Journal of Developmental Origins of Health and Disease

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Fetal programming by androgen excess impairs liver lipid content and PPARg expression in adult rats

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Original Article

Cite this article: Silva AF, Abruzzese GA, Ferrer MJ, Heber MF, Ferreira SR, Cerrone GE, and Motta AB. (2022) Fetal programming by androgen excess impairs liver lipid content and PPARg expression in adult rats. *Journal of Developmental Origins of Health and Disease* **13**: 300–309. doi: 10.1017/S2040174421000416

Received: 9 December 2020
Revised: 4 May 2021
Accepted: 14 June 2021
First published online: 19 July 2021

Keywords:
prenatal hyperandrogenization; liver lipid metabolism; PPARg; PPARa; chemerin

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Abstract

It is known that prenatal hyperandrogenization induces alterations since early stages of life, contributing to the development of polycystic ovary syndrome affecting the reproductive axis and the metabolic status, thus promoting others associated disorders, such as dyslipidemia, insulin resistance, liver dysfunction, and even steatosis. In this study, we aimed to evaluate the effect of fetal programming by androgen excess on the hepatic lipid content and metabolic mediators at adult life. Pregnant rats were hyperandrogenized with daily subcutaneous injections of 1 mg of free testosterone from days 16 to 19 of pregnancy. The prenatally hyperandrogenized (PH) female offspring displayed two phenotypes: irregular ovulatory phenotype (PHiov) and anovulatory phenotype (PHanov), with different metabolic and endocrine features. We evaluated the liver lipid content and the main aspect of the balance between fatty acid (FA) synthesis and oxidation. We investigated the status of the peroxisomal proliferator-activated receptors (PPARs) alpha and gamma, which act as lipid mediators, and the adipokine chemerin, one marker of liver alterations. We found that prenatal hyperandrogenization altered the liver lipid profile with increased FAs levels in the PHanov phenotype and decreased cholesterol content in the PHiov phenotype. FA metabolism was also disturbed, including decreased mRNA and protein PPARgamma levels and impaired gene expression of the main enzymes involved in lipid metabolism. Moreover, we found low chemerin protein levels in both PH phenotypes. In conclusion, these data suggest that prenatal hyperandrogenization exerts a negative effect on the liver and alters lipid content and metabolic mediators' expression at adult age.

Introduction

Fetal programming is a complex phenomenon in which different insults could lead to adaptive processes that negatively affect fetal development. When hostile factors disrupt the intrauterine environment, the development of the gestational organism could be altered and, consequently, generate future health problems in the offspring.[1] Although the exact mechanisms of fetal programming are not entirely understood, this phenomenon induces gene expression changes, involving epigenetic alterations, and thereby modifies tissue functions and, in some cases, predisposes to adult-onset diseases.[2]

The liver is a crucial organ in metabolic regulation that controls the body energy balance.[3] It is responsible for glucose and insulin metabolism, energy storage, and lipid availability,[4] and alterations in any of these pathways may lead to liver diseases.[5] In particular, it has been suggested that androgen excess contributes to hepatic dysfunctions. In the liver, this steroid hormone may act in a direct manner by the androgen receptor signaling,[6] and indirectly by both the modulation of insulin pathways[7] and the increased visceral adiposity,[8] or by a combination of these three actions. In this regard, some studies have reported that fetal programming by androgen excess affects fetal liver functions, leads to metabolic disorders, and deregulates lipid metabolism in adult offsprings.[9,10] However, the exact mechanisms by which androgen excess is able to program liver functions remain unknown.

The liver plays a major role in lipid metabolism and energy homeostasis, and it may produce energy using carbohydrates and triglycerides (TG).[3] Alterations in hepatic lipid metabolism affect not only metabolic homeostasis but also the immune response.[3] Under physiological conditions, there is a balance in the liver between the import and export of fatty acids (FAs) and lipid

metabolism. When this balance is altered, liver accumulation of lipid droplets can occur, leading to steatosis (TG accumulation in hepatocytes), and even non-alcoholic fatty liver disease (NAFLD).[5,11]

Lipid pathways are mainly controlled at the transcriptional level by multiple master regulators.[11,12] Particularly, the peroxisomal proliferator-activated receptors (PPARs) are a family of nuclear receptors, which present three isoforms: alpha (PPARa), beta/delta (PPARb/d), and gamma (PPARg), encoded by different genes with differential functions. They are involved in the gene regulation of multiple metabolic pathways, such as lipid metabolism, glucose and insulin homeostasis, and inflammatory processes, among others.[13,14] Regarding PPARa, it plays a crucial role in FA oxidation,[15] while PPARg participates in the synthesis and storage of FA.[16] Moreover, it has been described that several signaling molecules involved in lipid and energy metabolism, including adipokines, may be regulated by PPARg.[17]

The adipokines are polypeptides produced mainly, but not exclusively, by adipose tissue. They are involved in several processes, such as the regulation of insulin and glucose metabolism and inflammatory state.[18] It has been reported that adipokines influence lipid metabolism in several target tissues.[19] In this context, adipokines alterations contribute to the development of lipid disorders.[20] One of these adipokines is chemerin, which is mainly synthesized by the liver and adipose tissue.[21] It has been associated with some pathologies, such as obesity, NAFLD, metabolic syndrome, and polycystic ovary syndrome (PCOS).[22,23] Moreover, it has been described that chemerin promotes adipogenesis and regulates immunity as well as glucose metabolism.[24,25] Additionally, a sequence analysis revealed a PPARg response element within the chemerin promoter,[17] suggesting that this transcriptional factor regulates its expression.

