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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 pro-inflammatory 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 pro-inflammatory 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 affect 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 the 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 (Amalfi, et al. 2012; Demissie et al. 2008; 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 and Paletas 2009), all key factors in PCOS pathogenesis (Baranova, et al. 2011; den Boer, et al. 2004; Paschos and Paletas 2009; Vassilatou 2014). One of the most frequent hepatic affections, related to metabolic abnormalities, is non-alcoholic 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 and Horton 2004). NAFLD pathogenesis remains unknown and there are many hypotheses about its origin (Lee, et al. 2014; Yasui, et al. 2012). 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 and James 1998; Lin, et al. 2014), being the increase in oxidative stress one of the key factors in NAFLD pathogenesis (Lin et al. 2014; Madan, et al. 2006).

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 and Horton 2004; Kawano and 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 and Cohen 2013).

A common feature of NAFLD and PCOS is IR (Gambarin-Gelwan, et al. 2007; Schwimmer, et al. 2005). However, it remains controversial whether IR is the key cause in the development of NAFLD in women with PCOS (Gambarin-Gelwan et al. 2007; Liang and Ward 2006). 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 2014; Vassilatou, et al. 2010). 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 dark) and temperature (23-25 ºC). 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 previously described (Demissie et al. 2008), 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 that is observed in male rats when the reproductive axis in the fetus is established. Another group (N=15) received only 100 µl of sesame oil. The dose was selected based on previous findings of our lab (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 sacrificed 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 previously described (Amalfi et al. 2012). Serum estradiol levels were quantified by Cobase immunoassay analyzers using an Electro Chemiluminescence Immuno Assay (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 to 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 non-cycling (PHanov) (Karim, et al. 2003).

144 To evaluate ovarian histology, 10 ovaries from each group were removed and
145 immediately fixed in 4% (v/v) formaldehyde and analyzed by two different
146 investigators. Ovaries were embedded in paraffin wax and consecutively cut. To prevent
147 counting the same follicle twice, 6-µm step sections were mounted at 50-µm intervals
148 onto microscope slides. Then, slides were stained with hematoxylin and eosin
149 (Woodruff, et al. 1990).

150 **Hepatic enzymes**

151 Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and
152 gamma-glutamyl transferase (GGT) were quantified by colorimetric enzymatic methods
153 (Wiener Lab, Argentina) following the manufacturer´s instructions. The chromophoric
154 products were measured at 340 nm for ALT and AST and at 405 nm for GGT, all at
155 25°C. The intra- and interassay Cvs were 3.02% and 5.63% for ALT, 4.4% and 4.9%
156 for AST, and 1.62% and 5% for GGT.

157 **Liver lipid and TG content**

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

165 **Hepatic lipid metabolism**

166 The mRNA expression of Acetyl-CoA carboxylase (ACC) 1 and2 (*Acaca* and *Acacb*,
167 respectively), Fatty acid Synthase (*Fas*), stearoyl-CoA desaturase (*Scd1*), Sterol

168 regulatory element-binding protein1 (*Srebp1*), carbohydrate response element binding
169 protein (*Chrebp*), Peroxisome proliferator-activated receptor alpha and gamma
170 (*Pparalpha* and *Ppargamma*) and PPARgamma co-activator 1alpha (*Pgc1a*) was
171 evaluated by Real-Time PCR analysis in 15 different samples from each group. Total
172 mRNA from hepatic tissue was extracted using RNAzol RT (MRC gene, Molecular
173 Research Center, Cincinnati, OH, USA) following the manufacturer's instructions.
174 cDNA was synthesized from 500 ng mRNA by using random primers. Real-Time PCR
175 analysis was performed from this cDNA by means of the real mix B124-100
176 (Biodynamics SRL, USA). The amplified products were quantified by fluorescence
177 using the Rotor Gene 6000 Corbett and mRNA abundance was normalized to the 60s
178 Ribosomal protein L32 (*L32*) amount. L32 was validated as a reference gene because
179 the variance between treatments did not differ. Results are expressed as arbitrary units.
180 The primers are shown in Table 1.

