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A Simple Diet- and Chemical-Induced Murine NASH Model with Rapid Progression of Steatohepatitis, Fibrosis and Liver Cancer

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Abstract

Background and Aims—Although the majority of patients with nonalcoholic fatty liver disease (NAFLD) have only steatosis without progression, a sizable fraction develop non-alcoholic steatohepatitis (NASH), which can lead to cirrhosis and hepatocellular carcinoma (HCC). Many established diet-induced mouse models for NASH require 24–52 weeks, which makes testing for drug response costly and time consuming.

Methods—We have sought to establish a murine NASH model with rapid progression of extensive fibrosis and HCC by using a western diet (WD), which is high-fat, high-fructose and high-cholesterol, combined with low dose weekly intraperitoneal carbon tetrachloride (CCl₄), which served as an accelerator.

Results—C57BL/6J mice were fed a normal chow diet (ND) ± CCl₄ or WD ± CCl₄ for 12 and 24 weeks. Addition of CCl₄ exacerbated histological features of NASH, fibrosis, and tumor development induced by WD, which resulted in stage 3 fibrosis at 12 weeks and HCC development at 24 weeks. Furthermore, whole liver transcriptomic analysis indicated that

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dysregulated molecular pathways in WD/CCl₄ mice and immunologic features were closely similar to those of human NASH.

Conclusions—Our mouse NASH model exhibits rapid progression of advanced fibrosis and HCC, and mimics histological, immunological and transcriptomic features of human NASH, suggesting that it will be a useful experimental tool for preclinical drug testing.

Lay summary—A carefully characterized model has been developed in mice that recapitulates the progressive stages of human fatty liver disease, from simple steatosis, to inflammation, fibrosis and cancer. The functional pathways of gene expression and immune abnormalities in this model closely resemble human disease. The ease and reproducibility of this model makes it ideal to study disease pathogenesis and test new treatments.

Graphical Abstract

Keywords

NAFLD; NASH; steatohepatitis; fatty liver disease models; hepatic stellate cells; fibrosis; liver cancer; hepatocellular carcinoma; transcriptomics; insulin resistance; gene expression

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a rising cause of chronic liver disease worldwide. Although the majority of patients with NAFLD have only steatosis without progression, a sizable fraction develop non-alcoholic steatohepatitis (NASH), which can lead to cirrhosis, hepatocellular carcinoma (HCC), and increased liver-related mortality¹. The prevalence of NAFLD in the US population is estimated at ~24% (or ~65 million) and up to a third of these have NASH^{2,3}. The prevalence of NAFLD is steadily increasing in parallel to the rising prevalence of obesity. NAFLD/NASH is already the third leading indication for liver transplantation, and the second cause for HCC leading to liver transplantation in the US⁴, and it is likely to be the leading indication for transplantation by 2020.

Histologically, NASH is characterized by the presence of steatosis, inflammation, hepatocyte injury (ballooning), and/or fibrosis¹. Key risk factors include diabetes, obesity, age, ethnicity, gender, and genetic polymorphisms that can affect natural history and disease progression. These divergent risk factors reflect the complex and heterogeneous nature of the disease.

Despite the growing public health impact of NASH, treatment options remain limited and there are no FDA-approved therapies. One obstacle to drug development has been the paucity of standardized and relevant animal models. Although there are several dietary and

genetic models of NASH described, few, if any, replicate all the metabolic, histologic and genetic features of the human disease⁵. Mice are generally preferable because they are easy to handle and suitable for drug testing. Also, dietary models are preferred because genetic models often induce NASH by manipulating one specific molecule/pathway. For example, a methionine/choline deficient (MCD) diet has traditionally been used to induce histological features of NASH; however, mice treated with MCD diet lose body weight, and do not develop insulin resistance and related co-morbidities^{6,7}. A western diet (WD), which is high-fat, high-fructose and high-cholesterol, mimics fast food style diets that have been implicated in NASH pathogenesis in humans⁸. WD treatment induces obesity and insulin resistance in addition to NASH histology. However, the WD-based models do not fully progress to severe steatohepatitis and advanced fibrosis, even after long-term feeding for 25 to 52 weeks^{8,9}.

Carbon tetrachloride (CCl₄) has been widely used for decades to induce liver injury and fibrosis in mice¹⁰. A previous report has suggested that multiple administrations of CCl₄ with a high-fat diet induce oxidative stress that triggers inflammation and apoptosis, leading to the development of fibrosis in mice¹¹. Also, combined chronic treatment with CCl₄ and choline-deficient L-amino-acid-defined-diet for up to 9 months can induce NASH with fibrosis, and HCC¹². However, these reports have lacked blinded quantitative assessment of the three features of the NAFLD activity score, which is used routinely in human studies (steatosis, lobular inflammation, and hepatocyte ballooning); moreover, the fibrosis stage was not quantified or fully characterized. Importantly, the effects of chronic treatment with CCl₄ combined with a WD have not been assessed as a potential model for NASH.

Herein we have sought to establish a murine NASH model which leads to rapid and reproducible progression of steatohepatitis, with extensive fibrosis and hepatocellular carcinoma, and closely replicates the transcriptomic hallmarks of human NASH. Using a WD and CCl₄ we have generated all the key metabolic and histologic features of human NASH within 12 weeks, with consistent development of HCC by 24 weeks.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor). Five mice per cage were housed in a 12h light – 12 h dark cycle. All procedures were performed according to protocols approved by the Animal Care and Use Committee of Icahn School of Medicine at Mount Sinai (IACUC-2015-0112).

Dietary and CCl₄ treatment

Mice (9 weeks old) were fed a normal chow Diet (ND, Lab diet, Rodent diet20, #5053) and normal tap water or Western Diet (WD) containing 21.1% fat, 41% Sucrose, and 1.25% Cholesterol by weight (Teklad diets, TD. 120528) and a high sugar solution (23.1g/L d-fructose (Sigma-Aldrich, G8270) and 18.9 g/L d-glucose (Sigma-Aldrich, F0127)). CCl₄ (Sigma-Aldrich, 289116-100ML) at the dose of 0.2 µl (0.32 µg)/g of body weight, which is much lower than the dose that is usually given for fibrosis induction with CCl₄ alone¹⁰, or

its control vehicle, corn oil was injected intra-peritoneally once/week, starting simultaneously with the diet administration. Experimental groups were as follows: ND and oil (ND/Oil, 5 mice per group), WD and oil (WD/Oil, 10 mice per group), ND and CCl₄ (ND/CCl₄, 10 mice per group), and WD and CCl₄ (WD/CCl₄, 9 mice for 12 weeks and 10 mice for 24 weeks) treatment for 12 and 24 weeks. Mice were euthanized at 12 and 24 weeks by exsanguination after ketamine and xylazine anesthesia. Liver and serum samples were collected and processed for histological, serological and gene expression analysis.

