Prenatal hyperandrogenism induces alterations that affect liver lipid metabolism

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Abstract

Prenatal hyperandrogenism is hypothesized as one of the main factors contributing to the development of polycystic ovary syndrome (PCOS). PCOS patients have high risk of developing fatty liver and steatosis. This study aimed to evaluate the role of prenatal hyperandrogenism in liver lipid metabolism and fatty liver development. Pregnant rats were hyperandrogenized with testosterone. At pubertal age, the prenatally hyperandrogenized (PH) female offspring displayed both ovulatory (PHov) and anovulatory (PHanov) phenotypes that mimic human PCOS features. We evaluated hepatic transferases, liver lipid content, the balance between lipogenesis and fatty acid oxidation pathway, oxidant/antioxidant balance and proinflammatory status. We also evaluated the general metabolic status through growth rate curve, basal glucose and insulin levels, glucose tolerance test, HOMA-IR index and serum lipid profile. Although neither PH group showed signs of liver lipid content, the lipogenesis and fatty oxidation pathways were altered. The PH groups also showed impaired oxidant/antioxidant balance, a decrease in the proinflammatory pathway (measured by prostaglandin E2 and cyclooxygenase-2 levels), decreased glucose tolerance, imbalance of circulating lipids and increased risk of metabolic syndrome. We conclude that prenatal hyperandrogenism generates both PHov and PHanov phenotypes with signs of liver alterations, imbalance in lipid metabolism and increased risk of developing metabolic syndrome. The anovulatory phenotype showed more alterations in liver lipogenesis and a more impaired balance of insulin and glucose metabolism, being more susceptible to the development of steatosis.

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine and metabolic disorders that affects women in their reproductive age (Franks 2003) and its clinical manifestations often emerge during puberty (Rosenfield 2007, Yan *et al.* 2013). PCOS etiology remains controversial, and current theories emphasize on genetic and intrauterine origins coupled with environmental factors such as diet and altered lifestyle patterns (Franks 1995). It has been reported that prenatal androgen exposure is able to induce polycystic ovaries in rats (Demissie *et al.* 2008, Foecking *et al.* 2008), monkeys (Abbott *et al.* 2010) and sheep (Manikkam *et al.* 2006) and that fetal programming, mediated by prenatal hyperandrogenism, is related to hyperinsulinemia, dyslipidemia, insulin resistance (IR), cardiovascular disease and metabolic syndrome (Demissie *et al.* 2008, Amalfi *et al.* 2012, Heber *et al.* 2013). However, how fetal programming impacts on different tissues is unknown.

The liver is involved in lipid synthesis, transportation and storage, as well as in glucose and insulin metabolism (Paschos & Paletas 2009), all key factors in PCOS pathogenesis (den Boer *et al.* 2004, Paschos & Paletas 2009, Baranova *et al.* 2011, Vassilatou 2014). One of the most frequent hepatic affections, related to metabolic abnormalities, is nonalcoholic fatty liver disease (NAFLD), which affects 20–30% of the general population (Vassilatou 2014). NAFLD includes a clinicopathologic spectrum of conditions that encompass from simple steatosis (triglyceride (TG) accumulation in hepatocytes) to steatohepatitis with inflammation, fibrosis and even cirrhosis (Browning & Horton 2004). NAFLD pathogenesis remains unknown and there are many hypotheses about its origin (Yasui *et al.* 2012, Lee *et al.* 2014). Currently, the most accepted model proposes a multiple and parallel hits hypothesis. Fatty acids and their metabolites are the lipotoxic agents involved in NAFLD development (Day & James 1998, Lin *et al.* 2014), the increase in oxidative stress being one of the key factors in NAFLD pathogenesis (Madan *et al.* 2006, Lin *et al.* 2014).

In physiological conditions, in the liver, there is an equilibrium between the uptake and exportation of fatty acids (which in turn can be esterified or to be oxidized) (Browning & Horton 2004, Kawano & Cohen 2013). However, when the balance between lipolysis and lipogenesis is altered, or fatty acid influx to the liver is increased, lipid droplets could accumulate in the liver, leading to steatosis and even NAFLD (den Boer *et al.* 2004, Kawano & Cohen 2013).

A common feature of NAFLD and PCOS is IR (Schwimmer *et al.* 2005, Gambarin-Gelwan *et al.* 2007). However, it remains controversial whether IR is the key cause in the development of NAFLD in women with PCOS (Liang & Ward 2006, Gambarin-Gelwan *et al.* 2007). Since PCOS patients with hyperandrogenic phenotypes have a higher prevalence of developing NAFLD, it has been suggested that androgens could contribute to the development of the pathology (Vassilatou *et al.* 2010, Vassilatou 2014). We have previously demonstrated that the levels of androgen administered in pregnant rats are directly related to the PCOS-like phenotype displayed in the female offspring during the pubertal age (Amalfi *et al.* 2012) and that the fetal programming generated by androgens leads to metabolic alterations, particularly in lipid metabolism, which worsen through life (Heber *et al.* 2013). Based on the above, the aim of this study was to evaluate the effect of prenatal hyperandrogenism on the liver function and lipid metabolism.

Materials and methods

Animals and treatments

Virgin female rats 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 darkness cycle) and temperature (23–25°C). Animals received food and water *ad libitum*. Day 1 of pregnancy was defined as the morning on which spermatozoa were observed in the vaginal fluid. As described previously (Demissie *et al.* 2008), pregnant rats ($N=15$) received subcutaneous injections of 1 mg free testosterone (T-1500; Sigma) dissolved in 100 μL sesame oil from day 16 to day 19 of pregnancy. This hormonal paradigm mimics the fetal testosterone surge that is observed in male rats when the reproductive axis in the fetus is established. Another group ($N=15$) received only 100 μL sesame oil. The dose was selected based on previous findings of our laboratory (data not published) and other studies, which have shown that this dose leads to ovulatory and anovulatory phenotypes and that higher doses lead only to anovulatory phenotypes during the adult life and even to vaginal atresia (Wolf *et al.* 2002). 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 and killed during pubertal age at 60 days of age. Those from hyperandrogenized mothers

were the prenatally hyperandrogenized (PH) group and those from mothers injected with sesame oil were the control (Ctl) group. Animals were allowed free access to Purina rat chow (Cooperación SRL, Argentina) and water. 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. To establish the phenotype diversity, the above procedures were independently repeated three times.

