## Early-life endocrine regulation and neurodevelopmental outcomes

Hollanders, J.J.

2020

# 7

# No association between glucocorticoid diurnal rhythm in breastmilk and infant body composition at age 3 months

Jonneke Hollanders*,
Lisette R. Dijkstra*,
Bibian van der Voorn,
Stefanie M.P. Kouwenhoven,
Alyssa A. Toorop,
Johannes B. van Goudoever,
Joost Rotteveel,
Martijn J.J. Finken
* Authors contributed equally to this manuscript

Nutrients. 2019 Oct 2;11(10).

## ABSTRACT

### Objective

Glucocorticoids (GCs) in breastmilk have previously been associated with infant body growth and body composition. However, the diurnal rhythm of breastmilk GCs was not taken into account, and we therefore aimed to assess the assocations between breastmilk GC rhythmicity at age 1 month and growth and body composition at age 3 months.

### Methods

At one month postpartum, breastmilk GCs were collected over a 24-h period and analyzed by LC-MS/MS. Body composition was measured using air-displacement plethysmography at age 3 months. Length and weight were collected at age 1, 2 and 3 months.

### Results

39 healthy mother-infant pairs were included. No associations were found between breastmilk GC rhythmicity (area-under-the-curve increase and ground, maximum and delta) and infant growth trajectories or body composition (fat and fat free mass index, fat%) at age 3 months.

### Conclusions

This study did not find an association between breastmilk GC rhythmicity at 1 month and infant's growth or body composition at age 3 months. Therefore, this study suggests that previous observations linking breastmilk cortisol to changes in infant weight might be flawed by the lack of serial cortisol measurements and detailed information on body composition.

## INTRODUCTION

Growing attention is focused on the etiology of obesity, and it has been hypothesized that part of its origin can be traced back to events occurring in early life (i.e., the Developmental Origins of Health and Disease [DOHaD] hypothesis).[1]

Given its effects on fat disposition and metabolism, the hypothalamus-pituitary-adrenal (HPA) axis has been implicated to play a role in the pathway leading to obesity.[2,3] Not only endogenous, but also maternal glucocorticoids (GCs) appear to be involved. Evidence from animal experiments indicates that increased transplacental supply of maternal GCs may be associated with a lower birth weight and cardiovascular correlates such as hypertension and hyperglycemia.[4] In humans, fetal exposure to excess maternal cortisol, e.g., due to maternal anxiety or depression, has been associated with a higher risk of childhood adiposity.[5]

After birth, small amounts of maternal GCs appear to be transferred to the developing infant through breastmilk. Maternal GCs in breastmilk have been shown to cross the intestinal barrier in animals,[6] and have been associated with growth and body composition. Hinde et al. (2015)[7] found that cortisol in breastmilk of rhesus macaques was positively associated with weight gain in offspring. In humans, Hahn-Holbrook et al. (2016)[8] showed that cortisol in breastmilk at age 3 months was inversely associated with body mass index (BMI) percentile gains in the first 2 years of life. Whether the findings from these studies are contradictory is unclear, since length was not taken into account by Hinde et al.[7] Moreover, the effect of GCs on growth might change between the ages of 3 months and 2 years.

Our group has previously shown that GCs in breastmilk follow maternal HPA-axis activity, with a peak in the morning and a nadir at night.[9] Although previous studies have found associations between cortisol in breastmilk and growth of offspring, none of them took GC rhythmicity into account. However, obesity has previously been associated with a flatter diurnal cortisol slope in adults,[10] and there is also some evidence that a blunted GC rhythm is associated with obesity in children.[11,12] Both Hinde et al. (2015)[7] and Hahn-Holbrook et al. (2016)[8] did not collect samples around peak GC levels, while Hahn-Holbrook et al. (2016) also had a wide time window during which samples could be collected (11:30-16:00).

We therefore aimed to assess the associations between breastmilk GC rhythmicity and infant growth and body composition. We measured cortisol and cortisone in breastmilk at age 1 month over a 24-hour period, measured body composition using air-displacement plethysmography at age 3 months, and collected length and weight data monthly up to that age. Due to associations found between a blunted endogenous GC rhythm and obesity in both children and adults,[10-12] we hypothesized that less GC variability in breastmilk could be associated with a higher fat mass in the infants.

