The FASEB Journal

FASEB
Federation of American Societies for Experimental Biology

RESEARCH ARTICLE OPEN ACCESS

Fibrogenic Gene Signature as Early Prediction for the Efficacy of Pharmacological Interventions for MASH-Associated Fibrosis

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Received: 7 March 2025 | **Revised:** 2 May 2025 | **Accepted:** 9 June 2025

Funding: The authors received no specific funding for this work.

Keywords: fibrosis | gene signature | MASH | MASLD | transcriptomics

ABSTRACT

The incidence of metabolic dysfunction-associated steatohepatitis (MASH) and associated liver fibrosis is rapidly increasing, while pharmacological treatment options remain limited. Despite great efforts in developing novel MASH therapeutics, many investigative therapeutics that reduced fibrosis in preclinical models ultimately failed in clinical trials. To this end, we explored the possibility of predicting the efficacy of therapeutics by evaluating changes in the expression of a fibrogenic gene signature in the early stages of disease development and before effects on pathology become evident. Ldlr−/−.Leiden mice were fed a high-fat diet (HFD) to induce obesity and MASH. Mice were subsequently treated for 4 weeks with various therapeutics with established efficacy (obeticholic acid) or lack of efficacy (cenicriviroc and pioglitazone) to study their anti-fibrotic potential. Expression of a fibrogenic gene signature was evaluated, which predicts profibrotic processes before histopathologic fibrosis develops. The predictions were compared with a long-term experiment reaching histological fibrosis endpoints. Cenicriviroc and pioglitazone did not affect HFD-induced fibrosis signature, indicative of no effect of these treatments on active fibrosis processes. Consistently, in the long-term treatment study, both cenicriviroc and pioglitazone did not affect HFD-induced histologically measured fibrosis. In contrast, obeticholic acid improved the fibrogenic gene signature to a healthier state compared to untreated HFD controls. These early gene expression changes aligned with long-term histological fibrosis endpoints and clinical data on these investigative therapeutics. This study highlights the potential of using short-term studies and applying a fibrogenic gene signature as an early screening tool to investigate the efficacy of investigative drugs on MASH-associated fibrosis. This signature, which is based on the active fibrosis processes in humans, may allow rapid screening of therapeutics, or combinations thereof, when used in a translational mouse model.

1 | Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) is characterized by excess lipid accumulation in the liver and is often associated with obesity. When hepatic lipid accumulation is accompanied by hepatic inflammation, the disease progresses into metabolic dysfunction-associated steatohepatitis (MASH), which can ultimately result in the development of hepatic fibrosis as well. Alongside the rising worldwide prevalence of metabolic syndrome, the prevalence of MASH is rising as well [1].

Currently, pharmacological options for the treatment of MASH-associated fibrosis remain limited. Recently, a major breakthrough was achieved with resmetirom being the first drug for MASH-associated fibrosis receiving approval by the US Food and Drug Administration [2]. Despite great effort in developing novel anti-fibrotic therapeutics, many drugs with promising effects in preclinical and early clinical stages have ultimately failed in late-stage clinical trials, thereby questioning the credibility of the preclinical research trajectory and the translational value of models used. Several pharmacological compounds have been tested in the clinic, and while many improved hepatic steatosis and inflammation, the improvement of hepatic fibrosis has proven to be more difficult to achieve and may therefore involve combination therapy. Examples of tested anti-inflammatory therapeutics are the chemokine receptor type 2 (CCR2) and type 5 (CCR5) antagonist cenicriviroc and the nuclear receptor peroxisome proliferator-activated receptor alpha-gamma (PPAR-αγ) agonist pioglitazone. In many preclinical models, both cenicriviroc [3–6] and pioglitazone [7–9] have proven successful in alleviating hepatic fibrosis alongside improvements in hepatic steatosis and inflammation. In clinical phase III trials, both compounds showed promising effects on hepatic steatosis and inflammation yet failed to improve hepatic fibrosis [10, 11]. On the other hand, the farnesoid X receptor (FXR) agonist obeticholic acid had beneficial effects on both hepatic steatosis, inflammation and fibrosis in preclinical [12–14] and a phase III clinical study [15]. The discrepancies in the efficacy of therapeutics for MASH-associated fibrosis between preclinical models and clinical studies highlight the need for better approaches to predict treatment success in later stages.

For the current study, we selected Ldlr−/−.Leiden mice that have been extensively characterized for recapitulating features of the metabolic syndrome and MASH-associated fibrosis when fed a high fat diet (HFD) [14, 16–20]. Diet-induced models in general have been shown previously to better reflect the histopathological and molecular characteristics of MASH pattients [21, 22] and diet-induced Ldlr−/−.Leiden mice show even more overlap in underlying disease pathways with human patients with MASH compared to other preclinical models [14, 16, 23, 24]. In contrast to many other diet-induced MASH models in which dietary cholesterol drives liver pathology, no cholesterol supplementation is used in the diet-induced Ldlr−/−.Leiden model. As such, de novo cholesterol synthesis and de novo lipogenesis are intact and contribute to the liver disease pathology reflective of disease development in MASH patients. In a previous study using Ldlr−/−.Leiden mice, a gene expression signature of the active fibrosis process was identified using deuterated water labeling of collagens. This fibrogenic gene signature consisted of 232 differentially expressed genes that reflected profibrotic processes before histological fibrosis could be detected [25]. In the current study, we investigated whether this fibrogenic gene signature can be used as an early screening tool to rapidly assess the efficacy of MASH therapeutics. To this end, the effects of well-described therapeutics with different efficacy on MASH, namely cenicriviroc, pioglitazone, and obeticholic acid were predicted in a short-term experiment using this fibrogenic gene signature. Subsequently, these predictions were compared with the effects of the same compounds on histological fibrosis endpoint in independent long-term experiments.

2 | Materials and Methods

2.1 | Experimental Design

Experimental procedures were approved by The Netherlands Central Authority for Scientific Procedures on Animals (CCD; project license AVD5010020172064). Additionally, studies were approved by an independent Animal Welfare Body of The Netherlands Organization for Applied Scientific Research (IvD TNO; TNO-193, TNO-504, and TNO-540). Animals were bred and housed at the SPF animal facility at TNO (TNO Metabolic Health Research, Leiden, the Netherlands). Male mice were chosen because of their increased susceptibility for developing obesity and insulin resistance in comparison with female mice, and as such, male Ldlr−/−.Leiden mice are more translational to MASH patients [20].

