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Expression of core circadian clock genes BMAL1 and PER2 in the buccal epithelium in anxiety disorders and its association with poor sleep

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Summary

Background: *The circadian system is important for understanding the complex effects of the interaction of genes and the environment on daily human behavior and mental health. Our study aimed to determine the expression of the core circadian clock genes BMAL1 and PER2 in buccal epithelial cells of patients with anxiety and sleep disorders.*

Subjects and Methods: *The study involved 20 patients with anxiety disorders, aged 26 to 55 years. Research methods included HADS, HAM-A, PSQI, MEQ, and real-time PCR quantitative genetic analysis. All respondents have been assigned into two groups: group 1 – 10 individuals with good sleep quality, and group 2 – 10 individuals with poor sleep.*

Results: *The results of the study showed more severe anxiety syndrome, prolonged latency and more severe sleep continuity disturbances in patients with anxiety disorders and poor sleep quality, but not more depression. The level of expression of the peripheral circadian clock genes BMAL1 and PER2 in the oral mucosa does not differ in individuals with anxiety disorders with good and poor sleep quality both in the morning and in the evening. The BMAL1 expression in patients with anxiety disorders and good sleep quality was higher in the evening than in the morning, while it did not differ in those with poor sleep quality. We have found between morning BMAL1 expression and sleep quality.*

Conclusions: *We have demonstrated that increased BMAL1 expression in the morning may be associated with poorer sleep quality in patients with anxiety disorders.*

Keywords: *anxiety, sleep disturbances, chronotype, gene expression, circadian clock*

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INTRODUCTION

Epidemiological studies show that individuals with anxiety frequently report sleep disturbances (Bragantini et al., 2019). These disturbances encompass poor sleep quality, insufficient sleep duration and alterations in sleep structure, varying across different anxiety disorders (Baglioni et al., 2016; Cox & Olatunji, 2020). Furthermore, irregularities in the sleep-wake cycle may disrupt the function of responding to stimuli without external reinforcement, contributing to the onset and perpetuation of anxious states (Horenstein et al., 2019). Recently, the scientific community has paid significant attention to studying the mechanisms of the molecular clock, as disruptions in the synchronization between the circadian system and the sleep-wake cycle have been linked to clinical symptoms of anxiety disorders (Lamont et al., 2007; Boiko, Skrypnikov et al., 2022).

Numerical evidence have reported a genetic influence in the development of sleep disorders (Juli et al., 2018). In humans, sleep regulation is driven by circadian oscillator that synchronize the timing of sleep and wakefulness with the light-dark cycle. It is located in the suprachiasmatic nucleus of the hypothalamus, also known as the main circadian pacemaker of the internal molecular clock (Taillard et al., 2021). At the molecular level, the rhythmicity of the biological clock in oral mucosa is regulated by transcription-translation feedback loops, comprising a set of specific clock genes that oscillate over approximately a 24-hour period (Bjarnason et al., 2001). The proteins BMAL1 (Brain Muscle Arnt-Like Protein-1) and CLOCK (Circadian locomotor output cycles kaput), two transcription factors, activate the transcription of PER1, PER2, PER3 (PERIOD) and CRY1, CRY2 (Cryptochrome) proteins in the morning. These proteins are accumulated in the cytoplasm during the second half of the day and the first half of the night, inhibiting the transcriptional function of *BMAL1* and *CLOCK*. They negatively regulate their own transcription, resulting in the circadian expression of *PER* and *CRY* transcripts. This intricate rhythmic

cellular-molecular process ensures circadian behavior in humans (Andreani et al., 2015).

