are derived from pancreatic β-cells, glucagon from pancreatic α-cells, and incretins from the L-cells of the intestine. At any given time, the plasma glucose levels are maintained by striking a balance between glucose entry into and exit from circulation through various regulatory mechanisms. Circulating substrates (primarily glucose but also includes amino acids, ketone bodies and free fatty acids), which are results of insulin on intermediary metabolism signals the pancreatic α- and β- cells for the production of glucagon and insulin, respectively. Target tissues are gated with insulin and glucagon receptors for glucose regulation. Additionally, the metabolic hormones also act in a paracrine fashion on the pancreatic islets.

Under normal homeostasis, following an overnight fast of 10-14 hours, endogenous glucose production (EGP) occurs in the liver (Eleuterio Ferrannini & Groop, 1989) and kidneys (Meyer et al., 2002), with the liver being the major contributor. Under the influence of glucagon, a key catabolic hormone, the hepatic glucose production is initiated through contributions from primarily two metabolic pathways: glycogenolysis and gluconeogenesis (DeFronzo, Ferrannini, Hendler, Felig, & Wahren, 1983; DeFronzo & Ferrannini, 1987). Following a glucose load or mixed meal, insulin, a key anabolic hormone is secreted in response to high postprandial blood glucose. It regulates glucose clearance through insulin-mediated glucose uptake in peripheral tissues, predominantly in the skeletal muscles (DeFronzo et al., 1983). Its functions include inhibition of hepatic glucose production, inhibition of glucagon production by α-cells (Gerich et al., 2009) and stimulation of fat synthesis and triglyceride storage in the adipose tissue (Dimitriadis, Mitrou, Lambadiari, Maratou, & Raptis, 2011). Amylin another glucoregulatory

hormone works alongside insulin in decreasing blood glucose postprandially (Testa et al., 1996). It suppresses postprandial glucagon levels in the absence of hypoglycemia (Gedulin, Rink, & Young, 1997; Gedulin & Young, 1998) and slows the rate of gastric emptying (Samsom et al., 2000), thus slowing the rate of nutrient delivery to the intestine and subsequent rate of intestinal glucose absorption. Its action is primarily orchestrated through the central nervous system (Gedulin et al., 1997; Schmitz, Brock, & Rungby, 2004). Incretins secreted by the gut also induces glucose clearance. GIP stimulates insulin secretion and regulates fat metabolism (Yip & Wolfe, 2000), whereas GLP-1 stimulates glucose-dependent insulin secretion (Nauck, Holst, Willms, & Schmiegel, 1997), inhibits glucagon secretion (Perfetti & Merkel, 2000) and slows gastric emptying (Imeryüz et al., 1997).

Type 2 diabetes is characterized by impaired insulin (Guillausseau et al., 2008) and amylin secretion (Lutz, 2010; A. Young & Denaro, 1998). In addition, the suppression of glucagon is inadequate (Meier, Kjems, Veldhuis, Lefèbvre, & Butler, 2006). However, the secretion of GIP is higher in patients with diabetes compared with healthy control subjects (Theodorakis, Carlson, Muller, & Egan, 2004; Vollmer et al., 2008) but with severely impaired or absent GIP insulinotropic effect (Knop et al., 2007), whereas GLP-1 is normal or lower (Vilsbøll, Krarup, Deacon, Madsbad, & Holst, 2001). The characteristics of the glucotropic hormones changes during the pathology of diabetes, hence understanding the progression of these changes and the factors that induce them is essential to unraveling the pathophysiology of diabetes.

### a. (ii) PATHOPHYSIOLOGY OF TYPE 2 DIABETES

Diabetes is a state of abnormal glucose metabolism characterized by peripheral insulin

resistance, impaired hepatic glucose production and inadequate insulin secretion by pancreatic β-cells. The progression from homeostatic glucose regulation to dysfunction occurs in a continuum that is not necessarily linear, leading to the various stages of prediabetes before manifesting as overt diabetes. However, for clinical purposes prediabetes is categorized based on plasma glucose levels. During the course of the pathology of diabetes there is an excess release of glucose as evidenced by the loss of immediate insulin response to a meal or defects in glucose disposal as evidenced by progressive loss of β-cell decreasing the availability of insulin, amylin and GLP-1 or a combination of both, leading to an abnormal rise in glucose levels or hyperglycemia.

## Regulation Of Glucose Release In Type 2 Diabetes

Following an overnight fast, the endogenous glucose production (EGP) is initiated. The rate of EGP is positively linear with fasting plasma glucose in individuals with and without diabetes. Therefore, compared to normal controls (FPG < 6.105 mmol/L), prediabetes (6.105 - 6.993 mmol/L) and diabetes (>6.993 mmol/L) have higher EGP corresponding to higher fasting glycemia (Ferrannini et al., 2007). The inability of the EGP process to be adaptive to rising glucose levels is indicative of hepatic insulin resistance. Thus, during abnormal glucose tolerance, an increase in hepatic insulin resistance contributes to the increase in fasting blood glucose by defective insulinogenic suppression of EGP. This phenomenon worsens as overt diabetes ensues (DeFronzo, Simonson, & Ferrannini, 1982).

Following a glucose load or mixed meal, there is a significant suppression of EGP under normal glucose tolerance state, which is markedly less in diabetes (Ferrannini et al., 1988). This is due to impairment in the ability of a stimulated insulin response to adequately block

postprandial glucose output. In addition, the circulating glucagon levels are inadequately inhibited by the hyperglycemia and hyperinsulinemia following glucose or meal ingestion (Reaven, Chen, Golay, Swislocki, & Jaspan, 1987; Muscelli et al., 2008). Furthermore, the elevated fasting glucagon levels are independently associated with insulin resistance (Ferrannini et al., 2007). This indicates the poor responsiveness of insulin receptors of the pancreatic α-cells, to the inhibitory effect of insulin while the hepatic sensitivity to glucagon remains intact.

Additionally, alteration in free fatty acid (FFA) flux with increased uptake and oxidation competes with glycolysis and stimulates gluconeogenesis in the liver (Kahn & Goldfine, 1993). Accumulation of fat within the abdominal region and the liver is also associated with insulin resistance of gluconeogenesis with increasing EGP (Gastaldelli et al., 2004). This could be a result of increased metabolic stress-induced inflammation in the liver and adipose tissue (Olefsky & Glass, 2010).

## Regulation Of Glucose Uptake In Type 2 Diabetes

Peripheral insulin resistance is a hallmark of type 2 diabetes, with the skeletal muscle insulin resistance being the initiating step before overt diabetes develops (DeFronzo & Tripathy, 2009). It is well known that insulin sensitivity decreases with progression from normal to impaired glucose tolerance. The skeletal muscle is a significant site of glucose uptake (75% to 80% of insulin-stimulated glucose uptake) where glucose is converted to glycogen. Impairment in the glycogen synthesis pathway is the earliest step of insulin resistance (Bogardus, Lillioja, Stone, & Mott, 1984). Besides skeletal muscle insulin resistance, in individuals with high body fat, insulin resistance of the adipocytes limit the glucose uptake by decreasing the availability of substrates for free fatty acid (FFA) reesterification resulting in net excessive FFA release into the

bloodstream. In turn, the increase in circulating FFA causes its preferential uptake over that of circulating glucose (Randle, 1998). It is a demonstrated fact that as lipid oxidation rates increase, the glucose oxidation rates decrease correspondingly under insulin-resistant state and this phenomenon is called metabolic inflexibility (Kelley, 2005; Galgani, Moro, & Ravussin, 2008).

### Beta-Cell Function In Type 2 Diabetes

Increase in plasma glucose concentrations even in the presence of insulin resistance is minimal as long as the β-cell response is adequate; the hyperglycemia as seen in type-2 diabetes develops only when some critical aspect of β-cell function becomes defective. The cause of hyperglycemia is the reduced ability of the β-cell to respond to increasing glucose levels in a timely fashion during stimulation. Both insulin resistance and β-cell glucose insensitivity play a major role in progression through stages of dysglycemia to overt diabetes (Walker, Mari, Jayapaul, Bennett, & Ferrannini, 2005). Several mediators of glucose sensing in the pancreatic mitochondria target cell's excitability and insulin exocytosis (MacDonald, Joseph, & Rorsman, 2005). However the precise cellular mechanisms linking insulin exocytosis to glucose sensing in the β-cell remains unclear (Otani et al., 2004; Bouche et al., 2010).

## a. (iii) DIABETES RISK FACTORS AND THE EFFECT OF LIFESTYLE INTERVENTIONS

The lifetime risk factors for type 2 diabetes include age, race, sex, physical activity and body weight (Hanson et al., 1995). It has been established that although the incidence of diabetes can occur in young adults, individuals over 45 years are at a higher risk and this risk increases with age (Kirkman et al., 2012). Likewise, body weight is another well-established risk factor. Individuals with higher body mass index (BMI) especially those in the obese (BMI > 30 $kg/m^2$)

or morbidly obese range (BMI> 40 kg/$m^2$) are at a greater risk for diabetes and other comorbidities (Narayan, Boyle, Thompson, Gregg, & Williamson, 2007).

Studies evaluating lifestyle interventions in high-risk individuals have been promising in preventing or at the very least delaying the onset of diabetes and the success has been more far reaching than current pharmacological methods. These studies had emphasized increased physical activity and dietary modifications focused on consuming a healthy diet with the primary goal of weight loss among overweight participants. The key feature of these lifestyle modifications seems to be a comprehensive approach to correct several risk factors simultaneously. Furthermore, follow-up studies of lifestyle interventions suggests that there exists a carry-over effect on diabetes risk factors and incidence rates (Look AHEAD Research Group & Wing, 2010). Hence, it is evident an effective lifestyle intervention module can be an effective treatment option for high-risk individuals. A thorough understanding of human behaviors and their impact on various risk factors particularly modifiable factors is needed.

One of the first intervention studies to test lifestyle modification that included calorie restriction and increased physical activity demonstrated the impact of lifestyle on glucose tolerance. In over 50% of 181 subjects with impaired glucose tolerance, the glucose tolerance normalized. Improvement in glucose tolerance was correlated with increased weight loss and improved physical fitness. Both contributed equally and independently to the reduction in diabetes risk (Eriksson & Lindgärde, 1991). A subsequent study with a 2-year lifestyle intervention in 32 individuals with IGT also showed a significant decrease in BMI, HbA1c, fasting plasma glucose and 2-h postprandial glucose levels (Bourn, Mann, McSkimming, Waldron, & Wishart, 1994). Even though these studies suggested a combination of diet and exercise improved metabolic variables, the lack of control groups precluded drawing any firm

conclusions. However subsequent randomized clinical studies included appropriate control groups. A large cohort of over 100,000 people with diabetes and IGT (n=577) were randomized to either control group or one of three treatment groups: diet only, exercise only, or diet plus exercise (Pan et al., 1997). Follow-up evaluations were conducted at 2-year intervals over a 6-year period to identify subjects who developed diabetes. A decrease in the cumulative diabetes incidence was observed. Compared to the control, all three lifestyle treatments reduced diabetes risk by 31-46%, even after adjusting for BMI and fasting glucose. In another study of 522 overweight subjects with IGT, lifestyle intervention designed to produce weight loss by improving diet and physical activity also saw a reduction in diabetes risk by 58% (Tuomilehto et al., 2001). The first multi-centered randomized clinical trial in the US to directly compare lifestyle intervention and pharmacological treatment (metformin) in preventing the onset of diabetes in individuals with IGT was the Diabetes Prevention Program (DPP). The lifestyle intervention decreased the incidence of diabetes by 58% compared to 31% in the metformin-treated group. It was a behavioral intervention with the goal of achieving weight loss and increased physical activity (Diabetes Prevention Program (DPP) Research Group, 2002). Subsequently, multiple randomized clinical trials were designed to evaluate the effect of diet and physical activity with the primary outcome measure being weight loss and secondary outcomes being changes in diabetes risk factors and diabetes incidence rates (Tuomilehto, Schwarz, & Lindström, 2011).

Observational and interventional studies have consolidated the fact that diabetes prevention or prolongation of onset is primarily moderated by altering lifestyle factors, making a compelling case in point to unravel the risk factors that lead to disease onset and progression. Diet and physical activity have taken the center stage for decades, however in recent years other

behavioral traits such as sleep has been touted as behaviors that independently impact diabetes risk. More recently another phenomenon has been evolving, namely biological timing or circadian timing of behavior.

## b) CIRCADIAN RHYTHMS

### b. (i) OVERVIEW OF THE CIRCADIAN SYSTEM

The inception of circadian research is thought to have stemmed from a simple experiment performed by the French astronomer Jean Jacques d'Ortous De Marian in 1729. He observed that the opening and closing of the leaves of the mimosa plant mirrored its exposure to sunlight i.e. the leaves opened during the light period and closed during the dark period. To test whether this rhythmic movement of leaves is due to its exposure to sunlight or not, he placed the plant in a lightproof box. The rhythmic movement of leaves persisted even in the absence of light, suggesting the presence of an endogenous factor driving this observed rhythm. More than 200 years later, theses endogenous rhythms, almost mirroring the 24-hour light/dark cycle (solar day), called circadian rhythms, have been observed in many living forms from unicellular organisms to humans.

In animals, this endogenous timing system called the circadian system synchronizes physiology and behavior (sleep/wake, fasting/feeding) with the 24-h light/dark cycle (solar day). The main characteristics of the circadian system are: 1. Endogenous rhythmicity that cycles even in the absence of external cues, and 2. Capability of adjusting its timing in response to environmental factors such as sunlight. In diurnal species, the circadian biological day (active phase) corresponds to the habitual light period and the biological night (inactive phase) corresponds to the habitual dark period. The opposite is true for nocturnal species like rodents.

However melatonin, a key circadian hormone is only secreted during the dark period in both nocturnal and diurnal species (Van Cauter et al., 1994). The transitions between the biological day and biological night are accompanied by changes in several physiological factors such as body temperature and endocrine hormones (Wehr, Aeschbach, & Duncan, 2001). These clock-driven changes occurring with a periodicity of about 24 hours are called circadian rhythms. These rhythms are described primarily based on two properties phase and period. Circadian phase is typically identified by the time within the circadian cycle at which a particular physiological factor reaches its maximum (acrophase) or its minimum (nadir) level, and circadian period (cycle length) is the time between phase point on one day to the same phase point on the following day. The endogenous clock dictates the time of an individual's phase, preference or propensity for a biological function and this phenomenon is called chronotype. For instance, sleep chronotype defines an individual's actual sleep time or the time they prefer to sleep and this is a spectrum from early to late chronotypes.

### b. (ii) CIRCADIAN ENDOGENOUS RHYTHMICITY

The circadian genes regulate the precise oscillation of cell-autonomous circadian clocks (Ethan D. Buhr & Takahashi, 2013). These in turn, regulate the circadian oscillations of several cellular pathways. The core mechanism that generates these circadian oscillations is the transcriptional-translational feedback loop (TTFL) consisting of transcription factors: CLOCK (circadian locomoter output cycles kaput), BMAL1 (brain and muscle arnt-like protein-1), CRY (cryptochrome 1, 2), PER (period 1, 2, 3) and nuclear hormone receptors: ROR (α, β, γ) and REV-ERB (α, β). The positive feedback loop involves the heterodimerization of Clock and Bmal1 proteins to the cis-acting E-boxes of the promoters and subsequent activation of the

transcription of their own repressor genes: Pers, Crys, Rors and Rev-Erbs. Through Rev response elements (RRE) in the promoter of Bmal1, Rev-erb protein represses Bmal1 gene expression, whereas Per and Cry suppress clock gene expression. Rev and Ror proteins also affect the expression of Cry1, delaying its expression several hours relative to Cry2. Besides regulating each other, these proteins also regulate the rhythmic expression of thousands of target genes either through cis-regulated binding sites or through downstream transcriptional factors. By coupling the endogenous circadian clocks to expression of thousands of genes, the temporal oscillations of the core regulators synchronize with the rhythmic expressions of the genes, thereby enabling distinct peak expressions of the genes at distinct times of the day. The physiological role of each circadian component in circadian rhythmicity and homeostasis has been studied through deletions in rodent models (Ko & Takahashi, 2006).

