Integrative study of diet-induced mouse models of NAFLD identifies PPARα as a sexually dimorphic drug target

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Short title: PPARα as a sexually dimorphic target in NAFLD
ABSTRACT

Objective: We evaluated the influence of sex on the pathophysiology of non-alcoholic fatty liver disease (NAFLD). We investigated diet-induced phenotypic responses to define sex-specific regulation between healthy liver and NAFLD to identify influential pathways in different preclinical murine models and their relevance in humans.

Design: Different models of diet-induced NAFLD (high-fat diet, choline-deficient high-fat diet, Western diet, or Western diet supplemented with fructose and glucose in drinking water) were compared to a control diet in male and female mice. We performed metabolic phenotyping, including plasma biochemistry and liver histology, untargeted large-scale approaches (liver metabolome, lipidome, and transcriptome), gene expression profiling, and network analysis to identify sex-specific pathways in the mouse liver.

Results: The different diets induced sex-specific responses that illustrated an increased susceptibility to NAFLD in male mice. The most severe lipid accumulation and inflammation/fibrosis occurred in males receiving the high-fat diet and Western diet, respectively. Sex-biased hepatic gene signatures were identified for these different dietary challenges. The peroxisome proliferator-activated receptor α (PPARα) co-expression network was identified as sexually dimorphic, and in vivo experiments in mice demonstrated that hepatocyte PPARα determines a sex-specific response to fasting and treatment with pemafibrate, a selective PPARα agonist. Liver molecular signatures in humans also provided evidence of sexually dimorphic gene expression profiles and the sex-specific co-expression network for PPARα.

Conclusions: These findings underscore the sex-specificity of NAFLD pathophysiology in preclinical studies and identify PPARα as a pivotal, sexually dimorphic, pharmacological target.
What is already known about this subject?

The liver is a sexually dimorphic organ and non-alcoholic fatty liver disease (NAFLD) is sexually dimorphic.

Peroxisome proliferator-activated receptor α (PPARα) is a nuclear receptor expressed in many tissues and involved in the transcriptional control of metabolic and inflammatory responses.

PPARα is a target for hypolipidaemic drugs in the fibrate family that are under investigation for NAFLD treatment.

What are the new findings?

An integrated study of NAFLD in mice revealed sex-specific metabolic and inflammatory responses to four different dietary challenges.

Transcriptome analysis across these diet-induced NAFLD models identified robust sex-biased genes in mouse liver, including PPARα-dependent genes and correlation networks.

In human NAFLD, liver molecular signatures provide evidence of sexually dimorphic gene expression profiles and a sex-specific co-expression network for PPARα.

Hepatocyte-restricted deletion of Pparα highlights sex-specific and PPARα-dependent responses to fasting, to pemafibrate and to diet-induced NASH.

How might it impact on clinical practice in the foreseeable future?

This work emphasizes the importance of considering sex-specificity in NAFLD treatment, such as targeting hepatocyte PPARα.
INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide and is associated with obesity and type 2 diabetes.\cite{1} The prevalence of NAFLD is estimated to be 25% worldwide.\cite{2} NAFLD ranges from steatosis to non-alcoholic steatohepatitis (NASH), which can further progress to fibrosis, cirrhosis, and hepatocellular carcinoma.\cite{3} Diagnosis at early stages and monitoring of disease progression largely rely on histological analysis of liver biopsies. However, though NAFLD is a major public health problem, no medication has yet been approved for its treatment, but several drugs are currently being tested in clinical trials.\cite{4} In the era of personalized medicine, taking into account the molecular basis of sex differences is essential for comprehending the pathophysiology, advances in individualization of NAFLD management, and drug development.\cite{5,6} Epidemiological studies have revealed that premenopausal women are protected against cardiovascular diseases,\cite{7} as well as NAFLD,\cite{8–11} and a recent study demonstrated different molecular signatures in men and women with NAFLD.\cite{12} However, the potential sexually dimorphic traits leading to female protection against the progression of fibrosis progression are still unclear.\cite{13–15}

The molecular basis of sex-specific susceptibility to NAFLD has been investigated in rodents. Oestrogen deficiency promotes steatosis in ovariectomized mice fed a high-fat diet (HFD), and they are protected by 17β-oestradiol administration,\cite{16} indicating that oestrogens largely contribute to female protection. One of the mechanisms involves the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1A), a transcriptional coactivator of nuclear receptors. To modulate the expression of proteins involved in protecting against oxidative stress in hepatocytes, oestrogens signal through a mechanism dependent on oestrogen receptor alpha (ERα) and PGC1A.\cite{17} In mice, part of the liver sexual dimorphism is not dependent on hepatocyte ERα.\cite{18} Glucocorticoid-dependent signalling has also been suggested to play a significant role in liver sexual dimorphism.\cite{19,20} In the present study, we further investigated the basis of sex-specific NAFLD susceptibility through an integrative study of diet-induced mouse models of NAFLD.
Several models have recently been developed to investigate more advanced stages of NAFLD, including NASH in mice.[21,22] Though it is difficult to observe significant hepatocyte ballooning and fibrosis in mice, several groups have identified diets with specific formulations that promote NASH in male mice.[23–25] We took advantage of these different dietary challenges to identify influential pathways in the sexual dimorphism of the pathology. Using male and female mice, we determined diet-induced phenotypic responses using a combination of approaches integrating multiple layers of information across these different metabolic challenges. This allowed us to identify hepatocyte peroxisome proliferator-activated receptor α (PPARα) as being associated with sex-dependent susceptibility to NAFLD. Accordingly, sex-specificity in hepatocyte PPARα activity was characterized in response to fasting and to pharmacological activation by pemafibrate. Finally, we provide evidence of sex-specific molecular signatures in human NAFLD, including a sexually dimorphic co-expression PPARα network.
METHODS

Mice and diets

In vivo studies were performed in accordance with European guidelines for the use and care of laboratory animals and approved by an independent ethics committee (authorization 17430-2018110611093660 v3).

Thirteen-week-old male and female C57BL/6J mice (n=12 per group and per sex) were fed a chow diet (CTRL, D12450J, Research Diets), a high-fat diet (HFD, D12492, Research Diets), choline-deficient high-fat diet (CDHFD, D05010402, Research Diets), Western diet (WD, TD.88137, Envigo), or WD with glucose (18.9 g/L) and fructose (23.1 g/L) in drinking water (WD GF)[24] for 15 weeks.

