Research Article

The Relationship between Umbilical Cord Estrogens and Perinatal Characteristics

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Abstract

Background: Prenatal estrogen exposure is thought to contribute to later life diseases such as breast cancer. However, few studies have directly measured prenatal estrogens and most have relied on proposed "markers" of estrogen exposure. We used a large population-based birth cohort to directly measure the relationship between prenatal estrogens and perinatal characteristics, including putative markers of estrogen exposure.

Methods: Total estrone (E1), estradiol (E2), estriol (E3), and estetrol (E4) were assayed by liquid chromatography/tandem mass spectrometry from archived mixed arterial and venous serum from 860 umbilical cord blood samples.

Results: Values for all estrogens were strongly intercorrelated. Cord estrogen concentrations did not differ between males and females. Levels of all estrogens were reduced in twins and concentrations increased with gestational age. Neither E1 nor E2 was correlated with birth weight, but E3 and E4 levels correlated weakly, whereas onset of labor was associated with higher estrogen concentrations. E1 and E2 concentrations were not associated with preeclampsia in the current pregnancy, but E3 and E4 concentrations were lower in pregnancies complicated by preeclampsia and antepartum hemorrhage.

Conclusions: Umbilical cord estrogen concentrations vary with gestational age, mode of delivery, pregnancy complications, and twinning, but not with infant sex. Putative markers of prenatal estrogen exposure, preeclampsia, and birth weight did not correlate with direct fetal measures of the most potent estrogen (E2) but were associated with weaker estrogens (E3 and E4). Twins had lower concentrations of all estrogens.

Impact: This is the largest and best characterized dataset of prenatal estrogen concentrations, measured using highly accurate mass spectrometry/spectroscopy. These observations represent the new "gold standard" for umbilical cord estrogens, and will inform the interpretation of other datasets and the early life origins of health and disease. *Cancer Epidemiol Biomarkers Prev; 23(6); 946–52. ©2014 AACR.*

Introduction

The prenatal environment influences later life health. Specifically, the timing and magnitude of prenatal estrogen exposure are thought to affect later life risk for reproductive cancers such as breast cancer (1, 2). Experimental and human data demonstrate that supraphysiological prenatal estrogen exposure increases adult breast cancer risk (3, 4). Similarly, experimental data suggest that high prenatal estrogen exposure increases prostate cancer risk (5), but lack of information about normal ranges of prenatal estrogen exposure in females and males has limited translation to human studies. Lack of access to direct prenatal estrogen measurement has led to the widespread use of surrogate "markers" of estrogen exposure. Gestational factors such as preeclampsia, birth weight, and twinning are thought to modify adult breast cancer risk by modifying *in-utero* estrogen exposure (6, 7). Previous studies have questioned the association between these "markers" and direct measures of prenatal estrogens (8), but no previous studies have directly compared prenatal estrogen measures with surrogate markers in large community-based populations. Maternal circulating estrogens have also been used as surrogate markers of fetal estrogen exposure, but there is little correction between maternal and direct fetal measures (9, 10). Furthermore, umbilical cord sex steroids are most accurately measured using mass spectrometry because an abundance of interfering substances and cross-reacting steroids and their conjugates in cord blood interfere with conventional radioimmunoassay (11, 12).

To advance our understanding of the relationship between early life estrogen and later life health, more information is needed from direct fetal measures of estrogen exposure. In uncomplicated pregnancy, this information can only be obtained at birth from the umbilical cord

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as invasive investigators to access the fetal circulation or umbilical fluid during pregnancy cannot be justified for research purposes. Umbilical cord sex steroid concentrations at birth reflect fetal levels in late gestation with minimal maternal contamination (13, 14).

The human fetus is exposed to sex steroids from the mother and the placenta as well as endogenous gonadal and adrenal production. The placenta is a highly steroidogenic organ responsible for the production of large amounts of free and conjugated estrogens from fetal adrenal precursors (15). 17β-estradiol (E2) is the most bioactive estrogen. During pregnancy, the placenta makes considerable amounts of estrone (E1) and estriol (E3). A fourth estrogen, estetrol (E4, also known as 15α-hydroxyestriol), is synthesized exclusively by the fetal liver, primarily from E2 and E3 precursors (16). Accurate assessment of prenatal estrogens should include measurement of all four biologically active estrogens (11, 12, 14). To establish whether commonly used markers of prenatal estrogen exposure reflect circulating biologically active estrogens in the fetus, we conducted the first large population-based study of biologically active umbilical estrogen concentrations using liquid chromatography/tandem mass spectrometry (LC/MS-MS). In addition, we report the relationship between proposed markers of estrogen exposure, perinatal variables, fetal sex, and estrogen concentrations.

