Published in final edited form as:

J Sleep Res. 2016 February ; 25(1): 16–22. doi:10.1111/jsr.12345.

Pineal Hypoplasia, Reduced Melatonin, and Sleep Disturbance in Patients with *PAX6* Haploinsufficiency

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Summary

In rodent studies, paired box 6 (*PAX6*) appears to play an important role in the development of the pineal, the primary source of the circadian regulating hormone, melatonin. Pineal hypoplasia has been previously reported in patients with *PAX6* haploinsufficiency (+/-); however, pineal measurement, melatonin concentrations and sleep quality have not been reported. This cross-sectional descriptive study examined pineal volume, melatonin secretion, and sleep disturbance in 37 patients with *PAX6*+/- (age 15.3±9.9 years) and 17 healthy controls (16.0±7.2 years), within an inpatient setting at the Clinical Research Center of the National Institutes of Health, Bethesda, Maryland, USA. Pineal volume was evaluated by magnetic resonance imaging (MRI). Diurnal serum cortisol, serum melatonin, and urine 6-sulfatoxymelatonin (6SM) concentrations were measured by enzyme-linked immunosorbent assay. The Child Sleep Habits Questionnaire (CSHQ) was administered for patients <13y. Pineal volume was 5-fold lower in *PAX6*+/- vs. controls (mean±SD: 25±15 vs. 129±50 μ L, $p<0.001$). Midnight serum cortisol was similar in *PAX6*+/- vs. controls ($p=0.14$). Midnight serum melatonin was >2-fold lower in *PAX6*+/- vs. controls (median [25th–75th]: 28 [22–42] vs. 71 [46–88] pg/mL, $p<0.001$). First morning void urinary 6SM was 4-

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Disclosure Statement: The authors have no conflicts of interest.

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Supplemental data:

Supplemental data included in one table.

fold lower in *PAX6*^{+/-} vs. controls (11 [6–26] vs. 45 [34–61] ng/mgCr, *p*=0.001). CSHQ score was higher in *PAX6*^{+/-} vs. controls (48±6 vs. 41±5, *p*=0.03). Our findings suggest that *PAX6*^{+/-} is associated with smaller pineal size, lower melatonin secretion, and greater parental report of sleep disturbances in children. Further studies are needed to explore the potential use of melatonin replacement for improving sleep quality in patients with *PAX6*^{+/-}.

Key Terms

ELISA; WAGR syndrome; 11p deletion

Introduction

Heterozygous loss of function for the transcription factor paired box 6 (*PAX6*, located on chromosome 11p13) is the most common cause of aniridia, a rare developmental eye disorder (prevalence 1/50,000–100,000) associated with absence or hypoplasia of the iris and other ocular defects (Glaser et al., 1992, Ivanov et al., 1995). *PAX6* haploinsufficiency (*+/-*) can occur due to mutations involving only *PAX6* in patients with isolated aniridia or as part of contiguous 11p13 gene deletions that involve *PAX6* and adjacent genes in patients with WAGR (Wilms tumor, Aniridia, Genitourinary anomalies, cognitive impairment) syndrome (Fischbach et al., 2005, Lee et al., 2008).

In addition to its ocular role, *PAX6* also appears to be important for the development of the pineal, the primary source of the circadian regulating hormone, melatonin (Rath et al., 2009, Walther and Gruss, 1991, Estivill-Torrus et al., 2001). In wild-type mice, *Pax6* is expressed in the pineal during early embryologic developmental stages. Homozygous *Pax6*^{-/-} mice lack pineal-specific immunomarkers at all embryonic stages (Estivill-Torrus et al., 2001, Walther and Gruss, 1991). Heterozygous *Pax6*^{+/-} mice exhibit a present, but hypoplastic pineal gland (Estivill-Torrus et al., 2001).

In 2003, Mitchell et al. described the first observation of human pineal absence in a study of 24 subjects with *PAX6* mutations, 7 of whom had pineal hypoplasia and 13 had pineal absence on brain MRI (Mitchell et al., 2003). A subsequent study described 3 patients with *PAX6* mutations who underwent brain MRI, and all were found to have pineal absence (Abouzeid et al., 2009). Neither of these studies described sleep assessments or melatonin concentrations, and thus the functional consequences of pineal absence were not assessed.

