Steroid Metabolome in the Umbilical Cord: Is It Necessary To Differentiate Between Arterial and Venous Blood?

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Received July 25, 2013
Accepted September 12, 2013
On-line November 1, 2013

Summary

Steroids are important markers in pregnancy. Although estimating their levels separately in umbilical arterial (UA) and venous blood (UV) enable more precise insights into the functioning fetoplacental unit compared to using mixed umbilical blood (UM), selective aspiration of UA and UV is technically more demanding than collecting UM. We measured the levels of 67 unconjugated steroids and steroid polar conjugates in UA and UV using GC-MS in 80 women giving birth within weeks 28 to 42 of gestation. The samples were sorted into three groups: women entering labor within weeks 28-32 (group A, n=19), weeks 33-37 (group B, n=19), and weeks 38-42 (group C, n=42) of gestation, respectively. The preterm labors were due to pathologies unrelated to steroid status. Most unconjugated steroids exhibited pronounced arteriovenous differences (AVD). The AVD were less distinct in more stable steroid conjugates. Most steroids positively correlate with gestational age, but unconjugated 5β-reduced pregnanes show negative correlations, as do testosterone and androstenediol, substrates for the placental synthesis of estrogens. Tight correlations between steroids in UA and UV indicate that steroid measurements in UA, UV and UM can be accurately derived from each other, which is important for the diagnostics of steroid related diseases in newborns.

Key words

Steroids • Labor • Blood • Metabolome • GC-MS

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Introduction

Steroid biosynthesis in humans shows a number of differences compared to most laboratory animals. In addition, pregnancy is distinctively specific for humans (Power and Schulkin 2006), and investigations of steroid metabolism and the effects of steroids in pregnant women and fetuses *in vivo* are difficult due to ethical considerations. Therefore, steroid metabolomics may be an effective tool in studying the physiology and pathophysiology of human pregnancy. Besides the screening and diagnostics of numerous fetal, newborn and maternal pathologies (Cavarzere *et al.* 2009, Cheon 2011, Lykkesfeldt *et al.* 1984, Miller 2012, Mindnich *et al.* 2011, Nordenstrom *et al.* 2007, Shackleton and Malunowicz 2003, Shackleton *et al.* 2004) including preterm labor (Hill *et al.* 2010b, Mazor *et al.* 1994), there is growing evidence that multicomponent analyses of steroids in umbilical cord blood at labor may be also effective for predicting endocrine diseases during the subsequent life of the newborn (Baik *et al.* 2005, Bulun *et al.* 2010, Jari *et al.* 2004, Troisi *et al.* 2008, Yang *et al.* 2007).

As opposed to blood sampling in newborns and infants with the accompanying common problems (Fig. 1 and Fig. 2), the collection of umbilical cord blood during labor is not invasive, and cord blood analyses give prompt information about the immediate endocrine status of the newborn and may serve as an efficient tool for recognizing numerous steroid endocrinopathies. Concerning the proposed role of neuroactive and neuroprotective steroid effects in the fetal brain (Pluchino

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et al. 2009, Pluchino *et al.* 2013, Yawno *et al.* 2009), there might be also correlations between fetal steroid status and development of the central nervous system (CNS), hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes in the subsequent stages of human life (Hines 2011, Padmanabhan and Veiga-Lopez 2011, Todd *et al.* 2007, Ward *et al.* 2004). Therefore, steroid metabolomics can be particularly important in the diagnosis and prediction of multifactorial disorders with a polygenic background.

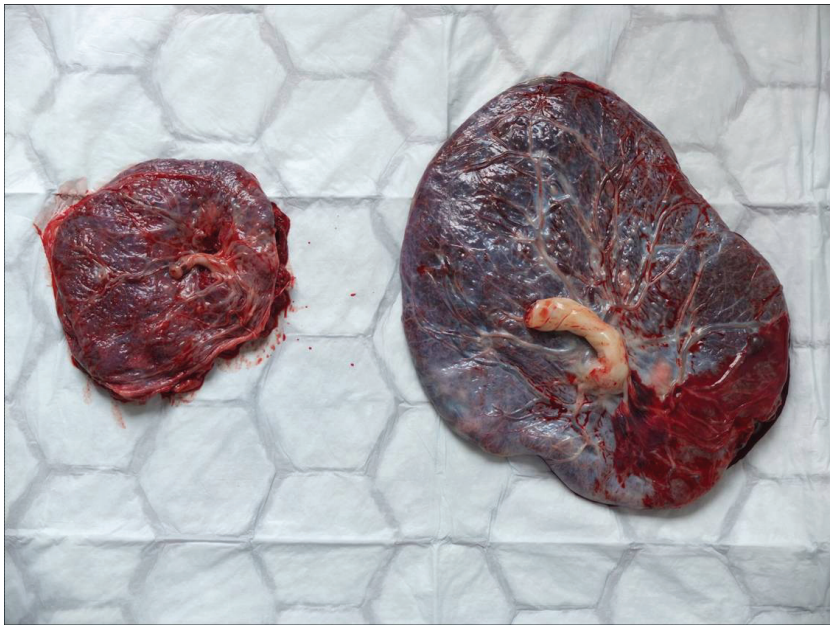

Fig. 1. Differences in placental size between a placenta at 23+5 weeks with a newborn weight of 550 g (left) and at 40+2 weeks of pregnancy with a newborn weight of 3560 g (right). This demonstrates the difficulty in separate arterial and venous sampling, especially in premature births.

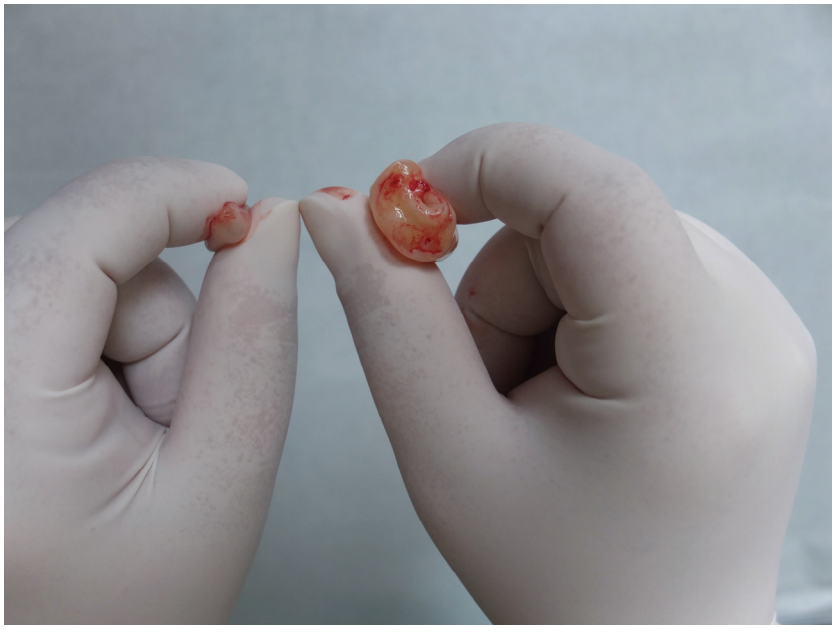

Fig. 2. Differences in the size and diameter of the umbilical cord at 23+5 weeks (left) and at 40+2 weeks of pregnancy (right), again demonstrating the difficulty of separate arterial and venous sampling in premature births.

