The maternal cortisol awakening response in human pregnancy is associated with the length of gestation

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OBJECTIVE: The purpose of this study was to examine the relationship between intraindividual changes in cortisol responsiveness over pregnancy and the length of human gestation.

STUDY DESIGN: Pregnancy-related changes in the cortisol awakening response (CAR), which is a measure of hypothalamic-pituitary-adrenal axis responsiveness, were assessed prospectively in 101 pregnant women at 16.8 ± 1.4 weeks’ and 31.4 ± 1.3 weeks’ (±SD) gestation. Cortisol was measured in saliva that was collected immediately and +30, +45 and +60 minutes after awakening.

RESULTS: The CAR was significant in pregnancy and exhibited progressive attenuation over the course of gestation. A larger CAR in late pregnancy and reduced attenuation of the CAR from early to late gestation were associated significantly with shorter gestational length.

CONCLUSION: The findings are the first to suggest that the hormonal (cortisol) response to a naturally occurring challenge (awakening) and the degree of attenuation of this response over the course of gestation may represent a novel biomarker of increased vulnerability for earlier birth.

Key words: cortisol, gestational length, hypothalamic-pituitary-adrenal, pregnancy, stress responsiveness

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Several lines of evidence converge to suggest that maternal-placental-fetal endocrine processes influence fetal development and birth outcomes. It is well-established that, toward the end of gestation, the shift from a progesterone-dominant to an estrogen-dominant milieu and functional progesterone withdrawal plays a key role in the sequence of events that promote labor.1,2 Earlier in gestation, hypothalamic-pituitary-adrenal (HPA) hormones (particularly cortisol) are known to regulate fetal growth and maturation in mammals3-4 and also the onset of parturition in sheep5-6; however, the potential role of cortisol in regulating the length of human gestation is less clear.

Maternal cortisol production increases 2- to 4-fold over the course of normal human gestation.4,7 Maternal cortisol acts on the developing fetus directly by passing through the placenta (the placental enzyme 11β-hydroxysteroid dehydrogenase type 2 acts only as a partial barrier) or indirectly through its effects on placental corticotrophin-releasing hormone (CRH) activity.4 In humans, some,8,9 but not all studies,10 suggest that variations in maternal cortisol concentrations during pregnancy predict outcomes that are related to the length of gestation. Most human studies of the effects of maternal cortisol in pregnancy, however, are limited by several methodologic concerns. First, although cortisol production and release into circulation follow a diurnal pattern over the course of the day (an average 2-fold difference between morning and evening levels),11 this variation is maintained in pregnancy.1,2,12 The time-of-day of sample collection is either not reported9 or samples have been collected at different times during the day,10 and time of collection has not been taken into consideration in the analyses. Although only 1 study assessed cortisol concentrations repeatedly over the day and related the diurnal cortisol decline over the day to size at birth,14 no study to date has assessed the relationship between diurnal changes in cortisol production and length of gestation. Second, cortisol production is known to increase 2- to 4-fold over the course of gestation.4,7 Although some studies have measured maternal cortisol concentrations at varying gestational ages, gestational age at testing is generally not taken into account in the analyses.9,10 Furthermore, despite this change in cortisol production over gestation, no study to date has examined the association between the rate of change (trajectory) of cortisol production over the course of pregnancy and birth outcomes. Third, although most total cortisol that is measured in plasma is biologically inactive because it is bound to cortisol binding globulin (which is known to increase substantially in pregnancy), all studies that have linked maternal cortisol concentration to the length of gestation have measured only...
total (bound + free) cortisol in the blood.\textsuperscript{8-10}

