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Fetal programming by androgen excess in rats affects ovarian fuel sensors and steroidogenesis

Giselle Adriana Abruzzese[1], Maria Florencia Heber[1], Fiorella Campo Verde Arbocco[2,3,4], Silvana Rocio Ferreira[1] and Alicia Beatriz Motta[1]

[1]Laboratorio de Fisio-patología ovárica, Centro de Estudios Farmacológicos y Botánicos (CEFYBO), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Facultad de Medicina, Universidad de Buenos Aires (UBA), Buenos Aires, Argentina; [2]Laboratorio de Hormonas y Biología del Cáncer, Instituto de Medicina y Biología Experimental de Cuyo (IMBECU), CONICET, 5500 Mendoza, Argentina; [3]Laboratorio de Reproducción y Lactancia, IMBECU, Mendoza, Argentina and [4]Facultad de Ciencias Médicas, Universidad de Mendoza, Mendoza, Argentina

Original Article

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Address for correspondence:
G. A. Abruzzese, CEFYBO – CONICET, School of Medicine, University of Buenos Aires, Paraguay 2155, 17th Floor, Sector M3, Buenos Aires, C1121 ABG, Argentina.
Email: giselleabruzzese@gmail.com

Abstract

Fetal programming by androgen excess is hypothesized as one of the main factors contributing to the development of polycystic ovary syndrome (PCOS). PCOS is more than a reproductive disorder, as women with PCOS also show metabolic and other endocrine alterations. Since both ovarian and reproductive functions depend on energy balance, the alterations in metabolism may be related to reproductive alterations. The present study aimed to evaluate the effect of androgen excess during prenatal life on ovarian fuel sensors and its consequences on steroidogenesis. To this end, pregnant rats were hyperandrogenized with testosterone and the following parameters were evaluated in their female offspring: follicular development, PPARG levels, adipokines (including leptin, adiponectin, and chemerin as ovarian fuel sensors), serum gonadotropins (LH and FSH), the mRNA of their ovarian receptors, and the expression of steroidogenic mediators. At 60 days of age, the prenatally hyperandrogenized (PH) female offspring displayed both an irregular ovulatory phenotype and an anovulatory phenotype with altered follicular development and the presence of cysts. Both PH groups showed altered levels of both proteins and mRNA of PPARG and a different expression pattern of the adipokines studied. Although serum gonadotropins were not impaired, there were alterations in the mRNA levels of their ovarian receptors. The steroidogenic mediators *Star*, *Cyp11a1*, *Cyp17a1*, and *Cyp19a1* were altered differently in each of the PH groups. We concluded that androgen excess during prenatal life leads to developmental programming effects that affect ovarian fuel sensors and steroidogenesis in a phenotype-specific way.

Introduction

An adverse environment during prenatal life programs alterations in the developing organs. In particular, since gametes and the growing fetal ovary are vulnerable to prenatal events,[1] an adverse environment during fetal life could affect the developing gonads and impact on postnatal fertility.

Polycystic ovary syndrome (PCOS) is one of the main causes of female ovarian infertility. Although the etiopathogenesis of PCOS is not yet fully understood, current theories emphasize on genetic and environmental factors during intrauterine life.[2] It has been reported that prenatal androgen exposure induces polycystic ovaries and metabolic alterations in several species.[3–9] In previous works, we found that androgen excess *in utero* programs two different phenotypes regarding the estrous cycle (anovulatory and irregular ovulatory) with biochemical hyperandrogenism and alterations in ovarian morphology.[7] These phenotypes show differences in metabolic features, being the anovulatory phenotype (PHanov) more affected.[5,7]

In female mammals, alterations in the metabolic status could impact on the energy balance of the ovary, affecting its functions and leading to fertility issues.[10]

Ovarian steroidogenesis is strictly regulated by both neuroendocrine negative feedback systems and by the response to the metabolic status.[11] Several molecules such as peroxisome proliferator-activated receptors (PPARs) and adipokines act signaling and responding to the metabolic status in ovaries.[12,13] Thus, they have been referred to as "fuel sensors" as they monitor energy status and regulate metabolic pathways and also those processes that depend on these.[14,15]

PPARs are a family of transcriptional nuclear factors with three isoforms—alpha, beta, and gamma—which regulate the gene expression of several molecules.[16,17] The three isoforms are present in the ovaries of different species, but PPAR gamma (PPARG) is the one that has been associated with female fertility and PCOS.[18,19] The activation of PPARG regulates the synthesis of steroid hormones in granulosa cells,[18,20] whereas the disruption of PPARG in the ovary leads

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to female subfertility.[21] In addition, PPARG has two isoforms (PPARG1 and PPARG2), both of which are found in the ovaries but whose regulation and action are still unknown.

Adipokines regulate reproductive functions in gonads and through the hypothalamic-pituitary axis.[22,23] The adipokines secreted by ovaries play autocrine and paracrine functions, directly affecting ovarian signaling.[24] In PCOS patients, the pattern of serum adipokines is altered independently of the body mass index and body weight. Thus, it has been proposed that they contribute to the pathology of PCOS as well as to the related metabolic abnormalities.[23,25]

The transcription of adipokines such as adiponectin and chemerin seems to be regulated by PPARG because they show PPAR response elements on their promoter.[24,26] Moreover, PPARG agonists decrease the levels of the adipokine leptin, another energy metabolism regulator also involved in the functionality of the hypothalamic-pituitary-gonadal axis.[27–30]

Based on this, we hypothesized that prenatal androgen excess programs alterations in the metabolic signaling within the ovary, which impact on ovarian functions. Thus, we aimed to study the effect of prenatal hyperandrogenization on the ovarian fuel sensors: PPARG and the adipokines leptin, adiponectin and chemerin, and on the steroidogenesis pathway.

Material and methods

Animals and treatments

Virgin female rats (90–110 days of age) of the Sprague Dawley strain were mated with fertile males of the same strain. Three females and one male were housed in each cage under controlled conditions of light (12 h light, 12 h dark) and temperature (23–25 °C). Animals received food and water *ad libitum*. Day 1 of pregnancy was defined as the day on which spermatozoa were observed in the vaginal fluid. To study the fetal programming effect of androgens, we used a rodent model already established in our laboratory.[7] Briefly, pregnant rats ($N = 15$) received subcutaneous injections of 1 mg of free testosterone (T-1500; Sigma, St. Louis, MO, USA) dissolved in 100 µl sesame oil from day 16 to day 19 of pregnancy. This hormonal paradigm mimics the fetal testosterone surge observed in male rats when the reproductive axis in the fetus is established. Another group ($N = 15$) received only 100 µl of sesame oil. Under the conditions of our animal facilities, spontaneous term labor occurs on day 22 of gestation. Female offspring were separated from males at 21 days of age. Those from hyperandrogenized mothers were assigned as the prenatally hyperandrogenized (PH) group, whereas those from mothers injected with sesame oil were the control group. Animals were allowed free access to Purina rat chow (Cooperación SRL, Argentina) and water.

At 60 days of age, female offspring were weighed and then anesthetized with carbon dioxide and killed by decapitation. Trunk blood was collected and serum and ovaries were separated, weighed, and kept at –80 °C for further studies. All animals were randomly assigned for each assay considering their litter precedence. Care was taken when assigning and equilibrating the number of animals from each littermate to all the assays, to prevent the maternal effect on the results. Most of the animals used for this work have been studied in previous projects from our laboratory.[7]

All the procedures involving animals were conducted in accordance with the Animal Care and Use Committee of Consejo Nacional de Investigaciones Científicas y Tecnicas (CONICET) 1996, Argentina, and the study was approved by the Ethics Committee of the School of Medicine of University of Buenos Aires, Argentina.

Determination of the phenotype according to the regularity of the estrous cycle

The estrous cycle of all the animals was determined by vaginal smears taken daily from 45 to 60 days of age and classified as previously reported[7,31] (Fig. 1).

The dose of testosterone used in the present study (1 mg) leads 123 to both the irregular ovulatory phenotype (PHiov) and the anovulatory phenotype (PHanov). As the PHanov group remained mainly in diestrous to allow the comparison between the phenotypes, all animals were sacrificed at this stage.

Follicle count and follicular growth

The ovaries were removed and immediately fixed in 4% (v/v) formaldehyde, dehydrated in ethanol, and embedded in paraffin. Serial 6-µm thick sections were mounted at 50 µm intervals onto microscope slides, to prevent counting the same structure twice,[32] and stained with hematoxylin-eosin. Histological serial sections were analyzed independently by two of the authors, and ovarian follicles were classified and quantified. Follicles were classified as primordial, primary, preantral, antral, corpora lutea, and cysts.[33,34] The number of different structures was determined in five ovaries per group.

Quantification of mRNA levels by real-time PCR

We assessed the mRNA expression of the *Pparg2* isoform and *Pparg1 + 2* isoforms, the adipokines (*Leptin*, *Chemerin*, and *Adiponectin*), the ovarian gonadotropin receptors *Lh-r* and *Fsh-r*, and the steroidogenic factors and enzymes: Steroidogenic acute regulatory protein (*Star*), cytochrome P450 family 11 subfamily A member 1 (*Cyp11a1*), 3B-Hydroxysteroid dehydrogenase (*3bhsd*), Cytochrome P450 family 17 subfamily A1 (*Cyp17a1*), and cytochrome P450 family 19 subfamily A1 and aromatase (*Cyp19a1*). All mRNA levels were evaluated by real-time PCR analysis. Briefly, total mRNA from ovarian tissue was extracted using RNAzol RT (MRC gene, Molecular Research Center, Cincinnati, OH, USA), following the manufacturer's instructions. cDNA was synthesized from 1 µg mRNA using random primer hexamers (Invitrogen-Life Technologies, Buenos Aires, Argentina). Real-time PCR analysis was performed from this cDNA (2.5 µL) in 10 µL reaction buffer containing a 20 mM dNTPs mix, GoTaqPolymerase (Promega), Eva Green 20x (Biotium Hayward, CA, USA), and gene-specific primers in a total volume of 12.5 µL. The qPCR conditions started with a denaturation step at 95 °C for 5 min and followed by up to 40 cycles of denaturation (95 °C), annealing (see temperature for each primer in Table 1) and primer extension (72 °C). The amplified products were quantified by fluorescence, using the Rotor Gene 6000 Corbett, and mRNA abundance was normalized to the amount of 60s Ribosomal protein L32 (*L32*). *L32* was validated as a reference gene as the variance between treatments did not differ. Gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method.[35] Results are expressed as a fold value of the controls.