We have previously demonstrated that fetal programming generated by prenatal androgen excess induces systemic insulin and glucose metabolism alterations at adulthood (90 days of age) in both prenatally hyperandrogenized (PH) phenotypes.[26] Besides, we have demonstrated the presence of insulin and glucose signaling disruption and a pro-oxidant state in the liver, although there was no evidence of TG accumulation.[26] Additionally, impaired hepatic lipogenesis was found at puberty (60 days of life) in the absence of obesity,[27] but it is known that the consequences of fetal programming on the affected organism could change over time.[28,29] Thereby, in the present study, we aimed to deepen the impact of prenatal hyperandrogenization in hepatic lipid metabolism during adulthood, focusing on lipid content, lipogenesis, and the main regulators of beta-oxidation (b-oxidation). In addition, we were interested in characterizing the effects of fetal androgen exposure on hepatic chemerin expression, a poorly understood adipokine.

Materials and methods

Animals and experimental designs

Virgin female rats of 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 (12h light:12h darkness cycle) and temperature (23°C–25°C). Animals received food and water *ad libitum*. Day 0 of pregnancy was defined as the morning on which spermatozoa were observed in the vaginal fluid. Between days 16 and 19 of pregnancy, one group of rats was hyperandrogenized, as described before.[27,30] Pregnant rats ($N = 10$) received daily subcutaneous injections of 1 mg free testosterone (T-1500; Sigma) dissolved in 100 µl sesame oil. The testosterone administered mimics the fetal testosterone surge observed in male rats when the reproductive axis in the fetus is established.[31] Another group ($N = 10$) received only 100 µl of sesame oil. Under the conditions of our animal facilities, spontaneous term labor occurs on day 22 of gestation. Pups were culled from litters to equalize group sizes (8 pups per mother). Female offspring were separated from males at 21 days of age and randomly chosen. Those from hyperandrogenized mothers formed the PH group, and the offspring from mothers who received the vehicle only formed the Control 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. The estrous cycle was determined by vaginal smears taken daily from 70 to 90 days of age. The Control animals had regular ovulatory cycles (100 %), presenting smears displaying the four stages of the estrous cycle in the following order: proestrus, estrus, metaestrus, diestrus, with cycles of 4–6 days.[32] The PH group showed two phenotypes: (i) irregular ovulatory phenotype (PHiov) (49%–50%), in which the animals showed some smears not following the progress of the cycle as described above, or whose cycles lasted 7 days or more; (ii) anovulatory phenotype (PHanov) (38%–43 %), with animals whose smears showed metaestrus, diestrus, or a combination of both for four consecutive days, and were thus considered to be non-cycling.[32] Animals from the Control, PHiov, and PHanov groups showed no significant differences in body weight (231 ± 15, 238 ± 22, and 231 ± 19 g, respectively).[26] As we previously reported, serum testosterone levels were increased in the PHanov group as compared to the PHiov and Control groups (Control = 67.76 ± 13.52 pg/ml; PHiov = 115.84 ± 34.82 pg/ml; and PHanov = 154.34 ± 32.00 pg/ml).[32] Moreover, serum estradiol levels were lower for both PH groups as compared to the Control group (Control = 12.25 ± 2.31 pg/ml; PHiov = 9.69 ± 1.93 pg/ml; and PHanov = 8.05 ± 1.24 pg/ml),[32] and the estradiol-to-testosterone ratio was also decreased in both PH phenotypes, showing that the PH groups present a hormonal imbalance.

At 90 days of age, female offspring from each group were anesthetized with carbon dioxide and killed by decapitation. As the PHanov group remained in diestrus, all animals were euthanized at this stage, to allow the comparison between phenotypes. The liver was separated and conserved at −80°C for further studies. All animals were randomly assigned to each assay considering the littermate. Thus, each assay was carried out with the same number of PHiov and PHanov animals from each randomly selected littermate.

Liver lipid content

To evaluate the TG content in the liver, six frozen samples of each group were saponified and the TG content was quantified by comparing it with a glycerol standard curve using a commercial kit (Wiener Lab, Argentina), as previously described.[33] The results are expressed as mg of TG/g tissue.

To analyze the FA, cholesterol, and cholesterol ester content, a lipid extraction was performed from six hepatic samples per group according to Blight and Dyer method.[34] Briefly, hepatic tissue was homogenized in 1 ml of phosphate-buffered saline (PBS),

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and protein content in the homogenates was measured by Bradford assay.[35] Tissue lipids were extracted from 500 µl of each homogenate by three rounds of organic extraction in methanol:chloroform (2:1), as previously performed.[36] The lipids extracted were developed by thin layer chromatography in 0.2 mm silica gel plates (Merck, Darmstadt, Germany). Along with the samples of each group, a known standard solution was developed. The plates were placed in a running buffer (hexane: ether: acetic acid [80:20:2, v:v:v] as the developing solvent mixture) for 30–45 min to allow polarity separation of the different lipid species. They were stained with iodine vapors and the plate was scanned. Densitometric analysis of the area intensity of each spot was performed with the Image J software. Quantification was performed by extrapolation from the standard curves built with the densitometric values of standards run in the same plate. The results are expressed as µg of lipid species/mg protein.

Liver lipid metabolism

Gene expression

The mRNA levels of *Ppara* and *Pparg*, PPAR co-activator 1a (*Pgc1a*), acetyl-CoA carboxylase (ACC) 1 and 2 (*Acaca* and *Acacb*, respectively), fatty acid synthase (*Fas*), stearoyl-CoA desaturase (*Scd1*), adipose triglyceride lipase (*Atgl*), carnitine palmitoyltransferase 1 (*Cpt1*), and *Chemerin* were evaluated by real-time polymerase chain reaction (real-time PCR) analysis. Total mRNA from hepatic tissue (six samples per group) 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. This cDNA (2.5 µl) was used for real-time PCR analysis in 10 µl reaction buffer containing a 20 mM dNTPs mix, $MgCl_2$, GoTaq Polymerase (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 4 min and followed by up to 50 cycles of denaturation (95 °C, for 20 s), annealing (see temperature for each primer in Table 1, for 25 s), and primer extension (72 °C, for 25 s). The amplified products were quantified by fluorescence using the Rotor Gene 6000 Corbett, and mRNA abundance was normalized to the 60s ribosomal protein L32 (*L32*) amount. *L32* was validated as a reference gene because the variance between treatments did not differ. Gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method.[37] The results are expressed as arbitrary units. The primers are given in Table 1.

