Research Article

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Validated HPLC-MS/MS quantification of melatonin in human breast milk from mothers of preterm infants confirms circadian rhythmicity

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Abstract: Melatonin in human breast milk follows a circadian rhythmicity and serves as a crucial chronobiological signal for neonatal development. Preterm infants are particularly vulnerable to melatonin deficiency due to the immature pineal gland function. Accurate analytical methods are essential for characterizing temporal patterns and assessing clinical relevance. We developed and validated a sensitive LC-MS/MS method for melatonin quantification in breast milk using liquid-liquid extraction with ethyl acetate. The method employed melatonin-d4 as internal standard and scheduled multiple reaction monitoring. Validation followed Eurachem and FDA guidelines and the method was applied to paired daytime and nighttime breast milk samples from mothers of preterm infants (n = 80). The method demonstrated excellent analytical performance with an LLOQ of 4.8 pg/ml, intraday and interday precision <5 %, and 97 % extraction efficiency. Nighttime melatonin concentrations (27.5 ± 16.8 pg/ml) were significantly higher than daytime levels (9.0 ± 10.0 pg/ml, $p < 0.0001$). Circadian variation was maintained across all lactational stages (colostrum, transitional, and mature milk). This validated LC-MS/MS method enables reliable quantification of physiological melatonin concentrations in breast milk and confirms circadian rhythmicity across lactation stages. The simplified sample preparation and robust performance make it suitable for clinical studies investigating chrononutrition and neonatal chronobiological development.

Keywords: melatonin; breast milk; antioxidant; LC-MS/MS

1 Introduction

Melatonin, an endogenous indoleamine hormone, primarily secreted by the pineal gland, plays crucial roles in regulating circadian rhythms, sleep patterns, and various physiological processes [1, 2]. Light-dark cycles regulate its production, with light inhibiting its synthesis and darkness promoting it [3]. Biosynthesis involves the transformation of tryptophan into serotonin, which is subsequently subjected to acetylation and methylation processes to produce melatonin [4]. Melatonin exerts its effects by binding to MT1, MT2 and MT3 receptors, which are present in multiple organs [3, 5, 6]. In addition, melatonin also functions as an antioxidant, anti-inflammatory agent, and regulator of mitochondrial homeostasis [7]. In humans, the fetus relies on maternal melatonin secretion during the prenatal period, as the pineal gland becomes functionally active after birth [8]. For the first 8–12 weeks after full-term birth, the initiation of pineal melatonin secretion seems to be impaired [9–12]. Preterm infants have even lower melatonin levels than term infants [13], and a longer period of melatonin deprivation due to immature neurological circuitry controlling melatonin production. They may lack chronobiological adherence and rhythmicity due to a desynchronized feeding schedule [9, 13]. Breastfeeding is crucial for newborn nutrition, and human breast milk is the main natural source of melatonin. However, there is a lack of research on factors that influence the melatonin content in human breast milk and their clinical significance in neonatal chronobiology, as reviewed in [14, 15].

