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Assessment of continuous positive airway pressure effect on the circadian clock signaling pathway in obstructive sleep apnea patients

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Obstructive sleep apnea (OSA) is associated with circadian rhythm dysregulation plausibly through affecting clock genes. The study's purpose was to investigate the effect of one-night continuous positive airway pressure treatment (CPAP treatment) on circadian clock genes: *BMAL1*, *CLOCK*, *CRY1*, and *PER1* at mRNA and protein levels. The study included 30 OSA patients, who underwent diagnostic polysomnography (PSG) and next a one-night effective CPAP treatment with PSG monitoring (CPAP). The blood was collected in the evening before and the morning after PSG and CPAP. Protein levels and mRNA expression were measured using ELISA and qRT-PCR, respectively. The increase in *PER1* expression was observed in the morning after compared to the evening before CPAP ($p = 0.005$); additionally, PER1 protein level decreased in the morning after CPAP compared to the morning after PSG ($p = 0.035$). In CLOCK protein levels significant changes were observed: an increase in the morning after CPAP compared to the morning after PSG ($p = 0.049$), an increase in the morning after CPAP compared to the evening before ($p = 0.006$), and an increase in difference between the morning after and evening before CPAP vs. difference between morning after and evening before PSG ($p = 0.012$). Obtained results suggest that even short-term effective CPAP treatment might reverse circadian clock signaling pathway disruption in OSA.

Keywords Obstructive sleep apnea (OSA), Circadian clock, Continuous positive airway pressure (CPAP), Polysomnography (PSG)

Circadian rhythm is a 24-h sleep–wake pattern driven by the master circadian pacemaker located in the suprachiasmatic nucleus of the hypothalamus[1]. In humans the main mechanism is regulated by two types of genes – activators, such as circadian locomotor output cycles kaput (*CLOCK*), and basic helix-loop-helix ARNT-like protein (*BMAL*), or repressors, such as period (*PER*) and cryptochrome (*CRY*) – working together as a negative feedback loop. CLOCK-BMAL1 complex promotes *PER* and *CRY* gene expression via binding to repressors' promoter regions. An increase of PER and CRY proteins results in the forming dimers PER-CRY, which translocate to the nucleus and in turn inhibit *CLOCK* and *BMAL* gene expression[2]. The rhythmic oscillations of repressor levels and BMAL1/CLOCK-dependent gene expression govern the circadian regulation of cellular processes, aligning them with the day/night cycle. In healthy individuals, the peak transcriptional activity of the BMAL1/CLOCK activator complex occurs around midday, coinciding with the nadir of repressor protein levels. The elevated activity of BMAL1/CLOCK during daylight hours drives the accumulation of repressors, reaching their maximum levels in the late evening. This, in turn, suppresses BMAL1/CLOCK activity, establishing a negative feedback loop essential for maintaining circadian rhythmicity[3].

Obstructive sleep apnea (OSA) is a prevalent sleep-related breathing disorder characterized by recurrent episodes of apneas or hypopneas during sleep[4] with the most popular treatment being continuous positive airway pressure (CPAP) treatment, which provides upper airway support and prevents collapse[5,6]. OSA results in chronic intermittent hypoxia, sleep fragmentation, and sympathetic activation[7]. These factors, especially hypoxia and multiple arousals during sleep, can contribute to circadian rhythm disruption among OSA patients, which might be involved in common OSA comorbidities including cardiovascular, metabolic, and psychiatric

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diseases[8–12]. For instance, major depressive disorder is characterized by disrupted expression of *PER1*, *PER2*, *CRY1*, and *BMAL1* genes[11], or hypertension, which through changes in circadian clock expression, may disrupt a lipid metabolism[12]. All those states are associated with OSA.

In general, literature shows that patients with OSA present with disruption of the circadian clock on protein and gene expression levels. However, the reports are not entirely consistent regarding specific circadian clock genes that are dysregulated in this group. For example, Yang et al. have shown that daily expression patterns of *CLOCK*, *BMAL1*, and *CRY2* gene expression levels were disrupted among OSA patients by its complete abolishing of expression daily patterns in comparison to the healthy control group. Moreover, only midnight *PER1* was not significantly downregulated out of 9 evaluated circadian clock genes that were evaluated in the study[13]. Additionally, analysis performed by this group selected expression levels of *CRY1* and *PER3* as independent risk factors for severe OSA[13]. On the other hand, Gaspar et al. reported only *BMAL1* expression was increased in the morning among OSA patients compared to healthy controls, while at night *PER1* and *CRY2* expression was decreased[14]. Other studies have presented upregulation of *BMAL1* and reduced expression of *PER1*[15] as well as dysregulation of *CLOCK* and *PER1*[16] expression levels in OSA patients. In our previous studies we found increased expression of *BMAL1*, *CLOCK*, *PER1* and *CRY1* genes and increased serum level of PER1 in OSA patients compared to controls[8,17]. Although the available studies contradict each other in some aspects, it can be generally concluded that patients with OSA are characterized by abolished normal expression of circadian cycle proteins and elevated levels of repressors, including PER1. The differences in results may be due to the selected study group, which, depending on the study, includes severe OSA or the entire spectrum of disease severity, not including the division.

One of the possible mechanisms involved in circadian clock dysregulation is suggested to involve interaction with hypoxia-inducible factor 1 α (HIF1-1 α), a key regulator of oxygen metabolism. This was reported on both the gene expression[18] and the protein levels[8].