Human liver samples from patients with NAFLD

Liver samples were selected from a cohort of biopsy-proven NAFLD patients established in the Hepatology Department of the Centre Hospitalier Universitaire de Toulouse. The 80 biopsies were from 48 men and 32 women (78% menopausal). This cohort was approved by the National Agency for the Safety of Medicines and Health Products (ANSM) and local ethics committee in 2015 (ClinicalTrial.gov: NCT02390232), and by the Minister of Higher Education, Research, and Innovation in 2017 (DC-2017-2984).

See Supplementary File 1 for further methods and experimental details regarding NAFLD classification in patients.

RESULTS

Sex-specific features in response to different dietary challenges

First, we investigated the responses of 13-week-old male and female C57BL/6J mice to five different dietary challenges (Figure 1A). They were fed either CTRL or one of four different hypercaloric diets for 15 weeks. All mice fed an hypercaloric diet significantly gained weight, except for females fed a CDHFD (Figure 1B). With all diets, males gained more body weight than females (Figure 1C). The food intake was higher in females regardless of the diet (Figure 1D). Perigonadal (PG) and sub-
cutaneous (SC) white adipose tissue (WAT) increased with all hypercaloric diets, especially in males with the exception of the PG WAT, which was higher in females fed a HFD (Figure 1E). The relative liver weight was increased in males in response to HFD, WD, and WD GF. Only WD and WD GF led to increased liver weight in females (Figure 1E).

Glucose tolerance, as assessed by the oral glucose tolerance test (OGTT), was impaired by all hypercaloric diets. Males were more affected than females by HFD and CDHFD, whereas glucose intolerance was similar between males and females in the WD groups (Figure 1F and G). Fasting insulineemia was higher in males fed any of the hypercaloric diets but not in females (Figure 1H). Plasma cholesterol, but not plasma triglycerides, increased with all hypercaloric diets, more severely in males (Figure S1).

**Sex-specific gene expression profile in response to different dietary challenges**

Next, we analysed hepatic gene expression. Principal component analysis of the transcriptome revealed a difference between male and female liver gene expression (Figure 2A) regardless of diet. However, discrimination seemed to be stronger in males than in females (Figure 2A). Differentially expressed genes (DEGs) were subjected to hierarchical clustering, further highlighting clusters of genes that are consistently sexually dimorphic with all diets (clusters 4, 5, 8, and 10) (Figure 2B). However, we identified four clusters that exhibited sex-dependent responses to the diets (clusters 1, 2, 3, and 7). We performed Gene Ontology analysis (Figure 2C) and revealed the upregulation of gene expression relative to fibrogenesis and PPAR signalling pathways (clusters 1 and 7) in males fed a HFD, WD, or WD GF. The higher expression of genes representative of these two clusters, such as Col1a1, Pparγ2, Ppara, Vnn1 and Fgf21 was confirmed by RT-qPCR (Figure 2D). Consistent with hepatic Fgf21 expression, circulating FGF21 was robustly increased in response to the different diets in males but not in females (Figure 2E). In addition, we observed that genes related to platelet activation and immune response (cluster 3) were consistently expressed at high levels in females for all five diets but were upregulated in males fed a WD or WD GF (Figure 2C). Finally, we noticed that WD and WD GF led to the upregulation of genes associated with the proteasome and biosynthesis of unsaturated fatty acids (cluster 2) in both sexes (Figure 2C). The hepatic expression of the rate-limiting enzyme in monounsaturated fatty acid synthesis (Scd1) was assessed by RT-
qPCR (Figure 2F). Finally, cluster 10 relates to genes related to complement and coagulation cascade as well as cytochrome P450s (KEGG function “Chemical carcinogenesis”), such as *Cyp4a14* (Figure 2G), which are more expressed in females liver whatever the diet. Overall, this liver gene expression profiling evidences some robust differences between sexes and sex-specific responses to diets enriched in metabolic pathways.

**Sex-specific susceptibility to diet-induced NAFLD**

To assess the correlation between changes in gene expression and the progression of liver steatosis, we performed histological analysis and hepatic lipid profiling. Males developed liver steatosis with all hypercaloric diets, but especially with HFD and WDs (Figure 3A and B). Compared to males, females were protected against steatosis regardless of the diet, but greater liver lipid accumulation was observed in females fed the WDs compared to HFDs (Figure 3A and B). The histological scores were confirmed by quantification of hepatic triglycerides (Figure 3C). Free and esterified cholesterol were similarly increased in both sexes in response to WDs (Figure 3C).

Next, we assessed liver inflammation. Lobular inflammatory foci were only detectable in male mice fed a WD or WD GF (Figure 3D). Females were robustly protected against inflammatory cell infiltration of the liver. The NAFLD activity score (NAS), including liver steatosis level, was sexually dimorphic with all hypercaloric diets (Figure 3E). In addition, the plasma alanine aminotransferase activity was elevated with all hypercaloric diets. This elevation was more severe in males (Figure 3F). Males developed significant liver fibrosis with all hypercaloric diets, whereas, in females, significant fibrosis was only detected in response to the CDHFD (Figure S2A-B). Therefore, the most dimorphic phenotype concerning fibrosis and inflammation was obtained with WD and WD GF (Figure S2B), and these data were confirmed by pathological scoring (Figure S2C). The main results obtained in mice of both sexes in response to the different diets are summarized in Figure 3G.

Taken together, our data suggest that HFD can be considered a representative model of sexually dimorphic steatosis, and WDs as a representative model of sexually dimorphic steatosis with early signs of NASH. Females are less prone to damages induced by either a HFD or WD, which we further investigated.

