Research article

JHEP|Reports

Hepatic estrogen receptor alpha drives masculinization in post-menopausal women with metabolic dysfunction-associated steatotic liver disease

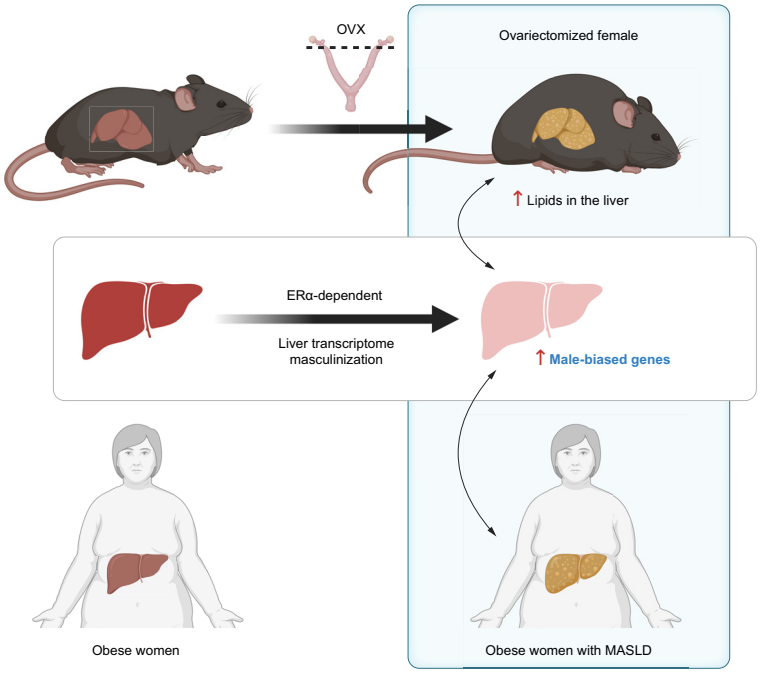
Authors

Clara Meda, Valeria Benedusi, Alessandro Cherubini, Luca Valenti, Adriana Maggi, Sara Della Torre

Correspondence

sara.dellatorre@unimi.it (S. Della Torre).

Graphical abstract



Highlights:

- Hepatic ERα is crucial for estrogen-depleted liver reprogramming.
- Hepatic ERα is needed for OVX-dependent induction of male-biased genes.
- MASLD development in obese women is associated with liver masculinization.
- Targeting hepatic ERα may tackle menopause-associated MASLD.

Impact and implications:

Despite the increased risk of developing MASLD after menopause, the specific contribution of impaired hepatic estrogen signaling in driving MASLD in females has not been a major research focus, and, thus, has limited the development of tailored strategies that address the specific mechanisms underlying MASLD in post-menopausal women. This study reveals the functional role of hepatic ERα in mediating liver metabolic changes in response to estrogens loss, leading to a shift in the liver transcriptome towards a male-like profile. In women with obesity, this shift is associated with the development of MASLD. These findings underscore the potential of targeting hepatic ERα as a promising approach for developing effective, sex-specific treatments to preserve liver health and prevent or limit the development and progression of MASLD in post-menopausal women.

https://doi.org/10.1016/j.jhepr.2024.101143

 JHEP Reports, 2024, 6, 1–11

Research article

JHEP|Reports

Hepatic estrogen receptor alpha drives masculinization in post-menopausal women with metabolic dysfunction-associated steatotic liver disease

Clara Meda[1], Valeria Benedusi[2], Alessandro Cherubini[3], Luca Valenti[3,4], Adriana Maggi[2], Sara Della Torre[2,*]

JHEP Reports **2024**. vol. 6 | 1–11

Background & Aims: The loss of ovarian functions defining menopause leads to profound metabolic changes and heightens the risk of developing metabolic dysfunction-associated steatotic liver disease (MASLD). Although estrogens primarily act on the female liver through estrogen receptor alpha (ERα), the specific contribution of impaired ERα signaling in triggering MASLD after menopause remains unclear.

Methods: To address this gap in knowledge, we compared the liver transcriptomes of sham-operated (SHAM) and ovariectomized (OVX) control and liver ERα knockout (LERKO) female mice by performing RNA-Seq analysis.

Results: OVX led to 1426 differentially expressed genes (DEGs) in the liver of control mice compared to 245 DEGs in LERKO mice. Gene ontology analysis revealed a distinct ovariectomy-induced modulation of the liver transcriptome in LERKO compared with controls, indicating that hepatic ERα is functional and necessary for the complete reprogramming of liver metabolism in response to estrogen depletion. Additionally, we observed an ovariectomy-dependent induction of male-biased genes, especially in the liver of control females, pointing to hepatic ERα involvement in the masculinization of the liver after estrogen loss. To investigate the translational relevance of such findings, we assessed liver samples from a cohort of 60 severely obese individuals (51 women; 9 men). Notably, a shift of the liver transcriptome toward a male-like profile was also observed only in obese women with MASLD (n = 43), especially in women ≥51 years old (15/15), suggesting that masculinization of the female liver contributes to MASLD development in obese women.

Conclusions: These results highlight the role of hepatic ERα in driving masculinization of the liver transcriptome following menopause, pointing to this receptor as a potential pharmacological target for preventing MASLD in post-menopausal women.

Introduction

In comparison with men, fertile women exhibit a lower risk of developing metabolic dysfunction-associated steatotic liver disease (MASLD). MASLD can progress to severe liver conditions and is closely linked with other cardiometabolic diseases.[1,2] However, after menopause, changes in estrogen levels predispose women to hepatic steatosis, nullifying sex differences in MASLD susceptibility.[3,4]

Estrogens predominantly exert their effects in the female liver through estrogen receptor alpha (ERα), whose signaling concurs to modulate the hepatic metabolism according to each reproductive stage.[5,6] Such a regulatory role of hepatic ERα in metabolism and reproduction,[5–8] has likely been acquired through evolution,[9,10] and contributes to sex differences in MASLD susceptibility. Indeed, when exposed to a diet rich in lipids, fertile female mice exhibit a greater ability, which is largely dependent on hepatic ERα, to limit liver lipid deposition compared with males.[11] Nevertheless, estrogen' deprivation impairs hepatic lipid metabolism and promotes lipid accumulation in mouse models of menopause.[5,12]

Given its relevance in the regulation of hepatic metabolism, estrogen supplementation has the potential to mitigate dysmetabolism and prevent liver lipid accumulation in women after menopause.[13] Although hormone replacement therapy (HRT) can be beneficial, it cannot be considered a primary approach to counteract MASLD in post-menopausal women. Indeed, the restricted window of opportunity (<10 years after menopause), the individual benefit:risk *ratio*, and the low HRT prescription rate and uptake,[14] limit this approach. Because women spend more than one-third of their lives in the post-menopausal state[15] and the global burden of metabolic diseases and MASLD,[16,17] the search for valuable alternative(s) to the classical estrogen-based HRT is of utmost importance for women's health. In this context, understanding the specific contribution of hepatic ERα to liver reprogramming after menopause may lead to the development of more targeted pharmacological approaches that focus on the

* Corresponding author. Address: Department of Pharmaceutical Sciences, Università degli Studi di Milano, via Balzaretti 9, 20133 Milan, Italy. Tel.: +390250318375.
E-mail address: sara.dellatorre@unimi.it (S. Della Torre).
https://doi.org/10.1016/j.jhepr.2024.101143

liver, thereby overcoming the potential, systemic side-effects of classical HRT.

Herein, we share our work aimed at investigating the impact of hepatic ERα signaling on menopause-associated changes by comparing the liver transcriptomes of sham-operated (SHAM) and ovariectomized (OVX) controls as well as that of liver ERα knockout (LERKO) female mice models.

