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Characterization of human fetal cord blood steroid profiles in relation to fetal sex and mode of delivery using temperature-dependent inclusion chromatography and principal component analysis (PCA)

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Abstract

In the present work, human male and female fetal cord blood samples were purified, selectively extracted and separated to examine a fraction of steroids ranging from polar estetrol to relatively non-polar progesterone using solid phase extraction based on C-18 tubes and β-cyclodextrin driven temperature dependent inclusion chromatography. Resulting UV diode array chromatographic patterns revealed the presence of 27 peaks. Chromatographic patterns of UV detected steroids were analyzed using principal components analysis which revealed differences between male/female and labour/not-in-labour clusters. Quantitative analysis of nine identified steroids including: estetrol, 17β-estradiol, estrone, estriol, cortisol, cortisone, progesterone, 20α-hydroxyprogesterone and 17α-hydroxyprogesterone were not significantly different between males and females. Significant differences between male and female fetuses were related to as yet unidentified compounds. Four peaks were significantly different with labour which corresponded with cortisol, cortisone and two unidentified compounds. This protocol may distinguish significant differences between clinical groups that are not readily identifiable using univariate measurements of single steroids or different low molecular mass biomarkers. Moreover, we have provided new evidence that despite the absence of testosterone there are number of steroids and low molecular mass compounds that differ between male and female fetuses.

Keywords: Human pregnancy; Fetal sex; Labour; Cord blood; Chromatography; Principle component analysis

1. Introduction

The sex of the human fetus is rarely considered in assessing the health of a pregnancy even though there are sexually dimorphic differences in morbidity and mortality. The male fetus is at greater risk of a poor outcome than the female fetus in association with complications such as placental insufficiency, intra-uterine growth restriction and pre-term delivery [1]. A Norwegian study that examined 1.7 million singleton births reported that preterm delivery and perinatal mortality was 21% greater in the male sex [2]. The presence of maternal asthma during pregnancy can result in a number of detrimental outcomes for the male fetus including intrauterine growth restriction and stillbirth [3–6]. This work highlights that there are significant differences in male and female fetal physiology and sexually dimorphic mechanisms associated with the response to a maternal stress.

The physiological differences between male and female fetuses may be mediated by differences in sex steroid concentrations and the metabolites of these steroids. Previous studies have indicated that testosterone concentrations in the maternal circulation during pregnancy [7] and in the cord blood [8] at delivery were higher when the fetus was a male. Sex-specific differences in estradiol concentrations are controversial with levels reported to be higher in male than female cord blood [8] or higher in female cord blood [9] and others report no difference [10]. There are no reported fetal sex differences in progesterone concentrations in the maternal or fetal circulation [11]. These studies used radioimmunoassay (RIA) techniques to measure the maternal and fetal cord blood steroids. RIAs are of high sensitivity and simple to perform. However, a major disadvantage

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of RIA techniques is cross reactivity. For example, a typical progesterone assay kit is affected by up to 10% cross reactivity from particular steroids including 5α-pregnane-3,20-dione, 11-deoxycorticosterone or 17α-hydroxyprogesterone. These cross reactive steroids are of similar size, three-dimensional structure and have other physico-chemical properties such as molecular weight, dipole moment or polarizability [12]. Moreover, RIA can only focus on the determination of a limited number of known compounds and cannot give information about as yet unidentified compounds present in biological samples. Such compounds can play an essential role in the characterization of biological samples.

Presently, information about a wide range of unknown compounds can be obtained with methods such as separation science techniques. The common separation techniques for quantification and identification of steroids other than RIA methods include gas chromatography (GC), thin-layer chromatography (TLC), over-pressured planar chromatography (OPLC), high-performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC) and capillary electrophoresis (CE). However, the diversity of steroid structures and their range of polarities present special problems for the separation and simultaneous analysis of steroids in one sample using both classical isocratic and gradient chromatographic methods. In our previous studies, we reported a simple strategy for the optimization of the isocratic separation of steroids using temperature as the critical parameter for selectivity when the mobile phase is modified with an inclusion agent [13]. Our results revealed that steroids including progesterone, testosterone, cortisol, cortisone as well as number of estrogens and progesterone derivatives can be efficiently and robustly separated under subambient and ambient temperatures using low carbon loaded C-18 column and an inexpensive macrocyclic mobile phase modifier: β-cyclodextrin. More recently we have optimized a solid phase extraction technique based on octadecylsilane tubes and binary methanol–water solvents that allowed us to isolate and concentrate a range of steroids of varying polarities from biological fluids [14]. The steroids were then separated and quantified at low nanogram levels using an isocratic HPLC method based on the temperature dependent inclusion chromatography [14].

