The effect of hormone therapy on serum melatonin concentrations in premenopausal and postmenopausal women: a randomized, double-blind, placebo-controlled study

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Abstract

Objectives. Melatonin levels decrease physiologically with age, and possibly with the transition to menopause. The plausible influence of hormone therapy (HT) on melatonin is poorly understood. The aim of this randomized, placebo-controlled, double-blind trial was to investigate the effect of HT administration on serum melatonin concentrations in late premenopausal and postmenopausal women. **Study design.** Analyses were carried out among 17 late premenopausal and 18 postmenopausal healthy women who participated in a prospective HT study in Finland. Serum melatonin was sampled at 20-minute (21:00-24:00 h; 06:00-09:00 h) and one-hour (24:00-06:00 h) intervals at baseline and after six months with HT or placebo. **Main outcome measures.** Melatonin levels and secretion profile after six months of HT compared to placebo. **Results.** Mean melatonin levels, mean melatonin exposure level (area under curve, AUC) and mean duration of melatonin secretion did not differ after six months with HT vs. placebo, irrespectively of the reproductive state. However, in postmenopausal women the melatonin peak time (acrophase) was delayed by 2.4 hours (2 h 21 min) on average after six months with HT vs. placebo ($p<0.05$). No interaction between time and group was detected when melatonin level was modelled before or after treatment. **Conclusions.** Administration of HT to postmenopausal women alters melatonin peak time, but not melatonin levels. Further research on larger clinical samples is needed to better understand the effects of HT on melatonin profile.

Keywords: acrophase, hormone replacement, late premenopause, postmenopause, reproduction

1. Introduction

Melatonin is a hormone produced by the pineal gland and, in smaller amounts, in peripheral sites including the retina, skin and the gastrointestinal tract [1]. Its synthesis starts from the serotonin precursor, tryptophan, and is strongly regulated by the light-dark transitions, with light having an inhibitory effect [2]. Specifically, melatonin production and secretion follow a circadian rhythm, increasing about two hours before the sleep onset, peaking during the night and decreasing in the early morning.

Although with conflicting results, animal and human studies suggest that female gonadal hormones contribute to the modulation of melatonin production [3,4]. Specifically, several animal studies have found a reduced melatonin synthesis and secretion in association with high oestrogen levels [5-9], while others have reported an oestrogen-mediated stimulation of melatonin receptor activation in rats and hamsters [10,11], and a stimulation of melatonin synthesis and release in rat pinealocytes following oestrogen exposure [4]. Similarly, high levels of progesterone (either endogenous, during the luteal phase of the menstrual cycle, or exogenous as in combined oral contraceptives) were associated with high melatonin levels in women [12,13]. In general melatonin levels seem to vary in connection with reproductive events. For example, despite inconsistencies regarding the associations with menstrual cycle phases [12,14-20], healthy pregnant women were found with higher melatonin levels than postpartum women [21]. Probably as a consequence of higher gonadal hormone levels, melatonin exposure levels increased with the number of weeks during pregnancy [21] and, contrary to the duration of secretion and the offset timing, positively correlated with oestrogen and progesterone levels [22]. As opposite associations were found in depressed pregnant women, the authors suggested that the sensitivity to the modulating effects of oestradiol on melatonin receptors may be impaired in depression [21,22].

Further indirect support to the hypothesis of potential associations between melatonin and reproductive hormones comes from research on the modulation of circadian rhythms by gonadal

steroids [23]. In fact, melatonin can be considered one of the best measures of circadian clock functions in humans [24]. On the basis of these studies, oestrogens are deemed to advance circadian rhythms (reflected in the timing of sleep onset) and shorten circadian periods [25-27], while progesterone may phase-delay [28] circadian rhythms. In this context, it would be plausible to hypothesize that in conditions of relatively high levels of gonadal steroids (such as in the premenopause), melatonin rhythms would be more phase-advanced, whereas in conditions, such as postmenopause, where there is a decline in gonadal steroids, rhythms would be more phase-delayed. However, as age is as such associated with a decreased hypothalamic sensitivity to oestrogens [29], it is possible that aging causes a reduced phase-shift response to gonadal steroids. In addition, peak levels, as well as the total amount of melatonin, are known to decrease physiologically with age [30,31]. Partly as a consequence of this, melatonin levels are lower in postmenopausal women when compared with both premenopausal and perimenopausal women [32,33]. It is likely that the menopause-related hormonal changes, alone or in combination with age, contribute to this decline. With this respect, a transient increase in melatonin levels has been described in connection with the transition to menopause, whether natural or surgical [34]. However, melatonin levels have been observed to subsequently decline after the beginning of menopause [34].

We have previously shown that the mean overnight melatonin concentration and exposure level (AUC, i.e. area under nocturnal melatonin curve), as well as the duration of secretion, are lower in postmenopausal than in perimenopausal women [33]. Administration of hormone therapy (HT) after the menopause is known to restore the female gonadal hormone levels and is commonly used to alleviate climacteric symptoms in peri- and postmenopausal women; additionally, it is also effective in controlling early symptoms in premenopausal women. Nevertheless, to date only a few studies have addressed the question whether HT, either as unopposed oestrogen treatment (ET) or combined oestrogen-progesterone treatment (EPT), may also influence serum melatonin levels.

Even with some inconsistencies [35], their main findings have been those of a reduction in nocturnal [34] or diurnal [36] melatonin levels after oestrogen or progesterone [5] administration in postmenopausal women.

