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Pemafibrate abrogates SLD in a rat experimental dietary model, inducing a shift in fecal bile acids and microbiota composition

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ABSTRACT

Background and aims: Drugs resolving steatotic liver disease (SLD) could prevent the evolution of metabolic dysfunction associated SLD (MASLD) to more aggressive forms but must show not only efficacy, but also a high safety profile. Repurposing of drugs in clinical use, such as pemafibrate and mirabegron, could facilitate the finding of an effective and safe drug-treatment for SLD.

Approach and results: The SLD High Fat High Fructose (HFHFr) rat model develops steatosis without the influence of other metabolic disturbances, such as obesity, inflammation, or type 2 diabetes. Further, liver fatty acids are provided, as in human pathology, both from dietary origin and *de novo* lipid synthesis. We used the HFHFr model to evaluate the efficacy of pemafibrate and mirabegron, alone or in combination, in the resolution of SLD, analyzing zoometric, biochemical, histological, transcriptomic, fecal metabolomic and microbiome data. We provide evidence showing that pemafibrate, but not mirabegron, completely reverted liver steatosis, due to a direct effect on liver PPARα-driven fatty acid catabolism, without changes in total energy consumption, subcutaneous, perigonadal and brown fat, blood lipids and body weight. Moreover, pemafibrate treatment showed a

List of abbreviations: ABCG5/8, ATP binding cassette G5/8; ACAT2, acyl-CoA:cholesterol O-acyl-transferase 2; ACO, acyl-CoA oxidase; ACLY, ATP-citrate lyase; AFLD, alcoholic fatty liver disease; αMCA, α-muricolic acid; ANGPTL3, angiopoietin-like protein 3; Apo AV, apolipoprotein AV; Apo B, apolipoprotein B; Apo CIII, apolipoprotein CIII; Apo E, apolipoprotein E; ALT, alanine aminotransferase; AST, aspartate aminotransferase; β-KT, β-klotho; BAT, brown adipose tissue; BA, bile acids; BMI, body mass index; BSEP, bile-salt export pump; CCT, CTP:phosphocholine cytidylyltransferase; ChREBPβ, carbohydrate response element binding protein β; CVD, cardiovascular disease; CSE, cystathionine γ-lyase; CT, control; CYP2C70P, cytochrome P450 2c70; CYP7A1, cytochrome P450 7α-hydroxylase; CYP8B1, cytochrome P450 8b1; DAG, diacylglycerols; DCA, deoxycholic acid; DNL, *de novo* lipid synthesis; EGR1, early growth response 1; FAS, fatty acid synthase; FGF21, fibroblast growth factor 21; FGFR1, fibroblast growth factor receptor 1; HDCA, hyodeoxycholic acid; HFHFr, high fat high fructose; HI, hydrophobicity index; HL, hepatic lipase; IRE-1, inositol requiring enzyme-1; ISI, insulin sensitivity index, KHK, ketohexokinase; LCA, lithocholic acid; LDLR, low density lipoprotein receptor; L-PK, liver-pyruvate kinase; L-CPT-I, liver-carnitine palmitoyl transferase I; LPL, lipoprotein lipase; MASH, metabolic associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; MBG, mirabegron; MTTP, microsomal triglyceride transfer protein; MDR 2/3, multidrug resistance protein 2/3; NASH, non-alcoholic steatohepatitis; NEFA, non-esterified fatty acids; NTCP, sodium-dependent taurocholate cotransporting peptide; OATP, organic anion transporter peptide; PCA, principal component analysis; PemA, pemafibrate; PERK, protein kinase RNA-like endoplasmic reticulum kinase; PNPLA3, patatin-like phospholipase domain-containing protein 3; PPARα, peroxisome proliferator activated receptor α; RCT, randomized clinical trial; SCFA, short chain fatty acids; SPPARMα, selective peroxisome proliferator activated receptor α modulator; SRBI, scavenger receptor BI; SLD, steatotic liver disease; TG, triglycerides; UCP1, uncoupling protein 1; UCP3, uncoupling protein 3; UDCA, ursodeoxycholic acid; VLDL, very-low density lipoprotein; VLDLr, VLDL receptor; WAT, white adipose tissue.

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neutral effect on whole-body glucose metabolism, but deeply modified fecal bile acid composition and microbiota.

Conclusions: Pemafibrate administration reverts liver steatosis in the HFHFr dietary rat SLD model without altering parameters related to metabolic or organ toxicity. Our results strongly support further clinical research to reposition pemafibrate for the treatment of SLD/MASLD.

1. Introduction

The detection in liver parenchyma of an abnormal accumulation of mostly neutral lipids (triglycerides and cholesteryl esters) is a landmark characteristic of Non-alcoholic fatty liver disease (NAFLD), diagnosed by the presence of steatosis in more than 5 % of hepatocytes. NAFLD encompasses several pathological entities, from steatosis (steatotic liver disease, SLD), to steatosis accompanied with mild inflammation, and necroinflammation with hepatocyte ballooning and fibrosis (non-alcoholic steatohepatitis, NASH). Approximately a quarter of patients affected of NASH can evolve to more aggressive liver pathologies (cirrhosis and hepatocellular carcinoma). NAFLD is considered the liver manifestation of the metabolic syndrome, a whole body metabolic dysregulation affecting, among others, skeletal and adipose tissues, which encompasses overweight and obesity, insulin resistance and pre-diabetic states, hypertension and dyslipidemia, directly responsible for an increased cardiovascular risk in affected individuals [1,2]. Thus, the term NAFLD has been replaced for metabolic dysfunction-associated steatotic liver disease (MASLD) (to avoid confusion, we will use hereafter the new terminology) [3]. A systematic review and meta-analysis by Riazi et al. [4] estimates a world-wide prevalence of 32.4 % for MASLD, with higher values in men than in women (39.7 % vs 25·6 %).

Recently, resmetirom has received the FDA approval for the treatment of NASH (metabolic associated steatohepatits MASH in the new terminology) with grade F2-F3 fibrosis. However, there is no approved drug-therapy for the treatment of SLD, the initial, reversible stage of MASLD [5]. Life-style modifications involving diet and physical activity changes are difficult to maintain over time, and are the only preventive/therapeutic interventions used to correct MASLD [6]. Although SLD is considered a benign-mild condition, up to date scientific data supports a causative role of liver triglyceride accretion in the progression to the more aggressive condition of MASH [7]. Theoretically, drug therapy for SLD could prevent the evolution to more aggressive forms but, given its prevalence in the general population and its basic asymptomatic nature, the drugs employed must show not only efficacy, but also a high safety profile. Repurposing of drugs in clinical use, alone or in combination, could facilitate the finding of an effective and safe drug-treatment for SLD. Combining drugs with different molecular targets at dosages lower than those used in monotherapy is an effective way to increase safety, while maintaining or even improving efficacy.

We have developed a SLD dietary model, the high fat-high fructose (HFHFr) female Sprague-Dawley rat model [8]. Rats differ from mice in their inflammatory responses and cholesterol metabolism, possessing characteristics in between those of humans and mice [9], share a majority of their biochemical capabilities at the genome level with humans, and are more susceptible to high-fat diets and show more severe NAFLD characteristics, including fibrosis, than mice [10]. In the HFHFr model, steatosis originates from dietary factors, without the influence of extrahepatic metabolic disturbances. Furthermore, liver fatty acids are provided, as it occurs in human pathology, both from dietary origin and *de novo* lipid synthesis (DNL) [8]. By using this model, we have demonstrated the efficacy in reversing SLD of bempedoic acid, an ACLY (ATP-citrate lyase) inhibitor recently approved for its clinical use in dyslipidemia [11].

Pemafibrate (PemA) is a selective peroxisome proliferator activated receptor α modulator (SPPARMα), already approved in Japan as an hypotriglyceridemic agent [11,12]. It displays a high affinity for peroxisome proliferator activated receptor α (PPARα) and a better safety profile than older fibrates [13]. PemA treatment was associated with a lower incidence of MASLD in a recently published multinational, double-blind, randomized, controlled trial (RCT) [14]. Mirabegron (MBG) is a β_3-adrenoreceptor agonist clinically used for the treatment of overactive bladder [15] which has been shown to increase brown adipose tissue (BAT) thermogenesis and blood adiponectin concentration in women [16], thus increasing the catabolic destruction of fatty acids. Among other effects, the activation of liver adiponectin receptors increases the fatty acid beta-oxidation activity. In this way, mirabegron can stimulate the catabolism of fatty acids in the organism, reducing their liver accretion in the form of triglycerides, and avoiding the appearance of SLD. Here, we use the HFHFr rat model to obtain zoometric, biochemical, histological, transcriptomic, metabolomic and gut microbiome data to evaluate the efficacy of PemA and MBG, alone or in combination, in the resolution of SLD.

