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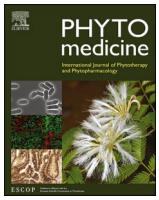

Original Article

Flavonoids from *Rhododendron nivale Hook. f* ameliorate alcohol-associated liver disease via activating the PPARα signaling pathway

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ABSTRACT

Background: Flavonoids are increasingly recognized for their potent antioxidant properties and potential therapeutic roles in the management of alcohol-associated liver disease (ALD). Extracts derived from *Rhododendron nivale Hook. f.* (FRN) have been shown to influence glutathione metabolism in aging animal models, exhibiting notable antioxidant effects. However, the specific impact of FRN on ALD remains insufficiently explored.

Hypothesis/Purpose: This study seeks to elucidate the efficacy of FRN in alleviating the pathology associated with ALD, delving into the underlying molecular mechanisms that facilitate its protective effects.

Study Design: We employed network pharmacology to predict the functional roles and pathway enrichments associated with FRN targets. Both a murine model of ALD and in vitro cellular models were utilized to clarify the mechanistic basis by which FRN mitigates ALD.

Methods: FRN was extracted and characterized according to well-established methodologies outlined in our previous studies. Potential functions and pathways implicated by FRN were predicted through network pharmacology analyses. A combination of liver transcriptomics, targeted lipidomics, molecular biology techniques, and antagonists of relevant targets were employed to investigate the mechanisms through which FRN exerts its protective effects in ALD.

Results: Network pharmacology identified multiple target genes modulated by FRN, particularly those within critical ALD-related signaling pathways, such as PPARα signaling and fatty acids (FAs) degradation. Notably, treatment with FRN in the ALD murine model led to a significant attenuation of hepatic lipid accumulation and a restoration of serum AST and ALT to baseline ranges. Subsequent validation through liver transcriptomics and molecular biology techniques revealed an upregulation of PPARα expression concomitant with a downregulation of ACSL1 in FRN-treated ALD mice. Targeted lipidomic and bioinformatic analyses demonstrated that FRN substantially reduced the accumulation of long-chain fatty acids in hepatocytes. Importantly, the reversal of FRN's protective effects on lipid accumulation through the PPARα antagonist GW6471 provides compelling evidence for the critical role of PPARα signaling modulation in mediating the beneficial impact of FRN on ALD.

Conclusion: Our research highlights FRN's capacity to alleviate ALD through PPARα pathway activation, paving the way for innovative treatment strategies. This underscores the significance of natural compounds in pharmacotherapy, suggesting that FRN may provide an effective alternative for managing ALD.

Abbreviations: ACSL1, acyl-CoA synthetase long chain family member 1; ALD, alcohol-associated liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FRN, flavonoids from *Rhododendron nivale Hook. f.*; GPX4, Glutathione peroxidase 4; H&E, Hematoxylin and eosin; NAFLD, non-alcoholic fatty liver disease; ORO, oil red o; PPARα, peroxisome proliferator activated receptor alpha; TAG-FA, triacylglycerols-fatty acid.

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Introduction

In recent times, given the rapidly evolving social dynamics, increased social activities, and the added pressure of the COVID-19 pandemic, there has been a slight but notable increase in individual alcohol consumption, as documented in smaller samples with consumption increases approaching 25% (Julien et al., 2022; Mackowiak et al., 2024). Consequently, a subtle yet significant increase in individual alcohol consumption has led to a rise in the prevalence of alcohol-related liver disease (ALD), an emerging public health concern. Currently, it is worth noting that there is no specific FDA-approved medication for ALD treatment and prevention, and abstinence remains one of the most effective approaches for ALD(Hernández-Évole et al., 2024; Jophlin et al., 2024). Therefore, to address this medical challenge, exploring innovative pharmaceutical interventions for treating and preventing ALD is imperative.

Flavonoids, celebrated for their powerful antioxidant capabilities, are gaining importance in ALD treatment (Kai et al., 2020; Park et al., 2013; Zhao et al., 2018), however, the specific molecular mechanism of its anti-ALD effect needs to be further elucidaed. As our previous research demonstrated, flavonoids from *Rhododendron nivale Hook. f.* (FRN) effectively modulate glutathione metabolism and the expression of Glutathione peroxidase 4 (GPX4) in an aging animal model, showcasing their antioxidative capacity (Guo et al., 2022). GPX4 is a pivotal protein that triggers ferroptosis through lipid peroxidation in both non-alcoholic fatty liver disease (NAFLD) and ALD (Luo et al., 2023; Shen et al., 2022; Zhang et al., 2023a,b). Interestingly, the present observations disclosed that while FRN did not markedly modify the alcohol-influenced GPX4 dysregulation in an ALD mouse model, it significantly improved hepatic lipid accumulation due to alcohol exposure. Therefore, identifying the mechanism of the FRN'S anti-ALD effect is essential for FRN to be a potential innovatic pharmaceutical intervention for ALD treatment.

Our network pharmacology analysis indicate that PPARα may hold considerable promise as a potential therapeutic target for FRN in the management of ALD. Peroxisome proliferator-activated receptors (PPARs) are a subset of ligand-activated nuclear receptors within the steroid/thyroid hormone receptor superfamily, comprising three isoforms: PPARα, PPARβ/δ, and PPARγ. Notably, PPARα is crucial for the regulation of fatty acids (FAs) transport and metabolism primarily in the liver (Aoyama et al., 1998). Research involing PPARα-null (*pparα*$^{-/-}$) mice subjected to a 4% ethanol-containing Lieber-DeCarli diet for six months has revealed significant pathological changes, inculding hepatomegaly, macrovesicular steatosis, mitochondrial swelling, hepatitis, and hepatic fibrosis-conditions that closely mirror the clinical and pathological manifestations of ALD (Meng et al., 2020; Rao and Reddy, 2004). Moreover, studies using PPARα ligands and *pparα*$^{-/-}$ mice has consistently revealed that PPARα is instrumental in promoting FAs catabolism. The resulting abnormalities exhibited high reproducibility and occurred independently of invasive procedures, such as gastric tube insertion, required to elevate alcohol levels (Okiyama et al., 2009; Wang, 2010). These findings underscore the potential of PPARα as a critical target for elucidating the pathogenesis of human ALD. However, the interraction between FRN and PPARα signaling remains unclear in the ALD treatment.

In this study, emerging evidences from network pharmacology, transcriptomics, and lipidomics indicates that FRN alleviates hepatic injury and steaosis by modulating PPARα signaling, positioning it as a promising therapeutic candidate for ALD. To futher investigate FRN'S mechanisms of action, we employed network pharmacology to delineate its potential target functions and enriched pathways. Utilizing liver transcriptomics, targeted lipidomics, molecular biology techniques, and PPARα antagonists, we aim to clarify the role of FRN in attenuating ALD. Importantly, our obesevations suggest that FRN may act as a ligand for PPARα, highlighting its potential application in the development of PPARα agonists for ALD prevention and treatment.

Material and methods

Chemicals and reagents

The preparation of FRN was carried out according to our established protocol (Guo et al., 2017). Dried shoots and leaves of Rhododendron nivale Hook f. were initially immersed in 70% ethanol for 12 hours, followed by extraction at 85 °C for 5 hours. The resultant extraction solution was subjected to filtration, concentration, evaporation, and subsequent drying at 60 °C under vacuum conditions. This ethanol extract underwent raw segmentation via Biorf-p60 column chromatography, employing sequential elution with water and methanol/water solutions at varying concentrations (30%, 60%, 90%). The fractions eluted with 30% and 60% methanol/water were processed through filtration, concentration, evaporation, and drying, ultimately yielding the dried FRN. The primary constituents of this dried FRN were characterized using thin-layer chromatography, liquid chromatography, and mass spectrometry, leading to the identification of four flavonoids: Myricetin-3-β-D-xylopyranoside, hyperin, goospeitin 8-methyl ether 3-β-D-galactoside, and diplomorphanin B. The chemical structure and composition of these extracts, based on an HPLC-based chemoprofile, were detailed in our previous study (Guo et al., 2022, 2017).