Protein expression analysis

Protein expression in ovarian tissue was determined by Western blot analysis. Briefly, ovarian tissue was lysed for 20 min at 4 °C in lysis buffer (20 mM Tris-HCl, pH = 8.0, 137 mM NaCl, 1% Nonidet

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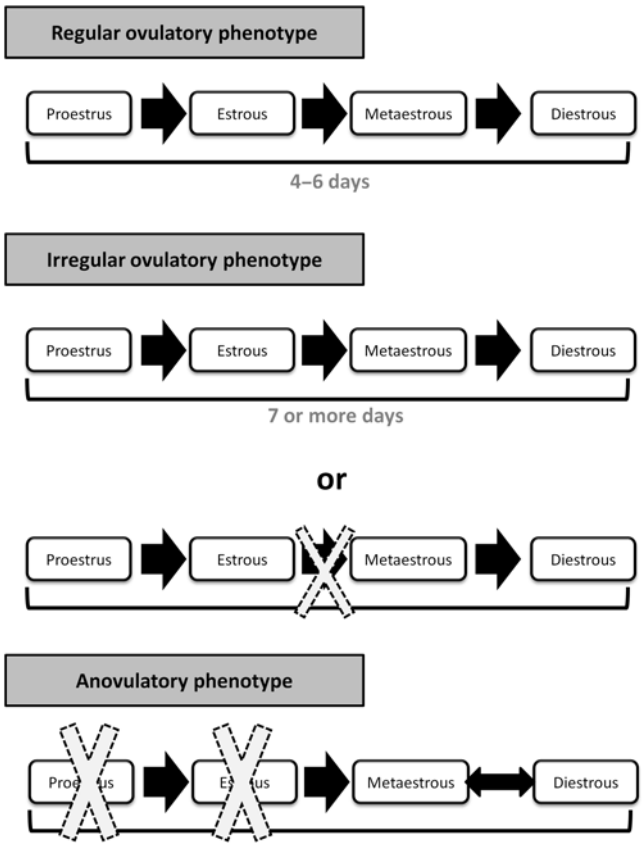

Fig. 1. Estrous cycle scheme for animals of each of the phenotypes defined from the postnatal day 45 to the time of sacrifice (around day 60). The animals defined as showing regular ovulatory phenotypes presented cycles that last between 4 and 6 days and respected the passage from proestrous, estrous, metaestrous, and diestrous. Rats, whose cycles lasted 7 days or more, showed some smears displaying an estrous stage, but other smears not following the progress of the cycle, as described above, were considered irregular ovulatory animals (PHiov). Rats that showed the metaestrous and diestrous stages or a combination of both for 4 consecutive days were considered to be noncycling and thus considered the anovulatory animals (PHanov).[7,31]

P-40, and 10% glycerol) supplemented with protease inhibitors (Sigma–Aldrich, St. Louis, MO, USA). The lysate was centrifuged at 4 °C for 10 min at 10,000 g and the pellet discarded. Protein concentrations in the supernatant were measured by the Bradford assay (Bio-Rad, Hercules, CA, USA). After boiling for 5 min, 50 µg of each protein was applied to an SDS-polyacrylamide gel (12% for PPARG and 3BHSD and 15% for adipokines) and electrophoresis was performed at 80 volts for 1.5 h. The separated proteins were transferred onto nitrocellulose membranes in transfer buffer (20% methanol, vol/vol; 0.19 M glycine; 0.025 M Tris-Base, pH = 8.3) for 1 h at 400 mA and 4 °C. Blocking was carried out for 1 h at room temperature in 5% (w/v) nonfat dry milk in phosphate-buffered saline (PBS) and membranes were incubated with the primary antibody (diluted in 1% (w/v) bovine serum albumin in PBS) overnight at 4 °C. Anti-PPARG 1 + 2 1:1000 (ab41928, Abcam, Cambridge, UK), anti-LEPTIN (sc-393043 1:400, Santa Cruz Biotechnology, CA, USA), anti-ADIPONECTIN (ab22554 1:1000, Abcam), anti-CHEMERIN 1:1000 (ab112520, Abcam), and anti-3BHSD 1:200 (sc-28206, Santa Cruz Biotechnology) were used as primary antibodies. The protein bands were visualized by incubating the blots with horseradish peroxidase-conjugated secondary antibody (Rabbit anti-mouse IgG H&L, HRP, 1:5000, ab6728, Abcam or Goat anti-rabbit IgG H&L, HRP 1:2000, 1706515, BioRad) for 1 h, followed by ECL Western Blotting Substrate (Thermo Scientific, IL, USA). Rainbow-colored protein mass markers (14.3-200 kDa, Bio-Rad) were applied to samples as molecular weight standards.

The consistency of protein loading was evaluated by staining the membranes with Ponceau-S and normalized applying the protein B-TUBULIN (ab131205, Abcam) or the protein B-ACTIN (Sigma. St. Louis, MO, USA). The intensities (area × density) of the individual bands were quantified by ImageQuant LAS 4000 (GE Healthcare Life Sciences, NJ, USA). Results are expressed as arbitrary units.

Serum determinations

LH and FSH were determined with radioimmunoassay (RIA) kits ($n = 7$ in duplicates for each of the phenotypes established), following the protocols previously described.[36,37] For these hormones, the intra- and interassay coefficients were less than 10% and 13%, respectively.

The estradiol to testosterone ratio (E2/T) was determined as a marker of ovarian function.[38] Serum testosterone and estradiol were already measured in the animal model and reported elsewhere before.[7] Animals from both the phenotypes (PHiov and PHanov) showed higher levels of testosterone than control group (Control = 35,9 ± 13,4 pg/mL; PHiov = 291,0 ± 82,7 pg/mL; PHanov = 344,5 ± 15,0 pg/mL), but only PHanov phenotype showed a significant difference in estradiol levels as compared with controls values (Control = 12,25 ± 2,31 pg/mL; PHiov = 9,69 ± 1,93 pg/mL; PHanov = 8,05 ± 1,24 pg/mL).[7]

Serum testosterone was quantified by RIA as previously described,[7] whereas serum estradiol levels were quantified by

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Table 1. List of primers used in real-time PCR

Primers used in real-time PCR		
Gene	Primers sequences	Temperature of annealing (°C)
Pparg1 + 2 F	TTT TCA AGG GTG CCA GTT TC	60
Pparg1 + 2 R	GAG GCC AGC ATG GTG TAG AT	
Pparg2 F	CTG TTA TGG GTG AAA CTC TGG	62
Pparg2 R	GAA ATC AAC CGT GGT AAA GG	
Leptin F	GGT GGC TGG TTT GTT TCT GT	62
Leptin R	TAT GTG GCT GCA GAG GTG AG	
Adiponectin F	TAG CCC CGA GAA TCA AAG AA	58
Adiponectin R	TGC AAT GTC AAA GTG GCA AT	
Chemerin F	GGC ACC TTT GTG AGG CTG GAA TTT	62
Chemerin R	ACC CTG TCC GGG GCT TAT TTG GAT	
L32 F	TGG TCC ACA ATG TCA AGG	58
L32 R	CAA AAC AGG CAC ACA AGC	
Lh-r F	AGG AGG CAC AGA TGC AAA CT	60
Lh-r R	AAC AAG ATG GGC TGA GAT GG	
Fsh-r F	CTC ATC AAG CGA CAC CAA GA	60
Fsh-r R	GGA AAG GAT TGG CAC AAG AA	
Star F	GCA GGG GGA TTT CTG AAT TT	62
Star R	GTC TCC GTC TCT GTG GCT TC	
Cyp11a1 F	TTC TCC TGC GAG GGT CCT AA	60
Cyp11a1 R	TTT GCT GCT TGA TGC GTC TG	
Cyp17a1 F	TCT CAT TAC ACC CAC GCA GA	60
Cyp17a1 R	CGG GGC AGT TGT TTA TCA TC	
Cyp19a1 F	CCT GGC AAG CAC TCC TTA TC	60
Cyp19a1 R	CCA CGT CTC TCA GCG AAA AT	
3bhsd F	GAC ACC CCT CAC CAA AGC TA	60
3bhsd R	TTG TAA AAT GGA CGC AGC AG	

F, forward sequence; R, reverse sequence

Cobase immunoassay analyzers using an Electro Chemiluminescence ImmunoAssay, following the manufacturer's instructions.[7] Progesterone serum levels were measured by RIA, as previously described.[39] Progesterone was extracted twice with diethyl ether. Progesterone antiserum was highly specific and showed low cross-reactivity. The intra- and interassay coefficients of variation were 10.9 and 12.8%, respectively. Values were expressed as ng/ml of serum progesterone.

Statistical analysis

Statistical analyses were carried out using the Instat program (GraphPad software, San Diego, CA, USA). *T*-student test or ANOVA with *post hoc* Tukey test were used to compare between two or three groups, respectively.

A generalized linear model (GLM) with binomial distribution was used to study the association between the proportion of anovulatory animals (classified as anovulatory or not) per female offspring per litter and the number of female dams, the number of male dams, and the total number of offspring per litter. These analyses were performed using the R environment (R Core team 2014).

Statistical significance was considered as $p < 0.05$.

Results

Litters analysis

The litters' details are shown in Table 2. Prenatal hyperandrogenization did not affect gestation time, the number of offspring neither the number of female nor male offspring. The prenatal hyperandrogenized animals showed alterations in their estrous cycles. When classifying the animals as anovulatory or not, the analysis showed that the proportion of the PHanov per females per litter was not associated with the total number of offspring (Fig. 2A, $p = 0.875$) nor with the number of males or females per offspring (Fig. 2B, $p = 0.4202$ and Fig. 2C, $p = 0.0581$, respectively).

Body and ovarian weight

Prenatal hyperandrogenization did not affect body weight or ovarian weight at 60 days of age. The PHanov phenotype showed a lower ovarian/body weight ratio (Table 3).

Ovarian follicle count and follicular development

To evaluate the effect of prenatal hyperandrogenism on folliculogenesis, we analyzed the ovarian morphology and the percentage of different follicular stages. No changes were found in the percentages of primordial follicles between groups (Fig. 3A, $p > 0.05$). The percentage of primary follicles was higher in the PHanov phenotype as compared with the Control group (Fig. 3B, $p < 0.05$). The percentage of preantral follicles was lower in the PHiov phenotype as compared with the Control group (Fig. 3C, $p < 0.05$). There were no differences between the percentages of antral follicles in the PH groups and the Control group (Fig. 3D, $p > 0.05$). The percentage of corpora lutea was lower in both the PH groups as compared with the Control group (Fig. 3E, $p < 0.05$). Regarding follicular cysts, we found that as expected control ovaries did not present follicular cysts, whereas both PH groups showed cysts (Fig. 3F, $p < 0.05$).

PPARG as an ovarian fuel sensor marker

Prenatal hyperandrogenization affected the PPARG system. Both PH phenotypes showed higher levels of mRNA of *Pparg1 + 2* (Fig. 4A, $p < 0.01$), but no alterations in the levels of *Pparg2* compared with the Control group (Fig. 4B, $p > 0.05$). Thus, as *Pparg* has two isoforms, the levels of *Pparg1* were the ones altered in the PH groups. To confirm this point, we evaluated the protein levels of both PPARG isoforms and found that the protein levels of PPARG1 were higher in both PH groups than in the Control group (Fig. 4C, $p < 0.05$) and that those of PPARG2 remained unaltered in the PH groups as compared with the Control group (Fig. 4D, $p > 0.05$).

