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Reference Genes for Circadian Profiling of Core Clock Genes in the Blood of Obstructive Sleep Apnea Patients

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Abstract

Circadian rhythm disruptions are increasingly recognized in disorders such as obstructive sleep apnea (OSA), yet analysis of 24-hour gene expression patterns remains challenging due to the lack of reliable reference genes for normalization. Even commonly used housekeeping genes may exhibit circadian oscillations, which can confound rhythmic gene expression analyses and hinder biomarker identification. To address this limitation, we evaluated the gene expression stability of 11 commonly used housekeeping genes in blood collected every 6 hours over 24-hour period from 40 adults with varying OSA severity and controls. Stability ranking by analytical tools RefFinder and EndoGeneAnalyzer identified *ACTB* (β-actin) and *RPL13A* (ribosomal protein L13a) as the most consistent reference genes, with minimal intra- and inter-individual variability across sampling times and disease groups. Their suitability was assessed by personalized cosinor analysis of core clock genes (*BMAL1, PER2, CRY1*), demonstrating that appropriate normalization enables detection of circadian oscillations in clinical samples. Using the optimal normalization, CosinorPy analysis of the core clock genes revealed significant circadian oscillations of at least one clock gene in the studied participants. These findings establish *ACTB* and *RPL13A* as robust reference genes for blood-based circadian studies of OSA and provide an important methodological framework for future circadian biomarker research.

Keywords: circadian rhythms; obstructive sleep apnea; reference genes; qRT-PCR normalization; gene expression stability; core clock genes

1. Introduction

Despite advances in transcriptomic technologies, quantitative reverse transcription polymerase chain reaction (qRT-PCR) remains the gold standard for low-throughput gene expression analysis. Accurate qRT-PCR measurements depend on RNA quality, reverse transcription and PCR efficiency, and particularly on the selection of appropriate reference genes for normalization [1]. Because reference gene suitability is context-dependent, circadian studies require genes with stable 24-hour expression profiles. However, this is challenging since the circadian clock regulates a wide range of physiological processes, including the expression of commonly used reference genes themselves.

Circadian rhythm is an adaptation of living organisms to the natural cycle of environmental light and darkness, with an intrinsic period of approximately 24 hours [2,3]. On a molecular level, it is controlled by the transcriptional and translational feedback loops of core clock components. The central clock involves transcription factors (e.g., BMAL1 and CLOCK) activating clock-controlled genes (*PER* and *CRY* gene families), which in turn inhibit the activators and produce self-sustained

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oscillations [2]. This biological clock is intrinsic to almost all life forms and has gained increasing interest, particularly due to its impact on physiology. Circadian rhythms influence a variety of physiological processes, from metabolism, regulation of body temperature, endocrine secretion and even labor onset [4]. Understanding the genetic mechanisms that drive circadian rhythms is crucial, as disruptions in these rhythms are linked to health conditions [5], such as metabolic disorders (e.g. metabolic dysfunction-associated steatotic liver disease [6–8], cardiovascular diseases (e.g. heart failure) [9], and sleep disturbances (e.g. obstructive sleep apnea) [10].

We aim to identify reliable circadian biomarkers in blood cells of patients with obstructive sleep apnea (OSA), a disorder characterized by repeated breathing interruptions during sleep, fragmented sleep, intermittent hypoxia, and multiple health complications [2,11] that overlap with other diseases associated with circadian rhythm disruption [3]. Accumulating evidence indicates that OSA disrupts the molecular circadian clock, and it has been hypothesized that OSA severity correlates with distinct patterns of clock gene expression in peripheral blood cells [2]. Therefore, a prerequisite for this investigation is the identification of stable reference genes from human blood cells for normalizing the circadian gene expression [12,13]. Since most validated datasets are still rodent-based, human circadian normalization studies remain scarce, which limits accurate and reproducible circadian gene expression studies in humans [12–18]. Therefore, the primary goal of this study was to determine which housekeeping genes exhibit the most stable expression profiles across different time points and different OSA severities. We present a 24-hour analysis of candidate reference gene expression of RNA, isolated from the buffy coat (the leukocyte-thrombocyte layer) of blood from patients with varying OSA severity. Our results identify two reference genes that are most suitable for normalizing circadian expression of core clock genes, paving the foundation for future circadian biomarker discovery in OSA.

2. Materials and Methods

2.1. Subjects and Blood Sample Collection

Forty participants (16 women and 24 men, aged 18–65 years) with clinically suspected OSA underwent overnight respiratory polygraphy (PG; Alice NightOne, Philips) in a hospital setting at the Clinical Institute of Clinical Neurophysiology, University Medical Centre Ljubljana, Slovenia. The study was approved by the Commission for Medical Ethics of the Republic of Slovenia (approval No. 0120-65/2023/3) and conducted in accordance with its guidelines. All participants were of the same ethnic background and provided written informed consent in the presence of the responsible physician. Participants were classified by apnea–hypopnea index (AHI) into four groups: control (AHI < 5), mild (AHI 5–14.9), moderate (AHI 15–29.9), and severe (AHI ≥ 30) OSA, each comprising 10 individuals.

Before the overnight respiratory PG, the participants were given instructions on timing and composition of the meal, and directions about sleep hygiene. Participants were asked to: eat dinner before 18:00 at home as fat-free as possible; not to eat before morning sampling (at 7:00); abstain from drinking coffee and alcohol throughout the study protocol. The PG started recording at 22:00 and ended at 6:00 the next day. During that time, patients were instructed not to use electronic devices, turn off the light, and to go to sleep as soon as possible. Any interaction between medical staff and patients during the night was performed using dim light.