Assay kits for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were sourced from the Nanjing Jiancheng Biotechnology Research Institute, based in Nanjing, China. Hematoxylin and eosin (H&E) and oil red O stain kits were acquired from Leagene, located in Anhui, China. The Bodipy fluorescent probe and anti-PPARα antibody (Cat. # AF5301) were supplied by Affinity Biosciences in Jiangsu, China. The anti-ACSL1 antibody (Cat. # ab177958) was procured from Abcam, based in Cambridge, USA. The Lieber-DeCarli EtOH liquid diet (5% vol/vol EtOH, TP 4030D) along with the Lieber-DeCarli control diet (TP 4030C) were provided by Trophic Animal Feed High-tech Co., Ltd, situated in Nantong, China.

FRN targeted ALD interaction network analysis based on network pharmacology

The network pharmacology analysis was performed in accordance with our established protocol (Liu et al., 2024). The identified compounds were rendered into simplified molecular-input line-entry systems (SMILES). Target gene predictions were conducted using online tools such as Swiss Target Prediction (Daina et al., 2019) and PharmMapper ("UniProt: the universal protein knowledgebase in 2021.," 2021), applying a threshold of probability > 0 for screening. Gene symbol data were retrieved from the Universal Protein Resource (UniProt) database (Stelzer et al., 2016). Therapeutic target genes relevant to alcoholic liver disease (ALD) were compiled from GeneCards®: The Human Gene Database version 5.5, with supplementary data obtained from the Online Mendelian Inheritance in Man® platform (Amberger et al., 2015).

To elucidate the overlap between the predicted targets of the compounds and the established targets linked to ALD, we constructed a Venn diagram utilizing R version 4.0.6. The intersecting targets were employed to build a protein-protein interaction (PPI) network via STRING (Szklarczyk et al., 2021), which was further analyzed using Cytoscape (Version 3.7.1). For the PPI network construction, the organism was designated as the common bovine (Bos taurus), with a minimum interaction score set at "medium confidence" (0.40), while unconnected protein nodes were excluded from the analysis. Enrichment analyses for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were facilitated by DAVID Bioinformatics Resources 6.8.

Animals, cell culture, and treatments

The Animal Care and Use Committee of Southwest Medical

University gave full approval to all the experimental plans and processes (Approval Number: XNYKDX202212 dated December 24, 2022). The study strictly adhered to the guidelines for animal welfare and experimental procedures outlined in the US National Institutes of Health's Guidelines for the Care and Use of Laboratory Animals. Male C57BL/6 mice, each weighing between 18 - 20 g, were sourced from Yinhui Experimental Animal Sales Ltd., Luzhou, China and housed under controlled conditions of temperature (22 ± 2 °C), relative humidity (45−65%), and a 12-hour light/dark cycle. Following this, the mice were randomly allocated to predetermined experimental groups, each containing 7 mice. The groups consisted of the following: A) Control group: mice received the LieberDeCarli liquid control diet over 26 days. B) Alcohol diet group: mice were first fed the Lieber-DeCarli liquid control diet for 5 days, followed by 21 days on the Lieber-DeCarli alcohol diet, which contains 5% alcohol. C) FRN intervention group: mice were pre-administered FRN (25 mg/ kg/day or 50 mg/kg/day body weight) by oral gavage, prior to being given the same regimen as the alcohol diet group. D) FRN + GW6471 intervention group: mice were pre-administered a daily dose of 50 mg/kg body weight FRN and 20 mg/kg GW6471 by gavage. E) GW6471 intervention group: mice were pre-administered a daily dose of 20 mg/kg GW6471 by gavage, then received the same regimen as the alcohol diet group.

Additionally, our study utilized stable mouse liver cell lines AML12 cells, obtained from the Type Culture Collection of the Chinese Academy of Sciences in Shanghai, China. Following the grouping, AML12 cells were grown in Roswell Park Memorial Institute 1640 medium (RPMI 1640; cat# 11,875,119, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS; cat# C04001–500, Shanghai XP Biomed Ltd., China). The cells were maintained in an incubator set at 37 °C with 5% carbon dioxide. Once the cells reached 80–90% confluency after the third generation, they were classified into five groups similar to the animal groupings, with variations in specific culture media and duration of exposure to 150mM ethanol for 48 hours.

The injury and lipid accumulation in liver and cellular analysis

The analysis was conducted in accordance with our previously established protocol (Liu et al., 2024). Brifly, liver tissues were initially preserved in 4% neutral paraformaldehyde, subsequently embedded in paraffin, and sectioned to a thickness of 5 μm for hematoxylin and eosin staining. To evaluate hepatic lipid accumulation, the liver sections were embedded in optimal cutting temperature (OCT) compound; the resulting frozen specimens, cut to a thickness of 10 μm, were then subjected to ORO staining and examined with a Bodipy fluorescent probe, in strict adherence to the manufacturers' protocols.

For the cellular analysis, AML12 cells cultured in 6-well plates were washed three times with PBS and then stained with the Bodipy fluorescent probe, following the manufacturer's instructions. The degree of steatosis in hepatocytes was assessed by quantifying lipid content using ImageJ software. Additionally, we measured serum and cellular supernatant levels of ALT and AST using commercially available detection kits, following the manufacturers' guidelines.

Liver transcriptome analysis

Following the mehods outlined to our previous publication (Cheng et al., 2022). Brifly, the extraction of complete RNA from the liver tissue samples was facilitated using TRIzol (Invitrogen). Any present genomic DNA within these samples was eliminated using the DNaseI (TaKara) technique. We determined both the concentration and purity of the RNA using NanoDrop 2000 (Thermo Fisher Scientific). Further, an assessment of RNA integrity was performed using the RNA Nano 6000 Assay Kit and the noted Agilent Bioanalyzer 2100 system (Agilent Technologies). Using 1 μg of RNA from each sample, sequencing libraries were developed using the NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB), according to the manufacturer's instructions. Index codes were incorporated to track sequences back to their respective samples.

The entire procedure encompassed several steps. These include mRNA purification from total RNA, fragmentation of mRNA, synthesis of two strands of cDNA, adenylation of DNA fragments at 3′ ends, and ligation of the NEBNext Adaptor, all in line with established protocols. The target cDNA fragments aimed to be 240 bp in length. The libraries were then set for sequencing using the Illumina HiSeq X Ten/NovaSeq6000 sequencing platform on PE150 read length. This enabled high-throughput sequencing while maintaining a stringent hold on temperature control and usage of specific reagents at every stage to ensure precision.

The resulting raw data underwent rigorous quality control and splicing to obtain high-quality sequencing data. This refined dataset was crucial for subsequent bioinformatics analyses, including target gene clustering, Venn analysis, gene set enrichment analysis (GSEA), and visualization.

AML12 cells targeted lipidomics analysis

Cell lipid extraction

To begin, AML12 cells are prepared for analysis. First, the culture medium is discarded and the culture dish is inverted onto an absorbent paper to carefully control drying. Then, pre-cooled PBS at 4 °C is added and gently shaken for a minute to thoroughly clean the cells. This cleaning step is repeated twice to ensure the removal of all residual culture medium. Next, the culture dish is placed on ice. After these preliminary steps, pre-cooled PBS is added to the dish. The cells are quickly scraped onto one side using a clean cell scraper and tilted on ice to facilitate the flow of buffer towards one side. All cells are meticulously collected into pre-cooled centrifuge tubes and centrifuged at low speed to remove the supernatant. The cell pellet is then quickly frozen in liquid nitrogen for one minute.

For sample preparation, three sequential thawing stages are conducted, followed by adding 200 μL in three batches. A new centrifuge tube receives a pre-cooled methanol-water solution (3:1, v/v) and 825 mL of pre-cooled MTBE (Methyl tert-butyl ether). The mixture is well shaken and extracted at 4 °C for 60 minutes. Subsequently, sonication in an ice bath is performed for 30 minutes. After this process, 200 μL of H_2O is added and left to stand at room temperature for 10 minutes. The sample is then centrifuged at 4 °C and 16,000 g for 20 minutes. The supernatant is retrieved and precipitated with 300 μL of LSDT lysis before quantitative analysis of BCA protein.

