

# Hepatic ER $\alpha$ accounts for sex differences in the ability to cope with an excess of dietary lipids



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## ABSTRACT

**Objective:** Among obesity-associated metabolic diseases, non-alcoholic fatty liver disease (NAFLD) represents an increasing public health issue due to its emerging association with atherogenic dyslipidemia and cardiovascular diseases (CVDs). The lower prevalence of NAFLD in premenopausal women compared with men or postmenopausal women led us to hypothesize that the female-inherent ability to counteract this pathology might strongly rely on estrogen signaling.

In female mammals, estrogen receptor alpha (ER $\alpha$ ) is highly expressed in the liver, where it acts as a sensor of the nutritional status and adapts the metabolism to the reproductive needs. As in the male liver this receptor is little expressed, we here hypothesize that hepatic ER $\alpha$  might account for sex differences in the ability of males and females to cope with an excess of dietary lipids and counteract the accumulation of lipids in the liver.

**Methods:** Through liver metabolomics and transcriptomics we analyzed the relevance of hepatic ER $\alpha$  in the metabolic response of males and females to a diet highly enriched in fats (HFD) as a model of diet-induced obesity.

**Results:** The study shows that the hepatic ER $\alpha$  strongly contributes to the sex-specific response to an HFD and its action accounts for opposite consequences for hepatic health in males and females.

**Conclusion:** This study identified hepatic ER $\alpha$  as a novel target for the design of sex-specific therapies against fatty liver and its cardio-metabolic consequences.

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**Keywords** Liver metabolism; Fatty liver; Sex differences; Estrogen; ER $\alpha$ ; Amino acids

## 1. INTRODUCTION

In female mammals, liver estrogen receptor alpha (ER $\alpha$ ) plays a central role in the adaptive response of hepatic metabolism to the energy requirements characterizing the different reproductive stages [1–3]. In addition, this receptor was reported to have a key role in the control of lipid and amino acid (AA) metabolism [1,4]. Unlike in females, the expression of ER $\alpha$  in the adult male liver is very low [2,4], suggesting that, in males, the role of this receptor in metabolic control might be extremely limited. In view of its prominent role in the female liver, ER $\alpha$  action might strongly contribute to the well-recognized, but often overlooked, hepatic sexual dimorphism [5–7] and to the sex-specific incidence of hepatic diseases [8,9]. Among them, non-alcoholic fatty liver disease (NAFLD), a syndrome characterized by excess triglyceride accumulation within hepatocytes [10], represents a paradigmatic example. Indeed, although obesity is prevalent in females, fertile women result to be, to some extent, protected from obesity-associated fatty liver/NAFLD and cardio-metabolic consequences [11–13], suggesting the involvement of estrogen signaling. This idea is further supported by studies showing that the lack of estrogens (ovariectomized, OVX, and aromatase knockout mice, ArKO) [1,14–17] or ER $\alpha$  (ER $\alpha$  knockout

mice, ER $\alpha$ KO) [16,18,19] is associated with the development of hepatic steatosis. However, the analysis of the lack of estrogen signaling in OVX, ArKO, and ER $\alpha$ KO mice did not allow to disentangle the systemic effects of estrogens from those occurring in the liver specifically, preventing the understanding of the contribution of hepatic estrogen signaling to the sex-specific prevalence of fatty liver/NAFLD.

Considering that, in fertile females, estrogens, acting through the hepatic ER $\alpha$ , might antagonize liver lipid deposition, we hypothesized that the low content of ER $\alpha$  and the lack of such a well-tuned regulation in males might be at the basis of the higher prevalence of hepatic steatosis/NAFLD in males, particularly under an unbalanced dietary regimen. Indeed, although the contribution of liver ER $\alpha$  in preventing or limiting the diet-induced hepatic steatosis/NAFLD in the two sexes has not been investigated, this hypothesis is supported by recent reports highlighting the negative correlation between hepatic ER $\alpha$  expression/activity and fatty liver [1,20,21].

Thus, the present study was undertaken to evaluate the extent to which the hepatic ER $\alpha$  might account for sex differences in the ability of the male and female liver to cope with an excess of dietary lipids and counteract the development of fatty liver/NAFLD in a model of diet-induced obesity.

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The data obtained show that, besides its sex-biased expression, the hepatic ER $\alpha$  contributes to the sex-specific metabolic adaptation to a fat-enriched diet, accounting for opposite consequences for health in males and females. This study, by unraveling the relevance of hepatic ER $\alpha$  in the liver of the two sexes, underlines the necessity to customize pharmacological and dietary interventions aimed to prevent obesity-associated fatty liver/NAFLD and the cardio-metabolic consequences.

## 2. MATERIAL AND METHODS

### 2.1. Animals

Syngenic (ER $\alpha$  floxed, ER $\alpha^{ff}$ ) and LERKO (liver ER $\alpha$  knockout) [3] mice were fed with a low fat diet (LFD, Research diet, D12450B) or a high fat diet (HFD, Research Diets, D12492) for 16 weeks starting at 4 months of age. At the end of the experiment, mice were 8 months old. Room temperature was maintained at 22 °C–25 °C, with a 12-hour light/dark cycle (lights on at 7:00 a.m.). For all the animal studies, both male and female mice were used. Female mice were collected when in the *Metestrus* phase of the estrous cycle; to do this, vaginal smears were performed at 9:00 a.m. To avoid any possible confounding effect due to the circadian rhythm or feeding status, the mice were euthanized in the early afternoon after 6 h of fasting [3]. All animal experimentation was performed in accordance with the ARRIVE guidelines and the European guidelines for animal care and the use of experimental animals, approved by the Italian Ministry of Research and University, and controlled by a departmental panel of experts.

### 2.2. Liver histology

The left lobe of the liver was fixed in 10% neutral formalin solution (Sigma–Aldrich) overnight at 4 °C, cryopreserved in a 30% (w/v) sucrose solution for 24 h at 4 °C, and stored at –80 °C. Liver sections 7- $\mu$ m thick were cut with a refrigerated microtome (Leica), collected on slides, and stored at –80 °C until staining. Hematoxylin–eosin (H&E) staining was performed on the frozen slides with Mayer hematoxylin (Bio–Optica) for 1 min and, after washing with water, with 1% eosin aqueous solution (Bio–Optica) for 4 min. Oil Red O staining was performed as previously described [1]. The Accustain Trichrome stain kit was used for Masson trichrome staining (Sigma–Aldrich). After staining, the slides were cleared in xylenes and cover slipped with xylene-based mounting medium (Eukitt, Bio–Optica). The liver sections were evaluated in a blinded fashion under a light microscope. Semi-quantitative grading of the hepatocellular vacuolar degeneration and portal inflammation (i.e., the infiltration of mononuclear inflammatory cells) was also performed in a blinded fashion. Images of the stained sections were captured using Microscope Axioscop2 mot plus (Zeiss).

### 2.3. Biochemical assays

The triglyceride (TG), free fatty acid (FFA), and cholesterol (CH) levels were measured with appropriate kits according to the manufacturer's protocols (Biovision).

