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Reference intervals for 6-Sulfatoxymelatonin in urine: a meta-analysis

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Conflicts of interest

The authors have no conflicts of interest to declare pertaining to this review.

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Summary

Despite the essential functions of melatonin in the human body, until now no norms of the amount of melatonin produced overnight have been established. Measuring the amount of the main urinary melatonin metabolite 6-sulfatoxymelatonin (aMT6s), corrected for creatinine, in the first morning void is the most simple as well as reliable method to evaluate the total amount of melatonin produced at night. We performed a meta-analysis to provide reference estimates and intervals by consolidating data from multiple studies. A total of 68 studies, representing 17847 subjects, were retained for the analysis. No gender differences could be found in aMT6s values in this meta-review. aMT6s excretion is very high during the first 5 years of life, flattens out in adolescence with gradual decline continuing to 50-60 years, after which the decline stagnates and a limited increase occurs around about 60 years of age. This late increase may suggest the premature death of individuals with low aMT6s levels, as lower aMT6s levels are found in various disorders, such as cardiovascular diseases, cancer and neurodegenerative disorders. Our aMT6s values can be used to identify individuals with a *possible* melatonin deficiency.

Key Words: meta-analysis, 6-sulfatoxymelatonin, 6-sulphatoxymelatonin, aMT6s, melatonin, reference interval, reference estimates, reference interval, melatonin deficiency, hypomelatoninemia.

Abbreviations: AANAT: arylalkylamine N-acetyltransferase; aMT6s: 6-sulfatoxymelatonin; ASMT: acetylserotonin O-methyltransferase; ELISA : enzyme-linked immunosorbent assay; GCMS: gas chromatography mass spectroscopy; LC–MS/MS: liquid chromatography and tandem mass spectrometry; RIA: radioimmunoassay; SCN: suprachiasmatic nuclei

Introduction

In human pathophysiology the abnormal amount of secretion of hormones results in well-known clinical syndromes.(1) Melatonin is a hormone with multiple functions. It is synthesised in the pineal gland during the dark phase of the day and involved in sleep induction and circadian rhythm regulation.(2) It is also involved in the daily rhythm of insulin sensitivity and glucose tolerance, and blood pressure.(1) Melatonin furthermore is considered highly effective in reducing the effects of oxidative stress throughout the body.(1) There are likewise indications that melatonin protects against oxidative stress-evoked DNA damage.(1) As the great importance of melatonin for human health becomes increasingly clear, as well as the consequences of a melatonin deficiency, it is important to determine when a melatonin deficiency exists. However, up to now there are no reference values for the daily melatonin production.

Although melatonin was originally discovered as a hormone synthesized by the pineal gland, it has become increasingly clear that melatonin is also synthesized in other organs (e.g. gastrointestinal tract), cells (e.g. leucocytes) and even in mitochondria.(1, 3) The amount of melatonin synthesized in the gastrointestinal tract can be more than a hundred times greater than in the pineal gland.(1) However, melatonin from extrapineal sites does not enter the circulation in substantial amounts. The amount of pineal melatonin has a clear circadian rhythm. Melatonin from extrapineal sites, however, often shows considerably lower amplitudes. This more continuous presence of melatonin is important for its physiological regulation of cell homeostasis.(4)

Melatonin is synthesised from serotonin in a two-step process, involving the enzymes arylalkylamine N-acetyltransferase (AANAT) and acetylserotonin O-methyltransferase (ASMT).(1) AANAT was generally considered to be the rate-limiting enzyme in the melatonin synthesis pathway. Later studies, however, indicate that AANAT is responsible for

the rapid activation of melatonin synthesis at the beginning of darkness.(5) The activity of the enzyme ASMT, the final step in melatonin synthesis, determines the amount of melatonin produced overnight.(5) Exposure to light rapidly decreases AANAT activity and subsequently melatonin synthesis.(1)

The rhythm of melatonin production is primarily regulated by the suprachiasmatic nuclei (SCN) of the anterior hypothalamus.(6) This 'internal clock' has a period which is not exactly 24 hour. Information on the 24-hour light-dark cycle is captured by retinal photoreceptors and passed on to the SCN to reset this internal clock to a 24 hours rhythm.(6)

There is a large inter-individual variation in the amount of melatonin synthesized in the glandula pinealis, as well as in the pineal volume.(7) But although the amount of melatonin secretion shows great inter-individual variability, its range within the same person is highly reproducible from day to day, justifying singular melatonin measurements.(2, 8)

Although several positive functions are attributed to melatonin, melatonin deficiency is not regarded as a risk factor or a disease yet.(1) In human pathophysiology the abnormal amount of secretion of hormones results in well-known clinical syndromes.(1) However, this does not seem to apply to melatonin. High or low melatonin levels are not associated with specific clinical syndromes yet. Given the functions of melatonin, a number of clinical syndromes can be presumed to exist. These are associated not only with hypo or hyper production of melatonin, but also with a temporal displacement of melatonin production, as well as a combination of these. Temporal displacement of melatonin production is mainly associated with sleep-wake rhythm disorders.(1)

Putatively, however, several clinical symptoms can be associated with low melatonin levels, given the important functions of melatonin. These are among others insulin resistance and glucose intolerance and metabolic syndrome, type II diabetes, hypertension, obesity,

Alzheimer's disease and other neurodegenerative disorders, as well as several types of cancer.(1)

Melatonin levels can be measured in plasma, saliva and urine.(1) Result of a measurement in plasma or saliva is a melatonin concentration at the time of collection. As melatonin levels follow a circadian rhythm, one single, salivary or plasma collection cannot serve as an estimation of melatonin production, as this requires several successive measurements. Also this is not a patient-friendly method in clinical practice or in clinical studies. The result of a measurement in urine is a reflection of the amount of melatonin that has been excreted over a certain period, namely the time lapse between urination and the preceding void.