## METHODS

### Population

Healthy mother-infant pairs were recruited at the maternity ward of the Amsterdam UMC, location VUMC (a tertiary hospital) in the Netherlands between March 2016 and July 2017. Subjects were eligible for inclusion when infants were born at term age (37-42 weeks) with a normal birth weight (-2 to +2 SDS), and when mothers had the intention to breastfeed for a minimum of three months. Exclusion criteria were: 1) major congenital anomalies, 2) multiple pregnancy, 3) pre-eclampsia or HELLP, 4) medication use other than "over the counter" drugs, 5) maternal alcohol consumption of >7 IU/week and/or 6) a maternal temperature of >38.5°C at the time of sampling. Approval of the Medical Ethics Committee of the VUMC was obtained (protocol number 2015.524), and written informed consent was obtained from all participating mothers.

### Data collection

#### *Peripartum*

Shortly after inclusion, within the first days postpartum, mothers filled in a questionnaire pertaining to their pregnancy and birth, as well as maternal and infant anthropometric and demographic data.

#### *One month postpartum*

At 30 days postpartum (± 5 days), mothers collected a portion of breastmilk (1-2 mL) prior to each feeding moment, over a 24h period (i.e., five to eight times). Although only foremilk was collected through this method, previous research has shown that GC concentrations are similar in fore- and hindmilk.[13] Mothers could follow their own feeding schedule and were therefore asked to report the exact time of sampling. Milk was collected manually or with a breast pump; we requested that mothers used the same method for all samples. Milk was stored in the mother's freezer, and subsequently in the laboratory at -20°C for less than 3 months prior to analysis.

At the time of sampling, maternal distress was quantified with the Hospital Anxiety and Depression Scale (HADS).[14] This questionnaire contains 14 questions scored from 0-3, which assess self-reported levels of depression and anxiety symptoms. Seven questions concern depressive symptoms (HDS) and seven anxiety symptoms (HAS). A score of ≥8 on a subscale is indicative of clinically relevant depression and/or anxiety symptoms.

#### *Three months postpartum*

At 3 months postpartum (± 2 wks), body composition of the infants was assessed with the Pea Pod, an air-displacement plethysmography (ADP) system (COSMED USA, Inc., Concord, CA, USA) [15] It is based on a bi-compartmental model, which uses pressure and

volume changes in the chamber through which body density were determined. Age- and sex-specific fat and fat free mass density values were subsequently used to calculate fat mass (FM) and fat free mass (FFM). [15]

As part of the national standard care, weight and length at 1, 2 and 3 months of age were measured by the staff of the child health clinic and were obtained through a questionnaire. Weight was measured fully undressed on a balance scale with an accuracy of 1 gr. Length was measured in supine position to the nearest 0.1 cm. Additionally, all mothers were asked if their infants were still breastfed for >80% at the age of 3 months.

### Laboratory analysis

Cortisol and cortisone concentrations in breastmilk were determined by isotope dilution liquid chromatography–tandem mass spectrometry (LC–MS/MS), as previously described.[16] In short, internal standards ($^{13}C_3$-labeled cortisol and $^{13}C_3$-labeled cortisone) were added to 200 µl of the samples. Then, breastmilk was washed 3 times with 2 mL hexane to remove lipids. Finally, samples were extracted and analyzed using Isolute plates (Biotage, Uppsala, Sweden) and analyzed by LC-MS/MS (Acquity with Quattro Premier XE, Milford MA, USA, Waters Corporation). The intra-assay coefficients of variation (CV%) were 4 and 5% for cortisol levels of 7 and 23 nmol/L, and 5% for cortisone levels of 8 and 33 nmol/L for LC-MS/MS measurements. The inter-assay CV% was <9% for both cortisol and cortisone. The Lower Limit of Quantitation (LLOQ) was 0.5 nmol/L for both cortisol and cortisone. All samples were measured in duplo.

### Statistics

First, data of GC concentrations in breastmilk were converted into the following rhythm parameters, in order to provide a full overview of GC rhythmicity:

- The maximum GC concentration, as a proxy for peak concentrations
- The delta between maximum and minimum GC concentrations, as a measure of rhythm variability
- Area Under the Curve (AUC) ground (g) and increase (i), using the trapezoid rule.[17] Calculations were corrected for total sampling time, since this differed between mothers. AUCg is a measure of total GC exposure, while AUCi provides information on GC variability.

