

The gonadal expression pattern of lipocalin-2 and 24p3 receptor is modified in the gonads of the offspring of obese rats

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Abstract. Obesity represents a global health and economic burden, affecting millions of individuals worldwide. This pathology is associated with a chronic low-grade inflammatory state that is partially responsible for the development of other cardiometabolic complications. Clinical studies have reported an association between high circulating levels of lipocalin-2 (Lcn2) and increased body weight. Additionally, there is scientific evidence demonstrating the impact of maternal obesity on fetal programming. The latter and the fact that the authors previously found that Lcn2 and its receptor (24p3R) are expressed in the gonads of wild-type rats, led to the analysis of their mRNA profile and cellular localization in gonads collected from the offspring of obese rats at 21 days postconception (dpc), and 0, 2, 4, 6, 12, 20 and 30 days postnatal (dpn) in the present study. Semi-quantitative PCR revealed a statistically significant downregulation of *Lcn2* and *24p3R* mRNA at 21 dpc in the ovaries ($P < 0.01$) and testicles ($P < 0.001$) of the offspring of obese mothers. At 30 dpn, the relative expression of *Lcn2* mRNA decreased significantly in the ovaries of the experimental group ($P < 0.05$), while *Lcn2* mRNA expression was not detectable in testicles. Regarding *24p3R*, its mRNA was only significantly decreased at 21 dpc in ovaries of pups of obese mothers. At 30 dpn, the change

in females was not significant. Conversely, in testicles, *24p3R* mRNA levels increased slightly in the experimental group at 30 dpn. The Lcn2 protein signal was less intense in gonadal tissue sections from 30 dpn offspring of obese rats ($P < 0.001$), whereas the 24p3R signal was downregulated in ovaries ($P < 0.001$) and slightly upregulated in testicles. It was concluded that maternal obesity changes the expression of Lcn2 and 24p3R in the gonads of the offspring of obese rats, possibly through fetal programming. The consequences of this dysregulation for the offspring's gonadal function remains to be determined.

Introduction

Obesity is currently considered to be a serious global health problem due to its high prevalence (39%), according to the World Health Organization 2016 survey (1). This pathological entity characterized by an excess of body fat is measured by body mass index (2). Moreover, it is now well established that maternal obesity plays an important role in early life programming. Obesity in pregnant females often leads to gestational diabetes, which results in the adaptation of the fetus to a hyperglycemic, metabolically altered intrauterine environment (3). However, during postnatal life, such adaptation becomes detrimental to the individual because it increases the susceptibility to excess body fat and subsequently, an impaired metabolic state during adulthood (4). In the first instance, a high percentage of body fat leads to a chronic low-grade inflammatory state caused by the dysregulation of adipokine synthesis and macrophage infiltration within adipocytes events that precede the development of other diseases, such as insulin resistance, diabetes mellitus, cardiovascular complications (5-8) and different types of cancer, such as lung, endometroid and breast cancer (9-11).

In previous years, it has been demonstrated that abnormal epigenetic regulation due to an adverse intrauterine environment is a robust explanation for the increased risk of developing metabolic diseases in postnatal life (12). Within this context, Castro *et al* (13) described a strong correlation between maternal blood concentrations of leptin and

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adiponectin, and the fat percentage of offspring. Likewise, studies have demonstrated that the administration of a high-fat diet to adiponectin knockout (KO) females had an impact on the offspring's body weight (14,15). Similarly, the administration of a diet with 30% sugar has proven effective in inducing obesity in experimental models, which imitates the amount of carbohydrates present in the diet that most individuals have today (16-19). Additionally, it has been reported that adipokines are not only secreted from adipose tissues, there are also several adipokines, like adiponectin and leptin that have been identified in reproductive organs of different species, including the hypothalamic-pituitary-gonadal axis (20,21). Furthermore, several research groups have identified that maternal metabolic disorders affect the expression of some adipokines at both the gene and protein level in reproductive organs of the offspring (9,22).

Due to its implications in different metabolic disorders, lipocalin-2 (*Lcn2*) has been widely studied (23,24). For example, circulating concentrations of *Lcn2* are higher in women with gestational diabetes and preeclampsia, which suggests that *Lcn2* may be of value as a possible marker of fetal programming in humans (25). Other studies conducted in humans and animal models indicated a sex specific regulation of *Lcn2* by estrogen (26-28); ovariectomized mice treated with estrogen showed an increase in *Lcn2* gene expression levels in white adipose tissue, liver and serum (29). This adipokine is a member of the lipocalin superfamily, characterized by the presence of three conserved motifs comprising a single eight-stranded antiparallel β -barrel similar to a calyx that is able to bind numerous ligands. These three specific features confer a vast functional diversity and lipocalins are therefore involved in a number of different processes, such as iron intake, cellular apoptosis and inflammation (30,31).

In 2008, by means of a DNA microarray assay, the present group identified the *Lcn2* gene within a cluster of DNA sequences whose expression profiles were increased in the perinatal murine ovary (32). Later, the current group also identified *Lcn2* and its receptor 24p3 (24p3R) mRNA and protein in the gonads of Sprague Dawley rats, and found their expression to be sexually dimorphic during the perinatal period (33). Based on these previous observations and the fact that several studies have demonstrated that adipokine synthesis can be altered by early life programming due to maternal obesity, the expression levels of *Lcn2* and 24p3R mRNA and their respective protein profiles were analyzed in the ovaries and testes of offspring of obese mothers in the present study. It should also be taken into account that only a few studies have addressed the expression of this adipokine in reproductive organs and even though it is well established that 24p3R participates in apoptosis and cellular iron intake (34), its expression in murine reproductive organs has not been documented until recently (33). Therefore, it is important to further investigate the role of *Lcn2* and its receptor in order to establish the effect of obesity in gonadal development during gestation.

Materials and methods

Animals. The animal experiments were conducted using 2-month-old Sprague Dawley rats obtained from an inbred colony at the National Medical Center, Mexican Social

Security Institute (Mexico City, Mexico). A total of 20 female (200 \pm 10 g) and 10 male rats (300 \pm 20 g) were housed under a controlled photoperiod (12-h light/dark cycle, lights on at 7:00 h) and temperature (21 \pm 2°C). Male rats had free access to rodent chow and tap water (5008 Formulab Diet; PMI Nutrition International). This diet is formulated to supply a complete life-cycle nutrition in rat breeding colonies, providing calories from 26.8% protein, 16.7% fat and 56.4% carbohydrates. Littermate female rats were assigned randomly to two nutritional groups (n=10) ~8 weeks before mating. The first group was fed standard chow *ad libitum* and had free access to tap water (control group); the second group received standard chow *ad libitum* and had free access to water with 30% sucrose, prepared by adding 30% w/v of commercial brown sugar (obese group) in order to induce obesity according to the model employed in previous studies (16-19). The experimental protocol was approved by the Research Ethics Committees of the National Medical Center of the Mexican Social Security Institute and the National Autonomous University of Mexico (approval nos. R-2011-3604-2 and UNAM-003-2013, respectively), and the study was conducted following the American Association for Accreditation of Laboratory Care and National Institutes of Health guidelines (35). All animal procedures complied with government published recommendations for the use of laboratory animals. In order to ensure a successful pregnancy, female rats were nulliparous, and had an average body weight of 200 \pm 10 g.

