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Sex differences in substrates and clearance products of cortisol and corticosterone synthesis in full-term human umbilical circulation without labor: Substrate depletion matches synthesis in males, but not females

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ABSTRACT

Background: Antenatal impacts on the hypothalamus- pituitary-adrenal axis affect health throughout later life and the impacts on developing males and females often differ. The female fetus at full-term (sampled as scheduled Caesarian section without antecedent labor) both receives more cortisol in umbilical venous blood and adds more cortisol to umbilical arterial circulation than the male. The current study was designed to expand our knowledge of sex-specific, fetal, adrenal steroid synthesis and clearance pathways.

Methods: Paired, full-term, arterial and venous umbilical cord samples were taken at the time of scheduled Caesarian delivery (N = 53, 33 male). Adrenal glucocorticoids (cortisol, corticosterone), cortisol precursor steroids (17-hydroxyprogesterone, 11-deoxycortisol), and cortisol and corticosterone metabolites (cortisone and 11-dehydrocorticosterone), as well as gonadal steroids (testosterone and androstenedione), were quantified by liquid chromatography coupled to tandem mass spectrometry.

Results: Both sexes preferentially added corticosterone. Males added more testosterone than females. The female fetus had higher umbilical cord (arterial and venous) concentrations of cortisol, as well as higher total steroid molarity summed across the six adrenal steroids, than males. Depletion of substrate pools of 17-hydroxyprogesterone, 11-deoxycortisol, and cortisone could account for only 20% of net female cortisol synthesis. In contrast, increased fetal synthesis of cortisol was balanced by equivalent molar depletion of substrate pools when the fetus was male.

Conclusions: Preferential fetal corticosterone synthesis in both sexes, and higher concentrations of cortisol in females were confirmed. Differences in adrenal steroidogenesis pathway function in full-term males and females might underlie antenatal programming of hypothalamic-pituitary-adrenal axis function in later life.

Even before birth, sex differences in fetal growth and fetal and neonatal morbidity and mortality are well known (Vatten and Skjaerven, 2004; Di Renzo et al., 2007; Engel et al., 2008). Sex-biased health challenges later in life have also been linked with antepartum maternal risk factors in pregnancy, including severe chronic asthma, pre-eclampsia, and preterm delivery (Vatten and Skjaerven, 2004; Clarke et al., 2007). In addition, there is considerable evidence that antenatal stress influences sex-specific risk for a wide range of developmental and mental health challenges throughout the lifespan (Davis and Pfaff, 2014; Sandman et al., 2013; Gluckman et al., 2008). It remains challenging, however, to identify developmental sex differences in the intra-uterine environment that might underlie the sex differences later in life (Stirrat and Reynolds, 2016).

In 2013, we published results comparing cortisol and corticosterone concentrations in umbilical arterial serum (returning from the fetus towards the placental circulation) and umbilical venous serum (inbound from the placenta towards the fetus) for a sample of 265 healthy, full-term deliveries (Wynne-Edwards et al., 2013). That analysis showed that the fetus preferentially secreted corticosterone, rather than cortisol, in response to fetal stress during vaginal delivery versus scheduled, elective Caesarian deliveries.

Those scheduled Caesarian deliveries (N = 52, 27 male) were

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assumed to be representative of the full-term, third trimester, uteroplacental environment, since neither mother nor baby had undergone the stress of antecedent labor, and regional spinal anesthetic, rather than general anesthesia, was used for all deliveries. A sex difference in the umbilical circulation was found with regards to cortisol, but not corticosterone (Giesbrecht et al., 2016). Term pregnancies with a female fetus had higher cortisol in both venous and arterial serum than pregnancies with a male fetus. Both sexes added cortisol to umbilical circulation but there was no evidence that the proportional increase in cortisol across the fetus was different for the male and female fetus. Thus, the full-term female fetus was exposed to higher overall circulating cortisol concentrations than the male, suggesting that placental influences on umbilical cord cortisol concentrations differed by sex (Clifton, 2010).

The current study had three objectives: First, to confirm the previously described sex difference in cortisol (Giesbrecht et al., 2016), in an independent replicate sample of scheduled, elective, Caesarian births without antecedent labor. Second, to confirm preferential corticosterone synthesis by the fetus. Third, to broaden the number of adrenal and gonadal steroid biosynthesis steroids quantified to include substrates, and clearance products. Specifically, steroids quantified were two substrates for cortisol,17-hydroxyprogesterone and 11-deoxycortisol, two immediate breakdown products of cortisol and corticosterone, cortisone and 11-dehydrocorticosterone, plus testosterone and its immediate precursor, androstenedione. This array was chosen to place the male-female difference in cortisol pool size and net cortisol addition into the context of its broader adrenal steroidogenesis pathway.

1. Material and methods

In all respects, sample collection and handling methods constituted an experimental replication of an earlier cohort (Wynne-Edwards et al., 2013). Additions to that method were the inclusion of multiple births and the inclusion of preterm births. A change in mass spectrometry steroid quantitation method is addressed in Section 1.7. Finally, the previous cohort had only cortisol and corticosterone quantified whereas the current steroid analysis method was expanded to include an additional four adrenal and two gonadal steroid hormones. Steroids quantified are highlighted in Fig. 1, in the context of their steroidogenic pathways, with the enzymes responsible for each conversion. In addition to cortisol and corticosterone, two cortisol precursor steroids were quantified. 17-hydroxyprogesterone is a direct precursor of cortisol, through 21-hydroxylase conversion to 11-deoxycortisol, followed by mitochondrial 11ß-hydroxylase conversion to cortisol. Progesterone saturated the mass spectra and could not be quantified as the internal standard peak could not be diluted and retain precision. Deoxycorticosterone and aldosterone methods were not available. Initial clearance products, which are the result of 11ß-hydroxysteroid dehydrogenase (11ß-HSD) activity, yield cortisone and 11-dehydrocorticosterone. 11-dehydrocorticosterone is also the substrate for aldosterone synthesis. Testosterone and its immediate precursor, androstenedione, were also quantified.

