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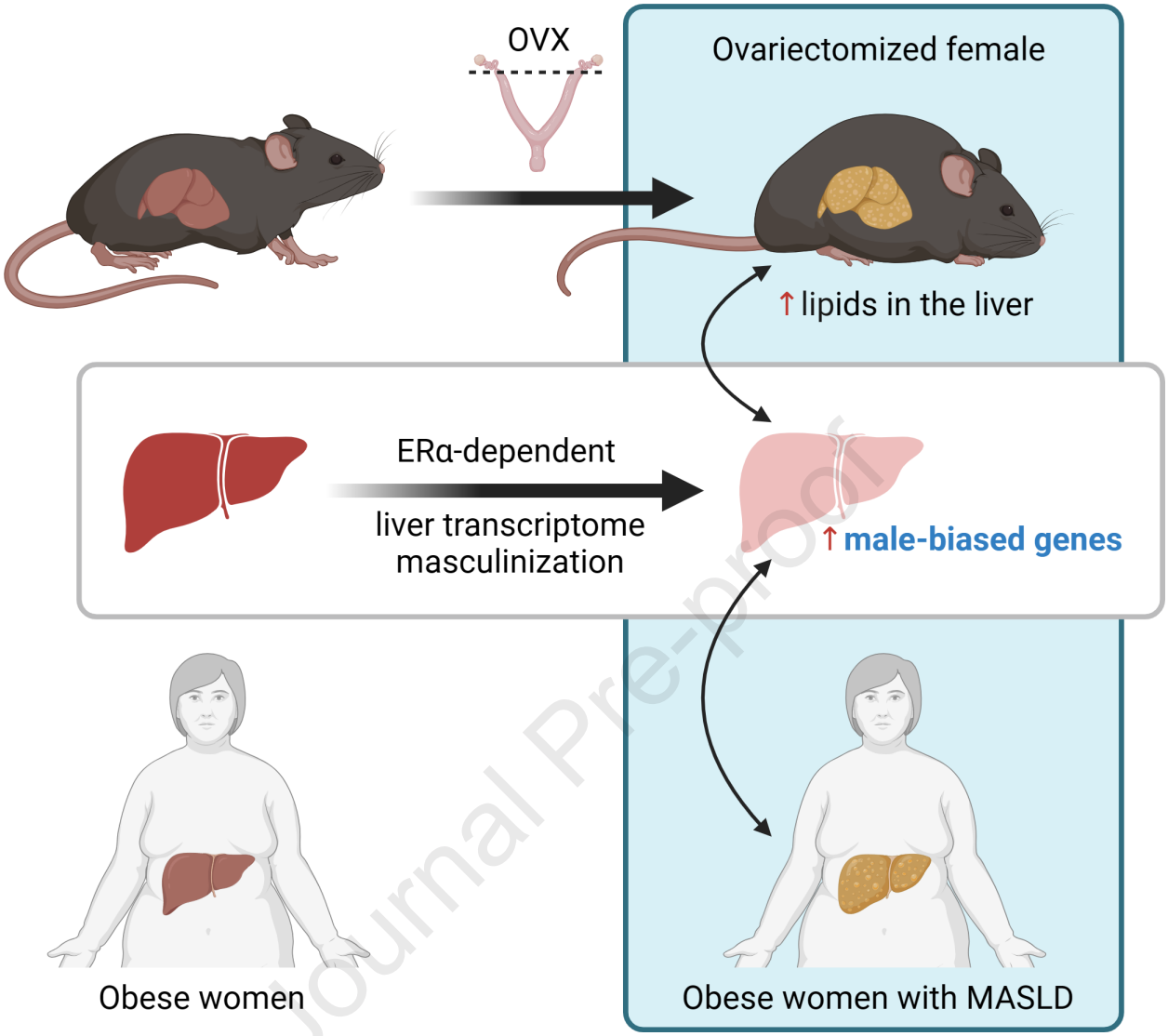

Title: Hepatic Estrogen Receptor alpha drives masculinization in post-menopausal women with MASLD

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Abstract

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 the estrogen receptor alpha (ERα), the specific contribution of impaired ERα signaling in triggering MASLD after menopause remains unclear.

Methods: To fulfill this gap of 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 OVX-induced modulation of the liver transcriptome in LERKO compared to 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 OVX-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 enquired 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 ≥51 years old women (15/15), suggesting that masculinization of 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.

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 been very few investigated, preventing, so far, the development of tailored strategies that address the specific mechanisms underlying MASLD in post-menopausal women.

This study reveals the functional role of hepatic estrogen receptor alpha (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 obese women, 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.

