CIRCADIAN PHASE ASESSMENT BY AMBULATORY MONITORING IN HUMANS: CORRELATION WITH DIM LIGHT MELATONIN ONSET

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Running Head: Circadian phase by ACM correlation with DLMO

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ABSTRACT

The increased prevalence of circadian disruptions due to abnormal coupling between internal and external time makes the detection of circadian phase in humans by ambulatory recordings a compelling need. Here, we propose an accurate practical procedure to estimate circadian phase with the least possible burden for the subject, that is, without the restraints of a constant routine protocol or laboratory techniques such as melatonin quantification, both of which are standard procedures.

In this validation study, subjects (N = 13) wore ambulatory monitoring devices, kept daily sleep diaries and went about their daily routine for 10 days. The devices measured skin temperature at wrist level (WT), motor activity and body position on the arm, and light exposure by means of a sensor placed on the chest. Dim light melatonin onset (DLMO) was used to compare and evaluate the accuracy of the ambulatory variables in assessing circadian phase. An evening increase in WT: WTOnset (WTOn) and "WT increase onset" (WTiO) was found to anticipate the evening increase in melatonin, while decreases in motor activity (Activity Offset or AcOff), body position (Position Offset (POff)), integrative TAP (a combination of WT, activity and body position) (TAPOffset or TAPOff) and an increase in declared sleep propensity were phase delayed with respect to DLMO. The phase markers obtained from subjective sleep (R=0.811), WT (R=0.756) and the composite variable TAP (R=0.720) were highly and significantly correlated with DLMO. The findings strongly support a new method to calculate circadian phase based on WT (WTiO) that accurately predicts and shows a temporal association with DLMO. WTiO is especially recommended due to its simplicity and applicability to clinical use under conditions where knowing endogenous circadian phase is important, such as in cancer chronotherapy and light therapy.

INTRODUCTION

The circadian timing system, a hierarchically organized network of structures responsible for generating circadian rhythms is driven, in mammals, by a circadian pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus. It allows organisms to adjust their physiology by anticipating daily environmental changes instead of just responding to them in a reactive manner. Thus, under natural conditions, endogenous circadian rhythms are entrained to the 24-h light-dark cycle (for a review, see Roenneberg et al., 2003).

In humans, daily rhythms can be observed in a variety of molecular, physiological, and psychological processes, such as gene expression, body temperature, heart rate, melatonin production as well as sleep, mood and higher cognitive functions (for a review, see Schmidt et al., 2007).The cycle of sunrise and sunset has provided a reliable time cue for many thousands of years, until recently when modern life and the "24-hour society" intensified exposure to artificial lighting environments, both during the day and at night, as people engage in shift work and leisure time is displaced towards the nighttime hours. These conditions can cause continuous phase shifts and favor the misalignment between the internal temporal organization of physiology and behavior with environmental cues. The repeated disruption of the circadian system in humans, or chronodisruption (CD) (Reiter et al., 2007; Erren et al., 2010), has been associated with several health impairments, such as metabolic syndrome (Reiter et al., 2011; Garaulet and Madrid, 2010), cardiovascular diseases (Knutsson & Boggild, 2000), cognitive impairments (Cho et al., 2000) and a higher incidence of breast (Schernhammer et al., 2001; Davis et al., 2001), colorectal (Schernhammer et al., 2003) and prostate (Kubo et al., 2006; Conlon et al., 2007) cancers, among other disorders.

The possibility of assessing circadian phase in an individual would allow us to evaluate the severity of CD and to diagnose circadian rhythm sleep disorders (Pandi-Perumal et al., 2007), and thus apply countermeasures to reduce the misalignment between internal and external time; for example, adapting timetable of shift-workers to their chronotype, personalizing chronotherapy in cancer patients (Lévi & Okyar, 2011), or optimizing the treatment and effectiveness of light therapy (Sharkey et al., 2011; Dewan et al., 2011; Zeitzer et al., 2012). Questionnaires have been developed that provide an estimate of chronotype (Munich ChronoType Questionnaire (Roenneberg et al., 2003) or diurnal preference (Horne-Östberg morningness-eveningness Questionnaire (Horne & Östberg, 1976). The accuracy of such questionnaires, however depends on self-report, good recall and the ability of the subject to complete them correctly and honestly (thus less useful for cognitively impaired and/or older individuals). Besides these subjective procedures, objective measurements of certain variables have been used as circadian rhythm markers to characterize a subject's circadian phase. Marker

