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ORIGINAL ARTICLE ***Reproductive endocrinology***

Placental STAT3 signaling is activated in women with polycystic ovary syndrome

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STUDY QUESTION: Does polycystic ovary syndrome (PCOS) in women without pregnancy complications affect placental signal transducer and activator of transcription 3 (STAT3) and mechanistic target of rapamycin (mTOR) signaling?

SUMMARY ANSWER: Placental STAT3 signaling is activated but mTOR signaling is unaffected in PCOS.

WHAT IS KNOWN ALREADY: Women with PCOS have increased risk of poor pregnancy outcomes (e.g. restricted or accelerated fetal growth), indicating placental dysfunction. Placental STAT3 and mTOR pathways regulate placental function and indirectly affect fetal growth.

STUDY DESIGN, SIZE, DURATION: In a case–control study, placental tissue and maternal blood were collected at delivery from 40 control pregnant women and 38 PCOS women with uncomplicated pregnancy.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Women with PCOS were recruited at two medical centers and pregnant controls were recruited at one of these centers. Placental mRNA expression of genes encoding proteins related to steroid action, metabolic pathways and cytokines was analyzed by quantitative RT–PCR. Phosphorylated placental STAT3 (P-STAT3) and mTOR targets was measured by western blot. Levels of sex steroids in serum were determined by mass spectrometry.

MAIN RESULTS AND THE ROLE OF CHANCE: Placental P-STAT3 (Tyr-705) was increased in women with PCOS ($P < 0.05$) versus controls. Placental mTOR signaling was not affected in PCOS women when compared with controls. Circulating levels of androstenedione, androst-5-ene-3β, 17β-diol, testosterone, 5α-dihydrotestosterone and etiocholanolone glucuronide were higher and estradiol lower in women with PCOS than in controls (all $P < 0.05$). No correlation between sex steroid levels in serum and P-STAT3 was observed.

LIMITATIONS, REASONS FOR CAUTION: Women with PCOS and pregnancy complications were excluded to avoid the confounding effects of placental pathologies, which could modify STAT3 and mTOR signaling. Moreover, 97.4% of women with PCOS in the study displayed oligoamenorrhea at diagnosis. Thus, the current findings could be restricted to PCOS women with the oligo-anovulatory phenotype without pregnancy complications.

WIDER IMPLICATIONS OF THE FINDINGS: Phosphorylation of STAT3 is increased in the placenta from women with PCOS and uncomplicated pregnancies, indicating that specific metabolic placental pathways are activated in the absence of obstetric and perinatal complications.

STUDY FUNDING/COMPETING INTEREST(S): The work was supported by the Swedish Medical Research Council (Project No. 2011-2732 and 2014-2775); Jane and Dan Olsson Foundation, Wilhelm and Martina Lundgrens's Science Fund; Hjalmar Svensson Foundation (E.S.-V and M.M.); Adlerbert Research Foundation; Swedish federal government under the LUA/ALF agreement ALFFGBG-136481 and 429501 and the Regional Research and Development agreement (VGFOUREG-5171, -11296 and -7861). MM thanks the Becas Chile Programme (Chile) and University of Chile for financial support through a postdoctoral fellowship. There are no competing interests.

Key words: polycystic ovary syndrome / pregnancy / hyperandrogenemia / signal transducer and activator of transcription 3 / mechanistic target of rapamycin

Introduction

Polycystic ovary syndrome (PCOS), a multifactorial disease associated with endocrine and metabolic disturbances, affects 4–15% of women of childbearing age (Azziz *et al.*, 2004a; Yildiz *et al.*, 2012). More than 80% of PCOS women have an endocrine imbalance characterized by hyperandrogenism (Azziz *et al.*, 2004a,b). PCOS is associated with obstetrical complications, including gestational diabetes, pre-eclampsia, preterm delivery and offspring small or large for gestational age (Boomsma *et al.*, 2008; Kjerulff *et al.*, 2011; Qin *et al.*, 2013). Circulating androgen levels are elevated in pregnant PCOS women (Sir-Petermann *et al.*, 2002; Falbo *et al.*, 2010), and prenatal exposure of experimental animals to androgens produces endocrine and metabolic alterations in offspring resembling those in PCOS (Abbott *et al.*, 2008; Wu *et al.*, 2010; Padmanabhan and Veiga-Lopez, 2013). In human pregnancy, androgens are metabolized to estrogens by placental P450 aromatase (Thompson and Siiteri, 1974). Thus, the effects induced by testosterone in pregnancy could partly be mediated by estrogen (Padmanabhan and Veiga-Lopez, 2011).

In rodents, prenatal testosterone exposure reduces fetal and placental growth (Sathishkumar *et al.*, 2011; Sun *et al.*, 2012), which in turn have been related to decreased amino acids transfer (Sathishkumar *et al.*, 2011). Moreover, placental expression of estrogen and androgen receptors is increased in prenatally androgenized rats (Sun *et al.*, 2012), suggesting higher placental sensitivity to sex steroids, which may directly or indirectly modulate signaling pathways associated with fetal growth.

