Nutrición Hospitalaria

Nutr Hosp. 2015;31(Supl. 3):279-289
ISSN 0212-1611 • CODEN NUHOEQ
S.V.R. 318

Methods for monitoring the functional status of the circadian system in dietary surveys studies: application criteria and interpretation of results

Marta Garaulet and Juan Antonio Madrid

Department of Physiology, University of Murcia. IMIB. Murcia. Spain.

Abstract

To evaluate the circadian system status of the subject may be of special interest in nutrition. Particularly for those studies related to the assessment of diseases related to malnutrition, as it is the case of most of the degenerative diseases such as obesity, cancer, or cardiovascular diseases. For this purpose, one of the approaches consists to measure a) the external synchronizers of the internal clock, such as light intensity, and changes from fasting to eating and from resting to activity. Indeed, "chronodisruptors" have been defined as "exogenous and endogenous exposures or effectors which are chronobiologically active and can thus disrupt the timing and order.

Another approach to assess the circadian system health is to measure the b) outputs of the internal clock (circadian marker rhythms). Among such outputs, the rhythm of body temperature, motor activity, melatonin, cortisol and clock gene expression are the most commonly used. From the genetic perspective, we are now able to measure failures in the internal clock, in order to assess c) the genetics of the molecular clock. Indeed, new nutrigenetics techniques are giving us the opportunity to measure the association between different genetic variants of our clock genes and several illnesses such as obesity, cardiovascular diseases, diabetes or cancer. In addition to these techniques, self-reported questionnaires based in the morning-evening preferences have been developed as complementary procedures to assess human chronotypes.

(*Nutr Hosp* 2015;31(Supl. 3):279-289)

DOI:10.3305/nh.2015.31.sup3.8776

Key words: *Circadian rhythm. Circadian dysregulation. Meal timing. Cosinor analyses. Body temperature.*

MÉTODOS CRONOBIOLÓGICOS EN LAS ENCUESTAS ALIMENTARIAS: CRITERIOS DE APLICACIÓN E INTERPRETACIÓN DE RESULTADOS

Resumen

Evaluar el estado del sistema circadiano del sujeto puede ser de especial interés en la nutrición. En particular, para los estudios relativos a la evaluación de las enfermedades relacionadas con la malnutrición como es el caso de la mayoría de las enfermedades degenerativas tales como la obesidad, cáncer, o enfermedades cardiovasculares. Para este propósito, uno de los enfoques consiste en medir a) los sincronizadores externos del reloj interno, tales como intensidad de la luz, y los cambios de ayuno/ingesta y de reposo/actividad. De hecho, se ha definido el término de "cronodisruptor" que se refiere a "exposiciones o efectores exógenos y endógenos que son cronobiológicamente activos y que por lo tanto pueden interrumpir el tiempo".

Otro enfoque para evaluar la salud del sistema circadiano es medir b) las salidas del reloj interno (ritmos circadianos). Entre ellos las más utilizadas son la medición del ritmo de la temperatura corporal, la actividad motora, la melatonina, el cortisol y la expresión de genes reloj. Desde el punto de vista genético, ahora somos capaces de medir c) las alteraciones del reloj interno, con el fin de evaluar la genética del reloj molecular. De hecho, las nuevas técnicas de nutrigenética nos están dando la oportunidad de medir la asociación entre las diferentes variantes genéticas de nuestros genes reloj y varias enfermedades como la obesidad, las enfermedades cardiovasculares, la diabetes o el cáncer. Además de estas técnicas, se han desarrollado cuestionarios basados en las preferencias de mañana-tarde como procedimientos complementarios para evaluar cronotipos humanos.

(*Nutr Hosp* 2015;31(Supl. 3):279-289)

DOI:10.3305/nh.2015.31.sup3.8776

Palabras clave: *Ritmos circadianos. Cronodisrupción. Hora de la comida. Análisis de cosinor. Temperatura corporal.*

Correspondence: Marta Garaulet.
Department of Physiology. Faculty of Biology.
University of Murcia.
Campus de Espinardo, s/n.
30100, Murcia, Spain.
E-mail: garaulet@um.es

Introduction

To evaluate the circadian system status of the subject may be of special interest in nutrition. Particularly for those studies related to the assessment of diseases related to malnutrition, as it is the case of most of the degenerative diseases such as obesity, cancer, or cardiovascular diseases. Current epidemiological studies have demonstrated that several situations in life such as shift working, night eating, jet lag and short sleep, are accompanied by a disruption of the individual circadian rhythms. Chronodisruption (CD) is a relevant disturbance of the circadian organization of physiology, endocrinology, metabolism and behavior, which links light, biological rhythms and the development of several diseases.

The International Agency for Research on Cancer (IARC) classified shift-work that involves circadian disruption as probably carcinogenic to humans in 2007 and this was the prelude to extensive experimental and epidemiological research in coming years[1]. Moreover, it was pointed out in Lancet Oncology that among the many different patterns of shift-work, those including night work were the most disruptive for the circadian clock. Indeed, with 20% of people worldwide being engaged in some work at unusual times, including the night, it is a relevant task in public health to clarify the biologically plausible links *via* circadian disruption with epidemic cancers such as of the breast or prostate.

The effect of CD on human health is an emerging issue. Many records link CD not only with cancer but also with cardiovascular, cognitive impairment and obesity, all of them conducive to premature aging. The precise mechanisms linking obesity to CD are not well known. It has been hypothesized that current habits, such as high snacking frequency, a reduction in total daily sleep and increased exposure to bright light during the night, and "inadequate" meal timing induce brain to lose its 'feeling' for internal and external rhythms. The lack of day–night environmental contrast may lead to CD and metabolic disturbances, including obesity. Conversely, studies performed using experimental models have shown that developing obesity and diabetes itself disrupts the molecular clock system. Both, as a cause or as a consequence, CD is closely linked to obesity.

Methods for monitoring the functional status of the circadian system

The role placed by the circadian system in maintaining health underlines the importance of developing techniques for its objective evaluation, just as there are techniques that evaluate the respiratory and cardiovascular systems. The main challenge is to be able to measure a process that develops over long periods of time, which implies multiple measurements, preferably ones that do not interfere with the subject's daily routine[2].

One of the approaches consists to measure a) the **external synchronizers** of the internal clock, such as light intensity, and changes from fasting to eating and from resting to activity. Indeed, **"chronodisruptors**" have been defined as "exogenous and endogenous exposures or effectors which are chronobiologically active and can thus disrupt the timing and order, i.e. temporal organization of physiologic functions and hierarchies"[1]. In principle, whatever allows the establishment of temporal organizational order in organisms should also be capable of disrupting such order when present or applied in excess or deficit and, most importantly, at unusual and inappropriate times. In this sense, apart from light, other external chronodisruptors are inadequate meal timing and exercise timing[3].