Characterization of the gene and protein expression of ovarian adipokines

The adipokine ovarian secretion pattern was found to be differentially deregulated in both PH phenotypes. *Leptin* mRNA levels were lower in the PHanov phenotype but not altered in the PHiov phenotype as compared with the Control group (Fig. 5A, $p < 0.01$). We found no alterations in the leptin protein levels

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Table 2. Litter characterization between control and hyperandrogenized groups, $n = 15$ litters analyzed per group. Statistical analyses were made by *t*-students test. No differences were found between Control and hyperandrogenized litters for gestation time or litter analysis $p > 0.05$

Parameter evaluated		Treatment	
		Control	Hyperandrogenized
Gestation time (days)		22,17 ± 0,41	22,00 ± 0,28
Litter analysis	Number of offspring	12,25 ± 0,50	12,4 ± 2,02
	Number female offspring	7,25 ± 1,89	6,05 ± 1,75
	Number of male offspring	5,20 ± 1,31	6,32 ± 1,76
Percentage of female offspring showing:			
Regular ovulatory phenotype		100% (106)	13,64% (12)
Anovulatory phenotype		0% (0)	42,04% (37)
Irregular ovulatory phenotype		0% (0)	44,32% (39)

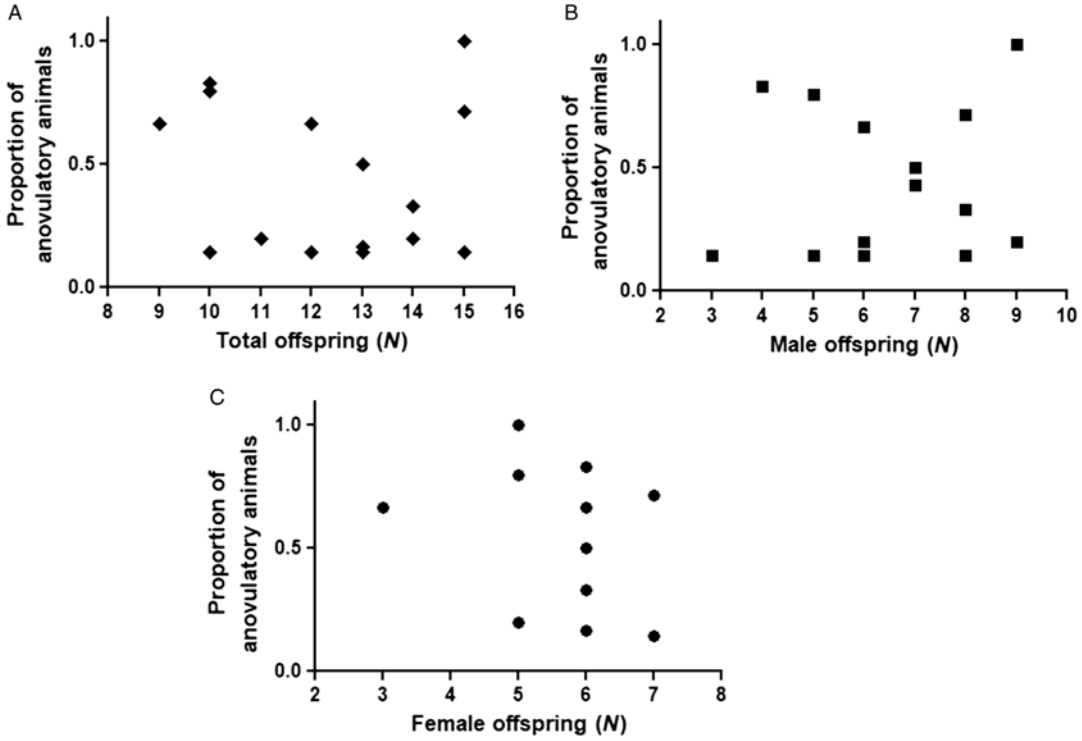

Fig. 2. Evaluation of the association between the proportion of anovulatory animals per litter and (A) the total number of offspring ($p > 0.05$), (B) the number of male dams ($p > 0.05$), and (C) the number of female dams ($p > 0.05$). Statistical analyses were made by a generalized linear model (GLM) with binomial distribution.

(Fig. 5B, $p > 0.05$). Regarding *Adiponectin* mRNA expression, the PHiov phenotype showed an increase in its levels as compared with both the PHanov and Control groups (Fig. 5C, $p < 0.01$). Adiponectin protein levels were lower in both of the PH phenotypes (Fig. 5D, $p < 0.01$). *Chemerin* mRNA and protein levels were only altered in the PHiov phenotype, showing higher levels than the Control and PHanov groups (Fig. 5E, 5F, $p < 0.05$).

Hormonal regulation of steroidogenesis

Neither of the PH groups showed altered levels of the gonadotropins FSH and LH as compared with the Control group (Fig. 6A, 6B, respectively, $p > 0.05$).

The PHiov phenotype showed lower levels of mRNA expression of *Fsh-r* than the Control group (Fig. 6C, $p < 0.05$), whereas the PHanov phenotype showed an increased expression of *Lh-r* mRNA levels as compared with the Control group (Fig. 6D, $p < 0.05$). The LH to FSH ratio, proposed as a marker of PCOS, presented no differences between the PH groups and the Control group (Fig. 6E, $p > 0.05$).

We have previously reported that testosterone levels are higher in both PH phenotypes and that estradiol levels are lower in the PHanov phenotype.[7] Here, we showed that both PH groups showed lower levels of the E2 to T ratio than the Control group (Fig. 6F, $p < 0.05$).

We analyzed progesterone serum levels and the expression of *3bhsd*, the limiting enzyme of its synthesis. Both, the mRNA and protein levels of *3bhsd*, were not affected in the PH groups as compared with the controls (Fig. 7A, 7B, $p > 0.05$). Progesterone serum levels were decreased in both the PH phenotypes as compared with the Control group levels (Fig. 7C, $p < 0.05$).

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Table 3. Body weight, ovarian weight, and ovarian weight/body weight ratio for the prenatally hyperandrogenized (PH) and Control groups for eight rats per group

Body weight, ovarian weight, and ovarian weight/body weight ratio				
	Control	PHiov	PHanov	*p value*
Body weight (g)	209.33 + 12.05[a]	208.00 ± 16.02[a]	222.00 ± 6.48[a]	$p = 0.20$
Average ovarian weight (g)	47.33 ± 1.53[a]	43.40 ± 3.97[a]	41.25 ± 3.20[a]	$p = 0.10$
Average ovarian weight (g) / body weight (g) ratio	0.22 ± 0.02[a]	0.21 ± 0.03[ab]	0.19 ± 0.02[b]	$p = 0.0103$

Statistical analyses were made by ANOVA, a vs. b, $p < 0.05$.

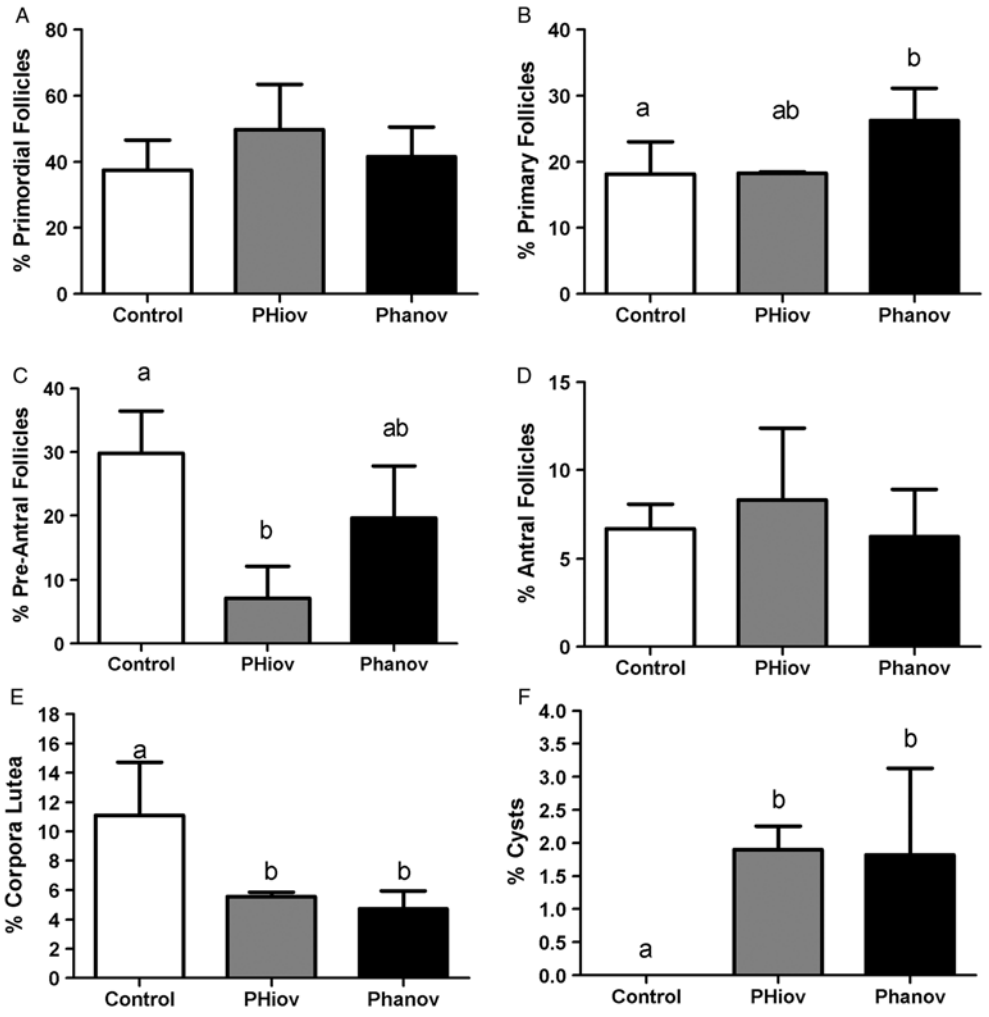

Fig. 3. Effects of prenatal hyperandrogenization on the quantification of the percentage of each follicular stage in the ovaries of the prenatally hyperandrogenized (PH) and Control groups. (A) Percentages of primordial follicles, (B) percentages of primary follicles, (C) percentages of preantral follicles, (D) percentages of antral follicles, (E) percentages of corpora lutea, and (F) percentage of cysts. Each column represents the mean ± SD from five animals per group. Statistical analyses were made by ANOVA; different letters mean statistically significant differences (a vs. b, $p < 0.05$).