Male Ldlr−/−.Leiden mice were group-housed in a temperature-controlled room on a 12 h light–dark cycle at 50%–60% humidity. Mice had access to food and heat-sterilized tap water ad libitum. Body weight, food intake at the cage level, and clinical signs were monitored regularly. After a run-in period on a high fat diet (HFD) containing 24% (*w/w*) fat from lard (D12451; Research Diets, New Brunswick, NJ, USA), mice were matched into comparable groups based on age, body weight, blood glucose, plasma cholesterol, and triglycerides. Mice either received no intervention (HFD control), 20 mg/kg/day cenicriviroc (Advanced ChemBlocks, Hayward, CA, USA), 10 mg/kg/day pioglitazone (Sigma Aldrich, Saint Louis, MO, USA) or 10 or 20 mg/kg/day obeticholic acid (OCA; Bio-Connect, Huissen, the Netherlands), all as admixture to the diet, or 20 mg/kg/day OCA via daily gavages. In a previous study in the same Ldlr−/−.Leiden mouse model, 10 mg/kg OCA reduced collagen deposition and de novo collagen synthesis but did not resolve already manifest fibrosis [14], while in a different study that also used 10 mg/kg OCA in Ldlr−/−.Leiden mice, we did not find a significant improvement on histological fibrosis [16]. These data suggest that the 10 mg/kg OCA dosage in this model is around the threshold of having a significant effect on hepatic fibrosis. For this reason, we also decided to test a higher dosage of OCA (20 mg/kg) in the current study. A group that received a chow diet (Ssniff Spezialdiäten, Soest, Germany) was included as a healthy reference.

The study was separated into two experiments. A short-term experiment was performed to evaluate the effects of the interventions on a fibrogenic gene signature, reflective of active fibrosis processes. A long-term experiment was conducted to evaluate the effects of the interventions on histological

endpoints, specifically to predict if the fibrogenic gene signature could predict the development of fibrosis pathology.

For the short-term experiment, 12–17 week old mice were first fed the HFD for 15 weeks to induce obesity-associated MASH. Then, at t = 0, treatment was started for 4 weeks, with $n = 8$ mice per group receiving either 20 mg/kg/d cenicriviroc, 10 mg/kg/d pioglitazone, 10 mg/kg/d OCA, 20 mg/kg/d OCA, or no treatment (HFD control). Appropriate group sizes were calculated a priori by power analysis (GPower [26]), using an average standard deviation/sigma of 15%, a minimal effect size of 25%, and a two-sided test with a 95% confidence interval, a power of 90%, and α of 0.05. After 4 weeks, mice were sacrificed non-fasted by gradual-fill CO_2 asphyxiation, and livers were weighed and collected for transcriptome analysis.

For the long-term experiment, 17–21 week old mice were first fed the HFD for 20 weeks to induce obesity and MASH. Then, at t = 0, treatment was started for 16 weeks with 20 mg/kg/d cenicriviroc ($n = 19$), 10 mg/kg/d pioglitazone ($n = 19$), 10 mg/kg/d OCA ($n = 18$) or no treatment (HFD control; $n = 20$). In addition, a healthy reference group of $n = 10$ mice on chow was included. Appropriate group sizes were calculated a priori by power analysis (GPower), using an average standard deviation/sigma of 23%, a minimal effect size of 25%, and a two-sided test with a 95% confidence interval, a power of 90%, and α of 0.05. After 16 weeks, mice were sacrificed after 5 h fasting by gradual-fill CO_2 asphyxiation, and livers were weighed and collected for histological analysis.

2.2 | Biochemical Analyses in Plasma and Liver

Blood was drawn at baseline and at the respective study endpoint after a 5 h fasting period from the tail vein and collected into EDTA-coated tubes for plasma isolation (Sarstedt, Nümbrecht, Germany). Blood glucose levels were measured at the time of blood sampling by using glucose strips and a hand analyzer (FreeStyle Lite, Abbott, Chicago, IL, USA). Plasma insulin was measured by enzyme-linked immunosorbent assay (ELISA) (#90080; Chrystal Chem, Elk Grove Village, IL, USA). Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was used to calculate insulin resistance. Five hours fasted plasma insulin and blood glucose values were used: HOMA-IR = ([insulin (ng/mL)] × [glucose (mM)])/22.5. Plasma cholesterol and triglyceride concentrations were determined using enzymatic colorimetric assays (Roche Diagnostics, Almere, the Netherlands). Plasma concentrations of alanine transaminase (ALT) and aspartate transaminase (AST) were measured using SimpleStep ELISA kits (Abcam, Cambridge, UK). Collagen content in the liver was measured in snap-frozen tissue of the left or medial liver lobe using a hydroxyproline-based colorimetric sensitive total collagen assay (Quickzyme, Leiden, the Netherlands) and expressed per mg total liver protein.

2.3 | Histological Assessment of MASH

Formalin-fixed and paraffin-embedded tissue of the left and medial liver lobe was cross-sectioned (3 μm) and stained with hematoxylin and eosin (H&E) or Sirius Red (SR). All histological analyses for the assessment of MASH were performed blindly by a board-certified pathologist using two liver slides per mouse and an adapted grading system for human MASH [27, 28]. H&E-stained cross-sections of the medial liver lobe were evaluated for macrovesicular and microvesicular steatosis at 40–100× magnification and expressed as a percentage of the total analyzed area. Inflammation in the liver was quantified by counting the number of aggregated inflammatory cells per field at 100× magnification (view size: 4.2 mm^2). For the scoring of inflammation, average scores of five random non-overlapping fields were taken and expressed per mm^2. SR-stained cross-sections of the left liver lobe were used to evaluate hepatic fibrosis. Two sections per mouse were evaluated by computerized analysis of collagen content and expressed as a percentage of the total liver surface area analyzed. Fibrosis stage was evaluated by the same board-certified pathologist that scored MASH in accordance with the protocol described by Tiniakos et al. [29]. This protocol classifies fibrosis stage as F0 (absence of fibrosis), F1 (fibrosis in the perisinusoidal or periportal area), F2 (fibrosis within both perisinusoidal and periportal areas), F3 (bridging fibrosis), and F4 (cirrhosis).

2.4 | Gene Expression Analysis in Liver Tissue

Liver tissue of the right liver lobe was used for transcriptomics analysis in accordance with previously described protocols [14]. In short, RNA was isolated using the RNA-Bee total-RNA isolation kit (Bio-Connect, Huissen, the Netherlands) and evaluated for quality with the 2100 Bioanalyzer (Agilent Technologies, Amstelveen, the Netherlands). Samples were processed using the NEBNext Ultra II Directional RNA Library Prep Kit (NEB #E7760S/L; New England Biolabs, Ipswich, MA, USA). mRNA was isolated from the total RNA with oligo-dT magnetic beads. Next, cDNA was synthesized and subsequently ligated with sequencing adapters and amplified by PCR. Quality and yield were assessed with a Fragment Analyzer (Agilent Technologies, Amstelveen, the Netherlands). The size of the resulting product was consistent with the expected size distribution (a broad peak between 300 and 500 bp). RNA sequencing was performed to determine (RNA expression using the NovaSeq6000 v1.5, Illumina, San Diego, CA, USA) in accordance with the manufacturer's protocol by the service provider GenomeScan B.V. (Leiden, the Netherlands). The genome reference and annotation file Mus_musculus.GRCm38.gencode.vM19 was used for analysis. RNA reads were aligned to the reference sequence using the STAR 2.5 algorithm with default settings. These counts served as input for statistical analysis using the DEseq2 package. Differentially expressed genes (DEGs) were used as input for pathway analysis (adjusted p-value < 0.01) through the Ingenuity Pathway Analysis suite (IPA, Ingenuity Systems Inc., Redwood City, CA, USA; www.ingenuity.com). The datasets obtained in this study are accessible at the NCBI Gene Expression Omnibus (GEO-IDs: GSE290235 and GSE290237).