Melatonin is metabolised principally by 6-hydroxylation to 6-hydroxymelatonin, which is conjugated to 6-sulfatoxymelatonin (aMT6s) and excreted in urine. CYP1A2, and to a lesser extent CYP1A1 and CYP2C19, are the main cytochrome isozymes to be responsible for the plasma clearance of melatonin in the liver.(1) Urinary aMT6s highly correlates with plasma melatonin levels.(9) It is not necessary to measure the volume of the discharged urine if the measured melatonin level is related to the urinary creatinine level. So first morning urinary aMT6s measurements, adjusted for creatinine, are an easy and reliable alternative to assess total melatonin production in epidemiological studies as well in clinical practice.(1) There are, however, no reference values available.

If it can be assumed that metabolism of extrapineal melatonin follows the identical pathway as pineal melatonin, urinary excretion of the metabolite 6-sulphatoxymelatonin (aMT6s) will reflect the sum of both pineal and extrapineal melatonin. However, since the aMT6s excretion in urine is low during the day(10), it may be assumed that, just as is the case with extrapineal melatonin, aMT6s from extrapineal melatonin sources also does not enter the circulation in substantial amounts. Given the important functions melatonin performs

throughout the body, this does not detract from the relevance of measuring aMT6s in the urine and establishing a lower limit for identifying people with a possible hypomelatoninemia.

There are only two studies that specifically aimed at establishing reference values for melatonin(11) and aMT6s excretion in urine.(10) Both studies confirmed an inverse relationship between melatonin excretion and age.

At this moment 59 clinical studies with melatonin therapy are registered in the ClinicalTrials.gov database. It concerns studies in patients with a variety of diseases, such as stroke, hypertension, diabetes, multiple sclerosis, cancer, as a neuroprotectant in asphyxiated or preterm born infants and even in prophylaxis and as a therapy in COVID-19 (<https://clinicaltrials.gov/> visited on 11-11-2021). This large number of clinical trials emphasizes the importance of establishing reference estimates and intervals for melatonin. Therefore, we performed a literature search to generate reference estimates and intervals based on current available data.

Methods

This study follows the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statements(12) and has the PROSPERO ID number CRD42020168826.

Data Sources and Searches

Relevant literature was identified via searches in PubMed, Scopus and Embase up to November 11th 2021. The MESH terms ‘6-sulfatoxymelatonin’, ‘6-sulphatoxymelatonin’ and ‘melatonin’, combined with ‘urine’, were used. Since the purpose of our study was to collect aMT6s values to establish reference estimates and intervals, only studies with apparently healthy individuals or studies with normal control groups (as opposed to patients) in humans were used. Data reported for males, females or both genders combined were extracted.

Studies in newborns and babies were removed because the endogenous melatonin production only starts after the age of about 4 months.(13) This is not an epidemiological study to establish norms based on prospectively collected data from the population.

We limited ourselves to publications in which aMT6s was measured in first morning urine, or in 8 or 12-hour overnight samples, corrected for creatinine (ng aMT6s/mg creatinine). Data presented in $\mu\text{mol}/\text{mmol}$ were converted to ng/mg applying the following formula: $\text{mmol creat} \times 113.12 = \text{mg creat}$ or $\mu\text{mol aMT6s} \times 382.34 = \mu\text{g aMT6s}$. Animal studies, review papers, congress proceedings, guidelines, editorials, abstracts, comments, and statements were excluded. If the same participants were included in more than one publication, then only the dataset with the most complete information was used. Publications other than in English language were excluded.

The years 1985 to 2021 were searched. Namely, from 1985, the radioimmunoassay (RIA) for urinary 6-sulphatoxymelatonin was used. The assay is highly specific and sensitive and in a comparison study(14) results with RIA had a good correlation with gas chromatography mass spectroscopy (GCMS). An advantage is that it records lower values. The same applies to the later developed enzyme-linked immunosorbent assay (ELISA) and the more recently introduced liquid chromatography and tandem mass spectrometry (LC–MS/MS).

Data Extraction and Quality Assessment

Data extraction was performed independently by both authors. Data extracted from all studies were converted into mean and standardized error with 95% Confidence Interval (95%CI). Hozo et al.(15) was applied in case recalculations were needed. That is, when results from different studies are presented in a non-consistent form Hozo et al. proposes an estimation method to generate them, provided adequate information is available.

The reference interval

The reference estimate is an aggregated mean per gender and age-categories. Age-categories were defined based on decades in analogy with most of the studies, with during childhood the distinction as 0-5, 5-10. In addition to tabulation, these point estimates will be plotted to represent the curve of reference estimates across the lifespan.