Thus, in the current study, we addressed the following issues: (1) cortisol was measured in saliva, where only the unbound, biologically active form of the hormone is present.\textsuperscript{15} (2) Subjects were instructed to collect saliva samples at fixed times during the day, and time of day of collection was verified electronically.\textsuperscript{16} The exact time of sample collection was monitored with a Medication Event Monitoring System (MEMS; APREX, a division of AARDEX, Union City, CA) that time-stamped every opening of the container wherein the swabs for saliva collection were stored. (3) To ensure reliable assessment of gestational age at assessment, all pregnancies were dated by early ultrasound. (4) In addition to the 3 aforementioned considerations, we included an additional measure, the cortisol awakening response (CAR). This measure represents the response of the HPA axis to the naturally occurring challenge of awakening from sleep state.\textsuperscript{17} We included this measure because it is known that early indications of dysregulation of a physiologic system can be assessed better by subjecting the system to challenge and assessing its response to challenge than by measuring only baseline function (eg, glucose levels after an oral glucose tolerance test are more sensitive for the detection of early metabolic alterations than baseline glucose concentrations). The CAR has been found to be useful in other contexts as a marker of HPA axis function and a predictor of adverse health outcomes,\textsuperscript{18-20} and it is maintained during pregnancy.\textsuperscript{12,13} Furthermore, because the state of pregnancy produces alterations in responsiveness to challenge that we and others have reported to be dampened progressively as gestation advances,\textsuperscript{21,22} we included a measure of degree of attenuation of the CAR over gestation as a marker of underlying HPA axis physiologic condition.

Thus, using a prospective, longitudinal design with serial samples, the objective of the present study was to assess the relationships between pregnancy-related changes in cortisol and cortisol responsiveness to challenge and the length of human gestation. The CAR was assessed at 2 time points in the second and third trimester of pregnancy. We hypothesized that the response to awakening would be dampened in late pregnancy and that a lack of dampening would be a marker of aberrant HPA function and thus would be associated with shorter pregnancy duration.

Materials and Methods

Participants

One hundred eighteen pregnant women who received prenatal care at the University of California, Irvine, were recruited for the study before their 20th week of gestation and provided written, informed consent. All study participants were English-speaking adult women (>18 years of age) with singleton, intrapartum pregnancies. Exclusion criteria included tobacco, alcohol, or other drug use in pregnancy; uterine or cervical abnormalities; or the presence of any condition that could be associated potentially with dysregulated neuroendocrine function (such as endocrine, hepatic or renal disorders, or corticosteroid medication use). Eligible subjects were recruited consecutively into the study. Women who delivered by elective cesarean section (n = 17) were excluded from the final sample because their delivery was not preceded by labor. Thus, the final sample consisted of 101 subjects.

For all subjects gestational age was determined by best obstetric estimate with standard clinical criteria.\textsuperscript{23} Information on birth outcomes was retrieved from medical charts after delivery. Length of gestation was assessed as a quantitative/continuous (completed weeks’ gestation) variable instead of a categoric (preterm/term) variable to assess effects across the entire distribution instead of only 1 tail of the distribution. Recent evidence suggests the effects of length of gestation on developmental and health outcomes extend continuously across the normal range of pregnancy duration instead of merely as a function of preterm birth.\textsuperscript{24}

Risk conditions were determined by a medical chart review and a medical interview that was conducted by our research nurse at each visit. Given our exclusion criteria, the vast majority of our study sample (83%) was at low-risk for adverse pregnancy outcomes. Detailed information on prevalence of obstetric complications and sociodemographic characteristics (which included maternal age, race/ethnicity, income, education, biophysical/clinical characteristics, parity, pregestational body mass index, fetal sex, type of labor onset, and mode of delivery and birthweight and gestational age at birth) of our study population are summarized in Table 1.

Study protocol

Pregnant women were invited to attend 2 office visits, 1 in the second and 1 in the third trimester of pregnancy. We obtained data for 79 women in early pregnancy (T1: 16.8 ± 0.9 1/2 weeks’ gestation) and in 73 women in late pregnancy (T2: 31.4 ± 1.3 weeks’ gestation); 51 women provided data at both time points. There were no differences in sociodemographic characteristics or birth outcomes in those women who provided data for both time points during gestation as opposed to those who provided data only once (either at T1 or at T2).

Home saliva collection

At each assessment, pregnant women collected saliva samples for cortisol assays at 7 time points over the course of the day: immediately, 30, 45, and 60 minutes after awakening (capturing the CAR) and at 12, 4, and 8 pm (capturing the diurnal change in cortisol concentrations). Exact time of saliva sampling was monitored with the use of the Medication Event Monitoring System (APREX). After saliva collection, each swab was stored in a plastic tube that was labeled with the designated sampling time by the experimenter.