Materials and Methods

Cord blood collection

The Western Australian Pregnancy Cohort (known as the Raine Study; ref. 17) consists of 2,868 unselected pregnancies recruited and sampled from the a public tertiary referral antenatal clinic of King Edward Memorial Hospital in Perth, Western Australia between 1989 and 1991 to study the effects of repeated ultrasound on fetal and postnatal growth and development and pregnancy outcomes. The study was approved by the King Edward Memorial Hospital/University of Western Australia ethics committees. As previously described (18), 50% of women attending the clinic were eligible for recruitment, of whom 90% agreed to participate in the study. Pregnant women completed a questionnaire at 18 and 34 weeks gestation, which provided information on a wide range of variables including ethnicity, social and economic circumstances, lifestyle, medical history, and environmental exposures. Pregnancy and neonatal outcome data were abstracted from hospital notes. Of the 2,868 participants in the cohort, 1,415 were randomized to the intensive arm of the study, which included repeated ultrasound measures of fetal growth and umbilical cord blood collection. This group forms the study population for the present analysis. There were no demographic differences between pregnancies in the control arm and ultrasound intensive arm. Immediately after delivery mixed umbilical arterial-venous (UA:UV) cord, blood was collected and allowed to clot, and serum was frozen at −80°C and stored without thawing until the present study was performed. Eight

hundred and sixty blood samples representing 431 male and 429 female infants had sufficient serum for steroid analysis and were included. This cohort contained 27 sets of twins and one set of triplets, from which samples were available for analysis from 32 female and 23 male neonates ($n = 55$ total, 54 with complete datasets). Eleven of these pregnancies had dizygotic twins of mixed sex.

Estrogen analysis

For the estrogen assays, cord serum samples were thawed, aliquoted, and refrozen, then shipped from Perth to South Australia for LC/MS-MS analysis (CPR Pharma Services Pty Ltd); in total samples were thawed and frozen less than three times following collection. A single 20 μL aliquot of each sample was solvent extracted following addition of internal standards [d4-estrone (IS-E1), d4-estradiol (IS-E2), and d3-estriol (IS-E3)] to control for extraction efficiency. The analytes are separated by high-performance liquid chromatography on a Phenyl-Hexyl column (run time approximately 7 minutes) and the eluates monitored by an API5500 MS/MS detector in negative multiple reaction monitoring (MRM) mode. The single charged Q1/Q3 transitions were 269.0/145.2, 271.0/145.0, 287.0/144.9, and 303.0/272.9 atomic mass units (amu) for E1, E2, E3, and E4, respectively. The single charged Q1/Q3 transitions for the internal standards were 273.0/147.2, 275.0/147.2, and 289.9/147.2 amu for IS-E1, IS-E2, and IS-E3, respectively. The data were processed by the data acquisition software Analyst linked directly to the API5500 MS/MS detector. The calibration curves ranged from 0.5 to 50 ng/mL for E1, E2, and E4, and 5 to 500 ng/mL for E3. The limits of quantitation of the E1, E2, E3, and E4 assays were 1.8, 1.8, 17.8, and 1.8 nmol/L, respectively. Within-study ($n = 42$) and prestudy ($n = 12$) precisions for E1 were 3.9% to 5.4% and 3.3% to 4.3%, respectively; 6.2% to 8.6% and 3.6% to 4.8% for E2; 4.4% to 6.9% and 1.5% to 5.1% for E3; and 7.4% to 8.5% and 4.2% to 7.4% for E4. Extraction efficiencies for E1, E2, E3, and E4 were $52.8 \pm 10.1\%$, $50.6 \pm 8.0\%$, $63.1 \pm 6.7\%$, and $67.2 \pm 7.9\%$, respectively, ($n = 18$). Assay validation was based on the U.S. Food and Drug Administration Guidelines for Bioanalytical Method Validation.

