Title:

Diurnal cortisol changes in newborn infants suggesting entrainment of peripheral circadian clock in utero and at birth

Short title:

Entrainment of circadian clock at birth

Authors:

Osuke Iwata[123*], Hisayoshi Okamura[1], Hiroki Saitsu[2], Mamoru Saikusa[2], Hiroshi Kanda[2], Nobuoki Eshima[4], Sachiko Iwata[12], Yasuki Maeno[2], Toyojiro Matsuishi[12]

1. Centre for Developmental & Cognitive Neuroscience, Kurume University School of Medicine, Kurume, Fukuoka, Japan
2. Department of Paediatrics and Child Health, Kurume University School of Medicine, Kurume, Fukuoka, Japan
3. Division of Neonatology, Department of Pediatrics, St. Mary's Hospital, Kurume, Fukuoka, Japan
4. Department of Biostatistics, Faculty of Medicine, Oita University, Oita, Japan.

*Corresponding author: Dr Osuke Iwata
Centre for Developmental & Cognitive Neuroscience
Department of Paediatrics and Child Health, Kurume University School of Medicine
67 Asahimachi, Kurume, Fukuoka 830-0011 Japan
Phone: 81-942-31-7565 Fax: 81-942-38-1792

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The authors declare no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years, no other relationships or activities that could appear to have influenced the submitted work.

Key words:

Circadian rhythm; salivary cortisol; newborn infant; entrainment; birth time

Abstract:

Background:

In the rodent and human foetus, a diurnal cortisol rhythm is observed that is entrained in antiphase to the maternal rhythm. However, after birth, the adrenal circadian rhythm becomes unsynchronised with the clock time, and an adult-type 24-hour rhythm is observed only after a few months. Little is known about when and how the foetal adrenal circadian rhythm is synchronised with the day-night cycle.

Methods:

To investigate the function of adrenal circadian clock in the newborn infant, 8 serial saliva samples were collected every 3 hours over 24 hours (starting at 09:00 in the morning) in 27 newborn infants.

Results:

Cortisol levels were higher during the period 15:00 to <21:00 than during 09:00 to <15:00 and 03:00 to <09:00 (both $p < 0.05$). Salivary cortisol levels collected during 0 to <6, 6 to <12, and 12 to <18 hours after the clock time at birth (birth time) were higher than those collected during 18 to <24 hours after the birth time ($p < 0.005$, 0.05 and 0.05 respectively). The acrophase of salivary cortisol was linearly correlated with the birth time within the first five days of life ($p < 0.005$), but not thereafter.

Discussion:

In the newborn infant, diurnal increase in cortisol was observed in the late afternoon and in correspondence with the birth time. The adrenal circadian rhythm acquired in utero may be re-entrained by endocrinological events at birth. Such complex regulation of the adrenal circadian clock may inhibit a swift synchronisation of the circadian clock to the day-night rhythm.

Introduction

Sleep problems are common in infants. Parents often report difficulties in initiating the infant's sleep, and settling them back to sleep after night wakings (1). Sleep problems during infancy and early childhood tend to persist or even worsen without proper treatment (2), affecting the cognitive functioning and behaviour of the infant (3) as well as the psychological condition of the parents (4). Early intervention to promote an optimal sleep environment for infants with early signs of sleep problem may help prevent the progress of symptoms when introduced during the critical age; precise understanding of when and how the circadian rhythm is established would be important to identify the infant at high risks of developing sleep disorders.

Diurnal rhythms of the heart rate and movement are recognised from the foetal period (5). A recent experimental study in rats demonstrated that, like in the adult species, the adrenal gland of the foetus serves as a peripheral circadian clock (6) A study in human cord blood showed a diurnal rhythm in foetal plasma cortisol (7). Interestingly, the foetal circadian clock observed in these studies was synchronised in almost antiphase to the maternal rhythm (6, 7). Information on the postnatal synchronisation of the circadian clock to the day-night rhythm is sparse. Studies that investigated the adrenal circadian rhythm of the newborn infant demonstrated the presence of both diurnal and non-24-hour changes in salivary and urinary cortisol (8-10). However, the phases of the observed rhythms were not synchronised with clock time, and the acrophase was random among subjects. The adult-type cortisol rhythm, with its acrophase in the early morning, becomes evident only about two months after birth (11). It remains unknown whether the circadian rhythm of maternal origin is temporally lost or if it is modified to be finally entrained to the day-night rhythm during the first few months of life. Detailed information on how a mature circadian rhythm is developed may help our understanding of how to induce a successful transition to extrauterine life and early establishment of a day-night sleep cycle in infants.

