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Original Article

# Degree of pineal calcification (DOC) is associated with polysomnographic sleep measures in primary insomnia patients

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**Abstract**

*Objective:* Melatonin plays a key role in the proper functioning of the circadian timing system (CTS), and exogenous melatonin has been shown to be beneficial in cases of CTS and sleep disturbances. Nevertheless, the concept of "melatonin deficit" has yet to be defined. The aim of our study was, therefore, to determine the relationship between the degree of pineal calcification (DOC) and a range of sleep parameters measured objectively using polysomnography (PSG).
*Methods:* A total of 31 outpatients (17 women, 14 men, mean age 45.9 years; SD 14.4) with primary insomnia were included in our study. Following an adaptation night, a PSG recording night was performed in the sleep laboratory. Urine samples were collected at predefined intervals over a 32-h period that included both PSG nights. The measurement of 6-sulphatoxymelatonin (aMT6s) levels was determined using ELISA. DOC and volume of calcified pineal tissue (CPT) and uncalcified pineal tissue (UPT) were estimated by means of cranial computed tomography.
*Results:* UPT was positively associated with 24-h aMT6s excretion ($r = 0.569$; $P = 0.002$), but CPT was not. After controlling for age, aMT6s parameters, CPT, and UPT did not correlate with any of the PSG parameters evaluated. In contrast, DOC was negatively associated with REM sleep percentage ($r = -0.567$, $P = 0.001$), total sleep time ($r = -0.463$, $P = 0.010$), and sleep efficiency ($r = -0.422$, $P = 0.020$).
*Conclusion:* DOC appears to be a superior indicator of melatonin deficit compared to the absolute amount of melatonin in the circulation. High DOC values indicate changes predominantly in the PSG parameters governed by the circadian timing system. DOC may thus serve as a marker of CTS instability.


*Keywords:* Sleep; Pineal gland; Melatonin; Degree of pineal calcification (DOC); Polysomnography; Rapid eye movements (REM); Age

## 1. Introduction

Studies on the role played by endogenous melatonin in humans have yielded some important contradictions [1]. On one hand, with the only relevant excretion site for circulating melatonin being the pineal gland [2], pinealectomy and pharmacological suppression of melatonin excretion have been shown

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to destabilize the circadian timing system (CTS) [3,4]. Furthermore, pharmacological suppression of melatonin excretion has also been shown to impair nighttime sleep [5]. Thus, endogenous melatonin clearly plays a key role in the proper functioning of the CTS and, as a consequence, in regulating the sleep–wake cycle [6]. On the other hand, attempts to define the concept of melatonin deficit by measuring melatonin excretion rates have failed to show any associations between these rates and the clinical phenomena studied to date [7–11]. As a result, melatonin deficit has yet to be defined as a clinical entity.

In light of these contradictions, it has been suggested that it is not the absolute amount of melatonin in the circulation that should serve as an indicator of melatonin deficit, but rather the subject-specific decline in melatonin excretion associated, for example, with advancing age [9,12,13]. This hypothesis is based on the fact that the size and weight of the pineal gland are genetically determined early in life and do not change as an individual grows older [14–17], but vary 20-fold within the general population. This means that the pineal gland's initial capacity to produce melatonin also varies considerably from individual to individual. Measuring the decline in this initial capacity on an individual basis could thus provide more valuable information than determining absolute melatonin excretion levels.

Based on this hypothesis, we previously introduced the concept that degree of pineal calcification (DOC) can be viewed as a subject-specific measure of decrease in the pineal gland's capacity to produce melatonin [18,19]. Preliminary findings have pointed to an association between DOC and the subjective perception of sleep-related disturbances and daytime tiredness [20].

The aim of the present study was to determine the relationship between DOC and a range of sleep parameters measured objectively using polysomnography in patients suffering from primary insomnia.