Another approach to assess the circadian system health is to measure the b) **outputs of the internal clock** (circadian marker rhythms). Among such outputs, the rhythm of body temperature, motor activity, melatonin, cortisol and clock gene expression are the most commonly used[4]. From the genetic perspective, we are now able to measure failures in the internal clock c) **the genetics of the molecular clock**. Indeed, new nutrigenetics techniques are giving us the opportunity to measure the association between different genetic variants of our clock genes and several illnesses such as obesity, cardiovascular diseases, diabetes or cancer. In addition to these techniques, **self-reported questionnaires** based in the morning-evening preferences have been developed as complementary procedures to assess human chronotypes[5-7].

Methods to measure external synchronizers

Light exposure

The circadian system is regulated by external signals, which are responsible for setting the clock each day. Given that the light-dark cycle is the most important synchroniser, it is of great interest to be able to quantify the light exposure of individuals. Low levels of illumination during the day lower the central temperature and state of awareness compared with the levels observed in high illumination[8]. However, during the night exposure to light, especially blue light, should be avoided in order to maintain melatonin secretion.

Indeed, one key external chronodisruptor is light at night. Under natural conditions, biological circadian and seasonal rhythms are synchronized to the regular 24-hr and seasonal light–dark cycles and the suprachiasmatic nuclei and melatonin have critical roles in these processes. In fact, light is a key Zeitgeber affecting melatonin rhythms and the circadian rhythms of melatonin can provide clock (24 hr) and calendar (seasonal and yearly) information for many species, including humans[1].

The light-darkness cycle to which subjects are exposed can be quantified by small data loggers, that contain a photosensitive cell, that periodically record the light

intensity received by the individual. Recently, sensors that differentiate between light wavelengths (blue, red and green) have become available, which means that the blue light, which has a greater capacity to synchronise the circadian pacemaker, can be accurately evaluated. The combination of these sensors with environmental temperature sensors provides complete information concerning the quality of the environmental synchronisers that act on the circadian system[8]. They also enable poor sleep hygiene habits to be identified; for example, sleeping in illuminated environmental conditions or in too high temperatures.

Meal timing (changes from fasting to eating)

The meal times (and number of meals consumed) differ greatly from culture to culture and through time. Indeed, timing of food intake is a modifiable behaviour that may influence energy regulation and consequently the risk of obesity. Several studies performed in experimental animals have demonstrated that when the animals eat at the "wrong time" they become obese, although they apparently eat and expend the same amount of energy[3].

Our group of research have demonstrated that the timing of the main meal (lunch) in a Mediterranean population from Spain, is predictive of the weight loss during a 20-week dietary intervention conducted in 420 obese and overweight individuals[9]. Another relevant result from this study was that insulin sensitivity was lower in late eaters as compared to early eaters. However, the physiological explanation for this novel discovery was unknown. In order to deep in these results we developed a randomized, crossover protocol in which we studied the same women (n=32) in two conditions: one week having lunch at 1 PM and the other at 4.30 PM. We demonstrated that eating late is associated with decreased resting-energy expenditure, decreased fasting carbohydrate oxidation, decreased glucose tolerance, blunted daily profile in free cortisol concentrations and decreased thermal effect of food on peripheral temperature. These results may be implicated in the differential effects of meal timing on metabolic health (Fig. 1).

Different methods may be used to assess habitual dietary intake and meal timing: For example, to evaluate food habits, initial nutrient intake can be determined by a 7 days dietary record. In each day subjects should record everything they eat and also the timing that they start or finish each meal. Patients can also record during one week the time at the day that they start every meal with the questionnaire developed by Bertéus et al.10 (Fig. 2).

Other questionnaire used to assess the circadian changes on hunger and appetite is the one developed by Flint *et al.*, of visual analogue scales (VAS) for measurement of appetite sensations. VAS are used to record hunger, satiety, fullness, prospective food consumption, desire to eat something fatty, salty, sweet or savory, and palatability of the meals[11]. These questionnaires may be completed before and after each meal everyday during one week.

Exercise timing (changes from activity to inactivity)

Actigraphy is a non-invasive method useful to measure the rest-activity cycle in humans. It is based on the principle that during periods when the individual

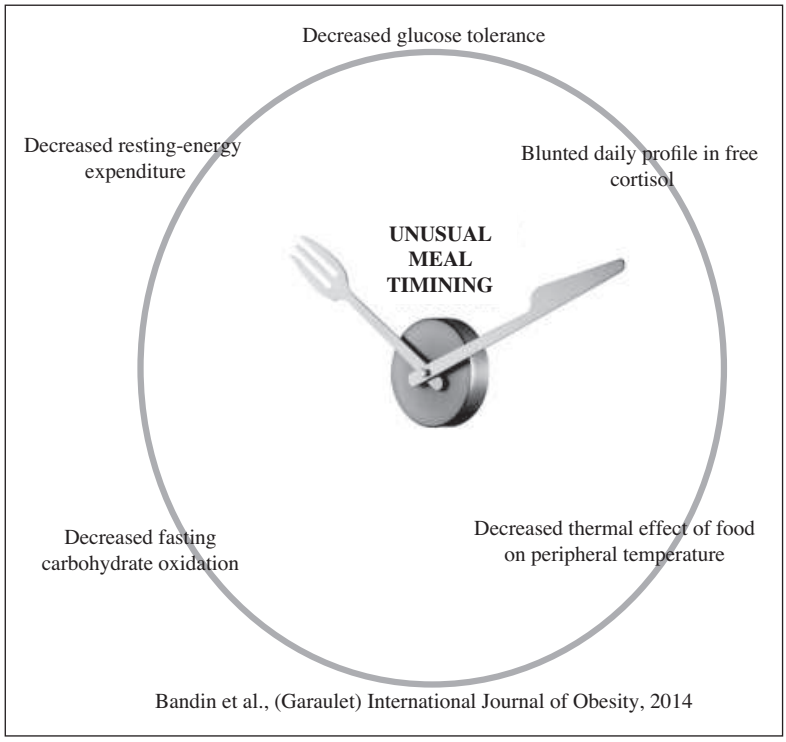


Fig. 1.—Effects of meal timing on metabolic health[34].