Subsequently, relative expression of fibrosis genes was evaluated based on a fibrogenic gene signature reflecting the active fibrosis process, which was identified by Van Koppen et al. [25]. These authors described a 232-gene expression signature that was selected based on correlation of hepatic gene expression

with newly formed collagens (measured by deuterated water labeling technique) synthesized the week before sacrifice in HFD-fed Ldlr−/−Leiden mice. Expression of all 232 genes of this signature was evaluated in the current study, and the relative expression of each gene was calculated for the treatment groups as compared to the HFD control group. Either the gene expression changes of HFD versus chow control group were set at 100% and compared with treatment group versus chow (Figure 4B) or the gene expression of HFD versus chow was compared with the gene expression of treatment versus HFD control group (Figure 4C).

2.5 | Statistical Analysis

Differences between groups were determined non-parametrically by Kruskal–Wallis testing followed by Mann–Whitney *U* testing for independent samples. All statistical analyses were performed in SPSS (version 25, ICM Corp., Armonk, NY, USA). Data are presented as mean ± standard error of the mean (SEM). Two-tailed *p*-values are reported, and a *p*-value <0.05 was considered statistically significant.

3 | Results

3.1 | Ldlr−/−.Leiden Mice Develop Obesity, Insulin Resistance and Hyperlipidemia in Response to High Fat Diet Feeding

Diet-induced obese Ldlr−/−.Leiden mice had a 30% higher body weight ($p<0.001$) compared to age-matched control mice fed a low-fat chow diet (Figure 1A). While blood glucose concentrations were similar in chow-fed and HFD-fed mice (Figure 1B), plasma insulin levels were significantly higher in the HFD-fed group (2.7-fold increase, $p<0.01$ vs. chow) (Figure 1C), resulting in a significantly higher HOMA-IR, reflective of insulin resistance on the HFD (HOMA-IR: 2.8-fold increase, $p<0.01$) (Figure 1D). In response to the HFD, mice developed hypercholesterolemia (2.7-fold increase, $p<0.001$ vs. chow) (Figure 1E) and hypertriglyceridemia (2.2-fold increase, $p<0.01$ vs. chow) (Figure 1F). Plasma concentrations of the hepatic damage marker ALT were significantly increased as well (3.4-fold increase, $p<0.01$ vs. chow) (Figure 1G) while the increase in plasma AST levels did not reach statistical significance (Figure 1H). These data indicate that 19 weeks of HFD feeding induced several characteristics of the metabolic syndrome, including obesity, insulin resistance, and hyperlipidemia in Ldlr−/−.Leiden mice.

3.2 | Prolonged High Fat Diet Feeding Induces MASH Development in Ldlr−/−.Leiden Mice

In parallel with the development of the obese phenotype and plasma characteristics of the metabolic syndrome, Ldlr−/−.Leiden mice on HFD developed MASH (Figure 2A). Nineteen weeks on the HFD resulted in a 2-fold increase in liver weight ($p<0.001$) compared to mice on the healthy chow diet (Figure 2B). Quantitative histological liver analysis revealed that while mice on the healthy chow diet did not develop any macrovesicular steatosis, it was abundantly present in mice on HFD (Figure 2C), as well as microvesicular steatosis (8.5-fold increase, $p<0.001$ vs. chow) (Figure 2D). In addition, while there was almost no inflammation in the livers of chow-fed mice, HFD feeding induced hepatic inflammation, as characterized by aggregates of inflammatory cells (Figure 2E). Hepatic fibrosis in chow-fed mice was largely absent while HFD feeding resulted in the onset of mild hepatic fibrosis (+71%, $p<0.001$ vs. chow) (Figure 2A,F). Further evaluation revealed that in HFD-fed mice, fibrosis was primarily located within the perisinusoidal and periportal areas (stage F2) with occasionally bridging fibrosis (stage F3) (Figure 2G).

3.3 | Long-Term Treatment Effects of Cenicriviroc, Pioglitazone, and Obeticholic Acid on MASH-Associated Fibrosis in Obese Ldlr−/−.Leiden Mice

In order to validate the accuracy of the fibrogenic gene signature as an early prediction for the efficacy of therapeutics against fibrosis, the long-term effects on histopathological endpoints of three therapeutics with ranging efficacy to improve MASH-associated fibrosis were evaluated. Thereto, Ldlr−/−.Leiden mice were fed a HFD for 20 weeks to induce obesity, MASH, and MASH-associated fibrosis, followed by 16 weeks of treatment with cenicriviroc, pioglitazone, or obeticholic acid (Figure 3A). HFD feeding induced a 1.8-fold increase ($p<0.001$) in liver weight compared to healthy chow controls (Figure 3B). Cenicriviroc tended to increase liver weight compared to HFD controls (+9%, $p=0.09$), while pioglitazone reduced liver weight (−10%, $p<0.05$) and obeticholic acid did not affect liver weight relative to untreated HFD controls (Figure 3B). The HFD induced a 9.9-fold increase ($p<0.001$) in macrovesicular steatosis and a 4.8-fold increase ($p<0.001$) in microvesicular steatosis (Figure 3C,D) compared to healthy chow-fed mice. Cenicriviroc did not affect HFD-induced macrovesicular steatosis, while pioglitazone and obeticholic acid significantly reduced macrovesicular steatosis (with −67% and −31%, respectively, both $p<0.01$ vs. HFD control) (Figure 3C). Cenicriviroc and obeticholic acid both significantly improved microvesicular steatosis (−12% and −12%, respectively, both $p<0.01$, vs. HFD control), while pioglitazone only tended to slightly reduce microvesicular steatosis (with 9%, $p=0.089$, vs. HFD control) (Figure 3D). The HFD feeding induced pronounced hepatic inflammation, as signified by the increased presence of aggregated inflammatory cells (Figure 3E). Cenicriviroc tended to increase hepatic inflammation (with +46%, $p=0.054$), while pioglitazone tended to reduce it (with −25%, $p=0.089$) and obeticholic acid significantly reduced it (with −69%, $p<0.001$) relative to HFD controls (Figure 3E).