## 2. Methods

### 2.1. Subjects

A total of 31 consecutive outpatients (17 women, 14 men; mean age 45.9 years) with primary insomnia according to the Diagnostic and Statistical Manual of Mental Disorders-IV were recruited for the study, none of whom had taken part in our recently reported investigation of melatonin excretion rates and PSG [13]. All 31 patients had contacted our outpatient sleep clinic while seeking help for unrestorative sleep. Polysomnography was performed in cases where the American Sleep Disorders Association's indications for diagnostic polysomnography were met [21]. Exclusion criteria were age <18 or >80; pregnancy; current shift work or shift work during the past year; transmeridian travel during or within one month of the study; psychiatric disorders; pathological findings in brain imaging; use of any medication within the past 4 weeks that might have influenced the excretion of melatonin or rapid eye movement (REM) sleep (e.g., β-blockers, benzodiazepines, antidepressants, anti-inflammatory agents); sleep disorders associated with specific REM sleep disturbances (e.g., narcolepsy, REM sleep behavior disorder); sleep state misperception; and psychophysiological insomnia. Urine samples were screened for benzodiazepines, barbiturates, cannabinoids, amphetamines, cocaine, and opiates. The study protocol was approved by the ethics committee of Charité – Universitätsmedizin Berlin. All participants provided written informed consent.

### 2.2. Study procedures

Sleep hygiene was monitored using actigraphy (ZAK; Simbach/Inn, Germany) and a sleep log, which patients kept for 9 days starting 7 days before the 2 nights (i.e., the adaptation night and the recording night) spent in the sleep laboratory. During these 9 days, bedtimes and rising times did not vary by more than 60 min in any of the participants. Between the adaptation and recording nights, all subjects left the clinical research center to attend to their normal activities. Participants were asked to refrain from napping (controlled for by actigraphy), exercise, and alcohol consumption during this time. As described in greater detail in the *Melatonin* section below, urine samples were collected at defined intervals starting at the beginning of the adaptation night and ending at the conclusion of the recording night.

### 2.3. Polysomnography

The procedures performed during the adaptation and recording nights were identical, with the exception that no electroencephalography (EEG), electromyography (EMG), or electrooculography (EOG) recordings were made during the adaptation night. All patients slept in windowless, completely dark, sound-attenuated, air-conditioned, single bedrooms. PSG included our standard 19-channel montage for scoring sleep stages: horizontal and vertical EOG; 5 frontal, central, and occipital EEG leads; 4 EMG leads (mental, submental, tibiales left, and right); electrocardiography; snore microphone; bed actometry; nasal/oral airflow; and thoracic respiratory effort. Signals were digitized and recorded using Walter Graphtek paperless EEG (Luebeck, Germany). All PSGs were scored visually (30-s epochs) by the same highly experienced scorer. The scorer was blinded to all patient data (e.g., age, sex, disease).

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### 2.4. Melatonin

The main melatonin metabolite 6-sulphatoxymelatonin (aMT6s) was used to estimate melatonin excretion rates. Subjects were asked to collect urine samples at the end of each of the following five consecutive urine collection periods: (1) 23:00–07:00 h (first nighttime period, $NTP_1$); (2) 07:00–11:00 h (morning period); (3) 11:00–18:00 h (daytime period, DTP); (4) 18:00–23:00 h (evening period); (5) 23:00–07:00 h (second nighttime period, $NTP_2$). Light exposure was not controlled for during the collection period. The urinary concentrations of aMT6s were measured in duplicate using a highly sensitive, competitive ELISA kit (IBL Hamburg, sensitivity: 1.7 ng/ml; intra-assay variation: 4–9%; inter-assay variation: 9–12%). Details on the urine collection protocol and biochemical assays have been described previously [9].

Nighttime excretion (aMT6s-NT) was calculated as the average of both NTP values; intra-subject variability was less than 15% in all subjects. Furthermore, 24-h excretion (aMT6s-24 h) was calculated as the sum of aMT6s-NT and the 3 other periods.

### 2.5. Degree of pineal calcification and uncalcified pineal tissue

Cranial CTs were performed in all subjects using a Siemens Somatom DRG or DRH with the pineal gland covered by 4-mm-thick adjacent slices. All CT evaluations were performed by the same investigator (RM), who was blinded to clinical data, including polysomnographic findings, diagnosis, sex, and age. The degree of pineal calcification was determined in each subject using our previously reported method [18]. ESCAPE Medical Viewer software (Escape OE, Thessaloniki, Greece; www.escape.gr) was used to take measurements. The pineal gland was outlined using the freehand selection tool, and the area of the outlined region was subsequently calculated by the computer software and given in square centimeters.