1.1. Ethics statement

This research was granted ethics approval by the University of Calgary Conjoint Health Research Ethics Board (CHREB) as Project E22197R2. Written consent, on a form approved by the CHREB, was obtained, witnessed, and a copy returned to the pregnant subject at the time of admission to the hospital. Consent addressed access to maternal labor and delivery chart records, and approval for umbilical cord blood sampling. No maternal blood was requested. No other access to the infant or maternal medical record was sought. Intra-partum chart data was coded by authors KL and HEE, the Obstetrician-Gynecologists who collected the samples. Hormone quantitation was blind to all chart data but samples were coded to pair arterial and venous umbilical cord samples within subjects.

1.2. Subject pool

Between March 2014 and the end of January 2015, written consent was obtained from 328 pregnant subjects at the time of admission to labor and delivery at the Rockyview General Hospital or the Foothills Medical Centre in Calgary, AB. No attempt was made to assess ethnicity or socioeconomic status. Five exclusions were applied. One mother was under active steroid treatment for Addison's disease, one infant had severe fetal acidosis, and three births had known collection errors. For the purposes of this analysis, all pre-term births (< 37 weeks), and all term births with antecedent labor were excluded. This filter yielded 53 births (33 male). One set of full-term female twins at 37 weeks was treated as two independent subjects because they had separate placentae and membranes (dichorionic and diamnionic). The delivery record provided sex of the baby, birth weight, gestational age, maternal risk factors, and infant Apgar score 5 min after delivery as a measure of fetal condition at delivery.

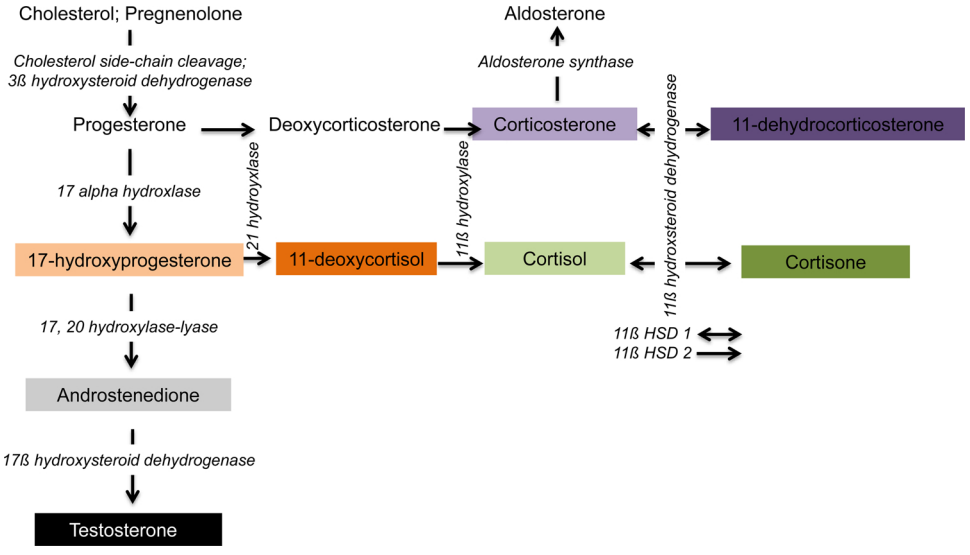

Fig. 1. Schematic representation of steroidogenic relationships between quantified steroids, showing the associated enzymes in italics. The pathway shown depicts the steroids involved in cortisol and corticosterone synthesis and clearance, plus the sex steroid pathway to testosterone. All highlighted steroids were quantified. Many steroidogenic enzymes are bidirectional in activity, most notably 11ß hydroxysteroid dehydrogenase (11ß-HSD) for which 11ß-HSD2 is unidirectional towards cortisone and 11-dehydrocorticosterone whereas 11ß-HSD1 is bidirectional, with both dehydrogenase and reductase activity.

1.3. Sample collection

There was no interference with normal post-partum protocol that routinely samples umbilical arterial and venous blood for pH and acid-base status. After that sampling, but prior to delivery of the placenta, one non-heparinized, 'red top', vial of umbilical venous and another of umbilical arterial whole blood were collected by needle aspiration (16 gauge). As per the initial study, 4.2% (or N = 2) of arterial samples obtained by needle aspiration were expected to be contaminated with venous blood, based on a decrease, rather than increase, between arterial [A] and venous [V] cortisol concentration (Wynne-Edwards et al., 2013). In the current study, the sampling error is likely to be even lower. Looking only at males, all [A-V] changes for testosterone were positive, and the sole cortisol [A-V] that was slightly negative (-0.24 nmol/l) was in a subject with a strong testosterone increase (3.38 nmol/l). Samples were immediately refrigerated and allowed to stand for at least one hour before being centrifuged (4000 xG for 5 min). Serum was separated and stored at -20 °C until delivery to the research laboratory.

1.4. Sample preparation

Cortisol, cortisone, corticosterone, 11-deoxycortisol, 11-dehydrocorticosterone, testosterone, 17-hydroxyprogesterone, and androstenedione calibrators were purchased from Steraloids Inc (Newport, RI). Deuterium labeled internal standards (Cortisol-d4, corticosterone-d8, testosterone-d2, and 17α-hydroxyprogesterone-d8) were obtained from CDN Isotopes Inc (Pointe-Claire, Quebec, Canada). HPLC grade methanol, Optima grade acetonitrile (ACN), and water were purchased from Fisher Scientific (Edmonton, AB, Canada). Protein precipitation solution consisted of $ZnSO_4$-$7H_2O$ solution (9 mg/mL) spiked with the mixture of deuterated internal standards. Pipetting was performed by a Hamilton Microstar liquid handling robot (Hamilton Company, Reno NV). Equal volumes of serum and protein precipitation solution (60 µL) were combined, vortexed for 30 s, then incubated at 4 °C for 20 min. Samples were centrifuged at 4000 x *g* for 15 min and 90 µl of supernatant was submitted to analysis.

