

Clock gene signature predicts Insomnia and links to sleep/circadian parameters

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Abstract

Chronic Insomnia is a prevalent sleep disorder that remains difficult to diagnose due to subjective symptoms and heterogeneous presentations. The most severe form, insomnia with short sleep duration (ISSD), is defined by a total sleep time of less than six hours on polysomnography. However, objective assessments are rarely recommended in diagnostic guidelines, highlighting the need for alternative biomarkers. Disruptions in the circadian clock system may contribute to chronic insomnia, though the extent of these effects remains unclear. In this study, we investigate sleep and circadian rhythm-related alterations in chronic insomnia and its subtypes, ISSD and insomnia with normal sleep duration (INSD), by assessing plasma cortisol, wrist and axillary temperature, and clock gene expression in peripheral blood mononuclear cells (PBMCs). Additionally, we use machine learning to identify the most relevant clock genes for detecting insomnia and classifying its subtypes. Chronic insomnia patients exhibited reduced body temperature rhythms, elevated nighttime cortisol levels, and significant alterations in clock genes expression, including in *BMAL1*, *PER1-2*, *REV-ERBa*, and *REV-ERBβ*, compared to controls. Most alterations were more significant in the ISSD. Moreover, associations between clock gene expression, sleep-related parameters and insomnia severity index (ISI) scores were identified. Using machine learning, we identified three genes as sensitive biomarkers distinguishing chronic insomnia from controls and differentiating between ISSD and INSD subtypes. Our findings suggest that circadian markers and machine learning could improve understanding of chronic insomnia and aid biomarker discovery for diagnosis.

Introduction

Chronic insomnia disorder is a prevalent condition affecting approximately 10% of the global population¹. It is defined by the Diagnostic and Statistical Manual of Mental Disorders, 5th edition, Text Revision (DSM-5-TR) and the International Classification of Sleep Disorders, 3rd edition, Text Revision (ICSD-3-TR) as persistent nighttime symptoms despite adequate sleep opportunities, accompanied by daytime impairments such as fatigue, occurring at least three nights *per week* for over three months^{2,3}. Diagnosis typically relies on self-reported symptoms, limiting objective assessment and standardization. Over time, varying definitions of insomnia and its subtypes, such as primary insomnia or classifications based on sleep duration or individual traits, have led to inconsistencies in research findings and diagnostic challenges⁴⁻⁸, hindering the identification of reliable biomarkers^{9,10}.

A clinically relevant subtype, insomnia with short sleep duration (ISSD), is characterized by a total sleep time (TST) of less than six hours, as measured by single-night polysomnography (PSG)⁷. ISSD is associated with an increased risk of significant medical and psychiatric comorbidities, adverse cardiometabolic outcomes and neurocognitive impairments^{7,11,12}. Moreover, individuals with ISSD respond less favorably to cognitive-behavioral therapy compared to those with insomnia with normal sleep duration (INSD, TST > 6 hours)⁷, highlighting the need for subtyping insomnia during diagnosis for tailored treatment approaches.

Despite the clinical relevance of ISSD and INSD, objective measures such as PSG and actigraphy are rarely incorporated into routine diagnosis of insomnia due to high costs, logistical challenges ¹³, and their inability to capture subjective wakefulness experiences ¹⁴. Additionally, European guidelines do not mandate objective sleep measures for diagnosing insomnia ⁹. To address these challenges, identifying reliable biological markers could enhance diagnostic precision and inform targeted treatment strategies.

Emerging evidence suggests that chronic insomnia may disrupt the circadian clock system, though the extent of these effects remains unclear ^{15–19}. Previous studies have proposed the analysis of clock gene expression profiles in human peripheral blood mononuclear cells (PBMCs) as a promising approach for assessing circadian rhythm ^{20–23}. Indeed, our prior study has shown through machine learning analysis, that patients with obstructive sleep apnea (OSA), another highly prevalent sleep disorder, can be distinguished from healthy controls based on altered expression patterns of several clock genes ²⁴, suggesting that clock gene expression may serve as potential biomarker for sleep disorders. In recent years, machine learning approaches have increasingly been utilized to identify biomarkers for different sleep disorders and to monitor treatment responses ^{24,25}.

Building on this concept, we investigated the impact of chronic insomnia on the biological clock by analyzing key physiological markers, including plasma cortisol, body temperature, and clock gene expression in PBMCs. We also characterized sleep using PSG, assessed actigraphy-derived circadian parameters, and machine learning-based analysis for identifying biomarkers that enable robust differentiation of insomnia patients from controls and between subtypes.

Our findings reveal significant circadian disruptions and altered clock gene expression in chronic insomnia, particularly in ISSD, underscoring the potential of circadian biomarkers for improved diagnosis and treatment. Machine learning analysis enabled robust differentiation between insomnia patients and controls, as well as between ISSD and INSD subtypes, based on clock gene expression. Overall, this work advances our understanding of insomnia's circadian disruption and highlights the utility of integrating circadian biomarkers with interpretable machine learning techniques to improve insomnia diagnosis, optimize treatment strategies based on subtype, and reduce the societal burden of insomnia, including work absenteeism and healthcare costs.

Methods

Ethical Approval

This study received approval from the Ethical Committees of the Faculty of Medicine, University of Coimbra (CE-162/2021) and the Coimbra Hospital and University Centre (ULSC, OBS.SF.55-2021) in Coimbra, Portugal. All experimental procedures adhered to the ethical guidelines and regulations established in the 1964 Declaration of Helsinki and its subsequent amendments. The study also complied with the provisions of Regulation (EU) 2016/679 of the European Parliament and Council, governing the General Data Protection Regulation (GDPR), as well as Portuguese Law n.º 12/2005 of

January 26 and its implementing regulations detailed in Decree-Law n.º 131/2014 of August 29, 2014. Informed consent was obtained from all participants prior to their inclusion in the study.

Study design

Female and male adult volunteers (age ≥ 18 years) suspected of having chronic insomnia were recruited for a sleep study at the Sleep Medicine Unit of CHUC, Coimbra, Portugal, between January 2022 and September 2023. All selected subjects underwent a detailed screening process, which included a clinical interview conducted by an experienced psychiatrist, validated questionnaires and continuous wrist actigraphy monitoring over two-weeks (ActTrust® 2, Condor Instruments Ltda, SP, Brazil).

Participants identified as potential chronic insomnia cases were asked to undergo a single-night, home-based polysomnography (PSG) study (type II) to determine sleep duration subtype and were categorized into two groups: insomnia patients with short sleep duration (ISSD) (TST < 6 hours) and insomnia patients with normal sleep duration (INSD) (TST ≥ 6 hours) ⁷. PSG was also used to rule out other sleep disorders. Sleep studies were staged and scored according to the American Academy of Sleep Medicine (AASM) scoring manual v2.4, 2012.

Healthy volunteers were invited to participate in a one-night, home-based sleep study using the WatchPAT™ 300 device (Itmar Medical, Gasoxmed, Portugal) to record their respiratory events and sleep parameters. Control subjects of similar age (± 5 years) and including both sexes, were classified as disease-free if met the following criteria: no lifetime history of significant insomnia-associated symptoms, TST ≥ 6 hours, absence of medical or psychiatric comorbidities that could interfere with sleep (e.g. major depressive disorder) verified through clinical history and a structured clinical interview using the SCID-IV; no history of other sleep disorders as confirmed by clinical evaluation and screening PSG; no engagement in shiftwork within the preceding 6 months; no current use of prescription medications or over-the-counter products that could affect sleep; not being pregnant or lactating within the past 6 months.

Data from the PSG test (PSG report) and WatchPAT™ 300 (report) were obtained from each participant. An ID was assigned to each subject to ensure data confidentiality and traceability.

On the morning following PSG or WatchPAT™ 300 examination, participants completed the following validated questionnaires to assess various sleep and mental health parameters: Insomnia Severity Index (ISI) ²⁶, Epworth sleepiness scale ²⁷, STOP-BANG ²⁸ and Hospital Anxiety and Depression Scale (HADS) ²⁹.

During this period, individual data was collected from all participants, including sex, age, body mass index (BMI), lifestyle (sleep/wake routine, diet, meal schedules, physical exercise), and clinical history (comorbidities and medication).