Liver histology

Formalin-fixed, paraffin-embedded liver sections were stained with hematoxylin and eosin (H&E) for assessment of liver histology, with Sirius Red (Sigma, 365548-5G)/Fast Green (Sigma, F258) for assessment of fibrosis, and with periodic acid-Schiff (PAS) for assessment of glycogen accumulation. NAFLD Activity Score (NAS) and fibrosis stage were evaluated by an expert pathologist according to the NASH CRN scoring system¹³. The histological scoring was performed blinded, with no knowledge by the pathologist of the treatment(s) received.

Liver histology of NASH patients

Anonymized and de-identified liver sections from four adult patients with NASH were analyzed. Pictures were taken from representative areas showing steatosis, lobular inflammation and hepatocyte ballooning with Mallory-Denk bodies in consultation with a pathologist.

Immunohistochemistry

Formalin-fixed, paraffin-embedded liver sections were incubated with primary antibodies against α SMA (Abcam, ab5694), desmin (Abcam, ab15200), CK-19 (Abcam, ab15463), and Ki67 (Abcam, ab15580). Quantitative morphometry was performed using computerized Life Science morphometry system (BIOQUANT) on equal number of pictures per mouse (10 or 20 images) under constant magnification (100 x or 200 x magnification) as indicated in the respective experiment. Immunofluorescence analysis for MIC1-1C3 (Thermoscientific #MA5-16136) was performed on fresh frozen liver sections which were fixed with acetone and incubated with MIC1-1C3 antibody and fluorescently-labeled secondary antibody. CD3 (Leica, PA0122), CD4 (Leica, PA0427), CD8 (Leica, PA0183), and CK8/18 (Dako, IR094) immunostainings were performed on randomly selected livers from 12-week and/or 24-week mice. Four-micron thick sections were stained using the Leica AutoStainer Universal Staining System.

Immunoblotting

Fresh frozen livers were homogenized using TissueLyser (Qiagen) in RIPA buffer containing protease inhibitors (Thermoscientific Pierce complete protease inhibitors, 1 tablet/10 ml RIPA buffer). Bradford protein quantification was performed (Biorad Protein assay) and 30 μ g protein analyzed by SDS-PAGE and immunoblotting with antibodies to Collagen 1 (Bioss, bs10423R), α -smooth muscle actin (Abcam, ab5694) and GAPDH (EMD Millipore, CB1001).

Serum analysis

Serum ALT, AST, total cholesterol and triglyceride levels were measured using VITROS 5,1 FS (Ortho Clinical Diagnostics). Non-fasting plasma insulin was measured with the Ultrasensitive Mouse Insulin ELISA kit (Crystal Chem, 90080) according to the manufacturer's instructions. Non-fasting blood glucose was assayed with the One Touch Ultra (Life Scan). In animals receiving CCl₄, blood was analyzed 7 days after the most recent administration. HOMA IR and QUICKI were calculated as described^{14,15}.

Quantitative Polymerase Chain Reaction

RNA was extracted from liver tissues and purified (RNeasy Kit, Qiagen), and 1 µg of total RNA was reverse-transcribed into complementary DNA using RNA to cDNA EcoDryTM (Clontech, 639548). Expression levels were determined by quantitative polymerase chain reaction (PCR) (iQTM SYBR Green Supermix, Bio-Rad Laboratories, 1708884) on the LightCycler 480 Real-Time PCR System (Roche). The relative expression of target genes was normalized by *β-actin* expression as an internal control. *β-actin* was measured by using the 5'-GCTGTATTCCCCTCCATCGTG-3', forward and 5'-CACGGTTGGCCTTAGGGTTCAG-3', reverse primers. *Tgf-β* was measured by using the 5'-TGACGTCAGTGGAGTTGTACGG-3', forward and 5'-GGTTCATGTCATGGATGGTGC-3', reverse primers. *β-Pdgfr* was measured by using the 5'-ACTACATCTCCAAAGGCAGCACCT-3', forward and 5'-TGTAGAACTGGTCGTTTCATGGGCA-3', reverse primers. *Collagen 1 α 1* was measured by using the 5'-GTCCCTGAAGTCAGCTGCATA-3', forward and 5'-TGGGACAGTCCAGTTCTTCAT-3', reverse primers. *Timp-1* was measured by using the 5'-CAGTAAGGCCTGTAGCTGTGC-3', forward and 5'-CTCGTTGATTTCTGGGGAAC-3', reverse primers.

Transcriptome profiling

Global transcriptome profiling of the mouse liver and HCC tumor tissues was performed by RNA-Seq. Briefly, sequencing library was prepared using 200 ng total RNA by TruSeq library prep kit (Illumina) following manufacturer's protocol, and resulting data were preprocessed by our custom preprocessing pipeline. Briefly, raw sequencing reads were aligned to mouse reference genome (mm10) by STAR 2-pass algorithm¹⁶, and gene expression levels were calculated as count per million (CPM) normalized by trimmed mean of M-values (TMM) method¹⁷. The dataset is available at NCBI Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) accession number GSE99010.

Bioinformatics data analysis

Molecular pathway dysregulation in the liver tissues was determined by Gene Set Enrichment Analysis (GSEA)¹⁸, surveying molecular pathway gene sets in Molecular Signature Database (MSigDB) (www.broadinstitute.org/msigdb) (Supplementary Table 1). Cross-species comparison of transcriptomic dysregulation was performed in the space of molecular pathway gene sets from Hallmark and KEGG databases¹⁹ and with statistically significant dysregulation defined as false discovery rate (FDR) <0.01 in either of the two human NASH liver transcriptome datasets: advanced (n=30) vs. mild (n=42) NAFLD

patients (GSE49541)²⁰, and NASH patients (n=18) vs. healthy individuals (n=14) (GSE48452)²¹. Dysregulation of the selected gene sets was similarly determined by GSEA in a panel of 16 previously published diet, chemical, and/or genetic NASH mouse models (Supplementary Table 2), and similarity to the human datasets was determined by Euclidean distance and visualized as previously described²². Similarity of HCC tumor transcriptome dysregulation to human HCC molecular subclasses was assessed by GSEA²³.