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

Primers used in real-time PCR		
Gene	Primers sequences	Temperature of melting (°C)
Acaca F	CCAGACCCTTTCTTCAGCAG	62
Acaca R	AGGACCGATGTGATGTTGCT	
Acacb F	CAAAGCCTCTGAAGGTGGAG	62
Acacb R	CTCGTCCAAACAGGGACACT	
Atgl F	AACCATCATTCTCGGCTCAC	62
Atlg R	CCCACCAGGAGTAGCATTGT	
Cpt1 F	AGGTCTGGCTCTACCACGAT	62
Cpt1 R	CGTCCAAAATAGGTCTGCGC	
Fas F	TCGAGACACATCGTTTGAGC	62
Fas R	CCCAGAGGGTGGTTGTTAGA	
L32 F	TGGTCCACAATGTCAAGG	58
L32 R	CAAAACAGGCACACAAGC	
Pgc1 F	AATGCAGCGGTCTTAGCACT	62
Pgc1 R	GTGTGAGGAGGGTCATCGTT	
Ppara F	TCACACGATGCAATCGGTTT	60
Ppara R	GGCCTTGACCTTGTTCATGT	
Pparg F	TTTTCAAGGGTGCCAGTTTC	60
Pparg R	GAGGCCAGCATGGTGTAGAT	
Chemerin F	GGCACCTTTGTGAGGCTGGAATTT	62
Chemerin R	ACCCTGTCCGGGGTCTATTTGGAT	
Scd1 F	GCTTCCAGATCCTCCCTACC	62
Scd1 R	CAACAACCAACCCTCTCGTT	

F, forward sequence; R, reverse sequence.

Protein expression

The hepatic protein levels of PPARa, PPARg, and CHEMERIN were analyzed by Western Blot, as previously described.[26] Briefly, 100 mg of liver tissue (six samples per group) was lysed at 4 °C in 500 µl lysis buffer (20 mM Tris-HCl, pH = 8.0, 137 mM NaCl, 1% Nonidet P-40 and 10% glycerol) and supplemented with protease inhibitors (Protease Inhibitor Cocktail P8340, Sigma Aldrich, St. Louis MO, USA). The lysate was centrifuged at 4 °C for 10 min at 10,000 g and the pellet was discarded. Protein concentrations in the supernatant were measured by the Bradford assay (Bio-Rad).[35] Total proteins (50 µg) were denatured and separated on a sodium dodecyl sulfate (SDS-polyacrylamide gel (12%) and transferred onto nitrocellulose membranes (GE Healthcare, Life Sciences). Membranes were blocked for 1 h at room temperature in 5% (w/v) nonfat dry milk in PBS and then incubated at 4 °C overnight with the primary antibody. Anti-PPARg 1 + 2 1:1000 (ab41928, Abcam, Cambridge, UK), anti-PPARa 1:400 (15540-1-AP, PROTEINTECH), and anti-CHEMERIN 1:800 (ab112540, Abcam) were used as primary antibodies. The membranes were washed three times for 7 min each in Tris buffered saline with Tween (TBST) (4 mM Tris-HCl, pH = 7.5, 100 mM NaCl, 0.1% Tween 20). Then, they were incubated at room temperature for 1 h with the secondary antibody, species-specific antirabbit and antimouse IgG (according to the primary antibody), linked to horse recombinant peroxidase (1: 3000, 1,706,515, BioRad or 1: 5000, ab6728, Abcam, respectively) while being rocked. After three washings of 7 min each with TBST, the band intensities were quantified by an image analyzed (GeneGnome XRQ, Syngene Synoptics Group, Cambridge, UK). The intensity of bands was analyzed using the Image J program and normalized relative to total protein relation, since tubulin protein levels changed in response to the experimental treatment.[38,39] Staining of the total protein on the blotting membrane has emerged as a better loading control than housekeeping proteins, since multiple advantages have been described: smaller technical and biological variations, unlikely signal saturation, no collision between the protein of interest and loading control signal, improvement of the sensitivity and accuracy of Western Blot results, among others.[40–42] Using the total protein method, a range between the certain protein weight was analyzed. The results are expressed as arbitrary units.

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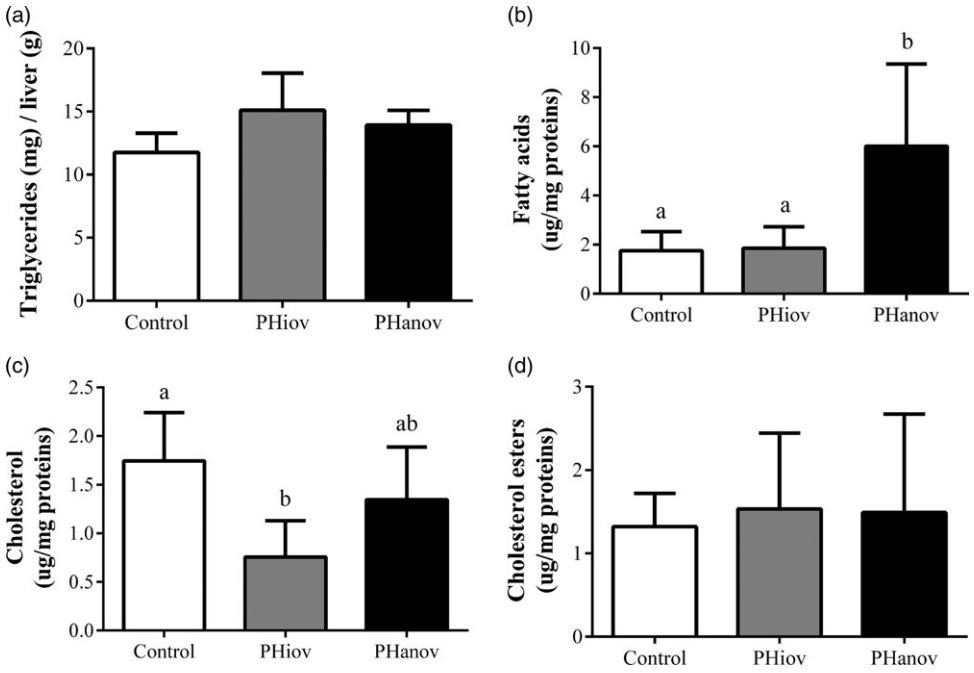