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Melatonin levels in breast milk follow a circadian rhythm, with higher levels at night and lower levels during the day [16–18]. This rhythm is present in both preterm and term breast milk and peaks during the nighttime [17]. As a result, breastfeeding at night increases the newborn's supply of melatonin. Furthermore, melatonin levels differ in breast milk as a function of time, with colostrum being the highest, followed by transitional and mature breast milk [17]. These differences have implications for neonatal nutrition and metabolism in various ways. It helps entrain the infant's circadian rhythm and improves sleep. Melatonin has potent antioxidant and anti-inflammatory properties, which support cardiovascular health and possibly healthy gut microbiome and immune responses in infants [15, 19, 20]. High melatonin levels in the early neonatal period may provide antioxidant protection to compensate for the high oxidative stress in the perinatal period. Therefore, melatonin levels were significantly higher in newborns with higher birthweights [21]. To study melatonin intake provided by breastfeeding, highly sensitive analytical methods are necessary, as melatonin levels range in the low pg/ml range. Several methods have been developed to quantify melatonin in human breast milk, including ELISA [16], RIA [22], high-performance liquid chromatography (HPLC) with fluorescence detection [23, 24] and various liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques [25–27]. To fully understand the clinical significance of melatonin in breast milk for infant health and development, further research is necessary. Melatonin alleviates inflammatory and oxidative stress, and its favorable safety profile in preterm settings highlights its therapeutic potential. However, pharmacokinetic/pharmacodynamic uncertainties require further investigation, including quantifications of melatonin levels in both maternal and neonatal blood and colostrum, transitional and mature breast milk during the perinatal period. Accurate quantification enables the investigation of the relationship between breast milk melatonin levels and infant outcomes, such as sleep patterns, growth, and neurodevelopment. Quantification is also crucial for evaluating the efficacy of interventions aimed at optimizing melatonin content in breast milk, particularly for mothers of preterm infants who may benefit most from its protective effects. We therefore aimed to develop a highly sensitive LC-MS/MS method and, upon its validation, apply it to a cohort of daytime and nighttime breast milk samples. The goal of this study was to preliminarily delineate variations in melatonin concentrations between paired daytime and overnight breast milk samples in the cohort.

2 Materials and methods

2.1 Chemicals and reagents for preparation of calibrators and quality controls

Melatonin was obtained from Caelo (Caesar & Loretz GmbH, Hilden, Germany), while melatonin-d4, used as an internal standard (IS), was obtained from Cayman Chemicals (Ann Arbor, MI, USA). All solvents and additives used were of LC-MS-grade quality. Water, acetonitrile, and formic acid were purchased from VWR Chemicals (Radnor, PA, USA), while ammonium acetate was obtained from Sigma Aldrich (Steinheim, Germany). A melatonin stock solution was prepared by dissolving the compound in LC-MS-grade water at a concentration of 2 mg/ml. Calibrators and quality control (QC) samples were generated from this stock, using pooled breast milk as the matrix. Calibrators were prepared at concentrations of 0, 3.2, 16, 80, 400, 2,000 pg/ml and QCs at 8 and 200 pg/ml, respectively. The IS was diluted in water to a concentration of 250 pg/ml. All prepared solutions were stored at −20 °C until analysis. To preserve the integrity of light-sensitive compounds, samples were thawed and handled under conditions that minimized light exposure.

2.2 Sample preparation

Human mature breast milk samples were obtained from the Division of Neonatology of the Department of Pediatrics at the Paracelsus Medical University Salzburg, Austria. Breast milk samples were collected either manually or using an electric breast pump. All mothers were asked to follow a strict day-night cycle for the collection of daytime and nighttime samples. Collected samples were immediately protected from light and stored at −80 °C. To prepare the samples, a liquid-liquid extraction method was developed. Both breastmilk samples and internal standard were thawed under the prevention of light exposure. From the homogenized breast milk, 1.5 ml was mixed with 200 µl of internal standard in a 4 ml conical tube, and 2 ml of ethyl acetate were added. After vortexing the sample, it was centrifuged at 4,402 rcf for 7 min. The supernatant was stored in a new tube, keeping it protected from light, and the extraction from the breast milk sample was repeated. The supernatants were pooled and the solvent was evaporated using nitrogen gas at a temperature of 45 °C. The dried pellet was reconstituted in 50 µl of mobile phase B. The resuspended sample was

centrifuged at 21,130 rcf for 2.5 min, and the upper phase was transferred into a glass vial for mass spectrometric analysis.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the local ethics committee in Salzburg, Austria.

Informed consent: Informed consent has been obtained from all individuals included in this study.

