Role of obesity and estrogen deficiency in non-alcoholic fatty liver disease: Insights from a mouse model

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Abstract. The prevalence of non-alcoholic fatty liver disease (NAFLD) increases in post-menopausal women, driven by obesity and metabolic syndrome (MS). However, the pathogenesis of this interaction remains poorly understood. The present study investigated the interplay between obesity, menopause and NAFLD in a C57BL6/J mouse model of diet-induced obesity. The study included male and female animals, in which a subgroup of females underwent ovariectomy to simulate menopause. Mice were fed a high-fat diet for 6 months which resulted in them becoming overweight, and developing hyperglycemia and insulin resistance. The present study analyzed liver histology, inflammatory markers and hepatic lipid profiles. All obese animals showed liver steatosis, hepatocyte ballooning and fibrosis. Sex-related differences were observed, including: i) Obese male mice developed increased expression of inflammatory markers and altered lipid profile; ii) obese female mice exhibited less severe steatosis, hepatic inflammation and lipotoxicity, and iii) ovariectomized obese female mice exhibited exacerbated hepatic lipotoxicity and tissue damage. Ovariectomized obese female mice also had reduced triacylglycerol and cholesteryl ester levels, but increased levels of toxic intermediaries, such as free fatty acids, diacylglycerols and free cholesterol, elevated expression of NF-κB in the liver and increased levels of serum transaminases, indicating liver damage. These findings suggested that estrogen may protect against NAFLD progression by regulating lipid droplet formation, especially in the context of insulin resistance. More studies in the field are clearly needed to achieve a complete understanding of these pathways, which may serve to improve current therapies.

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Abbreviations: CE, cholesteryl esters; Chol, free cholesterol; DAG, diacylglycerol; FA, fatty acids; FAMEs, fatty acid methyl esters; FFA, free fatty acids; GC, gas chromatography; HFD, high-fat diet; HFD-OVX, ovariectomized animals on high-fat diet; IR, insulin resistance; LC-PUFAs, long-chain polyunsaturated fatty acids; MS, metabolic syndrome; MUFAs, monounsaturated fatty acids; n-3, omega-3 fatty acids; n-6, omega-6 fatty acids; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis disease; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PUFAs, polyunsaturated fatty acids; SD, standard diet; SD-OVX, ovariectomized animals on standard diet; SFAs, saturated fatty acids; TAG, triacylglycerol; TLC, thin-layer chromatography

Key words: non-alcoholic fatty liver disease, estrogen, mice, lipotoxicity, menopause

Introduction

Non-alcoholic fatty liver disease (NAFLD) has reached alarming proportions, affecting ~40% of the world population (1,2). NAFLD is currently the main cause of cirrhosis, hepatocellular carcinoma (HCC) and liver transplantation in the western world (3,4). Therefore, understanding the pathogenesis of NAFLD is crucial to establish an improved prevention and management of the disease.

An excess of fat accumulation in the liver characterizes NAFLD. The disease progresses through different stages, beginning with simple steatosis, with the accumulation of lipid droplets within hepatocytes. This may evolve to non-alcoholic steatohepatitis (NASH), a more severe stage marked by inflammation, that might lead to fibrosis and cirrhosis (3,4). Between 2-5% of the cases with steatosis may develop NASH, fibrosis and chronic liver disease (2,4). The mechanisms behind the progression from simple steatosis to NASH remain unclear.

The risk factors for NAFLD and NASH include obesity, insulin resistance, diabetes and dyslipidemia, among others. The excess of lipid accumulation and dysregulation of lipid metabolism may unfold lipotoxic pathways (5). In this context, the formation of lipid droplets may serve as a protective mechanism by sequestering lipids and preventing lipotoxicity (6-8). Triacylglycerides (TAG) and cholesterol esters (CE) form the neutral core of lipid droplets. Conversely, charged intermediaries such as free fatty acids (FFA), diacylglycerols (DAG) and free cholesterol (Chol) are not able to enter the neutral core of lipid droplets. Thus, to be included into the droplets they must first be incorporated into TAG and CE (9-11). The inclusion of these lipid intermediaries in lipid droplets avoids its toxic effects such as inflammation and disruption of membrane dynamics. For instance, FFA can activate the Toll-like 4 (TLR4) receptors (12) and DAG can promote the activity of protein kinase C (PKC) (13). In the same manner, high levels of Chol may alter the fluidity of cell membranes (14,15), promoting endoplasmic reticulum (ER) stress and inflammation (16,17). Therefore, the esterification of Chol to form CE is also essential to avoid lipotoxicity (18).

The analysis of sex differences is crucial for understanding NAFLD and NASH. Whereas the prevalence of NAFLD is markedly higher in men than in women, the disease becomes more frequent in women following menopause. This is probably due to the higher prevalence of obesity and metabolic syndrome (MS) at this stage of life (19-21). Although the mechanisms of this sex difference remain unknown, the lack of estrogen may play an essential role in the increased incidence of NASH and NAFLD after menopause (22).

The present study aimed to evaluate the role of menopause in the pathogenesis of liver damage in the context of obesity and insulin resistance. Therefore, the present study used a well-established murine model of obesity, including males and females, where a subgroup was ovariectomized to mimic menopause.

Materials and methods

Animal model. The present study utilized a phenotypic mouse model of obesity and insulin resistance, described in our previous publication (21). A total of 79 C57BL/6J mice (57 females and 22 males; age, ~6 weeks; weight, 16-20 g), were acquired from Charles River Laboratories, Inc. These mice were then randomized to receive either a standard diet (SD) or a high fat diet (HFD) for six months. For males, 10 animals were assigned to the SD group and 12 to the HFD group. For females, 25 animals were assigned to the SD group (8 of which were ovariectomized: SD-OVX) and 32 to the HFD group (12 of which were ovariectomized: HFD-OVX). Animals were housed in cages at a constant temperature of 22˚C under a 12-h light/dark cycle and 50% relative humidity in the animal facility at the University of La Laguna (Tenerife, Spain). Ovariectomies were performed at 2 months of age, after diet-randomization. During the follow-up period, animals underwent intraperitoneal glucose tolerance test and insulin tolerance test during the follow-up period (21). The sample size was determined based on previous studies (21,23) using similar models. To effectively implement the 3Rs principles without compromising statistical power, 8-12 mice were initially included per group to mitigate potential losses during follow-up. Since female mice underwent a surgical procedure, more female mice were included at baseline than male to prevent a reduction in number due to surgical or post-surgical complications. All animals were euthanized by an overdose of sodium pentobarbital (100 mg/kg) and death was confirmed by cervical dislocation method. Animal care was performed in accordance with institutional guidelines in compliance with Spanish (Real Decreto 53/2013, February 1. BOE, February 8, 2013, n: 34, p. 11370-11421) and international laws and policies (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes). All procedures were approved by the Institutional Animal Care and Use Committee (Comité de Ética de la Investigación y de Bienestar Animal) of University of La Laguna (approval number CEIBA2021-3107).