The aim of this prospective, randomized, placebo-controlled, double-blind study was therefore to investigate the effect of HT (specifically EPT, which is the most commonly used form in clinical practice) on melatonin levels and secretion profile in late premenopausal and postmenopausal women. As gonadal steroids are known to influence the levels and secretion profile of melatonin, and as the menopausal-related reduction of gonadal hormones may be associated with a reduction in melatonin levels in postmenopause, we hypothesized that 6-month treatment with HT could restore serum melatonin levels of postmenopausal women to late premenopausal levels.

2. Methods

2.1 Subjects

Seventeen late premenopausal (mean age = 47.7 years; SD = 2.2; range = 43-51 years) and 18 postmenopausal (mean age = 63.4 years; SD = 3.6; range = 58-71 years) women were recruited to participate in a prospective study aimed to evaluate the effects of aging and HT on sleep and cognition as well as on melatonin secretion. The recruitment procedure consisted of advertisements in the local newspapers in the area of Turku, Finland. The reproductive state was defined as late premenopausal, if serum FSH levels were lower than 23 IU/ml and the subject had ongoing regular or irregular menstrual cycle, whereas postmenopausal women were defined by age (≥58 y) and chronic amenorrhea more than one year. Women having a mental, cardiovascular (except drug-treated balanced hypertension), endocrine (except drug-treated balanced hyperlipidaemia), pulmonary, neurological or specific sleep disorder (like sleep apnoea or restless legs); malignancies; or other conditions possibly affecting sleep (e.g. fibromyalgia, anaemia) were excluded. Alcohol

abuse, smoking, excessive caffeine intake (>5 cups per day) and use of other substances that affect the central nervous system were additional exclusion criteria. The subjects kept a sleep diary in the three weeks before and one week after the study to verify their sleep-wake schedules; all women had regular sleep-wake schedules (22:00-23:00 h to 6:00-7:00 h). Women were ensured to have normal levels of blood haemoglobin, leucocytes, thrombocytes and serum thyrotropin before enrolment on the study. One late premenopausal woman and 13 postmenopausal women had previously used HT. A washout period of at least 12 months was required. More details about the data collection and study design have already been described elsewhere [37]. After receiving oral and written information, all women gave written informed consent. The study was registered as a European Research Project (QLK6-CT-2000-00499) and approved by the Ethics Committee of Turku University Hospital and the University of Turku, Finland. The study was carried out in accordance with the Declaration of Helsinki.

2.2 Study design

The randomized, placebo-controlled, double-blind study consisted of a baseline phase followed by a 6-month follow-up assessment. At baseline, the women spent three nights (one adaptation night from 19:30 h to 8:00 h, and two sleep-recording nights, the first one from 19:30 h to 12:00 h and the second one from 19:30 h to 21:00 on the next day) in the sleep laboratory at the University of Turku, Sleep Research Unit. The women went to bed (lights-off) at 23:00 h, and were woken up (lights-on) at 7:00 h. During the night only red light was allowed for illumination if needed. During the third evening an intravenous catheter was inserted into the forearm and blood was drawn every 20 minutes for 24 hours, starting at 21:00 h. At night (21:00 h to 7:00 h.) the catheter was connected to a plastic tube extending into an adjacent room to allow repeated blood sampling with minimal disturbance of the subject's sleep. Between 21:00 h and midnight as well as between 6:00 h to 9:00 h melatonin measurements were available from 20-minute interval samples, and between midnight

and 6:00 h from one-hour interval samples. The blood samples were drawn into EDTA tubes, placed in the refrigerator for 20 minutes, centrifuged, frozen immediately and stored at -70°C until assayed. Samples were assayed for melatonin by radioimmunoassay with an iodinated melatonin tracer and a melatonin-specific antiserum [38]. The lowest detectable concentration by the method was 1.3 pg/ml (5.7 pmol/l), and the intra-assay and inter-assay coefficients of variation were from 6.7 to 9.5% and from 9.8 to 12.5%, respectively.

In the second step, the women were randomized to HT or placebo for a 6-month period. Randomization was performed in 6-person blocks at the pharmacy of the Turku Central University Hospital, where the randomization codes were kept until completion of the study, so that all the persons involved in the study were blinded to interventions. Nine late premenopausal women were given cyclic EPT (2 mg oestradiol valerate for 16 days and 2 mg oestradiol valerate + 1 mg norethisterone for 12 days, Mericomb®, Novartis, Basel, Switzerland), and 8 were allocated to placebo. Administration started on day 1 of their menstrual cycles. One of the late premenopausal women in the EPT group dropped-out for personal reasons after randomization. Nine postmenopausal women received continuous EPT (2 mg oestradiol valerate + 0.7 mg norethisterone, Merigest®, Novartis, Basel, Switzerland), and nine were allocated to placebo. All postmenopausal women completed the follow-up. The subjects underwent a 3-month check-up, where the compliance to the treatment was checked through an interview and assessment of serum FSH and E2 levels, and side-effects of the treatment were recorded. The blood tests showed that all women were compliant to the treatment. At the end of the 6-month treatment period the participants returned to the sleep laboratory to repeat the sleep studies and blood sampling protocol identically to baseline. The follow-up study was carried out at three months for one, at four months for two and at five months for a fourth postmenopausal woman of the HT group, mainly due to side-effects (bloating, uterine bleeding). One postmenopausal woman from the placebo group attended the

