



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Non-invasive assessment of liver disease in rats using multiparametric magnetic resonance imaging: a feasibility study

Citation for published version:

Hoy, AM, McDonald, N, Lennen, RJ, Milanesi, M, Herlihy, AH, Kendall, TJ, Mungall, W, Gyngell, M, Banerjee, R, Janiczek, RL, Murphy, PS, Jansen, MA & Fallowfield, JA 2018, 'Non-invasive assessment of liver disease in rats using multiparametric magnetic resonance imaging: a feasibility study' *Biology Open*, vol 7, no. 7, pp. bio033910. DOI: 10.1242/bio.033910

Digital Object Identifier (DOI):

[10.1242/bio.033910](https://doi.org/10.1242/bio.033910)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Biology Open

Publisher Rights Statement:

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/3.0>), which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Non-invasive Assessment of Liver Disease in Rats Using Multiparametric Magnetic Resonance Imaging: A Feasibility Study

Anna M. Hoy¹, Natasha McDonald¹, Ross J. Lennen^{2,3}, Matteo Milanesi⁴, Amy H. Herlihy⁴, Timothy J. Kendall^{1,5}, William Mungall⁶, Michael Gyngell⁴, Rajarshi Banerjee⁴, Robert L. Janiczek⁷, Philip S. Murphy⁷, Maurits A. Jansen^{2,3}, Jonathan A. Fallowfield^{1*}

¹MRC/University of Edinburgh Centre for Inflammation Research, Edinburgh, UK; ²BHF/University of Edinburgh Centre for Cardiovascular Science, Edinburgh, UK. ³Edinburgh Preclinical Imaging (EPI), University of Edinburgh, Edinburgh, UK; ⁴Perspectum Diagnostics, Oxford, UK; ⁵Division of Pathology, University of Edinburgh, Edinburgh, UK; ⁶Biomedical Research Resources, University of Edinburgh, Edinburgh, UK; ⁷GlaxoSmithKline (Experimental Medicine Imaging), Stevenage, UK.

***Corresponding Author Information:** Dr Jonathan A. Fallowfield MRC/University of Edinburgh Centre for Inflammation Research, Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh, EH16 4TJ, UK. E-mail: Jonathan.Fallowfield@ed.ac.uk, Tel: (+44)1312426655. Orcid ID: orcid.org/0000-0002-5741-1471.

KEYWORDS: chronic liver disease, rat, multiparametric MRI.

SUMMARY STATEMENT: Multiparametric liver MRI was feasible in experimental rat models and imaging parameters correlated with gold standard histopathological assessments, especially characteristics of fatty liver disease.

ABSTRACT

Non-invasive quantitation of liver disease using multiparametric MRI could refine clinical care pathways, trial design and preclinical drug development. The aim of the study was to evaluate the use of multiparametric MRI in experimental models of liver disease. Liver injury was induced in rats using 4 or 12 weeks carbon tetrachloride (CCl₄) intoxication and 4 or 8 weeks methionine and choline deficient (MCD) diet. Liver MRI was performed using a 7.0 Tesla small animal scanner at baseline and specified timepoints after liver injury. Multiparametric liver MRI parameters (T1 mapping, T2* mapping and proton density fat fraction (PDFF)) were correlated with gold standard histopathological measures. Mean hepatic T1 increased significantly in rats treated with CCl₄ for 12 weeks compared to controls (1122±78 ms vs. 959±114 ms; $d=162.7$, 95% CI (11.92, 313.4), $P=0.038$) and correlated strongly with histological collagen content ($r_s=0.717$, $P=0.037$). In MCD diet-treated rats, hepatic PDFF correlated strongly with histological fat content ($r_s=0.819$, $P<0.0001$), steatosis grade ($r_s=0.850$, $P<0.0001$) and steatohepatitis score ($r_s=0.818$, $P<0.0001$). Although there was minimal histological iron, progressive fat accumulation in MCD diet-treated liver significantly shortened T2*. In preclinical models, quantitative MRI markers correlated with histopathological assessments, especially for fatty liver disease. Validation in longitudinal studies is required.