### 2.4. Metabolomic analysis

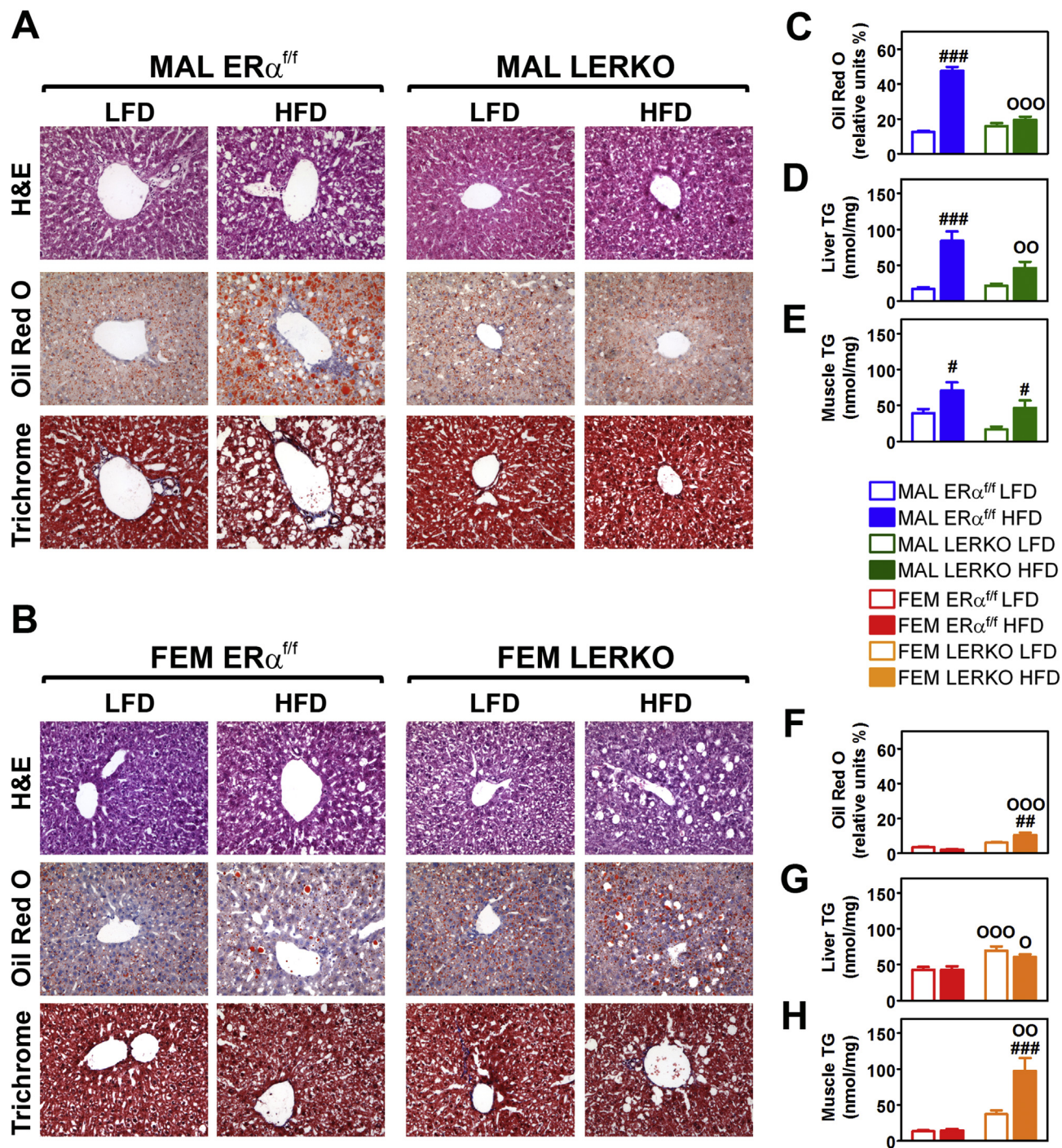
For metabolomic analyses, liver tissues were homogenized with a tissue lyser. Briefly, tissues were lysed in 250  $\mu$ L of methanol/ acetonitrile 1:1 (v/v) with D-Glucose-<sup>13</sup>C6 1 ng/ $\mu$ L (internal standard, Sigma Aldrich, 389374) and centrifuged at 4 °C. Supernatant was saved for subsequent analysis. Amino acid quantification was performed through previous derivatization. Samples were incubated with phenylisothiocyanate (PITC) solution for 20 min at room temperature, dried, and resuspended in 5 mM ammonium acetate in MeOH/H<sub>2</sub>O 1:1

(v/v). Metabolomic data were performed on an API-4000 triple quadrupole mass spectrometer (AB SCIEX) coupled with a high-performance liquid chromatography (HPLC) system (Agilent) and a CTC PAL HTS autosampler (PAL System). The identity of all metabolites was confirmed using pure standards. Quantification of different metabolites was performed with a liquid chromatography/tandem mass spectrometry (LC-MS/MS) method using a C18 column (Biocrates) for amino acids and a cyano-phase LUNA column (50 mm  $\times$  4.6 mm, 5  $\mu$ m; Phenomenex). The AAs were analyzed through a 10-minute run in positive ion mode, whereas other metabolites were run for 5 min in negative ion mode. Twenty multiple reaction monitoring (MRM) transitions in positive ion mode and 30 MRM transitions in negative ion mode (all other metabolites) were used, respectively. The mobile phases for positive ion mode analysis were phase A: 0.2% formic acid in water and phase B: 0.2% formic acid in acetonitrile. The gradient was T0 100% A, T5.5min 5%A, and T7min 100%A with a flow rate of 500  $\mu$ L/min. The mobile phase for negative ion mode analysis (all other metabolites) was phase A: 5 mM ammonium acetate pH 7.00 in MeOH. The gradient was 100% A for all the analysis with a flow rate of 500  $\mu$ L/min. MultiQuant™ software (version 3.0.2) was used for data analysis and peak review of chromatograms. Quantitative evaluation of all metabolites was performed based on calibration curves with pure standards, and data were normalized on micrograms of proteins. Metabolomic data analysis was performed using Metaboanalyst 3.0 software (<http://www.metaboanalyst.ca/>).

Plasma FFA extraction was conducted by Folch method and then as previously reported [22] using <sup>13</sup>C<sub>18</sub>-labeled linoleic acid and <sup>13</sup>C<sub>16</sub>-labeled palmitic acid (Sigma–Aldrich) as internal standards. Extracts were dried under nitrogen and resuspended in methanol/water (1:1) before submission for analyses. For FFA quantification, the MS analysis was conducted with a selective ion monitoring-tandem mass spectrometry (SIM-MS/MS) method. An electrospray ionization (ESI) source was used and connected with an API 4000 triple quadrupole instrument (AB Sciex, USA). The mobile phases were: water:methanol (97:3)/10 mM isopropylethylamine/15 mM acetic acid (phase A) and methanol (phase B). T0 20% A, T20min 1%A, T25min 1%A, T25.1min 20%A, T30min 20%A. The Hypersil GOLD PFP column (100 mm  $\times$  2.1 mm, 3  $\mu$ m) was maintained at 40 °C, and the flow rate was 500  $\mu$ L/min. Multi-Quant™ software version 3.0.2 was used for data analysis and peak review of chromatograms. The quantitative evaluation of different FFAs was performed based on standard curves. Quantitative data were normalized on microliter of plasma extracted.

### 2.5. Real-time PCR gene expression analysis

Total liver RNA extraction was isolated with TRIzol Reagent (Invitrogen) and purified using the RNeasy mini-kit protocol (Qiagen), according to the manufacturer's instructions. For the preparation of cDNA, 1  $\mu$ g RNA was denatured at 75 °C for 5 min in the presence of 1.5  $\mu$ g of random primers (Promega) in 15  $\mu$ L final volume. Deoxynucleotide triphosphate (GE Healthcare) and Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega) were added at 0.5 mM and 8 U/ $\mu$ L final concentration, respectively, in a final volume of 25  $\mu$ L. The RT reaction was performed at 37 °C for 1 h; the enzyme was inactivated at 75 °C for 5 min. Control reactions without the addition of the RT enzyme were performed for each sample. For the real-time polymerase chain reaction (rtPCR) experiments, the reaction mix for each sample was made up of 2  $\mu$ L of pre-diluted cDNA, 5  $\mu$ L of TaqMan 2 $\times$  Universal PCR Master Mix No AmpErase UNG (ThermoFisher/Life Technologies), 0.5  $\mu$ L of 20 $\times$  primers/probes mix, and 2.5  $\mu$ L of H<sub>2</sub>O. The primers used for the rtPCR reactions are listed in Table S4. The 36b4 primer was used as the



**Figure 1: Hepatic ER $\alpha$  deficiency limits HFD-induced lipid accumulation in males.** A-B. Histological analysis of the livers of ER $\alpha^{ff}$  and LERKO males (A) and females (B) fed with LFD (low fat diet) or HFD (high fat diet) for 16 weeks. Upper, hematoxylin and eosin (H&E) staining; center, Oil Red O staining plus H&E (orange-red: neutral fats; blue: nuclei); bottom, Masson's trichrome staining (blue: collagen deposits; red: hepatocyte cytoplasm; dark red-black: nuclei). Magnifications  $\times 200$ . C, F. Quantification of the lipid deposits in the liver tissues of males (C) and females (F) by Oil Red O staining. Data are expressed as percentages of the total section areas. D, G. Triglyceride (TG) content measured in the liver of male (D) and female (G) mice. E, H. Triglyceride (TG) content measured in the skeletal muscle of male (E) and female (H) mice. Data are presented as mean  $\pm$  SEM. In (C and F)  $n = 6$ ; in (D, E, G, H)  $n = 8-12$ .  $^{\circ}p < 0.05$ ,  $^{\circ\circ}p < 0.01$  and  $^{\circ\circ\circ}p < 0.001$  LERKO vs ER $\alpha^{ff}$ ;  $\#p < 0.05$ ,  $\#\#p < 0.01$  and  $\#\#\#p < 0.001$  HFD vs LFD (two-way ANOVA followed by Bonferroni's *post hoc* test).

reference gene assay. The reaction was conducted according to the manufacturer's protocol using QuantStudio™ 3 Real-Time PCR System with the following thermal profile: 2 min at 50 °C; 10 min 95 °C; 40 cycles (15 s 95 °C, 1 min at 60 °C). The data were analyzed using the  $2^{-\Delta\Delta Ct}$  method [23].