Using this technique, the aim of the present study was to determine if there are differences in the identified steroid profiles of plasma from cord blood of male and female fetuses at the time of delivery and in the presence and absence of labour. Moreover, using multivariate statistical methods particularly principal component analysis we also tried to assess the number of unknown peaks detected from the extracted steroids fraction as potential biomarkers that may determine the differences between male and female fetuses.

2. Experimental

2.1. Clinical subjects

Umbilical vein blood was collected following delivery from normal term pregnancies with informed consent and approved by the Hunter Area Health and The University of Newcastle human ethics committees. Blood was collected in lithium/heparin tubes and centrifuged at 2500 rpm for 15 min. Plasma was collected and frozen in 2 mL aliquots at −20 °C until required. Samples were collected from pregnancies with male ($n = 12$) and female fetuses ($n = 12$) that were delivered by elective Caesarean section with no labour (females $n = 4$, males $n = 6$) or spontaneous vaginal delivery (females $n = 8$, males $n = 6$).

2.2. Chromatography

2.2.1. Chromatographic standards

Estriol, 17β-estradiol, estrone, progesterone, 17α-hyroxyprogesterone (4-pregnen-17α-ol-3,20-dione), 20α-hydroxyprogesterone (4-pregnen-20α-ol-3-one), cortisone, cortisol, 7,8-dimethoxyflavone and β-cyclodextrin, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Estetrol and testosterone were products of Steraloids Inc. (Newport, RI, USA) and AppliChem GmbH (Darmstadt; Germany), respectively. Acetonitrile 99.7% HPLC grade was purchased from APS Ajax Finechem (NSW, Australia) and used as received. Sodium nitrate was obtained from a commercial supplier. Water was purified by double distillation.

2.2.2. Solid phase extraction

Raw biological samples (cord blood) were purified and concentrated using Supelclean^{TM} LC-18 SPE Tubes (6 mL, 0.5 g, Cat. no: 57054) obtained from Supelco, Bellefonte, PA, USA. Extraction procedure was optimised for efficient deproteinisation, purification and high recovery of wide range of steroids including estetrol, estriol, cortisol, testosterone, cortisone, 17β-estradiol, estrone, 20α-hydroxyprogesterone, 17α-hydroxyprogesterone, progesterone as well as internal standard (7,8-dimethoxyflavone). Cord blood samples (2 mL) were mixed with internal standard (1 μg/sample) and deproteinised using 4 mL of 100% methanol. Afterwards the supernatant was diluted with 36 mL of distilled water for a remaining methanol concentration of 10%. Washing solvent for SPE tubes was composed of 30% (v/v) methanol in water (5 mL). As an elution medium 2 mL of pure methanol was applied. Extracted samples were evaporated under vacuum at room temperature and reconstituted in 100 μl of 35% acetonitrile/water mixture. Average recovery of components of interest was 95.9%.

2.2.3. High-performance liquid chromatography

Chromatographic separation was carried out with the Supelcosil LC-18 (Cat. no.: 58298) HPLC analytical column that was obtained from Supelco Inc. (Bellefonte, PA, USA). The liquid chromatograph consisting of an analytical solvent pump (Programmable Solvent Module 126), UV-Vis spectrophotometer (Diode Array Detector Module 168) and System Gold (32 Karat^{TM} software; version 5.0) were products of Beckman Instruments Inc. (San Ramon, CA, USA). A Beckman 210A injection valve and a 20 μL loop were used for sample introduction.

Isocratic separations of steroid standards and cord blood extracts were done at 29 °C using a 30% (v/v) acetonitrile–water mobile phase modified with β-cyclodextrin at a concentration

of 12 mM. Due to the detection technique sodium nitrate was used for the determination of chromatographic system hold-up time, following the methods described elsewhere [1]. The column temperature was controlled with an accuracy of ±0.01 °C using an Alltech Water Jacket (Alltech Associates Inc., Deerfield, IL, USA) connected to a Polystat (Cole Parmer Instrument Co., Chicago, IL) digital circulating thermostat. The flow rate of the mobile phase was set at 1 mL min^{-1}. The chromatographic column and column inlet filter (Knauer, Berlin, Germany) were thermostated at least 1 h before the separation in order to obtain a proper temperature equilibrium.

