MEDICAL UNIVERSITY - PLOVDIV

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Epigenetic and neurofunctional profiles of insomnia phenotypes

ABSTRACT

OF DISSERTATION THESIS FOR ACQUISITION OF EDUCATIONAL AND SCIENTIFIC DEGREE "DOCTOR"

Field of higher education: **7. Health and sports**

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Scientific specialty: **Pathological physiology**

Scientific supervisor:

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More commonly used abbreviations

WBC – White blood cells

EDTA - Ethylenediaminetetraacetic acid

EEG - Electroencephalography

EC – Effective connectivity

INSD – Insomnia with normal sleep duration

ISSD - Insomnia with shortened sleep duration

SL – Sleep latency

MRI – Magnetic Resonance Imaging

NREM – non-REM sleep

PSG - Polysomnography

REM – Rapid Eye Movement Sleep

SCN – Suprachiasmatic nucleus

FC – Functional Connectivity

HAA – Hypothalamic-adrenal axis

AIL – Left anterior insula

AIR – Right anterior insula

BDI – Beck Depression Inventory

BDNF – Brain derived neurotrophic factor

DLPFC – Dorsolateral prefrontal cortex

DMN – Default mode network

DMPFC – Dorsomedial prefrontal cortex

ECN – Executive control network

ICSD – International Classification of Sleep Disorders

ISI – Insomnia Severity Index Scale

MPFC – Medial prefrontal cortex

NMDA – N-methyl-D-aspartate

PCC – Posterior Cingular Cortex

ROI – Regions of Interest

SN – Salience network

VMPFC – Ventromedial prefrontal cortex

Contents

Introduction

The latest, third edition of the International Classification of Sleep Disorders III (ICSD-III) encompasses 7 main categories of disorders, affecting between 20% and 64% of the world's population. Chronic insomnia is the most common sleep disorder in the population, and insomnia is the most common symptom in somnology.

Chronic insomnia is defined as a sleep disorder characterized by difficulty falling asleep (taking more than 30 minutes), inability to maintain consolidated sleep (there are multiple awakenings during the night), early awakening (before the desired awakening time), which leads to disturbances and difficulty in functioning of the individual during the day, with the described complaints being present at least 3 days a week and cannot be explained by another, underlying sleep disorder.

The ICSD-III classification of insomnia offers a simplified and clinically useful approach by defining three main categories – chronic, short-term and "other" insomnia. Although this classification unifies and facilitates the diagnostic process, it does not take into account the phenotypic heterogeneity of the condition. New data suggest the need to further differentiate chronic insomnia by objective parameters, such as sleep duration, which allows for a better understanding of the pathophysiology and prognostic value of the different forms of insomnia.

The variation in the clinical course and the multiple shades of the disorder have long been the basis of the difficulties in preparing a systematic classification that would encompass the variants of this disorder. The phenotypic manifestations of chronic insomnia still pose diagnostic and therapeutic challenges to medical specialists. The fact that insomnia often accompanies other medical and psychiatric conditions makes it difficult to make a correct and timely diagnosis, which can have serious consequences and increased health risks caused by chronic insomnia. Undoubtedly, the multiple pathophysiological mechanisms that explain the onset, progression and chronicity of insomnia also contribute to this heterogeneity.

These factors determine the need to search and develop new methods and approaches to diagnosing and classifying the individual manifestations of insomnia. The current view in scientific literature is that, additional stratification of patients with chronic insomnia is required based on objectively measured sleep duration by polysomnography. Two main phenotypes are distinguished:

1. **Insomnia with short sleep duration (ISSD)** – characterized by a total sleep duration of less than 6 hours and is associated with an increased risk of cardio-metabolic diseases, hypercortisolemia, and inflammatory conditions.

2. **Insomnia with normal sleep duration (INSD)** – despite subjective complaints, patients demonstrate normal total sleep duration. This phenotype is often associated with psychoemotional symptomatology and cognitive hyperarousal.

There is a need to update classification systems to account for the phenotypic heterogeneity of chronic insomnia and to include objective biomarkers. This will facilitate precise diagnosis, prognosis, and personalized therapeutic approaches. Two appropriate and promising modalities for such differentiation are:

- **MicroRNAs (miRNAs)** are small non-coding RNA molecules that regulate gene expression post-transcriptionally. In the present study, we identified specific miRNA expression profiles that differ between patients with ISSD, INSD, and healthy controls. These profiles have potential value as epigenetic biomarkers.
- **Functional Magnetic Resonance Imaging (fMRI)** – by analyzing the effective connectivity (EC) between brain regions associated with sleep, insomnia phenotypes have been found to demonstrate distinct patterns of neuronal activity and communication. Areas such as the medial prefrontal cortex and thalamus are particularly affected.

Finding new markers for profiling and phenotyping the manifestations of chronic insomnia is the main goal of this dissertation. The study of epigenetic and neurofunctional markers, as well as their correlation with standardized clinical methods, are innovative methods offering a new approach to clarifying the pathogenetic models of insomnia and defining its phenotypic manifestations.

Aim and objectives

The aim of this dissertation is to identify epigenetic and neurofunctional profiles of insomnia phenotypes (with normal and with shortened sleep duration) in order to correlate them with standard indicators from the clinical assessment of patients.

To achieve this goal, we set ourselves the following tasks:

1. Constructing macro- and microstructural characterization of sleep through polysomnographic analysis of patients with chronic insomnia and healthy controls.
2. Explore the transcriptional levels of microRNA expression in blood plasma and white blood cells in patients suffering from chronic insomnia and healthy controls.
3. Performing fMRI to determine effective connectivity of neuronal networks in the CNS in a resting state (resting state fMRI) in patients with chronic insomnia and healthy controls.
4. Correlation of data from the epigenetic and neurofunctional profile to the patient's clinical profile based on polysomnographic indicators and the results of the applied survey methods.

Materials and methods

To achieve the set goal and objectives, a cross-sectional, observational study was conducted, covering a population of Bulgarian citizens with chronic insomnia and healthy controls.

1. Study participants – For the purposes of the study, 37 patients and 29 healthy controls were examined. The study group was composed of a random sample of patients among those who self-sought help at the Sleep Research Laboratory at the Department of Pathophysiology at the Medical University of Plovdiv and the University Hospital "St. George" EAD for the period 2020-2024. The examinations and tests conducted according to the protocol of all participants in the study were performed upon their inclusion after prior signing of the "Informed Consent for Participation", in accordance with the Helsinki Convention on Human Rights. The study was approved by the Scientific Ethics Committee at the Medical University - Plovdiv (protocol No. 3/20.05.2021).

2. Patient selection criteria

Inclusion criteria:

- Outpatient patients with placed diagnosis Chronic insomnia
- Age between 18 and 70 years
- Signed informed consent

Exclusion criteria:

- Presence of concomitant sleep disorder.
- Presence of a psychiatric disorder with current clinically significant symptoms.
- Taking stimulants and other medications that significantly disrupt the quality and/or duration of sleep
- Contraindications for performing magnetic resonance imaging (e.g. claustrophobia, presence of metal implants, pacemakers, etc.).
- Shift work or other circumstances that significantly disrupt sleep hygiene
- Severe somatic diseases, chronic pain syndrome and other disorders that significantly disrupt the patient's sleep.
- Organic neurological diseases

The studies were conducted under outpatient conditions in the functional diagnostic laboratories at the Department of Pathological Physiology, at the Department of Medical Biology and the Scientific Research Institute (SRI) of the Medical University - Plovdiv (Figure 1.)

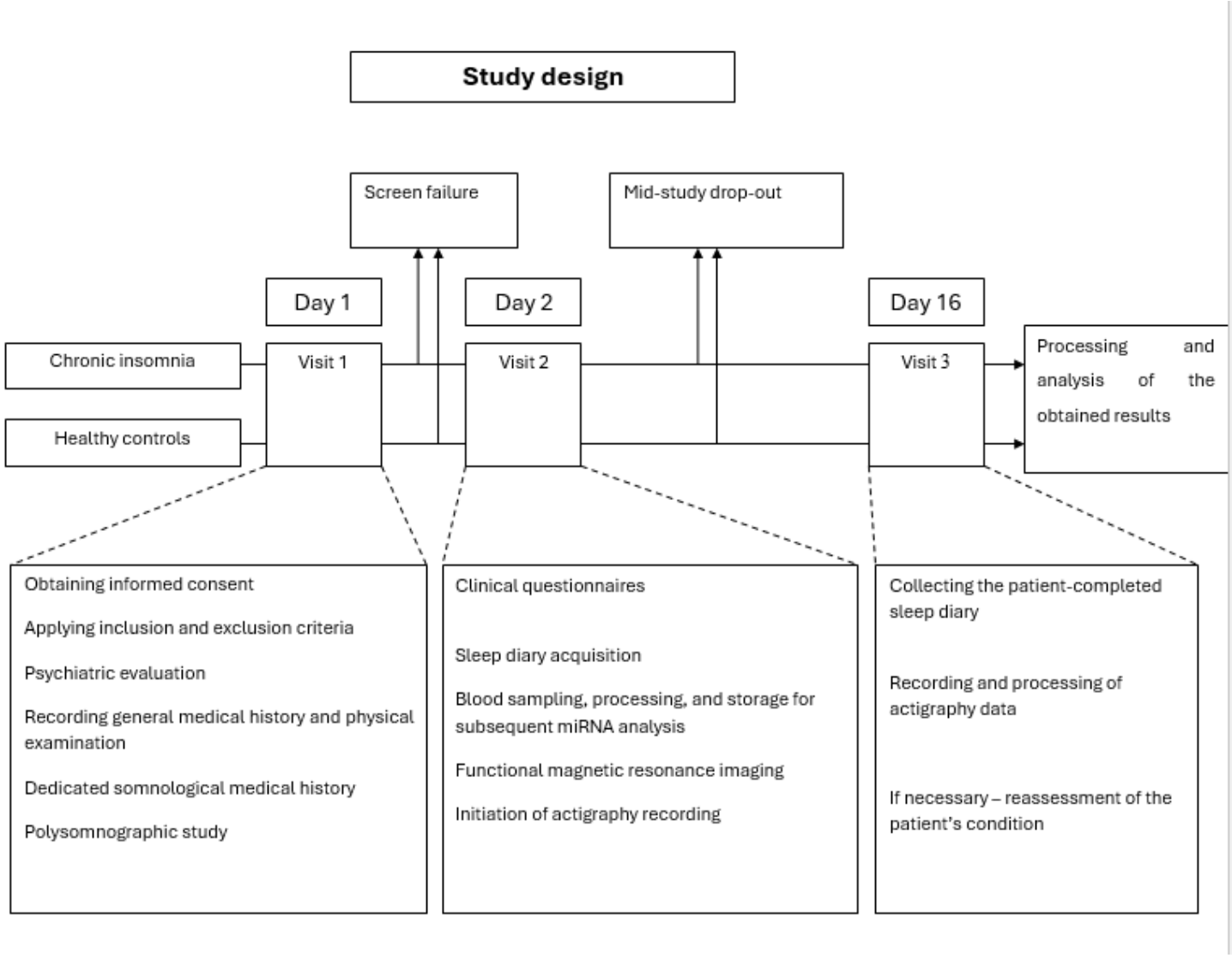

Figure 1. Study design

3. Clinical methods

General Medical History - The history taken included data on the main complaints related to sleep disorders, with a primary focus on chronic insomnia. Patients were asked about concomitant and past illnesses.

Survey methods – The following survey methods were administered to all study participants:

- **ISI – Insomnia Severity Index** . The Insomnia Severity Index scale consists of 7 questions for self-assessment of symptoms related to sleep disorders in patients and their effect on daily functioning.
- **ESS – Epworth Sleepiness Scale** . The Epworth Sleepiness Scale is a self-assessment tool for daytime sleepiness based on the probability (from 0 – never to 3 – almost always) of falling asleep in 8 monotonous everyday situations, with the total score ranging from 0 to 24. Values above 10 are considered indicative of increased daytime sleepiness.
- **Morningness-Eveningness Questionnaire** . The chronotype assessment scale (Morningness-Eveningness Questionnaire) consists of 19 questions that the patient answers independently. The resulting score has a numerical expression falling into one of 5 categories – “definitely morning type” (70-86), “moderately morning type” (59-69), “neutral type” (42-58), “moderately evening type” (31-41) and “definitely evening type” (16-30).
- **BDI – Beck Depression Inventory** . The Beck Depression Inventory is a self-assessment instrument consisting of 21 questions. The total number of points is interpreted in the following categories: 1-10 points – normal; 11-16 points – mild depression; 17-20 points – mild to moderate depression; 21-30 points – moderate to severe depression; over 31 points – severe depression.
- **Sleep diary** . A sleep diary (SD) is a useful and practical method for subjective assessment of sleep by the patient over a period of 14 days. The main indicators that can be obtained are: time to go to bed, time to fall asleep, number of nighttime awakenings, approximate duration of nighttime awakenings, time of last awakening from sleep, time of getting out of bed, naps and daytime sleepiness.

4. Polysomnography (PSG) – PSG is the gold standard for objective sleep assessment. The polysomnographic study was performed using an outpatient system (NOX A1 PSG System, Iceland) and monitored electroencephalogram (EEG), electrooculogram (EOG), submental electromyogram (EMG), lower limb movement, electrocardiogram (ECG), chest and abdominal wall movement, snoring, oronasal airflow, body position, arterial oxyhaemoglobin saturation (SpO2), pulse transit time (PTT). The placement of EEG electrodes was performed according to the international 10-20 system and

included 10 EEG electrodes. The determination of sleep phases was performed by scoring 30-second epochs. Electrooculography (EOG) was obtained from 2 electrodes – ROC and LOC, placed 1 cm above and lateral to the external canthus of the right eye (ROC) and 1 cm below and lateral to the external canthus of the left eye (LOC). Electromyography (EMG) was obtained from 2 electrodes placed submental. Electrocardiography (ECG) was obtained from 2 electrodes placed subclavianly (Lead 1). Respiratory effort was recorded by two piezoelectric belts placed around the chest and around the abdomen of the patient. Oronasal airflow was recorded using an oronasal cannula. PTT and SpO2 were measured using a Nonin 3012 pulse oximeter (Nonin, Plymouth, Minnesota, USA). One night of PSG study was conducted to rule out an underlying sleep disorder and determine objective sleep indicators (Distribution of the main sleep phases (N1; N2; N3; REM), total sleep time (TST), sleep efficiency (SE), sleep onset latency (SOL), wake after sleep onset (WASO), number of awakenings (arousal index; AI), etc.).