INTRODUCTION

Chronic liver disease (CLD) caused by obesity, excessive alcohol consumption and viral hepatitis represents a significant and increasing healthcare and socio-economic burden worldwide. Non-alcoholic fatty liver disease (NAFLD) is now the commonest form of CLD with a global prevalence of 25% (Younossi et al., 2016). Despite advances in our understanding of the pathogenic mechanisms that underlie NAFLD and liver fibrosis in general, there are still no FDA-approved drug therapies.

Important therapeutic targets and promising drug candidates have been identified in preclinical models, but reliable triage of efficacious therapies requires optimization and standardization of drug development strategies and consensus on acceptable endpoints (Torok et al., 2015). In particular, reproducible and scalable quantitative non-invasive markers of chronic liver injury that permit stratification and longitudinal monitoring of disease could refine preclinical testing, increase efficiency of trial design, and transform clinical care pathways.

Current clinical trials of potential anti-NASH or antifibrotic drugs are anchored to histopathological assessments of disease progression. However, liver biopsy is hampered by invasiveness and sampling variability and is unpopular with patients. Magnetic resonance imaging (MRI) has emerged as a promising new modality for improved understanding and characterization of *in vivo* liver pathophysiology. It is a versatile technique whereby structural and functional MRI can be performed in a single multiparametric scan session, depicting changes associated with inflammation, fibrosis, steatosis, siderosis, oxygenation, and tissue microstructure. Quantitative MRI parameters characterize these processes directly in the liver, rather than using downstream/upstream metabolic analytes. Compared to histopathology, MRI is non-invasive and avoids sampling bias by characterizing the whole organ with high spatial resolution. The MRI proton density fat fraction (PDFF) is a highly accurate and reproducible technique for the assessment of steatosis and can detect changes in hepatic fat as small as 1% (Noureddin et al., 2013). Magnetic resonance elastography (MRE) is a phase-contrast MR technique that measures liver stiffness as a surrogate of fibrosis. MRE has high accuracy for the diagnosis of advanced liver fibrosis and cirrhosis, but it is not yet known whether it is sufficiently sensitive or dynamic for the longitudinal monitoring of fibrosis progression/regression (Singh et al, 2015). LiverMultiScan™ (Perspectum Diagnostics, Oxford, UK) uses non-contrast multiparametric MRI to quantify hepatic

fibro-inflammatory injury (iron-corrected T1 mapping), steatosis (PDFF) and iron content (T2* mapping). In patients, LiverMultiScan™ can accurately quantify liver disease (Banerjee et al., 2014; Wilman et al., 2017; Pavlides et al., 2017), may predict clinical outcomes (Pavlides et al, 2016), and is now being adopted into clinical trial protocols as a surrogate endpoint. We hypothesized that multiparametric MRI could be developed and applied in experimental models of NASH and liver fibrosis using a small animal MR scanner system, with potential future utility in preclinical drug development studies whilst also addressing the principles of the 3Rs for humane animal research.

RESULTS

Assessment of hepatic fibro-inflammatory injury in rat CCl₄ model

To evaluate multiparametric MRI for the assessment of hepatic fibro-inflammatory injury in a preclinical setting we used the rat CCl₄ model. Compared to OO controls, animals receiving CCl₄ had higher serum transaminase levels and mild to moderate liver inflammation after both 4 and 12 weeks CCl₄ (Fig. S1). Hepatic fibrogenic activity increased progressively, with 12-fold greater α -SMA expression after 12 weeks in CCl₄-treated rats compared to controls ($P=0.006$) (Fig. S1). Hepatic collagen content (assessed by CPA) was increased, greater than 3-fold, after both 4 weeks ($P=0.043$) and 12 weeks ($P=0.013$) CCl₄ treatment relative to control (Fig. 1A).

After 4 weeks, liver T1 was 1025 ± 112 ms in CCl₄ rats compared to 984 ± 138 ms in OO controls ($d=40.68$, 95% CI (-141.7, 223.1), $P=0.621$) (Fig. 1B,C). There was a significant difference in mean liver T1 in 12 week CCl₄ rats (1122 ± 78 ms) compared to controls (959 ± 114 ms; $d=162.7$, 95% CI (11.92, 313.4), $P=0.038$) (Fig. 1B,C).

There was a strong positive correlation between liver T1 values and collagen content after 12 weeks CCl₄ (advanced fibrosis) ($r_s=0.717$, $P=0.037$) (Fig. 1D) but this correlation was absent after 4 weeks of CCl₄ ($r_s=-0.079$, $P=0.838$) (early bridging fibrosis).