Statistical analysis

Results were expressed as mean $\pm$ SEM, and were compared by two-way ANOVA with Bonferroni post-hoc test. Two-tailed *p*-values less than 0.05 were considered statistically significant.

RESULTS

Serum tests for liver injury and metabolism in mice treated with WD or WD/CCl₄

Mice treated with WD/Oil showed significant weight gain and adiposity compared to ND/Oil control mice (Figure 1A). CCl₄ treatment decreased food intake (data not shown) and attenuated the body weight gain induced by WD feeding (Figure 1A). Whereas the ND/Oil or ND/CCl₄ mouse groups did not develop increased liver weight, or liver to body weight ratio at 12 and 24 weeks, both these parameters were increased in the two WD treated groups (WD/Oil or WD/CCl₄) (Figure 1B and 1C). The increased liver weight in WD/Oil or WD/CCl₄ treated mice was accompanied by elevations of serum ALT and AST at 12 and 24 weeks. In contrast to WD fed animals, ALT and AST were only modestly but not significantly increased by ND/CCl₄ treatment at 12 and 24 weeks, indicating that while the NASH-related histologic features were amplified by the CCl₄ addition, the level of hepatocellular injury as reflected in AST and ALT was not (Figure 1D and 1E).

There were no differences in blood glucose levels among four groups at 12 weeks (Figure 1F). However, insulin was significantly increased in mice treated with WD/Oil vs ND/Oil at 12 weeks, suggesting that WD/Oil-treated mice might be insulin-resistant, although this was not tested formally with insulin-clamp studies. Although increased insulin was not observed in ND/CCl₄ or WD/CCl₄ treated mice (Figure 1G), HOMA IR and QUICKI¹⁴ were consistent with the presence of insulin resistance in all animals fed a Western diet. The abnormalities were partially attenuated when CCl₄ was added, yielding levels intermediate between Western Diet alone and CCl₄ alone (Supplementary Figure 1); these findings are consistent with transcriptomic data indicating engagement of insulin resistance pathways, described below. Total serum cholesterol was significantly increased in mice treated with WD/Oil and WD/CCl₄ at 12 and 24 weeks (Figure 1H). Caloric and food intake were stable across all groups through the duration of the study (Supplementary Figure 2A–B). However, serum triglycerides were decreased in groups fed a WD compared to their respective controls (Supplementary Figure 2C).

CCl₄ treatment accelerates WD-induced steatohepatitis and fibrosis

Representative pictures of H&E and Sirius Red staining from mice of each group are shown in Figure 2A and 2B. NAFLD activity score and fibrosis stage are described in Table 1.

WD/Oil or WD/CCl₄ treatment induced macrovesicular and microvesicular steatosis at 12 and 24 weeks. Most mice treated with WD/Oil, and all mice treated with WD/CCl₄ exhibited grade 3 steatosis, while ND/Oil and ND/CCl₄ treated mice did not develop steatosis. WD/Oil treatment induced lobular inflammation at 12 weeks and 24 weeks. CCl₄ treatment exacerbated WD-induced lobular inflammation, which led to grade 2.6 ($\pm$ 0.2) at 12 weeks and 2.1 ($\pm$ 0.3) at 24 weeks. Hepatocyte ballooning was observed at 12 weeks and 24 weeks by WD/Oil treatment. CCl₄ treatment promoted hepatocyte ballooning induced by WD, showing grade 2.0 ($\pm$ 0.0) at 12 weeks and 1.7 ($\pm$ 0.2) at 24 weeks. To confirm that the appearance of these cells was consistent with ballooning and not glycogen accumulation, PAS staining was negative in ballooned hepatocytes (not shown).

In mice treated with WD/Oil, NAFLD activity score was 5.5 ($\pm$ 0.4) at 12 weeks and 5.8 ($\pm$ 0.1) at 24 weeks. CCl₄ treatment increased further the NAS score attributable to WD, which resulted in 7.6 ($\pm$ 0.2) at 12 weeks and 6.8 ($\pm$ 0.4) at 24 weeks.

Based on Sirius Red staining, fibrosis stage was 1.1 ($\pm$ 0.2) at 12 weeks and progressed to stage 2.1 ($\pm$ 0.3) at 24 weeks in mice treated with WD/Oil. Remarkably, stage 3 bridging fibrosis developed at as early as 12 weeks in all mice treated WD/CCl₄. Some mice further developed to F4 cirrhosis at 24 weeks, with an average 3.6 ($\pm$ 0.2) of fibrosis stage. Per quantitative morphometry assessing fibrotic tissue area, there was a trend towards increased collagen deposition in WD mice when CCl₄ treatment was added (Figure 2C and 2D), which was consistent with the histological scoring. Overall, these results suggest that CCl₄ treatment accelerated WD-induced progression of steatohepatitis and fibrosis.

The liver histology of mice treated with WD/CCl₄ for 24 weeks was compared to human NASH patients. Steatosis (Figure 3A), lobular inflammation (Figure 3B), and ballooning hepatocytes with Mallory-Denk bodies (Figure 3C) resembled those of human NASH patients.

WD/CCl₄ treatment promotes activation of HSCs

Rapid and severe progression of fibrosis was a notable histological feature of mice treated with WD/CCl₄. Activated HSCs are major source of ECM in parenchymal liver disease including NASH²⁴, and CCl₄ treatment significantly increased IHC intensity for desmin, a HSC marker and α SMA, a marker of activated HSCs, following WD for 12 weeks (Figure 4A–D). At 24 weeks, there were no further increase in desmin- and α SMA-positive tissue area in WD/CCl₄ treated mice (Figure 4A–D), suggesting that HSCs might be activated earlier by WD/CCl₄ treatment compared to WD/Oil or ND/CCl₄ treatment. Quantitative PCR confirmed that WD/Oil or WD/CCl₄ treatment up-regulated fibrogenic genes such as *Collagen1 α 1*, *β -Pdgfr*, *Tgf- β* and *Timp-1* (Supplementary Figure 3A–D). These data were complemented by evidence of increased Collagen1 and α SMA expression by immunoblotting of whole liver lysates at 12 weeks (Supplementary Figure 4).