Diet composition. The HFD provides 60% of the calories from fat (cat. no. D12492; Research Diets Inc.). This HFD diet had 6-times more total lipid content than the SD. Furthermore, the fatty acid (FA) content was 7-fold higher in the HFD compared with SD (Table SI). The details of the diets have been described in a previous article (23).

Liver histology. Slides from liver tissue in paraffin were stained with hematoxylin-eosin and Sirius Red to evaluate steatosis, ballooning, inflammation and fibrosis. A pathologist specialized in liver diseases (SGH) evaluated all samples following the 'SAF index' published by Bedossa in 2012 (24).

Steatosis. The percentage of hepatocytes with lipid vacuoles was classified as S0 (minimal or non-existent): <5%; S1 (mild): 5-33%; S2 (moderate): 34-65%; and S3 (intense): >65%.

Lobular necroinflammatory activity and ballooning. Lobular inflammation was classified in 3 grades: I0, minimal lobular necroinflammation; I1, two lobular inflammatory foci in one field; I2, globular activity with multiple clusters in one field. Lobular inflammation was detected under a light microscope (magnification, x200; 20x objective). The pathologist examined a fraction of the liver and chose a field representing the sample. Hepatocyte ballooning was classified in 3 grades; B0: no ballooning, B1 when the hepatocytes had rounded edges while maintaining their size and B2 when the size of the cell is twice the size of a normal hepatocyte.

Liver fibrosis. Fibrosis in liver tissue was determined using Picrosirius Red staining. Liver sections (3 μm) were deparaffinized in xylene and rehydrated through a graded series of alcohol concentrations (100, 90 and 70%). The sections were covered in Picrosirius Red solution and incubated at room temperature for 60 min. Following incubation, the slides were

washed with 0.5% acetic acid solution and 100% ethanol. Finally, tissues were dehydrated and mounted. The slides were observed, and images were captured under a digital light microscope (magnification, x400; 40x objective). The area of positively red-stained collagen was quantified using ImageJ version 1.52k software (National Institutes of Health).

Serum analysis. Serum TAG and Chol were measured using an enzymatic colorimetric test. Hepatic aspartate (AST; cat. no. ACN 8687; Roche Diagnostics) and alanine aminotransferase (AST; cat. no. ACN 8685; Roche Diagnostics) transaminases were measured by spectrophotometric analysis. All analyzes were performed in a Cobas c711 Module (Roche Diagnostics).

Inflammation markers in liver tissue. SDS-PAGE and western blotting of liver proteins were performed as follows: Liver homogenates were prepared with RIPA buffer and disaggregated using the System Polytron PT 1200 E (Kinematica AG), followed by sonication for 3 min at 80% amplitude with a cycle of 15 sec on/off at a frequency of 20 kHz and 4˚C using a Qsonica Q800R3 sonicator (Thermo Fisher Scientific, Inc.). Homogenates were then centrifuged at 14,000 x g for 10 min at 4˚C. Supernatants were collected and protein concentration was determined by using a BCA protein assay kit (MilliporeSigma). Gels (10-12% acrylamide) were loaded with 50 μg of total protein per sample, then transferred to nitrocellulose membranes (Whatman-Protran; Merck KGaA). Membranes were blocked with 5% non-fat milk for 1 h at room temperature and incubated overnight with the respective primary antibody at 4˚C. The primary antibodies and dilutions used were TNF-α (1:1,000; BS2081R; BIOSS), IL-1β (1:500; P420B; Invitrogen, MA, USA) and NF-κβ p-65 (1:500; ab16502; Abcam). Membranes were washed in TBS-0.1% Tween and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000; cat. no. ab6721; Abcam) for 1 h at room temperature. Finally, membranes were developed with Clarity ECL Western Blotting Substrate (Bio-Rad Laboratories, Inc.) and images acquired using ImageQuant LAS 4000 mini digital imaging system (General Electric). α-Tubulin or β-Actin were used as controls.

Lipid analysis of liver tissue

Total lipid content. Lipids were extracted with chloroform/methanol (2:1 v/v) from a wedge of fresh liver tissue (~150/200 mg) following the Folch method adapted by Christie (25). Further details of the lipid extraction method were published previously (23).

Lipid classes. Aliquots of 30 μg from total lipid extracts were used for the analysis. Lipid classes were separated by high-performance thin-layer chromatography (HPTLC) in a single-dimensional double-development following the Olsen and Henderson method (26). Further details of the method were published previously (23).

Fatty acid methyl esters (FAMEs) from total lipids. 1 mg of the total lipid extract was subjected to acid-catalyzed transmethylation using toluene and sulfuric acid in methanol, and was incubated for 16 h at 50˚C to obtain the fatty acid methyl esters (FAMEs) profile. Subsequently, FAMEs were extracted and purified by thin-layer chromatography (TLC) and stored under a 100% nitrogen atmosphere to prevent sample oxidation. Finally, FAMEs were analyzed by gas chromatography-mass spectrometry (GC-MS; Agilent 7890A/7010B; Agilent Technologies, Inc.) and identification was performed according to retention times by library matching with NIST v.2.2 (Agilent Technologies, Inc.). Quantification was performed using the selected ion-monitoring mode and the results are expressed as relative percentage areas. Further details of the method are provided in a previously published article (23).

Composition of FAMEs in lipid classes. Aliquots of 2-4 mg of lipid extracts were loaded into TLC 20x20 cm silica plates. Lipid classes were isolated in a single-dimensional double-development following the Olsen and Henderson method (26). The lipid classes (LCs) were stained through spraying with dichlorofluorescein. Then, the samples were visualized under UV light and scraped from the silica plate. Finally, the LCs in the silica were transmethylated at 50˚C for 16 h to obtain FAMEs and the four major LCs were selected: Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and TAG. The quantification of the samples was performed by GC-MS following the aforementioned method. Further details can be found elsewhere (23).