Ovarian steroidogenic pathway

Prenatal hyperandrogenization affected the steroidogenic pathway. The mRNA levels of *Star* were higher in the PHanov phenotype than in the Control and PHiov groups (Fig. 8A, $p < 0.05$). Besides, the mRNA levels of *Cyp11a1* were higher in the PHanov phenotype than in the Control group (Fig. 8B, $p < 0.05$). The mRNA levels of *Cyp17a1*, a limiting enzyme in androgen synthesis, were lower in the PHiov phenotype than in the Control group but showed no alterations in the PHanov phenotype (Fig. 8C, $p < 0.05$). The mRNA levels of *Cyp19a1* (*Aromatase*) were lower in the PHanov phenotype than in the Control group (Fig. 8D, $p < 0.05$).

Discussion

Reproductive functions depend on the energy balance, and several metabolic misbalances may negatively impact on female reproduction.[10] Such as in the case of PCOS, these patients display not only reproductive alterations, but also metabolic disturbances.

We have previously reported that prenatal hyperandrogenization could lead to two different phenotypes regarding the estrous cycle and that both showed altered systemic metabolism and ovarian morphological alterations.[7] In the present study, we show that, after prenatal hyperandrogenization, the ovary signaling of fuel sensors is impaired and that each phenotype presents a different

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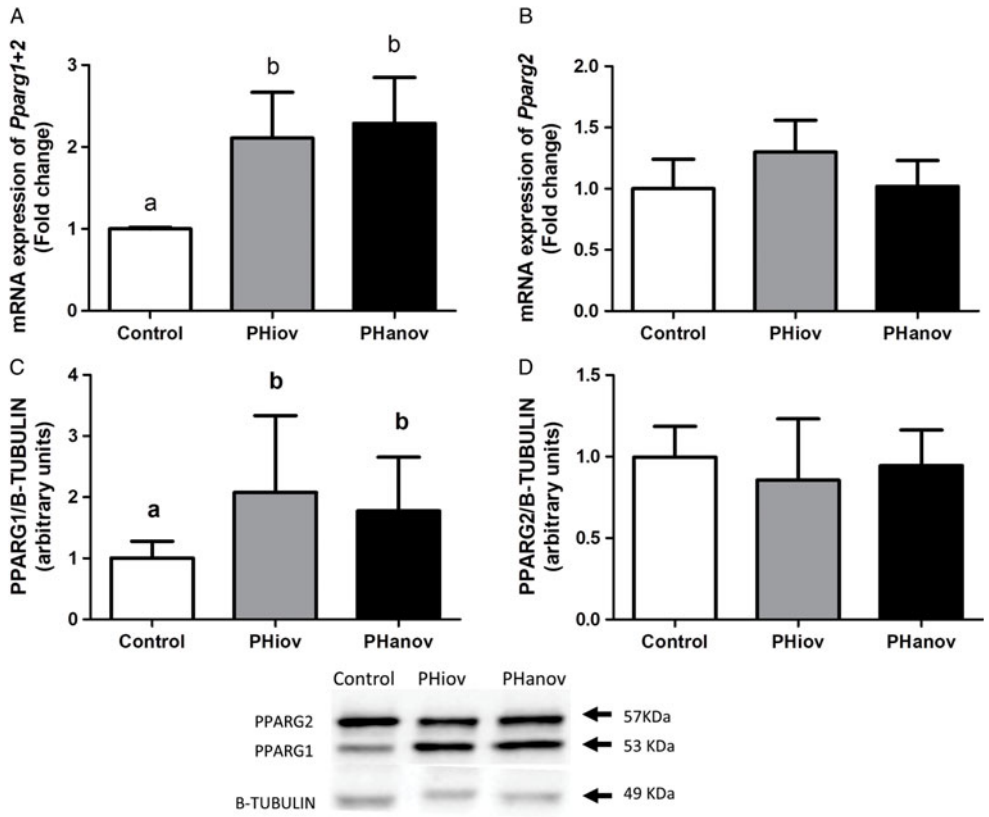

Fig. 4. Effects of prenatal hyperandrogenization on the PPARG ovarian system in the prenatally hyperandrogenized (PH) and Control groups. The graphs correspond to (A) mRNA abundance of *Pparg1 + 2* ($p < 0.01$) and (B) mRNA abundance of *Pparg2* ($p > 0.05$), both relative to *L32*. (C) Protein levels of PPARG1 ($p < 0.05$) and (D) protein levels of PPARG2 ($p > 0.05$), both relative to B-TUBULIN levels. Each column represents the mean ± SD from six animals per group. Statistical analyses were made by ANOVA; different letters mean statistically significant differences (a vs. b, $p < 0.05$).

deregulation that affects cyclicity and the steroidogenic pathway within the ovary.

In the present study, we evaluated a period of transition to adulthood (60 days of age).[40–42] Our results showed that the reproductive phenotype did not depend on the number of males or females per litter. In the same litter, we could detect animals that showed PHanov while their siblings not. Thus suggesting a plastic response to prenatal androgens. A plastic phenotype has also been described in PCOS patients and their relatives. Particularly, Jahanfar *et al.* (1995)[43] showed in a study of monozygotic and dizygotic twins that while one of the twins may show polycystic ovaries with hyperandrogenism and/or menstrual dysfunction, the other twin shows only some or none of the features. However, the complex network of mechanisms that leads to the described two phenotypes found in this work may involve not only developmental plasticity phenomena,[44] but also embryo position *in utero* and differential placental dysfunctions. The position of a fetus in relation to the sexes of its neighboring intrauterine littermates has been reported to influence its exposure to gonadal hormones and therefore its development.[45] Moreover, regarding placental dysfunction, it has been reported a deficit in placental aromatase in PCOS women, thus compromising placental protection mechanisms to androgen actions.[46] Sun *et al.* (2012) showed that prenatal hyperandrogenized rats presented altered placental steroidogenesis without affections on the maternal metabolic status or circulating estradiol, suggesting that the effect of testosterone on placental and fetal growth in rats is direct and not a consequence of alterations in metabolism and steroid hormones.[47] However, in other species, for example, in sheep and monkeys, the effects of testosterone on programming reproductive alterations might be caused by direct androgenic effects or by an indirect action via estrogenic pathways as a result of testosterone aromatization.[48–50] Thereby, the response to androgens in different species needs to be further studied.

The ovary weight/body weight ratio, usually used as a marker of prenatal growth when the gonads are developed, has been described as a better marker than organ weight.[51,52] Here, we found that this ratio was impaired in the PHanov phenotype but not in the PHiov phenotype. This lower ratio in the PHanov phenotype may reflect a harmful effect of the prenatal exposure of the developing organ to testosterone, which conditions its growth and functions during postnatal life. This may explain, at least in part, the differences in estrous cyclicity between the two PH phenotypes.

Ovarian energetic signaling involves two important master energetic fuel sensors: PPARG and adipokines. PPARG may regulate the gene expression of several adipokines and is in turn regulated by complex networks of adipokine pattern.[24,26,53,54]

In the present study, we found an altered expression of *Pparg* in both PH groups. Specifically, we found that both PPARG1 and PPARG2 were expressed in the ovaries of the PH groups and that PPARG1, the most abundant isoform in the ovary,[17,18] was impaired in both PH groups. It has been reported that PPARG may influence the development of granulosa cells and that alterations in its expression may affect oocyte developmental competence, thus compromising ovary functions.[17,18] Thus, the impairment of ovarian PPARG may be contributing to the dysregulation of the estrous cycle found in the PH groups. On the other

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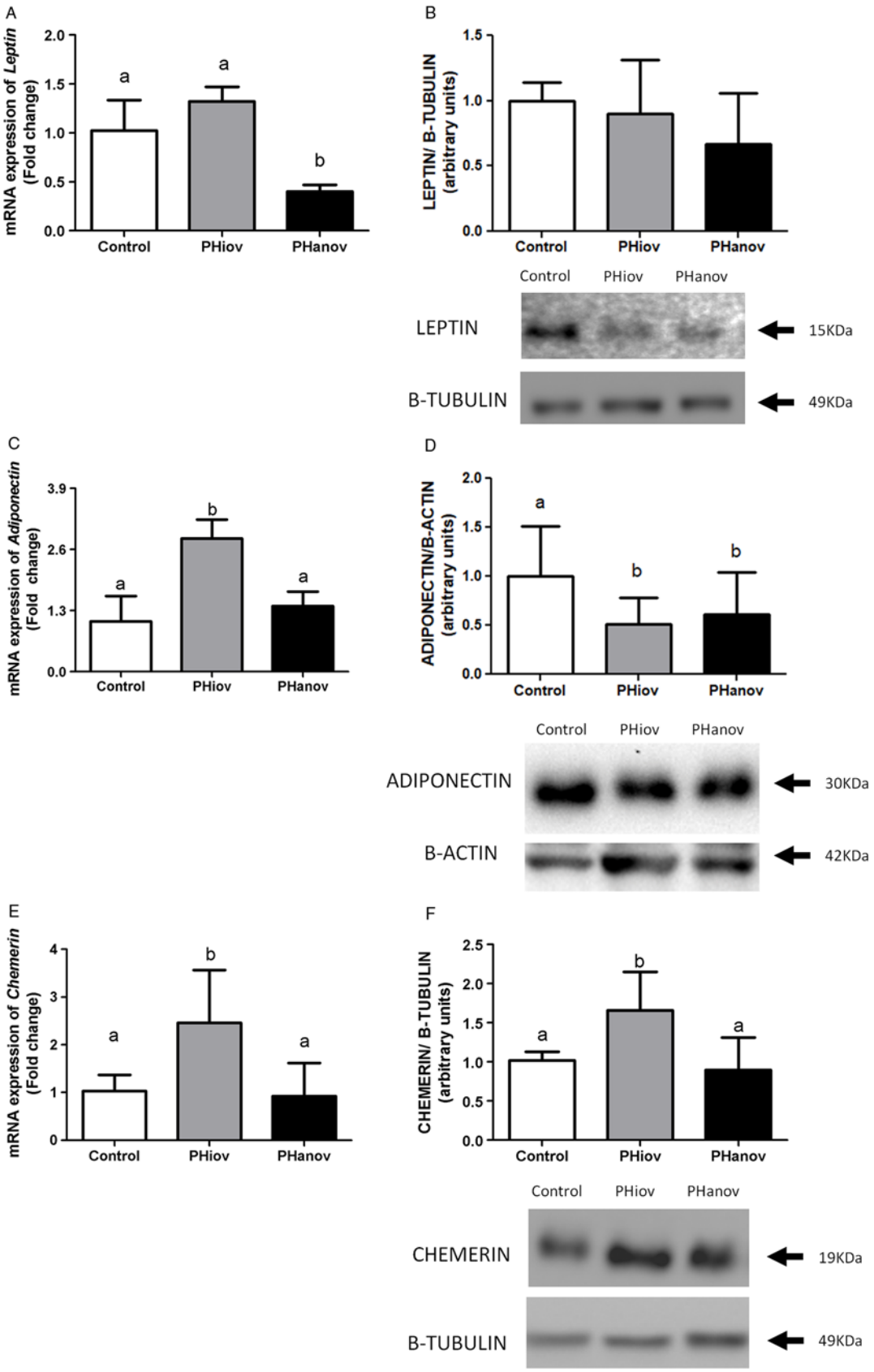

Fig. 5. Effects of prenatal hyperandrogenization on the adipokine ovarian pattern. The graphs correspond to (A) mRNA abundance of *leptin* ($p < 0.01$), (B) protein levels of *leptin* ($p > 0.05$), (C) mRNA levels of *adiponectin* ($p < 0.01$), (D) protein levels of *adiponectin* ($p < 0.01$), (E) mRNA levels of *chemerin* ($p < 0.05$), and (F) protein levels of *chemerin* ($p < 0.05$) of the prenatally hyperandrogenized (PH) and Control groups. Each column represents the mean ± SD from 6 animals per group for mRNA analysis and 10 animals per group for protein levels analysis. Statistical analyses were made by ANOVA; different letters mean statistically significant differences (a vs. b, $p < 0.05$).