To investigate how the foetal adrenal clock alters its rhythm within a short period after birth, we monitored the changes in salivary cortisol in medium-risk newborn infants throughout a 24-hour period. We hypothesised that the adrenal clock of the newborn infant, which has been synchronised in antiphase with the maternal rhythm, would be transiently re-entrained to the time of birth due to a range of hormonal events occurring at or shortly after birth.

Methods

This study was conducted at the neonatal intensive care unit of Kurume University Hospital (Kurume, Fukuoka, Japan) with the approval of the local ethics committee. Written informed consent was obtained from a parent of each infant.

Study Population

Between October 2009 and April 2010, we allocated 20 days for 24-hour saliva sample collection. On each of these days we recruited up to 3 near-term to term newborn infants who were older than one day and physiologically stable. Eventually, saliva samples were collected from 27 infants (33.7–41.8 weeks gestational age and 2–11 days postnatal age in range), who were hospitalised because of low birth weight (20 infants), maternal hyper- or hypothyroidism (2 infants), or maternal gestational diabetes mellitus (5 infants). Infants who underwent phototherapy during or within 24 hours prior to the study, and infants who required resuscitation

and/or intensive life support were not included because of the potential bias of stress on cortisol values.

Sample Collection

Sample collection was started at 09:00 in the morning to obtain eight saliva samples per infant with 3-hour intervals. In our unit, during the first few days of life, term and near-term infants are regularly fed every 3 hours starting at 01:00. Ad-lib feeding is started after the oral intake exceeds 140 mL/kg/day, usually after day 4. For infants who were regularly fed, samples were collected two hours after feeding. For infants who were ad-lib fed, the timing of feeding was adjusted so that samples were collected at least 90 minutes after feeding. The timing for the sample collection was adjusted by up to ± 20 minutes from the scheduled time to obtain samples during sleep or calmly awake states.

For the sample collection, an absorbent swab stick (Sorbette, Salimetrics LLC, PA, USA) was gently inserted into the infant's mouth for approximately 1 minute, allowing the swab end to absorb sufficient saliva. The sample was immediately centrifuged at 3000 rpm, and kept at 4°C until the eighth sample for the subject was collected; at this temperature range, salivary cortisol concentrations remain stable for up to 3 months (12). To better assess the pattern of diurnal cortisol changes, subjects with two or more invalid data points (mainly due to insufficient sample volume <50 µL) were excluded from the analysis.

Cortisol Assay

Saliva samples were frozen at -80°C until assayed. Concentrations of salivary cortisol were determined by enzyme immunoassay (High Sensitivity Salivary Cortisol ELISA Kit, Salimetrics LLC, PA, USA). The limit of detection of this assay in our laboratory was 0.19 nmol/L; intra-assay and inter-assay coefficients of variation were 5.43% and 6.41% respectively.

Clinical Data Collection

Background data were collected for both inter- and intra-subject variables, including antenatal steroid administration, multiple birth, intrauterine growth restriction, delivery mode (vaginal delivery, elective caesarean section or emergency caesarean section), age (gestational, corrected or postnatal), incubator type (open or closed), feeding mode (oral or tube), venipuncture or heel lance shortly before the sample collection, and the sleep status and the mode of lighting at the time of sample collection. In our unit, we provide cycled lighting aiming at 100-200 lux during the day (7:00 to <19:00) and 10-30 lux during the night (19:00 to <7:00). Quilt covers are used over the closed incubators.

Data Analysis

The influence of the infant's age on cortisol values (maximum, minimum, mean and standard deviation) were assessed using the Pearson's correlation coefficient. The potential influence of other variables on cortisol values was evaluated using the analysis of variance (ANOVA). To assess global trends in diurnal cortisol changes, samples obtained at 09:00 and 12:00, 15:00 and 18:00, 21:00 and 24:00, and 3:00 and 6:00 were averaged, and were compared using the repeated measures ANOVA and the Fisher's least significant difference test; the latter was chosen considering the exploratory nature of the analysis, where strict control for type-I error is unnecessary (13). Also to assess the influence of the clock time of birth (birth time), values were averaged for four time periods of 0 to <6, 6 to <12, 12 to <18, and 18 to <24 hours after the birth time.

