Patterns of Plasma Corticotropin-Releasing Hormone, Progesterone, Estradiol, and Estriol Change and the Onset of Human Labor

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Context: Clinical prediction of preterm delivery is largely ineffective, and the mechanism mediating progesterone (P) withdrawal and estrogen activation at the onset of human labor is unclear.

Objectives: Our objectives were to determine associations of rates of change of circulating maternal CRH in midpregnancy with preterm delivery, CRH with estriol (E3) concentrations in late pregnancy, and predelivery changes in the ratios of E3, estradiol (E2), and P.

Design and Setting: A cohort of 500 pregnant women was followed from first antenatal visits to delivery during the period 2000–2004 at John Hunter Hospital, New South Wales, Australia, a tertiary care obstetric hospital.

Patients: Unselected subjects were recruited (including women with multiple gestations) and serial blood samples obtained.

Main Outcome Measures: CRH daily percentage change in term and preterm singletons at 26 wk, ratios E3/E2, P/E3, and P/E2 and the association between E3 and CRH concentrations in the last month of pregnancy (with spontaneous labor onset) were assessed.

Results: CRH percentage daily change was significantly higher in preterm than term singletons at 26 wk (medians 3.09 and 2.73; $P = 0.003$). In late pregnancy, CRH and E3 concentrations were significantly positively associated ($P = 0.003$). E3/E2 increased, P/E3 decreased, and P/E2 was unchanged in the month before delivery (medians: E3/E2, 7.04 and 10.59, $P < 0.001$; P/E3, 1.55 and 0.98, $P < 0.001$; P/E2, 11.78 and 10.79, $P = 0.07$).

Conclusions: The very rapid rise of CRH in late pregnancy is associated with an E3 surge and critically altered P/E3 and E3/E2 ratios that create an estrogenic environment at the onset of labor. Our evidence provides a rationale for the use of CRH in predicting preterm birth and informs approaches to delaying labor using P supplementation. **(*J Clin Endocrinol Metab* 94: 2066–2074, 2009)**

In many animals, the process of parturition is initiated by a drop in the circulating concentrations of progesterone (P) in maternal plasma (1). Early efforts to confirm such a process in pregnant women were contradictory (2, 3); however, no dramatic drop in maternal plasma concentrations of P was observed in human pregnancies at the time of labor (4–6). Studies on the

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For editorial see page 1886

Abbreviations: CI, Confidence interval; CV, coefficient of variance; DHEAS, dehydroepiandrosterone sulfate; E2, estradiol; E3, estriol; P, progesterone; PTD, preterm delivery.

effect of administered androgen to pregnant rhesus monkeys have provided evidence that estrogen synthesized from androgens plays a central role in labor and delivery in monkeys (7).

In humans, the placenta synthesizes CRH, and the exponential rise of this hormone in maternal plasma correlates with the timing of birth (8). Recently, CRH has been shown to stimulate placental production of estrogens and to inhibit placental synthesis of P (9, 10). Placental CRH is also released into the fetal circulation, and *in vitro* CRH directly stimulates dehydroepiandrosterone sulfate (DHEAS) production from the fetal zone of the fetal adrenal (11, 12). DHEAS is the precursor for placental estriol (E3) synthesis. E3 is an inhibitor of the action of the potent estrogen estradiol (E2) at low concentrations but becomes an effective agonist when the ratio of E3 to E2 exceeds 10:1 (13). Placental CRH may therefore lead to increased E3 production and reduced P synthesis. Consequently, we hypothesized that before birth in human pregnancy, changes would occur in P and E3 trajectories generating an decrease in the P to E3 ratio and a rise in the E3 to E2 ratio in both normal and preterm birth.

To test these hypotheses we sought to 1) characterize P, E2, E3, and CRH trajectories through pregnancy to delivery, including pregnancies with singleton term deliveries, preterm delivery (PTD), and multiple gestations; 2) assess the potential for prediction of preterm birth (by comparing singleton preterm and term pregnancy groups) using P, E2, and E3 levels and ratios in early third trimester and CRH levels and rates of change in late second and early third trimester; 3) assess the association of CRH with E3 in the last month before spontaneous labor onset; and 4) assess changes in P, E2, and E3 levels and ratios leading up to spontaneous labor onset.

Subjects and Methods

Study design

The Human Ethics committee of the Hunter Area Health Service approved this study, and all subjects provided written informed consent. A cohort of unselected subjects was recruited by research midwives at their first antenatal visit and followed to delivery at the John Hunter Hospital in Newcastle, Australia, during the period 2000–2004. Maternal blood samples were taken at approximately monthly intervals until and including sampling at the time of labor and just after delivery where possible. Gestational age was defined by an early ultrasound scan.

Assay methods

Blood was obtained by venipuncture, transferred to heparin tubes, and centrifuged at 2000 × *g* at 4 C for 15 min. Plasma was separated and kept at −20 C until assayed. Samples for each subject were batched for assay.

CRH was measured using RIA, as previously described (14) and with the modifications of Livesey and Donald (15). Briefly, the plasma was extracted with methanol, dried, and reconstituted in assay buffer [0.05 mol/liter potassium phosphate (pH 7.45), 0.1% (wt/vol) alkali-treated casein, 0.01% (wt/vol) EDTA, 0.05% (vol/vol) Triton X-100, and 0.02% (wt/vol) sodium azide]. Synthetic human CRH (Peninsula, Belmont, CA) was used as the standard with the rabbit antiserum Y_2B0 (a gift from Prof. P. J. Lowry and Dr. E. Linton, University of Reading, Reading, UK). The limit of sensitivity was 2 pmol/liter. The intra- and interassay coefficients of variance (CV) were 7.6 and 9.8%, respectively.