We assessed pineal volume, diurnal concentrations of serum melatonin and its urinary metabolite, 6-sulfatoxymelatonin (6SM), and sleep disturbance in 37 patients with *PAX6*^{+/-} and 17 healthy control subjects matched for age, sex, race, and body mass index. We hypothesized that *PAX6*^{+/-} would be associated with smaller pineal volume, lower melatonin, lower 6SM, and greater sleep disturbance.

Materials and Methods

Study Population

Subjects in the *PAX6*^{+/-} group included patients with WAGR syndrome who had prior genetic testing showing 11p deletion (deletion boundaries, identified by oligonucleotide array comparative genomic hybridization as previously described (Han et al., 2008), are shown in Supplemental Table 1), and patients with isolated aniridia who had documented mutations or intragenic deletions affecting only *PAX6* (shown in Supplemental Table 1). Healthy control subjects included individuals who had no chronic medical conditions and were neurologically normal on physical examination. Subjects were excluded from analysis if they reported beta-blockers use (which could suppress melatonin secretion), renal insufficiency (which could affect urinary clearance of melatonin), or complete lack of light perception (which could lead to free-running circadian patterns). The study was approved by the institutional review board of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD and registered at ClinicalTrials.gov (NCT00758108). Written informed consent was obtained from adults who were competent to provide consent and from the parents or legal guardians of children and adults with cognitive impairment. Assent was obtained from patients who were unable to give consent.

Brain Imaging

Non-contrast brain MRI was performed using an 8 channel 12 element head array coil on a 3.0 tesla system (Philips Achieva, Netherlands) on all patients who could comply with the procedure or who were undergoing sedation for clinically indicated eye examination under anesthesia. In addition to routine clinical MRI sequences (e.g., T1-weighted, T2-weighted, fluid attenuated inversion recovery [FLAIR]), we performed two different dedicated high resolution sequences to evaluate the pineal gland. The first was a T2 weighted sequence using a 2D fast spin echo technique (performed in 34 *PAX6*^{+/-} patients and 16 control subjects). The second was a T2/T1 weighted 3D balanced fast field echo sequence (performed in 18 *PAX6*^{+/-} patients and 14 control subjects). Both methods generate high signal in CSF and low signal in pineal tissue.

Contrast parameters for the 2D FSE sequence were repetition time (TR) 2260 ms, effective echo time (TE) 180 ms, flip angle (FA) 90°, echo train length 32, bandwidth (BW) 218 Hz/pixel. Geometric parameters were field of view 150 mm anterior-to-posterior (AP) × 150 mm right-to-left (RL) × 38 mm superior-to-inferior (SI), sequence resolution 0.39 mm AP × 0.52 mm RL × 1.8 mm SI. Two averages were performed with an imaging time of 3m00s.

Contrast parameters for the 3D BFFE sequence were TR 5.5 ms, TE 2.2 ms, FA 30°, BW 389 Hz/pixel. Geometric parameters were field of view 80 mm anterior-to-posterior (AP) × 80 mm right-to-left (RL) × 88 mm superior-to-inferior (SI), sequence resolution 0.4 mm AP × 0.4 mm RL × 0.8 mm SI. Two averages were performed with an imaging time of 8m26s.

Two-dimensional analyses of pineal images were used to estimate volume by multiplication of the AP dimension with the square of the RL dimension. For the 3D measurements, pineal borders were manually traced slice-by-slice on the dedicated 3D BFFE images, using the

manual verification painting tool in RTHR CT-Slicer software, a custom-designed program developed at the NIH (software available upon request). The pixels inside the traced contour were summed to determine the total pineal volume.

Serum Cortisol and Melatonin

Venous blood was drawn from an indwelling intravenous catheter at 08:00 in the morning and at midnight (in a dark room while the subject was in bed). Serum cortisol concentrations were measured using a solid-phase, competitive chemiluminescent enzyme immunoassay on the Siemens Immulite 2500 Analyzer (NIH Clinical Center Chemistry Laboratory). Serum samples were stored at -80°C , and at the completion of the study, melatonin concentrations were measured by a commercially available competitive enzyme-linked immunosorbent assay (ELISA) (Genway, San Diego, CA) according to the manufacturer's protocol, with the following modification: serum melatonin samples were diluted 2.5-fold prior to assay. The intra-assay coefficient of variation was 3.0–11.4% and the inter-assay coefficient of variation was 6.4–19.3%. The detection limit of the assay was 1.6 pg/mL. Serum albumin was measured on the Siemens Dimension Vista (NIH Clinical Center Chemistry Laboratory) so that concentrations of serum melatonin could be adjusted for albumin concentration since endogenous melatonin is weakly protein bound (Kennaway and Voultsios, 1998).

