Mass spectrometry methods measured androgen and estrogen concentrations during pregnancy and in newborns of mothers with polycystic ovary syndrome

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Abstract

Objective: Little is known about the aetiology of polycystic ovary syndrome (PCOS). Some suggest that elevated maternal androgens during gestation play a causative role. This implies placental passage of androgens during pregnancy. The aim of this study is to compare androgen and estrogen concentrations in maternal serum during pregnancy and in umbilical cord blood, between mothers with PCOS and their offspring compared to controls.

Design: Prospective case–control study.

Methods: Maternal blood samples were collected around 20 weeks of gestation and at delivery. Umbilical cord blood was also taken at delivery. Androgens (testosterone (T), androstenedione (ADION), dehydroepiandrosterone (DHEA)) and estrogens (estrone (E₁), estradiol (E₂), estriol (E₃)) were measured using the liquid chromatography tandem mass spectrometry (LC-MS/MS) methods.

Results: At 20 weeks of gestation: T ($P=0.019$) and ADION ($P=0.034$) were higher in the PCOS mothers (pregnant with a girl), whereas DHEA, E₁, E₂, and E₃ were not different. Maternal concentration at birth: T ($P=0.004$) and ADION ($P=0.009$) were also higher in the subgroup of PCOS mothers that were pregnant with a girl compared to the girl pregnancy controls. DHEA, E₁, E₂ and E₃ were not different. In umbilical cord blood, no differences were found for T, ADION, DHEA, E₂, E₃, and AMH between the PCOS mothers and the controls respectively. E₁ was lower in girls from PCOS mothers ($P=0.007$).

Conclusions: Despite elevated maternal androgen concentrations during pregnancy in PCOS mothers, offspring showed no signs of elevated androgen concentrations in cord blood at birth using the latest highly specific LC-MS/MS methods.

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Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disorder affecting ~5–15% (1, 2, 3, 4, 5) of women in their reproductive lifespan according to the criteria used. PCOS is characterized by at least two of the following criteria: oligo- or amenorrhoea, signs of

hyperandrogenism (acne, hirsutism and/or biochemical hyperandrogenemia); and/or polycystic ovaries determined by ultrasound (6). The syndrome is associated with fertility problems, an increased risk of insulin resistance (type 2 diabetes) and cardiovascular disease (1, 2).

PCOS is a complex genetic disease and the phenotype is likely the result of the interplay of variation in a number of different genes with environmental factors. Twin (7) and genetic studies (8, 9, 10, 11) show high heritability of PCOS, and particularly hyperandrogenism (12). The pathophysiology of PCOS is still poorly understood. From animal studies it is known that prenatal exposure to supraphysiological androgen concentrations in mothers leads to PCOS phenotypes in the offspring (13, 14, 15). In addition, in cultured mice cells, androgens seem to stimulate growth of pre-antral and small follicles. In primate ovaries, androgens seem to enhance FSH receptor-expression (16, 17, 18). Whether this is the case in humans as well remains debatable.

The testosterone transfer theory suggests that androgens from the male in opposite-sex twins lead to masculinisation of the female co-twin (19). Androgen exposure in opposite-sex twin girls has been associated with reduced fecundity (20). However, Medland *et al.* (21) could not confirm this theory based on their observations. Furthermore, the prevalence of PCOS is not different in girls from opposite-sex compared to dizygotic girl-girl twins (22).

In relation to androgens in PCOS, the question remains whether prenatal exposure to androgens could cause PCOS and even more importantly can maternally elevated androgens play a role? There are studies that suggest that maternal androgens from PCOS women are passed on to their offspring during gestation through placental passage and could possibly contribute to higher androgen concentrations during gestation and at birth in PCOS offspring. Others speculate that more androgens are synthesized endogenously by the fetus of PCOS offspring (12, 23, 24, 25, 26). The aim of our study is to compare androgen and estrogen concentrations in maternal serum during gestation and in umbilical cord blood, between mothers with PCOS and their offspring compared to non-PCOS mothers and their offspring, using highly specific liquid chromatography tandem mass spectrometry (LC-MS/MS) methods.