Introduction

In comparison to men, fertile women show a lower risk of developing metabolic dysfunction-associated steatotic liver disease (MASLD), which 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 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], likely acquired through evolution [9,10], contributes to sex differences in MASLD susceptibility. Indeed, when exposed to a diet rich in lipids, fertile female mice exhibit a greater ability - largely dependent on hepatic ERα - to limit liver lipid deposition compared to 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]. However, while hormone replacement therapy (HRT) can be beneficial, it cannot be considered as a primary approach to counteract MASLD in post-menopausal women. Indeed, the restricted window of opportunity (<10 years after menopause), the individual benefits:risks *ratio*, and the low HRT prescriptions and uptake [14], constrain this approach. Given the fact that 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 paid in developing more targeted pharmacological approaches that focus on the liver, thereby overcoming the potential, systemic side-effects of classical HRT.

Here 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) control as well as liver ERα KO (LERKO) female mice.

Materials and methods

Animals and experimental design

Syngenic ERα floxed (CTRL) and LERKO mice [7] were both C57BL/6 J strain. At two months of age, mice were anesthetized with a s.c. injection of 70 μL solution of 109.2 mg/kg ketamine and 8.4 mg/kg xylazine and then OVX or SHAM operated (Fig. S1). Mice were fed *ad libitum* with a standard diet (ResearchDiets), provided with filtered water, and maintained 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 hrs 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 in [12]. Gene ontology (GO) and cluster analysis was performed using Cytoscape plug-in ClueGO, Genesis, Enrichr, and ShinyGO. Venn diagram was made with Bioinformatics & Evolutionary Genomics software (http://bioinformatics.psb.ugent.be/webtools/Venn/).

The human RNA sequencing analysis, count normalization and differential gene expression analysis were conducted as previously described [18] on RNA derived from liver biopsies from severely obese individuals from the Liver Biopsy Cohort who underwent a percutaneous liver biopsy performed during bariatric surgery at the Milan center 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 the female sex and the *PNPLA3 p.*I148M variant in determining the predisposition to develop MASLD [19], we excluded from the study the samples carrying such a variant, thus limiting our analysis to 60 samples (Table S1).

Statistical analysis

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

Results

Estrogen loss induces 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 padj<0.05, we identified 1426 differentially expressed genes (DEGs) between CTRL SHAM and CTRL OVX, with 667 (47.5%) up-regulated by OVX (Fig. 1A). Comparing CTRL SHAM and LERKO SHAM, we found 239 DEGs, with 57 (~24%) up-regulated in LERKO SHAM (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 systemic factors.

Comparing CTRL OVX/CTRL SHAM and LERKO SHAM/CTRL SHAM, we identified 157 shared genes between the two groups (Fig. 1C). Among these, 155 showed similar regulation patterns in response to both estrogen's deficiency and hepatic ERα's absence, while only 2 genes (Fig. S2A) exhibited opposite regulation.

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

We then explored the 1269 genes differentially regulated by OVX in CTRL but unaffected by the lack of hepatic ERα alone in LERKO SHAM. Among the 638 genes specifically up-regulated in CTRL OVX (Fig. 2A-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 (Fig. 2C-D). Estrogen deficiency can potentially trigger adipose tissue lipolysis, leading to the release of free FA, that, 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 but not in LERKO SHAM (Fig. S3C-D).

Among the 631 genes down-regulated by OVX but not affected by the mere lack of hepatic ERα (Fig. 2E-F), we identified those associated with steroid metabolic process, cholesterol homeostasis and trafficking, and several epoxygenase P450s (Fig. S4A-D). Other down-regulated genes belong to clusters involved in the regulation of signaling (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 exploits liver metabolic reprogramming in a mouse model of menopause

Since only a subset of genes differentially regulated by OVX is shared between the LERKO SHAM/CTRL SHAM comparison, it is reasonable to assume that the majority of changes found in OVX can be attributed to factors other than ERα in hepatocytes. In this perspective, if the role of ERα were marginal in driving the changes associated with estrogen deficiency, similar changes to those observed in CTRL would be expected in LERKO following OVX.

Analysing genes differentially regulated following OVX (Fig. 3A-E), we found fewer DEGs in LERKO (245) compared to CTRL (1426). Out of these, 146 were shared between CTRL OVX/CTRL SHAM and LERKO OVX/LERKO SHAM, with 56 being up-regulated and 90 down-regulated by OVX (Fig. 3C). Notably, this subset accounted for only ~10% of the genes differentially regulated by OVX in CTRL, 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. Among the 91 genes up-regulated by OVX in LERKO (Fig. 3A), there was a significant enrichment of genes involved in lipid synthesis, droplet organization and storage, with some genes (e.g. *Scd1,* stearoyl-CoA desaturase 1*; Plin5,* perilipin 5) specifically enhanced in LERKO OVX, that showed a greater increase in liver lipid deposition and body weight (Fig. S5A and S6A-C). Differently from CTRL, genes involved in FAO and lipid metabolism were not induced in LERKO following OVX (Fig. 3B), although *Pparα* was similarly enhanced by OVX (Fig. S5A).

