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Clinical Research Article

A Nested Case-Control Study of Allopregnanolone and Preterm Birth in the Healthy Start Cohort

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ABSTRACT

Context: Chronic stress is a risk factor for preterm birth; however, objective measures of stress in pregnancy are limited. Maternal stress biomarkers may fill this gap. Steroid hormones and neurosteroids such as allopregnanolone (ALLO) play important roles in stress physiology and pregnancy maintenance and therefore may be promising for preterm birth prediction.

Objective: We evaluated maternal serum ALLO, progesterone, cortisol, cortisone, pregnanolone, and epipregnanolone twice in gestation to evaluate associations with preterm birth.

Methods: We performed a nested case-control study using biobanked fasting serum samples from the Healthy Start prebirth cohort. We included healthy women with a singleton pregnancy and matched preterm cases with term controls (1:1; N = 27 per group). We used a new HPLC-tandem mass spectrometry assay to quantify ALLO and five related steroids. We used ANOVA, Fisher exact, χ^2, *t* test, and linear and logistic regression as statistical tests.

Results: Maternal serum ALLO did not associate with preterm birth nor differ between groups. Mean cortisol levels were significantly higher in the preterm group early in pregnancy (13w0d-18w0d; $P < 0.05$) and higher early pregnancy cortisol associated with increased odds of preterm birth (at 13w0d; odds ratio, 1.007; 95% CI, 1.0002-1.014). Progesterone, cortisone, pregnanolone, and epipregnanolone did not associate with preterm birth.

Conclusion: The findings from our pilot study suggest potential utility of cortisol as a maternal serum biomarker for preterm birth risk assessment in early pregnancy. Further evaluation using larger cohorts and additional gestational timepoints for ALLO and the other analytes may be informative.

Key Words: Cortisol, maternal stress, neuroendocrine, neurosteroids, neuroactive steroids, parturition

Abbreviations: ALLO, allopregnanolone; CRH, corticotropin-releasing hormone; GABA, γ-aminobutyric acid; MS/MS, tandem mass spectrometry.

Preterm birth (<37 weeks' gestational age) is a leading cause of neonatal morbidity and mortality (1). Chronic stress is a risk factor for preterm birth, but objective laboratory measures of stress in pregnancy remain elusive (2). Objective maternal stress biomarkers may enhance the clinician's diagnostic toolkit. Neurosteroids such as allopregnanolone (ALLO) (3α-hydroxy-5α-pregnan-20-one), a progesterone metabolite, are "stress-responsive" (3, 4). Neurosteroids are synthesized from cholesterol de novo in the brain and other steroidogenic organs including the placenta, and they may play a role in maintaining optimal pregnancy duration (5, 6). Both maternal and fetal neurosteroids increase during pregnancy reaching highest maternal and fetal concentrations near term gestation (≥37 weeks), then dramatically decrease after delivery of the placenta (7).

ALLO and other neurosteroids are indicated in numerous conditions related to stress dysregulation including anxiety, depression, and posttraumatic stress disorder (8-12). Maternal stress alters maternal steroid hormone profiles during pregnancy, most notably cortisol (13). The Food and Drug Administration recently approved synthetic ALLO (Brexanolone IV) as a novel treatment for postpartum depression (14). ALLO levels are lower under conditions of chronic stress, and animal models show lowered maternal ALLO, late in pregnancy, reduces the length of gestation (15, 16).

Neurosteroids during pregnancy could conceivably influence birth timing by several physiologic mechanisms. First, neurosteroids modulate γ-aminobutyric acid (GABA), which is present not only in the brain but also in uterine myometrium and endometrium (17, 18). ALLO and other GABAergic progesterone metabolites may induce uterine relaxation and

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inhibit uterine contractility (19). Second, ALLO inhibits maternal oxytocin release in late pregnancy, possibly preventing preterm oxytocin stimulation of the uterus (20).

Given the reported associations between neurosteroids, stress, and gestational length, we performed a nested case-control study to investigate maternal serum ALLO in preterm birth. We hypothesized women who delivered preterm would have lower serum ALLO compared with women who delivered at term. We evaluated associations between ALLO (and 5 related simultaneously quantified steroid hormones and neurosteroids) and preterm birth. In addition to ALLO, we evaluated progesterone, cortisone, cortisol, pregnanolone (3α-hydroxy-5β-pregnan-20-one), and epipregnanolone (3β-hydroxy-5β-pregnan-20-one). The biosynthetic pathways of the study analytes are summarized in Figure 1.