2. Methods

2.1. Animals and experimental design

Two-month-old female Sprague-Dawley rats weighing 147 ± 5 g (Envigo, Barcelona, Spain) were housed two per cage under conditions of constant humidity (40–60 %) and temperature (20–24 ºC), with a light/dark cycle of 12 h. Forty rats were randomly assigned into five groups (n = 8 each): (i) the control group (CT), fed a regular chow diet (2018 Teklad Global rodent diet, Envigo, Madison, USA), with free access to water; (ii) the HFHFr group fed a high-fat diet (Teklad Custom Diet 180456, Envigo, Madison, USA) [8] and free access to a 10 % w/v fructose solution; (iii) the PemA group (PemA at a dose of 1 mg/kg), (iv) the MBG group (MBG, at a dose of 10 mg/kg) and (v) the combination of PemA and MBG group (P+M, at a dose of 0.5 and 5 mg/kg, respectively). CT and HFHFr were fed for three months; PemA, MBG and P+M groups received the HFHFr diet for two months and, in the third month, were drug-treated by receiving the HFD solid food with the drugs incorporated on it, at a concentration as to provide the abovementioned daily dose. Solid food and liquid consumption were controlled three times a week, and body weight was recorded once a week. After adjusting for the actual HFD consumption during the last month of drug administration, the doses received by the rats in each experimental group, expressed as mg of drug/kg of rat weight/day, were as follows: PemA: 0.935, MBG: 10.2, and P+M: 0.53 and 5.34 for PemA and MBG, respectively. All animal experiments were carried out according to guidelines established by the Bioethics Committee of the University of Barcelona (Autonomous Government of Catalonia Act Biomedicines 2022, 10, 1517 3 of 19 5/21 July 1995), and were approved by the Animal Experimentation Ethics Committee of the University of Barcelona (approval no. 10106).

2.2. Sample collection

After 3-months, rats were fasted for 2 h, anesthetized as described [8] and samples of liver, BAT, subcutaneous and perigonadal WAT, and jejunum, as well as faecal samples were collected, immediately frozen in liquid nitrogen, and stored at −80 ºC. Blood was obtained from the tail vein before anesthesia, and serum through cardiac puncture and centrifugation at 10,000× g for 5 min at room temperature into micro-tubes (Sarstedt AG & Co., Nümbrecht, Germany). The Elisa kits for blood and serum determinations, RT-qPCR and Western blots

analysis, histological studies, and bile acid (BA) and microbiota measurements that have been used in this work are described in Supplementary Material. The *primers* used for qPCR and the antibodies for Western blot are listed in Supplementary Material.

2.3. Statistical analysis

Results are expressed as mean ± standard deviation (SD). Significance was established by one-way ANOVA and Šídák's multiple comparisons test (GraphPad Software version 10, San Diego, CA, USA). When the SD of the groups was different according to the Brown–Forsythe test, the data were log-transformed and ANOVA was rerun, or the corresponding non-parametric test (Kruskal–Wallis) was applied. For microbiota analysis, Grubb's test was applied to identify and eliminate outliers. Additionally, Levene's test was used to assess the homogeneity of variance and the Shapiro–Wilk test to verify data normality across groups. Post hoc analyses were performed using Fisher's Least Significant Difference (LSD) test. Correlation between fecal microbiota with SCFA, primary and secondary bile acids and liver triglycerides concentration was performed using the Pearson correlation, and the correlation between microbiota genera was visualized by a network plot using Cytoscape v 3.10.1. Statistical significance was considered when $P < 0.05$.

3. Results

3.1. PemA treatment abrogated SLD without significant alterations in energy consumption, body and WAT weight and glucose homeostasis

PemA, alone (1 mg/kd/day) or in combination (0.5 mg/kg/day) with MBG, reversed liver steatosis, histologically assessed by the Oil-Red O method, while MBG was ineffective (Fig. 1A). PemA almost halved the liver content of triglycerides (TG) (x0.46 and x0.42, for single or combined treatment, respectively), also significantly reducing the liver total, free and esterified cholesterol content (Fig. 1B). This effect was accompanied with a significant reduction in practically all the analyzed species of fatty acids present in liver TG (Fig. 1C). Similarly, PemA treatment reduced the liver concentrations of palmitic (C16:0), palmitoleic (C16:1 ω-7) and oleic acid (C18:1ω-9) diacylglycerols (DAG) (Fig. 1D). In doing so, PemA did not significantly altered calorie ingestion, either total or from solid or liquid origin, final body weight, nor the relative weights of subcutaneous and perigonadal WAT and BAT with respect to the values obtained in the HFHFr rats, although the liver weight percentage was significantly increased (x1.81 and x1.64, for single or combined treatment, respectively) (Fig. 1E). Neither PemA nor MBG significantly modified blood glucose, and serum concentrations of insulin, non-esterified fatty acids (NEFA) and glycerol, as well as the insulin sensitivity index (ISI) value (Fig. 1F), although, PemA administration induced an increase in the ISI value with respect to the HFHFr group ($p=0.07$, marginally significant), even suggesting an improvement of whole-body glucose metabolism in our model.

3.2. Drug-treatment did not change the expression of several serum, liver, WAT and jejunum parameters related to metabolic or organ toxicity

While PemA treatment (alone or in combination) practically resolved macro and micro vesicular steatosis, liver parenchyma architecture was not significantly modified by PemA, MBG treatment or their combination, as shown by the analysis of Hematoxylin-Eosin samples (Fig. 2A). Serum concentrations of alanine and aspartate aminotransferases (ALT and AST, respectively), as well as concentrations of major adipokines such as leptin and adiponectin, were also not significantly changed (Fig. 2B). Markers of the integrity of the intestinal barrier permeability, such as the mRNA levels of occludin and E-cadherin in jejunum, were not significantly modified neither by drug treatment nor by the HFHFr diet (Fig. 2C). The serum concentrations of total cholesterol and triglycerides were also not significantly modified by drug treatments, or even marginally increased ($p=0.08$) in the case of PemA (Fig. 2D), despite the reported hypotriglyceridemic effect of PPARα agonists and PemA in particular, either in preclinical as well as in clinical settings [13]. Accordingly, the increased levels of inositol requiring enzyme-1 (IRE1) at Ser^{724} induced by the HFHFr diet, whose activity has been related to VLDL assembly [17], were not significantly modified by PemA administration, while the reduction in liver phosphorylation of protein kinase RNA-like endoplasmic reticulum kinase (PERK) at Thr^{981} induced by the HFHFr diet was practically restored to control values (Fig. 2E). Given the lack of effect of MBG treatment on triglyceride levels, either in liver tissue or serum, we pursued our efforts in gaining further insight on PemA effects and the mechanisms involved.

3.3. PemA significantly increased liver fatty acid catabolism without affecting the expression of key enzymes involved in DNL

Liver samples from PemA-treated rats practically doubled their fatty acid β-oxidation activity (x1.86), with respect to HFHFr samples. This increased activity was both of mitochondrial and peroxisomal origin, as the mRNA levels of the two rate-limiting enzymes of fatty acid β-oxidation activity, mitochondrial liver-carnitine palmitoyl transferase I (L-CPT-I) and peroxisomal acyl-CoA oxidase (ACO), were markedly increased (Fig. 3A), and was accompanied with the characteristic liver hypertrophy induced by peroxisome-proliferators in rodents (see Fig. 1E). Of note, ACO expression was also significantly increased in jejunum samples of PemA-treated rats (see Fig. 2C), pointing to a PemA-mediated PPARα activation in the intestinal tissue. The liver expression of other well characterized PPARα-target genes, such as apolipoprotein CIII (*apocIII*), fibroblast growth factor 21 (*fgf21*), lipoprotein lipase (*lpl*), perilipin2 (*plin2*), VLDL receptor (*vldlr*), and uncoupling protein 3 (*ucp3*), at the level of mRNA/protein, were modified as expected (Fig. 3B), confirming that, in our model, the main effect of PemA administration was the activation of PPARα. Despite the huge increase in serum FGF21 concentrations induced by PemA, the lack of significant modifications of markers of FGF21 activity, besides the increased liver catabolism of fatty acids, such as reductions in WAT weight, serum concentrations of triglycerides and glucose, and increases in adiponectin concentrations and in the expression of uncoupling protein 1 (UCP1) in BAT and perigonadal WAT (Fig. 3C) [18], points to a state of FGF21 resistance. This resistance was not due, at least in liver, to changes in the expression of fibroblast growth factor receptor 1 (*fgfr1*) and β-klotho (β-*k*) genes, coding for the receptor proteins of FGF21 (Fig. 3D), and was probably due to the already high and sustained levels of serum FGF21 presented by HFHFr rats. In fact, the liver expression of early growth response 1 (*Egr1*), a gene commonly used as a readout for successful FGF21 intracellular signaling, was even significantly reduced in HFHFr and PemA samples.

As previously reported [8], livers of HFHFr rats showed increased expression of the lipogenic transcription factor carbohydrate response element binding protein β (ChREBPβ) and liver-pyruvate kinase (L-PK), as well as of key enzymes controlling *de novo lipid synthesis* (DNL), such as fatty acid synthase (FAS), ACLY, and acetyl-CoA carboxylase (ACC), (Fig. 3E). Although PemA administration significantly reduced the expression of ChREBPβ (x0.69) and L-PK (x0.41), this effect did not result in any significant reduction in the expression of lipogenic enzymes (Fig. 3E), pointing to a lack of effect of PemA treatment on DNL in our model.