All analyses were performed blinded. All four estrogens were present in detectable concentrations in almost all samples. There were two samples with E1 levels below the limit of detection (<0.5 ng/mL), one with undetectable E2 concentrations (<0.5 ng/mL), two with undetectable E3 levels (<5 ng/mL), and two with E4 levels below the limit of detection (<0.5 ng/mL); all of these samples had very low levels of all four estrogens.

Descriptive statistics and generation of charts and graphs were carried out using Microsoft Excel. Differences between singletons and twins were determined by the Mann–Whitney U test for nonparametric data or the Fisher exact test for categorical or discrete variables using InStat version 3.10 (GraphPad Software Inc). A P value <0.05 was considered statistically significant. Pearson correlations were performed on Instat 3.10 to explore the associations

between estrogen concentrations gestational age at delivery, birth weight, and placental weight; there were no significant departures from linearity. Multiple regression analysis was performed (using Instat 3) to determine the independent effects of fetal number and gestational age at delivery on cord estrogen concentrations.

Results

The demographic details of the entire cohort, segregated into singletons and twins, are shown in Table 1. The mothers of the twin and singleton pregnancies had similar ages, ethnicity, and smoking rates (Table 1). The relatively large number of twins likely reflects the tertiary referral centre. As expected, the twin pregnancies had significantly greater rates of preterm birth, intrauterine growth restriction (IUGR), and antenatal corticosteroid administration. The birth weights of the female and male infants were significantly lower in the twin group, whereas placental weight was significantly greater. The rates of labor induction and Caesarean-section delivery were also significantly greater in twin pregnancies compared with singletons (Table 1).

The mean ($\pm$SD) concentrations of E1, E2, E3, and E4 in the entire cohort (term and preterm singletons and twins) were lower than most previously reported concentrations (see Supplementary Table S1). Values in the subset of term singletons were similar to the levels in the entire cohort (Supplementary Table S1). Concentrations

of all four estrogens were strongly correlated with each other, with the strongest correlations shown between E1 and E2 ($r = 0.757$) and E3 and E4 ($r = 0.705$; Supplementary Fig. S1).

The effects of obstetric characteristics on cord estrogen levels were investigated after exclusion of twins due to the significant differences in estrogen levels and obstetric parameters between singletons and twins. E1, E2, E3, and E4 levels all showed a strong, significant positive association with gestational age at delivery (Fig. 1), with a consistent trend toward decline after 40 weeks' gestation. Estrogen concentrations in extremely preterm infants were markedly lower than those at term, with levels at 30 to 32 weeks' gestation around half those at term (Fig. 1A). The effect of gestation was seen in both singletons and twins (see Fig. 1B; gestational age profiles for E1-E3 are not shown but were very similar). Cord estrogen concentrations showed no association with placental weight. There were also no significant differences between males and females in umbilical cord estrogen concentrations (Fig. 2).

Cord blood E1 and E2 levels were not significantly correlated with birth weight, but a weak but highly significant correlation was seen with both E3 ($r = 0.140$, $P < 0.0001$) and E4 ($r = 0.129$; $P = 0.0003$) concentrations (data not shown). Concentrations of both E1 and E2 were weakly, but significantly, associated with maternal weight at delivery ($r = 0.138$ and 0.101, respectively; $P < 0.005$). Cord blood estrogen levels were not significantly

Table 1. Demographic and obstetric characteristics of the study cohort

	Singletons (*n* = 805)	Twins (*n* = 54)	*P*
Maternal age, y[a]	28.3 ± 5.9	29.3 ± 5.5	NS[f]
Caucasian ethnicity, %	82.0	87.0	NS
Smoker[b], %	25.1	33.3	NS
Gestational age at delivery, wks[a]	39.0 ± 2.6	35.0 ± 3.0	<0.0001
Birth weight, females, kg[a]	3.32 ± 0.59	2.09 ± 0.58	<0.0001
Birth weight, males, kg	3.31 ± 0.60	2.43 ± 0.65	<0.0001
Placental weight, g	587 ± 124	862 ± 228	<0.0001
Caesarean section[c], %	10.9	42.6	<0.0001
Delivery following labor[d], %	89.4	56.6	NS
Labor duration, h[a,e]	6.86 ± 4.3	5.38 ± 2.9	NS
Prelabor rupture of membranes, %	19.1	22.6	NS
Induction of labor, %	26.4	43.4	0.01
APH, %	8.5	3.8	NS
Antenatal steroids administered, %	2.75	20.8	<0.0001
Preterm birth, %	7.2	67.9	<0.0001
IUGR, %	3.0	24.1	<0.0001