Salivary cortisol assay

Saliva samples were collected with a Salivette sampling device (Sarstedt, Numbrecht, Germany). Samples were clarified, spun, and stored at –70°C until assayed. Thawed samples were centrifuged at 3000 rpm for 15 minutes before
assay. Salivary cortisol levels were determined by a competitive luminescence immunoassay (IBL-America, Minneapolis, MN) with reported detection limits of 0.015 μg/dL. The cross reactivity of the assay was <2.5% with cortisone, prednisone, and corticosterone and <0.1% with other naturally occurring steroids. The intra- and interassay coefficients of variance are 5.5% and 7.6%, respectively. Data reduction for the luminescence immunoassay was done by an automated 4-parameter logistics computer program (software Mikro Win 2000; Berthold Microplate Luminometer; Berthold Technologies, Oak Ridge, TN). All samples were assayed in duplicate and averaged.

**Statistical analysis**

Associations between the individual cortisol measures from saliva samples that were collected in early and late pregnancy and length of gestation at birth were computed with Pearson product-moment correlation coefficients. Hierarchical linear modeling (HLM) growth curve analyses were used to evaluate the CAR in early and late pregnancy and the changes in the CAR over gestation and their association with gestational age at birth. HLM, when used with repeated measures, treats the data in a hierarchic fashion with observations nested within persons. This approach allows variance to be modeled at multiple levels and provides several advantages over ordinary least squares regression: (1) assessment of within-person variability over time, (2) estimates of goodness of fit in modeling in which the most reliable data are given greater statistical weight, and (3) robust estimates of missing values for the repeated dependent measure. Cases with complete data are weighted more heavily, but all cases are included in the estimation of effects. Finally, HLM allows the use of precise measures of timing (ie, gestational age at assessment and time of day of sample collection) of data collection rather than nominal estimates of assessment intervals. The advantage of a repeated measures approach such as the 1 used in this study is that the statistical power is bolstered, not only by the number of participants, but also by the large number of observations that are obtained for each person.

A 3-level model was set up to predict cortisol concentrations with the effects of time-of-day in reference to awakening that was modeled on level 1, the effects of timing in pregnancy (2nd vs 3rd trimester) on level 2, and length of gestation, which presented a stable individual difference variable modeled on level 3.

The HLM analysis proceeded in 3 major steps:

**Step 1: modeling diurnal cortisol patterns**. Level 1 captured parameters that change within an individual and within an assessment period. To model the CAR (4 samples during the first hour after awakening) in reference to the course of cortisol changes over the rest of the day (diurnal change: 12, 4, and 8 PM), level 1 included 2 time parameters, 1 for the CAR effect and a second to capture within-the-day changes in cortisol (diurnal change). Based on known changes in the

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**TABLE 1**

| Variable | Measurement |
|----------|-------------|
| Maternal age, y | 27.6 ± 5.9 |
| Race/ethnicity, % | |
| Non-Hispanic white | 38.3 |
| Hispanic white | 36.2 |
| Asian | 5.3 |
| Education, % | |
| High school or equivalent | 94 |
| College graduate | 35 |
| Prepregnancy body mass index, kg/m² | 25.02 ± 6.05 |
| Fetal sex, % | |
| Male | 42.0 |
| Female | 58.0 |
| Obstetric complication, % | |
| Infection | 7 |
| Preeclampsia/hypertension | 3 |
| Vascular complications | 7 |
| Diabetes mellitus | 2 |
| Type of labor onset, % | |
| Spontaneous | 33 |
| Induced | 23 |
| Augmented | 44 |
| Mode of delivery, % | |
| Spontaneous | 72 |
| Assisted | 9 |
| Cesarean | 19 |
| Gestational age at birth, wk | 38.9 ± 1.96 |
| Birthweight, g | 3373.96 ± 564.9 |

*Data are given as mean ± SD; †Obstetric risk was defined as the presence of certain risk factors and medical conditions in the index pregnancy, which included hypertension, preeclampsia, vascular risk factors (eg, vascular bleeding, placenta abruption, anemia, and placenta previa), diabetes mellitus (gestational diabetes mellitus, diabetes mellitus types 1 and 2), and severe infection during pregnancy (chlamydia, syphilis, toxoplasmosis, bacterial vaginosis)."
awakening and daytime pattern of cortisol production, for both parameters linear (CAR, diurnal change) and quadratic effects of time were included (CAR², diurnal change²); this proved to be superior to linear modeling (P < .001). Quadratic modeling represents the initial cortisol increase and subsequent decline during the first hour after awakening (Figure 1). The quadratic solution produces 3 coefficients for comparison: (1) the mean level differences or the intercept at awakening, (2) the instantaneous rate of change (linear slope) at awakening, and (3) the overall acceleration (shape) of the curve. Time was centered at awakening so that the model intercept represents the mean log transformed cortisol levels at awakening. The 4 time parameters and the intercept were included as random factors because they are known to have substantial variability across individuals.