Describe how you cat during a usual 24-hour period. Give a time for cach cating episode and mark with a **cross** the type of meal which corresponds best.

Do not forget snacks, other "light meals" and drinks.

Note, you can have several main meals during a day.

	Type of meal			
Time	Main meal e.g. cooked dish, soup with bread, salad with bread, pizza	Light meal/ Breakfast e.g. porridge, cereals, sandwiches, soup, salad, omelette	Snack meal e.g. a sandwich, biscuit, bun, cake, fruit, sweets, ice cream (w/ or w/o a drink)	Drink, only e.g. coffee, tea, soft drink, juice, milk, beer, wine
Example: 2300				X

Fig. 2.—Questionnaire of Bertéus et al.,[10] in meal timing.

is awaken; activity levels are high compared to when the individual is asleep. For its measurement, an activity sensor (actimeter) is placed on the wrist of the non-dominant hand for not less than 5 days, the minimum period to obtain reliable data that reflect the characteristics of the subject[12].

Actigraphy has been used to study sleep/wake patterns for over 20 years. The advantage of actigraphy over traditional polysomnography (PSG) is that actigraphy can conveniently record continuously for 24-hours a day for days, weeks or even longer[13]. It is also considered the method of choice for evaluating and diagnosing circadian disorders such as chronodisruption in shift-workers, delayed and advanced sleep phase syndrome, free running syndrome and irregular circadian rhythms[14]. However, as with any other measurement, actigraphy is subject to masking and artefacts, for example, the difficulty in differentiating between the beginning of night rest and the removal of the sensor to shower just before going to bed, movements of one's bed partner, sleeping in a car or train, etc.[15].

Other methods are 7 days activity records, in which subject record the timing, type and intensity of the daily physical activity performed during one week, including weekdays and weekends.

Methods to measure "Outputs of the clock"

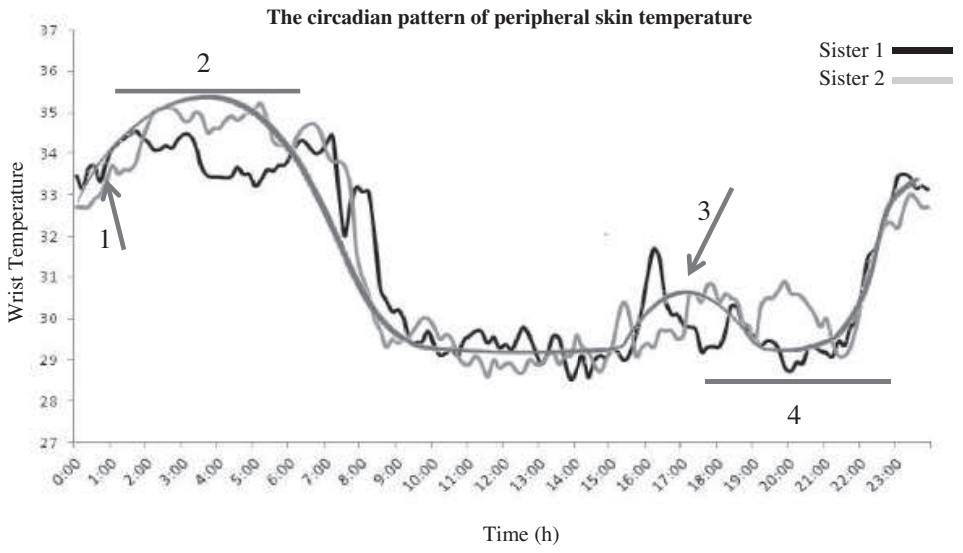
Central and peripheral thermometry

One of the most commonly used marker rhythms is the central temperature rhythm, whose profile has been widely described[16,17] and in which the highest values occur in the day and lowest at night.

In humans, the central temperature is usually measured by means of rectal probes that should be worn for several days, which is obviously uncomfortable. Recently as an alternative to measuring the central temperature, the rhythm of skin peripheral temperature has been proposed as a marker rhythm[18-21]. This rhythm is induced by the alternation between vasodilatation and vasoconstriction generated by the parasympathetic-sympathetic balance. The predominance of sympathetic activity during the day is associated with lower temperatures, while its inhibition and the simultaneous activation of the parasympathetic system are associated with higher temperature. Moreover, increased skin temperature constitutes a signal that favours the beginning of nocturnal sleep through stimulation of hypothalamic areas[22].

The circadian pattern of peripheral skin temperature exhibits some characteristics phases (Fig. 3)[18]. It increases prior to sleep and remains high during the night. Upon awakening, the temperature falls abruptly and remains low during the day. About 20-21h, when the peripheral temperature reaches its lowest value it is difficult to go to sleep in normal circumstances, this phase is known as wake maintenance zone.

The most used procedure to record skin temperature consists of a small autonomous data logger, placed on the internal surface of the wrist (over a radial artery) of the non-dominant hand and held in place by a bracelet or watch. The sensor can also be placed in any other peripheral region such as the arm, ankle or finger[18,19]. This easily obtained measure has been



1) Sharp increase in anticipation to sleep onset
2) High levels during nocturnal sleep
3) A secondary peak in the afternoon
4) A dip between 20:00 and 22:00h, a period known as the "wake maintenance zone"

Fig. 3.—24-h waveform of wrist skin temperature (WT) of two monozygotic sisters recorded continuously for seven days[59]. The standard skin temperature rhythm exhibits a sharp increase in anticipation to sleep onset, it maintains high levels during nocturnal sleep and shows a secondary peak in the afternoon.

shown to be useful to characterize biorhythms in a variety of population groups including babies[23] and young people[8,19] as well as to identify CD associated with different pathologies such as hypertension[24], metabolic syndrome and obesity[20,21,25,26]. Moreover, it has been shown that wrist skin temperature (WT) rhythm has a distinct endogenous component, even in the presence of multiple external influences. Therefore, WT has been proposed as an informative and minimally invasive technique to measure circadian rhythm in free-living subjects[27].

Melatonin

Melatonin is considered the best marker of the circadian system phase. However, its profile is strongly influenced by light exposure and, to a lesser extent, body position, physical activity, sleep, caffeine and drugs like beta-blockers[28-31]. Plasma levels of melatonin show a circadian profile, with low levels during the day and high levels during the night, the highest being between 02:00 and 04:00am. In humans, melatonin contributes to the body temperature rhythm since it is responsible for vasodilatation of the skin of the extremities through its activation of thermosensitive neurons present in brain areas involved in sleep regulation. The melatonin secretion schedule is closely related with the propensity to sleep and coincides with a fall in the central body temperature, arousal level and performance[32]. Indeed, since 1992 we know that the circadian rhythms of melatonin and body temperature are inversely coupled.