Upon achievement, calculate the relative number of cells in distinct samples by determining the protein content in each sample. Equivocally measure the supernatant from each sample and proceed with vacuum drying. Subsequently, prepare each sample for mass spectrometry analysis by adding 100μL, followed by re-dissolving in a dichloromethane/methanol solution (1:1, v:v) at 4 °C. Centrifuge the samples at 20,000 g for 15 minutes and collect the supernatant for further analysis. Wide-target lipidomics will be conducted by Bioprofile, employing identical instrument parameters and bioinformatics analysis procedures as delineated in prior investigations.

Protein expression analysis

To assess the expression levels of PPARα, CD36, Apoe, and ACSL1 in mouse liver following various treatments, we employed western blot analysis. Additionally, we evalutated the expression of PPARα and ACSL1 in AML12 cells subjected to specific treatments using the same technique. For protein extraction, the liver samples were processed using a tadioimmunoprecipitation assay (RIPA) buffer. The protein concentration was determined using the bicinchoninic acid method, followed by separation on SDS-PAGE (10% - 15%). Adhering to standard protocols, the separated proteins were transferred to polyvinylidene fluoride membranes. The membranes were then incubated with specific primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. The bands were visualized using a G:BOX

Chemi XRQ imaging system (Cambridge, United Kingdom). To ensure accurate results, the data was normalized to the internal control of β-actin, accounting for variations in total protein.

PPAR transcriptional activity level detection

To explore the impact of FRN on PPAR transcriptional activity, we utilized the PPAR luciferin reporter gene assay system. For this experiment, we selected AML12 cells that were transfected with a PPAR-Luc Reporter Lentivirus plasmid across five consecutive generations using a medium containing puromycin (1.5 μg/mL) to ensure their stability. Subsequently, these cells were exposed to FRN (36 μg/mL) or Rosiglitazone (a known PPARα, agonist at 0.5 μM) for 16 hours. As a control, cells were treated with an equivalent amount of dimethyl sulfoxide (DMSO), which is used to dissolve FRN and Rosiglitazone powders, for the same duration. Afterward, the cells were harvested and lysed using a buffer with an appropriate detergent, and any cellular debris was efficiently removed through microcentrifugation. The activity was then measured using a luminometer to assess the transcriptional activity induced by FRN on PPAR.

Statistical analyses

In our study, the execution of experimental procedures and the subsequent data analysis were both subject to stringent blinding principles, guaranteeing objectivity and minimizing potential biases. The statistical analysis was conducted using the sophisticated SAS 9.2 (Statistical Analysis System; version 9.2; RRID: SAS Institute Inc., NC, USA) to ensure accurate and reliable data interpretation. All recorded data were presented as mean values with their corresponding standard deviations (SD) for a clear and precise portrayal of the findings. To discern variations among different groups, we employed a one-way Analysis of Variance (ANOVA). Following this, we utilized Duncan's multiple comparisons test to pinpoint the specific sources of differences within the variations. To establish a threshold for statistical significance in our observations and tests, we set a criterion of $P < 0.05$, as is standard in most scientific investigation.

Results

The PPARα signaling pathway participates in mediating the interactive network between FRN and ALD

To elucidate the intricate therapeutic mechanism of FRN in addressing ALD, a sophisticated gene screening procedure was conducted encompassing 2645 ALD-related genes. This meticulous analysis resulted in the creation of an intricate topological network, which enabled the identification of 54 common target genes linking FRN and ALD (Fig. 1A). To enhance the precision and accuracy of our key target information, we employed the CytoHubba tool integrated within the Cytoscape software platform. Utilizing the Maximal Clique Centrality (MCC) algorithm, significant protein nodes were discernibly highlighted. Notably, TNF, PPARA, and AKR1B1 were found to occupy

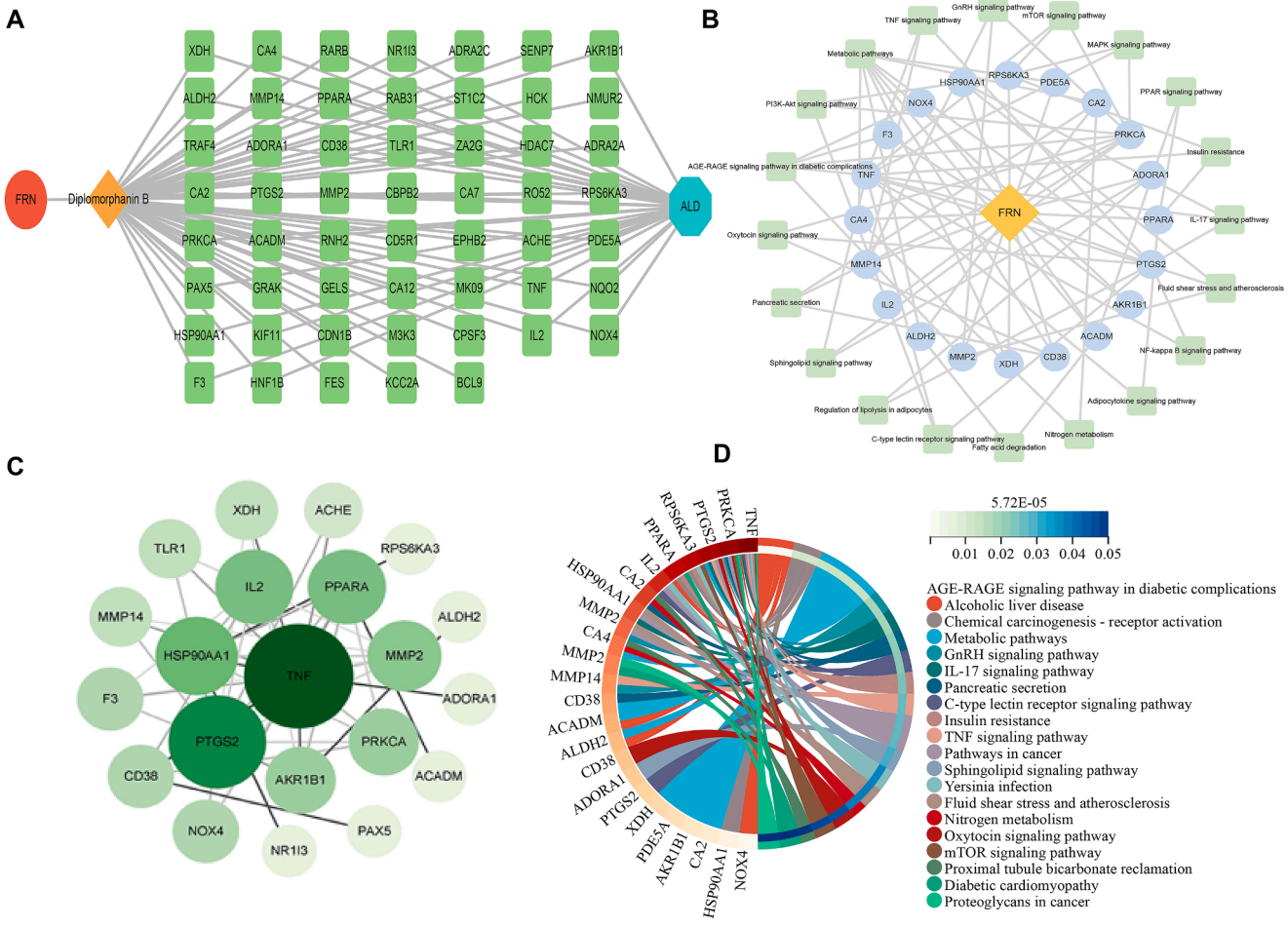


Fig. 1. The PPAR signaling pathway participates in mediating the interactive network between FRN and ALD diseases based on network pharmacology analysis. A: Component-target-disease network diagram of FRN. B: Component-target-signaling pathway network diagram of FRN. C: Protein targets interaction network of FRN involving in ALD. D: Chordal graph of KEGG enrichment analysis of targets.

pivotal positions within the PPI network (Fig. 1B, C).

We then embarked on a comprehensive GO and KEGG enrichment analysis, leveraging the sophisticated DAVID database. This rigorous analysis revealed a meticulous interplay of 52 distinct biological processes (BP), 12 cellular components (CC), and 12 molecular functions (MF), along with 25 KEGG pathways, all of which were statistically significant at $P < 0.05$. To facilitate a clear understanding of our data and enhance its practical applicability, we prioritized the most pertinent information, pinpointing the top 10 entries and the top 20 pathways (Fig. 1D). These findings suggest that PPARα may hold considerable promise as a potential therapeutic target for FRN in the management of ALD.