Blood samples were collected from all subjects following their sleep studies during hospital visits. Samples were collected at four different times: in the morning (8:00 and 11:00 h), afternoon (15:30 h) and night (21:30 h). Blood was collected for the analysis of cortisol plasma levels, and clock genes expression in PBMCs. Axillary temperature was monitored at the same four time points. Between sampling sessions, participants were allowed to leave the Sleep Unit to continue their usual daily activities, ensuring minimal disruption to their routine. The study design is summarized in Figure 1.

Measuring rest:activity and skin temperature rhythms

The structure and timing of daily rest:activity patterns were assessed using wrist-worn ActTrust 2 actigraphs (Condor Instruments, SP, Brazil). Participants wore the devices continuously on their non-dominant wrist for 14 consecutive days under normal ambulatory conditions. During this period, participants were instructed to follow their usual routines and record their sleep patterns in a sleep diary. They were advised to remove the actigraphs during activities involving water (e.g. bathing, dishwashing or swimming). The actigraphs were configured with a sampling frequency of 25 Hz to optimize battery life over the recording period. The devices continuously recorded motor activity (using the Proportional Integral Mode, PIM), skin temperature (°C), and light exposure (lux) in one-minute epochs. PIM measures movement intensity by calculating the area under the signal curve for each epoch³⁰. The raw actigraphy data was processed using the ActStudio software version 2.1.2 (Condor Instruments, SP, Brazil), which automatically detected sleep-wake cycles, off-wrist periods, sleep parameters, parametric (COSINOR) and non-parametric circadian rhythm analysis (NPCRA) calculations, and export of the study graphs. Off-wrist periods were automatically excluded from the analysis by the software.

Sleep parameters included: sleep efficiency (the percentage of time spent asleep while in bed), total sleep time (the cumulative amount of sleep), number of awakenings and wakefulness after sleep onset (WASO). COSINOR analysis included measures of amplitude (the difference between the peak and the mean value of the cosine function), midline estimating statistic of rhythm (MESOR, mean value of a rhythmic variable over a 24-hour period) and acrophase (timing of the peak activity in a circadian rhythm). NPCRA was conducted to assess the circadian structure of rest:activity rhythms, including intradaily variability (IV), interdaily stability (IS), and relative amplitude (RA). The analysis also examined average activity levels during the least active 5-hour period (L5) and the most active 10-hour period (M10). IV reflects rest:activity fragmentation, with higher values denoting greater consistency. IS measures rhythm stability across days, with higher values denoting greater consistency. RA quantifies rhythm strength by comparing activity levels between M10 and L5. Within-subject variability in L5, M10 and RA was assessed using the standard deviation of daily measures.

For generating the actigraphy plots for wrist temperature, PIM, TAT, and ZCM, actigraphy data was grouped by the mean per minute, for each participant and displayed on a 24-hour horizontal axis. Data transformations were performed using the Numpy and Pandas Python packages, within the Jupyter environment. The visualizations were generated using the Vega-Lite visualization grammar, with the

Altair Python binding. The layered chart has two components: an area chart for the SEM bounds and a line chart for the mean values of the data.

Axillary temperature

Axillary temperature was measured twice at each of the four time points within 24 h (8:00, 11:00, 15:30 and 21:30 h) in both chronic insomnia patients and control subjects, following the sleep unit routine, through an electronic thermometer. The average between the two temperature measurements was used.

Blood samples collection and processing

Blood samples (15–20 mL) were collected from chronic insomnia patients and control subjects at four time points within 24 hours (8:00, 11:00, 15:30, and 21:30 h) via the antecubital vein using a 21-gauge needle into K2EDTA tubes (#367839, BD Vacutainer) and subsequently processed as previously described²⁴. Plasma and PBMCs aliquots were stored at - 80 °C until further analysis.

Cortisol plasma levels assessment

Plasma cortisol levels were quantified using human-specific commercial ELISA assays (EH0641 respectively, Fine Test), according to the manufacturer's protocol. Absorbance was measured at 450 nm using a SpectraMax Plus 384 Microplate Reader (Molecular Devices). The concentrations were then calculated by interpolating the results from standard curves generated by plotting the concentration of the standards provided by each kit against their absorbance using a sigmoidal 4-parameter logistic (4-PL) curve, with concentration expressed logarithmically.

Clock gene expression analysis

Total RNA was extracted from PBMCs using the miRCURY RNA Isolation kit – Cell and Plant (#300,110, Exiqon) according to manufacturer's protocol, with DNase digestion to prevent genomic DNA contamination. Total RNA was quantified by optical density (OD) using the ND-1000 Nanodrop Spectrophotometer (Thermo Scientific), and purity was assessed by the OD ratio at 260 and 280 nm. RNA samples were converted into cDNA, using the iScript cDNA Synthesis Kit (Bio-Rad), according to manufacturer's instructions and as previously described²⁴.

The mRNA levels of *BMAL1*, *CLOCK*, *PER1–3*, *CRY1–2*, *REV-ERBa*, and *REV-ERBβ* of all the enrolled subjects were assessed by real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), using the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad). Primer sequences and qPCR conditions are listed in Table S1. Relative gene expression was calculated according to the $\Delta\Delta C_T$ method³¹. Each assay included a non-template control (NTC), a no reverse transcription (NRT) control, and a standard curve for each target gene that was used in all plates to normalize for interplate variability.

Hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and β -2-microglobulin (β -2 *M*) were used as housekeeping genes, as validated previously²⁴. Bio-Rad CFX Maestro software (Bio-Rad) was used to automatically determine amplification efficiencies and threshold cycles (CT). mRNA expression data is presented as $\Delta\Delta CT$ values, relative to the average of the ΔCT of all time points of all enrolled subjects, except for machine learning analysis, where the ΔCT values were used.

Statistical analysis

If not stated otherwise, statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, Inc). Data distribution was assessed using the Shapiro–Wilk test and the quartile–quartile plot (QQ-plot) was visually inspected. Based on data distribution, unpaired t-tests were performed to detect statistically significant differences between control and chronic insomnia group, at each time of the day. For correlation dot plot, pairwise Spearman correlation coefficients were calculated using Origin 2025 (OriginLab, New York, USA). All statistical tests performed were two-sided, with statistical significance set at 0.05. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. No values were excluded. Specific statistical details are described in the figure legends.

Rhythmicity analysis

For the detection of circadian oscillating genes, harmonic regression fitting was used as implemented in the **geoTS** R package using the **haRmonics** function³². The dataset was categorized into three groups: (1) controls, (2) ISSD and (3) INSD. Missing values were imputed using the respective group's mean $\Delta\Delta CT$ values normalized to the average of all participants. We then computed the mean gene expression $\Delta\Delta CT$ values normalized to the average of all participants values across four time points (8:00, 11:00, 15:30, and 21:30 hours) for each group and each gene. The summary plot using data from each participant of the study is depicted using the standard error of mean and the figures plotted in R programming software using ggplot2 package (v.3.3.2). A smooth harmonic regression curve was used for connecting the summary plot.

For each gene and each group, we fitted the harmonic regression model $y(t) = m + a \times \sin(2 \times \pi \times t/\omega) + b \times \cos(2 \times \pi \times t/\omega)$, with an ω being a period of 24 hours. The model parameters (m , a , b) were estimated using **haRmonics** function. The amplitude A and acrophase φ were derived using: $A = \sqrt{a^2 + b^2}$, $\varphi = \arctan(b, a)$. To visualize the fitted curves, a second-degree polynomial model of the form: $y(x) = b_0 + b_1x + b_2x^2$ was fitted to the harmonic regression outputs at the four sampling time points. We then computed the associated p-values via the F-test, which was obtained using the F-statistic components provided by the summary function in R. Error bars (standard errors) were obtained by dividing the sample standard deviation by the number of participants in each group.

For each gene, we generated separate plots illustrating the group-wise mean gene-expression values $\Delta\Delta\text{CT}$ at each sampling time, with error bars and the corresponding smoothed polynomial curves for each group.

Machine learning

To develop and validate a predictive model for classifying insomnia status based on clock gene expression, we used gene expression data from PBMCs collected at multiple time points throughout the day (8:00, 11:00, 15:30, and 21:30 h). For each participant, we derived a single feature vector by averaging gene expression levels across all time points for each gene.

We implemented a Support Vector Machine (SVM) using the Scikit-learn library in Python. In each training iteration, we selected a subset of the data containing three genes, representing each participant as a 3D data point with a feature vector of mean expression values for the selected genes. To identify the most predictive genes, we conducted multiple training iterations, testing all possible three-gene combinations from a set of nine genes.