rhythms, such as core body temperature (CBT), motor rest-activity or cortisol and melatonin secretion, are commonly used, although the melatonin rhythm has long been considered the "gold standard" to assess the circadian phase (Benloucif et al., 2008; Arendt, 2003). The most commonly used melatonin parameter is DLMO (Dim Light Melatonin Onset) (Lewy & Sack, 1989; Lewy et al., 1999) which although can be determined by an in-home saliva sampling protocol (Benloucif et al., 2008), requires the subject's active collaboration, and modification of the subject's normal routine (dim light conditions, posture control). Moreover, hormone (melatonin, cortisol) measurement is relatively expensive and requires access to laboratory facilities. Core body temperature monitoring is not problem-free, since it is more sensitive to masking effects than melatonin or cortisol measurements, and requires the use of probes that must be worn inside the body, such as rectal probes, or ingested, such as telemetry pills that record CBT (Teunissen et al., 2012). Alternatively, motor rest-activity has been also used to indirectly evaluate the sleep-wake cycle, but again, it presents its own masking and artifacts, and is difficult to differentiate between the onset of nocturnal rest and sensor removal for bathing before going to bed, bed partner movements, sleeping when travelling in a car, etc. (Sadeh & Acebo, 2002; Acebo & Lebourgeois, 2002).

Recently, our group proposed wrist skin temperature (WT) as a possible alternative method for evaluating circadian timing and phase in humans under normal living conditions, since WT increases during rest periods associated with sleep and decreases during activity periods in proportion to the level of arousal (Sarabia et al., 2008). This WT rhythm is (in part) the result of an alternating balance between parasympathetic (vasodilation) and sympathetic (vasoconstriction) actions on peripheral skin vessels, driven by the central SCN clock (Krauchi, 2007; Lack et al., 2008; Morris et al., 2009). Masking factors, such as environmental temperature (Sarabia et al., 2008) and posture (Blázquez et al., 2012), however, may reduce its accuracy when used by itself to evaluate circadian function. Considering that all single variables are affected to some extent by masking, new approaches to overcome potential artifacts during ambulatory measurements have been developed. These include circadian monitoring procedures using multivariable recordings (Kolodyazhniy et al., 2011), together with the use of integrated algorithms, such as TAP (a combination of WT, activity and body position) (Ortiz-Tudela et al., 2010). Finding an accurate, simple and inexpensive way to detect circadian phase in humans under free-living conditions for use in clinical practice, however, still poses an unresolved challenge.

Thus, the objective of the present work was to optimize a practical procedure to assess circadian phase in humans under free living conditions, using questionnaires and the ambulatory monitoring of light exposure, WT, motor activity (A), body position (P) and subjective sleep by comparison, for the first time, with the gold standard circadian phase marker, DLMO.

METHODS

Subjects

Study subjects were 13 healthy non-smoking volunteers (7 men, 6 women) between 19 and 35 years of age (27.2 ± 1.5 yrs.). All subjects displayed normal sleep patterns, had no medical or mental disorders, and were not taking any medication that could affect circadian rhythms, as determined from general health questionnaires completed during the screening period. None of the subjects were shift workers, had crossed more than two time zones in the two months prior to admission nor were color blind. Volunteers received appropriate information about the study protocol, signed a written informed consent form before being enrolled in the study and were compensated for their participation. This research project was approved by the University of Surrey Ethics Committee and abides by the principles set out by the Declaration of Helsinki. Experimental protocol is conform to international ethical standards (Portaluppi et al., 2010)

Ambulatory Monitoring Devices

The volunteers were instructed to maintain their habitual lifestyle for approximately 10 days parallel to the non-invasive circadian ambulatory monitoring of WT, A, P and light exposure (L) patterns. These ambulatory measurements always started on a Friday, between 12:00 h and 18:00 h. In addition, participants were instructed to complete a daily sleep diary designed by the Chronobiology team at the University of Surrey.

The WT rhythm was assessed continuously for 10 days, using a temperature data logger (Thermochron iButtonDS1921H, Dallas, Maxim) with a sensitivity of 0.125 °C and programmed to sample once every 10 minutes. It was attached to a double-sided cotton sport wristband, and the sensor surface was positioned over the inside of the wrist on the radial artery of the non-dominant hand, as previously described (Sarabia et al., 2008).

P and A rhythms were assessed using an actimeter (HOBO, Pendant G Acceleration Data Logger UA-004-64, Massachusetts, USA) placed on the non-dominant arm by means of a sports band, with its X-axis parallel to the humerus bone as already described (Ortiz-Tudela et al., 2010). The device was programmed to sample once every 30 seconds. The information stored in the actimeter was transferred through an optical USB Base Station (MAN-BASE-U-4, HOBO, Massachusetts, USA) to a personal computer, using the software provided by the manufacturer (HOBOware 2.2). The actimeter provided information on 2 variables: motor activity (A) and body position (P). Motor activity was expressed as the rate of change in degrees per minute, and body position was calculated as the angle between the X-axis of the actimeter

and the horizontal plane. Thus, P oscillated between 0 degrees for maximum horizontality and 90 degrees for maximum verticality (see (Ortiz-Tudela et al., 2010), for details).