This extensive and coordinated system of gene expression and function with precise timing mechanisms cannot be achieved entirely on the TTFL protein-protein interactions described above. Studies have shown that the core circadian transcription factors also interact with a number of coactivators, corepressors, and chromatin-associated factors to regulate gene transcription. For example, H3 histone acetylation exhibits rhythmicity at the promoter regions of Per1, Per2 and Cry1 genes. The histone acetyltransferase p300 associates with the Clock/Bmal1 complex and this is the target for Cry protein repression (Etchegaray, Lee, Wade, & Reppert, 2003). Similarly, rhythmicity of histone deacetylases at the Per gene promoter region represses Per gene transcription (Duong, Robles, Knutti, & Weitz, 2011). Rev-erbα protein binds directly to Bmal1 promoter and recruits the nuclear receptor corepressor (N-CoR)/histone deacetylase 3 (HDAC3) complex, with a subsequent decrease in histone acetylation (Yin & Lazar, 2005). ARID domain-containing histone lysine demethylase 1a (JARID1a) increases

histone acetylation by inhibiting histone deacetylase 1 function and activates transcription of Clock:Bmal1 (DiTacchio et al., 2011). Usf1, another transcription factor that regulates histone methylation, represses clock-mediated gene transcription by competing with the Clock:Bmal1 complex for binding to E-box sites on downstream target genes (Shimomura et al., 2013).

Besides these, another group of epigenetic regulators called microRNAs that are small non-coding RNA sequences of 22–24 nucleotides located in intra- or inter-regions of protein coding genes that act as specific inhibitors of their target genes, also exhibit circadian rhythmicity. These microRNA's oscillations could in turn regulate target gene rhythmicity. Two microRNAs, miR-181d and miR-191 target and suppress Bmal1 and Clock genes (Na et al., 2009). Similarly microRNAs miR-24, miR-29a and miR-30a regulate Per gene expression (R. Chen, D'Alessandro, & Lee, 2013). Furthermore circulating microRNAs miR-494, miR-152, and miR-142-3p predicted to target Bmal1 gene expressions have also been identified (Shende, Goldrick, Ramani, & Earnest, 2011).

b. (iii) CIRCADIAN ENTRAINMENT

In mammals, the circadian system is organized in a hierarchical manner with the master clock located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus in the brain. The SCN plays a key role in the generation of circadian rhythms and is demonstrated by 1. Neurons from isolated SCN of the brain tissue *in vivo*, or maintained as tissue explants *in vitro* maintain robust rhythmicity (Meijer, Schaap, Watanabe, & Albus, 1997), 2. SCN-lesioned animals have abolished rhythms (Moore & Eichler, 1972; Van den Pol & Powley, 1979), and 3. SCN-lesioned animals receiving SCN transplantation exhibit the same period length as that of the donor animal (Ralph, Foster, Davis, & Menaker, 1990). The circadian oscillators that autonomously generate

circadian rhythms through alternating gene expressions are also located in regions outside the brain, and these are called peripheral circadian clocks (Buhr & Takahashi, 2013; Dibner, Schibler, & Albrecht, 2010). Mammalian peripheral clocks were observed in retinal tissue (Tosini & Menaker, 1996), leukocytes (James, Cermakian, & Boivin, 2007), fibroblasts (Brown et al., 2005) and several metabolic tissues such as liver (Sakamoto et al., 1998), pancreas (Stamenkovic et al., 2012), gastrointestinal tract (Konturek, Brzozowski, & Konturek, 2011), adipose tissue (Gómez-Santos et al., 2009) and skeletal muscle (Lefta, Wolff, & Esser, 2011). Since each circadian clock can generate its own rhythms, it is essential that the rhythms be synchronized with one another to maintain normal homeostasis (Dibner, Schibler, & Albrecht, 2010). The SCN is synchronized to the external light/dark cycle through the photic signals it receives from the retina (Schmidt et al., 2011). It then disseminates this information to the peripheral clocks through neuronal and endocrine systems such as the secretion of glucocorticoids and the tone of the autonomic nervous system, thereby coordinating the various physiological mechanisms through careful orchestration of peripheral rhythms. The central circadian rhythms influence behaviors such as sleep, activity and feeding cycles. However, behaviors such as food intake can independently alter rhythms of the peripheral metabolic clocks. The external signal that conforms the period of circadian system to that of its environment is called '*zeitgeber'* and the process is called 'entrainment'. Examples include light, food intake, activity and temperature, with light being the strongest entrainer of the central clock and food intake a strong entrainer of the peripheral clocks (Till Roenneberg, Kantermann, Juda, Vetter, & Allebrandt, 2013; Johnston, 2014). Entrainment is fundamental to the circadian system since the period of endogenous autonomous rhythms is near but not exactly 24 hours and without

external stimuli the system will be free-running and dissociate from the natural cycle; hence a specific mechanism to reconcile them to a 24-hour cycle is needed on a day-to-day basis.

## Photic Entrainment

The photic signal is the most potent zeitgeber because the circadian system is extremely sensitive to light (Roenneberg & Foster, 1997). Two models can explain this mechanism of entrainment to the environmental light/dark cycle: the discrete (nonparametric or phasic) and the continuous (parametric or tonic) models (Johnson, Elliott, & Foster, 2003). The discrete model states that the circadian oscillator responds differently to light at different phases of its cycle and the induced phase shift is equal to the difference between the endogenous period and the period of the environment light/dark cycle. Discrete entrainment can be deduced from the free-running period and phase response curve (PRC) for that stimulus. For example, the PRC for light stimulus details the magnitude and direction of a phase shift in response to the stimulus at different circadian times. In other words, a PRC describes how a stimulus presented at various circadian phases influences the timing of the clock. The light PRC informs that light stimulus during the early biological night prior to the core body temperature minimum results in a phase delay, while light stimulus during the end of the biological night after the core body temperature minimum results in a phase advance (Khalsa, Jewett, Cajochen, & Czeisler, 2003). On the other hand, the continuous entrainment model is based on the observation that the length of the free running period is dependent on the intensities of continuous light exposure. In other words, light has a continuous effect on the clock to conform it to the light/dark cycle by accelerating or decelerating the free-running period.

Besides the circadian phase of light exposure, the intensity, spectral distribution, and duration of the light stimulus has a significant and unique effect on the circadian clock. Investigation of the relationship of each property with circadian phase is typically executed by keeping the other variables constant. Experiments involving 3 days of 5-hour light stimulus administered during the early biological day (phase advance portion of the PRC) of varying intensities (0-9500 lux) demonstrated that the greater the intensity of the light stimulus the greater the phase advancement (Jamie M Zeitzer et al., 2005). On the other hand, a significant phase-delay was observed after a single light exposure of 6.5 hours when administered during the early biological night (phase delay portion of the PRC). The degree of phase-delay increased with the intensity of light (Zeitzer, Dijk, Kronauer, Brown, & Czeisler, 2000). The light induced phase-shifts were independent of the timing of sleep/activity and meal consumed (Duffy, Kronauer, & Czeisler, 1996). The human circadian clock is more sensitive to shorter wavelength of light as shown in a study that compared the degree of phase-shift in participants receiving a single 6.5-hour exposure of monochromatic light of wavelength 460 nm or 555 nm (Lockley, Brainard, & Czeisler, 2003). Finally the pattern of exposure of the light pulse also induces phase change in humans. The first study compared a 3-cycle 46 minute light stimuli dispersed between equally spaced episodes of darkness or 13-cycle 5 minute stimuli dispersed between 20 minute episodes of darkness over a 5-hour period to 5-hour of continuous light stimulus or 5-hour of darkness stimuli (Rimmer et al., 2000). All the light stimuli were applied during the early biological day and showed significant phase advancement. Each of the stimuli pattern had 63% (3-cycle 46 minute light stimuli) or 31% (13-cycle 5 minute stimuli) of duration of light exposure as that of the continuous light group nevertheless induced 88% or 70% respectively of phase advances as the magnitude of that of the continuous light group. Likewise, when 6-cycle

15 minutes light stimuli separated by 60 minutes of dim light was compared to continuous light exposure for the same period administered during the early biological night; stimuli pattern with 23% duration of the continuous stimuli group resulted in phase-delay of magnitude 75% of the continuous light group (Gronfier, Wright, Kronauer, Jewett, & Czeisler, 2004). Taken together these results confirm the direct effects of light stimulus on the circadian phase.

Non-Photic Entrainment

Among non-photic entrainers of circadian rhythms, timing of feeding is an important synchronizer. Like light, when food availability is restricted to specific time window in a 24-hour day, this temporal restriction induces increased activity in search of food called food anticipatory activity (FAA) (Marchant & Mistlberger, 1997). This behavior exhibits properties similar to behaviors that are clock-controlled. For example, early studies have shown that SCN-lesioned mice entrained with one meal a day, exhibited both rise in core body temperature and corticosteroid in correspondence to FAA rhythm. When the animals were fed *ad libitum*, the FAA rhythms were dampened. However, food deprivation after an *ad libitum* feeding period induced the FAA at or near phase of entrainment to food timing prior to *ad libitum* feeding. Moreover, under constant darkness or constant light, free running activity couldn't be entrained to food timing. With the identification of clock genes in peripheral tissues, temporal changes in meal timing for rats under 24-hour light-dark cycle caused phase changes in peripheral oscillators without affecting the central oscillators. Two studies showed that when the animals were fed during the light phase or biological night for 12-hours or 4-hours (Stokkan, Yamazaki, Tei, Sakaki, & Menaker, 2001), they exhibited a phase shift in the peripheral tissues within one week with the faster phase resetting occurring in the liver than the kidneys and pancreas. A

similar phase resetting to meal timing was also observed in the adipose tissue (Zvonic et al., 2006) and gastrointestinal tract (Hoogerwerf et al., 2007). Among humans, FAA is primarily under the control of meal timing. Even though comparable studies in humans are limited, advancing meal times for 3-days advanced the phase of core body temperature and heart rate (Kräuchi, Cajochen, Werth, & Wirz-Justice, 2002). Similarly, delaying meal times, delayed the phase of leptin (satiety hormone) rhythm under controlled light-dark conditions (Schoeller, Cella, Sinha, & Caro, 1997). Therefore, it is evident that non-photic signals such as meal timing have a profound effect on the peripheral circadian clocks even in humans.

### b. (iv) CIRCADIAN SYNCHRONIZATION SIGNALS

We have discussed earlier that for effective functioning and time keeping, the biological clocks must adjust to external signals (example: light, meal time). However, the magnitude and response time to entrainment signals are tissue-specific. Thus, the orchestration of circadian timing between the central and peripheral clocks requires internal synchronizers, which are a combination of humoral and non-humoral signals.

#### Humoral Signaling

Effective internal timekeepers for peripheral clocks are hormones that are direct circadian outputs of the central clock and have target receptors on various tissues in the body. Two hormones that appropriately fit these criteria are cortisol and melatonin.

#### Cortisol Entrainment

The central clock drives the rhythm of cortisol, a glucocorticoid secreted by the adrenal

cortex (Kalsbeek et al., 2006). Cortisol secretion is gated by the stimulation of neuroendocrine neurons that control ACTH release, a multi-synaptic pathway from the SCN to the adrenal gland and the adrenal circadian clock (Dickmeis, 2009). The cortisol rhythm is measured in blood serum is shown to peak in the morning upon awakening and reach its nadir by late evening just prior to habitual bedtime (Debono et al., 2009). However, the daily variation of cortisol is blunted by stressful events that activate the hypothalamus–pituitary–adrenal axis and the autonomous nervous system (Stephens & Wand, 2012). Therefore, acute and chronic stressors alter the temporal cues mediated by circulating cortisol. Glucocorticoids are a potent peripheral clock synchronizer as demonstrated by studies using glucocorticoid analogues or exogenous glucocorticoid. One study showed that dexamethasone, an agonist of the glucocorticoid receptor, induces phase shifts in peripheral tissues such as liver, kidney, and heart but does not affect the SCN neurons (Balsalobre et al., 2000). This was further confirmed in humans, where the participants were administered oral synthetic hydrocortisone late in the afternoon which resulted in significant phase shifts in the clock genes of their peripheral blood mononuclear cells (Cuesta, Cermakian, & Boivin, 2015). Adrenalectomized animals displayed arrhythmicity of clock genes in peripheral organs such as liver, kidney, adipose tissue and cornea (Pezük, Mohawk, Wang, & Menaker, 2012). The glucocorticoid rhythm can also modulate the phase of other hormonal rhythms, as demonstrated by one study wherein adrenalectomized rats implanted with corticosterone pellets whose plasma leptin rhythm advances by 4 hours (Kalsbeek et al., 2001).

### Melatonin Entrainment

Melatonin, another central clock-controlled hormone is secreted predominantly by the pineal gland (Moore & Klein, 1974) and is regulated by the SCN via a multi-synaptic pathway

starting from GABAergic neurons of the SCN projecting in to the paraventricular nuclei of the hypothalamus, which then connects to the intermediolateral column of the spinal cord. From here, sympathetic neurons then project to the pineal gland via the superior cervical ganglion ( Kalsbeek et al., 2006). In humans, melatonin levels rise in the evening, peak during the middle of the night and finally return to baseline levels during the day (Laakso, Porkka-Heiskanen, Alila, Stenberg, & Johansson, 1994). Melatonin is secreted only during the night or dark period as it is suppressed by light (Lockley et al., 2003). It therefore reflects the dark period and helps integrate the measure of photoperiod with the SCN (Revel, Masson-Pévet, Pévet, Mikkelsen, & Simonneaux, 2009). Melatonin receptors are found in several tissues including the brain, retina, immune cells, adipose tissue, pancreas and intestine (Ekmekcioglu, 2006). *In vitro* treatment by exogenous melatonin established the temporal and rhythmic synchronization proteins in mouse adipocytes (Alonso-Vale et al., 2008), rat hepatocytes (Brodsky & Zvezdina, 2010) and human erythrocytes (Chakravarty & Rizvi, 2011). Melatonin administered daily in the early part of the dark cycle through infusion or drinking water entrains the locomotor rhythms in free-running animals (Pitrosky, Kirsch, Malan, Mocaer, & Pevet, 1999; Redman, Armstrong, & Ng, 1983). Further studies in nocturnal and diurnal animals confirm the entrainment property of melatonin, wherein exogenous melatonin in animals altered phase depending on the circadian period prior to induction (Slotten, Krekling, & Pévet, 2005; Slotten, Pitrosky, Krekling, & Pévet, 2002). It delayed phase when the period was shorter than 24-hour and advanced phase when the period was longer than 24-hours.

## Metabolic Entrainment

Besides the two central clock-controlled synchronizing hormones that signal the period of

activity and rest to the peripheral clocks, other metabolism-associated rhythms could also contribute to synchronization. For example, leptin, a satiety hormone, secreted by the adipocytes signals the inhibition of food intake to the hypothalamus (Patton & Mistlberger, 2013). Leptin oscillations increase after food ingestion, however it reaches it daily circulating peak at night (Bodosi et al., 2004). The liver and white adipose of mice lacking leptin (ob/ob mice) had altered clock gene expression (Ando et al., 2011). Moreover, the glucose rhythmicity was affected both in mice lacking leptin and in mice lacking leptin receptor (db/db mice) (Grosbellet et al., 2015). Even though *in vitro* experiments suggest that leptin could phase shift the master clock (Prosser & Bergeron, 2003) and modulate its firing rate properties (Inyushkin, Bhumbra, & Dyball, 2009), when tested *in vivo* it did not reset the free-running master clock (Martínez-Merlos et al., 2004). The data suggests that the leptin increase postprandially could act as a time cue for post-feeding for the central and peripheral clocks. Leptin is not the only possible post-feeding time cue; insulin secreted by the pancreatic β-cells could also regulate clock functioning. *In vitro* studies suggest that not only does pancreatic islets release insulin in a rhythmic manner (E Peschke & Peschke, 1998), insulin can also inhibit clock neuron firing rate in SCN slices (Shibata, Liou, Ueki, & Oomura, 1986). Additionally acute changes of Per2 and Rev-erbα gene expression mediated by feeding-induced insulin secretion were recorded during the early phase of feeding-based entrainment of the liver clocks (Tahara, Otsuka, Fuse, Hirao, & Shibata, 2011). Insulin can also regulate clock genes through insulin-activated insulin-phosphatidylinositol 3-kinase (PI3K)- and Forkhead box class O3 (FOXO3)- pathways in the liver (Chaves et al., 2014). Ghrelin, an orexinergic hormone, is secreted by the parietal cells of the stomach (Patton & Mistlberger, 2013). *In vitro* ghrelin can reset the master clock, while *in vivo* it can phase shift the locomotor rhythm in fasted animals (Yannielli, Molyneux, Harrington, & Golombek, 2007).

Together, these findings indicate that leptin, ghrelin and insulin rhythms are important, albeit not necessary, for modulation of circadian rhythmicity.

Non-humoral Signals

Autonomic innervation of peripheral tissues and the temperature rhythm also regulate physiological function through the circadian clocks. The SCN controls peripheral physiology through both sympathetic and parasympathetic pathways (Kalsbeek, la Fleur, & Fliers, 2014). The SCN's sympathetic innervation to the liver results in the daily glucose rhythm independent of liver clock gene expression (Cailotto et al., 2005; Kalsbeek, La Fleur, Van Heijningen, & Buijs, 2004; Vujovic, Davidson, & Menaker, 2008). Likewise, sympathetic innervation to the adrenal gland directly controls glucocorticoid release by modulating the sensitivity to ACTH (Buijs et al., 1999; Ishida et al., 2005). This is particularly interesting, given the role of glucocorticoids in the entrainment of peripheral clocks.