1.5. LC-ESI+/MRM acquisition method

Quantitation used an Agilent 1200 binary liquid chromatography (LC) system coupled to an AB SCIEX QTRAP® 5500 tandem mass spectrometer equipped with an electrospray (ESI) ionization source in positive mode. LC separation was performed on an Agilent ZORBAX Eclipse plus C18 column (100 x 2.1 mm, 1.8 µm particle size) at 40 °C. Mobile phase A was ACN/H_2O (5/95, v/v, 2 mM NaF) and the mobile phase B was 100% ACN (2 mM NaF). The 12 min gradient was 15–70% B (0–6 min), 70–100% B (6–7 min), 100% B (7–8.5 min), 100-15% B (8.5–9 min), held at 15% B for 3 min. Flow rate was 0.6 ml/min and injection volume was 15 µL. Acquisition parameters were: N2 curtain gas 45 psi, temperature 750 °C, ion source gas 1 18 psi, ion source gas 2 30 psi, collision gas medium, ionspray voltage 5000. Mass resolutions in Q1 and Q3 were set to unit resolution. Each analyte was monitored by two transitions, a quantifier and a qualifier (cortisol 363/121 363/327, cortisol-d4 367/121 367/331, corticosterone 347/329 347/121, corticosterone-d8 355/337 355/125, cortisone 361/163 361/121, 11-DHC 345/121 345/301, testosterone 289/91 289/109, testosterone-d2 291/99 291/111, 17-hydroxyprogesterone 331/97, 331/109, 17-hydroxyprogesterone-d8 339/100 315/109, 11-deoxycortisol 347/97 347/109, androstenedione 287/97 287/109). MRM conditions were: declustering potential = 100 V, entrance potential 3 V, collision energy 24–31 eV, and collision cell exit potential = 12.

1.6. Steroid quantitation

Calibration curves (r2 > 0.0995) used analyte concentration (x axis) vs analyte/IS peak area ratios (y axis), and a linear fit with 1/x weighting to improve precision at lower concentrations. Calibration curves (10 point) ranged from 0.1 ng/ml to 100 ng/ml for cortisol, corticosterone, cortisone and androstenedione. Other steroid calibrators ranged from 0.05 to 50 ng/ml. The lowest concentration that gave < 20% CV was deemed the lower limit of quantification (LLOQ), which was 0.1 ng/ml for all steroids except cortisol, at 0.2 ng/ml. Four quality control (QC) pools in MeOH, matched to ultrahigh (25 or 12.5 ng/ml), high (5 or 2.5 ng/ml), mid (0.5 or 0.25 ng/ml) or low (0.1 or 0.05 ng/ml), plus a triplicate serum pool, were quantified within each of 4 runs. Accuracy across all mid, high and ultrahigh QC from each of 8 steroids was 99.96% (range 88.0%–109.3%) with an average intra-run CV of 5.72%. Low QC were more variable across 8 steroids (average CV = 23.4%) but still accurate (95.88%, range 84.8%–108.0%). Serum pools delivered an average intra-run CV of 7.8% (across 8 steroids) and inter-run CV of 17.1% (max 22.6%). Paired venous and arterial samples were always in the same run.

1.7. Validation of quantitation method change

This study used a different steroid quantitation method than Wynne-Edwards et al. (2013). Therefore, a subset of 150 samples from the current dataset (75 matched pairs) were also quantified for cortisol and corticosterone using the APCI positive acquisition method of Wynne-Edwards et al. (2013). The methods were well-correlated for both cortisol and corticosterone (cortisol r = 0.9633 with 95% non-parametric density range from 0.9306 and 0.9807, slope 0.8145, intercept 5.0975; corticosterone r = 0.9854 with 95% non-parametric density range between 0.9720 and 0.9923, slope 0.9917, intercept 0.1844). Thus, cortisol and corticosterone concentrations are comparable, although reported in different units (nmol/l rather than ng/ml).

1.8. Analytical parameters

Prior to statistical analyses, all quantitation results in ng/mL were converted to nmol/l because molar concentrations were essential to compare the molecular pools of substrate, product, and breakdown product steroids, as each steroid has a different molecular weight (i.e 1 ng contains fewer molecules as the molecular weight increases). Although the units differ, definitions of analytical parameters from Wynne-Edwards et al. (2013) did not. Net fetal steroid synthesis or clearance was defined as the concentration difference between the steroid level in the umbilical artery ([A]: circulation that has passed the fetus and is returning to the placenta) and the umbilical vein ([V]: circulation from the maternal/ placental interface towards the fetus). This is represented in the results as [A-V] in nmol/l. In addition, a parameter representing the proportional change in steroid concentration (%) across to the fetus ([A-V]/V) was calculated for which negative values represent fetal clearance of steroid, zero represents no change, and a value of 100% represents a doubling in concentration. By definition, the steroid change across the materno-placental interface [V-A] is equal in magnitude, but opposite to, fetal change [A-V]. However, interpretation of the concentration change on the materno-placental side of the umbilical circulation is challenging because neither steroid exchange with maternal circulation nor placental steroid pools were assessed (Wynne-Edwards et al., 2013; Giesbrecht et al., 2016).

1.9. Statistical analyses

To assess molecular flows from substrates through to clearance products, concentrations (nmol/l) in arterial and venous samples were treated as matched pairs and change parameters [A-V] and [A-V]/[V] were calculated from untransformed concentrations. This quantitation method is linear throughout the calibration range (neither the x nor y axes are transformed before fitting the line). Parametric approaches were used throughout analyses. Non-parametric approaches yielded the

same conclusions. A parallel results section is available in supplemental materials with non-parametric equivalent statistical findings inserted (Supplemental 1). The cohort was homogenous with respect to gestational age, and the sample size was too small to partition by other demographic or intra-partum variables. All analyses were conducted using JMP version 13.1.0 (SAS Institute, North Carolina). For two sample male-female comparisons, t-tests are reported. For arterial-venous comparisons within a sex, paired t-tests are reported. For hormone changes across the fetus, [A-V] distributions were tested against a predicted mean of zero with no true standard deviation specified, yielding a t-statistic. For correlation, associations are reported as the F statistic, degrees of freedom, r, and p values with 95% non-parametric density ranges, as well as the equation for the line-fit, showing intercept and slope. Exact P values are reported throughout the results. Degrees of freedom for t-statistics were corrected for unequal variances. A critical alpha threshold of 0.005 was applied to avoid type II errors due to multiple comparisons within each steroid (4 parameters, two sexes). One statistical replication of a sex effect on cortisol applied an alpha threshold of 0.05, to match the previous statistical approach and is noted in the results.