The dataset was split into training, validation, and test sets. Hyperparameter tuning was performed using the training and validation sets, varying parameters such as kernel type and regularization through grid search to optimize performance. The ten models achieving the highest validation accuracy were selected.

Finally, we evaluated generalization performance by testing the best-performing SVM configuration on the test set. This approach ensured unbiased performance estimates on unseen data.

Results

Cohort profile: demographic and clinical characteristics

During recruitment, 30 potential insomnia patients and 15 potential controls completed the baseline visit. Of these, 14 patients with insomnia and 13 control subjects met the eligibility criteria and were included in the study (Figure 1A and B). The demographic and clinical characteristics are summarized in Table 1. The insomnia group comprised 1 male and 13 females, while the control group included 4 males and 9 females. All participants were Caucasian. There were no statistically significant differences between the groups in terms of age, BMI, STOP-BANG scores, or daytime sleepiness (Table 1). The ISI score was significantly higher in the insomnia group compared to controls. Similarly, Hospital Anxiety and Depression Scale (HADS) scores were significantly elevated in the insomnia group, relative to controls. It should be noted, none of the participants met the clinical criteria for anxiety or depression, as those with diagnosed psychiatric conditions were excluded. Insomnia patients also had higher rates of comorbid conditions, including hypertension, cardiovascular diseases, metabolic disorders (such as

diabetes and dyslipidemia), and gastrointestinal conditions. Details of medication use are provided in Table 1.

Accurate assessment of sleep duration and quality is essential for understanding insomnia, which is characterized by subjective complaints of poor sleep despite variations in objective measurements. While PSG remains the gold standard for evaluating sleep architecture and diagnosing sleep disorders, actigraphy provides a non-invasive, long-term alternative that also captures circadian parameters³³. However, the degree to which these methods detect sleep disturbances in chronic insomnia remains a key question.

This study aimed to characterize sleep parameters and stratify patients by objective sleep duration based on a single-night PSG and to determine if patients with chronic insomnia exhibited circadian parameter alterations by actigraphy (Figure 1B).

Based on TST duration, 8 patients were classified as ISSD, and 6 were categorized with INSD (Figure 1A and Table 2). PSG analysis shows that patients with chronic insomnia and the ISSD subgroup, but not the INSD, show significantly reduced TST compared to controls. Sleep latency was significantly prolonged in the ISSD subgroup compared to controls, while sleep efficiency was significantly reduced in the insomnia group and the ISSD subgroup compared to controls. Additionally, REM percentage (%) was significantly reduced and the number of awakenings was markedly higher in all insomnia groups compared to controls. Importantly, no significant differences were identified in apnea-hypopnea index (AHI) values across groups, confirming that sleep-disordered breathing was not a confounding factor in our sample (Table 2).

Our findings confirm that ISSD patients experience the most profound sleep disturbances, including significantly reduced TST, prolonged sleep latency, and lower sleep efficiency, while INSD patients show milder impairments.

Lower body temperatures suggest circadian dysfunction in patients with chronic insomnia

To further investigate sleep-wake patterns and circadian rhythm stability, we examined actograms, which provide a visual representation of rest-activity cycles over multiple days³³. These actograms allowed us to assess differences in sleep organization between groups, offering complementary insights to the PSG and actigraphy-derived sleep parameters. Representative actograms revealed marked differences between control participants and patients with insomnia. As expected, control group actograms showed well-organized entrainment to the day/night cycle, with stable phase and amplitude across days, aside from slight weekend phase shifts. In contrast, actograms from ISSD and INSD patients demonstrated increased sleep-onset latency, disrupted sleep maintenance and reduced sleep duration on most nights. Both controls and insomnia patients (ISSD and INSD) exhibited slightly increased sleep periods during weekends (Figure 2A).

Quantitative analysis of actigraphy-derived rest-activity rhythms further supported these findings. Insomnia patients demonstrated higher overall activity levels, as indicated by increased average daily activity (PIM) graphs (Figure 2B and 2C), time-above-threshold (TAT) and Zero Crossing Method (ZCM) values (Supplementary Figure S1A and B), suggesting prolonged wakefulness of chronic insomnia patients.

We next assessed circadian rhythm parameters, including parametric and non-parametric measures, wrist temperature measurements. However, most parametric and non-parametric measures, including M10, L5, RA, IS, circadian function index (CFI), MESOR, and amplitude did not significantly differ between groups. The only significant circadian rhythm alteration was lower intradaily variability in insomnia patients (Supplementary Figure S1 C-J). Skin temperature rhythms were shown to be disrupted in chronic insomnia, with patients exhibiting lower wrist temperatures compared to controls (Figure 2D), which could be indicative of circadian dysfunction. Despite these minimal differences in circadian rhythm robustness, a strong negative correlation was observed between CFI, IS and ISI scores (Figure 3 E), indicating that worse insomnia symptoms were associated with weaker circadian function.

Given the reduced wrist temperature observed in patients with chronic insomnia and the negative correlation observed between actigraphy-derived circadian measures (CFI and IS) and ISI scores, we hypothesized that chronic insomnia might be associated with circadian disruption in biological outputs. To further investigate this, we also assessed the impact of insomnia on the biological clock by measuring axillary temperature and plasma levels of cortisol at four time points across the day (08:00, 11:00, 15:30, and 21:30 h) in both insomnia patients (ISSD and INSD) and control subjects (Figure 3).

Significant differences in axillary temperature were identified between insomnia patients and controls. The insomnia group exhibited significantly lower axillary temperatures during the morning time points (08:00 and 11:00 h). Notably, the INSD subgroup mirrored this pattern, while the ISSD subgroup demonstrated significantly lower axillary temperature only at 08:00 h (Figure 3A-C). These diurnal differences in axillary temperature were consistent with wrist temperature data obtained via actigraphy (Figure 2D).

We also observed that cortisol levels had distinct circadian pattern of dysregulation in insomnia patients. The overall insomnia group showed significantly elevated cortisol levels at night (21:30 h). Further subgroup analysis indicated that the ISSD subgroup displayed heightened cortisol levels at both 15:30 and 21:30 h, while no significant cortisol alterations were observed in the INSD subgroup (Figure 3A-C). These results show that chronic insomnia patients, specially the ISSD group, show cortisol dysregulation.

These data suggest modest circadian dysfunction through actigraphy, as reflected by largely unchanged parametric and non-parametric measures and a significant correlation between circadian metrics and insomnia severity. Additionally, lower wrist and axillary temperatures, accompanied by elevated nighttime cortisol in insomnia patients suggest further circadian misalignment, warranting further investigation.

Chronic Insomnia, in particular ISSD, promotes alterations in expression levels and the temporal expression profiles of several clock genes

Given the overall impact of chronic insomnia on temperature and cortisol rhythms, we aimed to investigate whether clock genes were affected at the transcriptional level in peripheral blood samples, given their importance in circadian rhythm regulation. To this end, we analyzed the expression levels of nine core-clock genes (*BMAL1*, *CLOCK*, *PER1-3*, *CRY1-2*, *REV-ERBa*, and *REV-ERBβ*) at the four time points of the study (8:00, 11:00, 15:30 and 21:30 h) in PBMCs of patients with chronic insomnia and its subgroups, compared to control subjects.

Patients with insomnia showed higher expression levels of *PER1* (8:00 and 15:30 h), *PER2* (8:00 and 15:30 h), *REV-ERBa* (11:00 h), and *REV-ERBβ* (15:30 h), relative to control subjects. No significant changes were detected in the expression levels of *BMAL*, *CLOCK*, *PER1*, *CRY1* and *CRY2* in PBMCs of insomnia patients (Figure 4A). Subgroup analysis revealed that the ISSD subgroup exhibited similar alterations in clock gene expression to the total chronic insomnia group. By contrast, the INSD subgroup showed fewer differences, when compared with the insomnia group, with significantly higher expression levels of *BMAL*, *PER1*, and *CRY2* only at the morning timepoint (08:00 h) (Figure 4B and C).