Urine 6-sulfatoxymelatonin (6SM)

Urine was collected from the first morning void and during the evening. Melatonin secretion was estimated by measuring the urinary concentrations of 6-sulfatoxymelatonin (6SM) using a competitive ELISA (Genway, San Diego, CA) according to the manufacturer's protocol. The intra-assay coefficient of variation was 5.2–12.2% and the inter-assay coefficient of variation was 5.1–14.9%. The detection limit of the assay was 1.0 ng/mL. Urine 6SM concentrations were expressed in ng per mg of creatinine (ng/mg Cr) to control for renal function and dilution of urine. Urinary creatinine was measured using the Siemens Dimension Xpand (NIH Clinical Center Chemistry Laboratory). The first morning void concentration of 6SM, normalized to urinary creatinine, has been extensively used as an estimate of overnight melatonin secretion (McMullan et al., 2013).

Child Sleep Habits Questionnaire

The Child Sleep Habits Questionnaire (CSHQ) is a validated parent-report questionnaire that examines sleep disturbance in children (Goodlin-Jones et al., 2008, Owens et al., 2000). The CSHQ contains 33 items that measure sleep disturbance and 3 items collecting information about bedtime, wakeup time, and sleep duration. Parents rate the frequency of each item using a 3-point Likert scale: "usually" (5–7 times per week), "sometimes" (2–4 times per week) and "rarely" (0–1 time per week), with higher scores indicating more frequent sleep disturbances. The CSHQ was completed by parents of children <13 years of age.

Statistical Analyses

Statistical analyses were performed using SPSS version 19.0 (IBM Corp., Armonk, NY). Skewness, kurtosis, and Kolmogorov–Smirnov test for normality were assessed. BMI-Z scores were calculated from subject heights and weights using the modified LMS method

(Cole, 1990) of the Centers for Disease Control and Prevention 2000 growth charts (Kuczmarski et al., 2000). Parametric tests were used for normally distributed data, while skewed data were assessed using nonparametric tests. Independent samples *t*-tests, Mann-Whitney U tests, and Fisher's exact tests were used to compare unadjusted difference in demographic and clinical characteristics between patients with *PAX6*^{+/-} and healthy controls. Skewed data were also normalized by log-transformation so that parametric analyses of covariance could be used to assess differences in pineal volume, 6SM, and CSHQ (adjusting for age, sex, race, BMI-Z), and serum melatonin (additionally adjusting for albumin). Back-transformed adjusted means and 95% confidence intervals are reported in the results for variables that were log-transformed. Diurnal variation in morning and evening cortisol concentrations within subjects were compared using the Wilcoxon signed rank test. Pearson and Spearman correlations were calculated to assess the relationships among pineal volume, melatonin secretion, and CSHQ scores. P-values <0.05 were considered nominally significant, while P-values <0.0083 were considered significant after Bonferroni correction for multiple comparisons.

Results

Patients included 37 individuals with *PAX6*^{+/-} (age 2 – 40y) and 17 healthy controls (age 7 – 32y). Subject characteristics are shown in Table 1. Within the *PAX6*^{+/-} group, there were no significant differences between patients with WAGR syndrome versus isolated aniridia for pineal volume, cortisol, melatonin, and sleep questionnaire score (all *p*'s>0.1; data not shown). Due to these similarities, participants with WAGR/11p deletion syndrome and isolated aniridia were combined for all subsequent analyses.

***PAX6* Haploinsufficiency is Associated with Reduced Pineal Volume**

Pineal volume, estimate by 2D measurements, was approximately 5-fold lower in *PAX6*^{+/-} versus controls (*p*<0.001, Table 1). A subset of subjects also had 3D volume measurements, yielding a similar 5-fold lower volume in *PAX6*^{+/-} versus controls (*p*<0.001, Table 1). The 2D-calculated and 3D-measured volumes were well correlated (*r*=0.95, *p*<0.001). After controlling for age, sex, race, and BMI-Z, both 2D and 3D pineal volumes were still found to be significantly lower in patients with *PAX6*^{+/-} versus controls (*p*'s< 0.001, Figure 1A, 1B).