5. Functional Magnetic Resonance Imaging (fMRI) – Translational Neuroscience Complex – The participants underwent magnetic resonance imaging using a 3T MRI system (GE Discovery 750w) with 2 MRI sequences: high-resolution structural scan (Sag 3D T1 FSPGR sequence), TR (relaxation time) 7.2 ms, TE (echo time) 2.3 and flip angle 12 °, slice thickness 1 mm, matrix 256 × 256,; two functional series, resting-state (2D EPI echo planar imaging sequence) with eyes closed – slice thickness 3 mm, 36 slices, matrix 64 × 64, TR 2000 ms, TE 30 ms, flip angle 90 °, 192 volumes. Before the EPI sequence, participants are instructed to lie still, with their eyes closed, without thinking about anything in particular.

Data analysis was performed using SPM12 (Statistical Parametric Mapping; http://www.fil.ion.ucl.ac.uk/spm/) implemented in MATLAB R2020b (Windows version). Standard procedures were applied during preprocessing of the functional images: realignment, co-registration with the structural scans, spatial normalization to the Montreal Neurological Institute (MNI) template, and smoothing using a Gaussian kernel with a full width at half maximum of 6 mm.

Resting-state data were analyzed at the first level using a general linear model (GLM) applied to the time series. Regions of interest (ROIs), defined as spheres with a radius of 6 mm, were selected a priori based on their known involvement in the SN, DMN, and ECN. The MNI coordinates of these ROIs are presented in Table 1.

Spectral dynamic causal modeling (spDCM) was then applied to the selected regions of interest. A coupled model was used, in which each region is assumed to influence all others. Unlike stochastic DCM, spectral DCM estimates effective connectivity (EC) based on the spectral density of fluctuations in the neuronal state, rather than directly from the raw time series.

Table 1. Regions of interest, with their corresponding MNI coordinates

Region of interest	X	Y	Z	Brodmann's field
Ventromedial prefrontal cortex (VMPFC)	44	52	-2	25
Dorsomedial prefrontal cortex (DMPFC)	4	30	46	32
Medial prefrontal cortex (MPFC)	3	54	-2	14.11
Right anterior insula (AIR)	38	22	3	13
Right hippocampal formation	24	-12	-20	28
Dorsolateral prefrontal cortex (DLPFC)	-37	27	44	46
Posterior cingulate gyrus (PCC)	0	-52	26	23.31
Precuneus	-10	-64	24	7

Legend: MNI - Montreal Neurological Institute

Individual spDCM models were jointly estimated using the parametric empirical Bayes (PEB) approach available in SPM12. Finally, the A-matrix parameters—representing the strength of effective connectivity—were extracted and used for further statistical analyses in SPSS.

6. Laboratory methods - Department of Medical Biology, NIMU:

Sampling. After a preliminary explanation of the procedure and signing of an "Informed Consent" form, 4 ml of venous blood (2 x 2 ml) were collected by venipuncture using vacuum tubes - with a red cap for serum isolation and with a purple cap for plasma and WBC isolation.

Plasma isolation. After venipuncture, the red-capped vacuum tubes were left at room temperature for 30 minutes, then centrifuged at 4000 rpm for 10 minutes. The resulting serum was aliquoted (600 µl each) and stored in a freezer at –80 °C in Eppendorf tubes.

Purple-capped tubes containing anticoagulant were used to separate plasma and white blood cells (WBCs) for RNA analysis. The blood in the EDTA tubes was centrifuged at 4000 rpm for 10 minutes at room temperature.

The supernatant (plasma) was transferred to new 1.5 ml Eppendorf tubes, and the pellet (cell fraction) was stored on ice and used for WBC isolation. The remaining plasma was frozen at –80 °C for subsequent analyses.

Isolation of leukocytes. Isolation of WBC in patients and healthy controls was performed by the cold lysis method using a lysis solution containing ammonium carbonate and ethylenediaminetetraacetic acid (EDTA), according to the following sequence:

- In a 50 ml Falcon tube, 45 ml of 1X lysing solution were added, to which the cell fraction (approximately 4 ml) remaining after plasma isolation was added.
- The volume was made up with 1X lysing solution to a total of 50 ml.
- The sample was incubated on ice for 10 minutes to lyse the erythrocytes, then centrifuged at 4200 rpm for 10 minutes at 4 °C.
- The supernatant was removed, and the resulting pellet was washed with 25 ml of 1X PBS, homogenized by vortexing, and recentrifuged under the same conditions.
- After the second centrifugation, the supernatant was removed again. 800 µl of TriReagent was added to the WBC pellet, and the sample was homogenized by pipetting or vortexing until a homogeneous suspension was obtained.
- The homogenized sample was transferred to a 1.5 ml Eppendorf tube, shock-frozen by immersion in liquid nitrogen and subsequently stored at –80 °C for subsequent RNA isolation.

Analysis of the expression of selected miRNAs by TaqMan assay.

A specific TaqMan-based quantitative PCR (qPCR) assay was applied to study the expression levels of the selected miRNAs. Commercially available primers and probes – TaqMan (Thermo Scientific) – were used for miR-132, miR-212, miR-219, miR-146a, miR-126, miR-125b, miR-182a, miR-138, miR-30c and let-7. RNU48 and RNU6 were used as reference genes. The processing of the biological material was performed in the following sequence:

- **Total RNA isolation:** Total RNA, including miRNA, was isolated from plasma using a specialized miRNA extraction kit.
- **cDNA synthesis:** Complementary DNA (cDNA) synthesis was performed by reverse transcription using gene-specific stem-loop primers (Thermo Scientific) specific for the respective miRNAs.
- **Quantitative PCR analysis:** Quantitative PCR (qPCR) was performed with gene-specific TaqMan primers for each of the target miRNAs. The data were analyzed and presented as relative fold change in expression levels of the corresponding miRNA transcripts.

The analysis was performed using a RotorGene Q 6000 qPCR instrument, with the following thermocycling program:

- **Uracil-N glycosylase (UNG) incubation:** 2 minutes at 50 °C,
- **DNA polymerase activation:** 20 seconds at 95 °C,
- **Cycles (40):**

    - Denaturation: 1 second at 95 °C,

- Amplification: 20 seconds at 60 °C.

7. Statistical methods. Statistical analysis of the demographic and clinical characteristics of the participants was performed using IBM SPSS 25.0 for Windows. The level of statistical significance was set at $p < 0.05$ for all tests.

Methods used :

- Descriptive analysis – the frequency distribution of the considered features, broken down by study groups, is presented in tabular form.
- Analysis of variance – calculating estimates of central tendency and dispersion.
- Alternative analysis – to test hypotheses about differences in relative shares.
- Graphical analysis – for visualization of the obtained results.

Parametric methods:

- Student's t-test – for testing hypotheses about the presence of statistically significant differences between the studied indicators;
- Paired Student's t-test – for comparing mean values of dependent variables;
- Correlation analysis – to check for the presence of a linear relationship between quantitative traits.

Nonparametric methods:

- Kolmogorov-Smirnov test – to determine the type of distribution;
- Mann-Whiteney test – for comparing quantitative values in two independent samples with a distribution other than normal; when comparing groups with a total number of examined individuals under 30
- Kruskal-Wallis test – for more than two independent samples with a distribution other than normal;
- Binary logistic regression analysis – for quantitative assessment of the factors for the occurrence of a given disease.
- Chi-square test and Fisher's exact test – for testing hypotheses about the existence of a relationship between categorical variables.

The data is presented as:

- Mean ± standard deviation under normal distribution
- Median (minimum - maximum) in an irregular distribution
- Number of patients (percentage of group) for categorical variables

I. Results

1. Demographic and clinical characteristics of the subjects studied

For the period 2020-2024, 36 patients were examined in the Sleep Research Laboratory at the Department of Pathological Physiology at the Medical University of Plovdiv and the UMHAT "St. George" and 29 healthy controls. The two groups did not differ statistically in age, gender and level of education. Characteristics of basic demographic indicators are presented in Table 2.

The age range in both groups was similar, with the mean age showing no significant difference in patients 36.92 (±11.84) compared to healthy controls 32.93 (±11.61), p = 0.081.

In both groups, women predominated: 25 (69.40%) in patients and 20 (69.00%) in healthy controls, with no significant difference in gender distribution (p = 0.967).

The two groups did not differ significantly in terms of family history of insomnia - 17 (47.20%) of the patients and 7 (24.10%) of the healthy controls reported the presence of chronic insomnia in the family, compared to the absence of a family history in 19 (52.80%) of the patients and 22 (75.90%) of the healthy controls (p=0.055).

The distribution of the variable "Subjective sleep duration according to sleep diary data" between the two groups is also homogeneous - 17 (47.20%) of the patients and 11 (37.90%) of the healthy controls reported a shortened duration (< 6 hours), compared to 19 (52.80%) of the patients and 18 (62.10%) of the controls reporting a normal (> 6 hours) sleep duration (p=452).

Statistically significant differences between the two groups were evident in terms of the results of the questionnaires assessing the severity of insomnia and clinical depression. In terms of the ISI results, patients scored a significantly higher mean score - 18.36 (±4.01), compared to the control units - 3.59 (±2.63) (p<0.001). The distribution is similar for the BDI - a mean score of 13.47 (±7.95) for patients compared to 4.86 (±4.57) for healthy controls (p<0.001). The difference is presented in Figures 2 and 3.

Table 2 : Main demographic and clinical characteristics of patients and healthy controls

Variable	Patients (n = 36)	Controls (n = 29)	p-value
Age			
Mean value (±SD)	36.92 (±11.84)	32.93 (±11.61)	0.081
Minimum-Maximum	21 - 68	21 - 61	
Gender n (%)			
Women	25 (69.40%)	20 (69.00%)	0.967 χ^2
Men	11 (30.60%)	9 (31.00%)	
Education n (%)			
Higher	23 (63.90%)	22 (75.90%)	0.298 χ^2
Average	13 (36.10%)	7 (24.10%)	
Family history n (%)			
Yes	17 (47.20%)	7 (24.10%)	0.055 χ^2
No	19 (52.80%)	22 (75.90%)	
Sleep duration*			
Shortened (< 6h)	17 (47.20%)	11 (37.90%)	
Normal (> 6h)	19 (52.80%)	18 (62.10%)	0.452 χ^2
ISI			
Mean value (±SD)	18.36 (±4.01)	3.59 (±2.63)	<0.001 U
Minimum-Maximum	11 - 21	0 - 11	
BDI			
Mean value (±SD)	13.47 (±7.95)	4.86 (±4.57)	<0.001 U
Minimum-Maximum	2 - 33	0 - 20	
MEQ			
Mean value (±SD)	53.06 (±9.49)	52.83 (±7.41)	0.864 U
Minimum-Maximum	33 - 73	39 - 67	
ESS			
Mean value (±SD)	4.11 (±3.60)	5.41 (±3.33)	0.096 U
Minimum-Maximum	0 - 16	1 - 13	

Legend: t- independent-samples t-test; $\chi 2$ – Chi-square test; U – Mann-Whitney U test; SD – standard deviation; *subjectively reported duration from sleep diary data; ISI – Insomnia Severity Index; BDI – Depression Severity Questionnaire; MEQ - Chronotype Assessment Questionnaire; ESS Sleepiness Assessment Questionnaire.

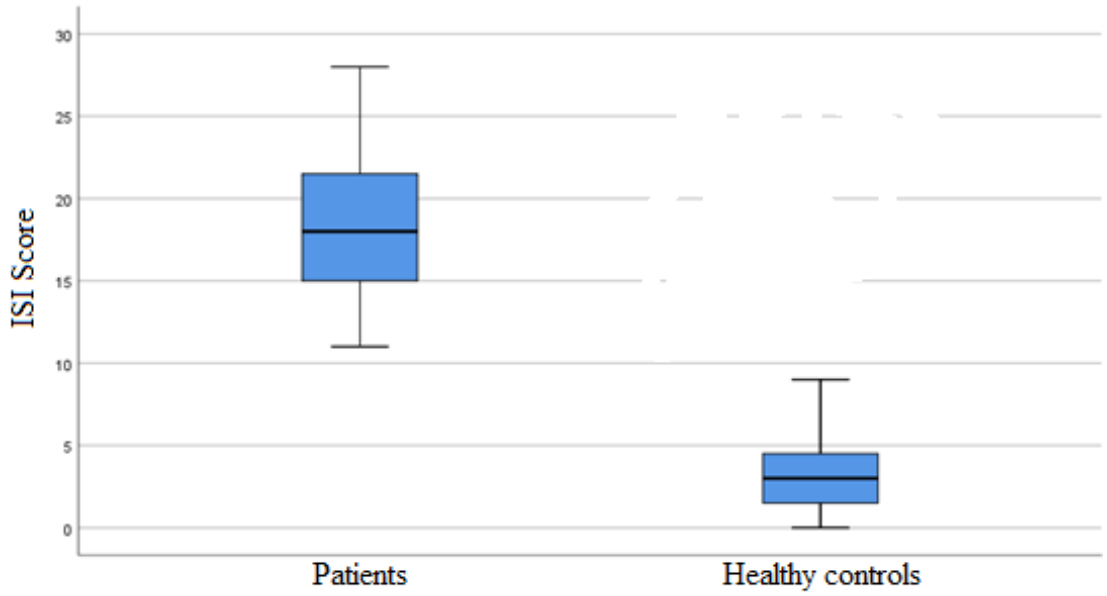

Figure 2. Distribution of the sum of ISI – Insomnia severity index between patients and healthy controls