**Sex differences in the liver metabolome associated with susceptibility to NAFLD**
We performed reporter metabolite analysis using the genome-scale metabolic model for liver tissue in the HFD and in the WD models, which are representative of sexually dimorphic steatosis and early NASH, respectively. This analysis is based on gene and predicts changes in hepatic metabolism. These predictions were sexually dimorphic and diet-dependent (Figure 4A). Their hierarchical classification highlighted sexually dimorphic soluble metabolites specific for HFD (cluster 1) and a large cluster combining soluble metabolites and lipids for WD (cluster 2). These predictions were validated by liver NMR profiling. In males fed a HFD, we identified an increase in the relative abundance of reduced glutathione (GSH) associated with a decrease in glutathione precursors (Figure 4B). Such markers of oxidative stress were not detected in females (Figure 4C). Moreover, the relative abundance of trimethylamine (TMA) and hypotaurine was lower in males fed a WD compared to CTRL (Figure 4D), whereas the hepatic glucose concentration was significantly increased in females in response to the WD (Figure 4E). Lactate, another marker of redox homeostasis was also significantly reduced in females when compared to males upon both HFD and WD (Figure 4F).

Lipids such as acyl-coAs were also predicted to be impacted significantly by both diet and sex (Figure 4A). To investigate whether fatty acid β-oxidation may be sensitive to either sex or diet, we measured plasma acylcarnitines of various chain lengths (Figure 4G and Figure S3D). While medium chain acylcarnitines were not significantly modified, short chain acylcarnitines and long chain acylcarnitines were diet-sensitive but not significantly different between sexes. Cluster 2 of metabolites predicted to be increased in males fed a WD also included eicosanoyl-coAs, suggesting remodelling of long chain fatty acids (Figure 4A). Therefore we measured the relative abundance of hepatic lipids (Figure S3A) that revealed a marked effect of both the diet and the sex on liver lipidome (Figure S3B). Consistent with metabolic modelling, lipidomic analysis revealed marked changes in the relative abundance of long chain fatty acids (Figure S3C) such as eicosenoic acid (C20:1n-9) (Figure 4H) and eicosapentaenoic acid (C20:5n-3) (Figure S3E). Changes in the relative abundance of long chain fatty acids correlates with marked changes in the relative abundance of complex lipids containing long acyl chains such as sphingolipids (Figure 4I and Figure S3F) and phospholipids (Figure S3C). Since long chain acylcarnitines were not different between sexes, we assessed whether changes in the expression of genes encoding
key enzymes in fatty acid synthesis, desaturation, elongation, storage, glycerolipid and sphingolipid homeostasis were sexually dimorphic (Figure 4J). Our analysis revealed major sex-specific profiles of genes such as Elovl3 and Cidec that are overexpressed in males while genes such as Pnpla3 and Acot3 are overexpressed in females.

Altogether, these results suggest that steatosis induced by either HFD or WD is associated with sex-specific rewiring of liver gene expression and lipid metabolism.

**Sex-biased hepatic transcriptome profile**

Beyond liver metabolism, we next analysed the sexually dimorphic changes in gene expression associated with susceptibility to NAFLD (HFD vs. CTRL and WD vs. CTRL). Only a few genes presented similar regulation in both sexes in response to HFD (Figure S4A-C) and WD (Figure S4D-F). We further analysed the sexually dimorphic gene expression profiles from CTRL, HFD, and WD-fed mice to define sex-biased genes, i.e., DEGs between males and females in these three conditions. First, we identified the genes that are differentially expressed between males and females by taking into account the magnitude of change in their expression in each diet group (Figure 5A). Genes highly expressed in females compared to males included CYP450 (Cyp2b13, Cyp3a44), sulfotransferases (Sult2a1, Sult2a3, Sult2a4, Sult3a1), Slc22a26, and Fmo3 regardless of the diet. Genes highly expressed in males compared to females include CYP450 (Cyp4a12, Cyp2d34), Elovl3, Slco1a1, and Hsd3b5, regardless of the diet. Moreover, KEGG categories associated with the main physiological activities of the liver were found to be sexually dimorphic (Figure S4G). As expected, steroid hormone biosynthesis was the most sex-biased category (50.7%). However, metabolism of xenobiotics, retinol metabolism, and complement and coagulation cascades were categories in which more than 30% of genes were sex-biased. In addition, we used a set of genes associated with either hepato-specific functions or NAFLD in databases (KEGG) and the literature (Table S1, FigS5A). Normalized expression of these genes revealed a clustering of genes Figure S5B regulated by both sex and diet in Figure S5C. We also presented the hierarchical clustering of the different groups based on the hepatic gene expression profile of this set of liver specific genes in Figure S5D. Males and females were highly segregated.

Next, we investigated the sex-biased genes robustly upregulated in the female (Figure 5B) or male liver (Figure 5C). Gene Ontology analysis performed on the 1017 female-
biased genes revealed KEGG categories related to complement and coagulation cascades, steroid hormone biosynthesis, linoleic acid metabolism, platelet activation, chemokine signalling, and bile secretion (Figure 5B). Gene Ontology analysis performed on the 1128 male-biased genes revealed KEGG categories related to metabolism, including xenobiotic and steroid hormone homeostasis, as well as the peroxisome (Figure 5C). This analysis further highlights the major differences between males and females in most of the physiological activities of the liver, which are not restricted to steroid hormone biosynthesis. Linoleic acid metabolism and Peroxisome are related to PPARs which are transcription factors activated by fatty acids and involved in the regulation of genes that determine hepatocyte peroxisome biogenesis.

We performed a correlation network analysis of the three PPAR isotypes, revealing dense networks, with liver PPARα being strongly sexually dimorphic (Figure 5D) and suggesting that PPARα contributes to hepatic sexual dimorphism.

**PPARα-associated hepatic gene network is sexually dimorphic in human NAFLD**

Because our data indicate that PPARα activity is sexually dimorphic in mice, we also questioned the influence of sex on PPARα activity in human disease. Therefore, we compared the liver gene expression profiles by microarray analyses in liver samples from 32 women and 48 men (mean age 58.1 ± 10.4 and 56.1 ± 11.9 years, respectively) included in a cohort of patients with biopsy-proven NAFLD (Table S2). Both in mouse (Figure S6A) and in human (Figure S6B), correlation matrix analysis of gene expression revealed sexually dimorphic pattern.