Statistical analysis. Quantitative variables are expressed as mean ± standard deviation. For males, continuous variables between groups were compared with unpaired Student's t-test, or non-parametric Mann-Whitney test if the conditions of normality were not met. For females, two-way ANOVA was performed, followed by Tukey's multiple comparisons test, Tamhane's T2 test or Dunnett's T3 test (if variables were not homoscedastic) as post-hoc tests. Pairwise comparisons were performed using Mann-Whitney test with Bonferroni correction if variables were not normally distributed. Fisher's exact test was used to compare proportions between groups. Sample size was calculated based on previous studies (21,23) using similar obesity and ovariectomized models in which 6 to 10 cases should reach the end of the study. The present used SPSS Statistics 20 (IBM Corp.) and GraphPad Prism 9 (Dotmatics) for statistical analyzes and data plots, respectively. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Obesity and metabolic syndrome. The model was reported previously (21). Briefly, i) all mice on HFD developed obesity and MS, ii) males reached higher weight than females and iii) only obese males and obese ovariectomized females developed hyperglycemia and insulin resistance (21).

Liver histology

Females. Of the animals on HFD-OVX and HFD, 70 and 100% showed steatosis, respectively ($P>0.05$), classified as severe (27 and 57%), moderate (27 and 15%) or mild steatosis (13 and 29%). Animals on HFD-OVX and HFD developed hepatocyte ballooning (67 and 85%), maintaining their size (60 and 57% in B1 stage) or increasing twice or more the hepatocyte size (7 and 28% in B2 stage, respectively; Table SII; Fig. 1). The HFD-OVX mice showed a mild increase in

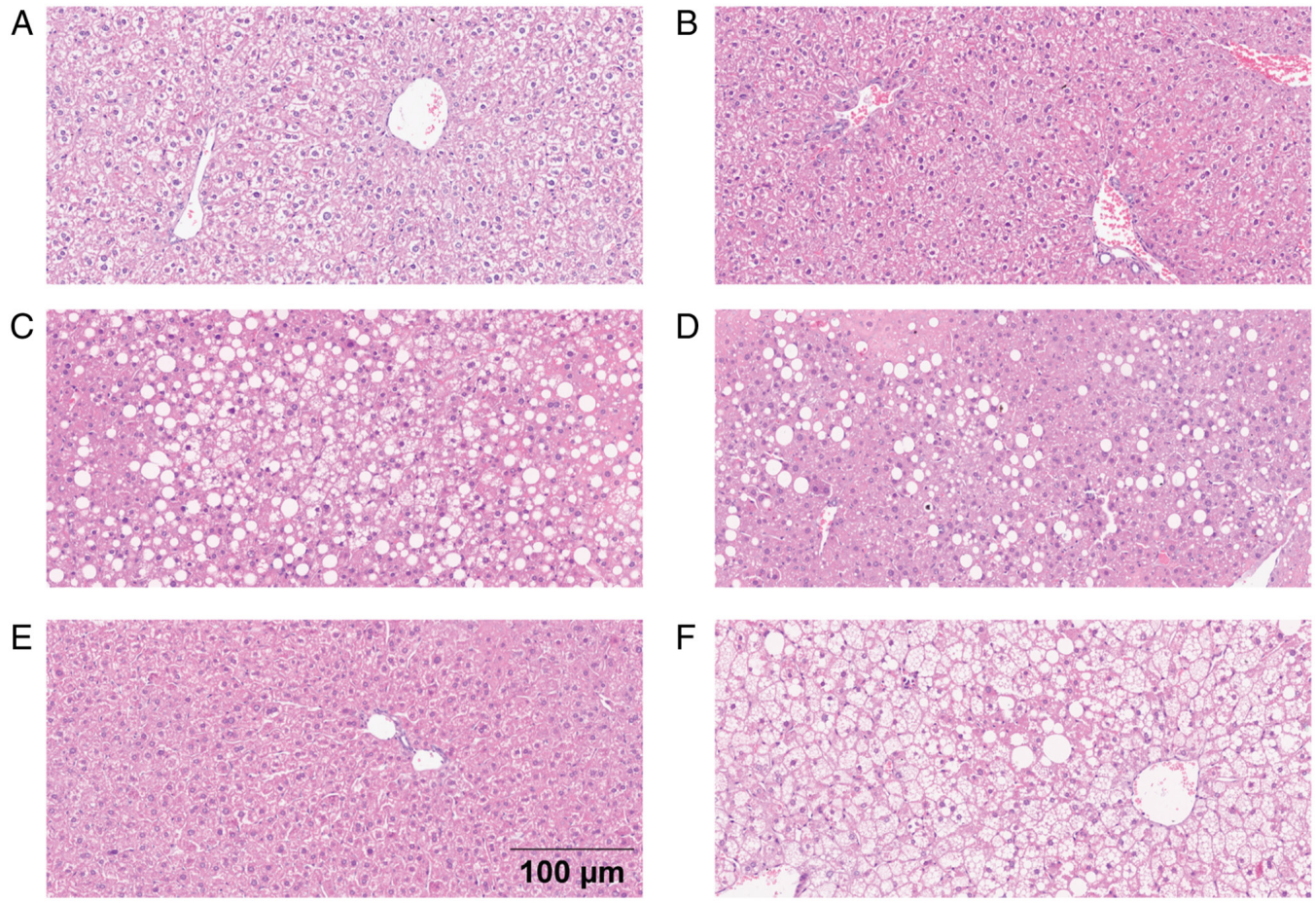


Figure 1. Representative images of liver sections of female and male mice. Females on (A) SD, (B) SD-OVX, (C) HFD and (D) HFD-OVX. Males on (E) SD and (F) HFD. SD, standard diet; HFD, high-fat diet; OVX, ovariectomized.

collagen staining compared with SD-OVX (1.28 vs. 0.99%; P=0.0109; Fig. S1). SD-OVX animals presented lower collagen staining in comparison with SD animals (0.99 vs. 1.61%; P=0.0106; Fig. S1). Despite the statistical differences between groups, the observed fibrotic area was minimal, representing only 1-2% of the total (Fig. S1). No differences were found between SD and SD-OVX whereas animals on HFD and HFD-OVX had higher steatosis and ballooning than those on SD and SD-OVX, respectively (Table SII).

Males. Most animals on HFD developed steatosis (94%, P<0.0001) with the following distribution: Severe (53%), moderate (29%) and mild (12%), and ballooning (94%; P<0.0001) where ~50% maintained the size of the hepatocyte and the other 50% doubled the size at least (Fig. 1). Male animals on HFD presented an average of 1.10% of area with positive collagen staining, while male animals in SD an average of 0.77% (P=0.0124; Fig. S1). Despite the statistical differences between groups, the observed fibrotic area was minimal, representing only 1-2% of the total (Fig. S1). Finally, animals had comparable low levels of necroinflammation between groups (Table SII).

Inflammation markers in liver tissue

Females. The HFD-OVX group showed upregulation of NF-κβ p-65 compared with HFD (P=0.013), SD-OVX (P=0.008) and SD (P=0.049) groups. The HFD-OVX also showed lower TNF-a compared with HFD (P=0.002) and SD-OVX (P=0.041). HFD-OVX mice showed lower levels of IL-1B compared with HFD (P=0.048) and SD-OVX (P=0.032). Finally, both HFD and SD-OVX animals had an increase in TNF-α compared with SD group (P<0.001, Fig. 2A).