MATERIALS AND METHODS

Study Design and Population

We performed a nested case-control study. The Colorado Multiple Institutional Review Board deemed this study exempt (COMIRB #19-2419) as a deidentified biobank investigation. We used morning fasting serum samples collected prospectively at the University of Colorado Hospital from December 2009 through May 2014 for the Lifecourse Epidemiology of Adiposity and Diabetes (LEAD) Center's Healthy Start Study (21). Healthy Start Study participants previously consented to the future use of blood samples for research. Fasting venous blood samples were collected twice during pregnancy from each participant in the morning between gestational weeks 11 and 34. Pregnant women were eligible for inclusion in the Healthy Start Study if they were at least aged 16 years with a singleton pregnancy at ≤23w0d at enrollment. Participants with history of cancer, psychiatric disease, steroid-dependent asthma, preexisting diabetes mellitus, previous preterm birth, or prior low birthweight delivery were excluded. Our nested cohort included healthy, low-risk women aged 18 to 34 years. We excluded subjects with hypertension, diabetes, preeclampsia, tobacco or alcohol use in pregnancy, any steroid administration before phlebotomy, or in vitro fertilization.

Our case-control design was based on outcomes of preterm (<37w0d) or full-term (≥37w0d) live birth. We matched preterm birth cases 1-to-1 with full-term controls using gestational age at first blood sample and the minimum absolute difference in gestational age between the first and second blood samples.

Neurosteroid/Steroid Hormone Quantification

We developed and validated a HPLC-tandem mass spectrometry (HPLC-MS/MS) assay to quantify maternal serum ALLO and related analytes in accordance with the Food and Drug Administration and the Clinical Laboratory Standards Institute guidelines for bioanalytical assays (22). Because neurosteroids increase significantly during pregnancy, we were able to extract analytes directly from serum using methanol precipitation. The simplicity of our methodology makes it attractive for future clinical research. The lower limit of detection for ALLO, pregnanolone, and epipregnanolone was 0.78 ng/mL. The lower limit of detection for cortisone and progesterone was 1.56 ng/mL and for cortisol was 3.91 ng/mL. The upper limit of quantification for ALLO, pregnanolone, and epipregnanolone was 100 ng/mL, for progesterone and cortisone it was 400 ng/mL, and for cortisol it was 1000 ng/mL. Inter- and intraday accuracy was between 90% and 110% and inter- and intraday imprecision was < 10%. There were no significant matrix interferences, no carryover, and no significant matrix effects (22).

Statistical Analyses

To evaluate relationships with preterm birth, we looked at the predictive value of these analytes by odds ratios for preterm birth and performed direct comparisons between the preterm and term groups. For odds ratios, we used logistic regression models. Model variables included the known serum analyte

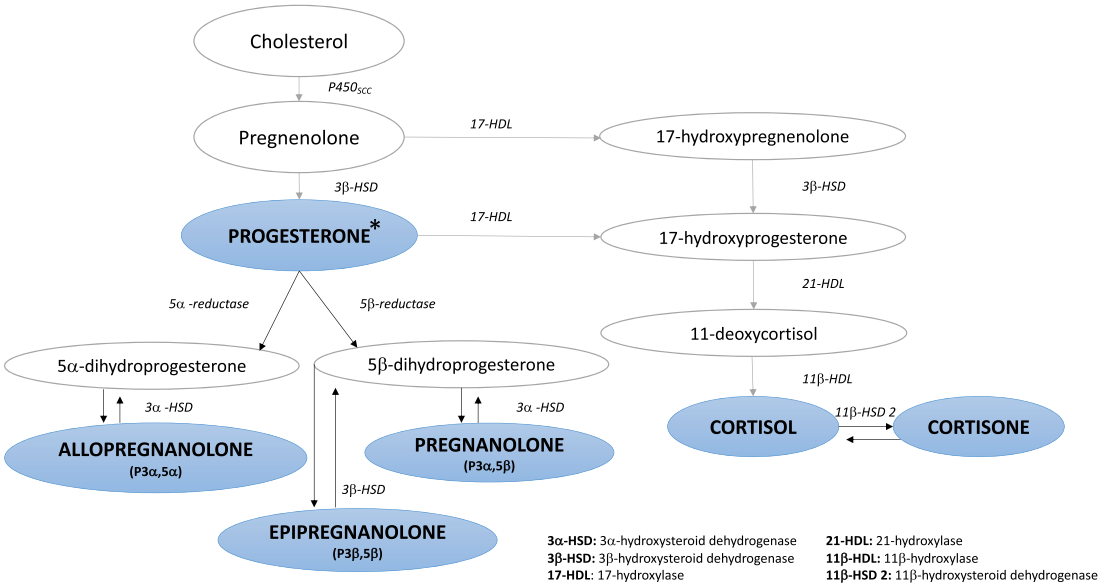

Figure 1. Biosynthetic pathways of study analytes. Study analytes are shaded. *Progesterone has previously been indicated in the biological pathway leading to spontaneous preterm birth.

