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Placenta DNA methylation levels of the promoter region of the leptin receptor gene are associated with infant cortisol

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Abstract

The intrauterine environment and early life stress regulation are widely recognized as an early foundation for lifelong physical and mental health. Methylation of CpG sites in the placenta represents an epigenetic modification that can potentially affect placental function, influence fetal development, and ultimately impact the health of offspring by programming the hypothalamic-pituitary-adrenal (HPA) axis stress response during prenatal development. Leptin, an adipokine produced by the placenta, is essential for energy homeostasis. It is also epigenetically regulated by promoter DNA methylation. Mounting evidence suggests that leptin also affects the stress response system. Though heterogeneity in the early stress response system may influence life-long mental and physical health, few studies explicitly examine the heterogeneity in the newborn stress response system. Less is known about leptin's association with the human hypothalamic-pituitary-adrenocortical (HPA) axis early in life. This study sought to serve as a proof of concept study investigating the relationship between newborn cortisol output trajectories and placental leptin DNA methylation in 117 healthy newborns from socioeconomically and racially- and ethnically-diverse families. We characterized heterogeneity in newborn cortisol output during the NICU Network Neurobehavioral Scales exam in the first week of life with latent growth mixture models. We then evaluated whether leptin promoter (LEP) methylation in placental samples was associated with newborn cortisol trajectories. Our findings suggest that increased placental LEP methylation, which corresponds to decreased leptin production, is associated with infant cortisol trajectories marked by increased cortisol output in the NNNS exam. These results provide important insights into the role of placental leptin DNA methylation in human newborn HPA axis development and subsequent developmental origins of health and disease processes.

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

Keywords

newborn; leptin; epigenetics; hypothalamic-pituitary-adrenocortical axis

1. Introduction

Developmental Origins of Health and Disease (DOHaD) research finds that developing tissues and organ systems *in utero* are vulnerable to perturbations that could affect long-term health and disease (journal of epidemiology, 2003). One key system susceptible to fetal programming is the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is key to short-term survival and lifespan health: it organizes the body's response to stressors and maintains homeostasis in the metabolic, cardiovascular, immune, reproductive, and central nervous systems. Dysregulation of the HPA axis is associated with many disorders, including cardiometabolic disease, gastrointestinal dysfunction, and affective disorders (Guidi et al., 2020). Humans are born with a well-functioning stress system that can react to changes in their internal and external environments. As infant stress responses may predict future development, understanding how variability in the physiology of the early HPA axis arises from fetal programming can elucidate future mechanisms of life-course health (Beauchaine et al., 2007; Gunnar & Quevedo, 2007; Howland et al., 2017).

The placenta is a sensitive, functional tissue that serves as an ideal fetal record of the intrauterine environment and mediates the effects of the intrauterine environment on the development and health of the offspring (Maccani & Marsit, 2009). The prenatal environment may affect health and HPA axis functioning through epigenetic processes, such as gene methylation, that have long-lasting effects on DNA expression and phenotypes (Smith & Ryckman, 2015). Much research has been done on the impact of psychosocial stress during pregnancy and newborn HPA axis development, focusing on placental mediators of glucocorticoid exposure (Harris & Seckl, 2011). Less research exists on placenta markers of metabolic functioning and how they affect infant HPA axis development, despite links between the systems that regulate metabolic stress responses and maintain energy homeostasis (Monk et al., 2013).

Leptin, an adipokine produced by the placenta and expressed centrally in the HPA axis, is an important regulator of energy homeostasis and is potentially involved in the developing stress response system. Though much evidence indicates that leptin inhibits the HPA axis, the reciprocal relationship between leptin and cortisol indicates that leptin may also upregulate HPA activity (Malendowicz et al., 2007). In support of a bidirectional relationship between leptin and glucocorticoids, leptin concentrations are lowest during the day and highest at night, acting in the opposite pattern of cortisol's circadian rhythms (Bouillon-Minois et al.). A recent meta-analysis on the relationship between leptin and cortisol reactivity found that in adult humans, leptin decreases after acute stressors (Bouillon-Minois et al., 2021). Leptin can dampen the HPA axis response to many kinds of stress (Malendowicz et al., 2007). In animal models, leptin reduces HPA activation to metabolic, physical, and social stressors (Collura et al., 2009; Fulton et al., 2006), and glucocorticoids are elevated in leptin-deficient mice (Giovambattista et al., 2000). Leptin

infusion in rhesus monkeys increased glucocorticoid negative feedback to the HPA axis, decreasing the neuroendocrine response to stressors (Wilson et al.).

Leptin can inhibit HPA activation in response to stress by inhibiting CRH release, disrupting the HPA axis's typical negative feedback loop (Heiman et al., 1997). Leptin can regulate corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) secretion through hormonal signals within cells and between adjacent cells (Malendowicz et al., 2007). Leptin can directly inhibit CRH release from the hypothalamus and can also inhibit CRH release indirectly through other neuropeptides (Heiman et al., 1997).

Regarding fetal programming, leptin is critical to neuroendocrine, immune, and reproductive functions during pregnancy (Alexe et al., 2006). The placenta produces leptin alongside maternal and fetal adipose tissue (Moschos et al., 2002). During pregnancy, placenta leptin regulates fetal growth and nutrient exchange (Tessier et al., 2013). Studies of leptin in pregnancy find that plasma leptin levels in individuals are twice as high in pregnant individuals compared to non-pregnant individuals (Masuzaki et al., 1997). The placenta is a key producer of maternal leptin during pregnancy: levels increase in the second and third trimesters and return to non-pregnant levels within 24 hours (Masuzaki et al., 1997). The placenta releases most of its leptin into the maternal circulation, but it also releases ~5% into fetal circulation (Lepercq et al., 2001; Linnemann et al., 2000). The amount of leptin released into fetal circulation from the placenta is comparable to the placental transfer of ~3-7% of cortisol into the fetal compartment (Stirrat et al., 2018). Leptin might impact fetal HPA axis development: a sheep model found evidence for a negative feedback loop between experimentally-introduced leptin, naturally-occuring leptin levels, and the fetal HPA axis (Yuen, 2004). Specifically, leptin suppressed the prepartum activation of the fetal HPA axis (Yuen, 2004). Yuen et al. (2004) found that leptin infusion suppressed activation of the fetal HPA axis and, in control animals, found a negative relationship between fetal plasma concentrations of cortisol and leptin.