Males. The HFD group showed higher levels of NF-κβ p-65 (P=0.032), TNF-α (P=0.005) and IL-1β compared with controls (P=0.006, Fig. 2B).

Serum analysis

Females. The HFD-OVX showed higher levels of AST (P=0.002) and ALT (P=0.024) compared with the SD, SD-OVX and HFD groups (Fig. 3A). HFD and HFD-OVX both showed higher total Chol levels compared with controls (Fig. 3B). No differences were observed in the TAG levels among groups.

Males. HFD and SD groups had comparable levels of AST, ALT and TAG (Fig. 3C and D). The HFD group displayed higher levels of serum total Chol (P=0.041; Fig. 3D).

Liver lipidomics

Total hepatic lipid content in females. HFD-OVX had higher total lipid content than SD-OVX (9.36±3.38 vs. 4.59±0.71; P<0.001) but lower than the HFD group (9.36±3.38 vs. 13.28±5.04; P=0.027; Fig. 4A; Table SIII).

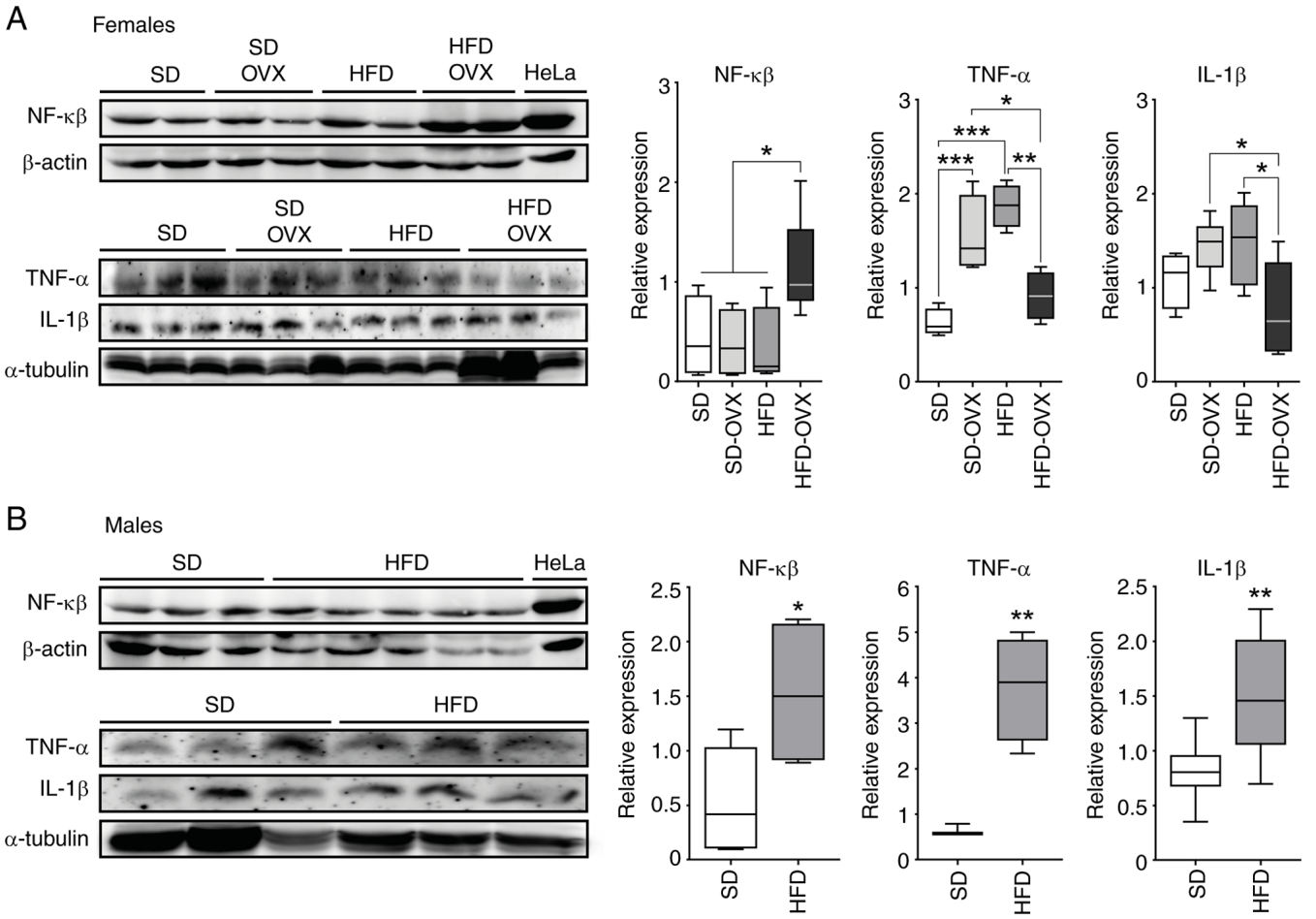


Figure 2. Western blot analysis of inflammatory markers in liver. The figure shows the relative expression of NF-κβ, TNF-α and IL-1β for (A) females and (B) males in liver homogenates as indicated. Tukey plots indicate the median as the horizontal line, the top and bottom of the box show the upper and lower quartiles, maximum and minimum values are displayed as whiskers. *P<0.05; **P<0.01; ***P<0.001. SD, standard diet; HFD, high-fat diet; OVX, ovariectomized.

Total hepatic lipid content in males. The HFD group showed higher total lipids compared with the SD (13.92±6.14 vs. 4.82±0.74, P<0.001; Fig. 5A; Table SIII). Values correspond to the mean mg total lipid/100 mg fresh tissue ± SD.

Hepatic lipid classes profile in females. HFD-OVX showed higher levels of FFA (0.54±0.12 vs. 0.36±0.08; P=0.044) and DAG (0.42±0.14 vs. 0.20±0.11; P=0.014) but lower CE (1.06±0.35 vs. 1.76±0.62; P=0.004) and TAG (3.95±2.02 vs. 7.91±4.77; P=0.045) compared with the HFD group (Fig. 4B and C; Table SIII). Animals on HFD showed higher levels of Chol (0.69±0.10 vs. 0.42±0.07; P=0.002), CE (1.76±0.62 vs. 0.57±0.12; P=0.002) and TAG (7.91±4.77 vs. 0.92±0.23; P=0.006) compared with the SD group.