Based on the finding from these exploratory analyses, a new hypothesis was formed that the acrophase of cortisol occurs with some delay after the birth time. To test the hypothesis, we first objectively estimated the acrophase in hours to the first decimal place for the range of 9:00 to <9:00 on the second day of the study by fitting a quartic polynomial to the eight-point time-series data (14); this polynomial was chosen to optimise curve fitting to the time-series data with up to two peaks (Online supplementary figure 1). Because of uncertainty around the presence of diurnal cortisol cycle in the newborn infant, periodic functions were not applied; for the analysis only, the sample collection time and the acrophase in cortisol were transformed into an interval scale of 9:00 to <33:00 by adding 24 hours to the clock time on the second day of the study. The dependence of the acrophase on the birth time was assessed using a linear regression model without adjusting value ranges for the intercept (Online supplementary figure 2). The presence of the linear correlation was assessed on the whole study population and on two subgroups of relatively younger infants (<day 5, n =13) and older infants (≥day 5, n =10) to evaluate the influence of the postnatal age on the pattern of diurnal cortisol changes. P-values were adjusted for subgroup analyses using the Bonferroni correction.

Results

Data Profile

Eight saliva samples were successfully collected from all 27 infants (224 samples in total), however, nine samples were of insufficient volume for the assay. Consequently, three infants had only six valid data sets; another infant started phototherapy halfway through the study; to minimise the bias, these four infants were excluded from further analyses. Physiological and clinical backgrounds of the study population are presented in the Table 1 and Online supplementary figure 3; 74% of subjects were born during the period 9:00 to <21:00 (Online supplementary figure 4).

Infant's Age, Body Weight and Salivary Cortisol

Maximum cortisol values per individual were dependent on the gestational age ($p < 0.005$) and corrected age ($p < 0.01$). Minimum cortisol values were only dependent on the postnatal age ($p < 0.05$). Mean cortisol values were associated with the gestational age ($p < 0.05$). Standard deviations of cortisol values per individual were dependent on the gestational age and corrected age (both $p < 0.005$) (Table 2). The influence of the delivery mode to the cortisol level and its individual standard deviation was not evident (Online supplementary figure 5).

Independent Variables of Salivary Cortisol

Infants who were orally fed had higher cortisol values than their peers ($p < 0.05$). The awake state was noted only 0 (1-3) times per subject (median (range)); the observation of the awake state did not vary between sample collection times except that no infant was noted to be awake at 12:00. The awake state was associated with higher cortisol levels than the sleep state ($p < 0.01$) (Table 3). No other clinical factors were identified as independent variables of salivary cortisol.

Clock Time, Birth Time and Diurnal Changes in Salivary Cortisol

Cortisol levels during the period from 15:00 to <21:00 were higher than those of 09:00 to <15:00 and 03:00 to <09:00 (both $p < 0.05$) for the whole study population; these trends were not seen for subgroups of younger and

older infants (Fig. 1 A-C). Cortisol levels were significantly higher during 0 to <6 hours, 6 to <12 hours, and 12 to <18 hours after the birth time than during 18 to <24 hours after the birth time for both the whole study population (p <0.005, 0.05 and 0.05 respectively) and the subgroup of younger infants (p <0.005, 0.005 and 0.05 respectively); these trends were not observed in the subgroup of older infants (Fig. 1 D-F).