Fig. 1. Liver lipid content at 90 days of age. (a) Triglycerides; (b) fatty acids; (c) cholesterol; (d) cholesterol esters. Each column represents the mean ± SD of Control and prenatally hyperandrogenized (PH) groups in six samples per group (a vs b $P < 0.05$, by ANOVA with post hoc Tukey test).

Statistical analysis

Statistical analyses were carried out using InfoStat program[43] and GraphPad Instat® software (GraphPad Software, San Diego, CA, USA). ANOVA with post hoc Tukey test was used to compare the three phenotypes. The normality of data (Shapiro–Wilks test) and homoscedasticity (Levene's test) were tested. The results were expressed as means ± standard deviation (SD). Statistical significance was considered as $P < 0.05$.

Results

Prenatal hyperandrogenization on liver lipid content

Prenatal hyperandrogenization did not affect the hepatic TG content (Fig. 1a); however, FA levels (Fig. 1b) were increased in PHanov animals as compared with both PHiov and Control groups. On the other hand, the cholesterol content (Fig. 1c) was decreased in the PHiov phenotype, while cholesterol ester levels (Fig. 1d) were not altered in the PH groups as compared with the Control group.

Prenatal hyperandrogenization on hepatic lipid mediators

The transcription factor involved in the FA synthesis regulation, PPARg, showed a lower expression in the PH phenotypes than in the Control group, in both mRNA (Fig. 2a) and protein levels (Fig. 2b). On the other hand, PPARa, a nuclear receptor isoform that regulates the FA oxidation pathways, presented no differences between groups in either mRNA (Fig. 3a) or protein levels (Fig. 3b).

Prenatal hyperandrogenization on hepatic lipid metabolism

To study the hepatic lipid metabolism, we analyzed whether prenatal hyperandrogenization altered the mRNA levels of the main enzymes that participate in these pathways. The mRNA levels of *Acaca* and *Acacb*, the two isoforms of the ACC enzymes, did not present significant differences with respect to the Control group (Fig. 4a and 4b). However, *Fas* (Fig. 4c) and *Scd1* (Fig. 4d) mRNA levels were increased in PHiov animals as compared with the PHanov and Control groups. In addition, regarding the *Atgl* mRNA levels, no significant differences were found between the PH and the Control groups (Fig. 4e). However, the *Atgl* mRNA levels of the PHiov phenotypes were increased when compared to those of the PHanov phenotype (Fig. 4e). Regarding the b-oxidation mediators, *Pgc1a* was not affected in the PH groups (Fig. 4f), but *Cpt1* mRNA levels were increased in the PHiov phenotype as compared with the PHanov and Control groups (Fig. 4g).

Prenatal hyperandrogenization on hepatic chemerin expression

We found that *Chemerin* mRNA levels did not show alterations in any of the phenotypes studied (Fig. 5a). However, CHEMERIN protein levels were decreased in the PHiov phenotype and were even lower in the PHanov phenotype as compared with the Control group (Fig. 5b).

Discussion

A hormonal imbalance, such as prenatal androgen excess, during the early stages of development can alter both endocrine and metabolic systems.[44] Recently, there has been a great interest in studying hepatic functionality in androgenic contexts, since the regulation of lipid and glucose pathways in this organ was altered in hyperandrogenic PCOS women.[10] In our present research, we reproduced a murine model of prenatal hyperandrogenization which displayed PCOS-like features,[32,45] leading to the development of two phenotypes from the same mother: PHiov and PHanov.[27]

The liver is an essential organ involved in the regulation of energy homeostasis.[46] It expresses sex steroid receptors, and both androgens and estrogens have direct as well as indirect effects on

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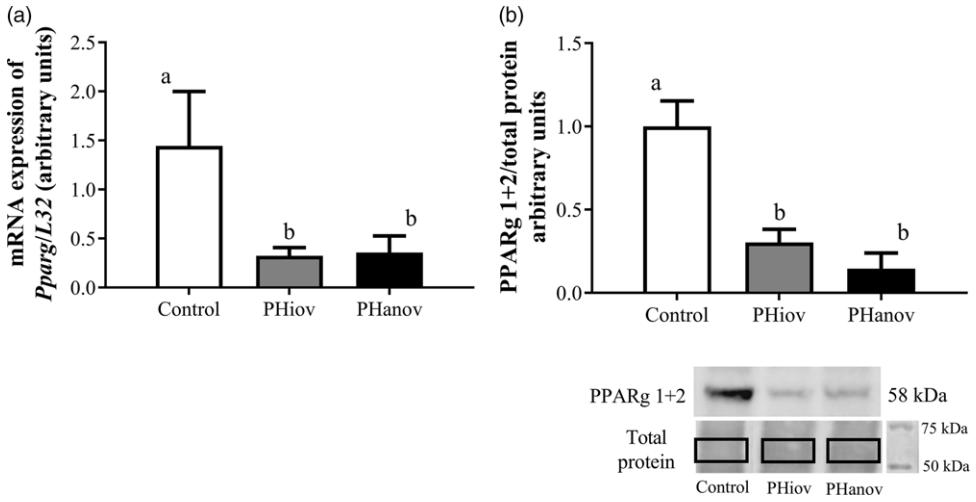