181 **Liver oxidant/antioxidant balance**

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

189 **Liver inflammatory status**

190 The inflammatory status in liver tissue was measured by evaluating the levels of
191 Prostaglandin E (PGE) and cyclooxygenase-2 (COX2), the limiting enzyme of its

192 synthesis. PGE was determined by RIA as previously reported (Motta, et al. 1999).
193 COX2 was measured by the Western blotting technique, using 200 mg of liver tissue
194 from 10 independent samples per group, as previously described (Amalfi et al. 2012).

195 **General metabolism imbalance**

196 The body weight of all the animals of all the groups was determined at 21, 28, 38, 45
197 and 60 days of age.

198 Basal insulin levels were measured by an ELISA kit, following the manufacturer's
199 instructions (Abcam Insulin Human ELISA Kit) and basal glucose levels were
200 quantified by colorimetric enzymatic methods (Wiener Lab, Argentina) (N=10 per
201 group). The intra- and interassay Cvs were 10% and 12% respectively for insulin and
202 1.39% and 1.92% respectively for glucose.

203 The glucose tolerance test (IPGTT) was performed in separate groups of ten female
204 offspring from each group, as previously described (Amalfi et al. 2012; Demissie et al.
205 2008). The homeostatic model assessment for IR (HOMA-IR) was determined (Yan et
206 al. 2013). The circulating lipid profile was evaluated as previously described and the
207 TG/HDL cholesterol ratio was taken as a marker of metabolic syndrome risk (Heber et
208 al. 2013).

209 **Statistical analysis**

210 Statistical analyses were carried out using the Instant program (GraphPad software, San
211 Diego, CA, USA). ANOVA with post-hoc Tukey test was used to compare the three
212 treatments. Statistical significance was considered as $p<0.05$.

213

214

215

Results

Characterization of the prenatally hyperandrogenized murine model

The PHov and PHanov groups showed higher serum testosterone levels than the control group, but only PHanov animals displayed lower estradiol levels than the control (Fig. 1A and 1B, $p<0.01$). 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 1D) 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 to the control group and small follicles could be seen invading the central medulla. The detail in figure 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 or GGT levels were affected in the PHov or PHanov groups as compared with the control group (Fig. 2B and 2C 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

240 2G) showed hepatic lipid droplets. In addition, no differences were found in the hepatic
241 TG content between groups (Fig. 2H, $p>0.05$).
242 Regarding the transcription factors that are mediators of lipogenesis, we found that
243 *Ppargamma* and *Srebp* mRNA levels were higher in both PH groups than in the control
244 group (Fig. 3A and 3B, $p<0.05$), whereas *Chrebp* levels were only altered in the
245 PHanov animals (Fig. 3C, $p<0.05$). Regarding the enzymes involved in lipogenesis, we
246 found that the mRNA levels of the genes encoding both isoforms of Acetyl-CoA
247 carboxylase (*Acaca* and *Acacb*) were decreased in both PH groups (Fig. 3 D and 3E,
248 $p<0.05$). *Fas* mRNA levels were increased in the PH groups (Fig. 3F, $p<0.05$), whereas
249 *Scd1* mRNA levels were only impaired in PHanov animals (Fig. 3G, $p<0.05$).
250 Regarding fatty acid oxidation pathways, we found that *Pparalpha* mRNA levels
251 showed no differences between the control and PHov groups but were decreased in
252 PHanov (Fig. 3H, $p<0.01$), and that *Pgc1a* levels were lower in both PH animals than in
253 controls (Fig. 3I, $p<0.01$).
254 *L32* was validated as a reference gene, obtaining no statistical difference in the stability
255 between treatments measured by the Ct (threshold cycle) (control=21.76 + 0.24; PHov=
256 21.36 + 0.30; PHanov= 21.70 + 0.34; $p=0.67$).
257 The lipid peroxidation index was higher in the PH groups than in the control (Fig. 4A,
258 $p<0.05$). GSH levels were altered in both PH groups as compared to the control. GSH
259 levels were increased in the PHov groups and decreased in the PHanov group (Fig. 4B,
260 $p<0.01$).
261 Both the levels of PGE and the protein expression of COX2 were lower in the PH
262 groups than in the control group. In addition, the PGE levels were lower in the PHanov
263 group than in the PHov animals (Fig. 4C and 4D, $p<0.01$).