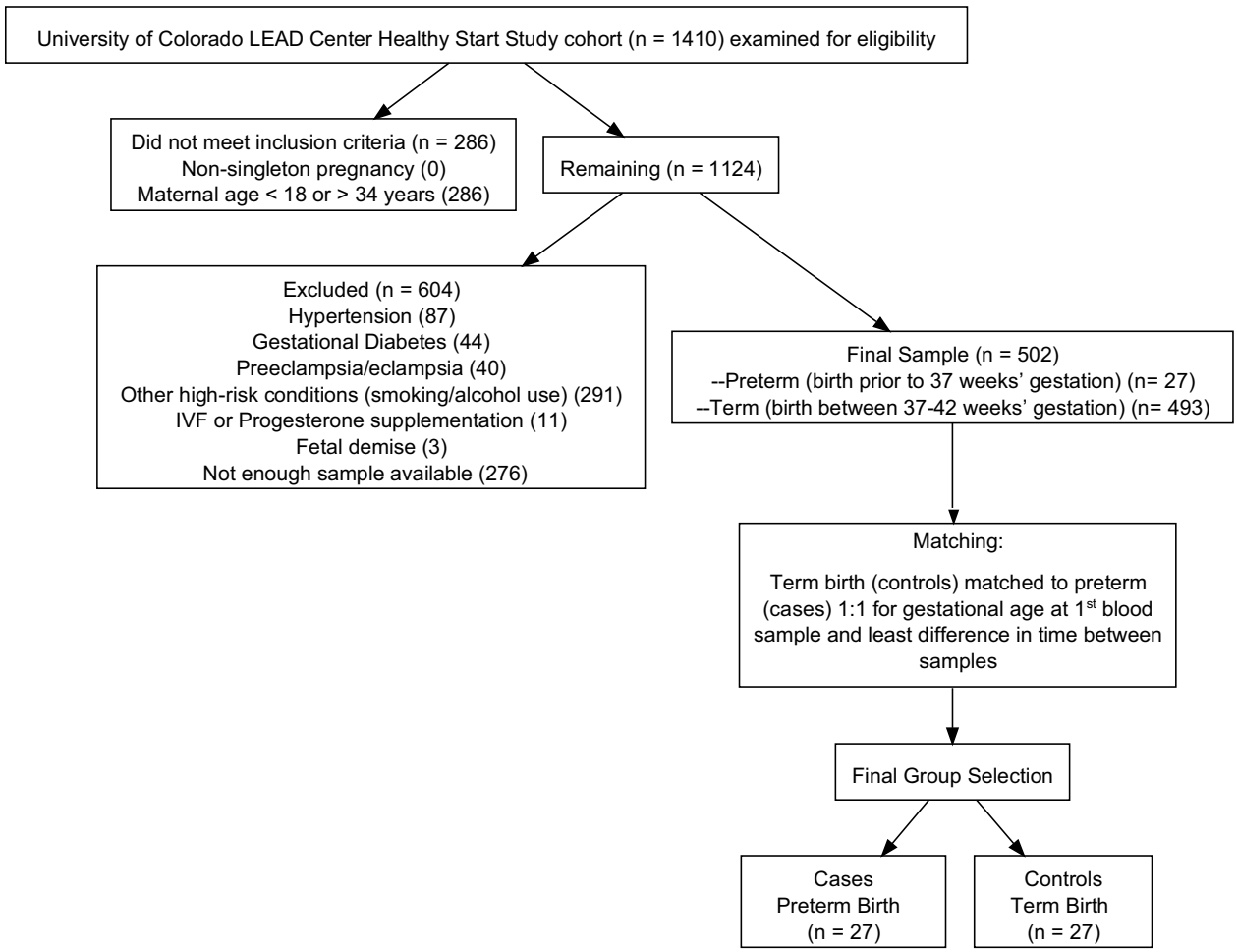

Figure 2. Subject selection flow diagram. Subject selection based on Lifetime Epidemiology of Adiposity and Diabetes (LEAD) Center's Healthy Start prebirth cohort enrollment and case-control assignments.