During the study, peripheral venous blood (3 mL per draw, EDTA anticoagulant) was collected via an indwelling catheter at five time points over 24 hours: T0, 13:00; T1, 19:00; T2, 01:00; T3, 07:00; T4, 13:00 (following day). Within 30 minutes of each draw, samples were centrifuged (2000 × g, 15 min, 4 °C), plasma was removed, and 300 µL of buffy coat was collected for RNA isolation.

2.2. RNA Isolation, cDNA Preparation, and qRT-PCR

Total RNA was extracted from buffy coat using TRI Reagent LS (Sigma-Aldrich); the detailed protocol is provided in the Appendix A1. RNA quantity and purity were assessed by

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spectrophotometry (NanoDrop 1000, Thermo Scientific). Complementary DNA was synthesized with the QuantiTect Reverse Transcription Kit (Qiagen). qRT-PCR was performed using PowerTrack SYBR Green Master Mix (Applied Biosystems) on a QuantStudio 5 instrument (Applied Biosystems), following the manufacturer's recommended cycling conditions. All reactions were run in triplicate with no-template controls, and amplification specificity was confirmed by melt-curve analysis (single peak per primer set).

2.3. Primer Design

Eleven candidate reference genes were selected based on published literature [1,9,13,14,16,17,19–24] and previous work from our group [15]: *GAPDH, SDHA, PPIB, ACTB, CDK4, HPRT1, RPL13A, TUBB2A, PPIA, UBC,* and *TBP*. Additional primer sets were tested for selected genes (denoted '#2') to evaluate alternative splice variants or amplicon targets (*SDHA* #2, *PPIB* #2, *CDK4* #2). Primers were designed with NCBI Primer-BLAST (Table S1). Amplification efficiency was determined by standard curve analysis; only primer pairs with efficiency between 90–110% were retained, and specificity was confirmed by gel electrophoresis. Primer sequences, amplicon lengths, and efficiencies are listed in Table S2. Raw quantification cycle (Cq) data were managed with QuantStudio Design and Analysis Software version 1.6.1.

2.4. Reference Gene Stability Analysis

Reference gene stability was assessed using two complementary tools. RefFinder [25] integrates four established algorithms: geNorm [26], NormFinder [27], BestKeeper [28], and the comparative ΔCt method [29]. It generates a geometric mean-based comprehensive ranking (lower score = higher stability). EndoGeneAnalyzer [30] complements RefFinder by accommodating complex, multi-factor designs. It handles multiple grouping variables (e.g., disease group, time point), removes outliers, and computes NormFinder-based stability metrics within each subgroup. This enabled evaluation not only across the full dataset but also within specific conditions (OSA severity, 24-hour intra-individual variation, and time of day). Reference gene selection followed a stepwise elimination approach guided by results from both tools (Section 3.1).

2.5. Cosinor Analysis of Core Gene Rhythmicity

To validate the selected reference genes, the rhythmic expression of core clock genes *BMAL1, CRY1,* and *PER2* was examined by cosinor rhythmometry, a statistical method that fits a cosine function to time-series data and tests for a 24-hour oscillation. The fitted model yields three biologically meaningful parameters: the mesor (rhythm-adjusted mean expression level), the amplitude (half the peak-to-trough difference, indicating oscillation magnitude), and the acrophase (time at which expression peaks). Cq values were normalized using an efficiency-corrected relative quantity method. For each gene, the relative quantity was calculated as E^(−Cq), where E denotes the amplification efficiency factor (E = 1 + efficiency/100). To obtain a sample-scaled relative expression, each value was divided by the maximum observed expression across all samples for the respective gene. The normalization factor (NF) was computed as the geometric mean of the scaled expression values of *ACTB* and *RPL13A*, further adjusted relative to the global average geometric mean across all samples, following the multi-reference gene normalization principle described by Vandesompele et al. [26]. Normalized expression (NE) was thus defined as $NE = (E_{target}^{(-Cq_{target})} / \max[E_{target}^{(-Cq)}]) / NF$. Cosine curve fitting was performed with CosinorPy [31]. Both population-level (group-averaged) and personalized (individual-level) cosinor models were constructed to capture group trends and inter-individual variation, respectively. For personalized cosinor analysis, rhythmicity was assessed separately for each participant and target gene. To account for multiple comparisons arising from testing multiple genes across multiple participants, p-values were adjusted using the false discovery rate (FDR) procedure. Adjusted p-values < 0.05 were considered statistically significant and reported as q-values.

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3. Results

3.1. Reference Gene Selection

Forty participants were allocated to three OSA severity and control groups (10 per group) based on AHI. Reference gene selection proceeded through three sequential steps (Figure 1), each expanding the analytical scope while narrowing the candidate pool.