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 (control = 14873.0 $\pm$ 119.7; PHov = 21045.0 $\pm$ 164.0; PHanov= 2090.0 $\pm$ 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 to controls (Fig. 5E, $p<0.05$).

Both PH groups showed an altered circulating lipid profile, displaying higher levels of circulating low-density lipoproein 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 6D, $p>0.05$). 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 $\pm$ 0.34; PHov= 2.42 $\pm$ 0.24; PHanov= 2.65 $\pm$ 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

288 a simple mechanism and emphasize genotype features (Franks and Berga 2012;
289 Gluckman and Hanson 2004). It is known that both the embryo development stages and
290 early postnatal life period are crucial to condition adult health life. Thus, currently used
291 PCOS animal models focus on these critical development time windows (Abbott et al.
292 2010; Amalfi et al. 2012; Demissie et al. 2008; Jang, et al. 2015; Manikkam et al.
293 2006). Furthermore, the metabolic and endocrine alterations found in the prenatal
294 models lead to several long-term effects, thus highlighting the importance of the *in*
295 *utero* environment.

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

301 Ovaries from PCOS women are known to contain an increased number of small follicles
302 that have and excessive early growth but with follicular arrest, thus preventing the
303 selection and further maturation of a dominant follicle. These data are in accordance
304 with our results showing that androgens play a role in follicle recruitment (Jonard and
305 Dewailly 2004).

306 We found that PH rats showed incipient liver damage. These data are in agreement with
307 previous reports that suggest that the intrauterine environment plays an important role in
308 the development of both NAFLD and PCOS during postnatal life (Baranova, et al.
309 2013; Brumbaugh and Friedman 2014).

310 Contrary to other reports (Demissie et al. 2008; Hogg et al. 2011), we found no lipid
311 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 (Figure 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 and Horton 2004; Strable and 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 and Horton 2004; Strable and Ntambi 2010). The third transcription factor studied, also involved in the regulation of lipogenesis, was PPARgamma. It has been shown that in animal models of IR, PPARgamma levels are increased, promoting lipid storage (Ables 2012; Kawano and Cohen 2013). In agreement with these data, our results showed increased *Pppargamma* 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 *Ppargamma.* Malonyl-CoA is an indirect inhibitor of fatty acid oxidation (Browning and 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 of *Acaca* and *Acacb* expression could be due to the effect of other regulatory mechanisms (independently of *Ppargamma, Srebp* and *Chrebp* action), which may be preventing lipid accumulation by decreasing Malonyl-CoA synthesis (Savage, et al. 2006; Strable and Ntambi 2010), thus favoring fatty acid oxidation.

Our results are in accordance with other studies (Mao, et al. 2006; Strable and Ntambi 2010) that show that *Acaca* levels are independent of the expression of the genes involved in fatty acid synthesis. In fact, the knock-out of *Acaca* shows increased levels of several genes that encode proteins involved in lipogenesis but without hepatic lipid accumulation (Mao, et al. 2006; Strable and 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, *Pparalpha* and *Ppargamma* regulate fat metabolism and while *Ppargamma* promotes lipid storage, *Pparalpha* promotes lipid utilization (Ables 2012; Kawano and Cohen 2013; Souza-Mello 2015). In the present study, prenatal hyperandrogenization decreased *Pparalpha* 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, *Pgc1a*, a key regulator of lipid metabolism, were decreased in both PH groups as compared with the control group. In fact, PGC1a 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 (Fernandez-Marcos and Auwerx 2011; Liang and Ward 2006). Taken together, our data suggest that dysregulation of lipogenesis and fatty acid oxidation could lead in the long-term to the development of hepatic damage, deepened by decreased insulin sensitivity.

Oxidative stress and the pro-inflammatory 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, here 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 pro-inflammatory process is involved in the development of NAFLD (Day and James 1998) and prostaglandins and COX2 play a role in lipid metabolism and lipid accumulation in the liver (Hsieh, et al. 2009; Ii, et al. 2009; Yu, et al. 2006). Increased levels of PGE correlate with TG accumulation in the liver (Henkel, et al. 2012; Hsieh et al. 2009; Ii et al. 2009). In agreement with these data, we found that decreased levels of PGE and COX2 in the PH groups evidenced an alteration in the pro-inflammatory 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 pro-inflammatory 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 most 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). 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 on the liver due to injury or damage. Unexpectedly, we found increased levels of ALT only in PHov animals.