Leptin is epigenetically regulated by promoter DNA methylation (Moschos et al., 2002) and, therefore, may be a candidate for understanding fetal programming of the HPA axis. DNA methylation of the promoter region of the leptin gene (LEP) is linked to pregnancy pathology and regulates placenta LEP expression (Bouchard et al., 2010). Namely, leptin expression is inversely correlated with LEP methylation: increased placental LEP methylation corresponds to decreased leptin production (Bouchard et al., 2010; Lesseur, Armstrong, Paquette, et al., 2014; Melzner et al., 2002; Noer et al., 2006). Recent studies have found evidence for associations between maternal exposures in pregnancy (e.g., diet, stress) and offspring LEP methylation in placenta ((Daniels et al., 2020; Tobi et al., 2009; Tian et al., 2019). Therefore, as outlined in Figure 1, maternal and environmental exposures (e.g., maternal diet and stress during pregnancy) may influence the heterogeneity of the newborn cortisol response to stress through changes in DNA methylation of the leptin promoter gene (LEP) in the placenta. Decreased placental LEP methylation, which corresponds to increased leptin production, would then be associated with the newborn stress response through leptin's actions on CRH and the HPA negative feedback loop. Concordantly, increased placental LEP methylation, corresponding to decreased leptin production, would be associated with the newborn stress response through similar

mechanisms. However, little is known about placenta leptin gene expression and early-life HPA axis function.

To our knowledge, no human studies have been conducted to ascertain the association between prenatal leptin and infant cortisol. The present study is a secondary analysis of extant data on infant cortisol reactivity and placental LEP methylation collected in a subset of a cohort of pregnant women and their offspring between 2007-2011. In these analyses, we first sought to characterize newborn HPA axis functioning and model intra-individual growth trajectories and inter-individual variation while accounting for unobserved population heterogeneity in outcome growth trajectory by identifying distinct latent classes (Wang & Wang, 2019). In this way, we aimed to determine if groups of newborns exhibited distinct trajectories of cortisol output during the NICU Network Neurobehavioral Scales (NNNS) exam. Many studies have focused on changes in infant salivary cortisol concentrations across an exam or heel stick stressor to assess HPA axis reactivity (Jansen et al., 2010). Furthermore, although many studies evaluate mean increases in salivary cortisol in response to a stressor, work by Gunnar and colleagues (Gunnar et al., 2009) suggests individual differences and variability in stress responses early in life. To our knowledge, no studies have examined heterogeneity in cortisol growth trajectories in the first week of life.

Subsequently, we sought to examine the associations between newborn HPA axis functioning and placenta DNA methylation of the promotor region of the leptin receptor gene (LEP) to explore whether the relationships between leptin and cortisol evident in adults would be evident very early in life. Based on the inverse relationship between leptin and cortisol reactivity in adults, we hypothesized that increased placental LEP methylation (representing lower leptin levels) would be associated with higher levels of newborn cortisol across the NNNS exam in the first week of life. The sample size for this study is modest (N=117), and this study uses methylation of a pre-specified region of the leptin receptor gene due to placenta collection and analysis constraints within the original cohort. Thus, our goal is to provide proof of concept data to support future work investigating heterogeneity in infant cortisol trajectories and examine early relationships between leptin and cortisol as they relate to the developmental origins of health and disease.

2. Method

2.1 Participants & Procedure Overview

Participants (N=117) were a subsample of mother-infant pairs recruited within Providence, Rhode Island, and surrounding areas as part of the 2007-2011 Behavior and Mood in Mothers and Behavior in Infants (BAMBI) Study with DNA methylation of the promotor region of the leptin receptor gene and infant cortisol data (Stroud et al., 2016). Briefly, prospective participants were screened via telephone and invited to the first session if they did not meet the exclusion criteria. Exclusion criteria included non-singleton pregnancy, maternal age < 18 years, life-threatening maternal complications, and infant congenital or chromosomal abnormalities. Additionally, one woman was excluded for binge drinking, one woman was excluded for a "major medical condition," and one infant was excluded who was extremely preterm.

2.2. Procedures

Maternal Interviews & Chart Review During Pregnancy & Delivery—Mothers were interviewed prospectively over the second and third trimesters of pregnancy and at delivery (*M*=3 interviews (range 2–4) between 24–42 weeks gestation). Mothers provided demographic information, including age, race, ethnicity, and household income. Mothers also reported pregnancy history (gravida, parity) and pre-pregnancy weight. Maternal depression was assessed by structured interview using the 21-item Hamilton Depression Rating Scale (Hamilton, 1986). In the first session, experimenters recorded self-reported pre-pregnancy weight and measured participants' height using a standing, wall-mounted stadiometer (Perspectives Enterprises, Portage, MI; accuracy ±.057kg). Maternal pre-pregnancy body mass index (weight(kg)/height(m)squared; PPBMI) was used as a continuous measure. Birth outcomes and hospital stay data, including gestational age at birth, fetal distress during labor, assisted delivery, birth weight, birth length, infant head circumference, small for gestational age (SGA; weight <10th percentile for the gestational age), large for gestational age (LGA; weight >90th percentile for gestational age), and circumcision date, were collected by medical chart review following delivery.

Infant Stress Response Assessment over the First Postnatal Month—Infant salivary cortisol output was assessed during the NICU Network Neurobehavioral Scale (NNNS) (Lester et al., 2004; Tronick & Lester, 2013). Certified examiners administered the NNNS in the hospital immediately after delivery or at home in the first week after delivery (mean age of NNNS in days: 1.2 ± 1.1). The NNNS assesses neurobehavioral development through observation, neurologic and behavioral tests, and exposure to auditory, visual, social, and non-social stimuli (Lester et al., 2004; Tronick & Lester, 2013). The NNNS lasts approximately 30 minutes. The NNNS is a mild stressor, as a stranger observes and handles the infant in a way similar to a routine medical exam. Four passive drool saliva samples were collected to quantify cortisol during and after the NNNS (baseline, end of the NNNS, 20 and 40 minutes post-NNNS; Salimetrics LLC, State College, Pennsylvania).

2.3 Bioassays

Infant Cortisol—After collection, infant saliva samples were frozen until assayed using a highly sensitive enzyme immunoassay (Salimetrics LLC (HS EIA)). The intra- and inter-assay coefficients of variation were 6.4% and 6.6%, respectively (Stroud et al., 2016).

Placenta Leptin Promoter Methylation

Sample collection and nucleic acid extraction.: Placenta samples were collected within two hours following delivery. Research assistants obtained placenta samples devoid of maternal decidua and comprised of twelve fragments, three from each quadrant, and two centimeters from the umbilical cord. After placenta tissue was excised, it was placed immediately in RNAlater solution (Life Technologies, Grand Island, NY, USA) and stored at 4°C. Afterward, tissue samples were blotted dry, snap frozen in liquid nitrogen, pulverized to homogeneity, and stored at −80°C until analysis. The lab of Dr. Carmen Marsit conducted the analysis. Placental genomic DNA was extracted using the DNAeasy Blood & Tissue Kit

(Qiagen, Inc, Valencia, CA, USA) and assessed for quantity and quality using an ND-1000 Spectrophotometer (Nanodrop, DE).