Figure 1

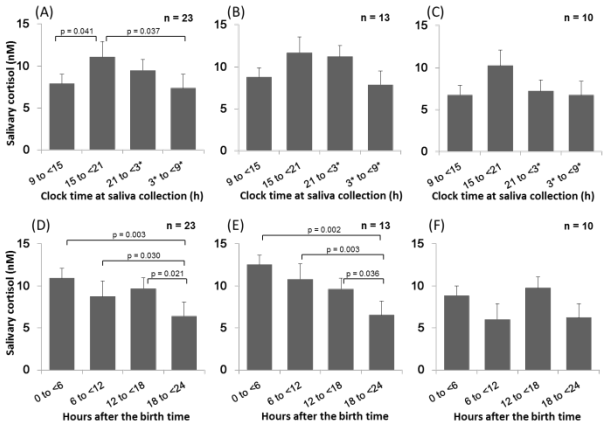

Figure 1: Dependence of diurnal changes in cortisol on clock time and birth time. Diurnal cortisol changes over four averaged bands of (A-C) the clock time and (D-F) the time after the birth time, for the (A; D) entire study population, (B; E) younger subjects <day 5, and (C; F) older subjects ≥day 5. Values are shown as mean and standard error. P-values are from repeated measures analysis of variance with the Fisher's least significant difference test. Label on the x-axis indicates (A-C) the sample collection time on the clock time and (D-F) the time interval after the birth time. Higher salivary cortisol values were observed during the periods of 15:00 to <21:00 and 0 to <6 hours after the birth time. *Clock time on the second day of the study.

Curve fitting to individual time-series data was satisfactory with mean (standard deviation) r^2 values of 0.63 (0.17) (Online supplementary figure 1). The acrophase of cortisol was positively correlated with the birth time in the whole study population (p <0.01) and the younger infant subgroup (p <0.005); the linear relationship was not observed in the subgroup of older infants (Fig. 2; also see Online supplementary figure 7-8 for findings in other subgroups of the delivery mode and individual variability in cortisol).

Figure 2

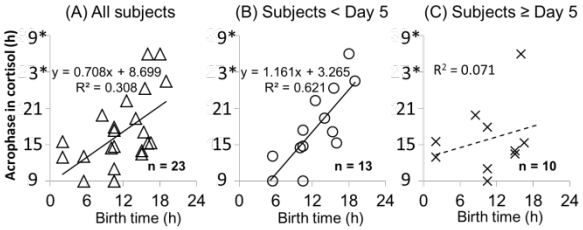

Figure 2: Association between the birth time and the acrophase of cortisol rhythm. Scatter plot demonstrating the association between the birth time and the acrophase in cortisol. The acrophase was positively correlated with the birth time in (A) the whole study population (p <0.01) and (B) the younger infant subgroup (p <0.005), but not in (C) the older infant subgroup. Solid lines are for regression with p-values <0.05 and broken lines are for reference from the Pearson's correlation coefficient and Bonferroni correction. *Clock time on the second day of the study.

Discussion

We confirmed the presence of a 24-hour adrenal circadian cycle in newborn infants. Increase in salivary cortisol was observed around 15:00 to <21:00 and 0 to <6 hours after the birth time. During the first five days of life, the acrophase of the cortisol rhythm was predominantly defined by the birth time, the influence of which disappeared thereafter. Our current findings suggest that, as previously demonstrated in the foetal species (15), the adrenal circadian rhythm of the newborn infant is first entrained by maternal stimuli in utero, and is then transiently re-entrained by strong but short lasting stimuli at birth, both of which appear to induce circadian cycles with different phases from the day-night cycle. Such complex regulation of the adrenal clock, while potentially relevant for protecting the infant

from life-threatening events during the transitional period, may disturb the swift synchronisation of the infant's circadian rhythm to the day-night cycle. Our current findings build on results from previous studies that addressed the developmental process of the foetal circadian rhythm, and may present a vital piece of the puzzle by providing information on the presently unclear function of the adrenal circadian clock during early neonatal period.

Monitoring the Adrenal Circadian Rhythm Using Salivary Cortisol

For adults, salivary cortisol has been proposed as a non-invasive surrogate for plasma cortisol (16). Salivary cortisol levels reflect those in the free plasma fraction without being affected by the salivary flow rate. Similar to serum cortisol, salivary cortisol in the adult has a well-documented diurnal pattern with peak levels in the early morning, typically decreasing over the day, and a nadir at night (17). This technique has successfully been applied in the newborn infant (18, 19). In our subjects, sufficient saliva was obtained in 96% of the cases. A tight linear relationship between plasma and salivary cortisol levels has been demonstrated even in very low birth weight infants (20), supporting the usefulness of this technique in the newborn infant.