The investigation of steroids in mixed umbilical blood (UM) involves composite information as concerns the functioning of the fetoplacental unit, but additional knowledge of steroid arteriovenous differences (AVD) in umbilical blood may bring more precise insight into the functioning of the individual compartments. However, the selective aspiration of umbilical arterial (UA) and venous (UV) blood is extremely demanding. This fact prompted us to evaluate whether individual bioactive steroids, their precursors and metabolites found in UA and UV have similar informative value. If steroid levels in UA and UV are interchangeable as regards their diagnostic use, then UM can be substituted. We addressed the following questions. 1) What are the steroid

levels in umbilical artery and umbilical vein at birth? 2) What are the AVD between umbilical arterial and umbilical venous blood? 3) How do the steroid levels and AVD depend on gestational age (GA) at labor? 4) How do steroid levels correlate between UA and UV? Although there are studies giving partial answers to some of the aforementioned questions (Hill *et al.* 2010a), studies based on complex steroid metabolomic data in human umbilical arterial and venous blood and including relationships to gestational age at labor are lacking.

Materials and Methods

Subjects

The study group consisted of 80 women (21-41 years of age) who entered labor from weeks 28 to 41 of gestation. The women were sorted into three categories according to GA at the onset of labor: within the period from weeks 28-32 (group A, n=19), weeks 33-37 (group B, n=19), and weeks 38-42 (group C, n=42).

The women giving birth after week 38 of gestation were without perinatological complications. Of the 38 women entering labor between weeks 28 and 37 of gestation, 29 pregnancies were terminated by caesarean section due to health risks to the mother or fetus, and 9 were vaginal deliveries with spontaneous uterine activity. In these women the reason for preterm uterine activity was infection in the mother as documented by high CRP levels, leukocytosis, and fever. In contrast to the group of healthy women entering labor after week 38 of gestation, all preterm births with spontaneous uterine activity were induced by sudden unexpected complications. Thus, gradual changes to the steroid metabolome were unlikely. Any dependence of preterm labors conducted by caesarean section on the steroid status was even less likely. Although we unable to verify our assumption that the reasons for preterm labors with spontaneous uterine activity were independent of steroid status, we strived to select women with the aim of providing maximum conformity of the steroid metabolome with the actual GA in both preterm and term labors.

The local Ethical Committee approved the study, and samples were taken only after signing written informed consent.

Sample collection

Samples of UA and UV were withdrawn immediately after the separation of the newborn from the umbilical cord. Each sample was collected into a cooled plastic tube containing 100 µl of 5 % EDTA. The plasma was obtained after centrifugation for 5 min at 2000g at 4 °C. The samples were stored at −20 °C until analysis.

Chemicals and reagents

Steroids were purchased from Steraloids (Newport, RI, USA), Sylon B from Supelco (Bellefonte, PA, USA), methoxylamine hydrochloride from Sigma (St. Louis, MO, USA) and solvents from Merck (Darmstadt, Germany).

Instruments

Measurements of steroid levels were done on a GCMS-QP2010 Plus system by Shimadzu (Kyoto, Japan) consisting of a gas chromatograph equipped with automatic flow control, AOC-20s autosampler and a single quadrupole detector with an adjustable electron voltage of 10-195 V. A capillary column with a medium polarity RESTEK Rxi (diameter 0.25 mm, length 15 m, film thickness 0.1 µm was used for analyses. Electron-impact ionization with electron voltage fixed at 70 V and emission current set to 160 µA was used. The temperatures of the injection port, ion source and interface were maintained at 220 °C, 300 °C, and 310 °C, respectively. Analyses were carried out in the splitless mode with a constant linear velocity of the carrier gas (He), which was maintained at 60 cm/s. The septum purge flow was set at 3 ml/min. The samples were injected using the high pressure mode, which was applied at 200 kPa and this pressure was maintained for 1 min. The detector voltage was set to 1.4 kV.

Steroids measured

The levels of 37 unconjugated steroids and 30 steroid polar conjugates after hydrolysis were concomitantly measured in blood from the umbilical artery and umbilical vein using the GC-MS method (Hill *et al.* 2010b).

Statistical data analysis

The relationships between arterial and venous steroid in umbilical cord blood were evaluated using Pearson's correlations after power transformation of the original data (to attain Gaussian distribution and constant variance) using the statistical software Statgraphics Centurion, version XVI by Statpoint Inc. (Herndon, Virginia, USA). Multivariate homogeneity of the transformed data before correlation analysis was checked

Table 1. Levels of unconjugated steroids in the umbilical artery in premature and normal births.