A mention deserves the effect of PemA treatment on the expression of fructokinase or ketohexokinase (KHK). KHK is an essential enzyme for fructose metabolism under the control of ChREBPβ, among other transcription factors, whose expression is induced by fructose ingestion [19] and downregulated by PPARα activation [20]. Our present data shows that PemA administration was able to significantly reduce the expression of KHK both in liver (x0.48 for) (Fig. 3F) and, marginally, in jejunum samples (see Fig. 2C). At least in liver, PemA significantly

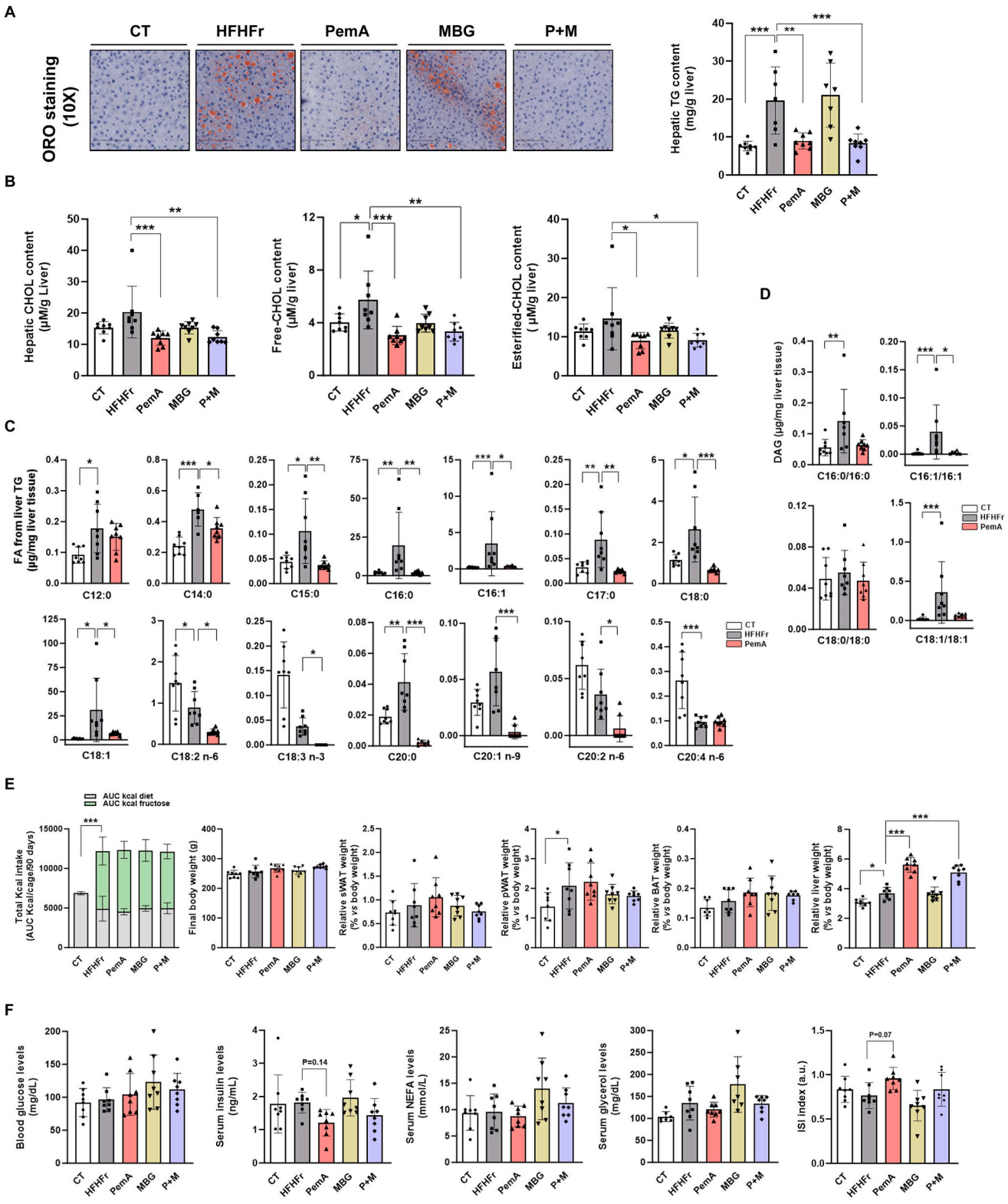

Fig. 1. PemA reduces liver steatosis and the amount of fatty acids from hepatic triglycerides without significant alterations in energy consumption, body and WAT weight and glucose homeostasis. (A) Representative images of Oil Red O-stained sections of liver samples from the five different experimental groups: CT, HFHFr, PemA, MBG, and P+M. On the right side of the images, liver content of triglycerides, expressed as mg/g tissue, are shown. (B) Content of total, free and esterified cholesterol, expressed as μM/g tissue, in liver samples from the five different experimental groups. (C) Amount of different species of fatty acids present in triglycerides, and (D) amount of different species of diacylglycerols, expressed as μg/mg tissue, in liver samples from CT, HFHFr and PemA experimental groups. (E) Total calorie intake, expressed as area under the curve (AUC) Kcal/cage/90 days, final body weight in g, and relative weight of subcutaneous (sWAT) and perigonadal (pWAT) WAT, BAT and liver tissue for the five different experimental groups. (F) Blood/serum concentrations of glucose, insulin, NEFA, glycerol and ISI (insulin sensitivity index) values for the five different experimental groups. Quantitative results are presented as bar plots with individual values, showing the mean ± SD of 7–8 animals/group. * P<0.05, ** P<0.01, ***P<0.001.

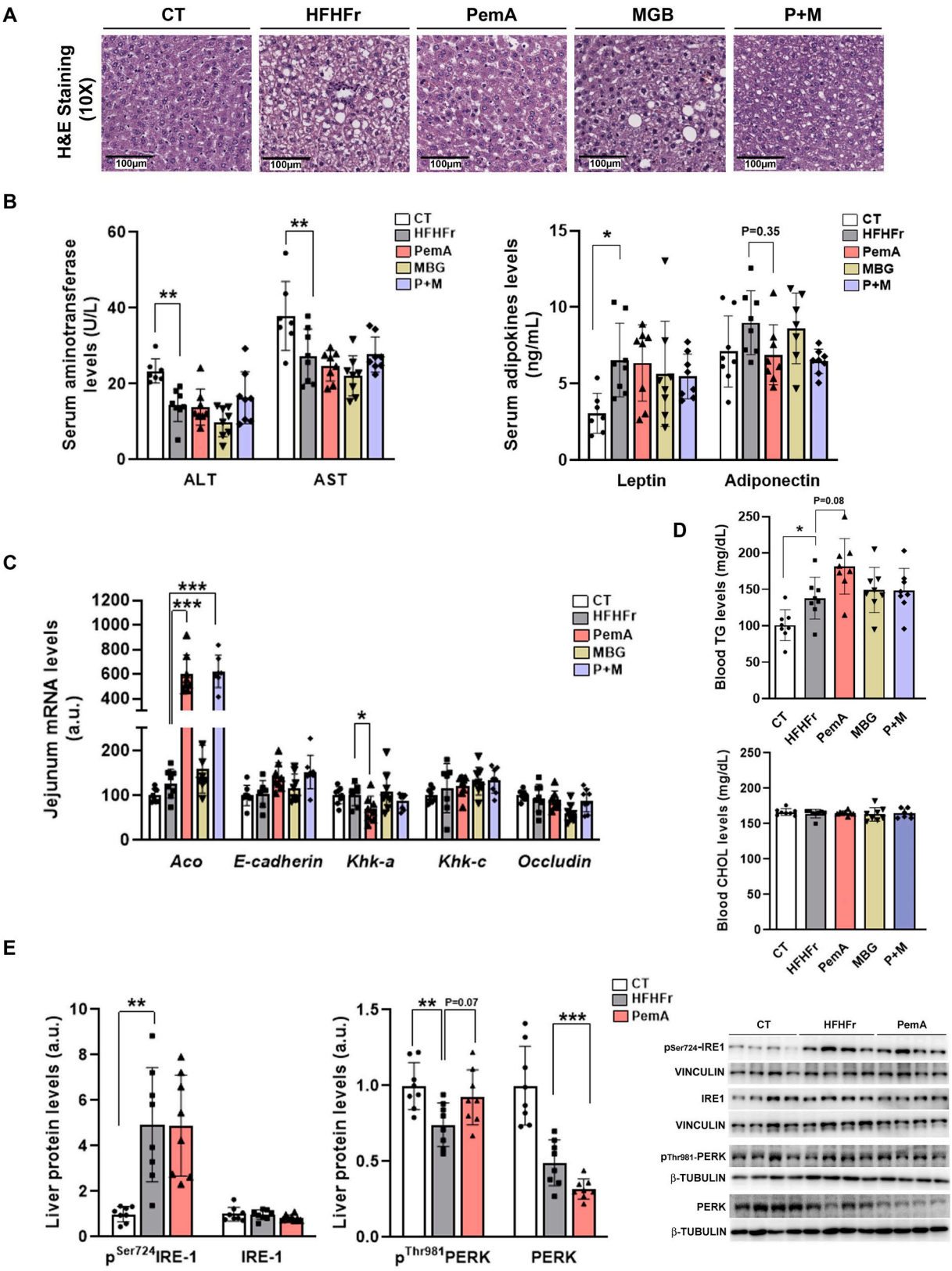

Fig. 2. Drug-treatment did not change the expression of several serum, liver, WAT and jejunum parameters related to metabolic or organ toxicity. (A) Representative images of Hematoxylin-Eosin stained sections of liver samples from the five different experimental groups: CT, HFHFr, PemA, MBG, and P+M. (B) Serum concentrations of ALT, AST, leptin and adiponectin in the five different experimental groups. (C) Relative mRNA levels of *aco*, *e-cadherin*, *khk-a*, *khk-c* and *occluding* genes in jejunum samples from the five different experimental groups. (D) Blood triglycerides and cholesterol concentrations in the five different experimental groups. (E) Relative content of phosphor Ser^{724}-IRE1, IRE1, phosphor Thr^{981}-PERK and PERK proteins in liver samples from the CT, HFHFr and PemA experimental groups. On the right side of the figure, representative western blot bands corresponding to the three different study groups are shown. Quantitative results are presented as bar plots with individual values, showing the mean ± SD of 7–8 animals/group. * P<0.05, ** P<0.01, ***P<0.001.