## 2.6. Western blot analysis

Samples of frozen mouse liver were homogenized in ice-cold buffer (20 mM HEPES, 5 mM MgCl<sub>2</sub>, 420 mM NaCl, 0.1 mM EDTA, and 20% glycerol) containing protease and phosphatase inhibitors according to the manufacturer's protocols (Phosphatase and Protease Inhibitor Mini

Tablets, Pierce). After three repeated cycles of freezing and thawing, the homogenate was centrifuged at 16,100 g for 15 min at 4 °C, and the supernatant was collected in a new tube. After appropriate quantitative analysis (Bradford assay, Pierce), equal amounts of the protein samples (25 µg of liver extracts) were resuspended in Laemmli sample buffer and separated in an 8%–10% sodium dodecylsulfate (SDS) polyacrylamide gel system (Biorad). After transfer, the nitrocellulose membranes were incubated with specific antibodies overnight at 4 °C and then with the secondary antibody conjugated with peroxidase for 1 h at RT. The primary antibodies used were the following: anti-LDLR (Novus Biological, NB110-57162), anti-CD36 (Abcam, ab133625), and anti-β-actin (Sigma, A1978). Immunoreactivity was detected with an ECL Western Blotting Analysis System (Amersham) and acquired and analyzed using an Odyssey Fc Imaging system and the Image Studio™ software (LiCor Biosciences).

### 2.7. Plasma CH profile

The CH distribution in the plasma lipoprotein fractions was determined *via* fast protein liquid chromatography (FPLC) using a Superose 6 column (Amersham Biosciences). Fractions of 500 µL were collected and assayed for CH with an enzymatic kit (Sentinel).

### 2.8. Quantification and statistical analysis

Statistical analyses were performed by the Student *t* test for the comparison of two different experimental groups, or two-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test for multiple testing comparisons. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software). All data are expressed as mean ± standard error of the mean (SEM). A *p* value less than 0.05 was considered statistically significant. The statistical parameters can be found in the figure legends.

## 3. RESULTS

### 3.1. The lack of hepatic ERα signaling has opposite consequences in the liver of males and females

To study the relevance of hepatic ERα in the response of male and female livers to an unhealthy, fat-enriched diet, syngenic (ERα<sup>fl/fl</sup>) and liver-specific ERα knockout (LERKO) [3] mice were fed with a low-fat diet (LFD) or a 60% high-fat diet (HFD) for 16 weeks starting at 4 months of age (see Methods and Tables S1 and S2). We focused on that time with the aim to study the sex-specific relevance of hepatic ERα in the metabolic adaptation to a diet enriched in fats and in counteracting lipid accumulation in the liver of the two sexes. At the end of the dietary treatment, the mice were euthanized in the early afternoon after 6 h of fasting [3], and the excised livers were processed for histological and biochemical analysis.

The HFD had a critical effect in ERα<sup>fl/fl</sup> males, leading to a major accumulation of lipids in the liver as shown by Oil Red O staining (+280% vs LFD) and by the measurement of TG content (+395% vs LFD) (Figure 1A, C, D). Lipid deposition was associated with hepatocellular vacuolar degeneration and portal infiltration of mononuclear leukocytes as indicated by H&E and Masson trichrome stainings.

Considering that in the male liver the content of ERα is quite low, we expected that the liver-specific ablation of this receptor had minimal effects in LERKO males with respect to their syngenic counterparts. Contrary to our expectations was the observation of a moderate lipid deposition in the liver of LERKO males after a HFD (Figure 1A, C, D). The effect of both diet and ERα deficiency was very different in females. In ERα<sup>fl/fl</sup> females, the HFD did not alter the hepatic lipid content, whereas the mere ablation of ERα was associated with a significant

increase in liver lipid deposition (+50% TG, irrespective of the diet) (Figure 1B, F, G).

In males, the HFD also led to lipid accumulation in the skeletal muscle of both ERα<sup>fl/fl</sup> (+81% vs LFD) and LERKO (+184% vs LFD). In females, the HFD enhanced fat deposition in the skeletal muscle of LERKO (+162% vs LFD) but not in ERα<sup>fl/fl</sup> females (Figure 1E–H). This suggested that, in females, the hepatic ERα plays a role in preventing ectopic lipid deposition.

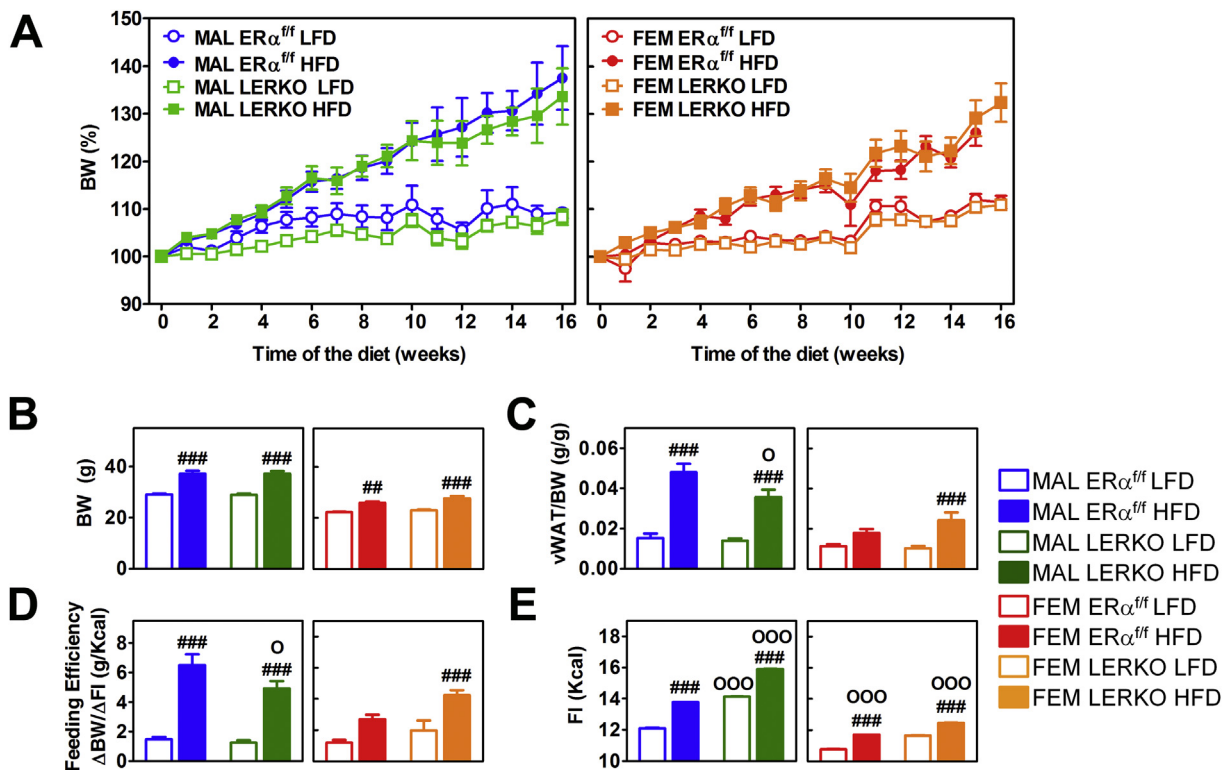
In both sexes, the described effects were associated with alterations of the body weight (BW) that were found to be independent from hepatic ERα. Indeed, in the course of the experiment, the changes in BW were superimposable in ERα<sup>fl/fl</sup> and LERKO mice (Figure 2A) and, at the end of the experiment, HFD-fed males and females weighed significantly more than their LFD-fed counterparts (Figure 2B).

The analysis of visceral white adipose tissue (vWAT) weight and feed efficiency (FE) further demonstrated a sex-specific response to HFD of ERα<sup>fl/fl</sup> and LERKO mice. HFD-fed ERα<sup>fl/fl</sup> males accumulated more vWAT than LERKO males, whereas the opposite was true for females (Figure 2C). With respect to males, ERα<sup>fl/fl</sup> females were able to better cope with the HFD; indeed, they kept FE relatively low and avoided the accumulation of fat tissue, an ability lost by LERKO females (Figure 2C–D). Differently from females, hepatic ERα deficiency in males limited FE after a HFD (−24% vs ERα<sup>fl/fl</sup> males) (Figure 2D). Nevertheless, the increased food intake (+15%) (Figure 2E) suggested an altered nutrient absorption and/or metabolism in LERKO males.