Adrenal Circadian Rhythm of the Foetus and Newborn Infant

In adult mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus serves as a master clock that regulates the biological rhythm of peripheral clocks and systemic organs (21). Until foetal monitoring techniques became available, it was long accepted that infants were born without a circadian rhythm. However, studies that used foetal physiological monitoring techniques demonstrated 24-hour rhythms of the foetal heart rate and respiratory and body movements (15). Recent studies that monitored maternal and foetal hormones and clock genes revealed that the foetal SCN is not involved in the modulation of circadian rhythm, whereas the foetal adrenal gland plays the central role in the entrainment of the foetal circadian clock (6, 15, 21, 22). A study in cord blood of infants born by scheduled elective caesarean section demonstrated the presence of a diurnal cortisol rhythm with its acrophase in the afternoon (7). A study in the rat confirmed that the foetal adrenal clock was entrained almost in antiphase to the maternal rhythm (6).

Although the function of the peripheral circadian clock in the adrenal gland is well established in utero, diurnal cortisol changes in the newborn infant are unsynchronised with the external clock time shortly after birth, presumably because of the loss of maternal hormonal stimuli and the immaturity of the SCN (8-10). This condition is likely to persist for several months until the adrenal clock is finally entrained to the day-night cycle (23, 24). Our study also suggested the presence of a diurnal cortisol rhythm in the newborn infant, which showed a modest trend towards increased cortisol in the late afternoon, suggesting the synchrony with the external clock. This observation contrasted with previous studies in the newborn infant (8-10), but was consistent with observations in the foetus (6, 7). Given that other circadian and ultradian rhythms are likely to be entrained in association with birth and subsequent extrinsic stimuli (e.g., cold stress, light-dark cycle, feeding and other cares) (25-27), it may not be surprising that previous studies failed to identify a cortisol rhythm of maternal or foetal origin shortly after birth (8-10). The exact mechanism and the relevance of the antiphase synchronisation of the foetal circadian clock are unknown.

However, this time difference may in part explain the prolonged period required for the entrainment of the circadian clock to the day-night rhythm.

Influence of the Birth Time on the Adrenal Circadian Clock

Thus far, the regulating factor for the adrenal circadian clock in the newborn infant has not been identified. In our current study, the influence of the birth time on the diurnal changes in cortisol was revealed when the acrophase of cortisol rhythm was studied relative to the birth time. For infants younger than five days, the acrophase was observed approximately 3 hours after the birth time, whereas such an association was not observed for the older infants. Given the series of hormonal events observed around delivery, which include the stimulation of hypothalamus, pituitary and their downstream systems (25, 26), birth itself may provide a sufficient stimulus to entrain the infant's circadian clock to the time of birth. However, the influence of the birth-related stimuli to the adrenal circadian clock was observed only during the first five days of life. Such transient synchronisation of the adrenal circadian clock with the birth time may only be relevant for the safe transition of the foetus to the extra-uterine life. However, given that natural onset of labour most frequently occurs in the early morning hours (28), entrainment of the adrenal clock to the morning may boost early synchronisation of the circadian clock to the day-night rhythm rather than introducing an additional, confusing signal to the foetal circadian clock. Future studies need to address the mechanism of and the relevance of re-entrainment of the adrenal clock at birth.

Determination Factors of Non-circadian Changes in Cortisol

Consistent with the observations in adults (29), salivary cortisol levels were dependent on the sleep or wake states in our current population. The rhythm of cortisol secretion in the newborn infant may include both diurnal and ultradian rhythms (30). In our subjects, oral feeding was also associated with higher cortisol levels as opposed to tube feeding. Spangler reported that the type and the timing of feeding affect salivary cortisol levels in the newborn infant (24). However, Shulman et al. reported that urinary cortisol levels were not associated with the mode of feeding in preterm infants (31). The difference between the feeding modes observed in our current study may merely be caused by the difference in the corrected age of the infants, which was another prominent independent variable of salivary cortisol. Stressful environments within an intensive care unit, represented by continuous bright lighting and painful procedures, may have negative effects on the development of the newborn infant (32, 33). In our current study, clinical variables such as blood sampling, lighting mode and type of incubator, were not recognised as independent variables of salivary cortisol, which may be in part because of the commitment of our unit to developmental care, including low-intensity cycled lighting and quilt covers.