2.3 High pressure liquid chromatography and mass spectrometry

The quantification was performed using an Exion LC (AbSciex, Darmstadt, Germany) connected to a TripleQuad 5500+ mass spectrometer (AbSciex, Darmstadt, Germany). The data was acquired using Analyst 1.7.1 (AbSciex, Darmstadt, Germany), and quantification was performed using SciexOS 1.7.0 (AbSciex, Darmstadt, Germany). Tuning of melatonin and the internal standard was performed in 30 % methanol solutions. Figure 1 shows MS/MS spectra as deduced from tuning experiments. Due to the absence of a melatonin-free matrix, we applied a standard addition quantification method. We used a Chromolith Performance RP-18e 100-3 mm HPLC column from Merck in Germany for reversed phase chromatography, along with a Polar-RP 4 × 2.0 mm Security Guard from Phenomenex in Germany and an injection volume of 15 µl. Eluent A consisted of water containing 0.1 % formic acid (FA) and 1 mM (mM) ammonium acetate. Eluent B consisted of 95 % acetonitrile (ACN), containing 0.1 % FA and 1 mM ammonium acetate. The gradient started with 100 % mobile phase A for 1.5 min and reached 70 % mobile phase B at 5.7 min, before reaching 99 % mobile phase B by 6.5 min. We set 100 % mobile phase A for 2.2 min after 7.8 min to re-equilibrate the column and maintained a flow rate of 0.65 ml/min throughout the 10-min acquisition period. The ion source parameters were optimized for the compounds in positive ionization mode. A scheduled multiple reaction monitoring (sMRM) system was used for quantification, along with an additional diverter valve and timed ionization to prevent contamination from the complicated mixture. We set the ion source parameters and gases at 5,200 V, 650 °C, CUR: 38, GS1: 70, and GS2: 60.