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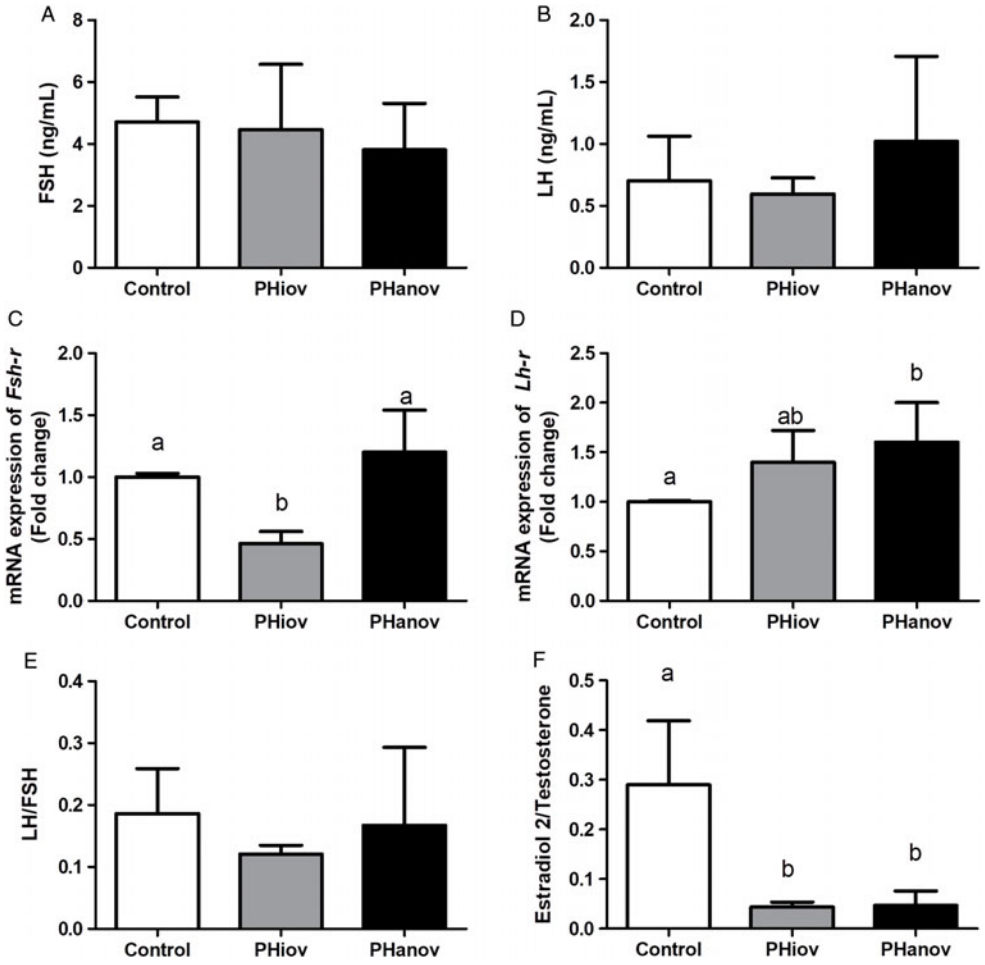

Fig. 6. Effect of prenatal hyperandrogenization on gonadotropin secrerum levels, ovarian receptors, and hormone ratios in the prenatally hyperandrogenized (PH) and control groups. (A) Serum FSH levels ($p > 0.05$) and (B) serum LH levels ($p > 0.05$); (C) mRNA abundance of ovarian *Fsh-r* ($p < 0.05$) and (D) mRNA abundance of ovarian *Lh-r* ($p < 0.05$), both relative to *L32*; (E) the LH to FSH ratio ($p > 0.05$), (F) estradiol to testosterone ratio ($p < 0.05$) of the prenatally hyperandrogenized (PH) and Control groups. Each column represents the mean ± SD from six animals per group for mRNA expression levels analysis and from seven animals per group for hormonal measurements. For the estradiol/testosterone ratio, seven animals per group were considered. Statistical analyses were made by ANOVA; different letters mean statistically significant differences (a vs. b, $p < 0.05$).

hand, PPARs may also modulate estradiol levels by regulating *Aromatase* expression.[55–58] In agreement with these findings, we have previously reported that estradiol levels were lower in the PHanov phenotype than in controls,[7] whereas here we found that *Aromatase* mRNA expression was also decreased in this phenotype. These findings can be due to the action of the increased PPARG levels that contribute to the hormonal imbalance of the estradiol to testosterone ratio. These results are in accordance with previous reports which have shown that, in a hyperandrogenic context, *Pparg* is upregulated, contributing to alterations in androgen production.[5,9] Our results add evidence to the idea that PPARG may be one of the mediators in the developmental programming of ovarian dysfunctions in a context of androgen excess.[9]

Adipokines can regulate ovarian steroidogenesis, oocyte maturation, and embryo development.[23] Our present research was focused on three adipokines: leptin, adiponectin, and chemerin, which are known to be involved in the regulation of ovarian steroidogenesis and related to the master metabolic regulator PPARG.[23,26,59–63] Regarding *Leptin* levels, the PHanov phenotype showed a decrease in its mRNA expression. In fact, decreased leptin levels are related to an infertility status.[64] In addition, leptin levels have been shown to be inversely correlated to PPARG levels in several tissues.[53,65,66] We did not find alterations in leptin protein expression, possibly due to a deregulation of its secretion. It has been shown that androgens might impact negatively on leptin exocytosis.[67] Thus, as in the PHanov phenotype, we found an increase in androgen production, which may impair leptin secretion. Regarding the other adipokines, in the PHanov phenotype, we found no alterations in chemerin levels, but a decrease in the protein levels of adiponectin without affecting the mRNA levels of this adipokine. It has been reported that adiponectin modulates follicle maturation and particularly preovulatory changes in the ovary, but also steroidogenesis.[68,69] These findings are in agreement with our results as low levels of both of these adipokines may be related to the increase of primary follicles found in this phenotype, thus contributing to the reproductive issues and ovulatory dysfunctions. It is also important to highlight that adiponectin acts as an insulin-sensitizing agent in several tissues,[28,70] and it has been found that insulin-resistant PCOS patients have lower adiponectin levels.[70] In accordance with these results, we have already reported that the PHanov phenotype showed insulin resistance.[7] Thereby, these results suggest that impaired ovarian adiponectin expression may be associated with metabolic alterations.

In the PHiov phenotype, the leptin levels remained unaltered compared with the Control group. Regarding adiponectin expression, we found that, although the protein levels of this adipokine

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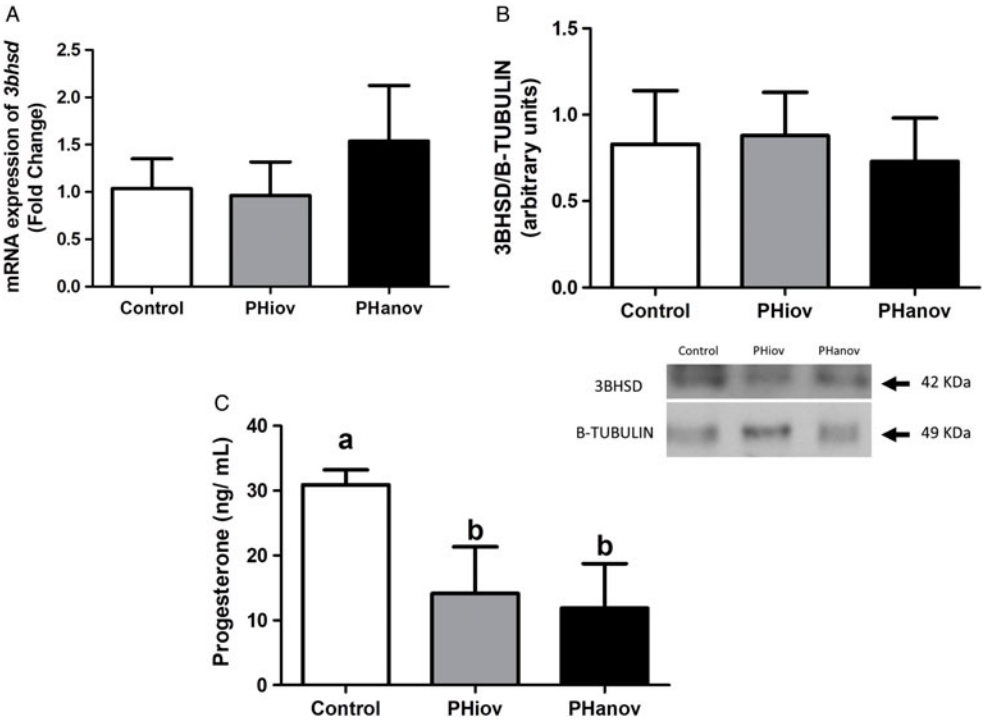

Fig. 7. Effect of prenatal hyperandrogenization on progesterone synthesis and serum levels in the prenatally hyperandrogenized (PH) and control groups. (A) mRNA abundance of ovarian *3bhsd* relative to *L32* ($p > 0.05$), (B) protein levels of ovarian 3BHSD relative to B-TUBULIN levels ($p > 0.05$), and (C) progesterone levels ($p < 0.05$) of the prenatally hyperandrogenized (PH) and Control groups. Each column represents the mean ± SD from 6 animals per group for mRNA expression, 10 animals per group for protein levels analysis, and from 7 animals per group for progesterone measurements. Statistical analyses were made by ANOVA; different letters mean statistically significant differences (a vs. b, $p < 0.05$).