To better characterize the clock genes expression along the day and part of the night, we then analyzed the alignment of the datasets with a 24-hour rhythmic oscillation using harmonic regression. Specifically, we applied a harmonic regression model with a fixed 24-hour period to the average data from all patients with insomnia, as well as to its subgroups (Supplementary Figure S2 A-C). The results indicate that *PER1* shows a marginally significant result ($p = 0.048$) in the INSD group, suggesting possible differences in circadian rhythms compared to the control group (Supplementary Table S2). Although trends in amplitude and acrophase are apparent, particularly the generally higher amplitudes in insomnia-related groups, the p -values indicated that these differences, except for *PER1* in the INSD group, are not statistically significant. Using this methodology of analysis, the results seem to suggest that circadian rhythms of clock genes are relatively stable across the groups in this dataset.

Explorative correlation analysis shows significant correlations between insomnia severity and circadian markers

To further investigate the relationship between insomnia severity and circadian regulation, we conducted an exploratory correlation analysis to assess the associations between insomnia sleep-related parameters (derived from PSG), actigraphy-derived circadian metrics, and biological circadian markers.

Interestingly, age emerged as a significant factor, positively correlating with the expression levels of *BMAL*, *CLOCK*, *PER1*, *CRY1*, and *REV-ERBa* in patients with insomnia and its subgroups, suggesting that clock gene expression may change with aging in individuals with chronic insomnia (Figure 5A).

Our analysis identified strong negative correlations between ISI and expression levels of *BMAL*, *CLOCK*, *PER2*, *CRY1-2*, and cortisol. These correlations were even more pronounced when examining the two

insomnia subgroups, ISSD and INSD, separately. Additionally, ISI showed significant positive correlations with axillary temperature and *PER1* expression, further supporting the involvement of circadian dysfunction in insomnia pathology.

TST was significantly negatively correlated with nighttime cortisol levels across all insomnia groups, reinforcing the well-established link between short sleep duration and increased hyperarousal. Moreover, modest associations were also observed between TST and *PER2* and *REV-ERB β* expression, indicating potential links between sleep duration and molecular circadian rhythms (Figure 5 A-C).

Among the actigraphy-derived circadian parameters, the strongest associations were observed between CFI and the expression levels of *CLOCK*, *PER1*, *PER2*, *CRY1*, and *CRY2* in the insomnia group. These findings suggest that molecular clock disruptions could be reflected in circadian activity patterns, reinforcing the role of intrinsic circadian misalignment in insomnia.

Subgroup analysis further highlighted distinct associations in INSD patients. Specifically, axillary temperature was negatively correlated with age, ISI, relative amplitude (RA), and CFI. This suggests that reduced circadian stability (as reflected by lower CFI and RA values) is linked to both insomnia severity and impaired thermoregulation, particularly in INSD patients. Interestingly, the negative correlation between axillary temperature and CFI further supports the role of altered thermoregulation in insomnia-related circadian disruption (Figure 5C).

Overall, these findings highlight the complex interplay between insomnia severity, circadian markers, and molecular clock regulation. The observed associations suggest that chronic insomnia, particularly its subtypes, is characterized by disruptions in both behavioral and molecular circadian rhythms.

Supervised machine learning predicts insomnia disorder using clock gene expression data

To determine whether the observed differences in clock gene expression could distinguish patients from controls and between insomnia subtypes, we applied a supervised machine learning using the clock gene expression dataset. We trained a linear support vector machine (SVM) with leave-one-out cross-validation, using mean gene expression levels as input features to classify samples as "control" or "patient". In each iteration, the model was trained on all subjects except one, whose health status (control or patient) was then predicted. This approach achieved robust classification performance, correctly identifying 13 insomnia subjects with an accuracy of 92%, along with 92% precision and 92% recall (Figure 6A and Supplementary Table S3).

Using the same SVM approach, we classified insomnia subgroups, achieving an overall accuracy of 92%, with 86% precision and 100% recall (Figure 6B and Supplementary Table S4).

These findings highlight a significant distinction between control and patient groups, identified solely from mean core-clock gene expression levels. Additionally, the model's ability to differentiate chronic insomnia subtypes underscores its potential for detecting nuanced molecular differences.

Discussion

Chronic insomnia is a complex disorder characterized by physiological and molecular alterations. Understanding its underlying mechanisms and identifying novel biomarkers is essential for improving diagnostic strategies and moving toward more objective approaches. Our findings support growing evidence that chronic insomnia disrupts circadian rhythms, with ISSD patients exhibiting the most pronounced impairments, including sleep disturbances, altered body temperature rhythms, elevated nighttime cortisol, and significant changes in clock gene expression. Additionally, machine learning identified key genes that may serve as biomarkers for diagnosing insomnia and distinguishing between subtypes.

Consistent with previous findings⁷, ISSD patients demonstrated the most profound sleep disturbances, including significantly reduced TST, prolonged sleep latency, and lower sleep efficiency, whereas INSD patients exhibited milder impairments. A key limitation of standard PSG is the reliance on single-night recordings, which may not fully capture the severity of insomnia due to night-to-night variability³⁴. Additionally, discrepancies between subjective and objective sleep assessments, often termed paradoxical insomnia or sleep state misperception, remain a challenge, as PSG often reveals less severe sleep disturbances than reported by patients^{34,35}. These limitations highlight the need for complementary biomarkers to improve diagnostic accuracy.

Increased nocturnal activity, as reflected by elevated PIM, TAT, and ZCM values, suggests that chronic insomnia patients experience more frequent or prolonged nighttime movements, potentially linked to physiological hyperarousal³⁶. Although actigraphy is increasingly recognized as a valuable tool for evaluating circadian rhythm integrity^{37,38}, our actigraphy-derived measures did not reveal significant differences in circadian robustness among chronic insomnia patients. However, a strong negative correlation between ISI scores and circadian robustness (IS and CFI) suggests that greater insomnia severity is associated with weaker circadian function.

We observed alterations in body temperature rhythms, with chronic insomnia patients with insomnia patients exhibiting attenuated amplitude and lower absolute values of wrist and axillary temperature. Given that skin temperature serves as both a marker and modulator of circadian rhythms^{39,40}, these findings suggest possible circadian misalignment. Prior studies have linked skin temperature dysregulation to insomnia symptoms, including difficulties with sleep onset and maintenance^{16 41}. While some research suggests impaired thermoregulation as a contributing factor in certain insomnia subtypes, findings remain inconsistent, warranting further investigation^{18,42}.

Elevated nighttime cortisol, a well-documented hallmark of hypothalamic-pituitary-adrenal (HPA) axis dysregulation^{43–46}, commonly observed in insomnia. In our study, ISSD patients exhibited significantly higher nocturnal cortisol levels compared to controls, but this effect was not observed in INSD patients, reinforcing the notion that hyperarousal is more pronounced in ISSD⁷. Prior research has reported positive correlations between nighttime cortisol and insomnia severity (ISI scores)⁴⁷, further supporting

HPA axis dysregulation in insomnia. However, cortisol levels may not always be significantly elevated in insomnia^{48,49}, which can be partly explained by the heterogeneity of insomnia subtypes and potential confounding factors such as medication use, highlighting the need for further research into the differential pathophysiology of ISSD and INSD.

Our study reveals significant alterations in clock gene expression in chronic insomnia patients, particularly in ISSD. *PER1* and *PER2* expression peaked during wakefulness and light exposure, while *BMAL1*, *REV-ERBa*, and *REV-ERBβ* showed modest but significant diurnal expression variations. These findings suggest that insomnia-associated circadian disruptions extend beyond behavioral and hormonal dysregulation to the molecular level.

Previous studies have linked polymorphisms in clock genes, particularly in *CLOCK* and *PER3*, to insomnia risk^{50–53}. However, research on clock gene expression patterns in chronic insomnia patients is limited. Prior work has demonstrated that acute sleep deprivation can disrupt *PER2* and *BMAL1* rhythms, indicating that sleep loss influences clock gene dynamics⁵⁴. Additionally, studies in sleep apnea patients have reported reversible alterations in clock gene expression following continuous positive airway pressure (CPAP) treatment, further supporting the role of clock gene expression as a potential biomarker for sleep disorders²⁴.

Our findings align with these observations, as *PER1* and *PER2* expression was significantly affected in insomnia patients, particularly in ISSD. This reinforces their potential as sensitive markers of sleep deprivation and circadian misalignment. However, it remains unclear whether these molecular disruptions contribute to insomnia onset or result from chronic sleep disturbances. Longitudinal studies are needed to clarify causality and further explore the molecular mechanisms underlying insomnia subtypes.