follow-up study after four months of treatment, and another developed a venous thrombosis of the eye, shortening the treatment period to five months. All late premenopausal women were examined in the beginning of their menstrual cycle both at baseline (in the follicular phase) and after treatment (on opposed oestrogen). Altogether thirty-four women completed the study; after the study three postmenopausal women from the placebo group were excluded from the analyses because of incomplete melatonin data (Figure 1). The study continued for 29 months. Blood samples were collected all throughout the year. In detail, 5 of the late premenopausal women in the HT group and 5 in the placebo group had their baseline evaluation during winter time (October to March) and the after-treatment assessment during summer time (April to September); additionally, 1 late premenopausal woman in the HT group had both baseline and after-treatment assessments during winter time, while the remaining 5 women were first studied during summer and re-assessed during winter time. Similarly, 5 of the postmenopausal women in the HT group and 3 in the placebo group were studied during winter time at baseline and in summer time at the end of the treatment. Of the 4 remaining women in the HT group, 2 were first studied during summer and re-assessed during winter time, 1 had both baseline and after-treatment assessments during winter time and 1 during summer time; in the placebo group, 2 were first studied during summer and re-assessed during winter time, and 1 had both baseline and after-treatment assessments during summer time. However, during the study period, the participants spent their time inside the building, in a dark room without windows, with strictly controlled night-time illumination levels; this limited the possible influence of different photoperiods on the participants.

2.3 Questionnaires

In order to guarantee that the groups were similar in their symptoms profiles, several questionnaires were included at baseline and follow-up assessments. Climacteric vasomotor symptoms were scored with two questions on the past six months (night sweats and hot flashes). The frequency of the

symptoms was determined on the following four-point scale: one (“seldom or never”), two (“approximately once a month”), three (“approximately once a week”), four (“almost every day”). Vasomotor symptom score was calculated as a sum of the two scores. Depression during the past four weeks was evaluated with the Beck Depression Inventory (BDI, a sum score, with the range of 0-63) [39], and current anxiety with the State-Trait Anxiety Inventory (STAI, a sum score, with the range of 20-80) [40]. Insomnia and sleepiness during the past three months were evaluated using the Basic Nordic Sleep Questionnaire (BNSQ) [41]. The variables were sum scores in the range of 5-25, where a low score indicated good sleep or a low level of sleeping problems and sleepiness. The subjective sleep quality of the preceding night (Subjective Sleep Score) was inquired in the morning by questions on sleep quality, sleep efficiency, sleep latency, number of awakenings, too early morning awakening and morning tiredness, with a lower score indicating better sleep or a low level of sleeping problems (range = 6-20). The quality of life (an index score, with the range of from -0.011 to +1) was assessed with the EuroQoL quality of life questionnaire (EQ-5D; an index score, ranging from -0.011 to +1) and the EQ-5D visual analogy scale (VAS, range = 1-100) [42]. The EQ-5D index was calculated through a specific algorithm which considers a weight for each dimension [43]. All the questionnaires were completed at baseline and at the end of the treatment period.

2.4 Statistical analysis

Normality of the distribution was tested with Kolmogorov-Smirnov test, after which bivariate analyses were performed to study the differences between the groups using Student's *t*-test or Wilcoxon rank-sum test. A *p*-value of <0.05 was considered as significant. The two-sample *t*-test or the Wilcoxon rank sum test was used to compare HT vs. placebo groups, both at baseline and after treatment, separately within late premenopausal and postmenopausal women. First, the nocturnal melatonin exposure curve was interpolated and smoothed curves were produced; thereafter, the area

under melatonin exposure curve (AUC) (from lights-off to lights-on) was calculated for each subject. For each group mean, quartiles and median values of melatonin exposure were calculated. Any change in melatonin exposure after HT/placebo was calculated by means of differences (after treatment vs. baseline), and analysis of variance was performed to test the significance of the changes in HT vs. placebo groups. Mixed regression models were used to disentangle the effect of age and reproductive state on melatonin exposure. The peak time of melatonin secretion (acrophase) and the duration of time when melatonin levels were ≥10 pg/ml were calculated for each group, and the differences between groups were tested by Wilcoxon rank-sum test. Additionally, correlations between the changes in melatonin peak time and in sleep quality after HT/placebo (after treatment vs. baseline) were calculated. In order to study the melatonin measurement profiles, repeated measurements of melatonin levels during night were modelled using a mixed-effect model with the individual as a random effect, and group (randomization) and time (starting from lights-off) as fixed explanatory variables [44]. Time was modelled using natural splines (with df=4). Interaction between time and group was tested using log-likelihood test. All the statistical analyses were performed using SPSS/PASW software (version 18.0) (SPSS Inc., Chicago, IL, USA) and R [45].

3. Results

At baseline melatonin levels were lower in postmenopausal women compared to late premenopausal women (mean serum melatonin levels: 16.9 pg/ml (SD 7.9) vs 24.6 pg/ml (SD 10.0), p=0.015) [33]. After randomization to HT or placebo, four groups were defined (late premenopausal HT and placebo groups, and postmenopausal HT and placebo groups). Within each reproductive group (late premenopausal and postmenopausal), at baseline the HT and placebo groups did not differ in respect to FSH levels, E2 levels, climacteric vasomotor symptoms, BDI scores, STAI scores, BNSQ insomnia or sleepiness scores, subjective sleep score or EQ-5D (Table 1). At baseline melatonin levels (mean, maximum, minimum), exposure levels (AUC) and peak

time (acrophase), as well as the duration of melatonin levels ≥10 pg/ml did not differ between HT and placebo within the reproductive groups (Table 2; Figures 2 and 3).