**Step 2: associations between gestational stage and awakening time on cortisol concentrations.** Level 2 captured potential changes in HPA physiologic condition from 1 assessment period to the next. Exact gestational age at each assessment was modeled (centered at mean gestational age at first visit) to capture change in HPA physiologic condition across pregnancy. Not all participants awoke at the same time at each assessment period. To control for fluctuations in wake-up time at each assessment, time of awakening also was entered at level 2. Thus, on level 2 interactions between gestational age at assessment and the level 1 π-coefficients and between time of awakening and the level 1 π-coefficients were assessed.

**Step 3: association between length of gestation and cortisol concentrations.** Person-level factors, which are variables that did not change from 1 assessment to the next, were introduced at level 3. These included completed weeks of gestation at delivery centered at the group’s mean and covariates of interest. The following covariates were tested for their impact on cortisol concentrations and for their impact on pregnancy duration: obstetric risk, race/ethnicity, maternal age, prepregnancy body mass index, and fetal sex. None of these variables had an influence on either cortisol concentrations over the day or cortisol changes over gestation and gestational length (P > .05), and therefore were not included in the final model. Thus, on level 3, interactions between length of gestation and the level 2 β-coefficients were assessed.

To test the association between the CAR and pregnancy duration for early and late pregnancy separately, 2 distinct 2-level models were computed, 1 model for the assessment in early gestation and 1 model for the assessment in late gestation. On level 1, these models captured cortisol change within an individual within an assessment period, and all time invariant variables were included on level 2: pregnancy duration, time of awakening, and gestational age at testing.

**RESULTS**

The mean cortisol concentrations at the 2 assessment time points are depicted in **Table 2**. All cortisol values were log-transformed by the following equation to yield an unskewed response variable: LnCort = ln(Cort + 1).

Most of our study participants (83%) had no obstetric risk conditions. In this sample, obstetric risk was not associated with any of the cortisol measures. Therefore, the reported findings are not mediated by obstetric risk conditions in this population. As previously mentioned, delivery was preceded by labor in all subjects in the final study sample (Table 1). The mean pregnancy duration (length of gestation) was 38.9 ± 1.96 (SD) weeks (range, 27.5–41.2 weeks), with 7 women delivering preterm (<37 completed weeks’ gestation).

None of the individual cortisol measures at either of the 2 study assessment periods was associated with gestational age at birth (all P > .35).
HLM estimates for salivary cortisol concentrations (log-transformed) during the first hour after awakening and the effects of gestational age at testing and pregnancy duration are depicted in Table 3. The main effect for “time” reflects changes in cortisol concentrations during the first hour after awakening at 17 and 31 weeks’ gestation. At both study assessments during pregnancy, there is a significant increase and subsequent decline in cortisol concentrations in response to awakening, as indicated by the significant linear and quadratic time slopes \((P < .001)\). Therefore, the results suggest that, during pregnancy, the cortisol increase to awakening is maintained. Interactions between the CAR early and late in pregnancy and length of gestation indicate whether HPA responsiveness at either time point is associated with length of gestation. The CAR assessed at 17 weeks’ gestation was not related to length of gestation, as indicated by the nonsignificant linear and quadratic time slopes \((P > .3)\). At 31 weeks gestation, a flatter CAR, which indicates less pronounced HPA responsiveness to awakening, was associated with longer length of gestation, as indicated by the significant quadratic time slope \((P < .05)\).

Significant changes in cortisol concentrations were observed from 17-31 weeks’ gestation, as indicated by the interactions between time and gestational age at testing-related changes in the CAR. Baseline cortisol concentrations significantly increased over the course of gestation \((P < .001)\), whereas the magnitude of the CAR decreased as gestation advanced \((P < .001)\).

