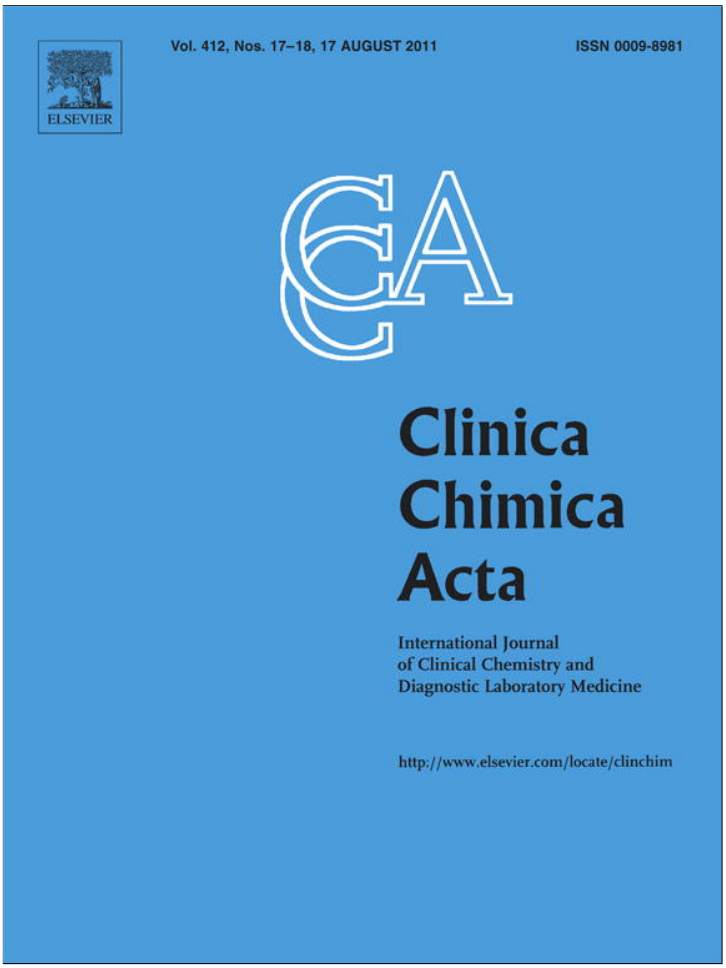

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Evaluation of salivary melatonin measurements for Dim Light Melatonin Onset calculations in patients with possible sleep–wake rhythm disorders

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ABSTRACT

Background: Dim Light Melatonin Onset (DLMO) can be calculated within a 5-point partial melatonin curve in saliva collected at home. We retrospectively analyzed the patient melatonin measurements sample size of the year 2008 to evaluate these DLMO calculations and studied the correlation between diary or polysomnography (PSG) sleep onset and DLMO.
Methods: Patients completed an online questionnaire. If this questionnaire pointed to a possible Delayed Sleep Phase Disorder (DSPD), saliva collection devices were sent to the patient. Collection occurred at 5 consecutive hours. Melatonin concentration was measured with a radioimmunoassay and DLMO was defined as the time at which the melatonin concentration in saliva reaches 4 pg/mL. Sleep onset time was retrieved from an online one-week sleep diary and/or one-night PSG.
Results: A total of 1848 diagnostic 5-point curves were obtained. DLMO could be determined in 76.2% (n = 1408). DLMO significantly differed between different age groups and increased with age. Pearson correlations (r) between DLMO and sleep onset measured with PSG or with a diary were 0.514 (p = <0.001, n = 54) and 0.653 (p = 0.002, n = 20) respectively.
Conclusion: DLMO can be reliably measured in saliva that is conveniently collected at home. DLMO correlates moderately with sleep onset.

1. Introduction

The endogenous 24-hour melatonin rhythm is one of the most reliable biological clock markers of circadian phase [1]. The time at which the melatonin concentration starts to rise in blood or saliva under dim light conditions (Dim Light Melatonin Onset or DLMO) is an important marker in assessing circadian phase [2].

Determining DLMO plays a key role in the diagnosis of Circadian Rhythm Sleep Disorders (CRSD) [3,4] and is needed for successful treatment of CRSD with exogenous melatonin, a potent chronobiotic drug [5]. Exogenous melatonin is able to shift the endogenous melatonin rhythm and its associated sleep and temperature rhythms. This induced advancement or delay depends on the time of melatonin administration [2,6,7] but also on the given dose [8].