Steroid	Group A (weeks 28-32)	B (weeks 33-37)	C (weeks 38-42)	Trend p-value	Multiple comparisons
Pregnenolone (nM)	18.5 (16.7, 27.1)	19.8 (16.9, 23)	29.1 (21.1, 35.8)	0.009	C>A*, C>B*
17-Hydroxypregnenolone (nM)	20.7 (11.3, 39.6)	14.4 (9.64, 29.3)	27.8 (17.5, 45.6)	0.092	
20α-Dihydropregnenolone (nM)	1.97 (1.38, 2.55)	2.42 (1.96, 3.06)	2.48 (1.61, 3.03)	0.190	
DHEA (nM)	7.63 (4.41, 10.1)	7.82 (3.68, 12.6)	9.85 (5.82, 15.6)	0.233	
7α-Hydroxy-DHEA (nM)	1.41 (1.03, 1.9)	1.55 (1.16, 2.09)	1.91 (1.45, 2.41)	0.076	
7β-Hydroxy-DHEA (nM)	0.185 (0.0795, 0.343)	0.156 (0.125, 0.221)	0.276 (0.21, 0.347)	0.002	C>A*, C>B**
Androstenediol (nM)	0.472 (0.409, 0.747)	0.363 (0.222, 0.469)	0.301 (0.184, 0.407)	0.009	C<A**
5-Androstene-3β,7α,17β-triol (pM)	8.92 (6.13, 19.4)	17.4 (12.7, 28.5)	25.6 (16.4, 46.3)	0.001	C>A***
5-Androstene-3β,7β,17β-triol (pM)	9.18 (5.55, 16.9)	10.4 (7.65, 13)	15.4 (8.93, 28.2)	0.023	C>A*
Progesterone (nM)	710 (404, 964)	510 (390, 622)	824 (533, 1070)	0.009	C>B**
17-Hydroxyprogesterone (nM)	27.1 (14.4, 33)	23.1 (18.2, 36.1)	52.2 (44, 73.1)	<0.001	C>A***, C>B***
20α-Dihydroprogesterone (nM)	91.7 (57.2, 118)	82.9 (66.8, 156)	88.1 (60.9, 123)	0.963	
Androstenedione (nM)	2.59 (0.984, 3.13)	1.32 (0.761, 2.27)	3.55 (2.67, 4.46)	<0.001	C>A**, C>B***
Testosterone (nM)	4.02 (1.39, 6.99)	1.04 (0.687, 2.24)	1.32 (0.751, 1.78)	0.003	B<A**, C<A**
Estrone (nM)	2.34 (1.61, 3.15)	4.64 (3.13, 8.92)	13.6 (7.55, 23.6)	<0.001	B>A**, C>A***, C>B**
Estradiol (nM)	0.915 (0.748, 1.38)	1.66 (1.02, 2.74)	3.05 (1.92, 6.17)	<0.001	C>A***, C>B**
16α-Hydroxyestrone (nM)	0.168 (0.0667, 0.554)	0.533 (0.376, 0.722)	1.62 (1.35, 2.27)	<0.001	C>A***, C>B***
Estriol (nM)	11.8 (6.68, 18.4)	21.2 (11.6, 39)	49 (34, 75.4)	<0.001	C>A***, C>B**
16α-Hydroxypregnenolone (nM)	8.04 (6.22, 11.9)	9.54 (5.64, 11.8)	10.8 (8.3, 19.1)	0.050	
16α-Hydroxy-DHEA (nM)	1.95 (0.863, 3.89)	2.52 (2.17, 3.77)	7.1 (3.79, 20.1)	<0.001	C>A***, C>B***
16α-Hydroxyprogesterone (nM)	53.5 (36.9, 82.8)	76.7 (40.6, 102)	163 (103, 234)	<0.001	C>A***, C>B***
16α-Hydroxyandrostenedione (nM)	2.47 (0.796, 3.08)	2.73 (1.61, 3.3)	11 (4.23, 18)	<0.001	C>A***, C>B***
16α-Hydroxytestosterone (nM)	6.54 (3.34, 12.2)	7.05 (4.96, 9.25)	13.6 (9.01, 22.5)	<0.001	C>A***, C>B***
5α-Dihydroprogesterone (nM)	31.9 (21.5, 50.6)	23.6 (16.7, 34.2)	45.1 (29.2, 59.7)	0.001	C>B***
Allopregnanolone (nM)	6.44 (4.14, 10)	4.93 (3.98, 6.38)	4.93 (3.61, 7.19)	0.361	
Isopregnanolone (nM)	11.6 (7.34, 17.5)	9.03 (6.66, 11.8)	9.71 (8.21, 13.7)	0.345	
5α,20α-Tetrahydroprogesterone (nM)	76.3 (58.1, 85.5)	60.6 (50.3, 102)	62.7 (41, 92.1)	0.674	
5α-Pregnane-3α,20α-diol (nM)	3.15 (1.81, 6.87)	2.73 (2.05, 3.13)	2.94 (2.57, 6.09)	0.235	
5α-Pregnane-3β,20α-diol (nM)	2.71 (2.12, 3.4)	2.31 (1.81, 3.16)	2.43 (1.78, 3.56)	0.657	
5β-Dihydroprogesterone (nM)	13.7 (8.81, 19.5)	7.4 (4.98, 10.2)	8.66 (5.81, 13.1)	0.013	B<A**
Pregnanolone (nM)	16.7 (12.6, 21.8)	12 (8.4, 13.6)	10.3 (7.04, 14.9)	0.005	B<A*, C<A**
Epipregnanolone (nM)	2.17 (1.39, 2.93)	1.35 (1.05, 1.67)	0.983 (0.699, 1.39)	0.001	C<A***
5β,20α-Tetrahydroprogesterone (nM)	27.9 (18.2, 41.6)	20.8 (16.8, 26.7)	14.8 (9.38, 28.7)	0.013	C<A**
5β-Pregnane-3α,20α-diol (nM)	25.6 (18.3, 34.6)	17.4 (12, 18.8)	12.4 (9.4, 15.3)	<0.001	B<A*, C<A***
5β-Pregnane-3β,20α-diol (nM)	0.775 (0.32, 0.964)	0.658 (0.454, 0.869)	0.419 (0.287, 0.595)	0.035	
Androsterone (pM)	139 (111, 191)	179 (91.3, 220)	158 (97.2, 213)	0.783	
Etiocholanolone (pM)	50.4 (36.7, 63.8)	38.3 (28.2, 48.9)	56.7 (27.8, 74)	0.232	

Kruskal-Wallis test followed by Dunn's multiple comparisons with Bonferroni correction was used for estimation of relationships between steroid levels and gestational age at birth, * p<0.05, ** p<0.01, *** p<0.001.

by Hotelling's statistics using NCSS 2007 statistical software by Number Cruncher Statistical Systems (Kaysville, Utah, USA). The principal axis as well as the 95 % confidence ellipsoid were retransformed to the original scale and shown together with experimental data points (UV).

The differences between groups according to the GA at labor were evaluated using the Kruskal-Wallis test followed by Dunn's multiple comparisons. Differences between umbilical and venous cord blood were tested using the Wilcoxon paired test.

Results

The steroid levels in UA for unconjugated steroids and steroid polar conjugates at birth for each group sorted according to GA are shown in Tables 1 and 2. Tables 3 and 4 demonstrate AVD for unconjugated and conjugated steroids, respectively, and the correlations between umbilical arterial and venous blood for unconjugated and conjugated steroids are illustrated in Table 5. Despite the significant difference in the levels of many steroids from UA versus UV blood, levels of

Table 2. Levels of conjugated steroids in the umbilical artery in premature and normal births.