2.3. Data analysis

All data analyses were performed with trial version of the XLSTAT software and the STATA statistical package with the help of the graphical capabilities of S plus. The levels of the chromatographic peaks were checked for normality using the norm test in STATA. This is appropriate for small samples. Median levels of individual peaks were compared in males and females. We assessed the appropriateness of factor analysis for the data by performing Bartlett's test of sphericity.

A principal components analysis (PCA) was then used to reduce the dimensions of the data matrix to fewer uncorrelated variables. The PCA method allows for data reduction and to determine latent information from the raw data set. PCA provides an approximation of an initial data matrix in terms of the product of two small matrices. These matrices may capture the essential data patterns of initial large raw data set. These principal components were then plotted against one another to see whether any obvious clusters could be identified corresponding to pre-specified clinical groups. We then inspected the composition of each principal component. Multiple comparisons were dealt with by Dunnett's test. The PCA technique has previously been tested in a number of chromatographic studies [15–18].

3. Results

Using our SPE protocol, a fraction of low molecular mass substances ranging from polar estrogens to relatively non-polar progesterone was extracted from cord blood samples [14]. A typical UV pattern of the extract separated under temperature-dependent inclusion chromatographic conditions involving β-cyclodextrin as the macrocyclic selector was composed of 27 chromatographic peaks and was observed in both male and female fetuses (Fig. 1). As can be seen from the top and middle section of the diode array profile more than 80% of the detected substances were eluted within the first 10 min and the total analysis time did not exceed 43 min (bottom part of