406 Thus, as the PHanov group presented a great depletion of the pro-inflammatory status,
407 then ALT serum levels would not be affected in these animals (Burgert et al. 2006;
408 Kerner, et al. 2005; Yamada, et al. 2006).
409 It should be pointed out that the incidence of NAFLD and liver fat accumulation are
410 associated with increasing age and body weight (Michaliszyn, et al. 2013; Park, et al.
411 2014). Here, we found no alterations in body weight or fatty liver presence related to the
412 decreased liver inflammation. However, studies are being carried out in adult rats, as we
413 found that derangements in the metabolic pathway of lipogenesis could worsen through
414 life (Heber et al. 2013).
415 Women with the more severe PCOS phenotype show increased prevalence of NAFLD
416 (Jones, et al. 2012). Here we found that the PHanov group was the most affected,
417 displaying decreased expression of *Pgc1a* and *Pparalpha* and over-expression of
418 *Ppargamma, Srebp, Chrebp*, thus being more susceptible to presenting signs of hepatic
419 steatosis and damage in the long-term (Browning and Horton 2004; Estall, et al. 2009).
420 Our data are in accordance to the multiple hits hypothesis (Day and James 1998; Lin et
421 al. 2014) to explain the origins of NAFLD. This involves IR, fatty acid signaling
422 impairment, oxidative stress and inflammation as contributing factors to NAFLD
423 development. As we found no lipid accumulation but alterations in the processes
424 described, steatosis may be a consequence of hepatic signaling derangements and
425 systemic metabolic detriment. We do not discard that the hepatic alterations found could
426 be due to an increased testosterone/estradiol ratio, worsened in the PHanov group.
427 In summary, our data show for the first time that both ovulatory and anovulatory
428 phenotypes that mimics PCOS features present, at pubertal age, signs of incipient liver
429 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.

Declaration of interests

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

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Author contributions

Conceived and designed the experiments AMB, GAA and MFH. Performed the experiments: GAA, MFH, SRF, LMV, RR and OPP. Analyzed the data: GAA and MFH. Contributed reagents/materials/analysis tools: GAA, MFH, SRF, LMV, OPP, ABM. Wrote the paper: GAA and ABM. Corrected and read the last version: GAA, MFH, SRF, LMV, RR, OPP and ABM.

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Legends of figures

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 ± SEM from fifteen different animals per group, a vs b $p<0.01$ by ANOVA test. **(B)** Serum estradiol levels. Each column represents the mean ± SEM from fifteen different animals per group, a vs b $p<0.05$ by ANOVA test. **(C)** A representative ovarian tissue section from the Ctl group (40X). **(D)** A representative ovarian tissue section from the PHov group (40X). **(E)** A representative ovarian tissue section from the PHanov group (40X). **(F)** Detail of a representative ovarian cyst from the PH groups (100X). Corpus luteum (CL), antral follicle (AF), cyst (Cy), granulosa cells (GC), theca cells (ThC) and preantral follicles (PaF).

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 ± SEM 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 (100X) 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).

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 *Ppargamma* (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)** *Pparalpha* (a vs b $p<0.01$), and (I) *Pgc1a* (a vs b $p<0.01$). Each column represents the mean ± SEM. 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 ± SEM. Different letters mean statistical significant differences ($a\neq b\neq c$, $p<0.05$ in both cases, by ANOVA test). **(C)** Liver PGE content was measured to evaluate the pro-inflammatory status ($a\neq b\neq c$, $p<0.01$ 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 ± SEM.

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

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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 + SEM 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.