The 3-way interactions among time, gestational age at testing, and length of gestation test whether pregnancy-related changes in cortisol concentrations during the first hour after awakening are associated with length of gestation. As in-

### Table 3

| Cortisol response to awakening | Parameter | SE | \(P\) value |
|-------------------------------|-----------|----|-------------|
| At 17 weeks’ gestation (T1)   |           |    |             |
| T1 intercept                  | 2.932     | 0.044 | < .001     |
| T1 time                       | 2.932     | 0.044 | < .001     |
| T1 time\(^2\)                 | -0.735    | 0.092 | < .001     |
| At 31 weeks’ gestation (T2)   |           |    |             |
| T2 intercept                  | 3.189     | 0.047 | < .001     |
| T2 time                       | 0.512     | 0.105 | < .001     |
| T2 time\(^2\)                 | -0.401    | 0.081 | < .001     |

**Association between CAR at 17 weeks’ gestation (T1) and length of gestation**

| Parameter | \(P\) value |
|-----------|-------------|
| T1 intercept \(\times\) length of gestation | -0.027 | .36 |
| T1 time \(\times\) length of gestation | -0.043 | .62 |
| T1 time\(^2\) \(\times\) length of gestation | 0.051 | .36 |

**Association between CAR at 31 weeks’ gestation (T2) and length of gestation**

| Parameter | \(P\) value |
|-----------|-------------|
| T2 intercept \(\times\) length of gestation | 0.008 | .72 |
| T2 time \(\times\) length of gestation | -0.113 | .08 |
| T2 time\(^2\) \(\times\) length of gestation | 0.121 | < .05 |

**Change in CAR from 17 (T1) to 31 (T2) weeks’ gestation**

| Parameter | \(P\) value |
|-----------|-------------|
| Intercept \(\times\) gestational age (T1, T2) | 0.017 | < .001 |
| Time \(\times\) gestational age (T1, T2) | -0.03 | .01 |
| Time\(^2\) \(\times\) gestational age (T1, T2) | 0.023 | < .01 |

**Association between change in CAR from T1-T2 and length of gestation**

| Parameter | \(P\) value |
|-----------|-------------|
| Intercept \(\times\) gestational age (T1, T2) \(\times\) length of gestation | 0.005 | .05 |
| Time \(\times\) gestational age (T1, T2) \(\times\) length of gestation | -0.0012 | .11 |
| Time\(^2\) \(\times\) gestational age (T1, T2) \(\times\) length of gestation | 0.0097 | < .05 |

\(\text{CAR, cortisol awakening response; } T, \text{ time point.}\)
The maternal endocrine milieu reflects, on the one hand, conditions in the maternal environment (eg, stress, infection); on the other hand, it also reflects processes inside the fetal compartment. Changes in neuroendocrine and HPA axis function during human pregnancy are triggered, in part, by the circadian rhythmicity of cortisol secretion that is maintained during human pregnancy. Previous studies have suggested that the circadian rhythmicity of cortisol secretion is disrupted in women who deliver at term compared to women who deliver preterm. This disruption is associated with shorter gestation, lower cortisol concentrations, and less reactivity in response to awakening. These findings may represent the underlying biologic basis for earlier findings of the association between psychologic stress and adverse pregnancy outcomes. Our findings of the association of the lack of dampening of physiologic responsiveness in later pregnancy with shorter pregnancy duration are in line with findings that suggest that the lack of psychologic stress dampening over the course of gestation is associated with adverse birth outcomes. The attenuation of the physiologic response to challenge in pregnancy may serve as a beneficial adaptation for maternal and fetal/newborn infant health and well-being. The findings of the current study also may represent the underlying biologic basis for earlier findings of the association between psychologic stress responsiveness and pregnancy outcomes because pregnancy-related changes in psychologic stress responses and perceived stress during human pregnancy may reflect changes in the biologic responsiveness of stress systems in pregnant woman.

Our findings replicate earlier observations that suggested that the circadian rhythmicity of cortisol secretion is maintained during human pregnancy. Furthermore, our findings demonstrate that systematic changes occur in the CAR during pregnancy that are characterized by progressively increasing baseline levels and reduced reactivity in response to awakening. Moreover, our findings suggest that the cortisol increase in response to awakening in the third trimester of pregnancy and the degree of attenuation of the CAR over the course of gestation are associated with gestational age at birth, such that a higher cortisol increase to awakening in late pregnancy and a less pronounced dampening of the CAR from early to late pregnancy are associated with a shorter pregnancy duration and earlier birth. There was an approximate 12% reduction in the dampening of the CAR response from 17-31 weeks for each week of shorter pregnancy duration.