Meta-analysis random-effects modelling was chosen, with reporting of z-value, its p-value and n being the number of subjects and k the number of samples included. The I-squared (I^2)-statistic, being the percentage of observed total variation across studies due to real heterogeneity rather than chance (e.g., sampling error) was used, with values indicating: 0% no, 25% low, 50% moderate, and 75% high heterogeneity. The tau-squared (τ^2) is the variance of true reference estimates or the between-study variance. More clinically intuitive, the prediction interval expresses (PI) the range of the common (shared) effect (95%PI) by the included studies or the expected range of true effects in 'similar' studies ($k > 2$).⁽¹⁶⁾ In other words, we would expect that a 'new' study's true effect size (or reference estimate) will fall in this 95%PI. The existence of publication bias is assessed via Funnel plot and Egger test, however, we additionally performed a sensitivity analysis by omitting studies with a poor methodological quality score (see further). The robustness of the reference estimates and intervals was further investigated by a Jackknife sensitivity analysis, i.e., a technique where one study at the time is removed, providing its estimate and 95% Upper-Lower Bound interval. (see also Supplemental material).

Difference tests were performed to assess significance across reference estimates, with a stringent two-sided p-value of 0.001 due to multiple comparisons. Descriptive statistics will be reported as mean \pm standard deviation and 95%CI, or as frequencies. We calculated the coefficient of variation (CV%), a measure of dispersion and the percentage change in pooled estimates when appropriate (see Supplemental material). Statistical analysis were performed

with Statistica TIBCO Software Inc (TIBCO, 2017) version 13 and Meta-analysis with Comprehensive Meta-Analysis version 3.3.070 (Biostat, Englewood, NJ).

Methodological quality assessment

Based on the National Institute of Health (NIH) quality assessment tool(17) each study was assessed on risk of bias and precision. The NIH quality assessment has slightly different sets of items per study design. For each study design tool, its items can be grouped to assess: A) study population, definition and selection e.g., reporting subject selection method, inclusion/exclusion criteria etc.; B) soundness of information e.g., reporting of (in)dependent variables, interventions, etc. C) analysis, comparability and outcomes e.g. reporting of statistical analyses, confounding variables, blinding etc.; D) interpretation, reporting e.g., results being well-described and in line with the hypothesis, design, etc.. We were interested in the ‘yes’ scores to each items across the tools. Depending on the number of items per group per tool, we applied the following grading: a grading of “poor” (0) is obtained when, in accordance with the appropriate study design, none of the items had an affirmative answer, a “fair” (1) grading when less than half of the items received a “yes” score, and “good” (2) when more than half the items obtained a “yes” score. This provides a 0,1,2 scoring per A-D, which eventually will generate a sumscore on 8. For the sensitivity analysis, a study scoring a sum score ≥ 5 on the four regrouped sections (max. score 8) was considered as qualitative appropriate.

Because the NIH quality assessment tools do not assess the quality features related to the biomarker, we have added the BIOCROSS(18) relevant items.

Results

Characteristics of the studies included

The search strategy initially resulted in 3027 references (see **Figure 1**) of which ultimately 68 studies remained. The earliest included study dates from 1998. 70.6% of the studies are published as of 2010. Almost half of the studies presented data from the USA (41.2%), followed by Japan (13.2%) and Italy (5.9%). Of the remainder (n=27) three or fewer studies presented data from various countries.

The included 68 studies presented aMT6s data of 17847 participants (see **Supplemental Figure 1-3**). In the majority of studies (86.8%) aMT6s was measured in a first morning void sample. In the other studies aMT6s data were from samples taken from urine that participants had collected in a container overnight during a specific length of time [7 (n=1)(9), 8 (n=3)(19-21) or 12 hours (n=5)(22-26)], depending on the study protocol. Because of urine sample collection at different times, we performed a sensitivity analysis excluding non-first morning voids. We also conducted a sensitivity analysis regarding assay method.

Gender distribution

In more than half of the studies (51.5%) only females participated. A smaller number of studies included only males (13.2%). Nineteen papers (27.9%) reported aMT6s values of both genders combined. Only four studies separately reported the aMT6s values by gender, and one study reported them separately and combined.

Age-category distribution

When ranking age-categories on their frequency of reporting values: 40-50 (18.3%); 30-40 and 50-60 (each 17.2%); 60-70 (12.9%); 20-30 (9.7%), and in decreasing order (5 or fewer studies) are 5-10, 10-20, 0-5, 70-80, >80 and one reporting categories of from 30 up to 70.

National Institute of Health quality scoring

aMT6s values were reported in 55 observational studies, with mostly a cross-sectional design. Data management was prospective in 55.9%, retrospective in 33.8% or a mixture (8.8%). In the majority of the included studies, a convenience healthy sample was used (52.9%; cohort 23.5% and snowball sampling 1.5%). Three case-control studies(24, 27, 28) were included reporting retrospective data(n=2) collected in convenience samples (n=2, and a cohort). The ten other study designs were: four randomized controlled trials,(19, 29-31) one case study,(32) one case series study(33), three cross-over designs (34-36) and a before-after study.(37) (see **Supplemental 2**)

Of the 68 studies on aMT6s values, the quality analysis of these studies indicated that the great majority of studies (n=56) had a “good” rating (see **Supplemental Table 2**, including the biomarker items) with the remainder especially being poor regarding “Analysis, comparability and outcomes”. The funnel plot (not printed) showed publication bias for especially studies in which the aMT6s values were reported in both genders combined, and some in female participants. To be more stringent, we excluded the studies with a poor NIH quality assessment score(25, 38-48) (score<5 on the regrouped sections; see also the references in the tables).