Steroid	Group A (weeks 28-32)	Group B (weeks 33-37)	Group C (weeks 38-42)	Trend p-value	Multiple comparisons
Pregnenolone (nM)	575 (341, 1060)	1970 (922, 3380)	2720 (2100, 3560)	<0.001	B>A***, C>A***
17-Hydroxypregnenolone (nM)	60 (43.4, 169)	913 (466, 1180)	469 (222, 934)	<0.001	B>A***, C>A***
20α-Dihydropregnenolone (nM)	447 (289, 1100)	1370 (882, 1900)	1600 (1110, 2070)	<0.001	B>A*, C>A***
DHEA (nM)	569 (185, 791)	1630 (702, 2340)	1510 (1150, 2530)	<0.001	B>A***, C>A***
Androstenediol (nM)	185 (149, 341)	1330 (715, 2840)	2610 (1730, 3780)	<0.001	B>A***, C>A***
5-Androstene-3β,7α,17β-triol (pM)	50.7 (15.3, 84)	353 (239, 637)	504 (380, 799)	<0.001	B>A***, C>A***
5-Androstene-3β,7β,17β-triol (pM)	23.9 (16.1, 59.8)	179 (107, 374)	600 (421, 993)	<0.001	B>A**, C>A***, C>B***
20α-Dihydroprogesterone (nM)	47.9 (29.1, 83.4)	84.1 (50.7, 118)	99.4 (77.8, 153)	<0.001	C>A***
Estrone (nM)	39.1 (19.5, 62.4)	191 (107, 231)	122 (87.7, 217)	<0.001	B>A***, C>A***
Estradiol (nM)	11.9 (8.91, 15.5)	26.4 (16.2, 34.6)	13.3 (8.3, 17.1)	<0.001	B>A***, C<B***
Estriol (nM)	1040 (602, 1250)	3940 (3230, 5100)	2870 (2350, 3940)	<0.001	B>A***, C>A***
16α-Hydroxypregnenolone (nM)	1.77 (1.38, 3.16)	6.88 (4.01, 15.1)	10.3 (6.03, 15.4)	<0.001	B>A**, C>A***
16α-Hydroxy-DHEA (nM)	603 (78.9, 1240)	549 (208, 1280)	1060 (281, 1920)	0.187	
Allopregnanolone (nM)	123 (69.4, 184)	421 (322, 671)	230 (179, 414)	<0.001	B>A***, C>A**, C<B**
Isopregnanolone (nM)	85.4 (56, 164)	289 (135, 482)	339 (255, 433)	<0.001	B>A***, C>A***
5α,20α-Tetrahydroprogesterone (nM)	70.7 (55.5, 118)	197 (171, 307)	122 (74.2, 181)	<0.001	B>A***, C>A*, C<B**
5α-Pregnane-3α,20α-diol (nM)	776 (426, 1160)	2790 (1830, 3690)	949 (706, 1470)	<0.001	B>A***, C<B***
5α-Pregnane-3β,20α-diol (nM)	919 (400, 1350)	2790 (1720, 4640)	1780 (1210, 2260)	<0.001	B>A***, C>A**
Pregnanolone (nM)	96.4 (67.6, 110)	259 (165, 355)	178 (144, 253)	<0.001	B>A***, C>A***
Epipregnanolone (nM)	33.8 (21.5, 50.5)	67.7 (44.8, 112)	48.4 (39.8, 66.1)	0.001	B>A***, C>A**
5β,20α-Tetrahydroprogesterone (nM)	40.7 (25.4, 74.7)	66.9 (45.2, 77.4)	45.6 (34.6, 80.9)	0.283	
5β-Pregnane-3α,20α-diol (nM)	1020 (749, 1500)	2520 (1810, 3760)	1390 (1040, 2250)	0.001	B>A***, C<B*
5β-Pregnane-3β,20α-diol (nM)	210 (124, 255)	449 (405, 727)	281 (202, 372)	<0.001	B>A***, C<B***
Androsterone (nM)	4.82 (2.5, 8.13)	14.8 (8.93, 20.4)	13.9 (10.9, 19.1)	<0.001	B>A***, C>A***
Epiandrosterone (nM)	24.9 (6.63, 39)	49.7 (22.6, 65.8)	52.9 (41.4, 90.3)	0.002	C>A***
5α-Androstane-3α,17β-diol (nM)	5.94 (3.66, 8.31)	25 (17.4, 37.7)	13.6 (8.34, 17.5)	<0.001	B>A***, C>A***, C<B**
5α-Androstane-3β,17β-diol (nM)	2.27 (1.48, 3.73)	7.97 (5.84, 10.9)	4.37 (2.77, 8.05)	<0.001	B>A***, C>A**, C<B*
Etiocholanolone (nM)	0.77 (0.578, 2.41)	2.96 (1.28, 4.02)	2.58 (1.5, 3.57)	0.013	C>A**
5β-Androstane-3α,17β-diol (nM)	1.81 (1.45, 2.4)	5.72 (2.28, 10.4)	3.83 (3.08, 4.73)	<0.001	B>A***, C>A***
5β-Androstane-3β,17β-diol (nM)	0.585 (0.258, 0.924)	1.93 (0.811, 3.18)	1.2 (0.888, 1.71)	<0.001	B>A***, C>A***

Kruskal-Wallis test followed by Dunn's multiple comparisons with Bonferroni correction was used for estimation of relationships between steroid levels and gestational age at birth, * p<0.05, ** p<0.01, *** p<0.001.

almost all free steroids and especially their polar conjugates were highly correlated between the two blood sources (Table 5). This indicates that levels from UA, UV and mixed umbilical cord blood can be accurately derived from each other. From the 67 steroids measured, 54 (80.6 %), 43 (64.2 %), and 24 (35.8 %) exhibit Pearson's correlation coefficients higher than 0.7, 0.8, and 0.9, respectively.

Discussion

The levels of steroids in the umbilical arterial blood (Tables 1 and 2) are mostly in accordance with our previous results and data from others (see our earlier study (Hill *et al.* 2010b)). The AVD (Tables 3 and 4) and steroid correlations UA and UV (Table 5) are also compatible with data in the literature (Hill *et al.* 2010b).

Steroid metabolome and gestational age at birth

While steroids in UA primarily reflect steroidogenesis in the fetal adrenal, liver, kidney and lungs and secondarily placental activity, those in UV predominantly mirror the steroidogenic activities in placental zones close to the fetal compartment (Hill *et al.* 2010a). Nevertheless, trends for steroid levels in UV, which are related to the GA, are mostly similar to the corresponding ones for the UA (data not shown) despite the pronounced AVD (Tables 3 and 4).

The data in the literature, including our previous studies, indicate a close (human-specific) association of the placental corticoliberin (CRH) boost with rising activity of the fetal zone of the fetal adrenal (FZ) in late

Table 3. Arteriovenous differences for unconjugated steroids expressed as a % of the steroid level in the umbilical artery.