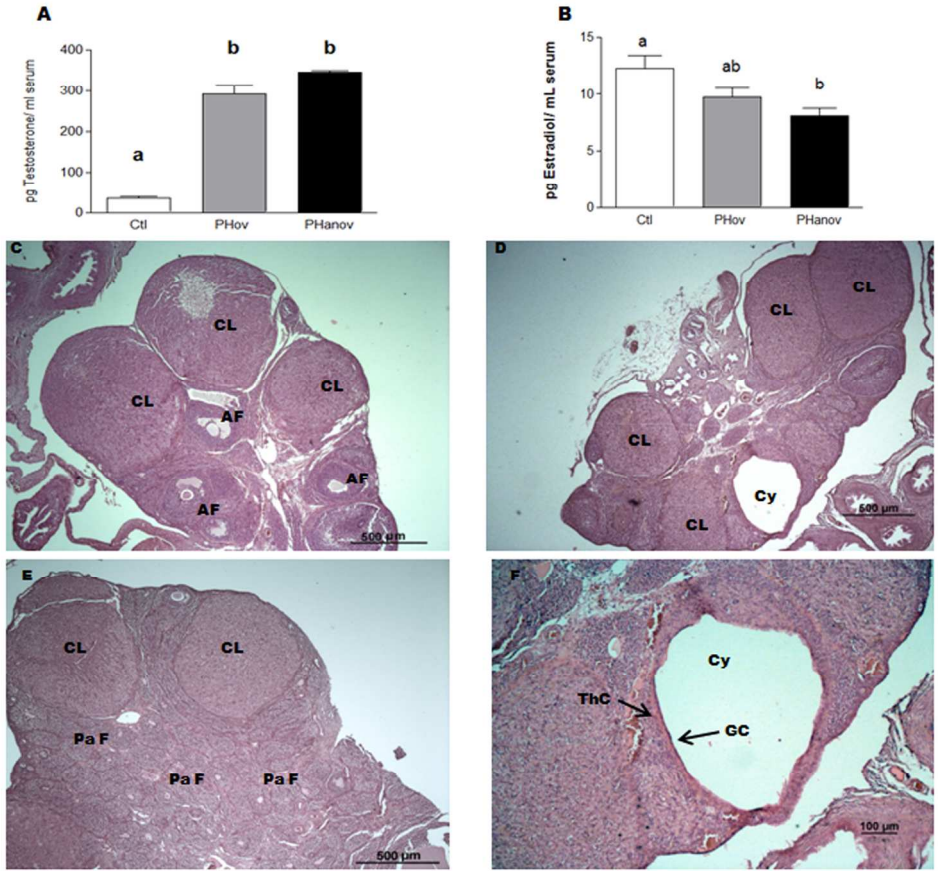
Figure 7. Mediators of hepatic lipogenesis and β-oxidation. SREBP, ChREBP and PPARgamma are transcription factors involved in the regulation of hepatic lipogenesis. Insulin and glucose modulate SREBP and ChREBP, respectively. These transcription factors and PPARgamma positively regulate *de novo* lipogenesis by modulating the expression of the enzymes involved in this pathway. On the other hand, PPARalpha and PGC1a are positively 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 up-regulation of ChREBP, SREBP-1c and PPARgamma and a decrease in fatty acid oxidation due to a negative regulation on PPARalpha.

Primers used in real-time PCR

Gene	Primers sequences	Temperature of melting(°C)
Ppargamma F	5'-TTTTCAAGGGTGCCAGTTTC-3'	60
Ppargamma 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'	
Pparalpha F	5'-TCACACGATGCAATCCGTTT-3'	60
Pparalpha R	5'-GGCCTTGACCTTGTTCATGT-3'	
Pgc1a F	5'-AATGCAGCGGTCTTAGCACT-3'	60
Pgc1a R	5'-GTGTGAGGAGGGTCATCGTT-3'	
L32 F	5'-TGGTCCACAATGTCAAGG-3'	58
L32 R	5'-CAAAACAGGCACACAAGC-3'	

Table 1 :List of primers used in real-time PCR. (F) forward sequence, (R) reverse sequence.