2. Results

2.1. Demographics

Within this full term (≥ 37 weeks) cohort of 53 births (Table 1), there were 13 births (24.5%) with antepartum risk factors: three IUGR (intrauterine growth restriction at 10th percentile), seven LGA (large for gestational age at 90th percentile), two diet-controlled gestational diabetes (+/- polyhydramnios), and one essential hypertension. Antepartum risk factors were summed to yield a risk profile score and did not differ by sex. All infants were healthy, with 51/53 babies having an Apgar score of 9/10 at five minutes after birth and the remaining two at Apgar 8. None of the infants were admitted to the Neonatal Intensive Care Unit. Male birthweight was significantly higher than female birthweight (Table 1).

2.2. Gonadal steroids: androstenedione and testosterone

The two gonadal steroids, testosterone and androstenedione, accounted for less than 2% of the total molarity across all eight steroids quantified. As expected, the male fetus had higher concentrations of testosterone than females in both the venous (0.72 ± 0.07 nmol/l vs 0.14 ± 0.03, $p < 0.0001$) and arterial umbilical cord samples (2.72 ± 0.23 nmol/l vs 0.23 ± 0.03, $p < 0.0001$). The male fetus also added more testosterone ([A-V]: 2.00 ± 0.21 nmol/l) than the female did ([A-V]: 0.09 ± 0.03), yielding a significant sex difference in [A-V] ($p < 0.0001$). There was also a larger proportional testosterone increase when the fetus was male ([A-V]/[V]: 346 ± 51 vs 55 ± 15, $p < 0.0001$). In other words, females added about 50% more net testosterone than they received in venous circulation, whereas male arterial circulation contained testosterone at a concentration 350% higher than venous blood.

Table 1
Description of the full-term (≥ 37.0 weeks), scheduled Caesarian section, study cohort.

	Male (N = 33)	Female (N = 20)	P
Gestational Age (wks)	38.50 ± 0.12	38.50 ± 0.21	0.890
Maternal Age (yr)	33.60 ± 3.80	31.40 ± 5.00	0.090
Birthweight (kg)	3.70 ± 0.11	3.25 ± 0.11	**0.0005**
Apgar (5 min)	8.90 ± 0.04	9.00 ± 0.00	0.520
Risk profile score[†]	0.42 ± 0.12	0.37 ± 0.16	0.770

[†] One point assigned for each antenatal maternal morbidity or fetal risk factor (Section 2.1).

In contrast, the immediate precursor of testosterone, androstenedione, did not differ by sex in venous (1.36 ± 0.08 vs 1.49 ± 0.23, $p = 0.294$) or arterial (2.00 ± 0.13 vs 2.64 ± 0.41, $p = 0.143$) umbilical cord blood. Both sexes added similar absolute amounts of androstenedione ([A-V]: 0.63 ± 0.12, females 1.15 ± 0.61, $p < 0.08$). Similarly, the proportional androstenedione changes ([A-V]/[V]) were not different between the sexes (0.51 ± 0.09, 0.95 ± 0.20, $p = 0.026$).

Finally, the two androgens, androstenedione and testosterone, linked together in the testosterone synthesis pathway, were similarly positively associated in the smaller cohort of 20 females (venous $F(1,19) = 14.869$, $r = 0.6726$ with 95% non-parametric density boundaries at 0.3276 and 0.8593, $p = 0.0012$; arterial $F(1,19) = 19.5943$, $r = 0.7220$ with 95% non-parametric density boundaries at 0.4106 and 0.8825, $p = 0.0003$). For females, both intercepts were close to zero (venous 0.685 ± 0.2724, $p = 0.0216$; arterial 0.6127 ± 0.5406 $p = 0.2719$) and slopes were strongly positive ($y = mx+b$; venous androstenedione = 0.658 + 5.932 venous testosterone; arterial androstenedione = 0.613 + 8.987 arterial testosterone). In contrast, the larger cohort of 33 males, with higher testosterone concentrations than the females, was not significantly correlated (venous $F(1,32) = 0.0211$, $r = -0.0261$ with 95% non-parametric density boundaries at -0.3661 and 0.3201, $p = 0.886$; arterial $F(1,32) = 3.752$, $r = 0.3286$ with 95% non-parametric density boundaries at -0.0166 and 0.6038, $p = 0.062$) and had positive intercepts (both $p < 0.0001$) and near-zero slopes ($y = mx+b$; venous androstenedione = 1.38 - 0.0306 venous testosterone; arterial androstenedione = 1.47 + 0.1929 arterial). Thus, androstenedione concentrations were similar in males and females, and positively associated with testosterone in females, whereas androstenedione and testosterone concentrations were independent in males.

2.3. Male and female glucocorticoid pathway steroid concentrations

In order of abundance in the venous circulation approaching the fetus, cortisone was at the highest concentration. Cortisol was the next most abundant steroid. In all cases, 17-hydroxyprogesterone was third in abundance, followed by the corticosterone analogue of cortisone, 11-dehydrocorticosterone, and then the precursor of cortisol, 11-deoxycortisol, and lastly, corticosterone.

Replicating the findings in Giesbrecht et al. (2016), where a critical alpha of 0.05 was applied, cortisol concentrations in venous umbilical cord were higher with a full-term female fetus ([V] = 100.3 ± 14.0) than with a full-term male fetus ([V] = 67.2 ± 5.75, $t = 2.18$, $p = 0.039$). The same pattern was observed with higher cortisol concentrations from female arterial samples ([A] = 172.6 ± 22.7) than from males ([A] = 106.2 ± 9.13, $t = 2.71$, $p = 0.012$). As seen in the previous study, corticosterone concentrations did not differ by sex (all $p > 0.11$).