Limitations

Our study was not powered to give a solid overview of the diurnal hormonal cycle in the newborn infant mainly because of the limited population size and study duration. Because of the lack of direct evidence to support the presence of diurnal cortisol cycles, we were unable to use routine statistical analysis such as periodic functions. Application of a linear regression model to the birth time (0:00 to <24:00) and acrophase (9:00 to <33:00) means the mandatory use of a coordinate plane with the birth time axis intersecting the acrophase axis at 9:00,

suggesting that the model is optimal only when the intercept for the acrophase is close to 9:00 (Online supplementary figure 2). However, we decided not to correct the intersection of the axis because (i) positive intercepts were expected for the acrophase from the exploratory analysis, and (ii) we aimed to keep the statistical model as simple as possible. Consequently, linear relationships were demonstrated in the whole study subjects and the subgroup of younger subjects without introducing complex corrections; calculated intercepts for these populations were 8.7 and 3.3 hours respectively (Figure 2). Only a few subjects in our population were born during 0:00 to <6:00, which might also help minimise the number of subject influenced by the fixed intercept. Future studies are required to confirm our findings using data collected over at least several days.

We observed that the sleep-wake state at the time of saliva collection affected the cortisol level, however, we were unable to monitor the sleep-wake rhythm of the infant over the whole study period; non-invasive continuous monitors such as actigraphy may enable the direct comparison of circadian cycles identified using serial salivary cortisol and limb motions (34). Our current study cohort consisted of infants who were hospitalised at a neonatal intensive care unit but are not under intensive life support. However, their clinical backgrounds and environmental factors were still different from their peers; the impact of high-risk birth may appear even long after weaning off intensive cares (35); caution is required when generalising the finding into normal newborn infants. Measurement of melatonin may provide additional information about the function of the master circadian clock. This information was not available for the current study because of the greater minimum sample volume (150 μL) required for the assay.

Clinical Implications and Conclusions:

A 24-hour adrenal circadian rhythm was present in the newborn infant; salivary cortisol increased around 15:00 to <21:00, and from 0 to <6 hours after the birth time. These presumably reflect the foetal circadian rhythm and the superimposed rhythm entrained at birth respectively. Given that the foetal circadian rhythm is entrained in antiphase to the maternal cycle (6, 7), and that the birth-induced re-entrainment may occur at any time of the day, the newborn infant is unlikely to acquire an optimal circadian rhythm for extrauterine life. Further studies are required to determine how the circadian rhythm is acquired. In addition, studies are needed to elucidate the relevance of (i) the antiphase entrainment of the foetal circadian rhythm to the maternal rhythm, (ii) the transient entrainment of the infant's adrenal clock to the birth time, and (iii) the subsequent disappearance of the overt adrenal rhythm. This information may help predict the time when a mature sleep-wake cycle is established, and promote early acquisition of a mature sleep-wake cycle.