Fig. 2. Effects of prenatal hyperandrogenism on PPARg expression. (a) mRNA abundance of *Pparg* relative to *L32* mRNA levels of Control and prenatally hyperandrogenized (PH) groups, (b) results of Western Blot analysis for PPARg 1 + 2 corresponding to Control and PH groups. Each column represents the mean ± SD in six replicates per group (a vs b $P < 0.01$, by ANOVA with post hoc Tukey test).

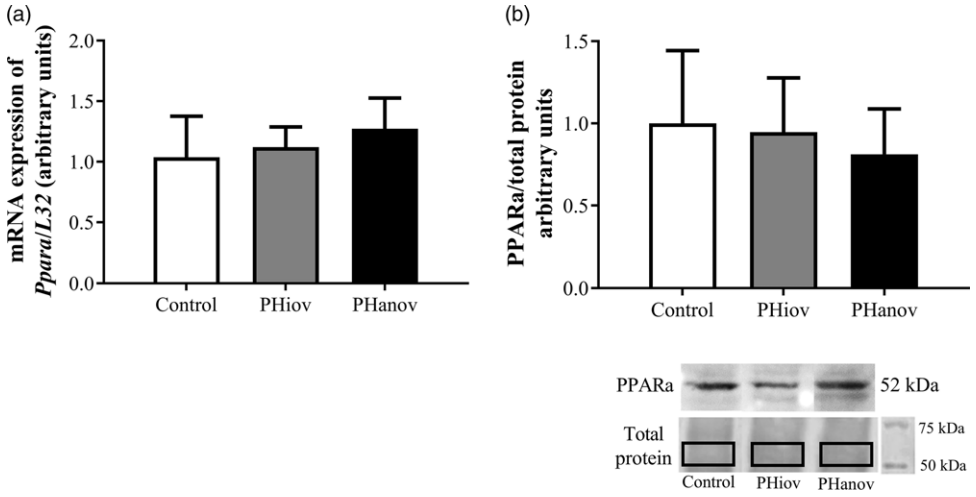

Fig. 3. Effects of prenatal hyperandrogenism on PPARa expression. (a) mRNA abundance of *Ppara* relative to *L32* mRNA levels of Control and prenatally hyperandrogenized (PH) groups, (b) results of Western Blot analysis for PPARa corresponding to Control and PH groups. Each column represents the mean ± SD in six replicates per group ($P > 0.05$, by ANOVA with post hoc Tukey test).

hepatic function.[47] Besides, sex steroids have been implicated in the transcriptional regulation of several hepatic genes that play key roles in lipid metabolism.[48] In that sense, in previous studies, we demonstrated that prenatal hyperandrogenization alters female hepatic functions and lipogenesis at pubertal stage.[27] However, there was no evidence of TG accumulation neither at puberty nor at adulthood.[26,27] Moreover, we found that the lipid content and a key metabolism mediator, PPARg, varied at different stages in the ovary.[28] Thereby, prenatal hyperandrogenization induces alterations in lipid metabolism that may change throughout life. Taken together, these considerations led us to deepen the study of hepatic lipid pathways on adulthood and analyze the reach of prenatal androgen exposure on the lipid content considering different lipid species.

We confirmed that there is no TG accumulation in the liver of PH animals. Contrary to other reports,[30,48] in which prenatally androgenized offspring that were fed with a high-fat diet exhibited significantly increased body weight and hepatic TG content, in our work, the animals were not subjected to a high-fat diet. In this regard, it was demonstrated that a high-fat diet increased the liver TG content considerably, both in control and PH rats.[30] Then, the regular feeding could have contributed to maintaining a normal weight, as we previously reported.[26] Additionally, it is important to consider that the studies performed with prenatal androgen exposure that showed hepatic TG accumulation presented differences in the strain and testosterone dose.[9,30,49] Such differences could account for those controversial results.

Despite the TG content was not impaired in the PH group, the FA levels did present alterations both in their hepatic amount and in the enzymes involved in their synthesis and regulation (Fig. 6). In the FA metabolism, control participates two important transcription factors: PPARg, which is involved in the regulation of the enzymes that participate in FA synthesis,[16] and PPARa, which regulates the enzymes involved in FA oxidation.[15] We found that the mRNA levels of *Pparg* were decreased in PH animals. These data are in agreement with our previous findings,[26] and in this