The cumulative steroid complement for male and female, in venous and arterial serum, is shown in Fig. 2. Total steroid molarity, summed across the six steroids, was not different for males and females in the arriving, venous, circulation ($t(36.7) = 0.77$, $p = 0.447$). However, there were sex differences in total molarity after the fetus, in arterial circulation ($t(26.6) = 2.72$, $p = 0.011$). The total nmol/l for venous serum arriving at the male fetus was not different from the paired arterial sample (-2.0 ± 13.0 nmol/l, paired $t(32) = 0.15$, $p = 0.8785$). In contrast, +65.2 ± 19.1 nmol/l were added by the female fetus (paired $t(19) = 3.42$, $p = 0.0029$). Thus, male total steroid molarity was stable whereas female total molarity increased.

2.4. Fetal steroid synthesis and clearance [A-V]

Widespread changes in the molarity of individual steroids were noted (Fig. 3). Within each sex, steroid molarity changed from venous to arterial samples as the circulation crossed the fetus. For males, net corticosterone and net cortisol synthesis was seen, while the substrates,

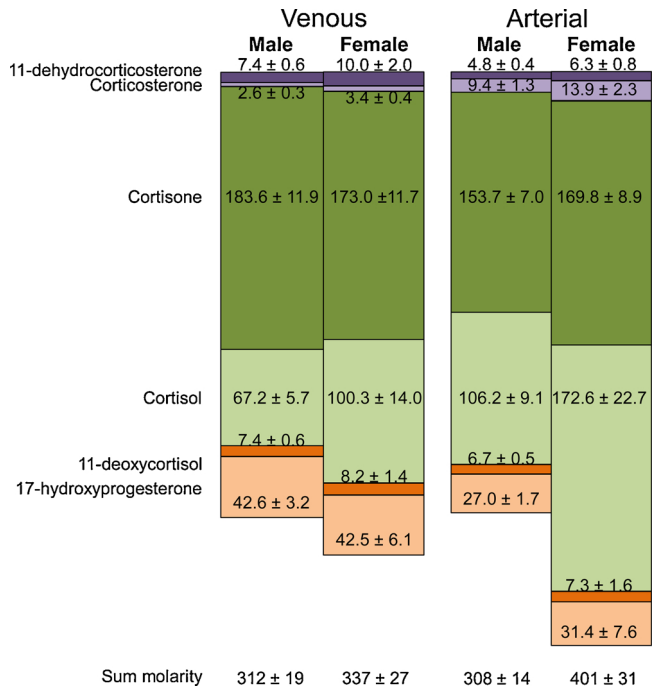

Fig. 2. Stacked concentrations for the 6 steroids quantified in male and female venous and arterial serum samples. Cross-sectional population concentrations are shown in nmol/l ± SE within each section of histogram. Venous umbilical cord steroid concentrations did not differ between males and females, although cortisol was higher in females at p = 0.04, but not the critical threshold of p < 0.0005.

11-dehydrocorticosterone, cortisone, 11-deoxycortisol and 17-hydroxyprogesterone, were depleted/cleared. When the fetus was female, corticosterone and cortisol were also actively added by the female fetus. However, decreases in cortisone, 11-dehydrocorticosterone and 17-hydroxyprogesterone were not significantly different from zero.

Replicating the previous finding (Giesbrecht et al., 2016), between-sex differences were seen for cortisol (male 39.0 ± 6.1, female 72.4 ± 13.1; t = 2.31, p = 0.029), but not corticosterone (male 6.78 ± 1.1, female 10.46 ± 6.78; t = 1.61, p = 0.12). Depletion of pools of the immediate precursor of cortisol, 11-deoxycortisol, were not responsible for this between-sex difference ([A-V] males (-2.30 ± 0.38, females -0.90 ± 0.53; t = 2.14, p = 0.039). At the critical alpha of p < 0.005, cortisone depletion also did not differ between the sexes (males -29.9 ± 8.1; females 3.16 ± 10.0; t = 2.07, p = 0.045).

Table 2
Proportional steroid concentration changes ([A-V]/[V] (nmol/l ± SE; 100 = doubling; -50 = 50% decrease).

	Male (N = 33)	Female (N = 20)	P
Corticosterone	277.64 ± 28.19	310.11 ± 46.12	0.552
11-dehydrocorticosterone	−29.23 ± 4.15	−16.98 ± 9.75	0.258
Cortisol	64.95 ± 12.34	79.58 ± 13.36	0.425
Cortisone	−11.69 ± 3.42	−4.39 ± 7.03	0.049
17-hydroxyprogesterone	−31.82 ± 3.74	−28.28 ± 5.43	0.594
11-deoxycortisol	−22.32 ± 3.14	−8.98 ± 7.49	0.113

2.5. Preferential fetal corticosterone synthesis [A-V]/[V]

As found for the Giesbrecht et al. (2016) cohort, there was no difference in proportional increase in cortisol between the sexes (80 ± 13, 65 ± 12, p = 0.425). Thus, cortisol steroidogenesis was proportionately similar for female and male, but female umbilical circulation consisted of higher arriving, and even higher departing, cortisol concentrations than males. Proportional steroid concentration changes for the other steroids also did not differ by sex (Table 2). As expected, the proportional change in corticosterone with the sexes pooled (290 ± 25) was significantly larger than the proportional change in cortisol (71 ± 9; Paired t(52) = 11.05, p < 0.0001). Fetal adrenal glucocorticoid synthesis at full-term was biased towards corticosterone.