There are only a few studies examining the potential effect of the CPAP treatment on circadian clock gene dysregulation among OSA patients. Burioka et al. assessed the expression of *PER1* mRNA in the peripheral blood of individuals with OSA both before and after CPAP treatment. The results revealed that the implementation of CPAP therapy significantly ameliorated the dysregulation of the *PER1* gene in OSA patients[19]. Other studies reported a lack of CPAP effect, Moreira et al. have shown that the expression level of *CLOCK* was lower compared to controls, but not improved by CPAP treatment[20]. Some data suggest that the length of CPAP treatment may be important, showing that long-term CPAP fully reversed circadian clock gene expressions, so they resembled those of controls[14]. There are no studies on the effect of one-night CPAP treatment on the circadian clock in OSA. However, existing literature provides valuable insights into the effects of one-night interventions on circadian clock gene expression. For example, Cedernaes et al. found that total sleep deprivation was associated with decreased expression of *BMAL1* and *CRY1* in comparison to the sleep group[21]. Also, the time-mealing has an impact on the circadian clock. The early time-restricted feeding (between 8 AM and 2 PM) was characterized by increased expression of BMAL1 in the morning, and *CRY1*, *CRY2*, and *RORA* in both time points[22]. Incorporating these findings into our study underscores the importance of examining the short-term effects of CPAP therapy on circadian rhythms and highlights the potential implications for therapeutic interventions targeting sleep disorders. To the best of our knowledge, no data on the effect of CPAP treatment on circadian clock proteins are available.

The aim of our study was to investigate the effect of one-night CPAP treatment on chosen activators (CLOCK, BMAL1) and repressors (PER1, CRY1) at the gene expression and protein level. We hypothesize that following CPAP changes in proteins and gene expressions will be observed and they will be mainly related to changes in respiratory-related PSG parameters. Based on the available literature, we hypothesized that the overexpression of repressors in OSA patients will be abolished by one-night CPAP treatment, both at the mRNA and protein level.

Methods

Sample

The study group consisted of 30 patients, diagnosed with OSA following a nocturnal PSG examination at the Sleep and Respiratory Disorders Centre in Lodz (Poland) and underwent a one-night effective CPAP treatment with PSG monitoring. Inclusion criteria for this study were: age within 18–75 years and body-mass index (BMI) between 20 and 45 kg/m^2. The exclusion criteria included inflammatory diseases (e.g., connective tissue diseases or inflammatory bowel diseases), chronic respiratory diseases (e.g., bronchial asthma or chronic obstructive pulmonary disease), any infection within one month of blood collection, diagnosis of cancer (in medical history), diagnosed major neurological conditions, diagnosed psychiatric disorders including insomnia and taking medications affecting sleep (e.g., benzodiazepines and melatonin). All experiments involving human subjects or tissue samples were conducted in accordance with the relevant guidelines and regulations, specifically the Act on the Medical Profession and Dentist Profession of Poland, as well as the guidelines and approval from the Bioethics Committee of the Medical University of Łódź. Prior to participation, all subjects provided written informed consent, and the study protocol was reviewed and approved by the Bioethics Committee of the Medical University of Łódź (RNN/432/18/KE).

Polysomnography and CPAP treatment

Participants were admitted to the sleep lab at 21:00 h (±0.5 h) and underwent physical examination (measurement of body mass, height, heart rate, and blood pressure). The following channels were used to perform nocturnal PSG: electroencephalography (C4\A1, C3\A2), chin muscles and anterior tibialis electromyography, electrooculography, measurements of oronasal airflow (a thermistor gauge), snoring, body position, respiratory movements of chest and abdomen (piezoelectric gauges), unipolar electrocardiogram and hemoglobin oxygen saturation (SpO2) (Alice 6, Phillips-Respironics). The criteria based on the 30-s epoch standard were used to score

sleep stages in the recorded PSG[23]. Apnea was defined as the reduction of airflow to less than 10% of the baseline for at least 10 s. Hypopnea was described as at least a 30% reduction of airflow for at least 10 s, accompanied by an over 3% decrease in SpO2 or arousal. The American Academy of Sleep Medicine guidelines were used to score the arousals[23]. The same setup and guidelines were used to monitor one-night CPAP treatment, which serves as an initial trial for long-term therapy (first night of CPAP treatment). During this night, patients are fitted with an appropriate mask, and optimal therapeutic pressures are calibrated. In this context, the minimum duration of CPAP usage is 4 h.

Material collection and assessment of protein and mRNA level

In the evening before and the morning following PSG, and CPAP treatment peripheral blood samples were collected into collection tubes with clot activator and with EDTA (at 21:00–21:30, 15 min before lights out, and 06:00–07:00, within 10 min from awakening, respectively). Blood samples with clot activator were centrifuged immediately following blood draws at 4 °C. The serum was collected and stored at − 80 °C. The utilization of blood in regulating the rhythms of clock gene expression, such as in the assessment of immediate fluctuations in circulating molecules influenced by CPAP, could be deemed suitable[24].

The serum BMAL1, CLOCK, PER1, and CRY1 protein concentration was assessed by ELISA kit (FineTest for CRY1, EIAab Science for BMAL1, CLOCK, and PER1 (Wuhan, China)). The absorbance was measured at λ = 450 nm wavelength by absorbance reader (BioTek 800 TS, Agilent Technologies, Santa Clara, CA, USA).

RNA isolation from PBLs was performed using TRIzol (Invitrogen). RNA Integrity Number (RIN) as well as the concentration of the isolated RNA was assessed using Nanodrop Colibri Microvolume Spectrometer (Titertek Berthold, Germany). The obtained material was reversely transcribed with a dedicated kit according to the protocol provided by the manufacturer (SuperScript IV First-Strand Synthesis System, Thermo Fisher Scientific Inc., California, United States). The process comprised 3 steps, assays underwent annealing at 60 °C in 60 s. The level of expression of chosen genes was determined by quantitative real-time polymerase chain reaction; the applied mixture consisted of nuclease-free water, Master Mix TaqMan Fast Advanced, cDNA, gene-specific probes (TaqMan assays for *BMAL1*, *CLOCK*, *CRY1*, *PER1*, reference gene: *β-Actin*). Three reactions were performed for each sample and the reference gene. For each sample, the cycle threshold (CT) was calculated. Then, ΔCt was calculated and used in mRNA expression analysis by the following Eq. 2^(− ΔCt).