Temperature exhibits a circadian rhythm with the lowest near the midpoint of the sleep period. The influence of temperature on the SCN clocks is insignificant owing to self-regulation of body temperature in mammals. However, temperature rhythms can influence peripheral clock activity (Buhr, Yoo, & Takahashi, 2010; Rensing & Ruoff, 2002). The likely mechanism of temperature regulation of peripheral clocks is through the heat shock pathway. *In vitro* studies have shown that Heat Shock factor 1 (HSF1) transcription factor activated by temperature changes Per2 rhythm by its interaction with Bmal1:Clock heterodimer (Buhr et al., 2010; Reinke et al., 2008).

It is clear that there is feedback at various levels of the circadian system. Several output signals of the central clock, such as body temperature, can become inputs to other clock

oscillators and transfer the information to various systems. This complex intertwined connectivity between the oscillators contributes to maintaining physiological functions and enables the system to adjust to changes in the environment.

b. (v) MOLECULAR MECHANISMS

The circadian system is intimately linked to health in that disturbances to the circadian system are associated with sleep and metabolic disorders. Circadian rhythms and physiological functions like sleep and metabolism have a reciprocal relationship, wherein the circadian clock regulates sleep and multiple metabolic pathways and in turn sleep behavior and feeding behavior regulate the circadian clock.

Two processes regulate sleep: the circadian clock and the sleep homeostatic process, and disturbances in one or both of these processes can lead to sleep and sleep-related disorders. Sleep is divided into two stages: non-rapid-eye-movement (NREM) and rapid-eye-movement (REM) and the differences between the two lie in the magnitude and frequency of the brain waves, the rate of breathing, heart rate and muscle tone. Role of clock genes in circadian sleep-wake timing is deduced from studies relating clock gene polymorphisms to sleep disorders in humans. For example, polymorphisms of Per3 promoter is associated with delayed sleep phase syndrome (DSPS) (Archer et al., 2010), whereas polymorphisms in Clock, Bmal1 and Per2 genes is linked to chronotype (Katzenberg et al., 1998; Mishima, Tozawa, Satoh, Saitoh, & Mishima, 2005; Song et al., 2016). Not only have these studies illustrated the role of clock genes affecting chronotype and sleep, a nurse's study showed the role of clock gene polymorphisms influencing chronotype which altered adaption to environmental stressors (Gamble et al., 2011). Furthermore, acute sleep loss also alters circadian clocks. In a randomized crossover study a

single night of pronged wakefulness when compared to normal sleep, altered the methylation profiles of Bmal1, Clock, Cry1 and Per1 genes in the adipose and skeletal tissue (Cedernaes et al., 2015). These epigenetic changes were accompanied by increased 2-hour postprandial glucose and elevated cortisol. Sleep deprivation and subsequent recovery alters clock gene expression in the brain as well, as illustrated in mice models (Franken, Thomason, Heller, & O'Hara, 2007). Another possible mechanism through which sleep interacts with the clock components is the activation of glucocorticoid signaling (Balsalobre et al., 2000). Corticosterone-bound glucocorticoid receptors activate Per gene expression through the glucocorticoid response elements (GRE), which then resets the phase of the clock genes.

Circadian regulation of metabolism is primarily managed through the core clock component's regulation of key enzymes in biosynthetic pathways, coordination with nuclear receptors, monitoring cell's energy status and modulation of metabolites. For example, HMG-CoA reductase, a rate-limiting enzyme in cholesterol biosynthesis, exhibits circadian variations with peaks during the biological night. Interestingly, cholesterol-lowering drugs like statin are found to be most effective when administered before bedtime. The circadian clocks directly control rhythmic expression of several nuclear receptors of which peroxisome proliferator-activated receptor (PPAR- α, β, γ) is a principal sensor for polyunsaturated fatty acids, regulation of fatty acid oxidation and apolipoprotein synthesis (Sonoda, Pei, & Evans, 2008). Clock and Bmal1 proteins regulate PPARα gene expression in the liver, while PPARα in turn regulates Bmal1 expression through its promoter (Canaple et al., 2006). PER2, a repressor of the Clock and Bmal1 genes, inhibits PPARγ recruitment to its target promoters in the adipocyte and thereby represses the downstream transcription of genes involved in adipogenesis, insulin sensitivity and inflammatory response (Grimaldi et al., 2010). Rhythmic expression Sirtuin 1

(SIRT1) and AMP-activated protein kinase (AMPK), the energy sensors of a cell are also clock-controlled (Nakahata, Sahar, Astarita, Kaluzova, & Sassone-Corsi, 2009). SIRT1 a deacetylase is activated by an increase in $NAD^+$/NADH ratio, a direct measure of a cell's energy status. It modulates acetylation of core clock protein such as BMAL1 and PER2 and also transcriptional cofactors critical for regulating gluconeogenesis like PPARγ-coactivator α (PGC1α) and Forkhead box O1 (FOXO1) in the liver (Asher et al., 2008; Nakahata et al., 2008; Schwer & Verdin, 2008). SIRT1 also regulates cholesterol metabolism by deacetylating and activating Liver X receptor (LXR) which induces the expression of the ATP-binding cassette transporter A1 (ABCA1) that then mediates cholesterol efflux from peripheral tissues to the blood (Li et al., 2007). An increase in AMP/ATP ratio, an indicator of low energy status activates the other energy sensor, AMPK. It modulates the phosphorylation of Cry1 and Per proteins, which leads to respective protein degradation through ubiquitination (Cantó et al., 2009; Lamia et al., 2009). Often metabolites that are outputs from clock-regulated pathways also counter-regulate the circadian clocks. For example, AMPK also increases $NAD^+$ levels and indirectly regulates rhythmic metabolic gene expression through clock-mediated SIRT1 pathway (Um et al., 2007).

b. (vi) CIRCADIAN PHASE MARKERS

In humans, it is challenging to study the physiology of the central and peripheral circadian clocks directly, thus, examining the physiological outputs of these clocks helps in deciphering its phase. For central clocks, the prominent output signals used are melatonin, cortisol, timing of sleep and core body temperature rhythms because they are robust and under the direct control of the central pacemaker. Of these, melatonin is stable and more reliable. It can be measured in blood, saliva and indirectly through its metabolite 6-sulphatoxymelatonin

(aMT6s) in urine (Benloucif et al., 2005; Dumont & Paquet, 2014; Voultsios, Kennaway, & Dawson, 1997). In humans, melatonin levels rise in the evening, peak during the middle of the night and finally return to baseline levels during the day (Laakso et al., 1994). Sleep timing regulated by sleep homeostasis and circadian clock, is correlated with melatonin onset ($R = 0.48$, $p < 0.05$ in young healthy adults) and used in epidemiological studies where biological sample collection is not possible (Sletten, Vincenzi, Redman, Lockley, & Rajaratnam, 2010). The central clock also drives the rhythm of cortisol, a hormone secreted by the adrenal cortex (Kalsbeek et al., 2006). Cortisol rhythm is measured in blood serum and is shown to peak in the morning upon awakening and reach its nadir by late evening (Debono et al., 2009). Core body temperature, yet another physiological output of the central pacemaker, peaks during the day and reaches its nadir in the night in humans (Rensing & Ruoff, 2002). In entrained individuals the midpoint of body temperature aligns with midpoint of sleep period (Roenneberg, 2012). Besides measurement of physiological rhythms, clock gene expression is mapped in human adipose tissue, white blood cells and hair follicles (Marta Garaulet, Ordovás, Gómez-Abellán, Martínez, & Madrid, 2011; Watanabe et al., 2012), but their phase relationships with the physiological rhythms is not fully understood.

### c) CIRCADIAN DESYNCHRONY IN TYPE 2 DIABETES

The shift to a modern industrial society introduces novel challenges, one of which is its impact on human health. The last century has seen a steady decline in infectious and nutrient deficient diseases while a parallel increase in degenerative and chronic diseases such as diabetes, CVD and cancer. One primary contributor to this change is behavioral changes like diet besides improvement in sanitation and modern medicine. Our behavioral drive evolved to perform

specific activities such as sleep/wake or fasting/feeding in a cyclic manner that mirrors the environmental dark/light cycle. However, today many people have unrestricted access to high caloric food, round the clock access to artificial light, providing us endless options to work, eat and function irrespective of the environmental photoperiod. These changes are reflected in the society as evidence from an increasing obesity epidemic, increasing proportion of short sleepers that are rampant among adolescents and adults alike. Although treatments for diabetes have improved substantially, it continues to remains a major cause of morbidity and mortality. The principle that prevention is better than cure holds good in case of metabolic disorders, particularly when pharmacological interventions or surgery is not universally accessible or without risks. Current modifiable factors for diabetes such as diet and physical activity are notoriously difficult to change and sustain. There is a pressing need to understand what are the other potential targets to help reduce the burden of the disease.

### c. (i) EXPERIMENTAL STUDIES OF CIRCADIAN MISALIGNMENT AND DIABETES RISK

Several studies have experimentally manipulated the circadian rhythms in healthy volunteers to stimulate shiftwork in a controlled laboratory setting to determine the effects of circadian disruption on glucose metabolism.

One methodology to accurately measure intrinsic circadian period is using the forced desynchrony protocol that disentangles the endogenous and activity-related effects on circadian rhythms. During the forced desynchrony protocol, subjects are placed on a short or longer day length (e.g. 20 or 28 h) and behavioral factors including sleep/wake patterns, feeding, and activity, are equally distributed across all circadian phases. The circadian system is unable to

entrain to these extreme light/dark conditions and allows researchers to tease apart rhythms that are due to behavioral factors (i.e. sleep/wake, feeding) and rhythms that are circadian in origin (Dijk & Czeisler, 1994; Wirz-Justice, 2007).

In one study of 10 young healthy participants (5 women) with mean age 25.5 years and mean BMI 25.1 kg/m2, circadian misalignment was induced by a 10-day forced desynchrony protocol with recurring 28-h day where the participants were given isocaloric meals (Scheer, Hilton, Mantzoros, & Shea, 2009). When the participants ate and slept about 12-h out of phase from their habitual times, reflecting the most severe circadian misalignment, there was a 6% increase in glucose, which was primarily due to an exaggerated postprandial glucose response with no changes in fasting glucose despite a 22% increase in insulin. In 3 out of the 8 subjects, the postprandial rise in glucose responses was in the prediabetic range. This is indicative of decrease in insulin sensitivity and insufficient β-cell compensation during misalignment. Also, a 17% decrease in leptin, the satiety hormone involved in appetite regulation, was observed. The cortisol rhythm was reversed and it was consistent with the dominant role of the circadian system relative to behavior.

In another study, a 3-week forced desynchrony protocol with a 28-h day, along with sleep restriction was conducted on 21 participants: 11 (5 women) with mean age 23 and 10 (5 women) with mean age 60 (Buxton et al., 2012). Circadian disruption with concurrent sleep restriction resulted in an 8% and 14% increase in both fasting and postprandial glucose levels. On the other hand, there was a 12% and 27% decrease in fasting and postprandial insulin. As a result, the relative hyperglycemia could be caused by inadequate pancreatic β-cell compensation. Both during the desynchrony protocol and post-recovery period there was no significant difference

between the young and old participants. During desynchrony, there were significant changes in appetite regulation hormones with a decrease in leptin and an increase in ghrelin. The resting metabolic rate also exhibited an 8% decrease.

To demonstrate the differences in the impact on glucose tolerance between endogenous circadian phase and circadian disruption independent of the behavioral cycle, one study used a cross-over design involving 14 healthy adults (6 women) with mean age of 28 years and BMI of 25.4 $kg/m^2$ (Morris et al., 2015). The protocol involved two 8-day studies where the behavioral cycles were aligned or misaligned (12-h shift) with their endogenous circadian system. Firstly, glucose tolerance assessed at 8 am and 8 pm in response to an identical mixed meal was lower in the biological evening (8 pm) than morning (8 am) along with a 17% increase in postprandial glucose in the biological evening. These differences illustrate the effect of circadian phase on glucose metabolism separate from the behavioral cycle effect. This may be partly explained by the decrease in β-cell function in the biological evening (27% lower early-phase insulin). Circadian misalignment (12-h behavioral cycle inversion) itself increased postprandial glucose by 6%, which may be due to decreased insulin sensitivity (elevated postprandial glucose despite 14% higher late-phase insulin) without change in early-phase insulin.

One study with a parallel group design (Leproult, Holmback, & Van Cauter, 2014) compared the effect of circadian misalignment independent of sleep loss by subjecting 26 healthy adults to 8 days of sleep restriction for 5 h with fixed nocturnal bedtimes (circadian alignment) or with bedtimes delayed by 8.5 h on 4 of the 8 days (circadian misalignment). Following an intravenous glucose tolerance test (IVGTT), measures on glucose metabolism were assessed. Among men, there was a decrease in insulin sensitivity (-32% (aligned group) vs. -58% (misaligned group)) without sufficient compensation in insulin response by the β-cells,

therefore resulting in an overall decrease in disposition index (a product of insulin sensitivity and β-cell responsivity) by (-19% (aligned group) vs. -48% (misaligned group)). There was a doubling of the percentage increase of high sensitivity c-reactive protein (hsCRP), a marker of low-grade inflammation in the misaligned group compared to the aligned, adding support to the reduced insulin sensitivity observed.

Another study assessed the changes in energy metabolism (McHill et al., 2014) in 14 participants (8 women) using a whole-room calorimeter with 3 days of simulated shiftwork by having the first night shift, which only allowed a brief 2-hour sleep opportunity and then two additional night shifts with 8-hour sleep opportunities during the day. Compared to baseline, there was a 4% increase in total daily energy expenditure on the first night shift, but 3% decrease on the 2 subsequent nightshifts. An average decrease in anorexigenic hormones leptin and PYY was observed despite a decrease in subjective appetite. There was also a decrease in the thermic effect of feeding (i.e. energy expenditure after food intake) in response to late dinner on the first night shift.

In summary, these experimental studies demonstrate the relative importance of the endogenous circadian system and the timing of behaviors such as eating in regulating metabolism and maintaining homeostasis.

### c. (ii) SHIFTWORK AND DIABETES

Observational studies assessing the impact of shiftwork on diabetes also support the role of circadian misalignment in diabetes. A real-life example of chronic misalignment is seen in shiftworkers whose active periods on work days align with their biological night and rest periods align with their biological day i.e. they are awake, active, and eating during their circadian night

and trying to sleep and fast during their circadian day. This switch from normal behavior (active during the day and inactive during the night) induces circadian misalignment.

One of the first cohort studies to establish the impact of shiftwork on diabetes risk was by Kawakami et al. (Kawakami, Araki, Takatsuka, Shimizu, & Ishibashi, 1999). They showed that in a cohort of 2194 males followed for 8 years the incidence rates for diabetes among workers who worked overtime (outside the regular dayshift) was 1.95 per 1000 person-years, with those who worked over 50 hours per month of overtime had 3.7 times increased risk of developing diabetes than those working 0–25 h per month. In another study of 2860 Japanese male workers after 8 years of follow-up, 87 new cases of diabetes were reported, resulting in an incidence rate of 4.41 per 1000 person-years (Morikawa et al., 2005). The relative risk for diabetes among two-shift workers compared with the fixed daytime workers was significantly higher after adjusting for confounders including age, BMI, family history, smoking, drinking and physical activity. Yet another study investigated the effect of alternating shiftwork on diabetes and reported a 1.35 odds ratio for the development of diabetes among those who worked alternating shifts compared to daytime shifts (Suwazono et al., 2006). Diabetes onset was confirmed if glycated hemoglobin A1c (HbA1c) was ≥ 6 during the 10-year long follow-up. In a prospective study on a population-based cohort of Swedish middle-aged men and women (35- 56 years), a positive association between shift work and diabetes was observed only in women (OR 2.2 [1.0–4.7] adjusted for age, education, and psychological distress) and not men (Eriksson, van den Donk, Hilding, & Östenson, 2013). This observation is partly explained by the incorporation of work stress in the model. It is postulated that unlike the previous studies where men and women were at the same occupational level, in this study this was not true. Moreover, it is suggested that recuperation period from work stress maybe less for men than women due to women bearing additional

household chores and family care and that could partly explain the observed results. Analysis of the data from the Nurses' Health study (NHS) I (1988-2008) and NHS II (1989-2007) that followed over a 150,000 women between the ages of 25 and 67 years without diabetes, cardiovascular disease, and cancer at baseline, established the impact of rotating night shifts on diabetes risk (Pan, Schernhammer, Sun, & Hu, 2011). Over the 18-20 years of follow-up a 6165 (NHS I) and 3961 (NHS II) incident type 2 diabetes cases were documented. Duration of shift work was associated with an increased risk of diabetes in both cohorts. Compared to those who reported no shift work, the pooled hazard ratios (95% confidence intervals) for participants with 1-2, 3-9, 10-19, and ≥20 years of shift work were 1.05 (1.00-1.11), 1.20 (1.14-1.26), 1.40 (1.30-1.51), and 1.58 (1.43-1.74), respectively. This positive association was attenuated after adjusting for BMI, suggesting that it is at least in part mediated by body weight.