Abbreviation: NS, not significant.
[a]Mean ± SD.
[b]Smoking defined as one or more cigarettes per day.
[c]Includes both elective and in-labor sections.
[d]All deliveries excluding elective Caesarean-section deliveries.
[e]Stage I and II of labor combined.
[f]*P* > 0.05 by the Mann–Whitney *U* test for group median comparisons or Fisher's exact test for categorical variables.

Figure 1. Influence of gestational age at delivery on umbilical cord estrogen concentrations. A, concentrations of estrogens (mean ± SD) in the entire cohort (singletons and twins) are shown in six gestational age categories. B, scatter plot comparing cord estetrol (E4) levels in singletons and twins from 26 to 42 weeks of gestation. The difference between twins and singletons remained significant after controlling for gestational age. Similar relationships existed in the other three estrogens.
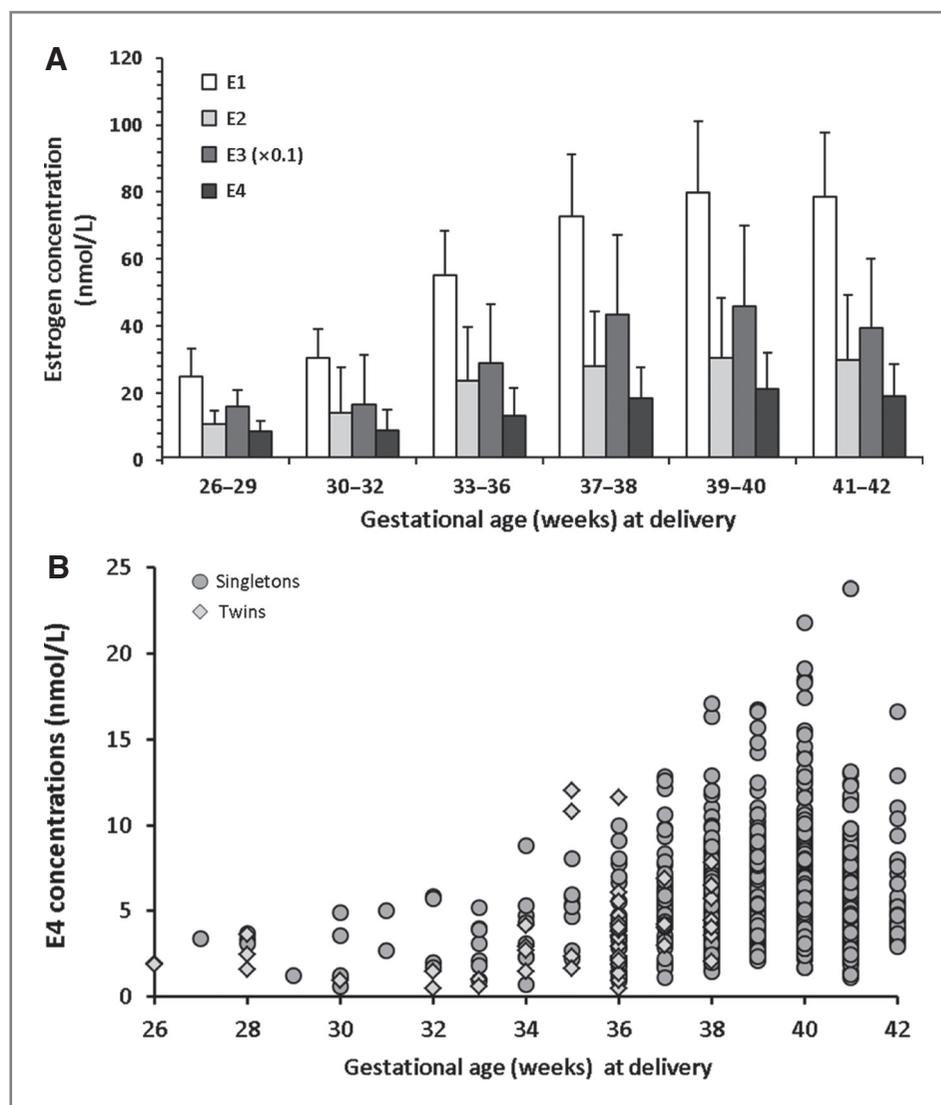
associated with smoking in pregnancy (smoking status or daily cigarette consumption).