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References

1. **Forsyth BW, Leventhal JM, McCarthy PL** 1985 Mothers' perceptions of problems of feeding and crying behaviors. A prospective study. Am J Dis Child 139:269-272
2. **Byars KC, Yolton K, Rausch J, Lanphear B, Beebe DW** 2012 Prevalence, patterns, and persistence of sleep problems in the first 3 years of life. Pediatrics 129:e276-284
3. **Iwata S, Iwata O, Iemura A, Iwasaki M, Matsuishi T** 2011 Determinants of sleep patterns in healthy Japanese 5-year-old children. Int J Dev Neurosci 29:57-62
4. **Smart J, Hiscock H** 2007 Early infant crying and sleeping problems: a pilot study of impact on parental well-being and parent-endorsed strategies for management. J Paediatr Child Health 43:284-290
5. **Seron-Ferre M, Torres-Farfan C, Forcelledo ML, Valenzuela GJ** 2001 The development of circadian rhythms in the fetus and neonate. Seminars in perinatology 25:363-370
6. **Torres-Farfan C, Mendez N, Abarzua-Catalan L, Vilches N, Valenzuela GJ, Seron-Ferre M** 2011 A circadian clock entrained by melatonin is ticking in the rat fetal adrenal. Endocrinology 152:1891-1900
7. **Seron-Ferre M, Riffo R, Valenzuela GJ, Germain AM** 2001 Twenty-four-hour pattern of cortisol in the human fetus at term. American journal of obstetrics and gynecology 184:1278-1283
8. **Bettendorf M, Albers N, Bauer J, Heinrich UE, Linderkamp O, Maser-Gluth C** 1998 Longitudinal evaluation of salivary cortisol levels in full-term and preterm neonates. Hormone research 50:303-308
9. **Valenzuela GJ, Hoffman A, Hess D, Seron-Ferre M** 1998 Does the Human Term Newborn have Circadian Rhythms at Birth? J Soc Gynecol Investig 5:153
10. **Zadik Z, Amer R, Dolfin Z, Arnon S, Cohen D, Mogilner B, Reifen R** 1999 Urinary free cortisol (UFC) values in newborns under stress. J Pediatr Endocrinol Metab 12:543-547
11. **de Weerth C, Zijl RH, Buitelaar JK** 2003 Development of cortisol circadian rhythm in infancy. Early Hum Dev 73:39-52
12. **Garde AH, Hansen AM** 2005 Long-term stability of salivary cortisol. Scandinavian journal of clinical and laboratory investigation 65:433-436
13. **Bender R, Lange S** 1999 Multiple test procedures other than Bonferroni's deserve wider use. BMJ 318:600-601
14. **Iwata O, Iwata S, Thornton JS, De Vita E, Bainbridge A, Herbert L, Scaravilli F, Peebles D, Wyatt JS, Cady EB, Robertson NJ** 2007 "Therapeutic time window" duration decreases with increasing severity of cerebral hypoxia-ischaemia under normothermia and delayed hypothermia in newborn piglets. Brain Res 1154:173-180
15. **Seron-Ferre M, Mendez N, Abarzua-Catalan L, Vilches N, Valenzuela FJ, Reynolds HE, Llanos AJ, Rojas A, Valenzuela GJ, Torres-Farfan C** 2012 Circadian rhythms in the fetus. Molecular and cellular endocrinology 349:68-75
16. **Kirschbaum C, Hellhammer DH** 1994 Salivary cortisol in psychoneuroendocrine research: recent developments and applications. Psychoneuroendocrinology 19:313-333
17. **Dockray S, Steptoe A** 2011 Chronotype and diurnal cortisol profile in working women: differences between work and leisure days. Psychoneuroendocrinology 36:649-655
18. **Harmon AG, Hibel LC, Rumyantseva O, Granger DA** 2007 Measuring salivary cortisol in studies of child development: watch out--what goes in may not

come out of saliva collection devices. Dev Psychobiol 49:495-500

19. **Shirtcliff EA, Granger DA, Schwartz E, Curran MJ** 2001 Use of salivary biomarkers in biobehavioral research: cotton-based sample collection methods can interfere with salivary immunoassay results. Psychoneuroendocrinology 26:165-173

20. **Matsukura T, Kawai M, Marumo C, Iwanaga K, Yoshida K, Shibata M, Niwa F, Hasegawa T, Heike T** 2012 Diagnostic Value of Salivary Cortisol in the CRH Stimulation Test in Premature Infants. J Clin Endocrinol Metab 97:890-896

21. **Edery I** 2000 Circadian rhythms in a nutshell. Physiological genomics 3:59-74

22. **Simonneaux V** 2011 Naughty melatonin: how mothers tick off their fetus. Endocrinology 152:1734-1738

23. **Price DA, Close GC, Fielding BA** 1983 Age of appearance of circadian rhythm in salivary cortisol values in infancy. Arch Dis Child 58:454-456

24. **Spangler G** 1991 The emergence of adrenocortical circadian function in newborns and infants and its relationship to sleep, feeding and maternal adrenocortical activity. Early Hum Dev 25:197-208

25. **Bolt RJ, van Weissenbruch MM, Lafeber HN, Delemarre-van de Waal HA** 2002 Development of the hypothalamic-pituitary-adrenal axis in the fetus and preterm infant. J Pediatr Endocrinol Metab 15:759-769

26. **Davis EP, Sandman CA** 2010 The timing of prenatal exposure to maternal cortisol and psychosocial stress is associated with human infant cognitive development. Child Dev 81:131-148

27. **Glotzbach SF, Edgar DM, Boeddiker M, Ariagno RL** 1994 Biological rhythmicity in normal infants during the first 3 months of life. Pediatrics 94:482-488

28. **Seron-Ferre M, Ducsay CA, Valenzuela GJ** 1993 Circadian rhythms during pregnancy. Endocr Rev 14:594-609