These data demonstrate that hepatic ERα action accounts for a sex-specific response to a HFD with different consequences for males and females, suggesting that, besides its dimorphic expression (Fig. S1), liver ERα exerts different metabolic functions in the two sexes.

### 3.2. HFD unmasks the sex-specific impact of hepatic ERα ablation on liver metabolome

To better comprehend the different roles exerted by ERα in the liver of the two sexes, we pursued our characterization of liver metabolism through a metabolomic approach. As shown in the heatmap of Figure 3A, the most relevant liver metabolites (Table S3) were affected by the diet and by the presence/absence of hepatic ERα in a sex-specific fashion. The principal component analysis (PCA) of liver metabolites clearly showed that the diet acts as a clear discriminating factor for LERKO males, but less for ERα<sup>fl/fl</sup> males that, conversely, are represented by partially superimposing ellipses (Figure 3B). In females, the overlap among the four groups indicated that the liver metabolome of females was less affected by the diet and by ERα deficiency (Figure 3C). Cluster analysis performed on liver metabolites (Figure 3D) clearly showed how the ablation of ERα in the liver of males is responsible for a metabolic signature that significantly diverged from that of all other experimental groups. HFD led to major changes in LERKO males: after the HFD, the metabolic fingerprint of LERKO males became more similar to that of the HFD-fed females, indicating that the hepatic ERα directs the male-specific response to a fat-enriched diet. By considering a fold change |FC| > 1.5 and a  $-\text{Log}_{10}(\text{p-value}) > 1.3$ , we found that, in the liver of ERα<sup>fl/fl</sup> males, HFD led to a significant decrease in the content of AA (in red) and of short-chain acyl-carnitine (C4) and to a greater content of medium-chain acyl-carnitines (C8 and C9) (Figure 4A and table S3). The lack of ERα by itself induced a metabolic rewiring in the liver of LERKO males, which showed low content of AA, intermediates of glycolysis/gluconeogenesis (Gly/Gln: glucose, glucose-6-phosphate, fructose-1,6-biphosphate, lactate), pentose phosphate pathway (PPP: erythrose-4-phosphate), and high content of intermediates of TCA cycle (acetyl-CoA, citrate, isocitrate, 2-



**Figure 2: Effect of HFD and hepatic ER $\alpha$  deficiency on body weight and food intake.** **A.** Body weight (BW) of ER $\alpha^{fl/fl}$  and LERKO mice measured weekly along the 16 weeks long experiment and expressed as percentage *versus* time 0. **B.** BW of ER $\alpha^{fl/fl}$  and LERKO mice measured at the end of the 16 weeks long experiment. **C.** Quantification of the visceral white adipose tissue (vWAT) normalized over the BW. **D.** Feeding efficiency expressed as  $\Delta$ BW/ $\Delta$ FI ( $\Delta$ Body weight/ $\Delta$ Food intake). **E.** Food intake (FI) expressed as kcal %. Data are presented as mean  $\pm$  SEM (n = 18).  $^{\circ}$ p<0.05,  $^{\circ\circ}$ p<0.01 and  $^{\circ\circ\circ}$ p<0.001 LERKO vs ER $\alpha^{fl/fl}$ ; #p<0.05, ##p<0.01 and ###p<0.001 HFD vs LFD (two-way ANOVA followed by Bonferroni's *post hoc* test).

oxoglutarate) and energy metabolism [oxidized nicotinamide adenine dinucleotide (NAD<sup>+</sup>), reduced nicotinamide adenine dinucleotide (NADH), oxidized nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>), and reduced nicotinamide adenine dinucleotide phosphate (NADPH)]. The already low content of AA further decreased in the liver of LERKO males after HFD; this change was associated with a decrease of intermediates of Gly/Gln (PEP, pyruvate), TCA cycle (acetyl-CoA, isocitrate, 2-oxoglutarate, succinyl-CoA, succinate), lipid synthesis (malonyl-CoA), and energy metabolism [NAD<sup>+</sup>, NADH, NADP<sup>+</sup>, NADPH, adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP)] and with an increase of short-(C4) and medium-chain (C8, C9) acyl-carnitines.

In ER $\alpha^{fl/fl}$  females, the effect of the HFD was circumscribed to a few changes (low content of glucose-6-phosphate, Ribulose-5-phosphate, citrate, isocitrate, fumarate, and malate) and, differently from males, did not affect the AA content. AA homeostasis in females was dependent on hepatic ER $\alpha$ ; indeed, compared with ER $\alpha^{fl/fl}$  females, LERKO females showed higher content of AA, some of which [alanine (Ala), threonine (Thr), glutamine (Gln), glutamate (Glu), methionine (Met), histidine (His), and arginine (Arg)] were significantly decreased after a HFD together with some intermediates of the TCA cycle (isocitrate, 2-oxoglutarate, succinate, fumarate, malate) and of energy metabolism (NADP<sup>+</sup>, NADPH).

A more detailed analysis based on the pathways with the most relevant impact (impact > 0.6 and  $-\text{Log}(p\text{-value}) > 3$ ) indicated that the metabolism of AA and, in particular, of branched-chain AA (BCAA: Val, Leu, Ile) was the most affected by the lack of hepatic ER $\alpha$  and by the nutritional challenge, especially in males (Figure 4B). Interestingly, only

the liver of ER $\alpha^{fl/fl}$  females was able to preserve the homeostasis of AA when challenged with a HFD, a feature associated with a healthy metabolic phenotype.

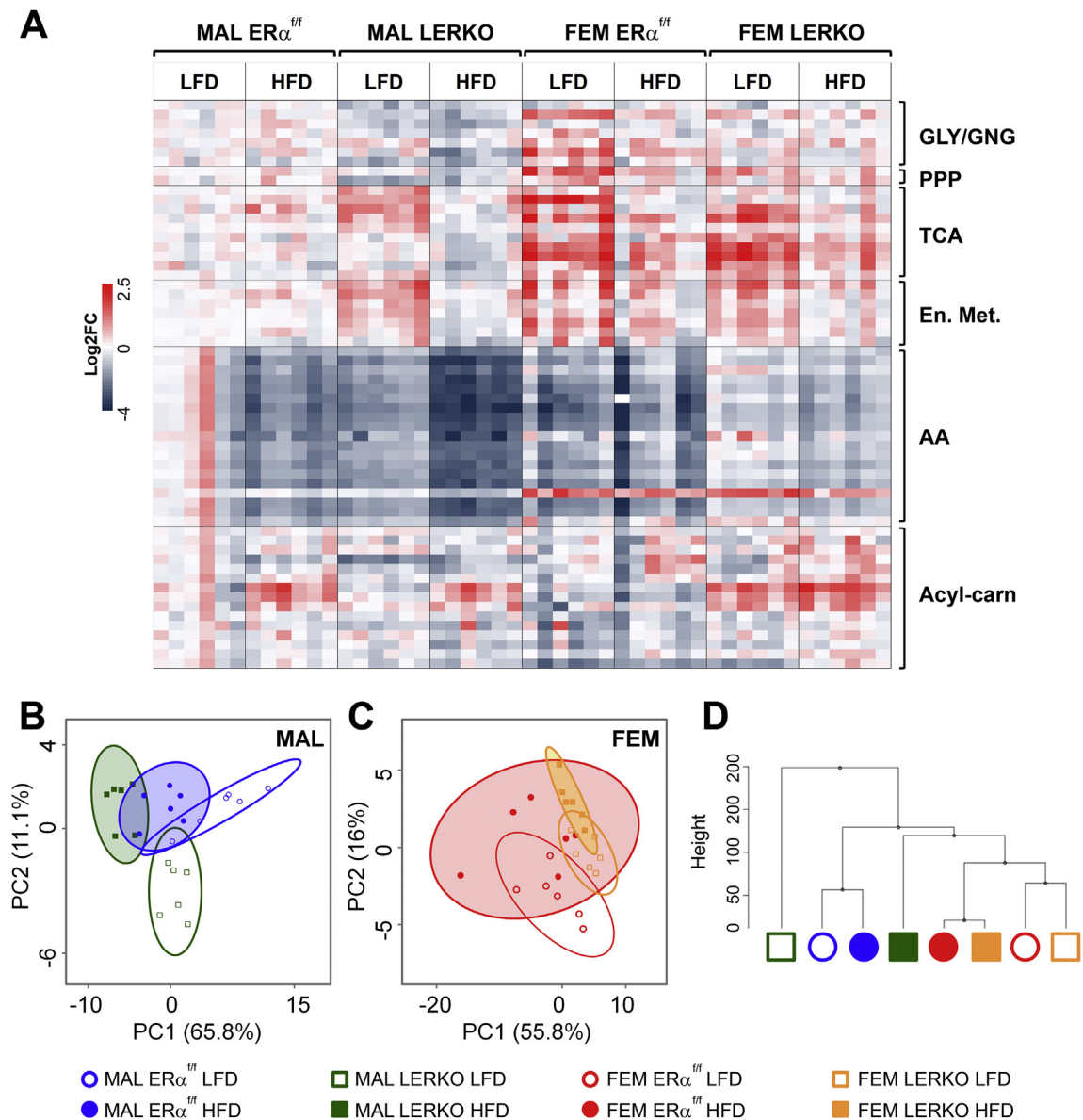
### 3.3. The lack of hepatic ER $\alpha$ in males, but not females, is responsible for an altered plasma lipid profile

Next, we examined the extent to which the lack of hepatic ER $\alpha$  could affect the homeostasis of circulating lipids (TG, FFA, and CH), particularly under a HFD regimen.