Regarding Biomarker features, 27.9% of the studies scored below average on the 9 BIOCROSS items. The two weakest items for all included studies were reporting of blinding and outlier or extreme value management.

aMT6s reference values

Female participants

There were 40 studies that reported on aMT6s data in females (n=12145). The pooled estimate is 30.8 ± 91.6 (k=52, 95%CI: 29.2-32.4; z-value=37.0 with p-value<0.00001, $I^2=99.9$, $\tau^2= 31.9$, CV%=297.5 and PI:19.3-42.3). Jackknife analysis estimate is 34.8 (95%CI: 26.1-43.5). Upon excluding studies with a poor quality score (**Table 1**), the pooled estimate did not

significantly differ ($p=0.8611$): 35.5 ± 163.5 ($n=9633$, $k=41$, 95%CI: 32.3-38.8; z -value= 21.3 with p -value <0.00001 and $I^2=99.9$, $\tau^2= 107.6$, and $CV\%= 460.2$). Excluding the non-first morning voids, the pooled point estimate of 32.6 ± 188.3 ($n=10445$, $k=45$, 95%CI: 29.0-36.2; z -value=17.7 with p -value <0.00001 and $I^2=99.9$, $\tau^2= 147.9$ and $CV\%=578.2$) was also not significantly different from the total female sample value ($p=0.35$). The RIA pooled estimate is 32.0 ± 102.3 ($k=15$, 95%CI: 25.9-38.2); ELISA pooled estimate is 32.7 ± 100.8 ($k=36$, 95%CI: 30.9-34.6) and LC-MS-MS pooled estimate is 10.4 ± 6.7 ($k=1$, 95%CI: 7.0-13.8), showing no differences related to assay method.

The aMT6s pooled estimates per age-categories in females are shown in **Table 1** (and **Figure 2**). The highest aMT6s values are found in girls <5 years of age (significantly different from all). Until the age group 5 - 10 years, there is a sharp significant decrease of 68.8%, after which there is a smaller decrease of 37.1% to the age group of 20 – 30 years. Between the 20-30 age group and the 70-80 age group, there is only a small decrease. The differences between each successive age groups are not significant. However, the difference between the 20-30 age group and the 70-80 age group is with 35.7% significant ($p=0.0005$). The aMT6s excretion in the >80 age group is significantly higher (224.1%) compared to the 70-80 age group ($p<0.001$).

Male participants

There were 14 studies that reported on aMT6s data in males ($n=4252$). The pooled estimate is 32.9 ± 153.5 ($k=16$, 95%CI: 28.3-37.6; z -value=14.0 with p -value <0.00001 , $I^2=99.9$, $\tau^2= 81.8$, $CV\%=466.0$ and $PI:12.9-53.0$). Jackknife analysis estimate is 38.2 (95%CI: 13.5-62.9). None of the studies had a NIH quality assessment score <5 . Excluding the non-first morning voids, a pooled point estimate of 38.4 ± 190.8 ($n=4064$, $k=11$, 95%CI: 32.5-44.2; z -value=12.8 with p -value <0.00001 and $I^2=99.9$, $\tau^2= 89.6$, and $CV\%= 497.2$) which was not significantly

different from the total male sample value ($p=0.15$). The RIA pooled estimate is 38.4 ± 92.5 ($k=9$, 95%CI: 32.7-44.2) and ELISA pooled estimate is 25.4 ± 334.3 ($k=7$, 95%CI: 13.9-36.9) showing no differences in assay methodology.

Levels of aMT6s in males decreased along age-categories (**Table 2** and **Figure 2**). The course of the curve from the 20-30 age group in males shows the same pattern as in females. The highest aMT6s values are found in boys <5 years of age (significantly different from all). Until the age group of 5 – 10 years there is a sharp significant decrease of 47.3% and an almost even significant decrease (32.0%) until the age group of 10 – 20 years. After this significant decrease, in adulthood the decline levels off until the 70 – 80 year age group. The differences between each successive age groups are small. The difference between the 20-30 age group and the 70-80 age group in males is 47.9%, but this is not significant ($p=0.77$). The aMT6s excretion in the >80 age group, however, was significantly higher compared to the 40-50 and 50-60 age group (both $p<0.00001$).

Combined gender data studies

There were 20 studies that did not give separate data on males and females (**Table 3**, $n=1450$). The pooled estimate is 44.9 ± 124.7 ($k=24$, 95%CI: 38.4-51.3; z -value=13.7 with p -value<0.00001, $I^2=99.4$, $\tau^2=233.5$, CV%= 278.0 and PI:12.5-77.3). Jackknife analysis estimate is 48.3 (95%CI: 32.2-64.4). Upon excluding studies with a poor quality score, the pooled estimate did not significantly differ ($p=0.8090$): 36.1 ± 89.1 ($n=836$, $k=16$, 95%CI: 30.0-42.1; z -value= 11.7 with p -value<0.00001 and $I^2=98.4$, $\tau^2=126.8$ and CV%= 247.3). Excluding the non-first morning voids, a pooled point estimate of 46.7 ± 131.8 ($n= 1434$, $k=23$, 95%CI: 39.8-53.5; z -value= 13.4 with p -value<0.00001 and $I^2=99.3$, $\tau^2=252.7$ and CV%= 282.4) which was also not significantly different from the combined gender sample value based on all void collections ($p=0.9618$). No differences were found between the RIA pooled

estimate of 74.4 ± 547.2 ($k=3$, 95%CI: 0-159.1), the ELISA pooled estimate of 38.0 ± 111.2 ($k=16$, 95%CI: 30.9-45.1) and the LC-MS-MS pooled estimate of 51.4 ± 222.6 ($k=5$, 95%CI: 27.8-74.9).