In addition to these longitudinal studies, cross-sectional studies have also reiterated the association between shiftwork and diabetes risk. In one study of 475 men who were either shift workers (seasonal shift work and continuous shift work) or not, presence of diabetes was defined as hemoglobin A1c ≥ 6.5% and fasting blood sugar ≥ 126 mg/dl (Ika, Suzuki, Mitsuhashi, Takao, & Doi, 2013). After adjusting for age, smoking status, frequency of alcohol consumption, and cohabitation status, odds ratios for diabetes mellitus were 0.98 (95% confidence interval [CI]: 0.28-4.81) and 2.10 (95% CI: 0.77-5.71) among seasonal shift workers and continuous shift workers, respectively, compared with non-shift workers. Following an age-stratified analysis (<45 years vs. ≥45 years), the association between continuous shift work and diabetes was more pronounced among older participants. In another study, the long-term persistent health effects after leaving shift work was studied (Guo et al., 2013). This study included 26463 retired shift workers among who significant effects of shift work on diabetes was observed with an OR

(95%CI) of 1.10 (1.03-1.17). The positive association of shiftwork with diabetes persisted even after adjusting for potential confounders including BMI.

All the observational studies more or less support the role of shiftwork in diabetes and future diabetes incidence. However, the published data should be interpreted with the understanding that the differences reported between studies arises due to a number of issues particularly non-comparable shift work patterns and insufficient model adjustments.

### c. (iii) OBSERVATIONAL STUDIES OF MILDER CIRCADIAN DISRUPTION

Shiftwork leads to extreme circadian misalignment that is associated with chronic metabolic disturbances, as illustrated previously. However, a milder form of circadian misalignment can also have detrimental consequences. The circadian preference of sleep timing for an individual dictated by the biological clock is referred to as chronotype. It is determined by genetic background, age, sex, and environment (such as light). An individual with early chronotype has a circadian preference for early sleep onset, whereas a late chronotype has a preference for late sleep onset. But, our social demands often are not in tune with the biological clock. Hence, a mistiming between biological and social clocks induces a mild circadian misalignment, referred to as social jet lag (Wittmann, Dinich, Merrow, & Roenneberg, 2006). This discrepancy between the internal timing and external timing is measured by subtracting each individual's midpoint of sleep on workdays from their midpoint of sleep on free days. Moreover, individuals with a later chronotype also often have greater social jetlag.

Few observational studies have explored the impact of social jet lag on markers of diabetes risk. In one such study of non-shift workers, social jet lag was associated with BMI and metabolic dysfunction phenotype after adjusting for sleep duration (Parsons et al., 2015). Further

examination revealed that SJL was positively associated with increased inflammation (hsCRP: OR = 1.3 (95% CI: 1.0–1.6)) and glycated hemoglobin (HbA1C: OR = 1.3 (95% CI: 1.0–1.6)), among metabolically unhealthy obese individuals. Another study of 145 healthy participants (78 women) concluded that those with ≥2 h social jetlag had higher fasting and 5-h area-under-the-curve cortisol levels, shorter sleep duration and were less physically active (Rutters et al., 2014). In a large epidemiological study, greater social jetlag was associated with being overweight (BMI ≥25 kg/$m^2$) (Till Roenneberg, Allebrandt, Merrow, & Vetter, 2012). There was a positive correlation between social jetlag and BMI beyond the effects of sleep duration. There were other studies that evaluated chronotype or the circadian preference of timing as a marker of mild circadian misalignment on diabetes risk. In one such 8-week prospective study among 137 college freshmen, evening chronotype had a significantly greater weight gain when compared with morning/neutral types, without any change in health behaviors (Culnan, Kloss, & Grandner, 2013). A population study of over 6000 participants, evening chronotypes had 2.5-fold odd ratio for type 2 diabetes as compared with morning types after adjusting for sleep duration and sleep sufficiency (Merikanto et al., 2013). In another study of over 1000 participants, a similar association between evening type and diabetes with odds ratio of 2.98 was reported (Yu et al., 2015). A large epidemiological study of over 60,000 women also showed that among day shift workers, evening chronotype was associated with increased diabetes risk (Vetter et al., 2015). Additionally, the association of evening preference in diabetic patients was associated with poor glycemic control, independent of sleep duration (Iwasaki et al., 2013; S. Reutrakul et al., 2013).

Together, these experimental and observational studies cement the role of circadian timing in diabetes; by illustrating the effect circadian misalignment has on glucose metabolism.

c. (iv) SUMMARY

This review discussed the impact of circadian desynchronization on metabolism with a focus on diabetes. It also summarized the molecular pathways that maybe be perturbed due to circadian desynchrony.

Circadian timing of physiology and behavior is important for synchronizing the central and peripheral clocks and maintaining homeostasis. A distinct shift in social behavior is evident over the past several decades with around-the-clock access to light sources and entertainment, which changed the timing of behaviors such as sleep and meals to periods that are not in synchrony with our endogenous clocks, thereby causing circadian desynchrony. Even though the consequences of circadian desynchrony in shift workers has been studied, chronic effects of circadian desynchrony on metabolic health in daytime workers is limited. Mapping habitual sleep and meal timings and evaluating its deviation from the endogenous signals controlling them, to eventually compare the impact of the degree of deviation on insulin sensitivity, will further elucidate the role of behavioral timing in diabetes risk. Devising metrics to assess the phase differences between behavioral timing and endogenous circadian clock and analyzing the relationship with diabetes risk factors will contribute to our understanding of the effect of altered social behaviors on metabolic health. Furthermore, understanding circadian desynchrony due to altered social behaviors will inform future research of behavioral interventions to eliminate or decrease circadian misalignment to reduce risk of metabolic disease incidence and progression.

# CHAPTER 2

# EXPERIMENTAL DESIGN

## a) STUDY PROTOCOL

Volunteers between the ages of 21 and 50 years living in the Chicago area were recruited to participate through flyers and online advertising. Inclusion criteria included African-American or Non-Hispanic White race, body mass index (BMI) between 19- 40 $kg/m^2$, premenopausal women and no major illness and no history of psychiatric, endocrine, cardiac or sleep disorders. Those with dyslipidemia and hypertension were included if these conditions were controlled by a stable treatment, such as lipid-lowering or antihypertensive medications (except beta-blockers). Additionally, those with moderate to severe sleep-disordered breathing (apnea-hypopnea index>15/hour) and those with diabetes were excluded. Other exclusion criteria included persons taking medications, including but not limited to antidepressants and hypnotics (but excluding lipid-lowering drugs and anti-hypertensive medications), persons taking exogenous melatonin or medications that affect endogenous melatonin (example: beta-blockers), persons who tested positive for common drugs of abuse, people with color blindness, people who have had Lasik eye surgery and persons who performed shiftwork. Participants who travelled across time zones were studied only after they remained in the Central Time Zone for one month prior to the study. All participants gave written informed consent. The institutional review board at The University of Chicago, Chicago, Illinois, approved the protocol. The study was divided into three parts: screening session, ambulatory session and laboratory session (Figure 1).

a. (i) SCREENING SESSION

Each participant underwent an overnight screening in the Sleep Laboratory (W4) or Clinical Research Center (W5) at the University of Chicago. The participants checked-in between 18:30 and 19:00, and were administered the drug urine test to exclude anyone who tests positive for common drugs of abuse. Each session included an overnight polysomnography (PSG) or sleep test (bedtime: 23:00 to 7:00) to rule out sleep disorders including moderate to severe sleep-disordered breathing (apnea-hypopnea index (AHI)>15/hour). PSG recordings included measures of airflow by nasal pressure transducer and oronasal thermocouples, chest and abdominal wall motion by piezo electrode belts and oxygen saturation by finger pulse oximeter, during sleep. These enabled the calculation of apneas defined as total cessation of airflow for at least 10 seconds and hyponeas defined as abnormally low respiratory rate, to determine AHI. The following morning a 2-hour oral glucose tolerance test (OGTT) to exclude those with diabetes and other abnormalities was administered. This session also included collection of anthropometric measures such as height, weight, blood pressure and heart rate. Participants who remained eligible after screening were then scheduled for the ambulatory session. They were given a 1-week brief sleep diary at screening to complete prior to the ambulatory session. This sleep diary included questions about their bed times and wake times, which was used later to set-up equipment for the saliva sampling.

a. (ii) AMBULATORY SESSION

Eligible participants completed a 10-day ambulatory session that assessed habitual behaviors such as sleep and meals consumed. The participants were required to wear a waterproof wrist actigraphy monitor continuously and complete sleep diaries to estimate habitual

sleep duration and timing. Saliva samples to estimate circadian phase was collected during the early evening of one preselected day. The participants were required to abstain from caffeine, alcohol and nonsteroidal anti-inflammatory drugs (NSAID) for 48 hours prior to the saliva collection because these components suppress melatonin (Murphy, Badia, Myers, Boecker, & Wright, 1994; Rupp, Acebo, & Carskadon, 2007; Shilo et al., 2002). A list of NSAIDs with both generic and commercial names was provided along with a sample of Tylenol to use if needed. On another evening after the saliva collection, a research team visited the participant's home to set up one night of unattended in-home PSG recording. Further, the participants also completed a 3-day food diary. The team preselected the three consecutive days so that it was comprised of a combination of weekday and weekend days, and excluded saliva sampling and PSG recording nights. The food diary included questions about the time, amount and types of food consumed through the entire recording period.

a. (iii) LABORATORY SESSION

Within a week from the completion of the ambulatory session, a 21-hour laboratory session was scheduled in the Sleep Laboratory or Clinical Research Center at the University of Chicago to obtain detailed characteristics of diabetes risk. The participants were admitted to the laboratory in the early evening (between 17:00 and 18:30) and began fasting at 20:30 when only water was allowed. Saliva samples were collected every 60 minutes beginning 19:50, up to 21:50 (total of 3 samples) for cortisol analysis. The participants were given a 10-hour bedtime opportunity from 22:00 to 8:00 the next day. Their self-reported age and sex were recorded and height and weight were measured in the morning. Actigraphy was recorded through the session and blood pressure was recorded every 30 minutes starting at 20:00 on the first day until 2:00,

which allowed for 6 hours of undisturbed sleep. Urine samples were collected from 6:00 PM onwards until the first morning void the next morning, to test for secretion of 6-sulfatoxymelatonin (aMT6-s). In the morning, a 5-hour frequently sampled oral glucose tolerance test (fsOGTT) was performed. Participants were given lunch after completion of the fsOGTT and then discharged.

A more detailed description of the measures pertinent to the analyses presented herein is provided below.

**b) SLEEP ASSESSMENT**

Habitual sleep timing and duration was estimated using 7-10 days of wrist activity monitoring. Participants wore waterproof wrist actigraphy monitors (Actiwatch-2 or Actiwatch Spectrum, Respironics/Philips) on their non-dominant wrist continuously. Actiwatch activity monitors contain highly sensitive omnidirectional accelerometers that counted movements in 30-second epochs and they feature digital integration, which is the most accurate measure of both movement level and intensity. The subjects were asked to press an event marker button that time-stamped when they went to bed and when they wake. Additionally, they completed a short sleep log each morning. Both the event marker and log were used to identify bedtime and waketime for analysis of sleep periods. Wrist actigraphy has been validated against polysomnography, demonstrating a correlation for sleep duration between .82 in insomniacs and .97 in healthy subjects (Jean-Louis et al., 1997). Estimates of habitual sleep duration and timing were calculated using the associated Actiware software (v. 6.0) and averaged across all days of recording. The sleep variables used in the analyses are sleep duration: the average sleep period from sleep start to sleep end, and sleep midpoint: midpoint of sleep duration.

## c) CIRCADIAN PHASE ASSESSMENT

From the saliva samples collected during the ambulatory session, salivary melatonin is measured to estimate circadian phase using the dim light melatonin onset (DLMO) phase marker, which is the gold standard measure of endogenous circadian phase (Pandi-Perumal et al., 2007). The at-home DLMOs is highly correlated with the laboratory DLMOs ($r = 0.91$, $P < 0.001$) using the following protocol that includes measures of compliance (Burgess, Wyatt, Park, & Fogg, 2015). Prior to the start of the ambulatory session, a member of the research team meets with the participant. During this appointment, the participants were given an Actiwatch ® (Philips Respironics, Bend OR), questionnaires, and an at-home saliva collection kit along with detailed and exhaustive verbal and written instructions about the procedures. On the day of the saliva collection, a research team member called the participant at a prearranged time and reminded them of the instructions for the saliva collection. Collection began 6 hours prior to bedtime (estimated from 1-week brief sleep diary provided during the screen session) and samples were collected every 30 minutes. The at-home saliva collection kit included a black messenger bag with a step-by-step checklist (with staff phone number), track cap bottle (with microchip in lid that records opening, MEMS, Aardex) containing 13 cotton inserts (salivettes) for saliva collection, 13 empty tubes for the salivettes, small test tube rack, timer (a personal data assistant, PDA) with preprogrammed alarms, label dispenser (pre-labeled and in order to avoid incorrect sample labeling), soft toothbrush (to avoid toothpaste and blood contamination), insulated travel bag for frozen samples, 8 Tylenol pills (to avoid inadvertent use of NSAIDs) and light sensor worn around the neck on top of clothing (Actiwatch Spectrum) through the evening of the saliva sampling session until bedtime (to reduce light intensity received by the eyes while allowing for comfortable vision). The participants were advised to the close blinds and/or curtains to avoid

outdoors light, and dim lights to a practical level (including bathroom lights). Minimizing light intensity is critical as light is a strong melatonin suppressor (Zeitzer et al., 2000). At the end of the sampling, the participants removed their light medallion and went to bed. The saliva samples were either picked up by a member of the research team or dropped off by the participant at the research center. Later the saliva samples were thawed, centrifuged and then refrozen and shipped in dry ice to Solidphase Inc. (Portland, ME) which radioimmunoassayed the samples for melatonin using commercially available kits (ALPCO, Inc, Salem, NH). The sensitivity of the assay is 0.3 pg/ml, intra-assay variance (<14%) and inter-assay variance (<17%). The DLMO threshold is calculated as the mean of 3 low daytime points plus 2 times the standard deviation of the 3 points (DLMO-3k) (Voultsios et al., 1997) or a fixed threshold of 3 pg/ml (DLMO-3pg) (Susan Benloucif et al., 2008).

**d) URINARY MELATONIN ASSESSMENT**

Urine was collected during the evening (18:00 to 22:00) and through the night (22:00 to 8:00) ending with the first morning void. Melatonin secretion was estimated by measuring the concentration of the major metabolite of melatonin, 6-sulfatoxymelatonin (aMT6s) in urine. Additionally, to account for differences in urine concentrations, 6-sulfatoxymelatonin levels were standardized to urinary creatinine (Cr) levels for all analyses (urinary aMT6s:Cr ratio). Urinary 6-sulfatoxymelatonin levels standardized to urinary creatinine level correlated with cumulative nocturnal plasma melatonin secretion (R = 0.76) independent of renal function (Baskett, Cockrem, & Antunovich, 1998; Graham, Cook, Kavet, Sastre, & Smith, 1998). Urinary aMT6s concentrations were measured using an enzyme-linked immunosorbant assay (ALPCO Diagnostics, Windham, New Hampshire) with interassay coefficient of variation (CV) of 5% and

urinary creatinine was measured using modified Jaffé method (interassay CV = 1%) using the Synergy H1 Hybrid Reader (Biotek Instruments Inc., Winooski, Vermont).