To investigate the effects of the three interventions on histopathological fibrosis endpoints in the long-term experiment, collagen deposition was evaluated in Sirius Red-stained liver cross-sections (Figure 3A,F). A total of 36 weeks of HFD feeding induced a 3.2-fold increase in hepatic fibrosis, and qualitative analysis demonstrated that HFD feeding induced pronounced stage F2/3 fibrosis relative to healthy chow controls (all $p<0.001$) (Figure 3F,G). Cenicriviroc non-significantly worsened HFD-induced hepatic fibrosis (+53%, $p=0.054$), while

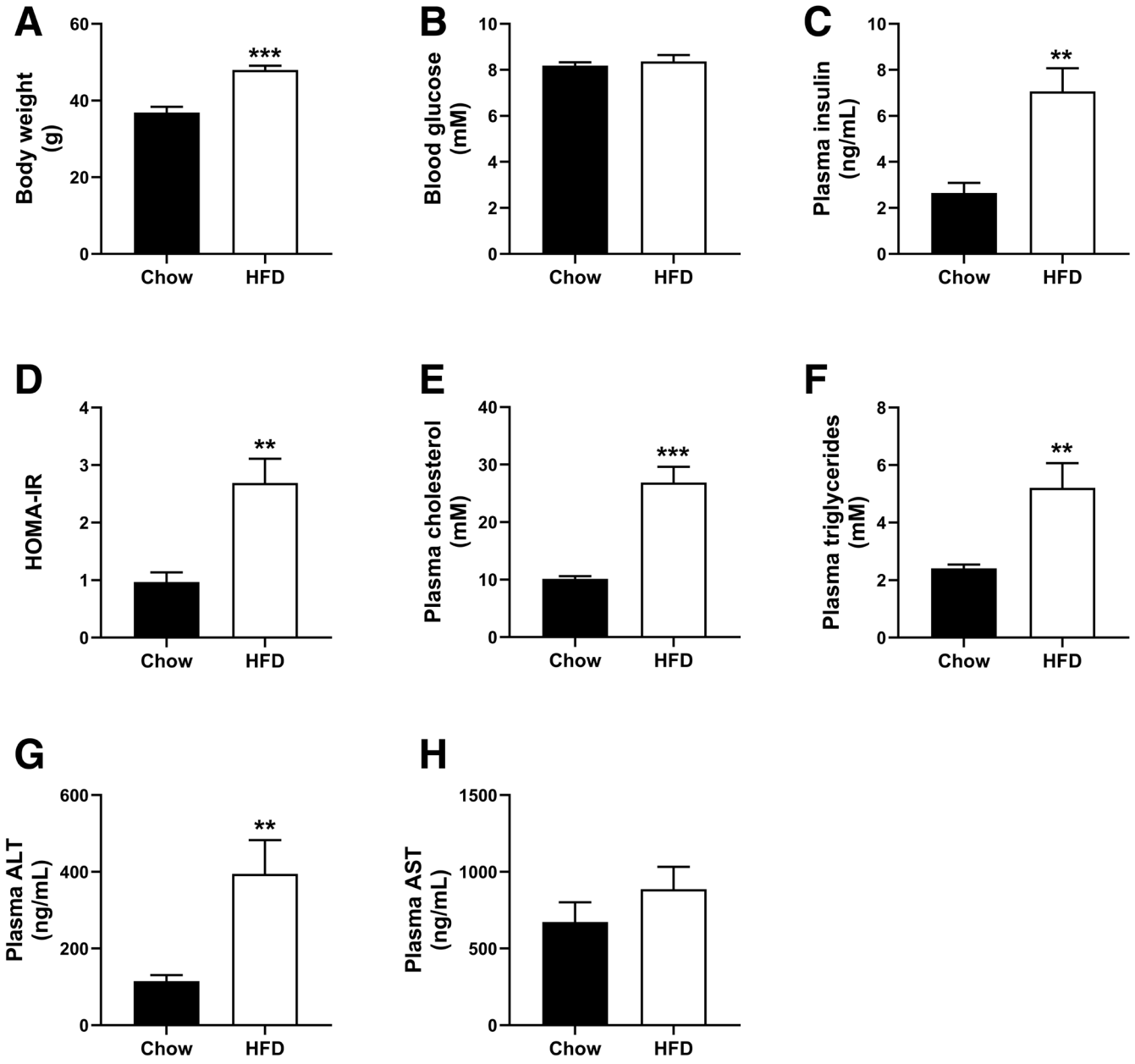


FIGURE 1 | High fat diet feeding in Ldlr−/−.Leiden mice induced development of obesity, insulin resistance and hyperlipidemia. Body weight (A), blood glucose (B), plasma insulin (C), homeostatic model assessment for insulin resistance (HOMA-IR) (D), plasma cholesterol (E), plasma triglycerides (F), plasma alanine transaminase (ALT) (G), and plasma aspartate transaminase (AST) (H) were determined after 19 weeks on the healthy chow diet ($n = 8$) or high fat diet (HFD; $n = 8$). Data are presented as mean ± SEM, $^{**}p < 0.01$, $^{***}p < 0.001$ versus chow.

pioglitazone and obeticholic acid did not significantly lower fibrosis measured as Sirius Red-positive percentage of surface area (Figure 3F). Subsequent evaluation of qualitative fibrosis analysis indicated that cenicriviroc-treated mice displayed more fibrosis in periportal and perisinusoidal areas (fibrosis stage +25%, $p < 0.05$) relative to untreated controls, while pioglitazone and obeticholic acid did not significantly affect fibrosis stage (Figure 3G). Subsequent biochemical measurement of hepatic collagen content revealed no significant effects of cenicriviroc and pioglitazone and a tendency towards a decrease in obeticholic acid-treated mice (−23%, $p = 0.082$) (Figure 3H). In aggregate, these results demonstrate a slight non-significant effect of obeticholic acid on hepatic fibrosis, no effect for pioglitazone, and rather adverse effect for cenicriviroc.

3.4 | Changes in Gene Expression After Short-Term Intervention With Cenicriviroc, Pioglitazone or Obeticholic Acid Are Predictive of Long-Term Histological Endpoints

In order to evaluate the accuracy of a fibrogenic gene signature as an early prediction for the efficacy of therapeutics for MASH-associated fibrosis, a short-term experiment was performed using cenicriviroc, pioglitazone, and obeticholic acid.

First, a pathway analysis was performed, demonstrating that HFD feeding induced 64 differentially expressed pathways (DEPs) compared to mice that received the healthy chow diet. After 4 weeks of treatment, the effects of the different