The global trend in the course of the curve is more or less equal to that of men and women separately. However, only for the >80 year age group significant group differences were found, which may start around the age of 30-40 years.

In the total sample

Here we combined the studies reporting on genders separately (49 studies) (**Table 4**, $n=16397$). The pooled estimate is 32.6 ± 151.5 ($k=68$ samples, 95%CI: 30.3-34.9; z -value= 27.5 with p -value < 0.00001, $I^2=99.9$, $\tau^2=89.7$, $CV\%=465.1$ and $PI:13.5-51.7$). Jackknife analysis estimate is 35.6 (95%CI: 27.2-44.1). Upon excluding studies with a poor quality score, the pooled estimate did not significantly differ ($p=0.186$): 35.0 ± 163.7 ($n=13885$, $k=57$, 95%CI: 32.3-37.7; z -value= 25.2 with p -value < 0.00001 and $I^2=98.4$, $\tau^2=103.6$, and $CV\%=468.1$). Excluding the non-first morning voids, a pooled point estimate of 34.3 ± 212.2 ($n=14509$, $k=56$, 95%CI: 30.8-37.7; z -value= 19.5 with p -value < 0.00001 and $I^2=99.9$, $\tau^2=167.9$, and $CV\%=619.3$) which was not significantly different from the total sample value ($p=0.41$). No differences in assay method were found: i.e., RIA pooled estimate is 34.6 ± 97.4 ($k=25$, 95%CI: 30.4-38.8); ELISA pooled estimate is 32.4 ± 175.7 ($k=43$, 95%CI: 29.4-35.3) and LC-MS-MS pooled estimate is 10.4 ± 6.7 ($k=1$, 95%CI: 7.0-13.8).

The highest aMT6s/creatinine ratio values are found in children <5 years of age. Until the age group 10 - 20 years, there is a 87.3% sharp decrease, after which there is a smaller decrease of 40.8% to the age group of 20 – 30 years. After this, the melatonin level stabilizes until the age group 70 - 80 years. The differences between each successive age groups are not significant. In addition, the difference between the 20 - 30 age group and the 70 - 80 age

group is not significant ($p=0.292$). However, the aMT6s excretion in the age group > 80 years is significantly higher than in the age group 70 - 80 years ($p=0.0004$).

Females versus Males comparison

Difference tests between males and females showed that there was a small, but not significant gender difference in our study ($p=0.288$). Compared to females the mean aMT6s excretion summarized from all age groups was 6.4% lower in males. However, the gender difference is not the same in all age groups. Only in three age groups: 0-5 (16.7% lower, $p=0.00044$), 30 - 40 (54.1% lower, $p =0.00005$) and >80 (13.8% lower, $p<0.00001$), mean aMT6s secretion in males was significantly lower than in females.

Discussion

Data from this meta-review provide a reasonable estimate with 95% confidence interval of healthy reference values for aMT6s in females and males. The lower limit of the confidence intervals of the age groups can be used to detect individuals with low melatonin excretion. Although aMT6s values are often used in observational studies to study the association between melatonin secretion and the risk of developing a specific disease, normative reference values from large samples of healthy individuals have not been published. Our meta-analysis suggest that the data from the included studies are heterogeneous and show a large spread across ages, particularly in females. Sensitivity analyses yet supports the robustness of our findings.

The aMT6s excretion shows a slow decline during adult life. This is in line with the generally accepted knowledge that the melatonin production in each individual shows a slow decrease from the age of approximately 20 year.(13) A surprising finding, however, is that the aMT6s level shows an upward trend after the 70-80 year group. This also does not fit with the

theory that the decrease in melatonin production is associated with a decrease with age in the volume of the pineal gland by calcification.(7) Longitudinal studies are required to investigate the individual course of melatonin production throughout life. Data in the included studies in our meta-analysis, however, come from research mainly in cross-sectional studies. This can yield different outcomes than measurements in longitudinal studies. Potential discrepancies in association with gender may therefore arise in observational studies. For instance, we similarly found that in the 0-5, 30-40 and >80 age groups the mean aMT6s secretion in males was significantly lower than in females, in absence of an overall gender difference. As assumed, there is a continuous downward trend in melatonin production in each individual.

Finding higher melatonin levels in age groups older than 70 or 80 years compared to age groups younger than 70 years seems to conflict with general knowledge, but has been described previously. Kennaway et al(49) evaluated 24h aMT6s excretion in 253 healthy subjects aged between 21 and 82 year. They found a very slow decline up to the penultimate age group and a small decrease in melatonin production from 20 years to 80 years. However, if the studies also included an age group older than 80 years, there was an increase again compared to the penultimate age cohort, which is in agreement with our findings.