Our findings highlight the intricate relationship between chronic insomnia and circadian dysregulation across molecular, physiological, and behavioral levels. Further supporting this, we show that ISI is negatively correlated with *BMAL*, *CLOCK*, *PER2*, *CRY1-2*, and cortisol, with more pronounced disruptions in ISSD and INSD subgroups, reinforcing the role of molecular circadian alterations in insomnia pathology. Additionally, ISI positively correlated with axillary temperature and *PER1* expression, linking sleep disturbances to thermoregulatory and circadian disruptions.

Actigraphy-based circadian parameters also reflected these disruptions, with CFI correlating strongly with *CLOCK*, *PER1*, *PER2*, *CRY1*, and *CRY2* expression. INSD patients exhibited distinct alterations, including negative correlations between axillary temperature and ISI, RA, and CFI, suggesting impaired thermoregulation and weakened circadian stability. The association between TST and nighttime cortisol, as well as *PER2* and *REV-ERBβ* further supports a link between hyperarousal, sleep duration, and molecular rhythms. Additionally, age correlated positively with *BMAL*, *CLOCK*, *PER1*, *CRY1*, and *REV-ERBa* expression, suggesting that aging may exacerbate circadian misalignment in insomnia.

Currently, chronic insomnia disorder diagnosis relies primarily on self-reported symptoms, with objective assessments used only in select cases⁹. Our study suggests that blood-based biomarkers analysis, combined with machine-learning, may provide a cost-effective alternative for identifying chronic insomnia cases and distinguishing between subtypes. Supervised machine-learning of clock gene expression profiles successfully differentiated chronic insomnia patients from control and further distinguished ISSD from INSD patients. Machine learning algorithms identified *PER2*, *CRY1* and *BMAL* as key classifiers to distinguishing insomnia patients from controls and *CLOCK*, *BMAL* and *PER2* for differentiating between insomnia subtypes. These findings highlight the potential of clock genes as a diagnostic biomarker and a tool for patient stratification.

In summary, our study provides further evidence that chronic insomnia, particularly ISSD, is associated with circadian dysfunction at behavioral, hormonal, and molecular levels. Altered clock gene expression, disrupted body temperature rhythms, and elevated nighttime cortisol underscore the role of circadian dysregulation in insomnia pathology. While actigraphy showed limited sensitivity in detecting circadian alterations, skin temperature changes and clock gene expression patterns emerged as promising markers of circadian dysfunction in insomnia. Moreover, while other studies have applied machine learning to assess circadian function^{24,55}, our study highlights the potential of machine learning approaches based on clock gene expression for identifying chronic insomnia cases and stratifying patients by subtype. Future research should validate these findings in larger cohorts, investigate causal mechanisms, and develop target interventions, particularly for ISSD patients, who exhibit more severe circadian disruptions. Recognizing ISSD and subtyping insomnia is important for personalized therapeutic approaches and should be integrated into clinical practice for more effective management.

Study Limitations

Although our sample size was relatively small, the robustness of our findings support the application of machine learning methodologies. The rigorous eligibility criteria and the stringent participant selection process help mitigate concerns related to sample size limitations. However, we did not assess the potential impact of individual clinical histories on clock gene expression. Nevertheless, the absence of predominant comorbidities within any study group suggests that this may not represent a significant confounder. Additionally, intrinsic inter-individual variability must be considered, as it may influence molecular and cellular responses to disease and contribute to the observed heterogeneity in outcomes. Despite this, the randomized and counterbalanced number of conditions across participants reduces the potential for systematic bias.

We did not specifically analyze the seasonal effects on clock gene expression, as patient recruitment occurred year-round. However, a previous study by our group with a similar design found no significant seasonal influence²⁴, suggesting minimal impact. Another consideration is the use of at-home sleep tests in healthy subjects rather than a type II PSG, which was impractical for routine clinical assessments due to cost, time demands, labor-intensive procedures, and long waiting lists¹³. To overcome these issues, we used the WatchPAT, a widely adopted sleep-screening tool, for the healthy

subject cohort ^{56,57}. However, it is important to note that the use of objective sleep measures remain optional in insomnia diagnosis ⁹.

Our study establishes a foundational framework for understanding the role of clock outputs and clock gene expression in chronic insomnia and its subtypes. However, given the complexity of clock-regulated signaling pathways, further research is needed to elucidate how circadian clocks interact with neuronal circuits and systemic physiology to better understand the pathophysiology of chronic insomnia.

Declarations

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Author contributions

CCA, CC and ARA conceived the study plan and design; CC and ARA supervised the work; CCA conducted the study and performed the sample processing, experiments for clock outputs evaluation and gene expression analysis, and PSG and actigraphy-derived data analysis; BS assisted in experiments, LSG and AS assisted in analysis and discussion, RR assisted with actigraphy evaluation; JM and JS recruited the patients; JS evaluated patients and all the necessary clinical data; MF gathered all clinical data from all patients; JA and TG were responsible for the design of the computational analysis. JA carried out the bioinformatics rhythmicity analysis in gene expression datasets and performed machine learning-based analysis with the obtained study datasets; TD analyzed the raw actigraphy data. CCA wrote the first draft of the manuscript. All co-authors reviewed the manuscript and approved the final version to be submitted.

Competing interests The authors declare no competing interests.

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Tables

Tables are available in the Supplementary Files section.