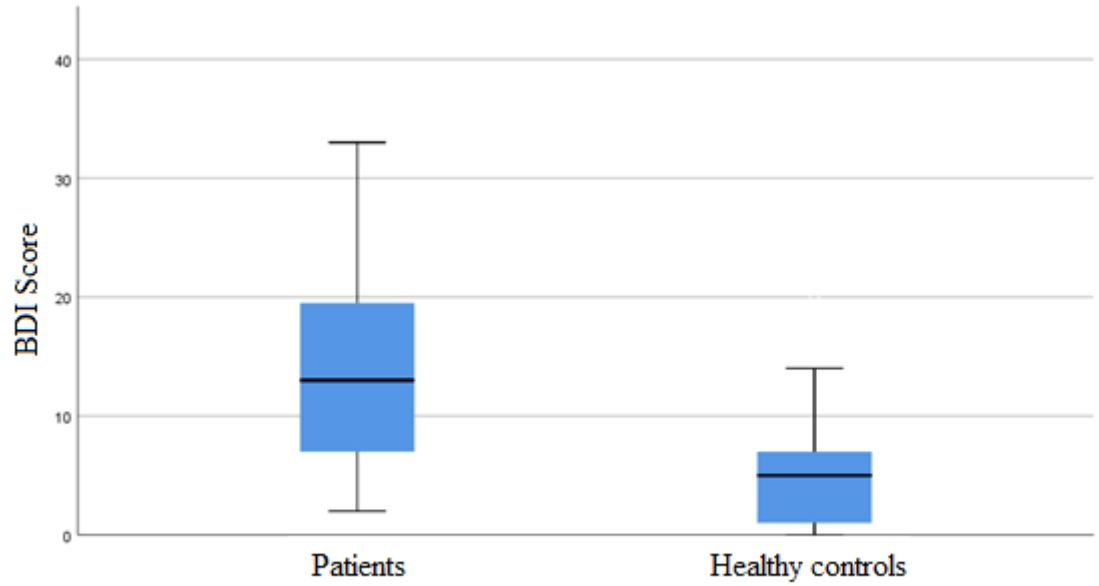

Figure 3 – Distribution of the BDI – Beck depression inventory score between patients and healthy controls

For further profiling and phenotyping, the patient group was divided into two subgroups based on subjectively reported sleep duration. The group with reduced sleep duration (SSD) had significantly higher ISI (p=0.033) and BDI (p=0.045) scores compared to the subgroup with normal sleep duration (NSD) (Table 3; Figures 4 and 5).

Table 3 : ISI and BDI results in patients with shortened and normal sleep duration

Variable	ISSD (n = 17)	INSD (n = 19)	p-value
ISI			
Mean value (±SD)	19.76 (±4.27)	17.11 (±3.43)	0.033 [U]
Minimum-Maximum	13 - 28	11 - 26	
BDI			
Mean value (±SD)	16.53 (±8.90)	10.74 (±5.99)	0.045 [U]
Minimum-Maximum	3 - 33	2 - 21	

Legend: U – Mann-Whitney U test; SD – standard deviation; ISI – Insomnia Severity Index; BDI – Depression Severity Questionnaire; MEQ – Chronotype Questionnaire; ESS – Sleepiness Assessment Questionnaire; ISSD – insomnia with reduced sleep duration; INSD – insomnia with normal sleep duration.

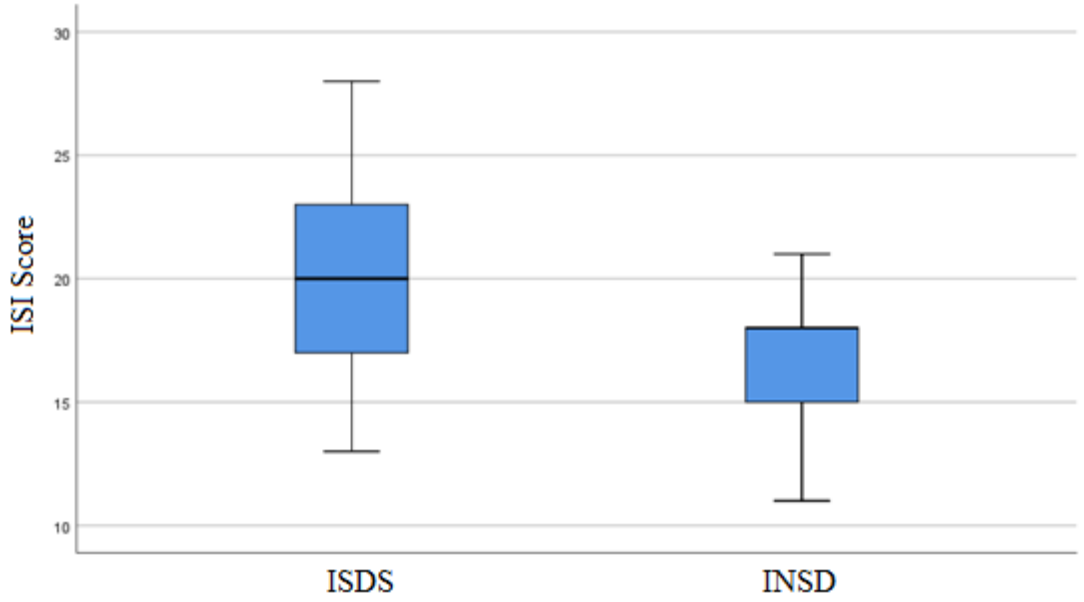

Figure 4. Distribution of the ISI score – Insomnia Severity Index Questionnaire, in the insomnia groups with shortened (ISSD) and normal (INSD) sleep duration.

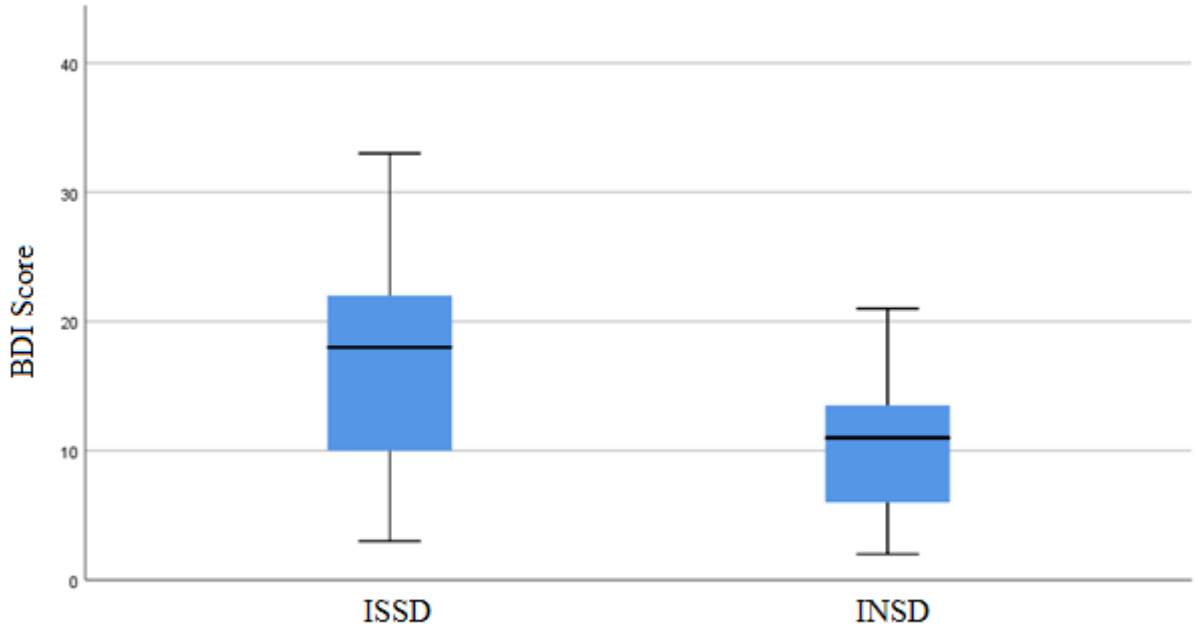

Figure 5. Distribution of the BDI score – Depression Assessment Questionnaire, in the insomnia groups with shortened (ISSD) and normal (INSD) sleep duration.

2. Polysomnographic results. To fulfil task 1 – establishing macro- and microstructural characteristics of sleep in all patients and healthy controls, PSG was performed. Due to technical failure and unsatisfactory data quality, the results of 1 patient and 2 healthy controls were removed. The data from PSG analysis of patients, healthy controls, as well as the two subgroups of patients are presented in Table 4.

The comparative analysis of the main parameters of the sleep microstructure revealed that there was no statistically significant difference in the total time spent sleeping between patients and healthy controls ($p=0.109$). The time spent awake during the study night was significantly longer among patients compared to healthy controls ($p<0.001$), as was the time spent awake after falling asleep ($p<0.001$). A statistically significant difference was also found in the distribution of sleep phases between the two groups (Figure 6). In the group with chronic insomnia, the relative share of N2 was significantly greater compared to healthy controls ($p=0.001$), while deep sleep was of longer duration in controls ($p=0.029$). When comparing the main parameters of PSG between ISSD and INSD, a significant difference in total sleep time is highlighted, as it is significantly shorter in ISSD ($p=0.004$). Regarding sleep phases, the only difference is found in the duration of REM, which is longer in INSD ($p=0.029$) - Figure 7.

Table 4. PSG Data analysis in patients, healthy controls, and patient subgroups.

Variable	Patients n = 35 Average value (±SD)	Controls n = 27 Average value (±SD)	Mann-Whitney U-test p-value	ISSD n = 17 Average value (±SD)	INSD n = 18 Average value (±SD)	Mann-Whitney U-test p-value
TST min	386.49 (±66.52)	360.71 (±66.29)	0.109	351.30 (±65.35)	419.72 (±49.22)	0.004*
Wakefulness (min)	88.76 (±52.92)	41.40 (±17.71)	<0.001**	102.92 (±63.38)	75.38 (±37.80)	0.241
Wakefulness %	18.61 (±10.63)	10.43 (±4.31)	<0.001**	22.31 (±12.51)	15.13 (±7.23)	0.089
N1 (min)	5.39 (±4.13)	4.52 (±4.27)	0.172	5.77 (±3.58)	5.03 (±4.67)	0.408
N1%	1.41 (±1.10)	1.24 (±1.19)	0.232	1.59 (±0.88)	1.24 (±0.900)	0.064
N2 (min)	220.27 (±47.76)	178.48 (±53.39)	0.001*	204.92 (±53.18)	234.78 (±37.97)	0.060
N2 %	57.36 (±9.35)	49.12 (±9.15)	0.002*	58.70 (±11.01)	56.09 (±7.57)	0.438
N3 (min)	87.80 (±38.40)	106.54 (±33.14)	0.029*	76.59 (±37.61)	98.39 (±37.05)	0.083
N3%	22.61 (±9.63)	30.04 (±9.23)	0.003*	22.28 (±10.79)	22.92 (±8.71)	0.921
REM (min)	72.27 (±30.25)	71.15 (±26.19)	0.870	62.47 (±26.94)	81.52 (±31.00)	0.029*
REM %	18.30 (±5.92)	19.68 (±6.22)	0.281	17.43 (±6.17)	19.12 (±5.73)	0.306
SL (min)	19.09 (±15.18)	12.81 (±8.01)	0.150	22.37 (±17.63)	15.98 (±12.13)	0.355
WASO (min)	69.77 (±46.15)	29.41 (±16.93)	<0.001**	80.71 (±53.69)	59.43 (±36.25)	0.187
REML (min)	110.19 (±59.44)	93.72 (±41.71)	0.317	115.15 (±69.88)	105.56 (±49.22)	0.908
SE %	80.95 (±11.65)	80.94 (±25.95)	0.011*	76.80 (±14.05)	84.87 (±7.23)	0.086
AHI (number/h)	2.79 (±2.44)	3.06 (±2.37)	0.191	2.45 (±2.94)	3.10 (±1.88)	0.023*
ODI (number/h)	1.94 (±1.87)	2.48 (±2.81)	0.374	2.17 (±2.38)	1.72 (±1.26)	0.947
PLMS Index (number/h)	3.28 (±5.36)	5.92 (±9.24)	0.218	3.10 (±6.52)	3.44 (±4.18)	0.528

Legend: TST – total sleep time; N1 – stage 1 NREM sleep; N2 – stage 2 NREM sleep; N3 stage 3 NREM sleep; REM – rapid eye movement sleep; SL – sleep onset latency; WASO – wakefulness after sleep onset; REML – REM latency; SE – sleep efficiency; AHI – apnea-hypopnea index; ODI – oxygen desaturation index; PLMS – periodic limb movement index during sleep; SD – standard deviation; * $p<0.05$; **$p<0.001$.