To explore the influence of fat on hepatic T1, we used 1H-MRS to re-analyse two distinct areas identified by different signal intensity on the T2-weighted structural scan. Although one area had a much higher fat content, the T1 values were similar (Fig. S2).

Assessment of hepatic steatosis and steatotic inflammatory injury in preclinical liver injury models

To explore the potential utility of multiparametric MRI as a non-invasive preclinical diagnostic method for the assessment and staging of NAFLD and evaluation of anti-NASH treatments, we used the rat MCD diet model. Rats fed a MCD diet developed florid micro- and macro-vesicular steatosis (Fig. S3). The proportionate area of Oil Red O-stained fat in the liver was <0.5% in control rats, but in comparison was grossly elevated after 4 weeks MCD diet ($22.9\pm 10.9\%$; $P=0.004$) and 8 weeks MCD diet ($30.2\pm 13.2\%$; $P=0.002$) (Fig. 2B) with no significant difference between both timepoints.

Quantification of hepatic fat by MRI (mean of 3 ROIs) showed a highly significant increase in PDFF after both 4 and 8 weeks of MCD diet, reaching $23.81\pm 6.00\%$ and $23.84\pm 5.64\%$ compared with control animals where PDFF was $1.73\pm 0.94\%$ ($P=0.0005$) and $2.09\pm 1.16\%$ ($P<0.0001$), respectively (Fig. 2A,C). The hepatic PDFF strongly correlated with fat proportionate area ($r_s=0.819$, $P<0.0001$) (Fig. 2F). Furthermore, hepatic PDFF correlated with both histological stage of steatosis and steatotic inflammatory injury ($r_s=0.850$, $P<0.0001$ and $r_s=0.818$, $P<0.0001$ respectively) (Fig. 2D,E).

In contrast to animals receiving MCD diet, CCl₄ treated rats displayed a smaller increase in histological liver fat compared with controls after 4 weeks ($8.94\pm 6.48\%$ vs. $0.26\pm 0.19\%$, $P=0.03$) and 12 weeks ($9.11\pm 9.6\%$ vs. $0.30\pm 0.13\%$, $P=0.11$) (Fig. 3B). Quantification of hepatic fat by MRI (Fig. 3A) showed an increase in PDFF compared to controls after 4 weeks CCl₄ ($3.86\pm 1.83\%$ vs. $0.91\pm 0.93\%$, $P=0.016$) and 12 weeks CCl₄ ($3.58\pm 0.33\%$ vs. $1.85\pm 0.87\%$, $P=0.0044$) (Fig. 3C). Moreover, PDFF was strongly correlated with histological grading of steatosis (Oil Red O staining) and steatotic inflammatory injury ($r_s=0.74$, $P=0.003$ and $r_s=0.84$, $P<0.0001$ respectively) (Fig. 3D,E).

It had previously been shown that administration of cilostazol for 16 weeks reduced hepatic steatosis, inflammation and fibrosis in rats fed a choline-deficient, l-amino acid-defined (CDAA) diet or a high-fat high-calorie diet (Fujita et al., 2008). We tested our MRI methodology in rats that were fed MCD diet for 4 weeks plus treatment with cilostazol or vehicle control between weeks 2-4. Animals underwent multiparametric MRI at baseline and after 4 weeks. On histological examination,

cilostazol treatment for 2 weeks only reduced hepatic steatosis by approximately 5% ($d=-5.19\%$, 95% CI (-13.91, 3.53), $P=0.21$), with a non-significant reduction in N(m)AS score (Fig. 4 A,B). Nevertheless, in a drug intervention setting multiparametric MRI methods performed similarly (Fig. 4C,D) and, importantly, strong correlations were observed between the histological assessment of steatotic inflammatory injury and MRI measures (PDFF; $r_s=0.675$, $P=0.019$) (Fig. 4E).