In singletons, onset of labor was associated with markedly higher umbilical cord levels of E1, E2, E3, and E4 (85%, 54%, 52%, and 55% greater, respectively; $P < 0.0001$) compared with deliveries by elective Caesarean section without labor (Fig. 3A). Preeclampsia was associated with lower estrogen levels (<20%), reaching statistical significance for E3 and E4 ($P < 0.05$ and 0.005, respectively; Fig. 3B); this difference was independent of gestational age at delivery or mode of delivery. Similarly, the presence of antepartum hemorrhage (APH) was associated with significantly (∼20%) lower E3 and E4 levels ($P = 0.0013$ and 0.0045, respectively) after controlling for gestational age and mode of delivery (data not shown).

Both male and female twins (from both single sex and mixed sex pregnancies) had lower cord estrogen concentrations compared with singletons: concentrations of E1, E2, E3 and E4 in twins were 44.1 ± 29.6, 19.9 ± 13.3, 296.2 ± 201.5, and 12.1 ± 8.4 nmol/L (mean ± SD), compared with singletons (73.4 ± 41.5, 29.3 ± 18.2, 417.1 ± 232.6, and 19.6 ± 10.24, respectively; Fig. 4A). This difference remained significant after accounting for gestational age at delivery in a multiple regression model, with the E4 levels in particular remaining highly significant. There was no statistically significant difference in estrogen concentrations between same sex twins, although levels in males tended to be slightly higher than females (Fig. 4B). In the 11 sets of male-female dizygotic twins, there were no significant differences between levels of any of the estrogens between the sexes (paired t test) and no gender differences as a group. Levels of E1 and E2 in the twin pairs were significantly correlated ($r = 0.836$ and 0.621, respectively; $P < 0.03$), whereas E3 and E4 were not correlated. Only two of the 27 sets of twins were from assisted reproduction pregnancies. Estrogen levels in these infants were within one SD of the mean of all the
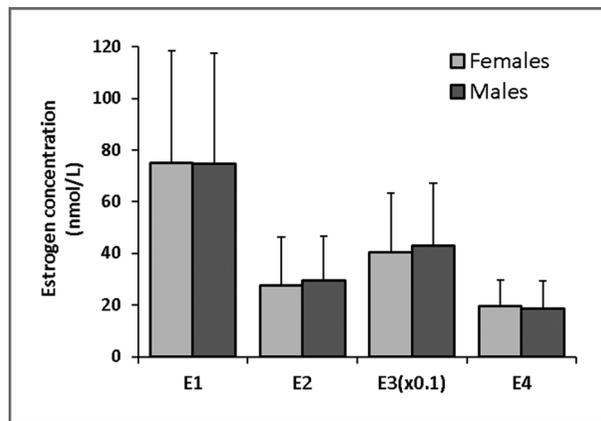
Figure 2. Comparison of umbilical cord blood estrogen concentrations (mean ± SD) in male and female infants. There were no significant differences.

twins. The one set of triplets (two girls, one boy) had very low levels of all estrogens (mean of 3.1, 3.0, 49.8, and 8.0 nmol/L for E1, E2, E3, and E4, respectively) and no obvious sex difference.

Discussion

This is the first study to report the relationship between direct measures of prenatal estrogen exposure from a large population-based pregnancy cohort and perinatal characteristics. It is also the first large study to compare direct measures of prenatal estrogens with proposed "markers" of prenatal estrogen exposure. The strengths of our data include large sample size (>10-fold larger than previous published studies and the first to have the power to measure these relationships), detailed prospective collection of obstetric and perinatal characteristics, mode of delivery and birth data, and high specificity LC/MS-MS assay. Cord blood was not separated into venous and arterial components, but sex steroids are closely correlated so separation of vessel source is not required for accurate measurement from the fetal circulation (19).