At 60 days of age, 75 female offspring from each group were anesthetized with carbon dioxide and killed by decapitation. Trunk blood was collected and serum was separated and kept at −80°C for further studies. Ovaries and liver were extracted and conserved at −80°C or fixed in 4% (v/v) formaldehyde for histological studies. All animals were randomly assigned for each assay.

Characterization of the prenatally hyperandrogenized murine model

Serum testosterone was quantified from 15 offspring from each group by radioimmunoassay (RIA) as described previously (Amalfi *et al*. 2012). Serum estradiol levels were quantified by Cobase immunoassay analyzers using an electrochemiluminescence immunoassay (ECLIA) following the manufacturer's instructions. The intra- and interassay coefficients of variation (CVs) were 7.3% and 13.2%, respectively, for testosterone and 3.93 and 7.08%, respectively for estradiol. The estrous cycle was determined by vaginal smears taken daily from 45 to 60 days of age in all the animals.

Regular ovulatory animals were those that showed smears displaying the four stages of the estrous cycle in the following order: proestrus, estrus, metaestrus, diestrus, with cycles of 4–6 days. Irregular ovulatory animals were both those that showed some smears displaying an estrous stage but further smears not following the progress of the cycle as described above, and those whose cycles lasted 7 days or more (PHov). Anovulatory animals were whose smears showed metaestrus, diestrus or a combination of both for four consecutive days, and were thus considered to be noncycling (PHanov) (Karim *et al*. 2003).

To evaluate ovarian histology, ten ovaries from each group were removed and immediately fixed in 4% (v/v) formaldehyde and analyzed by two different investigators. Ovaries were embedded in paraffin wax and

consecutively cut. To prevent counting the same follicle twice, 6 µm step sections were mounted at 50 µm intervals onto microscope slides. Then, slides were stained with hematoxylin and eosin (Woodruff *et al*. 1990).

Hepatic enzymes

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) were quantified by colorimetric enzymatic methods (Wiener Lab, Argentina) following the manufacturer's instructions. The chromophoric products were measured at 340 nm for ALT and AST and at 405 nm for GGT, all at 25°C. The intra- and interassay Cv values were 3.02 and 5.63% for ALT, 4.4 and 4.9% for AST, and 1.62 and 5% for GGT.

Liver lipid and TG content

Fragments of hepatic tissue randomly selected from 15 female offspring from each group were fixed in formaldehyde 4% (v/v), cut in cryostat and stained with SUDAN IV to visualize lipid droplets using hematoxylin as contrast stain. The intestine of tadpole was used as a positive control of the SUDAN IV technique (Regueira *et al*. 2016). To evaluate the TG content in the liver, 15 frozen samples of each group were saponified and TG content was quantified by comparing with a glycerol standard curve by using a commercial kit (Wiener Lab, Argentina), as described previously (Chow *et al*. 2011).

Hepatic lipid metabolism

The mRNA expression of acetyl-CoA carboxylase (ACC) 1 and 2 (*Acaca* and *Acacb*, respectively), fatty acid synthase (*Fas*), stearoyl-CoA desaturase (*Scd1*), sterol regulatory element-binding protein1 (*Srebp1*), carbohydrate response element-binding protein (*Chrebp*), peroxisome proliferator-activated receptor alpha and gamma (*Pparα* and *Pparγ*) and PPARγ co-activator 1α (*Pgc1α*) was evaluated by real-time PCR analysis in 15 different samples from each group. Total mRNA from hepatic tissue was extracted using RNAzol RT (MRC gene, Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's instructions. cDNA was synthesized from 500 ng mRNA using random primers. real-time PCR analysis was performed from this cDNA by means of the real mix B124-100 (Biodynamics SRL, USA). The amplified products were quantified by fluorescence using the Rotor Gene 6000 Corbett and mRNA abundance was normalized to the

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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 melting (°C)
$Ppar\gamma$ F	5′-TTTTCAAGGGTGCCAGTTTC-3′	60
$Ppar\gamma$ R	5′-GAGGCCAGCATGGTGTAGAT-3′	
Srebp F	5′-TAACCTGGCTGAGTGTGCAG-3′	60
Srebp R	5′-ATCCACGAAGAAACGGTGAC-3′	
Chrebp F	5′-GGTTGTCCCCAAAGCAGAGA-3′	62
Chrebp R	5′-TTGTTGTCTACACGACCCCG-3′	
Acaca F	5′-CCAGACCCTTTCTTCAGCAG-3′	62
Acaca R	5′-AGGACCGATGTGATGTTGCT-3′	
Acacb F	5′-CAAAGCCTCTGAAGGTGGAG-3′	62
Acacb R	5′-CTCGTCCAAACAGGGACACT-3′	
Fas F	5′-TCGAGACACATCGTTTGAGC-3′	62
Fas R	5′-CCCAGAGGGTGGTTGTTAGA-3′	
Scd1 F	5′-GCTTCCAGATCCTCCCTACC-3′	62
Scd1 R	5′-CAACAACCAACCCTCTCGTT-3′	
$Ppar\alpha$ F	5′-TCACACGATGCAATCCGTTT-3′	60
$Ppar\alpha$ R	5′-GGCCTTGACCTTGTTCATGT-3′	
$Pgc1\alpha$ F	5′-AATGCAGCGGTCTTAGCACT-3′	60
$Pgc1\alpha$ R	5′-GTGTGAGGAGGGTCATCGTT-3′	
L32 F	5′-TGGTCCACAATGTCAAGG-3′	58
L32 R	5′-CAAAACAGGCACACAAGC-3′	

F, forward sequence; R, reverse sequence.

60s ribosomal protein L32 (*L32*) amount. *L32* was validated as a reference gene because the variance between treatments did not differ. Results are expressed as arbitrary units. The primers are given in Table 1.

Liver oxidant/antioxidant balance

The oxidant–antioxidant balance in liver tissue was evaluated as the lipid peroxidation index and the content of the antioxidant glutathione (GSH) in 15 samples of each group. The amount of malondialdehyde formed from the breakdown of polyunsaturated fatty acids is taken as an index of the peroxidation reaction. The method used in this study was as that described previously (Amalfi *et al.* 2012, Heber *et al.* 2013). The reduced form of GSH, which comprises the bulk of cellular protein sulfhydryl groups, was quantified as described previously (Amalfi *et al.* 2012, Heber *et al.* 2013).