In addition, all subjects were required to wear a HOBO Pendant Temperature/Light Data Logger UA-002 64 (Onset Computer, Bourne, HOBO, Massachusetts, USA) on a necklace and over their clothing during waking hours, and to put it on the bedside table while sleeping in order to record light exposure as previously described (Martínez-Nicolás et al., 2011). According to the manufacturer's specifications, the data logger has a measurement range of 0 to 320,000 lux, a memory capacity for up to 28,000 values recorded at preprogrammed 30-second intervals, and a light spectrum wavelength recording capacity of 150–1200 nm, which is broader than the sensitivity of the human eye. In order to validate the HOBO light sensor, a LX 101 lux meter (3E NDT; Pasadena, Texas, USA) was used to make a set of simultaneous recordings in different environments (data not shown). Readings from both devices demonstrated a strong, significant positive correlation at different intensities ($R=0.997$, $p<0.01$). A high degree of repeatability was also observed when recordings were simultaneously performed with two different HOBO sensors ($R=0.998$, $p<0.01$).

DLMO test and melatonin quantification

One evening within the 10-day recording period (on a Wednesday), volunteers were required to stay at home and remain seated in dim light (e.g. a single table lamp on the other side of the room), starting at 18:30 h, while collecting saliva samples every 30 minutes starting at 19:00 h, until one hour after their usual bedtime (modified from (Pullman et al., 2012); for a review, see (Pandi-Perumal et al., 2007). Following saliva collection into labeled vials, subjects were asked to freeze the samples until they were brought to the laboratory in thermal ice-cooled bags (also provided). Melatonin concentrations in the subject's saliva were quantified by radioimmunoassay (Stockgrand Ltd., University of Surrey, Guildford, UK), with a detection limit of 0.44 pg/ml. The intra-assay coefficients of variation (CV) for the low (mean ± SD, 10.4 ± 0.6 pg/ml), medium (34.7 ± 0.1 pg/ml) and high (66.7 ± 4.6 pg/ml) pools were 6.1, 0.3 and 6.9 % respectively. All samples collected by each volunteer were measured in duplicate in a single assay.

Questionnaires

In order to compare the phase estimate obtained from the ambulatory physiological recordings, DLMO and the diurnal preference obtained from the subjective tests commonly used in literature, the subjects were required to complete the Horne-Östberg (HO) morningness eveningness questionnaire (Horne & Östberg, 1976). In addition, in order to compare the circadian phase assessment with other aspects related to the sleep-wake cycle, subjects also

completed the Pittsburgh Quality Sleep Index (PQSI) questionnaire (Buysse et al., 1988) and the Epworth Sleepiness Scale (EES) questionnaire (Jonhs et al., 1991), as the quality of sleep and daytime sleepiness have been proposed to be related to circadian phase and diurnal preference (Lázár et al., 2012; Reid et al., 2012; Tzischinsky & Shochat, 2011).

Data Analysis

Ambulatory circadian monitoring

Firstly, data from WT, A/P and L sensors were filtered in order to eliminate artifacts, such as those produced by temporarily removing the sensors.

The TAP algorithm was calculated as previously described (Ortiz-Tudela et al., 2010), in order to integrate the information provided by temperature, activity and position sensors. Thus, a score of 0 corresponds to complete rest and sleep (highest distal skin temperature, lowest activity and horizontal position), whereas a score of 1 corresponds to periods of high arousal and movement. In order to obtain the same sampling frequency for all variables for the purpose of computing TAP values, A and P data were added up and averaged, respectively, in 10-minute intervals to agree with the sampling rate of WT. The same procedure was carried out with the light exposure data (Martinez-Nicolas et al., 2011).

Sleep log data were converted into a binary code, in which 1 corresponded to a declared resting period and 0 to an activity period. Sleep probability indicates the percentage of individuals asleep at any given time (Sarabia et al., 2008). In the case of individual curves, sleep probability indicates the percentage of days when the subject was asleep at a given time. Mean WT, A, P, TAP, sleep, and L patterns were calculated per individual and for the whole group. All data are expressed as mean ± the standard error of the mean (SEM).

When daily mean waveforms were calculated, WT, A, P, L and sleep data obtained during the saliva sampling period (from 19:00 to 03:00 h) for assessment of DLMO were discarded, since subjects were forced to remain seated in dim light, and therefore were not under free-living conditions. To minimize intra-individual differences in melatonin production, individual melatonin concentrations were normalized as a percentage of the maximum melatonin level per subject, and then averaged for the whole group.

Phase marker calculation

Non-parametric analysis (Van Someren et al., 1999) was used to calculate the following phase markers: the central timing of the ten, five or two consecutive hours with the lowest values (L10, L5 and L2, respectively), and that of the ten, five or two consecutive hours with the highest values of each recorded variable (M10, M5 and M2, respectively).