The most striking observation was that cord estrogen concentrations were similar in males and females. Previous studies of cord estrogens have been limited to RIA with its attendant limitations, and have shown inconsis-

tent results, including no sex differences (20–23), higher estrogen concentrations in females (24), and higher estrogen concentrations in males (25, 26). Our data suggest that sex differences in estrogen exposure at late gestation are not responsible for sexually dimorphic outcomes in adult health or behavior.

Perinatal markers of estrogen exposure such as preeclampsia and twinning (thought to indicate lower prenatal estrogens) and birth weight (thought to indicate higher estrogens) have been widely used in studies of the early life origins of breast cancer (2). Our findings suggest that neither preeclampsia nor birth weight is consistently related to prenatal estradiol concentrations, but are associated with the weaker estrogens E3 and E4. However, our observations do support the validity of twinning as a "marker" of reduced prenatal estrogen exposure (27). Only one previous study has described the levels of E2 in cord blood of same-sex twins (28); ours is the first to report LC/MS-MS–derived umbilical concentrations of all estrogens from twin pregnancies. We found that cord estrogen concentrations were consistently lower in twins, regardless of fetal sex and gestational age at delivery. Our findings also showed that twins of opposite sexes have closely correlated umbilical E3 and E4 levels, but show no sex differences in estrogen concentrations; this further supports the view that the fetal adrenal-placental axis determines fetal estrogen levels in a sex-independent fashion.

Maternal cigarette smoking has been proposed to reduce prenatal estrogen exposure by action on aromatase activity (29). This is the largest study to report prospectively collected data on cigarette smoking in pregnancy and umbilical cord estrogen concentrations. Consistent with previous studies, we report no significant associations between cord estrogen levels and smoking status or the number of cigarettes smoked per day. Our data suggest that smoking in pregnancy does not affect fetal estrogen exposure at late gestation.

We found strong and consistent correlations between all four estrogens despite the large interindividual variations observed in all four analytes. This confirms observations in smaller studies of cord E1, E2, and E3 concentrations but the E4 report is novel. These correlations are not surprising considering the interconversion of androgen/estrogen precursors and end products by the placenta
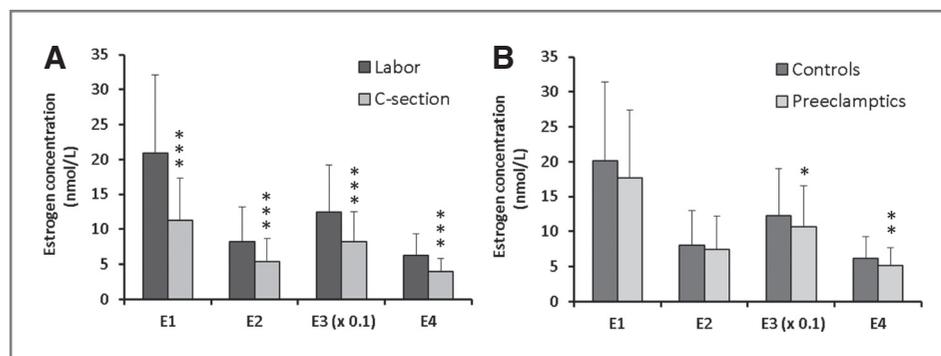

Figure 3. Comparison of umbilical cord blood estrogen concentrations (mean ± SD) in singletons delivered following spontaneous onset of labor versus elective Caesarean section before the onset of labor (A) or with or without preeclampsia (B). *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$ by the Mann–Whitney U test.