Statistical analysis

The level of statistical significance was set at $p < 0.05$. Statistical analysis was performed with SPSS 28.0 (IBM, Chicago, IL, USA). The distribution of variables was evaluated by the Shapiro–Wilk test. The parameters with normally distributed data were compared by paired t-test, otherwise Wilcoxon test was used to compare dependent variables. All continuous data is presented as median and interquartile range (IQR) to allow for comparison between variables. Spearman's rank correlation was used to assess correlations.

Results

The study group comprised 27 men (90%) and 3 women (10%) with a median age (years old) of 57.0 (46.8–62.3) and BMI (kg/m2) 35.1 (32.0–38.4). Comparison of PSG data from baseline examination and the first night with CPAP treatment are presented in Table 1.

	PSG	CPAP	*p*-Value	Δ PSG and CPAP
Sleep efficiency (%)	86.2 (74.8–89.7)	82.5 (73.5–88.7)	0.156	1.4 (− 2.9 to 18.5)
Sleep maintenance (%)	91.7 (80.0–93.2)	88.5 (83.7–93.2)	0.088	2.3 (− 1.1 to 10.6)
Sleep onset latency (min)	16.0 (9.0–27.0)	11.3 (7.4–26.9)	0.360	3.5 (− 4.8 to 8.8)
N1 (h)	2.10 (1.65–3.41)	0.97 (0.56–1.56)	**0.026**	1.26 (0.40–3.02)
N2 (h)	1.94 (1.07–2.78)	2.21 (1.59–2.58)	**0.025**	− 0.68 (− 1.13 to 0.82)
N3 (h)	0.56 (0.13–1.14)	1.07 (0.57–1.55)	**< 0.001**	− 0.28 (− 1.25 to 0.02)
TST (h)	6.55 (5.75–7.24)	6.20 (5.30–6.53)	**< 0.001**	0.21 (− 0.12 to 1.78)
REM (h)	1.17 (0.72–1.43)	1.64 (1.18–2.02)	0.218	− 0.43 (− 0.85 to 0.03)
nREM (h)	5.31 (4.88–5.81)	4.38 93.74–4.84)	**0.005**	0.85 (0.31–1.93)
Arousal Index (events/h)	22.3 (15.0–31.2)	8.7 (4.5–11.4)	**< 0.001**	10.9 (4.5–25.8)
AHI (events/h)	48.0 (24.8–67.2)	1.4 (0.4–4.2)	**< 0.001**	40.0 (24.3–61.1)
AHI REM (events/h)	40.1 (25.5–62.3)	0.3 (0.0–0.9)	**< 0.001**	40.9 (28.7–62.2)
AHI nREM (events/h)	42.3 (20.2–66.4)	1.1 (0.1–3.8)	**< 0.001**	38.6 (17.8–59.9)
Desaturation Index (events/h)	50.6 (27.1–78.7)	6.0 (1.0–12.5)	**< 0.001**	14.0 (0.0–34.3)
Total number of desaturations	303.0 (137.8–349.5)	29.5 (10.5–57.3)	**< 0.001**	15.1 (− 11.8 to 51.6)
Minimum Oxygen Saturation (%)	71.4 (65.0–76.0)	85.4 (83.4–86.9)	**< 0.001**	− 33.5 (− 53.4 to (− 4.3))

Table 1. Baseline characteristics of the study group. AHI – apnea–hypopnea index, BMI – body mass index, M – male, N1—non-rapid eye movement (nREM) sleep stage 1, N2—non-rapid eye movement (nREM) sleep stage 2, N3—non-rapid eye movement (nREM) sleep stage 3, nREM – non-rapid eye movement, OSA – obstructive sleep, REM – rapid eye movement, TST – total sleep time. Significant *p*-values are in bold.

Following CPAP treatment, a 54.9% (26.4–83.9%) and 95.6% (87.7–98.9%) reduction in arousal index and AHI was observed, respectively.

Comparisons between circadian gene expressions and protein levels

CLOCK gene expression increased in the morning after PSG compared to the evening before ($p = 0.037$). An increase in CLOCK protein level was observed in the morning after CPAP compared to the morning after PSG and the evening before CPAP ($p = 0.049$ and $p = 0.006$, respectively). Furthermore, the difference between morning after and evening before CPAP in CLOCK protein level was greater than the difference between morning after and evening before PSG ($p = 0.012$) (Table 2).

PER1 gene expression increased in the morning after CPAP in relation to the evening before the CPAP ($p = 0.005$) (Table 2). Moreover, the PER1 protein level was lower in the morning after and in the evening before CPAP compared with the same time points of PSG ($p = 0.035$ and $p = 0.045$, respectively). There were no statistically significant differences between morning after and evening before time points of both, PSG and CPAP.

CRY1 gene expression was greater in the morning after PSG compared to the evening before the PSG ($p = 0.028$), while no changes in CRY1 protein level were observed (Table 2).

No changes in gene *BMAL1* expression and protein level were observed (Table 2).

