## Early-life endocrine regulation and neurodevelopmental outcomes

Hollanders, J.J.

2020

# 8

# Diurnal rhythmicity in breast-milk glucocorticoids and infant behavior and sleep at age three months

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In progress.

## ABSTRACT

### Purpose

In previous studies, associations between breast-milk cortisol levels obtained on one occasion and infant neurodevelopment were demonstrated. However, more recent evidence indicates that breast-milk cortisol and cortisone concentrations follow a diurnal rhythm, and therefore these levels fluctuate throughout the day. We studied associations between breast-milk glucocorticoid (GC) rhythmicity and infant behavior and sleep.

### Methods

We included 59 mothers, and their infants, of whom 17 had consulted an expert center during pregnancy for an increased risk of psychological distress. At 1 month postpartum, breast milk was sampled (on average 6 times) over a 24h period for assessment of cortisol and cortisone using LC-MS/MS, and experienced maternal distress was assessed using the Hospital Anxiety and Depression Scale questionnaire. At 3 months postpartum, infant behavior was assessed using the Infant Behavior Questionnaire, and infant sleep was quantified by questionnaire. Associations between breast-milk GC rhythmicity (maximum, delta, and Area Under the Curve [AUC]) and infant behavior and sleep were tested with linear regression analyses.

### Results

No consistent associations between breast-milk GC rhythmicity or exposure and infant behavior or sleep were found.

### Conclusions

Breast-milk GC rhythmicity or exposure at 1 month postpartum was not associated with infant behavior or sleep at the age of 3 months. Findings from previous studies linking breast-milk cortisol to infant neurodevelopment might be biased by the lack of GC measurements across the full diurnal cycle, and should therefore be interpreted with caution.

## INTRODUCTION

Approximately 15% of pregnant women in Western countries are diagnosed with psychiatric conditions.[1] Depressive and anxiety disorders are associated with alterations in hypothalamic-pituitary-adrenal (HPA-) axis activity, such as a lower morning peak or less diurnal variability in cortisol level.[2,3] Maternal glucocorticoids (GCs) that cross the placenta may influence the fetal HPA-axis, possibly through alterations in the expression of GC and mineralocorticoid receptors in the developing hippocampus and amygdala.[4] In humans, fetal exposure to maternal depression or anxiety symptoms was associated with a more fearful temperament and disorganized sleep in infancy, along with a flattened cortisol rhythm.[5-7] The results of these studies suggest that fetal GC exposure may alter neurodevelopment and HPA-axis settings in later life.[8-10]

The development of an adult-type diurnal cortisol rhythm, characterized by cortisol concentrations that are higher in the morning than in the evening, is thought to start at approximately 1 month of age and continues to develop during the first year of life.[11] It has been hypothesized that the development of HPA-axis rhythmicity may serve as a modulator for the development of behavioral rhythms, such as the sleep-wake cycle.[12,13] Previous research has demonstrated that a diurnal GC rhythm develops before sleep rhythmicity is established around the age of 2-4 months.[14,15] Multiple factors may be involved in the development of HPA-axis rhythmicity, such as environmental time cues (e.g., daylight) and maternal care.[15] In addition, it has been proposed that non-nutritive bioactive compounds in breast milk might be involved in the development of sleep regulation in infants.[16]

Animal studies have shown that GCs in breast milk are able to cross the intestinal wall and to enter the circulation in offspring.[17,18] Among Rhesus monkeys, offspring exposed to higher levels of breast-milk cortisol were found to exhibit a more nervous, less confident behavior and impulsivity, albeit with few gender-specific differences.[19-21] In rats, exposure to physiological ranges of ingested GCs was associated with reduction of fearfulness and stress-induced corticosterone secretion throughout the lifespan.[22] In breastfed human infants, exposure to maternal cortisol has also been associated with behavior.[23-25] Two studies showed that higher breast-milk cortisol was associated with negative affectivity among girls, but not among boys.[24,26] Another study showed that higher plasma cortisol, which has strong correlation with breast-milk cortisol,[27] was associated with increased infant fear behavior [23]. However, none of these studies collected samples multiple times during the day or around the morning peak of cortisol secretion, in spite of evidence indicating that breast-milk GCs follow the diurnal rhythm of maternal HPA-axis activity.[27] Some of these studies statistically adjusted for inter-individual differences in collection time, which assumes that the cortisol slope barely differs between subjects. However, it has been demonstrated that *post-hoc* statistical

correction for time of sampling may not be able to provide an adequate representation of an individual's HPA-axis dynamics.[27,28]