***PAX6* Haploinsufficiency is Associated with a Normal Diurnal Pattern of Cortisol**

Midnight serum cortisol was similar in *PAX6*^{+/-} versus controls (*p*=0.14, Table 1). The 8AM serum cortisol values were also similar in *PAX6*^{+/-} versus controls (*p*=0.77, Table 1). After adjusting for age, sex, race, and BMI-Z, both midnight serum cortisol and 8AM serum cortisol values remained similar in *PAX6*^{+/-} versus controls (midnight serum cortisol adjusted mean [95% CI]: 2.2 [1.6 – 2.9] vs. 1.6 [1.1 – 2.3] µg/dL, *p*=0.19; 8AM serum cortisol: 11.7 [9.9 – 13.9] vs. 9.9 [7.9 – 12.5] µg/dL, *p*=0.26). Subjects in the *PAX6*^{+/-} and controls groups both had normal diurnal patterns of cortisol with higher levels at 8AM compared to midnight (*p*'s <0.001).

PAX6 Haploinsufficiency is Associated with Lower Serum Melatonin

Midnight serum melatonin was lower in *PAX6*^{+/-} versus controls ($p < 0.001$, Table 1). After controlling for age, sex, race, BMI-Z, and albumin, midnight serum melatonin remained lower in patients with *PAX6*^{+/-} versus controls ($p < 0.001$, Figure 2A). 8AM serum melatonin was also lower in *PAX6*^{+/-} versus controls ($p = 0.008$, Table 1). After controlling for age, sex, race, BMI-Z, and albumin, morning serum melatonin remained lower in patients with *PAX6*^{+/-} versus controls ($p = 0.006$, Figure 2B).

PAX6 Haploinsufficiency is Associated with Lower Morning Urinary 6SM

Morning urinary 6SM (reflecting nighttime melatonin production) was lower in *PAX6*^{+/-} versus controls ($p = 0.001$, Table 1). After controlling for age, sex, race, and BMI-Z, morning urinary 6SM melatonin remained lower in patients with *PAX6*^{+/-} versus controls ($p = 0.001$, Figure 2C). Evening urinary 6SM was not significantly different in *PAX6*^{+/-} versus controls ($p < 0.11$, Table 1). After controlling for age, sex, race, and BMI-Z, however, evening urinary 6SM melatonin was found to be nominally higher in patients with *PAX6*^{+/-} versus controls ($p = 0.04$, Figure 2D), but this difference did not remain significant after correction for multiple comparisons.

PAX6 Haploinsufficiency is Associated with Nominally Greater Reported Sleep Disturbances

Total CSHQ score was 15% higher (reflecting increased sleep disturbance) in children with *PAX6*^{+/-} versus controls ($p < 0.03$, Table 1), but this nominal difference was no longer significant after adjustment for multiple comparisons. After adjustment for age, sex, race, and BMI-Z, a trend toward higher CSHQ score was observed for the *PAX6*^{+/-} group as compared to the control group ($p = 0.06$).

Pineal Volume is Correlated with Serum Melatonin and Urine 6SM

Among all subjects combined, pineal volume was significantly correlated with midnight serum melatonin ($\rho = 0.57$, $p < 0.001$ for 2D-calculated; $\rho = 0.69$, $p < 0.001$ for 3D-measured) and first morning void 6SM ($\rho = 0.44$, $p = 0.006$ for 2D-calculated; $\rho = 0.39$, $p = 0.05$ for 3D).

Discussion

In patients with *PAX6*^{+/-} compared to controls, pineal volume, midnight serum melatonin, morning serum melatonin, and morning urinary 6SM were lower, while cortisol diurnal patterns were similar. Total CSHQ scores showed nominally greater parental report of sleep disturbances in children with *PAX6*^{+/-}. Pineal volume was significantly correlated with midnight serum melatonin and first morning void 6SM. Although pineal hypoplasia and absence have been previously reported in patients with various *PAX6* mutations (Abouzeid et al., 2009, Mitchell et al., 2003), this current study is the first to describe pineal volume, melatonin secretion, and sleep disturbance in this population. Our findings are consistent with animal studies that show correlation of pineal size with melatonin secretion. Melatonin in pinealectomized rats and humans is reduced and lacks circadian rhythmicity (Acuna-Castroviejo et al., 2014, Jan et al., 2001, Kocher et al., 2006, Ozaki and Lynch, 1976, Murata

et al., 1998, Lehmann et al., 1996). Furthermore, the size of the pineal has been correlated with melatonin concentrations in sheep (Coon et al., 1999).