Assessment of hepatic iron content in preclinical liver injury models

Hepatic iron may have a pathogenic role in NAFLD and, intriguingly, NAFLD itself may affect iron metabolism (Britton et al., 2016). From a technical standpoint, both hepatic steatosis and fibrosis can have confounding effects on $T2^*$ relaxation times. We showed histologically that CCl_4 and MCD diet induced injury models were not associated with significant accumulation of iron in the liver (Figs S1, S3), but we did observe variable effects on $T2^*$ in these models (Fig. 5A). Consistent with the lack of tissue iron staining, there was no change in liver $R2^*$ ($1/T2^*$) in CCl_4 treated rats compared with controls ($P>0.05$) (Fig. 5B). However, we observed a significant increase in $R2^*$ ($1/T2^*$) in MCD diet-treated rat livers compared with controls at 4 weeks ($177.9\pm 46.7s^{-1}$ vs. $76.2\pm 7.9s^{-1}$, $P<0.0001$) and 8 weeks ($213.6\pm 22.5s^{-1}$ vs. $83.6\pm 12.1s^{-1}$, $P<0.0001$) (Fig. 5C). Moreover, there was a strong positive association between $R2^*$ and PDFF ($r_s=0.790$, $P<0.001$) (Fig. 5D), indicating that steatosis had a cofounding effect on $T2^*$ relaxation time in this model.

DISCUSSION

Despite the identification of a large number of therapeutic targets from preclinical studies, there are currently no licensed drug treatments for liver fibrosis or for NASH. Drug development has been hindered by a reliance on liver biopsy and a lack of robust validated non-invasive biomarkers to diagnose and monitor changes in liver disease over time or in response to treatment. Multiparametric MRI using LiverMultiScan™ has shown promise as a clinical diagnostic tool and surrogate endpoint in therapeutic trials, especially in the assessment of NAFLD. Our objective was to develop and apply this non-invasive, contrast-free multiparametric MRI methodology in preclinical liver injury models that are commonly used in mechanistic and drug evaluation studies.

Here we showed that our multiparametric liver MRI protocol was feasible at 7.0 Tesla in experimental rats and scanning was completed within a realistic time frame (~40 minutes, compared to ~85 minutes for a combined preclinical MRE and collagen molecular imaging protocol) (Zhu et al., 2017). Both hepatic T1 and PDFF changed with increasing duration of liver injury and were strongly correlated with histological assessments of fibrosis and steatosis/steatotic inflammatory injury, respectively. Unfortunately, there was only a minimal effect of a short treatment course of cilostazol in the MCD diet model and even histological endpoints were not significantly altered within this abbreviated timeframe. Although a number of MRI techniques have been evaluated individually in animal models to assess hepatic fibrosis (Zhu et al., 2017), iron content (Britton et al., 2016) or fat (Runge et al., 2014), multiparametric MRI captures all of these parameters in a single contrast-free scan protocol. In a previous study in mice with fatty liver, non-invasive MR assessments (1H-MRS, PDFF and modified Dixon) had a higher correlation with liver triglyceride content than digital image analysis of Oil Red O stained liver sections (Runge et al., 2014). The authors concluded that MRI was the preferred method for fat quantification. The performance of LiverMultiScan™ in our experimental rat models was broadly similar to that observed in patients with CLD ($r_s=0.68$, $P<0.0001$ for fibrosis; $r_s=0.89$, $P<0.001$ for steatosis; $r_s=-0.69$, $P<0.0001$ for siderosis) (Banerjee et al., 2014).

Our findings indicate that multiparametric liver MRI could potentially refine the way we currently approach testing of therapeutic candidates in preclinical models. Firstly, multiparametric MRI could be used as a quantitative and objective method to stratify liver disease severity before exposing animals to a drug treatment. It is known that animals maintained on an experimental lipogenic diet develop NAFLD at differing rates and of varying severity and, similarly to human disease, fibrosis accumulates heterogeneously within the liver. A pretreatment liver (wedge) biopsy protocol was previously used to categorize NAFLD mice into three groups (steatosis without fibrosis, NASH with fibrosis, cirrhosis), in order to address the inherent and potentially confounding issue of variability in the study population (Clapper et al., 2013). Imaging with MRI could be used in a similar manner, especially where accurate stratification could permit a more precise evaluation of therapies that modulate different components of NAFLD pathogenesis (i.e. anti-steatotic, anti-