29. **Edwards S, Clow A, Evans P, Hucklebridge F** 2001 Exploration of the awakening cortisol response in relation to diurnal cortisol secretory activity. Life Sci 68:2093-2103

30. **Glotzbach SF, Edgar DM, Ariagno RL** 1995 Biological rhythmicity in preterm infants prior to discharge from neonatal intensive care. Pediatrics 95:231-237

31. **Shulman RJ, Heitkemper M, O'Brian Smith E, Lau C, Schanler RJ** 2001 Effects of age, feeding regimen, and glucocorticoids on catecholamine and cortisol excretion in preterm infants. JPEN J Parenter Enteral Nutr 25:254-259

32. **Blackburn S** 1998 Environmental impact of the NICU on developmental outcomes. J Pediatr Nurs 13:279-289

33. **Brandon DH, Holditch-Davis D, Belyea M** 2002 Preterm infants born at less than 31 weeks' gestation have improved growth in cycled light compared with continuous near darkness. J Pediatr 140:192-199

34. **Iwata S, Iwata O, Iemura A, Iwasaki M, Matsuishi T** 2012 Sleep architecture in healthy 5-year-old preschool children: associations between sleep schedule and quality variables. Acta Paediatr 101:e110-114

35. **Okada J, Iwata S, Hirose A, Kanda H, Yoshino M, Maeno Y, Matsuishi T, Iwata O** 2011 Levothyroxine replacement therapy and refractory hypotension out of transitional period in preterm infants. Clin Endocrinol (Oxf) 74:354-364

Tables:

Table 1. Background clinical variables.

Inter-subject variables	
Clinical background	
Female/ Male	7/16
Multiple births	14
Birth weight (g)	2344 (441)
Intrauterine growth restriction	5
Antenatal steroid	4
Vaginal delivery	8
Elective/ Emergency caesarean section	8/7
Age at birth and sample collection	
Gestational age (weeks)	36.6 (1.7)
Post-natal age (days)	5.3 (3.4)
Post-conceptional age (weeks)	37.1 (2.1)
Treatment and environment	
Continuous venous infusion	7
Tube feeding	4
Closed incubator	9
Intra-subject variables[†]	
Sleep state (asleep)	133
Blood sampling before saliva collection	14

Values are shown as the mean (standard deviation) or the number of incidence based on 182 salivary samples[†] from 23 subjects.

Table 2. Dependence of individual cortisol values on age.

	Cortisol (nM)		Correlation coefficients		
	Mean	(SD)	Gestational age	Post-natal age	Postconceptional age
Individual maximum	21.11	(8.45)	0.61**	-0.18	0.56**
Individual minimum	3.32	(1.32)	0.10	-0.46*	-0.02
Individual mean	9.08	(2.96)	0.47*	-0.41	0.36
Individual SD	6.47	(3.22)	0.65**	-0.06	0.64**

*p < 0.05 and *p < 0.01, Pearson correlation coefficient.

SD, standard deviation.

Table 3. Dependence of cortisol values on clinical variables.

Inter-subject variables		Mean (SD) nM
Gender	male	9.68 (0.69)
	female	7.71 (1.21)
Intrauterine growth restriction	no	8.96 (3.21)
	yes	9.51 (2.04)
Antenatal steroid	no	9.44 (2.74)
	yes	7.38 (3.84)
Caesarean delivery	no	10.69 (3.40)
	yes	8.38 (2.55)
Multiple births	no	9.88 (3.70)
	yes	8.57 (2.39)
Feeding mode	oral	9.66 (0.63)*
	tube	6.33 (2.71)
Incubator type	open	9.47 (3.07)
	closed	8.48 (2.85)
Continuous venous infusion	no	8.50 (3.06)
	yes	10.40 (2.41)
Intra-subject variables†		
Sleep status	sleep	8.13 (6.04)**
	awake	12.00 (9.99)
Blood sampling before saliva collection	no	8.99 (7.42)
	yes	9.68 (6.19)
Lighting mode	night	8.39 (7.39)
	day	9.38 (6.85)

**$p < 0.01$ and *$p < 0.05$ from the analysis of variance.

Values are based on 182 salivary samples† from 23 subjects.

SD, standard deviation.