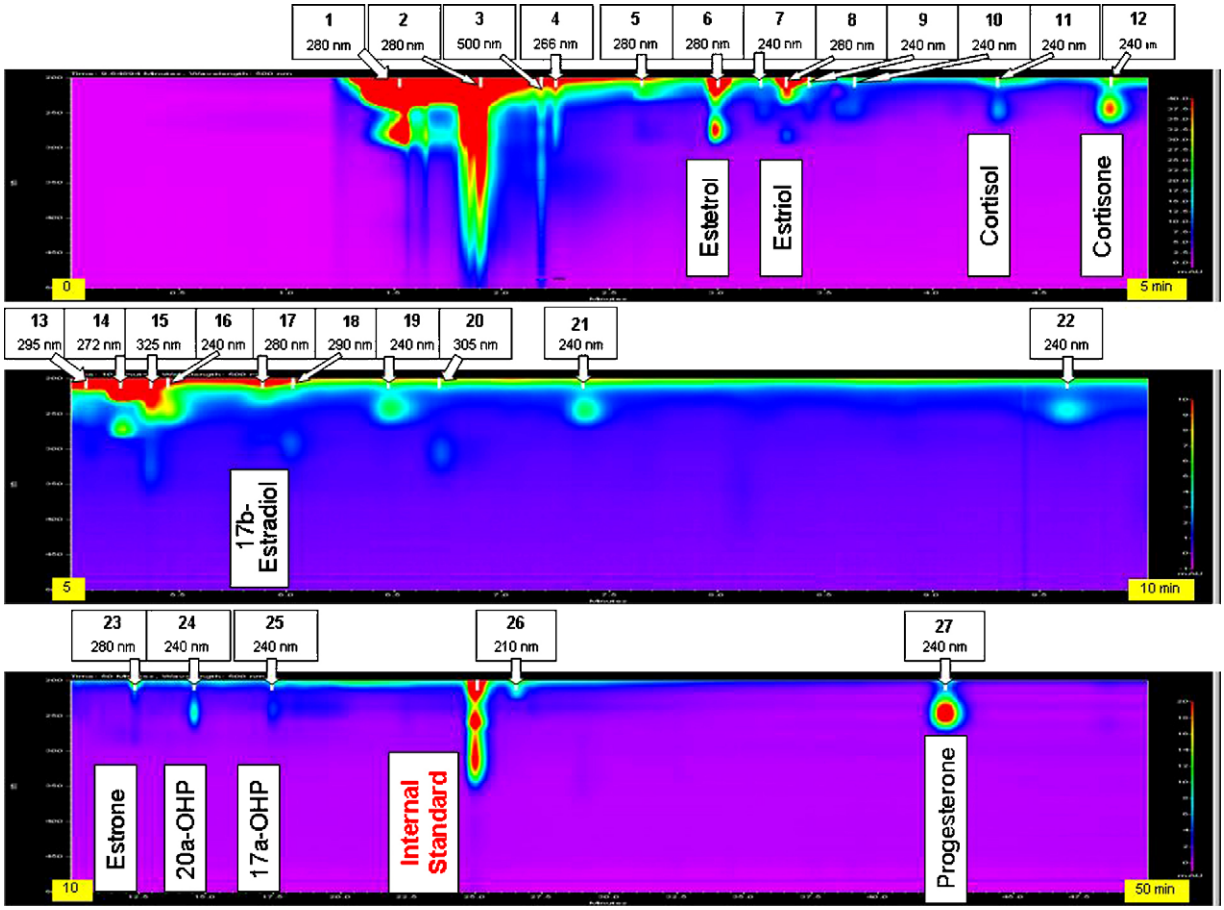

Fig. 1. Typical chromatogram of cord blood extract obtained from UV-Vis diode-array detector (λ = 200–500 nm) in presence of β-cyclodextrin in mobile phase. Selected regions correspond to the retention times 0–5 min (top), 5–10 min (middle) and 10–50 min (bottom).

Table 1
Comparison of chromatographic peak means and medians in cord blood of female and male fetuses in the presence and absence of spontaneous labour

Chromatographic peak [retention time (min)]	Male ($n=12$)		Female ($n=12$)		Non labour ($n=10$)		Labour ($n=14$)	
	Mean	Median	Mean	Median	Mean	Median	Mean	Median
1 [1.55]	2.699	2.819	3.696	3.508	3.489	3.333	2.913	3.100
2 [1.89]	*16.812	*13.904	*6.521	*5.841	11.383	14.246	9.834	9.824
3 [2.18]	0.249	0.273	0.300	0.298	0.277	0.240	0.274	0.299
4 [2.25]	0.100	0.000	0.170	0.133	0.000	0.179	0.000	0.104
5 [2.65]	*0.293	*0.251	*0.066	*0.057	0.181	0.225	0.134	0.147
6 [2.99] Estetrol	3.582	3.264	1.326	1.109	1.609	2.166	1.503	2.660
7 [3.21]	0.047	0.040	0.075	0.070	*0.019	*0.023	*0.079	*0.089
8 [3.32] Estriol	0.470	0.466	0.513	0.499	0.522	0.552	0.416	0.448
9 [3.43]	0.396	0.184	0.437	0.269	0.250	0.604	0.194	0.282
10 [3.63]	0.168	0.153	0.240	0.202	0.174	0.195	0.190	0.210
11 [4.30] Cortisol	0.215	0.159	0.186	0.168	*0.103	*0.111	*0.193	*0.265
12 [4.82] Cortisone	1.089	1.070	1.239	1.264	*0.739	*0.791	*1.406	*1.430
13 [5.09]	0.018	0.016	0.020	0.019	0.014	0.014	0.020	0.021
14 [5.31]	0.207	0.183	0.206	0.205	0.183	0.214	0.205	0.201
15 [5.37]	*0.182	*0.168	*0.102	*0.094	0.158	0.161	0.123	0.128
16 [5.44]	0.533	0.294	0.498	0.317	0.577	0.773	0.265	0.331
17 [5.89] 17β-Estradiol	0.049	0.040	0.039	0.039	0.045	0.050	0.035	0.040
18 [6.02]	*0.161	*0.149	*0.083	*0.079	0.119	0.147	0.100	0.104
19 [6.48]	0.107	0.107	0.104	0.102	*0.056	*0.067	*0.122	*0.133
20 [6.71]	*0.226	*0.222	*0.123	*0.127	0.176	0.198	0.153	0.158
21 [7.38]	0.431	0.364	0.357	0.311	0.329	0.489	0.340	0.326
22 [9.62]	0.052	0.043	0.075	0.072	0.043	0.061	0.063	0.066
23 [12.32] Estrone	0.027	0.025	0.031	0.031	0.025	0.027	0.028	0.030
24 [14.56] 20α-Hydroxypropyl progesterone	0.225	0.175	0.256	0.252	0.169	0.227	0.191	0.250
25 [17.49] 17α-Hydroxypropyl Progesterone	0.098	0.098	0.103	0.098	0.083	0.103	0.103	0.099
26 [26.56]	0.196	0.192	0.227	0.235	0.194	0.208	0.212	0.215
27 [42.50] Progesterone	0.961	0.849	1.141	1.050	0.813	0.841	1.060	1.200

The quantitative data is a ratio of the raw chromatographic peak to the internal standard peak.
* Values significantly different between clinical groups investigated; Significant values are $P<0.05$.

the chromatogram on Fig. 1). All of the peaks were well separated, especially the peaks corresponding to important known steroids which included 17β-estradiol, progesterone, estetrol, estriol, estradiol, cortisol and cortisone.