The aim of this study was to investigate associations between exposure to breast-milk GCs over a 24-hr period at 1 month postpartum, and infant behavior and sleep at 3 months postpartum. In this study, we oversampled mothers at risk of psychological distress during and after pregnancy in an attempt to capture a wide range of maternal HPA-axis activity, since depression and anxiety have previously been associated with GC rhythmicity.[2,3]

## METHODS

### Participants

From March 2016 to July 2017, mothers were approached within the first days after delivery at the maternity wards of the Amsterdam University Medical Center, location VUmc (Group 1, n=42), and the OLVG hospital (Group 2, n=17), The Netherlands. Mothers included at the OLVG hospital had an increased risk of psychological distress and therefore consulted the Psychiatric Obstetric Pediatric (POP) outpatient clinic during pregnancy. Breastfeeding mothers of infants born after full-term gestation (37-42 weeks of pregnancy) with a birth weight appropriate for gestational age (i.e., between -2 and 2 SD score) were eligible for inclusion. Exclusion criteria were preeclampsia/HELLP, multiple pregnancy, consumption of >7 IU of alcohol per week, fever >38.5 °C at the time of sampling, and major congenital anomalies. Additionally, mothers who used drugs other than 'over the counter' drugs were excluded, with the exception of Selective Antidepressants (SADs) use for mothers included at the OLVG. Approval of the Medical Ethics Committee of the Amsterdam University Medical Center, location VUmc was obtained (*protocol number 2015.524),* and written informed consent was obtained from all participating mothers.

### Data collection

#### *Infant and maternal characteristics*

During the first days postpartum, maternal and infant characteristics were obtained by questionnaire (Table 1). At the time of milk sample collection (1 month postpartum), mothers were asked to fill in the Hospital Anxiety and Depression Scale (HADS) for assessment of maternal psychological distress experienced during the past 2 weeks.[29] The HADS contains 14 items, including seven items for depressive symptoms (Hospital Depression Subscale [HDS]) and seven items for anxiety symptoms (Hospital Anxiety Subscale [HAS]). Items are scored as 0-3, and a score ≥8 on either subscale indicates clinically relevant depression and/or anxiety symptoms. Accordingly, we defined increased maternal stress as HDS score and/or HAS score ≥8.

**Table 1:** Maternal and infant characteristics of participants[1]

| Maternal characteristics | Group 1[2] (n=42) | Group 2[3] (n=17) |
|---|---|---|
| Maternal age, yrs | 33.6 ± 4.7 | 31.6 ± 4.7 |
| Maternal BMI, kg/m$^2$ | 22.3 ± 2.8 | 22.9 ± 2.2 |
| Social Economic Status (SES)[4] | 0.6 ± 1.2 | 0.4 ± 1.3 |
| Caucasian ethnicity | 34 (81) | 15 (88) |
| Primiparity | 23 (55) | 7 (41) |
| Selective antidepressant use | 0 | 12 (71)* |
| HAS/HDS score≥8 1 mo. pp (n=58)[5] | 6 (15) | 6 (35) |
| **Neonatal characteristics** | | |
| Male gender | 25 (60) | 11 (65) |
| Birth weight (grams) | 3389 ± 39 | 3561 ± 498 |
| Gestational age (weeks) | 39.1 ± 1.1 | 39.9 ± 1.3* |
| Vaginal birth (n=58)[5] | 20 (49) | 13 (77) |
| ≥80% breast milk 3 mo. pp (n=58)[5] | 37 (90) | 14 (82) |

[1] Values are presented as means ± SD or frequencies (%).
[2] Mothers included at the maternity ward of the Amsterdam UMC.
[3] Mothers included at the Psychiatry Obstetric Pediatric (POP) expert center, OLVG hospital
[4] Z-score based on average income, % low income, % low-skilled and % unemployed civilians per postal code area, based on data from the Dutch Social Cultural Project office [2014, The Netherlands]).
[5] Three participating mothers in group 1 did not provide these data
*p<.05

### *Breast-milk sample collection*

At 30±5 days postpartum, 1-2mL of breast milk was collected before every feed over a 24h-period, either manually or with a breast pump. Mothers were requested to report the exact time of the sample collection, since they were breastfeeding their child on demand. Mothers were asked to abstain from alcohol at least one day before and during the sample collection. Following collection, samples were stored in plastic tubes at -20°C until they were thawed for analysis.