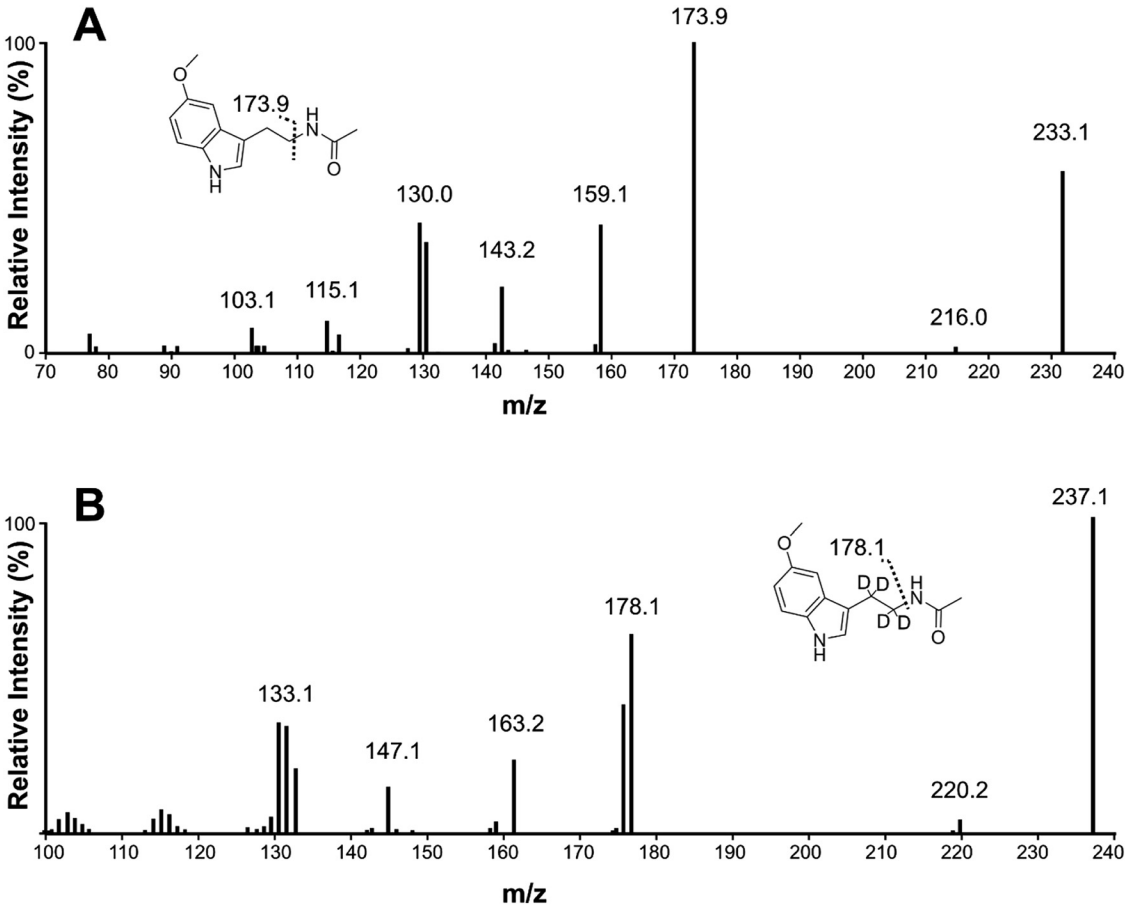

Figure 1: Chemical structures and MS/MS electrospray fragment spectra of melatonin (A) and the internal standard d4-melatonin (B).

Table 1: MRM parameters, including mass transitions, collision energy (CE) and declustering potential (DP).

Analyte	Precursor	Quantifier			Qualifier		
	m/z	*m/z*	CE (V)	DP (V)	*m/z*	CE (V)	DP (V)
Melatonin	233.1	173.9	21	76	159.1	35	76
Melatonin-d4	237.1	178.1	23	66	163.2	31	66

Table 1 provides the mass transitions for the quantifier and qualifier of melatonin, the internal standard melatonin-d4, and method-specific parameters.

2.4 Method validation

The validation was conducted according to the Eurachem guideline [28] to evaluate linearity, selectivity, sensitivity, precision, and accuracy. The FDA guideline [29] was applied to evaluate ion suppression and recovery. Briefly, to assess selectivity, blank matrix samples (n = 9) were compared to spiked melatonin. Linearity was evaluated using nine replicates based on the mean and standard deviation of each level. Over the linear range, calibration curves were created with a weighting factor of $1/x^2$. Furthermore, selectivity was enhanced by using the ion ratio of quantifier and qualifier areas. The lower limit of quantification (LLOQ) was used to define sensitivity. Six replicates of both quality control (QC) levels were used to assess intraday and interday precision and accuracy. To analyze ion suppression and enhancement, we introduced samples without melatonin after extraction and slowly added diluted melatonin with a syringe. The evaluation of analyte recovery was conducted by comparing samples spiked before preparation to samples spiked after preparation.

2.5 Test cohort

After successful validation, the method was used to quantify endogenous melatonin levels in breast milk with paired daytime and nighttime samples from mothers who gave preterm birth. The breast milk samples (n = 80) were transported by excluding light exposure and stored at −80 °C before analysis. Statistical calculations were performed with GraphPad Prism 10 (LLC) using a paired t-test and one-way ANOVA. Results with $p < 0.05$ were considered as statistically significant.

3 Results

3.1 Selectivity, sensitivity, linearity and carryover

Out of the three tested methods for sample extraction – protein precipitation, LLE, and SPE – LLE using acetyl acetate gave the best and most accurate results (data not shown). Melatonin devoid samples showed no additional signals for melatonin and melatonin-d4. Both blank matrix samples and analyte-spiked matrix showed specific signals at the expected retention time for the quantifier MRM transition for melatonin. Detected blank levels reflected endogenous levels in the matrix (Figure 2, left). Linearity ranged from the LLOQ of 4.8 pg/ml, which was detected as 10 times the standard deviation of the lowest calibrator calculated by nine replicates to the ULOQ of 2,000 pg/ml. The average coefficient of variation (CV, calculated as standard deviation/mean × 100) for all six standards was 4.3 %. The LLOD as derived from S/N ratios of 3 was 1.4 pg/ml. We observed a stable mean ion ratio of qualifier to quantifier mass transitions areas of 0.415 ± 0.05 (n = 50). Figure 2 displays representative chromatograms for a blank and a spiked sample. We observed no significant carryover exceeding 5 % of the LLOQ during validation.