The hypothermic properties of melatonin are accountable for the generation of at least 40% of the amplitude of the circadian body temperature rhythm. Manipulation of melatonin levels might be clinically useful to resynchronize the body temperature rhythm under conditions of body temperature rhythm desynchronization.

The levels of melatonin can be reliably measured in plasma, saliva and urine (in the last case as its metabolite, 6-sulfatoximelatonin). The best time to evaluate melatonin as a marker of the circadian rhythm coincides with its rapid increase at nightfall. Since its levels are altered by exposure to environmental light of a given intensity and spectrum, it is generally accepted that melatonin samples taken during the dark period should be collected under a dim light (< 50 lux), which is why this protocol is known as *DLMO* (*Dim Light Melatonin Onset*)[33]. It is sufficient to start sampling 2 to 3 hours before the subject's normal bedtime (around 19:30-22:00h), assuming that the individual shows no phase alterations.

Cortisol

Cortisol is a corticosteroid with a robust circadian profile peaking around the usual waking time and with much lowers values as the day progresses and reaching its lowest value about 2 hours after going to sleep. The physiological significance of this increase consists of preparing the body for the forthcoming days, increasing the blood pressure, plasma concentrations of glucose, cardiac output, etc. Because of its robustness,

this rhythm is also considered a good marker of the circadian system.

Similarly to the other variables mentioned above, cortisol levels can be affected by external factors such as food timing[34], stressful situations, light exposure at given moments of the day[35], hyperproteic meals or obesity[36]. Non-pathological situations such as aging also affect the cortisol profile. The sleep-wake profile can even modify cortisol rhythm. Sleep deprivation, the predominance of light sleep, and a certain number of nocturnal awakenings will increase cortisol levels[37].

Cortisol can be measured in serum or saliva, the most critical times for measuring its circadian profile being the increase just before waking up and its minimum level in blood at the end of the day/beginning of night.

Sleep and Wakefulness patterns (integrated variables)

Sleep is not a clear "output" of our internal clock. However, it is modifiable by the subject, and because it can also change the individual exposition to the external synchronizers such as light, it is able to influence the internal clock function. Moreover, it is clear that synchronization of the sleep wake schedule and the internal clock is essential to an individual's ability to maintain sleep and wakefulness when desired. For example, to fly across time zones or to work night shifts, desynchronize sleep and wake patterns from the internal clock's circadian rhythms and result in an alerting signal that is too low when an individual wishes to be awake and too high to allow for a consolidated period of sleep.

Therefore, to measure the circadian pattern of sleep and wakefulness may be relevant in chronobiological and nutritional studies. For this purpose, Laboratory-based polysomnography (PSG) is widely used. It is a comprehensive recording of the biophysiological changes that occur during sleep. The PSG monitors many body functions including brain (EEG), eye movements (EOG), muscle activity or skeletal muscle activation (EMG) and heart rhythm (ECG) during sleep.

Although PSG is the gold standard to measure sleep objectively it is impractical for long-term and home utilization. Therefore, alternative techniques have been developed. The validity of self-recording as a means of collecting data on sleep and wakefulness is still an open question, though it is probably as reliable as any other subjective method and is the most convenient way of accumulating data when a large sample of subjects must be used. Current evidence has shown that, overall, actigraphy is an excellent tool for unobtrusive documentation of sleep/wake activity in normal individuals. However, a number of methodological issues remain to be resolved to warrant its use in clinical research.

In order to increase the reliability of circadian monitoring, integrated variables obtained from processing individual variables have been recently proposed. For example, the TAP algorithm, proposed by Ortiz-Tudela et al.[19], is based on integrating, after normalisation, the following variables: skin temperature, motor activity and body position (Fig. 4). The first of these variables, skin temperature, is under endogenous control, while motor activity is modified voluntarily but it is also under endogenous control. Lastly, of the three variables used for the integration, body position is the most closely dependent on voluntary control. TAP is modular thus it can be amplified by incorporating new variables that complement the information even further. TAP variable permits us not only to determine how the individual's circadian system functions, but also to infer the sleep-wake rhythm with a precision higher than 90% according to polysomnographic recording. This technique constitutes the base of ambulatory circadian monitoring procedure (ACM), which recently has been applied to evaluating the circadian maturity in newborns[23], and pathologies like metabolic syndrome[21].

Methods to measure "failures in the internal clock"

Genetics of the molecular clock

The capacity to undergo rhythmic oscillations is a characteristic intrinsic to living matter. A fundamental statement of chronobiology states 'many rhythms persist even in complete isolation from the major known environmental cycles'. This concept supports that natural rhythms can exist independently of the periods defined by geophysical cycles; this means that living matter has its own time, i.e., the 'biological time'. In this sense, it has been hypothesized the existence of a Chronome within the Genome.

Over the past two decades, biochemical, genetic, and molecular studies have been making substantial advances towards the elucidation of the molecular bases of rhythmicity in living things. Riding on the wave generated by the seminal studies in the 1970's focusing on in the circadian variability of hormones such as cortisol, melatonin or growth hormone (GH), or those related to the discovering and description of the physiological bases of the suprachiasmatic nucleous (SCN), current chronobiology has dramatically evolved thanks to the new genetic and molecular biology techniques.

A major stride in understanding the molecular basis of circadian rhythms was the identification by Konopa and Benzer in 1971[38] of a chromosomal region controlling the period of eclosion time in Drosophila, followed by the cloning of the first clock genes in *Drosophila melanogaster* in 1984[39]. Today, thanks to these molecular techniques, we are able to study the expression of the known clock genes implicated in the circadian machinery. We already know that, in mammals, the core components of the clock molecular ma-