At the end of the treatment period FSH levels were lower and E2 levels higher in HT group compared to the placebo group; however, this finding was limited to the postmenopausal women (late premenopausal: FSH=10.4 vs. 9.4 IU/l, SD=5.5 vs. 5.0, p=0.674; and E2=226.4 vs. 247.8 pmol/l, SD=129.2 vs. 70.9, p=0.115; postmenopausal: FSH=11.6 vs. 72.3 IU/l, SD=11.1 vs. 19.4, p<0.001; and E2=193.0 vs. 29.6 pmol/l, SD=65.9 vs. 13.8, p<0.001). The symptom profiles did not differ between the groups (data not shown). No difference was found in mean melatonin levels, mean melatonin exposure level (AUC) and mean duration of melatonin secretion (Table 3; Figures 2 and 3). However, in postmenopausal women the melatonin peak time was delayed by 2.4 hours in the HT group compared to placebo group at the end of the treatment (05:12 h vs. 02:51 h, p=0.011); on the contrary, late premenopausal women had a non-significantly advanced acrophase after 6-month HT than after 6-month placebo (03:42 h vs. 04:45 h, p=0.195). Changes in melatonin exposure after six months of HT or placebo in comparison with baseline were calculated separately for late premenopausal and postmenopausal women: no significant difference emerged (Table 4). Further, an analysis of variance was performed to test whether the changes in melatonin exposure after six months of HT vs. six months of placebo differ, after controlling for age, body-mass index (BMI) and reproductive state, but the analysis produced no significant results. In additional linear mixed models (HT/placebo, age, BMI and reproductive state as predictors) no significant associations were gained. No significant interaction between time and group was detected when melatonin level was modelled before or after treatment, suggesting that HT did not affect the level of melatonin secretion (data not shown).

4. Discussion

The main finding of this study is the lack of significant influence of HT on serum melatonin levels in postmenopausal and in late premenopausal women. Furthermore, this is the first study to show that HT may alter melatonin peak time (acrophase), and in specific, that HT may delay the melatonin peak time after menopause without any other changes in the levels of melatonin secretion, and independently of age and BMI.

To date, the literature has produced sparse findings regarding the effects of HT on melatonin secretion. Bartsch et al. [35] found that the effect of unopposed ET on melatonin levels in postmenopausal women depended on the route of administration: after oral oestrogen administration there was a trend for higher melatonin levels, but after transdermal oestrogen administration the melatonin levels were lower. However, when analysing the individual melatonin secretion profiles, because of the high inter-individual variability, the route of oestrogen administration did not predict the profile of melatonin secretion. Similarly, Kerdelhué et al. [46] reported only a tendency toward a decline in melatonin levels after a single injection of conjugated oestrogen after menopause, and Kos-Kudla et al. [36] found a reduction of daily melatonin secretion after six months of EPT in postmenopausal women, with no effect on overall melatonin circadian rhythm. Also, ET has been found not to affect melatonin measures in healthy women [47], even though a combined EPT advanced melatonin onset in healthy women, and oestrogen and antidepressant in combination reduced melatonin levels in menopausal depressed women [47], who have generally higher melatonin secretion levels and delayed offset compared to non-depressed peri- and postmenopausal women [47,48]. This, though limited, lack of evidence for any strong influence of HT on melatonin measures is consistent with our results, which showed no difference in melatonin exposure levels. Moreover, even when controlling separately for the effect of age vs. reproductive state, we did not find any significant changes. Similarly, we did not find any interaction between time and group when the melatonin level was modelled before or after

treatment, providing more evidence for the lack of general effect of HT on serum melatonin concentrations.

However, we cannot rule out that this lack of association is a consequence of the high inter-individual variability in the levels of melatonin, typically found in middle and old age. In general, there seems to be a significant inter-individual variation in melatonin secretion in adulthood, while it is known that melatonin production and the amplitude of its rhythm decrease with age [30,49], as a consequence of higher daytime levels and lower night-time levels in the elderly. When analysing the profile of 24-hour salivary melatonin rhythm in different age groups, Zhou et al. [50] found the lowest melatonin amplitude in subjects aged 41-53 years, who also had the longest duration of melatonin secretion. However, they found higher levels of daytime salivary melatonin, and more variability in melatonin rhythms in old compared with young and middle-age subjects. These data suggest that the alteration in the profile of melatonin secretion starts already in middle age.

In general, HT has been shown to alleviate several climacteric symptoms, such as vasomotor symptoms, as well as sleep and mood problems [51,52]. Since in our study melatonin levels, exposure levels or duration of secretion were not altered by HT administration, it seems plausible to infer that the beneficial effects of HT are not mediated by influences on quantitative melatonin secretion. Rather, the benefits of HT could be related to its influence on melatonin timing (acrophase).

We have earlier found that the peak time of melatonin secretion is similar between postmenopausal women and perimenopausal women without HT [33]. However, in the current study we have shown that the effects of HT on melatonin acrophase differed in postmenopausal women compared to late premenopausal women. In specific, HT was associated with a delay in melatonin acrophase in postmenopausal women only. This finding is a novel one. It has been previously reported that the salivary melatonin acrophase is advanced in postmenopausal compared with premenopausal women

[53]. Thus, if melatonin acrophase tends to be advanced during menopause, we could speculate that administration of HT could, partly or totally, counteract this process. However, again the previously published data are inconsistent. While Sharma et al. [54] reported a positive correlation between the plasma melatonin acrophase and age, Zhou et al. [50] found no significant difference in the salivary melatonin acrophase in different age groups, even though middle-aged subjects (41-53 years) tended to have a delayed acrophase compared with both younger and older subjects. As according to Zhou et al. [50] the most relevant alterations in melatonin secretion profile are found in middle-aged subjects, it could be hypothesized that the menopausal transition is associated with a transient delay in the melatonin acrophase which would naturally return to more advanced values later after the menopause is entered. Thus, administration of HT to postmenopausal women would alter the melatonin acrophase towards values closer to those of the preceding reproductive phase, i.e. to a delayed acrophase as shown in women with own ovarian hormone production. On the contrary, administration of HT during late premenopause could affect the melatonin acrophase towards early premenopausal profiles. This is further supported by the evidence that in our study late premenopausal women had a tendency to an earlier acrophase after 6-month HT than after 6-month placebo. This would be in line with the hypothesis that the decline in gonadal steroids may be associated with delayed circadian rhythms, where HT could contribute to restore more advanced rhythms. However, as mentioned above, aging reduces the sensitivity to oestrogens, this possibly explaining our finding of delayed peak time in (old) postmenopausal women after HT. In this context, the delayed peak time in postmenopausal women could be mostly due to the progestogenic component of the HT. However, again it must be noticed that the high inter-individual variability in melatonin levels within the study subjects may have confounded the precise detection of the melatonin acrophase, in particular in late premenopausal women.