Signal transducer and activator of transcription 3 (STAT3) and mechanistic target of rapamycin (mTOR) are important pathways in the regulation of placental nutrient transport and fetal growth (Roos *et al.*, 2007; Maymo *et al.*, 2011). STAT3 is activated by multiple factors, including leptin and cytokines (Aggarwal *et al.*, 2009). mTOR is a central placental signaling pathway acting as nutrient sensor and regulated by an array of diverse signals, such as nutrients, oxygen, cytokines, growth factors and energy levels (Jansson *et al.*, 2012). However, it is not known if sex steroids can modulate these placental pathways, thereby indirectly affecting fetal growth.

Therefore, we measured the expression of total and phosphorylated STAT3 and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and S6 ribosomal protein as functional readouts of mTOR signaling in placentas of women with and without PCOS, and determined the mRNA expression of genes encoding proteins related to steroid action, metabolic pathways and cytokines. To correlate maternal sex steroid levels to placental signaling, we analyzed maternal circulating sex steroid precursors, estrogens, androgens, and glucuronidated androgen metabolites by gas or liquid chromatography/tandem mass spectrometry (GC-MS/MS and LC-MS/MS).

Materials and Methods

Ethical approval

These studies were performed according to the standards of the Declaration of Helsinki and were approved by the Committee for Research Ethics at Uppsala University and by the Committee for Research Ethics in Midt-Norge, Norway. All participants gave informed consent. After all the relevant clinical information was obtained, samples were coded and de-identified. All molecular analyses were performed at the University of Gothenburg.

Participants

Placental tissue and blood samples were obtained from pregnant women with PCOS ($n = 38$) and pregnant controls ($n = 40$) (see study flow chart Supplementary data, Fig. S1). Samples from PCOS women were obtained from two sources: (i) The PregMet study conducted at St. Olav's Hospital, University Hospital of Trondheim, Norway, which was a prospective, randomized, double-blind, multicenter trial comparing metformin 2000 mg daily with placebo (Vanky *et al.*, 2010). Only PCOS women randomized to placebo were eligible for this study. (ii) The BASIC biobank at Uppsala University, Sweden which is a longitudinal study investigating biological correlates of antenatal and post-natal depression (Hellgren *et al.*, 2013). Inclusion criteria for the study were: (i) Diagnosis of PCOS according to the Rotterdam criteria (Rotterdam, 2004) by a gynecologist based on documentation before the current pregnancy, and (ii) available placental tissue. Thirteen PCOS women (34.2%) had hyperandrogenism, oligoamenorrhea and polycystic ovary morphology (PCO) (full phenotype), 24 (63.2%) had oligoamenorrhea and PCO, one (2.6%) presented with hyperandrogenism and PCO.

Pregnant controls were from the BASIC Biobank and matched on a group level to the women with PCOS with regard to maternal age and BMI, parity, assisted reproduction requiring IVF, offspring birthweight and gestational length. None of the controls had anovulatory infertility. Given that the prevalence of PCOS (diagnosed by Rotterdam criteria) without menstrual disorder is around 3.4% in an unselected population (March *et al.*, 2010), it can be estimated that no more than one control woman was likely to suffer from PCOS.

All women were of Scandinavian heritage and had a single viable fetus. Women diagnosed during the current pregnancy with pre-eclampsia, gestational hypertension, gestational diabetes or women with severe chronic diseases, as chronic hypertension or kidney disease, were excluded.

Pre-eclampsia was diagnosed according to the guidelines of the International Society for the Study of Hypertension in Pregnancy (ISSHP), as blood pressure of 140/90 mm Hg or higher measured on two occasions after gestational Week 20 and albuminuria of at least +2 dipstick on one occasion or +1 dipstick on two occasions (Roberts *et al.*, 2003). Gestational hypertension was defined according to the ISSHP, as *de novo* hypertension alone, appearing after gestational Week 20 (Roberts *et al.*, 2003). Gestational diabetes was defined by The Expert Committee On The Diagnosis And Classification Of Diabetes Mellitus (1998) as fasting plasma glucose higher than 7.0 mmol/l and/or 2-h serum glucose higher than 7.8 mmol/l after an oral glucose tolerance test (75 g glucose solved in 300 ml water). The presence of babies born small or large for gestational age was not considered as exclusion criterion.

We included only women with uncomplicated pregnancies with the aim to avoid the confounding effect of pregnancy complications such as pre-eclampsia and gestational diabetes, which are known to alter the fetal growth and may affect placental STAT3 and mTOR signaling (Weber *et al.*, 2012; Pérez-Pérez *et al.*, 2013).

Clinical and anthropometric variables

Biometric variables, including height, weight, blood pressure and heart rate were recorded at inclusion and at each prescheduled maternity health care visit. Data on birth length, birthweight, Apgar score and the most common neonatal diagnoses were recorded. Small for gestational age was defined as a birthweight $\leq$2 SD below the mean for gestational age and sex and large for gestational age as a birthweight $\geq$2 SD above the mean for gestational age and sex.

Blood samples

In all women, a venous blood sample was obtained at delivery. After clotting, samples were centrifuged and the serum was frozen at −70°C.

Placental tissue

Placentas were obtained immediately after vaginal delivery or Cesarean section. Placental tissue was collected near to maternal side, avoiding the decidual layer of the placenta, and briefly washed in sterile phosphate-buffered saline. Samples for mRNA extraction were snap frozen within 60 min of delivery and stored at −70°C.