A high degree of variability of the aMT6s excretion data within age groups (coefficients of variation were approximately 60% across all ages) was also reported by others.(49) They suggested the possibility that in adult individuals melatonin production does not significantly decrease with aging, but that the lower production observed in the surviving elderly is simply a reflection of the people with the highest melatonin production not surviving to the same age.

Many diseases responsible for premature death have been associated with aMT6s levels that are lower than in healthy control groups.(50) This offers a possible explanation for the higher values we found for the aMT6s excretion in age groups above 80 years of age. The

higher melatonin excretion found in people older than about 80 years might simply reflect the survival of people with higher melatonin levels after the premature death of people with low melatonin excretion. This leads to the speculative assumption that people with a higher melatonin excretion are more likely to stay healthy and to live longer than people with a lower melatonin excretion. This bold hypothesis is supported by results from the Trelong study.(51) In this longitudinal study, 114 men and 146 women (mean age 85.4 ± 6.4 years, range 77-105.5 years) were followed for 7 years. Firstly, low levels of aMT6s were associated with a higher Charlson Co-morbidity Index score (list of 17 comorbidities). Secondly, at follow-up, participants who had developed a major illness during this period were found to have lower melatonin levels at baseline than those who had remained healthy for 7 years. This led the authors to suggest that melatonin may mediate an increase in susceptibility to the development of diseases in situations in which melatonin levels are reduced. The protective role of melatonin in the mitochondria against reactive oxygen species may explain this.(52)

The importance of physiological levels of pineal melatonin for human sleep is no longer an issue, but the relation between insomnia with low melatonin levels, as well as the effectiveness of exogenous melatonin in primary sleep disorders, is still debated. The latter is largely caused by the fact that in most double-blind studies with exogenous melatonin, no melatonin levels were measured prior to the start of the study and participants were prescribed a fixed melatonin dosage at a predetermined time. Dosage and timing of exogenous melatonin should be individually adjusted.(1) Since this is difficult to realize in double-blind research, failing to do so most likely led to an underestimation of the effect of melatonin. This raised doubts about the effectiveness of exogenous melatonin in primary insomnia, because it was not clear to what extent participants were prescribed a dosage that was right for them and was taken at an appropriate time. It was also unclear to what extent there was a relationship

between the degree of effectiveness of exogenous melatonin and the endogenous melatonin level prior to the study, as this was rarely measured.

We are not aware of any large studies in which the relationship between melatonin levels or urinary aMT6s excretion and the presence or absence of primary insomnia has been investigated. These data will be helpful in determining whether or not in patients with primary insomnia, this can be related to low melatonin secretion. However, there is a small number of studies in which lower melatonin levels correlate with the presence of insomnia.(53-56) On the other hand, there is also a small number of studies in which no relationship between melatonin levels and insomnia has been found.(57-59) Further research is therefore needed to clarify the relationship between low melatonin levels and primary insomnia.

A potential limitation of our study is that only few studies on very young and very old subjects are available. In addition, 17 studies are combined gender reports. However, we believe that, despite this, we were able to include data from a sufficient number of people in our analysis. A second limitation is that the included studies did not indicate in which season the data were collected. During the winter melatonin levels might be slightly higher than during the summer, but this difference occurs mainly in winter at high latitudes. Thirdly, the available data precluded investigation of potential ethnic differences(60, 61). Future studies aiming at establishing norms, and in particular epidemiological studies, should warrant transparency regarding specimen characteristics and assay methods.

Conclusion

This study provides reference estimates for aMT6s and shows that early and late in life but particularly in young adulthood females have significant higher levels of aMT6s. Our values can be used to identify individuals with a *possible* melatonin deficiency.

We found indications that, although the decrease in melatonin production is believed to be part of the aging process, people who have higher melatonin levels may live longer.

Practice points

1. This study provides reference estimates and intervals for aMT6s based on a healthy population as reported in the literature.
2. Compared to females the mean aMT6s excretion summarized from all age groups was 6.4% lower in males.
3. The aMT6s excretion shows a slow decline during adult life, but there is an upward trend after the 70-80 year group, suggesting that people with higher melatonin excretion in young adulthood may live longer than people with lower melatonin excretion.

Research agenda

1. In intervention studies with exogenous melatonin, aMT6s excretion should be measured at baseline to investigate the relationship between endogenous melatonin production, severity of insomnia and outcome of melatonin treatment.
2. Longitudinal studies are required to investigate the individual course of melatonin secretion throughout life.
3. Data from this study can be used to determine to what extent people with a disease that is known to be related to low melatonin levels can benefit from melatonin supplementation.