concentration of each participant, their gestational age at the time of sample collection, and the interaction between serum analyte concentration and gestational age. The odds ratios were calculated for each gestational week observed in our dataset (13w0d-32w0d). For direct comparison of the groups, we used independent samples *t* tests to compare the mean serum concentration at the first and second blood samples. Additionally, we used a linear regression model, accounting for each participant's precise gestational age at the time of sample collection, to estimate expected mean serum concentrations for preterm and term groups at each gestational week (13w0d-32w0d). Estimates and CIs for both the logistic and linear regression models were generated by interpolation of each respective regression model; no data were imputed. We generated CIs via a bootstrapping approach. Because of the small sample size, we chose not to adjust for other demographic variables. Although cases and controls were matched 1-to-1 to ensure close gestational ages at the time of blood collections, the data were analyzed as 2-sample collections and not by matched pair analysis. We performed analyses in R version 4.0.4 (R Core Team 2020) with mixed effects models fitted by the R package lme4 (23). We performed parametric ANOVA and post hoc *t* tests for differences in means. We used Fisher exact and χ^2 tests for categorical variables. We considered $P < 0.05$ significant.

RESULTS

Population

From the Healthy Start prebirth cohort of more than 1400 women, we selected 27 preterm cases and 27 matched term controls for our nested cohort (Figure 2) with banked blood samples between 12w4d and 32w0d. Mean gestational age in our cohort at the first blood sample was 16.9 weeks (range, 12.4-25 weeks) and mean gestational age at the second blood sample was 26.5 weeks (range, 22.3-32 weeks). Cases and controls were matched only on gestational age at the first blood sample and least difference in gestational age between the first and second blood samples. Maternal, pregnancy, and neonatal characteristics were similar between preterm cases and term controls except gestational age at delivery, infant birth weight, and maternal gestational weight gain, which were anticipated because of the difference in pregnancy duration (Table 1).

Odds Ratios for Preterm Birth

We observed no significant association between ALLO and preterm birth (Figure 3). The only significant association we observed in these data was a greater odds of preterm birth associated with higher cortisol levels from gestational weeks'

Table 1. Maternal/pregnancy/neonatal characteristics

	Overall N = 54, (%)	Term delivery N = 27, (%)	Preterm delivery N = 27, (%)	*P*
Maternal characteristics				
Maternal age, y				1.000
18-25	26 (48)	13 (48)	13 (48)	
25-35	28 (52)	14 (52)	14 (52)	
Maternal height, m				1.000
<1.5	2 (4)	1 (4)	1 (4)	
>1.5	52 (96)	26 (96)	26 (96)	
Body mass index, kg/m^2				
Pre-pregnancy	26.1 ± 6.4	26.1 ± 5.2	26.1 ± 7.5	0.992
At delivery (N; mean ± SD)	48; 30.5 ± 5.5	26; 31.3 ± 5.3	22; 29.6 ± 5.7	0.294
Self-identified race/ethnicity				0.922
White	20 (37)	10 (37)	10 (37)	
Black	9 (17)	4 (15)	5 (19)	
Hispanic	18 (33)	10 (37)	8 (30)	
Otherwise	7 (13)	3 (11)	4 (15)	
Household income				0.225
≤$70,000/y	29 (54)	11 (41)	18 (67)	
>$70,000/y	17 (31)	10 (37)	7 (26)	
Unknown/missing	8 (15)	6 (22)	2 (7)	
Education level				0.506
High school or GED	20 (37)	11 (41)	9 (34)	
Some college education or more	34 (63)	16 (59)	18 (66)	
Marital status				0.544
Married/partnered	39 (72)	21 (78)	18 (67)	
Unknown	1 (2)	0 (0)	1 (4)	
Pregnancy characteristics				
Gestational weight gain[a]				**0.049**
Adequate/inadequate	34 (63)	13 (48)	21 (78)	
Excessive	20 (37)	14 (52)	6 (22)	
Prenatal vitamins:				0.610
Yes	50 (93)	26 (96)	24 (89)	
Gravidity:				
Median (min, max)	2 (1, 7)	2 (1,7)	2 (1, 4)	0.352
Parity:				
Median (min, max)	0 (0, 3)	1 (0, 3)	0 (0, 2)	0.275
Spontaneous rupture of membranes				0.137
Yes	22 (41)	10 (37)	12 (44)	
Unknown	20 (37)	8 (30)	12 (44)	
Neonatal characteristics				
Gestational age at delivery				**< 0.001**
Mean (SD)	**37.1 ± 3.0**	**39.5 ± 1.0**	**34.7 ± 2.3**	
Infant birth weight (g)				**< 0.001**
Mean (SD)	**2784 ± 696**	**3193 ± 483**	**2375 ± 639**	
Infant sex				0.897
Female	28 (53)	15 (56)	13 (50)	
Unknown/missing	1 (2)	0 (0)	1 (4)	

Data are presented as means (standard deviations) unless stated otherwise. *P* values computed by χ^2, Fisher exact, and 2-sample *t* tests.
[a]Institute of Medicine recommendations for weight gain during pregnancy based on prepregnancy body mass index: 12.5-18 kg, underweight; 11.5-16 kg, normal; 7-11.5 kg, overweight; and 5-9 kg, obese.

