Pgc1a is responsible for the sex differences in hepatic *Cidec/Fsp27β* mRNA expression in hepatic steatosis of mice fed a Western diet

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Running title: Hepatic *Cidec/Fsp27* gene expression

Abbreviations: *Pgc1a***,** peroxisome proliferator-activated receptor gamma coactivator 1-alpha

Abstract

Hepatic fat-specific protein 27 (*Cidec/Fsp27*) mRNA levels have been associated with hepatic lipid droplet extent under certain circumstances. To address its hepatic expression under different dietary conditions and both sexes, *Apoe*-deficient mice were subjected to different experimental conditions for 11 weeks to test the influence of cholesterol, Western diet, squalene, oleanolic acid, sex and surgical castration on *Cidec/Fsp27* mRNA expression. Dietary cholesterol increased hepatic *Cidec/Fsp27β* expression, an effect that was suppressed when cholesterol was combined with saturated fat as represented by Western-diet feeding. Using the latter diet, oleanolic acid or squalene did not modify its expression. Females showed lower levels of hepatic *Cidec/Fsp27β* expression than males when they were fed Western diets. A result that was translated into lesser amount of CIDEC/FSP27 protein in lipid droplets and microsomes. A result also confirmed in *Ldlr*-deficient mice and in AML12 cells incubated with estradiol. While male surgical castration did not modify the expression, ovariectomized females did show increased levels compared to control females. Females also showed increased expression of *Pgc1a,* suppressed by ovariectomy, and the values were significantly and inversely associated with those of *Cidec/Fsp27β*. When *Pgc1a*-deficient mice were used, the sex-differences on *Cidec/Fsp27β* expression disappeared. Therefore, hepatic *Cidec/Fsp27β* expression has a complex regulation influenced by diet and sex hormonal milieu. The mRNA sex differences are controlled by Pgc1a.

Keywords:

Lipids/liver, lipid droplets, animal models, gene expression, non-alcoholic fatty liver disease.

Cidec/Fsp27, Pgc1a, apolipoprotein E deficient mice, high-fat diet, sex.

Introduction

Fat-specific protein 27 (*FSP27*) gene encodes a protein of 27 kDa with 238 amino acids, belonging to the cell-death-inducing DNA fragmentation effector (CIDE) family, composed of CIDEA, CIDEB, and CIDEC/CIDE-3/FSP27, all of which contain a conserved CIDE N-domain and a unique C-terminal domain. *Cidec*/*Fsp27* is expressed at high levels in white adipose tissue (26). By alternative splicing in HepG2, *CIDE-3* gene displays two transcripts, *CIDE-3* and *CIDE-3alpha*. While CIDE-3 comprises a full-length open reading frame, CIDE-3alpha encodes a truncated protein (29). In the liver, a third transcript, *FSP27β*, which contains 10 additional amino acids at the N-terminus of the original protein and is activated through the liver-enriched transcription factor cyclic-AMP-responsive-element-binding protein H (CREBH) but not by peroxisome proliferator-activated receptor gamma, has been described (11, 63). In this organ, CIDEC/CIDE-3/FSP27 contributes to triglyceride accumulation both in humans and pigs (28) and to the regulation of lipidation and maturation of very low-density lipoproteins (62). It is localized to lipid droplets (LD) and endoplasmic reticulum (55). The latter participates in the regulation of LD formation, expansion, and morphology under lipid-deficient conditions (25). To promote the formation of a unilocular droplet, the formation a ternary complex of AS160, the GTPase activating protein for RAB8a, FSP27 and RAB8a is required (59).

Fsp27-deficient mice show increased energy expenditure and lower levels of plasma triglycerides and free fatty acids (39). Only when they are crossed with leptin-deficient mice or BATless mice, or are fed them a high-fat diet, hepatic steatosis and insulin resistance are observed. Therefore, *Fsp27* deficiency requires further implication of genes to display hepatic insulin resistance (57, 68). In contrast, mice with adipocyte-

specific disruption of the *Fsp27* gene upon high-fat diet feeding are resistant to weight gain and fat-storing. This results in a lipid overflow from adipose tissue that generates hepatosteatosis, dyslipidemia, and systemic insulin resistance pointing out a role for this adipocyte protein to prevent lipodystrophies (56). An increased expression of *Cidec* has been found in a number of experimental or pathological conditions, such as in endoplasmic reticulum stress (24), spontaneous mouse insulin resistance (51) and hepatocellular carcinoma cells (37). Similar effects have been described in liver steatosis and in obese humans (13), being the latter increase reduced by weight loss (16). A homozygous human mutation of CIDEC has been reported to induce lipodystrophy and insulin-resistant diabetes (40, 48). Reduced expression of hepatic *Fsp27* abolished fasting-induced liver steatosis (23) and the former condition in combination with a PPARalpha agonist was also found to reduce hepatic steatosis (45) and even atherosclerosis (46) in *Ldlr*-deficient mice, a model of atherosclerosis and hepatosteatosis (49).

The expression of CIDEC is controlled at both transcriptional and posttranslational levels (5, 13). Different molecules seem to be involved in its expression, such as CD44 (17) or osteopontin, whose absences decrease its levels (22), while leptin absence displays the opposite (35). Ceramide (27) and TNF-alpha reduced its expression while insulin upregulated it. In the latter response, the activity of phosphatidylinositol 3-kinase was involved (21), so was the phosphatase and tensin homologue, an enzyme involved in degradation of phosphorylated phosphatidylinositol (50). Final effectors of these signaling pathways seem to be nuclear receptors such as TAK1/TR4/NR2C2, RORalpha and PPARalpha, nuclear proteins (CAAT-enhancer-binding proteins), LXRα and SREBP-1c (3, 7, 8, 18, 19, 23). Peroxisome proliferator-activated receptor gamma2 (PPARgamma2) also plays a role (34). Posttranslational regulation of FSP27 involves

stability through the proteasomal ubiquitin-dependent protein catabolic process (67), glycosylation (65) and acetylation (44).

A complex physiological regulation of CIDEC seems to exist, in which fasting and diet composition play important roles. In this regard, during the initial stages of fasting, *Fsp27* expression has been found dramatically increased by involvement of the PKA-CREB-CRTC2 signaling pathway (58). The fasting effect was not present in PPARalpha-deficient mice (66). However, after a long period of fasting, a decrease in *Fsp27* expression was observed (58). Despite the observed hepatic steatosis after a choline-deficient diet, no changes were observed for *Fsp27* (66). Nevertheless, a marked induction of its expression was found in the high-fat- or methionine- and choline-deficient diet-induced fatty liver, but not in alcohol-induced fatty liver. The induction of *Fsp27* mRNA was independent of peroxisome proliferator-activated receptor gamma (PPARgamma) levels and completely absent in the liver from PPARgamma-deficient mice (2). In less extreme dietary conditions, it has been reported that a high fat diet increased *Fsp27* expression through activation of PPARgamma (41). In vitro, a high supply of fatty acids stimulated hepatic expression (25). Using *Apoe*-deficient mice as a model of spontaneous hepatosteatosis, nature of fatty acids was important to increase its expression in these mice fed a Western-type diet enriched with linoleic acid isomers since only those mice receiving trans-10, cis-12-conjugated linoleic acid showed this effect. Furthermore, consuming olive oil-enriched diet reduced *Fsp27* expression (15). In addition, only one study has addressed the influence of sex on its expression in young mice (12). Growth hormone has also found to regulate this protein (52, 53). Therefore, influence of sex may be different in adult mice. Based on these facts, it was hypothesized that *Fsp27* hepatic regulation might be the result of complex interactions of dietary components and sex. To this end, the present work was undertaken to characterize the

147 influence of different dietary conditions and sex on *Fsp27* gene expression in adult liver
148 of several animal models.
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Material and methods

Animals

Charles River (Charles River Laboratories, Barcelona, Spain) was the source of *Apoe*-deficient mice on the C57BL/6J genetic background. Dr. Nobuyo Maeda (University of North Carolina at Chapel Hill, NC, USA) generously provided these mice on the C57BL/6JxOla129 genetic background. *Ldlr*-deficient mice on the C57BL/6J.SJL genetic background were obtained from Dr. Vicente Andrés from CNIC, (Madrid, Spain). C57BL/6J wild-type and *Pgc1a*-deficient mice were part of a colony established at the IIB animal facility (Madrid) and originally derived from mice provided by Dr. Bruce Spiegelman (DFCI, Boston, USA). Wistar rats were obtained from Charles River (Charles River Laboratories, Barcelona, Spain).

For all experiments, two-month-old mice were used. Blood samples were taken (after four-hour fasting) from the facial vein to determine plasma cholesterol and accordingly establish groups with similar initial values. Animals, housed in sterile filter-top cages, were maintained under a 12-h light/12-h dark cycle at the *CIBA, Universidad de Zaragoza*. *Pgc1a*-deficient mice were maintained at *Autónoma Universidad de Madrid*. Wistar rats were maintained at *Universidad de Córdoba*. Animals were handled and killed observing guidelines (Directive 2010/63/UE) from the European Union for care and use of laboratory animals in research. All had ad libitum access to food and water and study protocols were approved by the Ethics Committees for Animal Research of the Universities of Zaragoza, Madrid and Córdoba. After the diet intervention, and four-hour fast, the animals were killed by suffocation with CO_2. The livers were removed, weighed, frozen in liquid nitrogen, and stored at − 80 °C until analysis.

Experimental design

Table 1 provides detailed information of all experiments regarding characteristic

of animals, type of diets, number of animals and length of intervention. Since C57BL/6J mice do not express hepatic *Cidec/Fsp27* (35, 58), we decided to use *Apoe*-deficient mice which showed hepatic expression of this gene influenced by some dietary components (15). Using this model, we tested the effects on *Cidec/Fsp27* expression of dietary cholesterol, Western diet and sex. On Western diet, the influence of two modifiers of lipid droplet surface, oleanolic acid (10) and squalene (14) were tested. Likewise, this diet was used to analyze sex differences and its inhibition by surgical castration. A confirmation of the effects of ovariectomization on *Cidec/Fsp27* expression was carried out in female Wistar rats fed a Western diet. Since sex differences emerged on Western diet, this was also tested in another model of hepatic steatosis, *Ldlr*-deficient mice on C57BL/6J genetic background. All previous experiments were suggestive of an involvement of PGC1a, to confirm such issue; *Pgc1*-deficient mice on C57BL/6J genetic background fed a purified Western diet were used to analyze the sex differences on hepatic *Cidec/Fsp27* expression. Detailed compositions of purified diets are shown in supplementary Table 1.