Tables and figures

Table 1: Reference estimates of aMT6s secretion in females

Table 2: Reference estimates of aMT6s secretion in males

Table 3: Reference estimates of aMT6s secretion in both genders combined

Figure 1: Flowchart of included studies

Figure 2: Plot of point estimates and 95% Confidence Interval per age group

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Table 1: Reference estimates of aMT6s secretion in females

Age (group)	k	n	Reference estimate mean±SD	Reference interval 95% CI	Difference test (group)*	References
0-5 (1)	1	203	227.9±131.0	209.9-245.9	from all	Wada et al(62)
5-10 (2)	1	30	71.1±36.0	58.2-84.0	1, 3, 4, 9, 10	Lee et al(63)
10-20 (3)	1	55	44.7±22.7	38.7-50.7	1, 2, 4, 8, 9	Hersh et al(64)
20-30 (4)	4	154	22.6±12.0	20.7-24.5	1, 2, 3, 9, 10	McBean et al(65); Arikawa et al(19); <i>Cook et al(39)</i>
30-40 (5)	9	673	39.5±80.6	33.4-45.6	1	Bhatti et al(60); Davis et al(34, 66) ; Davanipour et al(20); Oba et al(29); Levallois et al(67); <i>Cook et al(39)</i> ; Hsu et al(68)
40-50 (6)	15	5378	27.2±154.1	23.1-31.3	1	Brown et al(69); Wang et al(70); Bracci et al(21) ; McMullan et al(71); Masue et al(72) ; <i>Schernhammer et al(25, 43, 44)</i> ;

						Schernhammer et al(73); <i>Forman et al (40)</i> ; Nagata et al(74) ; Levallois et al(67); <i>Cook et al(39)</i> ; Braam et al(75) ; Ji et al(76)
50-60 (7)	13	2690	28.6±141.7	23.3-34.0	1	Thrift et al(30); Davanipour et al(20) ; Nagata et al(74, 77, 78); Schernhammer et al(24) ; Travis et al(8) ; Levallois et al(67); <i>Cook et al(39)</i> ; Peplonska et al(79) ; Hurley et al(80) ; Razavi et al(81); Yang et al(31)
60-70 (8)	7	2889	24.7±168.0	18.6-30.8	1, 3	Devore et al(28) ; Sturgeon et al(82) ; McMullan et al(83) ; Schernhammer et al(73) ; <i>Schernhammer et al(42)</i> ; Levallois et al(67) ; <i>Cook et al(39)</i>
70-80 (9)	1	28	14.5±3.9	13.1-15.9	1, 2, 3, 4, 10	<i>Cook et al(39)</i>
>80 (10)	1	146	47.0±1.6	46.7-47.3	1, 2, 4	Gallucci et al(51)

*Adjusted P-value<0.001. *Italic* is poor rating on quality assessment, and excluded in the sensitivity analysis. Data presented as ng aMT6s/mg creatinine. Forest plot as Supplemental Figure 1. **95% CI**: 95% Confidence intervals of the pooled estimate; **k**=number of samples; **n**=number of subjects; **SD**: standard deviation

Table 2: Reference estimates of aMT6s secretion in males

Age (group)	k	n	Reference estimate mean±SD	Reference interval 95% CI	Difference test (group)*	References
0-5 (1)	1	235	189.8±92.9	177.9-201.7	all except 2	Wada et al(62)
5-10 (2)	1	8	100.1±27.1	81.4-118.9	3, 4, 6, 7, 8, 10	Stebelova et al(32)
10-20 (3)	1	44	39.3±26.1	31.6-47.0	1, 6, 7	Hersh et al(64)
20-30 (4)	2	31	30.3±37.3	17.1-43.4	1, 2, 6, 7	Hoffman et al(35); Coste et al(23)
30-40 (5)	5	408	18.1±88.7	9.5-26.7	1	Mirick et al(84); Touitou et al(26) ; Graham et al(9) ; Burch et al(85) ; Coste et al(23)
40-50 (6)	1	314	15.0±1.10	14.9-15.1	1, 2, 3, 4, 7, 8, 10	Burch et al(85)
50-60 (7)	1	100	8.0±1.6	7.7-8.3	1, 2, 3, 4, 8, 10	Thrift et al(30)
60-70 (8)	1	55	36.0±26.6	29.0-43.0	1, 2	Davanipour et al(20)
70-80 (9)	2	2943	15.8±278.0	5.7-25.8	1	Devore et al(86) ; Sigurdardottir et al(7)

>80 (10)	1	114	40.5±1.6	40.2-40.8	1, 2	Gallucci et al(51)
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*Adjusted P-value<0.001. *Italic* is poor rating on quality assessment, and excluded in the sensitivity analysis. Data presented as ng aMT6s/mg creatinine. Forest plot as Supplemental Figure 2.

95% CI: 95% Confidence intervals of the pooled estimate; **k**=number of samples; **n**=number of subjects; **SD:** standard deviation