Liver inflammatory status

The inflammatory status in liver tissue was measured by evaluating the levels of prostaglandin E (PGE) and cyclooxygenase-2 (COX2), the limiting enzyme of its synthesis. PGE was determined by RIA as reported
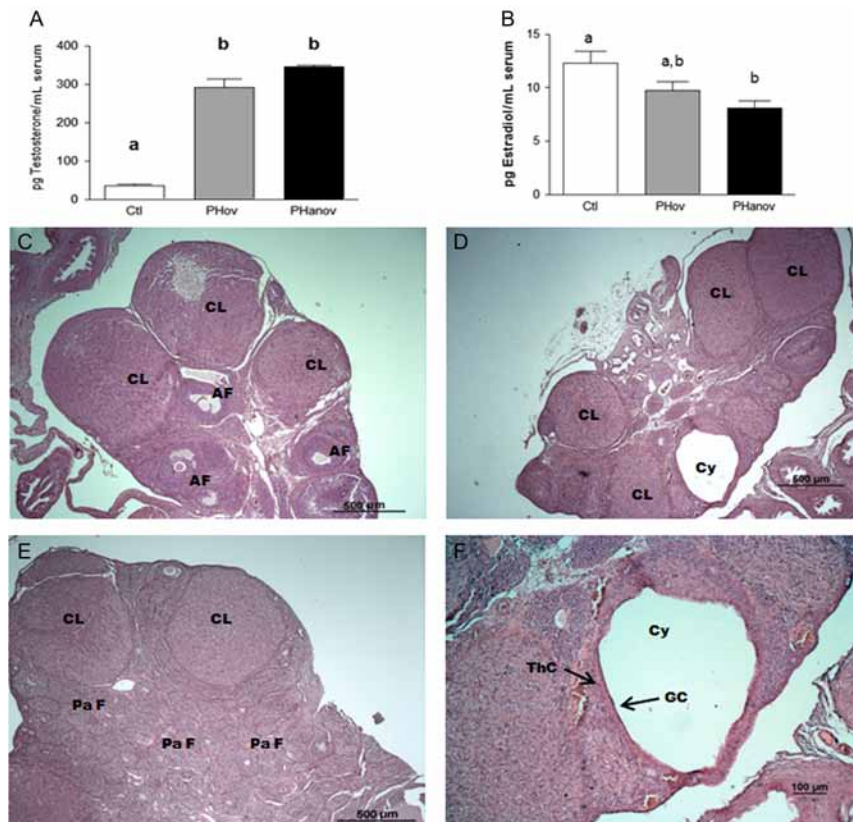
Figure 1
Prenatally hyperandrogenized murine model. Prenatal hyperandrogenism in female offspring of control (Ctl) and prenatally hyperandrogenized (PH) groups. (A) Serum testosterone levels. Each column represents the mean±s.e.m. from 15 different animals per group, a vs b $P < 0.01$ by ANOVA test. (B) Serum estradiol levels. Each column represents the mean±s.e.m. from 15 different animals per group, a vs b $P < 0.05$ by ANOVA test. (C) A representative ovarian tissue section from the Ctl group (40×). (D) A representative ovarian tissue section from the PHov group (40×). (E) A representative ovarian tissue section from the PHanov group (40×). (F) Detail of a representative ovarian cyst from the PH groups (100×). Corpus luteum (CL), antral follicle (AF), cyst (Cy), granulosa cells (GC), theca cells (ThC) and preantral follicles (PaF). A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-15-0471.

previously (Motta *et al.* 1999). COX2 was measured by the western blotting technique, using 200 mg liver tissue from 10 independent samples per group, as described previously (Amalfi *et al.* 2012).

General metabolism imbalance

The body weight of all the animals of all the groups was determined at 21, 28, 38, 45 and 60 days of age. Basal insulin levels were measured by an ELISA kit, following the manufacturer's instructions (Abcam Insulin Human ELISA Kit), and basal glucose levels were quantified by colorimetric enzymatic methods (Wiener Lab, Argentina) ($N = 10$ per group). The intra- and interassay Cv values were 10 and 12%, respectively, for insulin, and 1.39 and 1.92%, respectively, for glucose.

The glucose tolerance test (IPGTT) was performed in separate groups of 10 female offspring from each group, as described previously (Demissie *et al.* 2008,

Amalfi *et al.* 2012). The homeostatic model assessment for IR (HOMA-IR) was determined (Yan *et al.* 2013). The circulating lipid profile was evaluated as described previously and the TG/HDL cholesterol ratio was taken as a marker of metabolic syndrome risk (Heber *et al.* 2013).

Statistical analysis

Statistical analyses were carried out using Instant program (GraphPad Software). ANOVA with *post hoc* Tukey test was used to compare the three treatments. Statistical significance was considered as $P < 0.05$.

Results

Characterization of the prenatally hyperandrogenized murine model

The PHov and PHanov groups showed higher serum testosterone levels than the control group, but only
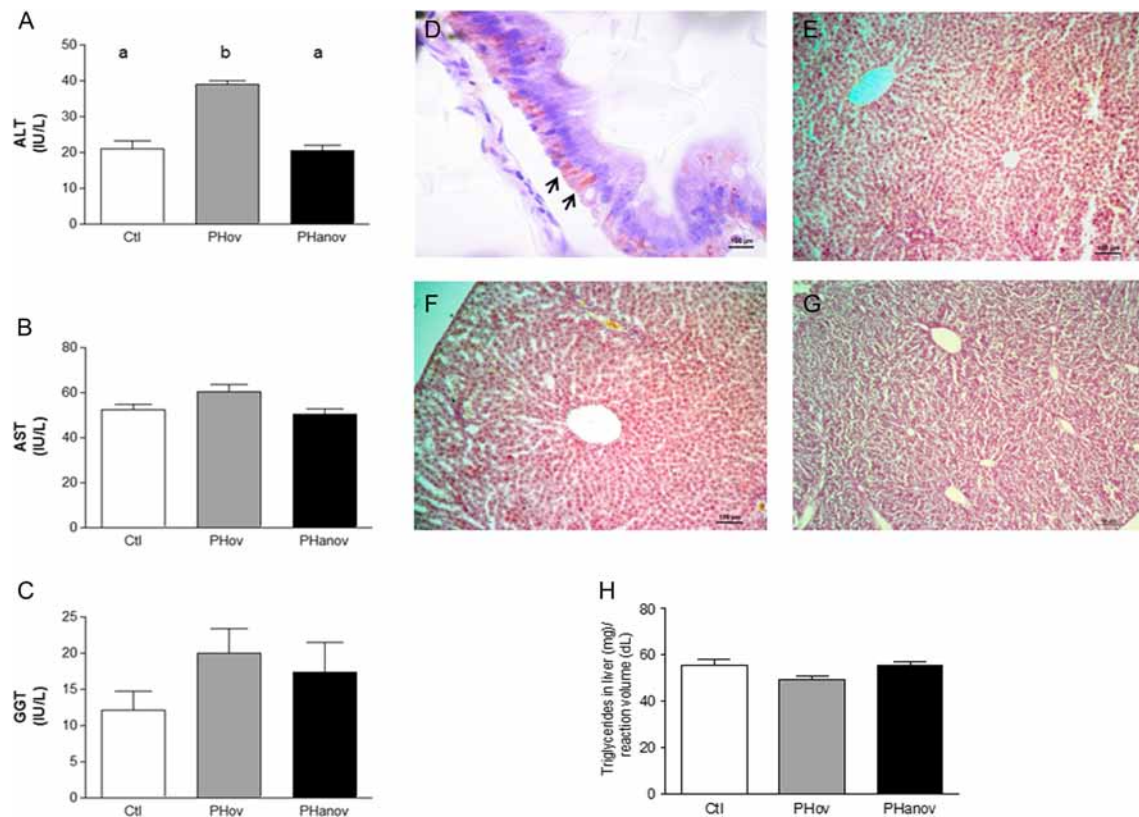
Figure 2