### e) DIETARY ASSESSMENT

Participants completed a self-administered food dairy completed on 3 consecutive days to determine habitual food intake. A food diary is a non-weighed food recording method that requires participants to record the description (brand name, recipes of home-cooked food) and the amount of food consumed (using common household measures such as cups, teaspoons, tablespoons and food units) along with meal times. Participants also classified each meal as breakfast, lunch, dinner or as a morning, afternoon or evening snack. The food diary included detailed instructions about the type of information the subject is required to record and these instructions were explained in-person by a trained study coordinator prior to the start of the food recording period. The food diary also included examples of a completed food log to serve as a reference for the details requested and 18-pages of photographs to help participants describe food portion size. The food log was then collected and reviewed by a trained study coordinator in the presence of the study participants to obtain additional explanations and details, if required. The food log information was entered into the Food Processor Nutrition Analysis Software ESHA (version 10.7.0, Salem, OR), which included over 30,000 foods and allowed for the addition of new recipes. The database sources include USDA Standard Reference database, manufacturer's data, restaurant data, and data from literature sources. Each food item on the food diary was matched to a corresponding food item on the Food Processor Nutrition Analysis database by name and nutrient composition. The software calculated the calories and macronutrient content

for each food item, which was then grouped by meal classification. Finally, the time of each meal and nutrient consumption was recorded.

The dietary variables calculated included total calories and macronutrients (carbohydrates, protein and fat) consumed. There were two measures to represent the timing of dietary intake. First, the proportions of total calories consumed were estimated for three periods in the day: morning (breakfast + morning snacks), afternoon (lunch + afternoon snacks) and evening (dinner + evening snacks). Similarly morning, afternoon and evening macronutrient content were also estimated. Second, we calculated the clock time at which the participant reached 25%, 50% and 75% of total daily caloric intake (TCI 25%, TCI 50%, TCI 75%). These calculations were conducted for each food diary day and then averaged across all days. Furthermore, we grouped participants based on whether they consumed more or less than the average morning and evening calories.

## f) GLUCOSE METABOLISM ASSESSMENT

The frequently sampled oral glucose tolerance test (fsOGTT) was initiated in the morning after a 12-hour overnight fast. An intravenous catheter was placed in the distal forearm and two baseline blood samples were collected at −10 and 0 minutes. Then the participant orally ingested 75 g dextrose dissolved in 296 ml of orange-flavored water (Trutol 75, Thermo Scientific) within 5 minutes. Additional blood samples were collected at 10, 20, 30, 60, 90, 120, 150, 180, 240 and 300 minutes post ingestion (Breda, Cavaghan, Toffolo, Polonsky, & Cobelli, 2001). Plasma glucose was measured with 2300 STAT PLUS analyzer (Yellow Springs Instruments), while serum insulin and C-peptide were measured using Immulite 2000 (Siemens) on all twelve blood samples. Fasting glucose and insulin concentrations were calculated as the average of the −10

and 0-min readings. We calculated insulin sensitivity by Matsuda Index (MI) calculated as 10,000 / sqrt [(fasting glucose (mg/dL) × fasting insulin (μU/mL)) × (mean glucose × mean insulin during the OGTT)] and QUICKI calculated as [1/ (log (fasting glucose) (mg/dL) + log (fasting insulin) (μU/mL))))] (Chen, Sullivan, & Quon, 2005; Matsuda & DeFronzo, 1999). We calculated two MI, one using 10 data points until the 180-minute time point and the other using 12 data points till the 300 minutes time point because 2 participants became hypoglycemic after the 180-minute time point and the OGTT was effectively terminated. Additionally, HOMA-IR and HOMA-B was computed as: [fasting insulin (μU/mL) x fasting glucose (mmol/mL)]/22.5 and [20 x fasting insulin (μU/mL)]/ [fasting glucose (mmol/mL) -3.5], respectively (Katsuki et al., 2001; Matthews et al., 1985).

## g) CARDIOVASCULAR ASSESSMENT

Systolic and diastolic arterial blood pressure was measured at 30-minute intervals from 18:00 to 2:00 the next day on the non-dominant arm using ambulatory monitoring equipment (Oscar II, SunTech Medical Instruments). Additionally plasma proinflammatory marker, high-sensitivity c-reactive protein (hsCRP) was assayed in the fasting blood samples taken during the fsOGTT. High-sensitivity CRP was measured from serum samples using the Immulite 1000 High-Sensitivity CRP enzyme immunoassay test kits (Siemens Medical Solution Diagnostics, Los Angeles CA, USA). The minimum reportable limit for the assay was 0.2 mg/mL.

## H) STATISTICAL ANALYSIS

Means and standard deviations of all continuous variables and proportions of categorical variables were calculated. Measures of whole-body insulin sensitivity such as Matsuda index-

180, Matsuda index-300, QUICKI, HOMA-IR from fsOGTT were the dependable variable or the primary outcome of interest for all regression analysis unless specified otherwise. The linear regressions were tested at the $\alpha = 0.05$ level.

The independent variables analyzed for sleep chronotype associations with diabetes risk included timing of melatonin onset (DLMO-3k, DLMO-3pg) and urinary melatonin (urinary aMT6s:Cr ratio). The associations were further adjusted for appropriate covariates including age, sex, race and BMI.

The independent variables analyzed for dietary associations with diabetes risk included morning and evening calorie proportions, TCI 25%, TCI 50% and TCI 75%. Analyses of variance were used to test for differences in measures of insulin sensitivity between the four groups defined by morning and evening calorie proportion. Potential confounders were identified by using Pearson's correlation analysis to test for significant associations between the measures of insulin sensitivity and continuous variables, including age, BMI, sex, race, total calories consumed, average sleep duration and average midpoint of the sleep period.

All statistical analyses were performed using STATA (version 14.0).

# CHAPTER 3

## ASSOCIATION BETWEEN MELATONIN AND INSULIN SENSITIVITY

### a) INTRODUCTION

Melatonin, an indoleamine, is predominately secreted by the pineal gland during the absence of light therefore acting as a hormonal messenger of the photoperiod (Cardinali & Pévet, 1998). It exhibits a diurnal rhythm typically peaking ~2 hours before sleep. In humans endogenous melatonin has a short half-life of 20 to 50 minutes (Di, Kadva, Johnston, & Silman, 1997). It gets converted to 6-hydroxymelatonin in the liver, then gets conjugated and excreted through urine (Young, Leone, Francis, Stovell, & Silman, 1985).

Chronotype is an individuals' circadian preference in behavioral rhythm in relation to the light/dark cycle or the circadian timing of behaviors such as sleep or diet. The circadian rhythm of melatonin in plasma or saliva or its metabolite in the urine is a functional output of the central circadian oscillator (Kalsbeek et al., 2004). Within the melatonin rhythm, the onset of melatonin secretion under dim lights (DLMO) is one of the accurate markers of circadian preference (Pandi-Perumal et al., 2007). The circulating melatonin levels are a preferred a circadian marker because of its stability compared to other core rhythms. For example, carbohydrate rich food can alter the rhythms of core body temperature and heart rate but not melatonin (Kräuchi et al., 2002). However, light is the only environmental signal capable of suppressing melatonin production (Cardinali & Pévet, 1998). So it is essential that melatonin measurements be conducted under dim light conditions (Gooley et al., 2011).

There are several lines of evidence that suggest a key role for melatonin in glucose metabolism. Although, melatonin was identified for its ability to induce phase shifts in the SCN leading to its use as a treatment for jetlag and other circadian rhythm disorders (Golombek,

Pandi-Perumal, Brown, & Cardinali, 2015), it was also found to regulate other functions including metabolism and inflammation. Melatonin signaling at the molecular level occurs through its G protein-coupled receptors that activate several signaling pathways, most notably the Gi/cAMP (cyclic adenosine monophosphate) and Gq/ PLC (Phospholipase C)/$Ca^{2+}$ pathways (Pandiperumal et al., 2008). Mammalian system has 2 melatonin receptor subtypes: MT1 and MT2 (1A and 1B in humans). These are located in the SCN and in several peripheral metabolic tissues including the pancreas, adipose tissue, skeletal muscle and gastrointestinal tract (Brydon, Petit, Delagrange, Strosberg, & Jockers, 2001; Lee & Pang, 1993; Mulder, Nagorny, Lyssenko, & Groop, 2009; Stratos et al., 2012).

The rhythmicity of insulin is contrasting to that of melatonin; when melatonin increases, insulin decreases (Boden, Ruiz, Urbain, & Chen, 1996). However, complete abolition of melatonin as seen in pinealectomized rats increased plasma glucose levels and reduced the adipose tissue response to insulin (Gorray & Quay, 1978; Lima et al., 1998). Pancreatic melatonin receptors reduce cAMP production and subsequently decrease insulin release. They can also increase calcium levels through phospholipase C / inositol trisphosphate to stimulate insulin release. Along with the pancreatic core clock mechanism they regulate the rhythmic insulin secretion (Elmar Peschke, 2007; Picinato et al., 2008). Furthermore, genome-wide association studies revealed the significance of melatonin in metabolic functions. Variants in the gene encoding melatonin receptors (1B) are associated with increased fasting plasma glucose, poor early insulin response, increased insulin concentrations and impaired β-cell function (Prokopenko et al., 2009; Staiger et al., 2008; Feero, Guttmacher, & McCarthy, 2010; Lyssenko et al., 2009).

Melatonin also confers protective effects against metabolic disorders. It was found that when melatonin was administered through drinking water in middle-aged rats, it decreased glucose, leptin and visceral fat mass (Rasmussen, Boldt, Wilkinson, Yellon, & Matsumoto, 1999). In a model of diet-induced obesity, mice treated with exogenous melatonin during the dark phase over 3-8 weeks showed a decrease in weight gain accompanied by decreases in glucose, leptin and triglycerides (Prunet-Marcassus et al., 2003). Additionally, there was a significant improvement in insulin sensitivity and glucose tolerance (Sartori et al., 2009).

Given all this evidence, there is a high likelihood that melatonin is involved in the pathogenesis of metabolic disorders like diabetes. The disruption or attenuation of melatonin can increase the risk for diabetes. In this study, we hypothesize that melatonin onset and melatonin secretion, markers of chronotype will be associated with insulin resistance in a cohort of healthy people without diabetes.

**b) RESULTS**

From 978 interested participants who went through an initial telephone screen followed by a laboratory screening, only 44 were enrolled in the study (Figure 2.). The salivary melatonin data in 29 participants was of acceptable quality. The main reasons for exclusion of salivary data were light-induced suppression and poor subject adherence to the instructions for sample collection detected by multiple measures of compliance (Burgess et al., 2015). Urine was collected only in a subset of the study population (N=18). Since the number of participants with both the urine and saliva data was limited (N=8), the analyses were performed separately in each subset.

The descriptive statistics for the subjects in the total population as well as those in each subset are listed in Table 2. The mean DLMO onset time based on the estimated threshold (DLMO-3k) was 20:52 ± 1:28 (N=29) and standard threshold (DLMO-3pg) was 21:32 ± 1:19 (N=27). In two participants the melatonin levels never reached the standard threshold of 3pg/mL. The average time interval between sleep start and DLMO-3k was 3:26 ± 1:2 (N=27), and between sleep start and DLMO-3pg was 2:38 ± 1:10 (N=25). Regression analysis between the DLMO variables and insulin sensitivity revealed significant associations (Table 3). DLMO-3k was negatively associated with Matsuda index of insulin sensitivity at 180 and 300 minutes. It was also positively correlated with HOMA-IR (an index of insulin resistance). These associations remained significant even after adjusting for potential confounders including age, sex, race and BMI. However, neither sleep onset time nor sleep duration or DLMO-sleep intervals showed any statistical significance.

The urinary melatonin was analyzed in the subset of 18 participants. The descriptive statistics for this group is listed in Table 2. The mean urinary aMT6s:Cr ratio was 18.22 ± 12.27 (N=18). Linear regression analysis between aMT6s:Cr ratio and glucose variables is listed in Table 4. Urinary aMT6s:Cr ratio was positively associated with markers of insulin sensitivity even after adjusting for potential confounders including age, sex, race and BMI. Furthermore, the models were unaffected when they were adjusted for sleep duration.

Melatonin is a known anti-inflammatory hormone. Since nocturnal melatonin was associated with insulin sensitivity, we checked if CRP, a marker of chronic inflammation was also associated with insulin sensitivity in this population. CRP was significantly associated with insulin sensitivity (MI-180: $\beta = -1.16$ [95% CI -2.19 to -0.14], $P = 0.027$) in the total study population (N=44). However the significance was moderate when this association was tested for

the urinary melatonin subset (MI-180: β = -1.07 [95% CI -2.37 to 0.22], *P* = 0.098, N=18). This could be due to small sample size, as the effect size remained similar. However, there was no significant association between urinary melatonin and CRP in this subset.

Insulin sensitivity exhibits diurnal variations that decrease during sleep (Van Cauter et al., 1991). Since, the timing of the morning OGTT in the study protocol was fixed in all individuals, we performed sensitivity analyses that excluded those who started their laboratory OGTT more than 30 minutes before their habitual sleep end time deduced from actigraphy. The entire data was reanalyzed and the significance of the associations remained unchanged.

## c) DISCUSSION

In this study, we obtained a reliable marker of circadian phase, the dim light melatonin onset (DLMO) in the home environment and urinary nocturnal melatonin data in adults between the ages of 21 and 50 years who do not have diabetes. This cohort was carefully chosen to exclude those who performed shiftwork and had other chronic illness, with the objective of deducing the role of chronotype in diabetes risk for daytime workers. With these data, a couple of key findings emerged. First, we found an independent inverse association between chronotype measured by dim light melatonin onset (DLMO) and insulin sensitivity. To our knowledge, this is the first report of an association between the objective measures of circadian phase and insulin sensitivity. Secondly, nocturnal urinary melatonin was positively and negatively associated with markers of insulin sensitivity (Matsuda index, QuickI) and insulin resistance (HOMA-IR), respectively. Finally, these associations persisted even after adjusting for confounders that included age, sex, race and BMI.

The determinants and outcomes of chronotype represent a complex interplay between circadian physiology, genetics, and behavior in the context of an individual's social and biological clocks. Inter-individual differences of melatonin onset and nocturnal melatonin secretion in our participants were associated with insulin sensitivity, suggesting the role of chronic mild circadian misalignment. Those with early circadian phase or early melatonin onset are grouped as individuals with preference for "morningness" (early chronotypes) and initiate their sleep phase before midnight (Roenneberg, 2012). On the other end of the spectrum, are those that have later melatonin onset and initiate sleep after midnight; referred to as individuals with preference for "eveningness" (later chronotypes). The rest of the population falls in between these two extremes. Studies have shown that later chronotypes have detrimental health, possibly due to circadian misalignment between their endogenous and social timing systems. For instance, a later chronotype individual will fall asleep later in the night due to their endogenous phase, but will wake up much earlier to begin their day due to social constraints like leaving to work or school. Later chronotypes have higher prevalence of short sleep duration, social jetlag (difference in sleep timing between working days and free days) that could possibly lead to misalignment (Vetter et al., 2015; Wittmann et al., 2006). Unlike previous studies that evaluated chronotype based on questionnaires, we utilized an objective marker of chronotype to evaluate its relationship with insulin sensitivity (Kantermann, Sung, & Burgess, 2015).

It is well established that short sleep duration (< 6 hours) leads to increased metabolic risk. Several mechanisms have been touted to contribute to this detrimental effect. Some of the significant ones include, changes in rhythms of hormones such as insulin, cortisol, leptin and adiponectin, activation of stress signaling through hypothalamus-pituitary-adrenal axis and activation of pro-inflammatory pathways leading to chronic inflammation. Through these

mechanisms short sleep duration alters glucose metabolism and causes insulin resistance (Rangaraj & Knutson, 2016). In our cohort, the mean sleep duration was about 6.6 hours. Since this is a representation of habitual sleep duration, we next checked if there was presence of chronic low-grade inflammation using the plasma marker: c reactive protein (CRP). We observed a significant inverse association between CRP and insulin sensitivity supporting the possibility of chronic low-grade inflammation co-existing with lower nocturnal melatonin levels. The association of CRP with diabetes risk has been established (Pradhan, Manson, Rifai, Buring, & Ridker, 2001). However its association with nocturnal melatonin wasn't significant in our cohort likely due to limited sample size. If low nocturnal melatonin mediates the effect of chronic inflammation on diabetes, then future interventional therapies can focus on improving melatonin secretion or substituting exogenous melatonin.