Analytical methods

Protein preparation

Proteins from placental samples (≈30 mg) were extracted from 25 PCOS women and controls matched pair-wise for age and BMI. Placental tissue were homogenized in cold RIPA lysis and extraction buffer (R0278, Sigma) containing 1.0 mM of phenylmethylsulfonyl fluoride, a cocktail of protease (P8340, Sigma), and phosphatase inhibitors (1.0 mM Na-ortovanadate), incubated on ice for 30 min, and centrifuged at 10 000*g* for 10 min. Supernatants were collected, and protein concentration was determined with a spectrometer (Direct Detect; Millipore, MA, USA).

Western blot

For western blot analyses, we used antibodies against total STAT3 (#9139, Cell Signaling, Beverly, MA, USA), suppressor of cytokine signaling (SOCS3) (sc-7010; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), S6 ribosomal protein (#2217, Cell Signaling), 4E-BP1 (#9452, Cell Signaling), estrogen receptor (ER) β (#PA1-310B, Pierce Biotechnology, Rockford, IL, USA) and phosphorylated fractions of STAT3 (Tyr-705) (P-STAT3) (#9145, Cell Signaling), S6 ribosomal protein (Ser-235/236) (#4858, Cell Signaling) and 4E-BP1 (Thr-37/46) (#9459, Cell Signaling). Moreover, the protein expression of ERβ was assessed because it is the main isoform expressed in placental tissue.

Protein (10–20 μg) was separated on precast 4–12% Bis–Tris gels NuPAGE Novex minigels (Invitrogen) and transferred to a nitrocellulose membrane in XCell II Blot Module (Invitrogen). Membranes were blocked in 5% milk in Tris-buffered saline—Tween, and incubated overnight with the primary antibody, washed and incubated in secondary antibody (#7074 or #7076, Cell Signaling) for 1 h at room temperature. Protein bands were developed with SuperSignal West Dura Extended Duration substrate (Pierce Biotechnology) and photographed with an LAS-1000 camera system (Fujifilm, Tokyo, Japan) or ChemiDoc XRS+ System (Bio-Rad Laboratories, Solna, Sweden). The blots were stripped in Restore Plus Western Blot Stripping Buffer (Pierce Biotechnology), blocked in 5% milk for 1 h, and reprobed for β-actin (A1978; Sigma). Protein signal intensity was quantified by densitometry with MultiGauge Software version 3.0 or Image Lab Software (Bio-rad). β-actin was used as a loading control and for normalization. For each protein target, all individual density values for controls and treated subjects were expressed relative to the mean density of the controls.

RNA isolation and quantitative real-time RT–PCR

Total RNA was isolated from placental tissue with a commercial kit (#74104, Qiagen, Hilden, Germany). Total RNA was treated with DNase I and the first-strand cDNA was prepared from 250 ng of total RNA with Superscript VILO (Life Technologies, Paisley, UK) following the manufacturer's protocol. For real-time PCR, 100 ng of cDNA was analyzed with custom TaqMan low-density arrays (Applied Biosystems, Carlsbad, CA, USA) covering genes encoding proteins related to steroid action, metabolic pathway and cytokines (Supplementary data, Table SI), an ABI Prism 7900HT Sequence Detection System, and ABI Prism 7900HT SDS Software 2.4 (Applied Biosystems). Eight samples were randomly analyzed per card in one run. Duplicates of samples were run on different cards to confirm the reproducibility of the method. Thermal cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Two putative reference genes—18S ribosomal RNA (*18S*) and β-actin (*ACTB*)—were included. Since these genes varied considerably between control and PCOS women, a wider range of genes was analyzed with NormFinder (Andersen *et al.*, 2004). Variability was lowest with the combination of catenin (cadherin-associated protein), beta 1 (*CTNNB1*) and hypoxia inducible factor 1, alpha subunit (*HIF1A*). Gene expression values were calculated with the ΔΔCq method (i.e. $RQ = 2^{-\Delta\Delta Cq}$; Livak and Schmittgen, 2001).

Circulating steroid concentrations

Plasma concentrations of dehydroepiandrosterone (DHEA), DHEA sulfate (DHEAS), androstenedione (4-DIONE), androst-5-ene-3β, 17β-diol (5-DIOL), testosterone, 5α-dihydrotestosterone (DHT), estrone (E1), estradiol (E2), E1 sulfate (E1-S), androsterone glucuronide (ADT-G), etiocholanolone glucuronide (ETIO-G) and androstane-3α, 17β-diol-17-glucuronide (17G) were measured with a validated GC-MS/MS or LC-MS/MS system at Endoceutics (Quebec City, Canada). The limit of detection was 100 ng/ml for DHEAS, 500 pg/ml for DHEA, 100 pg/ml for 4-DIONE, 100 pg/ml for 5-DIOL, 50 pg/ml for T, 10 pg/ml for DHT, 4.0 pg/ml for E1, 1.0 pg/ml for E2, 50 pg/ml E1-S, 4.0 ng/ml for ADT-G, 4.0 ng/ml for ETIO-G and 100 pg/ml for 17G. Sex hormone-binding globulin (SHBG) was analyzed on a Modular E170 (Roche Diagnostics, Mannheim, Germany). The total coefficient of variation was 1.5% at 43 nmol/l for SHBG.

Statistical analysis

Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS version 21.0; SPSS, Chicago, IL, USA) and Prism GraphPad (version 6.0, GraphPad Software, La Jolla, CA, USA). Differences between groups were analyzed by Fisher Permutation test (Oden and Wedel, 1975), and confidence interval for the difference between the means was calculated. Categorical data were analyzed by χ^2 test. Correlations between mRNA and protein expression and serum sex steroid concentrations were calculated by Spearman's Rho test. $P < 0.05$ was considered statistically significant.