Consistent with our hypothesis, although individual cortisol measures in pregnancy were not predictive of outcomes that were related to the length of gestation, measures of HPA response to challenge over gestation were associated significantly with gestational length. Women who preserve high HPA responsiveness in late gestation and show a less pronounced dampening of the CAR over gestation were more likely to deliver earlier than women whose condition is characterized by lower HPA responsiveness in later pregnancy and more pronounced dampening of the CAR over gestation.

To estimate the effect of the size of the degree of CAR dampening on the length of gestation, we calculated the percent decrease in the CAR by weeks of gestation. As shown in Table 2, the quadratic slope of the CAR gets more positive as gestation advances, which indicates a less dynamic and more dampened response (smaller increase and smaller decrease). In women delivering at approximately 39 weeks’ gestation (average gestational length in this sample), the change in the quadratic CAR slope from 17-31 weeks’ gestation is approximately 45.4% (T1, –0.735 to 0.051) = –0.786; T2, –0.401 to 0.121] = –0.522), which equates to a 2.4% decrease per week. Therefore, the results suggest that less dampening of the CAR over the course of gestation by approximately 1% per week are associated with reduction of pregnancy duration by 1 week. The modest sample size limited our ability to test the predictive ability of CAR dampening to differentiate preterm from term births. However, at a descriptive level, the direction of the difference between women who delivered preterm vs term is in the hypothesized direction (Figure 2).

**FIGURE 2**

Log-transformed cortisol concentrations during the first hour after awakening in early and late pregnancy in women who delivered A, preterm (<37 weeks of gestation; n = 7) vs B, term (≥37 weeks of gestation; n = 94). Descriptively, the data are in line with the cortisol concentrations that were predicted by the hierarchic linear model that was based on length of gestation entered as a continuous variable.

Buss. Maternal cortisol awakening response. Am J Obstet Gynecol 2009.
part, by an exponential increase in the expression of CRH in the placenta, which is an organ of fetal origin. Placental CRH is released into both the maternal and fetal compartment. This results in a progressive rise in maternal adrenocorticotropic hormone and cortisol levels over the course of gestation, with total and free plasma cortisol levels peaking during the third trimester at approximately 2-3 times that of non-pregnant values. The consequence of the elevated baseline levels in adrenocorticotropic hormone and cortisol is a reduced elevation of the maternal adrenocorticotrope axis during pregnancy.

There are some limitations of the current study. The sample size is relatively modest and therefore limited our ability to test the predictive ability of CAR dampening to differentiate preterm from term births. However, at a descriptive level, we note that the direction of the difference between women who delivered preterm vs term is in the hypothesized direction. Moreover, our study sample did not contain African American women. Racial/ethnic differences in endocrine characteristics during pregnancy have been reported previously, and a larger study is required to examine racial/ethnic differences in trajectories of stress responsiveness and whether these may account, in part, for the observed racial/ethnic disparities in adverse birth outcomes. Despite these limitations, to the best of our knowledge this is the first study to show in a prospective, longitudinal design that the magnitude in change of stress responsiveness over the course of gestation is associated with birth outcomes.

Our results therefore, may have implications for developing better risk assessment strategies for adverse birth outcomes and for a better understanding of the processes underlying the developmental programming of health and disease. Maternal stress may have more severe consequences in women who do not exhibit the expected physiologic dampening of the HPA axis in late gestation.

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**APPENDIX**

**Statistical analysis: hierarchic linear modeling equations**

**Step 1: modeling diurnal cortisol patterns**

Level 1 captured parameters that change within an individual and within an assessment period. To model the cortisol-awakening response (CAR; 4 samples during the first hour after awakening) in reference to the course of cortisol changes over the rest of the day (diurnal change at 12, and 8 PM), level 1 included 2 time parameters (1 for the CAR effect and 1 to capture within-the-day changes in cortisol [diurnal change]). Based on known changes in the awakening and daytime pattern of cortisol production, both parameters (linear [CAR and diurnal change] and quadratic effects of time) were included (CAR^2, diurnal change^2). The level 1 model predicted cortisol activity (where i represents the individual and j represents the repeated assessments over gestation and k represents the repeated sampling over the day):

\[
\text{LogCortisol}_{ijk} = \pi_0 + \pi_1 \times \text{diurnal change}_{ijk} + \pi_2 \times \text{CAR}_{ijk} + \pi_3 \times \text{diurnal change}_{ijk}^2 + \pi_4 \times \text{CAR}_{ijk} + e_{ijk}
\]