DNA bisulfite modification, pyrosequencing analysis, and SNP genotyping.: DNA samples (500 ng) were modified with sodium bisulfite using the EZ DNA methylation kit and manufacturer protocols (Zymo Research, Irvine, CA, USA). Bisulfite pyrosequencing was employed for methylation detection. Primers (Integrated DNA Technologies, Inc, Coralville, IA) were designed using the PyroMark Assay Design software version 2.0.1.15 (Qiagen) in a region previously associated with leptin expression (Bouchard et al., 2010; Melzner et al., 2002). The PyroMark PCR kit (Qiagen) and PCR primers were used to amplify a 383 base pair region in the LEP; cycling conditions were 94 °C for 15 min followed by 50 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min with a final extension of 10 min at 72 °C. Pyrosequencing was performed in triplicate using the Pyromark MD (Qiagen) instrument with two forward assays covering 23 CpG loci. Non-CpG cytosines within each read were internal controls to verify bisulfite DNA modification efficiency (> 95% in all samples), and each pyrosequencing run included a template-free control. The same operator sequenced all samples. The results of DNA methylation were analyzed with PyroMark CpG software, version 1.0.11 (Qiagen). LEP methylation was assessed by bisulfite pyrosequencing, which lead to percent DNA methylation at 12 CpG loci. The mean percent LEP methylation was taken across the 12 loci due to the high correlation between the loci (Daniels et al., 2020). The samples were also genotyped for rs2167270 (+19G > A), a common SNP in the LEP region. Genotype calls were made by analyzing the pyrograms and comparing peak heights for each allele; triplicate wells were called independently and compared for quality control.

2.3. Statistical Analyses

All analyses were performed using R (R Core Team). Upon visual examination of the data, infants exhibited variable trajectories of cortisol response to the NNNS at one week of life. Latent class mixed model (LCMM) analysis in the R package “lcmm” (Proust-Lima et al., 2017) was used to determine whether there were distinct groups of different cortisol response trajectories in response to the NNNS. Latent class mixed modeling (LCMM) was used to depict the course of infant salivary cortisol output during the NNNS, allowing for latent person-specific trajectory identification. Model estimation relied on robust maximum likelihood and full information methods. First, cortisol models depicting either linear or quadratic growth in the fixed and random components were examined. Afterward, models with an increasing number of trajectory classes were compared to identify the model with optimal class enumeration.

LCMM models with profiles ranging from two to four were examined. Several statistical criteria were used to determine the best-fitting model, including Bayesian information criterion (BIC), Akaike information criterion (AIC), sample-adjusted Bayesian information criterion (SABIC), and entropy. BIC, AIC, and SABIC are measures of the relative fit of different models, with lower values indicating better model fit. Entropy was used to inspect the separation between distinct profiles of one model, with higher values (at least 0.8) indicating better profile separation.

We used the classical three-step method to determine the association between placenta LEP promoter methylation and infant salivary cortisol latent classes. This three-step approach that starts with an unconditional model and uses the estimated latent class membership assignments for further analysis is a common practice in examining relationships from a latent class mixed modeling approach. Following that method, we first conducted the latent class analysis and classified the salivary cortisol observations based on their most likely posterior probabilities. We then extracted the latent class variable as an observed categorical variable based on those probabilities. Finally, we used this saved categorical variable as an outcome variable in the subsequent analysis that determined the association between placenta LEP promoter methylation and infant salivary cortisol classes (Wang, 2019).

Covariates.—Potential maternal, perinatal, and neonatal covariates were tested for associations with infant cortisol group membership. One-way ANOVAs (for continuous variables) or Pearson chi-square (for categorical variables) analyses were performed to ensure that infant cortisol group membership did not differ by key maternal, infant, and NNNS variables. Maternal variables included mother's age, marital status, maternal education, maternal depression (0 = none, 1 = past minor or major depression, 2 = current minor or major depression), maternal race/ethnicity, and household income. Infant variables included gestational age at delivery (weeks), fetal distress (1=yes, 0=no), whether labor was assisted (1=assisted, 0=vaginal and unassisted), birthweight, birth length, head circumference, and SGA or LGA status. NNNS covariates included infant age at cortisol assessment (days), time NNNS began, and circumcision within 24 hours preceding the NNNS exam (1=yes, 0=no). Two covariates, pre-pregnancy BMI and infant sex, were included a priori as past research in this sample and others have found these infant sex and maternal adiposity to be related to infant cortisol (Jones-Mason et al., 2018; Stroud et al., 2016).

3. Results

3.1. Sample Characteristics

Characteristics of the study population are listed in Table 1. The sample was racially/ethnically and socioeconomically diverse: participants reported an income range from < $10,000 per year to > $100,000 per year, and less than half self-identified as white. Mean LEP methylation was normally distributed and ranged from 8.9% to 51.7%. Genotype frequencies at rs2167270 were in Hardy-Weinberg equilibrium (Mayo, 2008), with 16.2% of the infants homozygous for the variant allele (A), 46.1% heterozygous, and 37.6% homozygous for the dominant allele (G).

3.2 Infant Cortisol

Three groups were identified using LCMM (Figure 2). The growth solution comprising three heterogeneous cortisol trajectories across the NNNS, with random intercepts and quadratic random slopes, demonstrated a better fit than the other nested models (Table 2). This model presented acceptable fit indexes (AIC = 79.05, BIC = 109.43, SABIC = 74.66) and a high mean of posterior probabilities belonging to each trajectory class (.90–.91). The model indicated the presence of 3 cortisol response groups: Group 1 was a "low cortisol throughout

NNNS" group (N = 81, 69.2% of sample, mean posterior probability = .91), Group 2 was a "start low and increase" group (N = 20, 17.1% of sample, mean posterior probability = .90), and Group 3 was a "start high and decrease" group (N = 16, 13.7% of sample, mean posterior probability = .91). Sensitivity analyses showed that none of the maternal (maternal age, marital status, education, race, ethnicity, household income, depression) or infant variables (baby's age, fetal distress, assisted labor, or circumcision) were significantly associated with infant cortisol group membership (Table 1).