P and E2 were measured using the Bayer ADVIA Centaur assay (Bayer Corp., Tarrytown, NY), a competitive immunoassay using direct chemiluminescent technology. For the P assay, sensitivity was 0.67 nmol/liter and intraassay CV 5.3%. For the E2 assay, sensitivity was 25.7 pmol/liter and intraassay CV 8.4%. Total E3 was measured using fluorescence polarization immunoassay technology and the Abbott TDx-FLx analyzer (Abbott Laboratories, Abbott Park, IL). Sensitivity was 22.9 nmol/liter and intraassay CV 2.3%.

Curve-fitting and statistical methods

The following exclusion criteria were applied to subjects for curve fitting: gestational length less than 26 wk; fewer than three blood samples taken in total; fewer than three measurements for P, E2, E3, or CRH available before the last 4 wk of pregnancy. It was assumed that a single equation type could be used to curve-fit the samples for each analyte. Lower-order equations were preferred. The samples in the last 4 wk of pregnancy were excluded from curve fitting for P, E2, and E3 to allow the detection of a late-gestation change in trajectory. Samples on (or after) the day before delivery were excluded for CRH to allow for the possibility of trajectory change in labor. Several plausible candidate equations were chosen for each analyte and fitted to the data for each subject by nonlinear least-squares estimation and goodness of fit evaluated. A final choice of equation was made for each analyte, primarily by consideration of normality and homoscedasticity in the residuals; further detail of curve fitting is provided in supplemental methods (published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

The equations and stored coefficients for each subject enabled analyte levels to be interpolated as required. The equation for CRH daily rate of change (CRH rate) was derived (the first derivative of CRH with respect to time) and CRH daily percentage change (CRH percentage change) was defined as CRH rate expressed as a percentage of CRH level. The ratios P/E2, P/E3, and E3/E2 were calculated by division. Estimated levels and derived variables were calculated weekly for each subject to graph median curves.

Stata 9.2 software (StataCorp, College Station, TX) was used for curve fitting and statistical analysis. Hypothesis tests of group medians or paired group medians were conducted using nonparametric statistical tests, as appropriate to the distribution of the data. A two-tailed significance level of 5% was used throughout. Means are reported with SD; medians are reported with bootstrapped 95% confidence intervals (CI) estimated by the bias-corrected method (16).

Results

Subjects

A total of 557 women were recruited, of whom 57 were withdrawn due to incomplete attendance, formal withdrawals for a variety of reasons, five spontaneous abortions before 20 wk, and two terminations of pregnancy for fetal anomalies. Minimum study requirements were two blood samples taken and delivery and fetal outcome data available. Gestational length and gestational age at sample were based on early ultrasound scans except for four subjects in whom last menstrual period dating was used. The characteristics of the 500 subjects included are provided in Table 1.

The women were predominately Caucasian (92%), with a small percentage of Aboriginal or Torres Strait Islander descent (3%) and others including Asians (5%). Preterm delivery rate was 7.2% in singleton and 77% in multiple gestations (PTD was defined as gestational length $<$ 37 wk).

TABLE 1. Maternal, fetal, and pregnancy characteristics

	Preterm pregnancies (n = 45)	Term Pregnancies (n = 455)
Study cohort (500 women)		
Plurality		
Singleton pregnancy	35	452
Twin pregnancy	9	3
Triplet pregnancy	1	
Maternal age (yr)[a]	28.7 ± 4.8 (21–41)	27.9 ± 5.4 (16–47)
Primiparous	14 (31%)	202 (44%)
No. of blood samples[a]	4.3 ± 1.3 (2–8)	5.6 ± 1.1 (2–9)
Sampling weeks[b]		
1st sample	13.2 ± 3.0	14.3 ± 2.6
2nd sample	19.6 ± 3.5	19.7 ± 2.6
3rd sample	24.6 ± 3.3	24.9 ± 3.2
4th sample	29.7 ± 3.1	30.3 ± 3.5
Smoking, self-reported		
At enrollment	18 (40%)	120 (26%)
At delivery	15 (33%)	113 (25%)
Maternal morbidities		
Essential hypertension	1	2
Gestational hypertension	2	10
Preeclampsia	2	4
Gestational diabetes	1	1
Labor onset		
Spontaneous	24	307
Induced	10	114
Cesarean	11	34
Fetal characteristics and outcomes	Preterm (n = 56)	Term (n = 458)
Fetal sex		
Females	29	223
Males	27	235
Neonatal outcomes		
Livebirths	48 (86%)	456 (>99%)
Stillbirths	7	2
Neonatal deaths	1	

[a] Mean ± SD (minimum − maximum).

[b] Mean ± SD.

TABLE 2. Gestational age at delivery

Cohort after exclusions (466 women)	Twins (n = 10)	Singletons: Spontaneous PTD (n = 15)	Singletons: Iatrogenic PTD (n = 10)	Term (n = 431)
Pregnancy numbers				
28 to < 32 wk	2	1		
32 to < 34 wk	1	2		
34 to < 37 wk	5	12	10	
37 to < 42 wk	2			402
>42 wk				29
Gestational age[a]	34.9 ± 2.7	35.2 ± 1.7[b]	35.9 ± 1.0[b]	40.1 ± 1.3

[a] Mean ± SD.