Changes in the synchronization of individual circadian rhythms result from the action of endogenous and exogenous factors. Therefore, the circadian system serves as a sensitive model for understanding the complex influences of gene-environment interactions on daily human behavior, as well as physiological and psychological functions. Dysregulation of the circadian system increases susceptibility to many pathological conditions including psychoemotional disorders (Vadon et al., 2022). Recent studies have shown that the expression of *CLOCK* gene in the evening was higher in individuals with an evening chronotype compared to a morning one. However, chronotype did not influence the expression of the core circadian genes *PER1, BMAL1* and *CRY1*. Moreover, this study demonstrated that *BMAL1* gene expression in the buccal epithelium of healthy individuals is higher in the evening compared to the morning, regardless of age and sex (Vasko et al., 2022).

It has been established that subjective differences in an individual's chronotype are associated with the level of psychological distress (Prat & Adan, 2013), influencing sleep-wake cycles, activity patterns and behavioral choices (Ferrante et al., 2015), as well as impacting lifespan (Duffy et al., 2015) and the development of diseases (Yu et al., 2015; Menculini et al., 2019; Makarem et al., 2020; Lokes et al., 2023). Manifestations of short-term circadian desynchronization, including social jet lag and the negative consequences of shift work, are quite common (Foster et al. 2013; Boivin & Boudreau, 2014). Moreover, prolonged disruptions of circadian rhythms are associated with serious health issues, including sleep disorders, metabolic disorders and mental health disturbances (Zelinski et al., 2014; Logan & McClung, 2019; Fishbein et al., 2021; Meyer et al., 2022). Sleep-wake cycle disturbances create a susceptibility to a higher risk of chronic diseases and the presence of a disease can influence the sleep-wake cycle and worsen sleep quality.

Understanding the peculiarities of the internal clock's functioning in patients with anxiety and sleep disorders at the molecular level is possible through the assessment of the expression of circadian genes. It has been demonstrated that gene expression in the tissues of the oral mucosa exhibits significant daily oscillations, and the biological sample can be considered a biomarker for determining circadian rhythms in patients (Dunlap, 1999; Gu et al., 2021). Recent publications contain insufficient data regarding the correlation between the expression of mRNA of key clock genes and anxiety, sleep disorders and chronotype. The aim of our study was to determine the expression of circadian clock genes *BMAL1* and *PER2* in buccal epithelial cells of patients with anxiety disorders and its association with sleep quality.

SUBJECTS AND METHODS

Participants and procedures

The study involved 20 patients with anxiety disorders, including 13 (65.0%) women and 7 (35.0%) men, aged 26 to 55 years, with a mean age of M=38.55, SD=9.62. Among them, 6 (30%) individuals sought outpatient assistance for the first time, and 14 (70%) sought assistance repeatedly. The duration of the illness ranged from 3 months to 4 years. All participants provided voluntary informed consent for the study before its commencement.

All patients had a confirmed diagnosis of neurotic anxiety disorder (F40.8, F41.1, F41.2, F41.3, and F43.22 according to ICD-10). Individuals with neurological disorders, mental and behavioral disorders caused by alcohol and psychoactive substances use, chronic somatic pathology in the medical history, as well as those with injuries or diseases of the periodontium and oral mucosa, and those working night shifts for at least two months prior to the study were not included in the study.

Measures

To confirm the predominance of anxiety symptoms over depressive symptoms, the Hospital Anxiety and Depression Scale (HADS), consisting of 14 items, was used (Zigmond & Snaith, 1983). The scale comprises 7 items for anxiety and 7 items for depression, focusing on the cognitive and emotional aspects of anxiety and depression. Each item is rated on a four-point Likert scale from 0 to 3. A higher score on the anxiety (HADS-A) and depression (HADS-D) subscales indicate more severe symptoms. Each subscale is interpreted as follows: 0-7 score – normal, 8-10 score – subclinical level, 11-21 score – clinically significant anxiety or depression. The internal consistency coefficient Cronbach's α is 0.78-0.93 for HADS-A and 0.82-0.90 for HADS-D.