Steroid	Group A (weeks 28-32)	Group B (weeks 33-37)	Group C (weeks 38-42)	Trend p-value	Multiple comparisons
Pregnenolone	6.47 (0.197, 24.4)	−5.7 (−29.7, 5.79)	−15.3 (−53.9, 16.2) +	0.038	C<A*
17-Hydroxypregnenolone	52.4 (41.5, 72) +++	60.3 (38.2, 77.5) +	75 (62.9, 81) +++	0.017	C>A**
20α-Dihydropregnenolone	36.8 (16.7, 48.8) ++	20.6 (2.17, 46.6)	27.8 (9.82, 46.9) +++	0.438	
DHEA	78.7 (69, 82.3) +++	67.8 (46, 82.5) ++	75.4 (62.6, 80.6) +++	0.192	
7α-Hydroxy-DHEA	41.2 (−2.97, 50.9) +	41.9 (14, 52.5)	13.6 (−21.7, 40.2)	0.175	
7β-Hydroxy-DHEA	37.6 (14.2, 62.6) +	33.1 (2.61, 44.9)	13.9 (0.556, 37.2) +++	0.142	
Androstenediol	61.4 (36.3, 76.9) ++	56.5 (29.7, 74.5) ++	68.4 (56.7, 74.7) +++	0.294	
5-Androstene-3β,7α,17β-triol	79 (57.3, 88.7) ++	74.5 (43.3, 83.1) +	76.8 (56.6, 89.5) +++	0.631	
5-Androstene-3β,7β,17β-triol	61.4 (17.6, 79.5) ++	37.5 (−7.04, 57.3)	56.4 (34.1, 67) +++	0.110	
Progesterone	−76.9 (−114, −41.6) ++	−116 (−156, −51.5) +++	−99 (−172, −65.6) +++	0.349	
17-Hydroxyprogesterone	−32 (−54.9, −0.398) +	−59.7 (−161, −35.9) +++	−18.9 (−35.4, −4.75) ++	0.011	C>B**
20α-Dihydroprogesterone	47 (37.6, 52.4) +++	38.2 (17.3, 44.7) +	28 (18.7, 45.8) +++	0.030	C<A*
Androstenedione	52.2 (36.1, 59) ++	29.4 (−3.22, 44.3)	37.5 (22.7, 51.4) +++	0.016	B<A**
Testosterone	52 (20.4, 64.2) +++	24.5 (−57.1, 53.3)	1.13 (−58.2, 45.1)	0.004	C<A**
Estrone	−529 (−730, −407) +++	−462 (−762, −284) +++	−430 (−619, −247) +++	0.362	
Estradiol	−264 (−380, −166) +++	−324 (−516, −161) +++	−244 (−325, −165) +++	0.431	
16α-Hydroxyestrone	−713 (−1650, −240) +++	−376 (−553, −227) +++	−318 (−487, −276) +++	0.205	
Estriol	−467 (−706, −333) +++	−373 (−776, −225) +++	−312 (−387, −148) +++	0.009	C>A**
16α-Hydroxypregnenolone	77.7 (74.8, 79.2) +++	73.2 (63.5, 79) ++	68.7 (60.4, 75) +++	0.003	C<A***
16α-Hydroxy-DHEA	−76.2 (−149, −43.9) +++	−82.3 (−202, −45.2) +++	−185 (−266, −121) +++	0.017	C<A*
16α-Hydroxyprogesterone	−29.6 (−46.3, −12.2) ++	−41.6 (−80.6, −23.8) +++	−73.5 (−118, −42.8) +++	0.007	C<A**
16α-Hydroxyandrostenedione	−38.6 (−76.7, −2.41) ++	4.12 (−20.1, 23)	5.34 (−27.6, 39.5)	0.014	C>A**
16α-Hydroxytestosterone	30.9 (13.1, 46.5)	24 (−16.5, 36.8)	6.76 (−29.6, 28)	0.102	
5α-Dihydroprogesterone	11.9 (−14, 33.5)	−11.2 (−36.3, 2.25)	5.11 (−24.5, 31.6)	0.140	
Allopregnanolone	7.33 (−13.7, 27.4)	−14.4 (−48.9, 10.9)	9.6 (−16.7, 33.2)	0.045	
Isopregnanolone	46.2 (27.9, 55.2) +++	33 (0.634, 39.8)	32.3 (11.7, 44) +++	0.079	
5α,20α-Tetrahydroprogesterone	39.4 (13.4, 48.9) +++	17.5 (−1.03, 30.3)	18.4 (0.801, 33.2) +++	0.113	
5α-Pregnane-3α,20α-diol	37.6 (25.5, 46.9) +++	24.8 (1.96, 42.6) +	30.1 (11.4, 53.9) +++	0.290	
5α-Pregnane-3β,20α-diol	18.5 (3.15, 41.3) ++	1.29 (−18.6, 18)	2.63 (−13.9, 20)	0.033	B<A*
5β-Dihydroprogesterone	14.8 (−4.74, 19.7)	−5.21 (−18.1, 35)	19.8 (−1.41, 38) ++	0.583	
Pregnanolone	57.7 (49.7, 66.2) +++	51.9 (34.5, 59.4) ++	58.3 (47.2, 63.9) +++	0.216	
Epipregnanolone	49.5 (38.1, 56.2) +++	34.2 (13.4, 53.8) ++	41.1 (11, 53) +++	0.185	
5β,20α-Tetrahydroprogesterone	26.8 (5.7, 32.9) ++	20.8 (1.9, 30.8)	17 (−0.453, 28.2) +++	0.517	
5β-Pregnane-3α,20α-diol	70.5 (61.9, 78.7) +++	64.7 (52, 73.7) +++	73.3 (66.6, 76.6) +++	0.036	C>B*
5β-Pregnane-3β,20α-diol	55.5 (31.1, 70.6)	44.9 (13.4, 56.4) ++	38.4 (15.8, 58.2) +++	0.463	
Androsterone	51.8 (30.1, 61.4) +++	52.2 (20.5, 69) +	53.3 (35.4, 73.9) +++	0.541	
Etiocholanolone	−11.5 (−117, 57.1)	−3.09 (−66.2, 30.6)	−8.96 (−46.5, 20.8)	0.949	

Significance of the arteriovenous differences: + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$; significance of the differences between groups according to the gestational age at birth: * $p<0.05$, ** $p<0.01$, *** $p<0.001$

gestation. However, CRH is unstable and poorly predicts the timing of the onset of labor, while CRH-induced changes in the steroid metabolome are substantially better at predicting the approaching onset. CRH directly stimulates the production of Δ^5 steroid sulfates in the FZ and cortisol synthesis in the transitional zone (TZ) *via* binding to specific type 1 CRH receptors and ACTH receptors. Early in gestation, only the Δ^5-steroid production occurs in the TZ and FZ, and ACTH does not influence steroidogenesis in the FZ. After mid-gestation, the TZ cells synthesize cortisol and by week 30 of gestation, the definitive zone of the fetal adrenal (DZ) and TZ begin to resemble the adult *zona glomerulosa* and *zona fasciculata*, respectively. The FZ still producing conjugated C19 Δ^5 steroids is similar to the adult *zona reticularis*, but unlike the adult *zona reticularis* it produces excessive amounts of conjugated C21 Δ^5 steroids (pregnenolone sulfate and 17-hydroxypregnenolone). The fetal C19 Δ^5 steroid sulfates are consumed for the placental production of estradiol

Table 4. Arteriovenous differences for conjugated steroids expressed as a % of the steroid level in the umbilical artery.