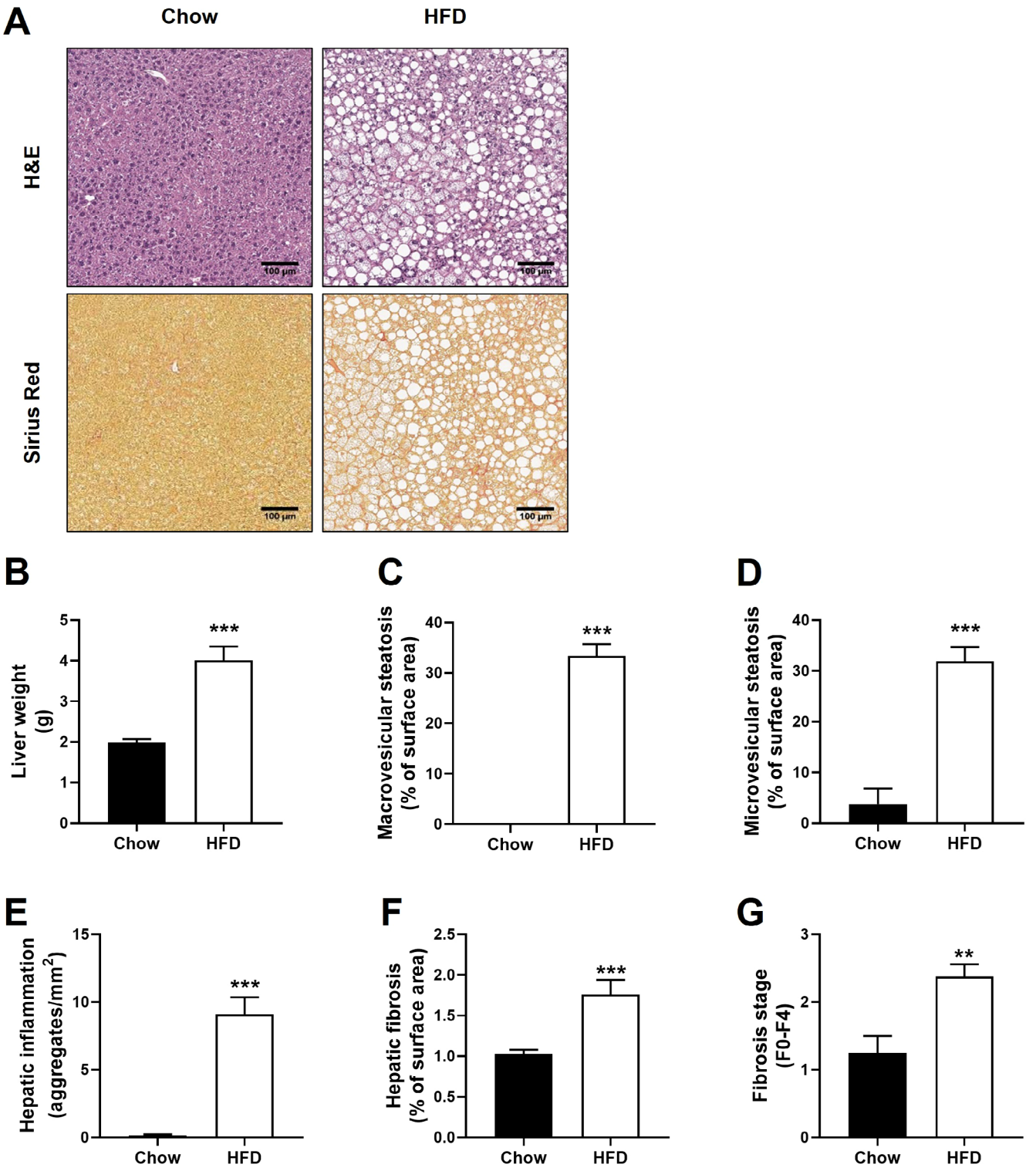


FIGURE 2 | High fat diet feeding induces development of MASH in Ldlr−/−.Leiden mice. Representative histological images of H&E-stained and Sirius Red-stained liver cross-sections (A), liver weight (B), macrovesicular steatosis (C), microvesicular steatosis (D), liver inflammation expressed as the number of inflammatory aggregates per mm^2 (E), hepatic fibrosis (F), and fibrosis stage (G) were determined after 19 weeks on the healthy chow diet ($n = 8$) or high fat diet (HFD; $n = 8$). Data are presented as mean ± SEM, $^{**}p < 0.01$, $^{***}p < 0.001$ versus chow.

therapeutics on DEPs were still limited compared to untreated HFD controls. For instance, there were only seven DEPs in cenicriviroc-treated mice (Figure 4A, blue circle), 14 DEPs in pioglitazone-treated mice (Figure 4A, gray circle) and 13 DEPs in obeticholic acid-treated mice (Figure 4A, green circle) compared to untreated HFD controls. For both cenicriviroc and pioglitazone, these pathways were involved in a variety of processes, including cholesterol biosynthesis and inflammation. For obeticholic acid, however, the most prominently affected pathway was the "bile acid and bile salt metabolism pathway," which was to be expected for a semi-synthetic bile acid analogue.

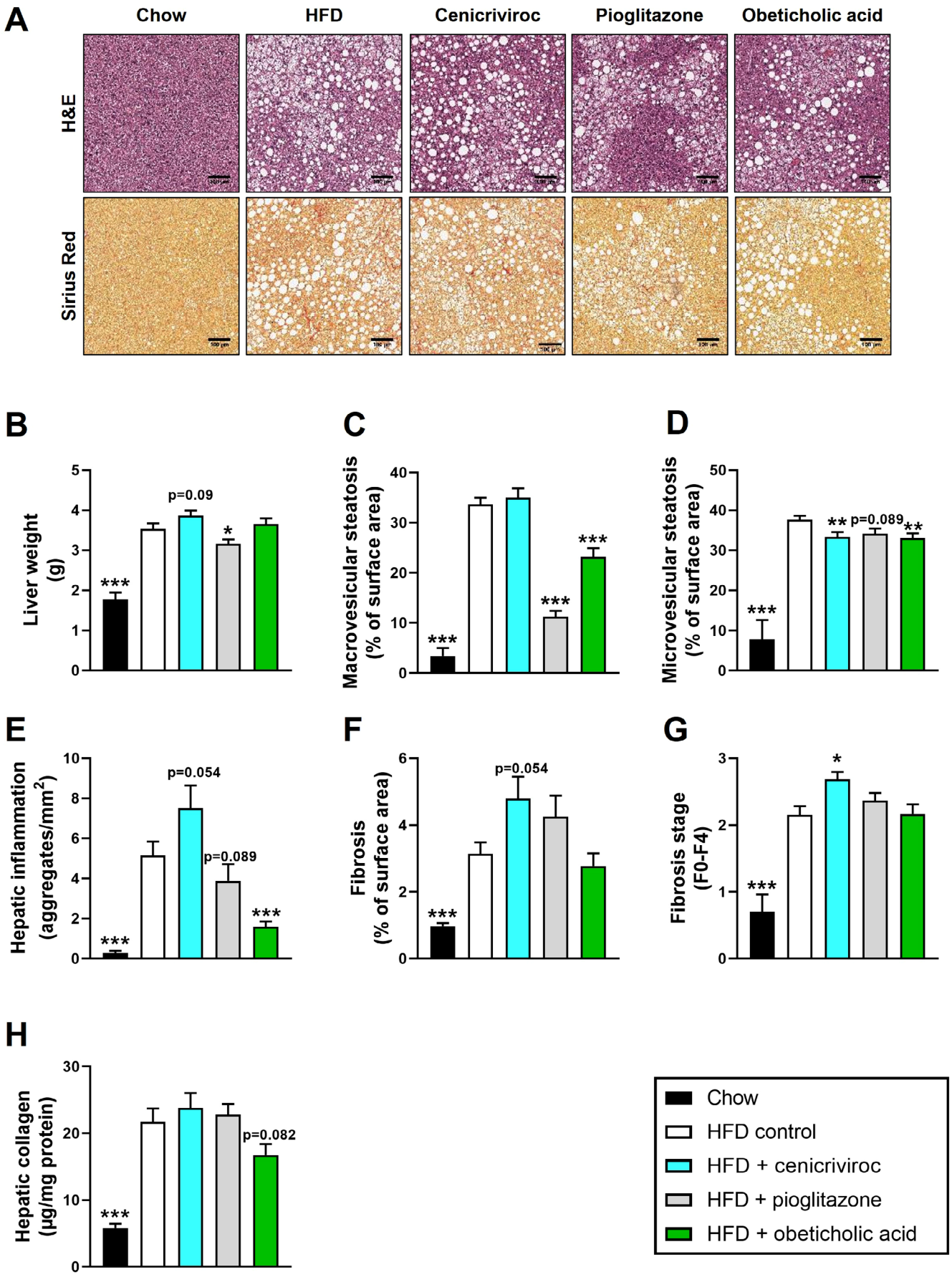


FIGURE 3 | Legend on next page.