The objective criterion for establishing the DLMO was that described by Voultsios et al. (1997) (Voultsios et al., 1997), i.e., determining a threshold from the mean plus 2 standard deviations of three baseline samples. To obtain a phase marker estimation similar to DLMO for ambulatory recorded variables, L2 for WT and sleep probability "onsets" (i.e., the mean value of two hours of the lowest values between 16:00 and 06:00 h for WT or sleep probability) plus 2 standard deviations was calculated. The onset time when these values were achieved was considered to be the WT (WTOn) and sleep (SleepOn) onset. The parameters M2 for A (AcOff), P (POff) and TAP (TAPOff) (i.e., the mean value of two hours of the highest values between 16:00 and 06:00 h for A, P and TAP) minus 2 standard deviations was also calculated. Again, the time when these values were achieved was considered to be the offset for the corresponding variable.

In addition, several composite phase indexes were calculated: $WT_{On}P_{Off}$ by averaging the time for WTOn and POff; $WT_{On}Ac_{Off}$ by averaging the time for WTOn and AcOff; AP_{Off} by averaging the time for AcOff and POff; and finally, $SleepWT_{On}$ by averaging the time of SleepOn and WTOn.

An additional phase marker, "wrist temperature increase onset" (WTiO), is also proposed. In order to determine WTiO, the percentage WT increase between L2 time (between 16:00 and 06:00 h) and the DLMO time with respect to the total WT increase between L2 and M5 was calculated for each subject and then averaged for the group. This mean percentage (35%) was then added to the individual temperature value for L2, and its corresponding time was determined to be WTiO.

Statistical analysis

Individual cross-correlations were performed between salivary melatonin evening levels and the rest of the variable patterns in order to obtain the phase relationship between the melatonin evening increase and the evening increase (or decrease) in the remaining variables (WT and sleep probability, A, P, TAP, and light exposure). An R-value for each phase difference and subject was obtained and these were then averaged for the entire group. The maximum averaged R-value corresponds to a specific phase difference for each variable. In addition, the individual phase relationships between the maximum individual R-values of each variable and the melatonin evening increase were also averaged.

Linear correlations were calculated between phase markers and DLMO.

The Effect size analysis (Cohen's d) was only performed on those phase markers that temporally coincided with DLMO. The formula used to calculate Cohen's d was:

$$d = \frac{|\bar{x}_1 - \bar{x}_2|}{\sqrt{\frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}}}$$

The accuracy of the predicted DLMO phase, obtained from the regression formulas previously calculated, was estimated for each phase marker and subject by calculating the prediction error as follows:

Error= absolute value (DLMOtime – Predicted phase)

Individual prediction errors were then averaged for each phase marker in terms of absolute values, and the error range was calculated and expressed in real values.

Data were analyzed using Excel® (Microsoft Office 2007) and SPSS® v15.0 software. Values of $p < 0.05$ were considered to be statistically significant. All results are expressed as mean ± standard error of the mean (SEM).

RESULTS

Ten-day average records for each variable captured by ambulatory circadian monitoring (ACM) are shown in Fig 1. As expected, WT (Fig. 1A) rose at the beginning of the night and remained high during sleep, decreasing immediately after waking. When the saliva sampling protocol for the DLMO test took place (grey area), the subjects went to bed later than usual, and WT started to increase as observed on other nights, but the nocturnal maximum values were only achieved at the end of the night. Motor activity, generally high during the day (Fig. 1B), also decreased during the evening when the saliva sampling for the DLMO test was performed, confirming that the subjects remained seated with low motor activity during that period. This was observed in the body position recordings (Fig. 1C), which showed more vertical positions during the saliva sampling period than on the other (normal) nights when the subjects remained lying down, although without reaching the vertical levels normally achieved during daytime hours. Similar results were obtained for the TAP composite (Fig. 1D), which showed a robust daily pattern, with high values during the daytime and the opposite during nighttime; the interference in the normal routine by the saliva sampling protocol was also observed on the sixth day. As expected, a slight delay in the onset of sleep (Fig. 1E) was observed on the evening of the saliva sampling, as compared to the remaining days, since the subjects were required to remain awake in order to collect their last saliva sample after their usual bed time.

Figure 1F represents the subjects' light exposure pattern. The highest values (>200 lux) were recorded during the day, decreasing slightly at sunset, and with a period of intermediate light intensity (<100 lux) until bedtime, at which point light exposure decreased to almost 0 lux. On the evening of the saliva sampling, light exposure was less than 5 lux, clearly lower than on the other days, which confirmed that the subjects remained in dim light during the saliva sampling period, as required. Since the saliva sampling protocol required that the subjects remain seated in dim light from 18:30 h until one hour after their habitual bed time, it specifically interfered with the usual patterns for WT, A, P, sleep and light exposure, and thus the day of the DLMO saliva sampling protocol was excluded from the remaining analyses.