3.3 Association between placenta DNA methylation of the promotor region of the leptin receptor gene and infant cortisol output

The ordinal logistic regression model predicting infant cortisol output group from mean LEP methylation with pre-specified covariates of pre-pregnancy BMI and infant sex demonstrated better fit indices than the model without the covariates and thus was retained (model with covariates: AIC = 187.829, BIC = 201.466, Nagelkerke's R^2 = 0.162. Model without covariates: AIC = 197.246, BIC = 205.533, Nagelkerke's R^2 = 0.028). As an additional check of the appropriateness of an ordinal logistic regression model compared to a multinomial logit model, we performed a likelihood ratio test to see if the ordinal logistic regression model and the same model using multinomial logistic regression are statistically different ($X^2(3) = 3.311$, $p = 0.346$). The high p-value indicated that the proportional odds model fit as well as the more complex multinomial logit model, so the more parsimonious proportional odds model was retained for parsimony and power (Hosmer et al., 2013). The ordinal logistic regression results showed a significant association between mean placental LEP methylation and infant cortisol group (p=.016), such that higher levels of DNA methylation of the promotor region of the leptin receptor gene were associated with increased odds of membership in cortisol groups typified by an increased cortisol output across the NNNS or a high starting output across the NNNS (Table 3, Figure 3). For every one unit (1%) increase in the mean methylation, the odds of being in a higher cortisol output group was 1.076, holding constant all other variables (pre-pregnancy BMI and infant sex). In other words, the odds of an infant belonging to an increased cortisol output group increase by 7.6% for every 1% increase in placenta LEP methylation.

Discussion

This study demonstrates that placenta LEP methylation was associated with infant salivary cortisol output during the NNNS exam in the first week of life. Lower levels of placenta LEP methylation were associated with a cortisol trajectory that was lower and did not rise throughout the stressor. Higher levels of placental LEP methylation were associated with an increased probability of exhibiting cortisol trajectories that either stayed high or increased throughout a stressor. To our knowledge, this is the first study to examine placenta LEP methylation and newborn cortisol.

This finding is consistent with adult and non-human animal model studies on the inverse association between leptin and cortisol in acute stress lab paradigms. In rodents, leptin inhibits steroid hormone secretion from the adrenal cortex. Leptin also dampens the HPA axis response to many kinds of stress. The current study did not collect leptin directly.

Instead, we measured placental LEP methylation as a proxy of potential leptin output and fetal programming. Nonetheless, just as studies in adults find that lower levels of leptin are associated with higher levels of cortisol after a lab stressor (Bouillon-Minois et al., 2021), we found that lower levels of methylation, associated with increased leptin output in other studies, were associated with a decreased cortisol output trajectory. Higher levels of placental LEP methylation, associated with reduced leptin output in other research, were associated with more elevated cortisol trajectories. Few studies have examined the dynamics between leptin and the HPA axis early in life. Relevant to the development of the HPA axis, an analysis of school-aged children (6-7 years) who were former extremely low birth weight newborns found that leptin methylation was positively correlated (modest, univariate correlation) with salivary cortisol output only in children born extremely preterm (24–27 weeks GA) (Padbury et al., 2022). This opposes what this study and studies in adults have found, but there are a few critical differences to note. First, genomic DNA was extracted from pooled saliva samples during the school-age follow-up visit rather than placental samples, which reflects different developmental timing and may reflect other developmental processes in a tissue-specific manner. Also, the current study consisted of healthy children born at term, while the sample size of comparison children born at term was small (N=38) in the Padbury (2022) study. It is, therefore, difficult to directly compare the two most comparable parts of each cohort. Overall, the results of the current study show that placental LEP methylation is associated with newborn HPA axis activity in a way consistent with adult and non-human animal research on leptin and cortisol dynamics. These results also suggest that the relationship between leptin and cortisol may affect fetal and newborn energy balance and HPA axis programming. Psychosocial stress and dysregulated HPA axis activity have been associated with cardiometabolic disease, eating disorders, and depression (Guidi et al., 2020). Leptin may play a role, as it plays multiple roles in controlling energy balance in adulthood, including body-weight regulation, metabolism, and neuroendocrine function (Ahima & Flier, 2000; Harris, 2000). In utero, circulating leptin is an endocrine signal of fetal and placental size. Circulating leptin may communicate the fetus's energy needs to the mother, the placenta, and other fetal tissues. Leptin concentration and methylation may signal nutrient availability relevant to controlling glucogenesis, tissue maturation during late gestation, and establishing neural pathways essential for energy balance in adulthood (Forhead & Fowden, 2009). Accordingly, leptin is linked to birth size and fat mass, and newborns with growth retardation have higher cord cortisol concentrations (Forhead & Fowden, 2009). Therefore, our findings may represent an early developmental pathway by which leptin and the HPA axis work in tandem to influence energy balance later in life.

However, leptin expression in utero and during early development may act differently than leptin in adult life. For instance, though leptin is colloquially understood as the “satiety hormone” in regulating appetite, the role of satiety or appetite in the intrauterine environment is less clear when the fetus has access to a continuous supply of nutrients (Forhead & Fowden, 2009). Nevertheless, the expression and regulation of leptin in utero seems to play a role in hormonal control of growth and maturation (Forhead & Fowden, 2009). The placenta expresses significant leptin mRNA in human and non-human primate pregnancies (Henson et al., 1999; Lea et al., 2000). Leptin may signal changes in the intrauterine environment and govern fetal growth and development (Forhead & Fowden,

2009). First, evidence from fetal sheep models suggest that leptin may mediate and regulate some of the glucocorticoids' maturational effects near term (Forhead et al., 2002; O'Connor et al., 2007). Fetal tissues undergo structural and functional changes to prepare for extrauterine life, many of which are triggered by fetal glucocorticoids (Fowden et al., 1998). Ovine models provide evidence that glucocorticoids and leptin synthesis act in a negative feedback loop (Howe et al., 2002; Yuen et al., 2004). This negative feedback loop between leptin and the hypothalamic–pituitary–adrenal axis may affect the glucocorticoid-dependent onset of parturition (Yuen et al., 2004). Therefore, the relationship between leptin and glucocorticoids may reflect an index of neonatal maturity, and our results may reflect fetal leptin-cortisol dynamics in late gestation.