Maximum organ density was measured in Hounsfield Units (HU) and scored on a five-point scale: 0–50 HU = 0; 51–150 HU = 1; 151–250 HU = 2; 251–350 HU = 3; 351–1000 HU = 4. The relative portion of calcified area was estimated by visual inspection on a four-category scale: 0–24% = 0; 25–49% = 1; 50–74% = 2; 75–100% = 3. We added both scores together and divided the sum by seven results in an estimation of the relative degree of pineal tissue calcification, or DOC. The size of uncalcified pineal tissue (UPT) was calculated by multiplying pineal gland area and 1-DOC, whereas the size of calcified pineal tissue (CPT) was calculated by multiplying pineal gland area and DOC.

### 2.6. Outcome measures and statistical analysis

Pineal outcome measures were DOC; UPT; CPT; aMT6s-24 h; and aMT6s-NT (as defined above). PSG outcome measures were sleep onset latency; slow-wave sleep latency; REM sleep latency; total sleep time (TST); sleep efficiency; wake after sleep onset (WASO); sleep stage percentage; non-REM sleep stage 1 (NREM-1); non-REM sleep stage 2 (NREM-2); slow-wave sleep (SWS); and REM sleep [22].

We used Pearson's $r$ to calculate the correlations of pineal parameters. Because age substantially influences PSG parameters, we calculated partial correlations (controlling for age) to examine the relationship between pineal parameters and PSG parameters. The level of significance was set at $P < 0.05$.

## 3. Results

Because urine samples were incomplete in five subjects, we calculated 24-h aMT6s excretion in only 26 of our study participants.

Table 1
Pineal parameters in patients with primary insomnia

| | $n$ | Mean | SD | Range | Age | DOC[a] | UPT[b] | CPT[c] |
|---|---|---|---|---|---|---|---|---|
| | | | | | Pearson's $r$ ($P$ values) | | | |
| Age [yrs] | 31 | 45.9 | 14.4 | 18–72 | | | | |
| DOC[a] [%] | 31 | 47.9 | 29.1 | 0–100 | 0.374* (0.038) | | | |
| UPT[b] [VU] | 31 | 35.2 | 23.7 | 0–84 | −0.400* (0.026) | −0.621*** (<0.001) | | |
| CPT[c] [VU] | 31 | 36.6 | 30.0 | 0–118 | 0.291 (0.113) | 0.748*** (<0.001) | −0.168 (0.368) | |
| aMT6s-24hr [d] [µg] | 26 | 22.5 | 12.4 | 1.9–53.3 | −0.725*** (<0.001) | −0.254 (0.210) | 0.569** (0.002) | 0.099 (0.629) |
| aMT6s-NT[e] [µg] | 31 | 14.6 | 8.6 | 0.3–32.4 | −0.683*** (<0.001) | −0.193 (0.298) | 0.427* (0.017) | 0.027 (0.884) |

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

[a] Degree of pineal calcification.

[b] Uncalcified pineal tissue [volume units].

[c] Calcified pineal tissue [volume units].

[d] Total 6-sulphatoxymelatonin 24-h excretion.

[e] 6-Sulphatoxymelatonin nighttime excretion per hour.

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Table 1 shows the range, mean, and standard deviation of the various pineal parameters, as well as the correlations between them. Table 2 shows the mean, standard deviation, and range of PSG parameters, as well as the correlations among PSG parameters, pineal parameters, and age.

Age was significantly and negatively correlated with melatonin excretion parameters and UPT, but was positively correlated with DOC. Uncalcified pineal tissue was significantly and positively associated with aMT6s-24 h ($r = 0.569$; $P = 0.002$) and aMT6s-NT ($r = 0.427$; $P = 0.017$), but calcified pineal tissue was not (Fig. 1).

Of the sleep parameters evaluated, TST, sleep efficiency, and NREM-2 declined significantly with age, whereas WASO increased. REM sleep percentage was not affected by age. There were no significant correlations between age and sleep latency parameters.

DOC was significantly and negatively associated with TST, sleep efficiency, and REM sleep percentage (see Fig. 2). In contrast, CPT, UPT, and none of the aMT6s parameters correlated with any of the PSG parameters we evaluated.

## 4. Discussion

The findings of the present study corroborate earlier data showing (1) that the amount of uncalcified pineal tissue predicts total melatonin excretion [18], whereas the amount of calcified pineal tissue does not; and (2) that melatonin excretion parameters are not associated with polysomnographic sleep parameters [13]. Moreover, our data prove for the first time that DOC is associated with changes in sleep.