Hepatic lipid classes profile in females. The HFD group showed increased FFA compared with SD group (0.86±0.21 vs. 0.38±0.10, P<0.001), DAG (0.55±0.16 vs. 0.13±0.04, P<0.001), TAG (9.06±4.93 vs. 1.02±0.35, P<0.001), Chol (0.95±0.17 vs. 0.57±0.09, P<0.001) and CE (0.96±0.44 vs. 0.41±0.12, P<0.05) (Fig. 5B and C; Table SIII).

FAMEs profile of total lipids

Females. The HFD-OVX had lower 16:0 but higher 18:2 n-6 and 18:3 n-6 compared with the SD group (Fig. 4D; Table SIV). The HFD group showed lower levels of monounsaturated fatty acids (MUFA), omega-6 long-chain polyunsaturated fatty acids (n-6 LC-PUFA) and 18:1 n-9/18:0 ratio compared with animals on SD (Table SIV). Finally, the total FAMEs profile of the HFD group showed lower 18:1 and 20:4 n-6 (arachidonic acid; ARA) but higher 18:0, 18:2 n-6, 18:3 n-6 and 18:3 n-3 compared with the SD group (Fig. 4D; Table SIV).

Males. The FA profile showed that the HFD group had lower SFA (53.36±5.83 vs. 59.64±2.13; P<0.001) and n-6 LC-PUFA (4.98±1.95 vs. 8.71±1.98; P<0.001) but also higher MUFA (25.17±4.68 vs. 17.37±4.77, P<0.001) and 18:1 n-9/18:0 ratio (2.88±1.44 vs. 1.12±0.47; P<0.001) compared with SD (Fig. 5D; Table SIV). The HFD group also showed lower 18:0, 20:4 n-6 and 22:6 n-3 (docosahexaenoic acid; DHA), and higher 14:0, 18:1, 20:1, 18:2 n-6, 18:3 n-6, 20:2 n-6, and 18:3 n-3 compared with the SD group (Fig. 5D; Table SIV).

FAMEs profile of lipid classes

Females. HFD-OVX showed lower 16:0 in PC and higher 18:0 dimethyl acetal (DMA) in PE compared with all other groups. The HFD group had lower 20:3 n-6 in PC, PI and PE) 16:1 and 18:1 (in PC and PE), and higher 18:0 (in PC and PE) and 22:6 n-3 (in PC) compared with the SD (Tables SV, SVI and SVII). HFD and HFD-OVX groups both had in TAG a lower SFA and MUFA (14:0, 16:1 and 18:1) and higher polyunsaturated (PUFAs) profile (20:3 n-6, 20:4 n-6, 22:4 n-6, 22:5 n-6 and 22:6 n-3) compared with SD and SD-OVX (Table SVIII).

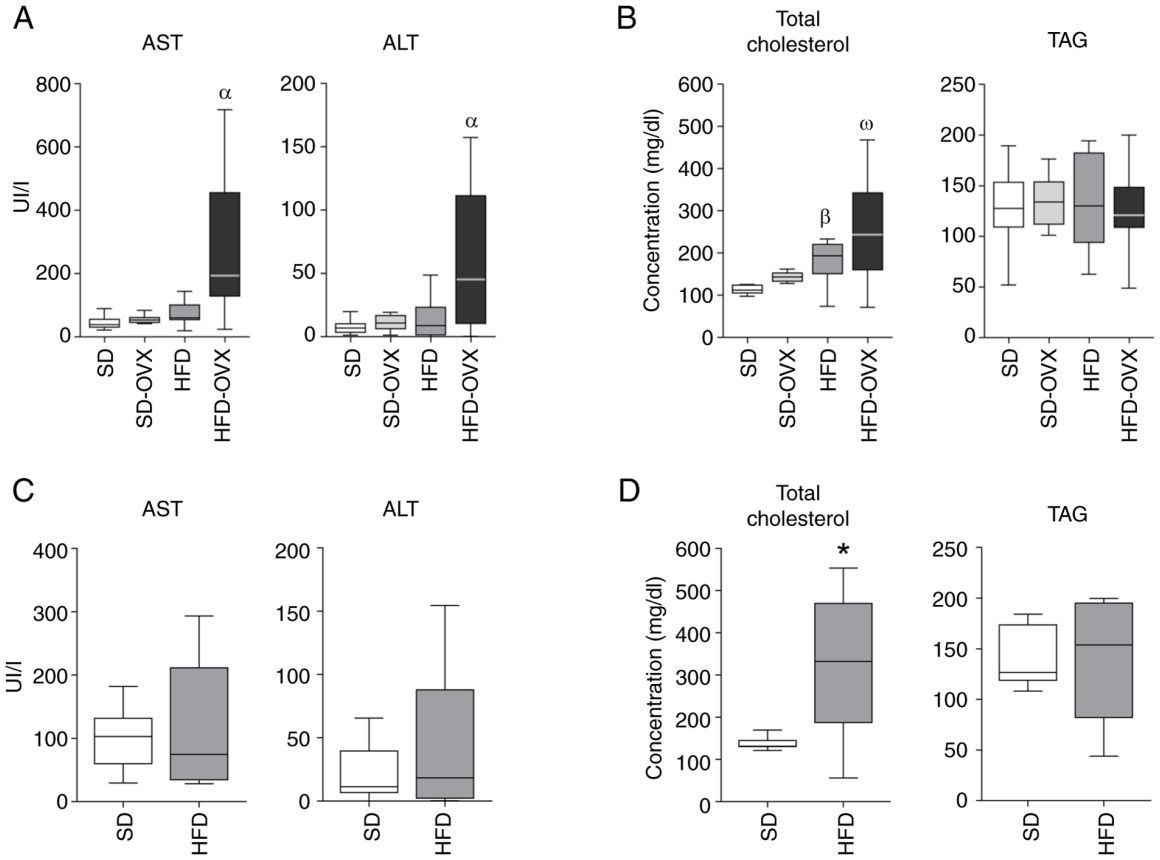


Figure 3. Serum biochemical analysis. (A) Serum transaminases in females. (B) Serum Chol and TAG levels in females. (C) Serum transaminases in males. (D) Serum Chol and TAG levels in males. Boxplots indicate the median as the horizontal line, the top and bottom of the box show the upper and lower quartiles, maximum and minimum values are displayed as whiskers. Female significance: $^{\alpha}$HFD-OVX vs. HFD, SD and SD-OVX, P<0.05. $^{\beta}$HFD vs. SD, P<0.05, $^{\omega}$HFD-OVX vs. SD-OVX and SD, P<0.05. Male significance: *HFD vs. SD, P<0.05. Chol, free cholesterol; TAG, triglyceride AST, aspartate aminotransferase; ALT, alanine aminotransferase; SD, standard diet; HFD, high-fat diet; OVX, ovariectomized.