WD/CCl₄ treatment induced ductular reaction and hepatocyte proliferation

Ductular reaction (DR) is a reactive lesion at the portal tract defined by the proliferation of small biliary ductules containing hepatic progenitor cells (HPC), which could be the source of regenerating hepatocytes, as well as cholangiocytes²⁵. In patients with NASH, expansion

of HPC and the extent of DR are correlated with fibrosis stage^{25,26}. IHC for CK-19, a marker for cholangiocytes which can also stain HPC, was more pronounced in WD/CCl₄-treated mice than in WD/Oil- or ND/CCl₄-treated groups at 12 weeks, suggesting that DR is increased by WD/CCl₄ treatment in accordance with histologic findings (Figure 5A and 5C). The number of Ki67 positive hepatocytes as assessed by IHC was increased by WD/Oil or WD/CCl₄ at 12 and 24 weeks (Figure 5B and 5D); these changes corresponded to a marked increase in MIC1-1C3 positive cells, consistent with expanded progenitors (Supplementary Figure 5). These results suggest that continuous liver damage and compensatory proliferation, a critical process in hepatocarcinogenesis²⁷, were promoted by WD/CCl₄.

WD/CCl₄ accelerates development of HCC

No tumor development was observed in mice at 12 weeks. However, at 24 weeks, 30%, 10%, and 100% of mice had tumors in the WD/Oil, ND/CCl₄, and WD/CCl₄ treated groups, respectively. Tumor numbers were increased further, and maximal tumor size was larger in WD/CCl₄ treated mice (Figure 6A and 6B). Histologically, some tumors were diagnosed as dysplastic nodules (Figure 6C–F). Notably, HCC developed in 10% and 30% of WD/Oil and WD/CCl₄ treated mice, respectively, but not in ND/CCl₄ treated mice, underscoring the pro-carcinogenic effect of the WD. In the WD/CCl₄ group, steatohepatic HCC²⁸(Figure 6G–J), and clear-cell HCC (lipid variant) (Figure 6K–N) were observed.

Global transcriptome dysregulation aligns between human and mouse NASH models

Our comparative transcriptomic analyses have revealed a close similarity between our murine model and human NAFLD by analyzing the functional pathways affected, in contrast to a previous report that only examined individual genes across several models, and which suggested a significant dissimilarity between the two species²⁹. Specifically, we compared dysregulated molecular pathways from KEGG and HALLMARK gene sets in two human NASH series to 16 previously published diet, chemical, and/or genetic NASH mouse models together with our mouse models (Figure 7 and Supplementary Table 2)^{9,20,21,30–39}. The mouse models were ranked according to their transcriptomic similarity to human NASH versus healthy liver by molecular pathway analysis from KEGG. Up-regulated pathways in both human and mouse NASH models included focal adhesion, ECM receptor interaction, chemokine signaling pathways, Toll-like receptor pathways, and epithelial mesenchymal transition. Down-regulated pathways were related to parenchymal functions including fatty acid metabolism, bile acid metabolism, and oxidative phosphorylation. Insulin signaling/insulin resistance-related genes sets and type II diabetes-related gene sets were induced over time, supporting the presence of dysregulated glucose metabolism in our model (Supplementary Figure 6). In contrast, this aberration in diabetes-related insulin signaling was not observed in MCD diet-fed mice, consistent with the notion that the MCD model lacks insulin resistance a feature observed in human NASH. Emerging candidate NASH therapeutic targets, FXR, FGF15 (mouse ortholog of human FGF19), and bile acid pathways are generally suppressed in our model, suggesting that our model can be utilized to test therapeutic strategies targeting these pathways (Supplementary Figure 6). An altered composition of infiltrating immune cell subsets has been linked to experimental NASH pathogenesis⁴⁰. Consistent with this previous report, there was a marked increase in CD3⁺, CD4⁺ and CD8⁺ positive cells in the livers of WD/CCl₄ treated mice compared to controls

as characterized by immunostaining and semi-quantitative assessment (Figure 8 and Supplementary Table 3). Moreover, gene sets associated with CD4-positive T helper cells, especially Th17 cells, were suppressed compared to other subsets in our model (Supplementary Figure 7). Confirmation for presence of ballooning degeneration of hepatocytes was determined by the lack of CK8/18 cytoplasmic staining in hepatocytes with foamy cytoplasm (Figure 8). Further confirmatory staining with CK18 alone showed similar results (data not shown). The lack of staining or otherwise known as CK18 deficiency is indicative of ballooning as compared to the neighboring hepatocytes with retained or positive cytoplasmic immunoreactivity. Overall, our transcriptomic analysis indicated that dysregulated molecular pathways in our NASH model induced by WD plus CCl₄ were similar to those of human NASH. Of note, WD and CCl₄ treatment for 12 weeks showed a higher similarity to human NASH versus healthy liver than 24 weeks based on KEGG pathway analysis. This is in agreement with the transcriptomic features of the tumors from mice treated with WD and CCl₄, which are similar to human HCC molecular subclasses 23 (Supplementary Table 4).

DISCUSSION

In this study, we report a simple murine NASH model by using WD and CCl₄ that develops histological features of NASH with rapid progression of extensive fibrosis and HCC, and closely replicates the transcriptomic hallmarks of human NASH. These features make the model unusually attractive as a platform for testing therapeutic agents for the disease. In particular, severe steatohepatitis and stage 3 bridging fibrosis developed as early as 12 weeks. Some of the mice treated with WD and CCl₄ further progressed to stage 4 cirrhosis and HCC development at 24 weeks. These histological phenotypes recapitulate the natural progression of human patients with NASH, albeit in a more telescoped time frame.

Another key feature of our NASH model is the activation of HSCs. HSCs are major source of activated myofibroblasts, and their activation is well known to drive fibrosis in chronic liver disease including NASH^{24,41,42}. WD and CCl₄ treatment induced proliferation and activation of HSCs earlier than WD treatment alone, suggesting that activation of HSCs was responsible for the rapid progression of fibrosis in our NASH model. Furthermore, ductular reaction (DR) was up-regulated in mice treated with WD and CCl₄ for 12 weeks. As expansion of HPC and the extent of DR is correlated with fibrosis stage in patients with NASH⁴³, these results also support the similarity of our NASH model to clinical setting of human patients.

Western style diets containing high-fat, high-fructose (or sucrose), and high-cholesterol have been widely used to establish mouse NASH models because such dietary features have been associated with NASH development in humans, and can induce not only steatohepatitis, but also obesity and insulin resistance in mice^{6,8,9,44,45}. However, the major disadvantage of WD-based NASH models is that they do not fully progress to severe steatohepatitis and advanced fibrosis even after long-term feeding^{6,8}. Such murine models are not ideal for drug testing because diet feeding and drug treatment are lengthy and costly.