Circadian phase can be assessed by measuring DLMO in serum or saliva [9,10] or by measuring of 6-sulfatoxymelatonin levels excreted in urine [11]. In healthy young adults, studied in sleep-labs, and in healthy adults without sleep time restrictions DLMO correlates well with sleep onset [12–14]. In contrast, in sleep onset insomniacs DLMO correlates only moderate with sleep onset time [15]. When melatonin measurements were not easily available physicians usually estimated DLMO based on the patient history [16].

Melatonin measurements in saliva are increasingly available worldwide. As far as we know, the success rate (percentage of cases where DLMO calculation was possible) of DLMO calculation out of melatonin measurements in patients with a sleeping difficulty has not yet been studied. In the Dutch National Referral Centre for sleep-wake disturbances and chronobiology at the Gelderse Vallei Hospital in Ede, the Netherlands, DLMO is measured in about 1500 patients annually. Patients collect saliva conveniently in their home environment and send it to the laboratory for analysis of endogenous melatonin concentration. To evaluate the success rate of DLMO measurements we retrospectively analyzed the melatonin measurements performed in the 2008 patients' sample size. Furthermore, in patients with a completed sleep diary and in patients where sleep architecture was assessed using ambulatory polysomnography (PSG), we studied whether DLMO can be correctly predicted by diary or PSG sleep-onset time.

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2. Materials and methods

Patients with a sleeping difficulty referred to the sleep center at the Gelderse Vallei Hospital completed an online questionnaire at www.slaapstoornissen.nl. The questionnaire assessed the time of falling asleep, trouble waking up in the morning, sleep maintenance problems, restless legs, snoring, apnoea and daytime sleepiness. If this questionnaire points toward a Delayed Sleep Phase Disorder (DSPD), i.e. falling asleep late or having trouble waking up at a conventional time in the morning, these patients received saliva collection devices for measurement of a 5-point partial melatonin profile. Sample collection occurred at the patient's home in dim light. Curtains were closed and no bright lights were used. Patients were instructed to rinse their mouths with water 15 min before sample collection. They were not allowed to brush their teeth, drink alcohol, coffee or beverages containing food color additives or caffeine, nor eat bananas. Exercise was not allowed either. Watching television was allowed. If the patient was taking exogenous melatonin, they were asked to stop treatment at least 1 week before saliva collection. Starting point for saliva collection was age dependent: <6 years at 6 PM; 6–12 years at 7 PM; 13–15 years at 8 PM; >16 years at 9 PM. Five samples were collected every sequential hour. Our routine diagnostic procedure was measurement of melatonin in these five consecutive samples. Additional samples were required if the 5-point curve was insufficient or for research purposes. After sampling the material was labeled by the patient and refrigerated. Within 2 days of collection material had to be sent by regular mail or brought to the laboratory for melatonin measurements. If sending within 2 days was impossible, the patients were instructed to freeze the samples. Sending samples was not allowed on Fridays or Saturdays. The 5-point partial profile was used as a diagnostic test to determine possible CRSD. Based on the 5-point profile results the sleep center physician started treatment or ordered additional saliva samples (i.e. a 24-hour profile), a sleep diary during at least 1 week or an ambulatory multichannel portable polysomnography (PSG) during one night [17].

2.1. Melatonin measurements

Saliva was collected with the Salivette® (Sarstedt, Nümbrecht, Germany). Melatonin was measured using a radioimmunoassay (Bühlmann laboratories, Schönenbuch, Switzerland), as described previously [9].

2.2. Data acquisition

Melatonin data from January 1st 2008 until December 31th 2008 were retrieved from the Laboratory Information Systems and exported in a Microsoft Excel datasheet. In addition to the melatonin results, the datasheet contained information about date of birth, gender, personal hospital number, date/time of sampling.

The correlation study was conducted in 130 randomly selected patients using SPSS (out of a total of 1272) with a sleeping difficulty. Their sleep onset time was retrieved from an online sleep diary and/or PSG data.

2.3. Statistical analysis

DLMO was defined as the time at which the increasing melatonin concentration in saliva reached 4 pg/mL [9]. DLMO was calculated by linear interpolation of 4 pg/mL melatonin value to the corresponding time. Extrapolation was done when the highest point was between 3 and 4 pg/mL.

Data were analyzed with SPSS version 17 (SPSS Inc, Chicago, Illinois) for descriptive statistics, independent *t*-test for comparison of means and Pearson correlation between DLMO vs. sleep onset.