FRN mitigates alcohol-induced liver injury

In order to substantiate the hypothesis formulated by network pharmacology concerning the association between FRN and ALD, we have conducted a thorough investigation into the effects of FRN on ALD models, both in vitro and in vivo. A key metric in this study has been the levels of AST and ALT, frequently utilized as reliable indicators of liver damage. Our analysis has demonstrated a conspicuous elevation in AST levels in both in vitro and in vivo ALD models compared to the control group (Fig. 2A, B). Remarkably, the administration of FRN has exhibited a dose-dependent and significant improvement in the normalization of AST and ALT levels towards their physiological ranges (Figs. 2A and B).

Furthermore, we have conducted a comprehensive histopathological evaluation of liver tissue across various treatment groups. This evaluation has revealed an apparent increase in liver vacuolization induced by an alcoholic diet. In the ALD mouse models, a notable reduction in this vacuolar change was observed with FRN exposure (Fig. 2C). Additionally, the accumulation of lipids, an early marker of ALD, was conspicuous in both in vitro and in vivo ALD models. However, FRN effectively mitigated this lipid buildup through a dose-dependent mechanism, a finding that was additionally supported by a series of staining outcomes (Fig. 2D, E). These findings suggest that FRN may have the potential to modulate hepatic histological changes, including hepatic lesions and steatosis, resulting from chronic alcohol consumption.

FRN reduces accumulation of TAG-FA lipid metabolites in the alcohol-induced liver cells

To better understand the effects of alcohol on lipid metabolism in hepatocytes, we have conducted a thorough analysis of the lipid profile. We have meticulously examined 912 lipids, presenting a nuanced picture of the lipid dynamics within the cellular system. Upon evaluating these lipids across various treatment groups, we have identified distinct alterations in their metabolic patterns (Fig. 3B). Using a one-way ANOVA analysis, we have quantitatively assessed the statistical significance of these changes, pinpointing key lipid molecules (Fig. 3C-F). These molecules exhibit a P-value < 0.05, a fold change > 1.5, and a VIP score from OPLS-DA > 1. Our findings indicate notable variations and significant differences in the lipid FAs profiles across 113 species, between normal and ethanol-induced AML12 cells, irrespective of FRN presence or absence (Fig. 3C-E).

An enrichment examination supported by the KEGG pathway depicted the lipids classes, like PE, sterols, TAG, Cer, DAG, FFA, LPC, LPE, LPG, PA, PC, PG, PS, SM, TAG-FA, and PI, participating in different biochemical processes, including Glycerophospholipid metabolism, Sphingolipid metabolism, Adipocytokine signaling pathway, Fat digestion, absorption, Sphingolipid signaling pathway, and Ferroptosis, as seen in Fig. 3I. The most noticeable alterations in lipid molecules were largely associated with the lipid class of TAG-FA, with significant increases noted in TAG53:5−FA20:4, TAG52:4−FA18:2, TAG55:7−FA22:6, TAG52:2−FA16:1, TAG52:3−FA16:0, among others, in ethanol-induced AML12 cells compared to normal (Fig. 3F-H).

Impressively, the correctional potential of FRN has been found to be commendably effective in addressing alcohol-induced alterations in the TAG-FA lipid class (Fig. 3F-H). These observations underscore the pivotal role of FRN in regulating hepatic lipid metabolism and preventing the accumulation of harmful lipid metabolites induced by alcohol in vitro.

FRN modulates gene expression along regulation of FAs biosynthetic in the liver of mice with ALD

To explore the intricate molecular mechanisms underlying FRN's role in mitigating FAs accumulation in the livers of ALD mice, we have employed cutting-edge liver transcriptomic techniques to meticulously assess gene expression variations across different treatment groups. Initial gene expression analysis, utilizing multivariate statistical tools like principal component analysis (PCA), has revealed notable deviations among the groups. Subsequent scrutiny of these differential genes has been conducted through a one-way ANOVA to determine statistical significance across various groups. Genes that meet the criteria of an adjusted P-value < 0.05 and a fold change > 2 have been flagged for advanced visualization and further investigation. This study has uncovered significant disparities in gene expression profiles involving 172 species, comparing control and ethanol-induced AML12 cells, irrespective of the presence of FRN (Fig. 4A-D).

GO-backed pathway enrichment assessment determined that gene expression disruptions were intricately linked with a range of biological processes including FAs biosynthesis regulation, sterol transport, cholesterol transport, and the positive regulation of phagocytosis, depicted in Fig. 4E, F. Our attention centered on genes integral to lipid metabolism, highlighting pronounced reductions in PPARα and Apoe in ALD models. Conversely, ACSL1 and CD36 levels were significantly higher in ALD models compared to controls (Fig. 4G-J). FRN was found to effectively counteract the alcohol-induced imbalances within the expressions of PPARα, CD36, Apoe, and ACSL1 (Fig. 4G-J). These findings collectively support the hypothesis that FRN exerts a profoundly corrective effect on alcohol-induced gene expression disruptions linked to the regulation of FAs biosynthesis in the hepatic tissue of ALD mice.

PPARα antagonists abolishes the effect of FRN on alleviating alcohol induced lipid accumulation in liver cells

To verify the role of biological processes in the identified genes with differential expression from liver transcriptomic screenings, we utilized esteemed academic techniques, including western blot and immunohistochemistry. We scrutinized the expression levels of PPARα, CD36, Apoe, and ACSL1 in the liver tissues of mice subjected to diverse treatment regimens. The inclusion of alcohol in the diet initiated an increase in the expression of CD36 and ACSL1, a decrease in PPARα expression, and Apoe expression unaffected in the liver. However, FRN administration markedly elevated PPARα expression and diminished ACSL1 expression in the livers of ALD mice. Nonetheless, FRN was not observed to significantly reverse the upregulated CD36 expression in the liver of ALD mice (Fig. 5A-G). Furthermore, the observation that FRN treatment considerably rectified PPARα and ACSL1 levels was also reconfirmed during in vitro experiments (Fig. 5H-J).

Based on the collective insights from our research and previous studies, we propose that PPARα and ACSL1 play a central role in FRN's beneficial effect on alcohol-induced lipid accumulation in liver cells. To substantiate this, we utilized an antagonist to delve into the role of PPARα. This approach has clarified PPARα's involvement in FRN's mechanism, which mitigates lipid accumulation in hepatic cells. Treatment with GW6471, a PPARα antagonist, resulted in significant changes in liver histopathology and lipid build-up. Furthermore, GW6471 was found to reduce the beneficial effect of FRN (Fig. 6A-D). Collectively, these results suggest that the activation of PPARα is critical for alleviating alcohol induced lipid accumulation in both in vivo and in vitro.