Materials and methods

Animals and experimental design

Syngenic ERα floxed (CTRL) and LERKO mice[7] both derived from the C57BL/6 J strain. At 2 months of age, mice were anesthetised with an s.c. injection of 70 μl volume of 109.2 mg/kg ketamine (Ketavet 100; Intervet, Milan, Italy) and 8.4 mg/kg xylazine (Rompun; Bayer, Milan, Italy) and were then OVX or SHAM operated (Fig. S1). Mice were fed *ad libitum* with a standard diet (#D12450B ResearchDiets; Broogarden, Lynge, Denmark), provided with filtered water, and housed within a temperature of 22–25 °C, relative humidity of 50 ± 10%, under an automatic 12-h light/dark cycle. Four months after surgery, animals were euthanized in the early afternoon after 6 h of fasting to avoid potential confounding effects due to the circadian rhythm or feeding status.[7] SHAM females were collected when in the *estrus* phase after vaginal smears analysis. All animal experimentation was done in accordance with the ARRIVE and European guidelines. The animal study protocol was approved by 'Istituto Superiore di Sanità - Ministero della Salute Italiano' (1272/2015-PR and 476/2015-PR).

RNA-sequencing and transcriptomic analysis

Mouse RNA sequencing and transcriptomics data analysis were performed as described previously.[12] Gene Ontology (GO) and cluster analysis was performed using the Cytoscape plug-in ClueGO, Genesis, Enrichr, and ShinyGO. A Venn diagram was constructed with Bioinformatics and Evolutionary Genomics software (http://bioinformatics.psb.ugent.be/webtools/Venn/).

The human RNA sequencing analysis, count normalization, and differential gene expression (DEG) analyses were conducted as previously described[18] using RNA derived from liver biopsies from severely obese individuals enrolled in the Liver Biopsy Cohort who underwent a percutaneous liver biopsy performed during bariatric surgery at the Milan centre for clinical staging of liver disease severity (n = 125). All research was conducted in accordance with both the Declarations of Helsinki and Istanbul. Individuals with at-risk alcohol intake (>30/20 g per day in men/women), viral autoimmune hepatitis, or other causes of liver disease were excluded. Given the interaction between female sex and the *PNPLA3 p*.I148M variant in determining the predisposition to develop MASLD,[19] we excluded all samples carrying such a variant from the study, thus limiting our analysis to 60 samples (Table S1).

Statistical analysis

Statistical analysis was conducted using GraphPad Prism v.8.0. Multiple testing comparisons were performed by one or two-way ANOVA followed by Bonferroni's *post hoc* test. The two-tailed Student's *t-test* was used for comparisons between two experimental groups. All data are expressed as mean ± SEM. A *p*-value <0.05 was considered statistically significant.

Results

Estrogen loss induced liver metabolic reprogramming in a mouse model of menopause

To investigate the impact of liver ERα on changes in hepatic gene expression associated with estrogen deficiency, we performed RNA-seq analysis comparing liver transcriptomes of CTRL SHAM, CTRL OVX, and LERKO SHAM female mice. Using a fold change |FC|>1.3 and a p-adj <0.05, we identified 1426 DEGs between CTRL SHAM and CTRL OVX, with 667 (47.5%) genes upregulated by ovariectomy (Fig. 1A). Comparing CTRL SHAM and LERKO SHAM mice, 239 DEGs were identified, with 57 (∼24%) upregulated in LERKO SHAM mice (Fig. 1B). These results suggest that estrogen deficiency surpasses the changes in the liver transcriptome associated with the mere absence of hepatic ERα, pointing to the involvement of other systemic factors.

Comparing CTRL OVX/CTRL SHAM with LERKO SHAM/CTRL SHAM mice, we identified 157 shared genes between the two groups (Fig. 1C). Of these, 155 had similar regulation patterns in response to both estrogen deficiency and hepatic absence of ERα, whereas only two genes (Fig. S2A) exhibited opposite regulation.

Cluster analysis revealed that of the 155 shared genes, 37 genes were upregulated and predominantly associated with the triglyceride (TG) biosynthesis (Fig. 1D and E and Fig. S2B). Among the 118 downregulated shared genes (Fig. 1F and G), several were associated with positive regulation of transcription (Fig. S2C), histone H3–K4 methylation, and chromatin organization (Fig. S2D), and the estrogen receptor signaling pathway (i.e. *Arid1a*, AT-rich interaction domain 1a; *Wbp2*, ww domain binding protein 2) (Fig. S2E). Notably, the downregulation of *Arid1a* and *Wbp2* has been associated with hepatic steatosis, insulin resistance, and inflammation.[20,21]

We then explored the 1269 genes differentially regulated in the OVX CTRL mice, which were unaffected by the hepatic knockout of ERα in LERKO SHAM mice. Among the 638 genes specifically upregulated in CTRL OVX mice (Fig. 2A and B), several were involved in fatty acid (FA) and lipid metabolism, particularly in mitochondrial and peroxisomal oxidation of FA (FAO) (Fig. S3A), FA uptake, very-long and long FA metabolic processes (Fig. S3B). Notably, several of these genes are targets of *Pparα,* peroxisome proliferator activated receptor alpha, the master regulator of FAO,[22] whose hepatic expression was also enhanced in CTRL OVX mice (Fig. 2C and D). Estrogen deficiency can potentially trigger adipose tissue lipolysis, leading to the release of free FA, which once taken up by the liver, may activate PPARα, thereby inducing its expression along with that of its target genes,[22] enhancing FAO and gluconeogenesis (GNG). Consistent with this hypothesis, mRNA levels of key genes involved in GNG (*Foxo1,* forkhead box O1; *Fbp1,* fructose-bisphosphatase 1; *G6pc,* glucose-6-phosphatase) and in the regulation of glucose metabolism (*Gk,* glycerol kinase; several major urinary proteins, *Mups*) were also increased in CTRL OVX mice but not in LERKO SHAM mice (Fig. S3C and D).

Among the 631 genes downregulated by ovariectomy but not affected by the knockout of hepatic ERα expression (Fig. 2E and F), we identified genes associated with steroid metabolic process, cholesterol homeostasis, and trafficking, and several epoxygenase P450s (Fig. S4A-D). Other downregulated genes

belonged to clusters involved in the regulation of intracellular signaling pathways (Fig. S4E), cell communication, and anatomical structure morphogenesis.

These findings highlight the complex interplay between systemic estrogen deficiency and hepatic ERα signaling in mediating metabolic reprogramming in the liver following estrogen loss.

Hepatic ERα fully exploited liver metabolic reprogramming in a mouse model of menopause

Since only a subset of genes differentially regulated by OVX was shared between the LERKO SHAM and CTRL SHAM mice, it is reasonable to assume that most changes in gene expression found in OVX mice can be attributed to factors other than ERα in hepatocytes. In this perspective, if the role of ERα were