Isolation and quantification of hepatic RNA

RNA was isolated using Tri-reagent (Ambion, Austin, TX, USA). Contaminant DNA was removed using the DNA removal kit from Ambion. Absorbance at $A_{260/280}$ served to quantify RNA concentrations and the ratio 28S/ 18S ribosomal RNAs used to estimate their quality. Changes in mRNA expression were determined by RT-qPCR. cDNA synthesis was carried out using the First Strand synthesis kit (Thermo Scientific, Madrid, Spain). The Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used to analyze gene expression by qPCR. Specific primers, designed and checked as previously described (33) were purchased from Applied Biosystems. Sequences are shown in supplementary Table 2. RT-qPCR reactions were performed on a Step One Real Time PCR System (Applied Biosystems) following the standard procedure and using

equal amounts of DNA-free RNA from each animal. The relative amount of all mRNAs was calculated using the comparative $2^{-\Delta\Delta Cq}$ method and *Cyclophilin B (Pipb)* mRNA expression as the reference gene.

Liver histology analyses

Aliquots of liver, stored in neutral formaldehyde, were used and processed as described (14).

Hepatic homogenate and lipid extraction

A piece of liver was homogenized in homogenization buffer (phosphate buffered solution with protease inhibitor cocktail (Roche, Mannheim, Germany) and used to assay protein concentration by the BioRad dye binding assay (BioRad, Madrid, Spain). Extracted lipids according to Folch's method (9) were evaporated under N_2 stream and dissolved in 100 µL of isopropanol. Infinity kits (Thermo Scientific) were used to measure total cholesterol and triglycerides.

Preparation of microsomal fractions

This fraction was prepared according to Osada et al. (43). Basically, 600 mg of pooled hepatic tissue of each group were homogenized in 2 mL of 0.25 M sucrose containing the Roche protease inhibitor cocktail at 4ºC and centrifuged at 280g for 5 min. Supernatants were centrifuged at 1500g for 10 min followed by another centrifugation at 19000g for 10 min to collect the supernatants containing cytosolic and microsomal proteins. After a centrifugation at 100000g for 60 min, the obtained pellets containing the microsomal fractions were resuspended in PBS containing 0.2% Triton X-100 and 10% glycerol and centrifuged at 12000 rpm 10 min in order to remove insoluble proteins. Protein concentrations were determined by BioRad dye binding assay.

Preparation of lipid droplets

They were prepared following the protocol of Ontko et al. (42). Briefly, pooled

hepatic tissue (600 mg) of each group were homogenized in 3 ml of 65% sucrose solution with protease inhibitor cocktail (Roche, Mannheim, Germany) at 4ºC. Discontinuous sucrose gradients were prepared as follows: 3 ml of liver homogenates in 65% sucrose were pipetted at the bottom of the centrifuge tubes kept in an ice bath. Then 2 ml of 60% sucrose solution were slowly added, followed by 2 ml of 52% sucrose, 2 ml of 44% sucrose and 2 ml of distilled water. The tubes with the gradients were centrifuged at 25000g for 30 min at 4ºC and the bands containing the different lipid droplets were collected. They were mixed with 3 volumes of acetone and kept at -80ºC for 10 min and then at -20ºC overnight. The tubes were centrifuged at 15000g for 15 min at 4ºC. The pellets were washed three times, firstly with acetone: diethyl ether 1:1 and then twice with diethyl ether. Dry pellets were resuspended in PBS containing 0.2% Triton X-100 and 10% glycerol and centrifuged at 12000 rpm 10 min in order to remove insoluble proteins. Protein concentrations were determined by BioRad dye binding assay.

Western blot

20 μg of protein were loaded onto a 10% SDS-polyacrylamide gel and electrophoresed for 120 min at 90V in a Bio-Rad Miniprotean cell (Hercules, CA). Proteins were transferred to PVDF membranes (GE Healthcare, Madrid, Spain). Membranes were blocked with PBS buffer containing 5% BSA for 1 h at room temperature. The primary antibodies, diluted in PBS buffer containing 2.5% BSA and 1% Tween 20, were added and the membranes were incubated 2 h at room temperature and then overnight at 4ºC. FSP27 protein expression was evidenced by using a rabbit polyclonal antibody (NB100-430 diluted 1/1,000, Novus Biologicals, Centennial, Colorado, USA). Equal loadings were confirmed by using a goat polyclonal anti-HSC70 (TA302666 diluted 1/500, OriGene, Rockville, MD, USA). Membranes were washed with PBS buffer containing 0.1% Tween 20. Conjugated goat anti-rabbit IgG (H&L)

DyLight 800 secondary antibody (SA5-35571, diluted 1/15,000, Thermo-scientific, Waltham, MA, USA) for FSP27 detection and a donkey anti-goat IRDye 680RD (926-68074, diluted 1/5,000, LI-COR Biosciences, Lincoln, NE, USA) for HSC70 detection were used and incubated for 1 h at room temperature in PBS buffer containing 2.5% BSA and 1% Tween 20. Images were captured using an Odyssey® Clx (LI-COR).

AML12 cell culture

The murine hepatocyte cell line was grown in a humidified atmosphere of 5% CO_2 at 37ºC in Dulbecco's modified Eagle's minimum essential medium (DMEM) (ThermoFisher Scientific, Waltham, MA, USA): F12-Ham's medium (GE Healthcare Life Science, South Logan, Utah) in 1:1 ratio supplemented with 10% foetal bovine serum (ThermoFisher Scientific), 1:500 insulin/transferrin/selenium (Corning, Bedford, MA, USA), 40 ng/ml dexamethasone (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) 1% nonessential amino acids (ThermoFisher Scientific), 1% penicillin (1000 U/ml) (ThermoFisher Scientific), 1% streptomycin (1000 mg/ml) (ThermoFisher Scientific) and 4 mM L-glutamine (ThermoFisher Scientific) in a 6 multiwell plate (in triplicate). Medium was changed every two days. After one week of growth, this medium was removed, and cells were washed twice with phosphate buffered saline (PBS) prior to the addition of the serum-free media supplemented with 200 μm stearic acid for 24 hours or 200 μM stearic acid for 24 hours and 50 nM estradiol dissolved in ethanol for 6 hours. Then, media were removed and cells collected with Tri-reagent solution (Ambion). RNA isolation and cDNA synthesis were performed as above described.

Reporter assays

The genomic region -2042 bp to 0 bp at 5' side of the starting transcription site of *CIDEC/FSP27β* (XM_024453700.1) from human genomic DNA was amplified by PCR using direct (5'-agaaccagatcttggCAAGTGATCCACCTGCCTCG-3) and reverse (5'-

gatatctgcagaattGAGCAGATAACCCAACTCAGGGC -3') primers. The 2-kb PCR product was cloned upstream a secreted Gaussia luciferase (GLUC) reporter gene using linearized pEZX-GAO1 (Genecopeia Rockville, Maryland, USA) according to In-Fusion® cloning protocol from Takara-Clontech (Cat No 638909, Kusatsu, Shiga, Japan). Restriction enzymes and DNA sequencing confirmed the resulting plasmid. This latter was transfected to AML12 cells alone or in combination with a plasmid containing *Pgc1a* (MN_008904) under the control of CMV promoter (MC204789, Origene) using lipofectamine 3000 (ThermoFisher) following manufacturer' instructions. Two days after, media were taken and secreted GLUC and alkaline phosphatase, also present in pEZX-GAO1, activities were evaluated. The ratio of GLUC/alkaline phosphatase was calculated.

Statistical analysis

The Statistical Package for Social Sciences version 15 (SPSS, Chicago, IL, USA) or Prism 5 for windows software for Windows (GraphPad, S. Diego, CA, USA) were used for statistical analyses. Variables, not showing normal distribution (according to the Shapiro-Wilk's test), or homology of variance, were analyzed with the Mann-Whitney's U test. Data are shown as medians and 10-90 percentile range of the values. Correlations between variables were tested using the Spearman's correlation test. The statistical significance was considered when $p < 0.05$.

Results

Dietary fat and hepatic Cidec/Fsp27β expression in Apoe-deficient mice.

To characterize the dietary regulation of the expression of this gene in mice, the supplement of dietary cholesterol to male mice was tested. Increased hepatic surface occupied by lipid droplets as well as hepatic total cholesterol and triglyceride contents (Fig 1a, b and c) were observed following dietary cholesterol supplementation. The latter induced a significant increase in the hepatic *Cidec/Fsp27β* expression as shown in Fig 1d. Hepatic cholesterol content was associated with hepatic *Cidec/Fsp27β* expression (Fig 1e).

In a second study, the influence of a Western diet (WD), containing cholesterol and palm oil as source of saturated fat, was explored in male *Apoe*-deficient mice on C57BL/6J genetic background (Fig 2). Significant increased hepatic areas occupied by lipid droplets (Fig 2a, b and c) as well as hepatic total cholesterol and triglyceride contents were also observed in mice on the Western diet. Unexpectedly, a significant decrease of hepatic *Cidec/Fsp27β* expression was found (Fig 2d). These expression changes were inversely associated with hepatic cholesterol (Fig 2e).

To further explore this dissociation between hepatic *Cidec/Fsp27β* and Western diet, its expression was tested in two dietary components that had been shown to influence dietary droplets without altering lipid content (oleanolic acid) or viceversa (squalene). In the first experiment and as expected, male mice receiving an oleanolic acid-enriched WD showed an increase in the hepatic area occupied by lipid droplets (Supplementary Fig 1a, b and c) without changes in hepatic cholesterol and triglyceride contents (Supplementary Fig 1c). In these conditions, no significant change was observed for hepatic *Cidec/Fsp27β* expression (Supplementary Fig 1d). In the second experiment, the effect of a squalene-enriched WD was explored, again in males. No significant changes were noted for the

percentage of hepatic surface occupied by lipid droplets despite the decreased liver cholesterol and triglyceride contents (Supplementary Fig 2a, b and c). Nor was there any significant change in the hepatic *Cidec/Fsp27β* expression by squalene (Supplementary Fig 2d). Overall, these nutritional experiments emphasize that hepatic *Cidec/Fsp27β* expression possesses a fine nutritional regulation at transcriptional level in *Apoe*-deficient mice, where cholesterol increased its levels and saturated fat reverted this finding, being the latter not influenced by minor dietary components, such as oleanolic acid or squalene, despite the changes in hepatic lipids.