Subjects and methods

The study population

Subjects were part of a large prospective cohort study that aimed to compare perinatal reproductive endocrinology in singleton vs twin pregnancies (27), performed between 2004 and 2009. Maternal reproductive hormones were measured during pregnancy as well as at time of delivery and from the neonates in umbilical cord

blood. We identified 20 PCOS mothers and 83 matched controls. PCOS was diagnosed according to the Rotterdam criteria (4). Clinical signs of hyperandrogenism were assessed by the presence of hirsutism (Ferriman-Gallwey score >8) (28) and/or acne. The controls were women pregnant after natural conception who had a regular menstrual cycle (<35 days) and no signs of androgen excess (hirsutism and/or acne). Controls were matched by age, parity and gestational age at delivery. The study was approved by the VU Medical Center ethical committee and written informed consent was obtained from all participants.

Blood samples

Maternal venous serum samples were taken around 20 weeks (18–22 weeks) of gestation and during labour. Umbilical cord blood was sampled immediately after delivery of the placenta (mixed arterial and venous blood). Blood samples were centrifuged (for 10 min, 3000 revolutions/min) and stored at -20°C degrees.

Samples were analysed for estrogens (estrone (E_1), estradiol (E_2), and estriol (E_3)) and androgens (testosterone (T), androstenedione (ADION), and dehydroepiandrosterone (DHEA)) using the LC-MS/MS methods at ARUP Laboratories (Salt Lake City, UT, USA). Samples were transported to ARUP Laboratories on dry ice. T, E_1 , E_2 , E_3 , hydroxylamine, formic acid, trifluoroacetic acid, dansyl chloride and sodium carbonate were purchased from Sigma Aldrich. ADION, DHEA were purchased from Steraloids, Inc. (Newport, RI, USA). The internal standards were deuterium labeled analogs of the steroids; d_3 -T was purchased from Cambridge Isotope Laboratories (Andover, MA, USA); d_4 - E_1 , d_3 - E_2 , d_3 - E_3 , d_7 -ADION and d_2 -DHEA were purchased from CDN Isotopes (Toronto, ON, USA). All other chemicals were of the highest purity commercially available. Samples were analyzed using the liquid chromatography tandem mass spectrometry methods (LC-MS/MS) as previously described (29, 30, 31, 32). Briefly, steroids were extracted from the samples. DHEA, ADION and T were derivatized with hydroxylamine to form oxime derivatives and E_1 , E_2 and E_3 were derivatized with dansyl chloride to form dansyl derivatives (29, 32). The lower limit of quantification (LOQ) for estrogens (E_1 , E_2 and E_3) was 1 pg/ml, for DHEA 0.05 ng/ml and 0.01 ng/ml for testosterone and ADION. The intra- and inter-assay coefficients of variation (CV) were <8% and <11% respectively. All steroids were analyzed in positive ion mode using electrospray ion source on a triple quadrupole mass spectrometer (API4000;

AB Sciex, Foster City, CA, USA). The HPLC system consisted of series 1260 HPLC pumps (Agilent Technologies, Santa Clara, CA, USA) and a HTC PAL autosampler (LEAP Technologies, Carrboro, NC, USA) equipped with a fast wash station. The quadrupoles Q1 and Q3 were tuned to unit resolution and the mass spectrometer conditions were optimized for maximum signal intensity of each steroid. Two mass transitions were monitored for each steroid and its internal standard (33). Quantitative data analysis was performed using Analyst 1.5.2 Software (AB/Sciex, Concord, Ontario, Canada). Calibration curves were generated with every set of samples using six calibrators and three quality control samples were included with every set of samples. Specificity of the analysis for each steroid in every sample was evaluated by comparing concentrations determined using the primary and the secondary mass transitions of each steroid and its internal standard.