Effects of prenatal hyperandrogenism on liver transaminase levels and lipid content. (A) Serum levels of ALT and (B) AST. (C) GGT. Each column represents the mean ± S.E.M. of control (Ctl) and prenatally hyperandrogenized (PH) groups; a vs b $P < 0.05$, by ANOVA test. Liver lipid content was quantified by SUDAN IV staining. (D) The cytoplasmic lipid droplets are shown in red, as shown in the positive control of the technique (arrows). Each photo shows the detail (100×) of a representative staining of the Ctl group (E) and PHov (F) and PHanov groups (G). There was no evidence of cytoplasmic lipid accumulation. (H) TG content in the liver. No significant differences were found between the Ctl and PH groups ($P < 0.05$, by ANOVA test). A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-15-0471.

PHanov animals displayed lower estradiol levels than the control (Fig. 1A and B, $P<0.01$). Figure 1C shows a representative ovarian tissue section from the control group. The general appearance of the tissue resembled normal histology: a central medulla consisting mainly of fibromuscular stroma and corpora lutea and antral follicles located in the peripheral cortex. Histological examination of ovaries from the PHov and PHanov groups (Fig. 1C and D) revealed the presence of corpora lutea, cysts and an excess of small antral follicles. In addition, in PHanov animals, the ovary was disorganized as compared with the control group and small follicles could be seen invading the central medulla. The detail in Fig. 1E shows that cysts present a layer of theca cells and a thin compacted formation of granulosa cells.

Regarding the estrous cycle, in the three independent repetitions of the animal procedure, always 100% of the control rats showed a regular estrous cycle. Within the PH group, 43–51% showed irregular estrous cycles and were considered as PHov, whereas 27–39% presented anovulatory cycles and were considered PHanov. Testosterone did not affect the age of vaginal opening.

Prenatal hyperandrogenism and hepatic alterations

ALT levels were increased in the PHov group (Fig. 2A, $P<0.01$). Neither AST nor GGT levels were affected in the PHov or PHanov groups as compared with the control group (Fig. 2B and C respectively, $P>0.05$).

As compared with the positive control of the SUDAN IV technique (Fig. 2D, arrows), neither the control group (Fig. 2E) nor the PHov or PHanov phenotypes (Fig. 2F and G) showed hepatic lipid droplets. In addition, no differences were found in the hepatic TG content between groups (Fig. 2H, $P>0.05$).
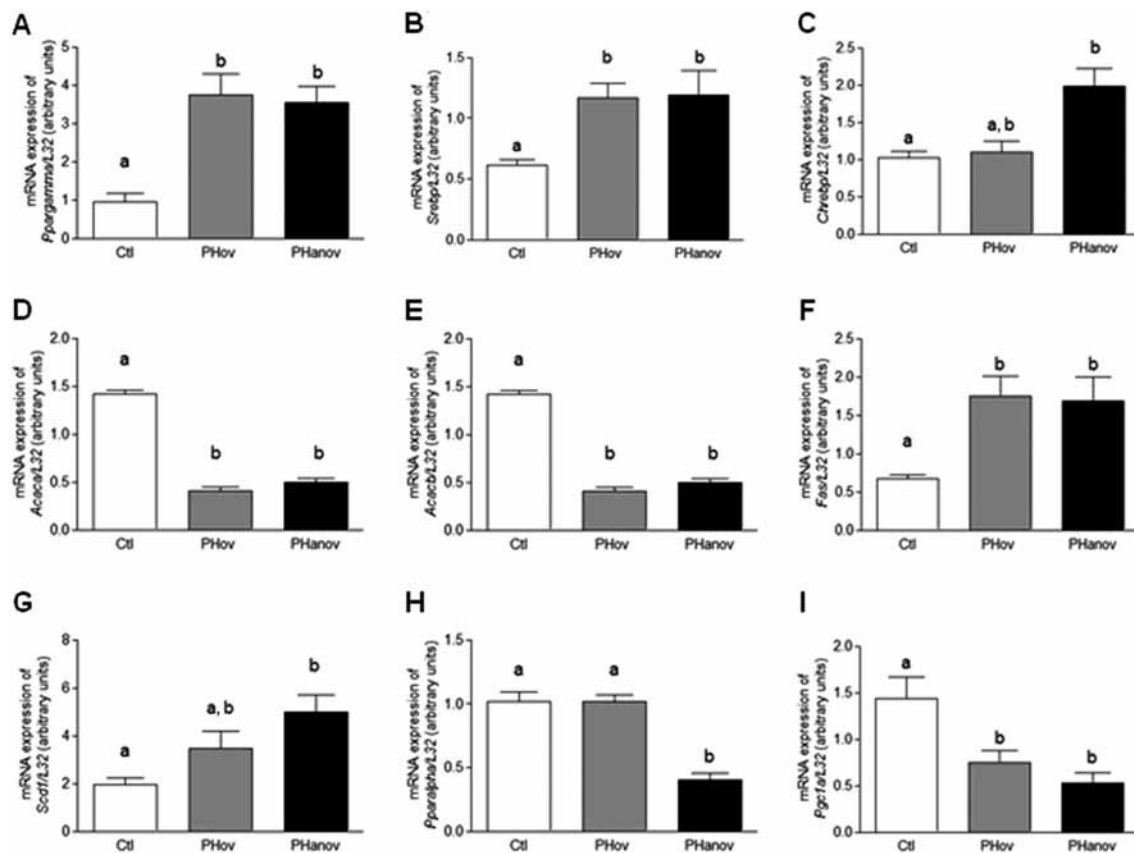
Figure 3