Steroid	Group A (weeks 28-32)	Group B (weeks 33-37)	Group C (weeks 38-42)	Trend p-value	Multiple comparisons
Pregnenolone	−6.97 (−15.9, 4.61)	15.4 (5.97, 20.9) ++	8.11 (−3.32, 24.2) +	0.007	B>A**, C>A**
17-Hydroxypregnenolone	3.77 (−1.83, 13.5)	7.4 (−3.24, 20)	−6.55 (−21.7, 18.3)	0.159	
20α-Dihydropregnenolone	4.92 (−6.62, 48.5)	4.12 (−2.78, 13.2)	−4.11 (−14.3, 4.23) +	0.023	
DHEA	−10 (−47.8, 3.59) +	12.9 (−7.6, 20.2)	12.9 (−2.45, 23.9) +	0.006	C>A**
Androstenediol	−10.5 (−18.4, −4.7) ++	2.89 (−5.22, 16.8)	−6.37 (−15.6, −1.07) +++	0.006	B>A**, C<B**
5-Androstene-3β,7α,17β-triol	−1.16 (−22.3, 24.4)	25.4 (−19.7, 46.9)	8.8 (−5.34, 27.6)	0.370	
5-Androstene-3β,7β,17β-triol	−0.446 (−35.3, 29.3)	27.8 (−33.6, 50.8)	14.5 (−7.43, 33.1) +	0.322	
20α-Dihydroprogesterone	−11.1 (−27.4, 9.7)	−29.1 (−87.7, 0.97) +	5.18 (−26.4, 18.8)	0.075	
Estrone	5.52 (−17.9, 32.5)	5.41 (−9.28, 42.6)	6.14 (−8.52, 17.2)	0.666	
Estradiol	15.2 (−2.96, 35.3)	14.3 (−15.9, 31.7)	−16.4 (−34.2, −8.95) +++	0.000	C<A***, C<B**
Estriol	16.4 (10.3, 27.7) +++	12.6 (−2.48, 29.5)	17.2 (−8.55, 30.2) +	0.754	
16α-Hydroxypregnenolone	−12.2 (−111, 8.75)	3.21 (−30.2, 31.3)	8.38 (−35.5, 40.6)	0.362	
16α-Hydroxy-DHEA	−68.9 (−196, 5.49) +	−44.1 (−177, −10.2) +	−112 (−375, −1.11) +++	0.474	
Allopregnanolone	−25.8 (−30.1, −16.2) +++	−11.2 (−31.8, 1.58) +	−13.7 (−21.6, −2.7) +++	0.039	C>A*
Isopregnanolone	−4.61 (−11.4, 4.51)	0.247 (−11.5, 14.5)	5.49 (−4.41, 13.6)	0.134	
5α,20α-Tetrahydroprogesterone	−7.22 (−34.6, 2.29) +	0.623 (−24.3, 14.2)	−0.422 (−31.7, 17.2)	0.466	
5α-Pregnane-3α,20α-diol	−13.8 (−17.8, −9.27) +++	−3.39 (−10.7, 8.33)	−7.11 (−12.5, 0.362) +++	0.010	B>A**
5α-Pregnane-3β,20α-diol	−13.3 (−20.1, −2.16) ++	−0.639 (−5.17, 3.23)	−6.36 (−14.4, 1.06) ++	0.136	
Pregnanolone	−8.45 (−14.7, 0.0729) +	−2.26 (−9.25, 9.53)	−0.591 (−12.1, 11.9)	0.285	
Epipregnanolone	−17.9 (−41.2, −5.99) ++	−4.73 (−23, 1.16) +	−12.8 (−42.4, −0.0517) ++	0.506	
5β,20α-Tetrahydroprogesterone	−14.7 (−20.9, 6.65)	14.1 (−18.6, 28.7)	3.94 (−28.7, 14.2)	0.416	
5β-Pregnane-3α,20α-diol	−0.925 (−7.78, 2.02)	3.88 (−8.85, 9.08)	−0.0373 (−8.16, 18.1)	0.875	
5β-Pregnane-3β,20α-diol	−8.76 (−12.4, −3.53) +	−1.24 (−9.94, 5.68)	−3 (−9.97, 4.54)	0.219	
Androsterone	−24.9 (−48.6, −11.3) ++	−3.44 (−34.3, 12)	0.976 (−15.3, 14.8)	0.014	C>A**
Epiandrosterone	−8.15 (−95.8, 30.3)	14.3 (−18.8, 18.9)	10.2 (−4.12, 22.2) +	0.254	
5α-Androstane-3α,17β-diol	−8.79 (−24.5, 0.525) +	2.91 (−33, 9.38)	−7.4 (−18, 8.24) +	0.515	
5α-Androstane-3β,17β-diol	−4.94 (−15, 6.39)	2.91 (−6.78, 20.5)	−1.79 (−16.5, 10.1)	0.431	
Etiocholanolone	−39.1 (−67.4, −15.1) ++	−3.39 (−70.7, 6.39)	−5.65 (−20.1, 16.6)	0.014	C>A**
5β-Androstane-3α,17β-diol	2.13 (−10.7, 18)	−20.6 (−40.6, 13.4)	−3.1 (−13.1, 20.4)	0.315	
5β-Androstane-3β,17β-diol	25.5 (−41.3, 43.4)	3.76 (−79.8, 41.7)	10.4 (−36.4, 32.6)	0.939	

Significance of the arteriovenous differences: + p<0.05, ++ p<0.01, +++ p<0.001; significance of the differences between groups according to the gestational age at birth: * p<0.05, ** p<0.01, *** p<0.001

and the pregnenolone sulfate is probably a substrate for progesterone synthesis. However, according to a different concept placental progesterone originates mainly from maternal LDL-cholesterol (Hill *et al.* 2010a). Our present data (Tables 1-4) are mostly compatible with the former mechanism, as discussed in detail in our previous study (Hill *et al.* 2010a). Almost all conjugated Δ^5 steroids and a number of unconjugated Δ^5 steroids positively correlate with advancing GA (Tables 1 and 2). In addition, some Δ^4 steroids (progesterone, 17-hydroxyprogesterone and androstenedione) also positively correlate with GA, like almost all estrogens (except conjugated estradiol), 5α-dihydroprogesterone and almost all conjugated 5α/β reduced pregnane metabolites, and conjugated 5α/β-reduced androstanes.