R version 2.9.1 (www.r-project.org) was used for calculating percentage ascent rate between sequential melatonin levels and plotted against age using the following syntax: logmodelperc<−lm(percpuur~log(AGE, data=mela).

The study was conducted in accordance with the ICH-GCP guidelines and local law. Ethical approval was not required; this study was a secondary analysis of a de-identified data set.

Table 1
Different DLMO outcomes from 5-point partial melatonin curve with frequencies (n) and mean age.

DLMO outcome	n	Percentage (%)	Mean age (year)	SD (year)
DLMO determined	1408	76.2		
Calculated	1316	71.2	27.9	19.2
Extrapolated (values ≥3–<4 pg/mL)	92	5.0	39.3	19.5
No DLMO determined	440	23.8		
Values ≥0.5–<3 pg/mL	162	8.8	43.4	19.7
All values <0.5 pg/mL	51	2.7	31.4	21.0
All values exceed 4 pg/mL	136	7.4	30.6	20.9
Unexplained curve fluctuation	53	2.9	27.5	20.6
Insufficient sample amount	38	2.1	18.7	21.1
Total	1848	100		

3. Results

In 2008 a total of 1848 5-point partial curves were analyzed and included is this study. Other curves (except the 24-hour curves) were omitted because these curves were not used in our clinical practice for diagnosing sleep problems.

DLMO was calculated in these 5-point partial curves (n=1848). DLMO could be determined in 76.2% (n=1408). Table 1 summarizes the results. Table 1 also shows a subdivision in cases where DLMO calculation was not possible. In 7.4% (n=136) DLMO could not be calculated because all values exceeded the 4 pg/mL threshold. Within this group 47.8% (n=65) had a 'normal' curve (ascending, descending or curving the top) and 43.4% (n=59) had an abnormal fluctuating curve. In the remaining patients all five sequential samples were above the linear range of 50 pg/mL (n=12).

In the "no-DLMO" group (values≥0.5–<3 pg/mL) (n=162) the mean age was significantly higher ($p<0.001$) compared to that of the "DLMO calculated" group. Mean age of the patients with all values above 4 pg/mL did not significantly differ ($p=0.14$) from the mean age of the "DLMO calculated" group.

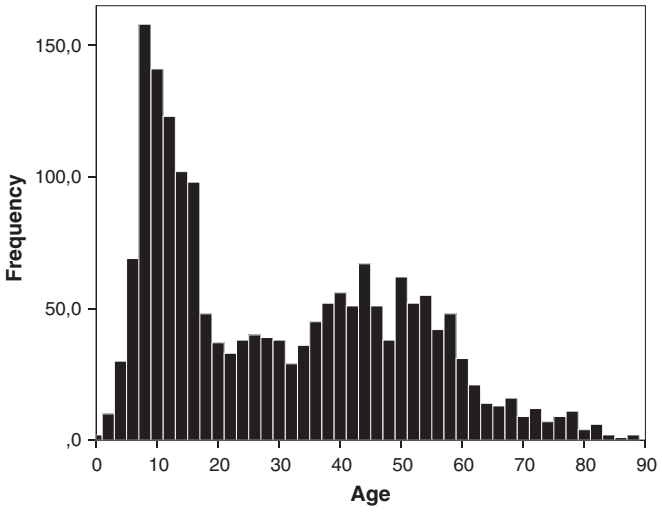

Fig. 1. Histogram of age distribution of the 2008 patient sample size where a diagnostic 5-point curve was measured (n=1848). Age is plotted on the X-axis while numbers of included patients are plotted on the Y-axis.

Table 2
Mean DLMO value in different age groups.

Age (year)	Mean DLMO (hh:mm)	SD (min)	Sample size
<6	20:19	68	40
6–12	20:43	64	364
13–15	21:32	71	120
16–50	22:11	66	541
>50	22:26	67	206
Total mean	21:41	79	1272

Table 3
Pearson correlation and means between DLMO and sleep onset determent by PSG or sleep diary.

DLMO PSG	Sleep onset time PSG	r	DLMO diary	Sleep onset time diary	r
22:26 (80) n = 54	23:49 (59) n = 54	0.514 p<0.001 n = 54	22:07 (112) n = 20	23:10 (89) n = 20	0.653 p = 0.002 n = 20

(SD in minutes).