[b] No difference; $P = 0.24$, Wilcoxon rank-sum test.

Curve fitting

After exclusions due to curve-fitting requirements, the cohort comprised 456 women with singleton pregnancies [subgroups of 15 spontaneous-onset PTD, 10 iatrogenic PTD, 89 normal spontaneous vaginal delivery at term, 313 other term deliveries (either induced or by cesarean section or spontaneous but associated with smoking or other pathology), 29 post-term] and a multiple gestation group of 10 women with twin pregnancies (four spontaneous PTD, four iatrogenic PTD, and two term pregnancies); a conservative definition of normal was used requiring spontaneous onset of labor, nonsmoking, and no pathology. The distribution of gestational ages at delivery is provided in Table 2.

The following equations were selected and curve-fitted to the data for each subject by nonlinear least-squares estimation: $\log_e P = a + bt^{1.5}$, where t is gestational age in days [coefficient of determination (R^2) > 0.85 for 95% of subjects; median for groups and subgroups range, 0.96–0.97; overall median, 0.97); $\log_e E2 = a + b/(\log t)$ ($R^2 > 0.67$ for 95% of subjects; median for groups and subgroups range, 0.93–0.98; overall median, 0.95); $\log_e E3 = a + b/\sqrt{t}$ ($R^2 > 0.74$ for 95% of subjects; median for groups and subgroups range, 0.89–0.97; overall median, 0.95); $\log_e CRH = a + bt^2$ ($R^2 > 0.95$ for 95% of subjects; median for groups and subgroups range, 0.98–0.99; overall median, 0.99).

Smoothed median curves for the twin group and preterm and term singleton groups are shown for P, E2, E3, CRH, and derived variables P/E2, P/E3, E3/E2, CRH rate of change (daily rate of change), and CRH percentage change (CRH rate expressed as a percentage of CRH level) in Fig. 1. (For this choice of CRH equation, CRH percentage change has the simple, linear equation 200bt.) Graphics showing examples of trajectories for three pregnancies with spontaneous onset of labor are provided in Fig. 2.

Comparisons of singletons with twins

Estimated median levels for P, E2, E3, and CRH at 26 wk in the twin-pregnancy group (n = 10) were compared with the singleton group (n = 456) using Wilcoxon rank-sum group median tests. Medians were significantly higher in the twin group for these analytes compared with the singleton group; medians were 1.8 times higher for P, 1.4 times higher for E2, 1.9 times higher for E3, and 3.4 times higher for CRH. (Results are provided in Table 3, and box and whisker graphs are provided in supplemental Fig. 1).

Potential for predicting PTD

Estimated levels for P, E2, E3, and CRH, and derived variables P/E2, P/E3, E3/E2, CRH rate, and CRH percentage change were interpolated at 26 wk gestation for each pregnancy and compared in the singleton term (n = 431) and PTD groups (n = 25) using Wilcoxon rank-sum group median tests. Additionally, CRH variables were compared at 18 wk gestation. None of the hypothesis tests for P, E2, E3, and ratios showed a significant difference, and all the tests for CRH variables at 26 wk showed significant increases in the PTD group. At 18 wk, neither CRH level nor rate of change was significantly different between groups; CRH percentage change provides the same *P* value (0.0027) and potential for prediction of PTD at any chosen time. Where the groups showed significant differences, receiver operator characteristics analysis was used to estimate the sensitivity for prediction of PTD pregnancies (*i.e.* delivery at <37 wk) with approximately 90% specificity; results indicated that in a screen-