Hepatic Cidec/Fsp27β expression is influenced by sex hormones in Apoe-deficient mice and in vitro.

The influence of sex on *Cidec/Fsp27β* expression was explored in *Apoe*-deficient mice on a chow diet of low fat content. As shown in Supplementary Fig 3, panels a,b,c, females showed lower surface occupied by lipid droplets despite a significant increase in hepatic cholesterol content and no changes in triglycerides. In this experiment, no significant changes were observed in hepatic *Cidec/Fsp27β* between sexes. In the second experiment, the differences between sexes were explored when both groups received a WD. As shown in Fig 3a, b and c, no significant change was observed in the percentage of liver surface occupied by lipid droplets. However, the levels of hepatic total cholesterol and triglycerides were significantly lower in females than in males. In this experimental approach, females showed significantly decreased hepatic *Fsp27β* expression (Fig 3d). The latter was significantly associated with hepatic triglyceride contents (Fig 3e). This mRNA decrease was translated in decreased amounts of CIDEC/FSP27 protein in lipid droplets and microsomes (Fig 3f and g). These data indicate that sex is playing an important role in hepatic *Cidec/Fsp27β* expression in the presence of WD and these

changes are reflected in a lesser amount of CIDEC in hepatic lipid droplets of female livers.

The involvement of hormonal changes on sex-differences was characterized in *Apoe*-deficient mice of both sexes that underwent surgical removal of gonads and were fed a purified Western diet. As shown in Supplementary Fig 4, no significant change in *Cidec/Fsp27β* expression was observed in orchiectomized males; nor was there any significant change in hepatic total cholesterol, or in hepatic triglycerides. However, there was a significant increase in the liver surface occupied by lipid droplets in orchiectomized males. In contrast, ovariectomy resulted in significant increases in hepatic cholesterol, triglycerides, and in the surface occupied by lipid droplets (Fig 4c). Ovariectomized females showed a significant increase in *Cidec/Fsp27β* expression compared to control females (Fig 4d). A positive significant association was also found between hepatic *Cidec/Fsp27β* values and those of hepatic triglycerides (Fig 4e). The increase in mRNA expression was translated into increased contents of CIDEC proteins in lipid droplets and microsomes (Fig 4f and g). These results indicate that ovarian hormones are responsible for the decreased hepatic *Cidec/Fsp27β* expression observed in females consuming WD. In fact, incubation of stearic-stimulated hepatic AML12 cells with estradiol resulted in a significant decrease in *Cidec/Fsp27β* expression (Supplementary Fig 5a).

Pgc1a is involved in hepatic Cidec/Fsp27β expression sex differences in vivo.

PKA and PPAR have been described in the regulation of hepatic *Cidec/Fsp27* expression (3, 7, 8, 18, 19, 23). To verify whether or not those agents were involved in the observed sex-dependent responses, hepatic *Prka2* expression was determined and no significant changes were observed (data not shown). Regarding PPARgamma, the hepatic expression of its regulator, *Pgc1a,* was significantly increased in females compared to males consuming the Western diet (Fig 5a) and an inverse significant relationship was

found between *Cidec/Fsp27β* expression and that of *Pgc1a* in both sexes (Fig 5b). While orchiectomy had no effect on *Pgc1a* expression (Fig 5c), ovariectomy induced a significant decrease in its expression in *Apoe*-deficient females (Fig 5d). Likewise, ovariectomized female rats also showed a trend to increase *Cidec/Fsp27β* expression (Supplementary Fig 6e) and decreased hepatic *Pgc1a* expression (Supplementary Fig 6f). Both effects were even more pronounced in rats neonatally androgenized by testosterone administration and then ovariectomized. In this model, hepatic fat, cholesterol and TG contents followed a similar pattern (Supplementary Fig 6d) and *Cidec/Fsp27β* expression was associated with hepatic TG content (data not shown). The sex differences in *Cidec/Fsp27β* expression were observed in *Ldlr*-deficient fed on WD as well (Supplementary Fig 7d). Concomitantly, a significant increase in *Pgc1a* expression was observed in these female mice (Supplementary Fig 7e). Decreased *Cidec/Fsp27β* gene expression in females was translated into lower amounts of CIDEC/FSP27 protein in lipid droplets and microsomes (Supplementary Fig 7f and g). Overall, these findings are suggestive of an inverse association between *Cidec/Fsp27β* and *Pgc1a* expressions as a general response, independent of absence of APOE. These mRNA changes are reflected in CIDEC/FSP27 protein present in lipid droplets from female livers.

According to this association, it was hypothesized that *Pgc1a* would reduce the transcriptional activity of a reporter gene under the control of CIDEC promoter. This was the case, as shown in Supplemental Fig 5b. The opposite hypothesis would be that sex-differences in hepatic *Cidec/Fsp27* would be abolished in the absence of *Pgc1a*. To test this, *Pgc1a*- deficient mice from both sexes were fed WD. In this model, female mice increased hepatic fat area, total cholesterol and TG contents (Fig 6a, b, c). As shown in Fig 6d, no differences were observed in hepatic *Cidec/Fsp27β* expression between sexes when using homozygous *Pgc1a*-deficient mice. However, the sex differences at the

CIDEC protein levels in lipid droplets and microsomes remained in absence of PGC1a (Fig 6e and f). These results suggest that absence of PGC1A abolishes the sex-induced mRNA changes of hepatic *Fsp27β* expression in response to WD, being the transcription factor a transcriptional repressor. However, the sex-induced differences in CIDEC present in lipid droplets and microsomes are independent of PGC1A.

Discussion

The present work explores the putative hepatic *Cidec/Fsp27β* transcriptional changes induced by dietary components and sex. Using *Apoe*-deficient mouse as a model of hepatic steatosis, dietary cholesterol increased hepatic *Cidec/Fsp27β,* which was repressed when combined with saturated fat. The latter was not influenced by dietary minor components such as oleanolic acid or squalene administered at pharmacological doses. Moreover, our study revealed a previously unnoticed sex regulation dependent on the prevailing diet, being the female sex a negative regulator. An effect observed in two models of genetic hepatic steatosis (*Apoe*- and *Ldlr*-deficient mice) and reflected in CIDEC/FSP27 content of lipid droplets. Using ovariectomized females, it was shown that ovarian hormones are crucial for the observed decrease in *Cidec/Fsp27β* expression noted in *Apoe*-deficient mice. This effect was also observed in Wistar female rats. An increased expression of *Pgc1a* inversely associated with that of *Cidec/Fsp27* and the lack of such effect after ovariectomy in *Apoe*-deficient mice allow us to infer that ovarian hormones are executing their action through *Pgc1a.* This was confirmed in mice lacking *Pgc1a* where the sex differences on hepatic *Cidec/Fsp27β* were erased providing further *in vivo* support for this role. However, the sex differences at the CIDEC/FSP27 content of lipid droplets and microsomes are independent of PGC1a.

As shown in Supplemental Figure 8, four set of primers were used to study hepatic *Cidec/Fsp27* m RNA expression in mice. With the exception of primers, named α, corresponding to exon 1, which showed no expression in the liver (data not shown), the remaining three sets gave concordant results in all experimental conditions. None of the selected primers amplified the truncated form. Thus, the observed changes corresponded to *Fsp27β*, a recently described isoform of the protein regulated by CREBH (63).

The present work has explored the influence of two main components of Western diet, cholesterol and saturated fat. Using the first dietary component, an increase in the

Cidec/*Fsp27β* expression was noted. Using information from ENCODE, it was observed that both SREBP1 and 2 bind to this gene (6). Recently, the involvement of SREBP-1c has been proved (7). Surprisingly, the combination of cholesterol and saturated diet decreased hepatic *Cidec/Fsp27β* expression. In this regard, variable effects of high fat diets have been described depending on the length of fat administration (36). While a short-term administration (3 weeks) increased the expression, a long-term administration of 12 weeks had the opposite effect. In this sense, our study lasted 11 weeks and would be in agreement with the latter finding. Similar results were observed in *Apoe*-deficient mouse males receiving an olive oil-enriched diet (15). Likewise, a decreased expression was found in a postprandial regimen after a virgin olive oil bolus in male Wistar rats and this decrease was inversely associated with hepatic triglyceride and cholesterol contents (32). In the latter case, the hepatic mRNA changes occurred just 4 hours after fat intake. In fasting rats, a rapid increase was equally observed four hours after its start (58). Elevations of *Cidec/Fsp27* mRNA expression by high fat diets required additional dietary deficiencies such those of methionine and choline (Table 2) or under certain metabolic derangements such as those posed by Db mice, PPAR-alpha-deficient mice (Table 2). In a previous study, using *Apoe*-deficient mice with C57BL/6JxOla129 genetic background and fed Western diets with different conjugated linoleic acid (CLA) isomers, we observed high hepatic *Cidec/Fsp27* mRNA expression in those mice receiving the trans-10,cis-12 CLA isomer and the levels were associated with the hepatic surface occupied by lipid droplets. In contrast, when the cis-9, trans-11 CLA isomer was provided resulted in decreased *Cidec/Fsp27* mRNA expression (15). Overall, regimen of administration and nutritional components are critical modulators of hepatic *Cidec/Fsp27* expression and this mRNA undergoes a rapid metabolic variation in few hours.

In the present study, the intake of oleanolic acid, a pentacyclic triterpene, and

454 squalene, a lineal triterpene, had no effect on *Cidec/Fsp27β* expression despite the
455 changes induced in lipid droplet area (10). Similar finding was reported by the
456 administration of a dietary supplement of *Boswellia serrata*, an extract rich in particular
457 derivatives of boswellic acid, also a pentacyclic triterpene-based compound (20). As
458 triterpenes tend to accumulate in the liver altering distribution of triglycerides in lipid
459 droplets (30, 31), it could be hypothesized that those lipid droplets would not need
460 changes in *Cidec/Fsp27β* expression or these are not executed at the mRNA level.