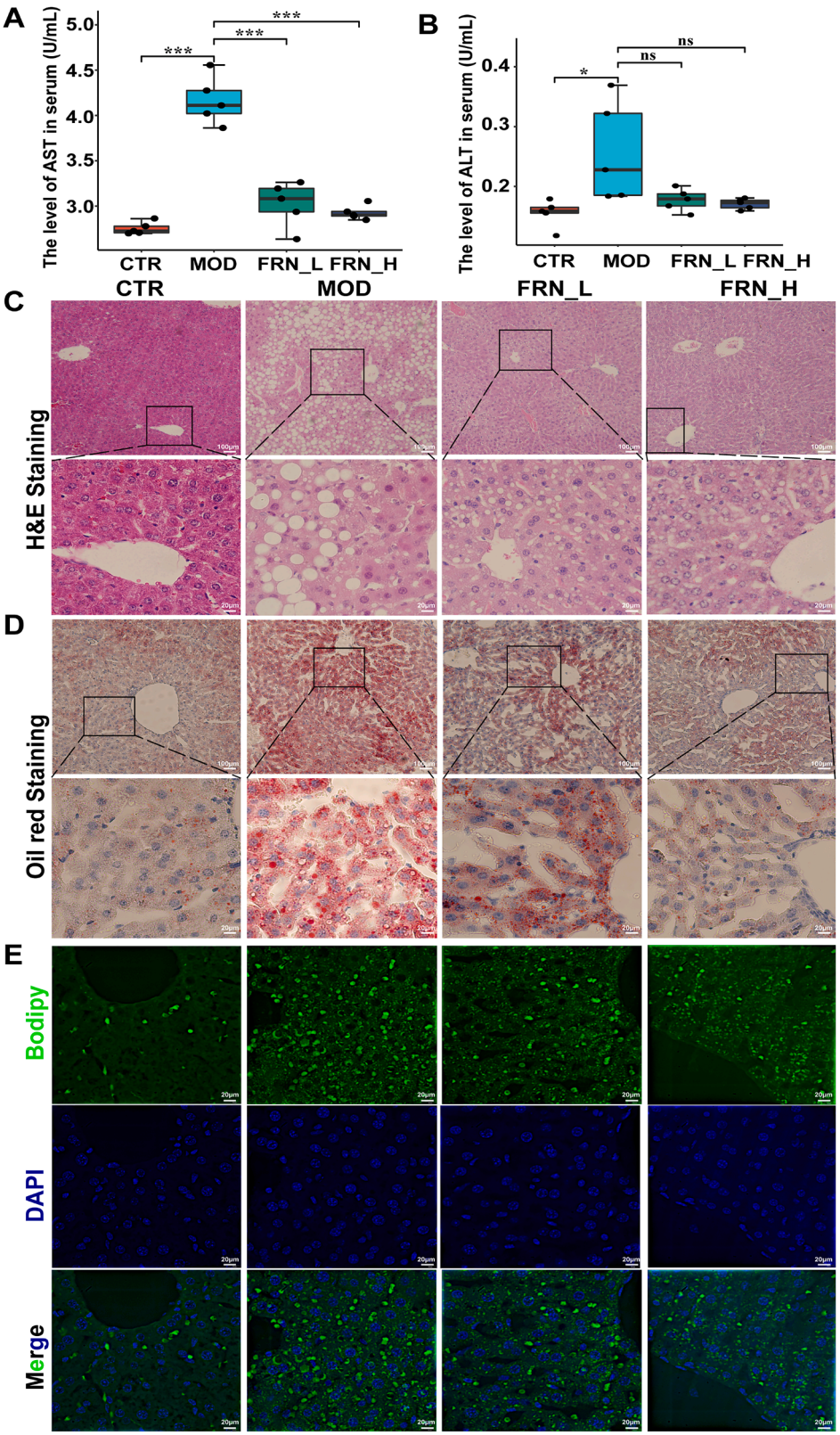


Fig. 2. FRN mitigates alcohol-induced liver injury. A-B: The difference in the AST and ALT level of mice subjected to different treatments. C The liver pathological changes in mice are subjected to different treatments. D-E: The changes of lipid accumulation in liver of mice are subjected to different treatments. 25 mg/ kg/day (FRN_L) or 50 mg/kg/day (FRN_H) body weight. *$P < 0.05$, compared with the MOD group, ***$P < 0.01$, compared with the MOD group.

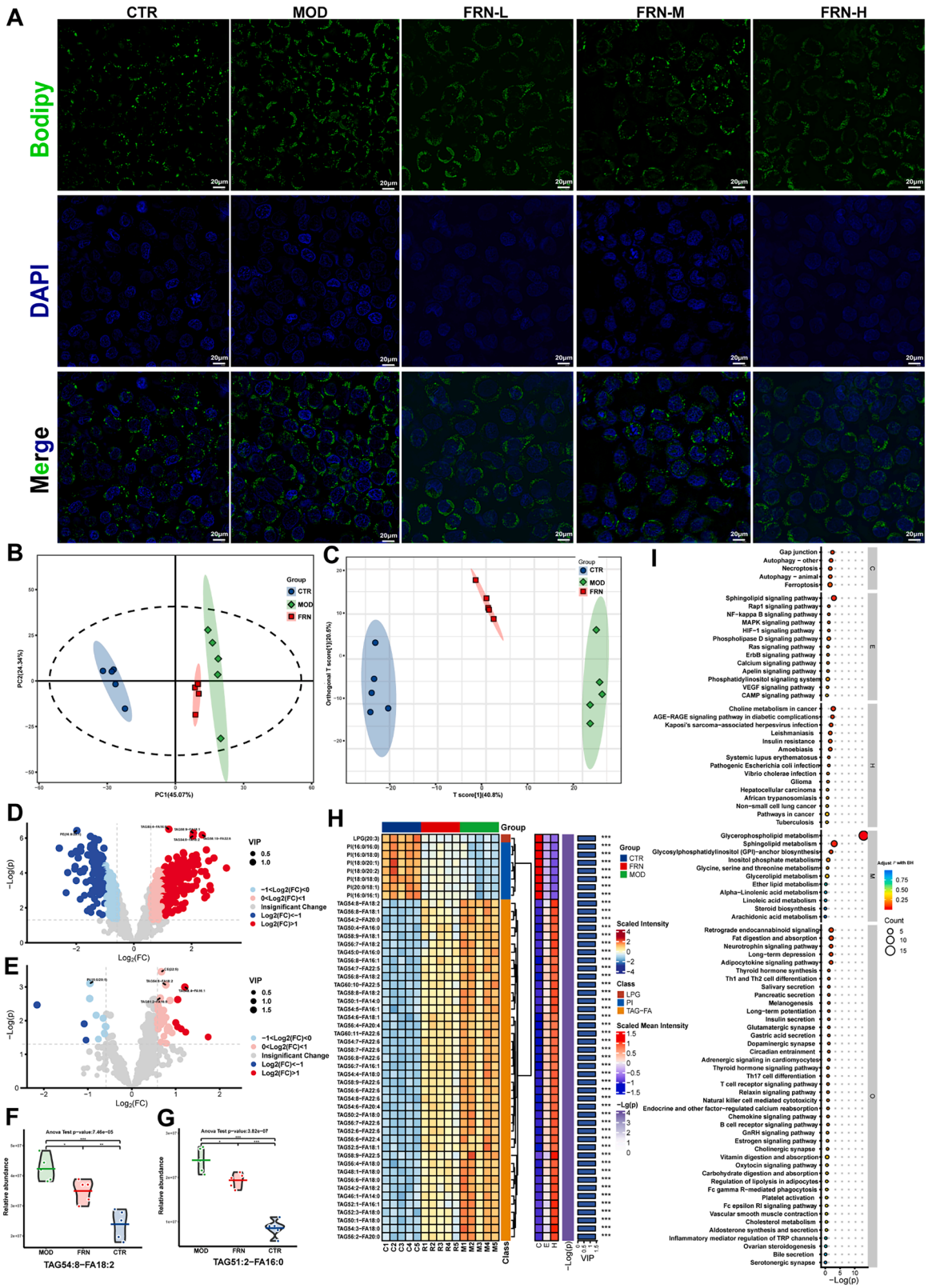
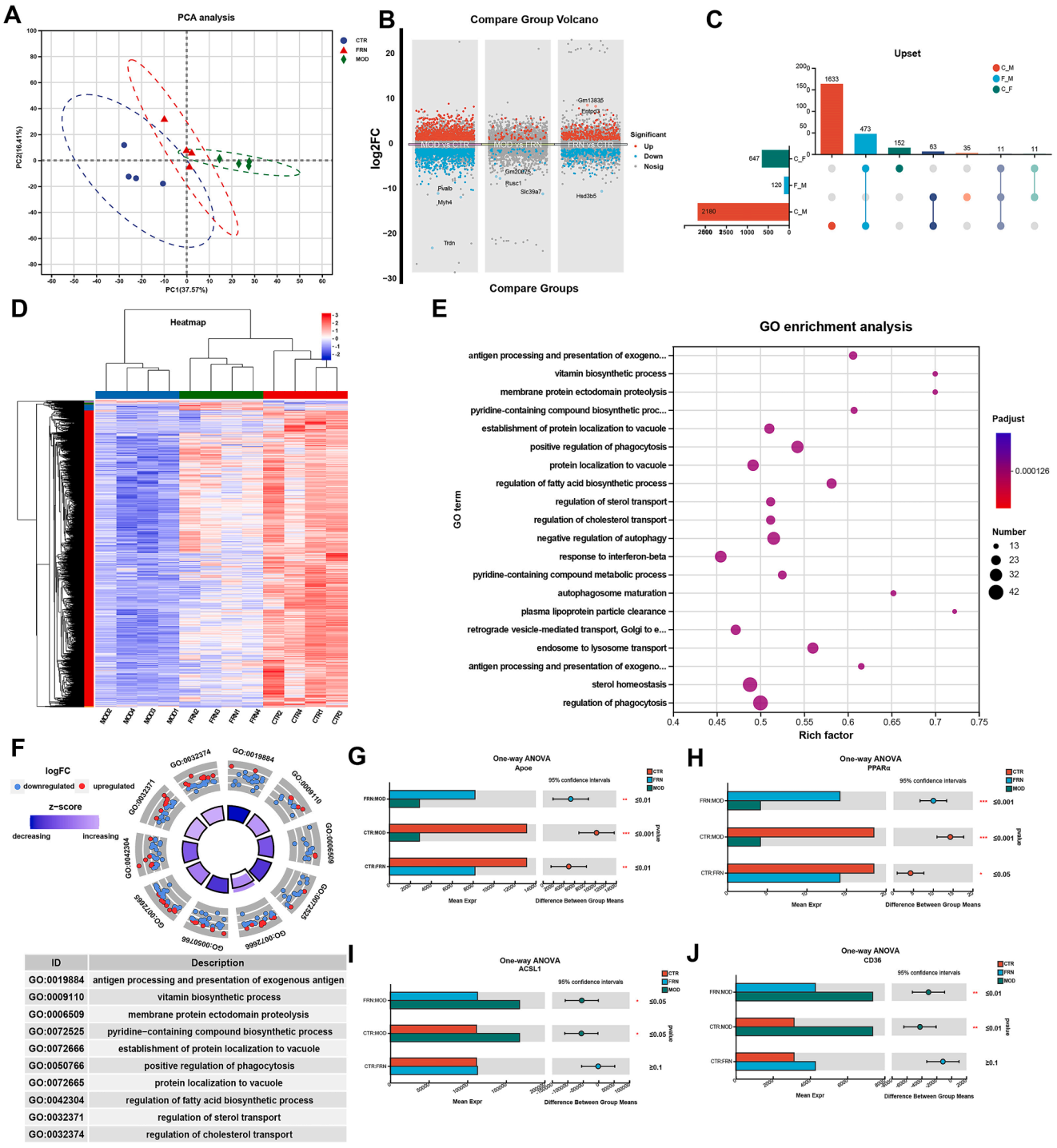