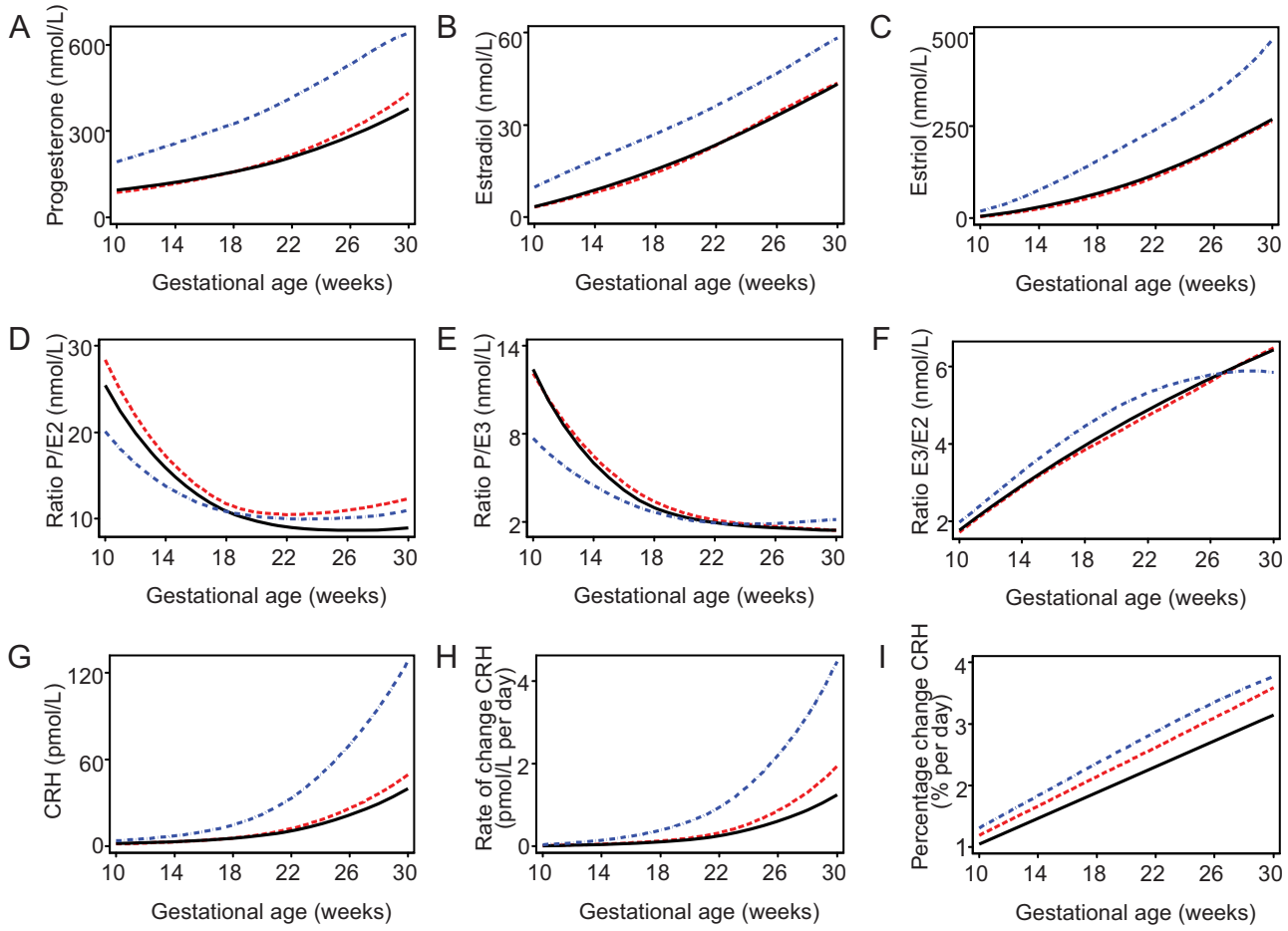

FIG. 1. Smoothed median curves for singleton term and PTD groups and twins. The medians were calculated weekly from trajectories, excluding pregnancies within 4 wk of delivery. A, P levels; B, E2 levels; C, E3 levels; D, ratio P/E2; E, ratio P/E3; F, ratio E3/E2; G, CRH levels; H, rate of change CRH; I, percent change CRH. *Blue dashed-dot lines*, Twins (n = 10); *red short dashed lines*, singletons PTD (n = 25); *black solid lines*, singletons term (n = 431).

ing test using CRH percentage change, 40% of the PTD group would be predicted correctly with 10% of the term group predicted incorrectly (false positive). Results provided in Table 3 indicate that CRH percentage change is a better predictor of PTD than CRH level or rate and potentially useful at an earlier timeframe. A comparison of the CRH variables estimated at the time of delivery revealed that the median CRH level and rate were significantly lower in the PTD group than the term group, whereas median CRH percentage change was not significantly different, indicating that a median daily percent change of approximately 4% is observed in both PTD and term groups at delivery. Additionally, a subgroup analysis (using Wilcoxon rank-sum tests) for singleton PTD pregnancies found no differences in P, E2, E3, CRH, CRH rate, or CRH percentage change between spontaneous onset labor and iatrogenic PTD subgroups at 26 wk (see Table 3).

Association of E3 with CRH in late-pregnancy samples

In 106 singleton term pregnancies with spontaneous labor onset, additional blood samples were taken either in the 24 h preceding delivery (labor group, n = 58) or in the first 4 h postpartum (postdelivery group, n = 48). Additionally, 172 blood samples were taken in the last 4 wk of pregnancy in 165 singleton term pregnancies with spontaneous labor onset (last-4-wk group). The association of E3 and CRH levels in the last 4 wk before spontaneous labor onset was assessed by multiple linear regression using one sample in each of these 165 pregnancies (with seven earlier samples excluded to provide independence of data). There was a significant positive association between E3 and CRH (both log-transformed) after adjustment for gestation days ($P = 0.003$; coefficient 0.17; 95% CI = 0.06–0.28; $R^2 = 0.13$). Possible confounders smoking, fetal sex, parity, maternal age, and days before delivery did not add significantly to the model, and the interaction term for CRH and gestation days was not significant.