As shown in Figure 5A, plasma TG levels were slightly increased by a HFD in males but not in females, a sex-specific effect that resulted independently from hepatic ER $\alpha$  presence/absence.

With the exception of docosahexaenoic acid (DHA, -40%), the levels of FFA were unchanged by HFD in the plasma of ER $\alpha^{fl/fl}$  males (Figure 5B). In males, this homeostasis was ensured by hepatic ER $\alpha$ ; indeed, the lack of this receptor in LERKO males led to a significant increase (or showed a trend to augment) in the majority of FFA, an effect boosted by the HFD (Figure 5B). In ER $\alpha^{fl/fl}$  females, the only significant changes driven by HFD were related to stearic acid (+48%) and to DHA (-41%), whereas no changes were observed in LERKO females independent of the diet (Figure 5C). These data suggest that the hepatic ER $\alpha$  deficiency impacts on the metabolism of FFA mainly in males.

Having found that hepatic ER $\alpha$  plays a pivotal role in the regulation of CH and lipoprotein metabolism in females [2], we pursued our study by measuring total CH levels and by analyzing the distribution of CH among plasma lipoproteins by fast protein liquid chromatography (FPLC) (Figure 6A–D). HFD induced a significant increase



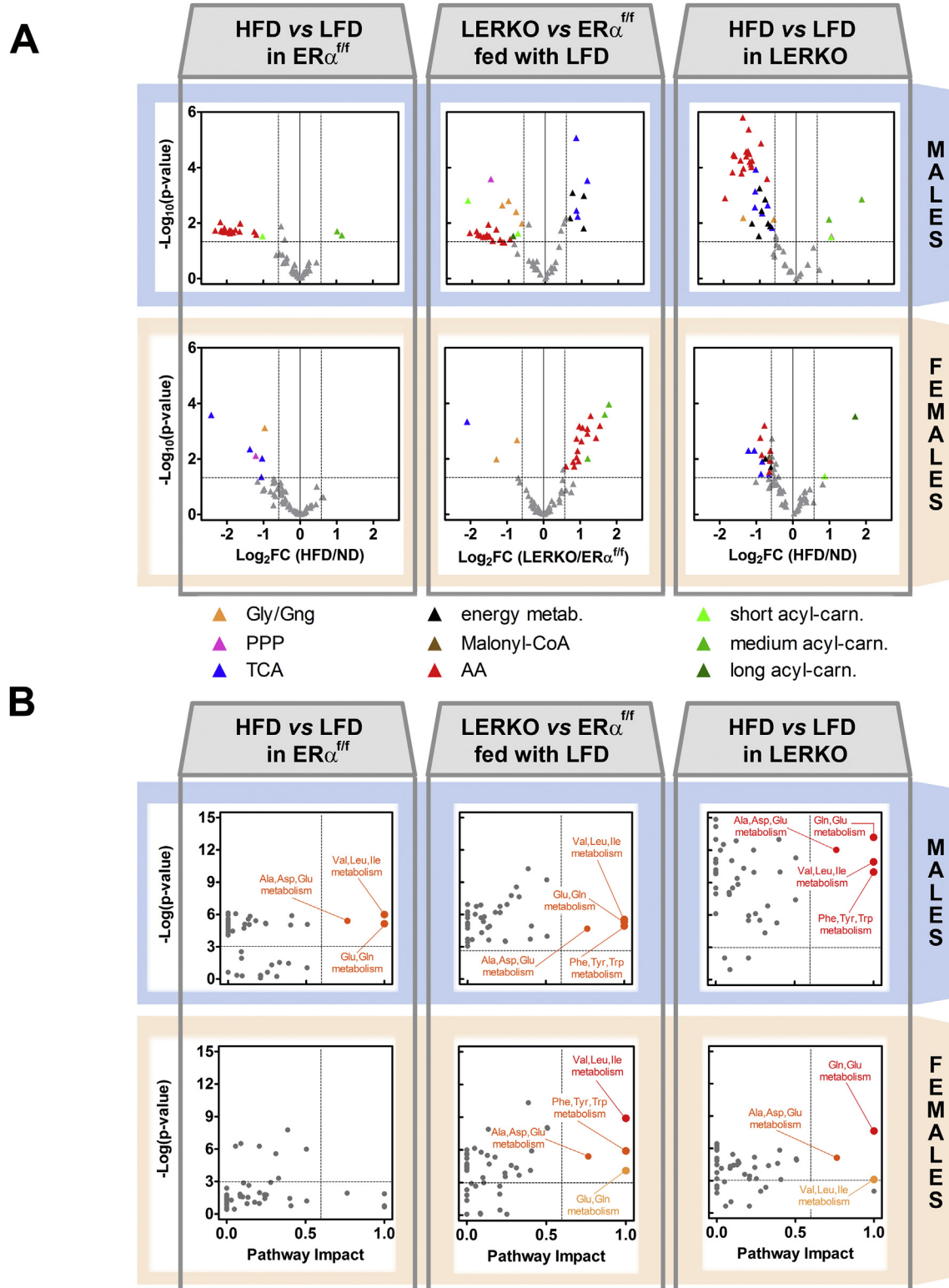
**Figure 3: HFD unmasks the sex-specific impact of hepatic ER $\alpha$  ablation on liver metabolome.** **A.** Hierarchical Clustering Heatmap of biologically major metabolites of the glycolysis/gluconeogenesis (GLY/GNG), pentose phosphate pathway (PPP), tricarboxylic acid cycle (TCA), energy metabolism (En. Met.), amino acids (AA), and acyl-carnitines (Acyl-Carn) measured in the liver. The color-coded scale on the left indicates the normalized metabolite expression. **B-C.** Principal Component Analysis (PCA) of the metabolites measured in the liver of ER $\alpha^{ff}$  and LERKO males (**B**) and females (**C**) fed with LFD and HFD. Colored ellipses - generated using MetaboAnalyst 3.0 software - represent 95% confidence intervals and highlight the basic clustering/separation between groups. Colored dots represent individual samples (n=6). **D.** Hierarchical Clustering (Dendrogram) of the data showed in A was done using a software available at the web page [http://www.wessa.net/rwasp\\_hierarchicalclustering.wasp](http://www.wessa.net/rwasp_hierarchicalclustering.wasp).

of total CH levels in the plasma of ER $\alpha^{ff}$  but not in LERKO males, whereas the opposite was true for females (Figure 6A). As a consequence of the HFD, the distribution of CH in the low-density lipoprotein (LDL) fraction increased, leading to a higher LDL/high-density lipoprotein (HDL) ratio in both ER $\alpha^{ff}$  males and females (Figure 6B–D). The sole ablation of liver ER $\alpha$  resulted in higher LDL-CH and LDL/HDL ratio in both LERKO males and females. However, only in LERKO males LDL-CH and LDL/HDL ratio were further augmented by HFD, whereas no changes were observed in LERKO females after HFD (Figure 6B–D), despite the higher total CH levels (Figure 6A). All together, these data suggest that ER $\alpha$  action in the liver strongly contributes to the different, sex-specific