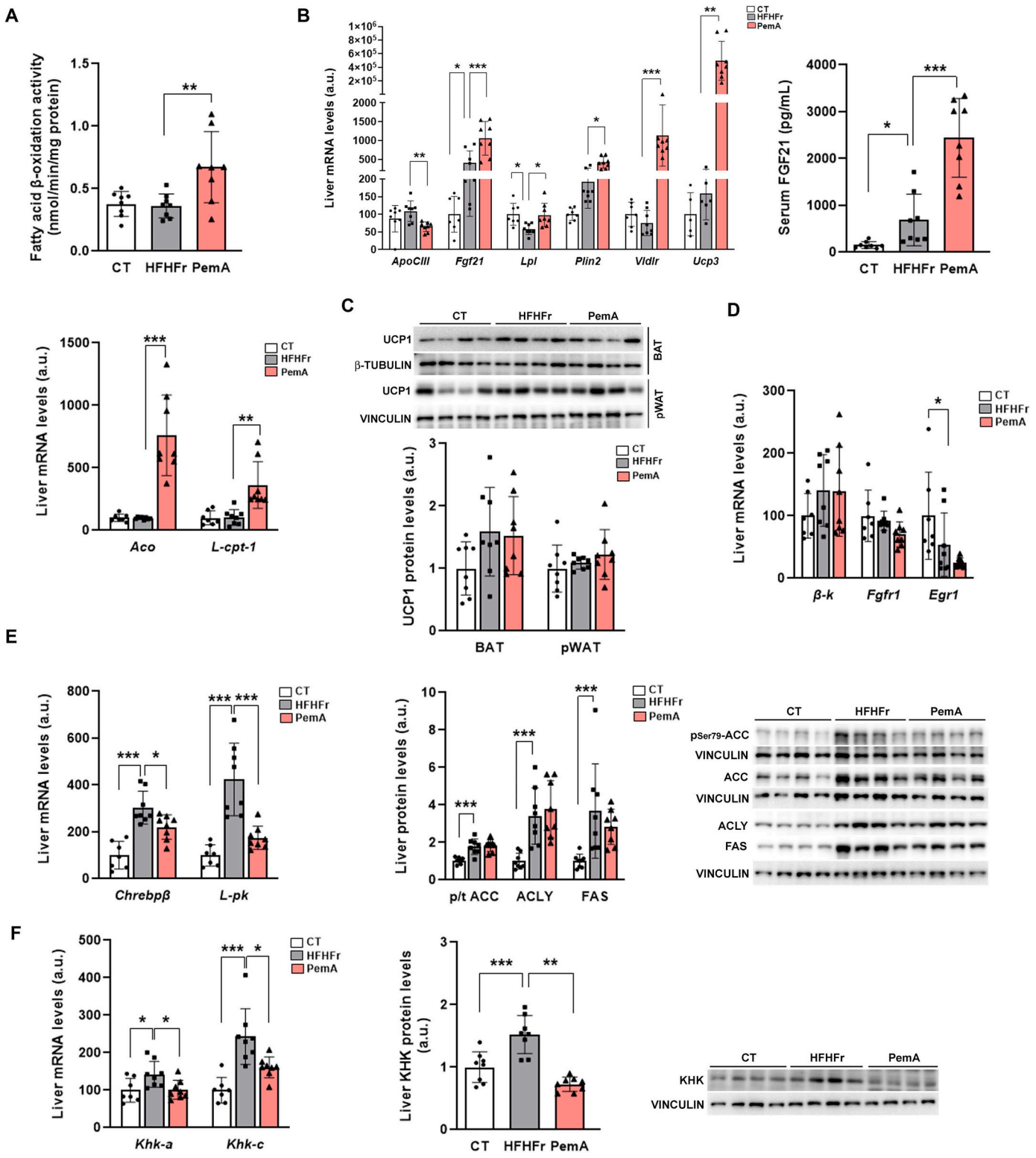

Fig. 3. PemA significantly increased liver fatty acid catabolism without affecting the expression of key enzymes involved in DNL. (A) Fatty acid β-oxidation activity and relative mRNA levels of *aco* and *l-cpt-1* genes in liver samples from the CT, HFHFr and PemA experimental groups. (B) Relative liver mRNA levels of *apo CIII*, *fgf21*, *lpl*, *plin2*, *vldlr* and *ucp3* genes, as well as serum concentrations of FGF21, in samples from the CT, HFHFr and BemA experimental groups. (C) Relative content of UCP1 protein in BAT and perigonadal WAT samples from the CT, HFHFr and PemA experimental groups. On the upper side of the figure, representative western blot bands corresponding to the three different study groups are shown. (D) Relative liver mRNA levels of *β-k*, *fgfr1* and *egr1* genes in samples from the CT, HFHFr and PemA experimental groups. (E) Relative mRNA levels of *chrebpβ*, and *l-pk* genes, as well as the ratio of phosphor Ser^{79}/total ACC, ACLY and FAS proteins in liver samples from the CT, HFHFr and PemA experimental groups. On the right side of the figure, representative western blot bands corresponding to the three different study groups are shown. (F) Relative mRNA levels of *khk-a* and *khk-c* genes, as well as fructokinase protein in liver samples from the CT, HFHFr and PemA experimental groups. On the right side of the figure, representative western blot bands corresponding to the three different study groups are shown. Quantitative results are presented as bar plots with individual values, showing the mean ± SD of 7–8 animals/group. * P<0.05, ** P<0.01, ***P<0.001.

reduced the expression of both isoforms of KHK, KHK-A (x0.71), ubiquitously expressed, and KHK-C (x0.66), expressed mainly in liver, small intestine and kidney [21]. The reduction of liver KHK-C activity seems to affect fructose metabolism without modifying its intake [21], thus reducing the SLD-promoting effect of fructose-consumption [19].

3.4. PemA treatment increased serum concentrations of angiopoietin-like protein 3 (ANGPTL3) in HFHFr rats

PemA, despite increasing liver fatty acid catabolism and *lpl* expression, and decreasing liver *apocIII* expression (see Figs. 3A and 3B), was unable to significantly reduce serum triglyceride concentrations of HFHFr rats (see Fig. 2E). To gain some insight on this subject, we determined the expression of several genes involved in VLDL production by the liver and its intravascular catabolism: apolipoprotein B (*apoB*), apolipoprotein AV (*apoAV*), apolipoprotein E (*apoE*), acyl-CoA: cholesterol O-acyl-transferase 2 (*acat2*), *angptl3*, CTP:phosphocholine cytidylyltransferase (*cct*), hepatic lipase (*hl*), microsomal triglyceride transfer protein (*mttp*), LDL receptor (*ldlr*), patatin-like phospholipase domain-containing protein 3 (*pnpla3*), and scavenger receptor BI (*srBI*) either at the liver mRNA (Fig. 4A) and/or protein level in serum (Fig. 4B) and in liver tissue (Fig. 4C). Although the mRNA expression of *angptl3* was not significantly modified, the protein concentration of ANGPTL3 in serum was significantly increased by PemA treatment, suggesting that, in the HFHFr rat model, PemA sustained high serum VLDL concentrations by the inhibition of circulating VLDL catabolism.