Analysis of samples at labor and after delivery

For the labor group (n = 58), P, P/E2, P/E3, and E3/E2 results at four time points are shown in box and whisker plots in Fig. 3. In this group, actual measured P, E2, E3, P/E2, P/E3, and E3/E2 results at labor were compared with the interpolated results 4 wk prior using Wilcoxon matched-pairs signed-rank tests. The paired hypothesis tests showed a significant increase at labor for E2, E3, and E3/E2 and a fall in P/E3 but no significant change in P levels and P/E2 ratio. Results are provided in Table 3. Samples at labor were also compared with measured penultimate samples; the mean time from the penultimate sample to the sample at labor was 24.2 d (SD 13.4). For P, 53% of measured levels at

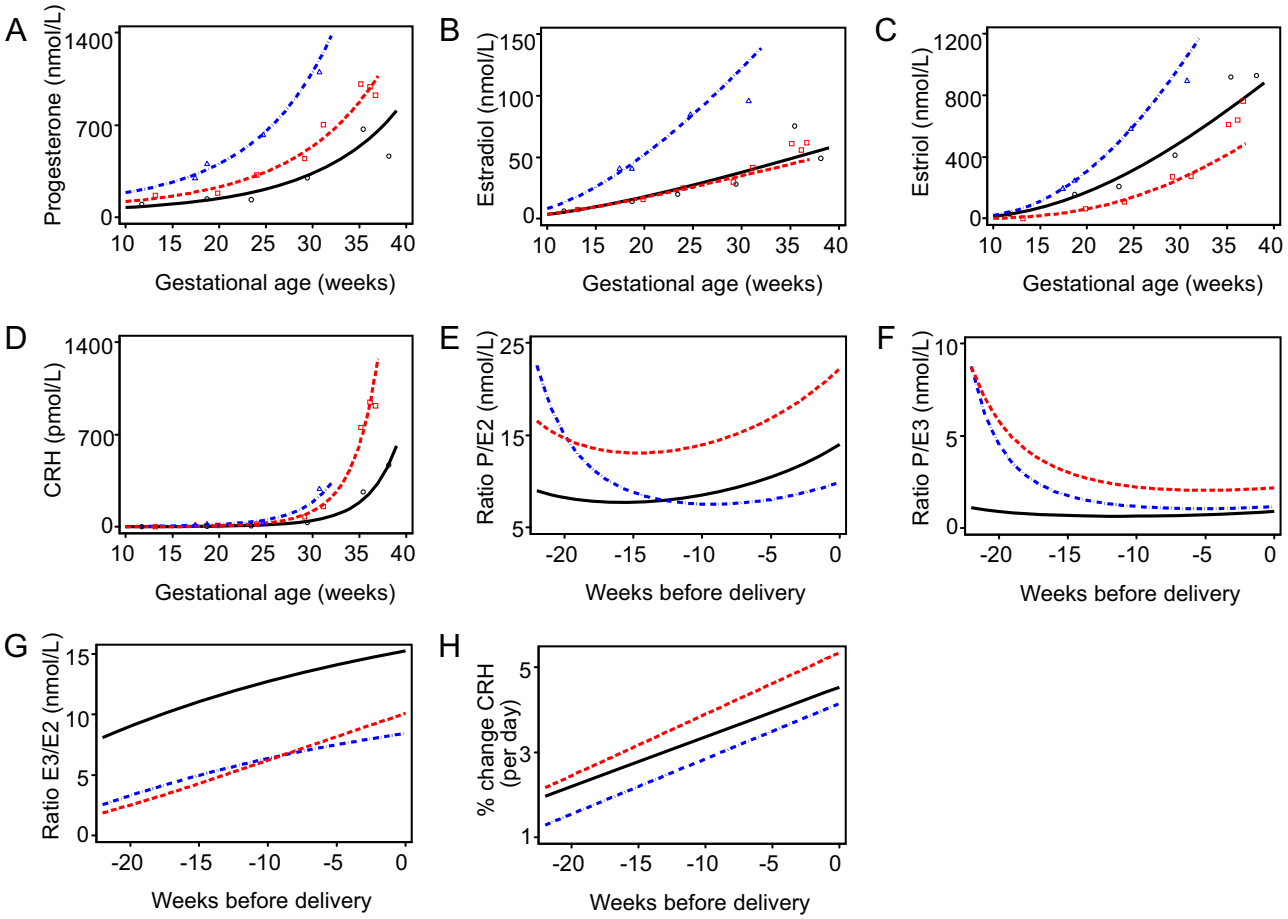

FIG. 2. Examples of estimated trajectories, extended to delivery, for three pregnancies with spontaneous onset of labor. A–D, Ten weeks to delivery, including measured samples; E–H, 20 wk before delivery. Note that some P/E3 ratios descend from very high levels in the early weeks of pregnancy. A, P; B, E2; C, E3; D, CRH; E, ratio P/E2; F, ratio P/E3; G, ratio E3/E2; H, percent daily change CRH. *Blue dashed-dot lines*, Twin pregnancy (31 wk); *red short dashed lines*, singleton pregnancy, last sample during labor (36 wk); *black solid lines*, singleton pregnancy (39 wk). *Small blue triangles*, *red squares*, and *black circles* represent actual measured samples.

labor were lower than the previous measured level, providing evidence that half of these trajectories had peaked before or during labor; however, the timing for this peak cannot be ascertained from these data. The predicted levels at labor were compared with measured levels for P, E2, and E3 using matched-pair sign tests (binomial distribution). For P, eight measured levels were above predicted and 50 below ($P < 0.001$); for E2, 29 measured results were above and 29 below ($P = \sim 1.0$); and for E3, 41 measured results were above predicted and 17 below ($P = 0.002$). Additional graphics are supplied in supplemental Figs. 2–4.