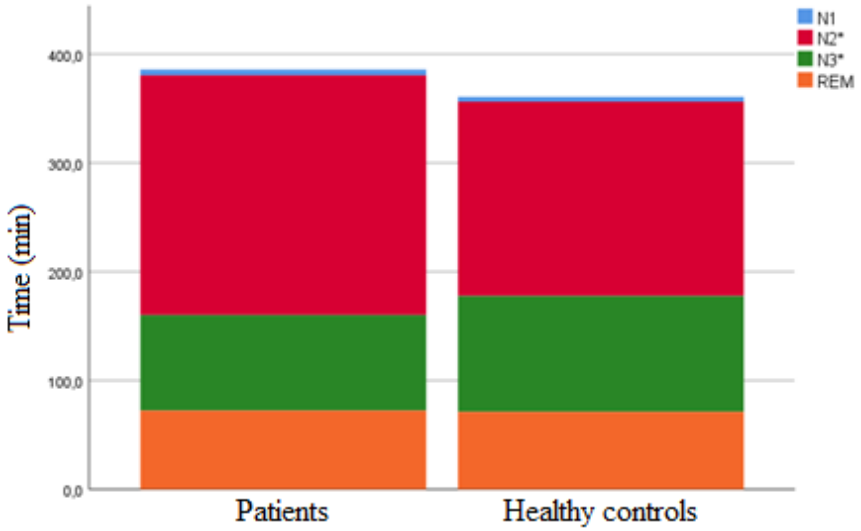

Figure 6. Macrostructure of sleep in patients and healthy controls. REM – Rapid Eye Movement (REM) sleep; N1 – First stage of NREM sleep; N2 – Second stage of NREM sleep; N3 – Third stage of NREM sleep. * p <0.05

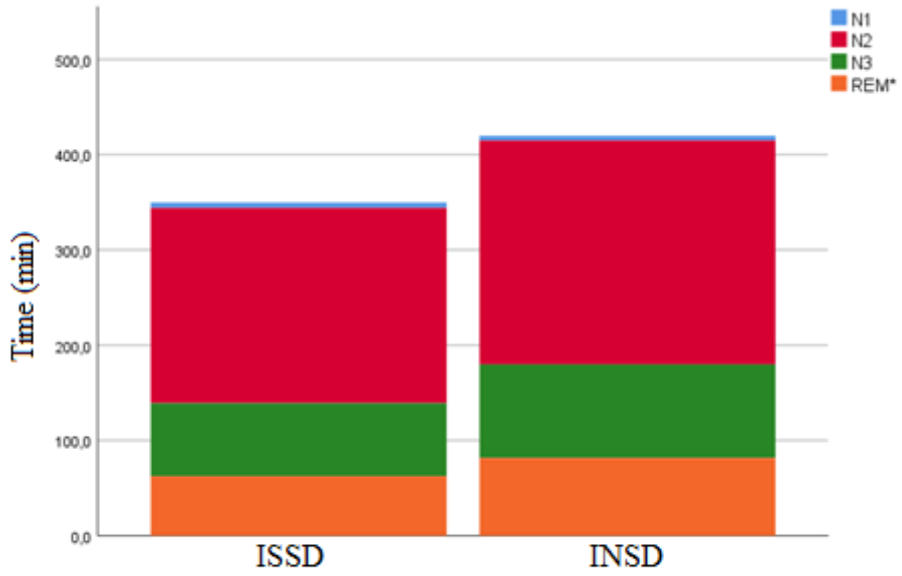

Figure 7. Sleep macrostructure in patients with reduced and normal sleep duration. ISSD – Insomnia with short sleep duration; INSD – Insomnia with normal sleep duration; REM – Rapid eye movement sleep; N1 – First phase of NREM sleep; N2 – Second phase of NREM sleep; N3 – Third phase of NREM sleep. *p<0.05

3. Laboratory results. Regarding task 2 - Study of transcriptional levels of microRNA expression in blood plasma and white blood cells in patients suffering from chronic insomnia and healthy controls. After a technical malfunction at the beginning of the study, samples from five patients were lost. When processing samples from the remaining 31 patients, 3 samples did not pass quality control, which left a total of 28 samples from patients with insomnia. The two newly formed groups did not differ significantly in terms of age and distribution by gender and level of education (table 5).

Table 5. Demographic characteristics of the patient groups and healthy controls studied for miRNA expression

Variable	Patients (n = 28)	Controls (n = 28)	p-value
Age			
Mean value (±SD)	36.46 (±11.14)	32.39 (±10.21)	0.156 ^{U}
Minimum-Maximum	21 - 61	21 - 61	
Sex n (%)			
Women	21 (75.00%)	19 (67.90%)	0.554 $^{\chi 2}$
Men	7 (25.00%)	9 (32.10%)	
Education n (%)			
Higher	15 (64.30%)	21 (75.00%)	0.383 $^{\chi 2}$
Average	10 (35.70%)	7 (25.00%)	

Legend: t- independent-samples t-test; $\chi 2$ – Chi-square test; U – Mann-Whitney U test; SD – standard deviation;

In the healthy control group, 1 sample did not meet the quality criteria and was removed, leaving 28 samples in the control group. In addition, due to the lack of a reagent for examining the expression levels of miRNA132 in plasma, 25 samples from patients and 28 from healthy controls were examined from the latter. Data from the analysis of the expression levels of the selected miRNAs in plasma and WBC, both between patients and healthy controls, and in ISSD and INSD, are presented in Table 6.

The main differences between miRNA expression levels in patients and healthy controls were found in the WBC samples. miRNA-132 (p=0.006), 212 (p=0.048) and 219 (0.036) were upregulated in patients, showing significantly higher expression levels compared to healthy controls, according to non-parametric analysis between the two groups (Figure 8).

Table 6. Expression levels of selected miRNAs in plasma and leukocytes in patients, healthy controls and patient subgroups.

Variable	Patients n = 28 Mean (±SD)	Controls n = 28 Mean (±SD)	Mann-Whitney U-test p-value	ISSD n = 17 Mean value (±SD)	INSD n = 18 Mean value (±SD)	Mann-Whitney U-test p-value
miR125b_P	1.55 (±1.20)	1.80 (±1.73)	0.974	1.52 (±1.39)	1.57 (±1.05)	,586
miR126_P	1.27 (±1.19)	1.71 (±1.40)	0.279	1.19 (±1.08)	1.35 (±1.32)	,786
miR138_P	1.45 (±1.10)	1.56 (±1.24)	0.799	1.41 (±1.20)	1.47 (±1.04)	,717
miR146a_P	1.33 (±1.24)	1.83 (±1.62)	0.310	1.26 (±1.22)	1.40 (±1.30)	,496
miR182_P	2.00 (±2.01)	3.14 (±4.57)	0.262	2.21 (±2.40)	1.81 (±1.67)	,821
let7_P	1.90 (±2.77)	1.78 (±1.48)	0.417	1.48 (±2.19)	2.27 (±3.23)	,185
miR30c_P	1.76 (±2.59)	1.92 (±1.66)	0.354	1.26 (±1.55)	2.19 (±3.23)	,185
miR132_P (n=25)	1.90 (±3.53)	1.53 (±1.11)	0.438	1.23 (±1.25)	2.43 (±4.59)	,434
miR212_P	2.86 (±4.38)	1.93 (±2.09)	0.310	2.39 (±1.71)	3.27 (±5.84)	,555
miR219_P	1.60 (±1.55)	1.65 (±1.47)	0.909	1.42 (±0.66)	1.75 (±2.05)	,751
miR125b_WBC	1.98 (±1.28)	1.52 (±1.38)	0.075	1.85 (±0.98)	2.10 (±1.51)	,821
miR126_WBC	1.76 (±2.11)	1.35 (±0.99)	0.774	2.57 (±2.84)	1.07 (±0.72)	,037*
miR138_WBC	0.84 (±0.56)	1.17 (±0.70)	0.053	0.74 (±0.34)	0.92 (±0.70)	,856
miR146a_WBC	0.96 (±0.45)	1.06 (±0.36)	0.134	1.07 (±0.60)	0.86 (±0.25)	,555
miR182_WBC	1.12 (±1.14)	1.83 (±2.93)	0.413	1.43 (±1.28)	0.86 (±0.97)	,019*
let7_WBC	1.51 (±0.91)	1.44 (±1.41)	0.390	1.85 (±1.11)	1.23 (±0.59)	,088
miR30c_WBC	1.32 (±0.52)	1.13 (±0.60)	0.116	1.49 (±0.58)	1.18 (±0.42)	,142
miR132_WBC	1.65 (±0.67)	1.14 (±0.63)	0.006*	1.59 (±0.65)	1.71 (±0.71)	,821
miR212_WBC	1.36 (±0.66)	1.10 (±0.58)	0.048*	1.46 (±0.77)	1.28 (±0.57)	,683
miR219_WBC	2.52 (±2.95)	1.84 (±0.21)	0.036*	3.71 (±3.96)	1.49 (±0.90)	,339

Legend: miR – micro RNA; P – expression levels in plasma; WBC – expression levels in white blood cells; SD – standard deviation; ISSD – insomnia with shortened sleep duration; INSD – insomnia with normal sleep duration; *$p<0.05$.

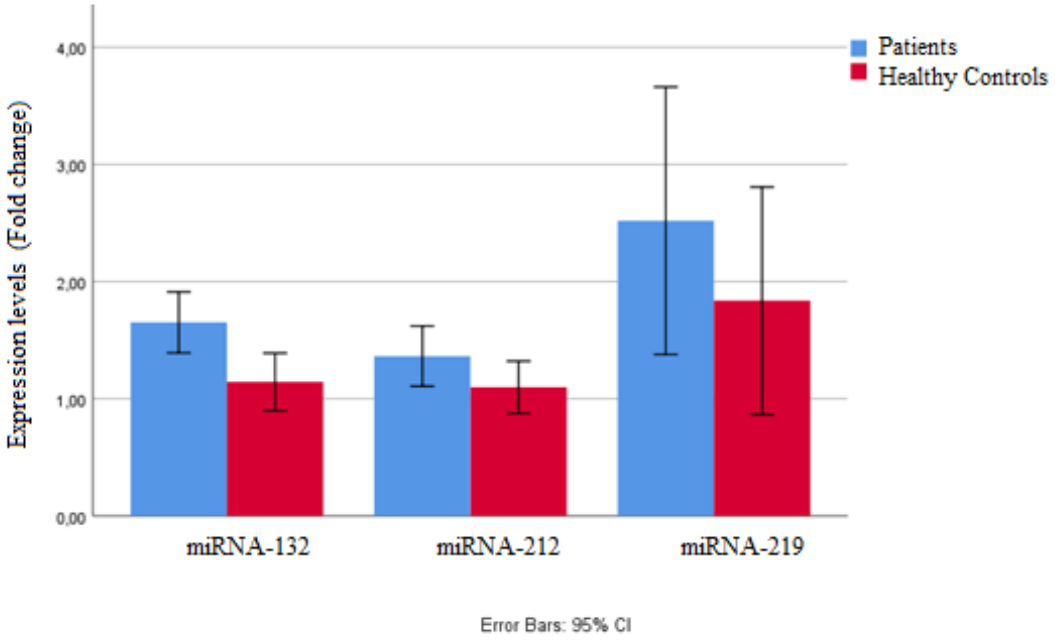

Figure 8. Expression levels of miRNA-132; miRNA-212 and miRNA-219 in leukocytes. WBC – white blood cells.

To assess the role of miRNAs as potential biomarkers in the diagnosis of insomnia, we performed ROC (Receiver operator characteristics) analysis and constructed AUC (area under curve) for the significantly expressed miRNAs between groups. The ROC analysis for miRNA-132 in EBC showed an AUC of 0.713. The optimal cut-off point was set at a value of 1.035, achieving a sensitivity of 75% and a specificity of 64.3% (Youden's Index = 0.393). This indicates a moderate diagnostic value in distinguishing patients with chronic insomnia from healthy controls (Figure 9).

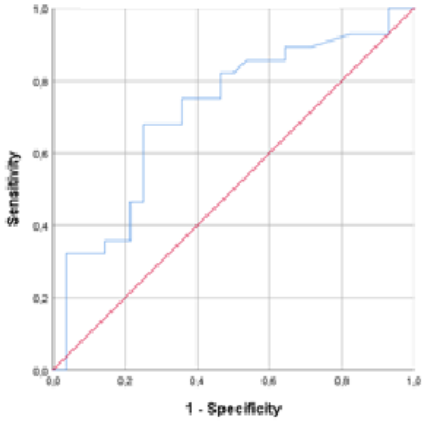

Figure 9. Receiver operator characteristics analysis of miRNA-132 in white blood cells from patients and healthy controls.

The ROC analysis for miRNA-212 in WBC showed an AUC of 0.654. The optimal cut-off point was set at a value of 0.865, achieving a sensitivity of 78.6% and a specificity of 42.9% (Youden's Index = 0.215) (Figure 10).

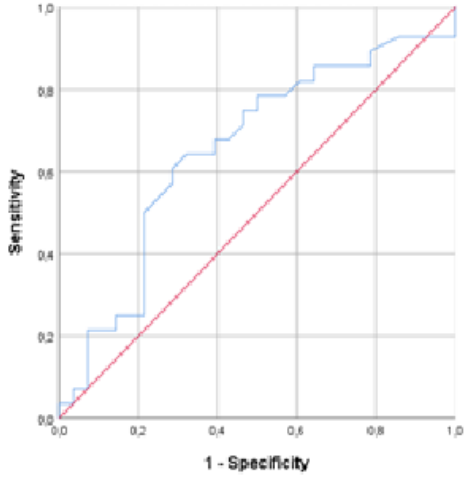

Figure 10. Receiver operator characteristics analysis of miRNA-212 in white blood cells from patients and healthy controls.

The ROC analysis for miRNA-219 in WBC showed an AUC of 0.663. The optimal cut-off point was set at 0.645, achieving a sensitivity of 89.3% and a specificity of 50% (Youden's Index = 0.393) (Figure 11). The data from the three miRNAs are summarized in Table 7. Logistic regression models combining the three significant miRNAs did not show improved predictive ability (combined AUC = 0.272) compared to the individual markers.

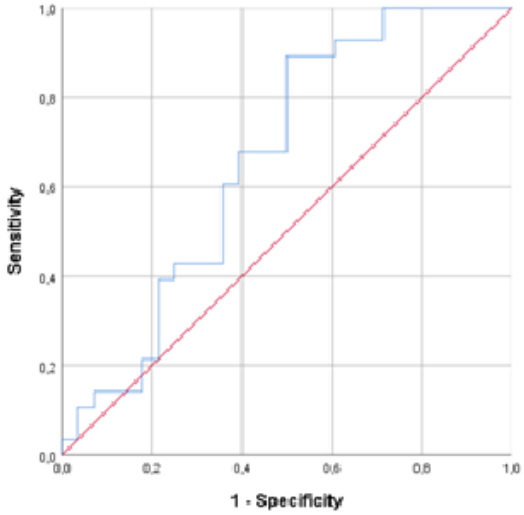

Figure 11. Receiver operator characteristics analysis of miRNA-219 in white blood cells from patients and healthy controls.