In LERKO, OVX 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 metabolic process (Fig. S5B), serine and glycine metabolism (Fig. S5C), and AA transmembrane transport (Fig. S5D) were inhibited by OVX in both genotypes. On the contrary, other genes involved in AA catabolism (*Ido2,* indoleamine 2,3-dioxygenase 2; *Kyat1,* kynurenine aminotransferase 1; *Bcat2,* branched chain amino acid

transaminase 2) resulted repressed only in LERKO OVX (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 CTRL after OVX (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 is essential to liver masculinization in a mouse model of menopause

The low overlap between CTRL OVX/CTRL SHAM and LERKO OVX/LERKO SHAM 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 (Fig. 4A). Among the 22 genes up-regulated in LERKO OVX, 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 down-regulated 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 were *Cyp4a12b* (40x less), *Cyp4a12a* (32x less), *Mup7* (18.4x less), *Hsd3b5* (16x less), and *Mup12* (9x less) (Fig. 4A). These genes, known to be associated with a male-specific liver transcriptome [24,25], were up-regulated by OVX in CTRL but either not or to a lesser extent in LERKO (Fig. S7-8).

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 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 up-regulated by OVX, while most female-biased genes (41/47, 87%) were down-regulated by OVX (Fig. 4B and S7A). In LERKO, 40 OVX-altered genes were identified as sex-biased genes; among these, 12/16 (75%) male-biased genes were up-regulated by OVX, while all female-biased genes were down-regulated by OVX (Fig. 4C and S7B). While these findings suggested that OVX reprograms the liver transcriptome towards a male-like pattern, the effect appeared to be slightly attenuated in LERKO compared to CTRL. To explore the contribution of hepatic ERα in the male-like liver reprogramming after estrogen deprivation, we focused our analysis on the 84 DEGs found in the comparison LERKO OVX/CTRL OVX. Among these, 26 (31%) were identified as sex-biased genes; notably, all the 21 male-biased genes were up-

regulated in CTRL OVX compared to LERKO OVX (Fig. 4D and 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 5b) 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 LERKO OVX/CTRL OVX that are also known sex-biased genes, and a list of STAT5 responsive genes [26]. Only 7 genes are unresponsive to STAT5, while 19 genes are altered in STAT5 KO mice (Fig. 4E-F and S8A-E). Among the STAT5 responsive genes, 13 male-biased genes (*Cyp4a12b*, *Cyp4a12a*, *Col27a1*, *Cyp7b1*, *Hsd3b5*, *Mup3*, *Mup7*, *Mup11*, *Mup12*, *Mup21*, *Nat8*, *Slco1a1*, *Slc22a28*) were up-regulated in CTRL OVX. Interestingly, all these genes were also expressed at low levels in LERKO OVX compared to CTRL OVX, as well as in STAT5 KO males compared to controls (Fig. 4F and S8A-B). Among the STAT5 responsive genes, two female-biased genes (*Serpina3h*, *Vldlr*) were up-regulated in LERKO OVX as well as in STAT5 KO compared to their counterparts (Fig. 4F and 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 is associated with liver masculinization

To investigate the translational relevance of such findings, we enquired liver samples from a cohort of 60 severely obese 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 OVX and hepatic ERα absence in mice was also altered in human liver samples. In particular, for most of the genes analyzed, the mRNA contents in the liver of women ≥51 years old became similar to those measured in men, suggesting a masculinization of the liver transcriptome profile after menopause, as observed for mice. Notably, for women <51 years old with MASLD the expression of these genes resulted to be intermediate between that of women <51 years without MASLD and that of women ≥51 years old women with MASLD, suggesting that - beyond obesity - an impaired hepatic estrogen signaling promotes liver masculinization and raises the risk of developing MASLD in women.

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 in OVX 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 SHAM and OVX, while most of OVX-induced changes in the liver transcriptome may be ascribable to factors other than ERα in hepatocytes. However, if OVX-induced changes were primarily hepatic ERα-independent, we would expect similar changes in CTRL and LERKO after OVX. Nonetheless, the OVX-induced changes were limited in LERKO and exhibited significant differences compared to CTRL (Fig. 3), arguing against this hypothesis.

In our study, OVX led to the over-expression of genes involved in lipid storage and deposition in the liver of both genotypes. However, OVX inhibits the expression of genes involved in AA catabolism, particularly in LERKO OVX (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 (Fig. S5A and S6A), suggesting a counteractive role of hepatic ERα in limiting lipid deposition even after estrogen loss. Accordingly, the up-regulation of PPARα target genes associated with FAO and lipid metabolism mainly occurred in CTRL OVX (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. Conversely, the comparison LERKO OVX/CTRL OVX confirms the unique role of hepatic ERα in reprogramming the liver transcriptome according to hormonal changes. In CTRL, OVX led to the over-expression of STAT5B-dependent male-biased genes, an effect greatly attenuated or nullified in LERKO OVX, highlighting the specific involvement of hepatic ERα in liver masculinization following estrogen depletion (Fig. 4 and S8).