The Hamilton Anxiety Rating Scale (HAM-A) (Hamilton, 1959) is one of the first rating scales developed with the primary purpose of assessing the severity of anxiety symptoms. The scale consists of 14 items, each of which contains a group of symptoms assessed on a Likert scale from 0 to 4, where 4 indicates the most severe symptoms. The scale determines the level of psychological anxiety (mental

agitation and psychological distress) and somatic anxiety (physical complaints related to anxiety). The overall score indicates the severity of an individual's anxiety with the interpretation of results as follows: 0-7 score – no anxiety, 8-14 score – mild anxiety symptoms, 15-23 score – moderate symptoms, the score of 24 and above – severe symptoms (Matza et al., 2010). The questionnaire has significant validity and reliability indicators, with Cronbach's $\alpha = 0.89$.

The Pittsburgh Sleep Quality Index (PSQI) was used to assess sleep quality over the past month. It comprises 19 items, with the first 4 items being open-ended questions, and items 5 to 19 being rated on a 4-point Likert scale from 0 to 3, where 0 indicates no sleep problems, and 3 indicates the most severe disruption. Scores for individual items yield seven components: subjective sleep quality, sleep latency, sleep duration, sleep efficiency, sleep disorders, use of sleep medication and daytime dysfunction. The global PSQI score indicates the degree of sleep quality disruption, ranging from 0 to 21. A total score > 5 indicates unsatisfactory sleep quality and provides a diagnostic sensitivity of 89.6% and specificity of 86.5% in distinguishing between good and poor sleepers (Buysse et al., 1989), with Cronbach's $\alpha = 0.83$.

The measurement of chronotype was conducted using the Morningness-Eveningness Questionnaire (MEQ), which consists of 19 items. The questionnaire explores general lifestyle patterns, such as sleep-wake cycles, optimal times for physical and mental activity and subjective activity preference, reflecting an individual's inclination toward being a morning or evening type (Horne & Ostberg, 1976). The assessment of belonging to a specific chronotype was determined by the sum of the acquired points. The total score in MEQ ranges from 16 to 86, where a score of 41 and below indicates an evening type, a score of 59 and above indicates a morning type and a score between 42 and 58 indicates an intermediate type.

Sampling, mRNA extraction and real-time PCR

The genetic study was conducted to determine the expression of *BMAL1* and *PER2* genes in biological samples taken from the buccal mucosa before the initiation of pharmacological therapy. Buccal epithelium samples were collected on a single day at 08:00 and 20:00 during the period of March-April 2023. Sample collection was designed to capture the peak expression of morning and evening genes in patients with anxiety disorders and sleep disturbances before and after sleep.

A minimally invasive buccal epithelium collection method was employed using Interdental bristles (Wild-pharma, Switzerland) equipped with a narrowed plastic bristle and a blunt end. To ensure optimal contact between the bristle and the buccal mucosa, the plastic handle was gently pressed against the mucous membrane. Subsequently, rotational movements in one direction were applied with simultaneous pressure for 10 seconds, effectively avoiding significant irritation to the mucous membrane. After collecting the material, the brush was immediately placed in an RNA stabilizing solution (ThermoFisher, USA) at room temperature. Stabilized samples were frozen at -70 °C for further use.

Total RNA was extracted from biological samples using a kit for RNA isolation and purification (Thermo Fisher Scientific, USA). To obtain cDNA, a set of reagents for reverse transcription reaction was utilized (New England Biolabs, USA). The mRNA expression of *BMAL1* and *PER2* genes in the biological sample was quantified using the real-time polymerase chain reaction (Real-time PCR) method with the CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, USA) and the Quantitec®sybr-Green I PCR Kit (QIAGEN, Germany). The oligonucleotide primer sequences (Li et al. 2019) are presented in Table 1. PCR was conducted under the following conditions: the first cycle at 95°C for 30 s, followed by 45 cycles of incubation at 95°C for 15 s and 60°C for 20 s. The β-actin gene was used as a reference gene. The 2-ΔCT relative method was applied for data analysis.

Grouping

All respondents have been assigned into two groups: the first group comprised 10 individuals with good sleep quality (group 1), and the second group included 10 individuals with poor sleep quality (group 2) (the Pittsburgh Sleep Quality Index score >5).