### *Infant Behavior and Sleep*

At 3 months (±2 wks) postpartum, mothers were asked to fill in the Infant Behavior Questionnaire (IBQ) and a questionnaire for the quantification of infant sleep. The IBQ is a validated instrument for the assessment of temperament in infants aged 3 months to 1 year.[30,31] The original IBQ contains 94 items on six scales of temperament dimensions (distress to limitations, approach to novel stimuli, soothability, duration of orienting, smiling and laughter, and activity) that show considerable stability over time.[32] The IBQ assesses behavior on a 7-point Likert scale, with answers ranging from 'never' to 'always', or 'does not apply'. To minimize recall bias, the answers pertain to the infant's behavior over the past 1-2 weeks. The mean scoring represents the outcome for each dimension

separately. The sleep questionnaire (see Supplementary File 1) included the total hours of night-time and day-time sleep, the number of daytime naps, and the number of nights with more than 6 hours of consecutive sleep during one week.

### Determination of cortisol and cortisone levels in milk

An isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was used to assess cortisol and cortisone concentrations in milk, as described previously.[33] In short, milk samples were washed with hexane after adding internal standards to the samples ($^{13}C_3$ labeled cortisol and cortisone). Samples were extracted using Isolute plates (Biotage, Uppsala, Sweden) and analyzed by LC-MS/MS (Acquity with Quattro Premier XE, Milford MA, USA, Waters Corporation). For cortisol, the intra-assay coefficient of variation (CV) was 4 to 5%, and for cortisone it was 5% at different levels. For both cortisol and cortisone, the inter-assay CV was <9%, and the Lower Limit of Quantitation was 0.5 nmol/L.

### Data analyses

Breast-milk GC parameters were recorded over a period of 24 hours, and were defined as: maximum (i.e., the maximum measured concentration), delta (Δ, i.e., the difference between the maximum and minimum measured concentrations), and Area Under the Curve increase (AUCi) and ground (AUCg) per hour collection. AUCi/h was used as an index for GC rhythmicity. AUCg/h was used to reflect total breast-milk GC exposure. Both AUCi/h and AUCg/h were calculated using the trapezoid rule.[34] Participants who did not provide morning samples (between 05:00-10:00 a.m.) or with <8 hours of total sample collection time were excluded from the analyses.

Maternal and infant characteristics were compared between mothers who had no increased risk of psychological distress (group 1) and those who had (group 2), using independent samples T-tests and Chi-square tests (Table 1). Of all characteristics, only maternal antidepressant use and gestational age were significantly different between the groups. Although one-third of the mothers monitored at the POP outpatient clinic reported increased psychological distress, no statistically significant differences were found between group 1 and group 2 with regard to HADS-score as well as breast-milk GC parameters. Therefore, all mother-infant pairs were analyzed as one group, while considering gestational age and maternal use of antidepressants as potential confounders. Subsequently, associations between breast-milk GC rhythmicity or total GC exposure, and IBQ scores or infant sleep were tested using linear or logistic regression, as appropriate. Second, we performed multivariate analyses correcting for a set of potential confounders based on previous literature (infant gender, socio-economic status and maternal stress) or statistical impact (maternal use of antidepressants and gestational age).[23,24,26] Results were presented as beta or Odds Ratio (OR) [95% confident interval (CI)]. A p-value <.05 was considered statistically significant.

## RESULTS

Figure 1 shows the stepwise inclusion procedure for the study. A total of 303 mothers were approached, of whom 110 gave written informed consent. Of these, 59 completed the study. Main reasons for drop-out were switching to formula feeding or withdrawal of consent. Characteristics of the participating mothers and their infants are presented in Table 1. Breast-milk GC parameters, IBQ scores and sleep outcomes are shown in Table 2. Mothers collected on average 6 (Range: 4 to 8) milk samples over a 24-hour period.

### Breast-milk glucocorticoids and infant behavior

Tables 3 and 4 show the multivariate associations between breast-milk GC parameters and infant behavior. An association between total breast-milk cortisol exposure and more infant soothability was found (β= 0.15 [0.02 to 0.29], p<.05). Other breast-milk GC parameters were not associated with infant behavior.