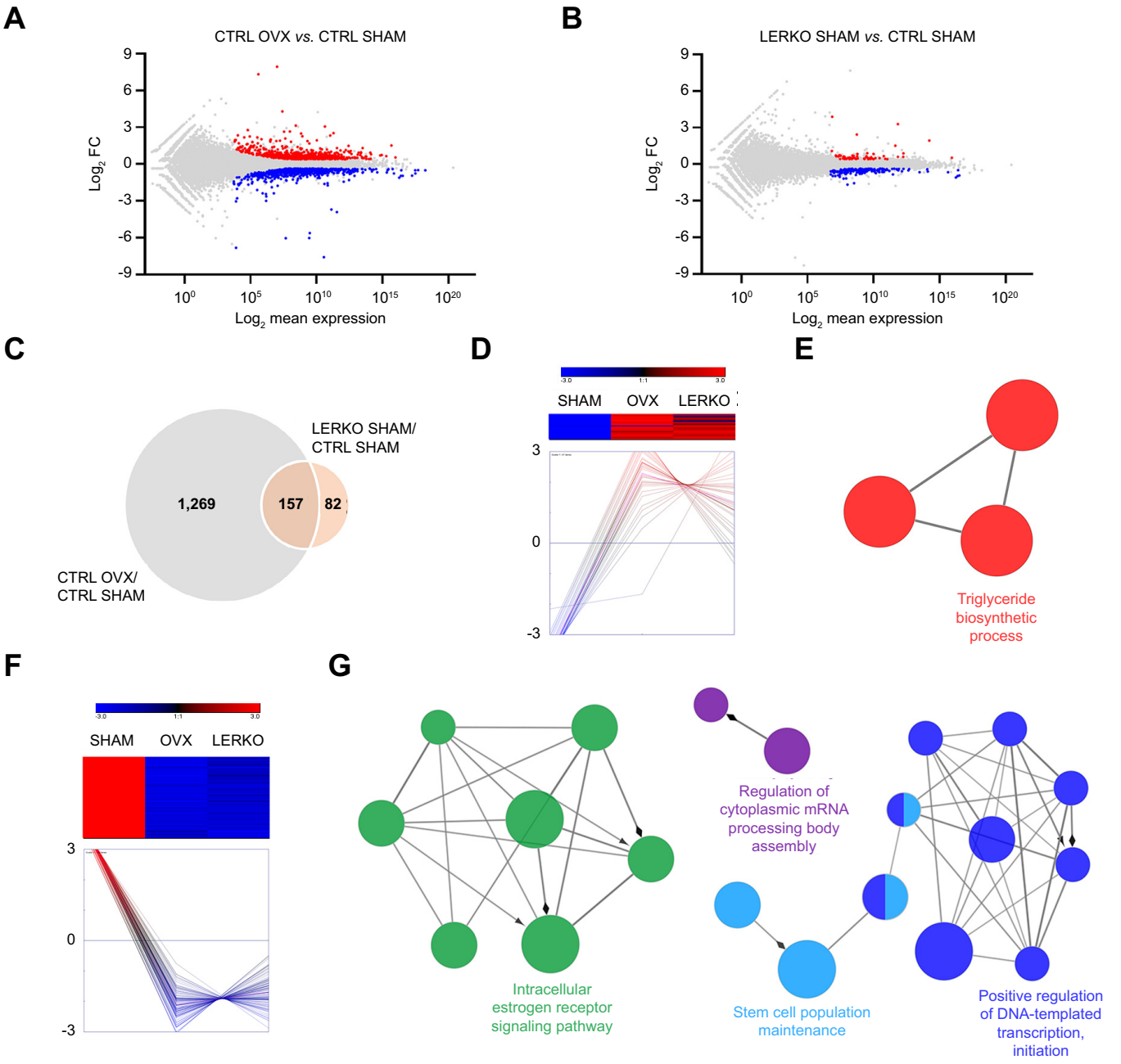


Fig. 1. The lack of hepatic ERα partially mimics changes in liver transcriptome observed in ovariectomized female mice. Distribution of genes upregulated (red) and downregulated (blue) by ovariectomy (A) or by lack of hepatic ERα (B) measured by RNA-Seq in the liver of CTRL SHAM, CTRL OVX, and LERKO SHAM (n = 4). (C) Venn diagram showing differentially expressed genes found in the comparisons between CTRL OVX/CTRL SHAM (grey) or LERKO SHAM/CTRL SHAM (orange). Heatmaps, clusters and Gene Ontology analysis of functional networks significantly upregulated (D, E) or downregulated (F, G) in CTRL OVX as well as LERKO SHAM with respect to CTRL SHAM mice.

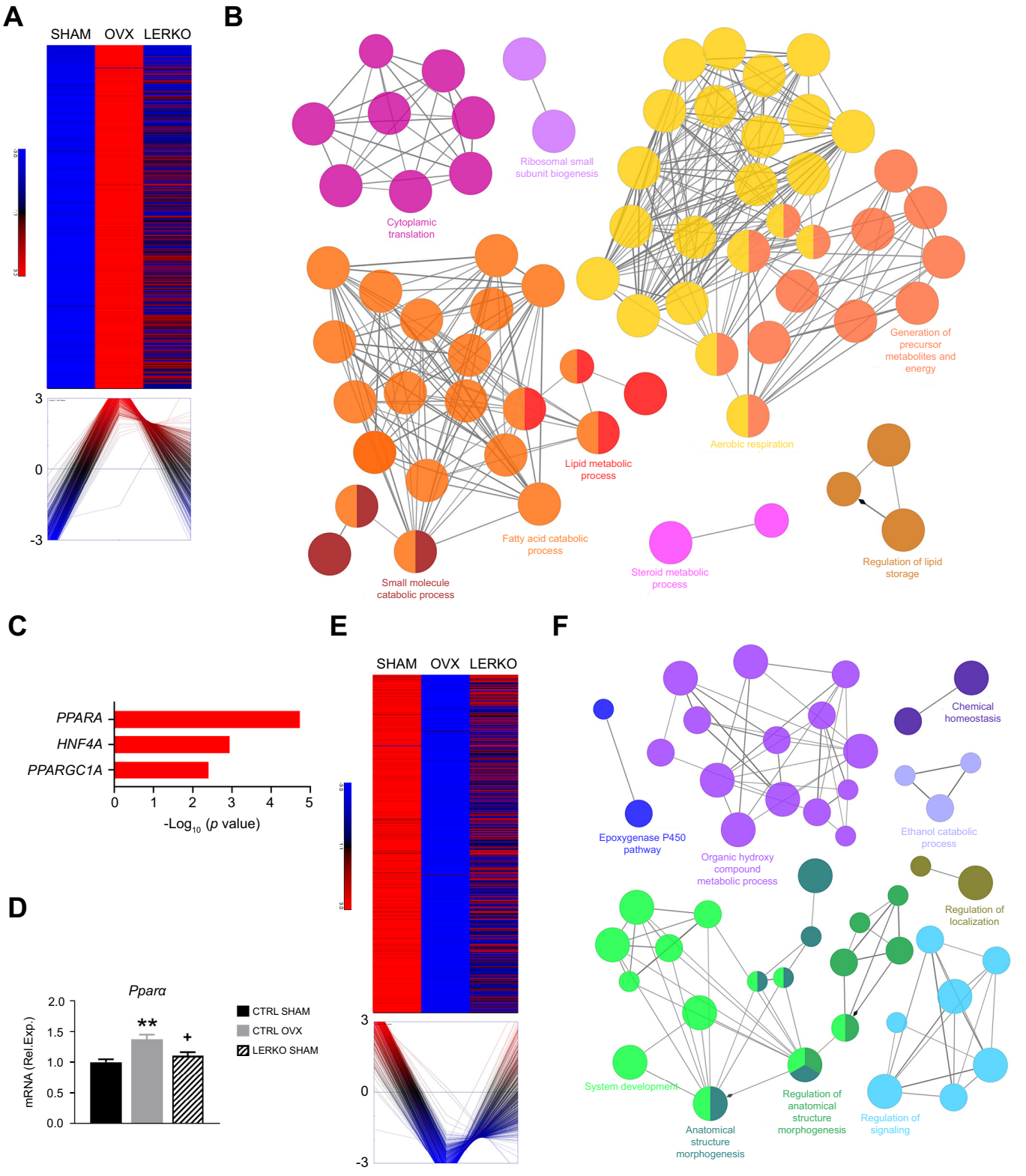


Fig. 2. Lack of estrogens reprograms liver metabolism in a mouse model of menopause. Heatmap and cluster (A), Gene Ontology (GO) analysis of functional networks (B), and most enriched motifs (C) associated with genes upregulated in CTRL OVX but not LERKO SHAM with respect to CTRL SHAM. (D) *Pparα* mRNA measured in the liver of CTRL SHAM, CTRL OVX and LERKO SHAM. Data from RNA-Seq analysis are represented as mean ± SEM (n=4). ***p* <0.01 CTRL OVX *vs.* CTRL SHAM; +*p* <0.05 LERKO SHAM *vs.* CTRL SHAM by one-way ANOVA followed by Bonferroni's *post hoc* test. Heatmap, cluster (E) and GO analysis of functional networks (F) associated with genes downregulated in CTRL OVX but not LERKO SHAM with respect to CTRL SHAM.