The mean waveforms (average of 9 days) for all ACM variables and self-reported sleep from either a representative subject or the whole population are presented in Figures 2A and 2B, respectively. Melatonin and WT began to increase prior to lights out at approximately the same time. Motor activity, P and TAP, however, started to decrease after melatonin levels had already increased. In the case of sleep probability, only once melatonin had approximately reached its maximum value, were 100% of the subjects sleeping. Light exposure showed a bimodal pattern, with two declines that probably corresponded to the change from indoor lighting influenced by sunlight to dimmer artificial light. Light levels finally decreased to zero, coinciding with the

beginning of the sleep period in darkness. Melatonin started to increase once the subjects were exposed to the dimmer artificial light conditions.

In order to evaluate the phase relationship between evening melatonin production and ACM variable rhythms, cross-correlations were calculated. Mean R-values resulting from the different individual phase differences were obtained and are presented in Fig. 3. In addition, maximum R-values were selected for each subject, and the corresponding individual phase differences were then averaged for the entire group. WT was slightly phase advanced with respect to the increase in evening melatonin increase (-0.69 ± 0.59 h) (Fig. 3A), while A (Fig. 3B), P (Fig. 3C) and sleep (Fig. 3E) were delayed by about two hours with respect to melatonin evening increase (A, 2.04 ± 0.36 h; P, 2.42 ± 0.48 h; sleep, 1.36 ± 0.25 h, respectively). The composite variable TAP was phase-delayed by approximately one hour (0.88 ± 0.45 h, Fig. 3D).

Correlations between the different phase markers, obtained from ACM and DLMO, are shown in Table 1. Each individual variable exhibited at least one phase marker that was significantly correlated with DLMO. For WT, the highest correlation was found for L5 and WTiO. L5 was also the best phase marker for activity, whereas M5 was the best phase marker for body position, TAP and sleep. Considering the composite indexes, $SleepWT_{On}$ showed the highest correlation with DLMO.

To evaluate the temporal relationship between the phase markers and DLMO, the effect size (Cohen's d) was calculated for the differences among them. All phase markers shown in Figure 4 associated with DLMO at statistically significant levels, except for AcOff ($p<0.05$). The highest temporal relationship was observed for $WT_{On}P_{Off}$ (d = 0.02), $SleepWT_{On}$ (d = 0.36) and WTiO (d = 0.37). Thus, the phase markers that met both criteria (a strong correlation with DLMO and a high temporal association with DLMO) were WTiO, as an individual variable, and $SleepWT_{On}$ and $WT_{On}P_{Off}$, as composite indexes.

A comparison of the accuracy of the different methods at predicting circadian phase (ACM *vs*. DLMO) is shown in Table 2. Using the equation of linear correlation between each ACM phase marker and DLMO, four parameters yielded an error range lower than 200 min: WTiO (154 min), Sleep M5 (157 min), WT L5 (188 min) and TAP M5 (195 min).

In our population, the frequency of diurnal preference was: 54% for indefinite, 38% for moderate morningness and 8% for moderate eveningness. Despite its subjectivity, scores on the Horne & Östberg (HO) morningness/eveningness test correlated well with DLMO. Although the Sleep Quality Index (PSQI) is not a circadian phase predictor, its score was also well correlated with DLMO, with the later DLMO correlating with worse sleep quality (higher PSQI

scores). However, the Epworth Sleepiness Scale (ESS), which provides a widely accepted daytime sleepiness score, was not correlated with DLMO (Table 3).

DISCUSSION

Our results indicate that circadian phase in humans can be reliably assessed by ambulatory circadian monitoring (ACM), while subjects maintain their normal life style with minimal discomfort and, unlike other studies (Kolodyazhniy et al., 2011), without the complication of having to wear many sensors. Although all the individual variables recorded using ACM could be used to predict DLMO, the phase markers for sleep, WT and the composite variable TAP showed the highest correlation with DLMO. Moreover, phase-markers from WT (SleepWT_{On}, WTiO and $WT_{On}P_{Off}$) showed a high temporal association with the gold standard DLMO.