Fig. 3. FRN reduces accumulation of TAG-FA lipid metabolites in the alcohol-induced liver cells. A: The changes of lipid accumulation in the AML12 cells subjected to different treatments. B-H Differential lipid metabolites profile of AML12 cells are subjected to different treatments. I: Bubble chart of KEGG enrichment analysis of differential lipid metabolites. AML12 cells were treated with 18 μg/mL (FRN_L), 36 μg/mL (FRN_M), and 72 μg/mL (FRN_H) for 24 hours.



ID	Description
GO:0019884	antigen processing and presentation of exogenous antigen
GO:0009110	vitamin biosynthetic process
GO:0006509	membrane protein ectodomain proteolysis
GO:0072525	pyridine–containing compound biosynthetic process
GO:0072666	establishment of protein localization to vacuole
GO:0050766	positive regulation of phagocytosis
GO:0072665	protein localization to vacuole
GO:0042304	regulation of fatty acid biosynthetic process
GO:0032371	regulation of sterol transport
GO:0032374	regulation of cholesterol transport

Fig. 4. FRN modulates gene expression along regulation of fatty acid biosynthetic in the liver of mice with ALD. A-D: Liver differential gene expression profile of mice are subjected to different treatments. E-F: KEGG and GO enrichment analysis of differential genes. G-J: the liver difference in the expression of Apoe, PPARα, CD36 and ACSL1 with different treatments. $^{*}P < 0.05$, compared with the MOD group,$^{***}P < 0.01$, compared with the MOD group.

FRN actives PPAR transcriptional activity

Accordance with our findings, we observe that FRN proficiently regulates PPARα expression, a vital component in the treatment of ALD. To further elucidate the nuanced mechanisms of FRN's regulation on PPARα, we conducted an in-depth analysis of its impact on PPARα's transcriptional activity. Our results, corroborated by advanced techniques such as ORO and Bodipy staining, along with an extensive analysis of the PPARα luciferin reporter gene evaluation system, unequivocally demonstrate that FRN significantly augments this activity (Fig. 6). These findings indicate that FRN possesses a remarkable capacity to regulate PPARα transcription activity, highlighting FRN's potential as a key player in mitigating the harmful effects of alcohol on the liver.

Discussion

The prevalence of alcohol-associated liver disease (ALD) has escalated as a global health concern, intensified by the escalating alcohol consumption patterns worldwide(Mackowiak et al., 2024; Wu et al.,

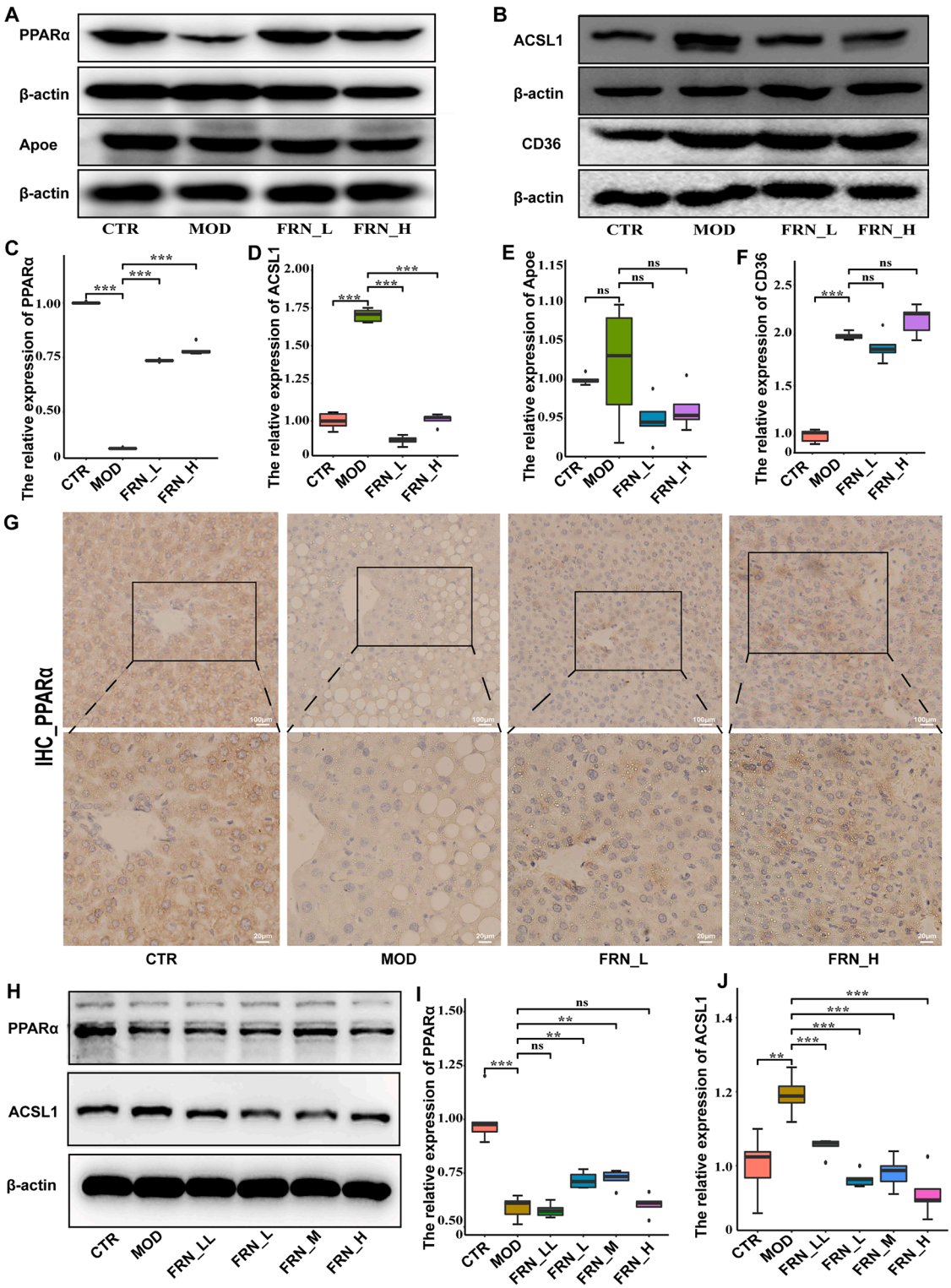


Fig. 5. FRN ameliorates alcohol-reduced expression of PPARα in the liver and AML12 cells. A-G: FRN ameliorated alcohol-reduced expression of PPARα in the liver. H-J: FRN ameliorates alcohol-reduced expression of PPARα in the AML12 cells. AML12 cells were treated with 9 μg/mL (FRN_LL), 18 μg/mL (FRN_L), 36 μg/mL (FRN_M), and 72 μg/mL (FRN_H) for 24 hours. *$P < 0.05$, compared with the MOD group. ***$P < 0.01$, compared with the MOD group.