Gene Ontology biological function analysis revealed that the most significant functional categories differentiating men and women in regards to the liver were related to inflammation and immune response, but also metabolism (Figure 6A). We analysed sexual differences in gene expression that correlated with PPARα and found that genes correlating with PPARα expression were more abundant in men (n=598) than in women (n=248) (Figure 6B). Focusing on the 50 best correlations with PPARα expression (Figure 6C) further highlighted differences between men and women. The correlation network of FGF21, a well-recognized PPARα target gene (Figure 6D), was also sexually dimorphic. In addition, the correlation network of several coregulators (NCOR2, TBL1X, TBL1XR) of PPARα was dimorphic (Figure S7). In contrast, the correlation network of other PPAR isotypes were not dimorphic (Figure S7). Finally,
our analysis revealed differences in the enrichment networks of genes that significantly correlated with PPARα expression in men (Figure 6E) and women (Figure 6F). Taken together, these results suggest that liver PPARα activity contributes to the sexual dimorphism of human NAFLD.

**Sexually dimorphic responses to fasting and drug treatment dependent on hepatocyte PPARα**

We next tested whether physiological and pharmacological challenges that acutely regulate hepatocyte PPARα activity may induce sexually dimorphic responses. Fasting robustly regulates hepatocyte PPARα activity in vivo in male mice.[26–28] Therefore we evaluated hepatocyte PPARα-dependent responses to fasting in both sexes. Upon fasting male and female Pparαhep−/− mice were hypoglycaemic compared to Pparαhep+/+ mice (Figure 7A), and the fasting-induced increase in ketonaemia was blunted in Pparαhep−/− mice of both sexes (Figure 7B). Compared to Pparαhep+/+ mice, fasted Pparαhep−/− males, but not females, had increased hepatic triglycerides (Figure 7C). We also analysed hepatic gene expression in mice fasted for 16h. Our results revealed that, in the absence of hepatocyte PPARα, the fasting response was different between male and female mice (Figure 7D). PPARα invalidation induced the overexpression of 2438 hepatic genes (p<0.01), including 255 exclusively in males, 1737 exclusively in females, and 446 common to both sexes (Figure 7D). This result indicated that the deletion of hepatocyte PPARα induces more extensive genomic response in females than males upon fasting. In females, these 1737 upregulated genes in Pparαhep−/− fasted mice were mainly associated with functions related to NFκB or TNF signalling (Figure 7E-F) suggesting higher inflammation.

Finally, male and female Pparαhep+/+ and Pparαhep−/− mice fed a standard diet were treated with pemafibrate, a selective PPARα agonist. Body weight and glycaemia were not influenced by pemafibrate treatment (Figure 7G). However, pemafibrate treatment induced significant hepatomegaly in both male and female wild-type mice but not in Pparαhep−/− mice (Figure 7G). Gene expression influenced by pemafibrate was then analysed in males (Figure 7H) and females (Figure 7I). Regulation of liver gene expression in response to pemafibrate was strictly dependent on hepatocyte Pparα in
both males and females (Figure S8A-D). In Pparαhep+/+ mice, the hepatic response to pemafibrate was sexually dimorphic (Figure 7J). Several genes were only sensitive to pemafibrate in females (1000 upregulated and 802 downregulated genes), suggesting that the female liver is much more responsive to pemafibrate than the male liver. We analysed the interaction between sex and pemafibrate treatment and found that the sex-specific response was mostly related to gene functions relative to lipid homeostasis (Figure 7K).

Sexually dimorphic effect of hepatocyte PPARα in diet-induced NAFLD

To further investigate the potential sexual dimorphism of PPARα activity in the liver, we fed male and female Pparαhep-/- mice and their Pparαhep+/+ littermates a WD for 15 weeks. Male, but not female Pparαhep-/- mice were protected against body weight gain induced by the WD (Figure 8A and B) while food intake, circulating leptin, adipose tissue weight, circulating free fatty acids were not modified by WD feeding (Figure S9A), only adiponectin was increased in response to WD feeding in female Pparαhep-/- mice (Figure S9A). In contrast to changes in body weight gain, glucose tolerance measured by the OGTT was not influenced by genotype either in males or females (Figure 8C and D).

Next, we evaluated liver steatosis by histological and liver lipid profile analyses. As expected, Pparα deletion in hepatocytes was sufficient to induce steatosis in male mice fed CTRL diet, but female Pparαhep-/- mice did not develop spontaneous steatosis (Figure 8E-G and Figure S9B). WD-induced liver steatosis was not exacerbated by Pparα invalidation in either sex (Figure 8E-G). Though males and females exhibited the same level of steatosis and alanine aminotransferase (Figure S9C), female Pparαhep-/- mice developed more severe inflammation (Figure 8H and Figure S9D) and fibrosis (Figure 8I) than Pparαhep+/+ females. This difference was not observed in males, except for the αSMA staining (Figure S9E).

Associated with these changes, we observed several sex-specific regulations of genes well-known to be direct PPARα targets such as Cyp4a10 and Fgf21 (Figure 8J) as well as Cyp4a14 and Cidec (Figure S9F).[28] Interestingly, the expression of genes involved in lipid metabolism such as Elovl3, Pnpla3 and Mogat1 previously identified as sexually dimorphic were also dependent on hepatocyte PPARα (Figure S9F).
However, other PPARα target genes such as *Ehhadh* or *Vnn1* showed similar expression pattern in both sexes (*Figure S9F*). Importantly, we observed that circulating FGF21 levels increased in response to WD feeding through a mechanism requiring hepatocyte PPARα in both sexes (*Figure 8K*). Circulating concentrations of plasma FGF21 was twice higher in male mice than in females.

Taken together, these results indicate sexually dimorphic activity of hepatocyte PPARα that controls liver lipid homeostasis upon standard diet. Moreover in response to WD sexually dimorphic activity of hepatocyte PPARα influences body weight gain, hepatic inflammation and the regulation of genes involved in lipid homeostasis as well as FGF21 level.
DISCUSSION

In order to investigate the sex-specific susceptibility to steatosis in both mice and humans,[29,30] we performed a large-scale integrative systems analysis to investigate the effect of diets promoting NAFLD in male and female mice. Our analysis revealed distinct metabolic and hepatic phenotypes with consistent sexual dimorphism. In agreement with this, steatosis severity was estimated by combining histological analysis and biochemical measurements, revealing that steatosis is consistently more severe in males than females. None of the preclinical models we used fully recapitulate the human disease as even the highest fibrosis score in WD-fed males was moderate and no ballooning was observed. In addition, we cannot exclude that, in mice, the differences observed between sexes are due to different time courses in the development of the disease that may take much longer time in females than in males.