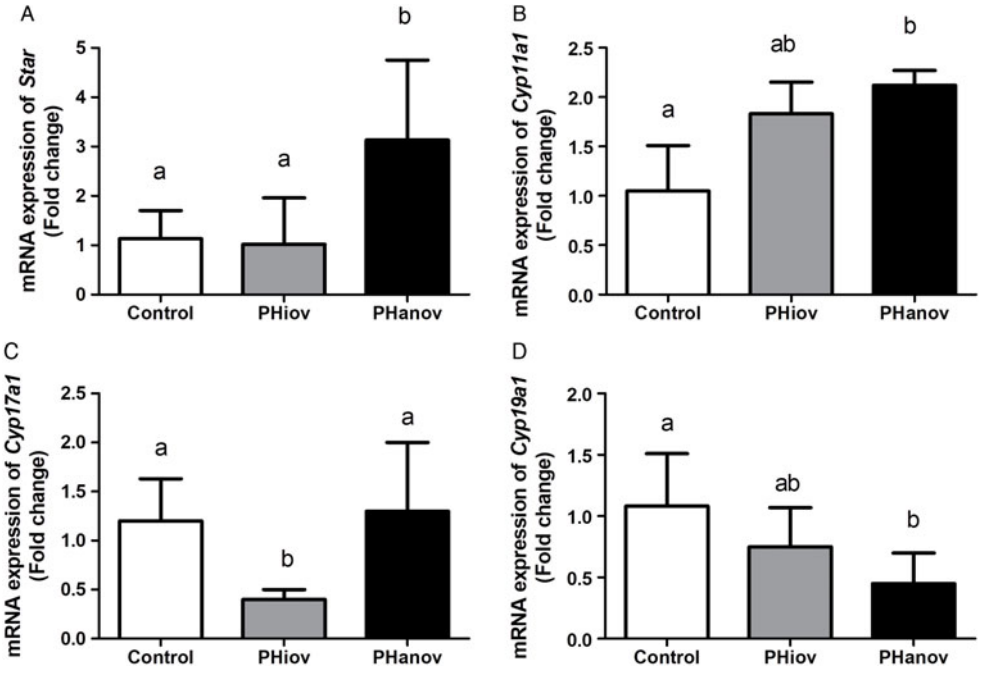

Fig. 8. Effect of prenatal hyperandrogenization on ovarian steroidogenic factors and enzymes. The graphs correspond to mRNA abundance of (A) *Star* ($p < 0.05$), (B) *Cyp11a1* ($p < 0.05$), (C) *Cyp17a1* ($p < 0.05$), and (D) *Cyp19a1 (aromatase)* ($p < 0.05$), all of them relative to mRNA abundance of *L32* of the prenatally hyperandrogenized (PH) and Control groups. Each column represents the mean ± SD from six animals per group. Statistical analyses were made by ANOVA; different letters mean statistically significant differences (a vs. b, $p < 0.05$).

were low, there was a high expression of the mRNA levels. These results suggest an increase in *Adiponectin* mRNA transcription, possibly in response to the low ovarian protein levels. It is important to highlight that many post-transcriptional mechanisms may modulate mRNA translation. In accordance to this, miR-378 may modulate *Adiponectin* expression in adipose tissue[71]; this miRNA is expressed in ovary and also downregulates *Aromatase* expression,[72] but to our knowledge, there have been no studies on

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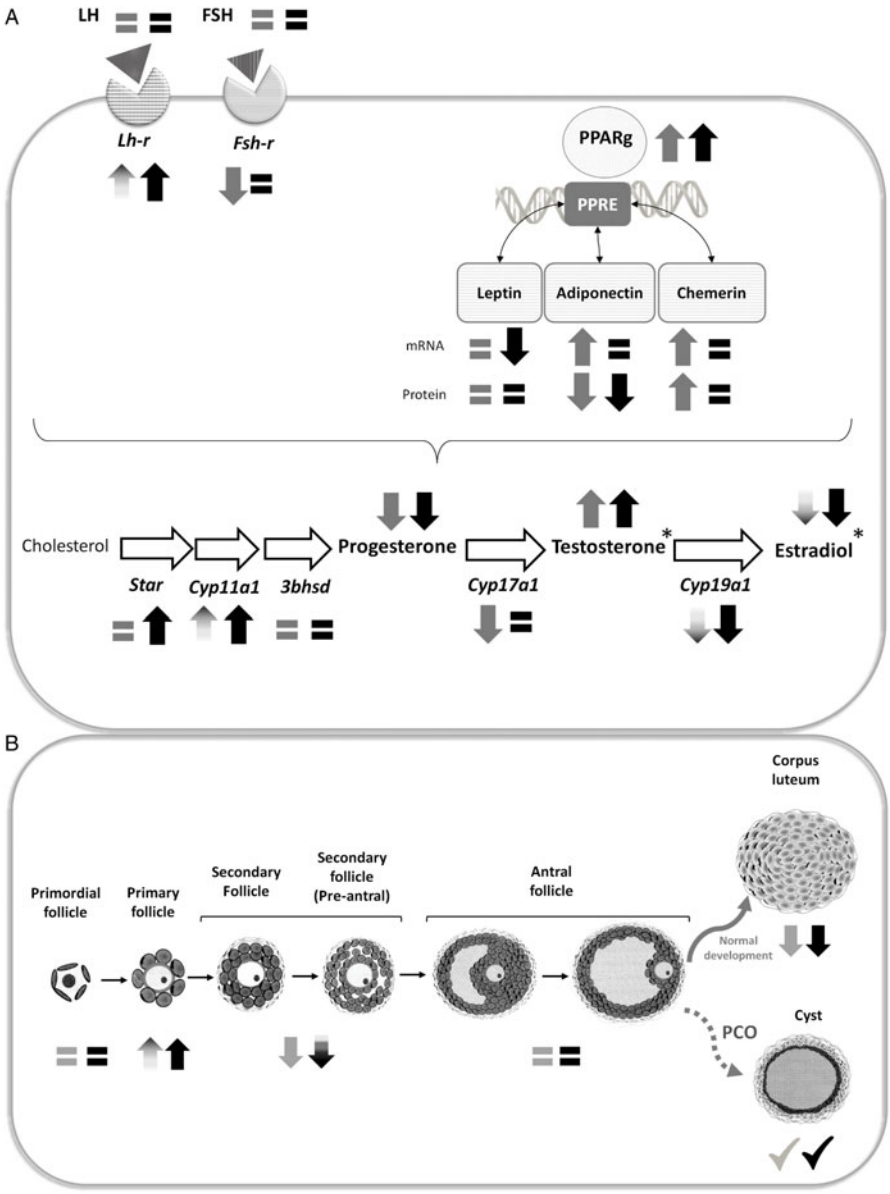

Fig. 9. Effects of prenatal hyperandrogenization on ovarian folliculogenesis and steroidogenesis. Ovarian folliculogenesis and steroidogenesis depend on gonadotropins (LH and FSH) and fuel sensors such as PPARG, leptin, adiponectin, and chemerin. An impaired profile of ovarian steroidogenic factors and enzymes as well as altered steroid production was found on PH groups. Moreover, prenatal hyperandrogenization also led to alterations in folliculogenesis. Gray arrows and equals signs correspond to PHiov group. Black arrows and equals signs correspond to PHanov group. Gray and black gradient arrows show the tendency to decrease (down arrow) or to increase (up arrow) for the different parameters in the PHiov and PHanov phenotypes, respectively. Check marks indicate the presence of cysts. PCO = polycystic ovaries. *Serum testosterone and estradiol were already measured in the animal model and reported elsewhere.

miR-378 in PCOS and neither on ovarian adiponectin regulation. Thus, the regulation of this adipokine in the ovary in an androgenic context needs further study.

The PHiov phenotype also showed increased levels of chemerin expression. It is known that this adipokine may inhibit steroidogenesis.[62] Therefore, adiponectin and chemerin may jointly contribute to the ovarian alterations observed in the PHiov phenotype.

Ovarian steroidogenesis is a process that depends on energy expenditure and neuroendocrine control and is regulated by autocrine signaling. Our results showed that the levels of both pituitary hormones LH and FSH were not altered in the PH groups as compared with the Control group. However, we found altered expression of the ovarian receptors *Fsh-r* and *Lh-r*, suggesting a possible impairment of the LH and FSH signaling in the ovaries. These data are in agreement with those of other authors who showed that in a hyperandrogenic context, gonadotropin levels may remain unaltered, although alterations in their pulsatility may be contributing to neuroendocrine dysfunction.[73,74] Here, we found that although LH and FSH secretion may not be impaired by prenatal hyperandrogenization, the ovarian response is affected, a fact also reflected in steroidogenic alterations and cyclicity deregulations found. In addition, it has to be considered that the secretion of these gonadotropins is pulsatile and that we only evaluated the levels at the diestrous stage. It is important to point out that within the ovary, FSH is necessary to induce the maturation of ovarian follicles and that together with estrogen it leads to ovulation.[75] Here, we found that the PHiov phenotype showed a decreased expression of *Fsh-r*, which affected the ovarian response to this

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hormone and low levels of the estradiol to testosterone ratio, which contributed to the irregular estrous cycles present in this phenotype. On the other hand, we found that the PHanov phenotype showed high levels of *Lh-r*, which allowed increased response to this hormone and contributed to an exacerbated androgen production,[75,76] which is reflected on the high serum testosterone levels and an impaired E2/T ratio found.

Alterations in hormone and metabolic sensors levels may lead to defects in follicular development. To further characterize these changes, we evaluated the ovarian percentage of each follicular stage, corpora lutea, and cysts. In ovaries from PH rats, we found more primary follicles and less preantral follicles than in ovaries from control rats. Moreover, PH ovaries showed an increase in the percentage of cysts. These results suggest that follicular development would be impaired in PH rats. Contrary to our findings, in PCOS women it has been shown that preantral follicles are increased.[77] However, the decrease of preantral follicles has also been reported in other androgenic contexts and PCOS models in rodents (Reviewed in Noroozzadeh *et al.* (2017)[78]). It is likely that preantral follicles in rats start to grow, although this development is abnormal and tend to form cysts. Surprisingly, we found corpora lutea in the PHanov animals. This has already been reported by other authors, who described the presence of corpora lutea in rats that were acyclic and did not present vaginal opening.[5,79] This phenomenon may be explained, as these animals showed their vaginal opening around day 33 of life, which matches the first ovulation.[80] However, as we started following the vaginal smears at day 45 of life, the observed corpora lutea may belong to those previous cycles that were not assessed and can be detected because they persist for three consecutive cycles (12–14 days).[81] In accordance with our findings, in young PCOS patients, the menarche usually occurs, but then develops secondary amenorrhea as they grow up.[82] It is important to highlight that we found the corpora lutea of PH animals show alterations in their structure (see Abruzzese *et al.* 2016[7]). Moreover, in a previous work from our group,[5] we found that acyclic rats also showed corpora lutea with abnormal luteinization; here, we found a decrease in progesterone levels of PH animals, thus suggesting a dysfunction in corpora lutea.