Likely through epigenetic mechanism, 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 (i.e. *Cyp7b1*, *Hsd3b5*, *Slco1a1*) resulted unmethylated in the liver of masculinized female mice [30]. In CTRL OVX, the hepatic over-expression of *Cyp7b1*, *Hsd3b5*, and *Slc10a1* may represent a counter-regulatory adaptation aimed at minimizing 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 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*) up-regulated by OVX in CTRL but not or to a lesser extent in LERKO 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*, *Slco1a1*) up-regulated by OVX in CTRL, but not or to a lesser extent in LERKO under short-term fasting, were also among the most down-regulated 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 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 OVX 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 OVX *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.

Besides all these limitations, of utmost importance liver transcriptomics confirmed that MASLD in obese women is 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 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 is 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 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 for the post menopause-associated MASLD.

Author Contributions

Conceptualization: A.M., S.D.T and V.B. Investigation: A.C., C.M., S.D.T and V.B. Resources: L.V. and S.D.T. Formal analysis: S.D.T. Original draft: S.D.T. Review and editing: all authors. Visualization: S.D.T. Supervision: A.M. and S.D.T. Funding acquisition: A.M., L.V. and S.D.T. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest: A.C., A.M., C.M., S.D.T and V.B. declare no conflict of interest. L.V. 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.

Data availability statement: Raw RNA-Seq mouse data have been deposited to Bioproject (PRJNA778593).

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Figure Legends

Fig. 1. The simple lack of hepatic ERα partially mimics changes in liver transcriptome observed in ovariectomized females. (**A-B**) Distribution of genes up-regulated (red) and down-regulated (blue) by OVX (**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 DEGs found in the comparisons CTRL OVX/CTRL SHAM (grey) or LERKO SHAM/CTRL SHAM (orange). (**D-G**) Heatmaps, clusters and GO analysis of functional networks significantly up-regulated (**D-E**) or down-regulated (**F-G**) in CTRL OVX as well as LERKO SHAM with respect to CTRL SHAM.

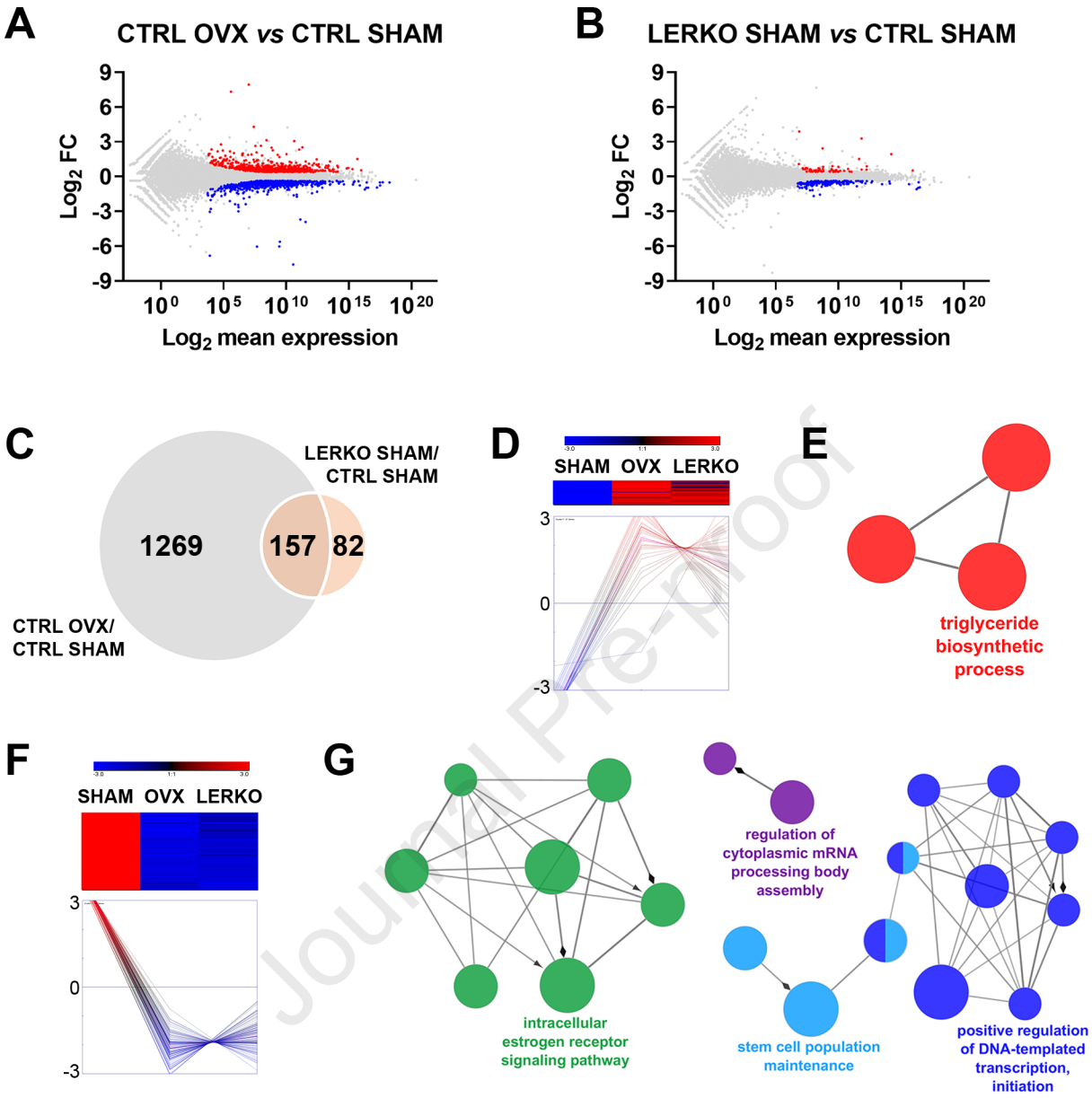
Fig. 2. Lack of estrogens reprograms liver metabolism in a mouse model of menopause. (**A-C**) Heatmap and cluster (**A**), GO analysis of functional networks (**B**), and most enriched motifs (**C**) associated with genes up-regulated in CTRL OVX but not LERKO SHAM with respect to CTRL SHAM. (**D**) *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. (**E-F**) Heatmap, cluster (**E**) and GO analysis of functional networks (**F**) associated with genes down-regulated in CTRL OVX but not LERKO SHAM with respect to CTRL SHAM.