Although a daily report on dietary and liquid intake of female dams was not included, these observations were made daily during the experiment. The amount of water and food consumption was not different between the control and the experimental groups. The weight of the female dams from both groups was measured weekly. Once the females of the experimental group reached a weight 30% higher than that of control females and following what is described elsewhere for the generation of a murine model of obesity (16-19), both control and experimental female rats were mated with a 3-month-old male (2 females with 1 male). The next morning, males were separated from the females and returned to their original cages (2 per cage) in order to be employed in further breeding. Females were examined for the presence of vaginal plugs and this was considered the first day of gestation. At 21 days postconception (dpc), three pregnant females of the control and experimental groups were anesthetized with sodium pentobarbital (25 mg/kg, i.p.) until they were unconscious (36). To verify the state of unconsciousness, which implies a reversible insensitivity to external stimuli, a minimum pinch in the tail of the rat was made to ensure the absence of pain in the presence of such stimulus. Immediately after, rats were culled by cervical dislocation following the National Institutes of Health guide for the care and use of Laboratory Animals (NIH Publications no. 8023, revised 1978) and the Mexican regulations for the use and care of laboratory animals (policy no. NOM-062-ZOO-1999). Euthanasia after cervical dislocation was confirmed by corroborating the separation of the cervical vertebrae from the skull manually, also the cessation of breathing, heartbeat, eye movements and the absence of response to external stimuli were verified before performing an abdominal incision to retrieve the fetuses from each mother. Upon retrieval, 27 fetuses (17 females and

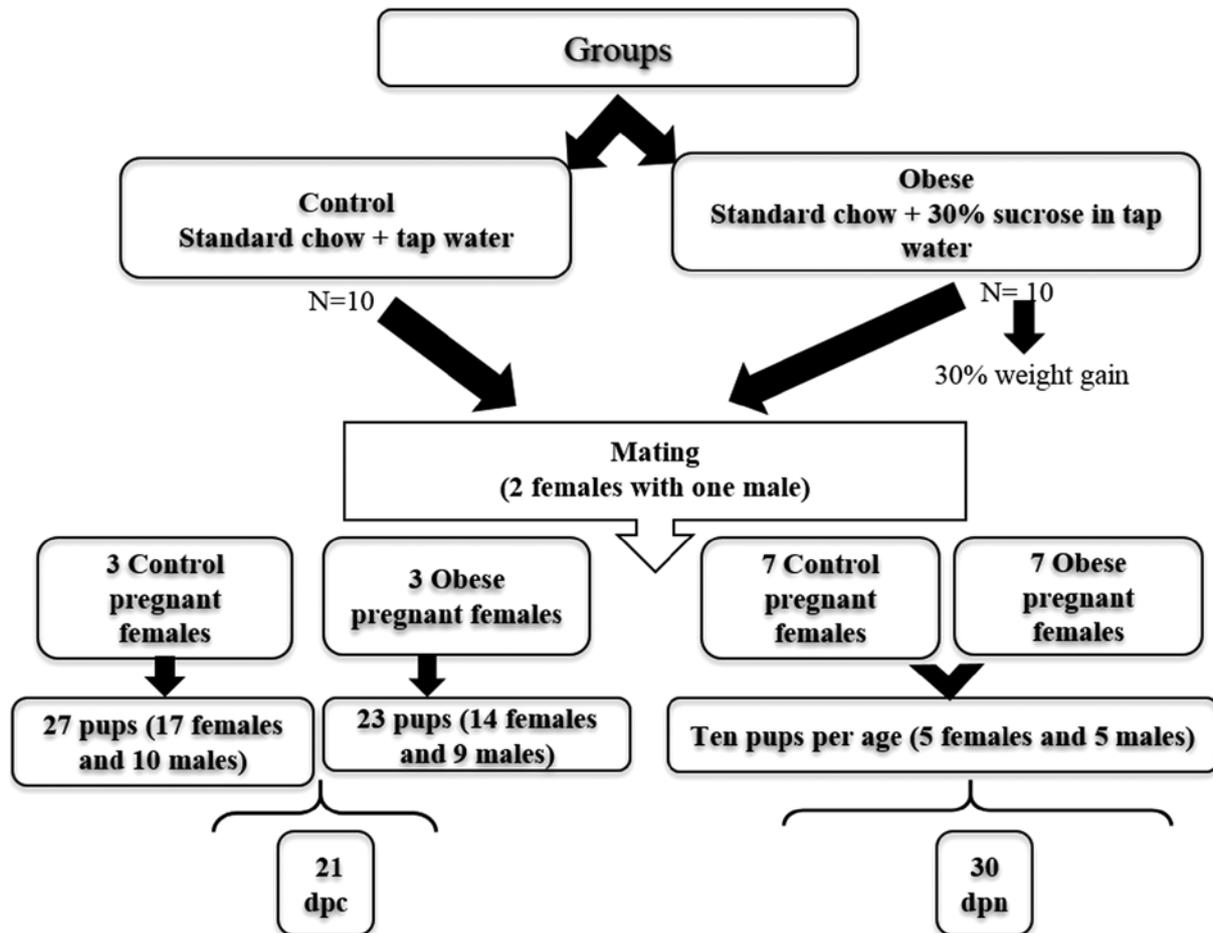


Figure 1. Schematic representation of the experimental design employed in this study. A total of 20 female dams were assigned randomly into either the control or the experimental group. After confirming the pregnancy of the females from both groups, the gonads from fetuses and pups were retrieved at 21 days dpc and 30 days dpn. Upon collection, gonadal tissue was processed for subsequent molecular and immunohistochemical experiments. Dpc, days postconception; dpn, days postnatal.

10 males) from the control group, and 23 fetuses (14 females and 9 males) from the experimental group were decapitated and their gonads were dissected and frozen on dry ice for RNA isolation (Fig. 1).

For the remaining pregnant rats (7 in total for each group, 1 per age), the day of birth was designated as postnatal day 0. In order to ensure adequate and standardized nutrition until weaning, litter sizes were standardized to 10 pups per litter (5 females and 5 males in each group; Fig. 1), the remaining pups were culled by decapitation. During lactation, the mothers were fed standard chow *ad libitum* and either tap water (control group) or water plus 30% sucrose (obese group) accordingly. The weight and glucose blood levels of both litters were recorded at each time point before sacrifice.