Results

Clinical characteristics

Control and PCOS women were comparable in age and BMI. Nine of 40 controls (22.5%) and 7 of 38 PCOS women (18.4%) had a BMI ≥ 30.0 kg/m^2 ($P = 0.413$; Table I). In PCOS group, seven women (18.4%) became pregnant after ovulation induction with clomiphene citrate and two (5.3%) after FSH stimulation. None from control group received drugs to induce the ovulation. Women with PCOS and controls

Table I Clinical characteristics of the control and women with PCOS and their newborns.

	Controls ($n = 40$)	PCOS ($n = 38$)	CI
Mothers			
Age (years)	30.5 (26.0–34.0)	29.0 (27.0–34.3)	−1.79 to 2.36
BMI (kg/m^2)	25.7 (21.3–29.9) ($n = 39$)	25.6 (22.1–29.0)	−2.42 to 2.06
Primipara, *n* (%)	27 (67.5)	23 (60.5)	
IVF pregnancy, *n* (%)	9 (22.5)	9 (23.7)	
Smokers, *n* (%)	3 (7.5)	0 (0)	
Cesarean section, *n* (%)	14 (35.0)	9 (23.7)	
Labor induction, *n* (%)	7 (17.5)	7 (20.0) ($n = 35$)	
Newborns			
Fetal sex (F/M)	20/20	14/24	
Gestational age (weeks)	40 (39–41)	40 (39–41)	−0.20 to 1.68
Birthweight (kg)	3.57 (3.39–3.86)	3.74 (3.35–4.04)	−0.27 to 0.18
Weight SDS	0.30 (−0.19–0.62)	0.45 (−0.3 to 1.0)	−0.99 to 0.18
Birth length (cm)	50.5 (50.0–51.1)	50.2 (49.6–50.6)	−0.32 to 1.38
Length SDS	0.31 (−0.07–0.90)	0.43 (−0.21 to 1.17)	−0.74 to 0.28

Values are median (25th–75th interquartile ranges). Differences were calculated by Fisher Permutation test or χ^2 test. There were no significant differences between the groups. CI, confidence interval for the difference between the means; F, female; M, male; SDS, standard deviation score.

did not differ in parity, the number of pregnancies achieved by of IVF, smoking or Cesarean section rate and induction of labor. The gestational age was comparable between groups. One PCOS and one control woman had a preterm delivery (in gestational Week 34 and 35, respectively). The distribution of sexes of newborns, birthweight, length and standard deviation scores for weight and length were comparable between groups. However, three newborns in the PCOS group (7.9%) and one in the control group (2.5%) were large for gestational age ($P = 0.189$). No babies were born small for gestational age in either group.

Placental protein expression

Total STAT3 and SOCS3 protein expression did not differ between the groups, but P-STAT3 (Tyr-705) was higher in PCOS women (Fig. 1). Total expression and phosphorylation of S6 ribosomal protein and 4E-BP1 (Fig 2A and B) or expression of ERβ (Fig. 3) did not differ between women with PCOS and controls. P-STAT3 (Tyr-705) was higher in PCOS women with full phenotype (hyperandrogenism + anovulation + PCO) compared with those who had anovulation + PCO (3.11 ± 0.88 versus 0.96 ± 0.20 relative density, $P = 0.014$). No differences were observed in total and phosphorylated S6 ribosomal protein, 4E-BP1 and ERβ between the PCOS phenotypes.

Placental gene expression

mRNA expression of leptin (*LEP*), leptin receptor (*LEPR*), solute carrier family 2 (facilitated glucose transporter), member 4 (*SCL2A4*) and cytochrome P450, family 11, subfamily A, polypeptide 1 (*CYP11A1*) was lower and mRNA expression of estrogen receptor 1 (*ESR1*) and aldo-keto reductase family 1, member C3 (*AKR1C3*) (Fig. 4A and B) was higher in women with PCOS than in controls. The mRNA expression for hydroxysteroid (11-beta) dehydrogenase 1 (*HSD11B1*), hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (*HSD3B1*), cytochrome P450, family 19, subfamily A, polypeptide 1 (*CYP19A1*), androgen receptor (*AR*), adiponectin receptor 1 (*ADIPOR1*), adiponectin receptor 2 (*ADIPOR2*), insulin-like growth factor 1 (*IGF1*), resistin (*RETN*), and for the cytokines, tumor necrosis factor (*TNF*), interleukin 6 (*IL6*), *IL11*, *IL1B*, *IL8*, leukemia inhibitory factor (*LIF*) and macrophage migration inhibitory factor (*MIF*) were similar between groups. No mRNA expression was observed for adiponectin (*ADIPOQ*), aldo-keto reductase family 1, member C2 (*AKR1C2*) and steroid-5-alpha-reductase, alpha polypeptide 2 (*SRD5A2*) and wingless-type MMTV integration site family, member 10B (*WNT10B*). No differences were observed in mRNA expression according to PCOS phenotype.

Circulating sex steroids and SHBG concentrations

Women with PCOS had higher circulating levels of 4-DIONE, 5-DIOL, testosterone, DHT, ADT-G and ETIO-G and lower levels of E2 ($P < 0.05$; Table II). Circulating DHEAS, DHEA, E1-S, 17G and SHBG did not differ between groups. No differences were observed in circulating sex steroid or SHBG between the PCOS phenotypes. No correlations between sex steroids and P-STAT3 or P-S6 ribosomal protein or P-4E-BP1 were observed.