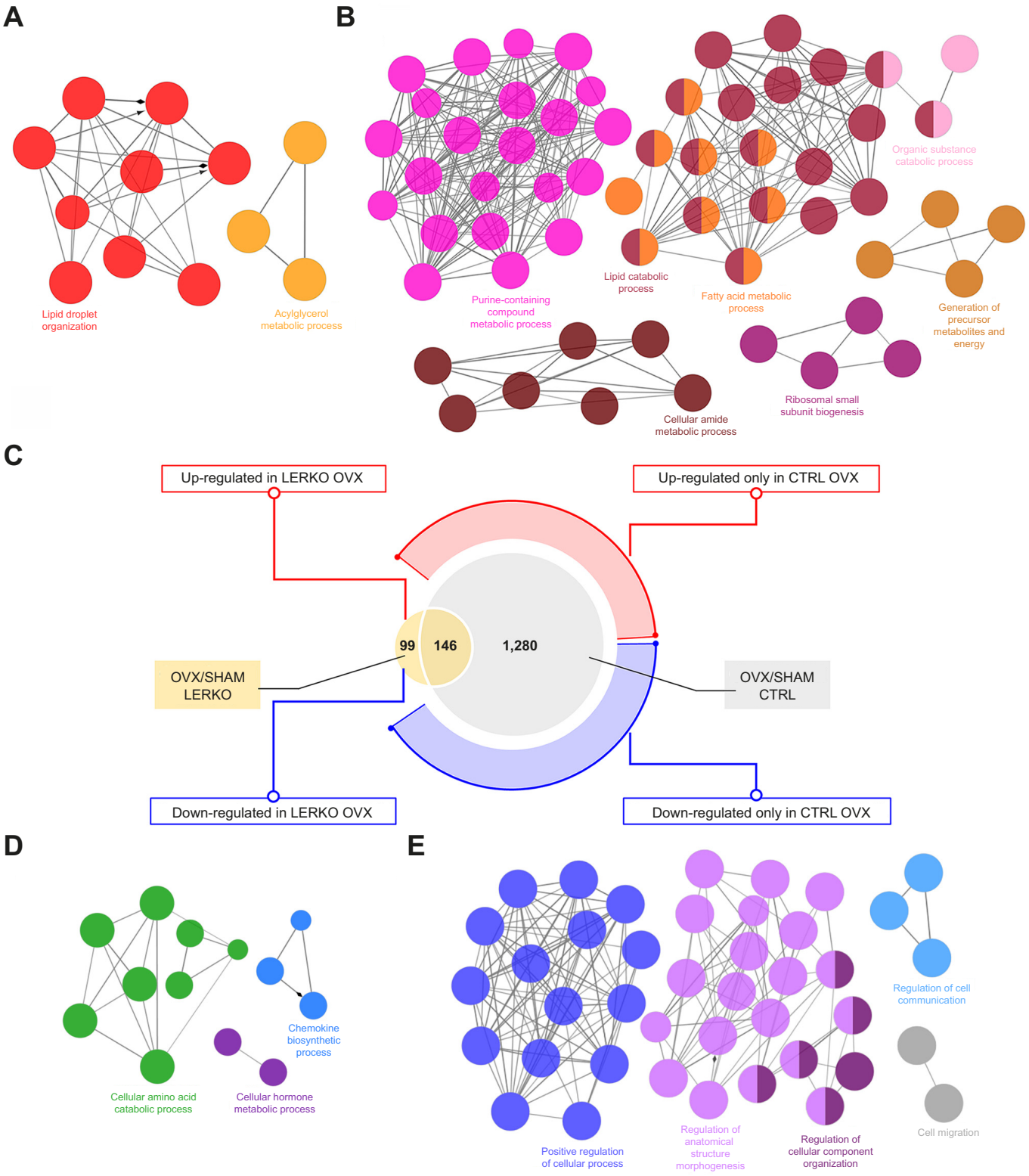


Fig. 3. Hepatic ERα profoundly impacts on ovariectomy-induced changes in liver transcriptome. GO analysis of functional networks significantly upregulated in LERKO (A) or uniquely in CTRL (B) mice following ovariectomy. (C) Venn diagram showing the overlapping among genes differentially regulated by ovariectomy in CTRL (grey) or LERKO (orange). GO analysis of functional networks significantly downregulated in LERKO (D) or uniquely in CTRL (E) mice by ovariectomy.

marginal in driving the changes associated with estrogen deficiency, similar changes to those observed in CTRL would be expected in LERKO following ovariectomy.

Analysing genes differentially regulated following ovariectomy (Fig. 3A-E), we found fewer DEGs in LERKO (245) compared with CTRL (1426) mice. Of these, 146 were shared between CTRL OVX/CTRL SHAM and LERKO OVX/LERKO SHAM mice, with 56 genes being upregulated and 90 downregulated by ovariectomy (Fig. 3C). Notably, this subset accounted for only ∼10% of the genes differentially regulated by ovariectomy in CTRL mice, arguing against the null hypothesis that hepatic ERα may have a marginal role in mediating changes associated with estrogen loss.

GO analysis revealed a distinct modulation of the liver transcriptome in LERKO OVX mice. Among the 91 genes upregulated by ovariectomy in LERKO (Fig. 3A), significant gene enrichment was associated with lipid synthesis, droplet organisation and storage, with some genes (e.g. *Scd1,* stearoyl-CoA desaturase 1*; Plin5,* perilipin 5) specifically enhanced in LERKO OVX mice, associated with a greater increase in liver lipid deposition and body weight (Fig. S5A and S6A-C). Differently from CTRL mice, genes involved in FAO and lipid metabolism were not induced in LERKO mice following ovariectomy (Fig. 3B), although *Pparα* was similarly enhanced by ovariectomy (Fig. S5A).

In LERKO mice, ovariectomy repressed 154 genes, including those associated with amino acid (AA) catabolism and transmembrane transport, regulation of chemokine biosynthesis, and hormone metabolic process (Fig. 3D). Among them, certain genes associated with steroid metabolism (Fig. S5B), serine and glycine metabolism (Fig. S5C), and AA transmembrane transport (Fig. S5D) were inhibited by ovariectomy in both genotypes. In contrast, other genes involved in AA catabolism (*Ido2,* indoleamine 2,3-dioxygenase 2; *Kyat1*, kynurenine aminotransferase 1; *Bcat2,* branched chain amino acid transaminase 2) were repressed only in LERKO OVX mice (Fig. S5E). In contrast to LERKO, we observed a repression of genes involved in the positive regulation of transcription, anatomical structure morphogenesis, and regulation of cell communication only in OVX CTRL mice (Fig. 3E).

These findings indicate that hepatic ERα is crucial for the majority of adaptative changes in the liver transcriptome in response to estrogen deprivation.

Hepatic ERα was essential for liver masculinization in a mouse model of menopause

The minimal overlap in gene expression between CTRL OVX/CTRL SHAM and LERKO OVX/LERKO SHAM mice indicates that estrogen deficiency leads to distinct transcriptome outcomes for CTRL and LERKO. We focused on the subset of 84 DEGs between CTRL OVX and LERKO OVX mice (Fig. 4A). Among the 22 genes upregulated in LERKO OVX mice, we identified *Gdpd3* (glycerophosphodiester phosphodiesterase domain containing 3, known to promote hepatic steatosis[23]), *Lars2* (leucyl-tRNA synthetase 2), and *Vldlr* (very-low density lipoprotein receptor). In addition to *Esr1,* which encodes for ERα, among the 62 downregulated genes in LERKO OVX, we found several cytochrome P450s, MUPs, and genes involved in steroid metabolism. Specifically, the genes exhibiting the most significant repression in the liver of LERKO OVX mice were *Cyp4a12b* (40 × less), *Cyp4a12a* (32 × less), *Mup7* (18.4 × less), *Hsd3b5* (16 × less), and *Mup12* (9 × less) (Fig. 4A). These genes, known to be associated with a male-specific liver transcriptome,[24,25] were upregulated by ovariectomy in CTRL but either not or to a lesser extent in LERKO mice (Fig. S7 and S8).

To investigate the extent to which estrogen loss may reprogram liver transcriptome towards a male-like pattern, we cross-referenced the DEGs obtained from the comparisons CTRL OVX/CTRL SHAM and LERKO OVX/CTRL OVX mice with a list of 274 well-established sex-biased genes.[26] In the liver of CTRL, 97 OVX-altered genes were identified as known sex-biased genes; notably, the majority of male-biased genes (46/50, 92%) were upregulated by ovariectomy, whereas most female-biased genes (41/47, 87%) were downregulated by ovariectomy (Fig. 4B and Fig. S7A). In LERKO, 40 OVX-altered genes were identified as sex-biased genes; among these, 12/16 (75%) male-biased genes were upregulated by ovariectomy, while all female-biased genes were downregulated by ovariectomy (Fig. 4C and Fig. S7B).