Our results of lowered nocturnal urinary melatonin associated with higher insulin resistance, is consistent with other similar studies. In a one study that compared nocturnal plasma melatonin levels in 6 people with diabetes to 5 controls, the melatonin levels were significantly decreased (McMullan, Schernhammer, Rimm, Hu, Forman, et al., 2013). In another study that compared nocturnal plasma melatonin between the diabetic group (n=30) and control (n=26), the nocturnal melatonin was significantly lowered in the population with diabetes (Hikichi, Tateda, & Miura, 2011). Consistent with these findings, two cross-sectional studies depicted the negative correlation between metabolic syndrome and plasma melatonin (Robeva, Kirilov, Tomova, & Kumanov, 2006; Robeva, Kirilov, Tomova, & Kumanov, 2008). All these studies involved cohorts that had diabetes or metabolic syndrome. Two studies evaluated the urinary melatonin metabolite levels to diabetes risk in the Nurses' Health Study cohort. One was a prospective study that evaluated the levels of melatonin metabolites and incidence of diabetes concluded that

the estimated diabetes incidence rate for the lower category of melatonin (aMT6s:cr ratio) were of 9.27 cases/1000 person-years and twice that of the highest category (McMullan, Schernhammer, Rimm, Hu, & Forman, 2013). The second study concluded that the nocturnal melatonin secretion is independently and inversely associated with insulin resistance. They reported the odds ratio for insulin resistance to be 0.45 among women in the highest quartile of urinary aMT6s:cr ratio compared with women in the lowest quartile (McMullan, Curhan, Schernhammer, & Forman, 2013). In other words, higher the nocturnal melatonin, the lower the risk for diabetes. However both these studies were based on the Nurses' Health cohort, which included women who had prior exposure to shiftwork. This could have independently increased the risk for diabetes. In our study we showed that nocturnal melatonin decreased with increase in insulin resistance, under dim light conditions in a healthy cohort without diabetes or prior exposure to shiftwork, suggesting a possibility of milder circadian disruption driven by mistiming between circadian and behavioral rhythms. Thus, future studies, should evaluate nocturnal melatonin as a marker of less severe circadian disruption in daytime workers (e.g. social jetlag).

Plasma melatonin between obese individuals with and without diabetes revealed lower melatonin levels in the group with diabetes, under controlled laboratory conditions of sleep, light and feeding cycles (Mäntele et al., 2012). However, there wasn't any difference in melatonin onset, suggesting that melatonin onset marker is independent of existing diabetes and obesity. Hence the associations between DLMO and insulin sensitivity that we observe in our study could be the result of chronotype altering insulin sensitivity through circadian misalignment.

Besides this, melatonin is known to confer protective effect in animal models of obesity and diabetes. These suggest that melatonin may have a chronobiological and therapeutic effect against insulin resistance.

It is important to acknowledge the limitations of the analysis. The primary limitation is the cross-sectional design because it limits the determination of causality. Secondly, we were limited by the small sample size with fewer people having both melatonin onset and urinary melatonin data. In the future increasing the sample size of individuals with both melatonin onset and urinary melatonin data, will help analyze the relationship between these circadian markers and insulin sensitivity. One of the primary strengths of the study was obtaining home –DLMO data to access circadian phase and evaluate its relationship with insulin sensitivity. The dim light below 100 lux was maintained (measured by a light sensor) through the course of salivary and urinary melatonin collection. Since light is a potent melatonin suppressor, this ensured the reliability of the data compared to previous studies.

# CHAPTER 4

## ASSOCIATION BETWEEN DIETARY CHRONOTYPE AND INSULIN SENSITIVITY

### a) INTRODUCTION

Insulin resistance is a central aspect in the etiology of type 2 diabetes and a strong predictor of future development of diabetes (Lorenzo et al., 2010). Major causes of insulin resistance include excess weight and physical inactivity (Balkau et al., 2008; Preis et al., 2010), however, identifying novel predictors of insulin resistance may help better understand diabetes etiology and ultimately reduce risk of the disease. One such novel predictor may be the timing of food intake. Although total energy intake and macronutrient composition have been extensively studied in relation to metabolic health, the distribution of energy intake across the day may also be important because feeding is a major synchronizer of the circadian clocks in peripheral organs and tissues (Damiola et al., 2000). Therefore, the timing of feeding may disrupt circadian physiology and impair glucose metabolism.

Circadian disruption induced in controlled laboratory studies in healthy volunteers resulted in impaired glucose tolerance and increased insulin resistance (Buxton et al., 2012; Leproult et al., 2014). In these experiments, feeding at the “wrong" time was likely a key-contributing factor because the feeding opposed the central circadian signal of food anticipation. These findings are supported by animal experiments in which mice fed a high fat diet only during their inactive period (the "wrong" time) gained significantly more weight than controls although both groups of mice consumed equal amount of calories (Arble, Bass, Laposky, Vitaterna, & Turek, 2009). Further, in a randomized crossover study of 6 individuals, consuming 60% of their daily calories in the evening lead to lower insulin sensitivity than when they consumed 60% calories in the morning (Morgan LM, 2011.). This combined evidence clearly

suggests that consuming calories during an inappropriate circadian time can lead to adverse metabolic dysfunction. Whether or not the timing of food intake in habitual settings is also associated with metabolic impairments is not known. Thus, the aims of this study were to examine the association between meal timing and insulin sensitivity in a group of free-living individuals without diabetes.

## b) RESULTS

The study enrolled 44 participants of whom 41 had completed both food dairy and oral glucose tolerance test. They were screened from 978 interested participants through an initial telephone screen followed by a laboratory screening (Figure 3). The participants were adults with mean age 29.6 ± 8.1 years, BMI 26.8 ± 5.3 kg/$m^2$ and average sleep duration of 401.5 ± 34.5 minutes measured by actigraphy (Table 5). However, sleep duration data was not available in three participants due to instrument failure. The average daily calorie intake for the participants was 2512.6 ± 731.5 Kcal. The calories were distributed across the day and documented as eating events: a time-stamp associated with calorie consumption (>5 kCal) through wakefulness and at least 15 minutes apart from the previous eating event. Only 17% (N=7) of the participants had 3 eating events or less each day, while the remaining participants on average had 5 eating events each day. When the eating events (main meals and snacks) were grouped into three categories: morning, afternoon and evening (as indicated by the participant), the proportion of morning calories consumed was 23.8 ± 12.3 %, whereas the proportion of evening calories was 36.0 ± 11.4%. A similar pattern for the proportions of macronutrients was also observed (Table 6).

Relationship Between Caloric Distribution And Insulin Sensitivity

To determine calorie distribution patterns, the caloric intake data was analyzed as 1. Dietary proportion variables and 2. Dietary timing variables. The participants were grouped into four groups based on their mean morning and evening caloric proportions (Figure 4). One-way ANOVA analysis showed a significant difference in the insulin sensitivity quantified by Matsuda index-180 [F (1,37) =4.42, $P$<0.01] and QUICKI [F (1,37) =3.61, $P$=0.02] between the groups. Post-hoc comparisons revealed that participants with greater than average morning proportion (24%) and lesser than average evening proportion (36%) had a significantly higher insulin sensitivity (Matsuda index-180 mean difference = -13.77, $P$<=0.01 and QUICKI mean difference =- 0.03, $P$=0.015) than those with lesser than average morning and evening proportions. Regression analysis of the proportion groups revealed a significant association between participants with greater than average morning proportion and lesser than average evening proportion and insulin sensitivity (MI-180: β = -13.77 [95% CI -21.46 to -6.08], $P$ < 0.01, and QUICKI: β = -0.03 [95% CI -0.05 to -0.01], $P$ < 0.01). After adjustments for multiple covariates (age, sex, BMI, race, daily calories and sleep midpoint) the association between insulin sensitivity and the proportion groups with greater than average morning proportion and lesser than average evening proportion (MI-180: β = -12.8 [95% CI -22.5 to -3.1], $P$ = 0.01, and QUICKI: β = -0.03 [95% CI -0.05 to -0.004], $P$ = 0.02), were largely unchanged.

Additionally the regression analysis of ungrouped dietary proportion variables (Table 7) showed the proportion of morning calories was significantly associated with insulin sensitivity and this association remained significant even after adjusting for covariates including age, sex, BMI, race and daily calories. When sleep midpoint was included in the model, the strength of the association persisted. Similarly, macronutrient analysis revealed that the proportion of

carbohydrates consumed in the morning meal had the most significant association with insulin sensitivity (Table 7), which remained unchanged in the multicovariate model.

When the dietary timing variables were analyzed by Pearson's correlation analysis, TCI 25% had the strongest and most significant correlation with both indices of insulin sensitivity (Figure 5). Further these associations were examined in detail using linear regression models adjusting for key covariates. Table 8 shows TCI 25% to be most significantly associated with insulin sensitivity (MI-180: β = -1.73 [95% CI -3.18 to -0.28], $P = 0.02$ and QUICKI: β = -0.004 [95% CI -0.008 to -0.001], $P = 0.015$). This association persisted after adjusting for multiple covariates (MI-180: β = -1.66 [95% CI -3.11 to -0.21], $P = 0.026$ and QUICKI: β = -0.005 [95% CI -0.008 to -0.001], $P < 0.01$). Consuming 25% of the daily calorie intake earlier in the day was consistently significant with higher insulin sensitivity in the unadjusted and adjusted models.

Two measure of sleep were used in the models as covariates: sleep midpoint, an indirect measure of circadian chronotype(r = - 0.73) (Zavada, Gordijn, Beersma, Daan, & Roenneberg, 2005) and sleep duration, an independent risk factor for diabetes (Rangaraj & Knutson, 2016). Since participants with early chronotypes tend to wake up early and are likely to eat sooner than late chronotypes, adjusting for chronotype negated any bias in the associations between dietary timing and insulin sensitivity. In the multivariable regression models when midpoint of sleep was replaced by sleep duration the significance of the associations persisted. Moreover, the glucose tolerance test was terminated prematurely for 2 participants due to hypoglycemia. However, the results after excluding the 2 participants remained unchanged.

Insulin sensitivity exhibits diurnal variations that decrease during sleep (Van Cauter et al., 1991). Since, the timing of the morning OGTT in the study protocol was fixed in all individuals, we performed additional sensitivity analyses that excluded those who started their

laboratory OGTT more than 30 minutes before their habitual sleep end time deduced from actigraphy. The entire data was reanalyzed and the significance of the associations remained unchanged.

## c) DISCUSSION

The present study illustrates that the distribution of calories towards the earlier part of the biological day is positively associated with increased insulin sensitivity after controlling for confounders such as age, race, sex, BMI, average 24-hour calories and chronotype. In other words, the higher the calories consumed by lunch, the greater the insulin sensitivity. Moreover, we defined a new metric to map temporal caloric distribution and based on this metric the sooner one consumes 25% of their daily caloric consumption, the higher the insulin sensitivity even after adjusting for sleep and 24-hour total caloric intake. These findings are consistent with literature reporting the association between breakfast skipping and increased insulin resistance (Farshchi, Taylor, & Macdonald, 2005; Smith et al., 2010) and type 2 diabetes (Mekary et al., 2013; Mekary, Giovannucci, Willett, Dam, & Hu, 2012). For example, in a randomized crossover study of 8 young men an increase in 24-hour glucose levels without changes in energy expenditure was observed following breakfast skipping when compared with breakfast consumption (Kobayashi et al., 2014). Despite all these reports, there are some inconsistencies in evidence that adults who skip breakfast are at increased risk for diabetes. These are primarily due to the absence of a standard definition of breakfast in the literature. Studies often include questionnaires that access meal consumption frequency to classify a participant as breakfast skipper (consumes breakfast 3 or less days a week) or not (consumes breakfast 5 or more days a week) (Odegaard et al., 2013; Thomas, Higgins, Bessesen, McNair, & Cornier, 2015). In others,

definition of breakfast included the first meal of the day or meal consumed within 2 hours of waking or calories consumed anywhere between 5 am to 10 am (Asao, Marekani, VanCleave, & Rothberg, 2016; Timlin & Pereira, 2007). Furthermore, these studies do not control for sleep duration or chronotype. Sleep duration is an important factor in timing of the first meal. For example, an individual who wakes up at 10 am will eat his first meal for the day after 10 m. So by definition this individual will be a breakfast skipper when in reality he could consume the first meal at 11 am or within an hour of his wake time. Collectively these discrepancies could result in differences in the degree of association between diabetes risk and breakfast skipping. To minimize the discrepancies in the definition of the morning calories consumed, and develop a holistic measure that captures the times calories are consumed, we defined 25%, 50%, 75% TCI metric or the time taken to consume 25%, 50% or 75% of daily caloric intake, respectively. This is an unbiased measure of temporal and quantitative calorie distribution because it is independent of frequency of eating events and subjectivity of participants to classify an eating event as a meal or a snack. Our results indicate that 25% TCI is the strongest predictor of insulin sensitivity and the earlier the consumption of 25% of daily calories relative to sleep time the higher the insulin sensitivity and lower the diabetes risk among individuals who have normal glycaemia.

TCI 50% was moderately associated with insulin sensitivity with the average clock time around 15:00. This data aligns with results from a previous study on weight loss, where participants who ate their main meal before 15:00 displayed significantly more weight loss than those who consumed their main meal later in the day, despite no differences in dietary composition, calories consumed, energy expenditure, appetite hormones and sleep duration between the two groups (M Garaulet et al., 2013). In general, those who consumed their meals

later had the tendency to skip breakfast or eat low calorie breakfast more frequently and also eat late night meals, each of which has a negative impact on metabolic homeostasis.

The effect of temporal distribution of calories on metabolism is best explained by circadian misalignment. The central circadian clock in the SCN controls sleeping and feeding behavior. Through its control of metabolism and feeding, it regulates energy balance. It is known that glucose metabolism exhibits clear diurnal variations with higher glucose tolerance and insulin sensitivity during the early part of the biological day that gradually tapers to reach its nadir during the biological night (Van Cauter et al., 1991). The plasma glucose exhibits a diurnal rhythm, independent of its acute rise following a meal. On the other hand, feeding also has a profound effect on glucose metabolism. But this effect is differential and dependent on the time of day. This is evident from the few controlled human studies that showed the effect of caloric load in the morning versus the evening (Jakubowicz, Barnea, Wainstein, & Froy, 2013; Morgan, Shi, Hampton, & Frost, 2012). In a randomized study of overweight and obese women who underwent 12 weeks of caloric restricted diet where one group ate 50% of their daily calories in the morning while the other group ate 50% of their calories in the evening (Jakubowicz, Barnea, Wainstein, & Froy, 2013). The morning group had higher insulin sensitivity than the evening group with a parallel decrease in 24-hour glucose, insulin and ghrelin. Decrease in satiety scores was also observed in the morning group. This was also observed in another randomized crossover study of patients with type 2 diabetes, where consuming the daily calories earlier in the day than later in the evening, improved fasting glucose and insulin levels (Kahleova et al., 2014). Changes in appetite and appetite regulating hormones like leptin and ghrelin could mediate the association between dietary timing and glucose metabolism (Thomas et al., 2015). Leptin secreted by the white adipose tissue relative to adiposity, exhibits diurnal rhythms with peaks

during the biological night (Radić et al., 2003). Leptin is a reflection of the energy needs of the body, with energy restriction as seen in fasting lowering its circulating levels and energy surplus increasing its circulating levels. A large cohort study showed an association of higher leptin among those who skip breakfast after adjusting of several potential confounders including age BMI, diabetes, smoking status (Asao et al., 2016). But this was not replicated by a controlled study limited by its sample size (Reeves et al., 2015). However, in another randomized crossover study, breakfast omission in obese individuals compared to those who consumed a carbohydrate rich breakfast had lower leptin levels post an ad libitum lunch (Chowdhury, Richardson, Tsintzas, Thompson, & Betts, 2016). It is unclear if the effect was solely based on breakfast consumption or a combined effect of carbohydrate-rich breakfast. Data from our study shows that consuming carbohydrates earlier in the day was associated with higher insulin sensitivity. This could be due to its effect on appetite regulation later in the day.

Timing of eating has emerged as a dietary behavior with pronounced effect on metabolic health. Mechanistic evidence comes from animal studies of time-restricted feeding. One study showed that when feeding is restricted to the nocturnal phase (biological day or active phase) in diet-induced obese rodents, several key regulators of metabolic pathways such as AMPK, CREB, mTOR, along with core circadian clock gene oscillations dampened by obesity were restored (Hatori et al., 2012). Additionally, when mice fed high fat diet (HFD) ad libitum were compared with those that were on time-restricted feeding (TRF), the TRF mice gained significantly less weight even though both groups consumed the same amount of calories. The TRF mice also had low adiposity, improved glucose and lipid metabolism (Sherman et al., 2012). This phenomenon of regulating the timing of food to protect against metabolic disorders was later studied in other types of nutritional challenges like high sucrose and high fructose diets in mice. The protective

effects of TRF persisted irrespective of the type of nutritional challenge (Chaix, Zarrinpar, Miu, & Panda, 2014). It is important to remember that TRF aligned during the active phase or biological day rather than inactive phase is beneficial. As one study in mice noted that restricted feeding during the inactive phase induced weight gain when compared to mice fed during the active phase, even though both groups consumed same amount of calories (Arble et al., 2009).