The interpretation of our present finding and its clinical implications are not unambiguous. The earlier body of literature has produced inconsistent findings on the association between the melatonin acrophase and health status. It seems that the melatonin acrophase correlates with mood in women, and that those with a more phase-advanced acrophase have more positive affects [55]. With respect to mood disorders the associations are even more inconsistent. In the case of seasonal affective disorder [56], Checkley et al. [57] found no acrophase differences, while, according to the "phase-shift hypothesis", seasonal affective disorder associates with a phase delay of circadian rhythms, including that of melatonin [58]. Similarly, a trend toward a delayed serum melatonin acrophase was reported in depressed patients [59], while other studies have observed an advanced (or a trend toward an advanced) plasma/serum melatonin peak in depression [60,61], or no associations between peak time of the urinary melatonin metabolite 6-sulfatoxymelatonin and current depression in the elderly (60-78 years) [62]. Finally, postmenopausal women with MDD (major depressive disorder) exhibited a tendency for delayed urinary melatonin metabolite acrophase compared with healthy postmenopausal women [63]. In the same study, an association was detected between delayed acrophase and lifetime MDD. Our results show that HT in postmenopausal women may contribute to change the melatonin peak time; this suggests that the beneficial effects of HT may partly be mediated by alterations (possibly normalization) of circadian rhythms, as melatonin is among the best measures of circadian rhythms in humans. If this is the case, it would have specific clinical implications for women suffering from sleep problems or depression during the menopause, especially with seasonal features. However, further research with longitudinal design is needed to better understand the effects of delayed or advanced melatonin peak time on mood and its relationships with HT.

4.1 Strengths and limitations of the study

In our study melatonin assessment was based on a repeated serum sampling technique, which is the best technique to measure melatonin phase, duration and amplitude, in particular when frequent, i.e. every 20-30 minutes, samples are taken [64]. Additionally, the high-frequency collection of serum samples under strictly controlled sleep laboratory conditions ensured the good quality of the samples. Furthermore, the randomized, double-blind, prospective design conferred additional validity to the study, and the strict exclusion criteria allowed us to exclude several confounding factors. As melatonin levels decrease with aging [30,31] and may be affected by BMI [65], we controlled our results by age and BMI. The time effect, which is crucial in repeated analyses, was also ruled out. In addition, we controlled the symptom profiles of the women by a large set of questionnaires in order to guarantee that the possible effects of HT on melatonin secretion were not influenced by differences in symptom profiles.

The main limitation of our study is the small sample size. Also, as mentioned above, the study was carried out on a generally healthy population, preventing the generalization of the results to larger populations with common chronic diseases. The melatonin sampling took place throughout the year, thus possibly influencing the results (a seasonal effect). However, during their visit in the sleep laboratory the women spent their time inside the building and the night-time illumination levels were strictly controlled. Also, the high inter-individual variability in melatonin levels prevented from calculating additional measures of melatonin secretion profile, such as the synthesis offset and midpoint time.

4.2 Conclusions

Our results suggest that HT may delay the melatonin peak time (acrophase) in postmenopausal women, without other effect on the melatonin rhythm. Further research on larger clinical

populations is needed to better understand the effects of HT on melatonin secretion profile and its possible interaction especially with mood and sleep quality.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

NK and PP-K contributed to the conception and design of the study, acquisition of data and interpretation of results. TP contributed to the conception and design of the study and interpretation of results. OV carried out the immunoassays and JH contributed to the analyses of the data and the interpretation of the results. ET contributed to the analyses of the data, interpretation of the results and wrote the first draft of the manuscript. All the authors revised the manuscript critically for important intellectual content, read and approved the final manuscript.

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Figure 1. Flow chart of the study design.

Figure 2. Overnight mean melatonin levels at baseline and at the end of the treatment period in late premenopausal women: HT vs. placebo.

Figure 3. Overnight mean melatonin levels at baseline and at the end of the treatment period in postmenopausal women: HT vs. placebo.

Table 1. Baseline hormone and health score values of late premenopausal and postmenopausal HT vs. placebo groups.

	Late premenopausal[a]		Postmenopausal[a]	
	HT	Placebo	HT	Placebo
	mean (SD)	**mean (SD)**	**mean (SD)**	**mean (SD)**
s-FSH **(IU/l)**	**12.3 (5.4)**	**9.9 (3.9)**	**69.9 (41.0)**	**78.9 (29.4)**
s-E2 **(pmol/l)**	**442.9 (442.9)**	**159.0 (102.1)**	**34.5 (12.4)**	**33.2 (12.6)**
Climacteric symptoms **score**	**2.7 (1.0)**	**2.9 (1.0)**	**4.9 (2.6)**	**3.4 (2.2)**
BDI **score**	**3.7 (2.6)**	**4.0 (3.6)**	**5.7 (4.1)**	**6.9 (4.3)**
STAI **score**	**44.8 (3.0)**	**42.8 (4.4)**	**43.8 (2.9)**	**44.1 (3.6)**
BNSQ insomnia **score**	**13.0 (4.0)**	**14.0 (2.6)**	**15.2 (4.9)**	**16.8 (2.8)**
BNSQ sleepiness **score**	**9.0 (2.7)**	**12.6 (4.8)**	**9.4 (3.3)**	**12.0 (2.3)**
Subjective sleep score	**12.1 (2.4)**	**11.5 (2.7)**	**12.2 (2.4)**	**11.1 (1.6)**
EQ-5D **index score**	**0.94 (0.1)**	**0.90 (0.1)**	**0.82 (0.2)**	**0.85 (0.1)**
EQ-5D VAS **score**	**89.5 (5.8)**	**89.8 (6.5)**	**80.2 (16.2)**	**80.3 (7.6)**