461 In a previous study, we observed that hepatic *Cidec/Fsp27* gene expression was
462 significantly associated with hepatic surface occupied by lipid droplets in *Apoe*-deficient
463 mice fed different conjugated linoleic acid isomers, in *Cbs*-deficient mice and in olive
464 oil-fed *Apoe*-deficient mice (15). This was not the case in the present study. Notably,
465 *Cidec/Fsp27β* expression was associated with hepatic triglyceride (Figures 3 and 4) or
466 cholesterol contents (Figures 1 and 2). The genetic background and the diet composition
467 are main differences between the previous and the current study. The former one used
468 Ola129xC57BL/6J mixed genetic background mice while the present study has been
469 carried out using C57BL/6J mice. Due to both strains do have important differences in
470 hepatic fat content (54), the experimental setting may have influenced the outcome. The
471 second aspect is the use of AIN-93 purified diet (47) in the present study compared to
472 commercial ones in the previous one. This choice was forced by the high variability noted
473 in our lab among control mice for years in atherosclerotic lesions when using commercial
474 chows and the failure of obtaining the same batch throughout years. Indeed, source of
475 protein has also been shown to induce changes in *Cidec/Fsp27* expression (60, 64). By
476 and large, dietary components are an important source of variation (49), and our current
477 study, in well-defined conditions of mouse strains and purified diets, adds further
478 evidence supporting this contention.

A striking result observed in this work was the decreased hepatic *Cidec/Fsp27β* expression in female mice consuming WD in *Apoe*- and in Ldlr-deficient mice. As consequence of this decrease, the amount of CIDEC/FSP27 protein in lipid droplets was decreased in females. This fact points to a sex-difference in hepatic regulation of lipid droplet enlargement considering the role of CIDEC/FSP27 in this process. An effect that was abolished when ovariectomy was performed in *Apoe*-deficient mice and Wistar rats. Interestingly, female mice lacking steroid receptor coactivator-2 showed increased hepatic expression of this gene (Table 2). Steroid receptor coactivator-2 promotes the transcriptional activation of estrogen receptor in some tissues (61). These results are indicating a negative regulation of the gene by the influence of female hormones. This could be executed through *Pgc1a* as the significant inverse association noted between *Pgc1a* and *Cidec/Fsp27β* suggests. Further evidences to this suggestion comes from the binding of PGC1a to this gene as evidenced by ChIP assays reported by the ENCODE consortium (6). Indeed, estradiol action has been found to be modulated by *Pgc1a* (4) and Pgc1a decreased *CIDEC* promoter activity. When we carried out ovariectomy, the decrease in *Cidec/Fsp27β* expression was lost. Deficiency of *Pgc1a* as the case of the experiment carried out in *Pgc1a*-deficient mice is also supporting the role of *Pgc1a* in the *in vivo* sex differences but only at the mRNA levels.

In conclusion, the present report evidences two axes of hepatic *Cidec/Fsp27β* regulation defined by diet and sex. Regarding the first one, cholesterol and the nature of fatty acids are a key component. On the other hand, the fact that the female decrease in hepatic gene expression was not observed in ovariectomized mice strongly suggests that ovarian hormones are involved in the control of hepatic *Cidec/Fsp27β* mRNA expression and this is modulated by *Pgc1a*. However, the sex-differences at the CIDEC/FSP27 protein levels observed in lipid droplets and microsomes are independent of PGC1a.

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752 Table 1. Summary of experimental conditions

Experiment	Genetic background	Diet	Sex	Groups and sample size	Influence
	***Apoe*-deficient mice**				
1	C57BL/6J x OLA 129	Commercial chow (B & K Universal Ltd, Humberside, UK) w/wo 0.1% cholesterol for 10 weeks (1)	Males	Control (n=7) Cholesterol (n=7)	Cholesterol
2	C57BL/6J	Purified chow and Western diets for 11 weeks	Males	Chow (n=13) Western (n=9)	Western diet
3	C57BL/6J	Purified Western w/wo 0.01% oleanolic acid (OA) (Extrasynthese, Genay, France) for 11 weeks (10)	Males	Western (n=8) Western + OA (n=9)	Oleanolic acid
4	C57BL/6J	Purified Western w/wo 1% squalene (Sigma, Madrid, Spain) for 10 weeks (14)	Males	Western (n=9) Western + Squalene (n=10)	Squalene
5	C57BL/6J	Purified chow for 11 weeks	Both sexes	Males (n=13) Females (n=13)	Sex in chow diet
6	C57BL/6J	Purified Western for 11 weeks	Both sexes	Males (n=9) Females (n=10)	Sex in Western diet
7	C57BL/6J	Purified Western for 11 weeks	Orchiectomized and non-orchiectomized males	Control (n=9) Orchiectomized on postnatal day 30 (n=9)	Testicular contribution in males
8	C57BL/6J	Purified Western for 11 weeks	Ovariectomized and non-ovariectomized females	Control (n=9) Ovariectomized on postnatal day 30 (n=9)	Ovarian contribution in females
***Ldlr*-deficient mice**	C57BL/6J.SJL	Purified Western for 11 weeks	Both sexes	Males (n=17) Females (n=18)	Sex in Western diet

***Pgc1a*-deficient mice**	C57BL/6J	Purified Western for 11 weeks	Both sexes	Males (n=8) Females (n=8)	Sex in Western diet
Rats	Wistar	Purified Western for 100 post-weaning days(38)	Ovariectomized and non-ovariectomized females	Control (n=6) Ovariectomized (n=6) Ovariectomized + a single injection of 1250 µg of testosterone propionate on postnatal day 1 (n=6)	Ovarian contribution and neonatal androgenization in females

753 w/wo, with or without
754

755 Table 2. Changes in hepatic *Cidec/Fsp27* expression according to Genome Expressed
756 Omnibus data bank and Array express.

Experimental condition	Type of change	Signal log_2 ratio	Accession number
Caspase 1 deficient mice	Increased	0.3	GDS4922
Glycerol kinase knockout	Increased	1.9	GDS1555
NADH-cytochrome P450 reductase deletion effect on liver	Increased	1.3	GDS1093
Stearoyl-CoA desaturase 1-deficient mutants on a very low-fat, high-carbohydrate diet	Increased	2.2	GDS1517
Steroid receptor coactivator-2-deficient female mice	Increased	0.8	GDS4785
Thioredoxin reductase 1-null liver	Increased	1.1	GDS4928
Fasting	Increased	0.5	GDS4918
Fasting and LPS in male BL6/SV129 mice	Increased	5.5	GDS4546
Alcoholic hepatitis	Increased	0.7	GDS4389
Sebacic acid supplemented diet effect on db/db liver	Increased	0.7	GDS3807
Ketogenic diet effect on the liver	Increased	5.7	GDS2738
High-fat high-calorie diet effect on liver	Increased	1.3	GDS2413
Liver response to a high fat diet deficient in methionine and choline	Increased	5.3	GDS4883
Liver response to a high fat diet: time course	Increased 12 h	2.4	GDS4783
Western diet induced changes in liver	Increased	4.9	GDS279
Perfluorooctanoic acid effect on livers lacking PPAR-alpha	Increased	7.1	GDS3407
Peroxisome proliferator-activated receptor subtype activation effect	Increased by PPARg2	0.6	GDS1373

on liver cell			
Female receiving dexamethasone	Increased	1.7	GDS5036
Hepatocyte nuclear factor 4 alpha depletion on hepatocellular carcinoma cell line	Decreased	-0.6	GDS4798
Transcriptional coactivator PGC-1beta hypomorphic mutation effect on the liver	Decreased	-1.3	GDS3197
RORα-deficient staggerer mice fed high fat diet	Decreased	-5.7	GSE23736
SIRT3 deficient liver response to a high fat diet	Decreased	-0.1	GDS4817
GPR120-deficient liver response to a high fat diet	Decreased	-1.3	GDS4830
TAK1/TR4-deficient mice	Decreased	-18	GSE21903
Conditional GBA1 deletion model of Type 1 Gaucher disease	Decreased	-1.4	GDS4162
Atherogenic diet effect on the liver: time course	Decreased long term	-4.8	GDS2292
Streptozotocin induced type 1 diabetes	Decreased	-0.3	GDS4845
Adrenalectomized liver at light and dark periods of the circadian cycle	Variable	Dark 1.0 Light -1.4	GDS1870
Sex specific transcription in somatic and reproductive tissues	Decreased	-0.6	GDS565

757 http://www.ncbi.nlm.nih.gov/gds/
758 https://www.ebi.ac.uk/arrayexpress/.
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760

761 Fig. 1. **Effect of dietary cholesterol on hepatic steatosis and *Cidec/Fsp27β* expression**
762 **in male *Apoe*-deficient mice.** Representative liver micrographs at x600 magnification
763 from *Apoe*-deficient mice consuming the chow (a) and cholesterol-enriched (b) diets.
764 Morphometric evaluation of surface of liver section occupied by fat, total cholesterol and
765 triglyceride contents (c). Hepatic *Cidec/Fsp27β* expressions determined by RT-qPCR
766 normalized to *Cyclophilin B* (d). Data are medians and 10-90 percentile range for control
767 (n=7) and cholesterol (n=7) groups. Statistical analyses were done according to Mann-
768 Whitney's U test. [a], $P < 0.05$ vs control. Association between hepatic cholesterol content
769 and *Cidec/Fsp27β* expression (f). Open squares correspond to control and striped squares
770 to cholesterol-fed mice. Spearman's correlation is shown.

771

772

773

774

775 Fig. 2. **Effect of Western diet on hepatic steatosis and *Cidec/Fsp27β* expression in**
776 **male *Apoe*-deficient mice.** Representative liver micrographs at x600 magnification from
777 *Apoe*-deficient mice consuming the chow (a) and Western (b) diets. Morphometric
778 evaluation of surface of hepatocyte occupied by fat and hepatic total cholesterol and
779 triglyceride contents (c). Analysis of hepatic *Cidec/Fsp27β* expression determined by RT-
780 qPCR normalized to *Cyclophilin B* (d). Data are medians and 10-90 percentile range for
781 chow (n=13) and Western (n=9) groups. Statistical analyses were done according to
782 Mann-Whitney's U test. [a], $P < 0.05$ vs chow. Association between hepatic cholesterol
783 content and *Cidec/Fsp27β* expression (e). Spearman's correlation is shown. Open squares
784 correspond to control and striped squares to Western-fed mice.