The size of the pineal gland is genetically determined and remains unchanged over a person's lifetime; between individuals, however, it has been shown to vary in size by some 20-fold. During childhood, when the pineal gland is completely uncalcified, individuals with a large pineal gland produce more melatonin than individuals with a small pineal gland. It is conceivable that the number of melatonin receptors in the central nervous system and peripheral vasculature during childhood may differ between individuals as well. As an individual ages, the pineal gland calcifies and its ability to produce melatonin decreases; at the same time, however, the number of receptors that needs to be stimulated for the circadian signal remains unchanged (i.e., the same as during an individual's childhood). Therefore, identical amounts of melatonin in the circulation of different individuals will result in different responses. DOC, however, reflects the degree of mismatch between the number of receptors and circulating melatonin. In other words, it may not be the absolute amount of melatonin that is important when investigating the effects of melatonin on sleep, but rather the DOC, which serves as

Table 2
Polysomnographic data and their age-controlled correlation to pineal parameters in patients with primary insomnia

| | Mean | SD | Range | Age $n = 31$ | DOC[a] $n = 31$ | UPT[b] $n = 31$ | CPT[c] $n = 31$ | aMT6s-24hr[d] $n = 26$ | aMT6s-NT[e] $n = 31$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | (*P* values) Pearson's *r* | Partial correlation controlling for age (*P* values) | | | | |
| *Sleep parameters* | | | | | | | | | |
| Sleep latency (NREM-2) [min] | 29.2 | 18.1 | 5.5–90.0 | −0.199 (0.282) | 0.277 (0.138) | −0.242 (0.197) | 0.013 (0.946) | −0.375 (0.064) | −0.119 (0.530) |
| SWS latency [min] | 26.3 | 36.7 | 4.0–201.0 | 0.049 (0.794) | 0.256 (0.172) | −0.144 (0.446) | 0.052 (0.785) | −0.271 (0.190) | −0.241 (0.200) |
| REM latency [min] | 90.4 | 44.7 | 35.5–186.5 | −0.090 (0.631) | 0.247 (0.187) | −0.113 (0.552) | 0.231 (0.220) | −0.083 (0.695) | −0.252 (0.178) |
| Total sleep time [min] | 412.7 | 66.9 | 252.5–497.0 | −0.482** (0.006) | −0.463* (0.010) | 0.218 (0.246) | −0.168 (0.374) | 0.231 (0.266) | −0.249 (0.185) |
| Sleep efficiency [%] | 79.5 | 13.1 | 50.7–96.4 | −0.514** (0.003) | −0.422* (0.020) | 0.109 (0.565) | −0.204 (0.279) | −0.175 (0.402) | −0.211 (0.263) |
| Wake after sleep onset [%] | 14.2 | 11.8 | 0.3–41.2 | 0.580** (0.001) | 0.360 (0.051) | −0.059 (0.757) | 0.170 (0.369) | 0.147 (0.484) | −0.197 (0.297) |
| NREM-1 [%] | 11.6 | 5.4 | 3.4–22.9 | 0.194 (0.296) | 0.116 (0.552) | −0.089 (0.641) | 0.184 (0.330) | 0.166 (0.428) | −0.002 (0.991) |
| NREM-2 [%] | 44.2 | 9.6 | 22.0–64.4 | −0.536** (0.002) | −0.155 (0.415) | 0.011 (0.953) | 0.050 (0.794) | 0.147 (0.483) | 0.336 (0.069) |
| Slow-wave sleep [%] | 11.2 | 6.0 | 1.1–20.4 | −0.342 (0.060) | 0.084 (0.661) | 0.151 (0.426) | −0.114 (0.550) | 0.053 (0.801) | −0.049 (0.798) |
| REM sleep [%] | 17.2 | 4.7 | 7.8–28.0 | −0.235 (0.202) | −0.567** (0.001) | 0.046 (0.809) | −0.330 (0.113) | −0.047 (0.823) | −0.002 (0.993) |

*$P < 0.05$, **$P < 0.01$.
[a] Degree of pineal calcification.
[b] Uncalcified pineal tissue [volume units].
[c] Calcified pineal tissue [volume units].
[d] Total 6-sulphatoxymelatonin 24-h excretion.
[e] 6-Sulphatoxymelatonin nighttime excretion per hour.