While these findings suggested that ovariectomy reprograms the liver transcriptome towards a male-like pattern, the effect appeared to be slightly attenuated in LERKO compared with CTRL mice. To explore the contribution of hepatic ERα in the male-like liver reprogramming after estrogen deprivation, we focused our analysis on the 84 DEGs identified in the comparison of LERKO OVX and CTRL OVX mice. Among these, 26 (31%) were identified as sex-biased genes; notably, all the 21 male-biased genes were upregulated in CTRL OVX mice compared with LERKO OVX mice (Fig. 4D and Fig. S8A-C), confirming hepatic ERα relevance in liver masculinization following estrogen loss.

In the liver, growth hormone (GH) stimulates the nuclear translocation of STAT5B (signal transducer and activator of transcription 5 b) in a sex-specific manner, strongly contributing to sex differences in gene expression.[24,27] To gain further insights into STAT5 signaling, we examined the overlap between DEGs found in the comparison of LERKO OVX and CTRL OVX that are also known sex-biased genes, and a list of STAT5 responsive genes.[26] Only seven genes were unresponsive to STAT5, while 19 genes were altered in the STAT5 KO mice (Fig. 4E and F and Fig. S8A-E). Among the STAT5 responsive genes, 13 male-biased genes (*Cyp4a12b*, *Cyp4a12a, Col27a1, Cyp7b1*, *Hsd3b5*, *Mup3*, *Mup7*, *Mup11*, *Mup12*, *Mup21*, *Nat8*, *Slco1a1*, and *Slc22a28*) were upregulated in CTRL OVX mice. Interestingly, all these genes were also expressed at low levels in LERKO OVX mice compared with CTRL OVX mice, as well as in STAT5 KO males compared with controls (Fig. 4F and Fig. S8A and B). Among the STAT5 responsive genes, two female-biased genes (*Serpina3h*, *Vldlr*) were upregulated in LERKO OVX mice as well as in STAT5 KO mice compared with their counterparts (Fig. 4F and Fig. S8D).

These findings underscore the crucial role of hepatic ERα in fully reprogramming the hepatic transcriptome of OVX females towards a male-like profile.

MASLD development in women was associated with liver masculinization

To investigate the translational relevance of such findings, we enquired liver samples from a cohort of 60 severely obese

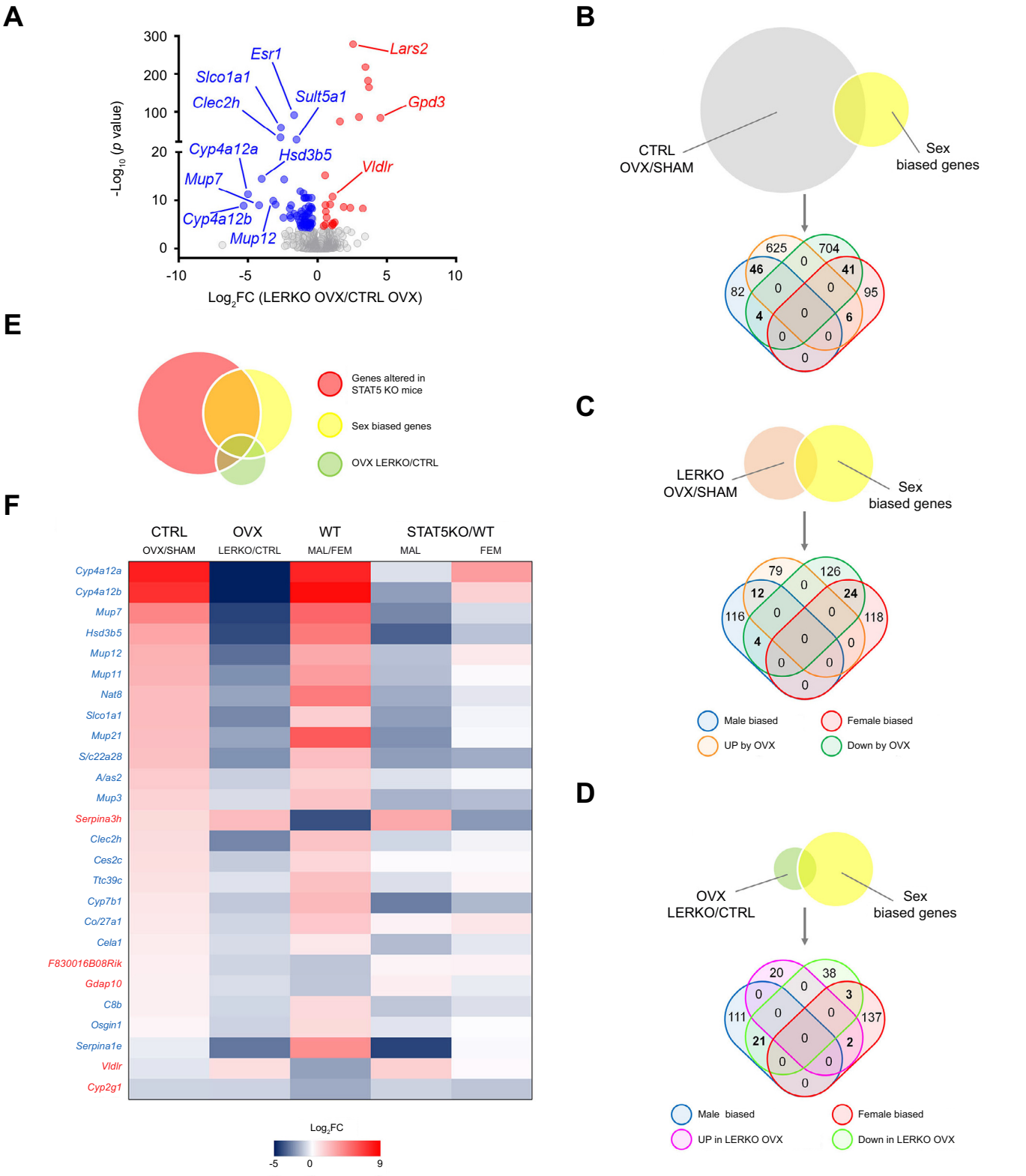


Fig. 4. Hepatic ERα mediates liver transcriptome reprogramming towards a male-like profile after ovariectomy. (A) Volcano plot of differentially expressed genes (DEGs) in the liver identified in LERKO OVX and CTRL OVX mice. Venn diagram showing the overlap between DEGs in CTRL OVX/SHAM (B), LERKO OVX/SHAM (C), and OVX LERKO/CTRL (D) with well-known sex-biased genes. (E) Venn diagram showing the overlay among DEGs in OVX LERKO/CTRL, sex-biased genes and genes known to be altered in STAT5 KO mice. (F) Heatmap reporting the expression of selected genes in CTRL OVX/SHAM, OVX LERKO/CTRL, wild-type males *vs.* females (WT MAL/FEM), STAT5 KO males *vs.* wild-type males (STAT5 KO/WT MAL), and STAT5 KO females *vs.* wild-type females (STAT5 KO/WT FEM). Male-biased and female-biased genes are shown in blue and red, respectively.

individuals not carrying the *PNPLA3* genetic risk variant, who underwent a percutaneous liver biopsy for confirming and staging MASLD. We classified women according to age, assuming 51 years as the discriminant age between pre-menopausal and post-menopausal stages, as suggested by the Endocrine Society guidelines.[28] We also differentiated women according to MASLD status.

As shown in Fig. 5, the expression of genes affected by ovariectomy and hepatic ERα absence in mice was also altered in human liver samples. In particular, for most of the genes analysed, the mRNA expression profile in the liver of women aged ≥51 years old was similar to that exhibited in men, suggesting a masculinization of the liver transcriptome profile after menopause, as observed for mice. Notably, for women aged <51 years with MASLD the expression of these genes was intermediate between that of women aged <51 years without MASLD and that of women aged ≥51 years women with MASLD, suggesting that beyond obesity an impaired hepatic estrogen signaling promotes liver masculinization and raises the risk of developing MASLD in women.