The ambulatory circadian monitoring (ACM) proposed here provides a 10-day record that permits evaluating the subjects' habits while they carry on their normal lives. Furthermore, it is sensitive enough to discriminate non-representative days, such as when a fixed protocol (like the DLMO protocol) disrupts the normal circadian pattern, or on weekends. Although the nature of the saliva sampling protocol required an alteration of the subjects' normal lives, this alteration only affected the day of melatonin sampling, while the subsequent days of the study showed a high degree of stability and were similar to those prior to the sampling. Moreover, saliva sampling during the study period itself represents an advantage over other studies, in which saliva sampling took place outside of the study period (Kolodyazhniy et al., 2011); firstly, because protocol compliance can be monitored; and secondly and most importantly, because DLMO and ACM phase markers are obtained during the same period, thus allowing any phase shifts to be discarded. Although in our protocol subjects were not strictly controlled as under laboratory conditions, the ACM data revealed that the stipulated conditions of light exposure, activity and body position for DLMO assessment (Deacon & Arendt, 1994), were followed by the volunteers. Ambulatory DLMO determinations under similar conditions (illuminance < 20 lux *vs*. <10 lux in our study) have been previously validated by other researchers (Pullman et al., 2012). Moreover the fact that the subjects were living at home implies that they were not exposed any laboratory stress factors.

All subjects showed a very stable wrist skin temperature rhythm with a rise in the evening and a sharp decrease near waking time, thus remaining high during the night. This periodic pattern throughout the 24 h day has been partially explained as a result of an alternating balance between parasympathetic (vasodilatation) and sympathetic (vasoconstriction) actions on peripheral skin vessels, driven by the SCN (Krauchi et al., 2007; Lack et al., 2008; Morris et al., 2009; Blázquez et al., 2012). Distal skin temperature has been previously described as being correlated and phase-advanced with respect to CBT, suggesting that heat loss from the extremities may drive the CBT circadian rhythm (Gradisar and Lack, 2004; Krauchi & Wirz-Justice, 1994; Van Someren, 2003; Raymann et al., 2005). However, the existence of masking

factors such as physical activity, body position, light exposure, environmental temperature and sleep have also been described as acting on this variable (Scheer et al., 1999; Waterhouse et al., 1999; Cajochen et al., 2000; Krauchi and Wirz-Justice, 2001; Wakamura & Tokura, 2001; Krauchi, 2007; Reilly & Waterhouse, 2009; Martinez-Nicolas, 2011).

Motor activity measured by a portable accelerometer has long been considered an important marker of rest-activity rhythms, and constitutes a standard technique for non-invasive measurements of chronotype-related rest-activity cycles, as well as for use in the diagnosis of patients with sleep-wake disturbances (Ancoli-Israel et al., 2003; Wirz-Justice, 2007). Body position (P) and the integrative variable TAP have recently been proposed as variables to assess the timing of the circadian system (Ortiz-Tudela et al., 2010). In the present results, each variable exhibited the expected periodic pattern, with high levels during the day, in the case of A (Kushida et al., 2001; Lötjönen et al., 2003), P and TAP (Ortiz-Tudela et al., 2010), or during the night, in the case of declared sleep probability (Sarabia et al., 2008; Kolodyazhniy et al., 2011).

To be considered as reliable circadian rhythm marker, candidate variables have to accurately predict circadian phase, as in the case of DLMO. However, to achieve clinical relevance, such variables should be able to be measured in a simple way, non-invasively, under ambulatory conditions and they should estimate circadian phase directly, with no or minimal mathematical algorithms required. Since the main goal of this study was to evaluate the usefulness of ambulatory circadian monitoring (ACM) to assess human circadian phase under ambulatory conditions, we used four different methods to determine the relationship between ACM variables and DLMO: 1) cross-correlation analysis; 2) evaluation of phase markers in the ACM non-parametric indexes; 3) a proposed equivalent phase marker for each variable, based on how DLMO is calculated from the nocturnal increase in melatonin (variable-onset for WT and sleep and variable-offset for A, P and TAP); 4) the timing of a 35% increase in WT (WTiO, using L2 to M5 as reference). Finally, all these methods were compared in terms of accuracy in predicting DLMO.

Among all the ACM variables investigated, only the evening increase in WT showed any anticipation with respect to the melatonin increase (in dim light), while the evening drop of A, P and L were phase delayed with DLMO. The close correlation between the phase of these rhythmic variables recorded by ACM, sleep logs and melatonin was consistent with the findings of previous studies (Dijk & Czeisler, 1995; Duffy et al., 1997; Wyatt et al., 1999). However, no previous studies have reported a phase relationship between WT, P and the composite variable TAP and DLMO. Furthermore, it is especially interesting that skin WT was the only variable that preceded the rise in melatonin. This would contradict the results obtained by Krauchi et al. in 2000, in which the increase in distal temperature took place shortly after DLMO. This

discrepancy, however, could be explained by the different temperature sensor locations used in the two studies (the center of the back of both hands in their case, *vs*. the ventral side of the wrist in ours). The rest of the decreases (A, P, TAP and L) and the increased sleep probability were delayed with respect to the evening increase in melatonin. According to our results, this could indicate that, while influenced by masking factors, wrist skin temperature is predictive of, and not only reactive to, melatonin phase.