Discussion

This study suggests that STAT3 signaling is increased in placentas of women with PCOS. Moreover, using mass spectrometry, we confirmed and expanded previous observations of abnormal steroidogenesis in pregnant women with PCOS. By design, infant birthweights were similar in control and PCOS women in our study. Moreover, 97.4% of women with PCOS displayed oligoamenorrhea when diagnosed. Thus, the current findings could be representative for PCOS women with oligo-anovulatory phenotype without pregnancy complications.

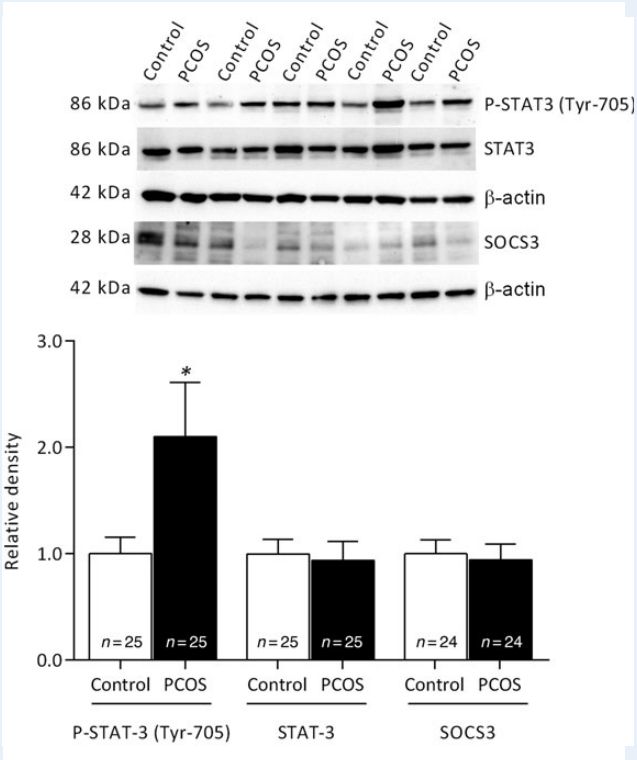

Figure 1 Placental protein expression of total and phosphorylated signal transducer and activator of transcription 3 (P-STAT3) (Tyr-705) and SOCS3 in controls and women with PCOS. P-values were calculated by Fisher Permutation test. Values are mean ± SEM. *$P < 0.05$ control versus PCOS.

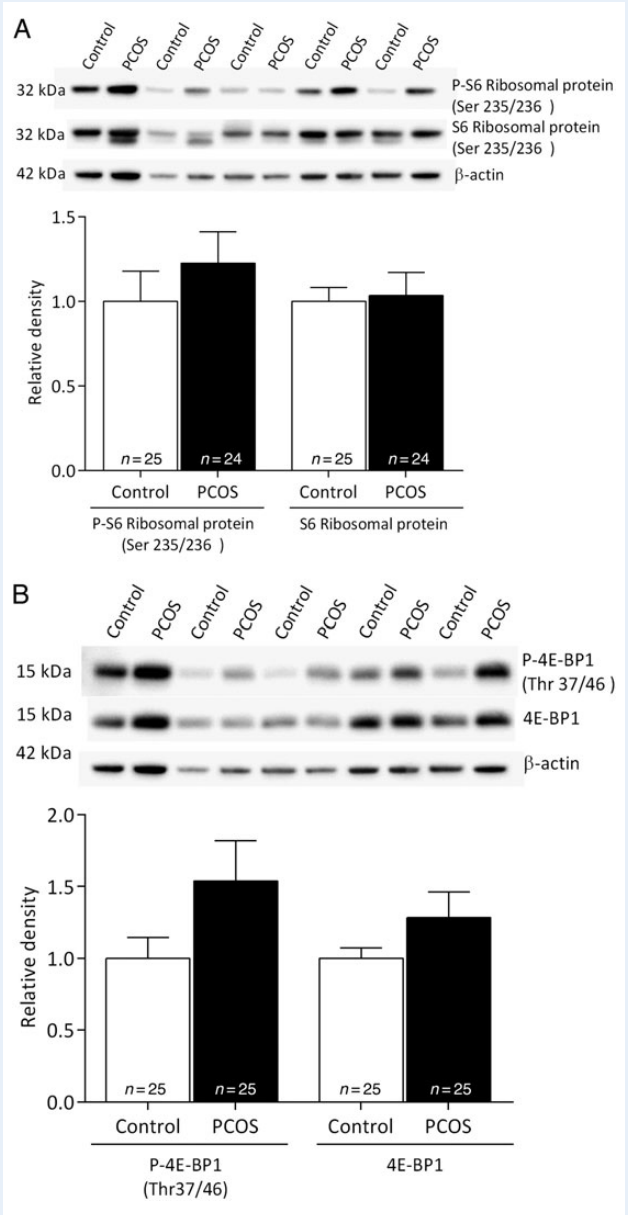

Figure 2 Protein expression of downstream mTOR effectors in placentas from controls and women with PCOS. (**A**) Phosphorylated (Ser-235/236) and total expression of S6 ribosomal protein. (**B**) Phosphorylated (Thr-37/46) and total expression of 4E-BP1. Data in A and B were compared by Fisher Permutation test. All comparisons were not significant. Values are mean ± SEM.