Mother-infant pairs were excluded from analyses when no valid GC data was available around the time of the expected morning peak (5:00-10:00) and/or when total sample collection was <8 hours.

Fat% was determined from FM and FFM values. Fat Mass Index (FMI) and Fat Free Mass Index (FFMI) were calculated by dividing FM and FFM values (in kg) respectively, by infant length squared ($m^2$), since fat mass and fat free mass are known to change with

length.[18] Length and weight data were converted to SDS.[19,20] Body mass index (BMI) was calculated for age 3 months only, and converted to SDS.[19]

Linear regressions were used to assess the associations between GC rhythm parameters at age 1 month and length SDS, weight SDS, BMI SDS, FMI and FFMI at age 3 months. First, unadjusted regression analyses were performed. Next, the following potential confounders were tested: sex, HADS-score (HAS and/or HDS ≥8), prepregnancy BMI, ethnicity (Caucasian vs. non-Caucasian), socio-economic status, birth weight SDS, gestational age, weight gain during pregnancy, parity (1 vs. >1), mode of delivery (vaginal vs. caesarian section), and %breastmilk at age 3 months (< or >80%). Due to our sample size, the three variables with the largest confounding effect (i.e., largest change in β of the independent variable) were used for the multiple linear regression analyses. Thus, weight gain during pregnancy, % breastmilk at age 3 months and ethnicity were included in the final model assessing the association between GC rhythm parameters and body composition outcomes. No effect modification was found for infant sex, and analyses were therefore not stratified.

Lastly, length and weight SDS growth trajectories between age 1 to 3 months were plotted against AUCi and AUCg values by using generalized estimating equations (GEEs), and 95% confidence intervals were calculated according to the method described by Figueiras et al. [21] AUCi and AUCg outcomes for cortisol and cortisone were categorized as ≤p25, p25-75 and ≥p75.

## RESULTS

### Population

Forty-four mother-infant pairs were included in the study. One mother-infant pair was lost to follow-up, three mother-infant pairs returned the growth questionnaires but did not consent to the Pea Pod measurement and one pair was excluded because no samples were collected between 5:00-10:00 and/or because total sampling time was <8 hours. Therefore, a total of 42 mother-infant pairs were included in the growth trajectory analyses, whereas 39 mother-infants pairs were included in the body composition analyses at age 3 months. Of the included mother-infant pairs, 59.5% were mother-son pairs. Table 1 shows the characteristics of the population. Supplementary Table 1 shows the cortisol and cortisone concentrations in breastmilk in 4-hour intervals.

### Linear regression analyses

No associations were found between the GC rhythm parameters (AUCi, AUCg, maximum and delta) and body composition in the unadjusted analyses. Adjusting the analyses

**Table 1:** Characteristics of the study population (n=42)

| | | |
|---|---|---|
| Gestational age | wks | 39.9±1.3 |
| Birth weight | grams | 3561±498 |
| | SDS | 0.2±1.0 |
| Birth length* | cm | 52.0±2.6 |
| | SDS | 1.0±1.6 |
| Male sex | | 25 (59.5) |
| Primiparity | | 23 (54.8) |
| Caesarian section | | 21 (51.2) |
| HAS and/or HDS ≥8 at 1 month pp | | 6 (14.6) |
| Prepregnancy maternal BMI | $kg/m^2$ | 22.3±2.8 |
| Weight gain during pregnancy | kg | 13.1±3.2 |
| Maternal age | Yrs | 36.0±4.7 |
| Non-Caucasian ethnicity | | 8 (20.0) |
| Socioeconomic status | SDS | 0.6±1.2 |
| >80% breastfed at age 3 months | | 35 (87.5) |
| Age at breastmilk sampling | days | 30.8±2.6 |
| Age at Pea Pod measurement** | days | 90.5±7.0 |

Values represent mean±SD or n (%); pp= postpartum
HAS, Hospital Anxiety Score; HDS, Hospital Depression Score
* n=31
** n=39

for weight gain during pregnancy, % breastmilk at age 3 months, and ethnicity did not change the results (Table 2).

### Growth trajectories

Figure 1 shows the growth trajectories for length and weight SDS according to breastmilk cortisone AUCi and AUCg outcomes. No differences were found between the categories ≤p25, p25-75 and ≥p75. Results for breastmilk cortisol AUCi and AUCg were similar [data not shown].