2023). The rising incidence rates of ALD have compelled the need for the development of effective prevention and treatment strategies (Hernández-Évole et al., 2024; Julien et al., 2022; Wu et al., 2023). Consequently, researchers have turned their focus to natural interventions for ALD, with a specific emphasis on flavonoids, which are abundant metabolites found in a wide range of fruits and vegetables (Shang et al., 2022; Zhang et al., 2023a). Flavonoids are widely recognized for their remarkable biological functions, including their antioxidant, anti-inflammatory, and anti-carcinogenic properties(Bangar et al., 2023; Yang et al., 2023; Zhang et al., 2023a). Recent studies have highlighted their protective role in mitigating liver damage caused by alcohol, thereby suggesting potential for their use in managing ALD.

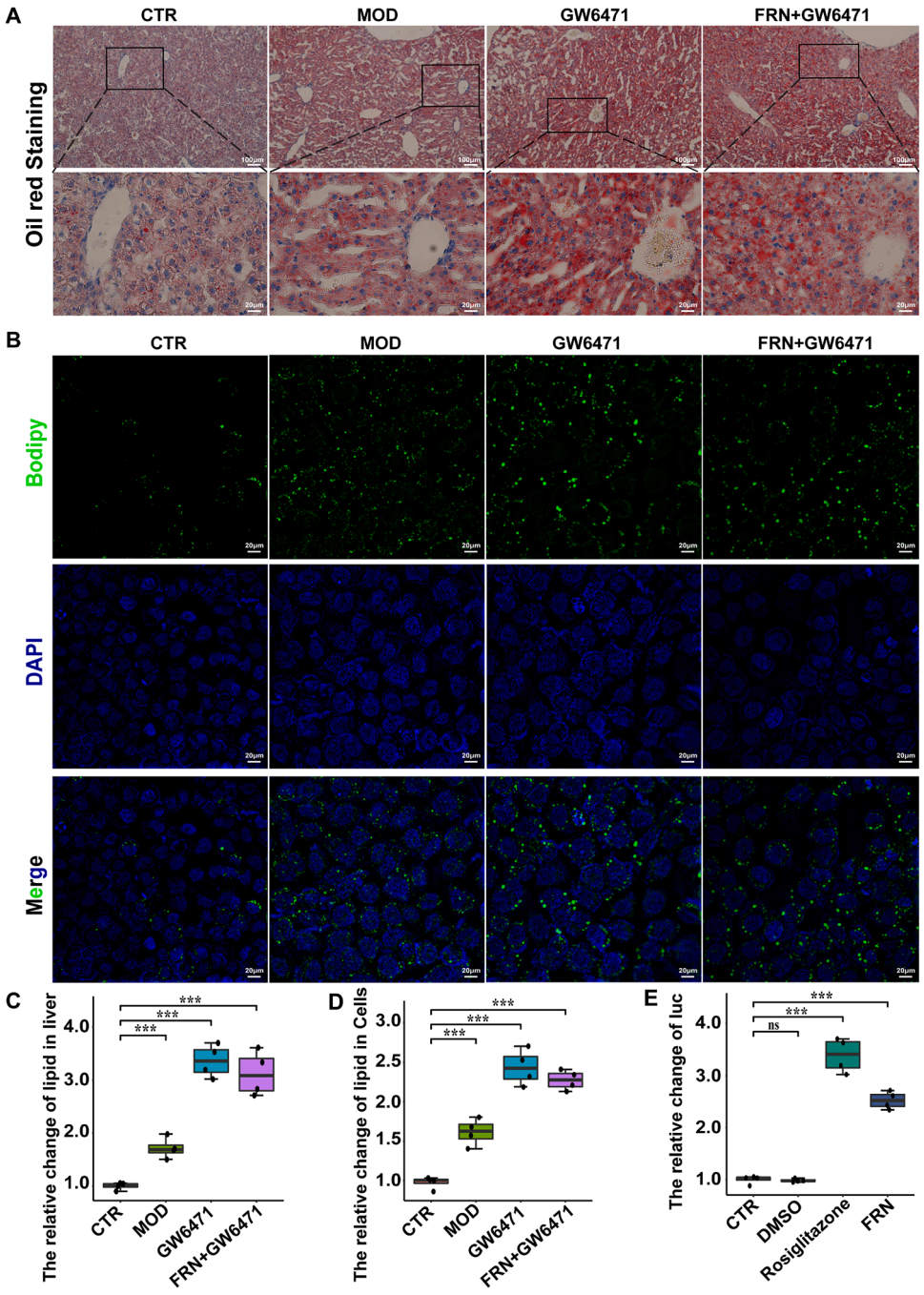


Fig. 6. PPARα antagonists abolishes the effect of FRN on alleviating alcohol induced lipid accumulation in liver cells. A-D: Treatment with the PPARα antagonist, GW6471, induces notable alterations in liver histopathology and lipid build-up in both liver tissues and AML12 cells. AML12 cells were firstly treated with 20M GW6471 for 3 hours, and then treated with 36 μg/mL FRN for 24 hours. E: FRN actives PPAR transcriptional activity. *$P < 0.05$, compared with the MOD group. ***$P < 0.01$, compared with the MOD group.

Specifically, flavonoids derived from Rhododendron Adamsii have demonstrated impressive hepatoprotective effects in various studies (Olennikov et al., 2021; Wang et al., 2024). These compounds' therapeutic applications extend beyond ALD, encompassing a range of disorders characterized by inflammation and oxidative stress (Kim et al., 2024; Wang et al., 2024; Yan et al., 2023). In our current study, we observed that FRN exhibited a notable ability to alleviate ethanol-induced lipid accumulation in the liver and promote the normalization of AST and ALT levels towards physiological ranges. This suggests a potential role for FRN in the management of ALD and its associated hepatic disorders, which underscores the importance of further exploring natural compounds as potential therapeutic agents in the management of ALD.

The advent of network pharmacology has profoundly transformed the realm of natural plant extract pharmacology, recognizing the intricacies of biological systems and diseases. Contrary to the conventional "one target, one drug" paradigm, network pharmacology emphasizes a more comprehensive "network target, multi-component therapeutics" approach, effectively tapping into the synergistic effects of various compounds (Nogales et al., 2022; Zhu et al., 2022). This methodology was used in our study to understand how different FRN constituents simultaneously modulate multiple biological targets to exert an extensive therapeutic effect on ALD. This has permitted us to identify potential active ingredients and therapeutic targets by constructing a

detailed interaction network relating FRN to ALD, based on chemical, genomic, and pharmacological data. From this analysis, the PPARα signaling pathway emerged as a vital link between FRN and ALD, suggesting its potential as a therapeutic target for ALD treatment using FRN.

Network pharmacology posits a sophisticated relational model between diseases and drugs, particularly accounting for the intricate, multi-targeted properties of flavonoids (Lazzara et al., 2024; Zheng et al., 2024). However, the implementation of network pharmacology in the context of natural plant extracts poses unique challenges. These challenges encompass determining appropriate thresholds, selecting an extensive array of targets, and discerning novel active components. Overcoming these obstacles is imperative for accelerating the pace of pharmacological research on natural plant extracts, as it enables a more comprehensive understanding of their complex interactions and potential therapeutic applications.