Table 1 Primer sequences used for quantitative RT-PCR analysis (Li et al. 2019)

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
BMAL1	CTGGCTAGAGTGTATACGTTTGG	GGTCACCTCAAAGCGATTTTC
PER2	CCCTTCCGCATGACGCCCTACCTG	GACCGCCCTTTCATCCACATCCTG
β-actin	TCCACCTTCCAGCAGATGTG	GCATTTGCGGTGGACGAT

Data analysis

Statistical analysis was performed using SPSS 27.0 software (SPSS Inc., IL, USA). Normality distribution was assessed using the Shapiro-Wilk test. The Mann-Whitney U test was applied for comparing parameters between groups, while the Wilcoxon signed-rank test was used for comparing parameters within the same group. All $p < 0,05$ values were considered statistically significant. The Spearman correlation method was employed to investigate the statistical dependence between two variables, and logistic regression analysis using the backward Wald method was used for the selection of a minimal set of factors and predicting the odds of poor sleep quality. Figures were generated using GraphPad Prism 8.0 (GraphPad Software, San Diego, USA).

RESULTS

The groups were representative in terms of gender and age (38.8±10.0 years and 38.3±9.8 years respectively, Z=0.076, p=0.940). Demographic information about the subjects, including age, gender and chronotype is presented in Table 2.

Table 2 General information about the subjects

Variables	Group 1 (n=10)	Group 2 (n=10)
Age (years)		
26-44	6 (60%)	6 (60%)
45-55	4 (40%)	4 (40%)
Gender		
Man	4 (40%)	3 (30%)
Woman	6 (60%)	7 (70%)
Chronotype (by MEQ)		
Morning	3 (30%)	4 (40%)
Intermediate	4 (40%)	3 (30%)
Evening	3 (30%)	3 (30%)

A comparative overview of indicators based on the diagnostic scales HADS, HAMA, MEQ and PSQI between group 1 and group 2 is presented in Table 3. The

Table 3 The average values of diagnostic scale indicators for patients with anxiety disorders depends on sleep quality

	Scales	Group 1 (n=10)	Group 2 (n=10)	Mann-Whitney U-test
		M±SD	M±SD	
Scales for Assessing Anxiety and Depressive Symptoms	HADS-A	10.8±1.69	12.6±1.84	**Z=-2.101, p=0.036**
	HADS-D	5.1±2.03	6.5±3.71	Z=-1.034, p=0.301
	HAMA total score	12.5±1.72	19.0±3.68	**Z=-3.396, p=0.001**
	HAMA psychic domain	6.6±1.58	10.5±1.43	**Z=-3.557, p<0.001**
	HAMA somatic domain	5.9±1.0	8.5±3.2	**Z=-2.103, p=0.035**
	Chronotype Questionnaire			
	MEQ	51.7±12.9	53.0±15.97	Z=-0.076, p=0.940
		ME (Q1; Q3)	ME (Q1; Q3)	
Sleep Quality Assessment Questionnaire	PSQI SSQ	1 (0; 1)	2 (2; 3)	**Z=-3.938, p<0.001**
	PSQI SL	1 (0.75; 2.0)	2.5 (2; 3)	**Z=-3.375, p=0.001**
	PSQI SD	0.5 (0; 1)	1 (1; 2)	**Z=-2.805, p=0.005**
	PSQI SE	0 (0; 0)	1 (0; 1)	**Z=-2.854, p=0.004**
	PSQI SDS	1 (1; 1)	2 (1.75; 3)	**Z=-3.446, p<0.001**
	PSQI USM	0 (0; 0)	0.5 (0; 1)	**Z=-2.517, p=0.012**
	PSQI DD	0.5 (0; 1)	2 (1; 2)	**Z=-3.425. p=0.001**
	PSQI GS	4 (2.75; 5)	11 (9; 13)	**Z=-3.807, p<0.001**

Note: HADS-A – anxiety subscale of Hospital Anxiety and Depression Scale, HADS-D – depression subscale of Hospital Anxiety and Depression Scale, MEQ – Morningness-Eveningness questionnaire score, PSQI SSQ – subjective sleep quality, PSQI SL – sleep latency, PSQI SD – sleep duration, PQSI SE – sleep efficiency, PSQI SDS – sleep disturbance, PSQI USM – use of sleep medication, PSQI DD – daytime dysfunction, PSQI GS – global score.