Upon collection, tissues were either frozen on dry ice for RNA extraction or fixed for immunohistochemistry. A total of 10 ovaries and 10 testicles were collected from Sprague Dawley rats at 0, 2, 4, 6, 12, 20 and 30 days postnatal (dpn; 10 per group) immediately after sacrifice. These specific time-points were selected based on the authors' previous results (32,33).

RNA isolation. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Inc.), following the manufacturer's protocol and as described previously (33). Briefly, the tissue was homogenized

in TRIzol reagent (Molecular Research Center, Inc.), and the aqueous and organic phases were separated by the addition of one volume of bromo-3-chloropropane (Sigma-Aldrich; Merck KGaA), followed by centrifugation in a MiniSpin® eppendorf centrifuge at 13,800 x g for 15 min at 4°C. Next, 350 µl of 70% ethanol (Sigma-Aldrich; Merck KGaA) were added to all samples and each sample was applied to an RNeasy mini-column. The columns were washed by centrifugation at 735 x g for 2 min at room temperature with buffers containing guanidine and ethanol. In order to elute the RNA, RNase-free water (30 µl) was added directly onto the silica-gel membrane of the columns and each column was centrifuged for 1 min at 13,800 x g at room temperature. The RNA was quantified in a SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, Inc.) by measuring absorbance at 260 nm and was stored at -85°C until use. The quality of each RNA sample was assessed on 2% formaldehyde denaturing agarose gels.

Semi-quantitative reverse transcription PCR. Total RNA from all samples was reverse transcribed using the Superscript™ First-Strand Synthesis system (Invitrogen; Thermo Fisher Scientific, Inc.), according to manufacturer's protocol and as described previously (28). All reactions were carried out in a total volume of 20 µl. First, 300 ng of total RNA, isolated

Table I. Primer sequences used for semi-quantitative PCR.

Gene	Primer sequence (5'→3')	Length, bp
<i>Lcn2</i>	F: TCTCGATTCCGTCGGGTGGTGG	592
	R: CCTGGGTGTCCTGTGTCTG	
24p3R	F: AGGACTGGGACTACAACGGA	507
	R: GTGCGGACTCCAGAAACAGA	
GAPDH	F: CAAGGTCATCCATGACAACCTTG	496
	R: GTCCACCACCCTGTTGCTGTAG	

F, forward; R, reverse; bp, base pairs; *Lcn2*, lipocalin-2; 24p3R, 24p3 receptor.

from the gonads collected at 21 dpc and 0, 2, 4, 6, 12, 20 and 30 dpn, were annealed at 65°C for 5 min to 0.5 µg of oligo (dT)12-18 primer (0.5 µg/µl) and 1 µl of a dNTP cocktail (10 mM). The annealed RNA-primer samples were incubated for 1 h at 42°C with the following components of the First-Strand Synthesis system (Invitrogen; Thermo Fisher Scientific, Inc.): RT buffer (10X), MgCl₂ (25 mM), RNaseOUT (40 U/µl), and Superscript II reverse transcriptase (50 U/µl). The reactions were terminated by incubation at 70°C for 15 min, followed by incubation at 37°C for 20 min with 2 U of *Escherichia coli* RNase H (2 U/µl) (Invitrogen; Thermo Fisher Scientific, Inc.).

The PCR amplification of the reverse-transcribed products was carried out in a total volume of 20 µl, using 10 µl of 2X KAPA Taq ReadyMix (Kapa Biosystems; Roche Diagnostics) and 1 µl of cDNA template annealed to 10 pmol of the *Lcn2*, *24p3R* or *GAPDH* specific primers (Table I). The PCR conditions used were 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing for 30 sec at 60°C for *Lcn2* and at 58°C for *24p3R* and *GAPDH*, and 1 min of extension at 72°C, with a 10 min final extension at 72°C. Each sample was amplified in triplicate in a Biometra TProfessional thermocycler (Biometra Ltd, Jena Analytic). A total of 20 µl of the PCR reactions were electrophoresed on 2% agarose gels stained with ethidium bromide. The gels were scanned electronically and the images were quantitated by densitometry using image-analysis software (Kodak Molecular Imaging Software 4.0, Kodak Image Station 4000R; Carestream Health, Inc.). Relative expression levels of *Lcn2* and its receptor were obtained by dividing the average number of pixels of the experimental transcript (*Lcn2* or *24p3R*) by the average number of pixels of the control transcript (*GAPDH*).

Immunohistochemistry. In order to determine *Lcn2* and 24p3R protein signaling within gonads of the offspring of wild-type and obese rats, 5-µm sections were obtained from formalin-fixed, paraffin-embedded gonadal samples collected from four 30 dpn rats (2 females and 2 males from each group) and mounted on glass slides previously coated with poly-L-lysine. The sections were then deparaffinized and rehydrated in a decreasing alcohol series (100, 90, 70 and 30%) until water. The sections were microwave heated in high mode at 60°C with antigen retrieval solution (Vector Laboratories, Inc.), rinsed in 1X phosphate buffered saline (PBS; pH 7.4), incubated for 30 min with four drops of the background sniper, which is an endogenous peroxidase-inactivation solution of the

Starr Trek Universal HRP detection system (Biocare Medical, LLC), and subsequently blocked with 10% bovine serum albumin (BSA) in 1X PBS for 30 min at room temperature. Tissues were then incubated with either primary rat polyclonal antibody against *Lcn2* or against 24p3R antibody (1:150; cat. no. ab41105 and ab124506; Abcam), at 4°C overnight.

The sections were washed in PBS, incubated at room temperature for 2 h with the Starr Trek Universal HRP detection system (Biocare Medical, LLC), and washed with 1X PBS. The peroxidase reaction was developed with diaminobenzidine and H₂O₂, generating a brown precipitate. Finally, the slides were counterstained with hematoxylin for 5 sec at room temperature, dehydrated and mounted with synthetic resin. The positive control consisted of sections of uterus collected from the same wild-type female (sections of rat uterus were used following immunohistochemistry protocol's recommendation in which it is suggested to be used as positive control, the latter because it is well established that lipocalin 2 is highly expressed in uterus). The negative control consisted of replacing the primary antibody with BSA in PBS. The brown precipitate signal was analyzed semi-quantitatively using the integrated optical density (IOD) provided by the Image-Pro Plus software 7 (Media Cybernetics, Inc.). A total of five sections of each of the three slides were analyzed at high magnification (x40) under a light microscope. Percentage was used to express the relative changes in the IODs of the gonadal tissue of experimental rats compared with the IODs of control rats.