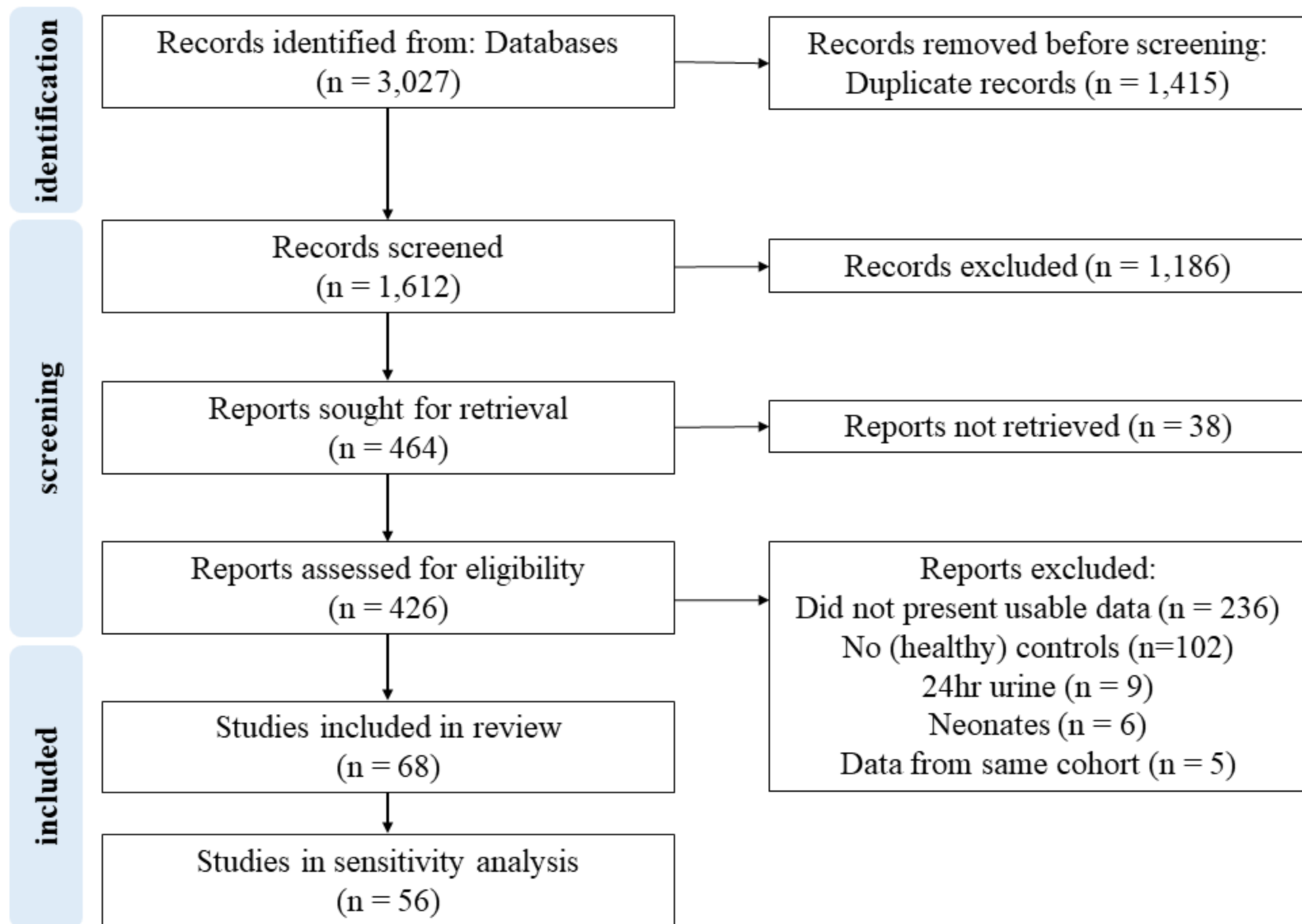
Table 3: Reference estimates of aMT6s secretion in both genders combined

Age (group)	k	n	Reference estimate mean±SD	Reference interval 95% CI	Difference test (group)*	References
0-5 (1)	2	44	65.3± 171.7	14.6 - 116.1	-	<i>Batllori et al(38)</i> ; Rensen et al(87)
5-10 (2)	5	256	89.5 ± 463.2	32.8 - 146.2	-	<i>Santoro et al(41)</i> ; <i>Batllori et al(38)</i> ; <i>Babinska et al(46)</i> ; <i>Steuer et al(88)</i> ; Rensen et al(87)
10-20 (3)	4	69	34.2 ± 59.6	20.1 - 48.3	-	<i>Okoshi et al(33)</i> ; <i>Batllori et al(38)</i> ; Rensen et al(87) ; <i>Barlow et al(89)</i>
20-30 (4)	3	68	46.9 ± 69.7	30.3 - 63.4	5	<i>Kondo et al(90)</i> ; <i>Slyepchenko et al(45)</i> ; <i>Stebelova et al(37)</i>
30-40 (5)	2	192	16.5 ± 58.9	8.2 - 24.9	4, 10	<i>Schobersberger et al(36)</i> ; <i>He et al(91)</i>
40-50 (6)	1	59	25.9 ± 7.6	24.0 - 27.8	7, 10	<i>Gobba et al(92)</i>
50-60 (7)	2	25	23.0 ± 59.1	0 - 46.2		<i>Reutrakul et al(93)</i> ; <i>Sirisreetreerux et al(48)</i>
60-70 (8)	4	477	22.7 ± 134.1	10.7 - 34.7		<i>Chen et al(22)</i> ; <i>Akdogan et al(27)</i> ; <i>Kim et al(94)</i> ;

						<i>Yoshikawa et al(47)</i>
70-80 (9)	0					
>80 (10)	1	260	44.2 ± 42.7	39.0 - 49.	5, 6, 8	Gallucci et al(51)

*Adjusted P-value<0.001. *Italic* is poor rating on quality assessment, and excluded in the sensitivity analysis. Data presented as ng aMT6s/mg creatinine. Forest plot as Supplemental Figure 3.

95% CI: 95% Confidence intervals of the pooled estimate; **k**=number of samples; **n**=number of subjects; **SD:** standard deviation;



Pooled Estimate of aMT6s (ng/mg)

gender
female
male

200
100
0

0-5 5-10 10-20 20-30 30-40 40-50 50-60 60-70 70-80 >80

Age Category (years)