Figure 1



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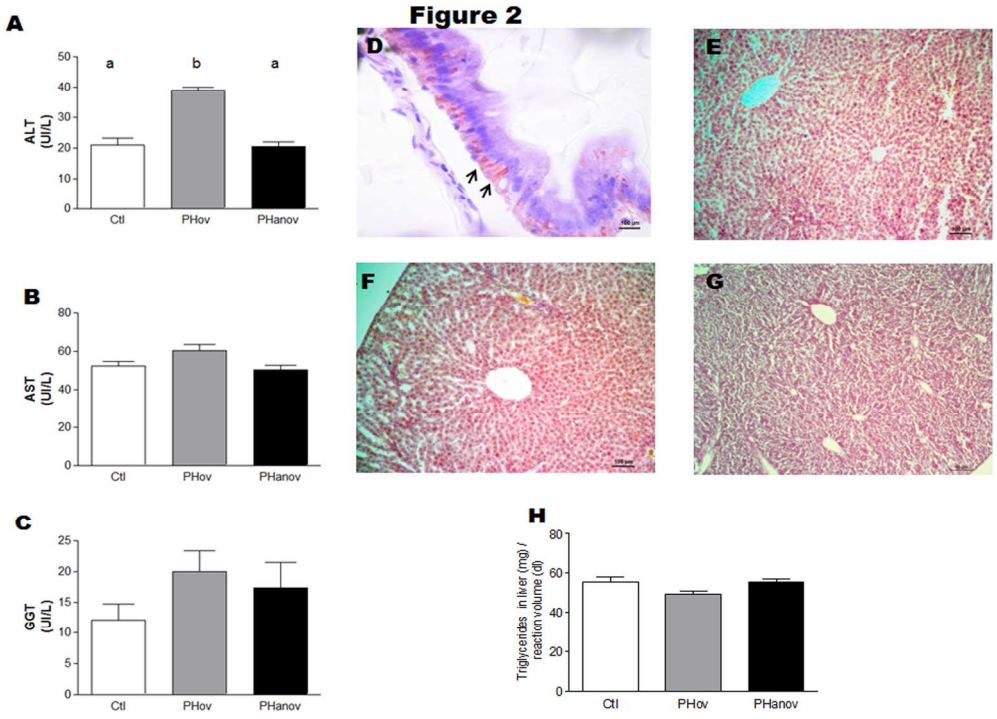

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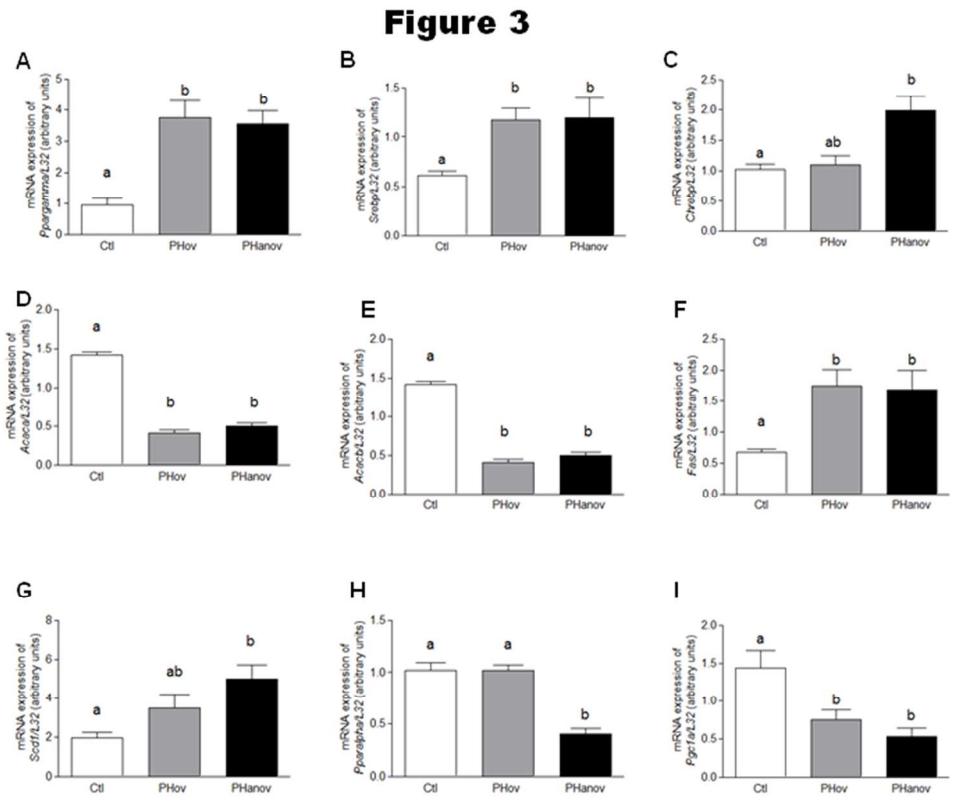