[a]*t*-test/Wilcoxon rank sum test not significant

BDI: Beck Depression Inventory; BNSQ: Basic Nordic Sleep Questionnaire; EQ-5D: EuroQoL quality of life questionnaire; E2: estradiol; FSH: Follicle Stimulating Hormone; HT: hormone therapy; SD: Standard Deviation; STAI: State-Trait Anxiety Inventory.

Table 2. Melatonin levels at baseline in late premenopausal and postmenopausal women: HT vs. placebo.

	Late premenopausal		Postmenopausal	
	HT	Placebo	HT	Placebo
	n=8	**n=8**	**n=9**	**n=8**
Melatonin levels **(pg/ml)**				
mean (SD)	**21.0 (9.8)**	**27.0 (9.7)[a]**	**17.1 (5.8)**	**16.8 (8.7)[a]**
maximum range	**10.5-63.3**	**11.0-73.5**	**18.6-47.0**	**9.7-56.3**
maximum, mean (SD)	**35.1 (18.6)**	**48.0 (19.5)[a]**	**29.1 (9.9)**	**29.5 (15.2)[a]**
minimum range	**2.4-10.0**	**4.0-15.4**	**3.0-8.8**	**2.5-10.6**
minimum, mean (SD)	**6.1 (2.7)**	**8.1 (3.5)[a]**	**5.4 (1.8)**	**5.3 (2.7)[a]**
Melatonin exposure (AUC**, pg/ml x h)**				
1st quartile	**5.2**	**9.0**	**5.1**	**3.9**
median	**7.4**	**11.3**	**5.7**	**6.5**
mean	**8.1**	**11.5[a]**	**6.3**	**6.6[a]**
3rd quartile	**11.9**	**14.0**	**7.4**	**7.3**
Melatonin peak time **(h:min)**	**04:08**	**04:28[a]**	**04:09**	**03:50[a]**
Duration of melatonin levels ≥10 pg/ml **(hours)**				
mean (SD)	**6.6 (2.4)**	**6.9 (2.4)[a]**	**6.8 (1.2)**	**6.0 (2.5)[a]**

[a] *t*-test/Wilcoxon rank sum test not significant

1

Table 3. Melatonin levels at the end of the treatment period in late premenopausal and postmenopausal women: HT vs. placebo.

	Late premenopausal		Postmenopausal	
	HT	Placebo	HT	Placebo
	n=8	**n=8**	**n=9**	**n=6**
Melatonin levels **(pg/ml)**				
mean (SD)	**22.1 (10.7)**	**31.7 (23.9)[a]**	**15.3 (6.0)**	**19.0 (13.4)[a]**
maximum range	**6.9-63.7**	**12.7-175.8**	**8.8-36.9**	**7.6-60.6**
maximum, mean (SD)	**35.3 (17.8)**	**52.7 (51.4)[a]**	**24.7 (10.6)**	**30.1 (19.1)[a]**
minimum range	**3.1-13.2**	**2.7-23.7**	**2.3-7.0**	**2.7-12.9**
minimum, mean (SD)	**8.9 (3.8)**	**11.6 (7.3)[a]**	**4.4 (1.6)**	**6.3 (3.7)[a]**
Melatonin exposure (AUC**, pg/ml x h)**				
1st quartile	**6.4**	**8.0**	**4.7**	**4.2**
median	**7.4**	**10.4**	**6.5**	**6.3**
mean	**8.4**	**13.1[a]**	**6.1**	**7.4[a]**
3rd quartile	**12.2**	**12.0**	**8.1**	**9.5**
Melatonin peak time **(h:min)**	**03:42**	**04:45[a]**	***05:12***	***02:51[b]***
Duration of melatonin levels ≥10 pg/ml **(hours)**				
mean (SD)	**6.7 (2.8)**	**7.1 (2.0)[a]**	**5.6 (2.9)**	**5.5 (3.2)[a]**