3.5. In HFHFr rats, PemA administration restored the expression of cytochrome P450 7α-hydroxylase (cyp7a1) and changed the fecal concentration of several primary and secondary bile acids (BA)

We have previously described that the HFHFr diet reduces the expression of the cyp7a1, the rate-limiting enzyme in liver BA biosynthesis [22]. Our present data shows that the HFHFr diet reduced also the liver expression of the ATP binding cassette G5/G8 (*abcg5/8*) genes, involved in the efflux of cholesterol, without significant changes in other genes involved in liver bile composition, such as bile-salt export pump (*bsep*), multidrug resistance protein 2/3 (*mdr2/3*), sodium-dependent taurocholate co-transporting peptide (*ntcp*), and organic anion transported peptide (*oatp*) (Fig. 5A). The HFHFr diet substantially increased the amount of fecal total BA (x2.38 *vs* CT) (Fig. 5B), mainly due to an increase in the concentration of secondary BA (x2.27 *vs* CT), specifically that of hyodeoxycholic acid (HDCA, x2.91 *vs* CT) (Fig. 5C), reducing the global hydrophobicity of total BA (Fig. 5B). PemA administration to female HFHFr rats recovered the decreased expression of the *abcg5/8* genes and cyp7a1 (Figs. 5A and D), and marginally decreased ($p=0.08$) the expression of *Oatp*, contributing to the reduction in liver cholesterol contents. These changes did not alter the total concentration of fecal BA, but they did alter the fecal concentration of several individual BA, either primary, such as α-muricholic acid (αMCA, x3.28), or secondary, such as deoxycholic (DCA, x1.99), lithocholic (LCA, x0.30), and ursodeoxycholic acids (UDCA, x0.06) (Figs. 5B and C). These quantitative changes were paralleled by similar changes in the relative proportion of individual BA (Fig. 5E), but did not result in a significant change in the hydrophobicity index (HI) of total fecal BA with regard to HFHFr rats (Fig. 5B). Changes in the expression of liver genes coding for enzymes involved in the synthesis of primary BA are shown in Fig. 5F. PemA treatment increased the expression of the cytochrome P450 2c70 gene (*cyp2c70p*; x3.26), in accordance with the observed increase in the concentration of fecal αMCA [23], as well as that of cytochrome P450 8b1 (*cyp8b1*; x2.56). Although the concentration of the direct product of cyp8b1 activity, cholic acid (CA) was not significantly modified in feces of PemA-treated rats, the concentration of DCA, the secondary BA produced by the microbiota-mediated metabolism of CA [23], was markedly increased. Thus, these changes could imply the production of a PemA-related modification in the intestinal microflora.

3.6. PemA treatment imprints a particular signature in rat fecal microbiota that significantly differentiates from those obtained from control and HFHFr samples

The HFHFr diet significantly reduced fecal-microbiota biodiversity and evenness distribution *vs* CT values; these qualitative changes were not reverted by PemA treatment (Fig. 6A) and resulted in a significant reduction in the fecal concentration of short-chain fatty acids (SCFA), except for formic acid (Fig. 6B). The Principal component analysis (PCA) shows that PemA treatment significantly changed rat fecal microbiota with respect both to CT and HFHFr values (Fig. 6C), altering the relative abundance of the principal microbiota phylum (Fig. 6D). PemA treatment partly restituted the HFHFr-increased Firmicutes to Bacteroidota ratio to CT values, basically by reducing the relative abundance of Firmicutes in fecal samples without significantly affecting Bacteroidota (Fig. 6E). At the family level, the main significant changes induced by the consumption of the HFHFr diet were increases in the relative abundance of Akkermansiaceae (Verrucomicrobiota), Erysipelotrichaceae, Lactobacillaceae (Firmicutes D), and Clostridiaceae (Firmicutes A), accompanied by reductions in the relative abundance of Lachnospiraceae (Firmicutes A), Muribaculaceae, and Bacteroidaceae (Bacteroidota). PemA treatment with respect to HFHFr samples significantly increased the relative abundance of Akkermansiaceae (Verrucomicrobiota), Erysipelotrichaceae (Firmicutes D) and Bacteroidaceae (Bacteroidota), while reducing those of Lactobacillaceae (Firmicutes D), Clostridiaceae, and Lachnospiraceae (Firmicutes A) (Fig. 7A). A detailed cladogram up to the genus level of the observed changes is shown in Fig. 7B.

Fig. 8 illustrates the correlations between metabolites present in feces and hepatic triglycerides with bacterial genera, as well as a network analysis that elucidates these relationships, for PemA *vs* HFHFr values. The results indicate that the relative abundance of several genera is correlated with SCFA concentration, thus highlighting their role in the production of beneficial SCFA that contribute to intestinal health. Moreover, several bacterial genera also show significant correlations with primary and secondary bile acids, indicating their involvement in bile acid metabolism. A similar analysis for HFHFr *vs* CT values is shown in Supplementary Material.

4. Discussion

In this preclinical study, we show that PemA, but not MBG, reverts liver steatosis in female Sprague-Dawley rats fed a diet rich in vegetable fat and liquid fructose. The anti-steatotic effect of PemA occurs mainly through a direct effect on liver PPARα-driven fatty acid catabolism, without changes in total energy consumption, blood lipids, body weight, and weight of subcutaneous and perigonadal WAT and BAT. Moreover, PemA has a neutral effect on whole-body glucose metabolism, but significantly modifies fecal BA composition and microbiota.

In a recent consensus statement on new nomenclature for liver disease, SLD has been proposed as the umbrella characteristic for several liver pathologies previously categorized as NAFLD and AFLD (alcoholic fatty liver disease), among others [3]. A drug that increases liver fatty acid catabolism is a good candidate for controlling or even reversing SLD development. Hypolipidemic fibrates (e.g., fenofibrate and gemfibrozil), are ligands and activators of PPARα, a transcription factor that controls the expression of the two genes encoding the rate-limiting enzymes of mitochondrial (L-CPTI) and peroxisomal (ACO) fatty acid β-oxidation. Thus, these drugs are well positioned to manage SLD. Unfortunately, differences in the expression and activity of human and rodent PPARα, as well as the fact that fibrates are weak PPARα activators have meant that fibrates show low efficacy in the treatment of human MASLD [24]. PemA, a potent and selective SPPARMα that is already in clinical use, could overcome these shortcomings.

The PROMINENT study, which investigated the efficacy of PemA in reducing cardiovascular outcomes in more than 10,000 patients with

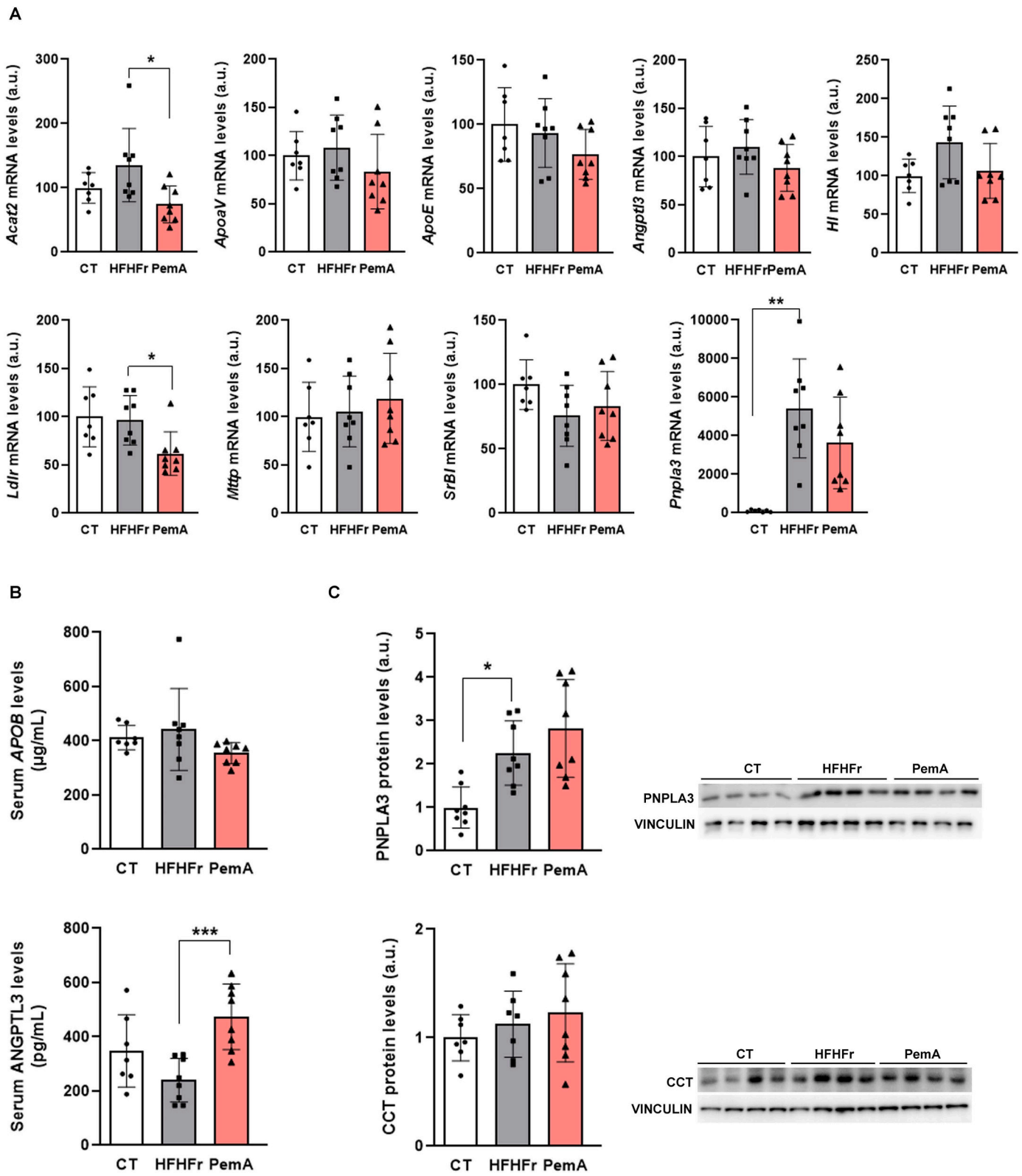

Fig. 4. PemA treatment increased serum concentrations of ANGPTL3 in HFHFr rats. (A) Relative liver mRNA levels of *acat2*, *apo AV*, *apo E*, *angptl3*, *hl*, *ldlr*, *mttp*, *srBI* and *pnpla3* genes in liver samples from the CT, HFHFr and PemA experimental groups. (B) Levels of apo B and ANGPTL3 proteins in serum samples from the CT, HFHFr and PemA experimental groups. (C) Relative content of PNPLA3 and CCT proteins in liver samples from the CT, HFHFr and PemA experimental groups. On the right side of the figure, representative western blot bands corresponding to the three different study groups are shown. Quantitative results are presented as bar plots with individual values, showing the mean ± SD of 7–8 animals/group. * $P<0.05$, ** $P<0.01$, ***$P<0.001$.