inflammatory, antifibrotic), but would have the advantages of being non-invasive and sampling the whole liver. Secondly, multiparametric MRI could potentially be used longitudinally to evaluate the effect of an antifibrotic or anti-NASH intervention in individual experimental animals, allowing each to serve as its own control, and thereby allowing determination of the distribution of effects in an experimental population (Clapper et al., 2013). This approach would also address the 3Rs (refinement, reduction, replacement) of humane animal experimentation. We acknowledge the limitations of this study. Firstly, this was an initial small feasibility study to determine whether a multiparametric MRI protocol could be successfully scaled down and applied in rats with chronic liver injury within a realistic timeframe. Our findings require further validation and confirmation of reproducibility, in order to use this test in longitudinal/interventional studies. Secondly, technical refinements to the protocol may increase robustness and reliability. The small organ size of rodents necessitates the use of high-field MR imaging systems to obtain adequate temporal and spatial resolution and poses major challenges to imaging due to shortened relaxation times and accentuated field inhomogeneity. Additionally, hepatic T1 values could potentially be influenced by the fat fraction, thus confounding its use as a quantitative marker of hepatic fibro-inflammatory injury. To explore this, we re-analyzed one rat using 1H-MRS that clearly had two distinct areas in the liver, one with a much higher fat content. However, the T1 value for both areas was the same. Nevertheless, in contrast to histological collagen quantification, we found that T1 mapping was not sufficiently sensitive to discriminate between normal liver and mild fibrosis (after 4 weeks CCl₄). This remains the Achilles' heel of non-invasive serum and imaging biomarkers in the clinical setting. Interestingly, a recent study in rats with liver fibrosis showed that MRE was only sensitive for the detection of late-stage fibrosis, whereas a collagen-specific molecular imaging probe could only distinguish between earlier stages of fibrosis (Zhu et al., 2017). The differing sensitivity of various MRI methods indicates complementary staging capabilities and the potential for developing clinically-relevant multiparametric composite MRI models. The utility of T2* as a quantifiable marker for iron overload is well established (Tanner et al., 2006) and T2* is currently the clinical standard for the non-invasive assessment of iron overload (Hernando et al., 2014). However, low tissue iron content in our rat liver injury models meant that we were unable to confirm the expected inverse relationship between increasing

iron load and decreased T2 and T2* relaxation time in liver tissue. Furthermore, as demonstrated in this study, T2* is influenced by a number of factors independent of tissue iron concentration. In particular, we observed a significant shortening of T2* in the lipogenic MCD diet model, despite very low hepatic iron levels. This finding is consistent with previous human studies that confirm a short apparent T2* of fat (Bydder et al., 2008; O'Regan et al., 2008).

Using multiparametric MRI to quantify liver injury in experimental animal models could be a powerful new tool to assist in the development of new therapies for CLD, and is directly translatable to clinical practice.

MATERIALS AND METHODS

Animal models

Animal experiments (Fig. 6) were conducted with local and Government ethical approval and in compliance with the Use of Animals in Scientific Procedures Act 1986. Experiments were designed and implemented according to NC3R ARRIVE guidelines. We calculated group sizes prospectively for the animal model experiments based upon our previously published data using the carbon tetrachloride (CCl₄) model (Fallowfield et al., 2014). A sample size of ≥ 5 rats per group was computed using G*Power version 3.1.2 given $\alpha = 0.05$, power $(1 - \beta) = 0.9$, and effect size $(d) = 2.64$. Cage-mates were randomized to treatment or control groups, and all surviving rats at designated endpoints were included in the data analysis. No animals or potential outliers were excluded from the datasets presented. Biochemical and histological assessments were performed in a blinded manner. Animals were housed under standard conditions (12:12h light-dark cycle, temperature $23 \pm 2^\circ\text{C}$, with free access to chow and water), 3 rats per cage, and were acclimatized to the room for 1 week before the beginning of the experiments.

Rat carbon tetrachloride (CCl₄) model

Chronic carbon tetrachloride (CCl₄) intoxication in rodents is a reproducible, well-characterized model that induces liver fibrosis (after ~4 weeks) and cirrhosis (after ~12 weeks). Liver injury was induced in 8-week old (250-300g) male wild-type Sprague-Dawley rats by twice-weekly intraperitoneal (i.p.) injection of 0.2 mL/100g CCl₄ (Sigma-Aldrich, Gillingham, UK) in a 1:1 ratio with sterile olive oil vehicle (OO; Sigma-Aldrich, Gillingham, UK), for either 4 or 12 weeks (n=6 per group). Littermate control rats (n=4 per group) were injected with an identical volume of OO. Animals

underwent MRI at the end of the 4- or 12-week treatment course under inhalational general anesthesia (1–2% isoflurane in oxygen) without recovery. Body weight, liver and spleen weights were recorded and liver tissue and blood were collected for histological and biochemical analysis. One of the 12-week CCl₄-treated animals was euthanized prematurely due to poor health and excluded from analysis.