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Figure 4

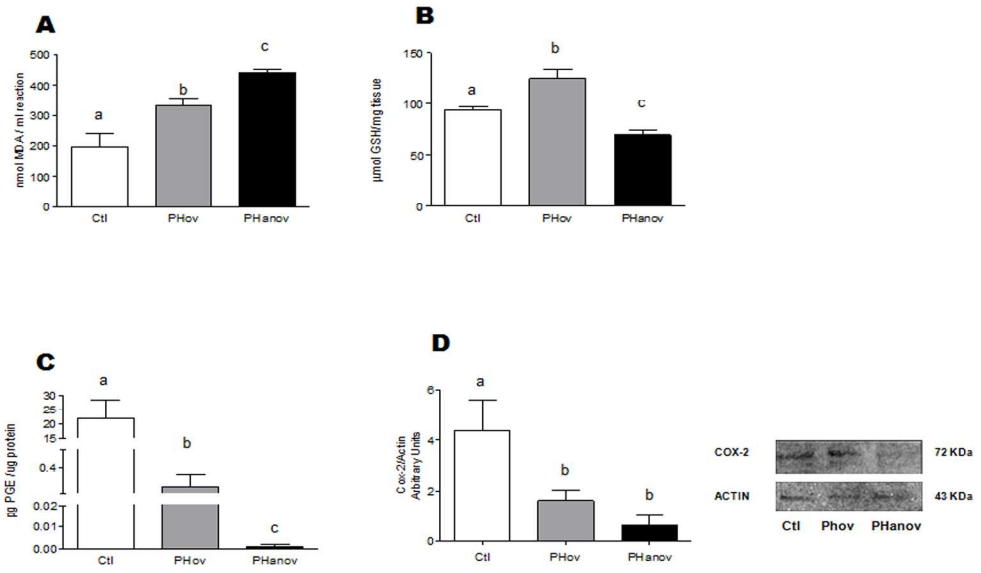

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Figure 5

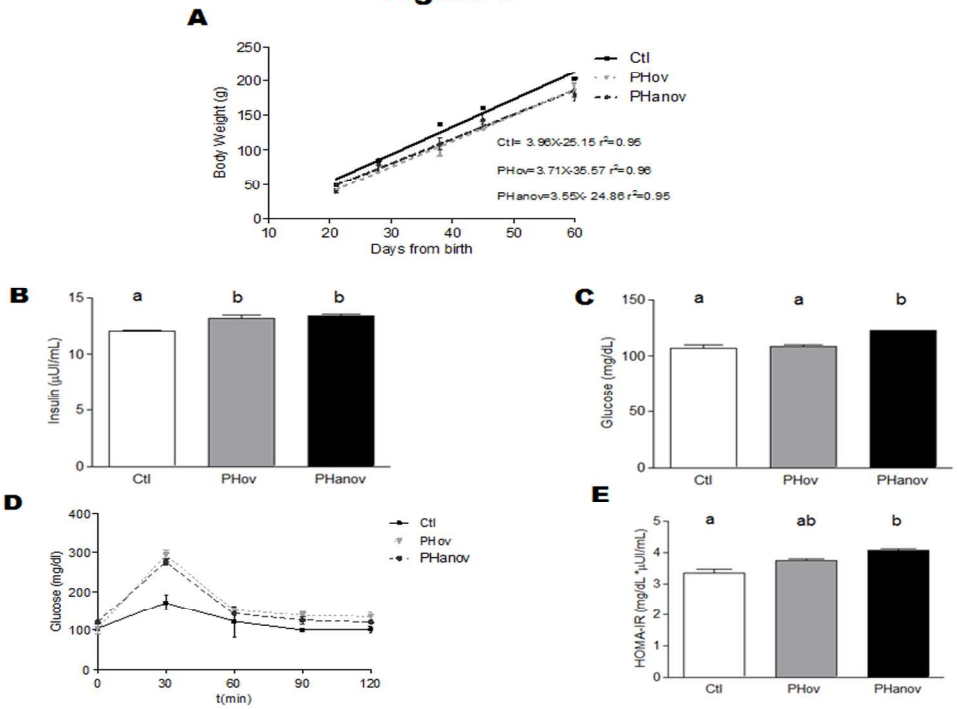