13w0d through 22w0d. Odds ratios ranged from 1.007 (95% CI, 1.0002-1.014) at 13w0d to 1.004 (95% CI, 1.0001-1.007) at 22w0d. Odds ratios for each analyte are provided in Supplemental Table S1 and model results are provided in Supplemental Table S2 (24). Supplemental Figure S1 graphically depicts the estimated probabilities for the model (24).

Direct Comparison of Preterm and Term Groups

We observed no significant difference in the mean ALLO serum level between the term and preterm groups. In *t* test comparisons, the first maternal blood sample mean ALLO concentration in preterm cases was 4.5 ± 1.7 ng/mL and for term controls it was 4.4 ± 1.7 ng/mL ($P = 0.87$). The second maternal blood sample mean ALLO concentrations were 7.4 ± 3.0 ng/mL and 7.8 ± 3.8 ng/mL for preterm cases and term controls ($P = 0.67$) (Supplemental Tables S3 and S4) (24). For secondary analytes, mean cortisol and cortisone concentrations were different between preterm cases and term controls. The first maternal serum sample mean cortisol concentration in preterm cases was higher (325 ± 120 ng/mL) compared with term controls (253 ± 107 ng/mL; $P = 0.02$). The absolute change in mean cortisol concentration was not significantly different across gestation; however, the percent change in mean cortisol between first and second blood samples was lower in preterm cases compared with term controls ($53 \pm 63\%$ vs $103 \pm 96\%$; $P = 0.03$). Mean cortisone, on the other hand, was significantly lower in preterm cases compared with term controls. Mean cortisone was 9 ± 9.9 ng/mL in first blood samples from pregnancies ending preterm and 15 ± 10.6 ng/mL for pregnancies ending at term ($P = 0.03$). The percent change in mean cortisone was $21 \pm 22\%$ vs. $36 \pm 29\%$ in preterm and term subjects ($P = 0.04$). Mean serum concentrations for each analyte at the first and second blood samples and absolute and percent change between samples are provided in Supplemental Tables S3 and S4 (24).

To address limitations in our *t* test comparisons resulting from overlapping gestational ages between the first and second blood samples, we used linear regression to model the expected mean analyte serum level at each gestational week (13-32 weeks) for the groups (Figure 4). From these estimates, we find only cortisol was statistically different by estimated mean value. The estimated mean cortisol concentration in the preterm group was higher in gestational weeks' 13w0d through 18wd0 than the estimated mean cortisol concentration for the term group ($P < 0.05$), whereas at later gestational ages, the estimated mean cortisol was similar for the two groups. The estimated mean maternal serum ALLO concentration was similar between preterm cases and term controls across the gestational timepoints observed in our dataset (13w0d-32w0d). All six study analyte concentrations increased with gestational age (Figure 4).

DISCUSSION

We used a multianalyte LC-MS/MS assay to assess several neurosteroids and steroid hormones in gestational weeks' 13 through 32 weeks of pregnancy and their association with preterm birth. In this pilot study, we found no association between maternal serum ALLO levels and preterm birth. In direct comparisons between the groups, mean ALLO levels were not significantly different. Maternal mean cortisol levels were significantly higher in the preterm group earlier in pregnancy (13w0d-18w0d). We also found small significant associations with preterm birth and higher cortisol earlier in pregnancy (13w0d-22w0d). Given the lack of effective tools in this area, these data show some promise in identifying women at higher risk for preterm delivery and who may benefit from psychosocial therapies earlier in pregnancy, which have been found to regulate maternal cortisol levels (25, 26).