Discussion

Many small trials of P for the treatment of preterm birth were conducted in the middle of the last century with conflicting results; however, two recent larger, blinded, randomized trials of P therapy for the prevention of PTD (17, 18) have reported marked reductions of preterm birth rates in high-risk women. These results have reawakened interest in the role of P in the onset of human labor (19). This issue was extensively examined in the period between 1950 and 1990 by many different investigators (20). Although several studies in small groups of patients demonstrated late gestational falls in maternal plasma P (2, 3), many studies at term comparing samples taken in labor with those in the absence of labor failed to demonstrate a clear difference related to labor (4, 5, 21–23), and the general conclusion emerged that human labor, in contrast to that in most mammals, is not associated with a fall in maternal plasma P concentrations (6). This has provoked a search for a mechanism for a functional withdrawal of P action. Several mechanisms have been suggested, including a change in P receptor subtypes (24–26), a change in concentrations of P receptor coactivators (27), and P antagonism by the transcription factor nuclear factor-κB (28). Most recently, a trial of P therapy in twin gestations has reported no benefit compared with placebo in reducing preterm birth (29), whereas in subjects with a shortened cervix, P pessaries were effective (30). There is current controversy on which type of P to give and on who would benefit; underlying this discussion is an uncertainty on the potential mechanism of action of P supplements. In this setting, we sought to reexamine the changes in P, estrogen, and CRH in human pregnancy to determine whether subjects with preterm birth differed from term births and to compare the results in multiple gestations with those in singletons.

A challenging aspect of the endocrinology of human pregnancy is the marked variability in absolute levels of placentally

TABLE 3. Results of hypothesis tests

	Median (95% CI)		Group median test: *P* value	ROC: sensitivity with ~ 90% specificity	Notes
Comparison of singleton and twin groups at 26 wk	Singleton group (n = 456)	Twin group (n = 10)			
P (nmol/liter)	275 (267, 281)	504 (395, 731)	< 0.001		
E2 (nmol/liter)	32.9 (31.0, 34.4)	45.6 (37.2, 93.4)	0.001		
E3 (nmol/liter)	183.3 (178.7, 190.8)	351 (292, 601)	< 0.001		
CRH (pmol/liter)	19.2 (18.0, 19.8)	65.8 (47.7, 101.7)	< 0.001		
Comparison of singleton term and PTD groups at 26 wk	Term group (n = 431)	PTD group (n = 25)			
P (nmol/liter)	274 (268, 281)	288 (231, 363)	0.63		
E2 (nmol/liter)	32.56 (31.57, 34.66)	34.3 (27.64, 40.04)	0.96		
E3 (nmol/liter)	183 (179, 191)	180 (135, 204)	0.29		
P/E2 ratio	8.37 (8.1, 8.9)	10.94 (7.85, 13.37)	0.26		
P/E3 ratio	1.53 (1.47, 1.61)	1.64 (1.31, 1.95)	0.23		
E3/E2 ratio	5.73 (5.48, 6.07)	5.67 (4.81, 7.86)	0.74		
CRH (pmol/liter)	19.09 (17.72, 19.83)	24.21 (18.64, 30.27)	0.029	24%	
CRH rate	0.5 (0.48, 0.54)	0.66 (0.53, 1.08)	0.0063	36%	Daily rate of change
CRH % change	2.73 (2.69, 2.78)	3.09 (2.78, 3.5)	0.0027	40%	Daily % change
Comparison of singleton term and PTD groups at 18 wk					
CRH (pmol/liter)	5.13 (4.77, 5.41)	5.31 (4.33, 6.5)	0.61		
CRH rate	0.096 (0.091, 0.1)	0.11 (0.092, 0.14)	0.069		
CRH % change	1.89 (1.86, 1.93)	2.14 (1.93, 2.42)	0.0027	40%	
Comparison of singleton term and PTD groups at delivery					
CRH (pmol/liter)	573.7 (526.9, 638.8)	316.7 (184.6, 527.8)	< 0.001		
CRH rate	24.93 (21.9, 27.28)	14.4 (7.3, 23.46)	0.0018		
CRH % change	4.23 (4.12, 4.32)	4.27 (3.86, 4.83)	0.89		
Comparison of singleton PTD groups at 26 wk	Spontaneous labor onset group (n = 15)	Iatrogenic group (n = 10)	Group median test: *P* value		
P (nmol/liter)	288 (218, 319)	305 (231, 447)	0.32		
E2 (nmol/liter)	37.9 (28.3, 43.0)	24.9 (19.4, 38.8)	0.12		
E3 (nmol/liter)	162 (135, 203)	199 (128, 280)	0.37		
CRH (pmol/liter)	25.49 (19.25, 38.47)	19.16 (17.55, 36.76)	0.44		
CRH rate	0.91 (0.57, 1.26)	0.59 (0.50, 1.28)	0.44		
CRH % change	3.10 (2.70, 3.56)	3.10 (2.64, 3.43)	0.91		
Comparison of measured samples at labor with respective estimates 4 wk before delivery, measured penultimate samples, and predicted results at labor (n = 58)	Estimated 4 wk before delivery	Measured at labor	Paired test: *P* value	No. of samples at labor decreased from penultimate (with %)	Predicted median at labor (95% CI)
P (nmol/liter)	632 (596, 746)	751 (682, 801)	0.14	31 (53%)	915 (835, 1073)
E2 (nmol/liter)	54.93 (50.43, 67.45)	70.86 (63.39, 74.38)	0.003	21 (36%)	64.87 (57.08, 81.08)
E3 (nmol/liter)	412 (380, 475)	750 (658, 854)	< 0.001	15 (26%)	528 (466, 605)
P/E2 ratio	11.78 (9.57, 14.29)	10.79 (9.76, 12.07)	0.07	39 (67%)	13.98 (11.33, 17.46)
P/E3 ratio	1.55 (1.46, 1.76)	0.98 (0.88, 1.11)	< 0.001	47 (81%)	1.75 (1.58, 2.07)
E3/E2 ratio	7.04 (5.71, 8.24)	10.59 (8.65, 13.4)	< 0.001	19 (33%)	7.76 (6.07, 9.39)

ROC, Receiver operator characteristics.