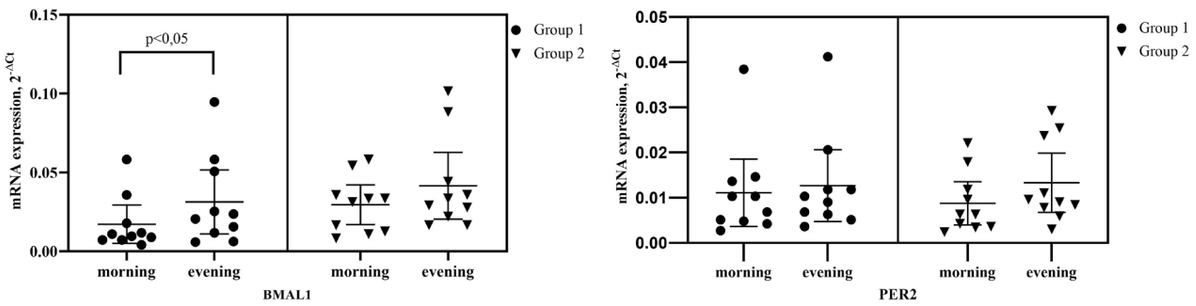

Figure 1. Comparison of the mRNA expression levels of circadian clock genes *BMAL1* and *PER2* in the oral mucosa in the morning and evening in patients with anxiety disorders with good and poor sleep quality.
Note: Group 1 – group with good sleep quality, Group 2 – group with poor sleep quality.

comparison between the two groups has shown a significant difference for the HADS-A (p=0.036), HAMA somatic domain (p=0.035), HAMA psychic domain (p<0.01) and HAMA total score (p=0.001) indicators. No significant difference between the groups according to the MEQ questionnaire was found. The PSQI questionnaire revealed a significant difference for all indicators, with the most remarkable differences noted for sleep latency, subjective sleep quality, sleep disturbance and global score (p<0.001).

The levels of expression of peripheral circadian clock genes *BMAL1* and *PER2* in the oral mucosa were determined through genetic study in group 1 and group 2, as shown in Figure 1.

In group 1, we observed statistically significant differences between the mRNA expression level for the regulatory circadian gene *BMAL1* in the morning and the evening (Z=-1.988, p=0.047), while in group 2 we did not find such differences between the two time points. However, the expression levels of the circadian clock gene *PER2* in the morning and evening did not show significant differences in both groups.

The expression levels of the circadian clock genes *BMAL1* and *PER2* in the buccal mucosa in morning-evening comparison and between the groups are presented in Table 4. There were no differences between the two groups when comparing morning samples and evening samples of both genes.

The correlation between the expression levels of circadian genes *BMAL1* and *PER2* in the morning and evening with sleep quality indicators in patients of both groups is presented in Table 5. The analysis revealed a positive correlation between the morning expression level of the *BMAL1* gene and the indicators of the PSQI

Table 4 The expression levels of the *BMAL1* and *PER2* genes in the buccal mucosa in the morning and evening in patients with anxiety disorders depends on sleep quality, M±SD [ME (Q1;Q3)]