On the other hand, testosterone decreases along with its Δ^5 precursor androstenediol, most probably due to increasing metabolism of the latter substances to placental estrogens. Besides androstenediol and testosterone, all unconjugated 5β-reduced pregnanes also decrease with advancing gestation, which is in line with previously published data, including ours (Hill *et al.* 2010a) demonstrating a decreasing activity of 5β-reductase (AKR1D1) in this period. Byrns (2011) suggested that inhibition of 5β-reductase by Δ^4 steroids and estrogens may participate in the induction of labor.

All 16α-hydroxy-metabolites of estrogens and their precursors show a rising trend from week 28 of gestation to the normal term of pregnancy, which indicates an increasing antiestrogenic effect of 16α-hydroxylase (CYP3A7). This enzyme, which is extensively expressed in fetal livers, most probably

Table 5. Correlations between levels of steroids in umbilical arterial and umbilical venous blood at preterm and term deliveries in premature and normal births.

	Unconjugated steroids			Steroid polar conjugates		
Steroid	**r**	**p-value**	**n**	**r**	**p-value**	**n**
Pregnenolone	0.689	<0.001	76	0.975	<0.001	70
17-Hydroxypregnenolone	0.711	<0.001	79	0.980	<0.001	66
20α-Dihydropregnenolone	0.781	<0.001	74	0.982	<0.001	69
DHEA	0.770	<0.001	75	0.954	<0.001	72
7α-Hydroxy-DHEA	0.474	<0.001	79		NA	
7β-Hydroxy-DHEA	0.652	<0.001	76		NA	
Androstenediol	0.502	<0.001	79	0.992	<0.001	74
5-Androstene-3β,7α,17β-triol	0.422	<0.001	78	0.818	<0.001	73
5-Androstene-3β,7β,17β-triol	0.411	<0.001	75	0.930	<0.001	73
Progesterone	0.593	<0.001	75		-----	
17-Hydroxyprogesterone	0.865	<0.001	46		NA	
20α-Dihydroprogesterone	0.853	<0.001	75	0.875	<0.001	71
Androstenedione	0.831	<0.001	73		-----	
Testosterone	0.668	<0.001	77		NA	
Estrone	0.912	<0.001	75	0.931	<0.001	73
Estradiol	0.895	<0.001	73	0.870	<0.001	74
16α-Hydroxyestrone	0.740	<0.001	52			
Estriol	0.809	<0.001	77	0.929	<0.001	73
16α-Hydroxypregnenolone	0.851	<0.001	74	0.669	<0.001	75
16α-Hydroxy-DHEA	0.846	<0.001	77	0.423	<0.001	76
16α-Hydroxyprogesterone	0.924	<0.001	78		NA	
16α-Hydroxyandrostenedione	0.833	<0.001	76		NA	
16α-Hydroxytestosterone	0.794	<0.001	78		NA	
5α-Dihydroprogesterone	0.776	<0.001	76		-----	
Allopregnanolone	0.704	<0.001	74	0.982	<0.001	74
Isopregnanolone	0.803	<0.001	76	0.978	<0.001	75
5α,20α-Tetrahydroprogesterone	0.781	<0.001	75	0.892	<0.001	72
5α-Pregnane-3α,20α-diol	0.542	<0.001	75	0.987	<0.001	74
5α-Pregnane-3β,20α-diol	0.872	<0.001	77	0.982	<0.001	73
5β-Dihydroprogesterone	0.847	<0.001	74		-----	
Pregnanolone	0.733	<0.001	77	0.966	<0.001	74
Epipregnanolone	0.755	<0.001	78	0.931	<0.001	72
5β,20α-Tetrahydroprogesterone	0.936	<0.001	74	0.864	<0.001	71
5β-Pregnane-3α,20α-diol	0.866	<0.001	75	0.974	<0.001	65
5β-Pregnane-3β,20α-diol	0.737	<0.001	73	0.974	<0.001	73
Androsterone	0.501	<0.001	76	0.914	<0.001	73
Epiandrosterone		NA		0.884	<0.001	74
5α-Androstane-3α,17β-diol		NA		0.970	<0.001	74
5α-Androstane-3β,17β-diol		NA		0.909	<0.001	73
Etiocholanolone	0.379	<0.001	77	0.930	<0.001	72
5β-Androstane-3α,17β-diol		NA		0.874	<0.001	73
5β-Androstane-3β,17β-diol		NA		0.559	<0.001	72

r = Pearson's correlation coefficient after power transformation of original data for attaining data symmetry and constant variance, p-value = statistical significance, n = number of subjects investigated, NA = not available

protects the fetus against hyperandrogenization in cooperation with fetal sulfotransferases (SULT2A1 and SULT1E1) providing sulfation of estrogens, their precursors and 16α-hydroxy-metabolites.

Arteriovenous differences and gestational age at birth

Unconjugated steroids

The decreasing AVD with advancing gestation and slightly but significantly negative AVD for

unconjugated pregnenolone in period C (Table 3) indicate that the increasing proportion of fetal pregnenolone sulfate is returned from the placenta after desulfation by placental sulfatase (STS) and unconverted to progesterone. Whereas the activities of both STS and type1 3β-hydroxysteroid dehydrogenase (HSD3B1) in the placenta are independent of GA, the aforementioned finding may be ascribed to the accelerating activity of the FZ near term, which is induced by the escalating production of placental CRH (Hill *et al.* 2010a).

In contrast to pregnenolone, 17-hydroxypregnenolone shows a pronounced positive AVD in all periods, which indicates its fetal origin. The increasing trend in 17-hydroxypregnenolone AVD with advancing gestation is most probably linked to increasing activity of the FZ.

The AVDs for 20α-dihydropregnenolone and C19 Δ^5 steroids do not significantly change with GA, which may indicate a sufficient capacity of placental enzymes to further metabolize these steroids even during increasing fetal production of the corresponding sulfated precursors (Hill *et al.* 2010a).

Progesterone and 17-hydroxyprogesterone exhibit pronouncedly negative AVD, which demonstrates their placental origin. While the progesterone AVD was independent of GA, the 17-hydroxyprogesterone AVD decreased from the period represented by group B to the period of group C (Table 3), which may be associated with increasing production of 17-hydroxyprogesterone in the fetus near term and a limited capacity of placental enzymes for its metabolism.

In contrast to progesterone and 17-hydroxyprogesterone, the 20α-dihydroprogesterone and further Δ^4 steroids like androstenedione and testosterone showed positive AVD, which demonstrates their fetal origin.