Anti-Müllerian Hormone (AMH) was measured using GEN II AMH elisa (Beckman Coulter) with a lower limit of detection of 0.2 µg/l (intra-assay CV: 7–10%, inter-assay CV: 8%).

Statistical analysis

All statistical procedures were performed using SPSS version 20.0 (SPSS, Inc.). Baseline characteristics are reported as means ($\pm$ s.d.) or number (percentages). Independent samples *t*-tests and χ^2 -tests were performed to compare baseline characteristics. Hormone concentrations are reported as mean ($\pm$ s.d.) and the non-parametric Mann–Whitney test was performed to compare the data. Hormone concentrations were positively skewed and therefore logarithmically transformed data were used for the regression analyses. Linear regression analyses were conducted to compare hormone concentrations between the mothers with and without PCOS. Data were corrected for age, BMI, parity, smoking and mode of delivery. A *P* value of <0.05 was considered significant.

Results

Maternal and neonatal baseline characteristics are shown in Table 1.

Concerning the mothers: age, BMI at 20 weeks of gestation, age of menarche, time trying to conceive, ethnicity, smoking status, parity and mode of delivery did not differ between the groups. However, by definition and as a result of our study design cycle duration, mode of conception, acne and signs of hirsutism differed

Table 1 Baseline characteristics. Data are presented as mean ($\pm$ s.d.) or number (percentages) of the group.

Characteristics	PCOS (n=20)	Non-PCOS (n=83)	P value
Mother			
Age (years)	32.0 (4.0)	33.8 (3.9)	0.061
Cycle duration (days)	140.1 (132.8)	27.7 (2.9)	0.002*
BMI (kg/m ²) ^a	24.2 (3.6)	25.1 (4.0)	0.375
Age of menarche (years)	13.3 (2.0)	13.3 (1.6)	0.911
Time trying to conceive (months)	8.3 (9.0)	7 (13.0)	0.698
Mode of conception			0.000*
Natural	5 (25%)	83 (100%)	
Ovulation induction	11 (55%)	0	
IVF	1 (5%)	0	
ICSI	3 (15%)	0	
Ethnicity			0.784
Caucasian	18 (90%)	73 (88%)	
Asian		1 (1.2%)	
Mediterranean	2 (10%)	4 (4.8%)	
Creole		4 (4.8%)	
Other		1 (1.2%)	
Acne			0.000*
Yes	11 (55%)	0	
No	9 (45%)	83 (100%)	
Hirsutism			0.000*
Yes	12 (60%)	0	
No	8 (40%)	83 (100%)	
Smoking			0.216
Yes	9 (45%)	51 (61.4%)	
No	11 (55%)	32 (38.6%)	
Para			0.051
0	6 (30%)	33 (39.8%)	
1	13 (65%)	31 (37.3%)	
> 1	1 (5%)	19 (22.9%)	
Mode of delivery			0.492
Natural	14 (70%)	52 (62.7%)	
Assisted delivery ^b	6 (30%)	31 (37.3%)	
Offspring			
Neonatal sex			0.052
Girl	14 (70%)	38 (45.8%)	
Boy	6 (30%)	45 (54.2%)	
Gestational age at birth (weeks+days)	39+6 (3)	39+4 (2)	0.652
Birth weight (grams)	3552 (451)	3410 (649)	0.385

n, number of subjects; *indicates a significant difference.

^aBMI at 20 weeks of gestation.

^bAssisted delivery includes: cesarean section and ventouse delivery.

significantly between the groups. Ninety percent of the cases demonstrated clinical and/or biochemical signs of hyperandrogenism (45% of the cases clinical and biochemical signs of hyperandrogenism, 40% clinical signs (acne and/or hirsutism), 5% biochemical, 10% none). Most of the women were multiparae and of Caucasian origin. Concerning the offspring: remarkably 70% of the offspring in the PCOS group were girls vs 45.8% in the

control group ($P=0.052$), whereas gestational age at birth and birth weight did not differ between the groups.