	Evening before PSG	Morning after PSG	Evening before CPAP	Morning after CPAP	Difference between morning after and evening before PSG	Difference between morning after and evening before CPAP	Difference between morning after CPAP and morning after PSG	Difference between evening before CPAP and evening before PSG	*p*-value
Gene expression									
BMAL1	6.09 (2.14–28.41)	8.43 (3.69–12.53)	6.45 (1.51–27.42)	10.77 (3.46–20.20)	1.81 (−8.86–5.89)	−0.00 (−7.67–4.25)	1.72 (-2.80–16.07)	−0.70 (-6.21–13.95)	0.285[a] 0.375[b] 0.738[c] 0.319[d] 0.268[e] **0.037[a]** 0.561[b]
CLOCK	1.95 (1.16–3.26)	3.72 (1.23–6.62)	1.80 (1.22–7.53)	4.18 (1.60–6.41)	2.38 (0.00–5.60)	−0.08 (−3.53–3.87)	−0.69 (-3.88–3.02)	0.75 (-2.67–7.09)	0.330[c] 0.763[d] 0.864[e] **0.028[a]** 0.816[b]
CRY1	1.43 (0.77–4.12)	2.95 (1.56–5.04)	2.57 (0.82–4.58)	3.68 (0.94–7.21)	1.50 (0.23–2.88)	0.48 (−2.28–1.32)	1.06 (-0.71–3.16)	0.02 (-2.40–5.78)	0.195[c] 0.329[d] 0.199[e] 0.340[a] **0.005[b]**
PER1	2.97 (0.54–12.86)	7.37 (2.08–16.65)	4.08 (0.87–7.62)	18.50 (2.86–55.75)	1.72 (0.50–7.56)	7.38 (0.01–49.45)	4.43 (-5.32–49.35)	−0.71 (-5.42–5.90)	0.492[c] 0.327[d] 0.630[e]
Proteins [ng/ml]									
BMAL1	17.58 (16.90–18.53)	17.75 (17.47–18.02)	17.50 (17.25–8.37)	17.75 (17.25–18.59)	0.17 (−0.94–0.87)	0.10 (−0.66–0.74)	0.02 (-0.50–0.99)	0.24 (-0.50–0.63)	0.571[a] 0.420[b] 0.870[c] 0.105[d] 0.231[e] 0.389[a] **0.006[b]**
CLOCK	3.55 (3.29–3.67)	3.59 (3.50–3.71)	3.53 (3.09–3.65)	3.64 (3.54–3.98)	0.00 (−0.08–0.16)	0.06 (−0.04–0.49)	0.03 (-0.14–0.34)	−0.03 (-0.13–0.07)	0.221[c] **0.049[d]** **0.012[e]** 0.370[a] 0.592[b]
CRY1	37.96 (29.24–43.44)	36.54 (27.13–43.78)	38.93 (25.65–45.53)	36.45 (24.83–46.82)	1.67 (−9.20–7.87)	1.82 (−7.84–8.26)	−1.49 (−7.90–5.85)	−1.22 (-13.22–6.13)	0.164[c] 1.000[d] 0.310[e] 0.125[a] 0.611[b] **0.045[c]**
PER1	300.84 (268.85–388.59)	311.59 (279.46–384.95)	282.18 (226.45–329.16)	286.92 (219.68–346.62)	8.95 (−18.40–68.48)	−3.68 (−34.02–45.37)	−8.72 (−138.33–18.88)	−22.50 (-79.68–14.99)	**0.035[d]** 0.125[a] 0.611[b] 0.548[e]

Table 2. Comparison of circadian gene expressions and protein levels. Data presented as median (IQR); *p*-values: a – the evening before vs. the morning after PSG, b – the evening before vs. the morning after CPAP, c – the evening before PSG vs. the evening before CPAP, d – the morning after PSG vs. the morning after CPAP, e - Difference between the morning after and the evening before PSG vs. Difference between the morning after and the evening before CPAP. Abbreviations: BMAL1—brain and muscle ARNT-like 1, CPAP – continuous positive airway pressure, CLOCK – circadian locomoto1r output cycles kaput, CRY1 – cryptochrome 1, PER1 – period 1, PSG – polysomnography. Statistically significant *p*-values are denoted in the bold.

The summary of circadian gene expression and protein level changes is shown in Fig. 1.

Correlation between circadian gene expressions and protein level

In evaluated relationships, *BMAL1* gene expression in the evening before and morning after PSG was positively correlated (R = 0.472; $p = 0.020$; Fig. 2A) as well as evening before and morning after CPAP were associated (R = 0.582; $p = 0.023$; Fig. 2B).There were no correlations between evening and morning BMAL1 protein level in both time points.

The *CLOCK* gene expression levels in the evening before and morning after PSG were associated (R = 0.466; $p = 0.033$; Fig. 2C). Additionally, *CLOCK* gene expression in the evening before PSG was positively correlated with its protein level at the same time point (R = 0.415; $p = 0.049$).

The CLOCK protein level in the evening before PSG was associated with its protein level the morning after PSG (R = 0.412; $p = 0.024$; Fig. 2D). During the CPAP treatment, there is no such association.

The *PER1* gene expression in the evening before and morning after PSG was associated (R = 0.596; $p < 0.001$; Fig. 2E). *PER1* gene expression in the morning after CPAP negatively correlated with its protein level at the same time point (R = − 0.550; $p = 0.005$).

The PER1 protein level in the evening before PSG correlated with the protein level in the morning after PSG (R = 0.429; $p = 0.018$; Fig. 2F). During the CPAP treatment, this association was lifted.

No correlations were found between either *CRY1* gene expression at different time points or with CRY1 protein level. CRY1 protein levels in the evening before and morning after PSG were associated (R = 0.410; $p = 0.024$; Fig. 2G), similarly the evening before and morning after CPAP (R = 0.505; $p = 0.004$; Fig. 2H).

A summary of the evaluated correlation between circadian gene expressions and protein levels is shown in Supplementary Table S1.