However, this large set of experiments allowed us to investigate the interaction between diet and sex in the regulation of gene expression in the liver. Beyond the direct effects of micronutrients and macronutrients on metabolism and the progression of steatosis,[31] the composition of the diet may also lead to changes in gut microbiota composition, with distinct dysbiosis between HFD and WD, and subsequently influencing hepatic homeostasis through bacterial metabolites and endotoxins.[32,33] Our analysis suggests the existence of complex biological regulatory levels controlling sex-specific liver homeostasis that is highly applicable to the network of genes associated with or involved in NAFLD. Using network analysis, we identified hepatocyte PPARα as a sexually dimorphic player in mouse liver homeostasis. PPARα is a nuclear receptor highly expressed in hepatocytes that plays a central role in fatty acid homeostasis. It is activated by long chain fatty acids and controls hundreds of genes involved in metabolism and inflammation. It is particularly active during adipose tissue lipolysis,[34] for instance in response to starvation.[26]. During adipose tissue lipolysis, PPARα is also essential to drive systemic responses through the transcriptional control of FGF21, [35] a liver-derived hormone controlling many aspects of metabolism and behaviour.[36].

Our observation agrees with a previous study [37] showing that whole-body deletion of Ppara leads to sexually dimorphic changes in lipid metabolism and the sex-specific role of liver PPARα in protection from oestrogen-induced intrahepatic
cholestasis. Preclinical and clinical studies have shown that PPARα is a pharmacological target in NAFLD.

To further explore the relationship between PPARα activity and sexual dimorphism in the liver, we used mice lacking hepatocyte PPARα in different experimental settings. Our work confirms previous findings showing that male mice develop steatosis in the absence of hepatocyte PPARα, but this was not observed in female mice. In addition, hepatocyte PPARα influences both body weight gain in response to WD, as well as FGF21 production and liver fibrosis, in a sex-specific manner.

To investigate whether hepatocyte PPARα may also control sexually dimorphic responses to an acute physiological challenge, we investigated the hepatic response to fasting and provide evidence that hepatocyte PPARα determines sex-specific regulation of liver gene expression. In NAFLD, a large part of hepatic lipid accumulation are derived from adipose tissue. Moreover, several lines of evidence suggest that adipose tissue lipolysis controls liver activity through mechanisms including PPARα-dependent responses. During fasting, this adipose-to-liver response is challenged. Therefore, our data support the possibility that part of sex-specific responses involving PPARα might relate to modification of this inter-organ communication.

To question the pharmacological relevance of our findings, we determined the changes in hepatic gene expression in response to an acute challenge with pemafibrate, a PPAR agonist that is currently being tested in clinical trials for the treatment of dyslipidaemia and NAFLD (ClinicalTrials.gov Identifier: NCT04079530 and NCT03350165, respectively). Our analysis revealed the very high specificity of the molecule for PPARα and that pemafibrate induces a sex-specific genomic response in the liver. Whether these sex-specific responses would be sufficient to alter the drug response to pemafibrate and other drugs currently in clinical trials remain to be investigated. Given the protection from NAFLD observed in female mice, experiments assessing the efficiency of drugs in both sexes cannot easily been tested.

In our experimental settings, the severity of steatosis observed in males depended on the type of diet. Both HFD and WD induced obesity and steatosis. In line with several previous reports, we found that females were protected from steatosis induced by HFD. In males fed a HFD, metabolomic profiling revealed changes in hepatic glutathione metabolism that were not observed in females. These results agree with
studies highlighting that oxidative stress contributes to the progression of NAFLD.[48,49] The hepatoprotective action of oestradiol, involving ERα and PGC1A in the transcriptional control of antioxidant proteins, has been demonstrated in hepatocytes from female mice.[17] Moreover, supplementation of precursors of glutathione and NAD+ has been shown to decrease steatosis in mice.[50] Finally, metabolic modelling of human samples showed that altered GSH and NAD+ metabolism is a prevailing feature in NAFLD.[50] Therefore, it would be important to consider the potential sexually dimorphic differences in the oxidative stress response that account for the protection of women from steatosis and whether it declines with menopause.

Mice fed a WD developed more severe steatosis than those exposed to a HFD. In addition, WDs increased liver inflammation and induced significant fibrosis in males. Moreover, though modest steatosis occurred in WD-fed female mice, they remained protected from inflammation and fibrosis. Our metabolic modelling and lipidome analysis highlighted additional sexually dimorphic metabolites including a marked shift in the relative abundance of long chain fatty acids and complex lipids containing long acyl chains such as ceramides and phospholipids. Since we did not detect sex-specific change in the relative abundance of plasma acylcarnitines, we thought that our observations result from sex differences in lipid anabolism rather than catabolism. Our data support this hypothesis, as genes involved in fatty acid homeostasis are highly sexually dimorphic. Hepatic lipogenesis is known to be transcriptionally regulated[51] and different between males and females. [52] Our analysis highlights that not only the core of lipogenic genes but also a set of genes involved in fatty acid elongation, traffic, fatty acid storage, acyl-coA synthesis and complex lipid synthesis (glycerolipids, sphingolipids) are differentially regulated in males and females. Importantly, some of these genes such as Elovl3,[53] Cidec,[54] and Pnpla3[55] have previously been linked to steatosis. Moreover, these data are in line with the finding that PPARα is an important regulator of hepatic sexual dimorphism since PPARα activity might be sensitive to the metabolism of fatty acids which are recognized ligands for this nuclear receptor. In addition, some of these sexually dimorphic genes have been described as directly regulated by PPARα in hepatocytes.[27,28]

Finally, we also questioned the relevance of our finding in human NAFLD. A recent study reported sexually dimorphic gene expression profiles in human NAFLD,[12] and
our analysis performed in an independent cohort extends and further confirms distinct molecular signatures in men and women with NAFLD. Using correlation analysis, we more specifically report sexually dimorphic PPARα-related and FGF21-related gene networks as well as PPARα-related functions in patients with NAFLD. Some limitations must be acknowledged in the interpretation of these data due to phenotypic differences between men and women with NAFLD. First, as observed in our cohort, BMI is usually higher in women than in men in individuals with metabolic diseases such as NAFLD.[15] Second, since most of the women in our cohort are post-menopausal, it is likely that the sexual dimorphism observed here does not rely on the direct influence of female gonadal hormones. Analysis of larger cohorts are thus needed to further examine the influence of BMI as well as the hormonal status in sexually dimorphic liver gene expression in human NAFLD.