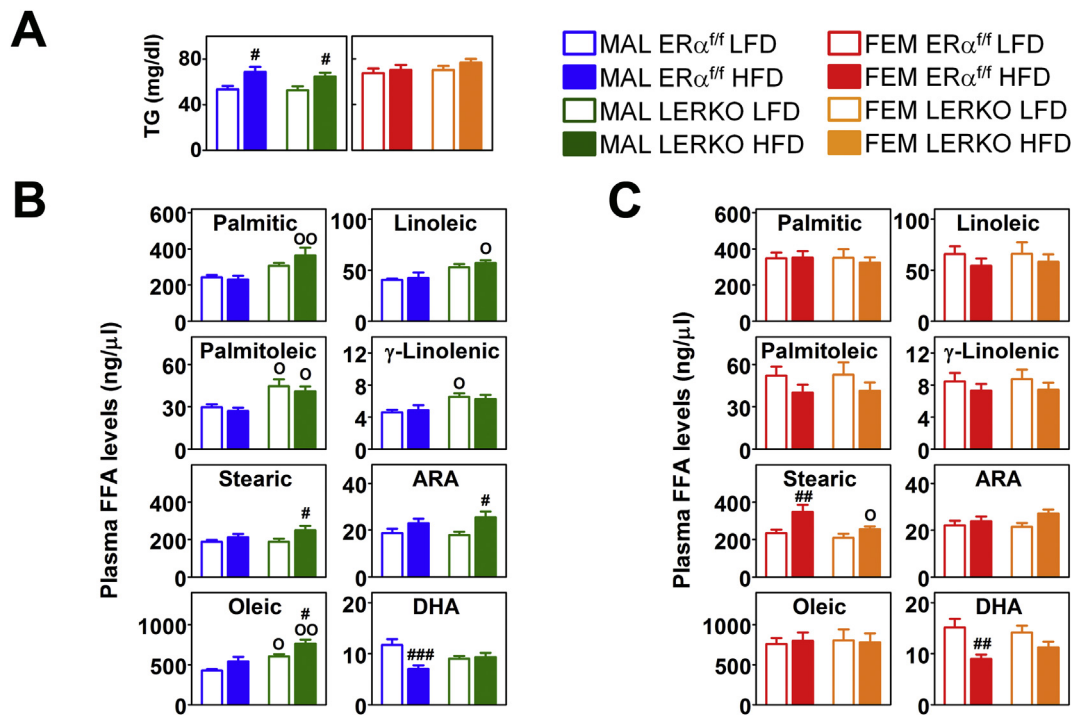
regulation of circulating lipids (FFA and CH, in particular) in response to a fat-enriched diet.

#### 3.4. Liver ER $\alpha$ regulates the hepatic lipid metabolism in a sex-specific fashion

Lipid deposition in the liver might be a consequence of an imbalance between the lipids that are taken up or synthesized *de novo* and those being catabolized or exported [24]. The fact that the lack of hepatic ER $\alpha$  prevented lipid deposition in the liver and increased the circulating lipids in males, but did the opposite in females, led us to hypothesize that the ER $\alpha$  action may regulate the uptake of lipids differently in the two sexes. To investigate on that, we first evaluated the expression of



**Figure 4: Hepatic ER $\alpha$  preserves AA homeostasis in the liver of females but not of males challenged with HFD.** **A.** Volcano plot of biologically relevant metabolites measured in the liver of ER $\alpha^{ff}$  and LERKO males and females fed with LFD and HFD. Only metabolites with a  $|FC| > 1.5$  and  $-\log_{10}(p\text{-value}) > 1.3$  were considered as significant and displayed with triangles of different colors depending on the class they belong to (see legend). **B.** Pathway impact analysis of the biological pathways regulated in the liver of ER $\alpha^{ff}$  and LERKO males and females fed with LFD and HFD. Only metabolic pathway with an impact  $> 0.6$  and a  $-\log(p\text{-value}) > 3$  were considered as significant and displayed as colored circles (varying from yellow to red).



**Figure 5: Lack of hepatic ER $\alpha$  affects the circulating lipids especially in males. A-C.** Levels of triglyceride (TG) (A) and free fatty acid (FFA) (B-C) measured in the plasma. Data information: In (A-C), data are presented as mean  $\pm$  SEM (n= 8-12). °p<0.05, °°p<0.01 and °°°p<0.001 LERKO vs ER $\alpha^{ff}$ ; #p<0.05, ##p<0.01 and ###p<0.001 HFD vs LFD (two-way ANOVA followed by Bonferroni's *post hoc* test).

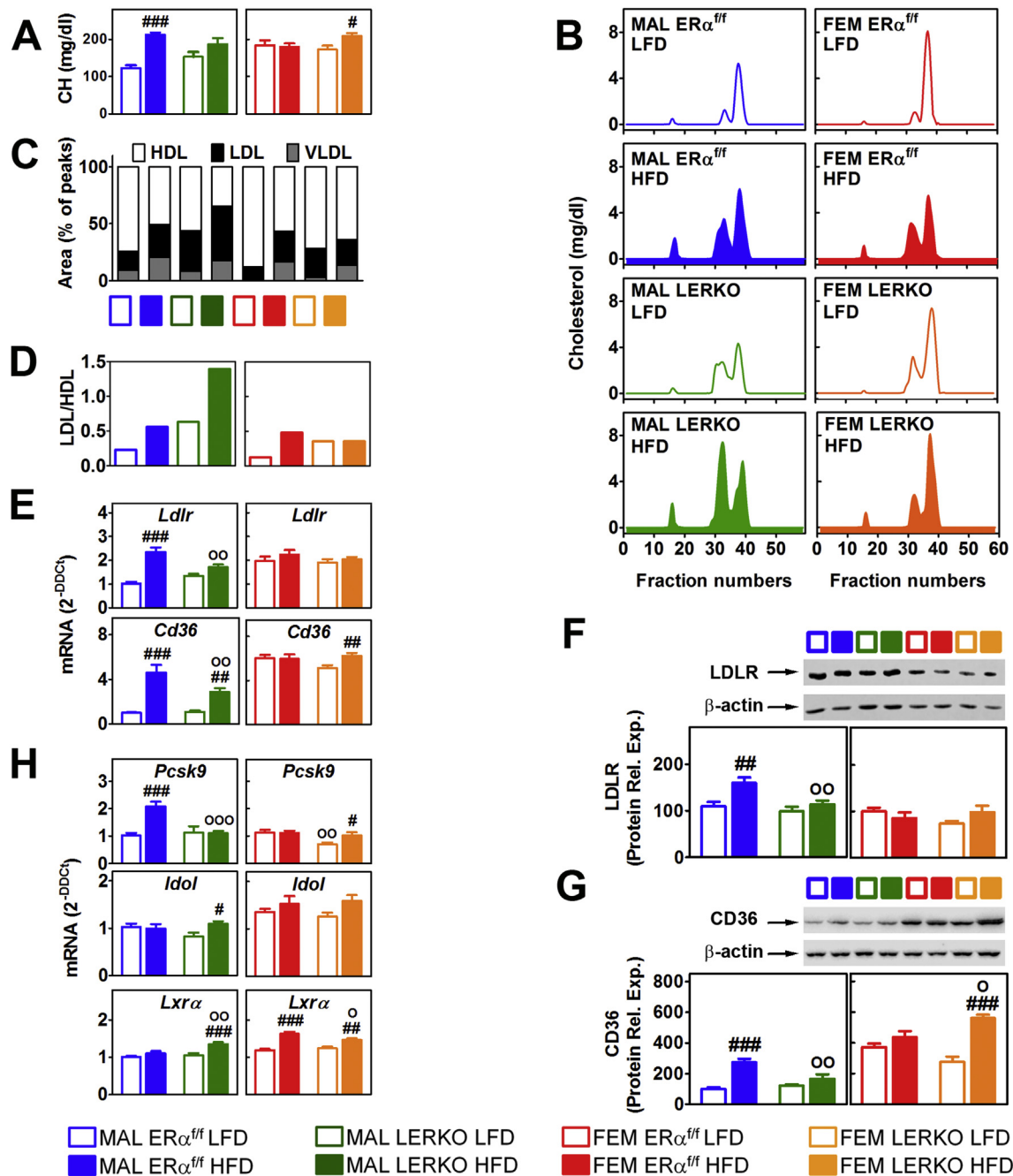
receptors relevant for the uptake of lipids, such as low-density lipoprotein receptor (LDLR) and cluster of differentiation 36 (CD36). LDLR and CD36 were induced in the liver of ER $\alpha^{ff}$  but not LERKO males when exposed to HFD, indicating that, in the absence of hepatic ER $\alpha$ , the male liver lost its ability to promote the lipid uptake (Figure 6E–G). Compared with their male counterparts, ER $\alpha^{ff}$  and LERKO females showed high basal *Ldlr* and *Cd36* mRNAs and followed a different strategy when exposed to HFD: the lipid uptake was unchanged in the liver of ER $\alpha^{ff}$  females (Figure 6E–G) and promoted in the liver of LERKO females (+103% CD36, Figure 6G). These data suggest that ER $\alpha$  might have a sex-specific role in the control of lipid uptake in response to HFD.