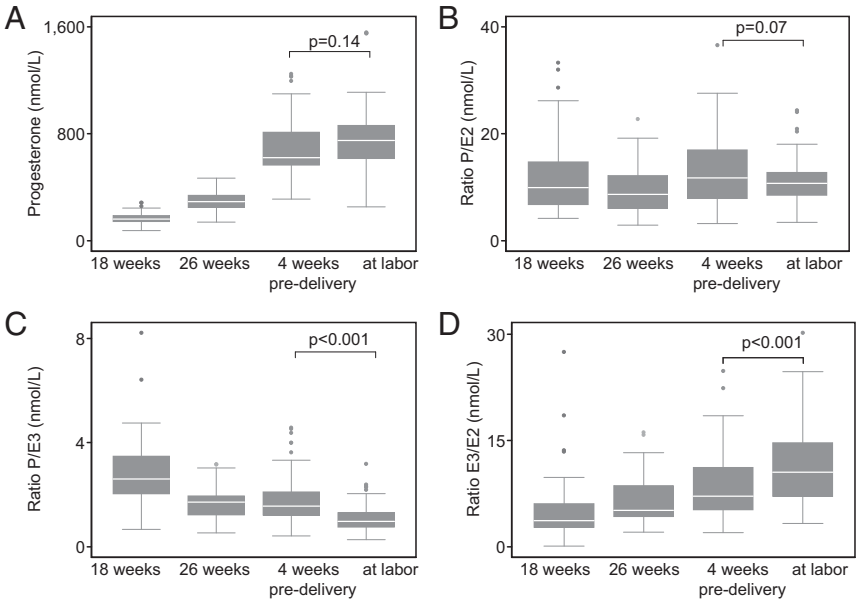

FIG. 3. Results at 18, 26, and 4 wk before delivery, estimated from trajectories and measured results at labor for 58 term pregnancies with spontaneous onset of labor. A, P levels; B, ratio P/E2; C, ratio P/E3; D, ratio E3/E2.

derived hormones between individual women, often by several orders of magnitude. We reasoned that target cells may respond more to relative changes in hormone concentrations rather than to absolute concentrations, and for this reason, the hormonal data may best be viewed in the form of trajectories across gestation. We have therefore used serial data on individual pregnancies to develop equations that describe the dynamic change in hormonal levels, which allow results of individual women to be effectively compared with each other to find common patterns. Other advantages of this method are to partially reduce the effect of measurement error for individual samples and assay results and to enable comparison between pregnancies where samples were not taken at identical gestational ages and would otherwise need adjustment. Interestingly, the equations derived from normal singleton pregnancies also provided good estimates of the results obtained in subjects who delivered preterm and in subjects pregnant with twins. This result supports the hypothesis that the patterns of change described by the equations are common to most pregnancies and are more important physiologically than absolute concentrations.

Comparison of P results at 26 wk obtained in the group of subjects with a singleton pregnancy who delivered preterm with the group who delivered at term did not identify a significant difference. This result argues that subjects delivering preterm do not have a deficiency in placental production of P relative to those who deliver at term. Similar results have been obtained by Klebanoff *et al.* (31). Additional P may provide benefit by opposing an increased drive to parturition that may arise due to inflammation (32, 33) or other altered biology associated with the pregnancy, such as an increased production of CRH (8, 34) or an increased intrauterine volume producing increased stretch (2, 35). Similarly, comparisons of E2, E3, and P/E2, P/E3, and E3/E2 ratios between groups did not reveal significant differences. Comparisons of CRH and derived variables CRH rate and CRH percentage change all showed significant differences between groups, confirming previous results (34, 36–39) and providing new evidence that rates of change of CRH, particularly CRH percentage change, are more useful predictors of PTD than CRH levels.

A comparison of P results in subjects pregnant with twins with singleton pregnancies confirmed previous reports that concentrations in twin pregnancies are close to double those of singletons (40). Similarly, for E2 and E3, twin gestations were associated with higher concentrations. For CRH, concentrations in twin pregnancies at 26 wk were more than three times those of singletons. However, the ratios P/E2, P/E3, and E3/E2 are surprisingly similar in singleton and twin groups up to the early third trimester of pregnancy. A clinical consequence for P supplementation in twin pregnancies is that to provide a similar P/E3 ratio to that seen in term singleton pregnancies, a higher dose of P may be required, perhaps double, and that reevaluation of dosage may be worthwhile. This may explain the recent negative results of P treatment in twin pregnancies (29). Nevertheless, the mechanism and site at which P supplementation exerts its action remains the subject of speculation, and it seems likely that the substantially increased PTD rate in twins is largely caused by factors such as the increased intrauterine volume (2) and increased uterine wall tension or by increased placental CRH. Notably, 17 α-hydroxyprogesterone caproate injections and P vaginal suppositories are likely to have different effects on maternal steroid ratios and trajectories. The causes of preterm birth are multifactorial with infection/inflammation predominating before 28–30 wk gestation, whereas maternal/fetal stress-related mechanisms appear to predominate thereafter. Our data particularly apply to mid-third-trimester PTD and not necessarily earlier. Subgroup analysis of the study (17) using 17 α-hydroxyprogesterone caproate injections for the prevention of preterm birth indicates that P supplementation by this method is more effective in reducing later preterm births than the early ones.