Table 7. Discriminatory ability of significantly regulated miRNAs in distinguishing patients with chronic insomnia from healthy controls

miRNA	Cut-off point	Sensitivity (%)	Specificity (%)	Youden's Index	AUC
miRNA-132	1.035	75.0	64.3	0.393	0.713
miRNA-212	0.865	78.6	42.9	0.215	0.654
miRNA-219	0.645	89.3	50.0	0.393	0.663

Legend: AUC – Area Under the Curve

Regarding the phenotypic profiling of insomnia, significantly higher expression levels of miRNA-126 (p=0.037) and miRNA-182 (p=0.019) were found in the ISSD group compared to the INSD (Figure 12). The two subgroups did not differ significantly in terms of age, gender and level of education (Table 8). Regarding the remaining miRNAs in WBC and blood plasma, no statistically significant difference was found in the levels of regulation between patients and the control group.

Table 8. Demographic characteristics of the groups with shortened and normal sleep duration studied for miRNA expression

Variable	ISSD (n = 13)	INSD (n = 15)	p-value
Age			
Mean value (±SD)	37.62 (±11.32)	35.47 (±11.27)	0.586 ^{U}
Minimum-Maximum	22 - 55	21 - 61	
Sex n (%)			
Women	9 (69.20%)	12 (80.00%)	0.512 $^{\chi 2}$
Men	4 (30.80%)	3 (20.00%)	
Education n (%)			
Higher	7 (53.80%)	11 (73.30%)	0.283 $^{\chi 2}$
Average	6 (46.20%)	4 (26.70%)	

Legend: t- independent-samples t-test; $\chi 2$ – Chi-square test; U – Mann-Whitney U test; SD – standard deviation; ISSD – Insomnia with reduced sleep duration; INSD – Insomnia with normal sleep duration.

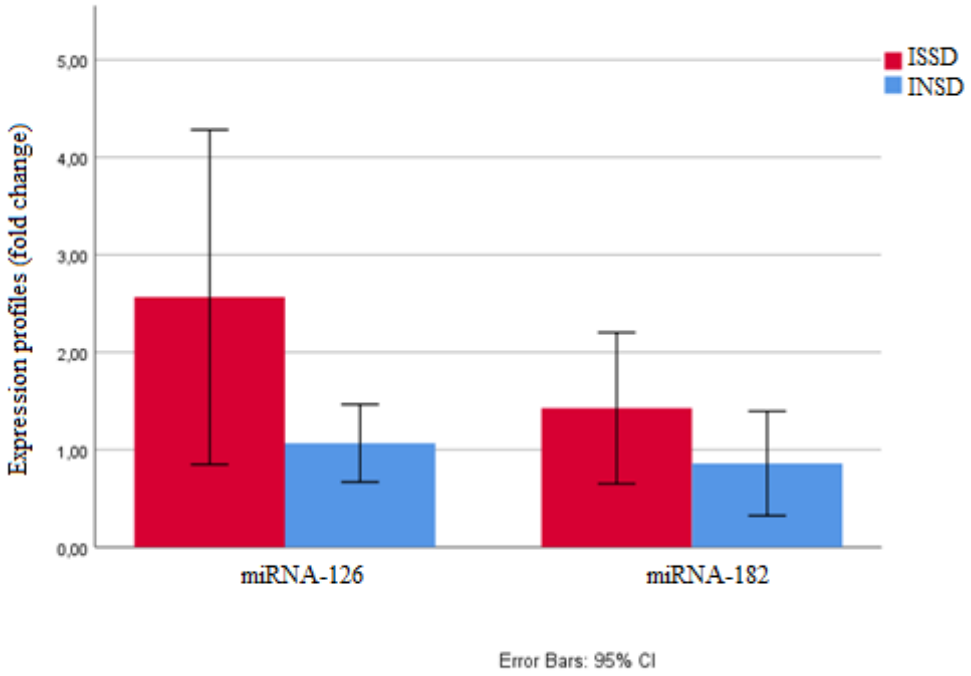

Figure 12. Expression levels of miRNA-126 and miRNA-182 in leukocytes in patients with shortened (ISSD) and normal sleep duration (INSD). WBC – white blood cells.

To consider the role of miRNAs as potential biomarkers in profiling insomnia phenotypes, we performed ROC analysis and constructed AUC for the significantly expressed miRNAs between the ISSD and INSD groups. The ROC analysis of miRNA-126 in WBC showed an AUC of 0.731. The optimal cutoff point between ISSD and INSD was set at 1.075 (Figure 13).

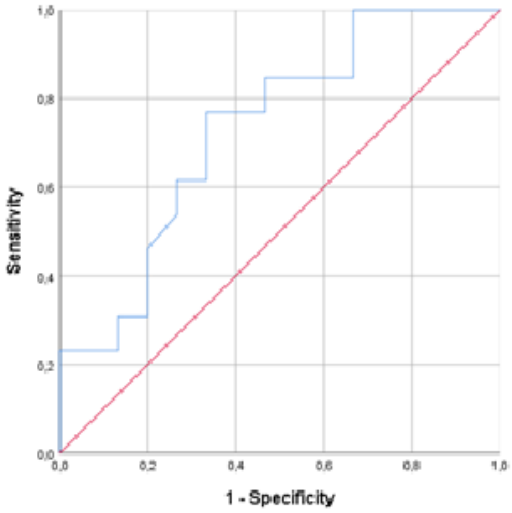

Figure 13. Receiver operator characteristics analysis of miRNA-126 in white blood cells in patients with shortened and normal sleep duration.

ROC analysis of miRNA-182 in WBC showed an AUC of 0.756. The optimal cut-off point between ISSD and INSD was set at 0.530, achieving a sensitivity of 78.6% and a specificity of 100% (Youden's Index = 0.467). This suggests that miRNA-182 may have potential as a specific, albeit less sensitive, marker for distinguishing between insomnia subtypes (Figure 14).

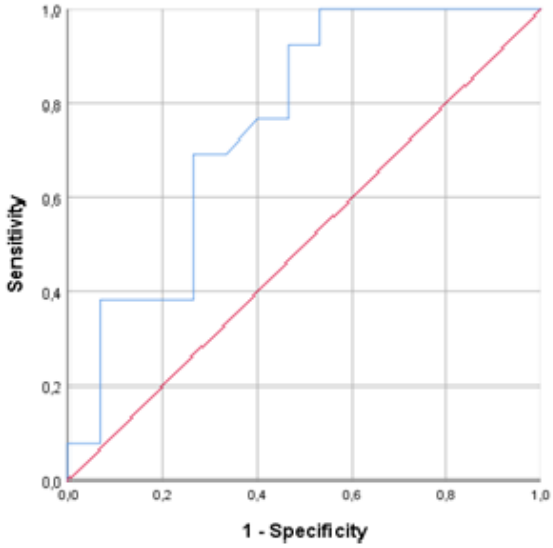

Figure 14. Receiver operator characteristics analysis of miRNA-182 in white blood cells in patients with shortened and normal sleep duration.

The data from both miRNAs are summarized in Table 9. Logistic regression models combining the two significant miRNAs did not show improved predictive ability (combined AUC = 0.267) compared to the individual markers. Therefore, miRNA-182 was retained as the most promising individual biomarker (AUC = 0.756).

Table 9. Discriminatory ability of significantly regulated miRNAs in distinguishing patients with shortened from normal sleep duration.

miRNA	Cut-off point	Sensitivity (%)	Specificity (%)	Youden's Index	AUC
miRNA-126	1.075	76.9	66.7	0.436	0.731
miRNA-182	0.530	78.6	100.0	0.467	0.756

Legend: AUC – Area Under the Curve

In the subsequent data processing, an additional analysis was performed, dividing the patient group into two subgroups according to the objectively measured sleep duration during PSG. A duration of 360 min (6h) was again used as a cut-off value – insomnia with objectively shortened (IOSSD) and objectively normal sleep

duration (IONSD). The two newly formed subgroups did not differ statistically in age, gender and level of education (table 10).

Table 10. Demographic characteristics of patients with objectively shortened and objectively normal sleep duration.

Variable	IOSSD (n = 8)	IONSD (n = 19)	p-value
Age			
Mean value (±SD)	37.88 (±11.42)	36.21 (±11.49)	0.734 tons
Sex n (%)			
Women	6 (75.00%)	14 (73.70%)	0.943 χ^2
Men	2 (25.00%)	5 (26.30%)	
Education n (%)			
Higher	4 (50.00%)	13 (68.40%)	0.365 χ^2
Average	4 (50.00%)	6 (31.60%)	

Legend: t- independent-samples t-test; $\chi 2$ – Chi-square test; U – Mann-Whitney U test; SD – standard deviation; IOSSD – insomnia with objectively shortened sleep duration, IONSD – insomnia with objectively normal sleep duration.

Comparative analysis of the expression levels of selected miRNAs in plasma and WBC in patients with objectively shortened and normal sleep duration revealed statistically significant differences for miRNA-182 WBC (p=0.039) and miRNA-219 in WBC (p=0.011). All data on expression levels are presented in Table 15, and the two significantly expressed miRNAs are graphically presented in Figure 11.

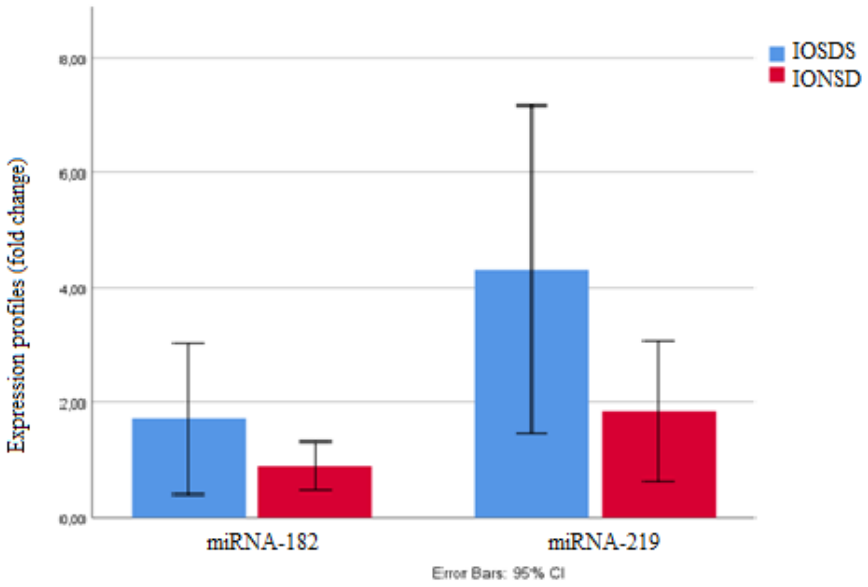

Figure 15. Expression levels of miRNA-182 and miRNA-219 in white blood cells in patients with objectively shortened (IOSSD) and objectively normal (IONSD) sleep duration.

Table 11 . Expression levels of selected miRNAs in plasma and leukocytes in IOSSD and IONSD.

Variable	IOSSD n = 8 Average value (±SD)	IONSD n = 19 Average value (±SD)	Mann-Whitney U-test p-value
miR125b_P	1.17 (±0.46)	1.63 (±1.37)	,815
miR126_P	1.43 (±1.07)	1.23 (±1.29)	,418
miR138_P	1.87 (±1.33)	1.23 (±0.98)	,307
miR146a_P	1.48 (±1.28)	1.28 (±1.29)	,696
miR182_P	2.85 (±2.75)	1.66 (±1.64)	,333
let7_P	1.22 (±1.28)	2.21 (±3.25)	,481
miR30c_P	1.36 (±1.23)	1.92 (±3.06)	,938
miR132_P (n=24)	1.22 (±1.06)	2.15 (±4.12)	1,000
miR212_P	2.53 (±1.78)	2.96 (±5.24)	,449
miR219_P	1.68 (±0.62)	1.53 (±1.85)	,132
miR125b_WBC	1.88 (±1.06)	1.80 (±0.98)	,696
miR126_WBC	3.28 (±3.49)	1.16 (±0.70)	,066
miR138_WBC	0.92 (±0.31)	0.84 (±0.64)	,360
miR146a_WBC	1.20 (±0.71)	0.87 (±0.27)	,481
miR182_WBC	1.71 (±1.57)	0.89 (±0.88)	,039*
let7_WBC	2.00 (±1.31)	1.34 (±0.65)	,333
miR30c_WBC	1.54 (±0.69)	1.25 (±0.42)	,238
miR132_WBC	1.55 (±0.69)	1.68 (±0.69)	,775
miR212_WBC	1.36 (±0.56)	1.38 (±0.73)	,815
miR219_WBC	4.31 (±3.41)	1.85 (±2.53)	,011*

Legend: miR – micro RNA; P – expression levels in plasma; WBC – expression levels in white blood cells; SD – standard deviation; IOSSD – insomnia with objectively shortened sleep duration; IONSD – insomnia with objectively normal sleep duration; *p<0.05.

4. fMRI data. To perform task 3, determining the effective connectivity of neuronal networks in the CNS in patients with chronic insomnia and healthy controls, resting-state functional magnetic resonance imaging (rs-fMRI) was performed. During the scanning process and in the processing stage, 6 individuals dropped out of the patient group and 5 from the control group. The main demographic and clinical characteristics of the study groups are presented in Table 12.

Table 12. Demographic and clinical characteristics of patients and controls who underwent fMRI.

Variable	Patients (n=31) Mean (±SD)	Controls (n=24) Mean value (±SD)	p-value
Age			
Mean value (±SD)	34.00 (±9.08)	30.00 (±7.01)	0. ,092 tons
Sex n (%)			
Women	22 (75.00%)	15 (73.70%)	0.579 χ^2
Men	9 (25.00%)	9 (26.30%)	
Education n (%)			
Higher	10 (50.00%)	6 (68.40%)	0.496 χ^2
Average	21 (50.00%)	19 (31.60%)	

Legend: t- independent-samples t-test; $\chi 2$ – Chi-square test; U – Mann-Whitney U test; SD – standard deviation;

The analysis of the strength of effective connectivity was conducted based on 3 connectivity models constructed from 6 to 8 ROIs. The analysis revealed multiple significant connections (non-zero) between regions of interest (ROIs) in each group. The total number of connections showing statistically significant differences between groups was five. Of these, three were present (non-zero) only in the patient group, while the remaining two were observed only in the control group and were absent in the patients (Table 13).