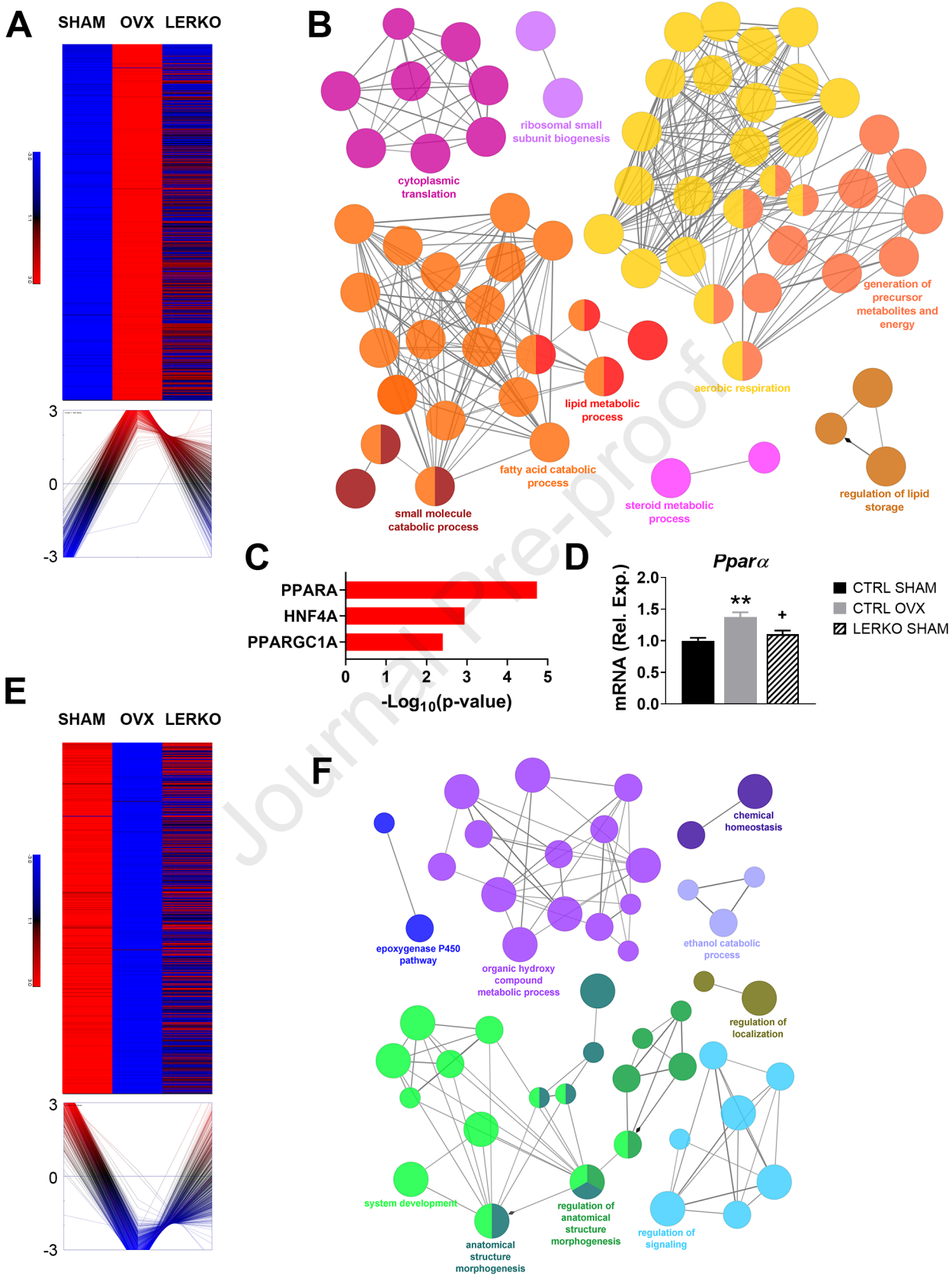
Fig. 3. Hepatic ERα profoundly impacts on OVX-induced liver transcriptome. (**A-B**) GO analysis of functional networks significantly up-regulated in LERKO (**A**) or uniquely in CTRL (**B**) by OVX. (**C**) Venn diagram showing the overlapping among genes differentially regulated by OVX in CTRL (grey)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