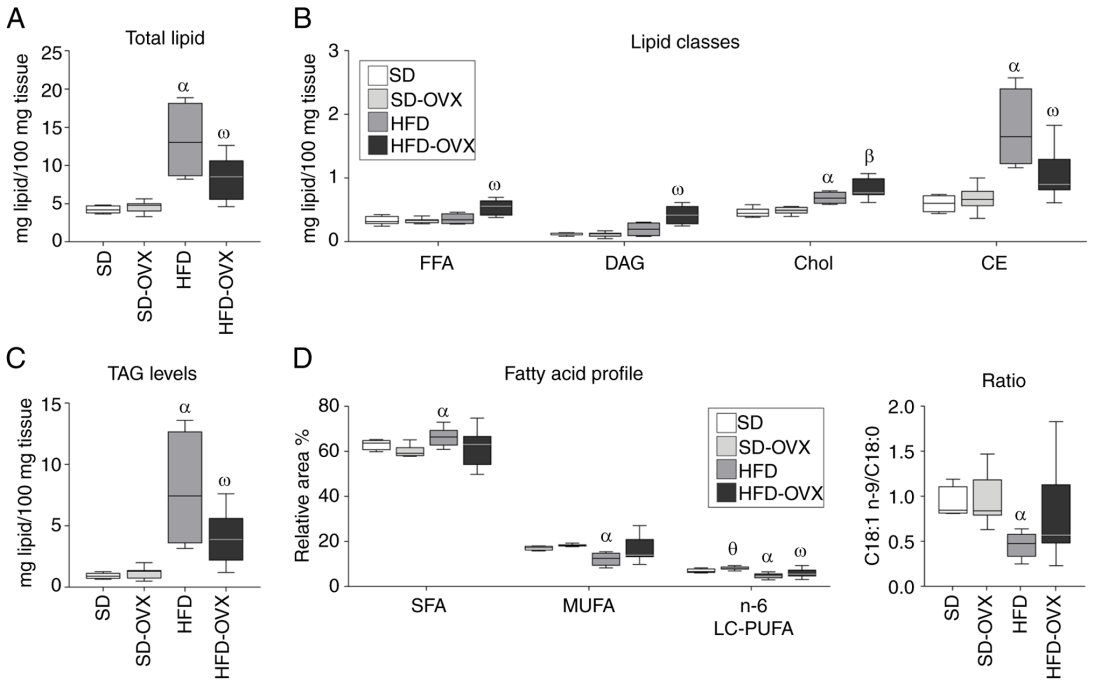
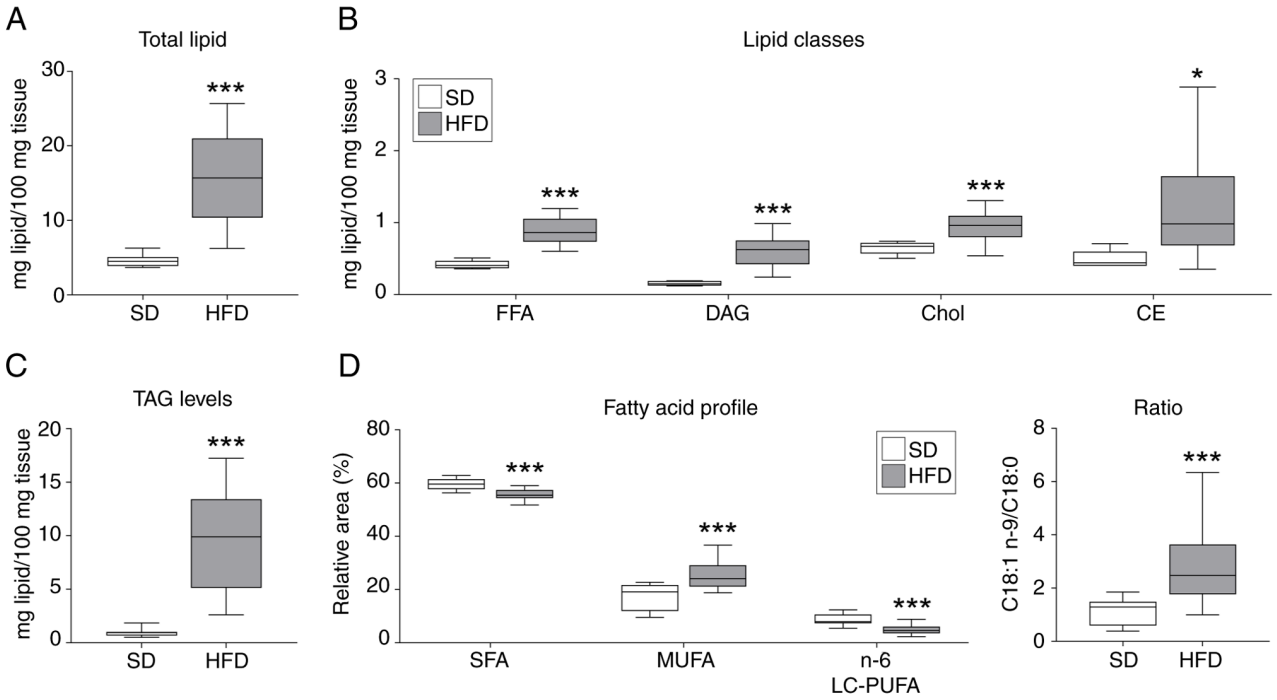


Figure 4. Lipidomic profile of liver tissue in female mice. (A) Total lipid content. (B) Lipid classes profile. (C) TAG levels. (D) Fatty acid profile of females as indicated in the figure. Boxplots show the median as the horizontal line, the top and bottom of the box show the upper and lower quartiles, maximum and minimum values are displayed as whiskers. Statistical significance: $^{\alpha}$HFD vs. SD, P<0.05, $^{\beta}$HFD-OVX vs. SD-OVX and SD, P<0.05. $^{\omega}$HFD-OVX vs. SD, SD-OVX and HFD, P<0.05. $^{\theta}$SD-OVX vs. SD, P<0.05. TAG, triglycerides; FFA, free fatty acid; DAG, diglycerides; Chol, free cholesterol; CE, cholesterol esters; SD, standard diet; HFD, high-fat diet; OVX, ovariectomized.



Males. The HFD had lower 20:1 (in PC, PE and PI), 16:0, 16:1, 18:2 n-6 and 22:5 n-3 (in PC and PE) and higher 18:0, 20:4 n-6, 22:6 n-3 (in PC and PE) and 18:0 DMA (in PE) compared with SD (Table SV, SVI and SVII). In TAG, the HFD group had lower SFAs and MUFAs (14:0, 16:0, 16:1) and higher PUFAs (18:3 n-3, 20:2 n-6, 20:4 n-6, 22:4 n-6 and 22:6 n-3) compared with SD (Table SVIII).