## DISCUSSION

In this study, despite increased evidence for associations between blunted endogenous GC rhythms and obesity in both children and adults,[10-12] no associations were found between GC rhythmicity in breastmilk sampled at 1 month and infant body composition or growth at age 3 months. Therefore, our study could not confirm previous observations in animals and humans. Hinde et al. (2015)[7] measured cortisol in breastmilk of 108

**Table 2:** Adjusted associations between breastmilk GC rhythmicity at age 1 month and infant body composition at age 3 months (n=39)

| | | Length | | Weight | | BMI | | FMI | | FFMI | | Fat % | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | B | 95% CI | B | 95% CI | B | 95% CI | B | 95% CI | B | 95% CI | B | 95% CI |
| Cortisol | Maximum | 0.006 | (-0.04 to 0.05) | 0.022 | (-0.02 to 0.07) | 0.022 | (-0.02 to 0.06) | 0.003 | (-0.04 to 0.05) | -0.006 | (-0.04 to 0.03) | 0.048 | (-0.17 to 0.27) |
| | Delta | 0.006 | (-0.04 to 0.05) | 0.024 | (-0.02 to 0.07) | 0.025 | (-0.02 to 0.07) | 0.003 | (-0.04 to 0.05) | -0.004 | (-0.04 to 0.03) | 0.043 | (-0.18 to 0.26) |
| | AUCi | 0.025 | (-0.15 to 0.20 | 0.101 | (-0.07 to 0.28) | 0.1 | (-0.06 to 0.26) | 0.06 | (-0.10 to 0.23) | -0.095 | (-0.23 to 0.04) | 0.53 | (-0.34 to 1.39) |
| | AUCg | 0.029 | (-0.13 to 0.19) | 0.06 | (-0.11 to 0.23) | 0.046 | (-0.11 to 0.20) | 0.06 | (-0.10 to 0.22) | -0.107 | (-0.24 to 0.02) | 0.53 | (-0.28 to 1.33) |
| Cortison | Maximum | -0.002 | (-0.04 to 0.03) | 0.01 | (-0.03 to 0.05) | 0.014 | (-0.02 to 0.05) | -0.006 | (-0.04 to 0.03) | -0.006 | (-0.04 to 0.02) | -0.007 | (-0.19 to 0.18) |
| | Delta | -0.002 | (-0.04 to 0.04) | 0.018 | (-0.02 to 0.06) | 0.024 | (-0.01 to 0.06) | -0.003 | (-0.04 to 0.03) | -0.001 | -0.03 to 0.03) | 0.005 | (-0.19 to 0.20) |
| | AUCi | -0.005 | (-0.09 to 0.08) | 0.042 | (-0.05 to 0.13) | 0.055 | (-0.02 to 0.14) | 0.002 | (-0.08 to 0.09) | -0.008 | (-0.08 to 0.06) | 0.034 | (-0.41 to 0.48) |
| | AUCg | -0.001 | (-0.07 to 0.07) | 0.003 | (-0.07 to 0.07) | 0.004 | (-0.06 to 0.07) | -0.013 | (-0.08 to 0.05) | -0.02 | (-0.08 to 0.04) | -0.02 | (-0.37 to 0.33) |

Values represent β (95% CI) as analyzed with linear regression

Analyses were adjusted for weight gain during pregnancy, % breastmilk at age 3 months and ethnicity

AUCi or g, Area Under the Curve increase or ground; FMI, fat mass index; FFMI, fat free mass index