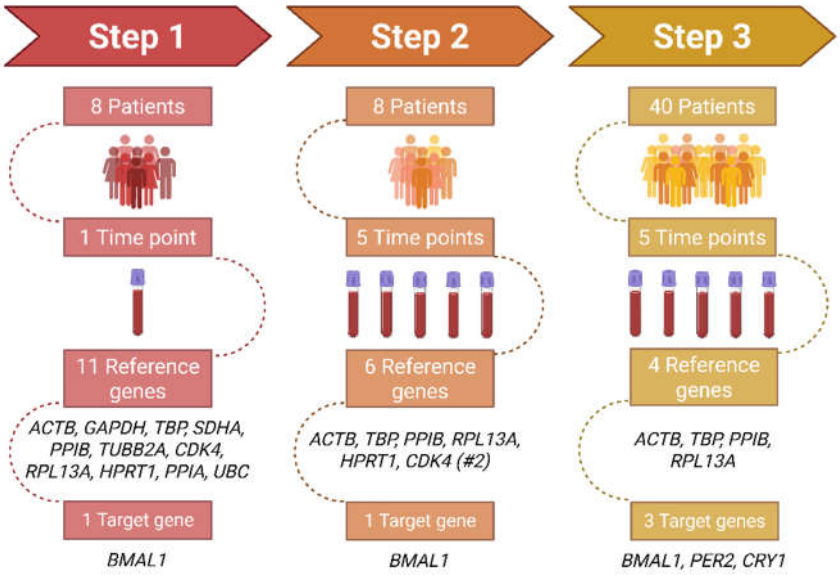

Figure 1. Schematic overview of the three-step reference gene selection process in this study. The study was conducted in three sequential steps. In each step, we increased the scope of analysis (either the number of participants, the number of blood sampling time points, and/or the number of target genes) while correspondingly narrowing down the list of candidate reference genes. At each step, the elimination of reference gene candidates was based on the stability results obtained with algorithms implemented in online tools RefFinder and EndoGeneAnalyzer. Step 1 involved 8 participants sampled at a single time-point to evaluate the expression stability of 11 candidate reference genes, using *BMAL1* as a representative target gene. In step 2, we used the same 8 patients and included samples from all 5 time-points to assess circadian expression patterns and refine the selection to the 6 most stable reference genes. In step 3, the final analysis was expanded on a cohort of 40 patients, sampled at 5 time-points, using the 4 most stable reference genes (*ACTB, TBP, PPIB, RPL13A*) from previous steps for normalization. Expression of three core clock genes (*BMAL1, PER2*, and *CRY1*) was evaluated in this final phase. "#2" denotes an alternative primer sequence targeting a different transcript of the same gene.

Step 1: Single time-point screening (n = 8). Eleven candidate reference genes (*ACTB, GAPDH, TBP, SDHA, PPIB, TUBB2A, CDK4, RPL13A, HPRT1, PPIA, UBC*), three alternative primer sets (*SDHA* #2, *PPIB* #2, *CDK4* #2), and *BMAL1* (representative target gene) were evaluated in 8 participants (2 per OSA group) at T0 (13:00). Because all samples were collected at the same circadian time, data were grouped by OSA severity and analyzed primarily with RefFinder. Six genes (*GAPDH, SDHA, TUBB2A, CDK4, PPIA, UBC*) and the *SDHA* #2 and *PPIB* #2 variants were eliminated due to high variability (Figures S1–S2, Tables S3–S4). Although the small sample size (n = 2 per OSA severity group) limits the statistical power of this screening step, Step 1 was intentionally designed as a rapid preliminary filter to exclude genes with clearly high variability before proceeding to the more resource-intensive multi-timepoint analysis in Steps 2 and 3.

Step 2: Circadian time-series assessment (n = 8, five time points). The six best-performing genes (*ACTB, TBP, PPIB, CDK4* #2, *RPL13A, HPRT1*) were re-evaluated across all five time points in the same 8 participants using EndoGeneAnalyzer with three grouping factors: individual patient, OSA severity, and time of day. The results (Figure S3, Table S5) showed that some genes had consistently

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low stability in all scenarios. In particular, *HPRT1* and *CDK4 #2* emerged as less stable (higher variability) across conditions. RefFinder (Figure S4, Table S6) also placed these two genes in mid-to-low stability range. Therefore, we eliminated *HPRT1* and *CDK4 #2* at this stage.

Step 3: Full cohort validation (n = 40, five time points). The four remaining genes (*ACTB*, *TBP*, *PPIB*, *RPL13A*) were assessed across all 40 participants at five time points. Three clock genes (*BMAL1*, *CRY1*, *PER2*) were measured concurrently for downstream rhythmicity analysis. EndoGeneAnalyzer results (Figure 2) showed that *ACTB* had the lowest standard deviation across all three grouping conditions (Figure 2a), while *RPL13A* achieved the lowest stability values (Figure 2b; lower = more stable), followed closely by *ACTB. TBP* ranked lowest on both metrics. Detailed values are in Table S7.

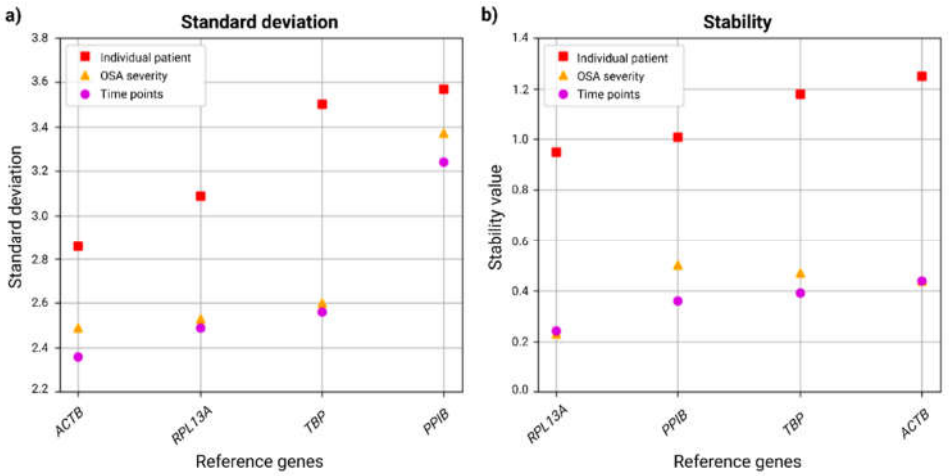

Figure 2. EndoGeneAnalyzer results of the four candidate reference genes in Step 3. Standard deviation (a) and stability (b) of the four best-performing reference genes (*ACTB*, *RPL13A*, *TBP*, *PPIB*) are shown based on three grouping criteria: individual patient (red squares), OSA severity (orange triangles), and time points (purple circles). Lower standard deviation and stability values indicate more consistent gene expression. *ACTB* consistently exhibited the lowest variability (a) within all tested conditions, while *RPL13A* showed the lowest stability values (b) across all grouping conditions.