What prenatal exposures contribute to changes in DNA methylation of the promotor region of the leptin receptor gene and corresponding HPA axis trajectories is still an open question. The placenta is a unique endocrine and metabolic organ that mediates the relationships of environmental signals, nutrition, endocrine signals, and immune signals with fetal development (Maccani & Marsit, 2009). There were no differences in delivery type or complications between infants in the three cortisol trajectories. Thus, differences in obstetric outcomes cannot explain the neuroendocrine findings. Specific aspects of the maternal diet or HPA axis functioning may be at play, though more research is needed. In a study on the Dutch Hunger Winter cohort, LEP methylation was associated with prenatal famine exposure (Tobi et al., 2009). A recent study using the Born in Bradford multi-ethnic birth cohort found that maternal mental health symptoms predicted lower infant cord blood leptin (Scott & Manczak, 2021), possibly associated with an increased placental LEP methylation. Our study did not find cortisol group differences in maternal depressive symptoms. However, previous work in the larger cohort found sex-specific effects on infant cortisol moderated by placenta glucocorticoid and serotonergic pathways (Stroud et al., 2016). Indices of the maternal stress response and HPA axis regulation in pregnancy may influence placenta LEP methylation. In a recent epigenome-wide study in 257 mother-infant pairs, maternal CRH in pregnancy was associated with increased LEP promoter DNA methylation levels in offspring cord blood (Tian, 2019). Interestingly, the association between maternal CRH and LEP DNA methylation levels persisted into mid-childhood (Tian, 2019). Maternal diet in pregnancy has also been associated with placenta LEP methylation (Daniels et al., 2020), and infants from mothers with gestational diabetes mellitus exhibited higher placental LEP methylation levels (Lesseur, Armstrong, Paquette, et al., 2014). In contrast, our participant population was relatively healthy, and we do not have measures of maternal diet during pregnancy. Future work should consider the combined effects of prenatal and postnatal stress and malnutrition.

Although many studies evaluate mean increases in salivary cortisol in response to a stressor (Jansen et al., 2010), this analysis uncovered three distinct trajectories of newborn cortisol reactivity and subsequently found that placental LEP promoter methylation is associated with these trajectories. These differences may help identify infants who are more sensitive to cortisol elevations than their peers, which may correspond to different risk or resilience patterns for poor health and psychopathology (Gunnar et al., 2009). Most of the sample fell into the "low cortisol, low reactivity" group, with salivary cortisol values that started low and continued to be low across the sampling time. Though physical examinations such as

the NNNS are reliable methods of provoking a cortisol response in infants (Bruce et al., 2009; Jansen et al., 2010), two foundational studies of newborn cortisol reactivity in the first week of life found increases in salivary cortisol +25 minutes after a physical examination but found no response to the same stressor 24 hours later (Gunnar, 1989). Therefore, as the NNNS were conducted in the hospital immediately following delivery, the group characterized by a low reactivity response could indicate newborns who had habituated to the physical examination while in the hospital, as hospital examinations from doctors and nurses are standard within the first week of life. Suppose this is the response expected in typically developing newborns. In that case, the relative cortisol reactivity in groups 2 and 3 during the NNNS may be less typical or more indicative of differential susceptibility to environmental context. Accordingly, infants with lower cortisol reactivity trajectories were also most likely to exhibit lower levels of placenta LEP methylation. As the placenta is generally hypo-methylated, this association provides some additional preliminary evidence that lower cortisol levels and placenta LEP methylation may be more typical. In contrast, higher levels of placenta LEP methylation and higher cortisol reactivity profiles may reflect individual differences in the developmental origins of HPA axis development that may be particularly salient for later health and psychopathology.

Previous studies have linked placenta leptin DNA methylation with neurobehavioral changes characterized by lethargy and hypotonicity (Lesseur, 2014). Increased methylation of the leptin gene in the human placenta is associated with lower levels of motor, state, and physiologic reactivity in male newborns (Lesseur, 2014). Though we did not find sex-specific effects, we found that increased methylation of the leptin gene in the human placenta was associated with newborn cortisol reactivity phenotypes characterized by higher cortisol levels across the NNNS exam. Future research should consider whether placental leptin DNA methylation is independently associated with these neurobehavioral and HPA axis phenotypes or if cortisol reactivity trajectories mediate the relationship between lethargy and hypotonicity characteristics found in newborn males. It remains to be determined whether placental LEP methylation could program HPA-axis dysregulation risk later in life or help explain the relationship between prenatal exposures and offspring development.

Limitations

Despite the importance and clarity of our findings, there are limitations. The ability of this study to clarify the mechanisms underlying the observed changes in DNA methylation of the promotor region of the leptin receptor gene as they relate to newborn cortisol output is limited. First, we do not have methylation data from the same timepoint or the same tissues as the salivary cortisol data. Our samples were collected from placentas at term, reducing the ability to infer how variability in leptin-associated methylation plays a role in HPA axis functioning throughout development. As epigenetic regulation is tissue-specific and time-dependent, associations between these variables must be interpreted with caution. We also do not have measures of maternal or infant leptin. Interpretations of the association between LEP methylation and leptin output related to infant cortisol levels, though supported by previous research, are hypothetical. While maternal and newborn serum leptin levels may

help contextualize the findings, maternal and umbilical blood leptin levels at delivery are unrelated (Schubring et al., 1997).

A second limitation is using a single stressor to represent cortisol output and reactivity. In contrast to the multiple days of measurement required to capture a prototypical diurnal cortisol response, it is common to accept a single stressor on one day as a proxy for the prototypical stress response. Accordingly, multiple stressors to fully capture the stress response in the first week of life may provide a clearer picture of the latent trajectories of cortisol during the NNNS exam and the association between cortisol trajectories and placenta LEP methylation. However, the stress response system is sensitive to habituation, such that repeated stressor exposures across multiple days may fail to activate the HPA axis in a way that makes it possible to capture a prototypical stress response. In newborn infants, foundational work by Gunnar et al. found that repeat stressors on multiple days failed to activate a newborn stress response after the first stressor exposure (1989). Future work that assessed stress reactivity to multiple stressors and carefully considered the rapid development of the HPA axis over the first few months of life may clarify longitudinal cortisol trajectories and their association with LEP methylation in hypothesis-generating ways.

There are also several methodological limitations in the statistical approach that must be acknowledged. Although this is the first study to date linking placenta leptin promotor methylation to newborn HPA axis activity, we are still limited by our sample size in our ability to observe robust associations with greater variability or small effect sizes. The sample, while well-characterized and diverse, is relatively small (N=117). This can create issues both in estimating latent class membership in infant cortisol trajectories and in the methylation analysis. Regarding latent class membership, power analysis and sample size estimation for latent growth mixture modeling is still challenging (Wang, 2019). The current recommendation for mixture model sample size is to generally avoid high data dimensionality, or to reduce the numbers of indicators and items (Wang, 2019). To this end, we have focused on estimating a latent class growth mixture modeling with low data dimensionality focused on infant salivary cortisol samples. While growth mixture modeling has been conducted on cortisol reactivity paradigms with adult samples as small as N=34 (Ram & Grimm, 2009) and in childhood samples close to N=200 (Koss et al., 2013), sample size estimation remains a concern and future work could incorporate the estimates presented from the current study population to conduct Monte Carlo studies. The model fit also suggests high “quality” classification using the entropy statistic (≥.80, with 1.0 representing “perfect” classification) (Connell & Frye, 2006). Entropy ≥ .80, which the selected model exhibits, is considered good (Clark, 2010). Further, the mean salivary cortisol levels across samples are statistically significant between latent groups (Table 1). In addition, the model selected had the lowest BIC fit index, which has the noted strength of being largely insensitive to sample size, though it can underestimate the number of groups (D'Unger et al., 1998). Nevertheless, the clusters presented herein should be interpreted with caution. The sample distribution may be informative for future data simulations, and future research should seek to replicate the analysis in larger datasets.