**Step 2: associations between gestational stage and awakening time on cortisol concentrations**

Level 2 captured potential changes in hypothalamic-pituitary-adrenal hormone physiologic condition from 1 assessment period to the next. Exact gestational age (GA) at each assessment was modeled (centered at mean gestational age at first visit). To control for fluctuations in wake-up time at each assessment, time of awakening (awak) also was entered at level 2. Thus, level 2 interactions between gestational age at assessment and the level 1 \(\pi\)-coefficients and between time of awakening and the level 1 \(\pi\)-coefficients were assessed with the following level 2 model:

\[
\pi_0 = \beta_{00} + \beta_{01} \times (GA_{ij}) + \beta_{02} \times (awak_{ij}) + r_{0ij}
\]

\[
\pi_1 = \beta_{10} + \beta_{11} \times (GA_{ij}) + \beta_{12} \times (awak_{ij}) + r_{1ij}
\]

\[
\pi_2 = \beta_{20} + \beta_{21} \times (GA_{ij}) + \beta_{22} \times (awak_{ij}) + r_{2ij}
\]

\[
\pi_3 = \beta_{30} + \beta_{31} \times (GA_{ij}) + \beta_{32} \times (awak_{ij}) + r_{3ij}
\]

\[
\pi_4 = \beta_{40} + \beta_{41} \times (GA_{ij}) + \beta_{42} \times (awak_{ij}) + r_{4ij}
\]

**Step 3: association between length of gestation and cortisol concentrations**

Person-level factors, which are variables that did not change from 1 assessment to the next, were introduced at level 3. These included completed weeks of gestation at delivery centered at the group’s mean and covariates of interest. Thus, on level 3, interactions between length of gestation and the level 2 \(\beta\)-coefficients were assessed and resulted in the following level 3 model:

\[
\beta_{00} = \gamma_{000} + \gamma_{001}(\text{length of gestation}_i) + \mu_{00}
\]

\[
\beta_{01} = \gamma_{010} + \gamma_{011}(\text{length of gestation}_i) + \mu_{01}
\]

\[
\beta_{02} = \gamma_{020} + \gamma_{021}(\text{length of gestation}_i) + \mu_{02}
\]

\[
\beta_{10} = \gamma_{100} + \gamma_{101}(\text{length of gestation}_i) + \mu_{10}
\]

\[
\beta_{11} = \gamma_{110} + \gamma_{111}(\text{length of gestation}_i) + \mu_{11}
\]

\[
\beta_{12} = \gamma_{120} + \gamma_{121}(\text{length of gestation}_i) + \mu_{12}
\]

\[
\beta_{20} = \gamma_{200} + \gamma_{201}(\text{length of gestation}_i) + \mu_{20}
\]

\[
\beta_{21} = \gamma_{210} + \gamma_{211}(\text{length of gestation}_i) + \mu_{21}
\]

\[
\beta_{22} = \gamma_{220} + \gamma_{221}(\text{length of gestation}_i) + \mu_{22}
\]

\[
\beta_{30} = \gamma_{300} + \gamma_{301}(\text{length of gestation}_i) + \mu_{30}
\]

\[
\beta_{31} = \gamma_{310} + \gamma_{311}(\text{length of gestation}_i) + \mu_{31}
\]

\[
\beta_{32} = \gamma_{320} + \gamma_{321}(\text{length of gestation}_i) + \mu_{32}
\]

\[
\beta_{40} = \gamma_{400} + \gamma_{401}(\text{length of gestation}_i) + \mu_{40}
\]

\[
\beta_{41} = \gamma_{410} + \gamma_{411}(\text{length of gestation}_i) + \mu_{41}
\]

\[
\beta_{42} = \gamma_{420} + \gamma_{421}(\text{length of gestation}_i) + \mu_{42}
\]