Neurosteroids significantly increase across pregnancy (7, 27, 28) with a marked decrease in the ratios of 3α progesterone metabolites (ALLO and pregnanolone) to the 3ß metabolites (epipregnanolone) around parturition, supporting a possible functional role for neurosteroids in parturition (29). Prior investigation of the steroid metabolome during spontaneous labor found strong correlation between gestational age and conjugated progesterone metabolites (ALLO and pregnanolone) demonstrating increased sulfation to inactive forms at labor onset (30). ALLO has high affinity for $GABA_A$ receptors, which demonstrate varying subunit expression in the uterus throughout pregnancy and could be an ALLO target for parturition signaling (31). Additionally, ALLO restrains maternal pituitary oxytocin production in late pregnancy, potentially delaying the onset of regular uterine contractions (20). Our results are consistent with a recent study that found no difference in maternal serum ALLO concentrations late in pregnancy between women with or without possible preterm labor (32). However, that work relied on quantification of a single blood sample at the time of hospital admission for preterm labor evaluation without considering preterm birth outcomes. Although mean maternal serum ALLO concentrations were similar between groups in our study and we found no association between ALLO and preterm birth for gestational weeks' 13 through 32, future translational studies may focus on the functional and mechanistic influence of ALLO in pregnancy maintenance later in pregnancy. Assessment of gestational time points after 32w0d is needed to evaluate associations at later gestational ages.

Our finding of elevated early pregnancy cortisol in preterm birth cases has also been reported for serum, plasma, and hair and could be a potential mechanism for psychosocial regulation of parturition onset (33-36). The trends we observed in cortisol may be influenced by conversion between cortisol/cortisone and future studies may look at these analytes together, as has been done in prior research (37). The maternal-fetal-placental neuroendocrine stress axis plays a key role in mediating maternal stress as well as the timing of parturition onset (38). Altered maternal-fetal-placental neuroendocrine function may represent a mechanism for preterm birth where stress-induced developmental plasticity, in utero, accelerates the timing of birth (39). A precocious rise in maternal corticotropin-releasing hormone (CRH) has been linked to preterm parturition (40-42). High levels of cortisol in early pregnancy in our study may suggest elevated pregnancy CRH, although CRH testing was beyond the scope of this work (36). Rapid detection of maternal steroid hormones early in pregnancy could represent a new target for prediction and may coincide with other diagnostic tools including self-reported maternal mood and stress data. The relationship between maternal vs fetal production of steroids and neurosteroids remains to be determined; however, placental production appears to be the primary driver of increasing levels across gestation. Prior research has demonstrated blunted maternal cortisol and combined ALLO +