The hepatic content of LDLR [25,26] and CD36 [27] can be regulated through the degradation promoted by proprotein convertase subtilisin/kexin type 9 (PCSK9), whose expression is affected by estrogens [28]. *Pcsk9* mRNA was upregulated by HFD in the liver of ER $\alpha^{ff}$  but not in LERKO males, whereas the opposite was true for females (Figure 6H). Although its relevance in the mouse liver is still debated [29–31], inducible degrader of the low-density lipoprotein receptor (IDOL), the expression of which is controlled at the transcriptional level by liver X receptor alpha (LXR $\alpha$ ), may mediate the degradation of LDLR, thereby limiting the uptake of CH through the LDLR pathway. The over-expression of *Idol* (+32%) and *Lxr $\alpha$*  (+29%) in the liver of LERKO males treated with HFD suggested that the low expression of LDLR protein might be a consequence of an enhanced LDLR degradation due to the over-activation of the LXR $\alpha$ -IDOL pathway (Figure 6H). Interestingly, such regulation was found to be male-specific, as we did not observe a similar impairment of the modulation of LDLR and of *Idol* expression in the liver of females (Figure 6F,H).

Next, we evaluated the relevance of sex and hepatic ER $\alpha$  in the regulation of lipid metabolism by measuring the changes in the expression of some key genes involved in: *i*) the synthesis of FA (*Fasn*, fatty acid synthase; *Elovl6*, ELOVL fatty acid elongase 6) and CH (*Hmgcr*, 3-hydroxy-3-methylglutaryl-CoA reductase; *Pmvk*, phosphomevalonate kinase); *ii*) generation of lipid droplets (*Plin2*, perilipin-2); *iii*) mitochondrial FA  $\beta$ -oxidation (mtFAO: *Cpt1 $\alpha$* , carnitine palmitoyl-transferase 1A; *Hadha*, hydroxyacyl-Co A dehydrogenase trifunctional multienzyme complex subunit alpha, and *Acadl*, acyl-CoA dehydrogenase long chain); and *iv*) conversion of CH into bile acids (*Cyp7a1* and *Cyp27a1*, cytochrome P450 7A1 and P450 27A1); *v*) esterification, assembly, and export of lipids (*Acat2*, acetyl-CoA acetyltransferase 2; *Mttp*, microsomal triglyceride transfer protein) (Figure 7).

In ER $\alpha^{ff}$  males, HFD feeding was associated with an increased lipid synthesis (+188% *Fasn*, +21% *Elovl6*, +158% *Hmgcr*, +76% *Pmvk* vs LFD) and with the generation of lipid droplets (+66% *Plin2* vs LFD), thus contributing to lipid accumulation in the liver (Figure 7). The mtFAO (+35% *Cpt1 $\alpha$* , +35% *Hadha*, +39% *Acadl* vs LFD) and the catabolism of CH (+73% *Cyp7a1* and +37% *Cyp27a1* vs LFD) were increased by HFD. However, mtFAO was found to be inefficient, leading to the accumulation of medium-chain acyl-carnitines (Figure 3A and Table S3) that, in turn, could further promote the degeneration of hepatic tissue. In the attempt to manage lipid excess, the male liver promoted the export of lipids by enhancing the expression of *Acat2* (+115% vs LFD) and *Mttp* (+22% vs LFD), a metabolic adaptation that might contribute to the high levels of TG and CH measured in the plasma (Figures 5A and 6A). These data suggested that the fat deposition in the liver of ER $\alpha^{ff}$  males might be a consequence of increased import and synthesis of lipids and altered mtFAO. In the liver of LERKO males, the synthesis of lipids was increased, although to a lesser extent with respect to ER $\alpha^{ff}$  males (–99% *Fasn*, –119%



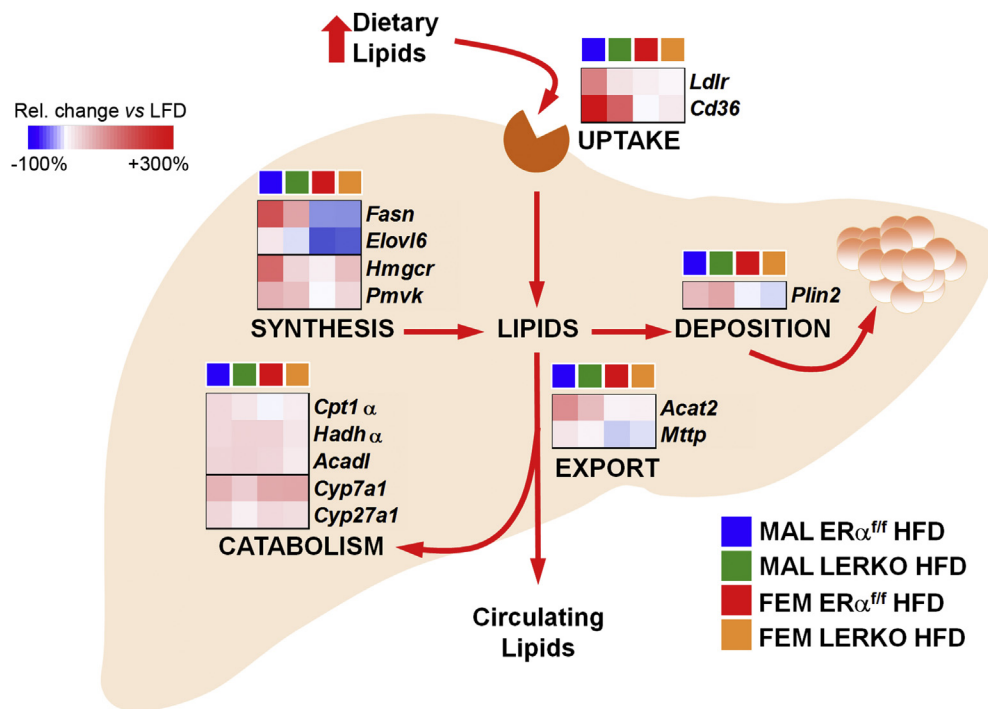


**Figure 6: Lack of hepatic ER $\alpha$  in males leads to an impaired cholesterol profile.** **A.** Levels of cholesterol (CH) measured in the plasma. **B.** Representative profile of the total CH content in the fractions of plasma separated by fast performance liquid chromatography (FPLC). **C.** Analysis of CH distribution in VLDL (very-low density lipoprotein), LDL (low density lipoprotein), HDL (high density lipoprotein) fraction in the plasma of ER $\alpha^{fl/fl}$  and LERKO males and females was done by integrating the area under the curve (AUC) of the CH profiles showed in **B**. **D.** Ratio between the LDL-CH and HDL-CH fraction values calculated as in **C**. **E.** mRNA contents of *Ldlr* (low-density lipoprotein receptor) and *Cd36* (cluster of differentiation 36) measured in the liver by rtPCR. **F-G.** Representative western blotting analyses and semi-quantitative analyses of the contents of LDLR (**F**) and CD36 (**G**) in liver extracts. **H.** mRNA content of *Pcsk9* (proprotein convertase subtilisin/kexin type 9), *Idol* (inducible degrader of the LDLR) and *Lxr $\alpha$*  (liver X receptor alpha) measured in the liver by rtPCR. Data are presented as mean  $\pm$  SEM: in (**A-D**),  $n = 8-12$ ; in (**E, H**),  $n = 8-12$ ; in (**F, G**),  $n = 6$ .  $^*p < 0.05$ ,  $^{**}p < 0.01$  and  $^{***}p < 0.001$  LERKO vs ER $\alpha^{fl/fl}$ ;  $\#p < 0.05$ ,  $\#\#\#p < 0.01$  and  $\#\#\#\#p < 0.001$  HFD vs LFD (two-way ANOVA followed by Bonferroni's *post hoc* test).

*Hmgcr* vs ER $\alpha^{fl/fl}$  males). The HFD regimen induced the FA catabolism, as suggested by the upregulation of *Cpt1 $\alpha$* , *Hadha*, *Acadl*, and the 54% reduction of malonyl-CoA (Figure 3A and Table S3), a powerful inhibitor of mtFAO [32]. The reduced uptake and synthesis of CH was associated with a restrained induction of CH catabolism and esterification with respect to ER $\alpha^{fl/fl}$  males ( $-26\%$  *Cyp7a1*,  $-25\%$  *Cyp27a1* and  $-51\%$  *Acat2* vs ER $\alpha^{fl/fl}$

males) and with unchanged values of *Mttp*. These data suggested that the limited fat deposition in the liver and the altered levels of lipids in the plasma of LERKO males were a consequence of a limited import and synthesis of lipids (Figure 7).