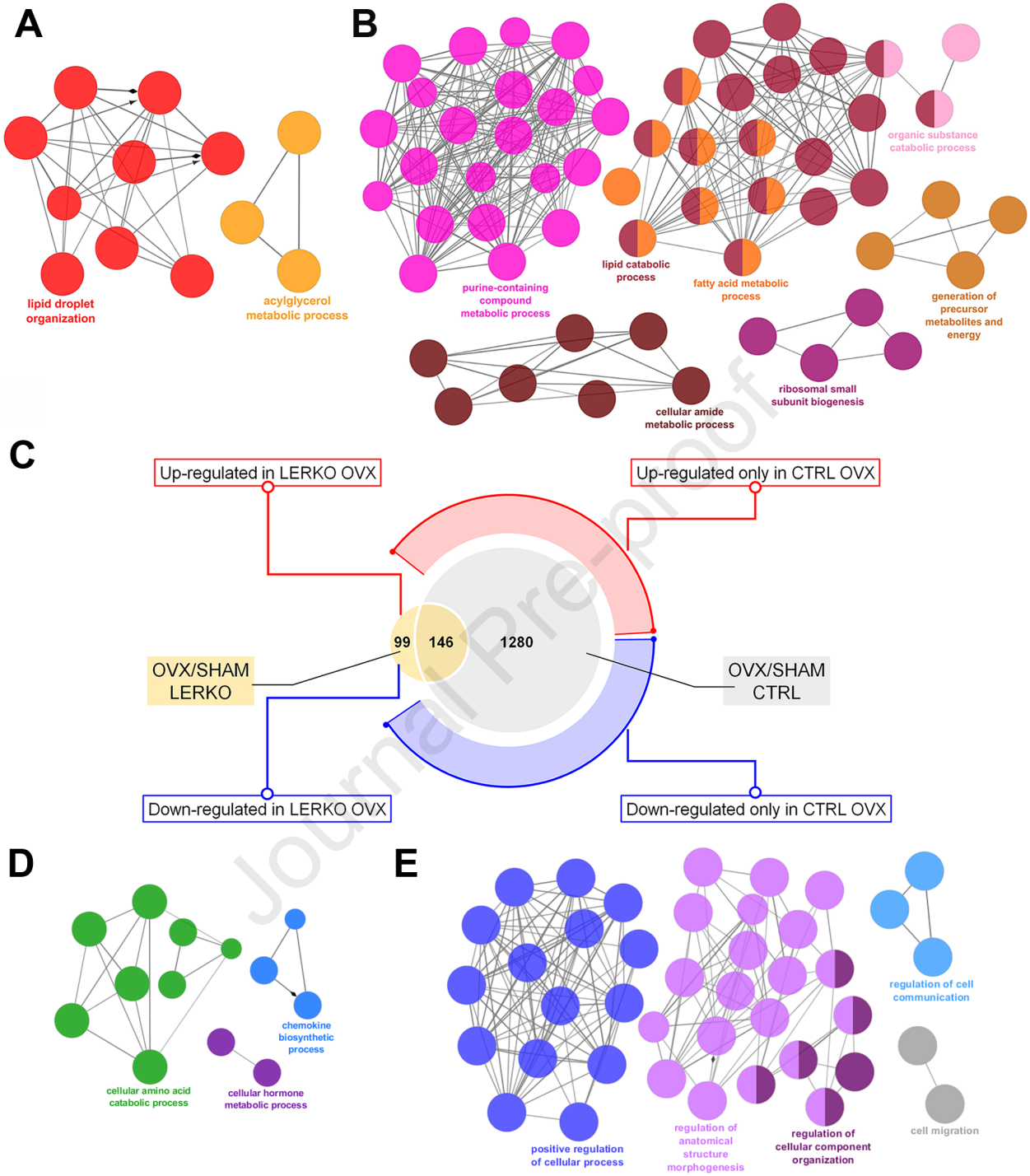
Fig. 4. Hepatic ERα mediates liver transcriptome reprogramming towards a male-like profile after ovariectomy. (**A**) Volcano plot of liver DEGs measured in LERKO OVX and CTRL OVX. (**B-D**) Venn diagram showing the overlap between DEGs in CTRL OVX/SHAM (**B**), LERKO OVX/SHAM (**C**), and OVX LERKO/CTRL (**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