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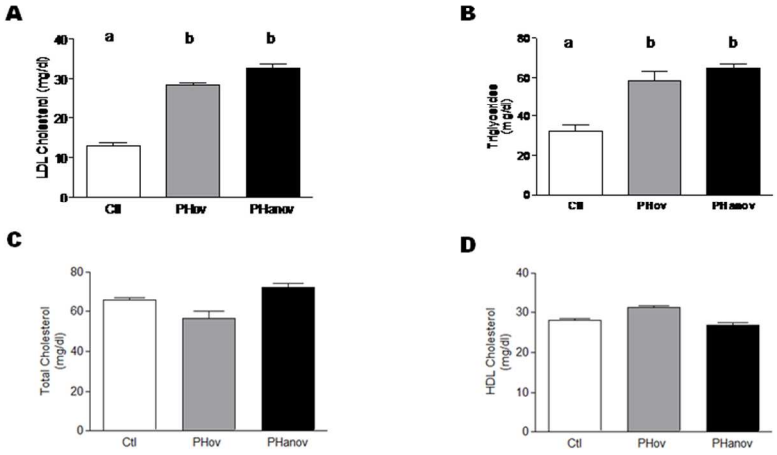
Figure 6



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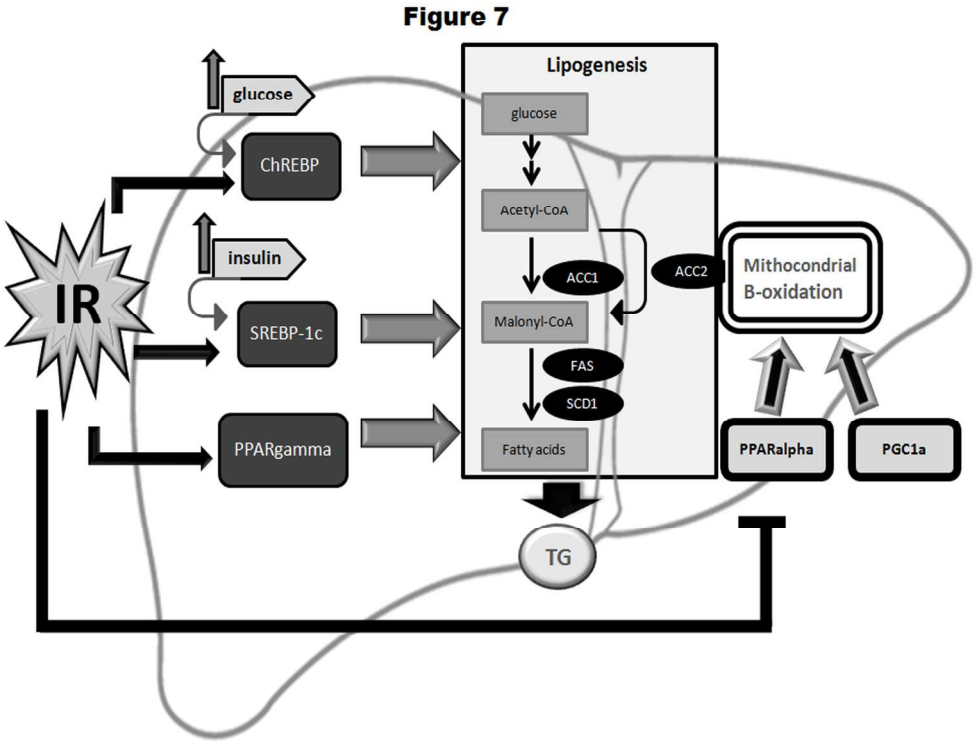

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