CCl₄ has been traditionally used for decades to induce liver injury and fibrosis in mice.¹⁰ In this study, we used CCl₄ as a “fibrosis accelerator” because fibrosis appears from zone 3, around central vein by chronic CCl₄ treatment. In adult patients with NASH, it has been known that steatosis and fibrosis are observed in zone 3 at first⁴⁶. In addition, CCl₄ treatment induces ballooning degeneration in mice⁴⁷, which is a typical hallmark of NASH. Thus, CCl₄ and WD could show synergistic effects to worsen pathophysiology of NASH. As expected, CCl₄ exacerbated hepatocyte ballooning, inflammation, fibrosis stage, HSC activation, ductular reaction, hepatocyte proliferation, and tumor development induced by WD. The histological components of our NASH model induced by WD and CCl₄ (steatosis, lobular inflammation, and ballooning) were similar to those of human patients. Of note, while the dietary cholesterol of this component is relatively high compared to other rodent models, cholesterol absorption in mice is much less efficient than in humans^{48,49}, and the hepatic levels of cholesterol following 1.2% dietary cholesterol approximate those of human NASH^{50–52}.

Besides liver histology, transcriptomic analysis also supports the similarity of our NASH model to human patients. KEGG transcriptomic analysis indicates that our WD and CCl₄ models are more similar to human NASH (versus healthy liver) than 18 other previously published murine NASH models induced by diet, chemical toxins and genetic modifications (Figure 7), whereas other models (e.g., MCD plus high fat diet) resemble advanced human NASH, when classical features of the disease are lost as the liver becomes cirrhotic. Up-regulated molecular pathways in both human and our mouse NASH model included focal adhesion, ECM receptor interaction, chemokine signaling pathway, Toll-like receptor pathway, epithelial mesenchymal transition. Down-regulated pathways were related to parenchymal functions including fatty acid metabolism, bile acid metabolism, and oxidative phosphorylation. These transcriptomic changes have been implicated in the progression of NASH with advanced fibrosis²⁰. Of note, WD and CCl₄ treatment for 12 weeks showed a higher similarity to human NASH than WD and CCl₄ treatment for 24 weeks in KEGG pathway analysis (Figure 7), suggesting that our model at 12 weeks replicates NASH with fibrosis, while the features progressed to NASH with cirrhosis and HCC at 24 weeks. The similarity was further supported by the presence of inflammatory cell infiltrates at the same time point (Figure 8 and Supplementary Table 3), a feature also associated with NASH⁴⁰.

There are also limitations in our NASH model induced by WD and CCl₄. First, animals are only maximally insulin resistant when WD was administered, typical of NAFLD patients³, whereas insulin resistance in the WD plus CCl₄ mice was at an intermediate level between WD alone and CCl₄ alone. The attenuated insulin resistance in the WD and CCl₄ mice may result from the blunted weight gain when CCl₄ was added to the Western Diet. However, only a subpopulation of NAFLD progresses to advanced fibrosis and HCC. Thus, it is still unclear whether obesity and insulin resistance are essential to drive steatohepatitis, advanced fibrosis, and HCC occurrence. Interestingly, obesity is not an independent risk factor of rapid fibrosis progression¹. Next, in HALLMARK transcriptomic analysis, the cholesterol homeostasis pathway was significantly down-regulated, but was up-regulated in human NASH, which likely reflects the fact that the ‘western diet’ used in our studies contains 1.25% of cholesterol, which as noted above, is necessary to lead to hepatic cholesterol levels that approximate those of human NASH as rodents do not absorb cholesterol well. These

transcriptomic changes are also observed in other NASH models induced by high-cholesterol containing Western diet⁵³.

In summary, by using WD and CCl₄, we have generated a murine NASH model that develops key histological and transcriptomic features of human NASH within 12 weeks, and with consistent development of HCC by 24 weeks. This could be a useful experimental tool for drug testing and to elucidate pathophysiology of NASH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- 1Singh S, et al. Fibrosis Progression in Nonalcoholic Fatty Liver vs Nonalcoholic Steatohepatitis: A Systematic Review and Meta-analysis of Paired-Biopsy Studies. *Clin Gastroenterol Hepatol*. 2015; 13:643–654 e649. DOI: 10.1016/j.cgh.2014.04.014 [PubMed: 24768810]
- 2Sayiner M, Koenig A, Henry L, Younossi ZM. Epidemiology of Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis in the United States and the Rest of the World. *Clin Liver Dis*. 2016; 20:205–214. DOI: 10.1016/j.cld.2015.10.001 [PubMed: 27063264]
- 3Younossi ZM, et al. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology*. 2016; 64:73–84. DOI: 10.1002/hep.28431 [PubMed: 26707365]
- 4Wong RJ, Cheung R, Ahmed A. Nonalcoholic steatohepatitis is the most rapidly growing indication for liver transplantation in patients with hepatocellular carcinoma in the U.S. *Hepatology*. 2014; 59:2188–2195. DOI: 10.1002/hep.26986 [PubMed: 24375711]
- 5Ibrahim SH, Hirsova P, Malhi H, Gores GJ. Animal Models of Nonalcoholic Steatohepatitis: Eat, Delete, and Inflammation. *Dig Dis Sci*. 2016; 61:1325–1336. DOI: 10.1007/s10620-015-3977-1 [PubMed: 26626909]
- 6Machado MV, et al. Mouse models of diet-induced nonalcoholic steatohepatitis reproduce the heterogeneity of the human disease. *PLoS One*. 2015; 10:e0127991. [PubMed: 26017539]
- 7Itagaki H, Shimizu K, Morikawa S, Ogawa K, Ezaki T. Morphological and functional characterization of non-alcoholic fatty liver disease induced by a methionine-choline-deficient diet in C57BL/6 mice. *Int J Clin Exp Pathol*. 2013; 6:2683–2696. [PubMed: 24294355]
- 8Charlton M, et al. Fast food diet mouse: novel small animal model of NASH with ballooning, progressive fibrosis, and high physiological fidelity to the human condition. *Am J Physiol Gastrointest Liver Physiol*. 2011; 301:G825–834. DOI: 10.1152/ajpgi.00145.2011 [PubMed: 21836057]
- 9Asgharpour A, et al. A diet-induced animal model of non-alcoholic fatty liver disease and hepatocellular cancer. *J Hepatol*. 2016; 65:579–588. DOI: 10.1016/j.jhep.2016.05.005 [PubMed: 27261415]
- 10Scholten D, Trebicka J, Liedtke C, Weiskirchen R. The carbon tetrachloride model in mice. *Laboratory animals*. 2015; 49:4–11. DOI: 10.1177/0023677215571192 [PubMed: 25835733]