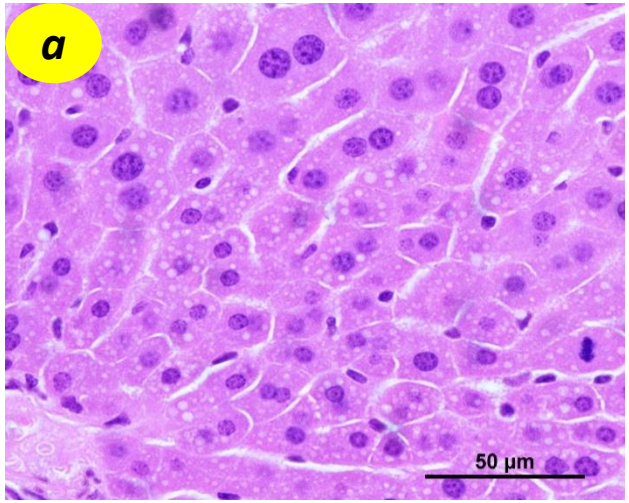
785

Fig. 3. **Effect of sex on hepatic steatosis, *Cidec/Fsp27β* expression and CIDEC/FSP27 content in lipid droplets and microsomes in *Apoe*-deficient mice fed on a Western diet.** Representative liver micrographs at x600 magnification from male (a) and female (b) *Apoe*-deficient mice consuming Western diets. Morphometric evaluation of surface of hepatocyte occupied by fat and hepatic total cholesterol and triglyceride contents (c). Analysis of hepatic *Cidec/Fsp27β* expression was determined by RT-qPCR normalized to *Cyclophilin B* (d). Data are medians and 10-90 percentile range for male (n=9) and female (n=10) groups. Relationship between hepatic triglyceride content and *Cidec/Fsp27β* gene expression (e). Open squares correspond to males and striped squares to females. Correlations were calculated according to Spearman's test. FSP27 protein levels normalized to HSC70 in lipid droplets (f) and microsomes (g), inserts show representative Western blots. Statistical analyses were done according to Mann-Whitney's U test. [a], $P < 0.05$ vs male.

Fig. 4. **Effect of ovariectomy on hepatic steatosis, *Cidec/Fsp27β* expression and CIDEC/FSP27 content in lipid droplets and microsomes in female *Apoe*-deficient mice fed on a Western diet.** Representative liver micrographs at x600 magnification from mock (a) and surgically castrated (b) female *Apoe*-deficient mice consuming Western diets. Morphometric evaluation of surface of hepatocyte occupied by fat and hepatic total cholesterol and triglyceride contents (c). Analysis of hepatic *Cidec/Fsp27β* expression determined by RT-qPCR normalized to *Cyclophilin B* (d). Data are medians and 10-90 percentile range for control (n=9) and castrated (n=9) groups. Relationship between hepatic triglyceride content and *Cidec/Fsp27* gene expression (e). Open squares correspond to control and striped squares to ovariectomized females. Correlations were calculated according to Spearman's test. FSP27 protein levels normalized to HSC70 in lipid droplets (f) and microsomes (g), inserts show representative Western blots. Statistical analyses were done according to Mann-Whitney's U test. [a], $P < 0.05$ vs control.

Fig. 5. **Effect of sex and castration on hepatic *Ppargc1a*/*Pgc1a* expression in *Apoe*-deficient mice fed on a Western diet.** Influence of sex on hepatic *Pgc1a* expression in *Apoe*-deficient mice (a). Relationship between *Cidec/Fsp27β* and *Pgc1a* gene expression levels (b). Open squares correspond to males and striped squares to females. Correlations were calculated according to Spearman's test. Effect of orchiectomy on hepatic *Pgc1a* expression in male *Apoe*-deficient mice (c). Effect of ovariectomy on *Pgc1a* expression in female *Apoe*-deficient mice (d)**.** Analysis of hepatic *Pgc1a* expression was determined by RT-qPCR normalized to *Cyclophilin B*. Data are medians and 10-90 percentile range for each group. Statistical analyses were done according to Mann-Whitney's U test. [a], P< 0.05 vs control.

Fig. 6. **Effect of sex on hepatic steatosis, *Cidec/Fsp27β* expression and CIDEC/FSP27 content in lipid droplets and microsomes in *Pgc1a*-deficient mice fed on a Western diet.** Representative liver micrographs at x400 magnification from male (a) and female (b) *Pgc1a*-deficient mice consuming Western diets. Morphometric evaluation of surface of hepatocytes occupied by fat and hepatic total cholesterol and triglyceride contents (c). Analysis of hepatic *Cidec/Fsp27β* expression was determined by RT-qPCR normalized to *Cyclophilin B* (d). Data are medians and 10-90 percentile range for male (n=8) and female (n=8) groups. FSP27 protein levels normalized to HSC70 in lipid droplets (e) and microsomes (f), inserts show representative Western blots. Statistical analyses were done according to Mann-Whitney's U test. [a], $P < 0.05$ vs male.


a
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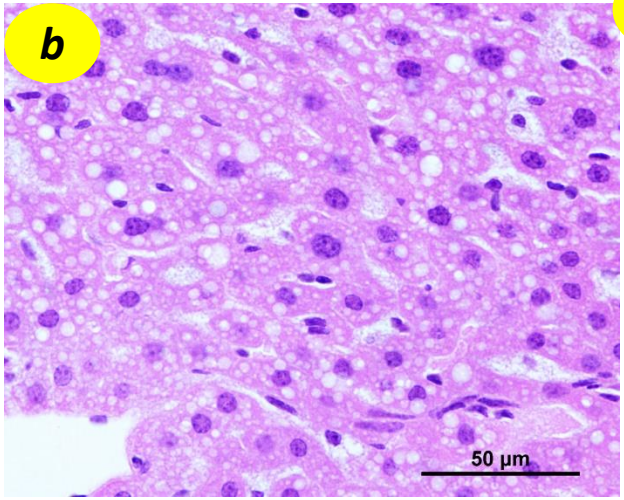
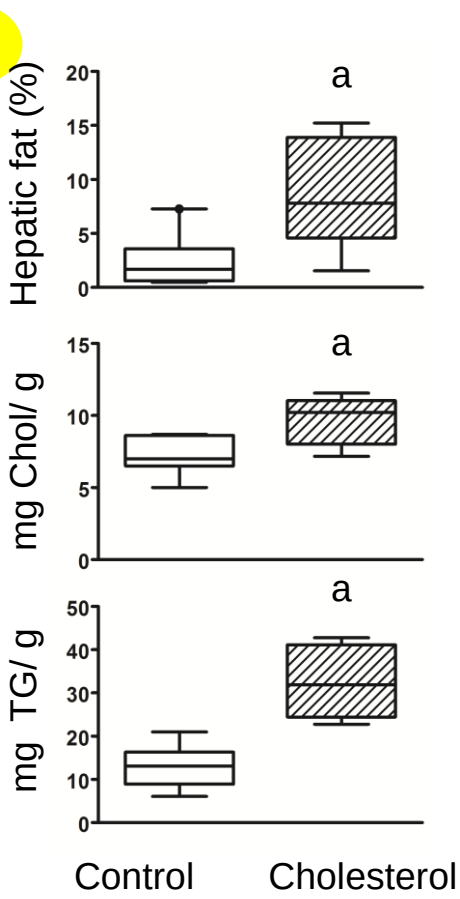
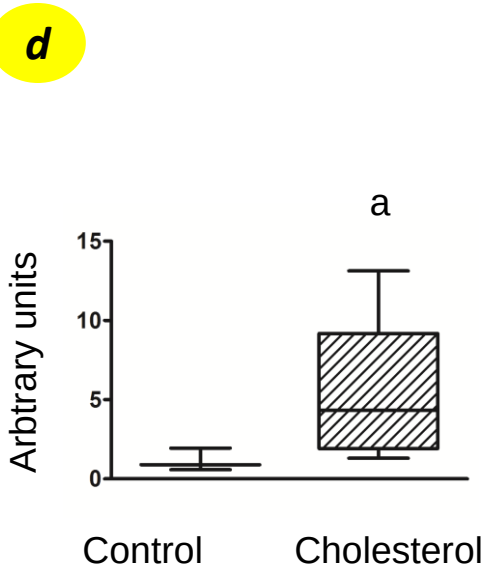
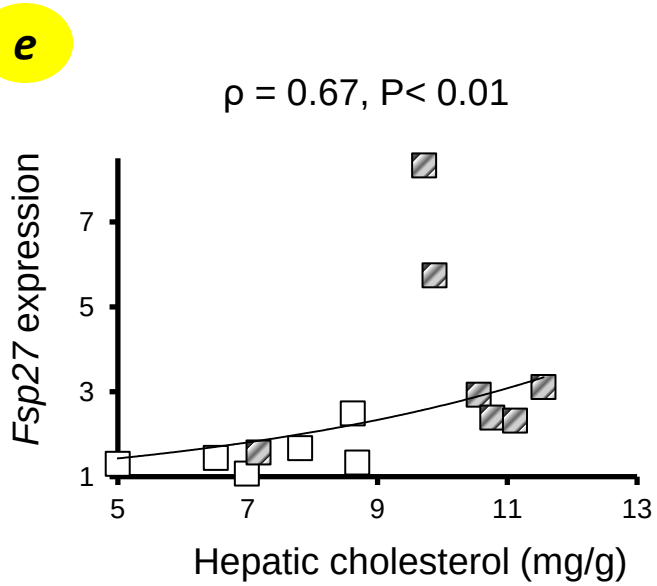
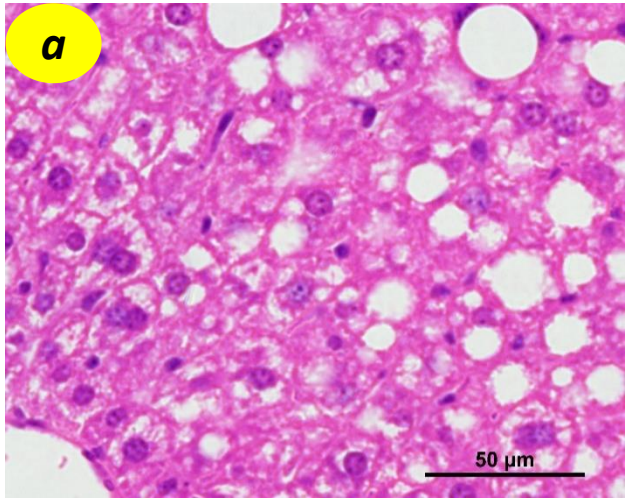
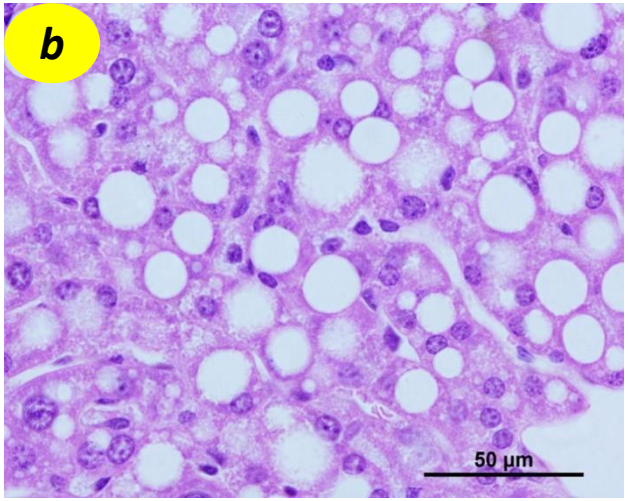