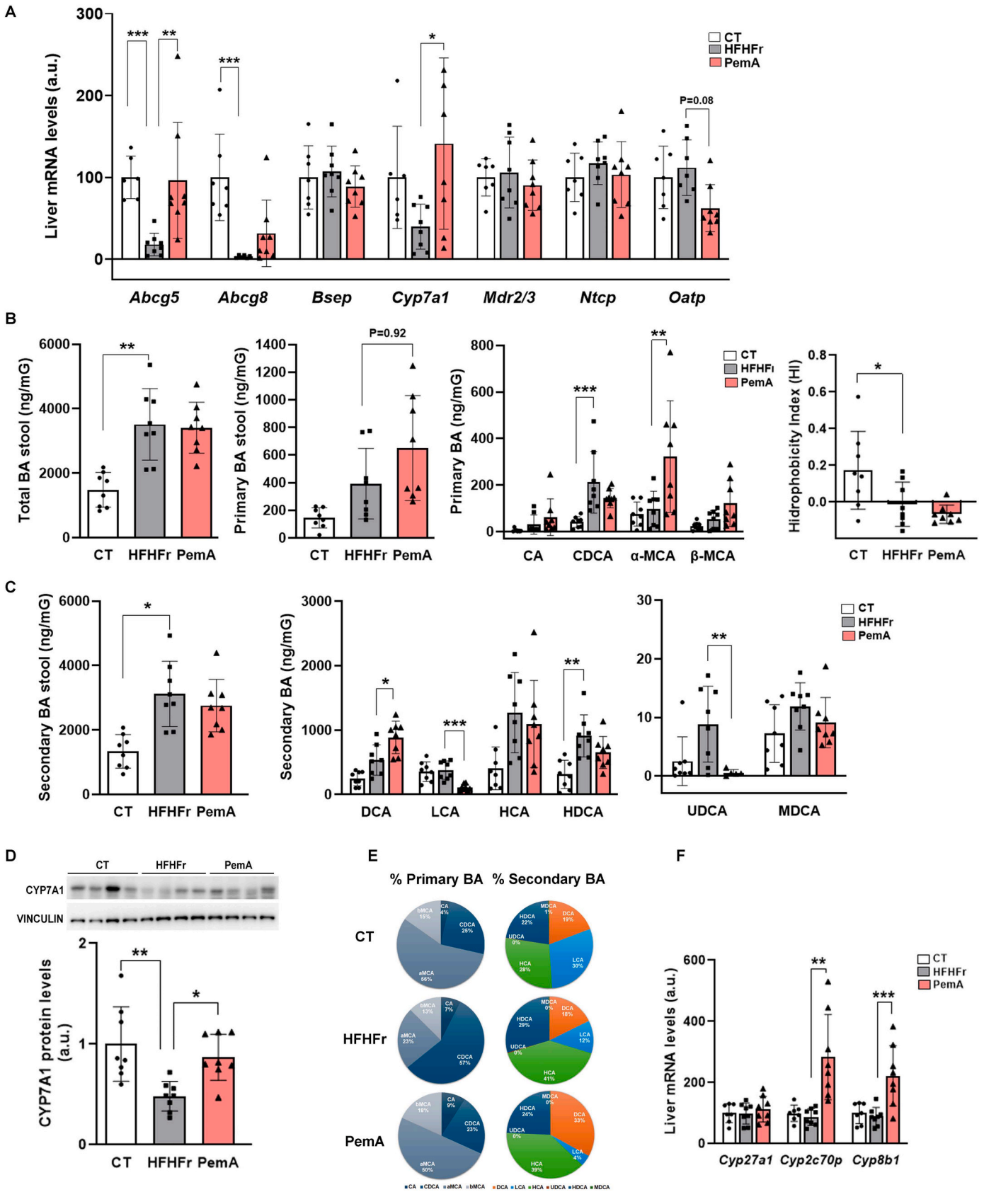

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Fig. 5. PemA administration restored the expression of cytochrome P450 7α-hydroxylase (cyp7a1) and changed the fecal concentration of several primary and secondary bile acids (BA) in HFHFr rats. (A) Relative liver mRNA levels of *abcg 5*, *abcg 8*, *bsep*, *cyp7a1*, *mdr 2/3*, *ntcp* and *oatp* genes in liver samples from the CT, HFHFr and PemA experimental groups. (B) Concentrations of total BA, total primary-BA and individual primary-BA (CA, CDCA, α-MCA and β-MCA), as well as the HI of total BA, in stool samples from the CT, HFHFr and PemA experimental groups. (C) Concentrations of total secondary-BA and individual secondary-BA (DCA, LCA, HCA, HDCA, UDCA and MDCA) in stool samples from the CT, HFHFr and PemA experimental groups. (D) Relative content of cyp7a1 protein in liver samples from the CT, HFHFr and PemA experimental groups. On the right side of the figure, representative western blot bands corresponding to the three different study groups are shown. (E) Cyclograms representing the relative proportion of individual BA in stool samples from the CT, HFHFr and PemA experimental groups. (F) Relative liver mRNA levels of cyp27a1, cyp2c70p and cyp8b1 genes in liver samples from the CT, HFHFr and PemA experimental groups. Quantitative results are presented as bar plots with individual values, showing the mean ± SD of 7–8 animals/group. * $P<0.05$, ** $P<0.01$, ***$P<0.001$.

type 2 diabetes mellitus, reported significant reductions in any hepatic adverse event and, specifically, in the MASLD incidence in the PemA-arm of the study [14]. However, a recently published pilot RCT of PemA treatment conducted on 118 MASLD patients showed a significant reduction in a pre-specified secondary endpoint (liver stiffness), although the primary endpoint, related to liver fat content [25], was not met. Preclinical studies using experimental mouse models of MASLD, give similarly mixed results that show improvements in the markers of MASH and associated metabolic alterations (obesity, insulin resistance, and inflammatory markers, etc.), but variable outcomes for liver steatosis [26-28]. Since rats, especially Sprague-Dawley rats, appear to be more sensitive than mice to diet-induced MASLD [29], our HFHFr rat model of simple non-inflammatory SLD [8] appears to be well suited for the assessment of the efficacy of PemA in treating SLD. Indeed, pemafibrate at the doses used in the present study, that are lower than those used in humans (0.5/1.0 *vs* 6.7 mg/kg/day for an average human weight of 60 kg [14]), prevented the histological manifestation of SLD and reduced liver triglyceride and cholesterol contents without significantly altering the markers of DNL, probably as a direct consequence of a PPARα-driven increase in liver fatty acid β-oxidation and cholesterol excretion into bile. Furthermore, the reduced liver expression of fructokinase, another PPARα-mediated effect, could reinforce the anti-steatotic effect of PemA, as fructose ingestion is a recognized risk factor for MASLD [19].

The anti-steatotic effect of PemA was not accompanied by any signs of liver or whole-body toxicity, apart from the characteristic liver hypertrophy induced in rodents by PPARα agonists. The main limitation of our study was the lack of a hypolipidemic effect of pemafibrate, probably related to the markedly increased expression in our model of PNPLA3, a lipase that mobilizes monounsaturated-rich TG stored in lipid droplets for VLDL formation and secretion [8] and whose liver expression is not modified by PemA. Furthermore, PemA hindered the intravascular catabolism of triglyceride-rich lipoproteins by increasing ANGPTL3 serum levels without changing its liver expression, precluding a direct PPARα-related effect. As PemA shows a strong hypotriglyceridemic effect in other rat models [30,31] and in its clinical use [13,14], this particular behavior of pemafibrate should not have any clinical/-translational implication.

PemA, as a SPPARMα [32], shares a core PPARα-mediated response with classical fibrates, modulating the expression of genes involved in the metabolism of TG-rich lipoproteins (*apoCIII* and *lpl*) and fatty acid catabolism (*cptI* and *aco*). Due to its high affinity and potency as a PPARα agonist, PemA can induce the expression of the *aco* gene not only in rodents, but also in human hepatocytes [33], an effect not observed with the classical fibrates [34]. This further contributes to the beneficial clinical effects of PemA on liver metabolism. Moreno-Fernández et al. recently reported that a deficit in the ACO-driven peroxisomal oxidation of very long-chain saturated fatty acids, in combination with an obesogenic diet, exacerbated hepatocellular damage and inflammation, upregulating the expression of several genes associated with HCC development [35].