- 11Kubota N, et al. A high-fat diet and multiple administration of carbon tetrachloride induces liver injury and pathological features associated with non-alcoholic steatohepatitis in mice. *Clin Exp Pharmacol Physiol*. 2013; 40:422–430. DOI: 10.1111/1440-1681.12102 [PubMed: 23611112]
- 12De Minicis S, et al. HCC development is associated to peripheral insulin resistance in a mouse model of NASH. *PLoS One*. 2014; 9:e97136. [PubMed: 24853141]
- 13Kleiner DE, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. 2005; 41:1313–1321. DOI: 10.1002/hep.20701 [PubMed: 15915461]
- 14Lee S, et al. Comparison between surrogate indexes of insulin sensitivity and resistance and hyperinsulinemic euglycemic clamp estimates in mice. *Am J Physiol Endocrinol Metab*. 2008; 294:E261–270. DOI: 10.1152/ajpendo.00676.2007 [PubMed: 18003716]
- 15Bowe JE, et al. Metabolic phenotyping guidelines: assessing glucose homeostasis in rodent models. *J Endocrinol*. 2014; 222:G13–25. DOI: 10.1530/JOE-14-0182 [PubMed: 25056117]
- 16Engstrom PG, et al. Systematic evaluation of spliced alignment programs for RNA-seq data. *Nat Methods*. 2013; 10:1185–1191. DOI: 10.1038/nmeth.2722 [PubMed: 24185836]
- 17Robinson MD, Oshlack A. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol*. 2010; 11:R25. [PubMed: 20196867]
- 18Subramanian A, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*. 2005; 102:15545–15550. DOI: 10.1073/pnas.0506580102 [PubMed: 16199517]
- 19Liberzon A, et al. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell systems*. 2015; 1:417–425. DOI: 10.1016/j.cels.2015.12.004 [PubMed: 26771021]
- 20Moylan CA, et al. Hepatic gene expression profiles differentiate presymptomatic patients with mild versus severe nonalcoholic fatty liver disease. *Hepatology*. 2014; 59:471–482. DOI: 10.1002/hep.26661 [PubMed: 23913408]
- 21Ahrens M, et al. DNA methylation analysis in nonalcoholic fatty liver disease suggests distinct disease-specific and remodeling signatures after bariatric surgery. *Cell Metab*. 2013; 18:296–302. DOI: 10.1016/j.cmet.2013.07.004 [PubMed: 23931760]
- 22Nakagawa S, et al. Molecular Liver Cancer Prevention in Cirrhosis by Organ Transcriptome Analysis and Lysophosphatidic Acid Pathway Inhibition. *Cancer Cell*. 2016; 30:879–890. DOI: 10.1016/j.ccell.2016.11.004 [PubMed: 27960085]
- 23Hoshida Y, et al. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. *Cancer Res*. 2009; 69:7385–7392. 0008-5472.CAN-09-1089 [pii]. DOI: 10.1158/0008-5472.CAN-09-1089 [PubMed: 19723656]
- 24Mederacke I, et al. Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. *Nat Commun*. 2013; 4:2823. [PubMed: 24264436]
- 25Richardson MM, et al. Progressive fibrosis in nonalcoholic steatohepatitis: association with altered regeneration and a ductular reaction. *Gastroenterology*. 2007; 133:80–90. DOI: 10.1053/j.gastro.2007.05.012 [PubMed: 17631134]
- 26Gadd VL, et al. The portal inflammatory infiltrate and ductular reaction in human nonalcoholic fatty liver disease. *Hepatology*. 2014; 59:1393–1405. DOI: 10.1002/hep.26937 [PubMed: 24254368]
- 27Maeda S, Kamata H, Luo JL, Leffert H, Karin M. IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell*. 2005; 121:977–990. [PubMed: 15989949]
- 28Salomao M, Yu WM, Brown RS Jr, Emond JC, Lefkowitz JH. Steatohepatitic hepatocellular carcinoma (SH-HCC): a distinctive histological variant of HCC in hepatitis C virus-related cirrhosis with associated NAFLD/NASH. *Am J Surg Pathol*. 2010; 34:1630–1636. DOI: 10.1097/PAS.0b013e3181f31caa [PubMed: 20975341]
- 29Teufel A, et al. Comparison of Gene Expression Patterns Between Mouse Models of Nonalcoholic Fatty Liver Disease and Liver Tissues From Patients. *Gastroenterology*. 2016; 151:513–525e510. DOI: 10.1053/j.gastro.2016.05.051 [PubMed: 27318147]
- 30Kobori M, Masumoto S, Akimoto Y, Oike H. Chronic dietary intake of quercetin alleviates hepatic fat accumulation associated with consumption of a Western-style diet in C57/BL6J mice. *Mol Nutr Food Res*. 2011; 55:530–540. DOI: 10.1002/mnfr.201000392 [PubMed: 21462320]