RefFinder results (Figure 3, Table S8) were consistent: *ACTB* ranked first by BestKeeper and second by NormFinder, the ΔCt method, and the comprehensive score. *RPL13A* ranked first by geNorm, together with *PPIB*, which scored best by NormFinder, ΔCt, and comprehensive ranking. *TBP* was the least stable gene by all algorithms, except BestKeeper.

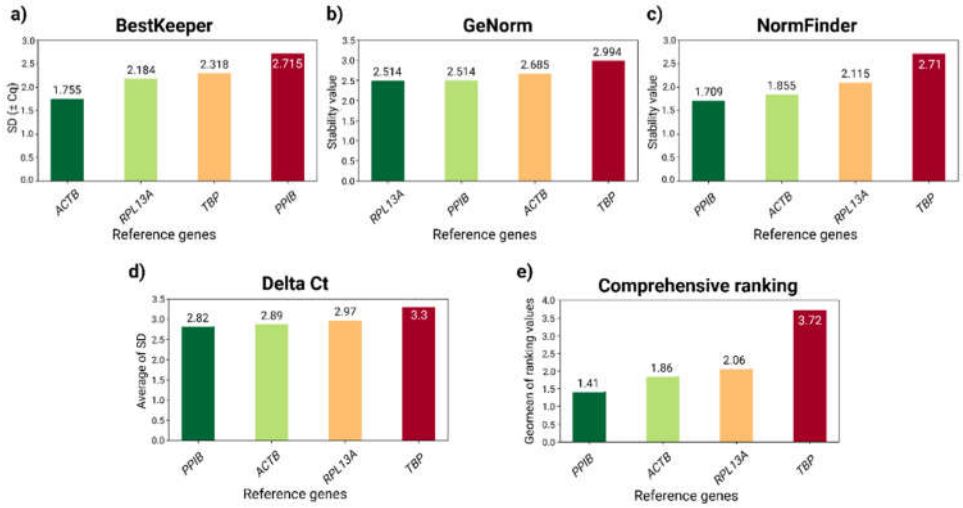

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Figure 3. RefFinder analysis of reference gene stability in Step 3. The figure shows results from four algorithms (BestKeeper, geNorm, NormFinder, and the Delta Ct method) with additional comprehensive ranking. Each graph (a-e) represents one analysis method, plotting the stability or variability measures for *ACTB*, *RPL13A*, *PPIB*, and *TBP*. In all cases, lower values (left side) correspond to greater expression stability. *ACTB* was identified as the top performer by BestKeeper (a), *RPL13A* ranked highest by geNorm (b), and *PPIB* showed the best stability by NormFinder (c), lowest average of SD by ΔCt method (d) and overall best geometric mean of ranking values by comprehensive ranking (e). *TBP* consistently showed the lowest stability in four algorithms (b-e).

Combining evidence from both tools across all grouping conditions, *ACTB* and *RPL13A* were selected as the optimal reference genes. Both showed low intra- and inter-group variability and stable expression across the circadian cycle, and were reliably detected in every sample on the first attempt. *PPIB* was excluded despite favourable algorithm scores because its Cq values were undetectable in a subset of 24 samples (all four groups and all time points) even after three repeated measurement, a practical limitation invisible to computational analysis alone. *TBP* was excluded due to poor stability across four out of five algorithms.

3.2. *Assessment of Core Clock Gene Rhythmicity*

BMAL1, *CRY1*, and *PER2* Cq values were normalised with *ACTB* and *RPL13A* using the comparative Ct method [26]. Group-specific cosinor models were built by averaging individual-level fits within each OSA severity group (Figure 4, Table S9).

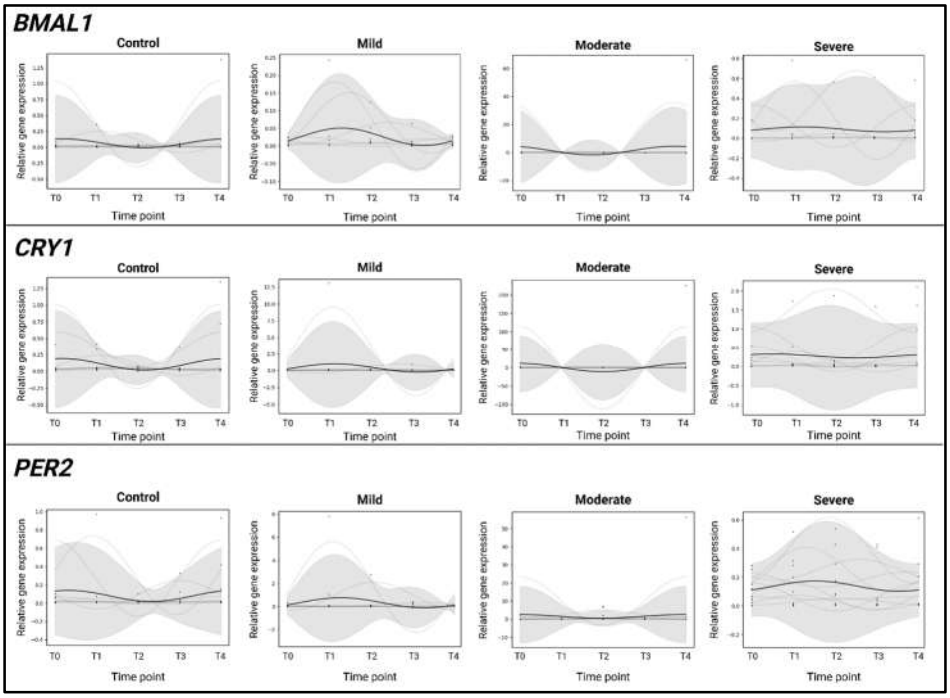

Figure 4. The circadian expression profiles of core clock genes (*BMAL1*, *CRY1*, and *PER2*) across OSA severity groups. Gene expression rhythms were assessed using a population-based cosinor for control, mild, moderate, and severe OSA groups. Cq values were normalized using *ACTB* and *RPL13A* as reference genes. Each subplot represents the fitted group-specific rhythm (black line), obtained by averaging individual-specific cosinor models (grey lines). Shaded areas denote 95% confidence intervals. No statistically significant rhythmicity was observed across severity groups for any of the genes analyzed. Sampling time (x-axis): T0 on day 1 at 13:00, T1 on day 1 at 19:00, T2 on day 2 at 1:00, T3 on day 2 at 7:00, and T4 on day 2 at 13:00.