Taken together, our data provide a resource for investigating hepatic genes and pathways involved in the development of steatosis that are useful for preclinical research on sex differences in NAFLD. Moreover, this study identifies hepatocyte PPARα as a relevant sexually dimorphic target in NAFLD, further supporting the need to consider sex as a crucial determinant of NAFLD progression, biomarker discovery and therapeutic responses.

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CONTRIBUTORS: SSG designed experiments, performed experiments, analysed the data and wrote the paper. AP, AF, SES, YB, YL, MR contributed to design experiments, perform experiments and to analyse the data. AL, MH, FL, AMarr, TAS, JW, CS, CN, AB, CL, TF, BT, PD, LS, JBM performed experiments and contributed to data analysis. MA and CZ contributed to analyze the data. NH, J-PP, BS, RB, FL, J-FA, TL, LGP, SLa, NL, SLo, CP, WW, CB provided critical materials, supervised experiments and contributed to data analysis. MG provided critical reagents, contributed to design the project and to data analysis. AMon and PG designed the project, supervised experiments, analysed the data and wrote the paper. HG designed the project, supervised experiments, performed experiments, analysed the data and wrote the paper.

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Figure 1. Sex-specific phenotype in response to different dietary challenges.

(A) C57BL/6J female and male mice were fed a control diet (CTRL), high-fat diet (HFD), choline-deficient HFD (CDHFD), Western diet (WD), or a WD provided with glucose and fructose in drinking water (WD GF) for 15 weeks (n=12/group). (B) Change in body weight in response to each diet compared to control (CTRL) in male (blue) and female (red) mice (n=12). (C) Relative weight gain after 15 weeks of diet. (D) Food intake expressed in grams based on mouse body weight for each diet. Food consumption was measured weekly. (E) Relative perigonadal (PG), subcutaneous (SC) white adipose tissue (WAT), and relative liver weight expressed in grams per gram body weight at the end of the experiment. (F) Oral glucose tolerance test (OGTT) assessed after 13 weeks of diet in males and females (n=12/group). Blue and red curves represent male and female glycaemia (mg/dL), respectively. (G) Area under the curve representing OGTT results. (H) Insulinaemia was measured after 6 hours of fasting. Results are the mean ± SEM. #diet effect, *sex effect. * or # p<0.05, ** or ## p<0.01, *** or ### p<0.001. Differential effects were analysed by analysis of variance followed by Student t-tests with a pooled variance estimate.

Figure 2. Sex-specific hepatic gene expression profiles associated with different dietary challenges.

(A) Principle component analysis (PCA) score plots of the whole transcriptomic dataset in the liver (n=6/group). Each dot represents an observation (animal) projected onto first (horizontal axis) and second (vertical axis) PCA variables. (B) Heatmap presenting data from a microarray experiment performed with liver samples (n=6/group). Hierarchical clustering is also shown, which allows the definition of 12 gene clusters (ps≤0.05). ECM: extracellular matrix. (C) Gene Ontology analysis of clusters 1, 2, 3, and 7. The false discovery rate is provided for each category. (D) Gene expression in the liver derived from complementary qPCR experiments (n=12/group) illustrating the selected clusters (Col1a1, Ppara, Pparγ2, Ppara, Vnn1 and Fgf21). (E) Plasma FGF21 was measured on samples collected at the end of the experiment. Gene expression in the liver derived from complementary qPCR experiments (n=12/group) illustrating the selected clusters, Scd1 (F) and Cyp4a14 (G). Results are the mean ± SEM. #diet effect, *sex effect. * or # p<0.05, ** or ## p<0.01, *** or ### p<0.001.

Figure 3. Sex-specific susceptibility to diet-induced steatosis and steatohepatitis.

(A) Representative histological sections of liver stained with haematoxylin and eosin (H&E) from males and females in each group. Magnification x100. (B) Liver steatosis estimated on histological liver sections. Scoring: parenchymal involvement by steatosis <5%, 0; 5-33%, 1; 33-66%, 2; >66%, 3 (n=12 per group). (C) Neutral lipids (triglycerides, free and esterified cholesterol) were extracted from the livers and analysed by gas-liquid chromatography. (D) Inflammatory score for histological liver sections. Each tissue section was analysed for 10 microscopic fields (200× magnification) to determine the mean number of inflammation foci per field (n=12 per group). (E) NAS score and (F) alanine aminotransferase (ALT) determined from plasma samples collected at the end of the experiment. (G) Table summarizing diet-induced NAFLD model characteristics and limitations. Results are the mean ± SEM. #diet effect, *sex effect. * or # p<0.05, ** or ## p<0.01, *** or ### p<0.001. Differential
effects were analysed by analysis of variance followed by Student t-tests with a pooled variance estimate. Histological scores were analysed by a non-parametric test (Kruskall-Wallis).

Figure 4. Sex-specific changes in the hepatic metabolome associated with susceptibility to steatosis and NASH.

(A) Reporter metabolites in the liver gene expression profile analysis of male and female mice fed a high-fat diet (HFD) or Western diet (WD) versus control (CTRL) (n=6/group) were investigated by referring to the liver genome-scale metabolic model (iHepatocytes2322). The adjusted p-values for each reporter metabolite were calculated for up- and down-regulated genes, and the minus Log10 of the p-values is presented. c: cytoplasm, m: mitochondria, n: nucleus, p: peroxisome, r: endoplasmic reticulum, FAME: fatty acid methyl ester. (B) Coefficient plots related to the O-PLS-DA models discriminating between CTRL and HFD in males and females (C) derived from the liver extract ¹H-NMR-based spectra of mice in the HFD versus CTRL group (n=12/group). The figure shows the discriminant metabolites that are higher or lower in the HFD versus CTRL group. Metabolites are colour-coded according to their correlation coefficient: red indicating a very strong positive correlation (R²>0.65). The direction of the metabolite indicates the group with which it is positively associated, as labelled on the diagram. (D) Coefficient plots related to the O-PLS-DA models discriminating between CTRL and WD in males and females (E) derived from the liver extract ¹H-NMR-based spectra of mice in the WD versus CTRL group (n=12/group). The figure shows the discriminant metabolites that are higher or lower in the WD versus CTRL group. Metabolites are colour-coded according to their correlation coefficient: red indicating a very strong positive correlation (R²>0.65). The direction of the metabolite indicates the group with which it is positively associated, as labelled on the diagram. (F) Area under the curve of the H1-NMR spectra was integrated for the lactate signals. (G) Plasma levels of long chain acylcarnitines measured after 6h of fasting. Relative abundance of C20:1n-9 (H), and Cer18:1/20:0 (I) in the liver measured by GC-FID (n=6/group). (J) Gene expression profile of genes involved in fatty acid synthesis, desaturation and elongation, traffic and storage in droplets, glycerolipid and sphingolipid metabolism. Data represent mean ± SEM, #diet effect, *sex effect. * or # p<0.05, ** or ## p<0.01, *** or ### p<0.001.