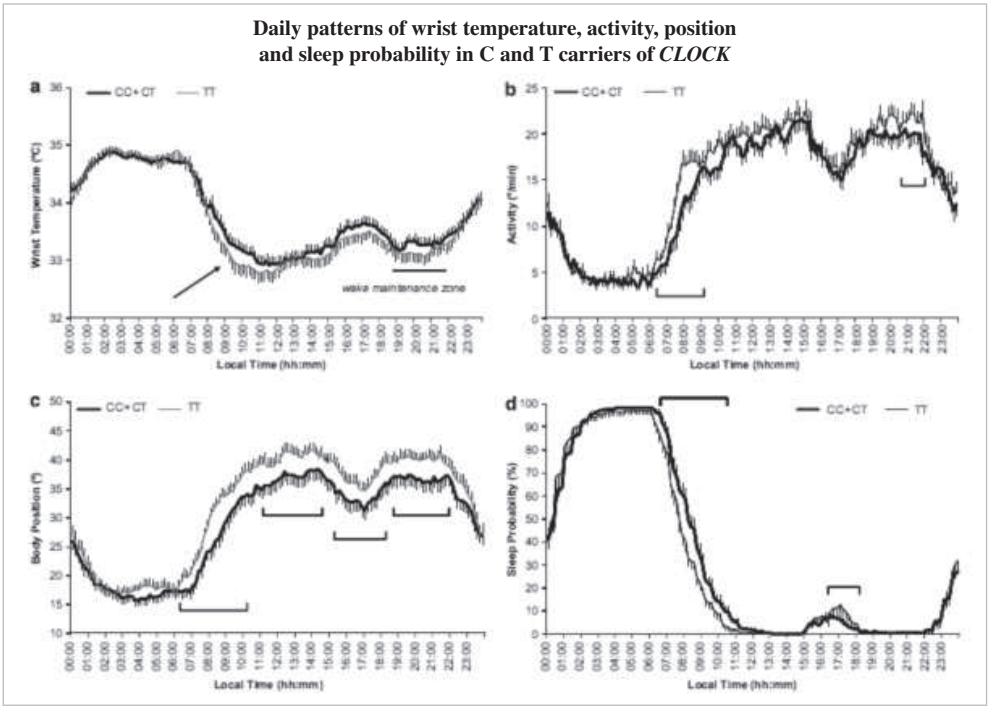


Fig. 4.—Skin temperature, motor activity and body position in CLOCK 3111TC[60].

chinery operate in almost all cells of the body through a complex network of transcriptional-translation loops and modulate the expression of specific target genes and their products to oscillate in 24-hour rhythm.

Nowadays, experimental models are allowing us to assess clock genes expression not only in the living animal but also outside of the body, (*in vitro* techniques), and we are also able to analyze the 24h fluctuations in gene expression and to assess the presence or absence of a peripheral clock in the different organs and tissues. Moreover, we can use experimental models to turn on and off specific components of the clock machinery to identify its effects on metabolic and disease phenotypes. From the genetic epidemiology point of view, the study of single nucleotide polymorphisms (SNPs), is contributing to the identification of the genetic background of chronotypes (morningness or eveningness), sleep alterations or seasonal mood disorders.

More recently, **epigenetic and nutrigenetic** approaches are also allowing us to study new interactions and layers of complexity that may have a significant impact on chronobiology as well as pathophysiology. *Epigenetics* is the study of heritable changes in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence. *CLOCK* and *SIRT* have both epigenetics effect in acetylation and deacetylation of histones respectively. MicroRNAs may become novel therapeutic targets for disorders in the circadian clock. The knowledge achieved in the circadian epigenome could give us new answers to the connections among genetics, circadian rhythmicity and obesity.

Moreover, the technological power of other "-omics" (i.e., metabolomics, proteomics) is becoming essential to our ability to "put-it-all-together" and we are fast learning about the timing of different metabolites such as aminoacids, lipids, xenobiotic, etc. in the liver in mice, and in plasma and saliva in humans, allowing us to achieve a more complete and refined knowledge of the circadian rhythm and its physiological effects. These advances have given to the science of chronobiology a renewed stimulus that makes this science increasingly robust and attractive.

Genetic variants associated to chronodisruption

Some examples of SNPs in chronobiology are *CLOCK* 3111TC which has been associated with eveningness, and different personality traits; *PERIOD2* (*PER2* rs2304672) polymorphism which moderates circadian-relevant reward circuitry; *CRY1* (rs8192440) related to psychological treatment effectiveness; *CRY2* associated with winter depression and S*IRT1* rs10997875 a good candidate gene for the pathophysiology for mood disorders.

Other candidate SNPs connected to obesity could be those associated to sleep disorders. Some examples are serotonin receptors, prepro-orexin or IL-6 SNPs which associate with obstructive sleep apnea syndrome. Others are SNPs residing in ROR1 and PLCB1which associated with insomnia.

Sleep disorders or short sleep duration, are both associated to several polymorphisms connected to obesity. In this regard, one of the best studied *CLOCK* SNPs (3111TC) has been significantly associated to short sleep duration, eveningness, several psychological traits, weight loss[40] and obesity[41,42].

Expression of clock genes in leukocytes and oral mucosa

The neurons that constitute the SCN and the cells of the peripheral oscillators show an autonomous rhythmicity that is controlled by the cyclic expression of the clock genes (*Clock, Bmal1, Per 1, Per 2, Per 3 y Cry 1 y Cry 2*). The involvement of these genes in numerous physiological processes (cell cycle regulation, adipogenesis, glucocorticoid synthesis, B cell maturation, etc.) and their probable misalignment in certain pathologies increase the interest of being able to quantify their expression. For this, polymerase chain reaction (PCR) techniques are usually used[43]. The most straightforward is RT-PCR, which enables us to qualitatively evaluate which genes are being expressed at the time of sampling. To know which genes are being expressed and its quantification, a quantitative PCR (Q-PCR) or a real time PCR is normally used.

Since it is not possible to evaluate clock gene expression in the SCN *in vivo*, samples obtained from peripheral tissues are used. In this case, there are two main options: evaluate gene expression in leukocytes or in the oral mucosa. In the first case, blood samples are periodically taken, the leukocytes are isolated from the rest of the blood cells and one of the above techniques is applied. In the case of oral mucosa, the most common practice has been to take small biopsies under local anaesthetic[44], although, more recently, pipette tips have been used to make scrape off a small amount of the mucosa, which provides sufficient tissue to be obtained[45].

Clock gene expression in adipose tissue (peripheral clock)

In the last years, one of the most influential discoveries relevant for this area of research is the presence of an active circadian clock in adipose tissue (Fig. 5). In particular, our group has recently demonstrated that the circadian clockwork can oscillate accurately and independently of the SCN in AT explants[46]. Moreover, we have provided an overall view of the internal temporal order of circadian rhythms in human AT including genes implicated in metabolic processes such as energy[47]. Thus, a specific temporal order in the daily patterns of these genes appears to be crucial for adipose tissue to exclusively either accumulate fat or to mobilize fat at the proper time, a phenomenon known as temporal compartmentalization.

Fig. 5.—Presence of an active circadian clock in adipose tissue[47].