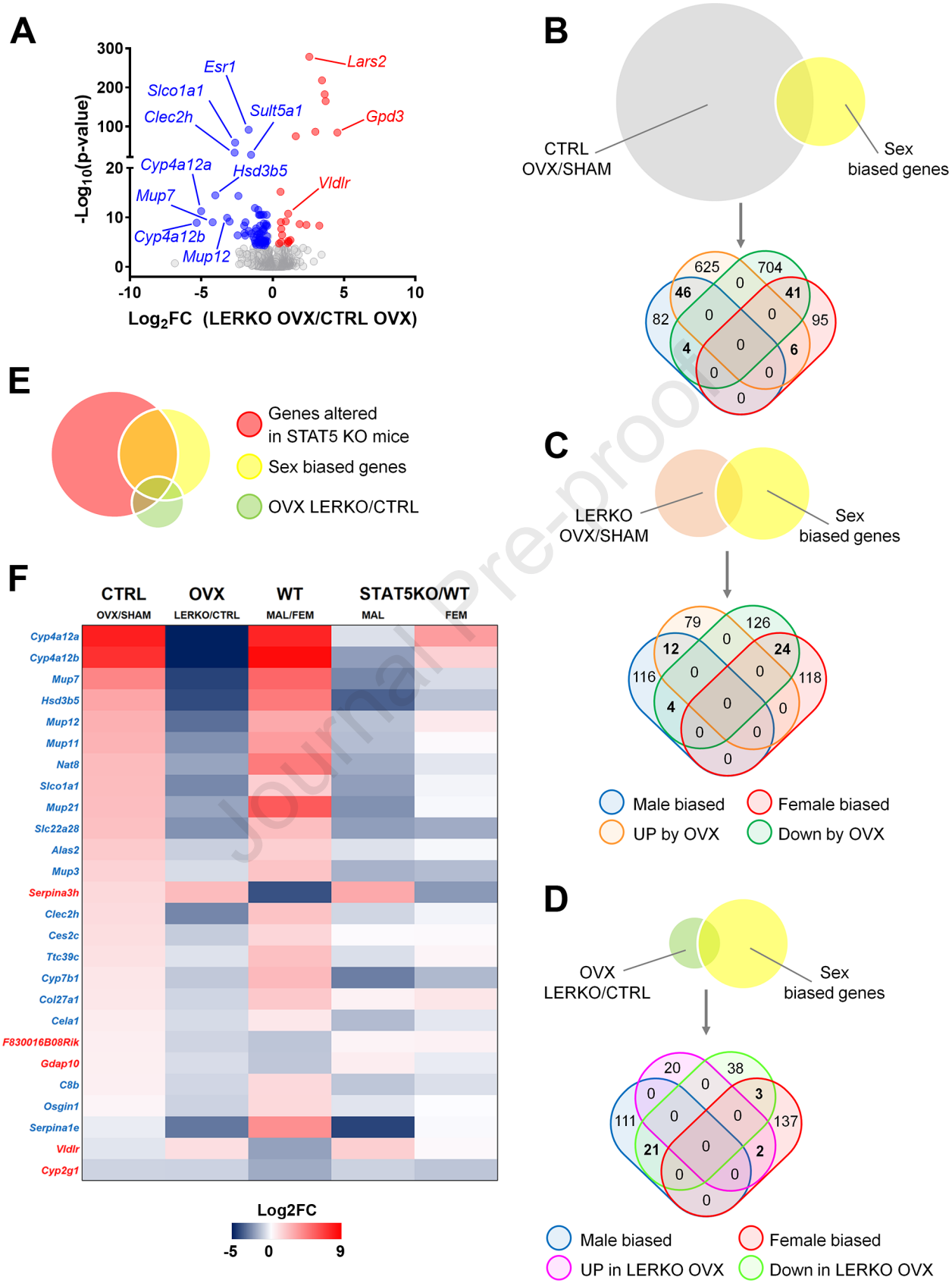
(STAT5 KO/WT FEM). Male-biased and female-biased genes are shown in blue and red, respectively.