Regarding analytical limitations of LEP methylation in the placenta, we used a candidate gene approach as it was what was available due to cost constraints within the existing cohort (collected 2007-2011). The power to detect true differences and false positives is a major concern with this approach and the small sample size. These factors may affect the generalizability of the present study's findings. More recently, studies of fetal DNA methylation on a genome-wide scale within the placenta with large sample sizes and replication samples are understood to be required to understand the relationship between methylation patterns and behavioral or physiological phenotypes. Unfortunately, very large studies with this level of placenta DNA methylation data may not be able to provide rich data on infant cortisol reactivity. Future work in this area with the ability to collect and analyze large numbers of methylation patterns in the placenta may be able to add to this area by conducting stress reactivity paradigms on a subsample of newborns with a planned-missingness design to more robustly examine whether the relationship between infant cortisol dynamics and placenta LEP methylation replicates in studies of fetal DNA methylation on a genome-wide scale.

Statistically, the methods used may underestimate the relationship between infant salivary cortisol and mean LEP methylation. The three-step method, widely used in applied research, may underestimate the relationship between mean LEP promoter methylation and the latent cortisol classes (Bolck et al., 2004; Vermunt, 2010). However, if the classification of classes is accurate, as determined by a good entropy value (≥ 0.80), the results obtained through this approach are deemed acceptable (Clark, 2010). In the current study, we found that our approach, which involved a three-class model selection with high posterior probabilities (≥ 0.90) and an entropy value (≥ 0.80), can be deemed acceptable for this particular application, as suggested by Wang (2019) and Clark (2010).

We also acknowledge that the generalizability of these findings may be hampered by underlying biases in our study population, including the oversampling of mothers with a history of depression, mothers with limited prenatal exposure to tobacco, alcohol, or other drugs of abuse, and a focus on a healthy newborn population. We suggest future research examining our findings in other populations, including longitudinal studies on growth and neurobehavioral outcomes and in non-human animal models, to supplement and strengthen the current results.

Conclusion

This study represents the first assessment of placental DNA methylation of the promotor region of the leptin receptor gene and infant HPA axis functioning. Our study’s diverse population, focus on the placenta, newborn cortisol collection method, and statistical analysis techniques strengthen the findings of our analysis. The participants of the study members are racially, ethnically, and socioeconomically diverse in addition to healthy, and the in-utero environment is likely more representative of the general population. As the placenta regulates the fetal environment, placental methylation may be a better biomarker than other tissues. Our results are also strengthened by the NNNS assessment, which is a type of exam that reliably activates the newborn HPA axis (Gunnar et al., 2009), applies to a broader research setting (Tronick & Lester, 2013), and has identified both high-risk infants

and later life health outcomes (Sucharew et al., 2012). Our statistical approach uncovered underlying variations in newborn cortisol phenotypes and placental LEP methylation. In support of our hypothesis, our analyses suggest that placenta LEP methylation at birth and newborns' cortisol patterns mirror adult dynamics of serum leptin and cortisol reactivity. Future work should examine the heterogeneity of infant cortisol reactivity and placental markers of the intrauterine environment. This study provides promising preliminary data highlighting the link between newborn HPA-axis activity and placental DNA methylation of the promotor region of the leptin receptor gene and its potential role in infant development.

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Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Highlights

- First study to investigate newborn cortisol trajectories and placenta LEP methylation.
- LEP methylation positively associated with cortisol output in first week of life
- Patterns mirror adult serum leptin and cortisol reactivity dynamics.
- Suggests role for placenta LEP methylation in human newborn HPA axis development

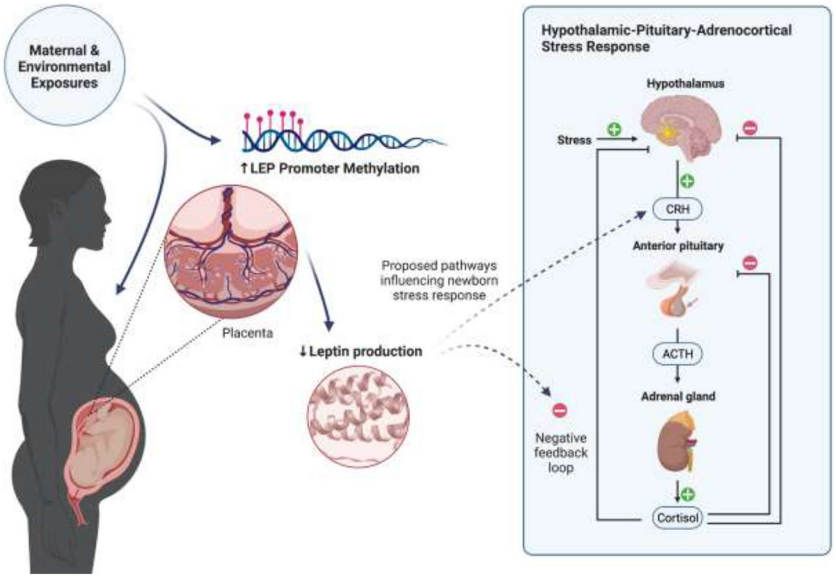

Figure 1. Hypothesized mechanisms of action from placenta leptin promoter methylation to newborn HPA axis functioning

Maternal and environmental exposures (e.g., maternal diet and stress during pregnancy) may influence heterogeneity of the newborn cortisol response to stress through changes in DNA methylation of the leptin promoter gene (LEP) in the placenta. Increased placental LEP methylation, which corresponds to decreased leptin production and, therefore, less leptin released into the maternal and fetal compartment, would then be associated with the newborn stress response through leptin's actions on CRH and the HPA negative feedback loop.