Protocols for measuring circadian rhythms

To evaluate the circadian system, techniques that eliminate or minimise the influence of external factors (denominated masking factors) are used. For this reason, measurements are normally made in subjects in conditions of constant routine, for example, lying in bed, without sleeping, under constant dim light and ingesting food at regular intervals over 24 hours[48]. This situation of constant routine is usually maintained for 24, 36 or 48 hours, although this, in itself, may introduce its own masking factors. One such factor is that the subject must go without sleep and fight against sleep pressure. To avoid the accumulation of sleep pressure, the multiple-nap protocol has been designed. This is a constant routine protocol with multiple naps scheduled over a 24-hour period or longer.

In an attempt to cut the link between environmental cycles and endogenous rhythms, alternative protocols (known as forced desynchronization) have been developed in which the subject lives 28-hour (or, less frequently, 20-hour) days[48,49]. Under these protocols, sleep episodes occurred at all phases of the endogenous periods. Circadian and sleep components can be distinguished very well in this way.

Analyses of circadian rhythm.

The analysis of rhythmic data requires its own methodology that differs from conventional statistical and mathematical techniques. Two procedures are basically used for this purpose, one based on fitting sinusoidal functions (the cosinor method) and the other based on a non-parametric analysis.

Cosinor analysis is a mathematical procedure based on least squares fitting of a cosine function to the original data. Three main parameters are defined from the cosinor fit: MESOR (Midline Estimating

Statistic of Rhythm), amplitude and acrophase[50]. Since it is applicable to unequidistant data, Mesor does not always coincide with the data mean. The amplitude is the difference between the Mesor and the maximum or the minimum value of the cosinusoidal function. The acrofase is the temporal localisation of the maximum value of the function. Given that the human rest-activity rhythm has a asymmetric distribution over 24 hr (about 8 rest:16 activity) and a shape that looks more like a square wave than a pure cosinusoid, the cosinor method only provides a rough and general description of the rest-activity rhythm. However, it is a relatively straightforward method that enables a great quantity of quantitative information to be obtained.

To give a more precise estimation of the rhythmic parameters of physiological functions that do no exhibit a symmetrical waveform, non-parametric procedures are increasingly used. Although these procedures were initially developed for actimetry data[51], it is also useful for analysing other biological variables. The most frequent parameters are interdaily stability (IS), intradaily variability (IV), least active 5 hr (L5), most active 10 hr (M10), L5 and M10 onset or midtime, amplitude (AMP) and relative amplitude (RA). IS quantifies the regularity of the rhythm, that is, the degree of resemblance between the rhythmic patterns on individual days. It ranges from 0 to 1, a typical value for human actimetry data being about 0.6 for healthy adults. IV determines the fragmentation of the rhythm. It ranges from 0 to 2, typical values in healthy subjects being below 1. L5 indicates the average values for the 5 least active consecutive hours in the 24 hr cycle. M10 is the average of the activity values for the ten most active consecutive hours in the 24-hr cycle. The midpoint of L5 and M10 gives reliable information about the phase of the rhythm, similar to that given by the acrophase and nadir of the cosinor method. AMP is the difference between M10 and L5, whereas RA is calculated by dividing AMP by the sum of L5 and M10. It ranges form 0 to 1, with higher values indicating higher amplitude of the rhythm.

Chronodisruption scores and biomarkers

Given the importance of a normal circadian rhythm in maintaining regular weight, it could be useful to define a biomarker of circadian deregulation that could be implemented in clinical practice. This biomarker could also be used as a tool for monitoring the effects of introducing a change in lifestyle designed to reduce the risk of obesity[52]. For this purpose and to detect the best biomarker to assess CD in obesity, our group of research tested in a female population several biomarkers of the circadian system previously used in different population types. In this regard, we used techniques that have been shown to be easily measurable and non-invasive, such as (a) sleep diaries, which examine the wake/sleep cycle and which have been demonstrated to be a convenient tool to assess sleep quality and duration[53]; (b) a feeding diary, an adequate tool to analyze the timing and duration of food intake[54]; (c) the Horne–Ostberg questionnaire, which is recommended to define the morningness-eveningness of the subject[55,56]; (d) salivary melatonin and cortisol defined as good markers to assess CD[57]; and finally (e) the measurement of the skin temperature rhythmicity, particularly wrist temperature (WT)[24].

Our results show that from the different biomarkers studied in the present work, the measurement of skin temperature rhythmicity, together with two questions of the sleep diary (sleep onset and offset times), and one morning salivary cortisol determination could be enough to characterize the chronobiology of obesity.

After including the several factors studied in a factor analyses we were able to define a Cronodisruption (CD) score. The results obtained showed that patients could be divided into two populations attending to circadian misalignment. Indeed, the cut-off point to divide the population was found in a value of 40.3 points, with a higher score indicating major risk of CD. The correlation analysis showed that patients with major CD scores had higher risk of obesity and MetS. Indeed, body fat percentage, plasma glucose values, and blood pressure were positively correlated with the final punctuation. With respect to triglycerides, total cholesterol and LDL cholesterol correlated with higher scores, while HDL cholesterol correlated with lower scores.

Morning eveningness questionnaires

The morningness–eveningness questionnaire (MEQ) is a self-assessment questionnaire developed by Horne and Östberg in 1976[58]. Its main purpose is to measure whether a person's peak alertness is in the morning, in the evening or in between. The original published study on the MEQ showed that subjective peak alertness time correlates with a time of peak body temperature; morning types have an earlier peak of oral temperature than evening types, with the intermediate types having temperature peaks between the two groups. The MEQ is widely used in many areas of psychological and medical research.

More recently, new questionnaires have been also developed, this is the case of the Munich Chronotype Questionnaire (MCTQ)[7]. Questions about work day and free day sleep schedules, work details, and lifestyle provide data to aid in the understanding of how biological clocks work in social life, such as Roenneberg's conclusions of social jetlag. The MCTQ categorizes each participant into one of seven chronotype groups, and utilizes data on participants' midsleep phase and sleep debt to survey what "type" of sleeper each person is.