b
50 µm

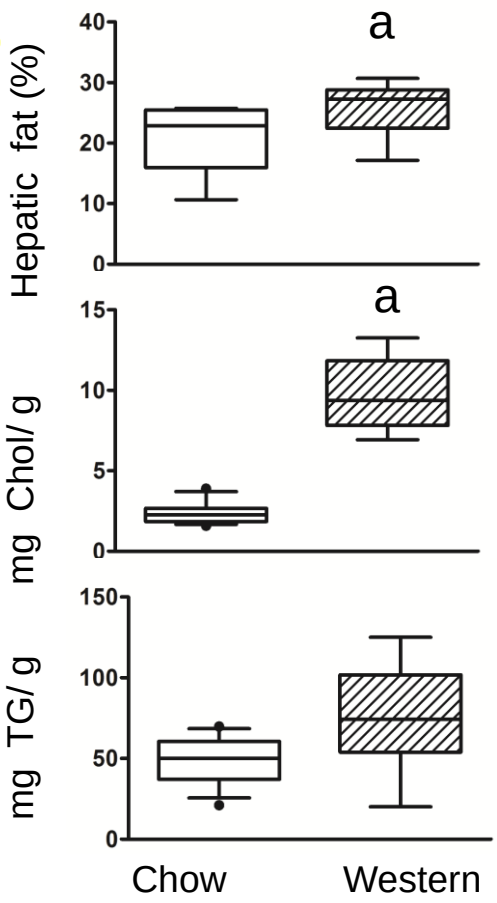

c
Hepatic fat (%)
20
15
10
5
0
a
mg Chol/ g
15
10
5
0
a
mg TG/ g
50
40
30
20
10
0
a
Control
Cholesterol

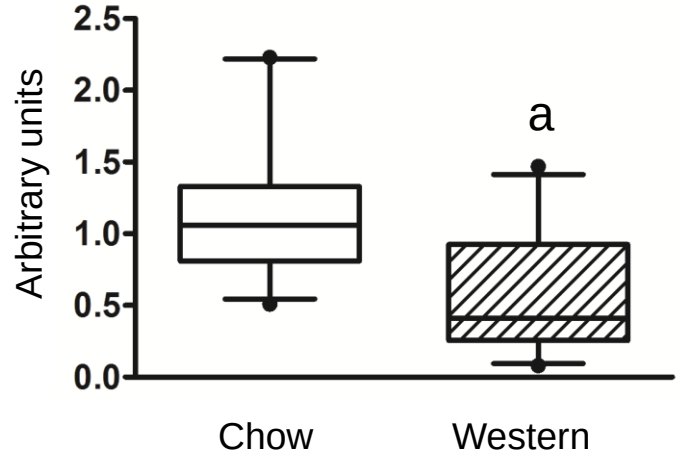

d
Arbtrary units
15
10
5
0
a
Control
Cholesterol

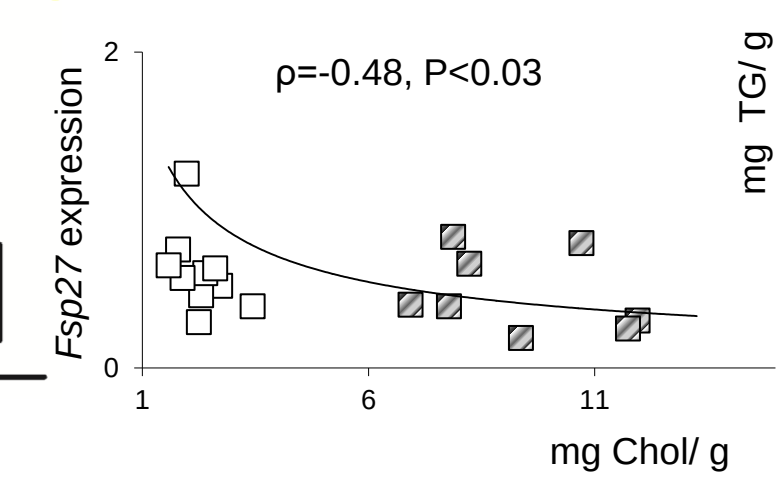

e
ρ = 0.67, P< 0.01
Fsp27 expression
7
5
3
1
5
7
9
11
13
Hepatic cholesterol (mg/g)

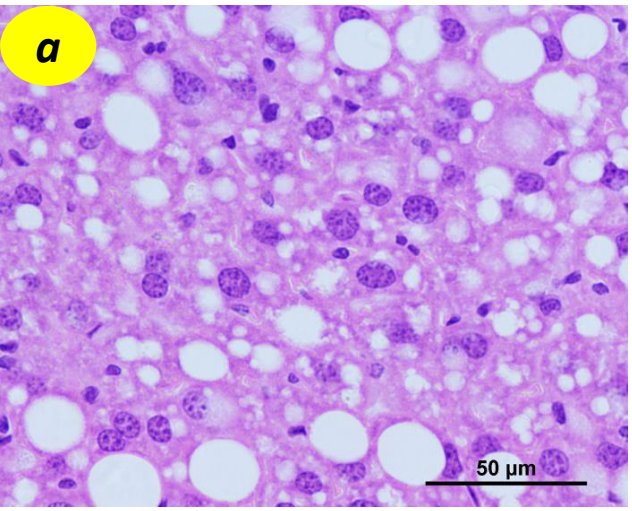

a
50 µm

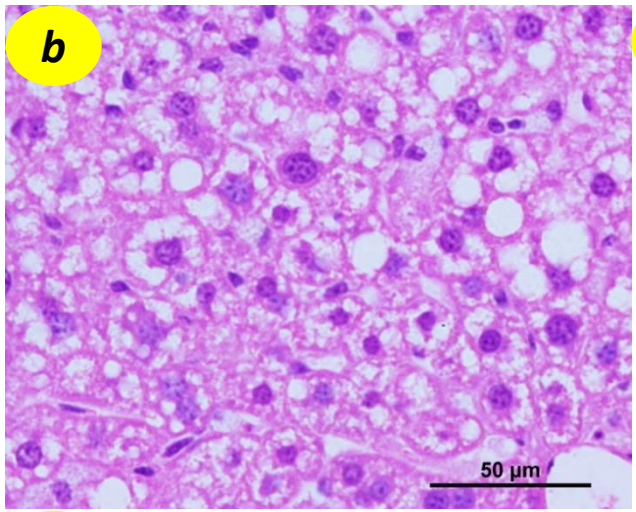

b
50 µm

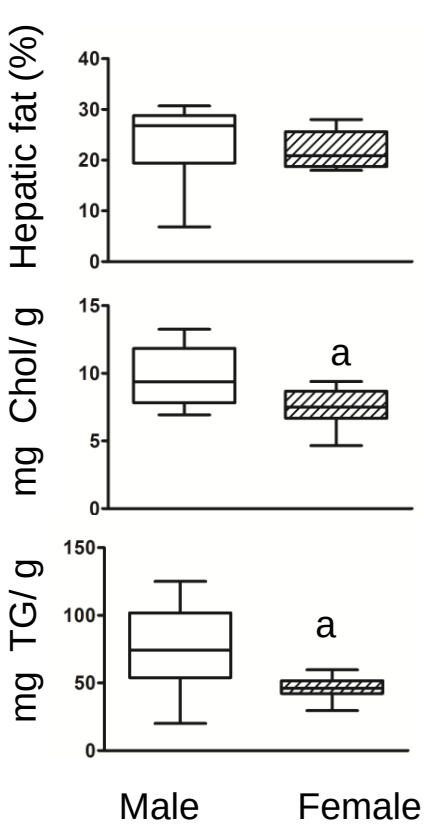

c
Hepatic fat (%)
40
30
20
10
0
a
mg Chol/ g
15
10
5
0
a
mg TG/ g
150
100
50
0
Chow
Western