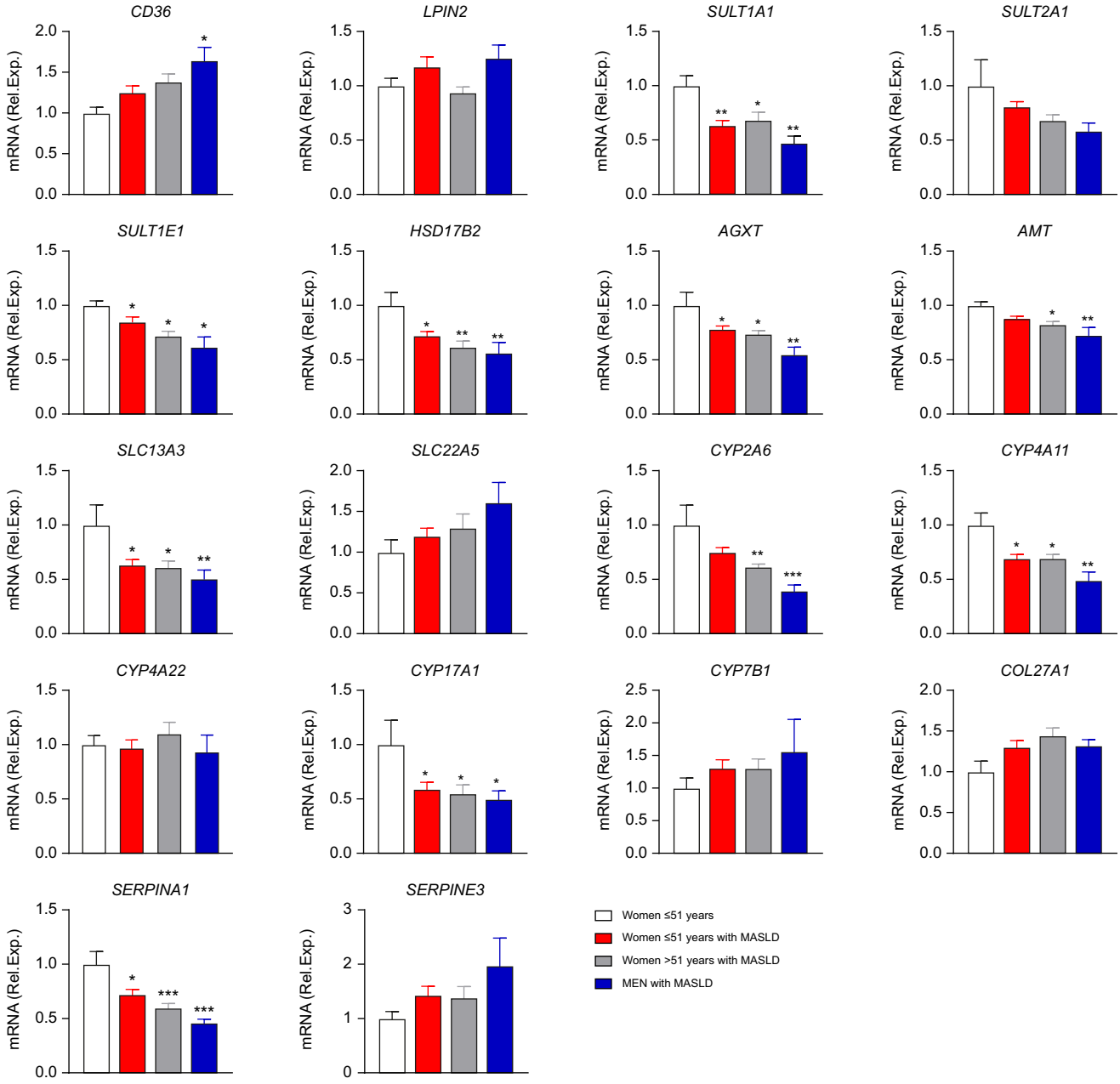


Fig. 5. MASLD in women is associated with a male-like liver transcriptome profile. mRNA levels of genes measured in the liver of women <51 years (open red bars), women <51 years with MASLD (red bars), women ≥51 years with MASLD (grey bars), and men with MASLD (blue bars). Data from RNA-Seq are represented as mean ± SEM (n = 8–25). *p <0.05, **p <0.01 and ***p <0.001 *vs.* women <51 years by one-way ANOVA followed by Bonferroni's *post hoc* test. Abbreviations: MASLD, metabolic dysfunction-associated steatotic liver disease; *CD36*, CD36 molecule; *LPIN2*, lipin 2; *SULT1E*, *SULT1A1*, *SULT2A1*, sulfotransferases; *HSD17B2*, hydroxysteroid 17-beta dehydrogenase 2; *AGXT,* alanine-glyoxylate aminotransferase; *AMT,* aminomethyltransferase; *SLC13A3* and *SLC22A5* (*Slc22a8* homolog), solute carrier family members; *CYP2A6* (*Cyp2a5* homolog), *CYP4A11* (*Cyp4a12b* homolog), *CYP4A22* (*Cyp4a12a* homolog), *CYP17A1*, *CYP7B1,* cytochrome P450 family members; *COL27A1*, collagen type XXVII alpha 1 chain; *SERPINA1* and *SERPINA3*, serpin family members.

Discussion

With this study, we found that hepatic ERα fully exploits liver metabolic reprogramming following estrogen loss, pointing to this receptor as a valuable pharmacological target for the post menopause-associated MASLD.

Estrogen loss following ovariectomy surpasses the changes observed in the hepatic transcriptome due to the simple lack of hepatic ERα, a finding not surprising, as estrogen deficiency affects the entire organism, including signaling pathways in organs cross-talking with the liver. It could be hypothesized that the absence of hepatic ERα represents an intermediate phenotype between the SHAM and OVX mice, while most of ovariectomy-induced changes in the liver transcriptome may be ascribable to factors other than ERα in hepatocytes. However, if ovariectomy-induced changes were primarily hepatic ERα-independent, we would expect similar changes in CTRL and LERKO after ovariectomy. Nonetheless, the ovariectomy-induced changes were limited in LERKO mice and exhibited significant differences compared with CTRL mice (Fig. 3), arguing against this hypothesis.

In our study, ovariectomy led to the over-expression of genes involved in lipid storage and deposition in the liver of both genotypes. However, ovariectomy inhibits the expression of genes involved in AA catabolism, particularly in LERKO OVX mice (Fig. 3D), an effect that may be linked to liver lipid deposition.[11,29] Notably, liver lipid sequestering and storage were particularly amplified in LERKO OVX mice (Fig. S5A), suggesting a counteractive role of hepatic ERα in limiting lipid deposition even after estrogen loss. Accordingly, the upregulation of PPARα target genes associated with FAO and lipid metabolism mainly occurred in CTRL OVX mice (Fig. 3B), suggesting that hepatic ERα may concur to the adaptive response to estrogen loss, possibly through interactions with other nuclear receptor signaling pathways.

If hepatic ERα were marginal in exploiting OVX effects, we would have expected similar transcriptome profiles for CTRL OVX and LERKO OVX mice. Conversely, the comparison between LERKO OVX/CTRL OVX mice confirms the unique role of hepatic ERα in reprogramming the liver transcriptome according to hormonal changes. In CTRL mice, ovariectomy led to the over-expression of STAT5B-dependent male-biased genes, an effect greatly attenuated or nullified in LERKO OVX mice, highlighting the specific involvement of hepatic ERα in liver masculinization following estrogen depletion (Fig. 4 and Fig. S8).