Although we originally hypothesized that serum melatonin concentrations and urinary melatonin metabolite levels would be lower in the morning and evening in patients with *PAX6*^{+/-} as compared to controls, we observed that evening urinary 6SM was similar after adjusting for multiple comparisons. This similarity may be due to release into circulation of small amounts of extrapineal sources of melatonin (e.g. gastrointestinal tract) (Acuna-Castroviejo et al., 2014).

One limitation of our study is that blood was drawn at midnight, which may have preceded the typical melatonin peak (median for healthy adults usually between 2–4AM) (Arendt, 1995). We found midnight serum melatonin was approximately 2-fold lower in *PAX6*^{+/-} vs. controls, whereas first morning void urinary 6SM was approximately 5-fold lower in *PAX6*^{+/-} vs. controls. It is possible that blood may have been collected prior to attaining peak serum melatonin levels, thus blunting the difference between groups. An additional limitation of our study was data on circadian rhythmicity (e.g. timing of melatonin rhythm) were not collected, and total, rather than free, melatonin concentration was measured because we did not have salivary samples available. Because melatonin is weakly protein bound in peripheral circulation (Kennaway and Voultsios, 1998), differences in albumin concentration could alter free melatonin concentrations. In our cohort, serum albumin was comparable between groups and did not alter the results when used as a covariate for analyses. Another limitation was that sleep disturbance was only assessed in children using parent report and no objective sleep measures were analyzed between groups. Although patients with *PAX6*^{+/-} had a higher mean CSHQ score compared to controls, this difference was only nominally significant, suggesting that melatonin may be beneficial for sleep but that diminished levels do not necessarily lead to extreme sleep disturbances. Of note, however, potential reasons for the lack of significant difference after correction for multiple comparisons and after adjustment for demographic characteristics include the small sample size due to rarity of the condition and use of a measure that only collected parent-reported sleep disturbances in their children. Further studies utilizing objective measures such as actigraphy monitoring in a home-based environment would be useful to examine sleep patterns, as well as provide insight into clinical assessment and sleep-related interventions in this population.

This is the first study to explore the functional consequences of pineal hypoplasia in patients with *PAX6*^{+/-}, and our findings support the hypothesis that *PAX6* plays an important role in human pineal development and sleep regulation. Further studies examining sleep disturbance and sleep patterns would be beneficial in elucidating the *PAX6*^{+/-} sleep-related phenotype, which will aid in establishing effective sleep management in these patients. Furthermore, common genetic polymorphisms that affect the function of *PAX6* may be associated with insomnia in the general population (Ban et al., 2011) and additional studies of these genetic variants and their impact on pineal size, melatonin secretion, and sleep patterns could lead to genotype-specific treatment of insomnia using melatonin as a targeted therapy in *PAX6*-insufficient individuals.

In conclusion, the present study is the first to demonstrate significantly reduced pineal volume, reduced melatonin secretion, and greater sleep disturbance in patients with *PAX6*^{+/−} – versus controls. Further research is needed to determine if melatonin replacement could be particularly beneficial in *PAX6*^{+/−} patients, and potentially also in individuals in the general population with common genetic variants affecting PAX6 functioning.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was funded by the Intramural Research Programs of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (1ZIAHD008898-02) and the National Institute of Nursing Research of the National Institutes of Health (NIH), and by the NIH Bench-to-Bedside Program. We thank the members of International WAGR Syndrome Association, Aniridia Foundation International, and Aniridia Network UK for assistance in identifying families who were willing to participate in this study. We thank Jack Yanovski and David Klein for helpful discussions. We are grateful to Amanda Preston, PhD, for editorial assistance. We thank the following individuals for their assistance: Sheila Brady, Kristen Danley, Shannon Fuhr, Amanda Huey, Melanie Hicks, Daniela Reyes-Capo, Lindsay Hunter, Matthew Tsang, Mark Lee, Tanvee Singh, Emily Yin, and Yael Caplan.