Table 13 Resting-state effective connectivity in patients with chronic insomnia. Statistically significant differences between the two groups.

Connections	Mean ± SD Patients	Mean ± SD Controls	Significance U
DMPFC → VMPFC	0.20608 ±0.342	0.35446 a ±0.369	0.049
MPFC → AIR	0.066696 ±0.172	-0.06238 to ±0.221	0.014
DLPFC → HippocampusR	-0.110019 ±0.258	0.18370 a ±0.204	<0.001
Precuneus → PCC	0.30175 a ±0.302	0.44117 ±0.388	0.040
PCC → AIR	-0.01701 a ±0.269	0.14839 ±0.242	0.039

Legend: U – Nonparametric Mann-Whitney analysis; DMPFC – Dorsomedial prefrontal cortex; VMPFC – Ventromedial prefrontal cortex; MPFC – Medial prefrontal cortex; AIR – Right anterior insula; DLPFC – Dorsolateral prefrontal cortex; HippocampusR – Right hippocampal formation. a – Not different from 0 in the respective group.

Aberrant connections in the patient group mainly involved the prefrontal cortex and the right hippocampus (Hippocamp R). Missing connections in the patient group (only present in healthy controls) involved the posterior cingulate gyrus (PCC), precuneus, and right anterior insula (Figure 16).

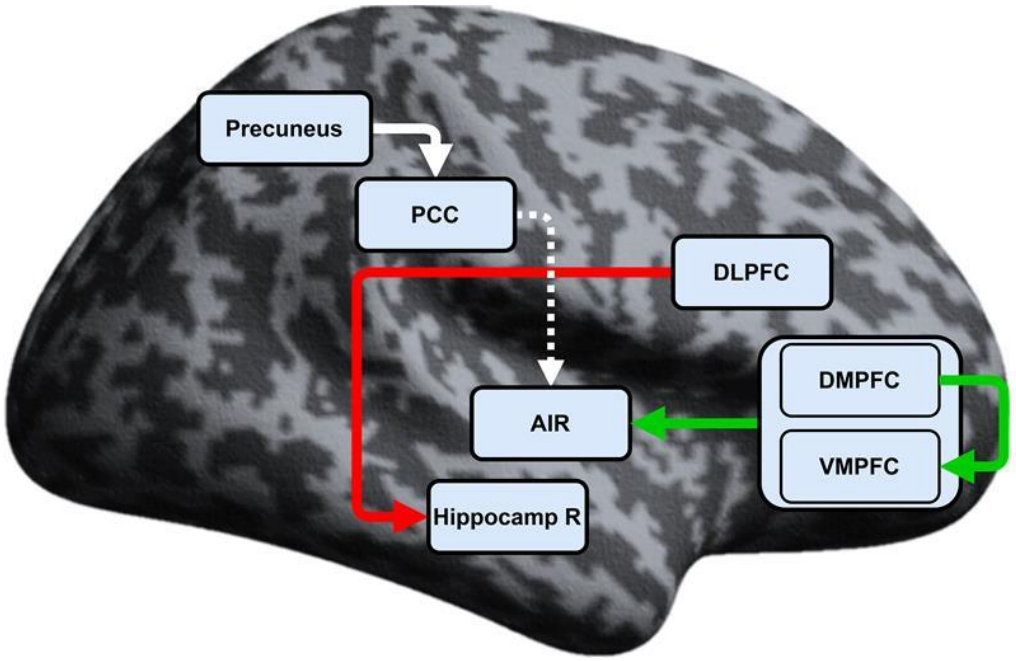

Figure 16. Resting-state effective connectivity in patients with chronic insomnia; DMPFC – dorsomedial prefrontal cortex; VMPFC – ventromedial prefrontal cortex; MPFC – medial prefrontal cortex; AIR – right anterior insula; DLPFC – dorsolateral prefrontal cortex; Hippocamp R – right hippocampal formation; PCC – posterior cingulate gyrus; Precuneus – precuneus; The total MPFC is represented as merged areas of DMPFC and VMPFC. Green arrows indicate an activating connection present only in the patient group. Red arrow indicates an inhibitory connection present only in the patient group. White arrow indicates significant connections present only in the control group (missing in patients).

5. **Correlation analysis** . To perform task 4, a Pearson correlation analysis was performed. The results of the objective clinical methods and the applied questionnaires were correlated with the expression level of significantly regulated miRNAs and with the degree of activation of neuroconnectome connections from the EU study in fMRI. The analysis revealed a significant positive correlation between the sum of the scores of the questionnaires assessing the severity of insomnia (ISI) and the risk of depression (BDI) ($r=0.702$, $p<0.001$), indicating a strong association between the severity of insomnia and the depressive profile of the individuals. Also, the ISI correlated positively with the total time awake ($r=0.527$, $p<0.001$), as well as with the time awake after falling asleep ($r=0.493$, $p<0.001$), indicating a strong association between the subjectively reported severity of insomnia and the objectively measured sleep time. The latter also correlated positively with the BDI score, indicating that the time awake was

associated with the depressive profile of the patients (r=0.340, p=0.007). The analysis of the degree of correlation of PSG parameters revealed a strong negative correlation between the total time awake and the percentage distribution of deep N3 (r = -0.425, p=0.001) and REM-phase (r = -0.387, p=0.002), while the positive correlation of time awake and N2% (r=0.391, p=0.002), indicating a disturbed sleep macroarchitectonics in the patients. The strength of the correlation is presented in Table 14.

Table 14. Correlation analysis between PSG data and questionnaires.

Correlation	Correlation coefficient (r)	Significance (p)
ISI-BDI	0.702	<0.001
ISI – Wakefulness (min)	0.527	<0.001
ISI – Wakefulness (%)	0.481	<0.001
ISI – WASO (min)	0.493	<0.001
BDI – Wakefulness (%)	0.293	0.021
BDI – WASO (min)	0.340	0.007
Wakefulness (min) – N3 (%)	-0.425	0.001
Wakefulness (min)–REM(%)	-0.387	0.002
Wakefulness (min) – N2 (%)	0.391	0.002

Legend: ISI – Insomnia Severity Index; BDI – Beck Depression Inventory; WASO – Wake, after sleep onset; N2 – Second Stage of Non-REM Sleep; N3 – Third Stage of Non-REM Sleep; REM – REM sleep; min – minutes; r – Pearson coefficient. PSG – Polysomnography.

Correlation analysis between miRNA expression levels and objective PSG data and questionnaire results revealed no significant correlations. The only positive correlation was found between the level of regulation of miRNA-132 WBC and miRNA-212 WBC (r=0.575, p<0.001), supporting the significance of both in the prediction of chronic insomnia.

When analyzing the relationship between the EC and the questionnaire results, a strong negative correlation between the DLPFC-HippocampR connection (available only in patients) and the ISI score (r=-0.507, p<0.001) is clearly evident, as well as a moderate negative correlation between the same connection and the BDI (r=-0.272, p=0.044). In addition, a moderate negative correlation was found between the DLPFC-HippocampR and the BDI (r=-0.304, p=0.025). Also striking is the strong negative correlation between the MPFC-AIR connections (available only in patients) and PCC-AIR (available only in controls) – r=-0.702, p<0.001, supporting the difference in the connectome between patients and healthy controls (Table 15; Figure 17)

Table 15. Correlation analysis between EC, PSG data and questionnaire results.

Correlation	Correlation coefficient (r)	Significance (p)
DLPFC-HippocampR → ISI	-0.507	<0.001
DLPFC-HippocampR → BDI	-0.304	0.025
DLPFC-HippocampR → WASO	-0.304	0.025
MPFC-AIR → PCC-AIR	-0.702	<0.001

Legend: EC – effective connectivity; PSG – polysomnography; DLPFC – dorsolateral prefrontal cortex; MPFC – medial prefrontal cortex; HippocampR – right hippocampal formation; AIR – anterior insula of the right; PCC – posterior cingulate gyrus; ISI – insomnia severity index; BDI – Beck Depression Inventory; WASO – Wake after sleep onset; r – Pearson coefficient.

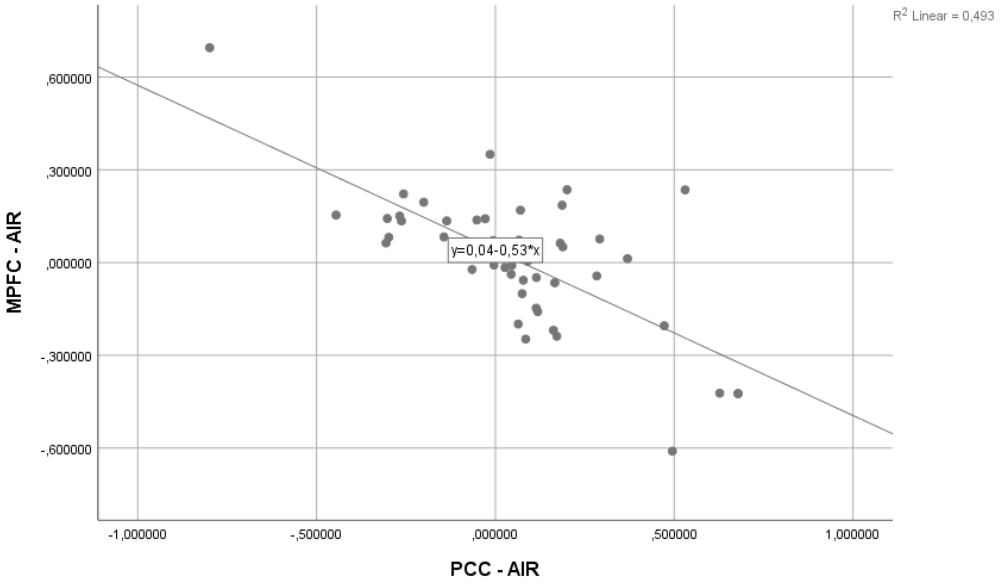

Figure 17. Correlation analysis between the strength of MPFC-AIR and PCC-AIR connections. MPFC – Medial prefrontal cortex; AIR – Right anterior insula; PCC – Posterior cingulate cortex.

At the next stage, the correlation between the level of miRNA expression and objective PSG data and the results of the questionnaires in the two subgroups of patients – ISSD and INSD (table 16). The analysis in the ISSD group revealed a strong positive correlation between the expression of miRNA-126 WBC and SL (r=0.689; p=0.009), as well as a moderate positive correlation between miRNA-182 WBC and SL (r=0.623, p=0.009). A moderate correlation was also found between the same miRNA and ISI (r=0.600, p=0.030). The strong positive correlation between the two significantly expressed miRNAs in ISSD (r=0.919, p<0.001) is impressive, pointing to a strong association between their expression and sleep duration in patients (Fig . 18).

Table 16. Correlation analysis between miRNA expression levels and objective PSG data and questionnaire results in the two subgroups of patients – ISSD and INSD

Correlation	Correlation coefficient (r)	Significance (p)
miRNA-126 WBC – SL	0.689	0.009
miRNA-182 WBC – SL	0.623	0.009
miRNA-182 WBC – ISI	0.600	0.030
MiRNA-126 WBC – MiRNA-182 WBC	0.919	<0.001

Legend: PSG – polysomnography; ISSD – insomnia with short sleep duration; INSD – insomnia with normal sleep duration; WBC – white blood cells; LS – sleep latency; ISI – Insomnia Severity Questionnaire.

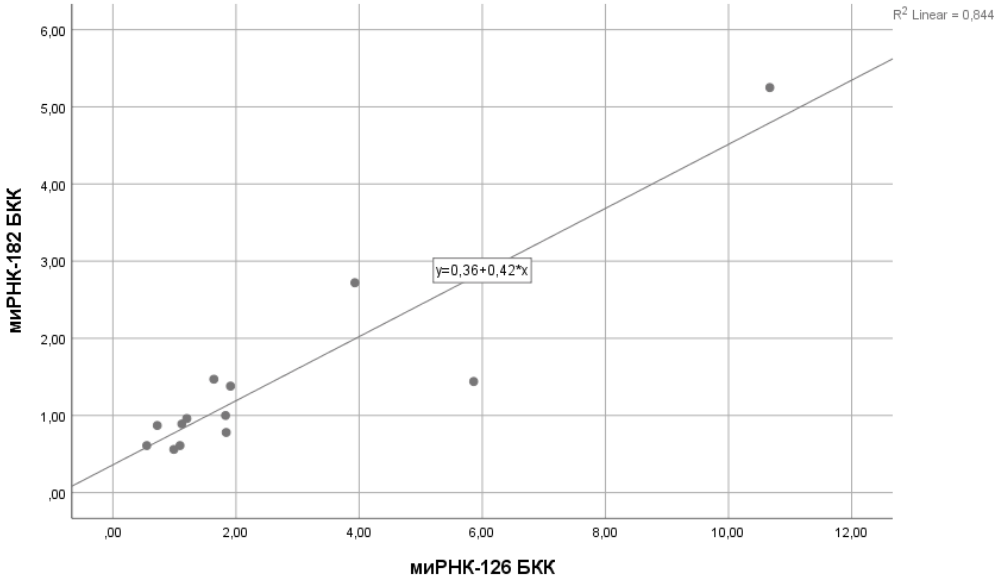

Figure 18. Correlation analysis between the expression levels of miRNA-182 and miRNA-126 in patients with shortened sleep duration; WBC – white blood cells.

Regarding the correlation between EC and clinical markers in the ISSD group, a strong negative association was found between the DMPFC-VMPFC connection, present only in patients, and the BDI score (r=-0.767, p<0.001).