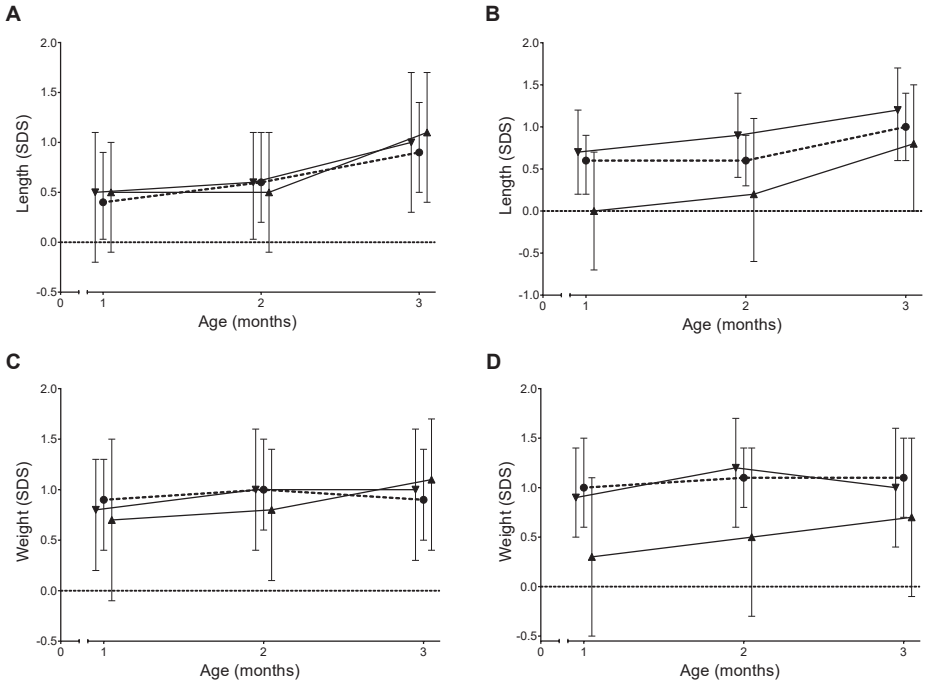


**Figure 1:** Growth trajectories between age 1 to 3 months for length and weight, according to breastmilk cortisone AUC outcomes (n=42). Results for breastmilk cortisol AUCi and AUCg were similar [data not shown]. A= length for AUCi, B= weight for AUCg, C= weight for AUCi, D= weight for AUCg; ▼ = AUCi or g <p25, ● = AUCi or g = p25-75, ▲ = AUCi or g >p75 ; AUCi or g, Area Under the Curve increase or ground

rhesus macaques at 1 month of age, and analyzed growth outcomes at 3.5 months of age. They found that higher cortisol concentrations were associated with greater weight gain over time. Hahn-Holbrook et al. (2016)[8] studied associations between breast-milk cortisol and BMI gains up until the age of 2 in 51 mother-infant pairs. They found that higher milk cortisol concentrations were associated with smaller BMI gains in offspring.

The different results between this study and previous studies could have several explanations. First, cortisol sampling in the previous studies did not take the diurnal rhythm of breastmilk GCs into account. Hinde et al.[7] sampled between 11:30-13:00, which did not capture peak GC concentration, since in Rhesus macaques, similar to humans, this occurs at around 8:00. Hahn-Holbrook et al.[8] collected a single breastmilk sample within a wide time window (11:30-16:00), which also did not capture peak concentrations. Analyses were corrected for time of collection, but it has previously been shown that correcting for time of sampling cannot account for all the variability observed in cortisol levels.[9,22] Second, in our study GC concentrations were determined by LC-MS/MS, which has been shown to be more sensitive and reliable than radioimmunoassay and chemiluminescent immunoassay,[23] which were used by Hinde et al. and Hahn-Holbrook et al. respectively.

Lastly, it has previously been shown that increases in fat mass specifically are associated with mid-childhood overweight and obesity[24]. Therefore, in this study, body composition was measured by ADP, which is able to differentiate between fat mass and fat free mass. In contrast, weight gain and changes in BMI were used as outcomes measured by Hinde et al.[7] and Hahn-Holbrook et al.,[8] respectively, both of which are less precise methods to determine body composition. Our more detailed methods when measuring GC concentrations in breastmilk as well as when determining body composition might therefore have led to more accurate conclusions.

Alternatively, the absence of associations might be due to the small sample size in this study, especially compared to Hinde et al.,[7] who included 108 mother-infant pairs, resulting in more power to detect small differences. However, this should be balanced against the use of of air-displacement plethysmography in this study, which is superior to weight gain for the assessment of body composition. Additionally, our follow-up until the age of 3 months was rather short. In contrast, follow-up took place up to 2 years of age in Hahn-Holbrook et al.'s study.[8] It is therefore possible that effects of GCs in breastmilk might only be noticeable at a later age. On the other hand, an increasing number of nutritional, life-style and family factors determine body composition with advancing age, and it is therefore progressively more difficult to determine to what extent breastmilk cortisol explains BMI gains.