Figure 5. Lipidomic profile of liver tissue in male mice. (A) Total lipid content. (B) Lipid classes profile. (C) TAG levels. (D) Fatty acid profile as indicated in the figure. Boxplots show the median as the horizontal line, the top and bottom of the box show the upper and lower quartiles, maximum and minimum values are displayed as whiskers. *P<0.05; ***P<0.001. TAG, triglycerides; FFA, free fatty acid; DAG, diglycerides; Chol, free cholesterol; CE, cholesterol esters; SD, standard diet; HFD, high-fat diet; OVX, ovariectomized; SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; LC-PUFAs, long-chain polyunsaturated fatty acids.

Discussion

The main finding of the present study was that ovariectomy in obese female mice caused severe hepatic lipotoxicity and liver damage. Only obese males and obese-ovariectomized females developed insulin resistance (21) and impaired lipid droplet formation with a consequent accumulation of toxic lipid intermediaries (FFA, DAG and Chol). These markers of hepatic lipotoxicity were associated with inflammation in both groups. Notably, only obese-ovariectomized females had signs of liver injury in serum analysis, evidenced by higher transaminases levels. These findings suggest that the lack of estrogen may lead to detrimental effects in the context of obesity and insulin resistance, thus potentially promoting chronic liver damage.

The present study used a well-established model of obesity and metabolic syndrome to investigate the effects of these conditions on hepatic lipid metabolism. Furthermore, to examine the role of estrogen in the progression of liver damage, the present study performed ovariectomy on a subgroup of female mice (21,23). As expected, obese female (both ovariectomized or not) and male mice exhibited liver steatosis, hepatocyte ballooning, mild fibrosis and expression of pro-inflammatory markers. Importantly, while chronic changes (especially fibrosis) were minimal, severe steatosis and ballooning were the most significant histological markers of damage in obese males. This suggested that the model reflected early-state damage, consistent with previous findings in preclinical and clinical models (27,28). Notably, sex markedly influenced the effect of obesity on the hepatic profile. Compared with obese males, obese female mice exhibited a lower degree of steatosis, hepatocyte ballooning and expression of inflammatory markers. Again, chronic fibrotic changes were minimal and the most important histological marker in ovariectomized obese females was a trend towards reduced steatosis. These findings in males suggested that the current model reflected an early stage of damage, thus, longer-term models might be necessary to fully characterize chronic damage progression. Similarly, the hepatic lipid metabolism was less affected in females, as they displayed high levels of Chol and CE whereas obese males had elevated Chol, CE, DAG and FFA. The hepatic accumulation of DAG and FFA is associated with the development of obesity, insulin resistance and NAFLD (29,30). Markedly, ovariectomy in obese female mice resulted in lower hepatic TAG and CE and higher levels of cytotoxic FFA, DAG and Chol. These differences reflected the protective role of estrogen on regulating the packaging of FFA and DAG into TAG to form lipid droplets in the liver (31).

In the context of lipotoxicity, the accumulation of lipids, particularly FFA and DAG, can lead to changes in the phospholipid profile, contributing to inflammation, cell dysfunction, and organ damage. The present study observed a significant reduction in total phospholipids in obese mice,

representing only 20% of total lipids, almost twice the value obtained in lean animals. The low proportion of phospholipids in relation to neutral lipids might impair membrane fluidity and cell functioning. Furthermore, inflammation in liver has been associated with alterations in phosphatidylserine and PE levels (32). The present study found a mild increment in these phospholipids in HFD-OVX mice as well as a markedly elevated Chol/PC ratio, which has been associated with liver injury (33). Altogether, these findings may indicate that the loss of estrogen in the context of obesity and insulin resistance may induce hepatic lipotoxicity, promoting inflammation and chronic liver damage. However, more studies are needed to clarify this pathway.

The complex interplay between insulin and estrogen may be key to understanding the results obtained in the current study, as they have a profound impact on lipid metabolism. On one hand, insulin modulates lipid metabolism in the hepatocyte by i) promoting lipogenesis from glucose and other metabolites (34), ii) mediating the packing of FFA and DAG into TAG (35), iii) promoting the esterification of Chol (36), and iv) regulating the correct lipid droplet formation via SREBP1 and mTORC1 (37). Given the toxicity of FFA, DAG, and Chol (11,12,38-41), insulin's promotion of lipogenesis and lipid droplet formation can be considered 'cell protective' in the context of lipid accumulation (8,35,42). However, obesity and MS can eventually induce insulin resistance in the hepatocytes. Consequently, reduced insulin responsiveness may impair lipid droplet synthesis and elevate FFA, DAG and Chol levels, thereby increasing hepatic toxicity, as observed in our model.

On the other hand, several studies have found that estrogen promotes insulin sensitivity and glucose uptake in muscle and liver (43,44). Estrogen and insulin can both regulate lipogenesis via E2-induced activation of PI3K-Akt-Foxo1 (45). Therefore, these hormones may facilitate neutral lipid droplet formation that protects from toxic intermediaries in obesity (46). This may explain the impaired hepatic lipid deposition associated with liver injury observed in obese ovariectomized mice (Fig. S2). Clinical trials have shown that estrogen replacement improved insulin sensitivity and glucose levels in diabetic postmenopausal women (47-49). Following ovariectomy in obese female mice, estrogen supplementation reduced DAG accumulation in the liver (44). In male mice with obesity, estrogen administration prevented diet-induced hepatic insulin resistance, hyperglycemia and the elevation in DAG levels (50). Furthermore, a study has shown that estrogen supplementation improved peripheral insulin sensitivity and increased TAG levels in the liver of obese turkeys (51). Notably, the metabolic changes induced by ovariectomy depend on the nutritional status. In murine models, ovariectomy in obese mice can result in a decreased expression of genes related to lipid metabolism in adipose tissue, liver and skeletal muscle, contributing to increased fat accumulation as well as toxic lipids intermediaries (52,53). By contrast, in non-obese mice, these effects are less pronounced, suggesting that obesity amplifies the metabolic alterations induced by estrogen deficiency (54). In humans, the transition to menopause, which involves a natural decline in estrogen levels, has been associated with increased central adiposity and metabolic alterations, indicating parallels to findings in animal models (55,56).