Figures

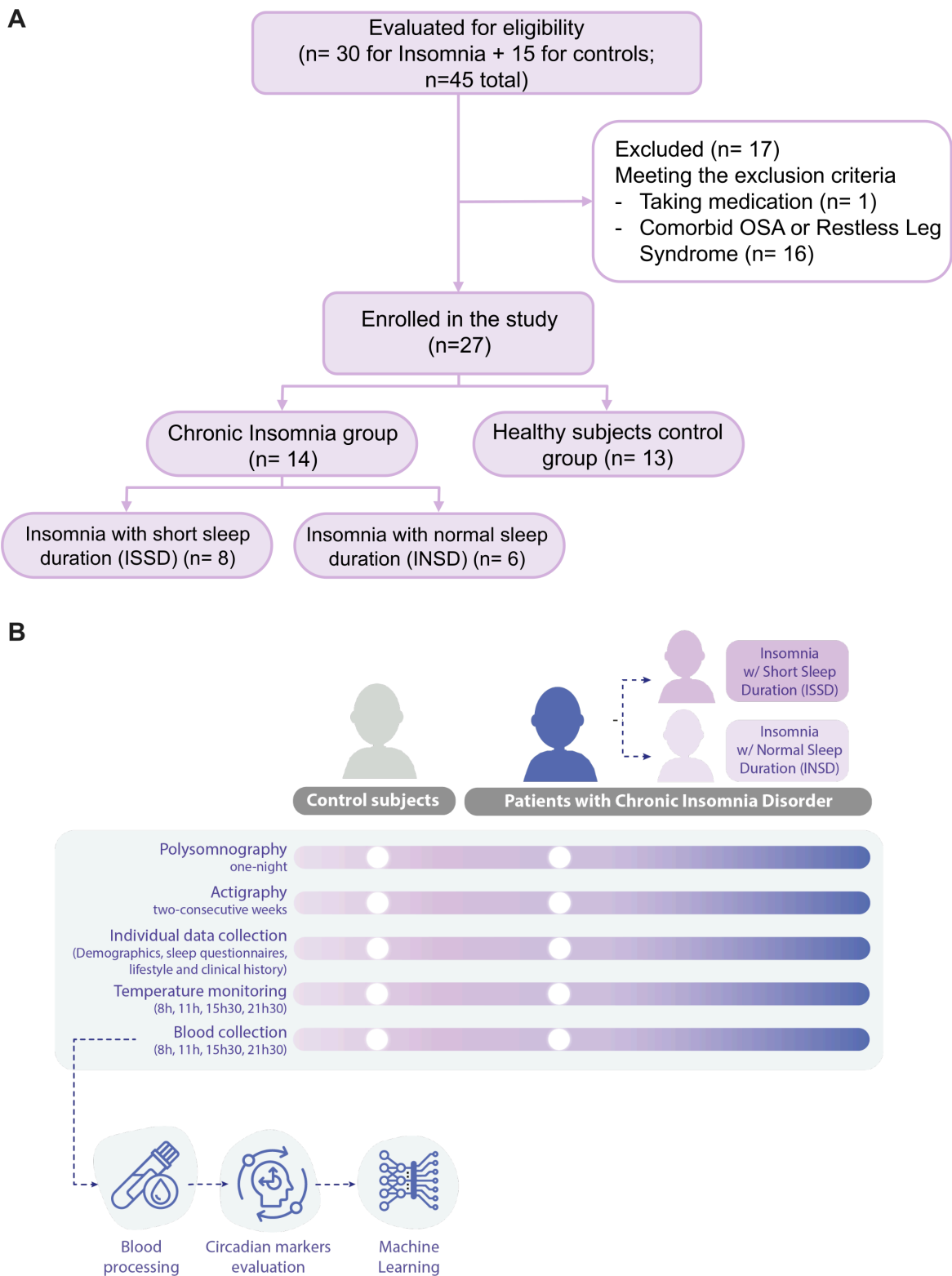


Figure 1

Study flow diagram and schematic of the study design. (A) The study flow diagram outlines subject selection and eligibility assessment. Of the 45 patients initially evaluated, 13 healthy control subjects and 14 patients with chronic insomnia disorder met the inclusion criteria. The chronic insomnia disorder group was further divided into two subgroups based on total sleep duration: 8 patients with insomnia with short sleep duration (ISSD) and 6 patients with insomnia with normal sleep duration (INSD). (B) Study design. All participants underwent a one-night polysomnography (PSG) and completed two weeks of actigraphy monitoring before PSG evaluation. Data collection included demographic and anthropometric measures (age, BMI), lifestyle and clinical history information, and sleep-related questionnaires. Additionally, body temperature was monitored, and blood samples were collected at four time points (8:00, 11:00, 15:30, and 21:30 h) for circadian marker analysis. Machine learning algorithms were applied to cluster groups based on clock gene expression profiles.

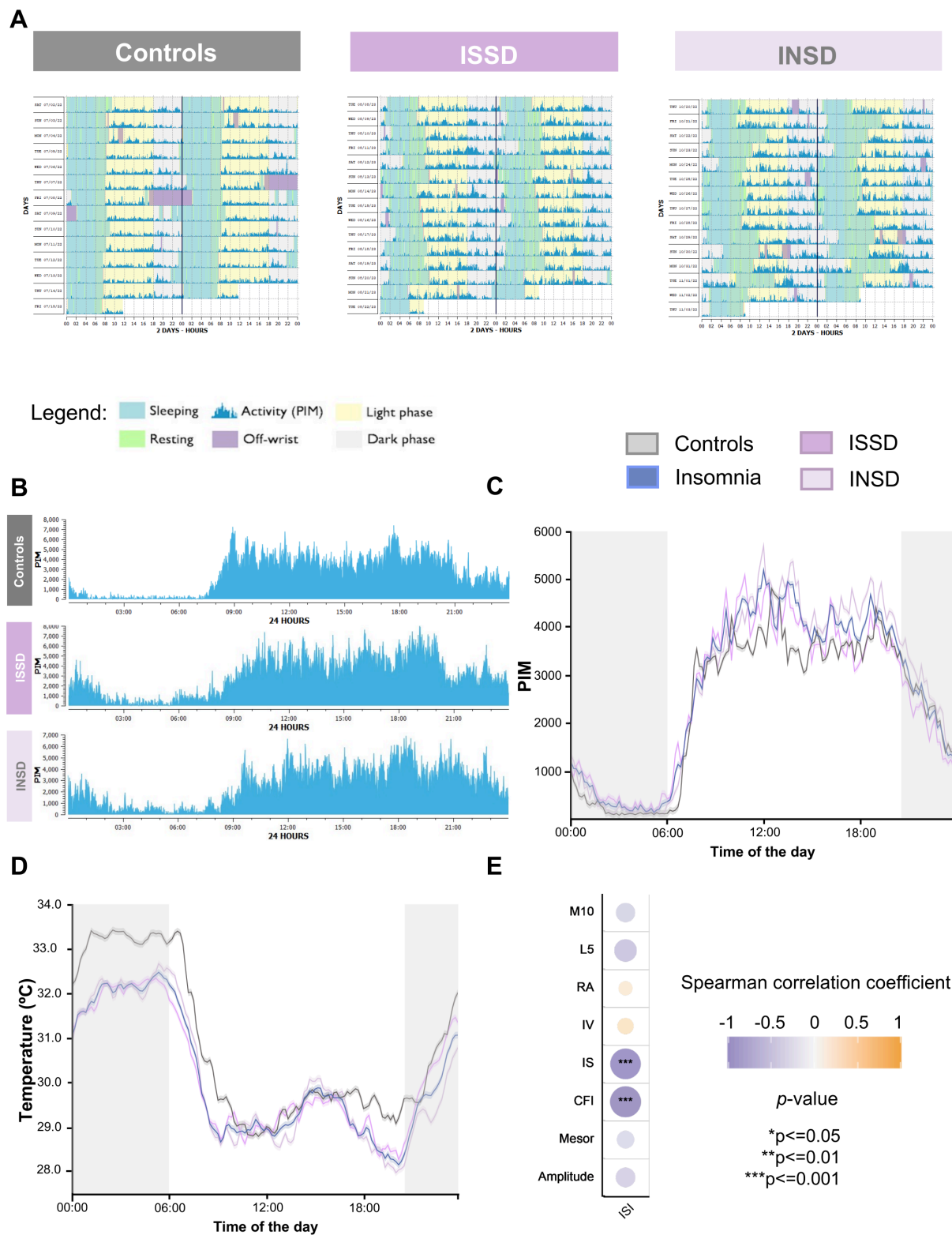


Figure 2

Rest:activity patterns, daily activity and temperature profiles assessed by actigraphy in healthy control subjects and patients with chronic Insomnia, stratified by short (ISSD) and normal (INSD) sleep duration.(A) Representative actograms illustrate rest:activity patterns over two weeks in control, ISSD and INSD groups. Double-plotted 24-h actograms generated from participants' rest:activity patterns reflect the characteristic differences detected between study groups with blue peaks representing

activity (PIM), light blue sleeping states, light green resting states, light yellow for light phases, light grey for dark phases, and purple for off-wrist periods. (B) Representative 24-hour actograms of the PIM activity for a healthy control subject, a patient with ISSD, and a patient with INSD. (C-D) Group-averaged 24-hour profiles of PIM activity and temperature rhythms over 14 days. Graphs are plotted as mean $\pm$ SEM. N = 6-14 per group. (E) Correlation of Insomnia severity index (ISI) score with circadian parametric and non-parametric measures, indicating a decline in circadian robustness with increasing insomnia severity. Colors and dot sizes represent the strength and direction of the correlations, evaluated using Spearman's test (*p < 0.05, **p < 0.01, ***p < 0.001), N = 14.