FIGURE 3 | Long-term effects of cenicriviroc, pioglitazone and obeticholic acid on MASH in Ldlr−/−.Leiden mice. Representative histological images of H&E-stained and Sirius Red-stained liver cross-sections (A), liver weight (B), macrovesicular steatosis (C), microvesicular steatosis (D), liver inflammation expressed as the number of inflammatory aggregates per mm^2 (E), hepatic fibrosis (F), fibrosis stage (G), and hepatic collagen content (H) were determined after 19 weeks on the healthy chow diet ($n = 8$) or high fat diet (HFD; $n = 8$). Mice on HFD received either no intervention (HFD control) or HFD supplemented with cenicriviroc, pioglitazone or obeticholic acid. Data are presented as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus HFD control.

Subsequently, the relative expression of fibrosis-associated genes was evaluated based on the fibrogenic gene signature described by Van Koppen et al. [25] that correlated with the onset of fibrosis in the context of MASH. Expression of all 232 genes comprised in this signature was evaluated in the current study and group average expression was plotted against the average expression of these genes in the HFD control group. First, for each gene the expression changes of HFD versus chow control group was set at 100% and subsequently compared with treatment group versus HFD control and results are shown as a box and whisker plot with 2.5–97.5 percentile whiskers (Figure 4B). Relative to HFD controls, cenicriviroc and pioglitazone did not have any effects on the fibrogenic gene signature. In contrast, obeticholic acid in either a low or high dose shifted the fibrogenic gene signature towards a state indicating less fibrosis compared to the HFD control group. As an example of how these signature genes were affected by the different interventions, a heatmap with 2log-fold-changes of the top genes upregulated by the HFD versus chow is shown (cut-off at 2logFC ≥ 2.0) and compared with the gene expression changes of the different interventions versus HFD controls (Figure 4C). Among these genes with increased expression were several genes related to fibrosis, including COL1A1, COL1A2, COL3A1, and COL5A2, all upregulated by HFD feeding. Effects of cenicriviroc and pioglitazone on expression of these collagens was minimal while obeticholic acid, both in low and high dose, reduced their expression compared to HFD controls. These data demonstrate that already after 4 weeks of treatment, changes in gene expression patterns are detectable and in line with the effects observed on histological fibrosis endpoints determined after long-term treatment.

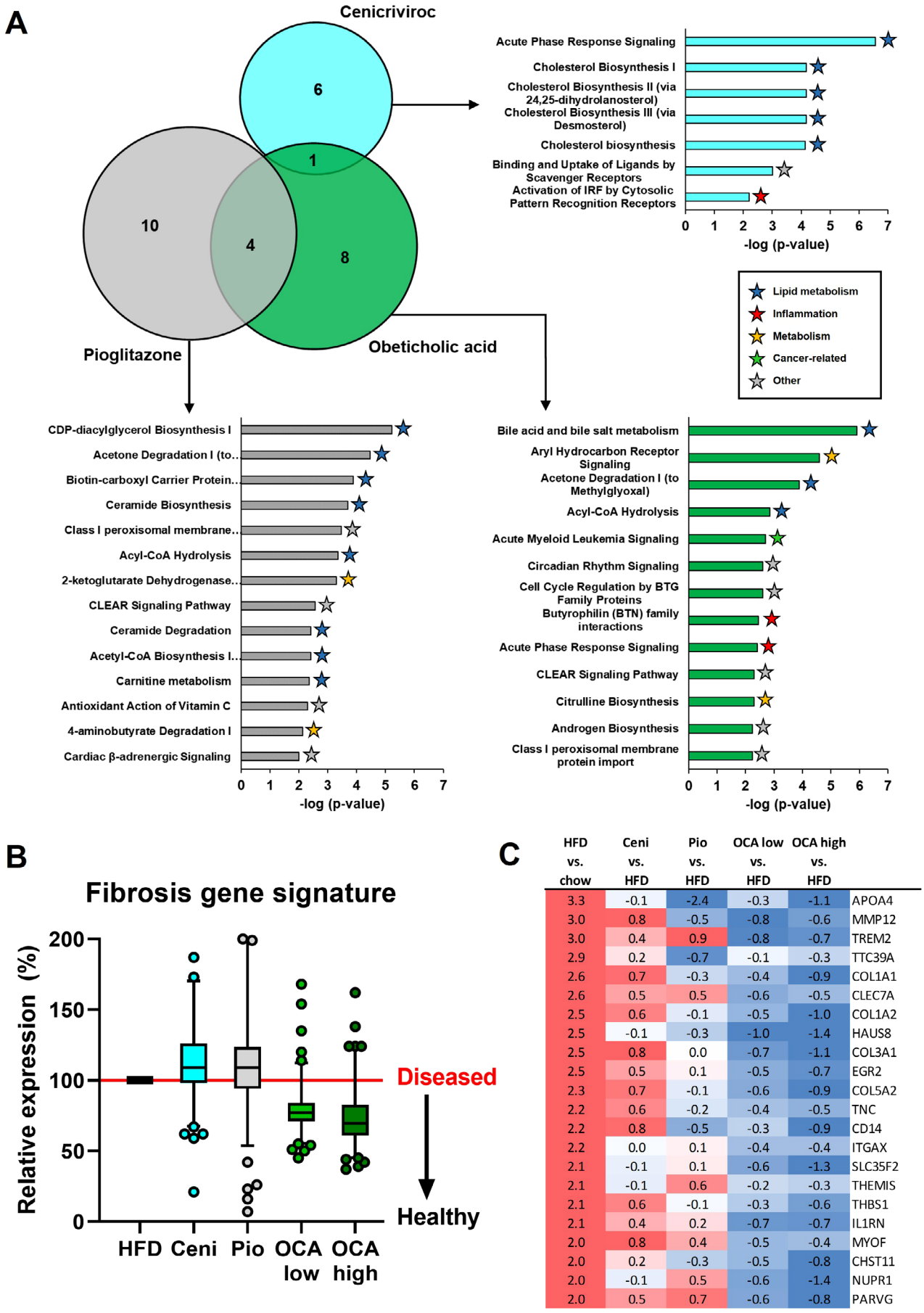
4 | Discussion

The increasing prevalence of MASH-associated liver fibrosis accentuates the need for the development of pharmaceutical interventions. To accelerate drug development, discrepancies between preclinical and clinical efficacy should be addressed to increase the therapeutic success rate. Despite numerous efforts in developing novel MASH compounds, many have failed in clinical stages, thereby raising concerns about the translatability of preclinical findings. In the context of MASH, improvements in fibrosis have been particularly difficult to achieve. In this study, we evaluated whether a previously established fibrogenic gene signature [25] can be used as a rapid and early tool for screening drug efficacy on liver fibrosis, using a translational MASH mouse model.