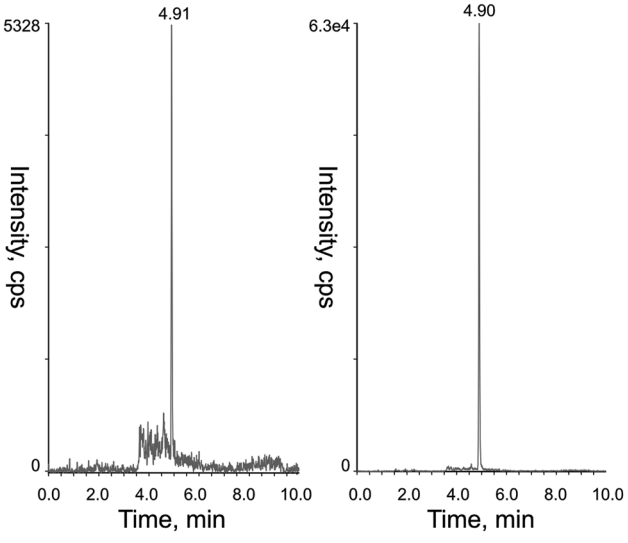

Figure 2: Extracted ion chromatogram (XIC) for melatonin (*m/z* 233.1/173.9) of blank sample with endogenous levels of 7 pg/ml (left) and calibrator 4 at 407 pg/ml sample (right).

3.2 Intraday and interday accuracy and precision, ion suppression, and recovery

Intraday precision CV was 4.7 % (mean accuracy 93.7 ± 4.4 %, n = 8) and interday precision CV was 3.6 % (mean accuracy 97.1 ± 3.46 %, n = 6) for the lower QC. For the higher QC, intraday precision CV was 3.3 % (mean accuracy 96.2 ± 3.8 %, n = 8) and interday precision CV was 3.2 % (mean accuracy 95.8 ± 3.87 %, n = 8). We detected no significant ion suppression or ion enhancement throughout the entire runtime upon analysis of analyte free samples with constant post-column infusion of melatonin via a tee connector (n = 6). Extraction efficiency reached 97 % (n = 6) over both QC levels, when analyzing recovery during sample preparation.

3.3 Cohort samples

In 26 of all 80 samples, the concentration of melatonin was below LLOQ. The average melatonin level in the breast milk samples was determined at 20.9 ± 17.2 pg/ml, whereas a difference between daytime and nighttime samples was noted. In nighttime samples, melatonin levels were significantly higher than in daytime samples ($p < 0.0001$). The mean concentration in nighttime samples was 27.5 ± 16.8 pg/ml, whereas in daytime samples it was 9.0 ± 10.0 pg/ml. In the next step, we compared the daytime and nighttime samples of the different stages of lactation (Figure 3). We observed mean melatonin day concentrations of 15.8 ± 16.7 pg/ml (n = 7), 10.9 ± 9.0 (n = 5) pg/ml, and 6.8 ± 3.9 (n = 8) pg/ml for colostrum, transitional, and mature milk, respectively. The mean melatonin levels in night samples were 28.4 pg/ml, 22.6 pg/ml, and 30.4 pg/ml for colostrum, transitional, and mature milk, respectively. Overall, in different lactational phases of breast milk, melatonin levels in daytime samples differed strongly, whereas the observed differences between the nighttime samples were less pronounced. Differences in nighttime samples were smaller, and levels of melatonin did not significantly differ between the lactational phases.