When we performed linear correlations between DLMO and the non-parametric phase markers calculated for the ACM recorded variables, the best correlation was obtained between declared sleep M5 timing (the central time for maximum sleep probability) and DLMO. This agrees with results previously obtained by Martin and Eastman (Martin & Eastman, 2002), which found the best correlation between DLMO and the midpoint of sleep. Kolodyazhniy et al. (Kolodyazhniy et al., 2011) also found a strong correlation between midsleep corrected for sleep deficit and melatonin phase, which would agree with the strong correlation found in our study between motor activity L5 (the timing of the midpoint of the 5 hours with the lowest activity levels). However, in the present study no significant correlation was found between DLMO and sleep onset, which also agrees with the findings of Burgess et al. (Burgess & Eastman, 2003). Regarding the rest of the variables (WT, P and the integrative TAP), DLMO seemed to be more closely correlated with phase markers representative of the wakefulness period. One possible explanation is that WT starts to increase immediately after its lowest value (L5 or L2) during the daytime. In previous studies (Wyatt et al., 1999), a stable phase relationship was found between melatonin onset and the initial decline of CBT. It should be noted that although the highest correlation was found between DLMO and a phase marker obtained from the sleep pattern, this was assessed by sleep logs, which must be completed correctly and honestly. Therefore, in some cases (especially clinical cases), an objective method would be required that does not rely on the subject´s active participation. In addition, it must be emphasized that this is the first time that a phase marker obtained from composite TAP (M5) has been correlated with DLMO.

Since DLMO is a well-established circadian phase marker, as is its calculation, we estimated an equivalent phase marker for each ACM variable in a similar manner to the calculation of DLMO. However, in spite of a high association in time between DLMO and WTOn, POff, TAPOff and SleepOn (the latter already reported by Lushington et al. (Lushington et al., 1996), only AcOff was significantly correlated with DLMO. This is in agreement with previous studies, in which the phase of actigraphically-derived sleep onset time was consistent with 6-sulphatometoxymelatonin (Middleton et al., 1996; Middleton et al., 1997) and salivary DLMO times (Carskadon et al., 1997). Contrary to what was observed for single variables, composite indexes obtained from the combination of two single ACM indexes, such

as SleepWT_{On}, $WT_{On}Ac_{Off}$, AP_{Off} and $WT_{On}P_{Off}$, were all significantly correlated with DLMO. It must be recognized, however, that only those indexes obtained from WT (WTOn and WTiO) preceded DLMO, which was consistent with the results obtained from the cross-correlation analysis.

In order to eliminate the influence of inter-individual variation in the slope of the evening increase in WT temperature when calculating phase markers based on WT, we propose another method based on the time when a 35% increase occurs in WT, from the moment of L2 to M5 (WTiO). This new parameter (WTiO) is closely correlated with and shows the best prediction for DLMO, while exhibiting a temporal coincidence with DLMO. This is in accordance with Kolodyazniy et al. (Kolodyazniy et al., 2011), who obtained CBT as the most accurate variable for determining melatonin phase, with an error range of 174 *vs*. 154 min in our study. While the M5 phase marker obtained from the integrative variable TAP also produced good accuracy rates for predicting DLMO (<200 min), WT (and thus WTiO) was chosen since it is the only variable that was phase advanced with respect to DLMO. Although WT is affected by masking factors, it would be the variable least influenced by behavior of those considered in this study (Ortiz-Tudela et al., 2010). Additional studies with more subjects of a range of chronotypes and ages would be needed to validate this calculation method.

Previous studies have investigated the relationship between the morningness-eveningness HO score (Horne & Östberg, 1976) and other phase markers, such as DLMO (Martin & Eastman, 2002; Mongrain et al., 2004; Goulet et al., 2007). As expected, our results showed a strong inverse correlation between the HO score and DLMO time, which implies that the more evening type the subject is, the later DLMO occurs. Although diurnal preference were not a criterion for recruitment into the current study, the correlation found agrees with previous studies (Duffy et al., 1999; Griefahn, 2002; Mongrain et al., 2004; Goulet et al., 2007) in which chronotype was an inclusion criterion. We also found a positive correlation between the PSQI score and DLMO, which indicates that subjects with an earlier DLMO have better sleep quality than those with a later DLMO, in agreement with previously cited works that described a delayed DLMO in poor sleepers, as compared to good sleepers (Olbrich & Dittmar, 2011) in an old woman population. Eveningness has also been previously described in young adults and adolescents as a predictor of short sleep duration, high levels of daytime sleepiness (Chung & Cheung, 2008; Tzischinsky & Shochat, 2011), and sleep-problem behaviors (Tzischinsky & Shochat, 2011). Along the same lines, evening types have been reported to present lower sleep efficiency (Lehnkering & Siegmund, 2007), higher levels of fatigue (Taylor et al., 2011), and shorter sleep duration (Tzischinsky & Shochat, 2011).