Methionine and choline deficient diet model

The methionine and choline deficient (MCD) diet is a frequently used rodent model of NASH. After 4 weeks of MCD diet, aminotransferase levels are elevated and hepatic histology shows florid centrilobular hepatocellular steatosis, whilst 8 weeks of MCD diet results in NASH and early fibrosis. Steatohepatitis was induced in 8-week old (250-300g) male wild-type Sprague-Dawley rats by feeding a lipogenic MCD diet (Research Diets, New Jersey, USA) for 4 or 8 weeks (n=6 per group). Littermate control rats received standard chow (n=4 per group). Animals underwent MRI at the end of the 4- or 8-week treatment course under inhalational general anesthesia (1–2% isoflurane in oxygen) without recovery. Body weight, liver and spleen weights were recorded and liver tissue and blood were collected for histological and biochemical analysis.

In a separate experiment, rats were fed an MCD diet for a total of 4 weeks. After 2 weeks, animals were randomized to treatment with either 100 mg/kg/day of cilostazol (a phosphodiesterase type-3 inhibitor previously shown to reduce steatohepatitis in experimental NAFLD models (Fujita et al., 2008)) or vehicle by daily gavage from week 2 to week 4 (n=6 per group). Animals underwent MRI at baseline and after 4 weeks.

In vivo MR imaging

All animals were continually monitored for vital signs and to maintain depth of anesthesia. Respiration rate was monitored by detection of breathing motion by compression of a respiratory sensor placed in contact with the abdomen and core temperature by a rectal thermometer.

Multiparametric liver MRI

Imaging studies were performed in anesthetized rats using a 7.0 Tesla preclinical MRI scanner (Agilent Technologies, Santa Clara, CA, USA), with a 72 mm internal diameter volume coil. At each timepoint, the following scans were run: two gradient-echo scans with arrayed echo times (repetition time (TR) 75 ms, 4 averages, flip angle 20°), one scan with 4 in-phase echoes (1.01, 2.02, 3.03, 4.04 ms) and one

with 4 out-of-phase echoes (1.52, 2.53, 3.54, 4.55 ms), to measure fat using a 3-point Dixon method, and T2* using the 4 in-phase echoes. An adapted gradient echo (fast low angle shot (FLASH)) Look-Locker sequence was used to measure T1 (acquired with 5 inversion times ranging from 270 to 5100 ms), 2 averages, flip angle 8°, echo train length 32). All scans were respiratory gated with a field of view (FOV) 60 mm x 60 mm, 2 mm thick single slice, 128 x 128 matrix. The pulse sequence was based on previous work (Li et al., 2010), but was adapted to use an inversion recovery module rather than a saturation module. The axial slice was positioned to cover an abdominal region containing primarily liver. Depending on respiration and anesthesia levels, the full set of scans including shimming and localization were completed in 40-50 min.

MR image analysis

Data was exported in phase and magnitude format. T2* was determined using the in-phase gradient echo data (Bonny et al., 1996). Pixels in which the magnitude failed to exceed the background noise by 2-fold (Bonny et al., 1996) were excluded from the analysis. A combination of the in-phase and out-of-phase gradient echo data were used to calculate proton density fat and water maps using the extended 3-point Dixon approach (Glover and Schneider, 1991). The PDFF was calculated from the fat (F) and water (W) maps as

$$PDFF = \frac{F}{F + W}$$

T1 maps were calculated from the Look-Locker data as described previously (Deichmann et al., 1992). From each of the T1, T2* and PDFF maps three circular regions of interest (ROI) were collected per time point per animal. ROI placement was in the left, right and central region of each liver in a location where there was no visible motion artefact or signal voids due to bowel gas. Vessels and non-liver regions were not included in ROIs.

To investigate the potential effect of parenchymal fat on hepatic T1, we also performed localized proton magnetic resonance spectroscopy (1H-MRS) using a point resolved spectroscopy (PRESS) sequence at two distinct locations depicting different signal intensity on the T2-weighted structural scan. The voxel dimensions were 4 x 4 x 4 mm, TR = 3 s and echo time (TE) = 23 ms. One signal average was used.