Statistical analysis. Values from three experimental repeats are expressed as the mean ± standard error. An unpaired two-tailed Student's t-test was used for comparisons between the two groups. Two-way ANOVA followed by Tukey's post hoc test was used to compare the relative expression of cDNA between gonads of the offspring of wild-type rats and offspring of obese mothers. Statistical analyses were performed using GraphPad Prism version 7 for Windows (GraphPad, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Taking into account that in murine species during the perinatal and prepubertal periods, key molecular processes for gonadal function take place and based on the fact that in 2008, our group identified the expression of the gene that encodes

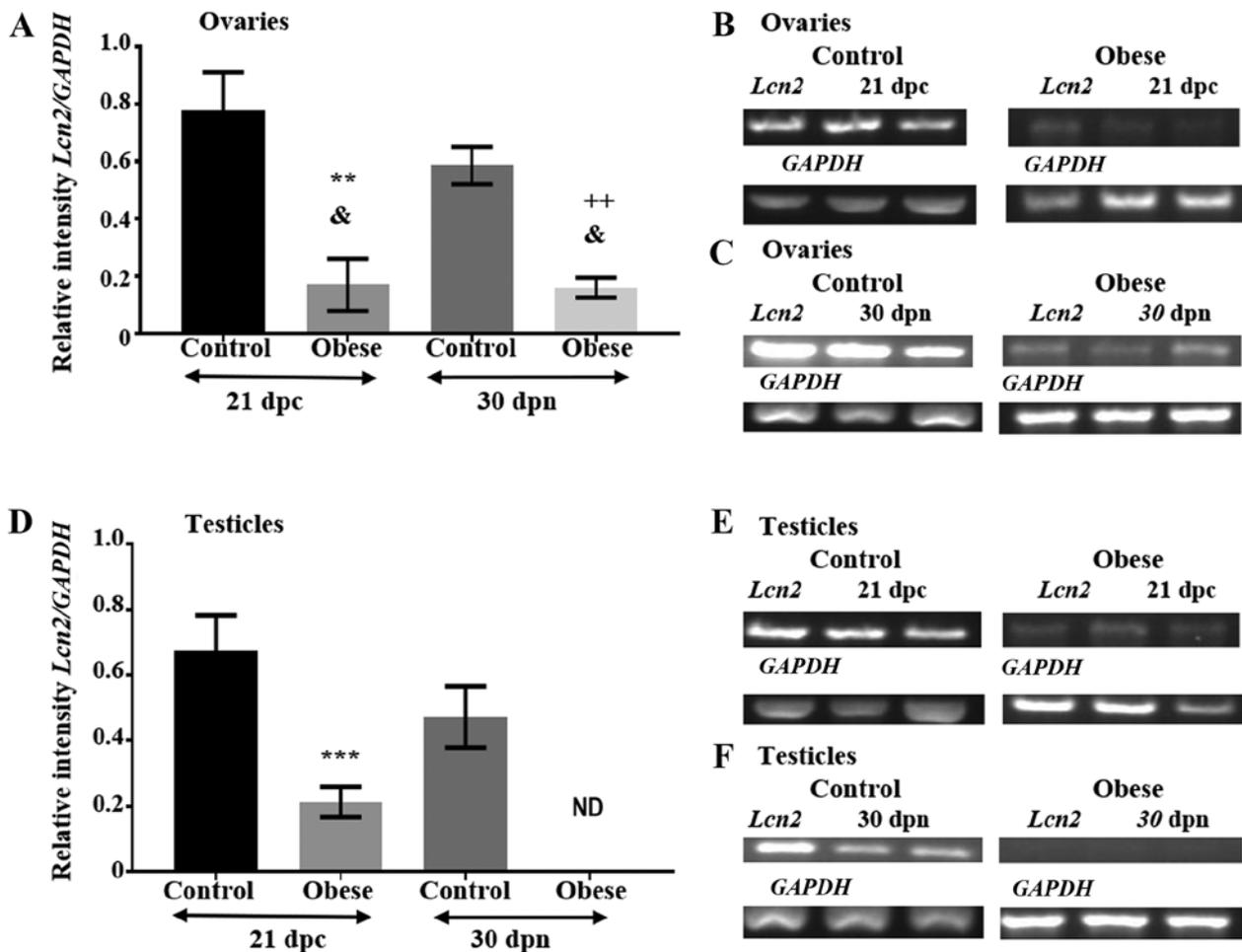


Figure 2. Relative expression levels of *Lcn2* mRNA are altered in the gonads of the offspring of obese rats. (A) Graphic presentation of the densitometric changes detected in the electrophoresis of PCR products amplified from the total RNA isolated from the ovaries collected at 21 dpc and at 30 dpn. The Y-axis indicates the expression levels of *Lcn2* cDNA in the ovaries of offspring of control and obese rats relative to the expression levels of the *GAPDH* gene. The X-axis indicates age in dpc and dpn. At 21 dpc and 30 dpn, mRNA expression of the adipokine is abundant in the ovaries of the control group, while *Lcn2* mRNA expression is downregulated in the ovaries of the experimental group. Values are expressed as the mean \pm standard error. (B) Representative image of the electrophoresis of PCR products, *Lcn2* is abundant in ovaries of the perinatal offspring of the control group and downregulated in the ovaries of the experimental group. The constitutively expressed *GAPDH* gene was used as the normalizing unit. (C) Representative image of the electrophoresis of PCR products, *Lcn2* is abundant in ovaries of the prepubertal offspring of the control group and downregulated in the ovaries of the experimental group. The constitutively expressed *GAPDH* gene was used as the normalizing unit. (D) Graphic presentation of the densitometric changes detected in the electrophoresis of PCR products amplified from the total RNA isolated from the testicles collected at 21 dpc and at 30 dpn. Y-axis indicates the expression levels of *Lcn2* cDNA in the testicles of offspring of control and obese rats relative to the expression levels of the *GAPDH* gene. The X-axis indicates age in dpc and dpn. At 21 dpc and 30 dpn, mRNA expression levels of the adipokine are abundant in the testicles of the control group, while *Lcn2* mRNA expression is either down regulated or absent in the testicles of the experimental group. Values are expressed as the mean \pm standard error. (E) Representative image of the electrophoresis of PCR products, *Lcn2* is abundant in testicles of the perinatal offspring of the control group and downregulated in the testicles of the experimental group. The constitutively expressed *GAPDH* gene was used as the normalizing unit. (F) Representative image of the electrophoresis of PCR products, *Lcn2* is abundant in testicles of the prepubertal offspring of the control group and not detected in the testicles of the experimental group. The constitutively expressed *GAPDH* gene was used as the normalizing unit. RNA was isolated from male and female gonads collected at 21 dpc and 30 dpn from both groups. At 21 dpc, n=27 animals in control group (17 females and 10 males), and n=23 animals in the experimental group (14 females and 9 males). At 30 dpn, n=20 animals (5 females and 5 males per group). Tukey's test was performed to compare all possible pair of means. [&]P=0.002; ^{**}P<0.01, obese female group vs. control female group at 21 dpc; ^{**}P<0.01, obese female group vs. control female group at 30 dpn; ^{***}P<0.001, obese male group vs. control male group at 21 dpc. *Lcn2*, lipocalin-2; dpc, days postconception; dpn, days postnatal; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ND, not detected.

for *Lcn2* in the mouse ovary (32), first we decided to analyze the expression levels of *Lcn2* and its corresponding receptor (24p3R) in female and male gonads from wild-type Sprague Dawley rats collected at different time-points (21 dpc, 0, 2, 4, 6, 12, 20 and 30 dpn), demonstrating that the relative expression of both *Lcn2* and *24p3R* is significantly different in female and male murine gonads at perinatal and prepubertal stages of development (33). In the present study, semi-quantitative PCR was used to assess possible changes in the relative expression levels of *Lcn2* and *24p3R* mRNA in the gonads of the offspring

of obese rats, by comparing these levels of expression with those observed in the gonads of offspring of the control group (Figs. 2,3). Immunohistochemistry was performed to determine if such changes were also present at the protein level. A significant difference in the expression level of this adipokine and its receptor was only found at two specific time points (21 dpc and 30 dpn) (Figs. 4,5).