Leptin and its signaling pathway are important regulators of placental metabolism (von Versen-Hoynck *et al.*, 2009; Maymo *et al.*, 2011). Leptin acts on its receptor to stimulate the phosphorylation of STAT3 at Tyr-705, which induces nuclear translocation and initiates transcription (Kaptein *et al.*, 1996). The termination of the signal is mediated by SOCS3 (Yoshimura *et al.*, 2007). Therefore, lower placental mRNA expression of leptin and leptin receptor could contribute to decreased activity of the placental STAT-3 signaling pathway in PCOS. In contrast, we found increased phosphorylation of STAT3 in placentas from PCOS women. It seems that these alterations are not associated with the circulating androgen concentrations. This finding is intriguing because it indicates that the activation of STAT3 is caused by other maternal factors, such as cytokines and/or hormones other than sex steroids (Aggarwal *et al.*, 2009). Another alternative is that cytokines synthesized by the placenta activate STAT-3 signaling in an autocrine/paracrine fashion. However, we did not observed differences in the mRNA expression of *TNF*, *IL6*, *IL11*, *IL1B*, *IL8*, *LIF* and *MIF* in placentas from women with PCOS, although we cannot exclude the action of others cytokines have not been included in our analysis. Another alternative is that signals produced by the fetus activate STAT3. In this regard, elevated leptin levels have been observed in cord blood from newborns of women with PCOS (Maliqueo *et al.*, 2009).

Because our design did not include PCOS women with pregnancy-related pathologies, we suggest that PCOS *per se* can modify the STAT3 signaling. In this regard, although in opposite way, granulosa cells of women with PCOS with failed IVF have showed a decreased phosphorylation of STAT3 and elevated leptin levels compared with those with successful IVF (Li *et al.*, 2007). The clinical consequences of increased placental STAT3 phosphorylation remain to be established, but may include activation of key placental amino acid transporters affecting fetal growth (Jones *et al.*, 2009), or reflect a proinflammatory state similar to those observed in placentas from women with maternal obesity, gestational diabetes and pre-eclampsia (Benyo *et al.*, 2001; Challier *et al.*, 2008; Aye *et al.*, 2014; Mrizak *et al.*, 2014). Moreover, metabolic abnormalities, such as insulin resistance, hyperinsulinemia and

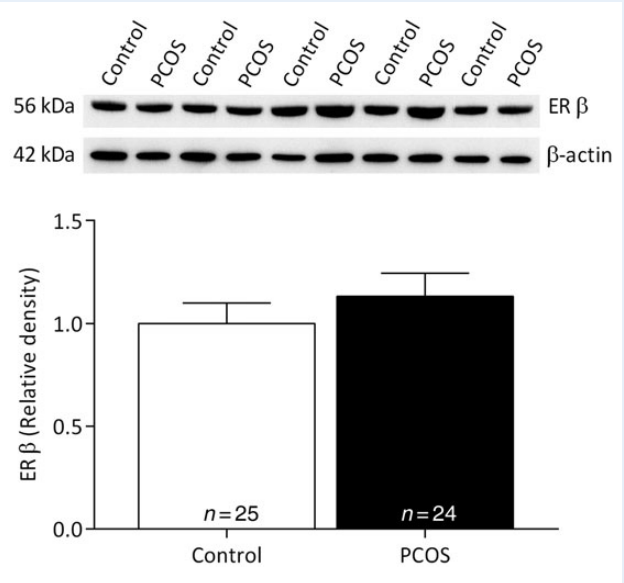

Figure 3 Protein expression of ERβ in placentas from controls and women with PCOS. The Fisher Permutation test showed no significant difference between groups. Values are mean ± SEM.

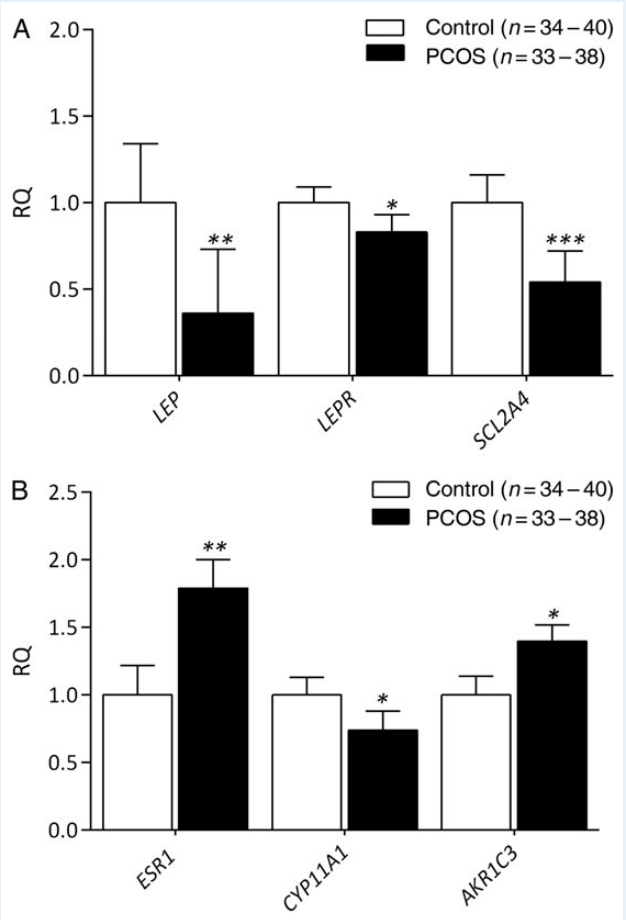

Figure 4 Gene mRNA expression in placental tissue from control and women with PCOS. (**A**) Metabolic gene expression. (**B**) Steroid synthesis and steroid receptor gene expression. *P*-values were calculated by Fisher Permutation test. Values are mean ± SEM. ***$P < 0.001$; **$P < 0.01$ and *$P < 0.05$ control versus PCOS.