Figure 5. Sex-biased hepatic gene expression profiles in control (CTRL), high-fat diet (HFD), and Western diet (WD).

(A) Volcano plot of differences in gene expression between males and females in CTRL, HFD, and WD. Colours indicate p< 0.05 and Log (base 2) fold change >2 (red), p <0.05 and Log (base 2) fold change <2 (blue), and non-significant (NS) (black). (B) Venn diagram representing the number of genes significantly upregulated in females compared to males receiving CTRL, HFD, and WD (p<0.05). Enrichment analysis of the 1017 genes consistently overexpressed in females compared to males in the three experimental groups (female-biased genes). (C) Venn diagram representing the number of genes significantly upregulated in males compared to females in CTRL, HFD, and WD (p<0.05). Enrichment analysis of the 1128 genes consistently overexpressed in males compared to females in the three experimental groups (male-biased genes). (D) Networks of the 50 genes showing the highest absolute correlation with each gene of interest (red node). Ppara, Pparb/δ, and Pparγ in males from the
CTRL, HFD, and WD groups are presented as circle plots (n=6/group). The edges corresponding to significant correlations are presented (Bonferroni-adjusted p<0.05). Another network circle plot based on these 50 genes is presented for female mice. Magenta nodes correspond to genes that significantly correlate with the gene of interest. Red and blue were used for positive and negative correlations, respectively.

**Figure 6** Gene expression profiling in human NAFLD highlights PPARα as a sexually dimorphic target.

Gene expression profiles were analysed by microarrays in 48 men and 32 women with biopsy-proven NAFLD. (A) Enriched terms across differentially expressed genes (p<0.05) between liver samples from men and women. (B) Venn diagram presenting the number of hepatic genes that correlated with PPARα mRNA expression in each sex (adjusted p value <0.05). (C) Networks of the 50 genes showing the highest absolute correlation with PPARα and FGF21 (D). The edges corresponding to significant correlations are presented (Bonferroni-adjusted p<0.05). The network circle plot based on the top 50 genes selected in men is presented on the left while the corresponding circle plot in women is on the right. Magenta nodes correspond to genes that significantly correlate with the gene of interest. Red and blue were used for positive and negative correlations, respectively. (E) Network of enriched terms across genes showing mRNA expression highly correlated with PPARα mRNA in men (adjusted p value <0.05). (F) Network of enriched terms across genes showing mRNA expression highly correlated with PPARα mRNA in women (adjusted p value <0.05).

**Figure 7** Sexually dimorphic hepatocyte Pparα activation by fasting and by pharmacological agonist.

Male and female C57BL/6J Pparαhep+/- and Pparαhep-/- mice were fasted for 24 hours. (A) Glycaemia (mg/dL) and (B) ketonaemia (mmol/L) evolution in each group (n=12/group). Mice were either sacrificed after 18 h of fasting or fed ad libitum. (C) Hepatic triglycerides were extracted from the livers and hepatic gene expression assessed by microarray analysis (n=6/group). (D) Venn diagram representing hepatic genes significantly upregulated (up) or downregulated (below) during fasting in Pparαhep-/- compared to Pparαhep+/- in female (red circle) and male (blue circle) mice (p <0.01). (E) Gene Ontology analysis of up and down regulated genes in the absence of hepatocyte Pparα during fasting, exclusively in females. The false discovery rate is provided for each category. (F) Gene expression in the liver derived from complementary qPCR experiments (n=6/group) illustrating Ccl2 and Cxcl10. Results are given as the mean ± SEM. #diet effect, *sex effect. * or # p<0.05, ** or ## p<0.01, *** or ### p<0.001. Male and female C57BL/6J Pparαhep+/- and Pparαhep-/- mice were treated with pemafibrate (0.1 mg/kg) or vehicle (CMC) for 14 days by daily oral gavage. (G) Body weight, glycaemia and relative liver weight were measured at the end of the experiment. Hepatic gene expression was assessed by microarray analysis (n=6/group). (H) Volcano plot of differences in gene expression between pemafibrate and vehicle in male and (I) female mice. Colours indicate p<0.05 and Log (base 2) fold change >2 (red), p<0.05 and Log (base 2) fold change <2 (blue), and non-significant (NS) (black). (J) Venn diagram representing hepatic genes significantly upregulated...
(up) or downregulated (below) by pemafibrate compared to vehicle in Pparαhep+/+ female (red circle) and male (blue circle) mice (p<0.01. (K) Gene Ontology analysis of genes with dimorphic response to pemafibrate. The false discovery rate is provided for each category.