The 2015 Dietary Guidelines for Americans (DGA) committee concluded that an overall healthy eating pattern is necessary to maintain good health and reduce chronic disease risk (U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015). The previous editions of the dietary guidelines focused primarily on individual dietary components such as food groups and nutrients (U.S. Department of Services and U.S. Department of Health and Human Agriculture, 2010). However, growing understanding and assessment of eating behaviors of people led to the conclusion that the food groups and nutrients are consumed together and not in isolation, and this together forms an overall food consumption or dietary pattern. The components of the dietary pattern can have a potentially cumulative effect on health. It is therefore necessary to identify the components of a healthy dietary pattern. Some of these components include quantity, quality and more recently timing of meals. In our study we show that consuming 25% of your calories earlier in the day relates to higher insulin sensitivity irrespective of the total calories consumed. A similar pattern was also established for carbohydrates consumed. There is overwhelming data on the health benefits of nutritional eating and reduced caloric intake. However, there is lack of guidance on recommended distribution of calories across a day. It is therefore necessary to examine eating behaviors beyond overall frequency meals and snacks, to provide information about distribution of calories throughout the day. Such information is necessary to understand which individual eating periods are integral to

healthy eating and reduction of chronic disease risk. This will also help identify those with poor eating patterns and selectively recommend behavioral changes in these individuals to improve their metabolism.

It is important to acknowledge the limitations of the analysis. The primary limitation is the cross-sectional design because it limits the determination of causality. Secondly, use of food records may introduce some bias due to underreporting, as it has with other dietary assessment methods in free-living humans. However, data suggesting underreporting has influence on meal timing is unknown. Our study has several strengths. One was the use of dietary timing variables that was independent of subjective definition of meals in the food records. Moreover, we used a rigorous protocol for habitual dietary assessments and insulin sensitivity estimations. Finally, we also adjusted for chronotype while evaluating the dietary associations for diabetes risk.

# CHAPTER 5

# CONCLUSION

## a) OVERVIEW OF FINDINGS

We demonstrated that early chronotype and larger percentage of daily calories consumed before noon were both associated with insulin sensitivity in people without type 2 diabetes. Moreover nocturnal melatonin secretion was also associated with diabetes risk, with lower nocturnal secretion associated with increased insulin resistance. These results support a role of circadian regulation in glucose metabolism in healthy free-living individuals.

Our results add to the ongoing discussions about the role of chronotype in diabetes risk. Controlled laboratory studies of circadian desynchrony demonstrated that circadian misalignment due to mistiming of sleep and feeding relative to the endogenous rhythm worsens glucose tolerance (Buxton et al., 2012). Late chronotypes have poorer health outcomes than early chronotypes, likely due to shorter sleep durations leading to adverse metabolic outcomes (Merikanto et al., 2013; Yu et al., 2015). The conflict between timing of endogenous preferences for sleeping and eating and social demands, causes circadian misalignment and in the long-term increases the risk for metabolic diseases. Besides chronotype, nocturnal melatonin was also independently associated with insulin sensitivity. Although the effects of sleep-wake timing on metabolism has been extensively studied, the extent to which dietary chronotype contributes to circadian misalignment is not clearly understood. More studies are needed to define the relationship between temporal eating and diabetes risk in humans.

## b) FUTURE DIRECTIONS

This study opens the possibility of conducting several interesting future projects. One that particularly stands out to me is the role of dietary chronotype in glucose metabolism. Although our study established an association between timing of food intake and glucose metabolism, several questions remain unanswered. Some of them include: Whether timed feeding synchronizes the circadian rhythms of peripheral metabolic tissues in humans? If so, what is the best feeding regimen to improve glucose metabolism? What are the possible mechanisms by which mistimed feeding affects glucose metabolism? To this end, controlled human experiments are needed to address these questions, which will be discussed below.

The effect of timed feeding on peripheral circadian clocks has only been demonstrated in mice. When mice fed *ad libitum* were switched to timed feeding regimen (restricted to either the active or inactive phase), the circadian oscillations of the peripheral metabolic tissues like liver, pancreas and adipose synchronized to the new schedule within a week (Stokkan, Yamazaki, Tei, Sakaki, & Menaker, 2001). This experiment modulated the eating interval and not the distribution of calories within the interval. It remains unclear if consistency of meal timing and/or the temporal distribution of calories through the day have a positive effect on peripheral circadian oscillations. In humans, the accessible metabolic tissue is the adipose tissue that has been previously shown to exhibit peripheral circadian oscillations (Gómez-Santos et al., 2009). Thus, designing a clinical study that includes 4 weeks of temporal feeding with subcutaneous adipose needle biopsies taken at the beginning and end of the intervention, would help determine if timed feeding can synchronize peripheral metabolic clocks in humans by comparing the core clock and metabolic gene expressions in the adipose tissue post-intervention with the baseline. Animal studies have shown that the liver is one of the first organs to get synchronized to timed

feeding, hence synchronization of subcutaneous adipose tissue in humans could be extrapolated as synchronization of circadian rhythms in all metabolic tissues.

The next step is deciphering the ideal feeding pattern for improving insulin sensitivity. Although literature suggests eating breakfast and lowering calories late at night is better, there are many inconsistencies in the studies conducted so far (discussed in Chapter 4). We need a comprehensive analysis of temporal feeding patterns and their effect on glucose metabolism. We have previously discussed that eating in the "wrong" time of day can lead to circadian misalignment. Hence a thorough analysis of calorie distributions relative to endogenous circadian phase is necessary. Melatonin is a robust endogenous marker of circadian timing in humans, which increases during the inactive phase (biological night). Acute melatonin rise that occurs on consuming an exogenous immediate release melatonin induces increased glucose tolerance (Rubio-Sastre, Scheer, Gómez-Abellán, Madrid, & Garaulet, 2014). In diurnal mammals like humans, glucose tolerance increases during the "biological" night parallel to the melatonin increase. Putting these two together, suggests that the melatonin rhythm maybe contributing to the increase in glucose intolerance during the night (fasting period). However, the opposite is true for nocturnal animals like rodents where the melatonin rise occurs during the metabolically active period. Thereby the role of melatonin and metabolism is likely different between diurnal and nocturnal mammals. Hence results from studies focusing on circadian rhythms and metabolism illustrated in nocturnal animals need to carefully examined and if possible repeated in humans. Given this data we could compare the impact of calorie consumption relative to melatonin rise. To do so, we can conduct randomized trials where one group is assigned their evening calorie meal 2 hours after their melatonin rise and the other assigned their evening meal 2 hours before their melatonin rise. To determine the melatonin rise,

we can use the protocol described in Chapter 2. Based on their endogenous melatonin rise, their daily meals can be scheduled. After 4 weeks of timed meals either before or after the melatonin rise, the glucose tolerance between groups can be compared. The data from this study will illustrate whether timing meals based on endogenous clock is critical. Furthermore, this will also help inform people who consume exogenous melatonin for jetlag that the timing of melatonin consumption and meal needs to be appropriately spaced to ensure it does not affect glucose metabolism. Similarly the time of consumption of morning meal in relation to melatonin fall needs to be analyzed.

To determine the mechanism through which timed feeding alters glucose metabolism, studying the role of feeding patterns on appetite regulating hormones in circulation and in the adipose tissue will provided useful information. Also, the effect feeding patterns have on the central clock outputs like temperature and glucocorticoid signaling also remains unclear. Moreover, it is not known which aspect: sleep/wakefulness or meal timings of circadian misalignment is most important. For example, does eating at a wrong circadian time have more adverse consequences than being awake at an unusual time? The results of such translational studies will open up the possibility of using timed dietary manipulations as an interventional therapy to help minimize or reverse the adverse effects of circadian desynchrony. Given the large number of individuals with disrupted rhythms due to shift work, social jet lag, such dietary manipulations could provide improvements in metabolic health.

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## APPENDIX A. FIGURES

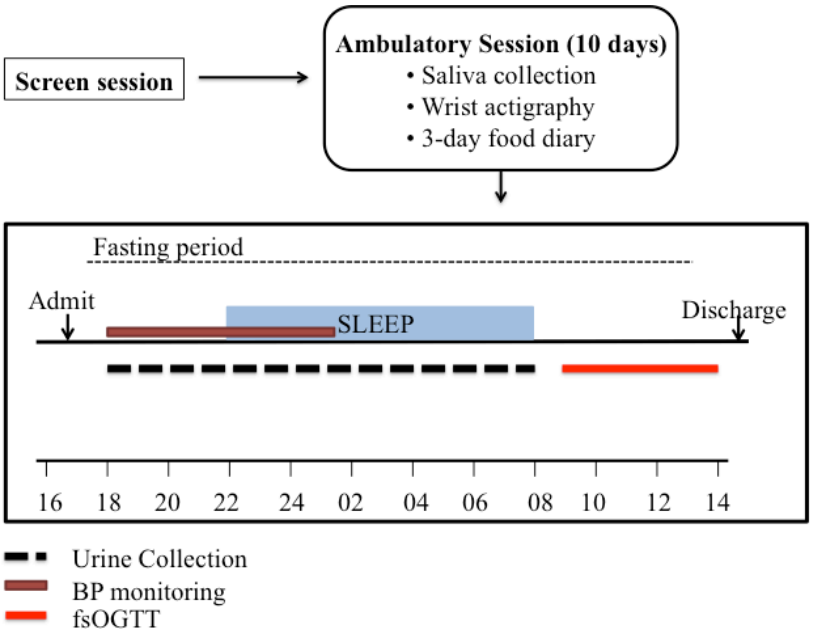


**Figure 1. Study Protocol**

Participants enrolled in the study begin with the 10-day ambulatory session that encompasses saliva collection to determine chronotype, actigraphy to determine habitual sleep patterns and food diary to determine dietary patterns. Within a week of completing the ambulatory session, they are enrolled in a 21-hour long laboratory session. This includes measurement of blood pressure (BP), overnight urine collection and a 5-hour frequently sampled oral glucose tolerance test (fsOGTT) the next morning. Participants are allowed to only consume water after 20:30 to ensure they have a minimum of 12-hour fast before the fsOGTT. They are provided a 10-hour sleep opportunity from 22:00 to 8:00.

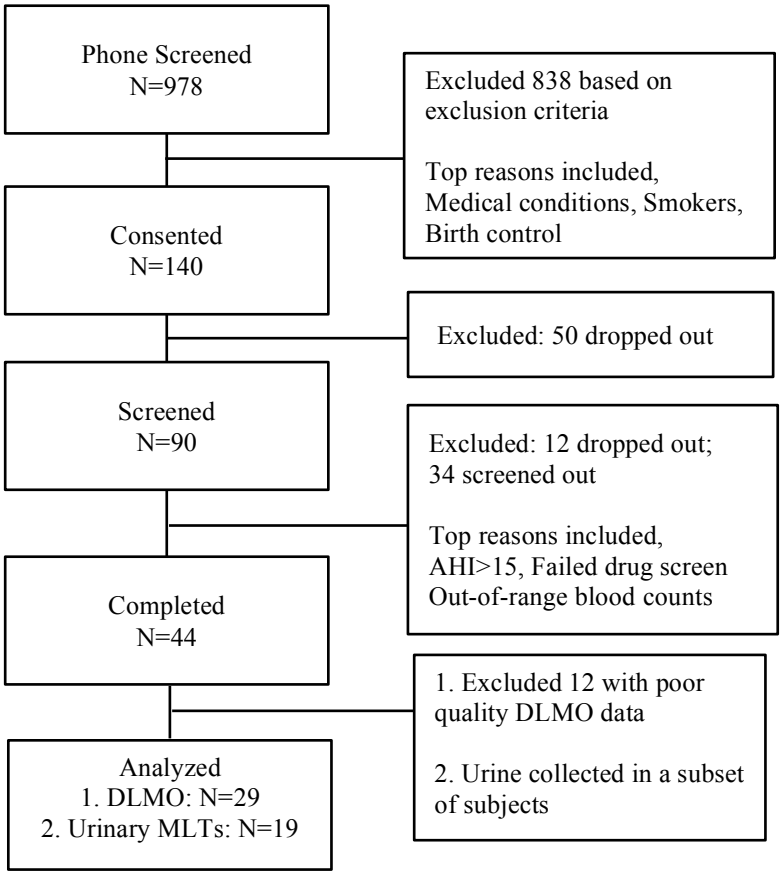


**Figure 2. Consort Diagram for Melatonin and Insulin Sensitivity Analysis**

Illustrating the flow of participants through each stage of the study. A total of 29 participants had both acceptable quality salivary DLMO and insulin sensitivity data and 19 participants out of the 44 had urinary melatonin data.

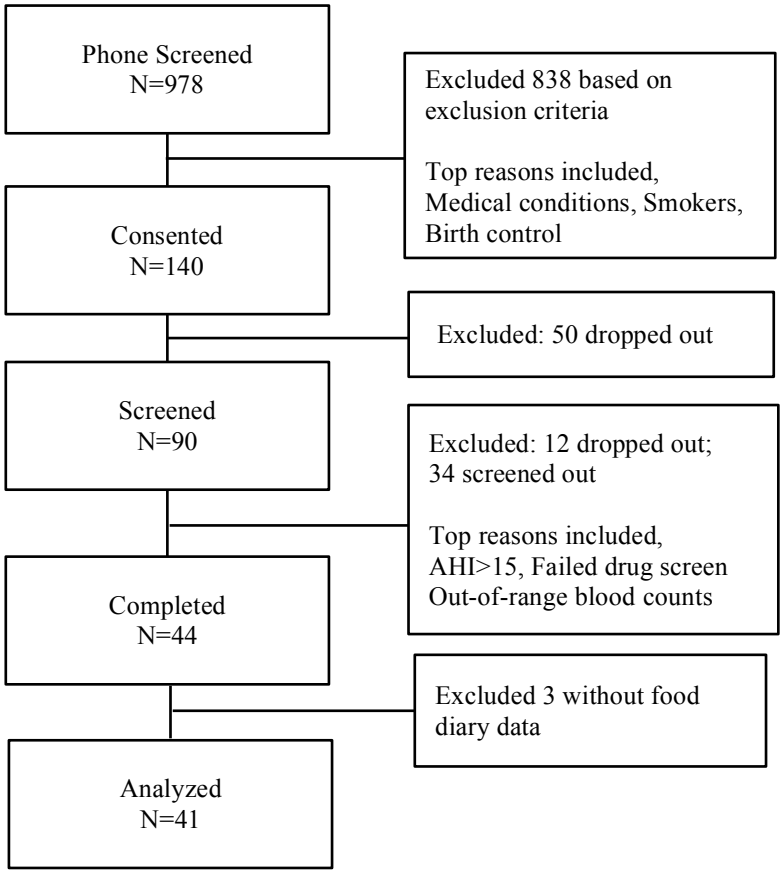


**Figure 3. Consort Diagram for Dietary Chronotype and Insulin Sensitivity Analysis**

Illustrating the flow of participants through each stage of the study. A total of 41 participants had both food records and glucose tolerance tests for the analysis of dietary behavior and its relationship with insulin sensitivity.