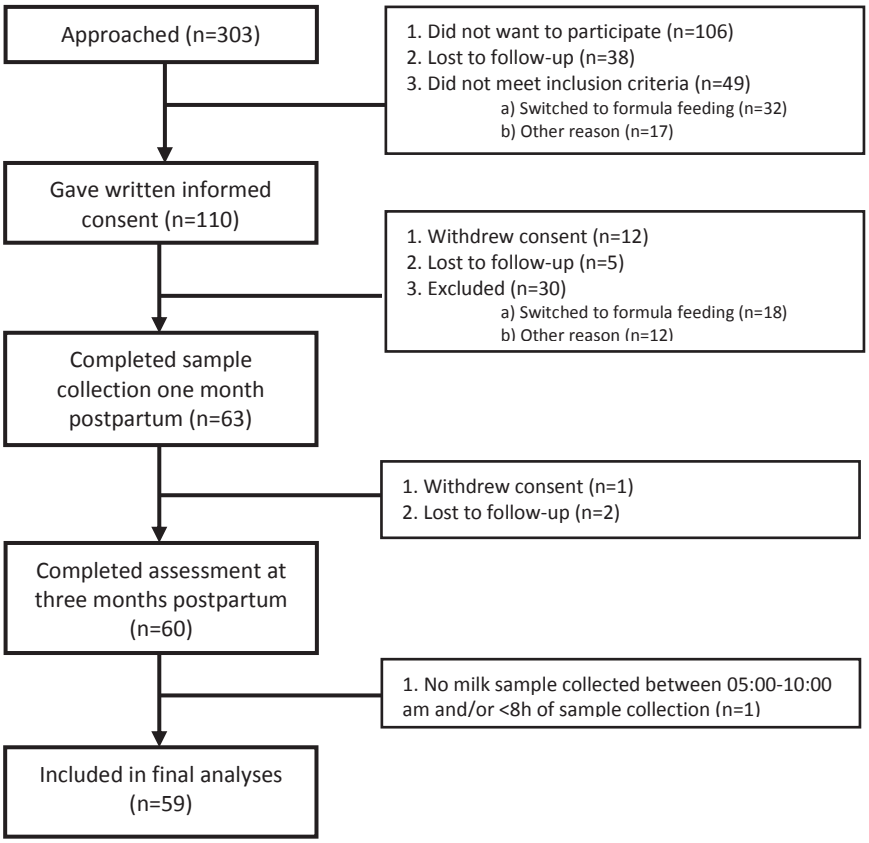


**Figure 1:** Flowchart of the inclusion of mother-infant pairs

**Table 2:** Measurements by group[1]

| | Group 1[2] (n=42) | Group 2[3] (n=17) | Total (n=59) |
|---|---|---|---|
| **Breast-milk GC levels** | | | |
| Cortisol maximum (nmol/L)<br>*Average time of maximum* | 15.8 ±8.9 [9.2 to 20.8]<br>*8:15h* | 13.6 ±8.2 [8.8 to 16.7]<br>*6:45h* | 15.2 ±8.7 [9.2 to 19.2]<br>*7:45h* |
| Δcortisol (nmol/L) | 14.7 ±9.0 [8.0 to 19.0] | 12.5 ±8.3 [8.3 to 16.0] | 14.2 ±8.8 [8.1 to 18.4] |
| AUCi/h of cortisol in 24h | 4.1 ±2.3 [2.6 to 5.0] | 3.0 ±1.5 [1.9 to 3.8] | 3.7 ±2.2 [2.2 to 4.7] |
| AUCg/h of cortisol in 24h | 5.2 ± 2.4 [3.6 to 6.2] * | 3.7 ± 1.5 [2.5 to 4.7] | 4.8 ± 2.3 [3.3 to 5.8] |
| Cortisone maximum (nmol/L)<br>*Average time of maximum* | 36.0±10.6 [27.7 to 42.7]<br>*08:00h* | 33.8 ±9.0 [26.6 to 40.2]<br>*8:30h* | 35.3 ±10.1 [27.4 to 42.3]<br>*8:15h* |
| Δcortisone (nmol/L) | 28.9 ±10.1 [23.5 to 35.6] | 27.8 ±8.7 [22.8 to 32.2] | 28.6 ±9.7 [23.3 to 34.2] |
| AUCi/h of cortisone in 24h | 12.2 ±4.4 [10.0 to 14.9] | 10.8 ±4.4 [8.0 to 14.5] | 11.8±4.4 [9.4 to 14.7] |
| AUCg/h of cortisone in 24h | 19.2 ± 5.7 [16.0 to 21.8] | 16.8 ± 4.5 [13.2 to 19.8] | 18.5 ± 5.4 [14.8 to 21.1] |
| **IBQ domain [4]** | | | |
| Activity | 3.2 ± 0.9 | 3.0 ± 0.7 | 3.1 ± 0.9 |
| Distress to limitations | 3.3 ± 0.9 | 3.2 ± 0.9 | 3.3 ± 0.9 |
| Approach to novel stimuli | 2.0 ± 1.0 | 2.1 ± 0.7 | 2.0 ±0.9 |
| Duration of orienting | 3.6 ± 1.2 | 4.0 ± 0.9 | 3.7 ± 1.2 |
| Smiling and laughter | 4.3 ± 1.0 | 4.5 ± 1.0 | 4.4 ± 1.0 |
| Soothability | 5.0 ± 1.2 | 4.9 ± 1.1 | 5.0 ± 1.1 |
| **Infant sleep parameters** | | | |
| Hours of night-time sleep | 8.2 ± 2.3 | 8.6 ± 2.3 | 8.3 ± 2.3 |
| Hours of daytime sleep | 4.3 ± 1.9 | 4.5 ±1.6 | 4.4 ± 1.8 |
| Number of naps during daytime (n=58) | 3.3 ± 0.8 | 3.3 ± 1.1 | 1.5 ± 1.0 |
| Number of nights per week with >6h sleep | 4.1 ± 3.0 | 4.4 ± 2.7 | 4.2 ± 2.9 |