Figure 4. Comparison of umbilical cord blood estrogen concentrations (mean ± SD) in singletons versus twins (A) and in male versus female twins (B). ***, $P < 0.0001$ by the Mann–Whitney test. The significant difference was maintained after controlling for gestational age at delivery.
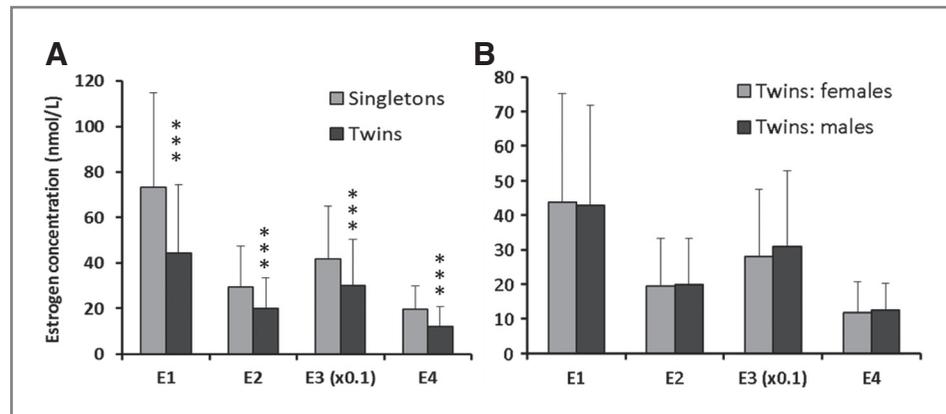
and E4 production via 15-α hydroxylation of E3 in the fetal adrenal gland (16, 30). We observed considerable variation (more than two orders of magnitude for E1 and E3) between individual concentrations of estrogens, suggesting that there is a wide range of estrogen exposure in normal pregnancy. We also documented a significant effect of labor on cord estrogen levels, presumably reflecting enhanced fetal adrenal steroid production due to stress. Consistent with previous small studies, we observed that estrogen levels increase with gestational age (12, 22, 31, 32) until around 38 weeks gestation (12). These findings suggest that cord sex steroid data cannot accurately be interpreted without information on gestation and mode of delivery. These modifying factors have not been considered in most previous publications of umbilical cord sex steroid concentrations. More robust studies have used organic solvent extraction with additional column purification techniques to eliminate interference from matrix factors and cross-reacting steroids (see Supplementary Table S1; refs. 13, 14). However, these assays are generally validated for use in adult female patients and their suitability for umbilical cord blood analysis is usually unverified. Cord estrogens concentrations were generally lower than those previously reported, with the exception of the gas chromatography tandem mass spectrometry (GC-MS/MS) studies of Hill and colleagues (12, 22) who reported concentrations of umbilical artery and vein steroids separately in a mixed group of normal (term) and complicated (preterm) pregnancies. The E2 levels described in our study are similar to those reported by Troisi and colleagues (23), who used a specific RIA using solvent extraction and column purification (Supplementary Table S1). However, our mean E1 and E3 values are about 25% to 50% of those described by Troisi and colleagues (10, 23).

This is the large first report of E4 concentrations in umbilical cord blood. Maternal plasma E4 concentrations increase steadily throughout the second and third trimesters of pregnancy. Fetal E4 concentrations at delivery are >10 times those in the maternal circulation. The biologic role and significance of E4 are unclear. Although its potency is only about 2% of that of E2, it is not bound by sex hormone-binding globulin, and hence is likely to have some significant estrogenic effects in the fetus. Previous studies of cord E4 concentrations from small selected populations described mean E4 values considerably higher (100%–200%) than those reported here. One published study using LC/MS-MS described E4 values in 12 samples that were closer to our concentrations, approximately 30 versus 20 nmol/L (33).

In summary, we report the largest, population-based study of umbilical cord estrogen concentrations using a highly specific LC/MS-MS assay and their relationship to pregnancy and perinatal factors including putative markers widely used to indicate prenatal estrogen exposure. We report that only twinning was consistently associated with lower concentrations of estradiol, the most bioactive estrogen. We also report that cord estrogens do not differ between males and females and that both gestation and labor affect cord estrogen concentrations and should be considered when interpreting these data. Our findings provide novel and definitive data for future studies evaluating the contribution of prenatal estrogen exposure to later life health.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Hickey, J.A. Keelan
Development of methodology: M. Hickey, J.A. Keelan
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Hickey, R. Hart, J.A. Keelan
Writing, review, and/or revision of the manuscript: M. Hickey, R. Hart, J.A. Keelan
Study supervision: M. Hickey, J.A. Keelan

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The Relationship between Umbilical Cord Estrogens and Perinatal Characteristics

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