References

1. Erren TC, Reiter RJ. Defining chronodisruption. *Journal of pineal research* 2009; 46:245-247.
2. Touitou Y, Haus E. Aging of the human endocrine and neuroendocrine time structure. *Annals of the New York Academy of Sciences* 1994; 719:378-397.
3. Garaulet M, Gomez-Abellan P. Timing of food intake and obesity: A novel association. *Physiology & behavior* 2014.
4. Hofstra WA, de Weerd AW. How to assess circadian rhythm in humans: a review of literature. *Epilepsy & behavior : E&B* 2008; 13:438-444.
5. Horne JA, Ostberg O. A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. *International journal of chronobiology* 1976; 4:97-110.
6. Smith CS, Reilly C, Midkiff K. Evaluation of three circadian rhythm questionnaires with suggestions for an improved measure of morningness. *The Journal of applied psychology* 1989; 74:728-738.
7. Roenneberg T, Wirz-Justice A, Merrow M. Life between clocks: daily temporal patterns of human chronotypes. *J Biol Rhythms* 2003; 18:80-90.
8. Martinez-Nicolas A, Ortiz-Tudela E, Madrid JA, Rol MA. Crosstalk between environmental light and internal time in humans. *Chronobiology international* 2011; 28:617-629.
9. Garaulet M, Gomez-Abellan P, Alburquerque-Bejar JJ, Lee YC, Ordovas JM, Scheer FA. Timing of food intake predicts weight loss effectiveness. *Int J Obes* (Lond) 2013; 37:604-611.
10. Berteus Forslund H, Lindroos AK, Sjostrom L, Lissner L. Meal patterns and obesity in Swedish women-a simple instrument describing usual meal types, frequency and temporal distribution. *European journal of clinical nutrition* 2002; 56:740-747.
11. Flint A, Raben A, Blundell JE, Astrup A. Reproducibility, power and validity of visual analogue scales in assessment of appetite sensations in single test meal studies. International journal of obesity and related metabolic disorders : journal of the *International Association for the Study of Obesity* 2000; 24:38-48.
12. Acebo C, Sadeh A, Seifer R, Tzischinsky O, Wolfson AR, Hafer A, Carskadon MA. Estimating sleep patterns with activity monitoring in children and adolescents: how many nights are necessary for reliable measures? *Sleep* 1999; 22:95-103.
13. Ancoli-Israel S, Cole R, Alessi C, Chambers M, Moorcroft W, Pollak CP. The role of actigraphy in the study of sleep and circadian rhythms. *Sleep* 2003; 26:342-392.
14. Morgenthaler TI, Lee-Chiong T, Alessi C, Friedman L, Aurora RN, Boehlecke B, Brown T, Chesson AL, Jr., Kapur V, Maganti R, Owens J, Pancer J, Swick TJ, Zak R. Practice parameters for the clinical evaluation and treatment of circadian rhythm sleep disorders. An American Academy of Sleep Medicine report. *Sleep* 2007; 30:1445-1459.
15. Sadeh A, Acebo C. The role of actigraphy in sleep medicine. *Sleep medicine reviews* 2002; 6:113-124.
16. Krauchi K. How is the circadian rhythm of core body temperature regulated? Clinical autonomic research : official journal of the *Clinical Autonomic Research Society* 2002; 12:147-149.
17. Waterhouse J, Drust B, Weinert D, Edwards B, Gregson W, Atkinson G, Kao S, Aizawa S, Reilly T. The circadian rhythm of core temperature: origin and some implications for exercise performance. *Chronobiology international* 2005; 22:207-225.
18. Sarabia JA, Rol MA, Mendiola P, Madrid JA. Circadian rhythm of wrist temperature in normal-living subjects A candidate of new index of the circadian system. *Physiology & behavior* 2008; 95:570-580.
19. Ortiz-Tudela E, Martinez-Nicolas A, Campos M, Rol MA, Madrid JA. A new integrated variable based on thermometry, actimetry and body position (TAP) to evaluate circadian system status in humans. *PLoS Comput Biol* 2010; 6:e1000996.
20. Corbalan-Tutau MD, Madrid JA, Ordovas JM, Smith CE, Nicolas F, Garaulet M. Differences in daily rhythms of wrist temperature between obese and normal-weight women: associations with metabolic syndrome features. *Chronobiology international* 2011; 28:425-433.
21. Corbalan-Tutau MD, Gomez-Abellan P, Madrid JA, Canteras M, Ordovas JM, Garaulet M. Toward a chronobiological characterization of obesity and metabolic syndrome in clinical practice. *Clin Nutr* 2014; .
22. Raymann RJ, Swaab DF, Van Someren EJ. Skin deep: enhanced sleep depth by cutaneous temperature manipulation. *Brain : a journal of neurology* 2008; 131:500-513.
23. Zornoza-Moreno M, Fuentes-Hernandez S, Sanchez-Solis M, Rol MA, Larque E, Madrid JA. Assessment of circadian rhythms of both skin temperature and motor activity in infants during the first 6 months of life. *Chronobiology international* 2011; 28:330-337.
24. Blazquez A, Martinez-Nicolas A, Salazar FJ, Rol MA, Madrid JA. Wrist skin temperature, motor activity, and body position as determinants of the circadian pattern of blood pressure. *Chronobiology international* 2012; 29:747-756.
25. Bandin C, Martinez-Nicolas A, Ordovas JM, Madrid JA, Garaulet M. Circadian rhythmicity as a predictor of weight-loss effectiveness. *Int J Obes* (Lond) 2013; .
26. Corbalán-Tutau MD G-AP, Madrid JA, Canteras M, Ordovás JM and Garaulet M. Toward a chronobiological characterization of obesity and metabolic syndrome in clinical practice. *Clinical Nutrition In Press*.
27. Martinez-Nicolas A, Ortiz-Tudela E, Rol MA, Madrid JA. Uncovering different masking factors on wrist skin temperature rhythm in free-living subjects. *PloS one* 2013; 8:e61142.
28. Monteleone P, Maj M, Fusco M, Orazzo C, Kemali D. Physical exercise at night blunts the nocturnal increase of plasma melatonin levels in healthy humans. *Life sciences* 1990; 47:1989-1995.
29. Shilo L, Sabbah H, Hadari R, Kovatz S, Weinberg U, Dolev S, Dagan Y, Shenkman L. The effects of coffee consumption on sleep and melatonin secretion. *Sleep medicine* 2002; 3:271-273.
30. Zeitzer JM, Duffy JF, Lockley SW, Dijk DJ, Czeisler CA. Plasma melatonin rhythms in young and older humans during sleep, sleep deprivation, and wake. *Sleep* 2007; 30:1437-1443.
31. Cardinali DP, Pevet P. Basic aspects of melatonin action. *Sleep medicine reviews* 1998; 2:175-190.
32. Dijk DJ, Cajochen C. Melatonin and the circadian regulation of sleep initiation, consolidation, structure, and the sleep EEG. J *Biol Rhythms* 1997; 12:627-635.
33. Van Someren EJ, Nagtegaal E. Improving melatonin circadian phase estimates. *Sleep medicine* 2007; 8:590-601.
34. Bandin C, Scheer FA, Luque AJ, Avila-Gandia V, Zamora S, Madrid JA, Gomez-Abellan P, Garaulet M. Meal timing affects glucose tolerance, substrate oxidation and circadian-related variables: A randomized, crossover trial. *Int J Obes* (Lond) 2014; .
35. Scheer FA, Buijs RM. Light affects morning salivary cortisol in humans. *The Journal of clinical endocrinology and metabolism* 1999; 84:3395-3398.
36. Garcia-Prieto MD, Tebar FJ, Nicolas F, Larque E, Zamora S, Garaulet M. Cortisol secretary pattern and glucocorticoid feedback sensitivity in women from a Mediterranean area: relationship with anthropometric characteristics, dietary intake and plasma fatty acid profile. *Clinical endocrinology* 2007; 66:185-191.
37. Caufriez A, Moreno-Reyes R, Leproult R, Vertongen F, Van Cauter E, Copinschi G. Immediate effects of an 8-h advance shift of the rest-activity cycle on 24-h profiles of cortisol. American journal of physiology *Endocrinology and metabolism* 2002; 282:E1147-1153.
38. Konopka RJ, Benzer S. Clock mutants of Drosophila melanogaster. *Proc Natl Acad Sci* USA 1971; 68:2112-2116.
39. Bargiello TA, Jackson FR, Young MW. Restoration of circadian behavioural rhythms by gene transfer in Drosophila. *Nature* 1984; 312:752-754.
40. Garaulet M, Corbalan MD, Madrid JA, Morales E, Baraza JC, Lee YC, Ordovas JM. CLOCK gene is implicated in weight reduction in obese patients participating in a dietary programme based on the Mediterranean diet. *Int J Obes* (Lond) 2010; 34:516-523.