It is known that steroidogenic enzymes are controlled by the ovarian fuel sensors PPARG and adipokines. Our results showed that *Star* and *Cyp11a1*, the two enzymes that participate in the first steps of the steroidogenic process, increased in the PHanov phenotype and slightly decreased in the PHiov phenotype. This indicates that in the PH groups, the steroidogenesis process is impaired from the very first steps. We also found a phenotype-specific pattern in the expression of *Cyp17a1* and *Cyp19a1*, the limiting enzymes of androgen and estradiol synthesis, respectively. The PHiov phenotype showed low levels of *Cyp17a1* and a tendency to low levels of *Cyp19a1*. It has been reported that adiponectin may regulate *Cyp17a1* by inhibiting androgen production in cattle,[83] but also PPARG system may modulate *Cyp17a1* expression.[20,84] Thus, in this phenotype, steroid synthesis may be partly buffered by the action of adiponectin and of PPARG. On the other hand, chemerin may act downregulating *Cyp19a1* expression and inhibiting estradiol secretion.[85] In accordance with these results, we found a tendency to a decrease in *Cyp19a1* levels, a result that agrees with our previous findings of a tendency to low levels of serum estradiol in this phenotype.[7]

The PHanov group showed no alterations in *Cyp17a1* but a decrease in *Cyp19a1* levels. It is known that leptin may regulate aromatase activity by stimulating estrogen production. As already mentioned, PPARG activation upregulates *Star* and downregulates *Cyp19a1*, and both PPARG and leptin are implicated in the dysregulation of the steroidogenic processes in the PHanov phenotype.[86,87]

These results show that, despite the absence of obesity or overweight, prenatal hyperandrogenization leads to an impaired ovarian energy status. We found that prenatal hyperandrogenism affects the energy expenditure within the ovary, altering the master fuel sensor PPARG, the adipokines leptin, adiponectin, and chemerin, and also impacts on ovarian steroidogenesis and the regulation of ovarian gonadotropin signaling leading to hormonal imbalances (Fig. 9).

Author ORCID. Giselle Adriana Abruzzese 0000-0003-3498-7010

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Conflicts of Interest. The authors have no conflicts of interest to declare.

Ethical Standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals committee of Consejo Nacional de Investigaciones Científicas y Tecnicas (CONICET) 1996, Argentina, and the study was approved by the Ethics Committee of the School of Medicine of University of Buenos Aires, Argentina.

References

1. Chan KA, Tsoulis MW, Sloboda DM. Early-life nutritional effects on the female reproductive system. *J Endocrinol.* 2015; 224, R45–R62.
2. Franks S. Polycystic ovary syndrome. *N Engl J Med.* 1995; 333, 853–861.
3. Demissie M, Lazic M, Foecking EM, Aird F, Dunaif A, Levine JE. Transient prenatal androgen exposure produces metabolic syndrome in adult female rats. *Am J Physiol Endocrinol Metab.* 2008; 295, E262–E268.
4. Foecking EM, McDevitt MA, Acosta-Martínez M, Horton TH, Levine JE. Neuroendocrine consequences of androgen excess in female rodents. *Horm Behav.* 2008; 53, 673–692.
5. Amalfi S, Velez LM, Heber MF, *et al.* Prenatal hyperandrogenization induces metabolic and endocrine alterations which depend on the levels of testosterone exposure. *PLoS One.* 2012; 7, e37658.
6. Ramezani Tehrani F, Noroozzadeh M, Zahediasl S, Piryaei A, Hashemi S, Azizi F. The time of prenatal androgen exposure affects development of polycystic ovary syndrome-like phenotype in adulthood in female rats. *Int J Endocrinol Metab.* 2014; 12, e16502. doi: 10.5812/ijem.16502.
7. Abruzzese GA, Heber MF, Ferreira SR, *et al.* Prenatal hyperandrogenism induces alterations that affect liver lipid metabolism. *J Endocrinol.* 2016; 230, 67–79.
8. Abbott DH, Barnett DK, Levine JE, *et al.* Endocrine antecedents of polycystic ovary syndrome in fetal and infant prenatally androgenized female rhesus monkeys1. *Biol Reprod.* 2008; 79, 154–163.
9. Ortega HH, Rey F, Velazquez MML, Padmanabhan V. Developmental programming: effect of prenatal steroid excess on intraovarian components of insulin signaling pathway and related proteins in sheep. *Biol Reprod.* 2010; 82, 1065–1075.
10. Torre SD, Benedusi V, Fontana R, Maggi A. Energy metabolism and fertility—a balance preserved for female health. *Nat Rev Endocrinol.* 2014; 10, 13–23.
11. Walker DM, Gore AC. Transgenerational neuroendocrine disruption of reproduction. *Nat Rev Endocrinol.* 2011; 7, 197–207.

https://doi.org/10.1017/S2040174419000126 Published online by Cambridge University Press

12. Fernandez-Fernandez R, Martini AC, Navarro VM, *et al*. Novel signals for the integration of energy balance and reproduction. *Mol Cell Endocrinol*. 2006; 254–255, 127–132.
13. Faut M, Elia EM, Parborell F, Cugnata NM, Tesone M, Motta AB. Peroxisome proliferator-activated receptor gamma and early folliculogenesis during an acute hyperandrogenism condition. *Fertil Steril*. 2011; 95, 333–337.
14. Sandoval D, Cota D, Seeley RJ. The integrative role of CNS fuel-sensing mechanisms in energy balance and glucose regulation. *Annu Rev Physiol*. 2008; 70, 513–535.
15. Shiue Y-L, Chen L-R, Tsai C-J, Yeh C-Y, Huang C-T. Emerging roles of peroxisome proliferator-activated receptors in the pituitary gland in female reproduction. *Biomarkers Genomic Med*. 2013; 5, 1–11.
16. Issemann I, Green S. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature*. 1990; 347, 645–650.
17. Komar CM. Peroxisome proliferator-activated receptors (PPARs) and ovarian function – implications for regulating steroidogenesis, differentiation, and tissue remodeling. *Reprod Biol Endocrinol*. 2005; 3, 41.
18. Froment P, Gizard F, Defever D, Staels B, Dupont J, Monget P. Peroxisome proliferator-activated receptors in reproductive tissues: from gametogenesis to parturition. *J Endocrinol*. 2006; 189, 199–209.
19. Vitti M, Di Emidio G, Di Carlo M, *et al*. Peroxisome proliferator-activated receptors in female reproduction and fertility. *PPAR Res*. 2016; 2016, 4612306. doi: 10.1155/2016/4612306.
20. Velez LM, Heber MF, Ferreira SR, Abruzzese GA, Reynoso RM, Motta AB. Effect of hyperandrogenism on ovarian function. *Reproduction*. 2015; 149, 577–585.
21. Cui Y, Miyoshi K, Claudio E, *et al*. Loss of the Peroxisome Proliferation-Activated Receptor gamma (PPARγ) does not affect mammary development and propensity for tumor formation but leads to reduced fertility. *J Biol Chem*. 2002; 277, 17830–17835.
22. Mitchell M, Armstrong DT, Robker RL, Norman RJ. Adipokines: implications for female fertility and obesity. *Reproduction*. 2005; 130, 583–597.
23. Reverchon M, Ramé C, Bertoldo M, Dupont J. Adipokines and the female reproductive tract. *Int J Endocrinol*. 2014; 2014, 10. doi: 10.1155/2014/232454.
24. Bharati J, Bharti MK, Kar D, Sahoo PR. Adipokines as metabolic modulators of ovarian functions in livestock: a mini-review. *J Adv Vet Anim Res*. 2016; 3, 206–213.
25. Chen X, Jia X, Qiao J, Guan Y, Kang J. Adipokines in reproductive function: a link between obesity and polycystic ovary syndrome. *J Mol Endocrinol*. 2013; 50, R21–R37.
26. Muruganandan S, Parlee SD, Rourke JL, Ernst MC, Goralski KB, Sinal CJ. Chemerin, a Novel Peroxisome Proliferator-activated Receptor γ (PPARγ) target gene that promotes mesenchymal stem cell adipogenesis. *J Biol Chem*. 2011; 286, 23982–23995.
27. Considine RV. Regulation of leptin production. *Rev Endocr Metab Disord*. 2001; 2, 357–363.
28. Dupont J, Chabrolle C, Ramé C, Tosca L, Coyral-Castel S. Role of the peroxisome proliferator-activated receptors, adenosine monophosphate-activated kinase, and adiponectin in the ovary. *PPAR Res*. 2008; 2008, 176275. doi: 10.1155/2008/176275.
29. Martos-Moreno GÁ, Chowen JA, Argente J. Metabolic signals in human puberty: effects of over and undernutrition. *Mol Cell Endocrinol*. 2010; 324, 70–81.
30. Elias CF, Purohit D. Leptin signaling and circuits in puberty and fertility. *Cell Mol Life Sci*. 2013; 70, 841–862.
31. Karim BO, Landolfi JA, Christian A, *et al*. Estrous cycle and ovarian changes in a rat mammary carcinogenesis model after irradiation, tamoxifen chemoprevention, and aging. *Comp Med*. 2003; 53, 532–538.
32. Woodruff TK, Lyon RJ, Hansen SE, Rice GC, Mather JP. Inhibin and activin locally regulate rat ovarian folliculogenesis. *Endocrinology*. 1990; 127, 3196–3205.
33. Paixão L, Velez LM, Santos BR, *et al*. Early ovarian follicular development in prepubertal Wistar rats acutely exposed to androgens. *J Dev Orig Health Dis*. 2016; 7, 384–390.
34. Abramovich D, Irusta G, Bas D, Cataldi NI, Parborell F, Tesone M. Angiopoietins/TIE2 system and VEGF are involved in ovarian function in a DHEA rat model of polycystic ovary syndrome. *Endocrinology*. 2012; 153, 3446–3456.
35. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods*. 2001; 25, 402–408.
36. Lacau de Mengido I, Becú-Villalobos D, Libertun C. Sexual differences in the dopaminergic control of luteinizing hormone secretion in the developing rat. *Dev Brain Res*. 1987; 35, 91–95.
37. Lacau-Mengido IM, Libertun C, Becú-Villalobos D. Different serotonin receptor types participate in 5-hydroxytryptophan-induced gonadotropins and prolactin release in the female infantile rat. *NEN*. 1996; 63, 415–421.
38. Horng S-G, Wang T-H, Wang H-S. Estradiol-to-testosterone ratio is associated with response to metformin treatment in women with clomiphene citrate-resistant polycystic ovary syndrome (PCOS). *Chang Gung Med J*. 2008; 31, 477–483.
39. Abraham GE, Swerdloff R, Tulchinsky D, Odell WD. Radioimmunoassay of plasma progesterone. *J Clin Endocrinol Metab*. 1971; 32, 619–624.
40. Sengupta P. The laboratory rat: relating its age with human's. *Int J Prev Med*. 2013; 4, 624–630.
41. McCutcheon JE, Marinelli M. Age matters. *Eur J Neurosci*. 2009; 29, 997–1014.
42. Spear LP. The adolescent brain and age-related behavioral manifestations. *Neurosci Biobehav Rev*. 2000; 24, 417–463.
43. Jahanfar S, Eden JA, Warren P, Seppälä M, Nguyen TV. A twin study of polycystic ovary syndrome. *Fertil Steril*. 1995; 63, 478–486.
44. Bateson P, Gluckman P, Hanson M. The biology of developmental plasticity and the predictive adaptive response hypothesis. *J Physiol*. 2014; 592, 2357–2368.
45. Zielinski WJ, Vandenbergh JG, Montano MM. Effects of social stress and intrauterine position on sexual phenotype in wild-type house mice (Mus musculus). *Physiol Behav*. 1991; 49, 117–123.
46. Maliqueo M, Lara HE, Sánchez F, Echiburú B, Crisosto N, Sir-Petermann T. Placental steroidogenesis in pregnant women with polycystic ovary syndrome. *Eur J Obstet Gynecol Reprod Biol*. 2013; 166, 151–155.
47. Sun M, Maliqueo M, Benrick A, *et al*. Maternal androgen excess reduces placental and fetal weights, increases placental steroidogenesis, and leads to long-term health effects in their female offspring. *Am J Physiol Endocrinol Metab*. 2012; 303, E1373–E1385.
48. Puttabyatappa M, Cardoso RC, Herkimer C, Veiga-Lopez A, Padmanabhan V. Developmental programming: postnatal estradiol modulation of prenatally organized reproductive neuroendocrine function in sheep. *Reproduction*. 2016; 152, 139–150.
49. Abbott DH, Bruns CR, Barnett DK, *et al*. Experimentally induced gestational androgen excess disrupts glucoregulation in rhesus monkey dams and their female offspring. *Am J Physiol Endocrinol Metab*. 2010; 299, E741–E751.
50. Luense LJ, Veiga-Lopez A, Padmanabhan V, Christenson LK. Developmental programming: gestational testosterone treatment alters fetal ovarian gene expression. *Endocrinology*. 2011; 152, 4974–4983.
51. Fujikura T, Froehlich LA. Organ-weight/brain-weight ratios as a parameter of prenatal growth: a balanced growth theory of visceras. *Am J Obstet Gynecol*. 1972; 112, 896–902.
52. Mitropoulos G., Scurry J., Cussen L. Organ weight/bodyweight ratios: growth rates of fetal organs in the latter half of pregnancy with a simple method for calculating mean organ weights. *J Paediatr Child Health*. 2008; 28, 236–239.
53. Cabrero A, Cubero M, Llaverías G, *et al*. Leptin down-regulates peroxisome proliferator-activated receptor gamma (PPAR-gamma) mRNA levels in primary human monocyte-derived macrophages. *Mol Cell Biochem*. 2005; 275, 173–179.
54. Mirzaei K, Hossein-Nezhad A, Keshavarz SA, *et al*. Crosstalk between circulating peroxisome proliferator-activated receptor gamma, adipokines and metabolic syndrome in obese subjects. *Diabetol Metab Syndr*. 2013; 5, 79.