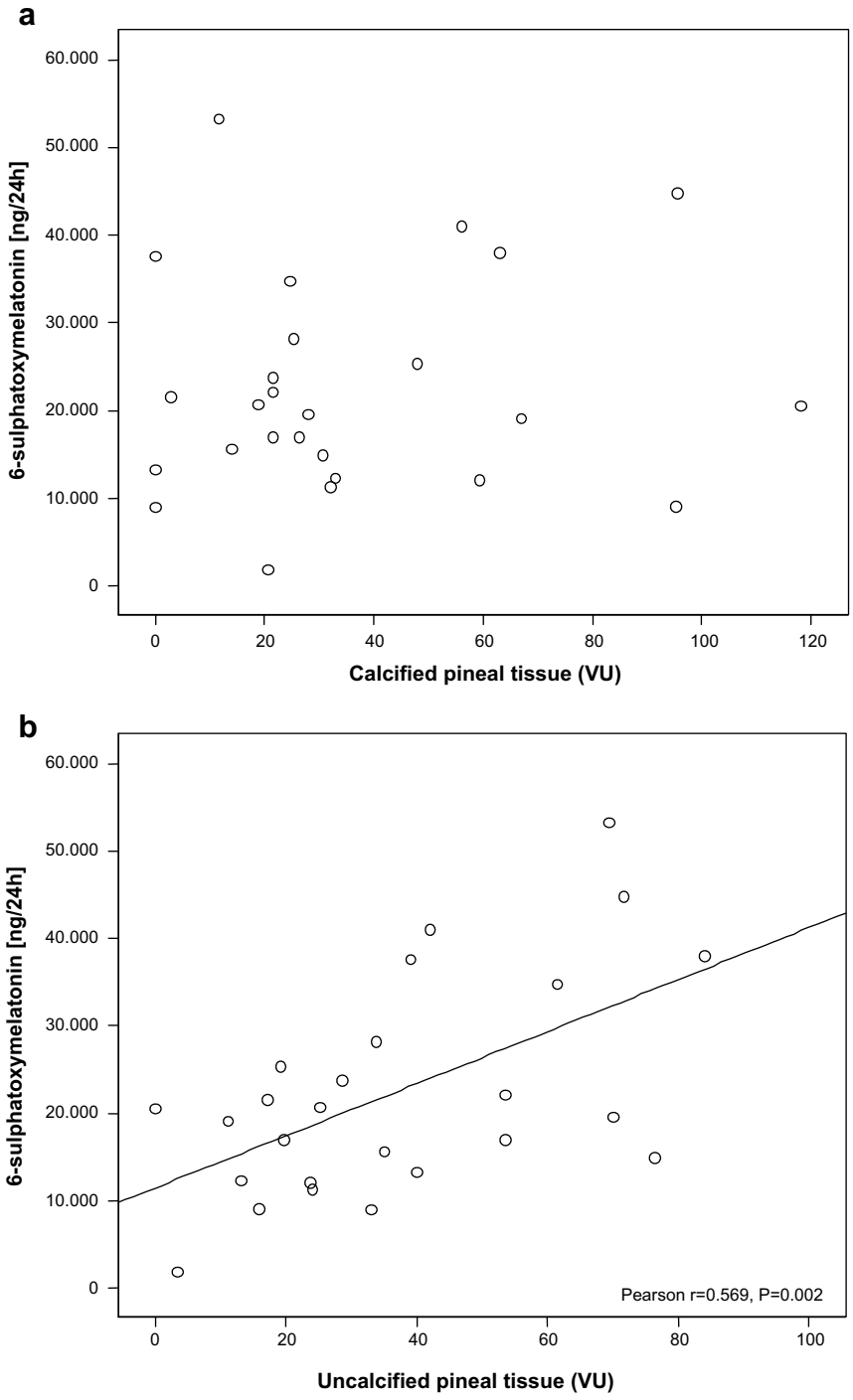


Fig. 1. (a and b) Calcified and uncalcified pineal tissue and melatonin excretion (urinary 6-sulphatoxymelatonin); VU, volume units.

a measure of an individual's ability to produce melatonin with advancing age.