The raw data matrix consisted of 648 data points made up of 27 variables (all chromatographic peaks recorded) and 24 objects (individual cord blood samples). All selected peaks were present in all the study subjects and their relative intensities were determined using an internal standard method involving 7,8-dimethoxyflavone as the quantitative marker. In the control chromatogram, in which appropriate volume of water instead of plasma was extracted consists of three "background interfering" peaks having retention times corresponding to peaks number 1, 3 and 26 on plasma chromatogram presented in Fig. 1.

Table 1 shows the mean and median values of the variables according to fetal sex and labour status. Quantitative analysis of nine identified steroids including: estetrol, 17β-estradiol, estrone, estriol, cortisol, cortisone, progesterone, 20α-hydroxyprogesterone and 17α-hydroxyprogesterone were not significantly different between males and females. Significant differences between male and female fetuses were related to as yet unidentified compounds corresponding to peaks 2, 5, 15, 18 and 20. Despite the excellent peak base separation of the testosterone standard from cortisol and cortisone, this steroid was not detectable in male or female fetal cord blood due to a detection limit of the diode array UV detector corresponding to a testosterone concentration in plasma of 0.75 pmol/mL.

Four peaks were significantly different with labour (Table 1). Two peaks corresponded with cortisol and cortisone which were increased with labour (Table 1). Peak 7 and 19 were unidentified compounds that were also increased with labour.

The starting point for PCA investigations was the raw data matrix listed above from which the appropriate standardized version and correlation matrix were calculated. The number of principal components characterizing our data set was determined by considering the eigenvalues. The following sequence of eigenvalues greater than 1 was calculated: 8.056; 5.720; 3.944; 1.847; 1.656 and 1.150. Therefore, instead of the Kaiser criterion, in which only factors with eigenvalues greater than 1 are retained, the Factorial Scree Test (Cattell Test) was used to graphically determine the optimal number of factors to retain. According to this criterion, the first three factors were selected and these explain over 65.6% of total variability. Particularly, factors 1, 2 and 3 account for 29.8, 21.2 and 14.6% of the variance. Graphs presented in Fig. 2 show the PC score plots for the objects investigated. It can be seen that male and female groups are clearly separated along *Y*-axis (Fig. 2A). There is also significant separation between labour and non labour groups however this clustering involves more than one factor (Fig. 2B). Such object separation on the principal component graphs indicates

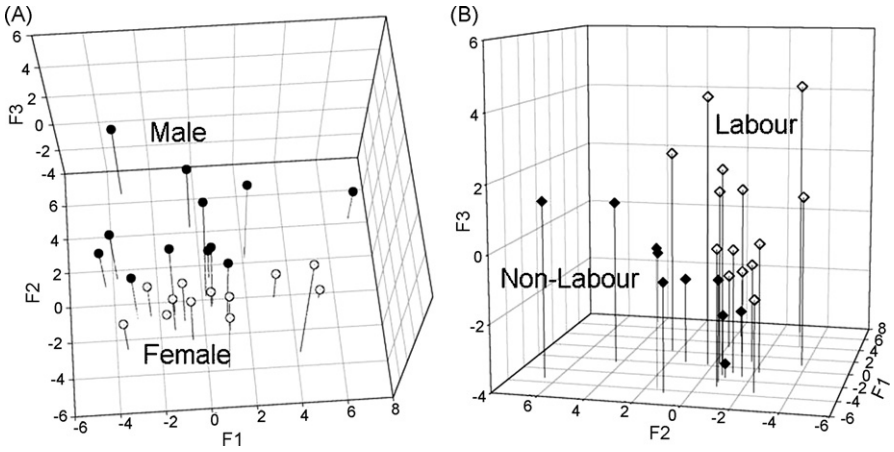

Fig. 2. Principal component 3D-plots showing relationships between objects investigated in respect to 1, 2 and 3 factor scores (*X* data, *Y* data and *Z* data axis, respectively). Graph (A) depicts the analysis based on fetal sex with males as black dots and females as open circles. Graph (B) depicts the analysis based on the presence and absence of labour (open diamonds for spontaneous labour and black diamonds for no labour).

that the chromatographic pattern of UV detected substances extracted from cord blood contains key information that may reveal the differences between important clinical groups. To investigate which variables may be responsible for the clinical group clustering, the factor loadings data was analyzed. According to data presented in Table 2 the high values of factor loading for 22 variables including all identified steroids suggest that they impart the most important information. The remaining five variables (No 1, 3, 4, 14 and 26) are characterized by low values of factor loadings. Interestingly this group includes all the variables corresponding to background interfering peaks (No 1, 3 and 26). This observation reveals that those variables may not contribute to the object clustering that was observed on Fig. 2A and B.