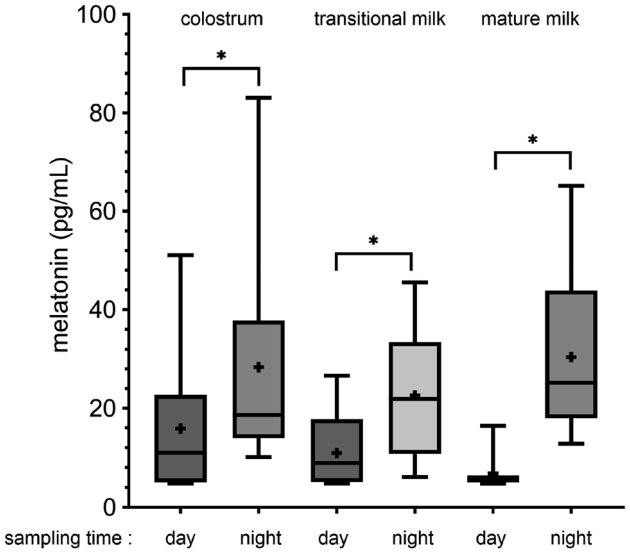

Figure 3: Day- and nighttime melatonin concentrations from the indicated lactational phases. * denotes $p < 0.01$.

4 Discussion

Given the importance of melatonin in newborn chronobiological development, accurate quantification methods are essential for understanding dose-response relationships, temporal secretion patterns, maternal and other factors affecting melatonin concentrations in breast milk. However, analytical methods for melatonin and studies investigating melatonin concentrations in breastmilk are limited. The available analytical repertoire includes ELISA [16], RIA and GC-MS [22], HPLC-FLD [30] and LC-MS [25–27]. However, each of the reported methods has inherent limitations. ELISA tests are limited by antibody specificity issues, including crossreactivity and inter-assay variability, lower analytical sensitivity, and a restricted dynamic range. ELISA LLOQ was reported to reach low pg/ml values (1.6 pg/ml for the Melatonin ELISA EIA-1431, DRG Instruments GmbH, Germany and 15.63 pg/ml for the Human Melatonin ELISA Kit EEL056 Thermofisher Scientific). These ELISA kits were validated for plasma and serum only and even weak cross-reactivities can cause strongly false-high results in the presence of abundant cross-reacting molecules [31]. A widely used commercial RIA kit (RK-MEL2, Novolytix, Switzerland) reports an LLOQ of 0.9 pg/ml for serum or plasma. RIA methods offer high sensitivity but reduced selectivity and there is the potential of cross-reactivity with various indolic compounds in the matrix, which may cause falsely elevated signals.

HPLC-FLD is limited by lower selectivity and sensitivity, a restricted dynamic range, and a high susceptibility to matrix interferences in comparison to LC-MS. Though methods that use the native fluorescence of melatonin for detection have been reported [23], derivatization may be needed to increase the sensitivity [32, 33], which is time-consuming and increases the analytical variability. Although HPLC–ECD have been reported [34], they remain uncommon in many laboratories and are limited by low selectivity, matrix interferences, and reduced robustness in complex biological samples. So far, no HPLC-ECD method for breast milk has been reported. Nevertheless,