**Figure 8 Sexually dimorphic effect of hepatocyte PPARα in diet-induced NAFLD**

Male and female C57BL/6J Pparαhep+/+ and Pparαhep/- mice were fed a control diet (CTRL) or Western diet (WD) for 15 weeks (n=6-12/group). (A) Change in body weight in response to WD compared to control (CTRL) male (blue) and female (red) mice (n=6-12). (B) Relative weight gain after 15 weeks of diet. (C) Oral glucose tolerance test (OGTT) assessed after 11 weeks of diet in males and females (n=6-12/group). Blue and red curves represent male and female glycaemia (mg/dL), respectively. (D) Area under the curve representing the OGTT results. (E) Representative histological sections of liver stained with haematoxylin and eosin (H&E) assessed in males and females from each group. Magnification x100. (F) Liver steatosis estimated on histological liver sections. Scoring: parenchymal involvement by steatosis <5%, 0; 5-33%, 1; 33-66%, 2; >66%, 3 (n=12 per group). (G) Hepatic triglycerides extracted from the livers. After extraction, lipids were analysed by gas-liquid chromatography. Results are given as the mean ± SEM. #diet effect, *sex effect. * or # p<0.05, ** or ## p<0.01, *** or ### p<0.001. Differential effects were analysed by analysis of variance followed by Student t-tests with a pooled variance estimate. (H) Inflammatory score for histological liver sections. Each tissue section was analysed for 10 microscopic fields (100× magnification) to determine the mean number of inflammation foci per field (n=6-12 per group). (I) Histological fibrosis score in CTRL and WD groups (males and females). Sirius red-stained was used to evaluate fibrosis as follows: 0, no fibrosis; 1, pericellular and perivenular fibrosis; 2, focal bridging fibrosis. Histological scores were analysed by a non-parametric test (Kruskall-Wallis). (J) Gene expression in the liver derived from complementary qPCR experiments illustrating Ppara, Cyp4a10 and Fgf21. (K) Plasma FGF21 was measured on samples collected at the end of the experiment Results are given as the mean ± SEM. #diet effect, *sex effect. * or # p<0.05, ** or ## p<0.01, *** or ### p<0.001. Differential effects were analysed by analysis of variance, followed by Student t-tests with a pooled variance estimate. ND: not detectable.
Figure 1.
Cluster 1
upregulated by WD, WD GF and HFD in males
484 genes, 9 KEGG functions
- DNA replication (0.00055)
- ECM-receptor interaction (0.0117)

Cluster 2
upregulated by WD and WD GF in both sexes
264 genes, 5 KEGG functions including
- Proteasome (9.2e-11)
- Biosynthesis of unsaturated fatty acids (0.0018)

Cluster 3
upregulated in females and only in males fed WD or WD GF
472 genes, 85 KEGG functions including
- Platelet activation (2.24e-05)
- Leukocyte transendothelial migration (5.60e-05)

Cluster 7
upregulated by HFD, CDHFD, WD and WD GF in males
548 genes, 18 KEGG functions including
- Peroxisome (5.69e-08)
- Pyruvate metabolism (0.00056)

Cluster 10
upregulated in females
446 genes, 18 KEGG functions including
- Complement and coagulation cascade (2.23e-09)
- Chemical Carcinogenesis (1.54e-06)
Figure 3.

**A**

Males Females

<table>
<thead>
<tr>
<th>Diet</th>
<th>Composition</th>
<th>Body Weight</th>
<th>Glucose Intolerance</th>
<th>Fasting Insulin</th>
<th>Steatosis</th>
<th>Lobular Inflammation</th>
<th>Fibrosis</th>
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<tr>
<td>CTRL</td>
<td>Prot 20%, Carbs 70%, Fat 10%, Sucrose 68.8 g/kg, Cholesterol 0.005%, 3.85 kcal/g</td>
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</tr>
<tr>
<td>CDHFD</td>
<td>Prot 20%, Carbs 35.1%, Fat 44.9%, Sucrose 178 g/kg, Cholesterol 0.03%, Choline deficient, 4.74 kcal/g</td>
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<tr>
<td>WD</td>
<td>Prot 15.2%, Carbs 42.7%, Fat 42%, Sucrose 341 g/kg, Cholesterol 0.2%, 4.5 kcal/g</td>
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<tr>
<td>WD GF</td>
<td>WD + glucose 18.9 g/L and fructose 23.1 g/L in drinking water</td>
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Figure 4.

**A** Heatmap showing the clustering of samples based on metabolite profiles.

**B** PCA biplot showing the separation of samples into clusters 1 and 2.

**C** Higher in HFD M.

**D** Higher in WD M.

**E** Higher in WD F.

**F** Graph showing the AUC for Lactate.

**G** Graph showing the LCAC.

**H** Graph showing the % total liver FAME.

**I** Graph showing the % total liver Cer.

**J** Graph showing the log2FC for males-females.
Figure 5.
Figure 6.

A. Enriched terms across differentially expressed genes (p<0.05) between man and woman liver samples

- GO:0170600: Cellular response to extracellular stimulus
- GO:0060369: Positive regulation of Fc receptor mediated signaling
- GO:0161247: Cell adhesion mediated by integrin
- GO:0043299: Leukocyte degranulation
- GO:0051186: Cofactor metabolic process
- GO:0071466: Cellular response to xenobiotic stimulus
- GO:0034341: Response to interferon-gamma

B. Genes correlated with PPARα in men (adj p<0.05) vs. women (adj p<0.05)

- 598 genes in men
- 141 genes in women
- 248 shared genes

C. Human liver PPARα correlation network

- Man
- Woman

D. Human liver FGF21 correlation network

- Man
- Woman

E. Network of enriched terms across gene expression correlated with PPARα mRNA in man NAFLD

- Regulation of small molecule metabolism
- Cellular amino acid metabolic process
- Carbohydrate biosynthetic process
- Regulation of polysaccharide metabolic process
- Steroid metabolic process
- Coenzyme metabolic process
- Lipid biosynthetic process
- Intracellular signaling by second messengers
- Negative regulation of phosphorylation
- FOXO-mediated transcription
- Asparagine N-linked glycosylation
- Prostate cancer
- HNF3B pathway
- Regulator of hormone levels
- Carbon metabolism
- Adaptable immune system
- Water-soluble vitamin metabolism

F. Network of enriched terms across gene expression correlated with PPARα mRNA in woman NAFLD

- Cellular response to cholesterol
- Transmembrane receptor protein tyrosine kinase
- Regulation of protein catabolic process
- Polycystin-1 multiprotein complex
- Actin cytoskeleton organization
- Negative regulation of dendrite development
- Positive regulation of organelle organization
- Epidermal growth factor receptor signaling
- Cell part morphogenesis
- Regulation of lipid metabolic process
- Positive regulation of transferase activity
- Regulation of protein binding
- Embryonic camera-type eye development
- Apoptosis
- Regulation of Wnt Signaling Pathway
Figure 7.
Figure 8.