Late gestation changes in P, E2, and E3 trajectories were assessed by comparing results at labor with the trajectory developed from samples before the last month of pregnancy. The majority of P results at labor were lower than predicted results, indicating that these trajectories had peaked in the last month, most measured E3 results were higher than predicted, indicating an E3 surge (41) in the last month in many pregnancies, and E2 results did not show a significant trend. The association between measured E3 and CRH levels was assessed in late-pregnancy samples for term pregnancies with spontaneous onset of labor; E3 was positively associated with CRH in the last month of pregnancy.

Differences in predicted and actual results at labor could arguably be due to deficiencies in our equations or a real effect due

to a late gestation alteration in the physiology of pregnancy. To distinguish these alternatives, we compared the results obtained by measurement at labor with those obtained by measurement of the penultimate sample for each individual. P levels observed in labor were decreased in 53% of subjects, supporting the view that late in pregnancy, P fails to continue to increase at the rate seen earlier in pregnancy and that in many subjects, P levels actually fall, contrary to most previous evidence (4, 5, 21). The alteration in trajectory implies a late gestational change in the physiological factors regulating P production. This change may also be a consequence of the exponentially increasing maternal plasma CRH concentrations that occur at this gestational age because *in vitro*, CRH has been shown to inhibit placental P synthesis (10). This inhibition of P in a setting where pro-parturient factors, such as increasing E3 and intrauterine volume and stretch, are occurring may shift the balance in favor of labor. Furthermore, the reduction in P trajectory while E3 levels are increasing leads to a change in the ratio of P to E3 from a median of 1.4:1 to 1:1 at the time of labor. Similar falls in the ratio of P to estrogen occur at the end of pregnancy in subjects delivering preterm and with twins, providing a rationale for the prophylactic use of P supplements to prevent or delay the altered ratio. Other researchers have previously noted similar changes in the ratio of salivary P to E3 as labor approaches (42, 43).

Our results suggest that alterations in the regulation of E3 occur only in late gestation as parturition proceeds, creating an acceleration in the rate of rise of this estrogen, perhaps explaining why CRH is a useful predictor of PTD from the second trimester of pregnancy and P, E2, and E3 are not. E3 is derived from fetal DHEAS precursors. Late in gestation, fetal concentrations of placentally derived CRH increase very rapidly, and at the same time, CRH binding protein decreases rapidly, leading to a substantial increase in bioavailable (free) CRH in the last month of pregnancy (8). CRH has been shown to have direct effects on the fetal zone of the fetal adrenal to increase production of DHEAS (11). CRH therefore may drive increased DHEAS, leading to increased placental E3 formation. CRH has also been shown to directly stimulate placental estrogen synthesis (9). E3 has relatively complex actions at the estrogen receptor, antagonizing the actions of E2 when present at ratios of E3/E2 less than 10:1 but becoming an agonist at greater than this ratio (13). Interestingly, the ratio of E3/E2 exceeds this critical point in late gestation, providing a mechanism for the increased drive to expression of labor associated genes such as connexin 43 and the oxytocin receptor and the consequent onset of generalized uterine contractions. The changes in maternal plasma may underestimate the changes occurring in the amniotic fluid and fetal membranes where the ratio of E3/E2 has been shown to increase to close to 700:1 by labor (44). Of note, E3 salivary concentrations show a marked diurnal variation with highest levels at night (45, 46), whereas no similar diurnal changes occur in E2 (46). This would lead to marked increases in the E3/E2 ratio at night when most spontaneous labors are initiated (47).

Examination of samples taken shortly after delivery revealed the expected postpartum fall in P and E2 concentrations as a consequence of placental separation. Surprisingly, however, the changes in E3 are far less dramatic, perhaps reflecting a longer half-life of this steroid in maternal plasma. It is not clear whether the resulting further decrease in the P to E3 ratio has biological consequences, although it is possible that this may promote the more vigorous contractions seen at this time that likely assist in reducing postpartum hemorrhage.

Intriguingly, in humans, a critical feature of parturition appears to be a change in the ratio of the two estrogens E2 and E3 as labor approaches, leading to a more than 10-fold excess of E3. The data demonstrate the absence of a difference in early third-trimester P levels in women who deliver prematurely compared with those who deliver at term and high concentrations of P concentrations in twin pregnancies. The results provide a rationale for P supplementation in the prevention of preterm birth by antagonizing the declining P to E3 ratio that occurs in late gestation as part of the normal progression toward labor. Our findings provide a basis and a methodology for investigating the rationale for P supplementation in preventing PTD. A counter-intuitive possibility is that E2 may delay the onset of labor when given in late gestation. CRH antagonists may also be useful by lowering the drive to E3 production. The data further suggest that effective P supplementation in multiple gestation may require a higher dose of P than that which is effective in singleton pregnancies.

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