For this study, we used a reported gene expression signature identified by Van Koppen et al. [25]. This signature was selected based on the correlation of hepatic mRNA with newly formed matrix proteins (including the collagens critical for fibrosis) measured using a deuterated water labeling technique and already detectable before fibrosis deposition could be noticed. This fibrogenic gene signature was tested herein to examine its utility as an early screening tool for drug efficacy using diet-induced obese Ldlr−/−.Leiden mice. More specifically, we evaluated whether cenicriviroc, pioglitazone, and obeticholic acid, that is, three compounds with varying efficacy in preclinical and clinical studies, would affect this fibrogenic gene signature after only 4 weeks of treatment. We demonstrate that after this relatively short treatment period, changes in gene expression patterns are predictive of the long-term outcomes of these three compounds on histological fibrosis endpoints, thus showing that a fibrogenic gene signature can be used for early efficacy analysis.

Cenicriviroc, a CCR2 and CCR5 antagonist, was originally hypothesized to alleviate MASH, as the CCR2 and CCR5 receptors are involved in the development of hepatic steatosis and inflammation [30, 31]. Indeed, in preclinical and clinical phase I and II studies, cenicriviroc improved liver steatosis and inflammation and in initial studies was shown to ameliorate fibrosis as well [4, 5, 32, 33]. Nevertheless, recently published results from the phase III AURORA trial showed that cenicriviroc lacked efficacy and did not reach the primary endpoint, that is, improved fibrosis of ≥ 1 stage [10]. In line with these data, long-term intervention with cenicriviroc in the Ldlr−/−.Leiden mouse model did not improve hepatic steatosis, inflammation, or fibrosis and even worsened some of these MASH histological parameters. In mice that received short-term cenicriviroc intervention, the relative expression of genes in the fibrogenic gene signature by Van Koppen et al. [25] was not changed in comparison to untreated HFD controls. These data demonstrate that the lack of efficacy of cenicriviroc on fibrosis could be predicted in a preclinical study after a short-term intervention period using a translational mouse model.

Pioglitazone is a PPAR-γ agonist and to a lesser extent an agonist of PPAR-α as well. Due to its effects on glycemic control and lipid metabolism, pioglitazone is used to treat type II diabetes mellitus [34]. Moreover, pioglitazone was demonstrated to improve hepatic steatosis in a MASLD patient population [11]. However, similar to cenicriviroc, pioglitazone did not improve hepatic fibrosis in these patients [11]. This is in line with our findings, where pioglitazone did not improve hepatic fibrosis relative to untreated controls after long-term intervention. When comparing the fibrosis gene expression signature after only 4 weeks of pioglitazone treatment, we found that the expression of this fibrogenic gene signature was similar to that of HFD controls. These data confirm that gene expression patterns after a short-term intervention with pioglitazone, similar to cenicriviroc, are



HFD vs. chow	Ceni vs. HFD	Pio vs. HFD	OCA low vs. HFD	OCA high vs. HFD	
3.3	-0.1	-2.4	-0.3	-1.1	APOA4
3.0	0.8	-0.5	-0.8	-0.6	MMP12
3.0	0.4	0.9	-0.8	-0.7	TREM2
2.9	0.2	-0.7	-0.1	-0.3	TTC39A
2.6	0.7	-0.3	-0.4	-0.9	COL1A1
2.6	0.5	0.5	-0.6	-0.5	CLEC7A
2.5	0.6	-0.1	-0.5	-1.0	COL1A2
2.5	-0.1	-0.3	-1.0	-1.4	HAUS8
2.5	0.8	0.0	-0.7	-1.1	COL3A1
2.5	0.5	0.1	-0.5	-0.7	EGR2
2.3	0.7	-0.1	-0.6	-0.9	COL5A2
2.2	0.6	-0.2	-0.4	-0.5	TNC
2.2	0.8	-0.5	-0.3	-0.9	CD14
2.2	0.0	0.1	-0.4	-0.4	ITGAX
2.1	-0.1	0.1	-0.6	-1.3	SLC35F2
2.1	-0.1	0.6	-0.2	-0.3	THEMIS
2.1	0.6	-0.1	-0.3	-0.6	THBS1
2.1	0.4	0.2	-0.7	-0.7	IL1RN
2.0	0.8	0.4	-0.5	-0.4	MYOF
2.0	0.2	-0.3	-0.5	-0.8	CHST11
2.0	-0.1	0.5	-0.6	-1.4	NUPR1
2.0	0.5	0.7	-0.6	-0.8	PARVG

FIGURE 4 | Legend on next page.

FIGURE 4 | Early changes in hepatic transcriptomics profile in Ldlr−/−.Leiden mice on HFD treated with cenicriviroc, pioglitazone or obeticholic acid. Venn diagram (A) showing the overlap of differentially expressed pathways (DEPs) in the liver. Diet-induced obese mice received 4 weeks of high fat diet (HFD) feeding supplemented with cenicriviroc (blue circle), pioglitazone (gray circle) or obeticholic acid (green circle). DEPs per intervention are specified in (A). A gene signature of Ldlr−/−.Leiden mice that received 19 weeks of HFD feeding was used, in which 232 genes are described that predict MASH-associated fibrosis [25]. Expression of the genes comprising this signature was evaluated and for each gene, expression changes of the HFD control group versus chow control group was set at 100%. Treatment groups were compared to HFD controls and shown as box and whisker plot with 2.5–97.5 percentile whiskers (B). A heatmap (C) showing 2log fold changes (2logFC) of the top genes upregulated in the HFD group versus chow control and 2logFC of the same genes in treatment groups. Red color indicates upregulation and blue color indicates downregulation. Ceni, cenicriviroc; OCA, obeticholic acid; pio, pioglitazone.

already predictive of the long-term effects of the same investigative drugs on histological fibrosis endpoints.

Like cenicriviroc and pioglitazone, the FXR antagonist obeticholic acid was shown to ameliorate hepatic steatosis and inflammation during preclinical [14, 35, 36] and clinical [15, 37, 38] stages. As a semi-synthetic bile acid analogue, obeticholic acid functions through FXR signaling in the liver and gut [39, 40]. In preclinical studies, the antifibrotic properties of obeticholic acid have been demonstrated [13, 35, 36, 41], leading to investigation of the investigative drug in clinical trials. In a phase II clinical study, MASH patients received obeticholic acid or placebo and after 72 weeks of treatment, there were significant improvements in hepatic steatosis and inflammation as well as fibrosis [38]. In contrast with results reported for cenicriviroc and pioglitazone, the antifibrotic effects of obeticholic acid were confirmed in a phase III clinical trial, where it resulted in a significant increase in the proportion of patients achieving a ≥ 1 stage improvement in qualitative fibrosis [15]. However, due to concerns such as increased LDL cholesterol levels, potential cardiovascular events and side effects including pruritus, obeticholic acid was disapproved as MASH therapeutic [38]. In the current study, Ldlr−/−.Leiden mice that received long-term intervention with obeticholic acid showed improvements in hepatic steatosis and inflammation. Compared to HFD controls, we did not find a significant effect of obeticholic acid on histologically measured fibrosis, while hepatic collagen content tended to be reduced. Nevertheless, after a short-term intervention period, relative expression of genes comprising the fibrosis gene signature showed a shift towards a less fibrogenic state compared to untreated controls. In line with these data, obeticholic acid was shown to reduce fibrosis progression and de novo collagen synthesis in a different study [14]. In summary, in contrast to cenicriviroc and pioglitazone that failed to improve fibrosis in phase III clinical studies, obeticholic acid has been shown in a phase III clinical trial to ameliorate hepatic fibrosis. Here, we have demonstrated that changes in expression of a fibrogenic gene signature after only 4 weeks of drug treatment and prior to manifestation of histological fibrosis predicted these ameliorative effects of obeticholic acid on fibrosis.