This study has several strengths and limitations. This was the first study to assess the association between GC rhythmicity in breastmilk and body composition in the offspring. Body composition and GC rhythmicity were analyzed in detail, respectively by use of ADP and by measuring both cortisol and cortisone in breastmilk using samples that were collected over a 24-hour period. Cortisone concentrations have been shown to be more reliable than cortisol measurements, at least in saliva and hair.[25,26] This is possibly due to the local conversion of cortisol by 11β-hydroxysteroid dehydrogenase type 2, which leads to higher concentrations of cortisone.[27] However, this study also has its limitations. The sample size of this study was relatively small, and it is therefore possible that modest effects could not be detected. It was also not possible to correct for all potential confounders. However, many confounders were considered, and the three variables with the largest confounding effect were included in the final model, which did not change the results compared to unadjusted analyses. It is therefore unlikely that adjusting for more variables would have altered the results. Second, the follow-up in this study was relatively short, and it is therefore possible that breastmilk GC rhythmicity has an effect only noticeable at a later age. Additionally, a selection bias cannot be ruled out, since we did not collect data on mothers who were eligible for inclusion but decided against participation and since we included mother-infant pairs at a (tertiary) hospital. The study population might therefore not reflect the general population; for example, 51% of the mothers have birth via Caesarian section, compared to approximately 17%

in the general population.[28] Lastly, the interplay between GCs and infant body composition is complex, and could be moderated by, for example, exposure to GCs and other conditions in utero, the number of feeds per day, and the extra-uterine environment of the infants, including synchrony in mother-infant interactions as well as stressful events. Unfortunately, we were not able to take these factors into account.

## CONCLUSIONS

This study did not find an association between breastmilk GC rhythmicity at 1 month and growth trajectories as well as body composition of the offspring at age 3 months. Therefore, this study suggests that previous observations linking breastmilk cortisol to changes in infant weight might be flawed by the lack of serial cortisol measurements and detailed information on body composition.

## REFERENCES


1. Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet 1986; 1:1077-1081
2. Finken MJ, van der Voorn B, Heijboer AC, de Waard M, van Goudoever JB, Rotteveel J. Glucocorticoid Programming in Very Preterm Birth. Horm Res Paediatr 2016; 85:221-231
3. Rosmond R, Bjorntorp P. The hypothalamic-pituitary-adrenal axis activity as a predictor of cardiovascular disease, type 2 diabetes and stroke. J Intern Med 2000; 247:188-197
4. Drake AJ, Tang JI, Nyirenda MJ. Mechanisms underlying the role of glucocorticoids in the early life programming of adult disease. Clin Sci (Lond) 2007; 113:219-232
5. Entringer S. Impact of stress and stress physiology during pregnancy on child metabolic function and obesity risk. Curr Opin Clin Nutr Metab Care 2013; 16:320-327
6. Angelucci L, Patacchioli FR, Scaccianoce S, Di Sciullo A, Cardillo A, Maccari S. A model for later-life effects of perinatal drug exposure: maternal hormone mediation. Neurobehav Toxicol Teratol 1985; 7:511-517
7. Hinde K, Skibiel AL, Foster AB, Rosso LD, Mendoza SP, Capitanio JP. Cortisol in mother's milk across lactation reflects maternal life history and predicts infant temperament. Behavioral Ecology 2015; 26:269-281
8. Hahn-Holbrook J, Le TB, Chung A, Davis EP, Glynn LM. Cortisol in human milk predicts child BMI. Obesity (Silver Spring) 2016; 24:2471-2474
9. van der Voorn B, de Waard M, van Goudoever JB, Rotteveel J, Heijboer AC, Finken MJ. Breast-Milk Cortisol and Cortisone Concentrations Follow the Diurnal Rhythm of Maternal Hypothalamus-Pituitary-Adrenal Axis Activity. J Nutr 2016; 146:2174-2179
10. Adam EK, Quinn ME, Tavernier R, McQuillan MT, Dahlke KA, Gilbert KE. Diurnal cortisol slopes and mental and physical health outcomes: A systematic review and meta-analysis. Psychoneuroendocrinology 2017; 83:25-41
11. Ruttle PL, Javaras KN, Klein MH, Armstrong JM, Burk LR, Essex MJ. Concurrent and longitudinal associations between diurnal cortisol and body mass index across adolescence. J Adolesc Health 2013; 52:731-737
12. Wirix AJ, Finken MJ, von Rosenstiel-Jadoul IA, Heijboer AC, Nauta J, Groothoff JW, Chinapaw MJ, Kist-van Holthe JE. Is There an Association Between Cortisol and Hypertension in Overweight or Obese Children? J Clin Res Pediatr Endocrinol 2017; 9:344-349
13. Patacchioli F, Cigliana G. Maternal plasma and milk free cortisol during the first 3 days of breastfeeding following spontaneous delivery or elective cesarean section. Gynecolog Obstet Invest 1992; 34:159-163
14. Zigmond AS, Snaith RP. The hospital anxiety and depression scale. Acta Psychiatr Scand 1983; 67:361-370
15. Ma G, Yao M, Liu Y, Lin A, Zou H, Urlando A, Wong WW, Nommsen-Rivers L, Dewey KG. Validation of a new pediatric air-displacement plethysmograph for assessing body composition in infants. Am J Clin Nutr 2004; 79:653-660
16. van der Voorn B, Martens F, Peppelman NS, Rotteveel J, Blankenstein MA, Finken MJ, Heijboer AC. Determination of cortisol and cortisone in human mother's milk. Clin Chim Acta 2015; 444:154-155
17. Pruessner JC, Kirschbaum C, Meinlschmid G, Hellhammer DH. Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change. Psychoneuroendocrinology 2003; 28:916-931