Under a HFD feeding regimen, the liver of ER $\alpha^{fl/fl}$  females inhibited *de novo* lipogenesis (DNL) ( $-43\%$  *Fasn*,  $-72\%$  *Elovl6* vs LFD), prevented



**Figure 7: Hepatic ER $\alpha$  confers on liver a sex-specific strategy to cope with the excess of dietary lipids.** Heatmaps representing the HFD-induced changes in the hepatic content of key genes involved in lipid metabolism. Data are expressed as percentage *versus* LFD.

the synthesis of CH, and promoted the catabolism of FA (+41% *Hadh $\alpha$* , +37% *Acadl* vs LFD) and the catabolism of CH (+84% *Cyp7a1* and +34% *Cyp27a1* vs LFD), without leading to an accumulation of medium-chain acyl-carnitines (Figure 3A and Table S3). The lipid export was unchanged. All together, these changes represented a concerted metabolic strategy useful to counteract the development of fatty liver and the rise of circulating lipids (Figure 7).

This female-biased ability to counteract the excess of dietary lipids by adopting protective metabolic adjustments was, at least in part, dependent on hepatic ER $\alpha$  (Figure 7). Indeed, with the lack of hepatic ER $\alpha$ , although the synthesis of FA was still inhibited DNL (−43% *Fasn*, −67% *Elovl6* vs LFD), the synthesis of CH increased (+58% *Hmgcr*, +36% *Pmvk* vs LFD). mtFAO was increased by HFD but to a lesser extent with respect to ER $\alpha^{fl/fl}$  females (−19% of *Hadh $\alpha$*  and *Acadl* vs ER $\alpha^{fl/fl}$  females) and led to the accumulation of medium- (C8, C9, C10) and long-chain (C12, C16, C18 and C18:1) acyl-carnitines (Figure 3A and Table S3). The lack of hepatic ER $\alpha$  did not affect the expression of genes involved in CH catabolism nor in lipid export (Figure 7). These changes suggested that the increased lipid import, the enhanced CH synthesis, and the impaired mtFAO might contribute to higher deposition of lipids in the liver and to higher CH levels in the plasma of LERKO females.

These data led us to conclude that hepatic ER $\alpha$  contributes to the sex-specific metabolic adaptation to a diet enriched in fats, by favoring and preventing lipid deposition in the liver of males and females, respectively.

#### 4. DISCUSSION

By directly comparing siblings of the two sexes, we here underline major, sex-specific, and ER $\alpha$ -dependent differences in male and female liver ability to cope with an excessive dietary intake of fats. The

male liver was unable to set up compensatory mechanisms to deal with the excess of dietary lipids in the long-term: after 16 weeks of HFD, enhanced lipid uptake, increased lipid synthesis, and impaired FA oxidation led to fatty liver and degeneration of hepatic metabolism, which favored the increase of circulating CH. On the contrary, female liver was able to manage the excess lipids, limiting lipid import, avoiding worthless DNL, and efficiently promoting the mtFAO. Such female metabolic flexibility was possibly a consequence of a sex-biased hepatic metabolism geared to satisfy the energy needs of reproduction [5]. Indeed, the necessity to adapt the hepatic metabolism to different and highly variable reproductive needs might provide the female liver with a greater ability to manage dietary stress, including lipid excess. In females, this metabolic flexibility was dependent on hepatic ER $\alpha$ ; indeed, LERKO females were unable to properly process dietary lipids and were affected by fatty liver, increased circulating lipids, and ectopic fat accumulation, all metabolic alterations generally occurring in females after menopause or ovariectomy [1,33,34].

Since hepatic ER $\alpha$  activity provides females with the metabolic flexibility necessary to antagonize the detrimental effects of a HFD, the finding of major metabolic changes in LERKO males was quite unexpected. Indeed, despite the low expression of ER $\alpha$  that led us to speculate that this receptor might be less relevant for the male hepatic metabolism, our results showed the opposite: the lack of hepatic ER $\alpha$  in LERKO males prevented lipid deposition and the progressive degeneration of hepatic tissue typical of ER $\alpha^{fl/fl}$  males exposed to HFD. Therefore, despite its abundance, ER $\alpha$  accomplishes a significant metabolic function in the male liver that is clearly not the same as in females. This is particularly relevant with regard to the uptake and *de novo* synthesis of lipids; indeed, under HFD conditions, the regulation of genes involved in these metabolic pathways is diametrically opposed in the liver of males and females, depending on the presence/absence of hepatic ER $\alpha$ .

Besides lipid catabolism, the hepatic content of AA (Figure 6A–B) was the pathway most affected by HFD regimen in ER $\alpha$ <sup>fl/fl</sup> males, confirming studies showing a correlation between low AA content and hepatic steatosis/NAFLD in obesogenic-like conditions [35]. The finding that the content of AA was decreased by HFD both in LERKO males and females, whereas it was unaffected in the liver of ER $\alpha$ <sup>fl/fl</sup> females, suggested that the homeostasis of AA under an unbalanced dietary regimen is a female-specific prerogative in charge of hepatic ER $\alpha$ . However, the lack of hepatic ER $\alpha$  did not decrease but, otherwise, increased the differences between the two sexes (Fig. S2), suggesting that ER $\alpha$  might strongly contribute to a different regulation of the hepatic metabolism in the two sexes.

How can we explain that the same receptor in the same tissue has a different function in the two sexes? In our previous reports, we showed that sex differences in the hepatic metabolism (and in particular in the AA content) depend on an estrogen-dependent sexual differentiation of the male liver that occurs right around birth; at that time, testis-derived testosterone is converted by the aromatase enzyme in estrogen that “organizes” and programs the liver in a male-specific manner, by acting through hepatic ER $\alpha$  [4]. The lack of such “organization” in the liver of LERKO males (that do not express hepatic ER $\alpha$  since embryonal day 18.5) might be responsible for a major metabolic rewiring of the liver, even when LERKO males are exposed to LFD (Figures 3A and 4A). As a consequence of the lack of ER $\alpha$ -dependent programming of male hepatic metabolism, the metabolic response of the LERKO male liver to HFD regimen was clearly different from that of the ER $\alpha$ <sup>fl/fl</sup> male liver and the LERKO female liver. In this view, liver ER $\alpha$  also has specific abilities in controlling hepatic metabolism in males, despite its low concentration.

## 5. CONCLUSION

This study highlights the essential role of hepatic ER $\alpha$  in the opposite regulation of lipid metabolism in the liver of the two sexes when dietary lipids are in excess: in males, hepatic ER $\alpha$  action contributes to liver lipid accumulation mainly by stimulating lipid import and synthesis; conversely, in females, ER $\alpha$  prevents the hepatic lipid deposition by keeping the lipid uptake low, by inhibiting lipid synthesis, and by efficiently promoting mtFAO.

All together, these data led us to conclude that hepatic ER $\alpha$  strongly contributes to the sex-specific response to a diet enriched in fats, and its action accounts for opposite consequences for hepatic health in males and females. In view of our observations, a proper liver ER $\alpha$  activity should be preserved in females to maintain the metabolic flexibility and counteract the metabolic impairments (fatty liver, increased circulating lipids) typical of the post-menopause period [1,33,34]. As the exposure to estrogenic compounds early in life may have severe consequences for the hepatic metabolism of males [4], our study questions whether the increased incidence of metabolic disturbances may be associated with an early exposure to estrogen-like endocrine disruptors [36,37], whose abundance is continuously growing as a result of contaminants of our environment.

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## AUTHOR CONTRIBUTIONS

C.M. performed most of the *in vivo* studies, biochemical assays and the liver histology analysis; C.M. revised the manuscript. M.B. performed

the western blotting analysis and real-time PCR analysis. N.M. conceived the metabolomic analysis and the FPLC analysis of CH profile; N.M. revised the manuscript. F.L. performed real-time PCR analysis and revised the manuscript. S.P. performed the metabolomic analysis and the FPLC analysis of CH profile. D.C. participated in the conception of the metabolomic analysis. A.M. participated in the conception of the study, discussed the results and revised the manuscript. S.D.T. conceived the project, performed the *in vivo* studies, the biochemical assays, the analysis of metabolomic data; S.D.T. wrote and revised the manuscript.

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## CONFLICT OF INTEREST

The authors do not have any conflicts of interest.

## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molmet.2019.12.009>.

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