[a] *t*-test/Wilcoxon rank sum test not significant

[b] *t*-test/Wilcoxon rank sum test significant at $p<0.05$

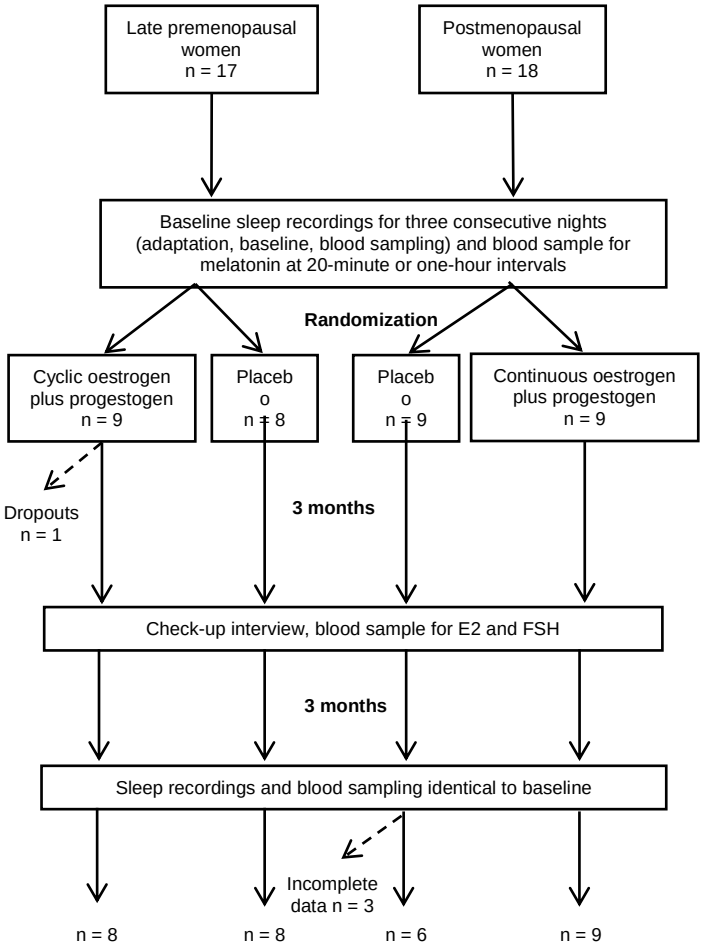
Table 4. Melatonin exposure (AUC) after HT/placebo, change from baseline.

	Late premenopausal		Postmenopausal	
	HT	placebo	HT	placebo
Melatonin exposure **(AUC, pg/ml x h, change from baseline)**				
1st quartile	**-0.2**	**-1.9**	**-1.2**	**0.1**
median	**0.1**	**-0.5**	**-0.5**	**0.4**
mean	**0.4**	**1.6[a]**	**-0.3**	**0.7[a]**
3rd quartile	**0.6**	**0.9**	**1.1**	**2.0**