Table 2
Factor loading values

Variable no.	F1	F2	F3
(1) (background interfering peak)	0.275	−0.215	−0.505
(2)	−0.216	*0.717	0.377
(3) (background interfering peak)	0.264	−0.372	0.222
(4)	0.052	0.111	−0.059
(5)	−0.183	*0.733	0.385
(6) (estetrol)	0.182	0.282	*0.621
(7)	0.130	−0.655	*0.551
(8) (estriol)	*0.876	0.223	−0.181
(9)	*0.737	0.297	−0.377
1(0)	*0.897	−0.121	−0.205
(11) (cortisol)	0.028	−0.370	*0.700
(12) (cortisone)	*0.618	−0.403	*0.596
(13)	*0.595	−0.425	*0.549
(14)	−0.076	0.442	0.172
(15)	−0.042	*0.818	0.403
(16)	*0.661	0.464	−0.392
(17) (17β-estradiol)	*0.542	*0.553	0.012
(18)	0.172	*0.874	0.259
(19)	*0.606	−0.334	*0.635
(20)	0.233	*0.701	0.246
(21)	*0.769	0.428	−0.222
(22)	*0.929	−0.107	−0.209
(23) (estrone)	*0.611	−0.187	0.112
(24) (20αOHProgesterone)	*0.854	0.050	-0.034
(25) (17αOHProgesterone)	*0.873	0.092	−0.092
(26) (background interfering peak)	−0.018	−0.477	−0.497
(27) (progesterone)	*0.647	−0.333	0.231

These are variable numbers corresponding to chromatographic peak on Fig. 1
* Factor loading values higher than 0.5.

4. Discussion

The use of chromatographic quantification of low molecular UV non-transparent substances provides an informative view of changes of steroidal and similar size compounds in biological fluids. In combination with robust statistical analyses there is the opportunity to distinguish significant differences between groups that not readily identifiable using univariate measurement such as a single RIA determination. These studies indicated that known steroids do not vary with fetal sex but a number of unknown compounds change significantly with the sex of the fetus. Unfortunately, testosterone was not detectable using this particular HPLC technique as the concentration of this sex steroid was below the limits of detection in both male and female cord blood samples. We subsequently identified that testosterone was present in our samples using a commercial RIA. Our study found that cortisol and cortisone were increased with spontaneous labour and these steroids have previously been reported to change in cord blood with labour using RIA [19,20] indicating that our findings are comparable to previous studies.

Sex specific differences in adult physiology are well characterized and include differences in cardiovascular function, stress response and metabolism [21,22]. These differences are mediated by the sex steroids: testosterone, estrogen and progesterone [22]. Sex specific differences in children are less well characterized but relate predominantly to differences in cognitive and motor function and have been proposed to be programmed *in utero* [23]. More recent studies have identified sex-specific differences in growth and fetal response to a maternal stress in pregnancies complicated by asthma [6].

There are very few studies that have identified the physiological mechanisms that control the differences between male and female fetuses [5,6,24]. It is assumed that testosterone has an important role in regulating the difference between human

male and female fetuses but the actual mechanisms have not been studied in the human. Proteomic studies from our laboratory have identified there are a number of cord blood and placental proteins that are significantly different between male and female fetuses [25]. Our present research suggests there are also a number of unidentified low-molecular mass compounds that are altered between male and female fetuses. These data indicate there are a range of steroids that may be central in determining sex-specific differences in male and female fetuses.

Diagnostically, this data has provided a potential technique for the identification of other complications during pregnancy using maternal serum, amniotic fluid or cord blood. For example the accurate detection of Down's syndrome and congenital adrenal hyperplasia could be defined using a steroid profile in maternal serum.

5. Conclusions

The use of temperature-dependent inclusion chromatography for separation of cord blood extracts provides the opportunity for steroids and low molecular mass compounds of differing polarities to be robustly profiled in biological samples and provides a great deal of information on a physiological situation without the need to perform multiple, individual assays. Based on principal components analysis these studies confirm that labour is associated with changes in a number of steroids and provide new evidence that despite of testosterone there are specific low molecular mass compounds that differ between male and female fetuses.

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