[1] Values are presented as means ± SD and range. Maximum= maximum value during 24h period. Δ= Difference between maximum and minimum value during 24h period. AUCi/h= Area under the curve for increase per hour collection. AUCg/h= Area under the curve with respect to ground per hour collection. Groups were compared using the independent samples T-test.

[2] Mothers included at the maternity ward of the Amsterdam UMC.

[3] Mothers included at the Psychiatry Obstetric Pediatric (POP) expert center, OLVG hospital.

[4] IBQ= Infant Behavior Questionnaire.

*p<.05

## Breast-milk glucocorticoids and infant sleep

Tables 3 and 4 show the multivariate associations between breast-milk GC parameters and infant sleep. A positive association between breast-milk delta cortisone and infant sleep at night-time was found (β= 0.07 [0.01 to 0.20], p<.05). Other breast-milk GC parameters were not associated with infant sleep.

**Table 3:** Associations between breast-milk cortisol parameters and infant behavior and sleep[1]

| **Infant behavior outcomes** | **Maximum cortisol** (nmol/L) | **Δcortisol** (nmol/L) | **Cortisol AUCi/h** (in 24h) | **Cortisol AUCg/h** (in 24h) |
|---|---|---|---|---|
| Distress to limitations | -0.02 [-0.05 to 0.01] | -0.02 [-0.04 to 0.01] | -0.02 [-0.13 to 0.09] | -0.03 [-0.13 to 0.08] |
| Approach to novel stimuli | -0.02 [-0.05 to 0.01] | -0.02 [-0.05 to 0.01] | -0.05 [-0.16 to 0.06] | -0.03 [-0.13 to 0.07] |
| Soothability | 0.02 [-0.02 to 0.04] | 0.02 [-0.02 to 0.05] | 0.13 [-0.02 to 0.27] | 0.15 [0.02 to 0.28] * |
| Smiling and laughter | 0.01 [-0.02 to 0.04] | 0.01 [-0.02 to 0.04] | 0.005 [-0.11 to 0.12] | 0.02 [-0.10 to 0.13] |
| Duration of orienting | -0.004 [-0.04 to 0.03] | -0.01 [-0.05 to 0.03] | -0.04 [-0.19 to 0.11] | 0.03 [-0.11 to 0.17] |
| Activity | 0.01 [-0.02 to 0.04] | 0.01 [-0.02 to 0.03] | 0.02 [-0.09 to 0.13] | 0.05 [-0.05 to 0.15] |
| **Infant sleep outcomes** | | | | |
| Hours of night-time sleep | 0.04 [-0.03 to 0.11] | 0.05 [-0.02 to 0.12] | 0.07 [-0.22 to 0.35] | -0.03 [-0.29 to 0.23] |
| Hours of daytime sleep | 0.01 [-0.05 to 0.07] | 0.01 [-0.05 to 0.07] | 0.01 [-0.23 to 0.25] | 0.01 [-0.23 to 0.22] |
| Number of nights per week with >6h sleep[2] (0-4 days=0, 5-7 days=1) | 0.98 [0.92 to 1.04] | 0.98 [0.92 to 1.05] | 0.92 [0.71 to 1.20] | 0.87 [0.67 to 1.12] |
| Number of naps during daytime[2] (<3 naps=0, ≥3 naps=1) | 0.93 [0.86 to 1.01] | 0.94 [0.88 to 1.02] | 0.87 [0.66 to 1.15] | 0.77 [0.57 to 1.04] |