Correlations between the differences in PSG variables between PSG and CPAP with circadian gene expression and protein levels

The change in total number of desaturations was associated with *BMAL1* gene expression in the morning after PSG (R = 0.413, p = 0.040), what was lifted during the CPAP. However, the repressors of the circadian clock genes in the morning after CPAP: *CRY1* and *PER1* (R = − 0.538, p = 0.031, and R = − 0.562, $p = 0.008$, respectively) were correlated (Table 3).

Out of protein level PER1 in the morning after PSG and CPAP positively correlated with the change in total number of desaturations (R = 0.427, $p = 0.030$, and R = 0.476, $p = 0.014$, respectively). What is more, relationships between the changes in total number of desaturations were found with BMAL1 protein level in the morning after CPAP (R = − 0.448, $p = 0.022$) (Table 3).

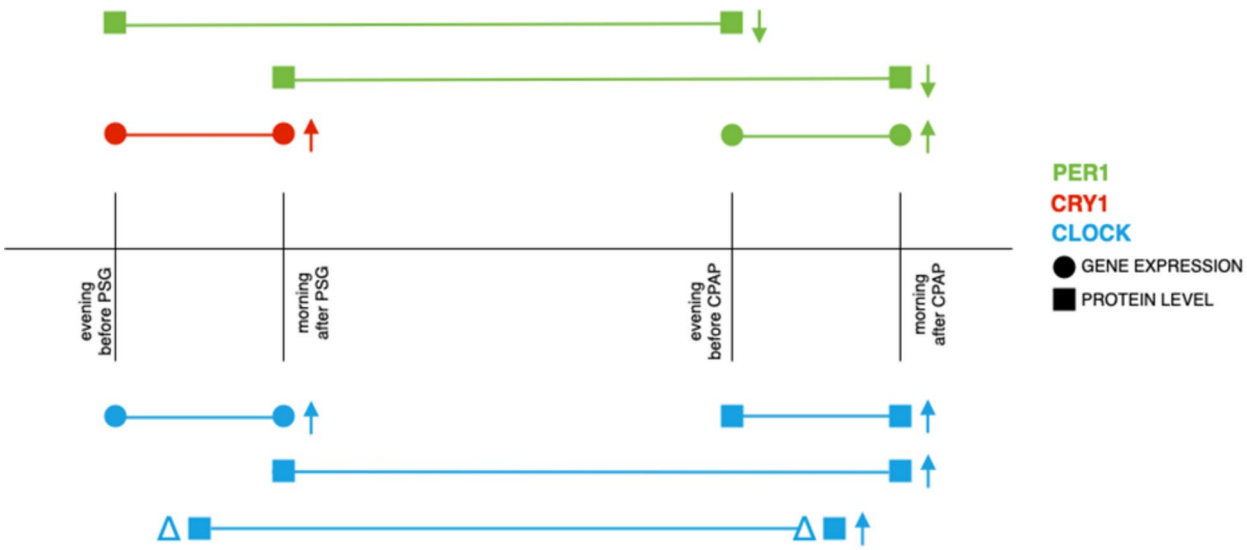

Fig. 1. Summary of the protein level and gene expression changes. Statistically significant changes: CLOCK – increase in gene expression in the morning after vs. the evening before PSG ($p = 0.037$), increase in protein level in the morning after CPAP compared to the evening before CPAP ($p = 0.006$) morning after PSG ($p = 0.049$), increase in the difference between protein level the morning after and the evening before CPAP vs. difference between the morning after and the evening before CPAP ($p = 0.012$); CRY1 – increase in gene expression in the morning after vs. evening before PSG ($p = 0.028$); PER1 – decrease in protein level in the evening before CPAP vs. evening before PSG ($p = 0.045$), decrease in protein level in the morning after CLOCK vs. morning after CPAP ($p = 0.035$), an increase in gene expression in the morning after vs. evening before CPAP ($p = 0.005$). No changes in BMAL1 expression and protein level were observed. Abbreviations: BMAL1—brain and muscle ARNT-like 1, CPAP – continuous positive airway pressure, CLOCK – circadian locomotor output cycles kaput, CRY1 – cryptochrome 1, PER1 – period 1, PSG – polysomnography.