Not surprisingly, REM sleep is associated with individual melatonin excretion levels, but NREM sleep is not. Though generated in an ultradian manner, REM sleep parameters such as REM sleep latency, REM sleep episode length, and REM continuity are all under strong circadian control [23,24]. In accordance with the data presented here, intervention studies conducted to date have failed to show an effect of melatonin on overall sleep parameters [25,26]. However, melatonin clearly influences REM sleep. Melatonin administered in the early evening hours to healthy subjects shortens REM latency and lengthens the first REM sleep episode [27]. Exogenous melatonin also restores REM sleep percentage to normal levels in patients with reduced REM sleep duration and reorganizes REM sleep episode length during nighttime sleep [28]. Moreover, exogenous melato-

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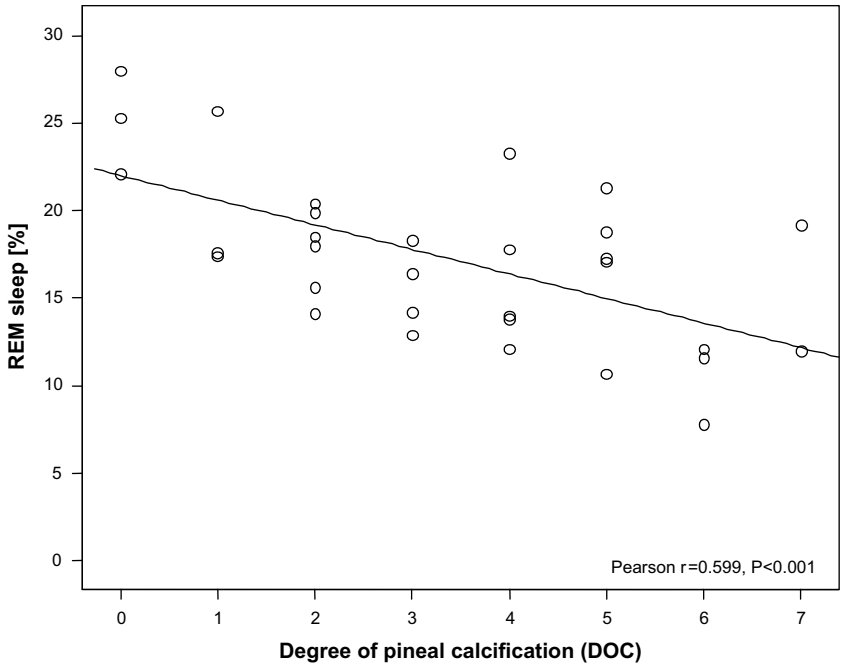


Fig. 2. Degree of pineal calcification (DOC) vs. REM sleep percentage.

nin restores REM sleep-associated muscle atonia in patients suffering from REM sleep behavior disorder [29]. β-Blockers decrease melatonin excretion and reduce the number of REM episodes simultaneously, which can be reversed by exogenous melatonin [5]. These findings suggest a close link among melatonin, the CTS, and the characteristics and occurrence of REM sleep.

Another PSG parameter associated with DOC in our study was sleep efficiency. Pinealectomy, which results in what could be described as the ultimate form of melatonin deficit, leads to instability in the CTS [3]. This kind of instability reduces the amplitude of the circadian sleep–wake propensity rhythm, thus leading to increased sleepiness during the day and increased wakefulness during the night [23,30]. Thus, high DOC scores may indicate instability in the CTS and, consequently, a pathological reduction in an individual's circadian sleep–wake propensity rhythm.

Although the structural and histochemical characteristics of pineal calcifications have been described in detail, little is known about their biogenesis [31]. There is some evidence that the calcification of pinealocytes results from the death or degeneration of the cells themselves, thus leading to an overall decrease in pineal activity [32,33]. In addition, it has been shown that, with advancing age, the number of light pinealocytes – cells which have been demonstrated to be active – decreases, leading to a significant reduction in both melatonin content and the total number of pinealocytes [33,34]. At the same time, advancing age leads to an increase in the number of dark pinealocytes, which are characterized by intra-nuclear deposits of calcium, as well as by many signs of degeneration [33,35]. The present study replicates the finding that uncalcified pineal volume is significantly associated with 24-h melatonin excretion [18]. This underscores the notion that pineal calcification is functionally relevant.

The clinical usefulness of DOC is now becoming increasingly apparent. Melatonin deficit leads to instability in the CTS, resulting in reduced REM sleep during nighttime sleep. The only known clinical entity with reduced REM sleep duration is Alzheimer's dementia. Indeed, we recently described an association between Alzheimer's dementia and high DOC scores [36]. Moreover, there is preliminary evidence that high DOC scores are related to reduced seasonality in humans [37]. Because of this, the concept of DOC may prove to be beneficial in melatonin replacement strategies.

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