Genes' expression	Group 1 (n=10)	Group 2 (n=10)	Mann-Whitney U-test
BMAL1 morning	0.017220±0.0169747 [0.010300 (0.007300; 0.22375)]	0.029550±0.0175027 [0.032350 (0.012275; 0.040450)]	Z=-1.779, p=0.075
BMAL1 evening	0.031300±0.0283409 [0.022150 (0.010425; 0.052600)]	0.041620±0.0283409 [0.031350 (0.020775; 0.055250)]	Z=-1.285, p=0.199
Wilcoxon signed-rank test	**Z=-1.988, p=0.047**	Z=-1.274, p=0,203	-
PER2 morning	0.011080±0.0104212 [0.008550 (0.004650; 0.013850)]	0.008760±0.0066679 [0.006300 (0.003550; 0.013325)]	Z=-0.643, p=0.520
PER2 evening	0.012650±0.0111158 [0.009650 (0.006000; 0.014000)]	0.013300±0.0091891 [0.009300 (0.007325; 0.024125)]	Z=-0.189, p=0.850
Wilcoxon signed-rank test	Z=-1.172, p=0.241	Z=-1.886, p=0.059	-

Table 5 The correlation between the expression levels of circadian genes *BMAL1* and *PER2* and the sleep quality indicators in patients with anxiety disorders

Genes	PSQI SSQ	PSQI SL	PSQI SD	PSQI SE	PSQI SDS	PSQI USM	PSQI DD	PSQI GS
BMAL1 morning	**0.47***	**0.49***	**0.46***	0.05	0.30	0.40	0.10	0.44
BMAL1 evening	0.44	0.39	0.43	0.22	0.12	0.27	0.01	0.39
PER2 morning	0.08	-0.01	0.15	-0.24	-0.01	0.06	-0.24	0.02
PER2 evening	0.17	0.29	0.30	-0.14	0.05	0.13	-0.29	0.15

Note: *p<0.05
PSQI SSQ – subjective sleep quality, PSQI SL – sleep latency, PSQI SD – sleep duration, PQSI SE – sleep efficiency, PSQI SDS – sleep disturbance, PSQI USM – use of sleep medication, PSQI DD – daytime dysfunction, PSQI GS – global score

questionnaire: subjective sleep quality (r=0.47, p<0.05), sleep latency (r=0.49, p<0.05) and sleep duration (r=0.46, p<0.05).

We constructed a logistic regression model for the analysis of the association between the probability of poor sleep quality in patients with anxiety disorders and circadian features, such as "*BMAL1* morning," "*BMAL1* evening," "*PER2* morning," "*PER2* evening," and "MEQ score." The analysis was carried out based on the examination results of all 20 individuals with anxiety disorders. It has been found that one factor feature, "*BMAL1* morning," is associated (p<0.05) with the probability of poor sleep quality. It has been found that with an increase in the "*BMAL1* morning" indicator, the odds of poor sleep quality increases in patients with anxiety disorders (p=0.046), OR = 5.372E+37 (95% CI 4.237 – 6.813E+74).

DISCUSSION

Behavioral and physiological aspects of life depend on circadian organization (Levi and Schibler, 2007). The central pacemaker, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, controls numerous clocks in most types of peripheral cells and ensures the circadian rhythmicity of processes at all levels of functioning—from gene expression to circadian patterns of behavior (Cox & Takahashi, 2019). A few scientific research on determining the role of clock genes in anxiety disorders have been found to date. However, recent studies demonstrate a bidirectional relationship between mood disorders and the circadian system (Gršković & Korać, 2023). Our study has shown that patients with anxiety disorders with poor sleep quality have more severe anxiety, but not depression. However, there is a strong link between depressive symptoms with sleep quality and the functioning of the circadian regulation system (Boiko, Shkodina et al., 2022). At the same time, poor sleep has a significant impact on the nervous system. Experimental studies have shown that sleep loss can increase inflammatory markers and affect metabolic processes (Shkodina, Zhyvotovska et al., 2022). Given the importance of sleep in maintaining adaptive emotional regulation and reactivity, poor sleep quality appears to be a risk factor for anxiety disorders (Shkodina, Iengalychev et al., 2022).