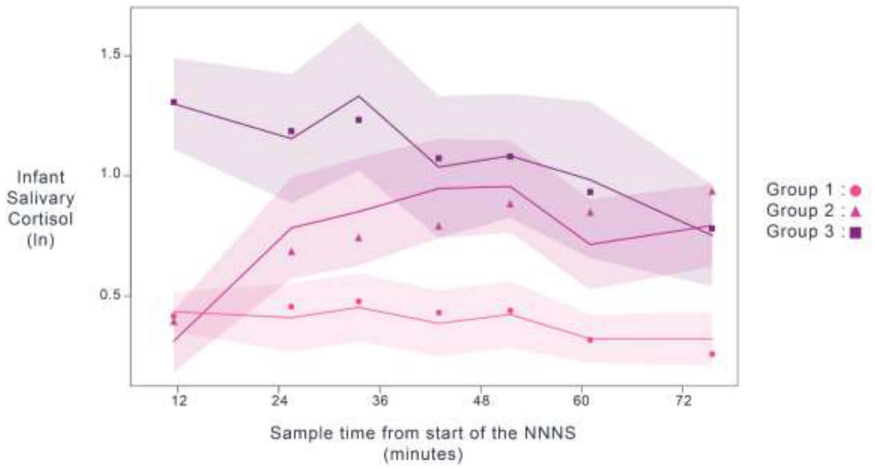

Figure 2. Weighted subject-specific predictions for infant salivary cortisol group membership

A latent class mixed model (LCMM) analysis determined that a quadratic random intercept & random slope model best fit the data and indicated the presence of 3 cortisol response groups (shown and color-coded below):

1. Group 1 [pink circles] The “low cortisol, low reactivity” group (N = 81, 69.2% of sample)
2. Group 2 [dark pink triangles] The “start low and increase” group (N = 20, 17.1% of sample)
3. Group 3 [purple square] The “start high and decrease” group (N = 16, 13.7% of sample)

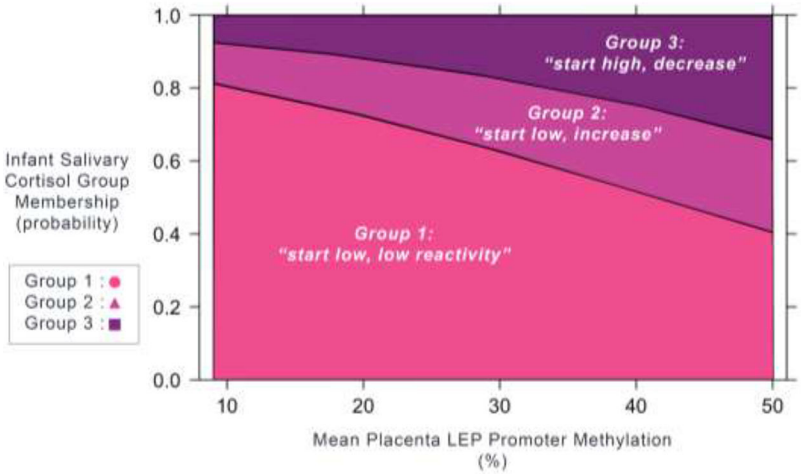

Figure 3. Predicting infant cortisol reactivity class from mean placental methylation

At lower levels of mean leptin methylation, the probability of being in the "low cortisol, low reactivity" group (0) is high; at increasing levels of mean leptin methylation, the likelihood of being in the other two classes ("1, start low, increase" and "2, start high, decrease") increases

Table 1.

Participant Demographic Information

	Infant Cortisol Group					
	Full Sample with Placenta & Cortisol (N=117)	**Group 1 (N=81, 69.2%)**	**Group 2 (N=20, 17.1%)**	**Group 3 (N=16, 13.7%)**	**Test of group differences**	
Variable	***Mean ± SD or N (%)***				***One-way ANOVA or p Pearson chi-square value***	***p-value***
Maternal Variables						
Mother's Age (years)	26.1 ± 5.3	26.3 ± 5.1	26 ± 6.2	25.7 ± 5.6	$F(2,114) = .095$	.910
Married	45 (28.5%)	36 (44.4%)	6 (30%)	3 (18.8%)	$X^2(2, 117) = 4.457$	.108
Maternal Education Above High School	73 (62.4%)	54 (66.7%)	9 (45%)	10 (62.5%)	$X^2(2, 117) = 3.209$	.201
Maternal Prenatal or Pre-conception Minor or Major Depression					$X^2(4, 116) = .680$	.954
None	36 (31%)	25 (30.9%)	7 (36.8%)	4 (25%)		
Past	22 (19%)	16 (19.8%)	3 (15.8%)	3 (18.8%)		
Current	58 (50%)	40 (49.4%)	9 (47.4%)	9 (56.3%)		
Hispanic or Latino	43 (37.1%)	27 (33.8%)	8 (40%)	8 (50%)	$X^2(2, 116) = 12.062$	.281
Self-reported race *					$X^2(2, 117) = 5.486$	.064
American Indian/Alaskan native	1 (.9%)	0 (0%)	0 (0%)	1 (6.3%)		
Asian	3 (2.6%)	1 (1.2%)	2 (10%)	0 (0%)		
Black or African American	14 (12%)	9 (11.1%)	2 (10%)	3 (18.8%)		
White	57 (48.7%)	43 (53.1%)	11 (55%)	3 (18.8%)		
More than once race	8 (6.8%)	7 (8.6%)	1 (5%)	0 (0%)		
Self-reported "don't know"[a]	34 (29.1%)	21 (25.9%)	4 (20%)	9 (56.3%)		
Total household income in past year					$X^2(6, 105) = 10.08$	.121
<$10,000	18 (17.1%)	11 (15.1%)	2 (11.8%)	5 (33.3%)		
$10,000 - $49,999	48 (45.7%)	29 (39.7%)	10 (58.8%)	9 (60%)		
$50,000 - $99,999	25 (23.8%)	21 (28.8%)	3 (17.6%)	1 (6.7%)		
≥$100,000	14 (13.3%)	12 (16.4%)	2 (11.8%)	0 (0%)		
Pre-Pregnancy BMI	25.4 ± 5.7	24.8 ± 5.2	25.7 ± 6.7	27.7 ± 6	$F(2,110) = 1.705$	.187
Leptin Variables						
Age of placenta sample (time of collection-time of delivery; hh:mm)	1:12 ± 0:32					
Mean leptin promoter methylation percent	22.7 ± 7.0	21.9 ± 7.0	25.8 ± 7.1	23 ± 5.8		
Genotype rs2167270 (%)					$X^2(4, 117) = 4.6$	.331
A/A	19 (16.2%)	10 (12.7%)	6 (20.1%)	3 (20%)		
G/A	54 (46.1%)	35 (44.3%)	12 (52.2%)	7 (46.7%)		