We have thus confirmed the usefulness of sleep logs and the Horne-Östberg morningness-eveningness questionnaire in estimating the circadian phase. However, despite their quality, their accuracy depends on the ability of the subject to complete them correctly and honestly. It is noteworthy that in this and other studies, volunteers were compensated for their participation, so they were highly motivated to complete the questionnaires correctly. In clinical cases, however, completing the questionnaires could be a problem for patients with cognitive abilities impairments, for example. Moreover, questionnaires do not provide information about the degree of adherence to a circadian treatment, while an ambulatory system that permits monitoring the subject over a long period does. We thus propose that these questionnaires be used to complement the more objective ambulatory variables evaluated under free living conditions.

The current findings show that a wide range of individual and composite variables exist that can be used in ambulatory studies to accurately and simply predict human circadian phase, with WT-based methods being the best phase predictors among the objectively recorded variables, and sleep phase markers the best indexes based on sleep logs. In conclusion, the proposed non-invasive measurement methods and their phase indexes are capable of accurately detecting the phase of the circadian system in subjects recorded under normal living conditions. However, we recommend the use of WTiO, due to its strong correlation, superior prediction capacity and temporal coincidence with DLMO, as well as its simple calculation (an important factor to be considered for clinical practice). Further studies are now needed to demonstrate its validity with other subject groups and specific pathologies. This method would be valuable for application in sleep medicine or for other clinical conditions where knowledge of the subject's endogenous circadian phase is important, such as in light therapy or cancer chronotherapy.

DECLARATION OF INTEREST

The authors report no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Ten-day ambulatory recording of wrist temperature (A), motor activity (B), body position (C), TAP (D), sleep probability (E) and light exposure (F), of 13 subjects. Grey areas indicate the period when saliva sampling for DLMO assessment took place. Data are expressed as mean ± SEM.

Figure 2. Mean waveform from a representative subject (2A) and the averaged mean waveform for all subjects (n=13) (2B) for wrist temperature, motor activity, body position, TAP, sleep probability and light exposure. Data are expressed as mean ± SEM. For purposes of comparison, melatonin levels are also represented, either expressed in pg/ml for individual values, as for subject #01 (2A), or as a percentage of the maximum concentration reached for each subject (2B).

Figure 3. Cross correlations between melatonin concentration and wrist temperature (A), activity (B), body position (C), TAP (D), sleep probability (E) and light exposure (F). Data are expressed as mean R-values ± SEM of the individual (n=13) cross correlations.

Figure 4. Mean time of dim light melatonin onset (DLMO), wrist temperature onset (WTOn), motor activity offset (AcOff), body position offset (POff), TAP offset (TAPOff), sleep onset (SleepOn), and the composite parameters $WT_{On}P_{Off}$ and SleepWTOn (see Methods for details), as well as the onset of the increase in wrist temperature (WTiO). Horizontal bars indicate the SEM for each parameter. (* $p<0.05$, ** $p<0.01$, ANOVA test).

Table 1. Linear correlations between DLMO and the non-parametric phase markers (NPI, expressed as mean ± SEM) for wrist temperature (WT), motor activity (A), body position (P), TAP and sleep probability (Sleep) and composite indexes. See Methods for details on NPI. The R coefficient, its probability, and the slope and intercept of the linear equation are also included. (* $p<0.05$, ** $p<0.01$).

Table 2. Accuracy of different methods used to predict ambulatory circadian phase *versus* phase determined by DLMO. The values are expressed as the phase difference in minutes (in absolute values) between DLMO and the predicted phase for each subject. WTiO: wrist temperature increase onset; SleepM5: timing of the midpoint of the five consecutive hours of highest values for sleep; WTL5: timing of the midpoint of the five consecutive hours of lowest values for wrist temperature; TAPM5: timing of the midpoint of the five consecutive hours of highest values for TAP; AL5: timing of the midpoint of the five consecutive hours of lowest values for activity; AcOff, timing for activity offset; AP_{Off}: timing for activity and position offset, TAPOff: timing for TAP offset; $WT_{on}Ac_{off}$: timing for WT onset and activity offset;

$WT_{on}P_{off}$: timing for WT onset and position offset; P_{off} : timing for position offset, WTOn: timing for WT onset, HO score: Horne-Östberg questionnaire score; SleepOn: the timing of sleep onset.

Table 3. Linear correlations between DLMO and the scores obtained from subjective questionnaires. HO: Horne-Östberg morningness - eveningness questionnaire; PSQI: Pittsburgh Quality Sleep Index; EES: Epworth Sleepiness Scale questionnaire. The R coefficient, its probability, and the slope and intercept of the linear equation are also included. *$p<0.05$, **$p<0.01$.