Fig. 1 shows the age distribution among included patients (n = 1848): 41.2% were children aged between 0 and 17; 6.9% were young adults aged between 18 and 24; 38.5% were adults aged between 26 and 54 and 13.4% were adults above the age of 55.

Further analysis was done in patients where a DLMO was calculated from the 5-point partial curve with a time range between 17:00 and 01:00. The patients where DLMO was extrapolated were excluded. The total number of patients is this group is 1272. In this group DLMO significantly differed (p<0.001) between age groups and increased with age (Table 2).

To assess possible age-related steepness of the curves, the percentage increase in melatonin between the different samples in the 5-point curve is shown in Fig. 2. Differences in melatonin concentrations between sequential samples were significant (p<0.001) and decreased with increasing age.

A gender difference was also observed. DLMO occurred 17 min later in women than in men (p<0.001). In women, mean DLMO was determined at 21:49 (n = 675; mean age: 31.4 years, SD 19.0) and in men at 21:32 (n = 597; mean age: 24.0 years, SD 18.6).

For the correlation study 130 patients were randomly selected (SPSS) out of a total of 1272. Of those 130 patients, 54 underwent a PSG (mean age: 40.5 years, SD 17.2 range 5–70 years and consisted of 33 women and 21 men) and 20 online diaries were available (mean age: 23.2 years, SD 20.5 range 4–63 years and consisted of 11 women and 9 men). Sleep onset time retrieved from PSG was a one day measurement and sleep onset from sleep diary was a 6.5 day average (range 1–12 days). The Pearson correlation was significant moderately positive between DLMO and the sleep onset time in PSG and sleep diary, as seen in Table 3.

A total of 51 full-profile curves (24 h) were analyzed in 2008 and in 82.4% (n = 42) DLMO could be calculated. In 15.7% (n = 8) melatonin values never reached 4 pg/ml and DLMO could not be calculated. In one patient (women, age 37) melatonin remained <0.5 pg/mL in all 24 measurements except for 1 measurement at 10:00 (i.e. 0.8 pg/mL).

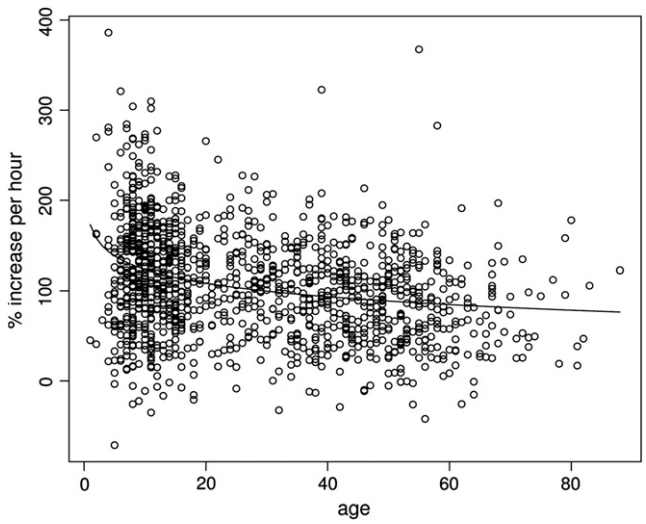

Fig. 2. Percentage increase of melatonin per hour (Y-axis) is plotted against age (X-axis). The trend line is shown.

4. Discussion

In this study we have calculated the success rate of DLMO measurements using a 5-point partial curve. DLMO was successfully determined in 76.2% (n = 1408). To our knowledge, this is the first study providing data on the success rate of DLMO measurements in an extensive patient sample size that collected saliva in their home environment.

Failure of DLMO determination was mainly due to profiles with melatonin values between 0.5 and 3 pg/mL (8.8%) or profiles where melatonin levels were already above the threshold of 4 pg/mL (7.4%). The low evening melatonin levels are consistent with late melatonin onset or with persistent low melatonin secretions. Mean age of the "no-DLMO" (values ≥ 0.5–<3 pg/mL) group was significantly higher compared to that of the "DLMO calculated" group. This can be explained by the fact that melatonin production declines with increasing age [18,19]. In these low melatonin secretors, DLMO threshold could be redefined at a lower fixed threshold [20] or at an individually calculated threshold: 2 standard deviations above the basal mean [21]. In our laboratory information system data was stored as <0.5 pg/mL if the value was between 0 and 0.5 pg/mL. Consequently we could not study the individually calculated DLMO threshold. High early-evening melatonin levels are consistent with advanced sleep phase syndrome (ASPS). However, the prevalence of this circadian rhythm disorder is very low [22]. High evening melatonin levels might also be caused by recent intake of high doses of melatonin or be due to slow melatonin metabolism [23]. Fluctuating curves were seen in 2.9% of the total sample size. This percentage increased to 43.7% in patients with high evening melatonin levels. These fluctuations are also often seen in patients taking exogenous melatonin. It may take about 3 months after stopping melatonin treatment before pre-treatment melatonin levels are reached again [24]. This might be caused by tissue storage of exogenous melatonin. In 2008 we advised stopping melatonin treatment at least 1 week before collecting saliva for DLMO measurement. Probably this time was too short. More research is necessary to make this observation evidence based and to provide an explanation. Other causes could be bleeding gums or mislabeling of tubes. However, patients were instructed not to brush their teeth or eat before sampling, thereby reducing the risk of gums bleeds.