- 31Dorn C, et al. Increased expression of c-Jun in nonalcoholic fatty liver disease. *Lab Invest.* 2014; 94:394–408. DOI: 10.1038/labinvest.2014.3 [PubMed: 24492282]
- 32Kim SC, et al. All-trans-retinoic acid ameliorates hepatic steatosis in mice by a novel transcriptional cascade. *Hepatology.* 2014; 59:1750–1760. DOI: 10.1002/hep.26699 [PubMed: 24038081]
- 33Kwon EY, et al. Time-course microarrays reveal early activation of the immune transcriptome and adipokine dysregulation leads to fibrosis in visceral adipose depots during diet-induced obesity. *BMC Genomics.* 2012; 13:450. [PubMed: 22947075]
- 34Hur W, et al. Downregulation of microRNA-451 in non-alcoholic steatohepatitis inhibits fatty acid-induced proinflammatory cytokine production through the AMPK/AKT pathway. *Int J Biochem Cell Biol.* 2015; 64:265–276. DOI: 10.1016/j.biocel.2015.04.016 [PubMed: 25957914]
- 35Kita Y, et al. Metformin prevents and reverses inflammation in a non-diabetic mouse model of nonalcoholic steatohepatitis. *PLoS One.* 2012; 7:e43056. PONE-D-11-24464 [pii]. [PubMed: 23028442]
- 36Tryndyak V, et al. Interstrain differences in the severity of liver injury induced by a choline- and folate-deficient diet in mice are associated with dysregulation of genes involved in lipid metabolism. *Faseb j.* 2012; 26:4592–4602. DOI: 10.1096/fj.12-209569 [PubMed: 22872676]
- 37Frades I, et al. Integrative genomic signatures of hepatocellular carcinoma derived from nonalcoholic Fatty liver disease. *PLoS One.* 2015; 10:e0124544. [PubMed: 25993042]
- 38Tsai WC, et al. MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. *J Clin Invest.* 2012; 122:2884–2897. DOI: 10.1172/jci63455 [PubMed: 22820290]
- 39Yang JS, et al. Changes in hepatic gene expression upon oral administration of taurine-conjugated ursodeoxycholic acid in ob/ob mice. *PLoS One.* 2010; 5:e13858. [PubMed: 21079772]
- 40Ma C, et al. NAFLD causes selective CD4(+) T lymphocyte loss and promotes hepatocarcinogenesis. *Nature.* 2016; 531:253–257. DOI: 10.1038/nature16969 [PubMed: 26934227]
- 41Wallace MC, Friedman SL, Mann DA. Emerging and disease-specific mechanisms of hepatic stellate cell activation. *Semin Liver Dis.* 2015; 35:107–118. DOI: 10.1055/s-0035-1550060 [PubMed: 25974897]
- 42Lee YA, Wallace MC, Friedman SL. Pathobiology of liver fibrosis: a translational success story. *Gut.* 2015; 64:830–841. DOI: 10.1136/gutjnl-2014-306842 [PubMed: 25681399]
- 43Machado MV, et al. Accumulation of duct cells with activated YAP parallels fibrosis progression in NonAlcoholic Fatty Liver Disease. *J Hepatol.* 2015
- 44Clapper JR, et al. Diet-induced mouse model of fatty liver disease and nonalcoholic steatohepatitis reflecting clinical disease progression and methods of assessment. *Am J Physiol Gastrointest Liver Physiol.* 2013; 305:G483–495. DOI: 10.1152/ajpgi.00079.2013 [PubMed: 23886860]
- 45Dowman JK, et al. Development of hepatocellular carcinoma in a murine model of nonalcoholic steatohepatitis induced by use of a high-fat/fructose diet and sedentary lifestyle. *Am J Pathol.* 2014; 184:1550–1561. DOI: 10.1016/j.ajpath.2014.01.034 [PubMed: 24650559]
- 46Kleiner DE, Makhlof HR. Histology of Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis in Adults and Children. *Clin Liver Dis.* 2016; 20:293–312. DOI: 10.1016/j.cld.2015.10.011 [PubMed: 27063270]
- 47Urtasun R, et al. Osteopontin, an oxidant stress sensitive cytokine, up-regulates collagen-I via integrin alpha(V)beta(3) engagement and PI3K/pAkt/NFkappaB signaling. *Hepatology.* 2012; 55:594–608. DOI: 10.1002/hep.24701 [PubMed: 21953216]
- 48Savard C, et al. Synergistic interaction of dietary cholesterol and dietary fat in inducing experimental steatohepatitis. *Hepatology.* 2013; 57:81–92. DOI: 10.1002/hep.25789 [PubMed: 22508243]
- 49Wang DQ, Lammert F, Cohen DE, Paigen B, Carey MC. Cholic acid aids absorption, biliary secretion, and phase transitions of cholesterol in murine cholelithogenesis. *Am J Physiol.* 1999; 276:G751–760. [PubMed: 10070053]
- 50Puri P, et al. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology.* 2007; 46:1081–1090. DOI: 10.1002/hep.21763 [PubMed: 17654743]
- 51Caballero F, et al. Enhanced free cholesterol, SREBP-2 and StAR expression in human NASH. *J Hepatol.* 2009; 50:789–796. DOI: 10.1016/j.jhep.2008.12.016 [PubMed: 19231010]

- 52Wang X, et al. Hepatocyte TAZ/WWTR1 Promotes Inflammation and Fibrosis in Nonalcoholic Steatohepatitis. *Cell Metab.* 2016; 24:848–862. DOI: 10.1016/j.cmet.2016.09.016 [PubMed: 28068223]
- 53Kristiansen MN, et al. Obese diet-induced mouse models of nonalcoholic steatohepatitis-tracking disease by liver biopsy. *World J Hepatol.* 2016; 8:673–684. DOI: 10.4254/wjh.v8.i16.673 [PubMed: 27326314]

- A carefully characterized model has been developed in mice that recapitulates the progressive stages of human fatty liver disease, from simple steatosis, to inflammation, fibrosis and cancer.
- The functional pathways of gene expression and immune abnormalities in this model closely resemble human disease.
- The ease and reproducibility of this model makes it ideal to study disease pathogenesis and test new treatments.

Figure 1. Metabolic profile of mice treated with diet and CCl₄

Mice were treated with ND/Oil, WD/Oil, ND/CCl₄, and WD/CCl₄ for up to 24 weeks. Body weight change for 24 weeks (A). From 0 to 11 weeks, ND/Oil: n = 10, WD/Oil: n = 20, ND/CCl₄: n = 20, WD/CCl₄: n = 19. From 12 to 24 weeks, ND/Oil: n = 5, WD/Oil: n = 10, ND/CCl₄: n = 10, WD/CCl₄: n = 10. Liver weight (B), liver per body weight ratio (C), serum ALT (D), AST (E), blood glucose (F), plasma insulin (G), and total cholesterol (H) were measured at 12 and 24 weeks. ND/Oil: n = 5, WD/Oil: n = 10, ND/CCl₄: n = 10, WD/CCl₄: n = 9 animals at 12 weeks. ND/Oil: n = 5, WD/Oil: n = 10, ND/CCl₄: n = 10, WD/CCl₄: n = 10 animals at 24 weeks. Results were expressed as mean $\pm$ SEM, and were compared by two-way ANOVA with Bonferroni post-hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ND, normal diet; WD, western diet; CCl₄, carbon tetrachloride.