Relative expression levels of Lcn2 mRNA changes in the gonads of the offspring of obese dams during the perinatal

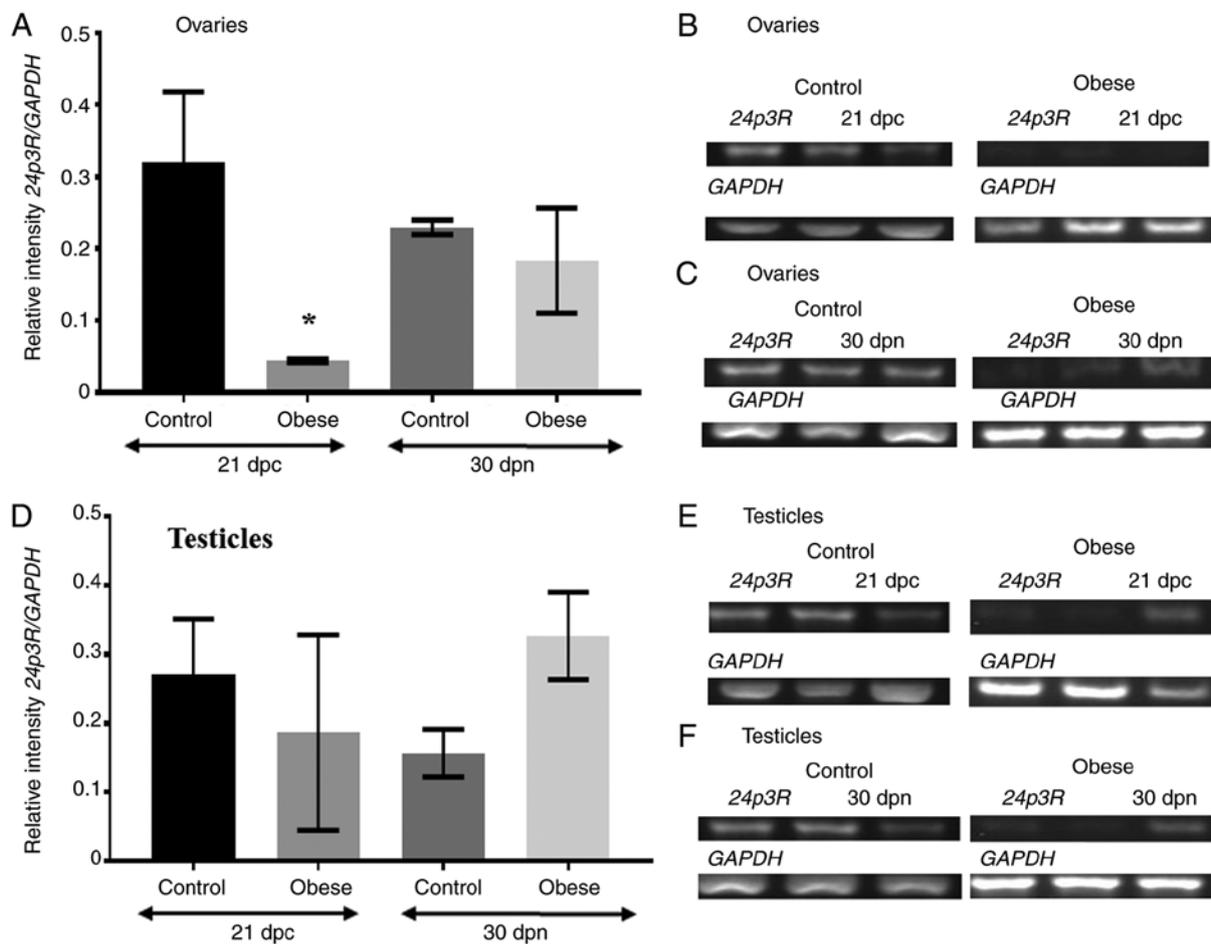


Figure 3. Differential expression level pattern of *24p3R* mRNA between the control and experimental groups at perinatal and prepubertal stages. (A and D) Graphic presentation of the densitometric changes quantitated in the electrophoresis of PCR products amplified from the total RNA isolated from the gonads collected at 21 dpc and from the gonads collected at 30 dpc. The Y-axis indicates the expression levels of *24p3R* cDNA in the gonads of offspring of control and obese rats relative to the expression levels of the *GAPDH* gene. The X-axis indicates age in dpc and dpc. Representative blots showing mRNA expression of the receptor is abundant in the ovaries of the control group but is downregulated in the ovaries of the experimental group at (B) 21 dpc and (C) 30 dpc. (E) Representative blots showing mRNA expression of the receptor is abundant in the testicles of the control group but is downregulated in the testicles of the experimental group at 21 dpc. (F) *24p3R* expression increased at 30 dpc in the testicles of the experimental group. Total RNA was isolated from male and female gonads collected at 21 dpc and 30 dpc from both groups. At 21 dpc, n=27 animals of control group (17 females and 10 males), and n=23 animals of the experimental group (14 females and 9 males). At 30 dpc, n=20 animals (5 females and 5 males per group). Values are expressed as the mean \pm standard error. Tukey's test was performed to compare all possible pair of means. * $P < 0.05$, obese female group vs. control female group at 21 dpc. *24pR*, *24p3* receptor; dpc, days postconception; dpc, days postnatal; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

period. During the perinatal period, the relative expression level of *Lcn2* mRNA was higher in the ovaries and testicles of offspring in the control group (0.78 ± 0.13 and 0.68 ± 0.11 , respectively) (Fig. 2A, B, D and E). However, at the same age, *Lcn2* mRNA expression in the obese group decreased by >50% in the ovaries (0.17 ± 0.09) and in the testicles (0.21 ± 0.05 ; Fig. 2A, B, D and E).