Lipid droplet formation is an essential metabolic mechanism to circumvent hepatic lipotoxicity in conditions of excessive energy intake (57). Sequestration of FFA and DAG into inert TAG and CE within lipid droplets is crucial for mitigating hepatic lipotoxicity. FFA, DAG and Chol cannot readily enter lipid droplets due to their polar groups (57). Thus, TAG and CE serve to sequester these cytotoxic intermediaries to form the neutral core of lipid droplets (58). Limiting lipid droplet formation can be deleterious for hepatocytes, as elevated levels of FFA, DAG and Chol are harmful to these cells (10-13,40). For instance, FFA can induce lipid peroxidation and the activation of TLR4 (12,59). DAG can induce inflammation and impaired autophagy via PKC activation (11,13). Furthermore, Chol can activate the unfolded protein response and promote ER stress (14,15). Notably, increased levels of FFA, DAG and Chol can promote the activation of NF-κβ and the initiation of inflammatory pathways, in accordance with the present results (53,60-62).

Differences in the hepatic FA profile between obese and lean mice are related to differences in the dietary FA composition. However, dysregulations of the metabolic pathway involved in the synthesis/utilization of FAs were pronounced in mice fed with HFD. On one hand, the present study found sexual dimorphism in the distribution of FA in the liver of female and male mice: Obese females (irrespective of ovariectomy) had higher SFAs, lower MUFAs and lower n-6 LC-PUFAs whereas obese males showed lower SFAs, higher MUFAs and lower n-6 LC-PUFAs. Taking into account that SFAs are more toxic than MUFAs (5), the FA profile of males may indicate a greater capacity to convert these SFAs into more inert MUFAs, as a protective pathway against SFA-induced toxicity. These differences might indicate sex-specific mechanisms to compensate metabolic insults that should be further explored in future experiments. Additionally, ARA (20:4 n-6) and consequently ARA/DHA ratio were depleted in hepatic total lipids of all obese animals. As ARA is the main precursor of proinflammatory prostanoids, this reduction could be related to its increased utilization for the synthesis of prostaglandins, thromboxanes and leukotrienes (63). Consistently, obese ovariectomized females had the highest levels of DMAs in PE (chiefly 18:0 DMA, see Table SVI). DMAs are precursors for *de novo* biosynthesis of plasmalogens which are receiving increasing attention due to their antioxidant role and their involvement in mammalian anti-inflammatory responses (64). This aligns well with the higher proportion of essential PUFAs, such as EPA and DHA in hepatic TAG of obese mice that might be attributed to the described ability of plasmalogens to reduce oxidative degradation (65).

NAFLD is associated with high SFA and Chol levels that inhibit desaturase activity (66,67). Notably, the present study revealed a decreased product 18C precursor ratios for both n-6 and n-3 pathways in hepatic total lipids, suggesting that delta-6 and delta-5-desaturase activities, which catalyze the conversion of shorter chain precursors linoleic acid (18:2 n-6) and linolenic acid (18:3 n-3) into their longer and more unsaturated counterparts, might be impaired. Consistently, the present study observed a decreased 18:1 n-9 proportion and 18:1 n-9/18:0 ratio in obese female mice; probably linked to the proinflammatory status of this group. Chena *et al* (68)

demonstrated the protective mechanism of oleic acid (18:1 n-9) against SFA-induced hepatocyte lipotoxicity in primary hepatocytes of rats with NASH, including apoptosis, oxidative stress, mitochondrial dysfunction, inflammation and fibrosis. Thus, an impairment of stearoyl-CoA desaturase (delta-9 desaturase) involved in the conversion of 18:0 to 18:1 n-9 could be responsible for this reduction. The connection between the observed lipid profile with the hepatic desaturase activities must be addressed in future studies.

Several therapeutic strategies that involve regulation of lipid metabolism and inflammation are being explored for NAFLD (69-75), including the use of SIRT1 activators (70), AMPK modulators (71), gut microbiota interventions (72) and hormone replacement therapy in the context of menopause (73). These approaches aim to reduce hepatic lipotoxicity, improve insulin sensitivity, and ameliorate inflammation. Among them, SIRT1 activation has gained attention for its role in suppressing NF-κβ signaling and mitigating disease progression (70). In the present study, NF-κβ upregulation in obese ovariectomized mice, along with increased transaminases, suggested an impairment in SIRT1 pathway, and highlights the need to explore its therapeutic potential in menopause-related liver injury. Finally, it is worth considering that the alterations in inflammation and lipid metabolism are related to cardiovascular disease, the main cause of death in patients with NASH and NAFLD (74,75).

An important strength of the present study is that it is among the few studies that analyze the interplay between estrogen deficiency and obesity in mice. Most previous studies have focused solely on males. Thus, sex-based comparisons may provide valuable insights that pave the way for more efficient treatments of metabolic disorders. One straightforward interpretation of the present results is that the lack of sex hormones might predispose females to more severe damage, mirroring the situation in males. Furthermore, the comparison revealed that obese females did not experience the same detrimental effects as obese males, which can be attributed to the protective effect of estrogen. These results demonstrated that ovariectomy generally worsened the hepatic profile in obese females. However, the present study has limitations. The first pertains to the applicability of pre-clinical models to humans. As mouse lipid metabolism differs from human metabolism, these findings should be confirmed in large animal models and human studies. The duration of the experiment is relatively short, and this may explain the mild fibrosis and moderate variations in the hepatic fatty acid profile observed in the model. Chronic animal models (small and large) are expensive, which may compromise their viability in research projects. In any case, larger periods of follow up could improve our understanding about the impact of menopause in MS and liver disease.

In conclusion, ovariectomy in obese animals exacerbated hepatic lipotoxicity, accelerating the progression of liver injury. This suggested that estrogen loss may contribute to the onset of hepatic damage in the context of obesity and insulin resistance. Furthermore, estrogen may exert its protective effect through the regulation of lipid droplet formation when insulin action is impaired. Further experiments are needed to unveil mechanistic insights, which may open therapeutic opportunities. Additional research in sex differences regarding liver disease may also contribute to achieve an improved understanding of the NAFLD and NASH pathogenesis.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

AERR, EP and MHG designed, discussed and supervised the study; AERR, EP, AAA, JDG, SLL, BAP, AHB, MIH, SGH, LDM and STT performed the experiments, data analyses and result plotting; AAA, JDG, JAPP, NGAG and CRG collaborated in the data interpretation, drafting and revising the manuscript; AERR, EP, MHG, AAA and JDG confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal care was performed in accordance with institutional guidelines in compliance with Spanish (Real Decreto 53/2013, February 1. BOE, February 8, 2013, n: 34, p. 11370-11421) and international laws and policies (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes) and were approved by the Institutional Animal Care and Use Committee (Comité de Ética de la Investigación y de Bienestar Animal of University of La Laguna, Spain). The ethics committee approval number was CEIBA2021-3107.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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