Likely through epigenetic mechanisms, the loss of estrogens may render 'male' chromatin regions more accessible to transcription factors such as STAT5B, thereby promoting the transcription of their target genes. In line with this, the promoters of several STAT5B-dependent male-biased genes over-expressed in CTRL OVX mice (i.e. *Cyp7b1*, *Hsd3b5*, *Slco1a1*) resulted unmethylated in the liver of masculinized female mice.[30] In CTRL OVX mice, the hepatic over-expression of *Cyp7b1*, *Hsd3b5*, and *Slc10a1* may represent a counter-regulatory adaptation aimed at minimising changes in the estrogen/androgen *ratio* and/or promoting cholesterol conversion into bile acids, therefore limiting MASLD progression to steatohepatitis.[31–33]

The incomplete liver masculinization in LERKO OVX mice underscores the essential role of hepatic ERα in liver sexual differentiation.[8,34] An intriguing hypothesis suggests that hepatic ERα might enhance the transcription of male-biased genes by facilitating the binding of STAT5B and coactivators, like the glucocorticoid receptor (GR), to DNA,[35] potentially through an 'assisted loading' mechanism.[36] Supporting this, some of the male-biased genes (*Cyp7b1*, *Hsd3b5, Mups*, *Serpin1e*) upregulated by OVX in CTRL mice but not or to a lesser extent in LERKO mice are known to be responsive to GR.[37,38]

In the absence of estrogens, hepatic ERα can be activated by various factors and extracellular signals, including AA, growth factors, and cytokines.[7,39] The cross-talk between ERα and the insulin signaling pathway (INS) represents one potential mechanism involved in this process. Besides classical activation, STAT5B can also be activated through phosphorylation by the insulin receptor (IR) or downstream components of INS.[40] Studies in mice treated with agonist/antagonist for GR and IR or in liver-specific KO mice for GR and IR demonstrated that GR and INS cooperate to regulate the hepatic metabolism in response to the feeding/fasting status.[41] Notably, several male-biased genes (*Alas2*, *Cyp4a12a*, *Cyp4a12b*, *Hsd3b5*, *Mup3*, *Mup21*, *Nat8*, and *Slco1a1*) upregulated by ovariectomy in CTRL mice, but not or to a lesser extent in LERKO under short-term fasting, were also among the most downregulated genes in liver-specific IR KO mice.[42] These data support the idea of a cross-talk among hepatic ERα, INS and GR in reprogramming female liver metabolism based on estrogen levels and nutritional status.

In this study, the comparison between OVX and SHAM mice was limited to the *estrus* phase characterized by low estrogen levels, to specifically assess the broader impact of long-term estrogen deficiency on hepatic gene expression. We cannot rule out that several other or more marked differences may exist during different phases of the estrous cycle, particularly at *proestrus* when estrogen levels are the highest. It is also possible that the timing and duration of ovariectomy may contribute to some of the observed differences, while other differences may be masked. Considering the role of hepatic ERα in sensing nutritional status, it is plausible to speculate that liver reprogramming may be influenced by various nutritional conditions. Nevertheless, we focused our investigation on control-fed mice to evaluate the extent to which estrogen loss alone induces alterations in hepatic gene expression that could predispose to MASLD development. Our data show that ovariectomy *per se* leads to a great reprogramming of liver transcriptome and to a significant increase (+45%) in liver lipid deposition even in control-fed mice. Finally, we could not examine the impact of gene expression on protein and lipid levels and metabolic fluxes. Despite all these limitations, it is most noteworthy that liver transcriptomics confirmed that MASLD in obese women was associated with a similar shift toward a male-like metabolic profile in key genes. These results are concordant with several clinical observations that have reported sexual hormone abnormalities and dysfunctions, including masculinization/defeminization, in women with liver diseases.[43–45] To our knowledge, however, this is the first study reporting a masculinization of liver transcriptome in obese women with MASLD.

Since the Liver Biopsy Cohort did not include information on the hormonal status of participants, we utilized the age of 51 years as a cutoff to distinguish between pre- or post-menopausal stages, following guidelines from the Endocrine Society. We observed a masculinization of the liver transcriptome specifically in women with MASLD, particularly pronounced in those aged ≥51 years. Notably, obese women

<51 years without MASLD did not exhibit liver masculinization. In our experimental mouse model, the complete liver masculinization was due to estrogen loss and relies on hepatic ERα. Consequently, our findings suggest that, besides obesity, the impairment of hepatic ERα signaling following menopause may serve as a primary factor driving liver masculinization and the development of MASLD in women.

In summary, this study unravels the peculiar role of hepatic ERα signaling in fully mediating the reprogramming of the female liver following estrogen loss toward a male-like profile, modelling MASLD development in women after menopause and, thereby, pointing to hepatic ERα as a valuable target for precision pharmacological therapy approach for post-menopause-associated MASLD.

Affiliations

[1]*Department of Health Sciences, Università degli Studi di Milano, Milan, Italy;* [2]*Department of Pharmaceutical Sciences, Università degli Studi di Milano, Milan, Italy;* [3]*Precision Medicine–Biological Resource Center and Department of Transfusion Medicine, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy;* [4]*Department of Pathophysiology and Transplantation, Università degli Studi di Milano, Milan, Italy*

Abbreviations

DEGs, differentially expressed genes; ERα, estrogen receptor alpha; FA, fatty acid; FAO, oxidation of FA; GR, glucocorticoid receptor; HRT, hormone replacement therapy; INS, insulin signaling pathway; IR, insulin receptor; LERKO, liver-specific ERα knockout; MASLD, metabolic dysfunction-associated steatotic liver disease; OVX, ovariectomized.

Financial support

Cariplo Foundation (Grant 2013-0786); the European Community (ERC-Advanced Grant 322977) to A.M. Italian Ministry of Health: Ricerca Finalizzata 2016 RF-2016-02364358, Ricerca Finalizzata 2021 RF-2021-12373889, Ricerca Finalizzata PNRR 2022 PNRR-MAD-2022-12375656; Rete Cardiologica "CV-PREVITAL"; Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Ricerca Corrente; Fondazione Patrimonio Ca' Granda, "Liver BIBLE" (PR-0361); Innovative Medicines Initiative 2 joint undertaking of European Union's Horizon 2020 research and innovation programme and EFPIA European Union (EU) Programme Horizon 2020 (Grant 777377 - LITMUS); The European Union, H2020-ICT-2018-20/H2020-ICT-2020-2 programme "Photonics" (Grant 1010 16726 – REVEAL); Gilead_IN-IT-989-5790; The European Union, HORIZON-MISS-2021-CANCER-02-03 (Grant 101096312 - Genial); Italian Ministry of University and Research: PNRR – M4 – C2 "National Center for Gene Therapy and Drugs based on RNA Technology" CN3, Spoke 4, ASSET: A sex-specific approach to NAFLD targeting; PRIN 2022, "DEFENDER" to L.V. Pfizer Global NASH ASPIRE Competitive Grant Program (Grant 77230651) to S.D.T.

Conflicts of interest

LV has received speaking fees from MSD, Gilead, AlfaSigma, and AbbVie, served as a consultant for Gilead, Pfizer, AstraZeneca, Novo Nordisk, Intercept, Diatech Pharmacogenetics, Ionis Pharmaceuticals, Boehringer Ingelheim, Resalis Therapeutics, and received unrestricted research grants from Gilead.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conceptualisation: SDT. Investigation: AC, CM, SDT, VB. Resources: LV and SDT. Formal analysis: SDT. Original draft: SDT. Review and editing: All authors. Visualisation: SDT. Supervision: AM and SDT. Funding acquisition: AM, LV, SDT. All authors have read and agreed to the published version of the manuscript.

Data availability statement

Raw RNA-Seq mouse data have been deposited to Bioproject (PRJNA778593).

Acknowledgements

Arianna Dolce and Elia Casirati for assistance with the study.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2024.101143.