Relative expression of Lcn2 mRNA decreases significantly in the gonads of 30 dpc offspring of obese dams. At 30 dpc, in the offspring of wild-type rats, expression of *Lcn2* was abundant in the ovaries (0.59 ± 0.06) and in the testicles (0.47 ± 0.09 ; Fig. 2A, C, D and F), while in the ovaries of the experimental group, the relative expression level of *Lcn2* decreased to 0.16 ± 0.03 (Fig. 2A and C). Notably, the mRNA expression level of *Lcn2* was null in the male gonads (Fig. 2D and F). Statistical analysis revealed a significant difference in the expression levels of *Lcn2* between control and experimental

groups at the two specific time points in ovaries ($P < 0.01$) and testicles ($P < 0.001$) (Fig. 2A and D).

Relative expression level of 24p3R mRNA is not modified in the gonads of the offspring of obese dams. The mRNA expression level of *24p3R* was also analyzed. Electrophoresis of the PCR products revealed a significant change in the relative expression level of this receptor in perinatal ovarian samples obtained from the offspring of obese mothers. As shown in Fig. 3A and B *24p3R* mRNA expression levels were downregulated in the experimental group (0.04 ± 0.001) compared with the control group (0.32 ± 0.09) ($P < 0.05$). As shown in Fig. 3D and E, the change in the relative expression level of *24p3R* in the perinatal testicles of the experimental group was not significant in the experimental group (0.19 ± 0.14) compared with the control group (0.27 ± 0.08).

At 30 dpc, the relative expression of *24p3R* in the ovaries of the experimental group did not change (0.18 ± 0.07) compared

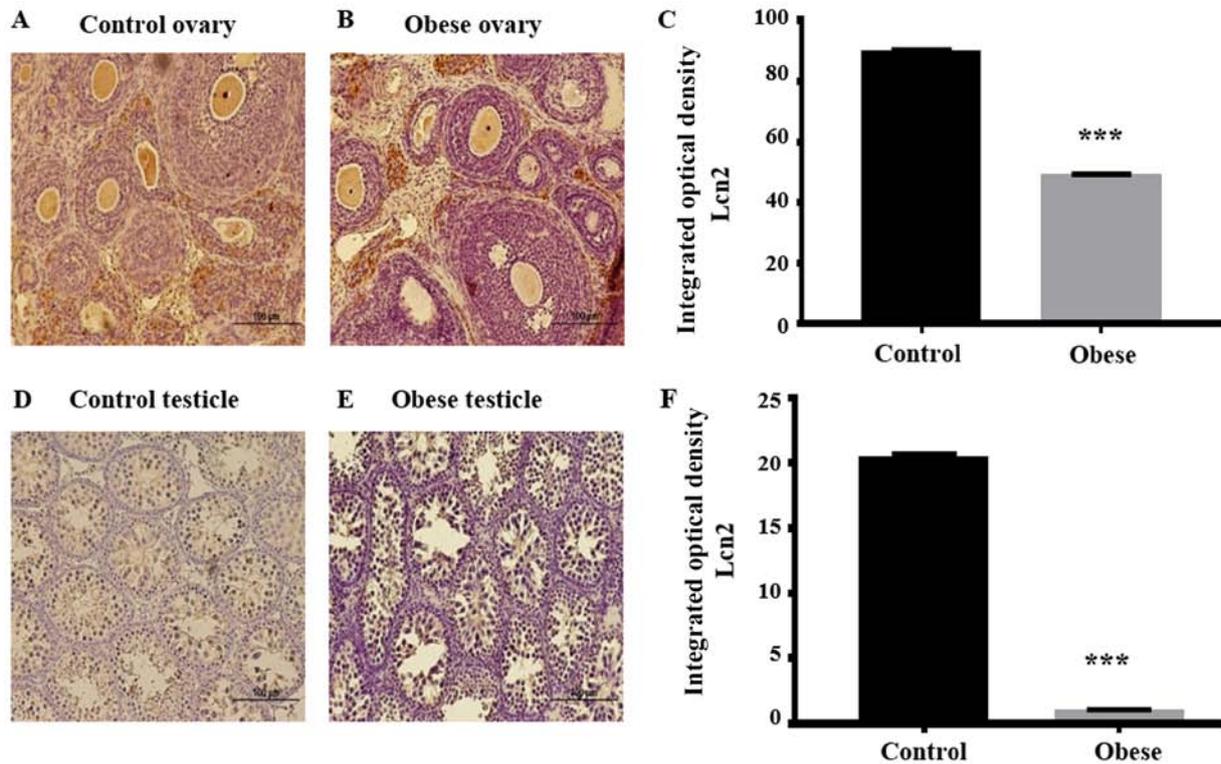


Figure 4. Lcn2 expression levels in the gonads of prepubertal offspring of control and obese rats. (A) Immunostaining for Lcn2 in serial sections of a control ovary, with the Lcn2 signal located in oocytes, epithelial cells and antrum of developing follicles. The signal is also observed in the stroma and corpus luteum. (B) A less intense signal can be seen in the same ovarian structures of the ovary from the experimental rat. (C) Graph depicting differences in overall signal intensity between the control and experimental ovarian samples assessed by means of the IOD tool of the Image-Pro Plus software. These differences were statistically significant. (D) Immunopositivity for Lcn2 in Sertoli and Leydig cells of a testicular sample from offspring of the control group. (E) A less intense signal was observed in the same testicular structures of the male gonad from the experimental group. (F) Graph depicting statistically significant differences in overall mean IOD values between the control and experimental testicular samples. Magnification, $\times 40$; scale bar, $100\ \mu\text{m}$. $n=3$ slides with serial sections from 2 ovaries and 2 testicles (1 gonad per group, control or experimental) were used in the assay. *** $P<0.001$ experimental vs. control group. Lcn2, lipocalin-2; IOD, integrated optical density.

with the control group (0.23 ± 0.01), as shown in Fig. 3A and C. Conversely, at 30 dpn, the relative expression of *24p3R* increased slightly in the testicles of pups of obese mothers (0.33 ± 0.06) compared with the control group (0.16 ± 0.03). These changes were not statistically significant (Fig. 3D and F).

As a positive control, a fragment (496 bp) of the ubiquitous *GAPDH* gene obtained from the same cDNA samples was also amplified. The signal intensity obtained from the PCRs of *GAPDH* was also used to normalize the signal intensity generated by the amplification of the experimental genes (*Lcn2* and *24p3R*).

Immunohistochemistry analysis of the *Lcn2* and *24p3R* proteins. Immunohistochemistry was performed to determine possible changes in protein synthesis or cellular localization of Lcn2 and 24p3R within the gonads of 30 dpn offspring of obese rats (Figs. 4 and 5) compared with the findings observed previously in the gonads of 30 dpn offspring of wild-type rats (33). In paraffin-embedded ovarian sections from offspring of the control group, Lcn2 immunostaining was strong in the oocytes, zona pellucida, antrum, corpus luteum and stroma of developed follicles. A signal of minor intensity was also present in the theca and granulosa cells of primary and growing follicles (Fig. 4A).