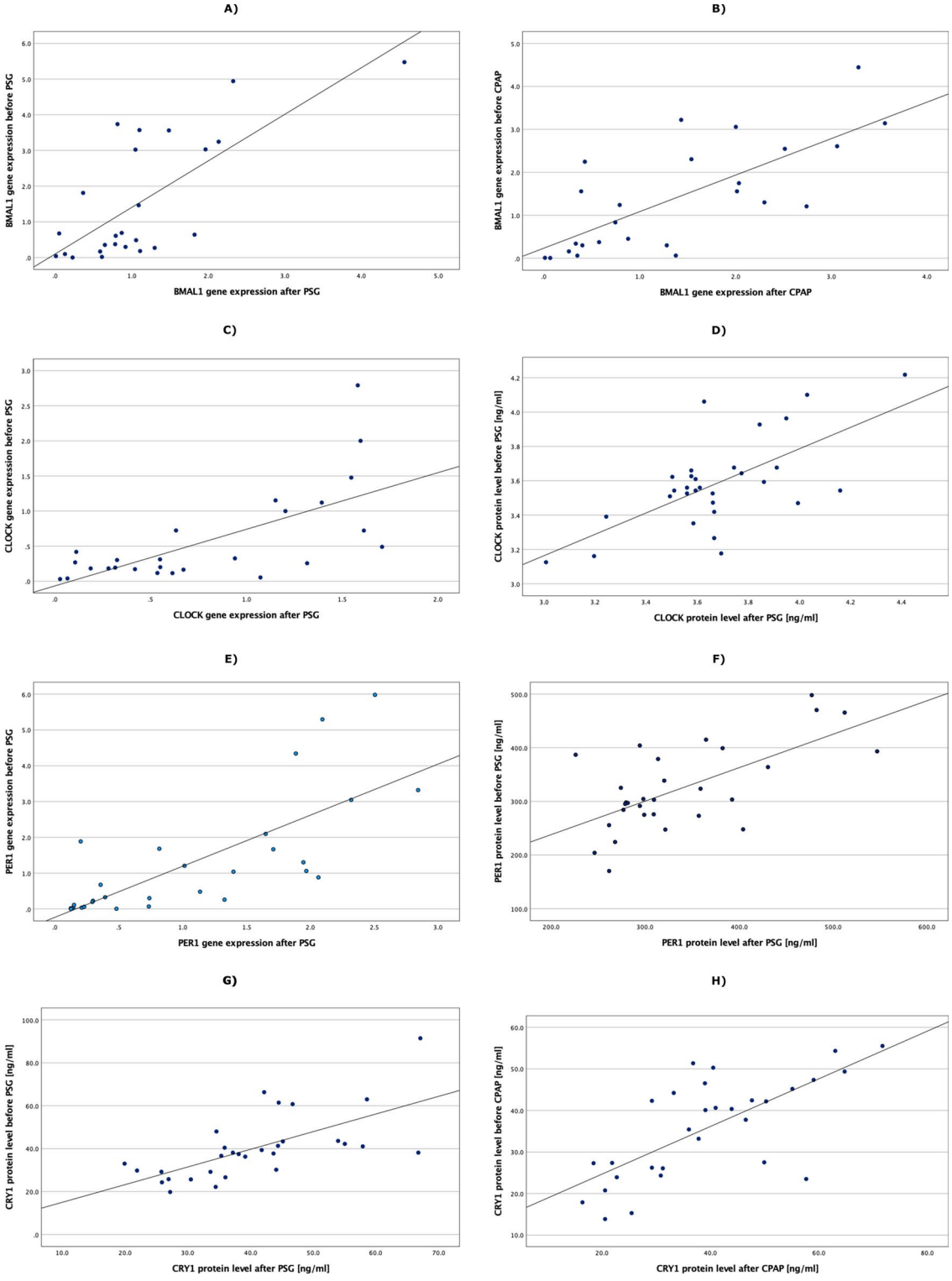

Fig. 2. Correlations between circadian clock gene expressions and protein levels. BMAL1—brain and muscle ARNT-like 1, CPAP – continuous positive airway pressure, CLOCK – circadian locomotor output cycles kaput, CRY1 – cryptochrome 1, PER1 – period 1, PSG – polysomnography.

Out of gene expressions *BMAL1* in the evening before CPAP and *CLOCK* in the morning after CPAP positively correlated with change in AHI (R = 0.407, $p = 0.044$, and R = 0.629, $p = 0.028$, respectively), while *PER1* in the morning after CPAP was associated with a percentage decrease in AHI (R = – 0.463, $p = 0.034$) (Table 3).

Furthermore, out of protein levels, correlations between the change in AHI with CLOCK and PER1 protein levels in the morning after CPAP were observed (R = – 0.400, $p = 0.043$ and R = 0.441, $p = 0.024$, respectively)

	Gene expression				Protein concentration			
	Morning after PSG	Morning after CPAP	Difference between morning after CPAP and evening before CPAP	Difference between morning after CPAP and morning after PSG	Morning after PSG	Morning after CPAP	Difference between morning after CPAP and evening before CPAP	Difference between morning after CPAP and morning after PSG
Δ AHI [events/h]	R = 0.407* p = 0.044*	R = 0.629# p = 0.028#	x	x	x	R = − 0.400# p = 0.043# R = 0.441† p = 0.024†	x	R = − 0.491* p = 0.011*
Decrease Percentage in AHI	x	R = − 0.463† p = 0.034†	x	x	x	R = − 0.589* p = 0.002* R = − 0.431# p = 0.028#	x	R = − 0.662* p < 0.001* R = − 0.517# p = 0.007#
Δ Desaturation Index [events/h]	x	x	R = − 0.646† p = 0.005†	x	R = 0.435† p = 0.030†	x	x	x
Δ Total Number of Desaturations	R = 0.413* p = 0.040*	R = − 0.538‡ p = 0.031‡ R = − 0.562† p = 0.008†	x	R = − 0.565‡ p = 0.035‡	R = 0.427† p = 0.030†	R = − 0.448* p = 0.022* R = 0.476† p = 0.014†	x	R = − 0.532* p = 0.005* R = − 0.506# p = 0.008#

Table 3. Correlation summary of the differences in chosen PSG variables between PSG and CPAP with circadian gene expression and protein levels. Δ—delta, AHI—apnea-hypopnea index, BMAL1—brain and muscle ARNT-like 1, BMI—body mass index, CPAP—continuous positive airway pressure, CLOCK—circadian locomotor output cycles kaput, CRY1—cryptochrome 1, PER1—period 1, REM—rapid eye movement, PSG—polysomnography. *BMAL1, #CLOCK, †PER1, ‡CRY1, x—no significant correlation for all assessed genes and proteins.

as well as with the difference between PER1 protein levels in the morning after CPAP and PSG ($R = -0.491$, $p = 0.011$). Additionally, the percentage decrease in AHI negatively correlated with both activator proteins: BMAL1 and CLOCK in the morning after CPAP ($R = -0.589$, $p = 0.002$ and $R = -0.431$, $p = 0.028$, respectively) as well as with their difference in the morning after CPAP and PSG ($R = -0.662$, $p < 0.001$ and $R = -0.517$, $p = 0.007$, respectively) (Table 3).

No correlations between circadian clock gene expression and protein levels with either a change in arousal index or a percentage decrease in arousal index were found.

All correlations between circadian clock gene expression and protein levels and PSG parameters between CPAP and PSG are presented in Supplementary Table S2.