2.6. Steroid correlations within individuals

As the synthesis (11ß-hydroxylase) and back-synthesis (11ß-HSD1) enzymes for cortisol and corticosterone are shared, there was a positive association between the two glucocorticoid concentrations for each sex in venous samples (male F (1,32) = 14.961, r = 0.5705 with 95% non-parametric density boundaries at 0.2826 and 0.7642, p = 0.0005; female F (1,19) = 24.502, r = 0.7593 with 95% non-parametric density boundaries at 0.4770 and 0.8996, p = 0.0001) and in arterial samples (male F (1,32) = 33.512, r = 0.7208 with 95% non-parametric density boundaries at 0.5015 and 0.8530, p < 0.0001; female F (1,19) = 50.378, r = 0.8584 with 95% non-parametric density

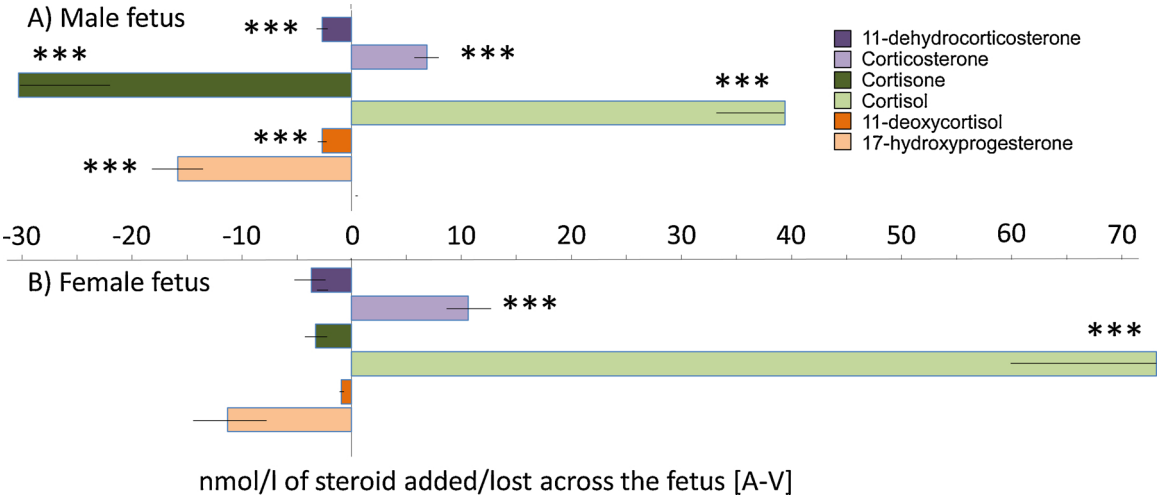

Fig. 3. Mean ± SE within-individual change in the concentration of each steroid hormone [A-V] (nmol/l) between venous and arterial samples for males (A) and females (B). Significant differences from zero are shown within each sex (*** p < 0.0001, *t*-test against a mean of zero). In males, the total substrate pool depletion for cortisol synthesis (sum of 17-hydroxyprogesterone + 11-deoxycortisol + cortisone) is an approximate molar equivalent to net cortisol synthesis, whereas depletions of the available female substrate pool would only provide about 20% of the cortisol synthesis across the female fetus.

boundaries at 0.6705 and 0.9428, $p < 0.0001$). Intercepts were not significant for either sex (all four $p \geq 0.1127$). The slope of the venous fitted line was similar in each sex (males 0.0297, females 0.0243) and less steep than the similar slopes in arterial samples (males 0.1026, females 0.0887). Thus, in spite of the differences in concentrations of cortisol and corticosterone between males and females, the relationship between cortisol and corticosterone was similar in males and females.

Sequential precursor steroids for cortisol synthesis, 17-hydroxyprogesterone and 11-deoxycortisol were positively associated. In venous serum approaching the fetus 17-hydroxyprogesterone and 11-deoxycortisol correlations were 0.94 in the smaller female cohort and 0.65 in the larger male cohort (male $F(1,32) = 23.190$, $r = 0.6541$ with 95% non-parametric density boundaries at 0.4009 and 0.8146, $p < 0.0001$; female $F(1,19) = 146.451$, $r = 0.9437$ with 95% non-parametric density boundaries at 0.8605 and 0.9779, $p < 0.0001$). The negative female intercept did not differ from zero ($p = 0.2721$), although the positive male intercept did ($p = 0.0479$). Slopes were positive (male 0.1446 ± 0.0300; female 0.2174 ± 0.0180). In arterial serum leaving the fetus, 17-hydroxyprogesterone and 11-deoxycortisol correlations were 0.98 in the smaller female cohort and 0.58 in the larger male cohort (male $F(1,32) = 15.676$, $r = 0.5795$ with 95% non-parametric density boundaries at 0.2949 and 0.7697, $p < 0.0004$; female $F(1,19) = 399.190$, $r = 0.9782$ with 95% non-parametric density boundaries at 0.9445 and 0.9915, $p < 0.0001$). The female intercept did not differ from zero ($p = 0.1647$), and neither did the male ($p = 0.0648$). Slopes were positive (male 0.1642 ± 0.0415; female 0.2110 ± 0.0106). Thus, the two sequential precursor steroids for cortisol synthesis were highly correlated (r˜ 0.95) in females that did not deplete these substrates and also well correlated (r ˜ 0.60) in males that did show net substrate depletion between venous and arterial samples.

For the 11ß-HSD breakdown products of cortisol and corticosterone, namely cortisone (abundant in umbilical circulation) and 11-dehydrocorticosterone (lower concentrations, like corticosterone), associations were also present. In venous serum approaching the fetus cortisone and 11-dehydrocorticosterone correlations were 0.67 in the smaller female cohort and 0.55 in the larger male cohort (male $F(1,32) = 13.406$, $r = 0.5496$ with 95% non-parametric density boundaries at 0.2541 and 0.7511, $p = 0.0009$; female $F(1,19) = 14.564$, $r = 0.6688$ with 95% non-parametric density boundaries at 0.3213 and 0.8575, $p = 0.0013$). The female intercept ($p = 0.0856$) and male intercept ($p = 0.1692$) did not differ from zero. Slopes were positive though steeper in the female venous serum (male 0.0288 ± 0.0079; female 0.1144 ± 0.0300). In arterial serum leaving the fetus, cortisone and 11-dehydrocorticosterone correlations were 0.49 in the smaller female cohort and 0.45 in the larger male cohort (male $F(1,32) = 7.731$, $r = 0.4468$ with 95% non-parametric density boundaries at 0.1222 and 0.6850, $p < 0.0091$; female $F(1,19) = 5.624$, $r = 0.4879$ with 95% non-parametric density boundaries at 0.0579 and 0.7652, $p = 0.0291$). The female intercept did not differ from zero ($p = 0.7147$), and neither did the male ($p = 0.3327$). Slopes were positive and more similar (male 0.0229 ± 0.0082; female 0.0442 ± 0.0187).