The Lcn2 signal in ovarian sections of the offspring of obese rats was less intense in all follicular structures, except

for the corpus luteum where the signal was slightly increased compared to the same cellular structure in the ovaries of control rat offspring (Fig. 4B). The IOD generated by each follicular structure was quantitated by employing the IOD tool of the Image-Pro Plus software. Differences between the sum of all IODs of the follicular structures of the control group (90 ± 0.4) and the corresponding IODs of the respective follicular structures of the experimental group (49.34 ± 0.2) were statistically significant, with the IOD being lower in the latter group ($P<0.001$) (Fig. 4C).

Regarding the 24p3R, intense staining was observed in the ovarian stroma of wild-type offspring, while the signal was barely visible in oocytes, granulosa and theca cells (Fig. 5A). The overall intensity decreased markedly in ovaries of the offspring of obese rats (Fig. 5B). IOD decreased in the experimental group (7.67 ± 0.5) compared with the control group (27.69 ± 1.5), this was statistically significant ($P<0.001$) (Fig. 5C).

In testicles of wild-type offspring, Lcn2 was only detected in Sertoli and Leydig cells. Neither germinal nor myoid cells presented immunopositivity for this protein (Fig. 4D). On the other hand, in the experimental group, this adipokine was only detected in Sertoli cells (Fig. 4E). Overall, the Lcn2 IOD was higher in the testicles of wild-type offspring (20.73 ± 0.2) compared with the IOD observed in Sertoli cells of testicles of the experimental group (1.08 ± 0.01), this was statistically significant ($P<0.001$) (Fig. 4F). The 24p3R signal was detected

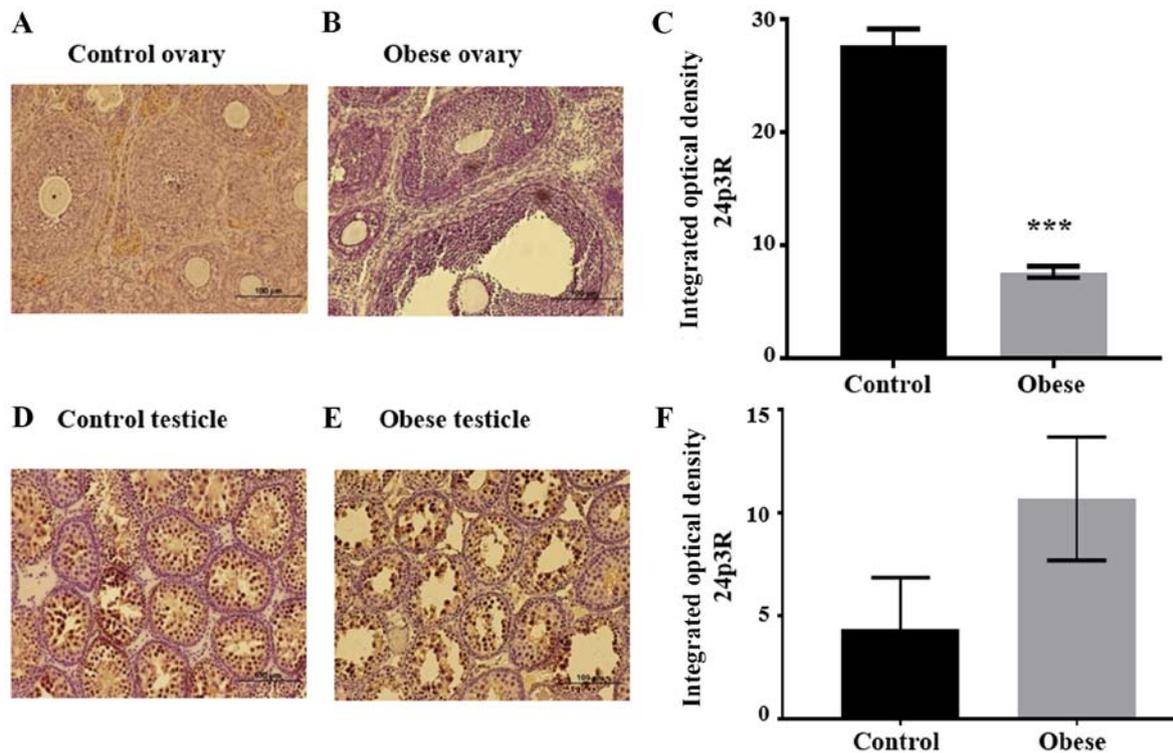


Figure 5. 24p3R expression levels in the gonads of prepubertal offspring of control and obese rats. (A) The 24p3R signal is strong in the stroma of ovary sections from the offspring of wild-type rats, while the signal was barely visible in the remaining follicular cells, including oocytes, granulosa, theca cells and the corpus luteum. (B) In paraffin-embedded ovarian sections from one ovary collected from the offspring of obese mothers, overall intensity decreased notably. (C) Graph depicting statistically significant differences in overall mean IOD values between ovarian samples of the control and experimental groups. (D) Immunoreactivity to 24p3R in a normal testicle, with staining observed in Sertoli and germinal cells of different developmental stages. (E) A stronger signal is observed in Sertoli and germinal cells of the testicle from the offspring of the experimental group. (F) Graph depicting higher mean IOD values in the testicular structures of the offspring of obese mothers. The increase in the IOD values was not statistically significant. Magnification, $\times 40$; scale bar, $100 \mu\text{m}$. $n=3$ slides with serial sections from 2 ovaries and 2 testicles (1 gonad per group, control or experimental), were used in the assay. *** $P<0.001$ experimental group vs. control group. 24p3R, 24p3 receptor; IOD, integrated optical density.

in Sertoli and germinal cells at different developmental stages (Fig. 5D and E) and, contrary to what was expected, the IOD was higher in the two testicular structures of the experimental group (10.7 ± 1.0) compared with the same structures of the control group (4.36 ± 1.5). However, the difference between groups was not statistically significant (Fig. 5F).

Discussion

At present, obesity is considered a serious global health problem to which a considerable number of human and economic resources are allocated. It is well established that a state of low-grade chronic inflammation is involved in obesity, affecting not only individuals but also their offspring through fetal programming (37). Furthermore, it has been demonstrated that there is an association between imbalanced concentrations of anti-inflammatory and pro-inflammatory adipokines, and the development of the chronic inflammatory state, which affects different organs, including those comprising the hypothalamus-pituitary-gonadal axis (9).

The present study describes the changes in mRNA and protein expression patterns of the adipokine *Lcn2* and its corresponding receptor (24p3R), in the gonads of the offspring of obese mothers during perinatal and prepubertal development. Even though the expression profiles of the two proteins

were analyzed at different time points starting from hours before birth to 30 dpn, a significant change in these expression profiles was only observed at 21 dpc and 30 dpn compared with those observed in the gonads of the offspring of control dams. The modification in the expression profile of both the adipokine and the receptor at these specific time points coincides with mechanisms that are essential for gonadal development, such as the onset of folliculogenesis and spermatogenesis in perinatal murine gonads or the beginning of the gonadotropin-dependent cascade in the prepubertal stage (38-40).