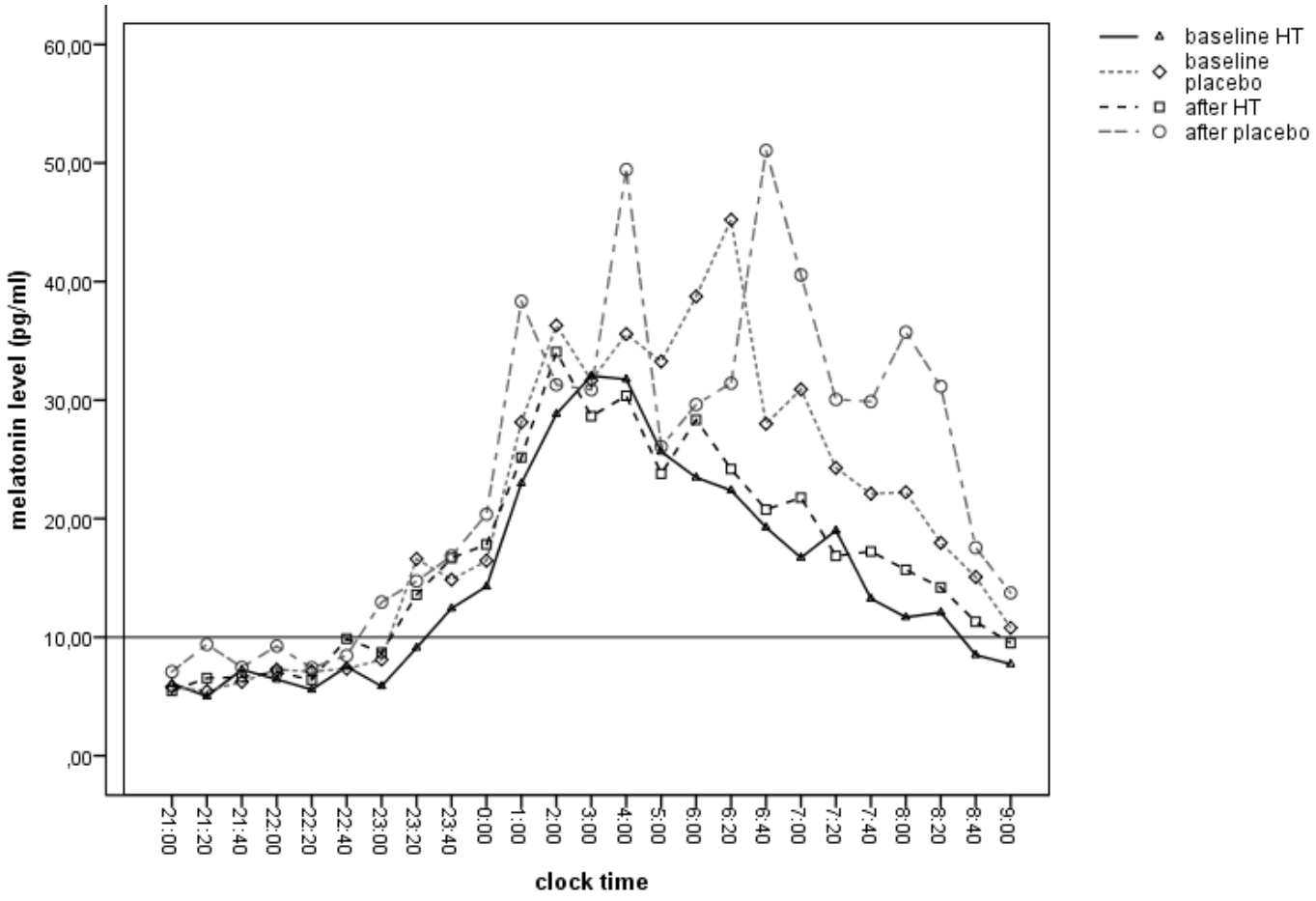
[a] *t*-test not significant

1 Figure 1

2


Late premenopausal women n = 17
Postmenopausal women n = 18
Baseline sleep recordings for three consecutive nights (adaptation, baseline, blood sampling) and blood sample for melatonin at 20-minute or one-hour intervals
Randomization
Cyclic oestrogen plus progestogen n = 9
Placebo n = 8
Placebo n = 9
Continuous oestrogen plus progestogen n = 9
Dropouts n = 1
3 months
Check-up interview, blood sample for E2 and FSH
3 months
Sleep recordings and blood sampling identical to baseline
Incomplete data n = 3
n = 8
n = 8
n = 6
n = 9

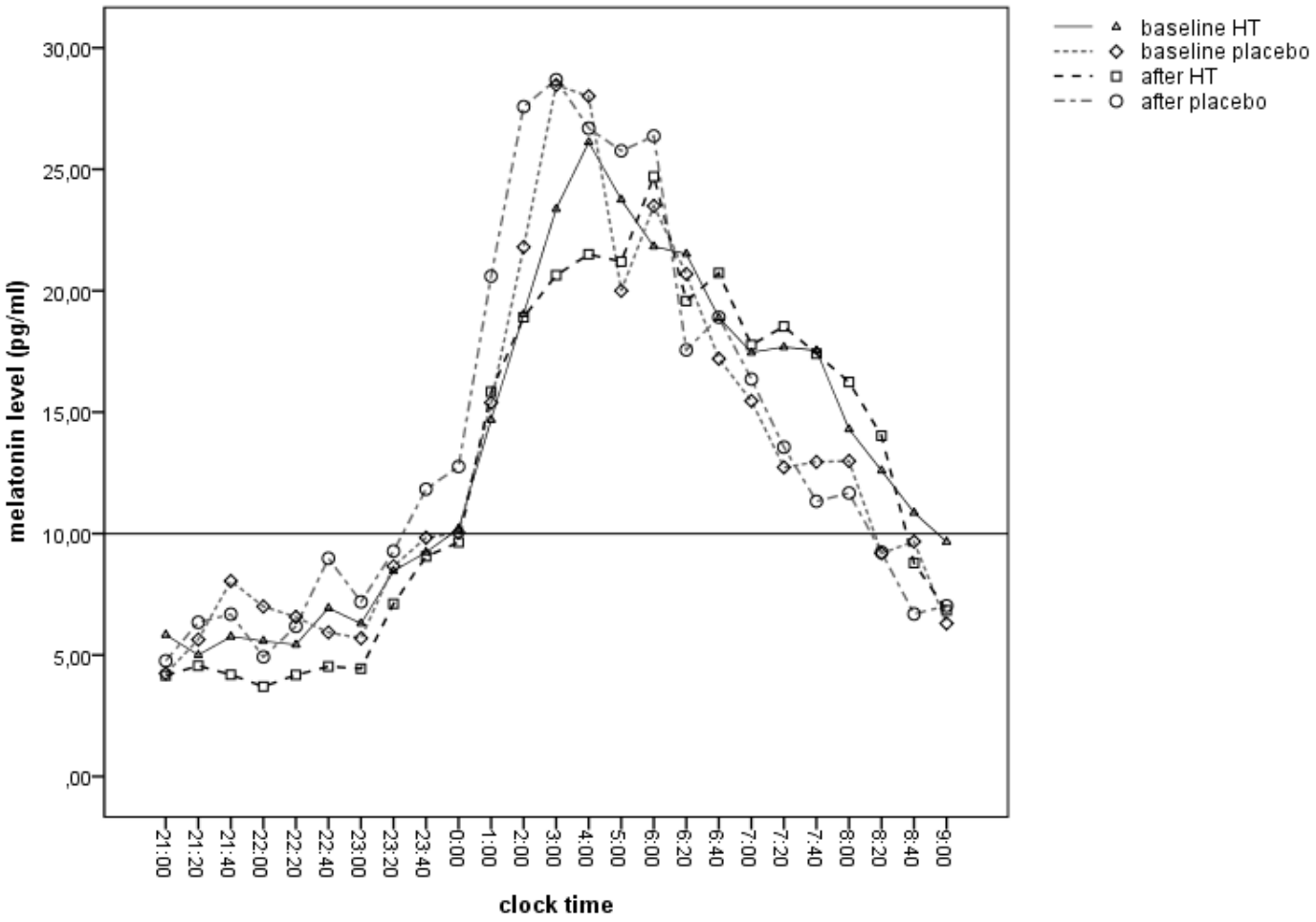
1 Figure 2


baseline HT
baseline placebo
after HT
after placebo
melatonin level (pg/ml)
60,00
50,00
40,00
30,00
20,00
10,00
,00
21:00
21:20
21:40
22:00
22:20
22:40
23:00
23:20
23:40
0:00
1:00
2:00
3:00
4:00
5:00
6:00
6:20
6:40
7:00
7:20
7:40
8:00
8:20
8:40
9:00
clock time

2

3

1 Figure 3


baseline HT
baseline placebo
after HT
after placebo
melatonin level (pg/ml)
30,00
25,00
20,00
15,00
10,00
5,00
,00
21:00
21:20
21:40
22:00
22:20
22:40
23:00
23:20
23:40
0:00
1:00
2:00
3:00
4:00
5:00
6:00
6:20
6:40
7:00
7:20
7:40
8:00
8:20
8:40
9:00
clock time

2