The decreasing AVD for the 20α-dihydroprogesterone with increasing GA may reflect decreasing activity of the reductive isoforms of 17β-hydroxysteroid dehydrogenases (HSD17B) and/or family 1 aldoketoreductases (AKR1C) with increasing GA, while the decrease of AVD in testosterone and partly also in androstenedione may be associated with increasing placental consumption of these steroids (serving as substrates for estrogen production) induced by rising placental CYP19A1 activity near term (Hill *et al.* 2010a).

Both 16-deoxy-estrogens and 16α-hydroxy-estrogens showed pronouncedly negative AVD due to their placental origin and intensive catabolism in the fetus. While the AVD for 16-deoxy-estrogens do not depend on the GA, the 16α-hydroxy- ones tend to have less pronounced AVD with increasing GA, which might be associated with higher CYP3A7 activity in the fetus at constant STS and HSD3B1 activities in placenta (Hill *et al.* 2010a).

Concerning the non-estrogenic C19 and C21 16α-hydroxy-steroids, 16α-hydroxypregnenolone and 16α-hydroxytestosterone showed positive AVD with a slightly decreasing trend for the 16α-hydroxypregnenolone and constant value for the 16α-hydroxytestosterone. The positive AVD indicate fetal origin of these substances.

On the other hand, 16α-hydroxy-DHEA and 16α-hydroxyprogesterone exhibited significantly negative AVD showing more pronounced differences with advancing gestation, which points to their placental origin. The levels 16α-hydroxyandrostenedione are significantly higher only in the period of group A and then do not differ. In general, the more pronounced negative AVD and the less pronounced positive AVD for the 16α-hydroxy-steroids may be associated with their lower avidity to their further placental metabolism (after desulfation by STS) when compared with the corresponding 16α-deoxy-counterparts.

From the 5α-reduced pregnanes and androstanes, the 5α-dihydroprogesterone, allopregnanolone and etiocholanolone levels do not significantly differ between UA and UV but isopregnanolone, 5α,20α-pregnanes and all 5β-reduced pregnanes show highly significant positive AVD, which demonstrates fetal origin of the latter substances. The dependence of AVD for both 5α- and 5β reduced pregnanes and androstanes on GA is weak or absent, which demonstrates stable balance between the primary fetal source of the 5α/β reduced pregnanes and androstanes and their further placental metabolism regardless the GA.

Steroid polar conjugates

In spite of huge STS activity in the placenta (Hill *et al.* 2010a), the AVD for the steroid polar conjugates are less pronounced due to their higher resistance to placental catabolism when compared with the unconjugated ones.

The polar conjugates of pregnenolone, DHEA, 5-androstene-3β,7β,17β-triol, estriol and epiandrosterone show positive AVD in the period of group C, which indicates their fetal origin. Pregnenolone sulfate and DHEAS show an increasing trend towards normal term of

pregnancy. This may be related to rising activity of FZ at constant activities of STS and HSD3B1 with increasing GA (Hill *et al.* 2010a).

Polar conjugates of androstenediol, 16α-hydroxy-DHEA, allopregnanolone, 5α-pregnane-3α,20α-diol, 5α-pregnane-3β,20α-diol, epipregnanolone, 5α-androstane-3α,17β-diol surprisingly exhibit a negative AVD in all periods and conjugated 20α-dihydropregnenolone showed a slightly negative AVD of borderline significance in the C-group period only. The similar situation is in conjugated estradiol but the respective difference is more pronounced and highly significant. AVD for estradiol polar conjugates show significantly decreasing trends from the group A to group C periods, as do the AVD for conjugates of allopregnanolone and 5α-pregnane-3α,20α-diol. Conjugates of pregnanolone, androsterone, etiocholanolone and 5α-androstane-3α,17β-diol showed negative AVD in the group A period but insignificant AVD in the group B period. While the placenta does not possess significant sulfotransferase activity, both maternal and fetal compartments provide extensive sulfation in the *zona reticularis* and ZF, respectively, as well as in the liver. Therefore, the explanation for negative AVD in some steroid polar conjugates (5α/β-reduced pregnane and androstane steroids and estradiol) might be just their extensive sulfation in the maternal compartment and penetration of some of these sulfates to the fetal compartment without their complete hydrolysis by the placental STS. This possibility is also supported by higher levels of various steroid conjugates in the maternal circulation when compared to the fetal, as documented in our previous reports (Hill *et al.* 2010a,b, 2011). Nevertheless, the negative AVD for androstenediol and 16α-hydroxy-DHEA still remains an enigma because their levels in the maternal circulation are substantially lower than in the fetus.

Steroid correlations between umbilical arterial and venous blood at birth

The strong correlations found between umbilical arterial and venous blood exhibit higher values for the conjugated steroids, which might be ascribed to a higher resistance of steroid conjugates to further metabolism (Table 5). In general, the steroid catabolites correlate more than the bioactive steroids and their precursors, despite the pronounced AVD in some steroids. These strong correlations indicate that steroid levels in UA, UV and most probably also in the UM can be derived from each other.

Conclusions

In conclusion, steroids in umbilical arterial and venous blood have very different concentrations, ranging from picomolar levels for unconjugated 5-androstene-3β,7α/β, 17β-triols and unconjugated 5α/β-reduced-androstanes, to micromolar levels for progesterone and for polar conjugates of Δ^5 steroids, estriol, and some 5α/β-reduced-pregnanes.

Most unconjugated steroids exhibit pronounced AVD, with positive ones indicating a fetal origin of the respective steroids, while negative ones indicate their placental or perhaps also maternal origin. AVD for the steroid conjugates are less pronounced when compared to unconjugated steroids, probably due to greater stability of the former substances in the circulation and placenta.

The majority of steroids positively correlate with GA, with the exception of unconjugated 5β-reduced pregnanes, which exhibit negative correlations with GA most probably due to the inhibition of AKR1D1 activity by Δ^4 steroids and estrogens (Byrns 2011). Furthermore, testosterone and its precursor androstenediol (being consumed for the placental synthesis of estrogens) also negatively correlate with GA, most probably due to rising placental CYP19A1 activity with increasing GA at constant activities of HSD3B1 and STS. Except for 5α-dihydroprogesterone that shows significantly higher levels in UA during the group C period (like its precursor progesterone), the levels of unconjugated 5α-reduced-pregnanes are independent of the GA. Some AVD also depend on GA, which might indicate a physiological importance of these changes as discussed above.

In spite of pronounced AVD in some steroids, generally strong correlations between steroid levels in UA and UV indicate that they can be accurately derived from each other, and most probably also from steroid levels in UM. Sampling UM blood only is more convenient than the complicated blood sampling separately from UA and UV, and would be particularly appropriate in very premature labors, where the diameter of the umbilical cord is low (see Fig. 1 and 2).

Conflict of Interest

There is no conflict of interest.

Acknowledgements

Grant IGA NT 12211-5/2011 from the Czech Ministry of Health supported the study.

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