Although different studies have associated an increase in serum *Lcn2* concentrations with obesity and related cardio-metabolic diseases (41,42), in the present study, this adipokine was downregulated in the ovary both at 21 dpc and 30 dpn and was downregulated to a greater extent in the testicle, where the expression level of this adipokine was non-existent at 30 dpn. Rees and Hay (43) observed that *Lcn2* mRNA expression level was decreased in the fetal liver of offspring exposed to a maternal low-protein soy oil diet compared with the relative expression observed in the fetal liver of offspring exposed to a high-protein diet. Nevertheless, the same was not observed when the diet was prepared with corn oil, which led the authors to suggest that a difference in fatty acid composition between the oil and corn diets rather than protein content could be driving the changes in *Lcn2* mRNA

expression level in the fetal liver. Moreover, these changes in the expression level of this adipokine persisted until adulthood, suggesting that *Lcn2* plays an essential role in fetal programming of hepatic metabolism. In the same manner, in the current study, the relative expression of *Lcn2* decreased in the gonads of fetal and prepubertal offspring from obese dams fed with a high sucrose diet. This type of diet was used because it has been demonstrated that its administration leads to the development of maternal insulin resistance, which elicits perinatal insulinemia, considered to be one of the causes of genetic programming (44). It has been observed that in order to survive an abnormal intrauterine environment, the offspring of obese mothers develop an adaptive response to such challenges via changes in epigenetic regulation (45). Therefore, the downregulation in *Lcn2* expression observed in the present study could also be a result of fetal programming.

It is now commonly known that an adverse fetal environment often leads to genetic reprogramming through DNA methylation (12). Houde *et al* (3) demonstrated that maternal hyperglycemia causes promoter hypermethylation of the leptin and adiponectin genes. Thus, DNA silencing could be a plausible explanation for the downregulation of *Lcn2* in the gonads of offspring of obese mothers. Further DNA methylation studies are needed to confirm this hypothesis.

On the other hand, Law *et al* (46) demonstrated that *Lcn2*-KO mice showed improved systemic energy homeostasis and insulin sensitivity under both basal and obesogenic conditions. From 11 weeks of age onwards, *Lcn2*-KO mice had lower fasting glucose and serum insulin levels than their wild-type counterparts. This improved metabolic condition is in line with the current findings observed in 30 dpn offspring of obese rats, which had normal serum glucose levels despite a considerable increase in body weight (data not shown). At 21 dpc, the offspring of these obese dams presented normal serum glucose levels and exhibited no weight alterations. Therefore, the present results indicated that maternal obesity is associated with molecular alterations in the fetal gonads, which are the result of genetic programming.

Jungheim *et al* (22) demonstrated that ovarian follicular function is severely affected in the presence of excess circulating glucose concentrations, including granulosa cell apoptosis, abnormal follicular development and delayed maturation. In the present study, the immunohistochemical results showed that, in the female gonads of 30 dpn obese offspring, exposure to a high-glucose diet until weaning led to a considerable number of atretic follicles characterized by an irregular shape and a reduced granulosa cell layer.

A specific role of *Lcn2* in the regulation of cell differentiation has been demonstrated in spermatogenesis, in which spermatogonia drive the expression levels of *Lcn2* in Sertoli cells via activation of the NF- κ B pathway (47). This pathway is also involved in apoptotic processes in pancreatic β -cells, where the endoplasmic reticulum stress-unfolded protein response is activated in order to ameliorate the effects of a metabolically adverse maternal environment (48). In the present study, the testicular seminiferous tubules of obese offspring were narrower and the number of gametic cells was reduced. However, these results should be interpreted with caution as they may be the result of dysregulation in the cell differentiation signaling pathway, in which *Lcn2* could be involved via

NF- κ B in the gonads, triggering activation of the apoptotic process. *Lcn2* also participates in the apoptotic pathway by binding to the 24p3R in order to internalize iron captured by the adipokine, thereby increasing intracellular iron levels that induce the mitochondrial pro-apoptotic cascade (49).

In 21 dpc ovaries of offspring of obese mothers, the expression of *24p3R* mRNA and protein were downregulated, while at 30 dpn, the expression level profile was similar to that observed in female gonads of the offspring of wild-type rats. In the fetal male gonads, the relative expression of this receptor was similar between the control and experimental groups; surprisingly, at the prepubertal stage, mRNA and protein abundance increased in the testes of the offspring of obese mothers. The reason for this difference in the expression pattern of 24p3R between ovaries and testicles is not clear. A recent study performed by Chella Krishnan *et al* (50) demonstrates that *Lcn2* in conjunction with megalin, not the 24p3R, exerts its metabolic function in liver and adipose tissue of mice in a sexually dimorphic pattern. The latter may account for the differences between female and male gonads regarding the expression of both *Lcn2* and 24p3R. As for 24p3R, the insignificant change during the fetal stage and the slight increase in its expression profile within the prepubertal testis, suggested that megalin could be the receptor participating in *Lcn2* signaling within the male gonad. Perhaps different regulation mechanisms take place in each gonad. Further experiments are necessary in order to elucidate this difference.

Finally, immunohistochemistry studies indicated that *Lcn2* and the 24p3R are expressed in both germinal and somatic cells, which is consistent with the localization of other adipokines in gonads (20,51). Even though the present study found that both the mRNA and the protein profiles of *Lcn2* and 24p3R are modified in the gonads of the offspring of obese mothers, it is limited since reverse transcription-quantitative PCR was not performed, therefore such modifications may not be that accurate, also it does not experimentally demonstrate the cause of this alteration or the subsequent consequences of a disturbed *Lcn2*/24p3R signaling pathway. It is now well established that this adipokine signals via its receptors in order to regulate gonadal cell differentiation. Therefore, assessing the effect that a change in the expression profile of either one might have in the fertility and/or the hormonal milieu of the offspring's gonads is essential. This is why in the first instance, the participation of this adipokine and its corresponding receptor in gonadal development, including cell differentiation, gametic cell maturation and steroidogenesis, needs to be determined.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

EdIC participated in the conception and design of the study, performed most of the semi-quantitative and immunohistochemistry experiments, participated in the analysis and interpretation of the data, and prepared the manuscript. LMA participated in the data analysis and revision of the manuscript. LD, RLB, and EC collected the biological samples and performed the remaining semi-quantitative reverse transcription PCR and immunohistochemistry experiments. MCRM participated in the analysis and interpretation of the data. JPM participated in the design of the study and prepared and revised the manuscript for its intellectual content. The final version of the manuscript was read and approved by all authors and each author believes that the manuscript represents honest work.

Ethics approval and consent to participate

The experimental protocol was approved by the Research Committees of both the National Medical Center and the National Autonomous University of México, México City, and was performed in accordance with the American Association for Accreditation of Laboratory Care and National Institutes of Health guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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