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Figure 1.

Lower pineal volume in patients with *PAX6* haploinsufficiency (*PAX6*^{+/-}) as compared to controls. (A) Pineal volume was calculated by brain magnetic resonance imaging (MRI) using 2D analysis. (B) Pineal volume was calculated by brain MRI using 3D analysis. Covariates for (A) and (B) were age, sex, race, & BMI-Z. Adjusted means ± SEMs and nominal p-values, with asterisk (*) indicating significance after adjustment for multiple comparisons, are shown.

Figure 2.

Melatonin in patients with *PAX6* haploinsufficiency (*PAX6*^{+/-}) as compared to controls. (A, B) Patients with *PAX6*^{+/-} had lower adjusted midnight and morning serum melatonin as compared to controls. Melatonin was measured using a competitive enzyme-linked immunosorbent assay (ELISA). Covariates were age, sex, race, BMI-Z, and serum albumin. (C, D) Patients with *PAX6*^{+/-} had lower adjusted first morning urinary 6-sulfatoxymelatonin (6SM) but no significant difference in evening urinary 6SM as compared to controls. 6SM was normalized to urinary creatinine (to control for differences in dilution) and adjusted for age, sex, race, and BMI-Z. For (A–D), skewed data were normalized by log transformation. Back-transformed adjusted means with ± 95% confidence intervals are shown. Nominal p-values, with asterisk (*) indicating significance after adjustment for multiple comparisons, are shown.

Table 1

Characteristics of patients with *PAX6*^{+/-} and healthy controls.

	<i>PAX6</i> ^{+/-} (n=37)	Healthy Controls (n=17)	P-Values
Age (y)	15.3 ± 9.9 (2.4 – 40.5)	16.0 ± 7.2 (7.8 – 32.3)	0.80
Sex (% Female)	57	65	0.77
BMI (kg/m ²)	25.3 ± 7.4 (14.0 – 44.0)	26.6 ± 8.0 (15.2 – 39.2)	0.56
BMI-Z	1.4 ± 1.0 (-1.4 to +2.7)	1.2 ± 1.2 (-1.3 to +2.5)	0.64
Race/Ethnicity (%)			
Non-Hispanic Caucasian	86	65	0.08
Other	14	35	
Pineal Volume by 2D Calculation (μL)	31 ± 22 [*]	156 ± 76 [†]	<0.001
Pineal Volume by 3D Measurement (μL)	25 ± 15 [‡]	129 ± 50 [§]	<0.001
Midnight Serum Melatonin (pg/mL)	28.6 [22.1 – 42.6]	71.6 [46.4 – 88.1]	<0.001
8AM Serum Melatonin (pg/mL)	25.4 [18.1 – 29.3]	36.8 [24.1 – 68.0]	0.008
Morning Urinary 6SM (ng/mL)	11.8 [6.6 – 26.1]	45.2 [34.0 – 61.4]	0.001
Evening Urinary 6SM (ng/mL)	8.6 [3.7 – 16.7]	4.7 [3.5 – 7.0]	0.11
Midnight Serum Cortisol (μg/dL)	1.7 [1.2 – 2.9] ^{//}	1.1 [1.0 – 2.6] [¶]	0.14
8AM Serum Cortisol (μg/dL)	11.9 [7.6 – 15.8] [#]	11.5 [7.6 – 16.1] ^{**}	0.77
Albumin (g/dL)	3.9 [3.7 – 4.0] ^{††}	4.0 [3.8 – 4.2] ^{‡‡}	0.15
CSHQ Total Score	48 ± 6 ^{§§}	41 ± 5 ^{///}	0.03

Normally distributed variables are shown as mean ± SD and were analyzed using independent samples t-test. Nonparametric data are shown as median [25th – 75th percentile] and were analyzed using Mann-Whitney U test. Percentages were compared using Fisher's exact test. Abbreviations: BMI=body mass index; 6SM=6-sulfatoxymelatonin; CSHQ=Child Sleep Habits Questionnaire.

^{*}
n=34;

[†]
n=16;

[‡]
n=18;

[§]
n=14;

^{//}
n=25;

[¶]
n=16;

[#]
n=31;

^{**}
n=17;

$\dagger\dagger$
n=28;

$\dagger\dagger$
n=17;

$\S\S$
n= 15;

$////$
n=6

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