Discussion

Circadian clock disruption, represented by alteration of the particular set of genes – *CLOCK*, *CRY1*, *BMAL1*, *PER1* – can be one of the OSA consequences, leading to further health complications[8,14]. CPAP treatment as the gold standard for OSA has been associated with the improvement of life quality, e.g. reducing the risk of premature death, metabolic and cardiovascular comorbidities, or cognitive impairment – major circadian clock disruption outcomes[25]. Our study demonstrated that one-night CPAP treatment has an impact on the circadian clock gene expression in moderate-to-severe OSA patients.

In our recent study, we found that OSA patients were characterized by elevated protein concentrations of CLOCK, PER1, and CRY1 in the evening and CLOCK, BMAL1, and PER1 in the morning, before and after PSG[8]. In this study, first, we assessed changes in concentrations of circadian clock proteins during PSG at both time points, which did not reveal any statistically significant. It would correctly expect a constant concentration of CLOCK and BMAL1 proteins, both in the morning and in the evening. In turn, repressors (PER1 and CRY1) should show circadian variability. Their concentrations should be highest in the evening and lowest in the morning. However, we observed an increase in *CLOCK* and *CRY1* gene expression in the morning after PSG, which is supported by results by Moreira et al. noted that the *CLOCK* gene is overexpressed in the OSA population[20]. BMAL1-CLOCK is an active transcription complex that leads to increased expression of repressors. Next, CRY and PER are translocated to the cytoplasm, where they are phosphorylated and make heterodimer, which inhibits activators in the nucleus[26]. At the same time, there is continuous ubiquitin-dependent degradation of repressors[26]. Repressor protein concentrations peak in the evening, which leads to stopping their expression, and further to the lowest levels in the morning[8,26]. Those oscillations may be altered in OSA patients, due to the increased activity of hypoxia-inducible factor 1 (HIF-1), which is a complex of helix-loop-helix (bHLH)—Per/Arnt/Sim (PAS) transcription factor family proteins[27]. Among its targets are circadian clock repressor genes, which have hypoxia response elements in their gene promotors. Thus, the hypoxia state in OSA can increase the expression of *PER1* and *CRY1* and might disable the negative feedback loop, which may diminish the differences between evening and morning repressor (PER1 and CRY1) levels and might explain the lack of protein concentration differences. It would be confirmed by increased protein levels and expression of *PER1* in the morning after PSG in comparison to the evening before PSG, which was shown in our study. However, those differences were not statistically significant.

The primary aim of our study was to evaluate the impact of one-day CPAP treatment on the circadian clock elements (such as CLOCK, BMAL1, PER1, and CRY1) at the protein and gene expression levels. We assessed

those parameters at both time points—in the evening before and the morning after CPAP. Interestingly, increased CLOCK protein concentration was observed in the morning after CPAP compared to the evening before CPAP, as well as in relation to the morning after PSG. Moreover, the difference between the morning after and evening before CPAP CLOCK protein level was greater than the difference between the same time points of PSG. It could indicate possible overexpression of *CLOCK*. Moreira et al. in their study, showed that one month of CPAP treatment was not enough to affect *CLOCK* gene expression[20]. We similarly did not observe any effect of one-day CPAP treatment on *CLOCK* gene expression level. It is conceivable that the CPAP treatment affects the translational process of CLOCK, potentially elucidating why an elevation in CLOCK was observed at the protein level while not manifesting at the gene expression level[28,29]. On the other hand, the analysis revealed a diurnal increase in *PER1* gene expression following CPAP. This observation is supported by results from a study by Burioka et al., in which the abolition of the daily pattern of *PER1* expression was noted among OSA patients, which next improved following the 3 months of CPAP treatment reverting the daily pattern of *PER1* to similar structure to one present in the control group. Their study also evaluated the role of noradrenaline (NA). NA was elevated in OSA patients, and what is more, administration of NA induced an increase of *PER1* expression in mice model[19], pointing to one of the plausible involved mechanisms—the overactivity of the sympathetic nervous system on circadian clock disruption. Moreover, similar outcomes of Tampakakis et al. showed that inhibition of sympathetic innervation in vivo leads to down-regulation of PER1 and PER2 in heart neurons, and administration of NA caused the opposite effect[30].

Interestingly, PER1 protein concentrations were reduced in the evening before and morning after CPAP compared to the same time points of PSG, respectively. It suggests instability or fluctuation of the protein itself. Moreover, *PER1* expression was negatively correlated with its protein product in the morning after CPAP. The illogical correlation between *PER1* expression and the concentrations of PER1 protein may result from newly activated repair mechanisms. One possible explanation is post-transcriptional or post-translational regulation[29]. For example, it turns out that the 3' untranslated region (3'UTR) of *PER2* mRNA with N6 methylation of adenosine negatively regulates its expression and promotes RNA degradation[29,31]. Unfortunately, this mechanism was not examined in the context of the *PER1* gene. However, there are three miRNAs (miR-24, miR29a, and miR30a) that target 3'UTR of *PERs* and destabilize their mRNA, including *PER1*[32,33]. As for the regulation of the stability of PERs proteins, it may depend on the binding of casein kinase 1 (CK1) subunits in its binding domain, which, depending on the site of modification, may stabilize or promote degradation. For example, phosphorylation at S478 in the phosphodegron promotes recruitment of the E3 ubiquitin ligase β-TrCP and thus degradation[29]. It's essential to consider the intricate network of molecular interactions and regulatory factors influencing gene expression and protein synthesis when explaining such negative correlations.