substantial progress has been made in the development of electrochemical and optical sensors for melatonin detection, these techniques are not yet fit for purpose in routine quantitative analysis [35]. GC-MS requires mandatory derivatization because melatonin is thermally labile, and this step is labour-intensive and prone to incomplete reactions [31, 36]. Therefore, additional LC-MS/MS methods without derivatization steps and straightforward sample preparation are clearly needed to facilitate most accurate melatonin quantifications in breastmilk. This study reports a rapid, reliable and sensitive LC-MS/MS method, which proved sufficient sensitivity and accuracy and was fit for purpose of quantifying low endogenous melatonin levels in human breast milk. Melatonin represents a critical chronobiological signal that plays a fundamental role in infant development and circadian entrainment. Therefore, its quantification is an essential analytical requirement in understanding chrononutrition, the temporal delivery of nutrients and bioactive compounds that may influence physiological rhythms in developing infants. The LC-MS/MS method demonstrates superior analytical performance compared to existing techniques and represents the gold standard. The presented method contributes a practical, validated approach specifically for human breast milk analysis. As such, the LLOQ of 4.8 pg/ml compares with other methods [23], that reported 1 pg/ml for cow milk and another method with higher sensitivity but using complex online enrichment [24]. The here reported simplified sample extraction procedure eliminates solid-phase extraction steps, reducing analysis time and costs while maintaining excellent precision and accuracy. The cohort findings of this study are in accordance with a previous study that reported peak melatonin levels in breast milk during nighttime [37, 38]. The observed mean peak value was 46.9 ± 4.2 pg/ml (mean ± SEM) as derived from four studies measuring at midnight [39–42]. This is in line with levels in the analyzed cohort, although mean nighttime levels were lower with a mean of 27.5 ± 16.8 pg/ml. This may reflect differences between cohorts, analytical methodology or sampling times. The samples included in this study were not all collected at suspected peak times. In contrast, the above-mentioned studies reported a lack of sensitivity as they could not quantify melatonin in daytime samples. With this newly developed method, we were able to quantify melatonin levels in daytime samples with a mean concentration of 9.0 ± 10.0 pg/ml. The sensitivity derived of the presented method is therefore in the range of a comparable existing LC-MS/MS method reporting an LLOQ of 1 pg/ml [25]. Differences in analytical equipment and sample preparation may cause the slightly reduced sensitivity of this method. The advantage of this method is that sample preparation does not require a solid phase extraction step and is therefore faster and more cost efficient. In addition, sensitivity might be increased by upscaling and using larger sample volumes. In the analyzed cohort, we observed larger differences between melatonin levels in daytime samples, whereas fluctuations between nighttime samples were less pronounced. The trend may reflect the relatively low number of samples included and needs further investigations. This maternal-infant chronobiological communication seems particularly crucial during the neonatal period when infants have not yet developed autonomous circadian rhythmicity and melatonin in breast milk is their primary source of the hormone. This temporal variation ensures that night-time breastfeeding results in increased melatonin supply to the newborn which, in addition to the entrainment of the infant's circadian rhythms [15], facilitates appropriate sleep-wake cycle development. The melatonin absorbed by the infant through breast milk has implications extending beyond immediate sleep patterns. Melatonin from breast milk influences weight gain in infants, limiting the development of obesity and comorbidities in the long term [20], highlighting the metabolic consequences of chronobiological programming during early life. To set melatonin levels in a better context, a validated method for the quantification of melatonin from plasma/serum samples is needed to correlate melatonin levels in mothers and infants. In addition, it would be of great advantage to include additional non-invasive biological matrices such as saliva, although its standardized acquisition from neonates is challenging. The development of an LC-MS/MS method for quantifying melatonin from plasma samples is currently in progress. The quantification of melatonin in breast milk and blood samples enables the investigation of the relationship between maternal circadian health, milk composition, and infant development outcomes. This may be of particular importance for preterm infants. The here reported method may be useful to advancing the understanding of chrononutrition and developing evidence-based recommendations for optimizing infant circadian health through breastfeeding practices and is applicable to other nutritional milk products for infants. The described method has limitations, although it seems adequate for physiological concentrations. Still, it was not possible to quantify melatonin in 26 of all 80 samples. To overcome this issue, sample extraction procedures may be optimized, or more adequate, the existing method can be upscaled to larger sample volumes. The study focused on preterm infant mothers and validation in term populations would strengthen generalizability. Future research should incorporate paired maternal-infant sample analysis and

investigate relationships between breast milk melatonin and infant developmental outcomes.

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