Group-specific cosinor analysis showed that the core clock genes *BMAL1*, *CRY1*, and *PER2* displayed detectable temporal variation across OSA severity groups, although rhythmicity did not

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reach statistical significance at the population level. For *BMAL1*, amplitudes were low (0.024–3.08) and mesors modest (0.026–1.43), indicating low-level, irregular expression. The acrophase, i.e. the time of peak expression, was later in mild (~20.8 h) and severe OSA (~20.5 h) groups than in controls (~13.9 h), suggesting a phase delay, though not statistically significant. For *CRY1*, amplitudes ranged from 0.05 to 11.20 (largest in the moderate OSA group) and acrophases from ~13.9 h to ~19.9 h, none significant. *PER2* amplitudes were uniformly low (0.036–1.02 in most groups), and the severe OSA group had the latest acrophase (~22.2 h), consistent with a progressive phase delay at higher disease severity, but without statistical support (Table S9).

The absence of group-level significance reflects substantial inter-individual variability rather than the universal absence of rhythmicity. Inspection of individual-level cosinor fits (Figure 5) revealed that many participants displayed clear oscillatory profiles that were obscured by group averaging, which is a known limitation of population-based chronobiology analyses in heterogeneous clinical cohorts. Detailed analysis of individual expression profiles further demonstrated substantial inter-individual variability and desynchronization of clock gene oscillations across OSA severity groups.

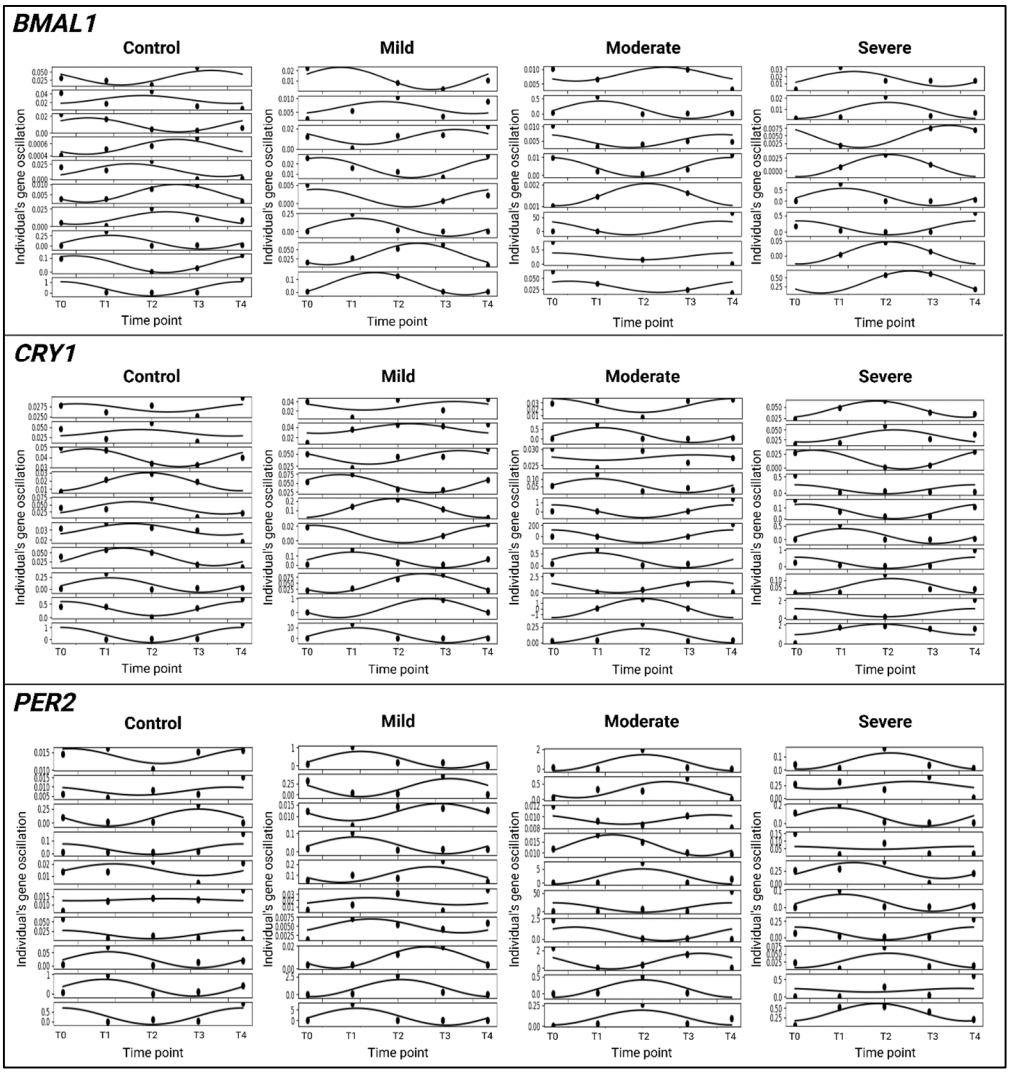

Figure 5. The circadian expression of core clock genes (*BMAL1*, *CRY1*, and *PER2*) of individual participants within each OSA severity group obtained from population-based cosinor analysis. Each panel represents the fitted cosinor curves (black lines) and raw expression data (black dots) for each individual participant. Rows correspond to different target genes, while columns correspond to OSA severity groups. Time points (x-axis) are plotted over a 24-hour period, and the y-axis represents normalized gene expression levels. Population-based cosinor was used to estimate individual mesor, amplitude, and acrophase parameters, highlighting inter-