[1]Values are presented as betas or ORs [95% CI] and adjusted for gender, socio-economic status, elevated HADS score at one mo. pp (HAS/HDS score ≥8), maternal use of antidepressants and gestational age.

[2]OR.

Maximum= maximum value during 24h period. Δ= Difference between maximum and minimum value during 24h period. AUCi/h= Area under the curve for increase per hour collection. AUCg/h= Area under the curve with respect to ground per hour collection. *p<0.05.

**Table 4:** Associations between breast-milk cortisone parameters and infant behavior and sleep[1]

| **Infant behavior outcomes** | **Maximum cortisone** (nmol/L) | **Δcortisone** (nmol/L) | **Cortisone AUCi/h** (in 24h) | **Cortisone AUCg/h** (in 24h) |
|---|---|---|---|---|
| Distress to limitations | -0.01 [-0.03 to 0.02] | -0.01 [-0.04 to 0.01] | -0.03 [-0.09 to 0.03] | -0.01 [-0.05 to 0.04] |
| Approach to novel stimuli | -0.02 [-0.04 to 0.01] | -0.02 [-0.05 to 0.003] | -0.03 [-0.09 to 0.03] | -0.004 [-0.05 to 0.04] |
| Soothability | 0.003 [-0.03 to 0.04] | 0.001 [-0.04 to 0.04] | 0.02 [-0.06 to 0.10] | 0.02 [-0.04 to 0.08] |
| Smiling and laughter | -0.01 [-0.04 to 0.02] | -0.01 [-0.04 to 0.02] | -0.04 [-0.10 to 0.02] | -0.03 [-0.08 to 0.02] |
| Duration of orienting | 0.002 [-0.03 to 0.04] | -0.01 [-0.04 to 0.03] | -0.04 [-0.12 to 0.03] | -0.001 [-0.06 to 0.06] |
| Activity | 0.001 [-0.02 to 0.03] | -0.006 [-0.03 to 0.02] | -0.02 [-0.08 to 0.03] | 0.01 [-0.04 to 0.05] |
| **Infant sleep outcomes** | | | | |
| Hours of night-time sleep | 0.06 [-0.001 to 0.12] | 0.07 [0.006 to 0.14] * | 0.11 [-0.04 to 0.25] | 0.06 [-0.06 to 0.18] |
| Hours of daytime sleep | -0.01 [-0.06 to 0.05] | 0,001 [-0.06 to 0.06] | 0.001 [-0.13 to 0.13] | -0.03 [-0.13 to 0.07] |
| Number of nights per week with >6h sleep[2] (0-4 days=0, 5-7 days=1) | 0.98 [0.92 to 1.04] | 0.99 [0.94 to 1.06] | 0.94 [0.82 to 1.08] | 0.89 [0.79 to 1.0] |
| Number of naps during daytime[2] (<3 naps=0, ≥3 naps=1) | 0.94 [0.88 to 1.00] | 0.97 [0.91 to 1.03] | 1.00 [0.87 to 1.15] | 0.88 [0.78 to 1.01] |

[1]Values are presented as betas or ORs [95% CI] adjusted for gender, socio-economic status, elevated HADS score at one mo. pp (HAS/HDS score ≥8), maternal use of antidepressants and gestational age.

[2]OR. Maximum= maximum value during 24h period.

Δ= Difference between maximum and minimum value during 24h period. AUCi/h= Area under the curve for increase per hour collection. AUCg/h= Area under the curve with respect to ground per hour collection.

*p<0.05.