Discussion

Task 1. Extensive published data show that the main PSG characteristics among patients with insomnia are shortened total sleep time, prolonged sleep latency, reduced REM and increased N2, and increased frequency of microarousals. Our results partially overlap with those described in the literature, mainly with respect to time awake and disturbances in NREM sleep. In light of the qualitative changes in REM, multiple arousals, and transitions from NREM to REM in insomniacs, the concept of REM instability is also considered, contributing to the understanding of the "misperception of sleep". REM instability contributes to the prolonged wakefulness after falling asleep and can be perceived as a substrate of PVS, since instability gives rise to a tendency to sleep fragmentation, making the corresponding phase more vulnerable and susceptible to arousing stimuli - external or internal.

Increased N2 accompanied by decreased N3 is among the most frequently reported macrostructural changes in patients. This characteristic feature of sleep structure is associated with the inability to deepen sleep in patients due to the lower arousal threshold and contributes to the feeling of non-restorative sleep, despite its normal duration. Interestingly, patients with acute insomnia and decreased N3 are more prone to chronicity compared to those with symptoms of acute insomnia but normal N3 duration. This supports the notion that the disturbed sleep in patients is predetermined, part of the so-called predisposing factors, according to the Spielman model.

Increased WASO is a characteristic PSG sign in chronic insomnia, also present in our study. WASO is an objective marker reflecting sleep fragmentation in patients, contributing to the non-restorative nature and lack of consolidated structural integrity of sleep in insomniacs. WASO is considered a marker of hyperarousal and a lowered threshold for awakening, reflecting cortical overactivity in patients, demonstrating increased beta-activity, correlating with objectively established WASO. An additional finding in our study is the increased total time in bed, which is a function of WBZ and LS. Patients with insomnia spend significantly more time in bed, reducing sleep efficiency and contributing to the formation of false associations between bed and activities other than sleep, according to the "Stimulus Control" model. This maladaptive behavior is supported by the need for more sleep by patients who mistakenly try to increase the amount by providing the opportunity to sleep, in the absence of sleep pressure.

At the next stage of data processing, we divided the patient group into two subgroups according to the subjectively reported sleep duration (based on sleep diary data) – ISSD and INSD. The comparative analysis of the two groups by PSG parameters showed a significantly shorter sleep time in ISSD (mean value 351.30 min) compared to INSD (mean value 419.22 min;

p=0.004). These results do not correspond to the more frequently reported discrepancy between objectively and subjectively reported sleep duration. Of particular interest is the significantly reduced REM sleep time in ISSD compared to INSD (p=0.029), which is the only difference in the macroarchitectonics of sleep between the two groups. The presence of a longer REM period in NREM contributes to the erroneous perception of sleep in patients from this group, as part of REM sleep, especially in cases with REM instability, can be perceived as conscious wakefulness, giving the appearance of paradoxical insomnia.

Task 2. The results of the analysis of miRNA expression levels revealed a statistically significant regulation of 3 miRNAs in WBC in the patients in our study – miRNA-132 (p=0.006), miRNA-212 (p=0.048) and miRNA-219 (p=0.036). miRNA-132 and miRNA-212 are often regulated simultaneously and their expression levels follow a common relationship, as is the case in our study. miRNA 132 is considered a factor modulating circadian rhythms and sleep structure. Reduced deep sleep time and prolongation of REM are among the main findings established in rodents affected with this miRNA. Additional evidence of its influence on sleep structure is its effect on the light-induced part of the SCN, as well as the effect of miRNA-132 and miRNA-212 on dendritic density in the SCN itself. At the same time, the upregulation of miRNA-132 in WBC can be considered as a function of a subthreshold inflammatory response in patients, compared to controls. The tandem miRNA-132/212, often called “neuro-miRs”, due to their high concentration in the CNS, show a relationship with inflammatory and immunological activity. A hypothesis about the influence of miRNA-132 on acetylcholinesterase activity in the CNS and peripheral inflammatory cells draws attention to an additional mechanism by which this miRNA is involved in the regulation of sleep and wakefulness. It is not clear why the interaction between miRNA-132 and AcH2 leads to circadian disorders in mice, the proven influence of this tandem in the processes of neuronal alteration and inflammation contributes to the change in periods of activity and rest in rodents.

Last but not least, miRNA-132 also shows a relationship with the manifestation of anxiety-depressive spectrum disorders, being associated with a disorder in emotional processing and the manifestation of clinical depression. 20% of individuals diagnosed with "Chronic Insomnia" also develop a clinically significant depressive disorder. At the same time, a relationship has been shown with the regulation of the expression of brain-derived neurotrophic factor (BDNF), which is key for neuronal survival and synaptic plasticity. Increased levels of miRNA-132 can lead to reduced expression of BDNF, which has been observed in patients with depression and may contribute to the symptoms of the disease. Inhibition of miRNA-132 in these regions leads to antidepressant effects, which emphasizes its role in mood regulation.

Based on the above, we hypothesize that miRNA-132 is involved in the pathogenesis and maintenance of chronic insomnia through coordinated dysregulation of circadian, neuronal, and inflammatory mechanisms. The observed decrease in local expression of miRNA-132 after REM deprivation suggests that sleep and miRNA regulation are interdependent and that chronic fragmentation may lead to long-term epigenetic changes. The demonstrated association between miRNA-132, BDNF, and disorders in emotional regulation creates an additional pathway by which stress and affective factors – major mechanisms supporting insomnia – can be integrated. Based on these data, we hypothesize that miRNA-132 represents a key epigenetic mediator that links circadian, cholinergic, inflammatory, and affective mechanisms in a stable pathological cycle characteristic of chronic insomnia.

miRNA-212 together with miRNA-132 has been shown to adapt to light periods and regulate circadian oscillations in the CNS. Potential disorders in this mechanism could be attributed to the inability to initiate sleep correctly and in a timely manner, contributing to the manifestation of insomnia-like disorders. Similar to the effects described above, overexpression of miRNA-212 is associated with neuronal damage and inflammation. In an animal model of depressive disorder, increased expression levels of miRNA-212 were found, significantly decreasing after therapy. Based on the described data, we suggest that miRNA-212 is involved in the pathogenesis of chronic insomnia through dysregulation of light-dependent circadian adaptation in the CNS, thus disrupting timely and effective sleep initiation. As a member of the neuromir cluster (miRNA-132/212), this miRNA likely contributes to altered light sensitivity and inadequate circadian synchronization, characteristic of patients with difficulties both in falling asleep and maintaining sleep.

Pre-miRNA-219 is a product sensitive to the E-box element in the SCN and is activated by dimerized CLOCK:BMAL1 in the SCN, i.e. the expression of miRNA-219 follows a circadian dependence. The miRNA itself is associated with modulation of the duration of the period of dimerization and disintegration of the complex responsible for maintaining circadian oscillations. Polymorphism of the first gene locus for miRNA-219 in patients with GDR is associated with their symptoms of disturbed sleep, such as difficulty falling asleep. On the other hand, downregulation of miRNA-219 was found in rats with sleep deprivation, indicating a bidirectional relationship between miRNA expression levels and sleep duration. Among the main targets of miRNA-219 is a signaling pathway related to the N-methyl-d-aspartate (NMDA) glutamate receptor, causing its translational repression, mainly through its influence on calcium/calmodulin-dependent protein kinase II. The NMDA receptor is essential for the maintenance of NREM and REM sleep in healthy individuals, is associated with the regulation of mood and emotions, and its damage in autoimmune encephalitides is associated with symptoms of

difficulty maintaining sleep and insomnia. In addition, miRNA-219 also modulates the effects of the NMDA receptor in some psychiatric disorders such as schizophrenia and affective-anxiety disorders.

Since NMDA receptors are crucial for the circadian action of glycine in the CNS, rhythmic regulation by miRNA-219 is likely necessary for the correct phase setting of the sleep-wake cycle. Therefore, altered regulation of miRNA-219 may destabilize NMDA-mediated mechanisms of synaptic plasticity and neuromodulation, creating a biological basis for the maintenance of chronically disturbed sleep patterns. In fact, the sleep-promoting effects of the neurotransmitter glycine are mediated in the CNS via NMDA receptors, whose activity is modulated by rhythmically expressed miRNA-219. These data give us reason to hypothesize that changes in the levels of miRNA-219 regulation may be associated with disturbances in the mechanisms controlling and regulating the sleep-wake cycle.

When analyzing the expression levels of miRNAs between ISSD and INSD, a significant difference was found in two of them – miRNA-182 in WBC ($p=0.019$) and miRNA-126 in WBC ($p=0.037$), showing higher regulation in the ISSD group. Disturbances in the expression of miRNA-182 are associated with retinal degeneration and impaired circadian control in SCD. Among the main targets of miRNA-182 is the CLOCK gene, as a polymorphism of pre-miRNA-182 is associated with late-onset insomnia in patients with GDR, showing altered function of CLOCK products. The expression of miRNA-182 is higher during the day, which suggests that its higher levels of regulation in the ISSD group can be interpreted as a disturbance in circadian control and manifestations characteristic of the daytime period. On the other hand, REM deprivation in mice results in downregulation of miRNA-182 in hippocampal tissue, supporting the bidirectional influence of circadian control on its expression. Overexpression of miRNA-182 is also associated with reduced levels of BDNF , which in turn enhances NREM sleep in rats, which suggests that sleep disruption in patients is due to dysregulation of miRNA-182 and the resulting reduced levels of BDNF. The interaction between miRNA-182, circadian imbalance, inflammatory signals and chronic stress activation (CHA) outlines this miRNA as a potential epigenetic mediator that integrates circadian, emotional and neuroinflammatory mechanisms in the maintenance of insomnia.

In contrast to our data on increased expression of miRNA-182, the authors reported downregulation of the corresponding miRNA in patients with insomnia. Their results were obtained by examining miRNA expression in exosomal structures, a methodological difference that could underlie the opposing results. Also, in patients with shortened sleep, the inflammatory response is more pronounced, which explains the increased levels of detected miRNA in WBC, an expression of the inflammatory response. It is also

necessary to consider the possibility of increased expression of miRNA-182 in a state of chronic stress and activation of the CAO, characteristic of patients with ISSD, which also contributes to its upregulation in this cohort.

Unlike previously described miRNAs, miRNA-126 is expressed mainly in endothelial tissue, without showing signs of neurospecificity. miRNA-126 is mainly associated with inflammatory response, and its expression levels are altered in neurodegenerative diseases, systemic inflammation, sleep deprivation and proatherogenic conditions. Its expression does not follow a circadian dependence, but is associated with factors leading to endothelial damage and inflammatory response. Its effect is linked to the vasodilatory capacity of the vessels, affecting nitric oxide levels and reducing the prothrombogenic profile in individuals. In addition, low levels of miRNA-126 are associated with increased levels of the atherogenic proteins SPRED1 and CXCL12. Several studies have shown reduced levels of miRNA-126 in shortened sleep. The interaction between miRNA-182, circadian imbalance, inflammatory signals and chronic stress activation (CHA) outlines this miRNA as a potential epigenetic mediator that integrates circadian, emotional and neuroinflammatory mechanisms in the maintenance of insomnia. The reported results differ from those in our cohort, in which we found an upregulation of miRNA-126 in the ISPA group. This fact deserves attention, as an expected compensatory manifestation of the organism to the systemic damage from the shortened sleep and the overactive CHAO. The data from our study can be interpreted as indicative of a preserved compensatory potential of the organism to the damaging effects of the shortened sleep on endothelial function and systemic inflammation. An important difference between the participants in our study and those in previous studies is that the patients in our cohort were selected after a strict analysis and rejection of an underlying sleep disorder or other somatic disease that would have clouded the data and contributed to a change in the expression levels of this endo-miRNA. Validation of the hypotheses presented requires an integrative approach, conducting experiments and clinical studies on the role of the described molecular structures, as well as assessing and accurately formulating the causal relationships between the primary dysregulation in miRNA expression and the development of chronic insomnia.

Task 3. The main findings of the EC study in patients with chronic insomnia indicate that there are distinct changes in the reciprocal connectivity patterns between central areas of the DMN, SN, and ECN in patients with insomnia compared to healthy individuals. We observed statistically significant changes in EC between patients and controls in five connections, namely from DMPFC to VMPFC (excitatory), from MPFC to AIR (excitatory), from PCC to AIR (excitatory), from Precuneus to PCC (excitatory), and from DLPFC to right hippocampus (inhibitory). Furthermore, the connectivity values for the

connections DMPFC - VMPFC, MPFC - AIR, and DLPFC - Hippocamp R were significantly different from zero only in the patient group, while the connections PCC - AIR and Precuneus - PCC were statistically different from zero only in the control group (missing in patients).

The MPFC is considered a key structure in the pathophysiology of insomnia. Given the major functions of the MPFC related to emotion processing, motor control, and decision-making, we can hypothesize that the observed arousal of the AIR leads to hyperstimulation of the SN, which prevents the individual from falling asleep and maintaining the sleep state. The increased arousal influence may also lead to disruption of the integrative function of the insula, leading to disorganization of the "switch" between the DMN and the ECN and an inability to suppress arousal, which is consistent with the hypothesized model of hyperarousal.

Another finding supporting the aforementioned impaired "switching" mechanism is the observed PCC–AIR excitatory coupling in controls that is not observed in the CID group. Impaired connectivity between these key nodes of the two networks was reported in a study investigating FS in patients with impaired cognitive control, highlighting the role of the SN in reducing DMN activity at rest. A potential explanation for the observed difference may lie in the methodology of their study, as the tests were conducted while CID patients were instructed to fall asleep during the scanning, suggesting active participation in the otherwise passive process of sleep initiation. This phenomenon, consistent with the attention–intention–effort hypothesis of insomnia, may be a cause of, rather than a consequence of, an overactivated insula.