Mean hormone concentrations

The mean hormone concentrations in maternal serum (mid-gestation and delivery) and cord blood, according to maternal PCOS status, according to fetal gender are reported in Table 2.

Non-parametric Mann–Whitney test showed similar results compared to the regression analyses; 20 weeks of gestation, pregnant with a girl: T ($P=0.059$), ADION ($P=0.026$), at delivery T ($P=0.003$) ADION ($P=0.005$), umbilical cord T ($P=0.31$), ADION ($P=0.057$), E_1 ($P=0.007$). Regression analyses were performed to rule out confounding.

Regression analyses

Linear regression analyses according to fetal gender were conducted with hormone concentrations used in the model as dependent variables and PCOS/non-PCOS as the independent variable (Table 2). The model was tested with age, BMI, smoking, parity and mode of delivery as possible confounders.

Maternal serum mid-gestation

Around 20 weeks of gestation PCOS mothers pregnant with a girl have significantly higher ADION and T concentrations compared to non-PCOS mothers pregnant with a girl. In boys ADION and T also tend to be higher.

Maternal serum at delivery

In accordance to mid-gestational concentrations, PCOS mothers pregnant with a girl show significantly higher ADION and T concentrations compared to the non-PCOS mothers. However, E_1 was significantly higher in the PCOS mothers pregnant with a boy, ADION and T tend to be higher.

Cord blood

Androgens in cord blood did not differ significantly between daughters from the PCOS and the non-PCOS mothers. E_1 was significantly lower in girls from the PCOS mothers and E_2 also tends to be lower. Cord blood from boys showed comparable estrogen and androgen concentrations between the PCOS and the non-PCOS mothers. Concentrations of AMH in girls and boys did not differ between the PCOS and the non-PCOS group.

Table 2 Mean hormone concentrations according to fetal gender. Hormone concentrations at 20 weeks of gestation and delivery in maternal serum and in cord blood, according to fetal gender and PCOS status of the mother. Data are presented as mean ($\pm$ s.d.).

	Girls (n=52)				Boys (n=51)			
	PCOS (n=14)	Non-PCOS (n=38)	β	P value	PCOS (n=6)	Non-PCOS (n=45)	β	P value
Mother mid-gestation								
E_1 (pg/ml)	3889.3 (1565)	4469.4 (2513)	−0.017	0.928	5433.3 (2823)	3728.7 (2079)	0.319	0.061
E_2 (pg/ml)	6441.4 (1909)	6942.2 (3833)	−0.113	0.559	7250 (2441)	6074.4 (2105)	0.158	0.300
E_3 (pg/ml)	2213.6 (491)	2106.2 (628)	−0.042	0.815	2345 (458)	1972.9 (627)	0.232	0.139
ADION (ng/ml)	2.13 (1.49)	1.08 (0.43)	0.365	0.034*	2.60 (2.67)	1.24 (0.81)	0.298	0.084
DHEA (ng/ml)	3.12 (1.44)	2.76 (1.25)	0.048	0.788	4.18 (4.79)	2.58 (1.3)	0.071	0.654
T (ng/ml)	1.04 (0.80)	0.51 (0.22)	0.391	0.019*	1.15 (1.04)	0.56 (0.32)	0.279	0.113
Mother delivery								
E_1 (pg/ml)	9786.7 (6111)	8845.3 (6929)	0.074	0.690	13625 (3754)	7454.5 (4834)	0.500	0.015*
E_2 (pg/ml)	8184.8 (6628)	9882.3 (6884)	−0.005	0.979	13850 (11036)	9460.0 (7084)	0.250	0.208
E_3 (pg/ml)	2342.3 (3034)	4392.9 (4248)	−0.130	0.461	19922.3 (26786)	7843.4 (15971)	0.182	0.341
ADION (ng/ml)	4.22 (3.17)	1.62 (0.71)	0.513	0.009*	4.48 (5.12)	2.04 (1.7)	0.112	0.537
DHEA (ng/ml)	9.11 (7.70)	6.85 (3.76)	0.199	0.360	5.16 (4.81)	6.09 (5)	0.014	0.940
T (ng/ml)	2.80 (2.32)	0.91 (0.64)	0.523	0.004*	2.57 (2.83)	1.10 (0.9)	0.078	0.675
Cord blood								
E_1 (pg/ml)	10511.1 (5717)	22025.7 (12366)	−0.552	0.007*	14734.5 (7707)	21123.9 (13559)	−0.065	0.718
E_2 (pg/ml)	5426.1 (2334)	8919.2 (5373)	−0.407	0.052	9601.5 (5044)	9196.4 (6586)	0.095	0.520
E_3 (pg/ml)	57412.1 (19263)	52729.2 (22145)	−0.041	0.836	51568 (10952)	55135.1 (24036)	0.160	0.378
ADION (ng/ml)	0.45 (0.25)	0.84 (1.11)	−0.374	0.094	0.49 (0.17)	0.54 (0.25)	0.036	0.834
DHEA (ng/ml)	4.90 (2.84)	5.65 (6.33)	0.123	0.577	2.98 (0.85)	2.9 (2.5)	0.255	0.162
T (ng/ml)	0.12 (0.04)	0.15 (0.21)	−0.010	0.959	0.18 (0.07)	0.20 (0.12)	−0.091	0.676
AMH (μ g/l)	2.11 (2.42)	1.26 (1.16)	0.178	0.412	46.8 (21.5)	44.31 (30.9)	0.013	0.947