Figure 2. Histological features of mice treated with diet and CCl₄

H&E (A) and Sirius Red (B) staining of representative mice treated with ND/Oil, WD/Oil, ND/CCl₄, and WD/CCl₄ for 12 and 24 weeks. Original magnification x 100. Quantification of Sirius Red-positive area at 12 (C) and 24 (D) weeks. Results were expressed as mean $\pm$ SEM, and were compared by two-way ANOVA with Bonferroni post-hoc test. ND/Oil: n = 5, WD/Oil: n = 10, ND/CCl₄: n = 10, WD/CCl₄: n = 9 animals at 12 weeks. ND/Oil: n = 5, WD/Oil: n = 10, ND/CCl₄: n = 10, WD/CCl₄: n = 10 animals at 24 weeks. * P < 0.05, ** P < 0.01, *** P < 0.001

Figure 3. Histological comparison of WD and CCl₄-treated mice with human NASH
H&E staining of liver sections from representative mice treated with WD/CCl₄ for 24 weeks (left) or human NASH (right) showing steatosis (A), lobular inflammation (B), hepatocyte ballooning with Mallory-Denk bodies (C). Original magnification x 1000.

Figure 4. Hepatic stellate cell activation in mice treated with diet and CCl₄

Immunostaining for desmin (A) and αSMA (B) in liver sections from representative mice treated with ND/Oil, WD/Oil, ND/CCl₄, and WD/CCl₄ for 12 and 24 weeks. Original magnification x 100. Quantification of desmin-positive tissue area (C) and αSMA-positive tissue area (D). Results were expressed as mean ± SEM, and were compared by two-way ANOVA with Bonferroni post-hoc test. ND/Oil: n = 3, WD/Oil: n = 5–8, ND/CCl₄: n = 5–6, WD/CCl₄: n = 5–10 animals at 12 and 24 weeks. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 5. Ductular reaction and hepatocyte proliferation in mice treated with diet and CCl₄
 Immunostaining for CK-19 (A) and Ki67 (B) in liver sections from representative mice treated with ND/Oil, WD/Oil, ND/CCl₄, and WD/CCl₄ for 12 and 24 weeks. Original magnification x 200. Quantification of CK-19-positive area (C) and the number of Ki67-positive hepatocytes per high power field (HPF) (D). Results were expressed as mean $\pm$ SEM, and were compared by two-way ANOVA with Bonferroni post-hoc test. ND/Oil: n = 3, WD/Oil: n = 5, ND/CCl₄: n = 5, WD/CCl₄: n = 5–6 animals at 12 and 24 weeks. * P <0.05, ** P <0.01, *** P <0.001.

Figure 6. Tumor development in mice treated with diet and CCl₄

Tumor numbers per mouse (A) and largest tumor size (B) in mice treated with ND/Oil, WD/Oil, ND/CCl₄, and WD/CCl₄ for 24 weeks. Representative pictures of dysplastic nodules (C–F), HCC with fat and ballooning (G–J), clear cell HCC of lipid rich variant (K–N) in mice treated with WD/CCl₄ for 24 weeks. Gross pictures (C, G, K). H&E staining (D, H, L, original magnification x 10). H&E staining showing interface between tumor and adjacent parenchyma (E, I, M, original magnification x 200). H&E staining showing tumor region of dysplastic nodules (F, J, N, original magnification x 50).

Figure 7. Cross-species comparison of global transcriptome dysregulation between human NASH and mouse NASH models

Dysregulated molecular pathways in either of two human NASH series were compared to 16 previously published diet, chemical, and/or genetic NASH mouse models together with our mouse models (see Methods and Supplementary Table 2 for details). The mouse models are ordered according to similarity to human NASH compared to healthy liver based on Euclidean distance. Orange and green colors in the heat map indicate statistical significance of induction and suppression of each gene set, respectively. HFChSuD: high fat/cholesterol/sugar diet, HFChD: high fat/cholesterol diet, HFD: high fat diet, MCD: methionine/choline-deficient diet, CFD: choline/folate-deficient diet (see Supplementary Table 2 for detailed references of datasets used).

Western Diet + CCl₄ – 12 wks

Figure 8. Immunostaining for T lymphocyte subtypes in mice treated with WD and CCl₄

Immunostaining was performed on liver sections from mice fed a WD/CCl₄ (12 weeks) for T lymphocyte subtypes CD3, CD4 and CD8 (black arrows). CK8/18 staining showed cells with CK8/18⁺ inclusions typical for human NASH (Black arrows). White arrows indicate ballooning degeneration of hepatocytes without CK8/18⁺ staining. Original magnification x 400. Average T lymphocyte subtype numbers were assessed by counting positive cells per field (200x magnification) in 20 random fields per liver section. Lymphocytes were identified by the dark staining nuclei with minimal cytoplasm (see Supplementary Table 3).

Table 1

NAFLD activity score and fibrosis stage of mice treated with diet and CCl₄

Duration	12 weeks				24 weeks			
	ND/Oil	ND/Oil	ND/CCl ₄	WD/CCl ₄	ND/Oil	ND/CCl ₄	WD/CCl ₄	WD/CCl ₄
Steatosis	0.0 (0.0)	2.8 (0.2)***	0.0 (0.0)	3.0 (0.0)***	0.0 (0.0)	2.9 (0.1)***	0.0 (0.0)	3.0 (0.0)***
Lobular inflammation	0.4 (0.2)	1.3 (0.2)*	1.1 (0.1)	2.6 (0.2)***, ###	0.0 (0.0)	1.1 (0.1)***	0.9 (0.2)	2.1 (0.3)***, ###
Ballooning	0.0 (0.0)	1.4 (0.2)***	0.4 (0.2)	2.0 (0.0)***, ###	0.0 (0.0)	1.8 (0.1)***	0.0 (0.0)	1.7 (0.2)***
NAFLD activity score	0.4 (0.2)	5.5 (0.4)***	1.5 (0.2)	7.6 (0.2)***, ##	0.0 (0.0)	5.8 (0.1)***	0.9 (0.2)	6.8 (0.4)***, #
Fibrosis stage	0.0 (0.0)	1.1 (0.2)**	3.0 (0.0)	3.0 (0.0)***, ###	0.2 (0.2)	2.1 (0.3)***	3.0 (0.0)	3.6 (0.2)***, ###

Results were expressed as mean ± SEM, and were compared by two-way ANOVA with Bonferroni post-hoc test. ND/Oil: n = 5, WD/Oil: n = 10, ND/CCl₄: n = 10, WD/CCl₄: n = 9 at 12 weeks. ND/Oil: n = 5, WD/Oil: n = 10, ND/CCl₄: n = 10, WD/CCl₄: n = 10 at 24 weeks.

- * $P < 0.05$,
- ** $P < 0.01$,
- *** $P < 0.001$ vs. ND/Oil.
- # $P < 0.05$,
- ## $P < 0.01$,
- ### $P < 0.001$ vs. WD/Oil