Of particular note is the previously reported right-lateralization of the AIR's ability to modulate and act as a switching mechanism, a phenomenon also observed in our study. This function involves detecting salient internal and/or external stimuli and initiating a shift from self-directed (DMN) to goal-directed (ECN) processing. In contrast, Long and colleagues report altered insular FS following sleep deprivation, particularly in young adults, suggesting that impaired signaling between control networks is a consequence of sleep disruption rather than a causal factor.

Furthermore, the precuneus–PCC excitatory connection, present only in controls, may be responsible for maintaining the functional integrity of the DMN, and we can hypothesize that its absence in the patient group reflects poor internal regulation and increased mind wandering or rumination in patients. Our hypothesis is based on previously reported data suggesting that reduced FC between key DMN nodes underlies CID and contributes to the inability to disengage from internal thoughts, which may underlie the state of hyperarousal.

The DLPFC, a core node of the ECN, is associated with working memory, attentional regulation, and inhibitory self-control. Our findings regarding the inhibitory connection between the DLPFC and Hippocampus R may provide further insight into the importance of these regions in patients. We hypothesize that this may lead to impaired emotional regulation and episodic memory consolidation. Both phenomena are common in patients with insomnia, potentially leading to fragmented sleep and rumination. Similarly, an overactivated DLPFC may induce excessive top-down control, reflecting a state of cognitive hyperarousal. On the other hand, this hypothesis is not consistent with previous findings describing reduced resting metabolism and prefrontal cortex activity in insomniacs, which highlights the complexity of this hypothesis and suggests that other factors may also contribute to hyperarousal in patients.

Our findings contribute to the understanding of insomnia as a disorder at the level of brain networks, rather than simply as a dysfunction of individual CNS regions. Altered EC in key nodes such as the MPFC, DLPFC, and anterior insula may serve as potential biomarkers of disease severity, treatment response, and prognosis.

Task 4. The correlation analysis performed revealed a significant positive correlation between the sum of the scores from the questionnaires assessing the severity of insomnia (ISI) and the risk of depression (BDI) ($r=0.702$, $p<0.001$). ISI also correlated positively with the total time awake ($r=0.527$, $p<0.001$) and with the time awake after falling asleep ($r=0.493$, $p<0.001$). These results confirm those reported in the literature so far. The presence of common characteristics in the profile of depressed patients and patients with chronic insomnia emphasizes the similarities in the manifestation of the two disorders. The relationship between chronic insomnia and mental disorders is well established. Polysomnographic data on disturbed sleep have been reported in almost all mental disorders. Insomnia is often an initial symptom of many mental illnesses, especially ADHD and GDD. The relationship between chronic insomnia and disorders from the anxiety-depressive spectrum and/or affective psychiatric continuum is bidirectional and multifactorial.

The importance of chronic insomnia for mental health is also highlighted by the fact that insufficient sleep is associated with an increased risk of developing depressive symptoms. Furthermore, depressive symptoms often recur after clinical remission in patients with a history of insomnia. Often, sleep-related complaints persist after remission from a depressive episode, which significantly increases the risk of relapse. One possible explanation, based on the classic 3-P model, is that stressful life events act as triggers for insomnia, establishing lasting changes in sleep. These same stressors also serve as determinants for the development of depressive disorders, mainly

through mechanisms that disrupt monoaminergic and serotonergic neurotransmission in the central nervous system, favoring wakefulness.

Triggers may also lead to dysregulation of the “zeitgebers” (internal “clocks”) that control circadian rhythms, thereby affecting sleep/wakefulness. This hypothesis underlies the “zeitgeber theory” of depression, which posits that depressive symptoms arise from disrupted environmental time signals, with further consequences on circadian regulation.

Polysomnographic data show similar disturbances in sleep architecture in patients with chronic insomnia and depression, which may illustrate common pathophysiological mechanisms. REM sleep instability, defined as fragmented REM with increased number of awakenings, is observed in patients with depression and/or insomnia. REM instability is associated with both subjectively disturbed sleep perception and impaired emotional processing. Disturbed REM structure interferes with normal sleep consolidation, disrupts wakefulness, and simultaneously impairs emotional regulation, which affects mental stability and leads to the manifestation of depressive symptoms in those affected. Disturbances in deep sleep can lead to increased fatigue, reduced cognitive function, and poor mood, creating a vicious cycle between poor sleep quality and depressive symptoms. Patients with depression often show reduced duration and percentage of N3 sleep, which is associated with impaired sleep architecture and poorer recovery. In addition, studies have shown that reduced N3 sleep may be associated with an increased risk of developing depressive symptoms. At the same time, some antidepressants, such as mirtazapine and trazodone, can increase the duration of N3 sleep, improving sleep quality in patients with depression, considered a sign of clinical improvement.

The analysis of the degree of correlation of PSG parameters revealed a strong negative correlation between the total time awake and the percentage distribution of deep N3 ($r = -0.425$, $p=0.001$) and REM phase ($r = -0.387$, $p=0.002$), and a positive correlation of the time awake and N2% ($r=0.391$, $p=0.002$). These data point to an altered macroarchitecture of sleep in insomniacs. The time spent awake at night in them is mainly at the expense of restorative deep sleep and the REM phase, responsible for cognitive and emotional control. The N3 phase is essential for physical recovery and memory consolidation, and its reduction is a sign of fragmented and poor-quality sleep. In addition, REM sleep plays an important role in emotional regulation and cognitive function. Reduced REM sleep, especially with frequent awakenings, is common in individuals with affective disorders, including depression.

A positive correlation was found between the degree of regulation of miRNA-132 WBC and miRNA-212 WBC ($r=0.575$, $p<0.001$), supporting the significance of both in the prediction of chronic insomnia. Both are significantly expressed in the patient group. The potential role of miRNA-132

as a marker of chronic insomnia is supported by the multiple functions performed by this miRNA in the CNS. As already mentioned, BDNF enhances NREM sleep and is a key regulator of synaptic plasticity and neuronal growth. miRNA-132 regulates synaptic plasticity by targeting genes such as PSD-95 and GluA1, which are essential for synaptic function. Dysregulation of miR-132 may disrupt synaptic integration and contribute to cognitive deficits observed in depression. Considering the significant overlap between depression and insomnia described above, we can speculate that miRNA-132 influences key pathways in the manifestation and development of both disorders.

Correlation analysis in the ISSD group revealed a strong positive correlation between the expression of miRNA-126 WBC and SL ($r=0.689$; $p=0.009$), as well as a moderate positive correlation between miRNA-182 WBC and SL ($r=0.623$, $p=0.009$). The above hypothesis of secondary upregulation of these miRNAs due to systemic inflammatory response and activation of HAA is complemented here as well, as we can speculate that the prolonged time to sleep initiation and the accompanying catastrophic thoughts and worries lead to potentiation of the sympathetic response, hypercortisolemia and secondarily increased levels of miRNA182 and 126. Moreover, the strong positive correlation between the two significantly expressed in ISSD miRNA126 and 182 ($r=0.919$, $p<0.001$), points to a strong association between their expression and sleep duration in patients. This strong correlation suggests that both miRNAs are co-regulated in the context of reduced sleep duration in chronic insomnia. On the one hand, this may be due to the fact that these miRNAs are associated with disrupted circadian, neurovascular, and inflammatory pathways that contribute to the development and maintenance of chronic insomnia. On the other hand, there is evidence that miRNA-182 and miRNA-126 can be transcriptionally co-regulated under the influence of stress-induced signals and inflammatory cytokines, which further supports the observed strong correlation. The increased expression level of both miRNAs may be a compensatory response to chronic hyperactivation of the HPA axis and disrupted circadian regulation in insomnia with short sleep.

Given these results, future studies should investigate whether miRNA-182 and miRNA-126 can serve as dual biomarkers for pathological adaptation in chronic insomnia. Their role as therapeutic targets to restore normal neuroplasticity and vascular response is also possible.

When analyzing the relationship between ES and the questionnaire results, a strong negative correlation between the DLPFC-HippocampR connection (available only in patients) and the ISI score ($r=-0.507$, $p<0.001$) is clearly evident. This provides a neurobiological hypothesis regarding the relationship between cognitive-emotional regulation and the severity of insomnia. ES, established in patients, reflects the ability of the prefrontal cortex to regulate

activity in limbic structures responsible for emotional memory and arousal. The negative correlation shows that with more severe insomnia (high ISI values), a weaker downregulation from the DLPFC to the hippocampus is observed. This suggests impaired cognitive control over emotional activation. This model supports the neurocognitive hypothesis of hyperarousal in insomnia, in which an imbalance between prefrontal control and limbic reactivity underlies impaired sleep initiation and maintenance.

Regarding the correlation between EC and clinical markers in the ISPA group, a strong negative association was found between the DMPFC-VMPFC connection, present only in patients, and the BDI score ($r=-0.767$, $p<0.001$). This highlights the importance of dysregulation in the prefrontal cortex for the severity of depressive symptoms.

The DMPFC and VMPFC are central components of the DMN, which is responsible for internal processing of signals at rest, appraisal of affective stimuli, and cognitive control of emotions. Reduced connectivity between these two areas may lead to impaired interaction between cognitive and emotional processes. This condition favors excessive auto-focus, ruminative thinking, and the inability to regulate negative affect—typical features of chronic insomnia and GDR. DMPFC–VMPFC connectivity can be considered as a potential neuromarker for the severity of insomnia and depressive symptoms, as well as a target for interventions aimed at restoring prefrontal integration.

Conclusions.

1. Patients with chronic insomnia exhibit a reduction in N3 (slow-wave) sleep accompanied by a compensatory increase in N2 sleep when compared with healthy control subjects.
2. Among individuals with chronic insomnia, those with objectively shortened sleep duration demonstrate a reduced proportion of REM sleep relative to patients with insomnia and normal sleep duration.
3. In patients with chronic insomnia, the subjective severity of depressive symptoms is significantly correlated with insomnia severity, a relationship not observed in healthy controls.
4. Chronic insomnia is associated with altered expression of miRNA-132, miRNA-212, and miRNA-219 in white blood cells, implicating molecular pathways involved in neuronal development and circadian rhythm regulation when compared with healthy controls.
5. Patients with chronic insomnia and shortened sleep duration exhibit differential white blood cell expression of miRNA-126 and miRNA-182 compared with patients with insomnia and normal sleep duration, suggesting involvement of inflammatory processes, neuronal development, and sleep regulation mechanisms.
6. Functional magnetic resonance imaging revealed aberrant effective connectivity between core regions of the default mode network (DMN), executive control network (ECN), and salience network (SN) in patients with chronic insomnia, a pattern absent in healthy controls.
7. Subjective assessments of insomnia severity show significant correlations with objective polysomnographic sleep parameters across the studied patient cohort.
8. Disrupted effective connectivity within DMN regions is associated with the presence and severity of depressive symptoms in patients with chronic insomnia and shortened sleep duration.
9. Sleep duration in patients with chronic insomnia demonstrates a strong correlation with white blood cell expression levels of miRNA-126 and miRNA-182.

Contributions.

Contributions of a scientific and theoretical nature

1. This dissertation proposes, for the first time, a hypothesis linking altered microRNA (miRNA) regulation to variations in sleep duration in patients with chronic insomnia, thereby extending current theoretical models of insomnia pathophysiology.
2. For the first time, a conceptual framework is introduced for the objective characterization of hyperarousal in chronic insomnia, based on disrupted effective connectivity within and between key brain networks, namely the default mode network (DMN), executive control network (ECN), and salience network (SN).
3. The findings provide further empirical support for the association between chronic insomnia and the presence of symptoms within the anxiety–depressive spectrum, reinforcing existing neurobiological and clinical models of comorbidity.

Contributions of a scientific and applied nature

1. This work presents, for the first time, a methodological approach for differentiating insomnia with shortened sleep duration (ISSD) from insomnia with normal sleep duration (INSD) based on distinct patterns of miRNA expression in white blood cells.
2. A novel method is proposed for distinguishing patients with chronic insomnia from healthy control subjects using differential miRNA expression profiles in white blood cells, highlighting the potential of miRNAs as peripheral biomarkers of insomnia.
3. For the first time, sleep architecture differences between patients with chronic insomnia and healthy controls have been systematically investigated within the Bulgarian population, providing population-specific normative and pathological data.

Scientific publications and communications related to the dissertation work

1. Georgiev T, Avramov K, Draganova A, Terziyski K. "Serum miRNA levels and fMRI-resting state functional connectivity as novel markers for assessing chronic insomnia subtypes" Scientific Works of the Union of Scientists Plovdiv. Series G. Medicine, Pharmacy and Dentistry Vol. XXVIII. ISSN 1311-9427 (Print), ISSN 2534-9392 (On-line). 2022.
2. Georgiev T, Draganova A, Avramov K, Terziyski K. Chronic insomnia–beyond the symptom of insufficient sleep. Folia Medica. 2025 May 16;67(3):e151493.
3. Georgiev T, Paunova R, Todeva-Radneva A, Avramov K, Draganova A, Kandilarova S, Terziyski K. Aberrant Effective Connectivity Within and Between the Default Mode, Executive Control, and Salience Networks in Chronic Insomnia Disorder—Toward Identifying the Hyperarousal State. Biomedicines. 2025 May 24;13(6):1293.

Participation in scientific forums.

1. T Georgiev, K Avramov, A Draganova, V Dichev, N Mehterov, K Terziyski Downregulation of miRNA-125b and let-7 provides a novel aspect to chronic insomnia disorder – a pilot study. Sleep and Breathing Conference, Prague, Czech Republic – 22-22 April 2023 – poster
2. T. Georgiev, R. Paunova, S. Kandilarova, T. Zdravkova, K. Avramov, A. Draganova, K. Terziyski. Aberrant effective connectivity in Default Mode Network and Salience Network may reflect the hyperarousal state in chronic insomnia disorder - World Sleep Congress 20-25. 10. 2023, Brazil - poster
3. Georgiev T, Avramov K, Draganova A, Terziyski K "Serum miRNA levels and fMRI-resting state functional connectivity as novel markers for assessing chronic insomnia subtypes" IXth International Conference of Young Scientists - Plovdiv 2022 - 14-15.07.2022 - poster