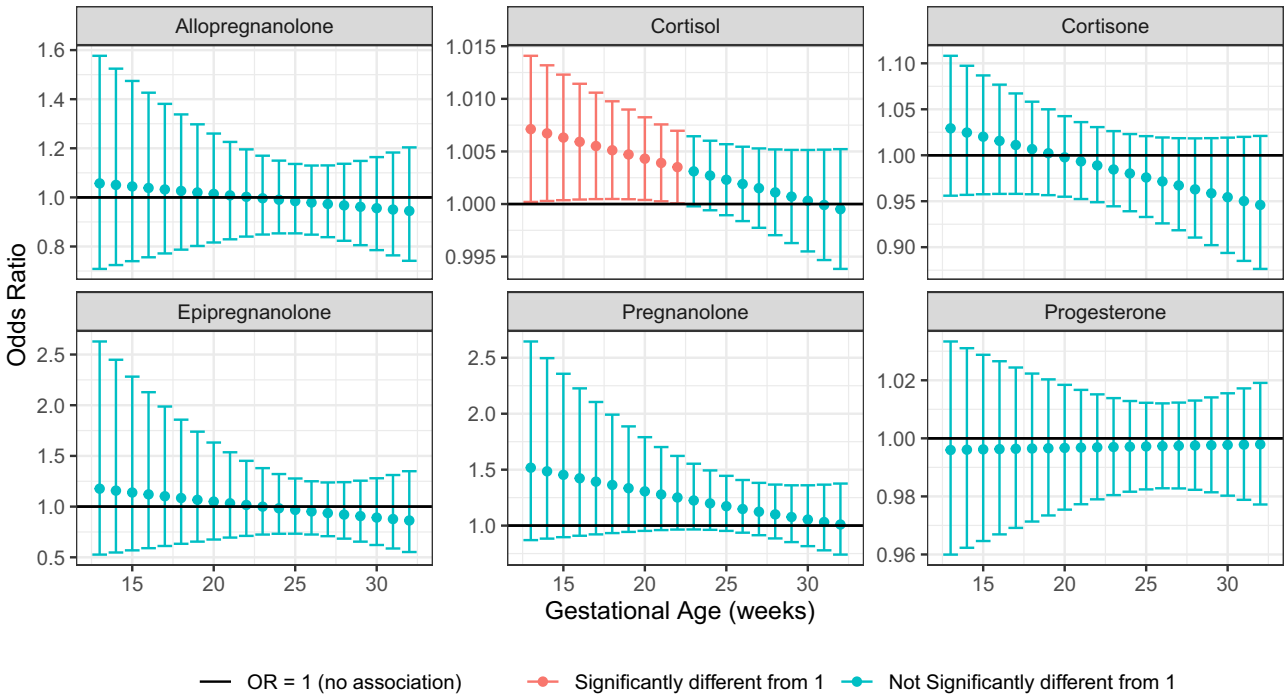

Figure 3. Odds ratios for preterm birth by serum concentration at a given gestational age. Odds ratios at 13 to 32 weeks of gestation with 95% CIs. Horizontal black bar is odds ratio of 1, indicating no association. Values above 1 indicate increased association with odds of preterm birth and values below 1 indicate reduced association with odds of preterm birth. Red indicates the association is significant, $P < 0.05$. Blue indicates the association is not significant. Estimates and CIs were generated by interpolation of the regression model. Regression model variables include serum analyte concentration, gestational age at the time of blood sample collection, and an interaction between serum analyte and gestational age. See Supplemental Table S1 for model results and Supplemental Table S2 for detailed reports of each analyte (24).