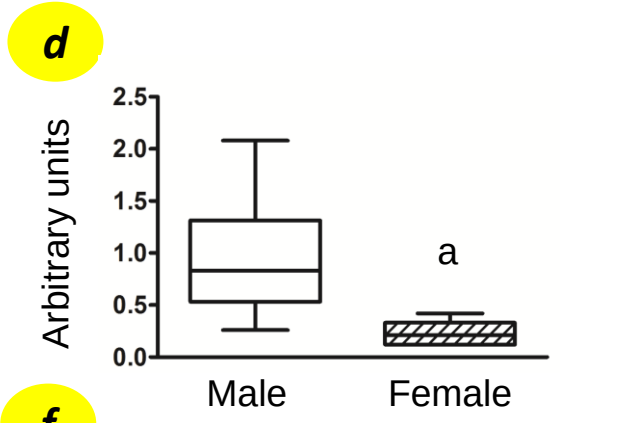

d
Arbitrary units
2.5
2.0
1.5
1.0
0.5
0.0
a
Chow
Western

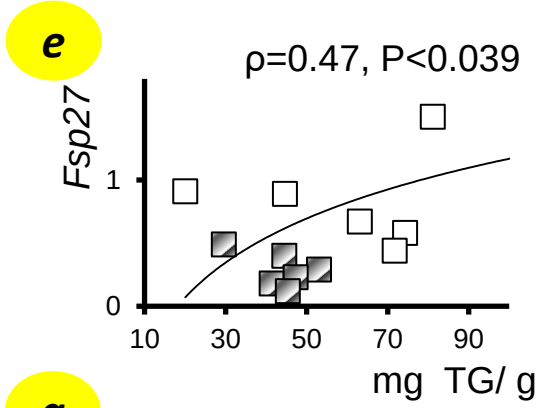

e
Fsp27 expression
2
0
ρ=-0.48, P<0.03
1
6
11
mg Chol/ g

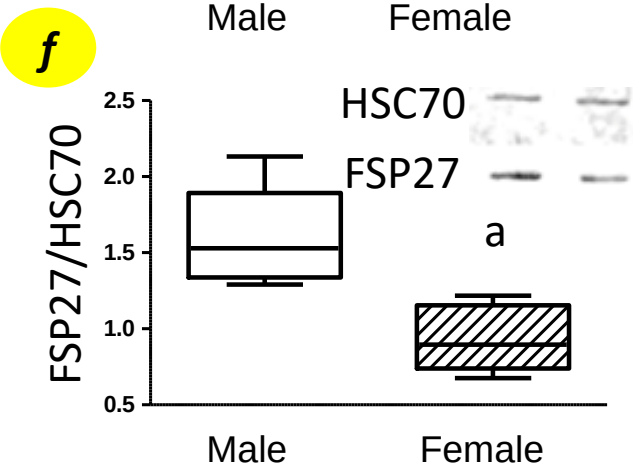

a
50 µm

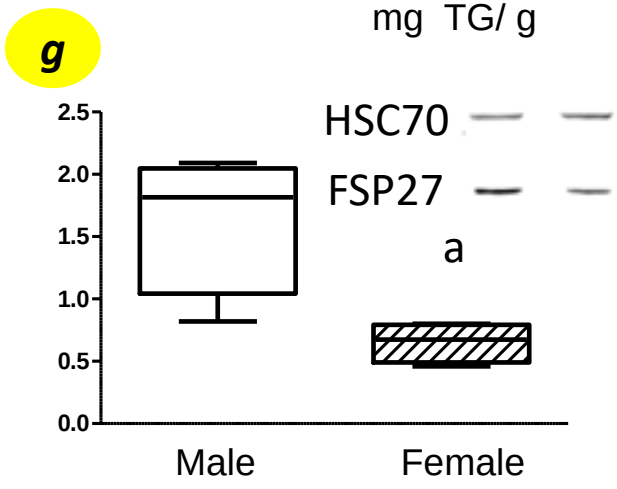

b
50 µm

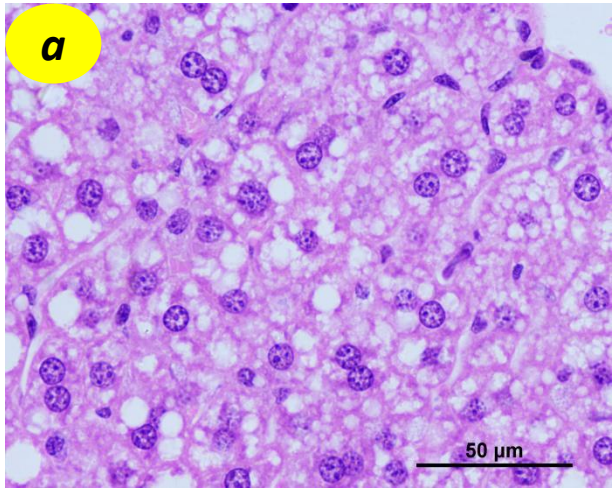

c
Hepatic fat (%)
40
30
20
10
0
mg Chol/ g
15
10
5
0
a
mg TG/ g
150
100
50
0
a
Male
Female