dyslipidemia, are prevalent in non-obese and obese pregnant women with PCOS increasing the risk of development pregnancy-related complications in these women (Sir-Petermann *et al.*, 2007; Palomba *et al.*, 2014a).

Of interest, it has been reported that women with PCOS with uncomplicated pregnancies have reductions in placental thickness, density, and volume associated with vascular lesions, chronic villitis and intervillositis, and abnormal villus maturity (Palomba *et al.*, 2013). Interestingly, these morphological changes are more common in women with PCOS presenting with the full phenotype than in those women with PCOS with other phenotypes (Palomba *et al.*, 2014b). These alterations might be associated with the release of inflammatory cytokines activating STAT3, which is also more evident in those women with the full PCOS phenotype. Indeed, it has been observed that pregnant women with PCOS exhibit an exacerbated low-grade chronic inflammation characterized by increased white blood cell count and C-reactive protein, which in turn are positively associated with circulating testosterone serum concentrations (Palomba *et al.*, 2014c).

The mTOR pathway is central in placental nutrient transfer and an altered mTOR signaling has been observed in pregnancy complications associated with altered fetal growth. The phosphorylation of mTOR effectors is reduced in fetal growth restriction (Roos *et al.*, 2007) but is increased in obese women giving birth to babies large for gestational age and in women with gestational diabetes (Jansson *et al.*, 2013; Pérez-Pérez *et al.*, 2013). However, in contrast to STAT3 pathway, mTOR signaling was unaffected in placenta from women with PCOS indicating these two pathways could have independent mechanisms of control in these women.

On the other hand, a reduction of mRNA expression of *SCL2A4* encoding GLUT4 was observed. Placental glucose transfer is mediated mainly by GLUT1 but it has been demonstrated that GLUT4 is expressed in intravillous stromal cells of term placenta and probably stimulated by fetal insulin (Xing *et al.*, 1998). Then, the lower expression of *SCL2A4* could indicate some degree of insulin resistance in these cells.

Previous observations showed that pregnant women with PCOS have elevated levels of androgens, including T, 4-DIONE and DHEAS (Sir-Petermann *et al.*, 2002; Falbo *et al.*, 2010). Using mass spectrometry, the gold standard for sex steroid measurements, we found higher circulating levels of T, 4-DIONE and 5-DIOL but no differences in circulating levels of DHEAS and DHEA in pregnant women with PCOS suggesting maternal and fetal adrenal androgen production and placental steroid sulfatase activity were normal in these women.

The elevated 4-DIONE levels are consistent with increased activity of 3β-hydroxysteroid dehydrogenase type (3β-HSD1), as previously suggested (Maliqueo *et al.*, 2013) because this enzyme catalyzes the conversion of DHEA to 4-DIONE. However, we did not find differences in the mRNA expression for 3β-HSD1 indicating that the increase in the activity of this enzyme is probably due to the modulation of kinetics parameters rather than changes in its gene expression.

Moreover, elevated activity of 17β-Hydroxysteroid dehydrogenase (17β-HSD) types 1 and 5 can explain the increased circulating levels of

Table II Serum sex steroid concentrations at delivery as measured by gas chromatography–mass spectrometry or liquid chromatography–mass spectrometry in control women and women with PCOS.

	Controls ($n = 38$)	PCOS ($n = 38$)	CI
DHEAS (μmol/l)	1.89 (1.13–2.84)	2.30 (1.38–3.62) ($n = 35$)	−1.02 to 0.43
DHEA (nmol/l)	21.23 (8.74–28.63)	22.01 (11.72–33.58)	−9.27 to 3.77
4-DIONE (nmol/l)	8.03 (5.37–12.04)	10.85 (7.57–17.21)*	−7.84 to 0.39
5-DIOL (nmol/l)	1.17 (0.57–1.68)	1.62 (0.59–3.01)*	−1.09 to −0.08
Testosterone (nmol/l)	2.85 (1.74–4.23)	3.89 (2.71–7.11)*	−3.84 to −0.23
DHT (nmol/l)	0.48 (0.27–0.68)	0.58 (0.45–0.93)*	−0.41 to −0.02
E1 (nmol/l)	24.41 (16.94–39.46)	40.39 (23.86–58.84)	−22.73 to 0.19
E2 (nmol/l)	79.09 (55.83–105.91)	50.57 (19.26–84.39)**	9.99 to 46.85
E1-S (nmol/l)	339.6 (209.4–696.3)	468.0 (292.4–643.5) ($n = 37$)	−209.3 to 83.80
ADT-G (nmol/l)	43.72 (30.65–63.12) ($n = 37$)	58.94 (42.22–78.87) ($n = 35$)*	−35.60 to −0.28
ETIO-G (nmol/l)	25.51 (15.35–37.93) ($n = 37$)	32.79 (17.81–57.87) ($n = 35$)*	−20.79 to −0.83
17G (nmol/l)	0.45 (0.30–0.70) ($n = 37$)	0.49 (0.30–0.94) ($n = 35$)	−0.36 to 0.09
SHBG (nmol/l)	399.5 (335.3–490.8)	436.0 (326.8–509.8)	−59.80 to 59.48