The high percentage of included children (41.2%) is probably due to parents that want to have direct medical attention if their children have sleep problems. We observed that a relatively low number of adolescents/young adults visited our clinic for sleep problems. Adolescents have a tendency for an evening preference [25]. This apparently does not result into a medical problem or sleep problems in adolescents are under diagnosed.

Gender differences in sleep are well-known. Men have a more pronounced evening preference [26,27]. Women have an earlier circadian phase (DLMO occurred earlier) and a higher melatonin amplitude compared to men [28,29]. The results of our study contradict

the circadian phase with those studies. In our study, DLMO occurred later in women compared to men. However, our sample size mainly consisted of patients with sleep problems.

DLMO can be predicted in healthy young adolescents and young adults using the sleep onset time in their diary. In these people a measured DLMO correlates well with sleep onset [12,13]. These good correlations also exist in adults with no restriction on their sleep schedule [14]. However, in patients with sleep onset insomnia the correlation is moderate [15] and caution is advised when predicting DLMO in these patients. Our results show that correlation between DLMO and PSG is moderately. PSG is not routinely used in diagnosing CRSD but to exclude other causes for sleep difficulties [30,31] and is usually a one day measurement. The correlation between DLMO and sleep diary is slightly better compared to the PSG. The sleep onset time in these patients was an average over multiple days and the lower mean age probably yielded the better results. Nevertheless with these moderate sleep diary results caution needs to be advised when estimating DLMO in patients with a sleeping difficulty. However, in DSPD patients the circadian phase angle does not differ compared to the control group [32].

Bright light, but also room light, and some drugs may suppress melatonin secretion and consequently influence DLMO [33–35]. We did not take into account these confounding variables in the present study. Measuring light during saliva collection (i.e. with an actigraph containing a light sensor) could be considered. Another drawback of the present study is that we did not check whether samples were sent to the laboratory within 2 days after collection. Low melatonin levels might be due to degradation of melatonin when sending the samples by regular mail. Samples can be stored at 27 °C for 4 days (communication Bühlmann laboratories). The sequential hourly sampling is also a limitation. A more accurate DLMO would be determined if sampling occurred every half hour.

In this present study we analyzed whether a 5-point partial melatonin curve can be used to calculate DLMO. Although we did not couple these DLMO data to clinical data, we experienced the clinical value of measuring DLMO in our sleep center. When DLMO is late in patients suffering from chronic idiopathic sleep onset insomnia, the first choice treatment is endogenous melatonin, administered 5 hours before DLMO and/or bright light treatment early in the morning. However, when DLMO is normal, the first choice treatment is cognitive behavior therapy or other psychiatric interventions. Furthermore we found that DLMO is frequently delayed in several disorders with “unclear” symptoms, e.g. Chronic Fatigue Syndrome and Chronic whiplash syndrome, and that well-timed treatment with exogenous melatonin remarkably improved patients’ wellbeing [36,37]. DLMO also predicts treatment effects, i.e. the later the DLMO, the more sleep onset and DLMO advance [38]. DLMO measurements as a clinical tool should be validated more extensively. For example: establishment of sensitivity and specificity of DLMO in relation to final diagnosis and treatment effects, age-specific reference values of DLMO and phase angle calculation within different age groups and sleep disorders should be determined. Future prospective studies should be performed to obtain this information.

Abbreviations

ASPS:	Advance Sleep Phase Syndrome
CRSD:	Circadian Rhythm Sleep Disorders
DLMO:	Dim Light Melatonin Onset
DSPD:	Delayed Sleep Phase Disorder
PSG:	Polysomnography

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