https://doi.org/10.1017/S2040174419000126 Published online by Cambridge University Press

55. Keller H, Givel F, Perroud M, Wahli W. Signaling cross-talk between peroxisome proliferator-activated receptor/retinoid X receptor and estrogen receptor through estrogen response elements. *Mol Endocrinol*. 1995; 9, 794–804.
56. Nuñez SB, Medin JA, Braissant O, *et al*. Retinoid X receptor and peroxisome proliferator-activated receptor activate an estrogen responsive gene independent of the estrogen receptor. *Mol Cell Endocrinol*. 1997; 127, 27–40.
57. Yanase T, Mu YM, Nishi Y, *et al*. Regulation of aromatase by nuclear receptors. *J Steroid Biochem Mol Biol*. 2001; 79, 187–192.
58. Lovekamp-Swan T, Jetten AM, Davis BJ. Dual activation of PPARα and PPARγ by mono-(2-ethylhexyl) phthalate in rat ovarian granulosa cells. *Mol Cell Endocrinol*. 2003; 201, 133–141.
59. Barkan D, Jia H, Dantes A, Vardimon L, Amsterdam A, Rubinstein M. Leptin modulates the glucocorticoid-induced ovarian steroidogenesis. *Endocrinology*. 1999; 140, 1731–1738.
60. Kendall NR, Gutierrez CG, Scaramuzzi RJ, Baird DT, Webb R, Campbell BK. Direct in vivo effects of leptin on ovarian steroidogenesis in sheep. *Reproduction*. 2004; 128, 757–765.
61. Karamouti M, Kollia P, Kallitsaris A, Vamvakopoulos N, Kollios G, Messinis IE. Modulating effect of leptin on basal and follicle stimulating hormone-stimulated steroidogenesis in cultured human lutein granulosa cells. *J Endocrinol Invest*. 2009; 32, 415–419.
62. Wang Q, Kim JY, Xue K, Liu J, Leader A, Tsang BK. Chemerin, a novel regulator of follicular steroidogenesis and its potential involvement in polycystic ovarian syndrome. *Endocrinology*. 2012; 153, 5600–5611.
63. Wang Q, Leader A, Tsang BK. Inhibitory roles of prohibitin and chemerin in FSH-induced rat granulosa cell steroidogenesis. *Endocrinology*. 2013; 154, 956–967.
64. Cornejo MP, Hentges ST, Maliqueo M, Coirini H, Becu-Villalobos D, Elias CF. Neuroendocrine regulation of metabolism. *J Neuroendocrinol*. 2016; 28. doi: 10.1111/jne.12395.
65. Wang L, Shao YY, Ballock RT. Leptin antagonizes peroxisome proliferator-activated receptor-γ signaling in growth plate chondrocytes. *PPAR Res*. 2012; 2012, 9. doi: 10.1155/2012/756198.
66. Abbasi A, Moghadam AA, Kahrarian Z, Abbsavaran R, Yari K, Alizadeh E. Molecular effects of leptin on peroxisome proliferator-activated receptor gamma (PPAR-γ) mRNA expression in rat's adipose and liver tissue. *Cell Mol Biol (Noisy-le-grand)*. 2017; 63, 89–93.
67. Jenks MZ, Fairfield HE, Johnson EC, Morrison RF, Muday GK. Sex steroid hormones regulate leptin transcript accumulation and protein secretion in 3T3-L1 cells. *Sci Rep*. 2017; 7, 8232.
68. Dobrzyn K, Smolinska N, Kiezun M, *et al*. Adiponectin: a new regulator of female reproductive system. *Int J Endocrinol*. 2018; 2018, 12. doi: 10.1155/2018/7965071.
69. Ledoux S, Campos DB, Lopes FL, Dobias-Goff M, Palin M-F, Murphy BD. Adiponectin induces periovulatory changes in ovarian follicular cells. *Endocrinology*. 2006; 147, 5178–5186.
70. Michalakis KG, Segars JH. The role of adiponectin in reproduction: from polycystic ovary syndrome to assisted reproduction. *Fertil Steril*. 2010; 94, 1949–1957.
71. Ishida M, Shimabukuro M, Yagi S, *et al*. MicroRNA-378 regulates adiponectin expression in adipose tissue: a new plausible mechanism. *PLoS One*. 2014; 9, e111537.
72. Xu S, Linher-Melville K, Yang BB, Wu D, Li J. Micro-RNA378 (miR-378) regulates ovarian estradiol production by targeting aromatase. *Endocrinology*. 2011; 152, 3941–3951.
73. Foecking EM, Szabo M, Schwartz NB, Levine JE. Neuroendocrine consequences of prenatal androgen exposure in the female rat: absence of luteinizing hormone surges, suppression of progesterone receptor gene expression, and acceleration of the gonadotropin-releasing hormone pulse generator. *Biol Reprod*. 2005; 72, 1475–1483.
74. Yan X, Yuan C, Zhao N, Cui Y, Liu J. Prenatal androgen excess enhances stimulation of the GNRH pulse in pubertal female rats. *J Endocrinol*. 2014; 222, 73–85.
75. Hunzicker-Dunn M, Maizels ET. FSH signaling pathways in immature granulosa cells that regulate target gene expression: branching out from protein kinase A. *Cell Signal*. 2006; 18, 1351–1359.
76. Raju GAR, Chavan R, Deenadayal M, *et al*. Luteinizing hormone and follicle stimulating hormone synergy: a review of role in controlled ovarian hyper-stimulation. *J Hum Reprod Sci*. 2013; 6, 227–234.
77. Chang RJ, Cook-Andersen H. Disordered follicle development. *Mol Cell Endocrinol*. 2013; 373, 51–60.
78. Noroozzadeh M, Behboudi-Gandevani S, Zadeh-Vakili A, Ramezani Tehrani F. Hormone-induced rat model of polycystic ovary syndrome: a systematic review. *Life Sci*. 2017; 191, 259–272.
79. Wu XY, Li ZL, Wu CY, *et al*. Endocrine traits of polycystic ovary syndrome in prenatally androgenized female Sprague–Dawley rats. *Endocr J*. 2010; 57, 201–209.
80. Caligioni CS. Assessing reproductive status/stages in mice. *Curr Protoc Neurosci*. 2009; 48: A.4I.1–A.4I.8. doi: 10.1002/0471142301.nsa04is48.
81. Greenwald GS, Rothchild I. Formation and maintenance of corpora lutea in laboratory animals. *J Anim Sci*. 1968; 27 Suppl 1, 139–162.
82. Carmina E, Oberfield SE, Lobo RA. The diagnosis of polycystic ovary syndrome in adolescents. *Am J Obstet Gynecol*. 2010; 203, 201.e1–201.e5.
83. Lagaly DV, Aad PY, Grado-Ahuir JA, Hulsey LB, Spicer LJ. Role of adiponectin in regulating ovarian theca and granulosa cell function. *Mol Cell Endocrinol*. 2008; 284, 38–45.
84. Veldhuis JD, Zhang G, Garmey JC. Troglitazone, an insulin-sensitizing thiazolidinedione, represses combined stimulation by LH and insulin of de Novo Androgen Biosynthesis by Thecal Cells in vitro. *J Clin Endocrinol Metab*. 2002; 87, 1129–1133.
85. Reverchon M, Cornuau M, Ramé C, Guerif F, Royère D, Dupont J. Chemerin inhibits IGF-1-induced progesterone and estradiol secretion in human granulosa cells. *Hum Reprod*. 2012; 27, 1790–1800.
86. Kitawaki J, Kusuki I, Koshiba H, Tsukamoto K, Honjo H. Leptin directly stimulates aromatase activity in human luteinized granulosa cells. *Mol Hum Reprod*. 1999; 5, 708–713.
87. Fan W, Yanase T, Morinaga H, *et al*. Activation of peroxisome proliferator-activated receptor-γ and retinoid X receptor inhibits aromatase transcription via nuclear factor-κB. *Endocrinology*. 2005; 146, 85–92.