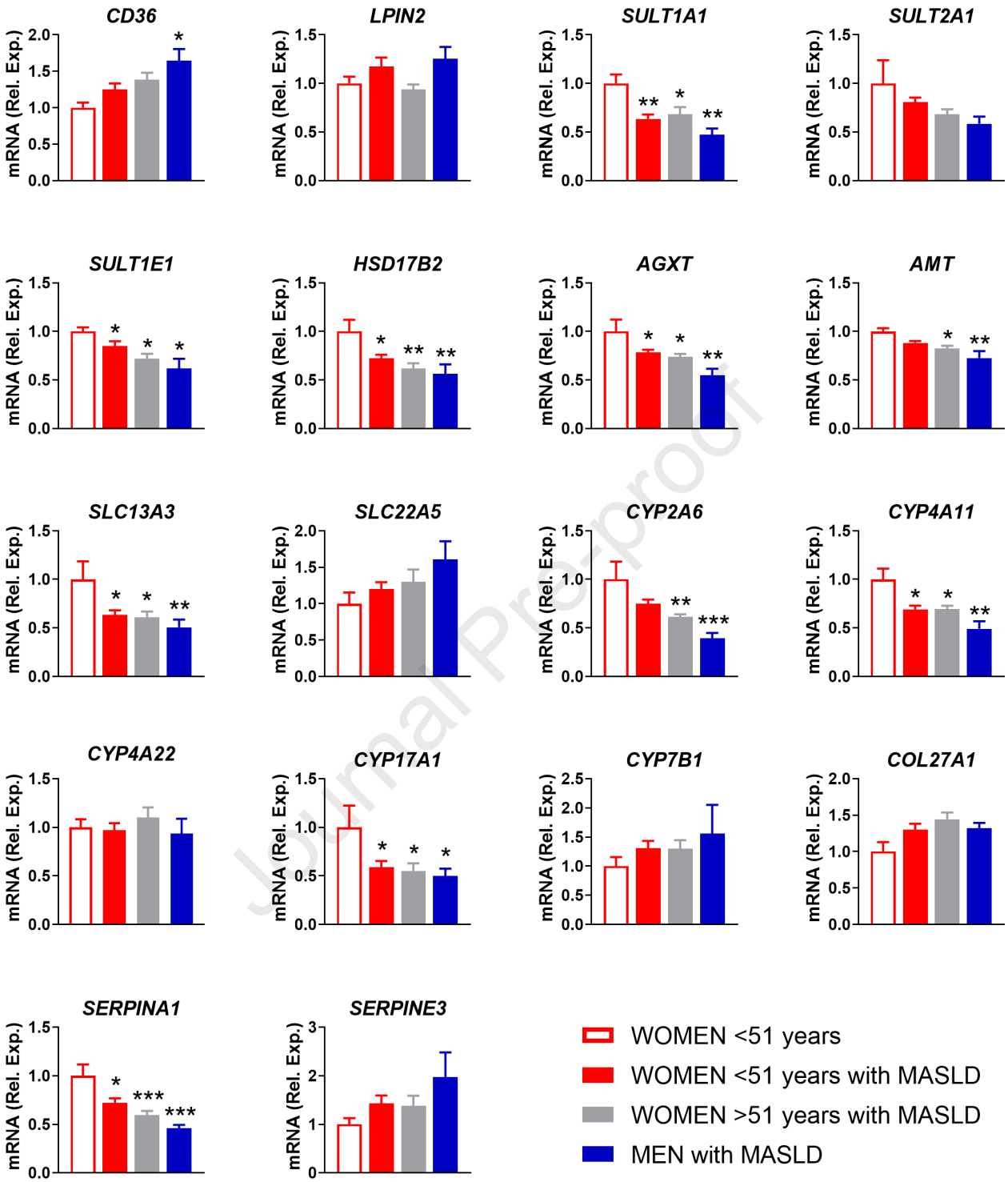
Fig. 5. MASLD in women is associated with a male-like liver transcriptome profile. mRNA levels of genes measured in the liver of women <51 years (open red bars), women <51 years with MASLD (red bars), women ≥51 years with MASLD (grey bars), and men with MASLD (blue bars). Data from RNA-Seq are represented 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: *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.











Highlights

- Hepatic ERα is crucial for estrogen-depleted liver reprogramming.
- Hepatic ERα is needed for the 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.