β indicates the regression coefficient and *indicates a statistically significant difference between PCOS and non-PCOS mothers.

Discussion

In this study, we have demonstrated that maternal hormone concentrations of ADION and T are significantly higher throughout pregnancy (mid-gestation and delivery) in PCOS mothers pregnant with a girl compared to non-PCOS mothers.

Remarkably, although the mothers had elevated concentrations of androgens throughout gestation, daughters of PCOS mothers showed no signs of increased androgen concentrations in umbilical cord blood at birth.

Our data do support less the idea that elevated concentrations of androgens in PCOS-mothers affect offspring, also that offspring themselves have increased production of androgens. This challenges data that support that a maternal hyperandrogenic environment in PCOS-mothers contributes to PCOS features in their daughters other than on the genetic basis according to animal studies.

Animal studies suggest that high maternal androgen concentrations during gestation result in PCOS-like phenotypes, in primates (14, 15, 34), sheep (35) and rodents (36). Especially studies by Abbott (13, 14, 15, 34, 37), in prenatally exposed rhesus monkeys, have strongly encouraged the hypothesis that the intra-uterine exposure to androgens can cause clinical and biochemical features of PCOS in later life, though these androgen concentrations are supraphysiological.

However, human data are scarce. Pathological conditions in humans that cause hyperandrogenism, for example, congenital adrenal hyperplasia (38) and studies in transsexual women treated with high doses of testosterone (39, 40, 41, 42) show conflicting outcomes.

The present and previous studies (43, 44, 45, 46) show that elevated androgen concentrations during pregnancy in PCOS women, do not contribute to elevated androgens in cord blood. Remarkably, Anderson and Maliqueo, as we did, also found significantly lower ADION concentrations in cord blood of female offspring of the PCOS women, as compared to the female offspring of non-PCOS women (43, 45). To our knowledge only Barry *et al.* (47) reported elevated T concentrations in cord blood in PCOS daughters compared to controls. Hickey *et al.* (44) even found no evidence for a relationship between prenatal androgen exposure and PCOS in adolescence in normal pregnancies in a large prospective cohort study. Therefore this study and previous studies fail to support the hypothesis that maternal androgen excess contributes to elevated androgen concentrations in cord blood, and therefore the development of PCOS. Although having close genomic

and phenotypic similarity to humans, primates cannot serve as a correct model for the human situation.