Effects of prenatal hyperandrogenism on liver lipid metabolism. The graphs correspond to the mRNA abundance of the gene of interest relative to *L32* mRNA levels of control (Ctl) and prenatally hyperandrogenized (PH) groups. *L32* was validated as a reference gene using Cts (cycle threshold) (Control=21.76+0.24; PHov=21.36+0.30; PHanov=21.70+0.34; $P=0.67$) (A) Gene expression of *Pparγ* (a vs b $P<0.01$), (B) *Srebp* (a vs b $P<0.05$) (C) *Chrebp* (a vs b $P<0.05$), (D) *Acaca* (a vs b $P<0.01$), (E) *Acacb* (a vs b $P<0.01$), (F) *Fas* (a vs b $P<0.01$), (G) *Scd1* (a vs b $P<0.05$), (H) *Pparα* (a vs b $P<0.01$) and (I) *Pgc1α* (a vs b $P<0.01$). Each column represents the mean±s.e.m. Statistical analyses were made by ANOVA test.
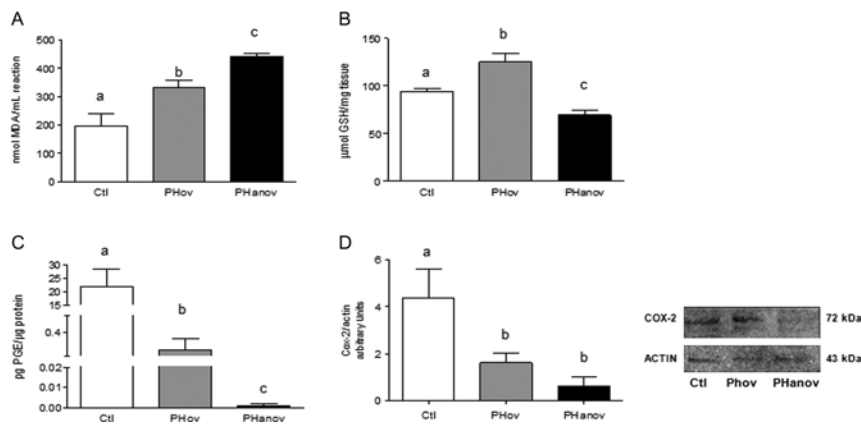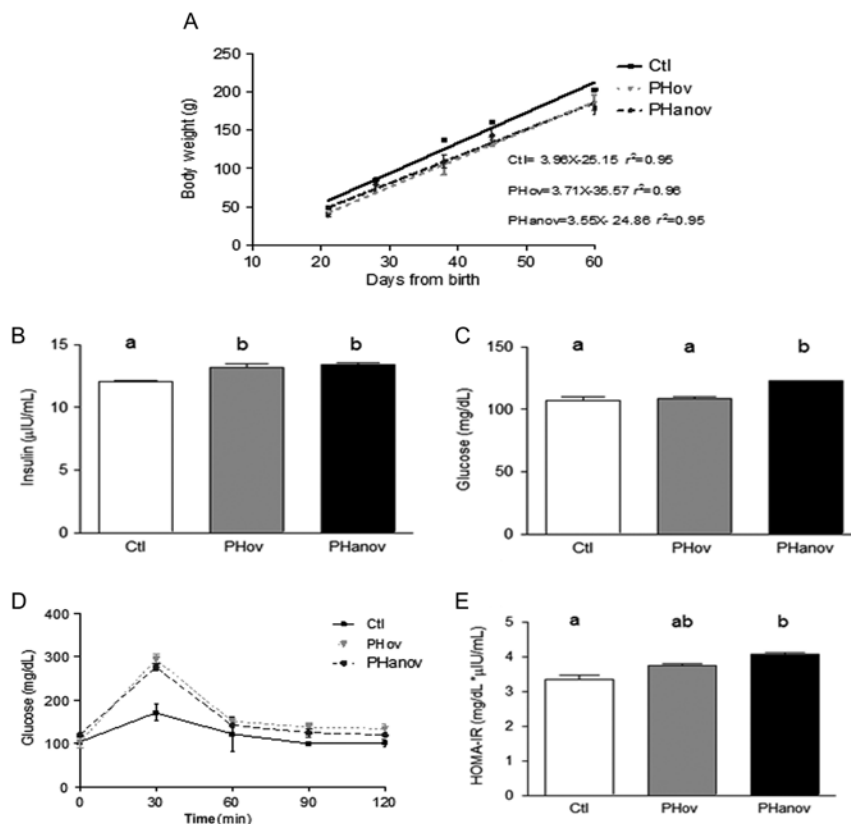
Figure 4

Effects of prenatal hyperandrogenism on liver oxidant/antioxidant balance and proinflammatory status. The oxidant–antioxidant balance in liver tissue was evaluated by measuring the lipid peroxidation index (A) and the content of the antioxidant glutathione (B). Each column represents the mean ± S.E.M. Different letters mean statistical significant differences (a≠b≠c, $P<0.05$ in both cases, by ANOVA test). (C) Liver PGE content was measured to evaluate the pro-inflammatory status (a≠b≠c, $P<0.01$ by ANOVA). (D) To evaluate whether PGE levels were influenced by the limiting enzyme of its synthesis, COX2 was measured, a vs b $P<0.05$, by ANOVA test. Each column represents the mean ± S.E.M.

Regarding the transcription factors that are mediators of lipogenesis, we found that *Pparγ* and *Srebp* mRNA levels were higher in both PH groups than in the control group (Fig. 3A and B, $P<0.05$), whereas *Chrebp* levels were only altered in the PHanov animals (Fig. 3C, $P<0.05$). Regarding the enzymes involved in lipogenesis, we found that the mRNA levels of the genes encoding both isoforms of acetyl-CoA carboxylase (*Acaca* and *Acacb*) were decreased in both PH groups (Fig. 3D and E, $P<0.05$). *Fas* mRNA levels were increased in the PH groups (Fig. 3F, $P<0.05$), whereas *Scd1* mRNA levels were only impaired in PHanov animals (Fig. 3G, $P<0.05$).