The expression level and epigenetic changes of peripheral circadian genes are directly related to sleep quality (Bukowska-Damska et al., 2017). In our study, we analyzed the expression of peripheral circadian genes in buccal mucosa samples from patients with anxiety disorders. Although this method is minimally invasive, it cannot be performed unnoticed by the patient, which results in longer intervals than usual. In our study, a 12-hour interval was chosen, considering the comfort and capabilities of patients, as well as their daily routine (Crnko et al., 2021).

Our study has shown that in patients with anxiety disorders, the levels of circadian gene *PER2* expression in the morning and evening demonstrated no significant oscillations or correlations with sleep quality. The *PER2* expression in human buccal epithelium peaks in the evening, demonstrating a clear circadian rhythm (Takata et al., 2002). This rhythmic pattern highlights the role of *PER2* in regulating daily physiological processes and its potential as a marker for studying circadian-related disorders. However, according to the results of the study by Cajochen and co-authors, the sinusoid line of the expression of the *PER2* gene may demonstrate no difference when comparing samples taken at 8:00 and 20:00 (Cajochen et al., 2006). Thus, we cannot state that the lack of differences we found indicates desynchronization. This requires a detailed evaluation with multiple repeated samples throughout the day in further studies. A team of authors from Poland and Norway investigated sleep quality and the methylation status of circadian clock genes

among 710 nurses and midwives aged 40-60. A significant association was found between shorter sleep duration and the methylation level of PER2 among daytime workers and individuals of morning chronotype. The work system and chronotype are presented in the study as modifying factors regarding the genetic data on sleep quality (Bukowska-Damska et al., 2017).

We have established a relationship between the circadian gene *BMAL1* and sleep quality, while a lack of morning-evening differences in *PER2* expression was observed in both groups of patients with anxiety disorders, regardless of good or poor sleep quality. The findings of the studies on the association of circadian genes *PER2* and *BMAL1* with sleep disorders are somewhat contradictory. The impact of acute sleep deprivation on the expression of circadian genes *PER2* and *BMAL1* in mononuclear cells of human peripheral blood has been estimated. Circadian variations in the expression of *PER2* were observed only under conditions of normal sleep and were disrupted after sleep deprivation. The *BMAL1* gene maintained circadian variations in expression both at the baseline and in short-term sleep deprivation, although the peak expression was slightly shifted (Kavcic al., 2011). A similar study was conducted on a mouse model, revealing that sleep deprivation led to a significant increase in anxiety and deterioration of basic cognitive functions. There was also a significant decrease in the expression level of the circadian gene *Bmal1.* Intracerebroventricular administration of human recombinant protein rhBMAL1 reduced anxiety, improved cognitive functions and reduced oxidative stress in the hippocampus of sleep-deprived mice (Qi et al., 2023).

The study of the impact of the circadian clock gene *Bmal1* in skeletal muscles on sleep was conducted on mice by a group of American researchers. They found that restoring the expression of *Bmal1* in the brains of *Bmal1*-knockout mice did not affect sleep. However, restoring the expression of *Bmal1* in skeletal muscles led to the restoration of sleep duration (Ehlen et al., 2017). A similar study was conducted by Italian researchers with *BMAL1*-knockout monkeys, which exhibited higher nocturnal activity, reduced sleep duration, increased anxiety and depression. It was established that the suppression of the expression of the *Bmal1* gene regulates transcriptional programs associated with stress reactions induced by sleep deprivation, depressive disorders and aging (Qiu et al., 2019).

The expression of circadian genes *BMAL1* and *PER2* was also studied among a cohort of patients with traumatic brain injury with and without sleep disturbances. The expression of *CLOCK, BMAL1* and *PER2* genes were determined in the buccal cell and blood samples at 06:00, 12:00, 18:00 and 24:00. In the cells of the oral mucosa and mononuclear cells, a significant difference between the groups with normal and disturbed sleep was identified regarding the expression level of *PER2* at 12:00 and 18:00. However, no significant differences between the groups were found in the expression level of *BMAL1* at any of the study time points (Zhanfeng et al., 2019). Contrastingly, in our study, we found that the expression level of the *BMAL1* gene in the morning was lower compared to the evening in the group of patients with good sleep quality and had a direct correlation with subjective sleep quality, latency and duration.