During pregnancy maternal androgen excess is unlikely to affect the fetus, because the placenta presents an effective barrier. Placental aromatase rapidly catalyzes the conversion of ADION to E₁, 16-hydroxytestosterone to E₃ and T to E₂ (4448). A recent study aimed to evaluate the placental activities of steroid sulfatase (STS), 3 β -hydroxy-steroid dehydrogenase type 1 (3 β -HSD-1) and P450 aromatase (P450arom) in PCOS compared to normal pregnant women. The data suggest that placental tissue from women with PCOS shows changes in the activities of two important enzymes for steroid synthesis: higher 3 β -HSD-1 and lower P450arom which would indicate increased androgen production during pregnancy (45). In line with our findings, Maliqueo *et al.* (45) found that cord blood of the female offspring from the PCOS mothers did not contain elevated androgens, and even had lower ADION and higher E₃ concentrations (although androgens during pregnancy were elevated). It remains unclear whether placental steroidogenesis is altered in PCOS.

Interestingly, E₁ concentrations differ between PCOS and non-PCOS women and in their offspring as well. E₁ concentrations at birth were significantly higher in PCOS mothers pregnant with a boy compared to controls. In the women pregnant with a girl, they were also higher but non-significantly. However, cord blood E₁ concentrations were lower in daughters of the PCOS women. Limited data are available on E₁ concentrations during gestation (27, 49) and post-partum. Data by Troisi *et al.* (50, 51, 52) demonstrated no gender related differences. Concentrations of E₁ in cord blood did not differ between boys and girls (51, 52). However these data did not report on hyperandrogenism or PCOS prevalence in the mothers. Remarkably Maliqueo *et al.* also found lower E₁ concentrations in PCOS cord blood from daughters compared to controls ($P=0.061$). It is possible that placental steroidogenesis follows different pathways in PCOS mothers which result in a different ratio of estrogen fractions during gestation and in their offspring (45).

Due to the different phenotypes of women with PCOS, not all mothers had biochemically proven hyperandrogenism, which could influence our results. However, 90% of the mothers did have biochemical and/or clinical signs of hyperandrogenism. Furthermore, PCOS mothers had higher androgen concentrations throughout pregnancy, which did not lead to higher androgens in cord blood. This suggests that maternal androgens are not transferred directly to their offspring and challenges the hypothesis that maternal androgens directly cause PCOS in their

daughters. Androgens might have their effect on PCOS offspring in a more indirect manner such as fetal programming. This is in line with twin (7, 22) and genetic (8, 9, 10, 11) studies that demonstrate high heritability of PCOS and particularly hyperandrogenism (12).

Furthermore, it remains questionable how closely cord blood reflects actual fetal androgen exposure during gestation (53). Androgens in girls could have been elevated during gestation and could have decreased at the time of birth resulting in normal androgen concentrations in cord blood. Although maternal hormones are almost unable to pass the placenta (54) or are metabolized along the way, it is not clear to what extent maternal androgens programme the fetus for future endogenous androgen biosynthesis.

Collected cord blood was mixed arterial and venous blood, which was inevitable because of the amount of hormones measured. Although there are indications that concentrations differ between arterial and venous cord blood samples (55, 56), there is sufficient data that measurements in mixed cord blood do not differ significantly (27, 57).

Despite these limitations, this study provides a complete overview of steroid hormone concentrations during gestation and in cord blood of the PCOS vs non-PCOS mothers. Concentrations of estrogens and androgens in this study were determined using highly specific LC-MS/MS methods, a technique that is considered state of the art for measurement of endogenous steroids (58, 59, 60, 61).

Conclusion

In conclusion, we found that daughters of PCOS mothers do not have elevated concentrations of androgens in cord blood despite the fact that their mothers had elevated serum androgen concentrations throughout gestation. This challenges the idea that a maternal hyperandrogenic environment in PCOS-mothers directly contributes to PCOS features in their offspring. Androgen exposure could, however, play a role in programming the fetus for endogenous androgen production later in life.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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