Values are median (25th–75th interquartile ranges). Two controls were not analyzed because samples were missing.
CI, confidence interval for the difference between the means; DHEA, Dehydroepiandrosterone; DHEAS, DHEA sulfate; 4-DIONE, androstenedione; 5-DIOL, androst-5-ene-3β, 17β-diol; DHT, 5α-dihydrotestosterone; E1, estrone; E1-S, E1 sulfate; E2, estradiol; ADT-G, androsterone glucuronide; ETIO-G, etiocholanolone glucuronide; 17G, androstan-3α, 17β-diol 17-glucuronide; SHBG, sex hormone-binding globulin.
$^{**}P < 0.01$ and $^{*}P < 0.05$ control versus PCOS. Differences were calculated by Fisher Permutation test.

5-DIOL and T in PCOS, because these isoforms catalyze the reduction of DHEA to 5-DIOL and of 4-DIONE to T (Mindnich *et al.*, 2004). Interestingly, mRNA expression of *ARK1C3*, which encodes 17β-HSD type 5, was increased in placenta from women with PCOS. Further, T can be 5α-reduced to produce DHT, and 4-DIONE can be 5β-reduced to produce 5β-4-DIONE. Androgens are inactivated by glucuronidation to 17G, ADT-G or ETIO-G (Sten *et al.*, 2009). Therefore, the higher circulating DHT, ADT-G and ETIO-G levels reflect the hyperandrogenic state in pregnant women with PCOS. Androgens levels increase throughout gestation and are important in maintaining pregnancy and in the initiation of labor (Makieva *et al.*, 2014). However, the impact of elevated androgen levels during pregnancy in women with PCOS remains to be fully established. There are multiple mechanisms limiting the biological effects of maternal androgen excess, including increase of circulating maternal SHBG and progesterone levels, which competes for androgen receptor binding and its affinity for 5α-reductase inhibiting the conversion of testosterone to DHT (Makieva *et al.*, 2014). Despite these protective mechanisms, the maternal hyperandrogenemia could contribute to the development of pre-eclampsia or premature delivery.

The low maternal E_2 levels in the women with PCOS are consistent with a previous observation of abnormal placental P450 aromatase activity in PCOS (Maliqueo *et al.*, 2013), although we did not observe differences in the expression of *CYP19A1*. However, we found that E1 (produced by aromatization of 4-DIONE) was elevated in pregnant women with PCOS (although not significantly compared with control women), which may be attributed to the higher efficiency of P450 aromatase in producing E1 than E2 (Gibb and Lavoie, 1980). On the other hand, higher activity of 17β-HSD type 2 increases oxidation of E2 to E1 (Mindnich *et al.*, 2004). In agreement with this observation, we previously observed increased placental expression of 17β-HSD type 2 protein in pregnant rats exposed to testosterone (Sun *et al.*, 2012). Interestingly, the lower placental leptin mRNA expression in women with PCOS could be explained by their decreased E2 levels, as E2 regulates, by genomic and non-genomic action on ERα, placental leptin production (Gambino *et al.*, 2012). Moreover, the higher mRNA expression of *ESR1*, which encodes for ERα, could be a compensatory mechanism of lower estradiol levels.

On the other hand, the mRNA expression of *CYP11A1*, encoding the enzyme P450 cholesterol side-chain cleavage (P450scc), was lower in placenta from women with PCOS. This enzyme catalyzes the conversion of cholesterol to pregnenolone, which is the rate-limiting step of placental progesterone synthesis (Tuckey, 2005). Interestingly, *CYP11A1* is positively regulated by estrogen in cultured primary human trophoblast cells (Babischkin *et al.*, 1997). Therefore, lower estradiol levels observed in women with PCOS could explain the reduced expression of *CYP11A1* in women with PCOS.

In summary, we found that phosphorylation of STAT3 is increased whereas total and phosphorylated fraction of mTOR effectors, 4E-BP1 and S6 ribosomal protein, remain normal in the placenta of women with PCOS with uncomplicated pregnancies indicating that specific metabolic pathways are activated independently of the presence of pregnancy-related pathologies. This phenomenon seems not to be directly related to altered circulating sex steroid concentrations. However, we cannot rule out a higher sensitivity of placental tissue to the steroid actions. Additional studies are needed to elucidate the clinical implications of increased activity in the STAT3 signaling pathway in placentas from women with PCOS.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

M.M., I.S.P., E.V., T.J. and E.S.-V. were involved in the study design. I.S.P., E.V., H.Å and S.S. recruited the patients and collected the placental and blood samples. M.M., R.F. and A.B. performed the molecular analysis. M.M., A.B., F.L., T.J. and E.S.-V. contributed to the analysis and interpretation of data. M.M. and E.S.-V. wrote the manuscript. All the authors revised critically and approved the manuscript.

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Conflict of interest

The authors have nothing to disclose.

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