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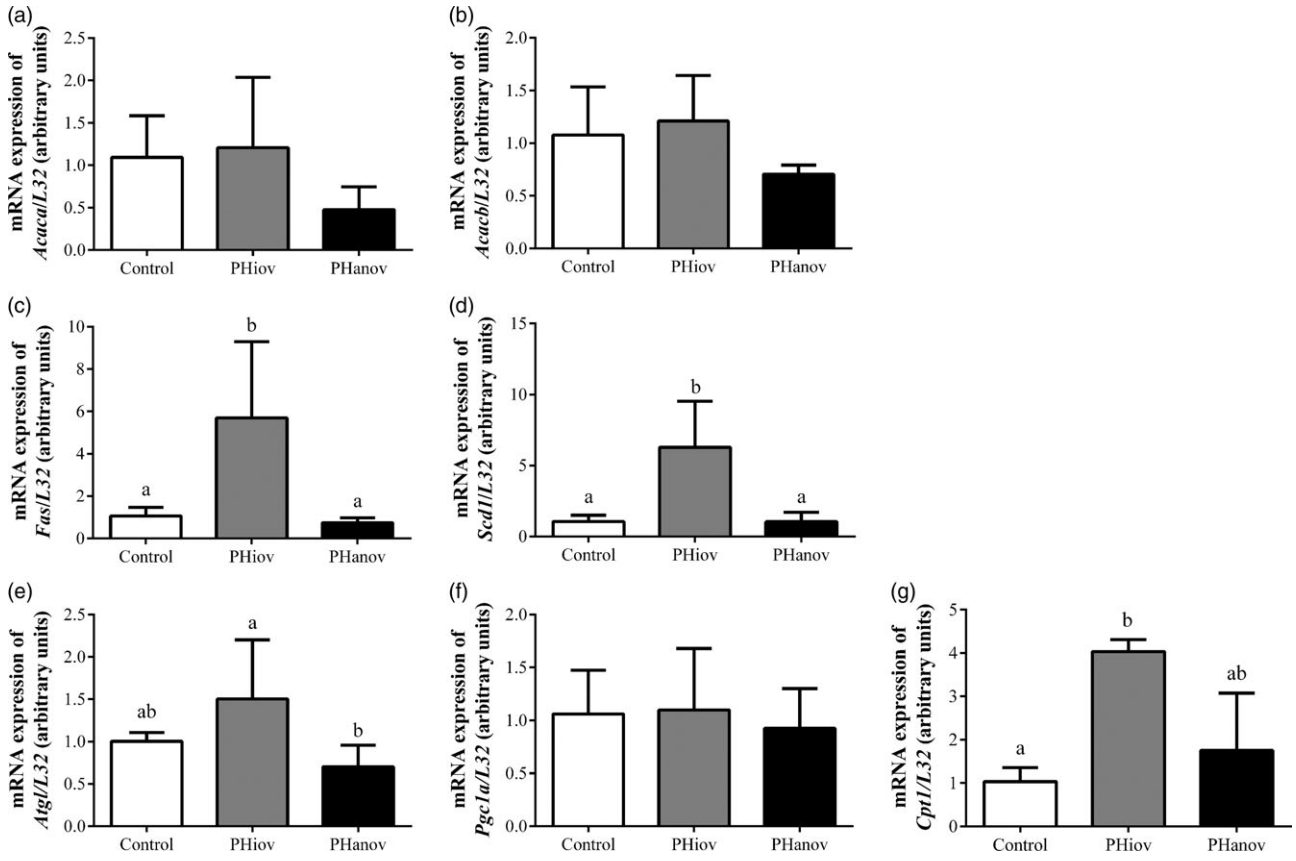

Fig. 4. Effects of prenatal hyperandrogenism on the gene expression of liver lipid metabolism mediators. The graphs correspond to the mRNA abundance of the gene of interest relative to *L32* mRNA levels of Control and prenatally hyperandrogenized (PH) groups. Gene expression of (a) *Acaca*, (b) *Acacb*, (c) *Fas*, (d) *Scd1*, (e) *Atgl*, (f) *Pgc1a*, and (g) *Cpt1*. Each column represents the mean ± SD in six replicates per group (a vs b $P < 0.05$, by ANOVA with post hoc Tukey test).

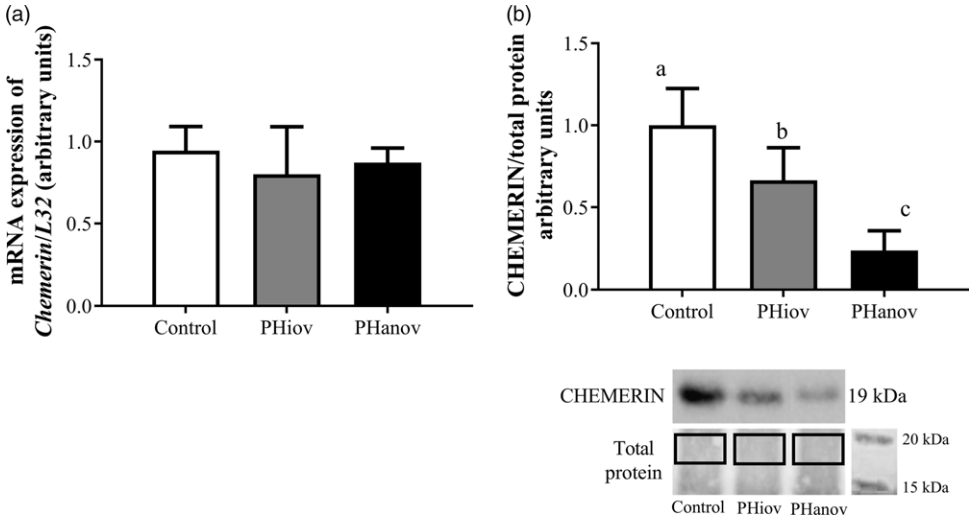

Fig. 5. Effects of prenatal hyperandrogenism on hepatic chemerin. (a) mRNA abundance of *Chemerin* relative to *L32* mRNA levels of Control and prenatally hyperandrogenized (PH) groups, (b) results of Western Blot analysis for CHEMERIN corresponding to Control and PH groups. Each column represents the mean ± SD in six replicates per group (a vs b vs c $P < 0.01$, by ANOVA with post hoc Tukey test).

work, we also found that PPARg protein levels are consistent with the mRNA levels, thus showing a dysregulation of this transcriptional factor. Moreover, considering that a liver deficiency of PPARg worsens the hyperglycemia and the systemic insulin resistance,[50,51] these results contribute to the understanding of the altered metabolic state that we have previously reported.[26] Taken together these findings, prenatal hyperandrogenization induces alterations in PPARg expression, contributing to disruptions in lipid homeostasis. On the other hand, we found no alterations in either the mRNA or protein levels of PPARa in any of the groups studied. Considering that this transcriptional factor is the master regulator of FA oxidation,[46] the result might indicate