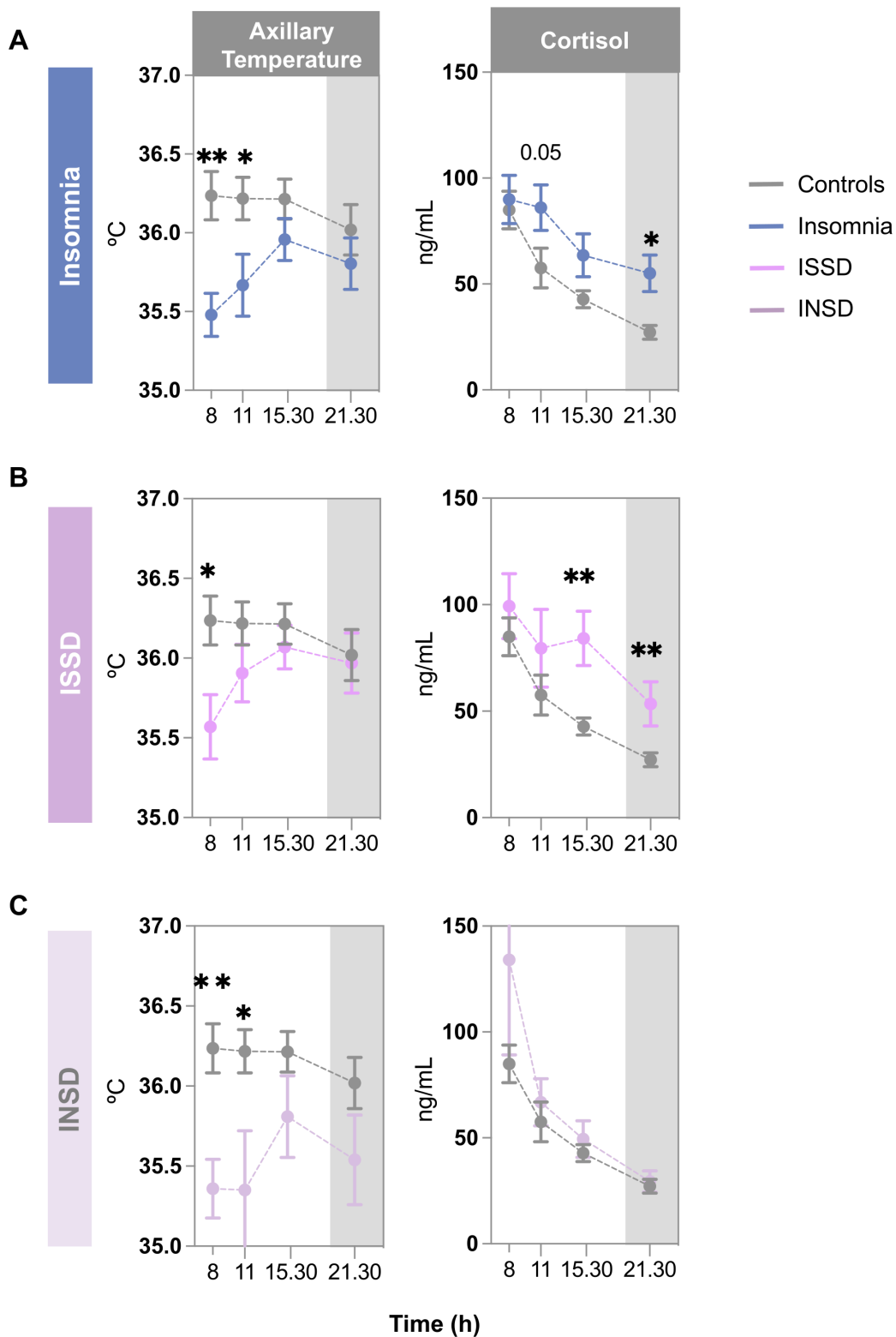


Figure 3

Chronic Insomnia alters axillary temperature, plasma cortisol levels and profiles. (A) Axillary temperature and plasma cortisol levels were measured at four time points (8:00, 11:00, 15:30, and 21:30 h) in patients with chronic insomnia and controls (grey line). (B-C) Data were further analyzed in subgroups: insomnia with short sleep duration (ISSD) and insomnia with normal sleep duration (INSD).

Graphs represent mean $\pm$ SEM. Significant differences relative to the control group at each timepoint were assessed using an unpaired parametric t-test (* $p < 0.05$, ** $p < 0.01$). N = 6–14 per group.

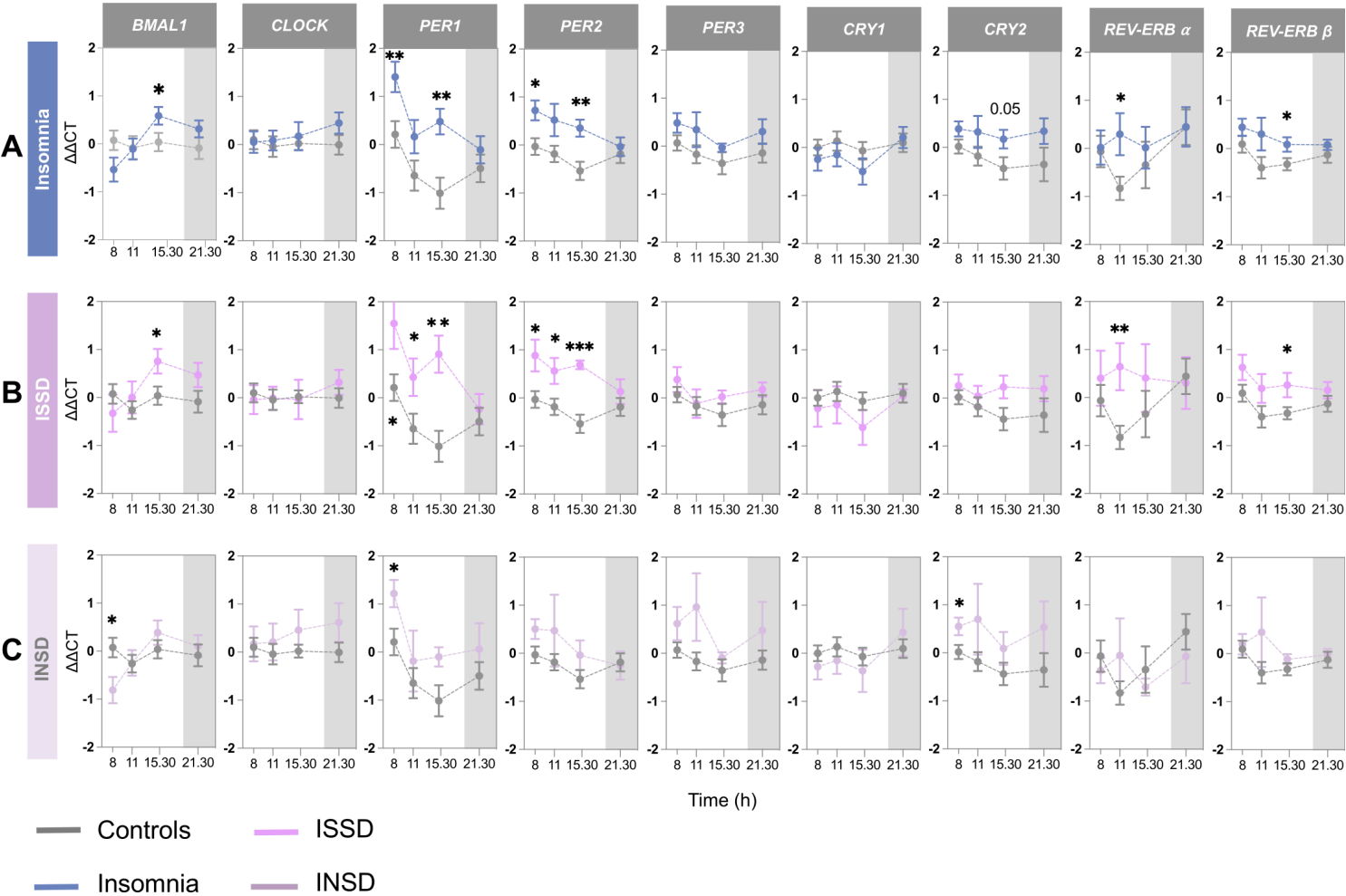


Figure 4

Chronic Insomnia alters the expression of core-clock genes in peripheral blood mononuclear cells (PBMCs), with more pronounced alterations in patients with short sleep duration (ISSD) patients. (A) Expression levels of nine clock genes (*BMAL1*, *CLOCK*, *PER1–3*, *CRY1–2*, *REV-ERBα*, and *REV-ERBβ*) were measured at four time points (8, 11, 15:30, and 21:30 h) in PBMCs of chronic insomnia patients and healthy controls(grey line). Data were further analyzed in subgroups: (B) insomnia with short sleep duration (ISSD) and (C) insomnia with normal sleep duration (INSD). Data are presented as mean $\Delta\Delta CT$ values $\pm$ SEM. Significant differences relative to controls at each timepoint were determined using an unpaired parametric t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). N = 6–14 per group.