3. Discussion

3.1. Independent confirmation of previous findings

Recently, we described full-term sex differences in umbilical cord glucocorticoid concentrations (immediately after scheduled Caesarean delivery) in the human fetus (Giesbrecht et al., 2016). In that study, females had higher concentrations of cortisol than males in both venous and arterial umbilical cord samples and added more cortisol than corticosterone. However, the proportionate increase ([A-V]/[V]) was similar. Without knowing whether substrates were at similar concentrations, that paper was unable to determine whether the combination of synthesis and clearance within the fetus was proportionate to the concentration of the substrate arriving in venous circulation (Giesbrecht et al., 2016). The current study replicated that study, collecting an independent set of matched arterial and venous umbilical cord samples at full-term (38.5 wks). Again, females had higher concentrations of cortisol than males in both venous and arterial cord samples, and a larger absolute increase across the fetus, but a similar proportionate increase. Thus, higher concentrations of cortisol in umbilical circulation when the fetus is female is confirmed.

Explicit paired comparison of venous and arterial samples was important to detecting this sex difference. Although Stark et al. (2009a) reported increased cortisol concentrations in neonatal female cord blood and urine at 24 h, relative to males, Clifton et al. (2007) found no sex difference in umbilical cord steroid profiles, using high-pressure liquid chromatography techniques on mixed cord blood from neonates at the time of elective Caesarean section. In human umbilical cord, venous circulation towards the fetus travels within a large, thin-walled vessel, whereas human umbilical arteries are smaller in diameter, and more thick-walled. Thus, arterial blood is a minor contributor to mixed cord blood sampling. A wide range of cortisol concentrations is typical of umbilical cord serum (Wynne-Edwards et al., 2013). When stress of delivery is added to the delivery experience (spontaneous vaginal deliveries and FTP 2 emergency Caesarian sections as compared to these elective Caesarian sections) fetal sex differences in cortisol were not detectable (Wynne-Edwards et al., 2013).

Wynne-Edwards et al. (2013) demonstrated that any fetus that had gone through stage 2 of labor (all vaginal deliveries plus emergency Caesarian sections for failure to progress in the second stage of labor (FTP2)) increased the preferential secretion of corticosterone, rather than cortisol. To aid in the interpretation of this finding, the current data set was specifically designed to yield a 'substrate to product' steroid snapshot of full-term umbilical circulation under the least-stressed fetal state possible. To achieve this, labor was excluded. During labor, maternal cortisol diffusing across the placental interface increases to the point of saturating the placental activity of 11ß-HSD2, allowing the excess cortisol to reach the fetus (Chapman et al., 2013). These elective scheduled Caesarian sections, in contrast, were conducted under regional spinal anesthetic, avoiding widespread physiological responses. Normal maternal anticipatory anxiety and excitement were expected to increase cortisol, but to be converted to cortisone by abundant placental 11ß-HSD2 before reaching umbilical venous circulation (Chapman et al., 2013; Stirrat et al., 2018), and were not expected to influence the umbilical steroid environment. Thus, the steroid concentrations and changes reported here were assumed to be representative of a 'baseline' full-term state, as opposed to the more stress-filled situation, for mother and baby, in vaginal deliveries with head compressions during stage 2 of labor.

3.2. Preferential corticosterone synthesis in both sexes

Nevertheless, despite the relative lack of stress in these elective Caesarian deliveries, preferential corticosterone secretion was clear, and confirms the finding in Giesbrecht et al. (2016). Sex differences in steroid pools and biosynthetic activity are known. For example, within the current data, we would expect male fetal 17ß-hydroxysteroid dehydrogenase enzyme activity in the testes to add testosterone, as seen. We would also expect female testosterone synthesis to be lower because females have no testes.

In contrast to testosterone, none of the quantified steroids in the adrenal steroid pathway showed sex differences in proportionate change, whether it was a depletion or an addition. This supports the null hypothesis that neither adrenal steroidogenic pathway enzymes, nor tissue distributions of those enzymes, differ between the sexes. It also suggests that the corticosteroid binding capacity of umbilical serum is not different between males and females. Corticosteroid binding was expected (Jeffray et al., 1999; Edwards and Boonstra,

2017), but not assessed.

Failure to reject that null hypothesis suggests that preferential corticosterone synthesis is achieved through competitive, differential, enzyme-substrate affinity. Alterations in the action of 17-alpha hydroxylase, which converts progesterone to 17-hydroxyprogesterone, largely determine whether the adrenal synthesis pathway leads to corticosterone or cortisol. If substrate pool size was responsible for the observed, higher, concentration of cortisol (in both sexes) than corticosterone, then we would also expect the proportionate difference to be shared rather than preferentially biased towards the lower concentration glucocorticoid.

Therefore, we hypothesize that preferential corticosterone synthesis, seen in both sexes, will involve higher affinity of at least one shared steroidogenic enzyme for the corticosterone rather than cortisol substrate. In other words, preferential corticosterone synthesis would arise if a) the human fetal 11ß-hydroxylase enzyme affinity for 11-deoxycorticosterone is higher than its affinity for 11-deoxycortisol; b) the 11ß-HSD1 and 2 enzymes have higher affinity for dehydrogenation of cortisol than corticosterone; or c) the reductase role of the 11ß-HSD1 enzyme has preferentially higher affinity for cortisone than 11-dehydrocorticosterone, One or more of these affinities would result in proportionally higher (i.e. preferential) corticosterone synthesis.

If the enzyme-substrate affinities do not differ, then an alternate mechanism to generate the observed preferential synthesis of corticosterone would be lower binding affinity, or residence time, of corticosterone on corticosteroid binding globulin (CBG), or albumin, under competitive conditions where higher concentrations of cortisol are competing with the rarer corticosterone molecules for binding sites. Evidence that corticosteroid binding is dynamically regulated during pregnancy is widespread (Edwards and Boonstra, 2017), and consistent with *in vitro* placental perfusion (Jeffray et al., 1999), and findings in pregnant women with genetic CBG deficiencies (Lei et al., 2015).