Regarding fatty acid oxidation pathways, we found that *Pparα* mRNA levels showed no differences between the control and PHov groups but were decreased in PHanov (Fig. 3H, $P<0.01$), and that *Pgc1α* levels were lower in both PH animals than in controls (Fig. 3I, $P<0.01$).

L32 was validated as a reference gene, obtaining no statistical difference in the stability between treatments measured by the Ct (threshold cycle) (control = 21.76 + 0.24; PHov = 21.36 + 0.30; PHanov = 21.70 + 0.34; $P=0.67$).

Figure 5

Prenatal hyperandrogenism and metabolic derangements. Metabolic features evaluated in female offspring of control (Ctl) and prenatally hyperandrogenized (PH) groups. (A) The curve represents the mean growth rates of the Ctl and PH groups. Differences between growth rates were not significant ($P<0.05$, by ANOVA test). (B) Basal insulin levels, a vs b $P<0.05$. (C) Basal glucose levels; a vs b $P<0.05$. (D) Blood samples followed by intraperitoneal injection of 2 g dextrose/kg body weight were collected at 0, 30, 60, 90, and 120 min post injection for IPGTT. (E) HOMA-IR index; a vs b $P<0.05$. Values are mean ± S.E.M. by ANOVA test.

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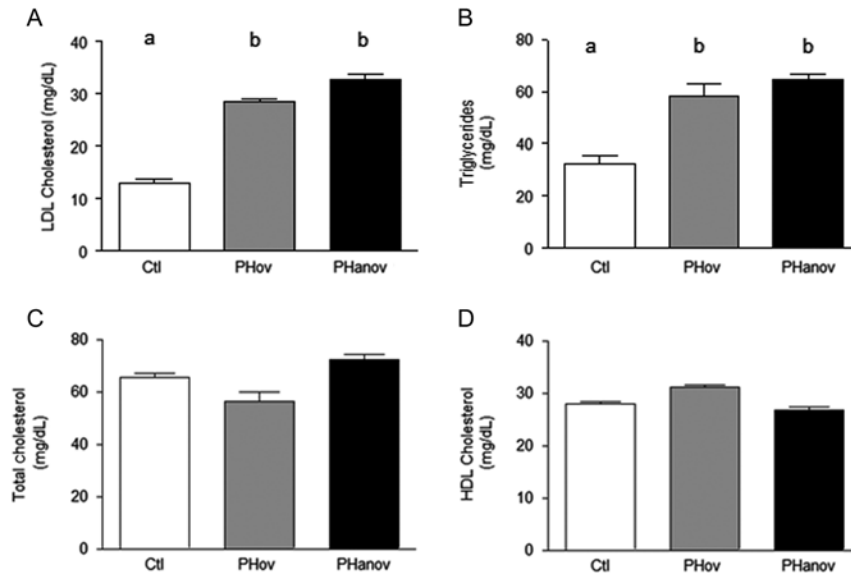
Figure 6
Effect of prenatal hyperandrogenism on the circulating lipid profile. (A) Serum levels of low-density lipoprotein cholesterol (LDL-cholesterol), (B) triglycerides, (C) total cholesterol and (D) high-density lipoprotein cholesterol (HDL-cholesterol). Each column represents the mean ± s.e.m. of control (Ctl) and prenatally hyperandrogenized (PH) groups; a vs b $P < 0.01$ panel A; $P < 0.05$ panel B. Data were analyzed by ANOVA test.

The lipid peroxidation index was higher in the PH groups than in the control (Fig. 4A, $P < 0.05$). GSH levels were altered in both PH groups as compared with the control. GSH levels were increased in the PHov groups and decreased in the PHanov group (Fig. 4B, $P < 0.01$).

Both the levels of PGE and the protein expression of COX2 were lower in the PH groups than in the control group. In addition, the PGE levels were lower in the PHanov group than in the PHov animals (Fig. 4C and D, $P < 0.01$).

Prenatal hyperandrogenism and metabolic derangements

Prenatal hyperandrogenism did not affect the body weight from prepubertal to pubertal age (Fig. 5A, $P = 0.41$). Insulin levels were increased in both PH groups as respect to controls (Fig. 5B, $P < 0.05$), and basal glucose levels were impaired in the PHanov group (Fig. 5C, $P < 0.05$). The IPGTT showed that prenatal hyperandrogenism induced increased levels of circulating glucose (Fig. 5D), represented by the area under the curve of glucose concentration
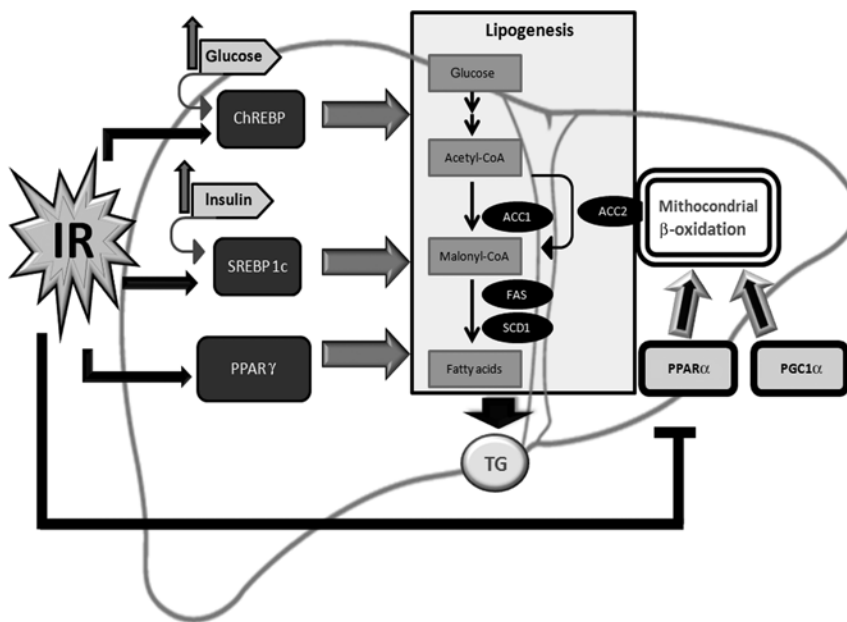
Figure 7
Mediators of hepatic lipogenesis and β-oxidation. SREBP, ChREBP and PPARγ are transcription factors involved in the regulation of hepatic lipogenesis. Insulin and glucose modulate SREBP and ChREBP, respectively. These transcription factors and PPARγ positively regulate *de novo* lipogenesis by modulating the expression of the enzymes involved in this pathway. On the other hand, PPARα and PGC1α are positive regulators of genes involved in fatty acid oxidation. A balance between these processes is needed to avoid hepatic liver accumulation leading to steatosis. Insulin resistance is associated with an increase in lipogenesis due to an upregulation of ChREBP, SREBP1c and PPARγ and a decrease in fatty acid oxidation due to a negative regulation on PPARα.