References

Author names in bold designate shared co-first authorship

[1] Powell EE, Wong VW-S, Rinella M. Non-alcoholic fatty liver disease. Lancet 2021;397:2212–2224.
[2] Li AA, Ahmed A, Kim D. Extrahepatic manifestations of nonalcoholic fatty liver disease. Gut Liver 2020;14:168–178.
[3] Della Torre S. Non-alcoholic fatty liver disease as a canonical example of metabolic inflammatory-based liver disease showing a sex-specific prevalence: relevance of estrogen signaling. Front Endocrinol (Lausanne) 2020;11:572490.
[4] Della Torre S. Beyond the X factor: relevance of sex hormones in NAFLD pathophysiology. Cells 2021;10:2502.
[5] **Villa A**, **Della Torre S**, Stell A, et al. Tetradian oscillation of estrogen receptor α is necessary to prevent liver lipid deposition. Proc Natl Acad Sci U S A 2012;109:11806–11811.
[6] Della Torre S, Mitro N, Fontana R, et al. An essential role for liver ERα in coupling hepatic metabolism to the reproductive cycle. Cell Rep 2016;15:360–371.
[7] Della Torre S, Rando G, Meda C, et al. Amino acid-dependent activation of liver estrogen receptor alpha integrates metabolic and reproductive functions via IGF-1. Cell Metab 2011;13:205–214.
[8] Della Torre S, Mitro N, Meda C, et al. Short-term fasting reveals amino acid metabolism as a major sex-discriminating factor in the liver. Cel Metab 2018;28. 256-267.e5.
[9] Della Torre S, Maggi A. Sex differences: a resultant of an evolutionary pressure? Cel Metab 2017;25:499–505.
[10] Maggi A, Della Torre S. Sex, metabolism and health. Mol Metab 2018;15:3–7.
[11] Meda C, Barone M, Mitro N, et al. Hepatic ERα accounts for sex differences in the ability to cope with an excess of dietary lipids. Mol Metab 2020;32:97–108.
[12] Della Torre S, Benedusi V, Pepe G, et al. Dietary essential amino acids restore liver metabolism in ovariectomized mice via hepatic estrogen receptor α. Nat Commun 2021;12:6883.
[13] DiStefano JK. NAFLD and NASH in postmenopausal women: implications for diagnosis and treatment. Endocrinology 2020;161:bqaa134.
[14] Flores VA, Pal L, Manson JE. Hormone therapy in menopause: concepts, controversies, and approach to treatment. Endocr Rev 2021;42:720–752.
[15] Appiah D, Nwabuo CC, Ebong IA, et al. Trends in age at natural menopause and reproductive life span among US women, 1959–2018. JAMA 2021;325:1328–1330.
[16] **Chew NWS**, **Ng CH**, Tan DJH, et al. The global burden of metabolic disease: data from 2000 to 2019. Cel Metab 2023;35. 414-428.e3.
[17] Younossi ZM, Golabi P, Paik JM, et al. The global epidemiology of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH): a systematic review. Hepatology 2023;77:1335–1347.
[18] Baselli GA, Dongiovanni P, Rametta R, et al. Liver transcriptomics highlights interleukin-32 as novel NAFLD-related cytokine and candidate biomarker. Gut 2020;69:1855–1866.
[19] **Cherubini A**, **Ostadreza M**, Jamialahmadi O, et al. Interaction between estrogen receptor-α and PNPLA3 p.I148M variant drives fatty liver disease susceptibility in women. Nat Med 2023;29:2643–2655.
[20] **Zheng Z**, **Li Y**, Fan S, et al. WW domain-binding protein 2 overexpression prevents diet-induced liver steatosis and insulin resistance through AMPKβ1. Cell Death Dis 2021;12:228.
[21] **Moore A**, **Wu L**, Chuang J-C, et al. Arid1a loss drives nonalcoholic steatohepatitis in mice through epigenetic dysregulation of hepatic lipogenesis and fatty acid oxidation. Hepatology 2019;69:1931–1945.
[22] Montagner A, Polizzi A, Fouché E, et al. Liver PPARα is crucial for whole-body fatty acid homeostasis and is protective against NAFLD. Gut 2016;65:1202–1214.
[23] Key C-CC, Bishop AC, Wang X, et al. Human GDPD3 overexpression promotes liver steatosis by increasing lysophosphatidic acid production and fatty acid uptake. J Lipid Res 2020;61:1075–1086.
[24] Clodfelter KH, Holloway MG, Hodor P, et al. Sex-dependent liver gene expression is extensive and largely dependent upon signal transducer and

activator of transcription 5b (STAT5b): STAT5b-dependent activation of male genes and repression of female genes revealed by microarray analysis. Mol Endocrinol 2006;20:1333–1351.
[25] Connerney J, Lau-Corona D, Rampersaud A, et al. Activation of male liver chromatin accessibility and stat5-dependent gene transcription by plasma growth hormone pulses. Endocrinology 2017;158:1386–1405.
[26] Hao P, Waxman DJ. STAT5 regulation of sex-dependent hepatic CpG methylation at distal regulatory elements mapping to sex-biased genes. Mol Cel Biol 2021;41:e00166-20.
[27] Oshida K, Vasani N, Waxman DJ, et al. Disruption of STAT5b-regulated sexual dimorphism of the liver transcriptome by diverse factors is a common event. PLoS One 2016;11:e0148308.
[28] Stuenkel CA, Davis SR, Gompel A, et al. Treatment of symptoms of the menopause: an endocrine society clinical practice guideline. J Clin Endocrinol Metab 2015;100:3975–4011.
[29] Gaggini M, Carli F, Rosso C, et al. Altered amino acid concentrations in NAFLD: impact of obesity and insulin resistance. Hepatology 2018;67:145–158.
[30] Dkhil MA, Al-Quraishy S, Abdel-Baki A-A, et al. Epigenetic modifications of gene promoter DNA in the liver of adult female mice masculinized by testosterone. J Steroid Biochem Mol Biol 2015;145:121–130.
[31] Kakiyama G, Marques D, Martin R, et al. Insulin resistance dysregulates CYP7B1 leading to oxysterol accumulation: a pathway for NAFL to NASH transition. J Lipid Res 2020;61:1629–1644.
[32] **Yang N**, **Sun R**, Zhang X, et al. Alternative pathway of bile acid biosynthesis contributes to ameliorate NASH after induction of NAMPT/NAD+/SIRT1 axis. Biomed Pharmacother 2023;164:114987.
[33] Tanaka N, Matsubara T, Krausz KW, et al. Disruption of phospholipid and bile acid homeostasis in mice with nonalcoholic steatohepatitis. Hepatology 2012;56:118–129.
[34] Della Torre S, Rando G, Meda C, et al. Transcriptional activity of oestrogen receptors in the course of embryo development. J Endocrinol 2018;238:165–176.
[35] Stöcklin E, Wissler M, Gouilleux F, et al. Functional interactions between Stat5 and the glucocorticoid receptor. Nature 1996;383:726–728.
[36] Voss TC, Schiltz RL, Sung M-H, et al. Dynamic exchange at regulatory elements during chromatin remodeling underlies assisted loading mechanism. Cell 2011;146:544–554.
[37] **Engblom D**, **Kornfeld J-W**, Schwake L, et al. Direct glucocorticoid receptor-Stat5 interaction in hepatocytes controls body size and maturation-related gene expression. Genes Dev 2007;21:1157–1162.
[38] Præstholm SM, Correia CM, Goitea VE, et al. Impaired glucocorticoid receptor expression in liver disrupts feeding-induced gene expression, glucose uptake, and glycogen storage. Cel Rep 2021;37:109938.
[39] Stellato C, Porreca I, Cuomo D, et al. The "busy life" of unliganded estrogen receptors. Proteomics 2016;16:288–300.
[40] Le MN, Kohanski RA, Wang L-H, et al. Dual mechanism of signal transducer and activator of transcription 5 activation by the insulin receptor. Mol Endocrinol 2002;16:2764–2779.
[41] **Kalvisa A**, **Siersbæk MS**, Præstholm SM, et al. Insulin signaling and reduced glucocorticoid receptor activity attenuate postprandial gene expression in liver. PLoS Biol 2018;16:e2006249.
[42] **Fougeray T**, **Polizzi A**, Régnier M, et al. The hepatocyte insulin receptor is required to program the liver clock and rhythmic gene expression. Cel Rep 2022;39:110674.
[43] Neong SF, Billington EO, Congly SE. Sexual dysfunction and sex hormone abnormalities in patients with cirrhosis: review of pathogenesis and management. Hepatology 2019;69:2683–2695.
[44] Di Stasi V, Maseroli E, Rastrelli G, et al. SHBG as a marker of NAFLD and metabolic impairments in women referred for oligomenorrhea and/or hirsutism and in women with sexual dysfunction. Front Endocrinol (Lausanne) 2021;12:641446.
[45] **Lee JY**, **Shin DW**, Oh JW, et al. Non-alcoholic fatty liver disease as a risk factor for female sexual dysfunction in premenopausal women. PLoS One 2017;12:e0182708.

Keywords: Female; Menopause; Estrogens; Liver metabolic programming.
Received 5 February 2024; received in revised form 24 May 2024; accepted 7 June 2024; Available online 13 June 2024