The PPARα signaling pathway plays a pivotal role in the pathogenesis of ALD, finely regulating lipid and FAs homeostasis (Meng et al., 2020; Szántó et al., 2021). Chronic alcohol consumption disrupts this dilicate balance, leading to fatty liver formation, an integral component of ALD (Rehman and Mehta, 2022; Villarroel-Vicente et al., 2021). The hepatic PPARα activity is dependent on the dosage of genes involved (Lalloyer et al., 2011) and its expression and function are reduced as ALD progression (Xu et al., 2021), potentially contributing to the limited clinical efficacy of single PPARα agonist treatment. Futhermore, in *pparα*-null mice subjected to chronic alcohol feeding, severe histological changes such as severe steatosis are observed, which are commonly found in ALD patients albeit with a lesser severity in our mouse model (Okiyama et al., 2009; Xu et al., 2021). PPARs are lipid sensors, activated by FAs and their derivatives or pharmacological agonists, moudulating the gene expression profiles of target tissues, enabling adaptation to changing nutritional environments. And PPARs regulate gene expression through two primary mechanisms: transactivation and transrepression. Through transactivation, ligand-activated PPARs form heterodimers with the retinoid X receptor, binding to specific regulatory DNA sequences known as PPAR response elements, thereby modulating the expression of target genes. Importantly, PPARs can also influence with immune-inflammatory pathways without direct DNA binding, utilizing an incompletely understood process called transrepression (Jones, 2010; Gross et al., 2017; Dubois et al., 2017). It is well established that PPARα is a key transcriptional regulator of FAs metabolism in the liver, with downstream genes such as cd36, acsl1, and fasn, etc., playing crucial roles in lipid metabolism (Park et al., 2021; van der Meer et al., 2010). Our study underscores the significant role of alcohol in enhancing hepatic lipid accumulation and reducing PPARα expression in both in vivo and in vitro. Futhermore, we have discovered that the dysregulation of TGA-FA lipids appears to play a pivotal role in mediating lipid accumulation observed in this condition. And our findings indicate that FRN treatment effectively ameliorates hepatic PPARα and ACSL1 expression. Moreover, the central role of PPARα in lipid homeostasis regulation is of paramount importance, as it not only modifies gene transcription but also acts as a liaison for multiple signaling pathways related to lipid metabolism. This suggests that there are additional interactive signaling pathways, beyond PPARα, which may be involved in the development and progression of ALD (Fig. 7). In conclusion, our current study highlights the complex interplay between alcohol consumption, lipid metabolism, and the crucial role of PPARα in maintaining hepatic health.

In our study, it remains challenging to pinpoint the exact FRN ingredient that modifies the PPARα signaling pathway. While nine flavonoids have been identified within FRN, their commercialization remains limited due to extraction and isolation constraints. Utilizing network pharmacology, potential targets for these identified FRN-

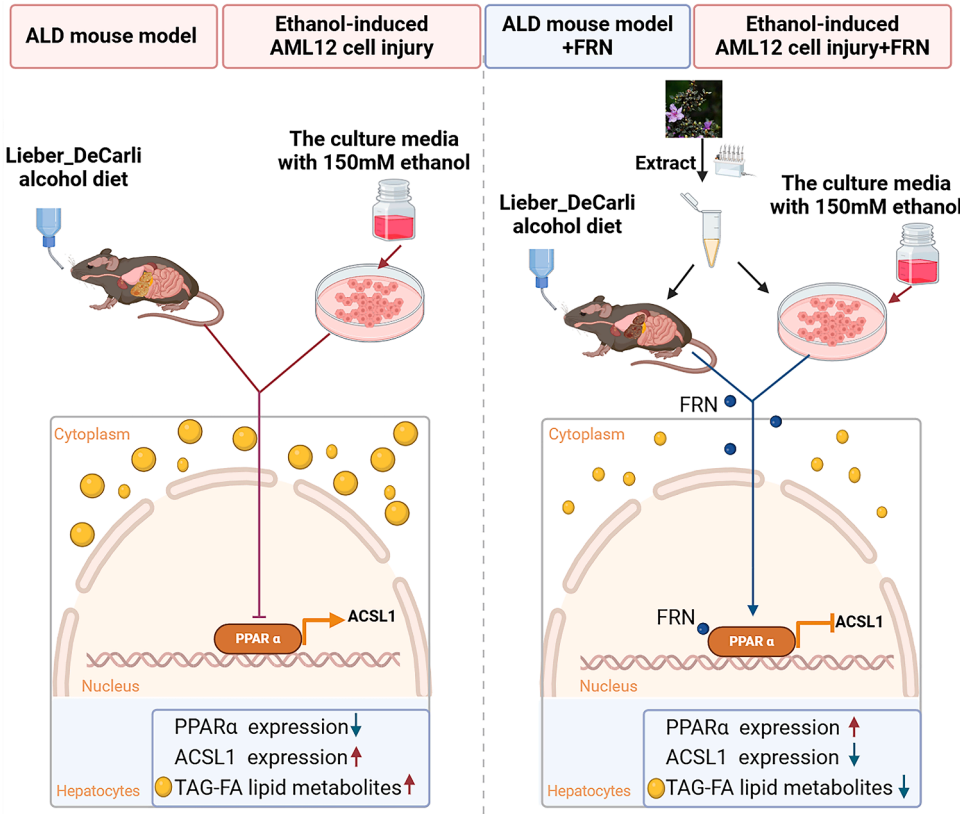


Fig. 7. A working model of FRN modulates activation of PPARα singaling to alleviate alcohol-associated liver injury. FRN regulates PPARα expression in an orchestrated effort alongside the reduction of ACSL1, ultimately leading to a marked reduction in long-chain fatty acid accumulation within hepatocytes. This substantial discovery contributes significantly to the comprehension of ALD pathogenesis. In this context, FRN emerges as a crucial ligand for PPARα, delicately steering the modulation of TGA-FA lipids, a pivotal role anticipated to play a key part in the effective management of lipid accumulation in ALD.

derived flavonoids can be predicted. However, the intricacies of body absorption, metabolism, and interaction with intestinal microorganisms may alter their efficacy or specificity. Therefore, a comprehensive investigation is warranted to pinpoint the specific FRN components that impact PPARα signaling. Furthermore, the accumulation of FAs in hepatocytes may act as a PPARα ligand, suggesting that alterations in TAG-FA class, particularly elevations in specific TAGs like TAG53:5-FA20:4 and other (Fig. 3F-H), are of significant concern. This underscores the need to elucidate the unique role and mechanism of FRN and free fatty acids in regulating PPARα activation. It highlights the critical importance of futher exploring the underlying mechanisms driving ALD developoment and exploring the potential therapeutic benefits of targeting PPARα and its related pathawys. The complexity of these interactions underscores the importance of meticulous research to elucidate the precise role of FRN and its components in regulating PPARα signaling, as well as the potential therapeutic applications in treatiing ALD.

Conclusion

The focus of our study was to meticulously evaluate the role of FRN in providing a defensive mechanism against alcohol-induced liver damage. Leveraging diverse techniques such as metabolomics and transcriptomics, along with experimental validations in vivo and in vitro, we have gained a deeper understanding of the anti-ALD effect and mechanism associated with FRN, and its dependency on PPARα signaling. Moreover, our findings underscore the pivotal part played by PPARα in potential ALD remedies and contributes to the development of pharmaceutical interventions utilizing natural compounds, contributing to the growing body of research exploring the use of natural compounds in pharmaceutical development. We believe this improved comprehension may lead to more alternatives for treating patients with ALD.

CRediT authorship contribution statement

Xiao Guo: Supervision, Resources, Methodology, Data curation. **Chen Liu:** Project administration, Methodology, Investigation, Formal analysis, Data curation. **Zhen Dong:** Visualization, Validation, Project administration. **Gang Luo:** Conceptualization, Software, Writing – review & editing. **Qien Li:** Data curation. **Meizhou Huang:** Writing – original draft, Validation, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data availability statement

The authors confirm that Liver transcriptome data that support the findings of this study are available in NCBI databases at https://submit.ncbi.nlm.nih.gov/subs/sra/SUB14323675/overview

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2024.156215.

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