It should be emphasized that in our study we examined the effect of one-day CPAP therapy. Gaspar et al. have shown that the duration of CPAP treatment has an impact on the circadian clock gene expression. In their study, short-term (four months) CPAP treatment promoted an evident re-establishment of *BMAL1* expression and decreased *PER1* and *CRY2* expression[14] in comparison to a control group (before the treatment). Oppositely, after long-term (two years) CPAP treatment they revealed overexpression of many circadian clock genes, such as *BMAL1*, *DEC1* (deleted in esophageal cancer 1 protein), *PER2*, *PER3*, *CRY1*, *REV-ERBβ* (nuclear receptor subfamily 1 group D member 2[14]) at specific time points. It points to a variety of effects depending on the duration of CPAP treatment. The observed changes may indicate an improvement in health conditions following the administration of appropriate treatment, serving as an indicator of the treatment's effectiveness. The alterations noted during our study decreased after discontinuation of the primary factor in OSA – episodes of apneas and hypopneas.

Our study also indicates some relations between changes in the studied genes expression and PSG parameters after one-day of CPAP treatment. Firstly, it is necessary to emphasize that all patients were characterized by good compliance with the one-night CPAP treatment, as seen in the improvement in AHI, arousal index, desaturation index, and total number of desaturations. There are few interesting relationships between those parameters and circadian clock elements. The higher the AHI during PSG relative to CPAP was, the higher the *BMAL1* expression. Moreover, the same difference in AHI correlated negatively with *CLOCK* expression and positively with CLOCK protein in the morning after CPAP. This may mean that the treatment simultaneously impacts the expression of some proteins, but also somehow regulates post-transcriptional and post-translational processes. Also, greater improvement in AHI was associated with increased *PER1* expression at the mRNA and protein level in the morning after CPAP and decreased BMAL1 and CLOCK protein concentrations. A similar effect of CPAP treatment was observed in increased expression at the protein and mRNA level of PER1 in relation to a change in the total number of desaturations in the morning after CPAP. In the available literature evaluation of the relationship between changes in PSG parameters and circadian clock elements was not described, which does not provide us with a reference for comparisons.

All the above results indicate that an important factor influencing the circadian clock elements in patients with OSA treated with CPAP is the improvement of hypoxia, which may affect both expression and post-transcriptional or post-translational modifications. However, it is not clear how exactly this would happen. One possible mechanism could be mRNA polyadenylation, resulting in an extension of the poly(A) tail, and further in a reduced translation[34]. A potential factor that could influence this process is Nocturnin deadenylase, which is characterized by circadian expression variability and regulates the transcriptomes of circadian genes[35,36]. However, this protein has never been studied in the context of hypoxia or OSA. What seems interesting is the involvement of an RNA-binding protein in the polyadenylation process—cold inducible RNA binding protein (Cirbp). It shows increased activity under stress caused by, among others, hypoxia, binding to 3'UTR transcriptomes, thereby limiting polyadenylation and increasing the translation of circadian clock components such as BMAL1, CLOCK, and PER1[37,38]. Moreover, an equally interesting hypothesis seems to be the role of

miRNAs in the regulation of the circadian cycle. One of the potential targets may be the miR-192/194 cluster, which is sensitive to hypoxia and binds PERs transcriptomes[34,39,40].

There was no association between a change in arousal index and any gene expression or protein concentration.

The OSA treatment is known for its effect on prolonging REM sleep[20]. Moreover, it turns out that the very first night of CPAP treatment resulted in a rebound of slow-wave sleep and REM sleep, which was associated with subjective improvement in sleep quality[41]. However, Moreira et al. argue that the improvement in REM sleep after CPAP does not cause changes in circadian gene expression, due to persistently reduced slow-wave sleep even after the treatment. Interestingly, we found an increase in REM sleep duration after the one-night CPAP treatment, and what is more the difference between PSG and CPAP was positively correlated with CLOCK protein concentration in the morning after CPAP. Moreover, slow-wave sleep was also prolonged, and its change was associated with the same protein level.

It is worth mentioning some limitations of the study. First, the study included a small group of participants, who suffered from OSA. A healthy control group was not included. Second, we acknowledge that our study sample consisted predominantly of male patients (90% male, 10% female), which may limit the generalizability of our findings to a broader population. The circadian system and its responses can vary between genders due to differences in hormonal profiles and genetic expression patterns. Third, as gene expression can be regulated through multiple factors, such as hormones, metabolic issues, or environmental conditions, these circadian genes' line of action might depend on more intricate mechanisms, which were not included in the study. Fourth, we investigated only the effect of one-day CPAP treatment, not long-term. Fifth, our study included determinations only from single blood samples evening before and morning after PSG, and not multiple measurements during the day allowing for full daily profiles of circadian clock elements. Lastly, the biological material used in the study came from peripheral blood, which can differ from cerebral expression and form the principal circadian clock located in the suprachiasmatic nucleus, however, studies investigating the issue of humans based on leucocytes from peripheral blood as a model, which also applies to our study.

Conclusion

Results of the study indicate that circadian clock disruption among patients with OSA can be at least partially changed by CPAP application, even after one-night of intervention. The main genes involved in these changes include CLOCK and PER1, while no changes in CRY1 and BMAL1 in response to treatment were observed pointing to complex mechanisms involved in circadian clock elements' reaction to alternating oxygen levels.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

Conceptualization, A.Gab.; methodology, A.Gab. and M.S.; formal analysis, A.Gab.; investigation, A.Gab., S.T., A.Gaj., M.S..; writing—original draft preparation, A.Gab., S.T., J.J.; writing—review and editing, A.Gab., S.T., A.Gaj., J.J., D.S., P.B., M.C., M.S.; funding acquisition, A.Gab.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

The study was approved by the Ethics Committee of the Medical University of Lodz (RNN/432/18/KE, approval date: 10/12/2018). All patients provided written informed consent to participate in the study.

Additional information

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