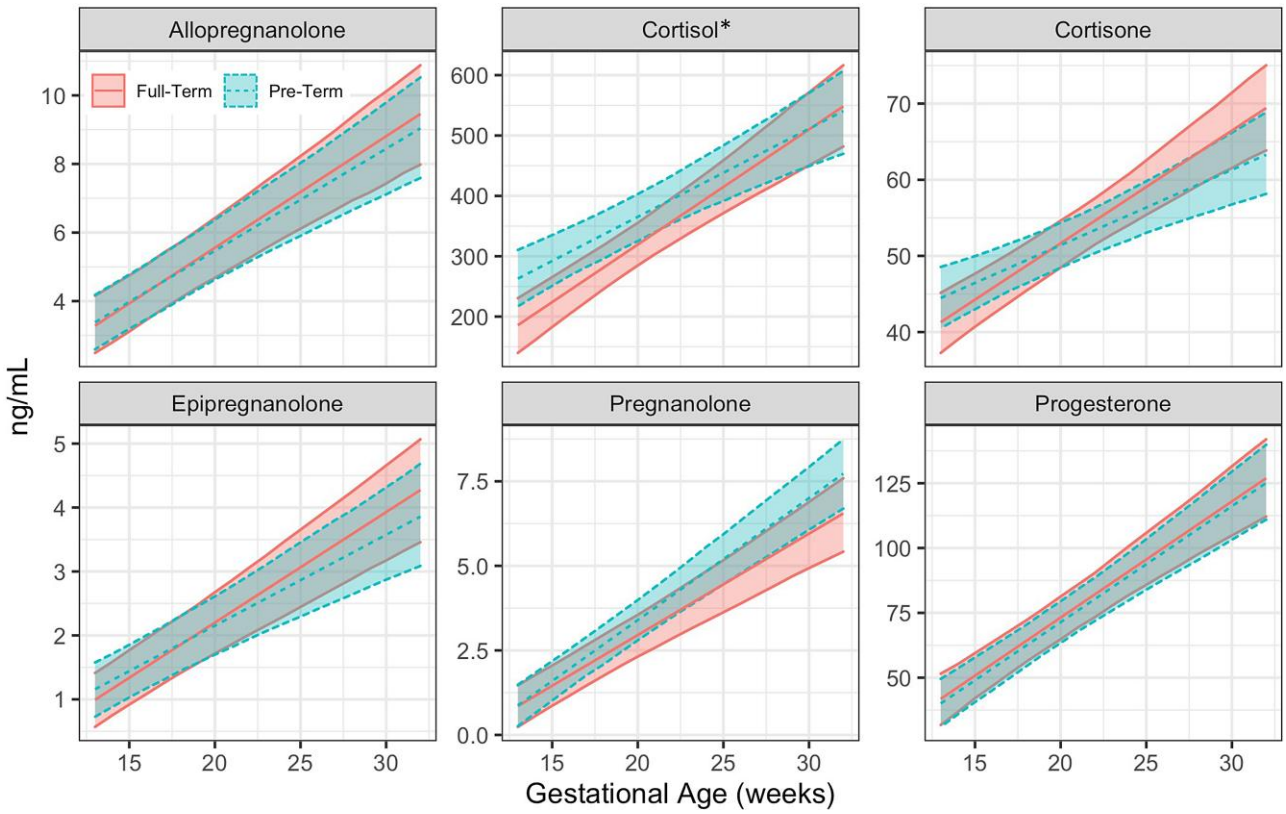

Figure 4. Comparison of estimated mean serum analyte concentrations in preterm and term cohorts across gestation. Estimated mean analyte concentrations reported for gestational ages 13 to 32 weeks with 95% CIs. Estimates and CIs were generated by interpolation of the regression model.
*****Estimated mean maternal cortisol was significantly higher in the preterm group at gestational ages 13w0d, 14wd0, 15w0d, 16w0d, 17w0d, and 18wd0.

pregnanolone levels in mid-pregnancy concurrent with self-reported poor maternal sleep and negative affect, which can influence birth timing (8). Mitigating maternal psychosocial stressors is important for addressing preterm birth socioeconomic and racial disparities. Simple, nontechnical interventions, such as doulas and increased social support, reduce maternal stress during pregnancy and correlate with lower cortisol levels, better birth outcomes, and reduced disparities (25, 26, 39).

Human pregnancy duration and the onset of functional labor may rely on several overlapping physiologic clocks and redundant signaling mechanisms (43, 44). For example, CRH (45), progesterone receptor isoform expression (46), placental cell-free DNA release (47), gasotransmitters (48), epigenetic (49), and metabolic signals (50-52) can influence cervical ripening and uterine contractions leading to labor. Identifying additional unknown physiologic mechanisms could enhance our understanding of human parturition onset. There is conflicting evidence ALLO inhibits uterine contractility through its action at the GABA receptors; this may suggest ALLO's role in parturition is better characterized by a shift from an increase in ALLO synthesis and GABA receptor sensitivity late in pregnancy to a decrease at labor onset (19, 31, 53). The specific actions of GABA receptors in cervical, placental, and uterine tissues and the role of ALLO in uterine GABA signaling are not well studied.

All analyte concentrations fell within the expected ranges for pregnancy in comparison to other reported values in the literature (reviewed in Mayne et al., 2021) (22). General metabolomic characterization of biochemical signatures associated with preterm birth is a promising approach (54-57). Both broad metabolomics and restricted specific metabolite studies such as ours may be needed to identify acute biomarkers for preterm birth as well as more general biological pathways such as inflammatory, neuroendocrine, metabolic, and/or infectious. Recent metabolomics studies have demonstrated general associations between cortisol and steroid derivatives for preterm birth, consistent with our findings (33, 58).

This study has several strengths, including a well-characterized, healthy young cohort with matched gestational age fasting blood samples. Our case-control approach permitted us to use biobank specimens to understand spontaneous labor physiology in a low-risk population for whom it is difficult to predict preterm birth. We performed analysis using a new LC-MS/MS assay to simultaneously quantify ALLO and five related steroid hormones as part of a pilot study to determine the relationship and predictive value of these analytes with preterm birth. The simplicity of our validated LC-MS/MS assay offers an attractive tool for future translational research linking subjective with objective markers of stress (22). This study also has limitations. The number of preterm cases was low, but adequate to meet our a priori sample size. The significant odds ratios for cortisol and preterm birth were small, so the clinical significance is unclear. The overlap in gestational age ranges between the first and second blood sample weakens the interpretations of the direct comparison *t* tests, and we were only able to analyze available samples between 12w4d and 32w0d. Finally, the field lacks detailed knowledge of ALLO's mechanisms in the reproductive tract and during pregnancy.

In conclusion, we found no association between maternal serum ALLO levels and preterm birth in gestational weeks' 13 through 32. Maternal serum mean ALLO levels were not significantly different in direct comparison between women who delivered preterm vs full term. Early pregnancy maternal serum mean cortisol levels were significantly higher in women who gave birth preterm (13w0d-18w0d) and these higher levels early in pregnancy associated with preterm delivery (13w0d-22w0d). Progesterone, pregnanolone, and epipregnanolone did not associate with preterm birth or differ between groups. Future studies may benefit by larger samples sizes and additional gestational time points to detect group differences for ALLO and the other neurosteroids. Further work determining the clinical utility of maternal cortisol levels in early pregnancy may benefit new diagnostic approaches to identify women at greater risk for preterm birth.

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Conflict of Interest

The authors declare they have no conflicts of interest.

Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Prior Presentation

Portions of this study were presented at the 68th annual meeting for the Society for Reproductive Investigation, poster session; Boston, MA, July 6-9, 2021.

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