Circadian genes are not only influenced by changes in the light-dark cycle. For instance, peripheral clock genes in the liver can be entrained by the rhythmicity of food intake. This natural adaptation ensures activity during the same period as the sleep-wake cycle when a food source is available. The ability to obtain food products at any time of day for contemporary humans can alter the phase relationship of the circadian system and lead to desynchronization and disruptions in circadian health (Mistlberger, 2020). Research conducted on mouse models showed an increase in the expression levels of genes *Per1, Per2, Cry1, Cry2* and *Arntl* at midnight, and an increase in the expression of *Per2, Cry1, Cry2* and *Nr1d1* at noon in the kidney cortex in mice with nighttime feeding (Izmailova et al., 2022). Similar results in lung tissue and liver tissue of mice were found for mRNA expression of genes *Per1, Per2, Clock* and *Arntl*, with levels decreasing at midnight and increasing at noon when subjected to a daytime feeding schedule (Fedchenko et al., 2022; Shlykova et al., 2023). The social clock has also been demonstrated to impact the expression of circadian genes. Work and education in modern society are often constrained by working hours, which are typically standardized for a given locality and culture. These temporal constraints may conflict with an individual's internal clock and may be too early for individuals with an evening chronotype or coincide with their circadian sleep time, such as when working a night shift. The mismatch between the social clock and the internal biological clock, known as "social jet lag," can lead to sleep disturbances, circadian misalignments and deterioration of overall health (Roenneberg et al., 2012).

Limitations

Our study has several limitations. The small sample size in this study limited the statistical power for exploring circadian genes with daily rhythmicity and assessing the correlation between the expression of clock genes

and the clinical symptoms of patients. Prospective research with a larger sample size is needed to confirm our findings. Additionally, sample collection was performed only twice for each patient. It was due to financial constraints and patient convenience. Since the participants were outpatients, we could only monitor morning and evening pickups when they attended the clinic. It would be important to compare the expression of circadian genes in patients with anxiety disorders with poor and good sleep quality over a 24-hour period in shorter time intervals, because the RT-PCR method, while useful, requires more biological repeats to draw robust conclusions. Moreover, the limited sampling times (morning and evening) do not capture the full oscillation patterns of circadian genes, particularly *PER2*. The inclusion of a control group and considering individual chronotypes, different age categories and a focus on lifestyle, dietary behavior or the intensity of physical activities among the subjects, data about medication use could provide more comprehensive and detailed results. Lastly, the short duration of the study does not allow for observation of chronic effects, highlighting the need for longitudinal studies.

CONCLUSIONS

The findings of the study demonstrate more severe anxiety syndrome, prolonged latency and more severe sleep continuity disturbances in patients with anxiety disorders and poor sleep quality, but not more depression. The level of expression of the peripheral circadian clock genes *BMAL1* and *PER2* in the oral mucosa does not differ in individuals with anxiety disorders with good and poor sleep quality both in the morning and in the evening. However, there is a physiological fluctuation in *BMAL1* expression in patients with anxiety disorders and good sleep quality, while *BMAL1* expression does not differ in the morning and evening in patients with anxiety disorders with poor sleep quality, which may indicate a desynchronization of the circadian system. Given the associations found between morning *BMAL1* expression and sleep quality, it can be assumed that these changes play an important role in clinical features of anxiety disorders, which requires a more detailed study, considering chronotype, behavioral characteristics, and medication use in a longitudinal design. In particular, we have demonstrated that increased *BMAL1* expression in the morning may be associated with poorer sleep quality in patients with anxiety disorders.

Ethical Considerations: Does this study include human subjects? YES
Authors confirmed the compliance with all relevant ethical regulations.
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