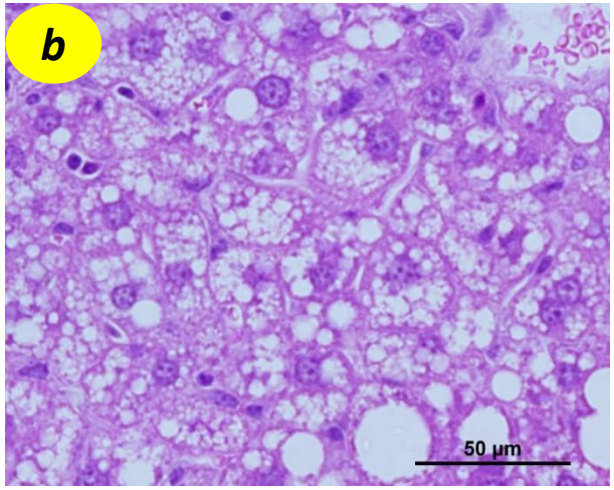

d
Arbitrary units
2.5
2.0
1.5
1.0
0.5
0.0
a
Male
Female

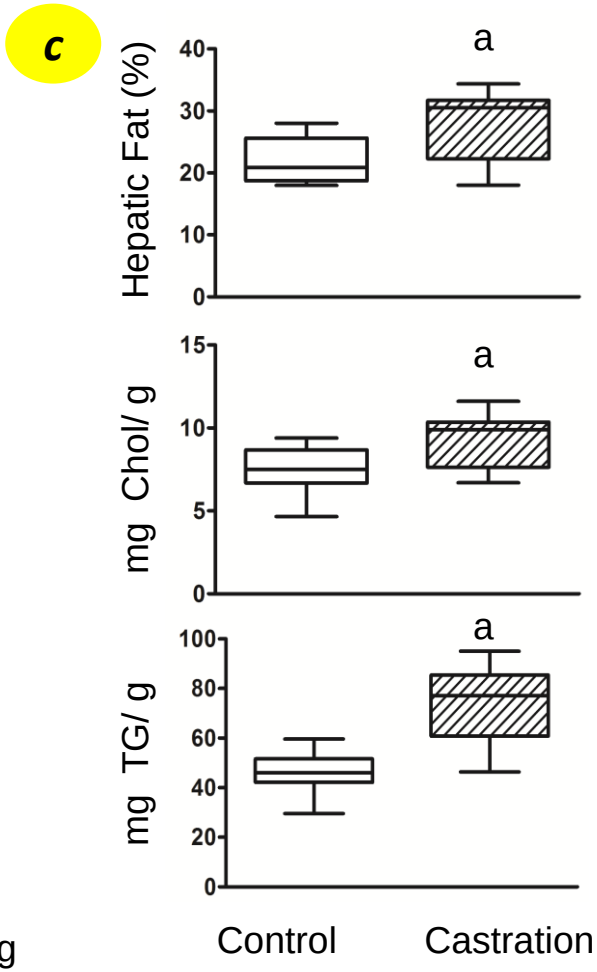

e
ρ=0.47, P<0.039
Fsp27
1
0
10
30
50
70
90
mg TG/ g

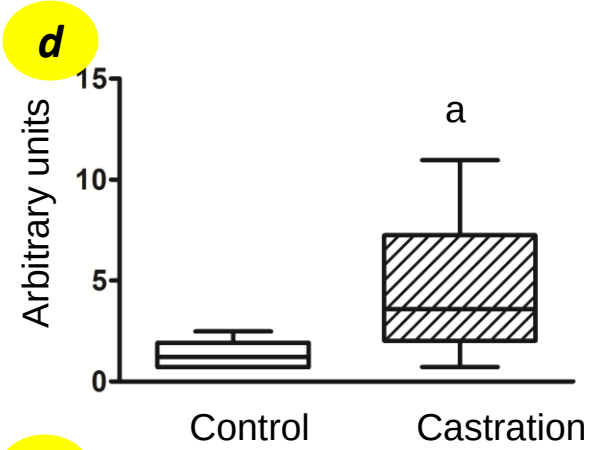

f
FSP27/HSC70
2.5
2.0
1.5
1.0
0.5
HSC70
FSP27
a
Male
Female

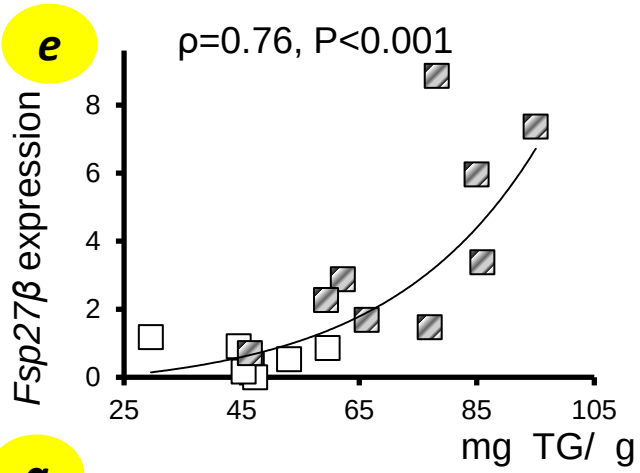

g
2.5
2.0
1.5
1.0
0.5
0.0
HSC70
FSP27
a
Male
Female

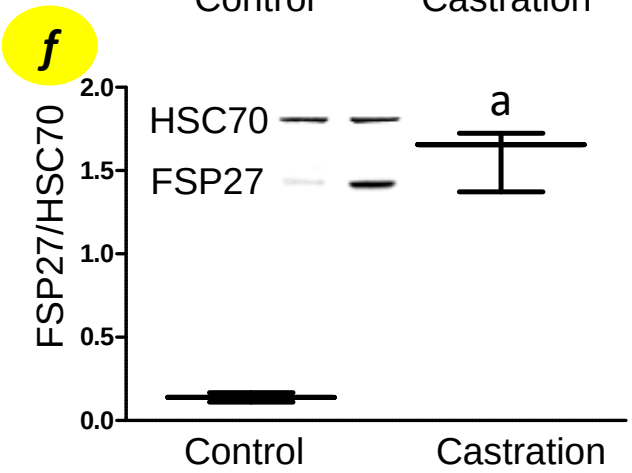

a
50 µm

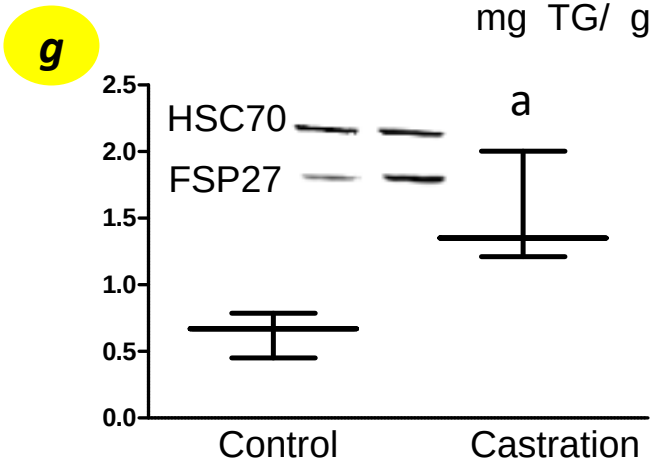

b
50 µm

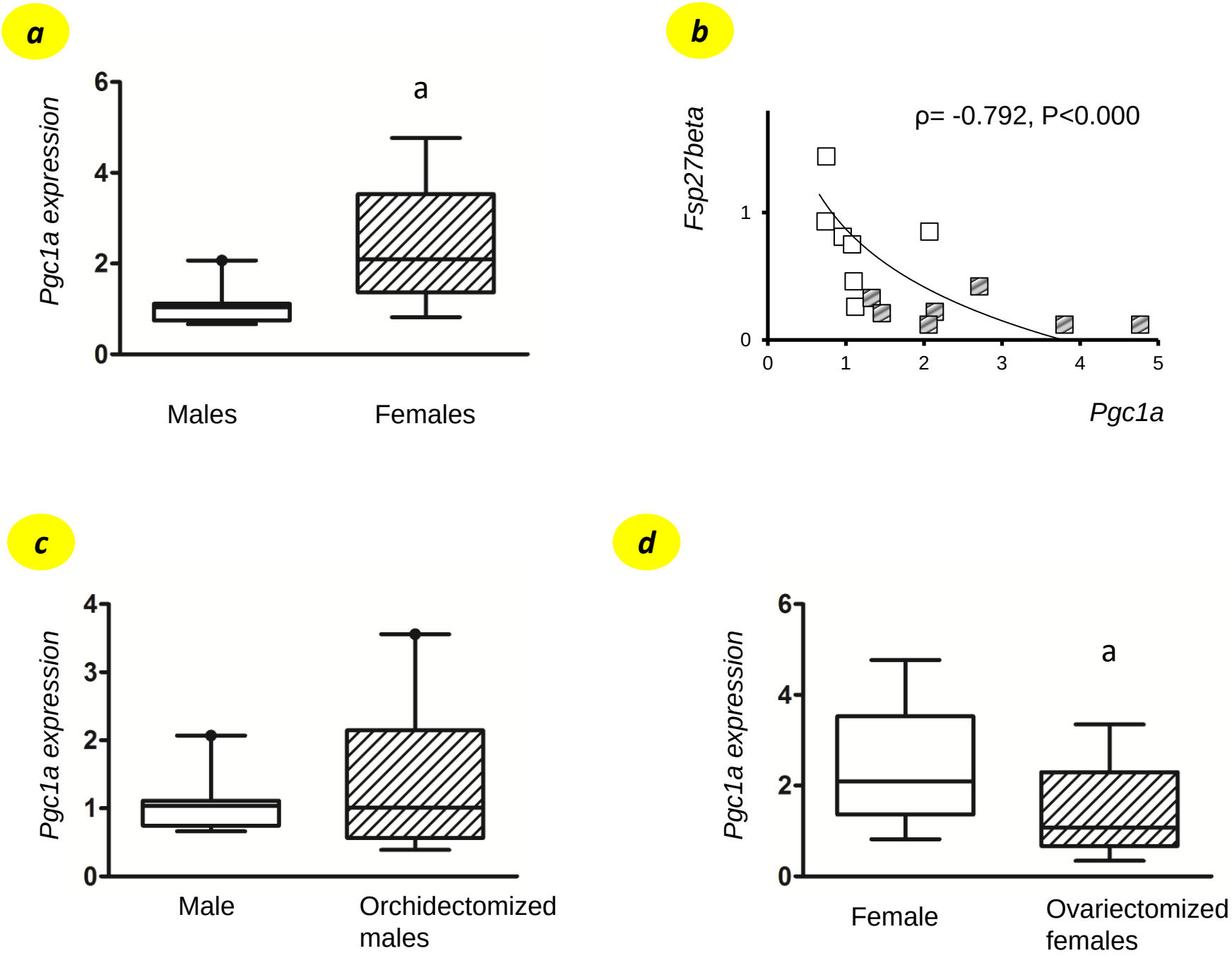

c
Hepatic Fat (%)
40
30
20
10
0
a
mg Chol/ g
15
10
5
0
a
mg TG/ g
100
80
60
40
20
0
a
Control
Castration

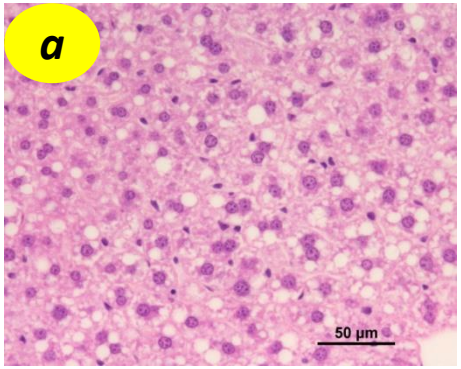

d
Arbitrary units
15
10
5
0
a
Control
Castration

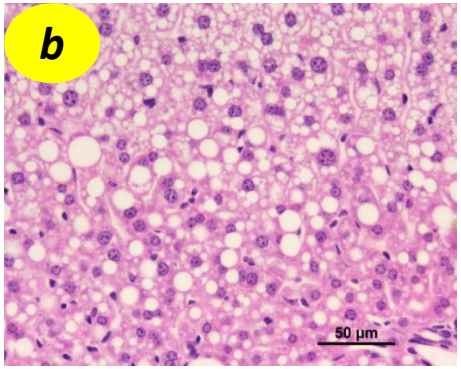

e
ρ=0.76, P<0.001
Fsp27β expression
8
6
4
2
0
25
45
65
85
105
mg TG/ g

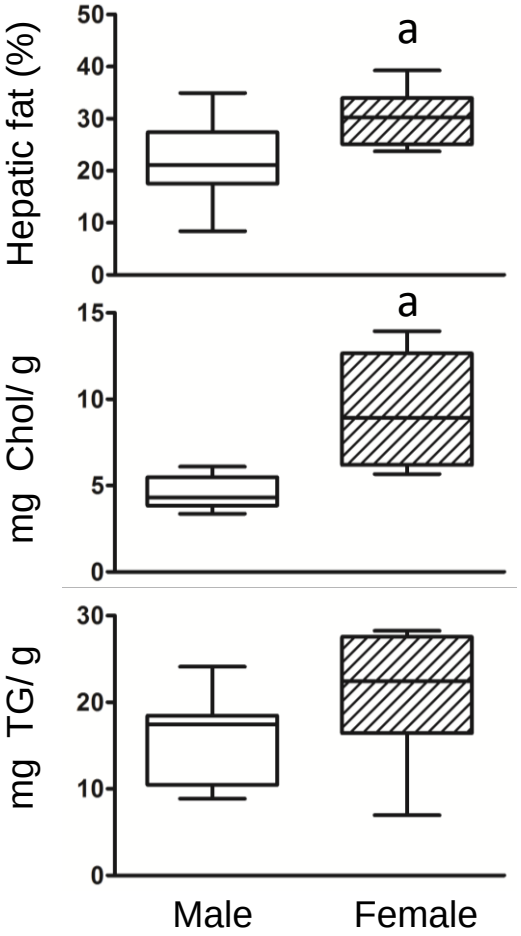

f
FSP27/HSC70
2.0
1.5
1.0
0.5
0.0
HSC70
FSP27
a
Control
Castration

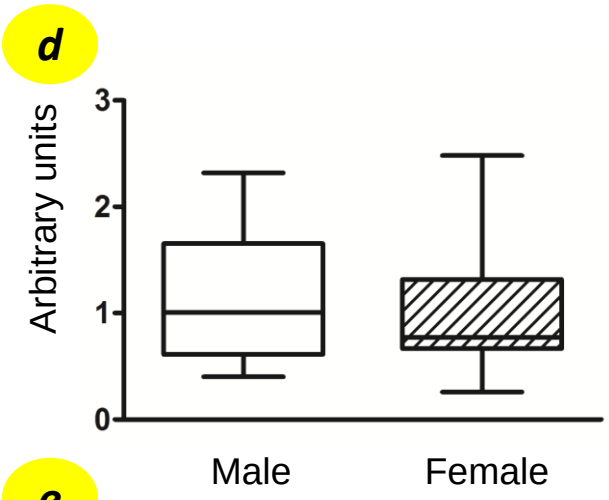

g
2.5
2.0
1.5
1.0
0.5
0.0
HSC70
FSP27
a
Control
Castration

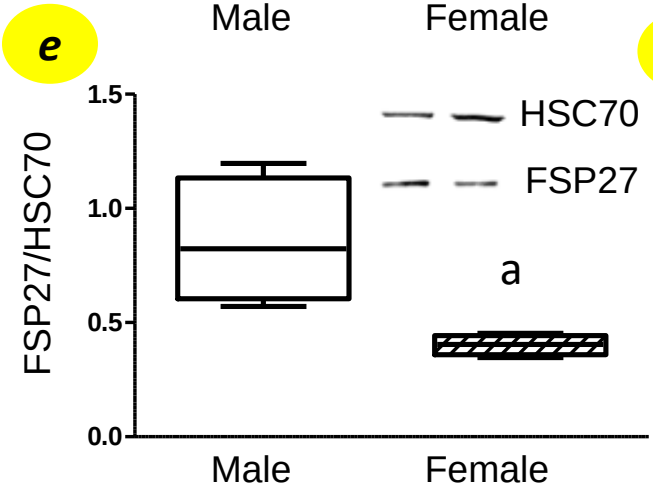

a
Pgc1a expression
6
4
2
0
a
Males
Females
b
Fsp27beta
ρ= -0.792, P<0.000
1
0
0
1
2
3
4
5
Pgc1a
c
Pgc1a expression
4
3
2
1
0
Male
Orchidectomized males
d
Pgc1a expression
6
4
2
0
a
Female
Ovariectomized females

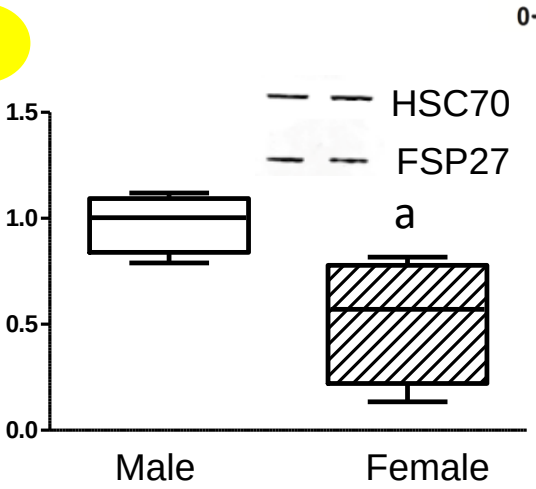

a
50 µm


b
50 µm


c
Hepatic fat (%)
50
40
30
20
10
0
a
mg Chol/ g
15
10
5
0
a
mg TG/ g
30
20
10
0
Male
Female


d
Arbitrary units
3
2
1
0
Male
Female


e
FSP27/HSC70
1.5
1.0
0.5
0.0
HSC70
FSP27
a
Male
Female


f
1.5
1.0
0.5
0.0
HSC70
FSP27
a
Male
Female