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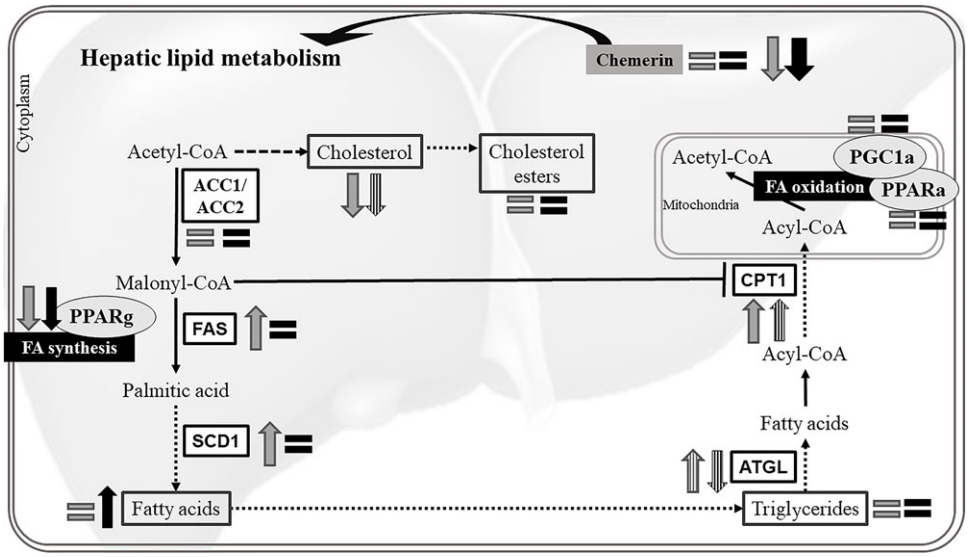

Fig. 6. Liver lipid metabolism studied in this work. The different steps of the metabolic pathways are linked by thin black arrows. The dotted thin black arrows represent the presence of multiple intermediate steps. The results obtained in this work are showed as follows: wide solid arrows indicate dysregulation in PH phenotypes (increased or decreased levels) with respect to the Control group. Wide striped arrows indicate tendency without significant statistical differences with respect to the Control group. Equal sings indicate no alterations with respect to the Control group. PHiov phenotype is represented in grey and PHanov in black. Concerning chemerin, the results of the mRNA levels are indicated first, and the protein levels are indicated second, following the phenotype colors previously mentioned. ACC, acetyl-CoA carboxylase; ATGL, adipose triglyceride lipase; CPT1, carnitine palmitoyltransferase 1; FA, fatty acids; FAS, fatty acid synthase; PGC1a, PPAR co-activator 1a; PPARa, peroxisomal proliferator-activated receptor alpha; PPARg, peroxisomal proliferator-activated receptor gamma; SCD1, stearoyl-CoA desaturase 1.

that the nuclear receptor does not affect the FA oxidation pathways. However, PPARs bind to DNA in a PPAR response element (PPRE) as a heterodimer containing PPARa and retinoic X receptor alpha. The heterodimer acts as ligand-activated transcription factor, affecting the interactions with co-activators or co-repressors to stimulate or inhibit its functions.[15,52] Thus, failures in the heterodimer interactions with co-activators or co-repressors or in the binding of the PPAR to DNA could alter PPARa-dependent gene regulation.[53] In fact, previous studies have demonstrated that a nuclear receptor co-activator is essential in PPARa functionality,[15] and that a PPARa-driven activation depends on PPRE binding *in vitro* and *in vivo* in a PPARa-deficient mice model.[53] Moreover, since polyunsaturated FAs are endogenous ligands of PPARa,[54] changes in the concentration of these lipids directly impact on the PPARa activity. Thereby, although we found no variation in PPARa expression, its activity may be impaired. Then, further studies are needed to clarify this point.

To further analyze FA metabolism, we evaluated the expression of key enzymes of lipogenesis and FA oxidation (Fig. 6). The FA synthesis starts with acetyl-CoA, which is used to get malonyl-CoA by ACC, an enzyme that presents two isoforms: ACC1 and ACC2, encoded by the *Acaca* and *Acacb* genes, respectively. Surprisingly, our results showed that the mRNA levels of both isoforms were not affected in the PH phenotypes, although high levels of FA content in the PHanov phenotype and decreased levels of PPARg were obtained in both PH phenotypes. In that sense, since the synthesis of malonyl-CoA is a crucial step in lipid metabolism, as it determinates whether FAs are synthesized or oxidized, the ACC enzymes are highly controlled[55] and, although PPARg is a master regulator of ACC, other factors that could be influencing the lipogenic enzymes expression must be considered. ACC expression depends on a variety of factors including glucose, insulin, thyroid hormone, catabolic hormones, and leptin.[56] Among the key transcription factors that regulate the ACC gene expression, it has been demonstrated that Liver X receptors is important not only in the regulation of FA synthesis but also in mediating the glucose and insulin response of other transcription factors.[55]

To deepen the study of the lipogenic genes, we analyzed the mRNA levels of the genes encoding FAS and SCD1, both enzymes involved in lipogenesis. We found higher mRNA levels of *Fas* and *Scd1* in the PHiov phenotype than in the Control group. These enzymes catalyze rate-limiting steps in the synthesis of FA.[55] *Fas* utilizes malonyl-CoA as substrate, which inhibits CPT1. Thus, the high *Fas* mRNA levels seem to favor the FA oxidation, as the enzyme removes the malonyl-CoA and the transporter could act without any inhibition.[55] In fact, high mRNA levels of *Cpt1*, which mediates the acyl-CoA translocation into the mitochondria, were found. Thereby, the transporter is enabling the FA availability for oxidation. Moreover, high hepatic SCD1 activity has been associated with the prevention of liver fat accumulation,[57] consistent with the unaffected hepatic TG content. Since, no differences were found in the FA amounts in the PHiov group, this phenotype seems to present a balance between these two lipid species.