The particular SPPARMα gene expression signature of PemA involves several genes associated with bile composition and BA synthesis. Our previous data [36], and the comprehensive review by Ghonem et al. [37] indicated that classical fibrates increase the liver expression of CYP8B1 and MRD 2/3, decrease the expression of CYP7A1, CYP27A1, BSEP, NTCP, and OATP, and have a neutral effect on ABCG5. These changes have been related to a reduction in total fecal BA and an increased risk of lithogenicity, especially with clofibrate [38]. In contrast, the present study indicated that PemA did not significantly affect the expression of MRD 2, CYP27A1, BSEP, NTCP, and OATP, but increased the expression of CYPA71, ABCG5, CYP8B1, and CYP2C70P. These modifications in gene expression resulted in no significant changes in the total amount of fecal BA, both primary and secondary, although the amount of several species of primary and secondary BA was significantly altered, in accordance with the changes in the expression of the genes encoding the enzymes involved in their biosynthesis. Safety data from the PROMINENT study did not indicate an increased risk of lithogenicity with PemA [14]. Tamai and Okomura reported very recently the efficacy and safety of PemA in patients with cholestatic liver disease [39], suggesting that the changes induced by PemA in bile-related gene expression and fecal BA composition are not harmful and could even contribute to the beneficial effects of PemA on liver metabolism.

The HFHFr diet induced changes in the rat fecal microbiota similar to those reported previously in humans with MASLD, such as an increased Firmicutes/Bacteroidota ratio, an increased Lactobacillaceae prevalence, and a reduced Bacteroidota prevalence [40-44]. These changes were associated with a general decrease in the concentration of SCFA, which was directly related to reductions in Lachnospiraceae, Muribaculaceae, and Bacteroidaceae, as well as increases in Erysipelotrichaceae and Clostridaceae (Fig. 8 C). Since the SCFA absorbed in the gut are an appreciable energy source [45], their reduction could be a contributing factor to the maintenance of body weight in the HFHFr rats, despite their increased calorie intake. PemA administration induced specific changes in the rat fecal microbiota that were clearly different from those induced by the HFHFr diet. The changes in Akkermansiaceae, Erysipelotrichaceae, and Lachnospiraceae induced by the HFHFr diet were further magnified by PemA. Meanwhile, PemA partially reverted the reduction in Bacteroidaceae and the increases in Lactobacillaceae and Clostridiaceae produced by the HFHFr diet. Moreover, the changes induced by PemA in Akkermansiaceae, Erysipelotrichaceae, Bacteroidaceae and Clostridiaceae were significantly associated with the PemA-related changes in the primary and secondary BA (Fig. 8 C). These changes, although differentiated from those induced by the HFHFr diet, were very different from the pattern of fecal microbiota in the control rats (Fig. 6C), precluding any inference about their involvement in the PemA-induced remission of SLD. In any case, our data indicate that PemA directly affects the composition of the fecal microbiota and also modifies the fecal BA composition, which is probably related to the fact that biliary excretion is the main way of PemA exit from the organism [13].

In summary, PemA abrogates SLD development with an optimal safety profile. Liver fat, independently of the BMI, is directly related to the risk of developing of cardiovascular disease (CVD) [46], and MASLD is associated with a higher risk of non-fatal CVD [47]. Given that PemA is already in clinical use, our results strongly support further clinical research for the repurposing of PemA in the treatment of SLD/MASLD and its associated CV burden.

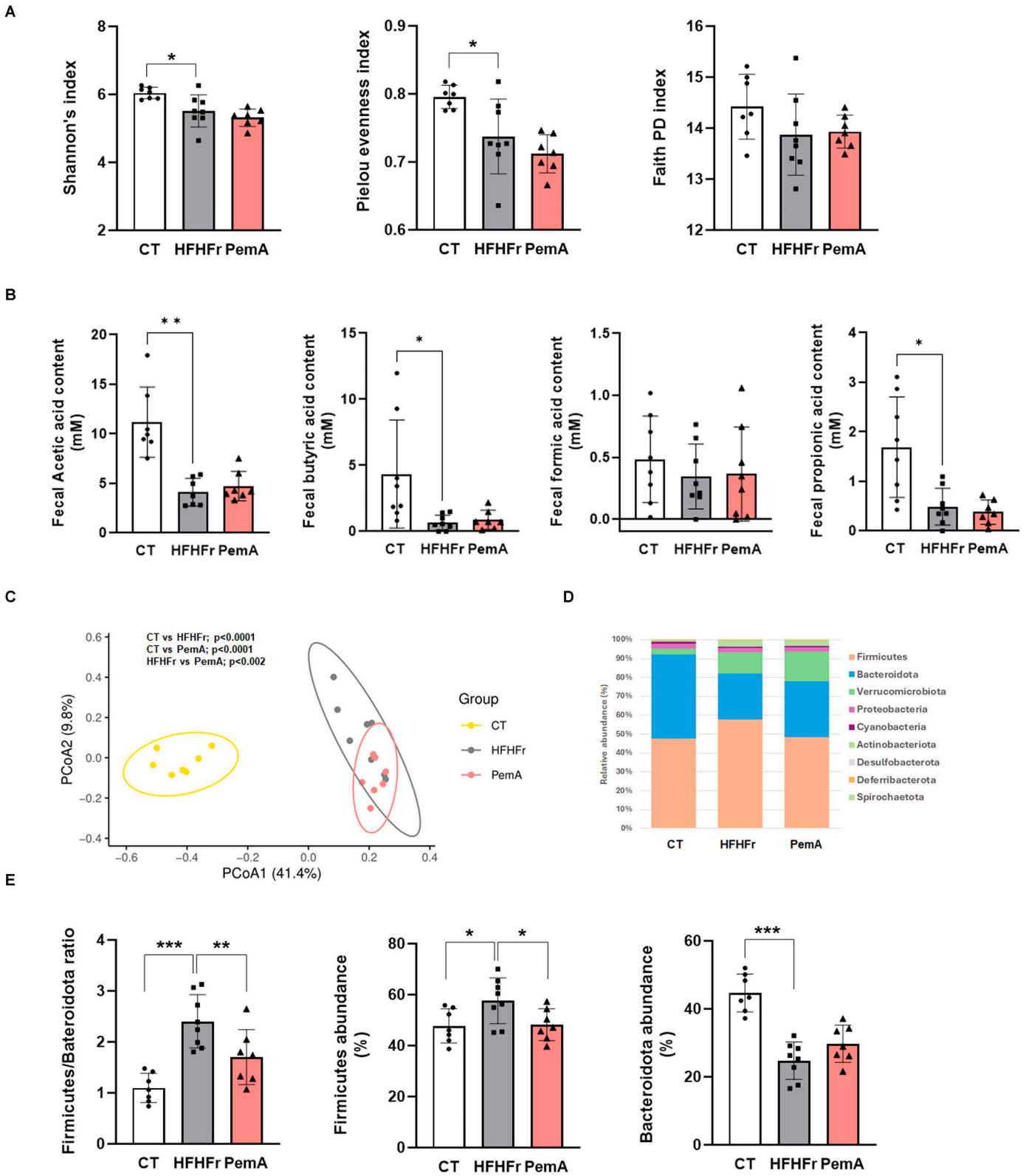

Fig. 6. PemA treatment imprints a particular signature in rat fecal microbiota that significantly differentiates from those obtained from control and HFHFr samples. (A) Alpha diversity indices: Shannon's index, Pielou Evenness index and Faith Phylogenetic Diversity (PD) index. (B) Concentrations of SCFA. (C) Beta diversity analysis: Principal component analysis. The significant p-values displayed in the plot were derived from PERMANOVA (Permutational Multivariate Analysis of Variance) and PERMDISP (Permutational Analysis of Multivariate Dispersions) tests. The PERMANOVA analysis revealed significant differences between the groups, particularly between HFHFr and PemA, with a p-value of 0.0027 and an R^2 of 0.153. The PERMDISP test demonstrated a significant disparity in dispersion between HFHFr and PemA, with a p-value of 0.009. (D) Phylum-level relative abundance. (E) The ratio of Firmicutes to Bacteroidota and the relative abundance of Firmicutes and Bacteroidota. Data are presented for fecal samples from CT, HFHFr, and PemA groups. In panels (A), (B), and (E), results are shown as bar plots with individual values, showing the mean ± SD of 7–8 animals/group. * P<0.05, ** P<0.01, ***P<0.001.