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individual variability in circadian rhythmicity within each group. This approach reveals both well-defined oscillations and flattened or phase-shifted profiles, underscoring the heterogeneity of circadian rhythms in patients with OSA. Sampling time (x-axis): T0 on day 1 at 13:00, T1 on day 1 at 19:00, T2 on day 2 at 1:00, T3 on day 2 at 7:00, and T4 on day 2 at 13:00.

After FDR correction, personalized cosinor analysis (Figure 6; Tables S10–S11) detected statistically significant rhythmicity of *BMAL1* in 7 participants, *CRY1* in 13, and *PER2* in 5, totaling 25 individuals with rhythmicity in at least one gene, largely independent of OSA severity, highlighting the highly individualized nature of circadian regulation in OSA. Acrophase distributions (Figure 6d–f) showed wide inter-individual spread with no consistent phase alignment within any OSA severity group, indicating that circadian disruption in OSA is not simply a graded function of disease severity. These findings emphasize the importance of patient-specific circadian assessment and suggest that personalized approaches may provide greater sensitivity for detecting circadian alterations in OSA.

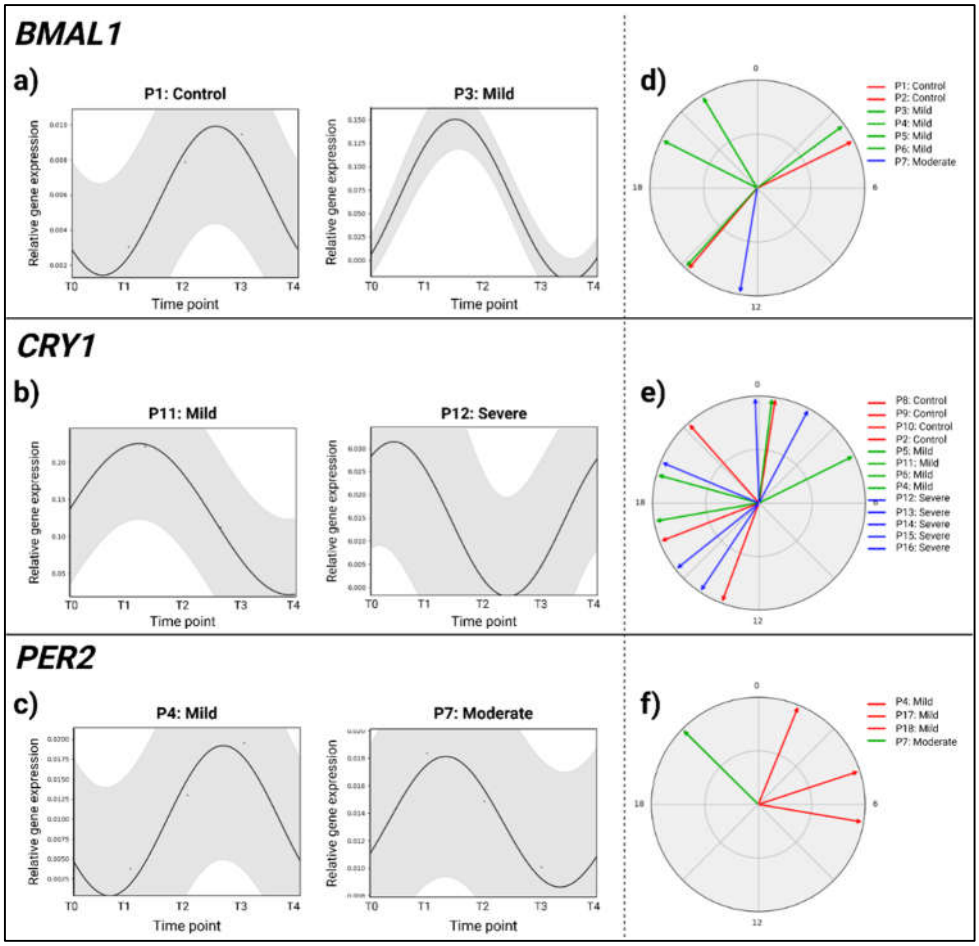

Figure 6. Personalized cosinor analysis of core clock genes in OSA patients and controls. Panels a–c show individual cosinor fits (black lines) with 95% confidence intervals (grey areas) for representative participants, illustrating gene-specific oscillatory patterns of *BMAL1* (a), *CRY1* (b), and *PER2* (c). Each subplot corresponds to one participant, where "P" followed by a number denotes a specific individual (e.g., P1 = Participant 1). Examples were selected as best-case examples. Panels d–f display acrophase distributions derived from CosinorPy for *BMAL1* (d), *CRY1* (e), and *PER2* (f). Each line represents different participant denoted with P and a number. Sampling time (x-axis): T0 on day 1 at 13:00, T1 on day 1 at 19:00, T2 on day 2 at 1:00, T3 on day 2 at 7:00, and T4 on day 2 at 13:00.