The placenta expresses high levels of 11ß-HSD2 throughout gestation on the fetal side, and 11ß-HSD1 on the maternal side (Stirrat et al., 2018). The fetus also has developmental changes at full-term in tissue 11ß-HSD1 reductase expression (Chapman et al., 2013; Hirasawa et al., 1999; Salvante et al., 2017), but sex differences have not been explored. Correlations were significant for cortisol and corticosterone as subject to the same synthesis and degradation enzymes, 11-hydroxyprogesterone and 11-deoxycortisol as sequential substrates on the pathway to cortisol, plus cortisone and 11-dehydrocorticosterone as the shared enzyme/ initial breakdown products of cortisol and corticosterone. However, although the female sample size was considerably smaller than the male sample size (20 vs 33), reducing statistical power, female associations between steroids were typically stronger, including correlations over 0.95 than male associations. This sex difference might reflect a developmental asynchrony between males and females at full term, particularly in light of the existing literature suggesting that placental steroidogenesis differs when the fetus is female (Clifton, 2010).

Full-term maturation of fetal lungs and gross motor function also requires sufficient amniotic fluid (oligohydramnios is a recognized, serious, antenatal risk factor), and developmentally-appropriate kidney function. Thus, an alternative pathway to explain preferential corticosterone synthesis in the fetus is that an interaction with aldosterone synthesis and/or clearance is driving the preferential increase in arterial corticosterone pool size. Future research will be needed to identify the basis for preferential corticosterone secretion.

3.3. Different adrenal steroidogenic patterns in males and females

None of the hypotheses about mechanisms underlying preferential corticosterone synthesis are able to explain the observed sex differences in the function of the adrenal steroid pathway. Simultaneous quantitation of 8 steroids, six of which were in the adrenal pathway, combined with paired arterial and venous samples, revealed an unanticipated sex difference. Within males, each steroid changed significantly. Molarity for both products (cortisol and corticosterone) increased and all three substrates for cortisol synthesis (17-hydroxyprogesterone, 11-deoxycortisol and cortisone) decreased. With regards to corticosterone synthesis, 11-dehydrocorticosterone also decreased, but the two glucocorticoid pathways could not be directly compared because deoxycorticosterone and progesterone substrates were not quantified. When the fetus was male, the total molarity of steroid was so stable that the sum of individual concentration changes was not different from zero. Increases in cortisol were matched by equivalent molar depletions in cortisol substrate pools.

The situation differed considerably when the fetus was female. As noted above, increases in cortisol were even larger than those in males, with corticosterone also increasing. However, unlike the situation in males, there was little evidence of substrate depletion and no evidence of molar equivalency. Decreases in cortisone and 11-deoxycortisol and 17-hydroxyprogesterone and 11-dehydrocorticosterone substrates were not significantly different from zero. Thus, unlike the situation in the male, molar conversions do not explain the steroid pathway through which the female fetus adds an average of 65 nmol of new steroid to arterial umbilical cord circulation. Adrenal steroidogenic function differs depending on fetal sex.

The synthesis pathway from progesterone/ 17-hydroxyprogesterone to deoxycorticosterone/11-dexoycortisol, through the 21-hydroxylase enzyme, functioned similarly in each sex. Specifically, correlation between 17-hydroxyprogesterone and 11-deoxycortisol in arterial samples was notably high in males and in females, suggesting similar linkage activity of the enzyme. Therefore, the most likely possibility is that the sexes differ in flux (rate of steroid conversion). A higher flux of steroid substrates from cholesterol all the way through to cortisol and corticosterone, would sustain net cortisol synthesis in the female without depleting substrate pools. The human fetus is more active in *de novo* conversion of acetate into cholesterol, and in the synthesis of 21-carbon progestagens and 19-carbon androgens from cholesterol, than the placenta (Pasqualini and Chetrite, 2016). In other words, if female fetal steroidogenic enzymes are rapidly converting substrate to product, and that product is being rapidly converted to another product, static measures of substrate pool size (concentration) will not reveal that difference. Nevertheless, it does represent a developmental sex difference in adrenal steroidogenesis. Pregnancies with a male fetus are not the same as pregnancies with a female fetus (Clifton, 2010).

Without data earlier in the pregnancy than full-term, and in the immediate postnatal window, the persistence of this sex difference is unknown. It is possible that this sex difference simply represents a developmental asynchrony in hypothalamic-pituitary-adrenal axis function between male and female. Further research will be necessary to clarify the temporal persistence of this sex difference during development.

Nevertheless, this clear sex difference in HPA axis function has the potential to play a significant role in establishing the early-life programming caused by stressors during early life that influence later life (Lorente-Pozo et al., 2018; Clifton, 2010). Male birthweight was higher than female birthweight, and maternal salivary cortisol is linked to birthweight (Cherak et al., 2018). Male neonates also suffer more growth restriction from a repeated betamethasone treatment antenatally than females (Lorente-Pozo et al., 2018; Carson et al., 2016), are more susceptible to hypoxic brain injury than females at birth (Fellman, 2015; Lehtonen et al., 2017), and are more susceptible to intrauterine growth restriction, preterm delivery and stillbirth (Wilms et al., 2016). Each of these risks increase in prevalence when there are maternal comorbidities such as severe asthma (Murphy et al., 2003), pre-eclampsia (Stark et al., 2006, 2009b), diabetes (Engel et al., 2008), or exposure to environmental pollutants (Sakamoto et al., 2001). It is therefore critical that antenatal sex differences in adrenal steroidogenesis pathway functioning are explored further.

CRediT authorship contribution statement

Katherine E. Wynne-Edwards: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Kovid Lee:** Investigation, Validation, Data curation, Writing - review & editing. **Ruokun Zhou:** Validation, Data curation, Writing - review & editing. **Heather E. Edwards:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2019.104381.

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