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(control=14873.0±119.7; PHov=21045.0±164.0; PHanov=2090.0±156.6 in arbitrary units, control vs PHov $P<0.01$; control vs PHanov $P<0.05$; PHov vs PHanov $P>0.05$). The HOMA-IR index was increased in the PHanov group as compared with controls (Fig. 5E, $P<0.05$).

Both PH groups showed an altered circulating lipid profile, displaying higher levels of circulating low-density lipoprotein cholesterol (LDL) (Fig. 6A, $P<0.05$) and TG than the control group (Fig. 6B, $P<0.01$). No significant differences were found in the levels of total cholesterol or high-density lipoprotein (HDL) cholesterol (Fig. 6C and D, $P>0.05$). The TG/HDL cholesterol ratio, as a marker of metabolic syndrome risk, was higher in both PH groups than in the control group (control=1.13±0.34; PHov=2.42±0.24; PHanov=2.65±0.39, $P<0.05$; PHov vs control and PHanov vs control, $P>0.05$ PHov vs PHanov).

Discussion

The developmental origins of PCOS are controversial. Some authors have reported that an altered *in utero* environment could be responsible for metabolic diseases and PCOS features development in different species (Abbott *et al.* 2005, Demissie *et al.* 2008, Hogg *et al.* 2011), whereas others propose PCOS as a multiplicity of etiologies and not a simple mechanism and emphasize genotype features (Gluckman & Hanson 2004, Franks & Berga 2012). It is known that both the embryo development stages and early postnatal life period are crucial to condition adult health life. Thus, currently used PCOS animal models focus on these critical development time windows (Manikkam *et al.* 2006, Demissie *et al.* 2008, Abbott *et al.* 2010, Amalfi *et al.* 2012, Jang *et al.* 2015). Furthermore, the metabolic and endocrine alterations found in the prenatal models lead to several long-term effects, thus highlighting the importance of the *in utero* environment.

In the present study, we reproduced a murine model which displayed PCOS features leading to two phenotypes: with both hyperandrogenism and ovarian alterations, such as an increase in the number of preantral follicles and the formation of cysts. Nearly 50% of the cases of the PH group presented irregular ovulatory estrous cycles, whereas around 40% of the cases in PH group presented the anovulatory phenotype.

Ovaries from PCOS women are known to contain an increased number of small follicles that have an excessive early growth but with follicular arrest, thus preventing

the selection and further maturation of a dominant follicle. These data are in accordance with our results showing that androgens play a role in follicle recruitment (Jonard & Dewailly 2004).

We found that PH rats showed incipient liver damage. These data are in agreement with previous reports that suggest that the intrauterine environment plays an important role in the development of both NAFLD and PCOS during postnatal life (Baranova *et al.* 2013, Brumbaugh & Friedman 2014).

Contrary to other reports (Demissie *et al.* 2008, Hogg *et al.* 2011), we found no lipid accumulation in the liver. This difference could be due to the higher doses of androgens used in those reports and is in agreement with our previous findings where higher doses of androgens induced a more severe PCOS-like phenotype as well as worse endocrine and metabolic disturbances (Amalfi *et al.* 2012). To deepen the study of lipid metabolism in the liver, we evaluated the status of the *de novo lipogenesis* pathway and β-fatty acid oxidation mediators (Fig. 7). To assess the lipogenic pathway, we evaluated the role of three transcription factors involved in the regulation of *de novo* lipogenesis. Two of them, SREBP and CHREBP, are regulated by insulin and glucose levels, respectively, and both regulate the expression of genes encoding lipogenic enzymes (Browning & Horton 2004, Strable & Ntambi 2010). Our results showed that the PH groups presented high levels of *Srebp*, consistent with the high levels of serum insulin found in PH animals. Moreover, *Chrebp* was impaired in the PHanov group, showing a correlation with basal glucose levels in those animals. These data are in accordance with that described by other authors showing that in the hyperinsulinemic and even more in the IR states, SREBP1-c transcription is stimulated and could lead to *de novo* fatty acid synthesis (Browning & Horton 2004, Strable & Ntambi 2010). The third transcription factor studied, also involved in the regulation of lipogenesis, was PPARγ. It has been shown that in animal models of IR, PPARγ levels are increased, promoting lipid storage (Ables 2012, Kawano & Cohen 2013). In agreement with these data, our results showed increased *Pppary* levels in the PH groups, thus highlighting the relationship between liver lipid metabolism and glucose and insulin metabolism. Although only the PHanov phenotype presented signs of IR, by the HOMA-IR index, both PH groups showed increased insulin levels and decreased glucose tolerance by the IPGGT test.

In addition to an altered expression of the transcription factors modulating lipogenic genes, we found alterations in the mRNA levels of the genes

encoding FAS and SCD1, both enzymes involved in lipogenesis. Both PH groups showed increased levels of *Fas*, but only the PHanov group showed altered levels of *Scd1*, the enzyme leading to monounsaturated fatty acids. Thus, although the lipogenic pathway was altered in both PH groups, the PHanov showed a deeper dysregulation of the lipogenesis system.

The first limiting factor in lipogenesis is the synthesis of malonyl-CoA, which is synthesized by ACC, which is present in both isoforms encoded by the *Acaca* and *Acacb* genes. Our results surprisingly showed that both mRNA levels were decreased in the PH groups, independently of the increased levels of *Srebp*, *Chrebp* and *Pparγ*. Malonyl-CoA is an indirect inhibitor of fatty acid oxidation (Browning & Horton 2004), and it has been described that when one of the ACC isoforms is reduced, fatty acid oxidation results increased. These findings suggest that the reduction in *Acaca* and *Acacb* expression could be due to the effect of other regulatory mechanisms (independently of *Pparγ*, *Srebp* and *Chrebp* action), which may be preventing lipid accumulation by decreasing malonyl-CoA synthesis (Savage *et al*. 2006, Strable & Ntambi 2010), thus favoring fatty acid oxidation.