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4. Discussion

In this study, we addressed an important methodological challenge in circadian biology research – the identification of suitable reference genes for normalization of 24-hour gene expression profiles. Accurate normalization of circadian qRT-PCR data requires reference genes that remain stable not only across disease conditions but also across the 24-hour cycle, a combination that had never been validated for peripheral blood in OSA patients. Using a three-step selection framework, two independent computational tools, and progressive narrowing of an initial panel of 11 candidate reference genes, together with three additional primer sets (*CDK4*, *SDHA*, and *PPIB*), we identified *ACTB* and *RPL13A* as the most stable reference genes in human buffy coat under conditions specifically relevant to OSA: 24-hour intra-patient variation, OSA severity, and the hypoxic milieu of intermittent nocturnal desaturation. This is, to our knowledge, one of the first time-resolved, blood-based reference gene validation for circadian studies in an OSA cohort.

The three-step design proved efficient. Single time-point screening (Step 1) identified and removed the most variable genes before more resource-intensive time-series work was undertaken. Extending evaluation to five time points in Step 2 revealed that *HPRT1* and *CDK4* #2, acceptable at a single time point, were in fact unstable across the circadian cycle. This underscores a general principle that reference genes for circadian studies must be validated under the same temporal conditions as the intended experiment. Final validation in 40 participants (Step 3) confirmed that *ACTB* and *RPL13A* maintained stable expression across individuals, disease severity groups, and time of day.

The stable performance of both genes is consistent with the existing literature [32–34]. *ACTB* has been validated as a reliable reference in blood-based circadian studies [21,35–37], and *RPL13A* has shown stable expression in human leukocytes under stimulation [38] and in blood under high-altitude hypoxia [39]. Particularly relevant to OSA, Wardaszka et al. (2025) demonstrated that *RPL13A* is among the most suitable reference genes in peripheral blood mononuclear cells under both normoxic and hypoxic conditions [38], which directly mirrors the intermittent hypoxia experienced by OSA patients. Together, these lines of evidence support the robustness of *RPL13A* in clinical blood-based settings characterized by disturbed oxygenation [38,39]. Conversely, both genes have shown instability in other contexts (*ACTB* in mouse lung [1] and under sleep deprivation [35]), reaffirming that reference gene suitability is tissue- and condition-specific.

Among the genes that proved unsuitable, *GAPDH* and *TBP* are particularly instructive. *GAPDH*, though widely used as a housekeeping gene [40], was eliminated in Step 1 due to high variability, and being consistent with reports that its expression is modulated by metabolic state and time of day [41–43]. *TBP* ranked as the least stable gene across four RefFinder algorithms in step 3 (Figure 3, Table S8), contrasting with reports of its stability in human mammary epithelial cells [12] and illustrating once more that housekeeping gene behavior cannot be assumed from the literature alone.

The case of *PPIB* illustrates a complementary pitfall, because it performed well computationally but was undetectable in 24 samples (in all triplicates) even after 3 repeated measurements. The number of undetected samples spread through all four groups and time points (3 controls, 4 mild, 4 moderate, and 3 severe OSA samples). This practical failure would have been invisible if selection had relied solely on algorithm output. We therefore recommend that computational ranking always be complemented by empirical quality checks (detection rate, Cq reproducibility across all samples) before finalizing the reference gene choice. Consistent with best-practice guidance [26], using two reference genes in combination (*ACTB* and *RPL13A*) further improves normalization robustness compared to a single gene.

A key methodological strength is the integration of two independent tools. RefFinder synthesizes four established algorithms into a consensus ranking, reducing reliance on any single method's assumptions. EndoGeneAnalyzer [30] was particularly well-suited to our multi-factor design, enabling simultaneous evaluation of stability across time of day, OSA severity, and intra-individual variation. The agreement between the two tools in selecting *ACTB* and *RPL13A* substantially strengthens confidence in the final gene pair.

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Using *ACTB* and *RPL13A* for normalization, we assessed the rhythmicity of *BMAL1*, *CRY1*, and *PER2*. None showed statistically significant 24-hour rhythmicity at the group level, in contrast to studies in healthy populations [20,21,35–37,44,45]. Several non-exclusive explanations are possible: (i) intermittent hypoxia and sleep fragmentation in OSA may dampen or desynchronise peripheral clock gene expression; (ii) the heterogeneous cellular composition of buffy coat may dilute oscillatory signals; (iii) group sizes of 10 may be insufficient to detect modest rhythmicity in a mixed clinical cohort; and (iv) five sampling points over 24 hours may provide inadequate temporal resolution for low-amplitude waveforms. Comparisons with existing literature are further complicated by methodological differences across studies [20,21,35–37,44,45].

Despite the absence of statistical significance, non-significant phase trends deserve note. The acrophase of *BMAL1* was earlier in moderate OSA (~11.3 h) than in controls (~13.9 h), and the acrophase of *PER2* was latest in severe OSA (~22.2 h), showing a pattern consistent with a severity-dependent phase delay in the peripheral clock. These trends, although not statistically significant, could point to a progressive disruption of the molecular circadian system with increasing disease severity. In addition, these observations reinforce the rationale for future longitudinal studies with pre- and post-treatment designs using the reference gene framework established here.

After the correction for multiple testing, the personalized cosinor analysis revealed that circadian gene expression in OSA is highly heterogeneous, given that 25 participants displayed significant individual oscillations, yet these did not cluster by disease severity. Notably, *CRY1* showed the greatest number of participants with significant rhythmicity (n = 13), while *PER2* showed the fewest (n = 5). This gene-specific pattern, with some clock components more affected than others, parallels observations in OSA-related depression, where selective upregulation of specific clock genes rather than uniform clock suppression characterizes the circadian phenotype [46]. The wide spread of acrophase values within each severity group (Figure 6d–f) further suggests that individual factors (e.g. metabolic status, lifestyle, chronotype, and genetic variation) shape circadian gene expression in OSA more than disease severity alone [47]. These findings highlight the limitations of group-averaged analyses and support the use of personalized chronobiological approaches in both research and clinical chronotherapy [2,46,48,49].