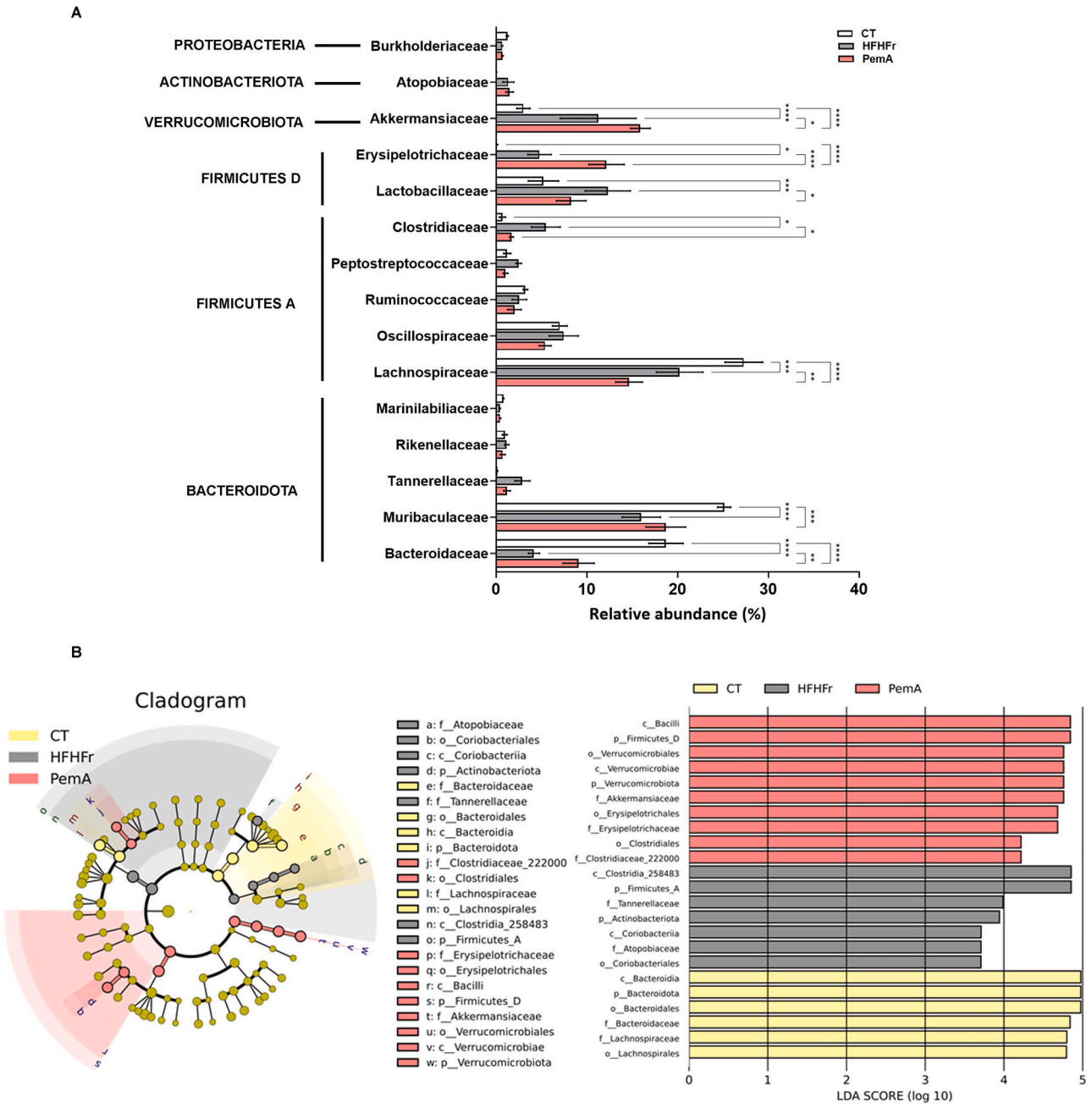

Fig. 7. Relative composition of the fecal microbiota of female Sprague-Dawley rats induced by the HFHFr diet and PemA treatment. (A) Relative abundance of bacterial families in fecal samples from CT, HFHFr and PemA experimental groups. Data are presented as bar plots, showing the mean ± SD of 7–8 animals/group. Statistical significance is indicated as follows: * P<0.05, ** P<0.01, ***P<0.001. (B) The cladogram (left panel) and the Linear Discriminant Analysis (LDA) Effect Size (LEfSe) score plot (right panel) demonstrate the differences in the relative abundance of bacterial taxa among the three groups. The phylogenetic tree illustrates the changes in the fecal microbiota down to the genus level induced by the HFHFr diet and PemA treatment. Colored nodes represent significantly enriched taxa in each treatment group: yellow for CT, gray for HFHFr, and red for PemA. The bar chart on the right displays the LDA scores (log 10) for each taxon, indicating the effect size of their differential abundance among the groups. The taxa are listed on the y-axis, while the x-axis shows the LDA score. Higher LDA scores indicate a more significant difference in abundance.

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Author contributions

Conception or design of the work: Marta Alegret, Juan C. Laguna. Drafting the work: Juan C. Laguna. Acquisition, analysis, or interpretation of data: Roger Bentanachs, Núria Roglans, Lluïsa Miró, Patricia Ramírez-Carrasco, Concepció Amat, Anna Pérez. Substantively revised the work: Rosa M. Sánchez, Marta Alegret, Núria Roglans, Anna Pérez. All authors have read and agreed to the published version of the manuscript

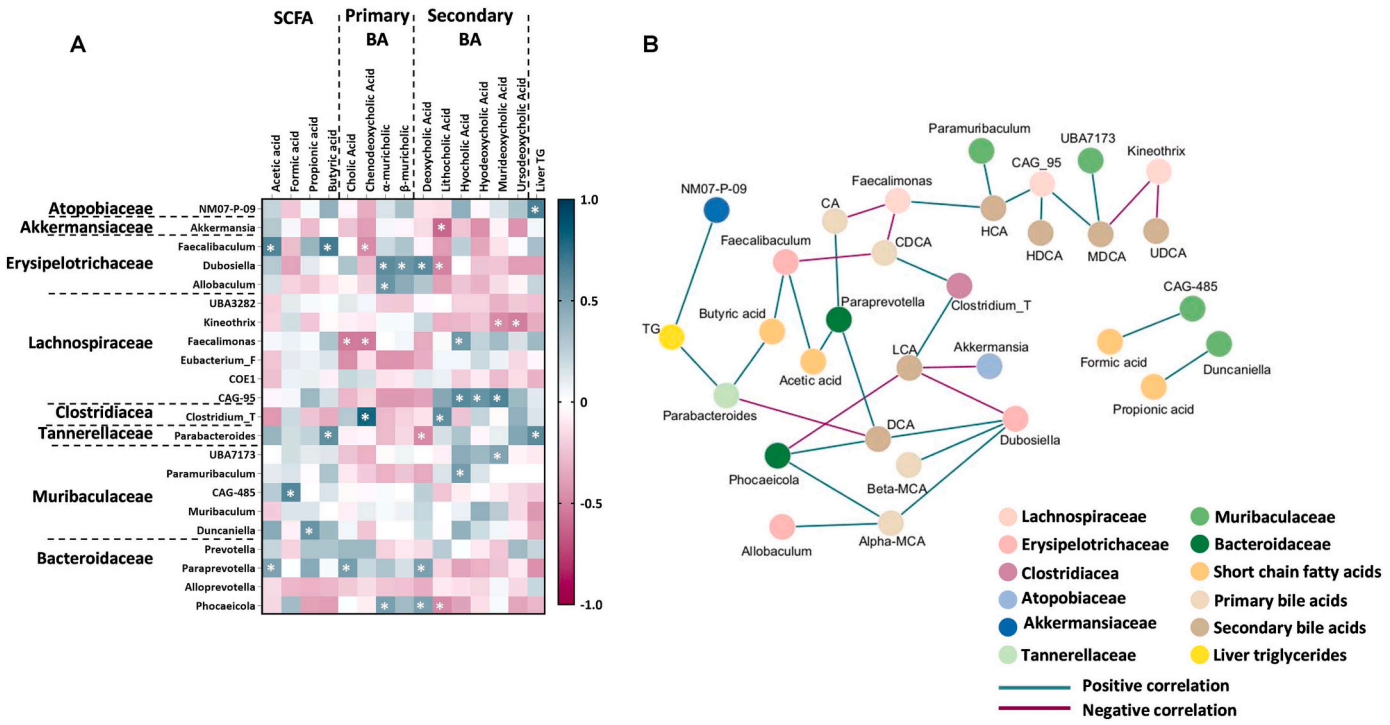

Fig. 8. (A) Heatmap displaying Spearman's rank-based correlations between fecal metabolites (SCFA and primary and secondary BA), liver triglycerides and bacterial genus for PemA vs HFHFr values. The color scale represents the strength and direction of the correlations, with blue indicating positive correlations, plum indicating negative correlations, and white indicating no correlation. (B) Network diagram illustrating the interactions between SCFA, primary and secondary BA with microbiota genera for PemA vs HFHFr groups. Nodes represent microbiota genera and metabolites, while edges represent significant correlations. Node colors correspond to different microbiota genus or metabolites, and edge colors indicate the type of correlation (positive or negative). Abbreviations: Alpha-MCA, alpha muricholic acid, BA, bile acids; Beta-MCA, beta muricholic acid; CA, cholic acid; CDCA, chenodeoxycholi acid; DCA, deoxycholic acid; HCA, hyocholic acid; HDCA, Hyodeoxycholic acid; LCA, lithocholic acid; MDCA, murideoxycholic acid; SCFA, short chain fatty acids; UDCA, ursodeoxycholic acid.

CRediT authorship contribution statement

Rosa M. Sánchez: Writing – review & editing, Supervision. **Lluïsa Miró:** Methodology, Investigation. **Concepció Amat:** Writing – review & editing, Resources, Funding acquisition. **Patricia Ramírez-Carrasco:** Investigation. **Juan C. Laguna:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Roger Bentanachs:** Methodology, Investigation. **Marta Alegret:** Writing – review & editing, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Núria Roglans:** Visualization, Software, Methodology, Investigation, Data curation. **Anna Pérez:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117067.

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