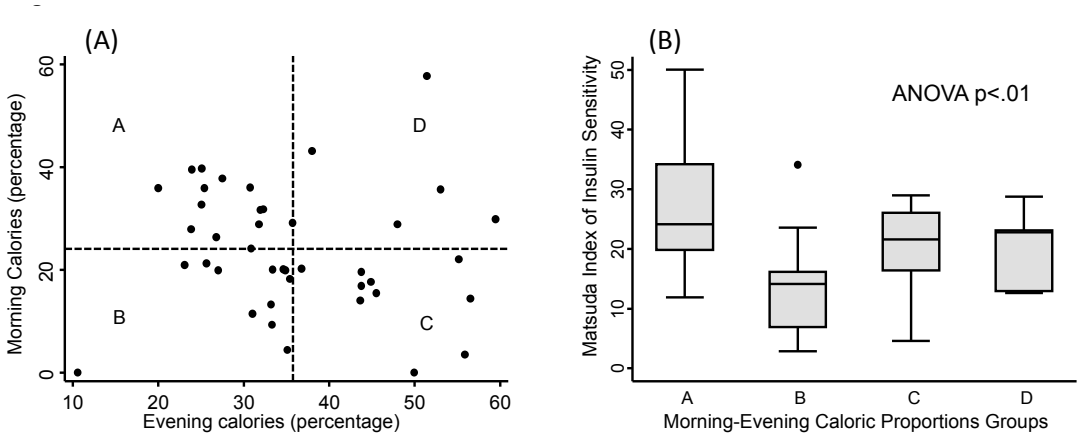


**Figure 4. Comparison of Insulin Sensitivity in Morning-Evening Calorie Distributions**

(A) Participants were classified in to 4 groups based on their average morning (24%) and evening (36%) calorie proportions.
(B) Subsequently the insulin sensitivity between the four groups revealed that participants with greater than average morning proportion and lesser than average evening proportion (group A) had a significantly higher Matsuda index of insulin sensitivity with mean difference of 13.77 between group A and group B

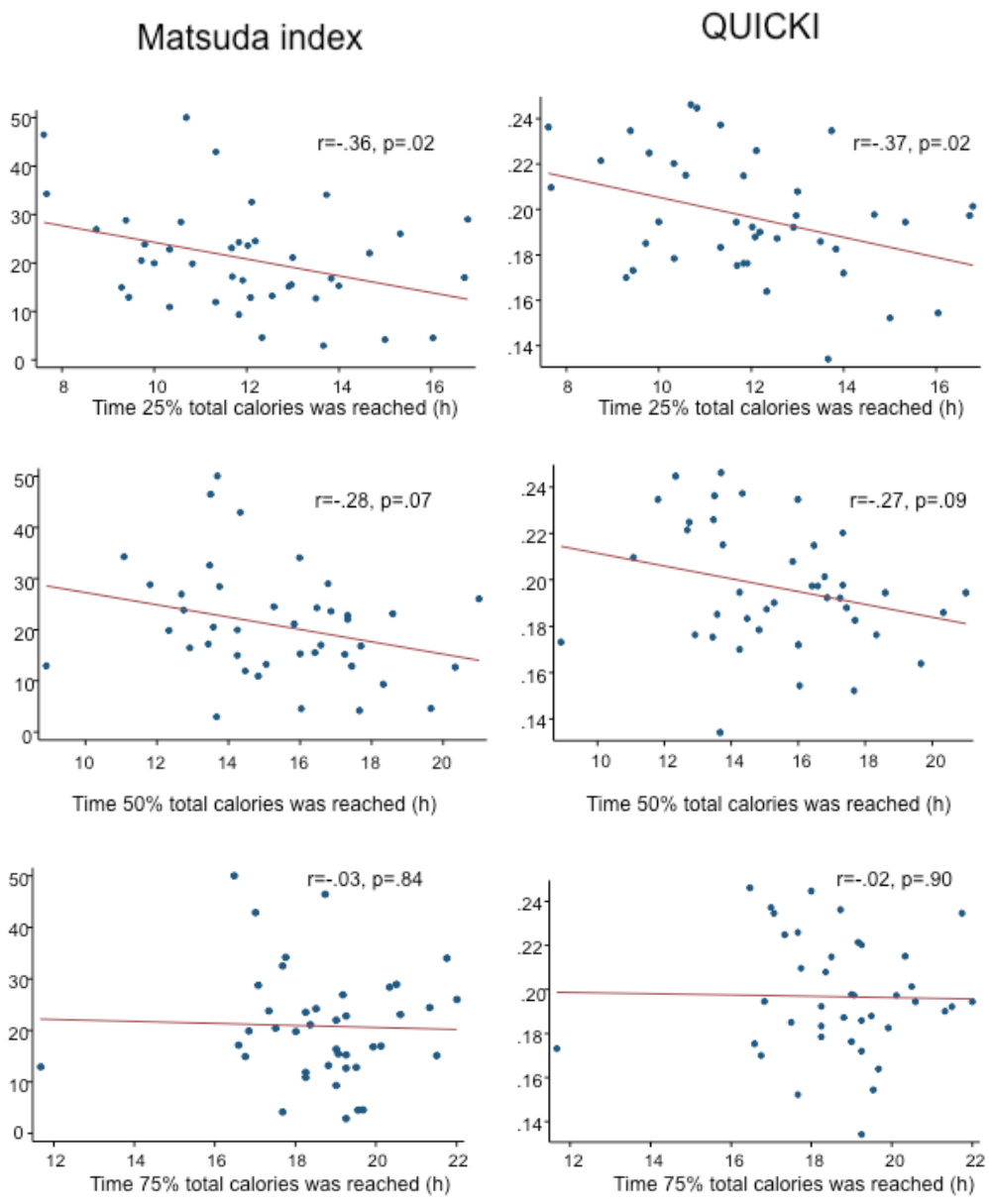


**Figure 5. Pearson's Correlation between Dietary Timing and Insulin Sensitivity**

Correlations were considered to be strongly and moderately significant if $p<0.05$ and $p<0.1$ respectively, where r is the correlation coefficient.

## APPENDIX B. TABLES

| Categories of glucose tolerance | Glycosylated hemoglobin HbA1C (%) | Fasting plasma glucose FPG (mg/dL) | 2-hour postprandial 2hPG (mg/dL) |
|---|---|---|---|
| Normal glucose tolerance | < 5.7 | <110 | <140 |
| Impaired fasting glucose | 5.7-6.4 | 110-125 | <140 |
| Impaired glucose tolerance | 5.7-6.4 | <110 | 140-199 |
| Combined glucose intolerance | 5.7-6.4 | 110-125 | 140-199 |
| Diabetes | ≥ 6.5 | ≥126 | ≥200 |

**Table 1. Classification of Prediabetes and Diabetes**

| Baseline characteristics | Mean ± SD or n (%) | | |
|---|---|---|---|
| | Total Population (N=44) | DLMO dataset (N=29) | Urinary MLTs dataset (N=18) |
| Age (years) | 29.54 ± 8.1 | 28 ± 7.2 | 30 ± 7.7 |
| Sex | | | |
| Male | 21 (51.2%) | 18 (62.1%) | 6 (33.3%) |
| Female | 20 (48.8%) | 11 (37.9%) | 10 (66.7%) |
| Race | | | |
| African-American | 16 (39.0%) | 9 (31%) | 9 (50%) |
| Non-Hispanic White | 25 (61.0%) | 20 (69%) | 9 (50%) |
| BMI ($kg/m^2$) | 26.8 ± 5.3 | 26.8 ± 4.6 | 26.1 ± 5.4 |
| | | | |
| Sleep variables* | | | |
| Sleep start | 00:17 ± 1:16 | 00:19 ± 1:13 | 23:56 ± 1:13 |
| Sleep end | 7:36 ± 1:20 | 7:40 ± 1:26 | 7:23 ± 10 |
| Sleep duration (minutes) | 401.5 ± 34.5 | 399.9 ± 33.2 | 410.2 ± 32.5 |
| | | | |
| OGTT variables | | | |
| Fasting glucose (mmol/L) | 4.8 ± 0.4 | 4.8 ± 0.4 | 4.9 ± 0.2 |
| Fasting Insulin (pmol/L) | 50.8 ± 60.6 | 39.1 ± 26.9 | 49.3 ± 26.7 |
| HOMA-IR | 1.8 ± 2 | 1.4 ± 1 | 1.8 ± 1 |
| HOMA-B | 176.9 ± 321.1 | 165.3 ± 330 | 118.1 ± 54.1 |
| Matsuda Index-180 min | 20.8 ± 10.8 | 23.5 ± 10.5 | 17.5 ± 7.2 |
| Matsuda Index-300 min# | 26.1 ± 14.2 | 29.7 ± 13.9 | 21.4 ± 8.9 |
| QuickI | 0.2 ± 0.03 | 0.2 ± 0.03 | 0.19 ± 0.02 |
| | | | |
| CRP (mg/L) | 2.05 ± 3.02 | 2 ± 3.12 | 1.6 ± 2.63 |

**Table 2. Clinical Characteristics of Study Participants in Melatonin Analysis**

Participant demographics are described, along with the means and standard deviations of their habitual sleep characteristics, and laboratory-tested glucose, insulin and derived insulin sensitivity. Actigraphy data (sleep variables) was collected for 41, 27 and 18 participants in the total population, DLMO dataset and urinary MLTs dataset, respectively. Matsuda index-300 minutes index for insulin sensitivity is available for 41, 28 and 16 participants in the total population, DLMO dataset and urinary MLTs dataset, respectively.

| Dependent Variable | Independent Variable | Unadjusted Model | | Adjusted Model | |
|---|---|---|---|---|---|
| | | β-coefficient (95% CI) | P-value | β-coefficient (95% CI) | P-value |
| Matsuda Index 180 | DLMO-3k (N=29) | -3.05 (-5.6 to -0.49) | 0.021 | -2.83 (-5.39 to -0.27) | 0.032 |
| | DLMO-3pg (N=27) | -3.03 (-5.96 to -0.09) | 0.044 | -2.52 (-5.68 to 0.64) | 0.112 |
| Matsuda Index 300 | DLMO-3k (N=28) | -4.58 (-7.97 to -1.2) | 0.01 | -4.45 (-7.76 to -1.13) | 0.011 |
| | DLMO-3pg (N=26) | -4.74 (-8.66 to -0.81) | 0.02 | -4.36 (-8.61 to -0.11) | 0.045 |
| HOMA-IR | DLMO-3k (N=29) | 0.26 (0.02 to 0.5) | 0.037 | 0.26 (0.04 to 0.49) | 0.023 |
| | DLMO-3pg (N=27) | 0.13 (-0.07 to 0.33) | 0.194 | 0.14 (-0.09 to 0.36) | 0.215 |
| QuickI | DLMO-3k (N=29) | -0.006 (-0.012 to -0.00004) | 0.052 | -0.006 (-0.013 to -0.002) | 0.056 |
| | DLMO-3pg (N=27) | -0.007 (-0.014 to 0.0004) | 0.065 | -0.007 (-0.015 to 0.0007) | 0.072 |

**Table 3. Regression Models for Dim Light Melatonin-Onset (DLMO)**

The linear regression models were considered significant if p-value<0.05
The models were adjusted for age, sex, race and BMI.

| Dependent Variable | Unadjusted Model | | Adjusted Model | |
|---|---|---|---|---|
| | β-coefficient (95% CI) | P-value | β-coefficient (95% CI) | P-value |
| Matsuda Index 180 (N=18) | 0.31 (0.05 to 0.57) | 0.024 | 0.28 (-0.007 to 0.56) | 0.055 |
| Matsuda Index 300 (N=16) | 0.38 (0.02 to 0.74) | 0.039 | 0.35 (-0.06 to 0.77) | 0.086 |
| HOMA-IR (N=18) | -0.04 (-0.08 to -0.005) | 0.027 | -0.02 (-0.047 to 0.004) | 0.096 |
| QuickI (N=18) | 0.001 (0.0005 to 0.002) | 0.001 | 0.0008 (0.0003 to 0.0014) | 0.009 |

**Table 4. Regression Models for Urinary aMT6s:Cr ratio**

The linear regression models were considered strongly significant if p-value<0.05 and moderately significant for p-value <0.1
The model was adjusted for age, sex, race, BMI and sleep duration

| Baseline characteristics | Mean ± SD or n (%) |
|---|---|
| Age (years) | 29.54 ± 8.1 |
| Sex | |
| Male | 21 (51.2%) |
| Female | 20 (48.8%) |
| Race | |
| African-American | 16 (39.0%) |
| Non-Hispanic White | 25 (61.0%) |
| BMI ($kg/m^2$) | 26.8 ± 5.3 |
| | |
| Sleep variables (n=38) | |
| Sleep start | 00:17 ± 1:16 |
| Sleep end | 7:36 ± 1:20 |
| Sleep duration (minutes) | 401.5 ± 34.5 |
| | |
| OGTT variables | |
| Fasting glucose (mmol/L) | 4.8 ± 0.4 |
| Fasting Insulin (pmol/L) | 50.8 ± 60.6 |
| Matsuda Index-180 min | 20.8 ± 10.8 |
| Matsuda Index-300 min (n=39) | 26.1 ± 14.2 |
| QuickI | 0.2 ± 0.03 |

**Table 5. Clinical Characteristics of Study Participants in Dietary Analysis**

Participant demographics are described, along with the means and standard deviations of their habitual sleep characteristics, and laboratory-tested glucose, insulin and derived insulin sensitivity.

| Dietary characteristics | Mean ± SD |
|---|---|
| *Dietary Proportion Measures* | |
| Total calories (Kcal) | 2512.6 ± 731.5 |
| Proportion of morning meal (% Kcal) | 23.8 ± 12.3 |
| Proportion of evening meal (% Kcal) | 36.0 ± 11.4 |
| Proportion of carbohydrates in morning meal (% Kcal) | 26.0 ± 13.7 |
| Proportion of carbohydrates in evening meal (% Kcal) | 35.3 ± 11.6 |
| Proportion of protein in morning meal (% Kcal) | 22.0 ± 10.9 |
| Proportion of protein in evening meal (% Kcal) | 36.5 ± 12.7 |
| Proportion of fat in morning meal (% Kcal) | 24.3 ± 14.8 |
| Proportion of fat in evening meal (% Kcal) | 33.8 ± 13.3 |
| | |
| *Dietary Timing Measures* | |
| Time of consumption of 25% total calorie | 11:59 ± 2:15 |
| Time of consumption of 75% total calorie | 18:41 ± 1:49 |
| Time of consumption of 25% carbohydrate | 11:49 ± 2:13 |
| Time of consumption of 75% carbohydrate | 18:29 ± 2:02 |
| Time of consumption of 25% protein | 12:30 ± 2:08 |
| Time of consumption of 75% protein | 18:26 ± 1:57 |
| Time of consumption of 25% fat | 12:26 ± 2:24 |
| Time of consumption of 75% fat | 17:49 ± 2:29 |

**Table 6. Descriptive Statistics for the Dietary Variables**

Means and standard deviations of calorie distribution variables

| Dependent Variable | Independent Variable | Unadjusted Model [a] | | Adjusted Model [b] | |
|---|---|---|---|---|---|
| | | β -coefficient (95% CI) | P-value | β -coefficient (95% CI) | P-value |
| Matsuda Index at180 minutes | Proportion of morning calories | 30.46 (3.66 to 57.26) | 0.027 | 33.93 (8.45 to 59.41) | 0.011 |
| | Proportion of morning carbohydrates | 37.25 (14.80 to 59.69) | 0.002 | 39.24 (18.37 to 60.11) | 0.001 |
| Matsuda Index at 300 minutes * | Proportion of morning calories | 40.93 (5.50 to 76.35) | 0.025 | 45.66 (11.12 to 80.20) | 0.011 |
| | Proportion of morning carbohydrates | 49.74 (19.79 to 79.69) | 0.002 | 52.51 (24.01 to 80.99) | 0.001 |
| QUICKI | Proportion of morning calories | 0.070 (0.004 to 0.136) | 0.038 | 0.08 (0.02 to 0.14) | 0.013 |
| | Proportion of morning carbohydrates | 0.085 (0.029 to 0.14) | 0.004 | 0.08 (0.04 to 0.14) | 0.001 |

**Table 7. Regression Models for Dietary Proportion Variables**

The linear regression models were considered significant if p-value<0.05. The confounding variables for the adjusted model are age, sex, BMI, race, average 24-hour calories and sleep midpoint

For the unadjusted model; N=41, adjusted model; N=38 with the exception of matsuda index 300 where, unadjusted model; N=39 and adjusted model; N=36. Two participants were hypoglycemic before the completion of the glucose tolerance test (fsOGTT).

| Dependent Variable | Independent Variable | Unadjusted Model [a] | | Adjusted Model [b] | |
|---|---|---|---|---|---|
| | | β-coefficient (95% CI) | P-value | β-coefficient (95% CI) | P-value |
| Matsuda Index 180 | TCI 25% | -1.73 (-3.18 to -0.28) | 0.021 | -1.66 (-3.11 to -0.21) | 0.026 |
| | TCI 50% | -1.2 (-2.52 to 0.12) | 0.074 | -1.16 (-2.51 to 0.18) | 0.088 |
| | TCI 75% | -0.2 (-2.13 to 1.73) | 0.835 | -1.35 (-3.4 to 0.7) | 0.188 |
| Matsuda Index 300 * | TCI 25% | -2.39 (-4.35 to -0.42) | 0.019 | -2.42 (-4.39 to -0.44) | 0.018 |
| | TCI 50% | -1.64 (-3.44 to 0.16) | 0.073 | -1.84 (-3.68 to 0.01) | 0.051 |
| | TCI 75% | -0.44 (-2.99 to 2.12) | 0.731 | -2.00 (-4.8 to 0.79) | 0.154 |
| QuickI | TCI 25% | -0.004 (-0.008 to -0.001) | 0.015 | -0.005 (-0.008 to -0.001) | 0.008 |
| | TCI 50% | -0.003 (-0.006 to 0.0004) | 0.092 | -0.003 (-0.006 to 0.0003) | 0.071 |
| | TCI 75% | -0.0003 (-0.005 to 0.004) | 0.897 | -0.003 (-0.008 to 0.002) | 0.21 |

**Table 8. Regression Models for Dietary Timing Variables**

The linear regression models were considered significant if p-value<0.05 and moderately significant if p-value<0.1. The confounding variables for the adjusted model are age, sex, BMI, race, average 24-hour calories and sleep midpoint.

For the unadjusted model; N=41, adjusted model; N=38 with the exception of Matsuda index 300 where, unadjusted model; N=39 and adjusted model; N=36. Two participants were hypoglycemic before the completion of the glucose tolerance test (fsOGTT).