OSA patients experience chronic intermittent hypoxia, sleep fragmentation, and sympathetic activation, all of which can perturb the molecular clock. Altered expression of core clock and clock-controlled genes in OSA blood is well documented [24,32,33,49], and these changes may mediate downstream comorbidities including cardiovascular disease, metabolic syndrome, and psychiatric disorders. The validated *ACTB*/*RPL13A* reference pair provides a reliable and practical tool for pursuing these questions in future studies.

Nevertheless, several limitations should be acknowledged. First, buffy coat is a heterogeneous mixture of cell types. Therefore, cell-type-specific circadian signals may be diluted or cancelled by this heterogeneity, and future studies using sorted leukocyte subsets or single-cell approaches may improve sensitivity. Second, the initial gene screening in Step 1 relied on only two participants per OSA severity group (n = 8 total), which limits the statistical rigor of that step in isolation. However, this was a deliberate design choice to maximize screening efficiency prior to full time-series analysis, and the final reference gene selection is grounded in the expanded cohorts of Steps 2 and 3. In addition, group sizes of 10 may limit the power to detect subtle rhythmicity. Consequently, larger cohorts are needed to characterize low-amplitude oscillations and to test whether the validated reference genes remain stable in more diverse populations. Third, five time points at six-hour intervals may insufficiently resolve waveform dynamics with non-sinusoidal shapes or shifted phases. While a 6-hour sampling interval (with an additional point at 13:00) is a logistically feasible approach for clinical cohorts, it inherently limits the statistical power of single-subject cosinor models. Consequently, the significant rhythmicity observed at the individual level in 25 out of 40 participants should be interpreted with caution, as low-density sampling schedules can elevate the risk of false-positive or false-negative rhythmic classifications. Finally, these reference genes are validated

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specifically for buffy coat. Hence, their stability in other blood fractions, sorted cell populations, or different tissues cannot be assumed without independent validation.

Despite these limitations, the multi-step, dual-algorithm framework presented here is transparent and reproducible, and should be directly applicable in future clinical chronobiology studies. The consistent performance of *ACTB* and *RPL13A* across all conditions tested (hypoxia, sleep fragmentation, disease severity variation) supports their adoption as reference genes for circadian qRT-PCR in peripheral blood, enabling rigorous investigation of molecular clock disruption in OSA and its comorbidities.

5. Conclusions

ACTB and *RPL13A* were identified as the most stable reference genes for normalizing circadian qRT-PCR data in the buffy coat of OSA patients across a full 24-hour sampling period. Their suitability was tested by personalized cosinor analysis of core clock genes (*BMAL1, CRY1, PER2*), which detected significant individual oscillations in 25 of 40 participants, consistent with inter-individual desynchrony that may obscure group-level rhythmicity. This underscores the value of personalized circadian assessment in heterogeneous clinical cohorts and the critical importance of validated, context-specific reference genes for such analyses. The reference gene pair and methodological framework established here lay the groundwork for future circadian biomarker studies in OSA, including pre- and post-treatment designs that may ultimately contribute to personalised chronotherapy in sleep medicine.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org; Table S1: qRT-PCR primer design criteria; Figure S1: Results of step 1 candidate reference genes using EndoGeneAnalyzer; Table S2: Primer sequences and efficiency of reference genes; Figure S2: Stability evaluation of candidate reference genes using RefFinder based on Step 1 data; Table S3: Results of standard deviation and stability from EndoGeneAnalyzer, comparing different OSA severity levels from step 1 of reference gene selection; Figure S3: Evaluation of step 2 reference gene performance under different conditions using EndoGeneAnalyzer; Table S4: Results of step 1 obtained from RefFinder representing stability values of different algorithms and standard deviation from the BestKeeper algorithm; Figure S4: Comparative assessment of reference gene stability using RefFinder based on data from Step 2; Table S5: Results of standard deviations and stability from EndoGeneAnalyzer analysis of the step 2; Table S6: Results of step 2 obtained from RefFinder representing stability values of different algorithms and standard deviation from BestKeeper algorithm; Table S7: Results of standard deviations and stabilities from EndoGeneAnalyzer analysis of the step 3; Table S8: Results of step 3 obtained from RefFinder representing stability values of different algorithms and standard deviation from BestKeeper algorithm; Table S9: The rhythmicity parameters obtained on group-specific cosinor models for each group of participants and for each of the observed genes; Table S10: The statistically significant rhythmicity parameters obtained on personalized cosinor models for each individual participant and for each of the observed genes; Table S11: Number of patients with statistically significant rhythmicity of core clock genes across OSA severity groups.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Commission for Medical Ethics of the Republic of Slovenia (approval No. 0120-65/2023/3, 14th of February 2023).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The original contributions presented in this study are included in the article and supplementary material. Further inquiries can be directed to the corresponding author.

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Abbreviations

The following abbreviations are used in this manuscript:

AHI	Apnea-hypopnea index
Cq	Quantification cycle
FDR	False discovery rate
OSA	Obstructive sleep apnea
PG	Polygraphy

Appendix A

RNA Isolation Protocol

Total RNA was extracted using TRI Reagent LS (Sigma-Aldrich) in a 1:1 ratio to the volume of thrombocyte-leukocyte layer samples. The samples were stored at -80 °C overnight. The next day, phase separation was done following the manufacturer's protocol. RNA precipitation was done by adding ice-cold isopropanol in a 1:1 ratio to the volume of the transferred aqueous phase. After washing the samples with 75% ethanol solution, we stored the samples at -80 °C overnight. The following day, we washed RNA two more times with a 75% ethanol solution. The RNA solubilisation was performed by drying the samples for approximately 20 minutes at room temperature and adding of appropriate volume (app. 20 µL) of RNA-free water to the RNA pellet. Then the samples were incubated for 5 minutes at 55 °C.

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