41. Bandin C, Martinez-Nicolas A, Ordovas JM, Ros Lucas JA, Castell P, Silvente T, Madrid JA, Garaulet M. Differences in circadian rhythmicity in CLOCK 3111T/C genetic variants in moderate obese women as assessed by thermometry, actimetry and body position. *Int J Obes* (Lond) 2012:1-7.
42. Garaulet M, Esteban Tardido A, Lee YC, Smith CE, Parnell LD, Ordovas JM. SIRT1 and CLOCK 3111T>C combined genotype is associated with evening preference and weight loss resistance in a behavioral therapy treatment for obesity. *Int J Obes* (Lond) 2012.
43. Johnson CH. Forty years of PRCs--what have we learned? *Chronobiology international* 1999; 16:711-743.
44. Bjarnason GA, Jordan RC, Wood PA, Li Q, Lincoln DW, Sothern RB, Hrushesky WJ, Ben-David Y. Circadian expression of clock genes in human oral mucosa and skin: association with specific cell-cycle phases. *The American journal of pathology* 2001; 158:1793-1801.
45. Cajochen C, Jud C, Munch M, Kobialka S, Wirz-Justice A, Albrecht U. Evening exposure to blue light stimulates the expression of the clock gene PER2 in humans. *The European journal of neuroscience* 2006; 23:1082-1086.
46. Gomez-Santos C, Gomez-Abellan P, Madrid JA, Hernandez-Morante JJ, Lujan JA, Ordovas JM, Garaulet M. Circadian rhythm of clock genes in human adipose explants. *Obesity* (Silver Spring) 2009; 17:1481-1485.
47. Garaulet M, Ordovas JM, Gomez-Abellan P, Martinez JA, Madrid JA. An approximation to the temporal order in endogenous circadian rhythms of genes implicated in human adipose tissue metabolism. *Journal of cellular physiology* 2011; 226:2075-2080.
48. Minors DS, Waterhouse JM. The use of constant routines in unmasking the endogenous component of human circadian rhythms. *Chronobiology international* 1984; 1:205-216.
49. Waterhouse J, Jones K, Edwards B, Harrison Y, Nevill A, Reilly T. Lack of evidence for a marked endogenous component determining food intake in humans during forced desynchrony. *Chronobiology international* 2004; 21:445-468.
50. Halberg F. Frequency spectra and cosinor for evaluating circadian rhythms in rodent data and in man during Gemini and Vostok flights. *Life sciences and space research* 1970; 8:188-214.
51. van Someren EJ, Hagebeuk EE, Lijzenga C, Scheltens P, de Rooij SE, Jonker C, Pot AM, Mirmiran M, Swaab DF. Circadian rest-activity rhythm disturbances in Alzheimer's disease. *Biol Psychiatry* 1996; 40:259-270.
52. Ko CH, Takahashi JS. Molecular components of the mammalian circadian clock. *Human molecular genetics* 2006; 15 Spec No 2:R271-277.
53. Naismith SL, Rogers NL, Mackenzie J, Hickie IB, Lewis SJ. The relationship between actigraphically defined sleep disturbance and REM sleep behaviour disorder in Parkinson's Disease. *Clinical neurology and neurosurgery* 2010; 112:420-423.
54. Baron KG, Reid KJ, Kern AS, Zee PC. Role of sleep timing in caloric intake and BMI. *Obesity* (Silver Spring) 2011; 19:1374-1381.
55. Eisenmann JC, Ekkekakis P, Holmes M. Sleep duration and overweight among Australian children and adolescents. *Acta paediatrica* (Oslo, Norway : 1992) 2006; 95:956-963.
56. Urban R, Magyarodi T, Rigo A. Morningness-eveningness, chronotypes and health-impairing behaviors in adolescents. *Chronobiology international* 2011; 28:238-247.
57. Erren TC, Reiter RJ. Axelrod, the pineal and the melatonin hypothesis: lessons of 50 years to shape chronodisruption research. *Neuro endocrinology letters* 2010; 31:585-587.
58. Horne JA, O O. A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. *International journal of chronobiology* 1976; 4.
59. Lopez-Minguez J, Ordonana JR, Sanchez-Romera JF, Madrid JA, Garaulet M. Circadian system heritability as assessed by wrist temperature: A twin study. *Chronobiology international* 2014:1-10.
60. Bandin C, Martinez-Nicolas A, Ordovas JM, Ros Lucas JA, Castell P, Silvente T, Madrid JA, Garaulet M. Differences in circadian rhythmicity in CLOCK 3111T/C genetic variants in moderate obese women as assessed by thermometry, actimetry and body position. *Int J Obes* (Lond) 2013; 37:1044-1050.