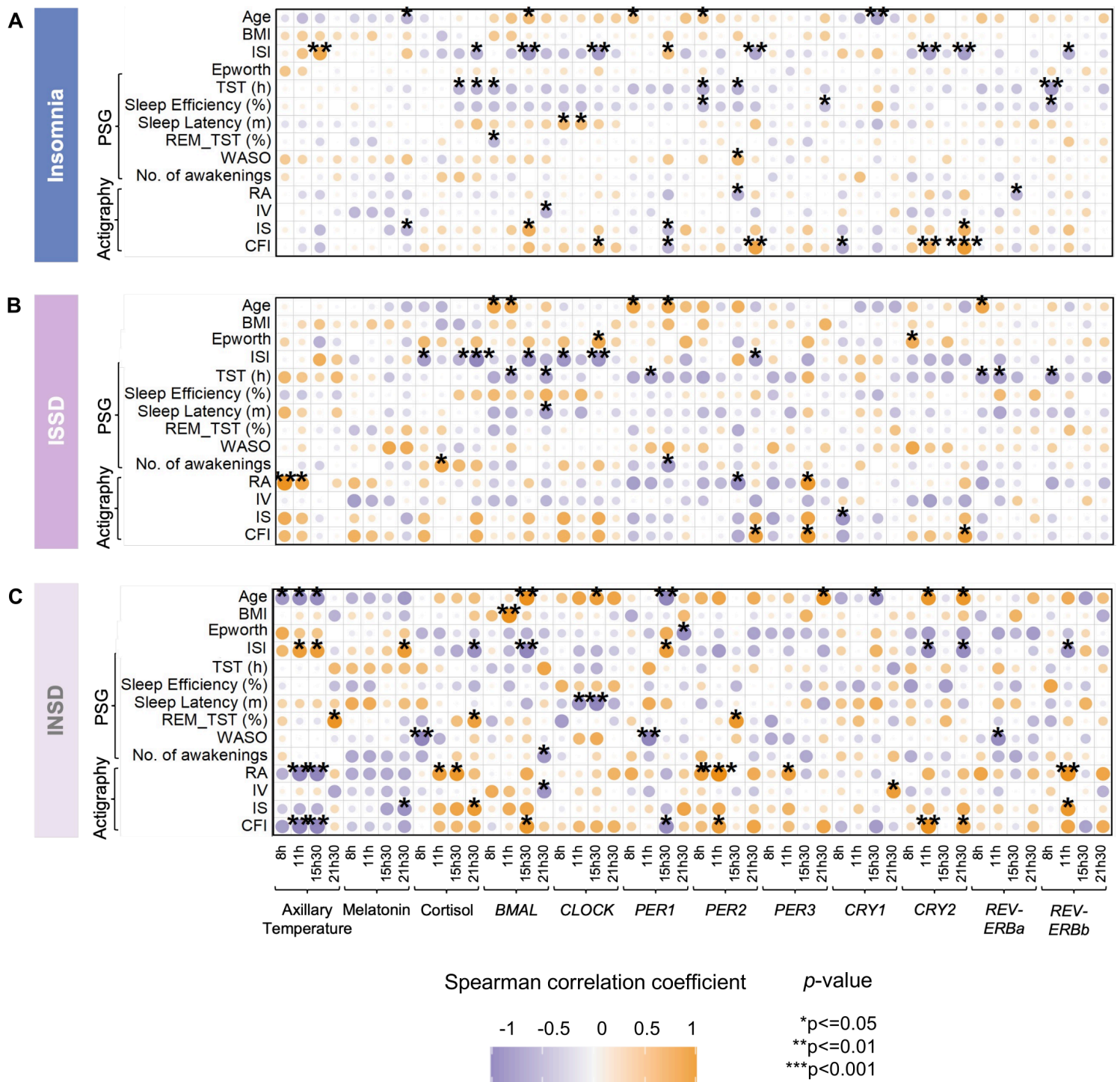


Figure 5

Correlation analyses of sleep metrics, circadian parameters, clinical symptom scores, clock outputs, and core-clock gene expression in chronic insomnia and its subgroups: insomnia with short sleep duration (ISSD) and insomnia with normal sleep duration (INSD). Variables include body mass index (BMI), Epworth sleepiness scale, insomnia severity index (ISI), total sleep time (TST), rapid eye movement (REM), wakefulness after sleep onset (WASO), relative amplitude (RA), intradaily variability (IV), intradaily stability (IS) and circadian function index (CFI). (A-C) Dot plots highlight significant correlations at different time points (8:00, 11:00, 15:30, and 21:30 h) in chronic insomnia patients (A),

ISSD (B), and INSD (C). Colors and dot sizes represent correlation strength and direction, assessed using Spearman's test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). N = 6–14 per group.

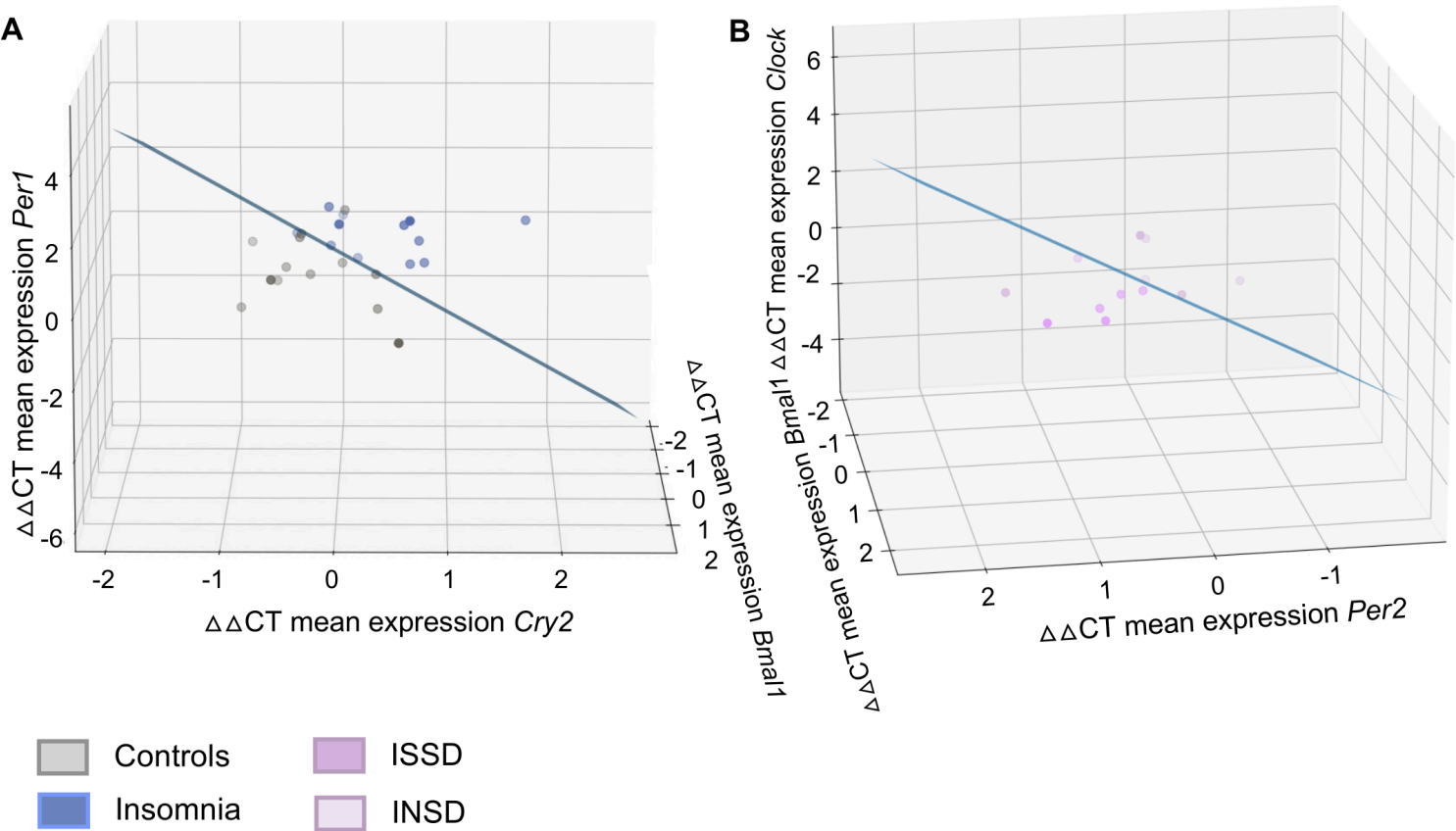


Figure 6

Differential expression of core clock genes in peripheral blood mononuclear cells (PBMCs) in chronic insomnia patients. Chronic insomnia patients exhibit distinct mean expression profiles of core-clock genes in PBMCs compared to control subjects. Significant differences are also observed between chronic insomnia subgroups with short sleep duration (ISSD) and normal sleep duration (INSD). Subjects were classified based on the mean expression levels of specific clock genes of the four time points across the day. (A) Classification of control subjects and chronic insomnia patients based on the mean mRNA expression levels of *PER1*, *CRY2*, and *BMAL1*. (B) Classification of ISSD and INSD subgroups based on the mean mRNA expression levels of *PER2*, *CLOCK*, and *BMAL1*.

Supplementary Files

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