TGs are hydrolyzed mainly by ATGL to release FA and diacylglycerol[46] (Fig. 6). We found that *Atgl* mRNA levels showed a tendency to increase in the PHiov phenotype and a tendency to decrease in the PHanov phenotype, although without significant statistical differences with respect to the Control group. However, we did observe differences in *Atgl* mRNA levels between the two PH phenotypes, being higher in the PHiov than in the PHanov phenotype. The increased *Atgl* mRNA levels in the PHiov phenotype could be contributing to the balance between the FA and TG content that was mentioned above. However, an increased level of FA was observed in the PHanov group. In this regard, we have previously found disturbances in the hepatic glucose and insulin signaling.[26] In the liver, glucose transport is carried out by GLUT2,[58] and, in previously findings, we obtained a decrease in the gene expression of *glut2* in both PH groups.[26] Moreover, it has been demonstrated that GLUT2 can be a direct target of

https://doi.org/10.1017/S2040174421000416 Published online by Cambridge University Press

PPARg.[59] We have shown in this work that PPARg, which plays an important role in glucose homeostasis,[60] presented decreased mRNA and protein levels in both PH phenotypes. Then, our results agree with the low gene expression of the glucose transporter. Considering that these alterations impair the liver glucose uptake,[61,62] and that the mRNA levels of the limited enzymes that participate in *de novo* lipogenesis are not affected, the high FA levels in the PHanov phenotype could be due to the entrance of this lipid species from circulation to the liver,[5] as a compensated pathway to the glucose deficiency. Nevertheless, further studies are needed to elucidate this aspect. Since no TG accumulation was found, the balance between the high FA levels and their oxidation may explain the results.[46,61] Actually, when analyzing the status of FA oxidation, we found that the expression of *Pgc1a* and *Ppara* was not altered. PGC1a not only acts as a PPARa co-activator but also modulates mitochondrial functions and glucose homeostasis.[63,64] Taken together these data, both master regulators are facilitating the b-oxidation.

It has been reported that in a hyperandrogenic state, the cholesterol efflux capacity, in which this lipid removal from peripheral tissues is delivered to the liver, is reduced.[65] In that sense, when evaluating the cholesterol content, it showed a decreased level in PHiov animals and a tendency to decrease in the PHanov group. These data are in agreement with those previous findings.[65] Moreover, cholesterol is a potential lipotoxic molecule that causes cellular toxicity or acts in a pro-inflammatory or pro-fibrotic manner.[66] One strategy for the cells to avoid cholesterol accumulation is its esterification, resulting in cholesterol esters.[67] In this work, we found no alterations in cholesterol ester levels between groups. Since there is no evidence of hepatic damage at adulthood in our animal model, as we have previously demonstrated,[26] our results are consistent with these findings.

Finally, we assessed the status of chemerin in the liver (Fig. 6). It is an adipokine associated with obesity, NAFLD, and metabolic syndrome, participating in glucose homeostasis and inflammatory processes.[21,68] In this work, we found that the liver mRNA levels of this adipokine showed no alterations between Controls and PH animals. Nevertheless, CHEMERIN protein levels were decreased in the PHiov phenotype and even lower in the PHanov phenotype. Despite the fact that PPARg has been described to regulate *Chemerin* mRNA levels in different tissues such as adipose tissue and ovary,[17,69] here, we found that the hepatic mRNA levels of *Chemerin* are not concordant with those obtained for PPARg. This could be due to a tissue-specific PPARg action as a transcription factor. In that sense, other results have demonstrated that PPARg activation increased chemerin expression in white adipose tissue but not liver.[17,68] Thus, further studies are needed to determine whether PPARg action actually regulates chemerin expression in the liver in androgenic conditions. Moreover, we found an inconsistency between chemerin mRNA and protein levels. This difference between the gene and protein expression may be explained due to the post-transcriptional and post-translational mechanisms that regulate the processing of mRNAs and proteins.[70]

Although the role of chemerin in the systemic metabolism is not yet clear, it has been shown that this adipokine affects insulin sensitivity, as it has been demonstrated in a chemerin knockout model.[25] However, these results for either hepatic or serum concentrations are controversial,[71,72] and more research is needed. The results so far suggest that changes in this adipokine levels reveal signs of alterations in liver functionality.[25] Furthermore, it has been shown a positive correlation between chemerin concentration and lipid content in the liver.[73]

In summary, we found that prenatal hyperandrogenization alters the liver lipid content. We also found a disturbance of FA metabolism mediated by PPARg as well as an imbalance of the mRNA levels of the lipid metabolism enzymes. Finally, we showed an alteration in the protein expression of CHEMERIN, indicating a liver dysfunction resulting from the androgen exposure during fetal development. In that sense, these results highlight the link between androgen actions and hepatic lipid metabolism. However, further studies are needed to elucidate the role of this sex steroid hormone in the pathogenesis of metabolic liver disease.

Acknowledgements. We thank Enzo Cuba and Marcela Marquez for their technical support in the animal care.

Financial support. This study was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) (Grant PICT 632/2016), Fundación Alberto Roemmers, Argentina, and Universidad de Buenos Aires (20720160100004BA/2017).

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 Técnicas (CONICET) 1996, Argentina, and the study was approved by the Ethics Committee of the School of Medicine of University of Buenos Aires, Argentina.

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https://doi.org/10.1017/S2040174421000416 Published online by Cambridge University Press

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