18. Kyle UG, Schutz Y, Dupertuis YM, Pichard C. Body composition interpretation. Contributions of the fat-free mass index and the body fat mass index. Nutrition 2003; 19:597-604
19. TNO. De Vijfde Landelijke Groeistudie. 2010;
20. Schonbeck Y, Talma H, van Dommelen P, Bakker B, Buitendijk SE, HiraSing RA, van Buuren S. The world's tallest nation has stopped growing taller: the height of Dutch children from 1955 to 2009. Pediatr Res 2013; 73:371-377
21. Figueiras A, Domenech-Massons JM, Cadarso C. Regression models: calculating the confidence interval of effects in the presence of interactions. Stat Med 1998; 17:2099-2105
22. de Weerth C, Zijl RH, Buitelaar JK. Development of cortisol circadian rhythm in infancy. Early Hum Dev 2003; 73:39-52
23. Ackermans MT, Endert E. LC-MS/MS in endocrinology: what is the profit of the last 5 years? Bioanalysis 2014; 6:43-57
24. Koontz MB, Gunzler DD, Presley L, Catalano PM. Longitudinal changes in infant body composition: association with childhood obesity. Pediatr Obes 2014; 9:e141-144
25. Blair J, Adaway J, Keevil B, Ross R. Salivary cortisol and cortisone in the clinical setting. Curr Opin Endocrinol Diabetes Obes 2017; 24:161-168
26. Savas M, Wester VL, de Rijke YB, Rubinstein G, Zopp S, Dorst K, van den Berg SAA, Beuschlein F, Feelders RA, Reincke M, van Rossum EFC. Hair glucocorticoids as biomarker for endogenous Cushing's syndrome: validation in two independent cohorts. Neuroendocrinology 2019;
27. Smith RE, Maguire JA, Stein-Oakley AN, Sasano H, Takahashi K, Fukushima K, Krozowski ZS. Localization of 11 beta-hydroxysteroid dehydrogenase type II in human epithelial tissues. J Clin Endocrinol Metab 1996; 81:3244-3248
28. Macfarlane AJ, Blondel B, Mohangoo AD, Cuttini M, Nijhuis J, Novak Z, Olafsdottir HS, Zeitlin J, Euro-Peristat Scientific C. Wide differences in mode of delivery within Europe: risk-stratified analyses of aggregated routine data from the Euro-Peristat study. BJOG 2016; 123:559-568

**Supplementary Table 1:** Cortisol and cortisone concentrations in breastmilk in 4-hour intervals.

| | **Cortisol (nmol/L)** | **Cortisone (nmol/L)** |
|---|---|---|
| 0:00-4:00 | 4.1±5.5 | 15.1±11.8 |
| 4:00-8:00 | 11.6±8.7 | 29.7±12.8 |
| 8:00-12:00 | 8.2±6.5 | 27.9±8.6 |
| 12:00-16:00 | 4.4±2.9 | 21.3±6.8 |
| 16:00-20:00 | 2.1±1.4 | 13.0±6.1 |
| 20:00-24:00 | 2.1±3.9 | 10.4±9.2 |

Values represent mean±SD