## DISCUSSION

In this study, with few exceptions, no associations were found between breast-milk GC rhythmicity and total exposure at 1 month postpartum and infant behavior or sleep at age 3 months. Therefore, our study could not confirm previous observations in animals and humans.[19,20,24,25]

The results of our study differed from those of previous studies. Importantly, cortisol sampling in the previous studies did not take the diurnal rhythm of breastmilk GCs into account. Although some of these studies corrected analyses for time of collection,[23,24,26] it has previously been demonstrated that correcting for sampling time cannot account for variability in cortisol levels over time.[27,35] There is no doubt that sampling fluctuation has a major impact on the interpretation of HPA-axis dynamics, which plausibly leads to false conclusions when only cross-sectional breast-milk GC levels are studied; e.g., an outcome might be associated with the height of the cortisol level, whereas it actually reflects the time of sampling.[28]

There is some evidence suggesting that breast-milk GCs influence infant neurodevelopment in a sex-specific manner. Among Rhesus monkeys, the associations between higher milk cortisol levels and more nervous, less confident temperament in offspring differed between males and females in such a way that male offspring appeared to be more sensitive to cortisol increments over time and female offspring to the absolute cortisol concentration.[20] In humans, milk cortisol was positively associated with negative affectivity among girls, but not among boys.[24,26] This might be attributed to differences in the developmental timing of GC sensitivity between males and females. Indeed, studies in rodents showed that forebrain and hippocampal GC receptor expression patterns developed in a sex-specific manner.[22,36,37] Due to the small sample size in our study, we were unable to perform sex-specific analyses.

This study has several strengths. It is the first to assess the association between GC diurnal rhythmicity in breast milk and infant behavior and sleep. Moreover, measurement of cortisone along with cortisol in breast milk carries the advantage of having a more precise estimate of GC exposure. Epithelial tissues have been demonstrated to harbor 11β HSD type 2, which converts cortisol into cortisone upon entrapment.[38] Consequently, cortisone may be a more accurate marker of the circulating cortisol level than cortisol itself, at least in saliva and hair.[38-40] This is corroborated by observations demonstrating that cortisone is less likely to have concentrations below the lower limit of quantification at the nadir.[33] Moreover, the presence of 11β-reductase activity in infants implies that cortisol can be regenerated from cortisone. Breast-milk cortisone may thus become a part of the biologically available GC pool.[35,41] Another strength is the use of mass spectrometry for the measurements of cortisol and cortisone. LC-MS/MS is superior to the immunoassays that were used by previous studies in terms of specificity.[42]

However, this study also has its limitations. First, the sample size of our study, although comparable to previous studies in this field,[23-26] might have been too small to detect subtle differences or to stratify for sex. However, this limitation must be balanced against having multiple measurements across the diurnal cycle. Second, we cannot exclude the possibility that distressed mothers were less likely to participate. Non-participants did not sign informed consent, and non-response analyses could therefore not be performed. This might offer an explanation for the observation that experienced maternal stress did not differ between the groups. Third, in view of the limited number of participants, it was not possible to correct for all potential confounders, such as parental temper and other environmental factors that might interfere with infant behavior or sleep. Fourth, infant temperament and sleep were self-reported by the mothers, while parenting behavior was not assessed. Even though behavior is still thought to be best reported by the infant's primary caregiver, distressed mothers may rate their infant's behavior as more difficult,[43-45] To account for this phenomenon, all outcomes were corrected for elevated HADS scores.[46,47]

In conclusion, in our study breast-milk GC rhythmicity at 1 month postpartum was not associated with infant behavior or sleep at 3 months postpartum. Therefore, this study suggests that findings from previous studies linking breast-milk cortisol to infant neurodevelopment might be biased by the lack of GC measurements across the full diurnal cycle or the choice for less reliable immunoassay measurements.

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## SUPPLEMENTARY FILE 1

### Sleep questionnaire

#### Amount of sleep

*These questions relate to how much sleep you and your child have had in the past week. We will also ask some short questions about the quality of your own sleep. The questions have to be answered differently than the previous questions of this questionnaire. This time the questions are open. Your answers can therefore be a rough estimate.*

*In the past week...*

1. On average, how many hours did your child sleep at night? ….. hour(s)

2. On average, how often did your child wake at night? ….. time(s)

3. How often did your child sleep more than 6 hours at night? ….. time(s)

4. On average, how many naps did your child take during daytime? ….. nap(s)

5. On average, how many hours of sleep did your child get during daytime ….. hour(s)