Because of the heterogeneity of the disease, it is challenging to accurately reflect human MASLD in a preclinical model. It has been shown that high fat diet-induced models are associated more closely with human fatty liver disease than other genetically modified models [21]. While ob/ob mice and db/db mice are obese and insulin resistant, they lack leptin or the leptin receptor and are therefore less representative for the human population. In contrast, the lack of Ldlr expression in the model used here more accurately recapitulates the hyperlipidemia that is often observed in human MASLD patients. The use of this model combined with high fat diet feeding results in disease progression that is well-documented, reflecting hyperlipidemia, liver histopathology and underlying pathways characteristic for human MASLD [14, 16, 17, 23]. In our view, the combination of the reported fibrogenic gene signature with this model-diet combination constitutes a powerful tool for rapid screening of novel MASH/fibrosis drugs and drug combinations regarding their efficacy on hepatic fibrosis. The gene signature was identified in this model using deuterated water labeling technique and was selected unbiased based on active fibrosis [25], which appears to be not present in standard mouse models of disease, like the Western-type diet-fed C57BL/6 mice [24]. Notably, the diet-induced obese Ldlr−/−.Leiden mouse model has been shown to represent the underlying disease pathways of human MASH patients and reflects patients on the level of the liver transcriptome and plasma metabolome [14, 16, 23, 24]. Furthermore, in contrast to many other MASH mouse models, Ldlr−/−.Leiden mice do not require supranormal levels of cholesterol supplemented in the diet to induce liver inflammation and fibrosis and it develops cardiovascular disease as well, the major cause of mortality in MASH patients [23]. We realize that each animal model has its limitations and we did not herein test the predictive value of this fibrogenic gene signature in other mouse models. In more classical MASLD mouse models, false positive effects of cenicriviroc have been reported. Therefore, we expect that the use of a translational MASH model that includes the key pathogenic mechanisms of MASLD, for example, lipid- and inflammation-induced insulin resistance and associated dyslipidemia (e.g., high triglycerides and high LDL cholesterol), is a prerequisite for the current screening approach. The ability to determine compound efficacy on fibrosis after a short treatment period holds great potential for both preclinical and clinical trials. The fibrogenic gene signature has recently been translated into a blood-based biomarker panel based on transcriptomics analysis of liver biopsies and protein levels in plasma samples of patients with MASLD-related fibrosis. This biomarker panel has been validated in two independent cohorts of patients with histologically confirmed MASLD [24]. This study indicated that the same approach as described in the current study in a preclinical setting might be applied in clinical trials, which will guide earlier decisions on continuation of the trial.

Unlike for example biomarkers, determination of a gene signature as the one used here does not have a clear cut-off value. In the current study, we deliberately chose to include compounds that failed in clinical trials to study how these would affect gene expression patterns. Since it is well known that gene expression

patterns can change long before physiological or histological changes become apparent, it might have been possible that these failed compounds still had a beneficial effect on this gene signature. Indeed, for individual genes, beneficial effects were observed upon treatment with cenicriviroc and pioglitazone, but clearly the signature as a whole (Figure 4B) did not shift towards a less fibrogenic state, indicative of a healthier condition. In the current study, long-term treatment with a relatively low dose of OCA resulted in a tendency to improved hepatic collagen content and failed to improve histologically measured fibrosis. In a previous study using 10 mg/kg OCA in Ldlr−/−.Leiden mice, we found that OCA reduced collagen deposition and de novo collagen synthesis, but again did not resolve already manifest fibrosis [14], while in another study that also used 10 mg/kg OCA in Ldlr−/−.Leiden mice we did find a significant improvement on histological fibrosis assessment [16]. This suggests that the 10 mg/kg OCA dose is around the threshold of having a significant effect on hepatic fibrosis after long-term treatment. Although OCA-treated mice may exhibit changes in collagen deposition, these changes might not yet translate into histological fibrosis effects. However, extending the treatment duration could potentially result in observable effects on histological fibrosis. Many other therapeutics with effects on MASH-associated fibrosis such as resmetirom and semaglutide are yet to be tested in a short-term study using this fibrogenic gene signature. Nevertheless, we expect that new therapeutics or combination therapies that show similar ameliorations in gene expression patterns after a short-term intervention as in the current study will be effective against MASH-associated fibrosis in the long term.

In summary, in the current study we evaluated whether changes in fibrogenesis-related gene expression patterns after a short-term intervention period could predict the long-term effects of cenicriviroc, pioglitazone, and obeticholic acid on histological fibrosis endpoints. In line with phase III clinical studies, we found no effects of cenicriviroc and pioglitazone on this fibrogenic gene signature, while obeticholic acid shifted gene expression patterns to a healthier, less fibrogenic state in line with histology. Application of this fibrogenic gene signature in the HFD-fed Ldlr−/−.Leiden mouse model can therefore be used as a rapid and early screening tool to estimate the efficacy of drugs and their combinations on liver fibrosis, prior to the manifestation of fibrosis on a histological level.

Author Contributions

José A. Inia, Martine C. Morrison, Robert Kleemann, and Anita M. van den Hoek: conceptualization. **José A. Inia and Anita M. van den Hoek:** formal analysis. **Martien P. M. Caspers, Aswin L. Menke, Nicole Worms, and Lars Verschuren:** investigation. **Martien P. M. Caspers and Lars Verschuren:** data curation. **José A. Inia and Anita M. van den Hoek:** writing – original draft. **José A. Inia, Martine C. Morrison, Arianne van Koppen, Eveline Gart, Martien P. M. Caspers, Aswin L. Menke, Nicole Worms, Robert Kleemann, Lars Verschuren, J. Wouter Jukema, Hans M. G. Princen, Roeland Hanemaaijer, and Anita M. van den Hoek:** writing – review and editing. **José A. Inia and Anita M. van den Hoek:** visualization. **José A. Inia and Anita M. van den Hoek:** supervision. **Anita M. van den Hoek:** project administration.

Acknowledgments

We thank Nikki van Trigt, Simone van der Drift, Marijke Voskuilen and Joline Attema for their excellent technical assistance. Furthermore, we would like to acknowledge that the graphical abstract was made possible by Biorender.com, accessed in February 2025.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Transcriptomics data are available at the NCBI Gene Expression Omnibus (GEO) under dataset numbers GSE290235 and GSE290237.

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