Our results are in accordance with other studies (Mao *et al*. 2006, Strable & Ntambi 2010) that show that *Acaca* levels are independent of the expression of the genes involved in fatty acid synthesis. In fact, the knockout of *Acaca* shows increased levels of several genes that encode proteins involved in lipogenesis but without hepatic lipid accumulation (Mao *et al*. 2006, Strable & Ntambi 2010). These data demonstrate that *Acaca* could be playing a protective role in the development of hepatic steatosis.

In addition to the alterations found in the lipogenesis pathways, we found derangements in fatty acid oxidation (Fig. 7). By having opposite functions, *Pparα* and *Pparγ* regulate fat metabolism and while *Pparγ* promotes lipid storage and *Pparα* promotes lipid utilization (Ables 2012, Kawano & Cohen 2013, Souza-Mello 2015). In the present study, prenatal hyperandrogenization decreased *Pparα* levels in the liver of PHanov animals, suggesting that this group could be more sensitive to hepatic steatosis. We also found that the levels of the PPARs coactivator, *Pgc1α*, a key regulator of lipid metabolism, were decreased in both PH groups as compared with the control group. In fact, PGC1α not only acts as a regulator of PPARs but also promotes oxidation of fatty acids by binding to specific transcription factors, modulates mitochondrial functions and controls glucose homeostasis (Liang & Ward 2006, Fernandez-Marcos & Auwerx 2011). Taken together, our

data suggest that dysregulation of lipogenesis and fatty acid oxidation could lead to the long-term development of hepatic damage, deepened by decreased insulin sensitivity.

Oxidative stress and the proinflammatory process are also involved in the pathogenesis of hepatic damage. Oxidative stress is involved in the regulation of very low-density lipoprotein (VLDL) production and its excretion by the liver (Pan *et al*. 2004). Moreover, the addition of GSH to rat hepatoma cells reverses steatosis and decreases lipid peroxidation levels (Pan *et al*. 2004). In the liver, GSH levels correlate with high levels of circulating LDL cholesterol, which reinforces the association between the oxidative balance and lipid metabolism (Lin *et al*. 2014). In agreement with these findings, in this study we found that GSH compensated the damage caused by lipid peroxidation in the PHov group but not in the PHanov group. We suggest that in the PHov group, GSH could be modulating VLDL secretion from the liver to the circulation, as manifested by the increased levels of serum LDL observed, and thus preventing the accumulation of VLDL, as evidenced by the lack of accumulation of lipid droplets.

As mentioned before, the proinflammatory process is involved in the development of NAFLD (Day & James 1998) and prostaglandins, and COX2 play a role in lipid metabolism and lipid accumulation in the liver (Yu *et al*. 2006, Hsieh *et al*. 2009, Ii *et al*. 2009). Increased levels of PGE correlate with TG accumulation in the liver (Hsieh *et al*. 2009, Ii *et al*. 2009, Henkel *et al*. 2012). In agreement with these data, we found that decreased levels of PGE and COX2 in the PH groups evidenced an alteration in the proinflammatory mediators that could be preventing liver fat accumulation. In fact, the decrease in the inflammatory pathway in the PH groups correlated with a lack of increase in TG content. These results contribute to the explanation of the absence of lipid droplets in the PHanov group. Although the liver PPAR system was altered and lipogenesis was favored, there was a systemic manifestation of these consequences seen by an altered circulating lipid profile. Thus, a depletion of the proinflammatory mediators could be acting as a compensatory system.

Hepatic transaminases have been described as markers of liver function and damage (Vassilatou 2014). Although ALT is one of the hepatic enzymes mostly used as a marker to detect hepatic injury, several studies have shown other causes for its increase, including growth spurs and looming diabetes (Burgert *et al*. 2006, Vassilatou 2014).

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Despite these findings, it remains controversial whether ALT is a good marker for liver function because some individuals have NAFLD but normal ALT levels and vice versa (Kim *et al*. 2008). It has been recommended that increased levels of these enzymes in blood may only be used to detect the inflammation that occurs in the liver due to injury or damage. Unexpectedly, we found increased levels of ALT only in PHov animals. Thus, as the PHanov group presented a great depletion of the proinflammatory status, then ALT serum levels would not be affected in these animals (Kerner *et al*. 2005, Burgert *et al*. 2006, Yamada *et al*. 2006).

It should be pointed out that the incidence of NAFLD and liver fat accumulation are associated with increasing age and body weight (Michaliszyn *et al*. 2013, Park *et al*. 2014). In this study, we found no alterations in body weight or fatty liver presence related to the decreased liver inflammation. However, studies are being carried out in adult rats, as we found that derangements in the metabolic pathway of lipogenesis could worsen through life (Heber *et al*. 2013).

Women with the more severe PCOS phenotype show increased prevalence of NAFLD (Jones *et al*. 2012). Herein, we found that the PHanov group was the most affected, displaying decreased expression of *Pgc1α* and *Pparα* and overexpression of *Pparγ*, *Srebp* and *Chrebp*, thus being more susceptible to presenting signs of hepatic steatosis and damage in the long term (Browning & Horton 2004, Estall *et al*. 2009).

Our data are in accordance to the multiple hits hypothesis (Day & James 1998, Lin *et al*. 2014) to explain the origins of NAFLD. This involves IR, fatty acid signaling impairment, oxidative stress and inflammation as contributing factors to NAFLD development. As we found no lipid accumulation but alterations in the processes described, steatosis may be a consequence of hepatic signaling derangements and systemic metabolic detriment. We do not discard that the hepatic alterations found could be due to an increased testosterone/estradiol ratio, worsened in the PHanov group.

In summary, our data show for the first time that both ovulatory and anovulatory phenotypes that mimics PCOS features present at pubertal age, signs of incipient liver injury and an imbalance of the fatty acid metabolism mediated by the PPAR system, SREBP and CHREBP, as well as an imbalance of the lipogenic enzymes but without development of NAFLD. These derangements are related to systemic effects, dyslipidemia and decreased glucose tolerance.

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