	Infant Cortisol Group					
	Full Sample with Placenta & Cortisol (N=117)	**Group 1 (N=81, 69.2%)**	**Group 2 (N=20, 17.1%)**	**Group 3 (N=16, 13.7%)**	**Test of group differences**	
Variable	***Mean ± SD or N (%)***				***One-way ANOVA or p Pearson chi-square value***	***p-value***
G/G	44 (37.6%)	34 (43%)	5 (21.7%)	5 (33.3%)		
Infant Variables						
Infant female (%)	60 (51.3%)	38 (46.9%)	12 (60%)	10 (62.5%)	$X^2(2, 117) = 2.033$	.362
Infant Gestational Age at Delivery (weeks)	39.6 ± 1.2	39.5 ± 1.3	40.1 ± 0.8	39.4 ± 1.0	$F(2,114) = 2.328$	.102
Fetal distress during labor (%)	20 (17.1%)	10 (12.3%)	7 (35%)	3 (18.8%)	$X^2(2, 117) = 5.844$	.054
Assisted Delivery (%)	6 (6.7%)	3 (4.9%)	1 (6.7%)	2 (14.3%)	$X^2(2, 90) = 1.606$	.448
Infant birthweight (grams)	3397.4 ± 461.3	3389.1 ± 474.2	3422.0 ± 375.3	3408.3 ± 516.1	$F(2,114) = .045$	.956
Infant birth length (cm)	50.6 ± 2.5	51 ± 2.4	50.3 ± 2.1	49.8 ± 2.1	$F(2,83) = 1.616$	.205
Infant head circumference at birth (cm)	34.6 ± 2.1	34.6 ± 2.3	34.6 ± 2.3	34.6 ± 1.4	$F(2,83) = .010$	.990
Infant large for gestational age (LGA >90th growth percentile, %)	6 (5.1%)	4 (4.9%)	1 (5%)	1 (6.3%)	$X^2(2, 117) = .048$	.976
Infant small for gestational age? (SGA <10th growth percentile, %)	6 (5.1%)	2 (2.5%)	2 (10%)	2 (12.5%)	$X^2(2, 117) = 3.84$	.139
Infant circumcision within 24 hours before NNNS session day (%)	15 (12.8%)	12 (14.8%)	1 (5%)	2 (12.5%)	$X^2(2, 117) = 1.384$	.501
NNNS Variables						
Infant age at cortisol assessment (days)	1.2 ± 1.1	1.3 ± 1.3	1.1 ± 0.4	1.2 ± .4	$F(2,114) = .355$	.702
Time NNNS Began (HH:MM)	10:08 ± 4:36	10:28 ± 4:53	9:33 ± 4:00	9:20 ± 4:01	$F(2,114) = .612$	.544
Infant salivary cortisol during NNNS (ug/dL)						
Sample 1	.83 ± .95	.57 ± .39	.29 ± .22	2.68 ± 1.12	$F(2,91) = 100.313$	**<.001**
Sample 2	1.02 ± .98	.57 ± .3	1.25 ± .68	2.72 ± 1.31	$F(2,102) = 74.493$	**<.001**
Sample 3	0.96 ± .76	.48 ± .28	1.69 ± .55	1.94 ± .7	$F(2,82) = 93.71$	**<.001**
Sample 4	0.73 ± .61	.4 ± .22	1.21 ± .49	1.47 ± .85	$F(2,74) = 42.582$	**<.001**
Infant salivary cortisol sampling time from sample 1 (minutes)						
Sample 2	29.88 ± 9.46	30.78 ± 7.5	26.09 ± 14.54	31.67 ± 6.02	$F(2,102) = 2.487$	.088
Sample 3	49.99 ± 9.81	51.19 ± 7.53	45 ± 15.31	52.5 ± 4.58	$F(2,82) = 3.443$	**.037**
Sample 4	69.29 ± 10.28	70.53 ± 7.85	64.61 ± 15.58	71.6 ± 6.88	$F(2,74) = 2.576$	.083

Group difference determined by t-test for continuous variables or chi-square for categorical variables.

[a]The proportion of participants reporting “don’t know” may reflect participants’ self-identification outside the pre-established categories. In approximately ⅓ of cases, respondents reported “don’t know” for their race and wrote in a specific country of origin or ethnicity (e.g., Dominican, Cape Verdean, Hispanic). The rest of the respondents reporting “don’t know” did not provide additional context for their self-identified race.

[*]Note that chi-square test was conducted comparing whether participants were non-Hispanic White (yes = “1”, no = “0”), as cell counts were too small to test for every racial group. Bolded *p* values represent statistical significance such that *p-values* < .05 (2-tailed).

Table 2.

Mixed model solutions for newborn cortisol output.

	LL	AIC	BIC	SABIC	entropy	Class %			
						1	2	3	4
One Trajectory Class									
Fixed intercept, fixed linear slope	−147.52	301.04	309.33	299.84	1	100			
Fixed intercept, fixed quadratic slope	−142.87	293.73	304.78	292.14	1	100			
Random intercept, fixed quadratic slope	−75.88	161.76	175.58	159.77	1	100			
Fixed intercept, random quadratic slope	−125.27	260.54	274.35	258.55	1	100			
Random intercept, random quadratic slope	−50.42	114.85	134.18	112.06	1	100			
Multiple Trajectory Classes									
2 classes: Random intercept, random quadratic slope	−54.06	124.12	146.21	120.92	0.52	38.5	61.5		
3 classes: Random intercept, random quadratic slope	**−28.52**	**79.05**	**109.43**	**74.66**	**0.80**	**13.7**	**17.1**	**69.2**	
4 classes: Random intercept, random quadratic slooc	−24.84	77.68	116.35	72.09	0.67	16.2	17.1	12.8	53.8

Note. Models in bold face showed the best fit.

LL = Maximum log-likelihood estimator for model convergence; AIC = Akaike information criterion; BIC = Bayesian information criterion; SABIC = Sample-adjusted Bayesian information criterion; Class % = percentages of participants by trajectory classes.

Table 3.

Placenta LEP methylation is associated with infant cortisol group membership

Variable	β	Std. Error	*t* value	*p* value	*Odds Ratio*	*Confidence* 2.5%	*Intervals* 97.5%
Mean LEP Methylation (%)	.073	.030	2.414	**.016**	1.076	1.015	1.145
Infant sex[a]	−.586	.425	−1.379	.168	.556	.237	1.269
Pre-pregnancy BMI	.057	.034	1.682	.093	1.059	.990	1.133
Intercepts							
"Low cortisol, low reactivity" group | "Start low and increase" group	3.705	1.227	3.019	**.003**			
"Start low and increase" group | "Start high and decrease" group	4.865	1.270	3.830	**<.001**			

Proportional Odds Linear Regression predicting infant cortisol group membership of increasing cortisol output over the course of the NICU Neurobehavioral Exam (NNNS) where 0 = "low cortisol, low reactivity" group, 1 = "start low and increase" group, and 2 = "start high and decrease" group. Model with covariates: AIC = 187.829, BIC = 201.466, Nagelkerke's R^2 = 0.162.

[a]Coded as 0 = female, 1 = male. Bolded *p* values represent statistical significance such that *p-values* < .05 (2-tailed).