Serum liver enzyme measurement

Serum alanine aminotransferase (ALT), was measured as described previously (Bergmeyer et al., 1978) utilizing a commercial kit (Alpha Laboratories Ltd., Eastleigh, UK). Aspartate aminotransferase (AST) was determined by a commercial kit (Randox Laboratories, Crumlin, UK). Total bilirubin was determined by the acid diazo method (Pearlman et al., 1974) using a commercial kit (Alpha Laboratories Ltd., Eastleigh, UK). All assays were adapted for use on a Cobas Fara centrifugal analyzer (Roche Diagnostics Ltd., Welwyn Garden City, UK). Within-run precision of these assays was calculated and expressed as coefficient of variation (CV) < 4%, while intra-batch precision was CV < 5%.

Liver histology assessments

Liver tissue was fixed in 4% phosphate-buffered formaldehyde and embedded in paraffin, or in OCT and frozen on dry ice and stored at -80°C until histological analysis. Five-micron sections were stained for activated hepatic stellate cells by alpha-smooth muscle actin (α -SMA) (A2547; Sigma-Aldrich, Gillingham, UK) and interstitial fibrosis by picrosirius red, as described previously (Fallowfield et al., 2014). Histology was assessed independently by an expert liver histopathologist, blinded to treatment allocation. Livers were staged for fibrosis by the injury-independent modified Ishak score (scale 0-6; Table S1). Both CCl_4 and MCD diet induce steatosis and lobular necroinflammation, modeling histological features characterizing NAFLD. In human disease, this is assessed by application of the NAFLD activity score (NAS). The same features of steatotic inflammatory injury in the rodent models were scored on a scale from 0 to 8 (steatosis 0-3, lobular inflammation 0-3 and hepatocyte ballooning 0-2) on hematoxylin and eosin (H&E) stained sections using the same criteria (and referred to as the NAFLD (model) Activity Score (N(m)AS) to reflect this application (Kleiner et al., 2005). Hepatic iron (ferric form) was detected using Perls' Prussian Blue and scored using the Scheuer method (Scheuer et al., 1962) with grade 0 being negative and grades 1, 2, 3, and 4 representing increasing amounts of stainable iron. Frozen sections were stained with Oil Red O to assess liver fat content. Sections were analyzed using an AxioScan Z1 slide scanner at x20 magnification and ImageJ software (NIH, Bethesda, USA) to quantify the collagen proportionate area (CPA), α -SMA and Oil Red O staining, assessed by a blinded assessor.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 6.0 (GraphPad Software Inc., San Diego, USA). One-way analysis of variance (ANOVA) with Tukey's post-hoc test was used to assess differences between groups. Spearman's rank-order correlation (r_s) was used to examine associations. For all tests, a P -value <0.05 was taken to indicate statistical significance. Graphical data is presented as mean \pm standard deviation (SD).

ACKNOWLEDGEMENTS: The work was carried out on a preclinical MRI scanner within the Edinburgh Imaging Facility, University of Edinburgh. Cilostazol was kindly provided by Dr Barret Kalindjian (Zebra Therapeutics Ltd.).

COMPETING INTERESTS: Amy Herlihy, Michael Gyngell, Matteo Milanesi and Rajarshi Banerjee are employees of Perspectum Diagnostics Ltd., the developer of LiverMultiScan™. Amy Herlihy, Michael Gyngell and Rajarshi Banerjee hold stock in the company. Robert Janiczek and Philip Murphy are employees of GlaxoSmithKline and hold stock in the company. The remaining authors have no relevant conflicts of interest to declare. This is an academic led and reported study, with industry engagement. The role of Perspectum Diagnostics Ltd. was the provision of access to multiparametric MRI methodology and blinded analysis of raw MRI data. Study design and potential conflicts do not affect adherence to policies on sharing data and materials. All study investigations, data analysis, manuscript preparation and decision to submit was undertaken by the academic centre.

FUNDING: This study was funded by an unrestricted research grant from GlaxoSmithKline. JAF was supported by a NHS Research Scotland/Universities Scottish Senior Clinical Fellowship. TJK was supported by a Wellcome Trust Intermediate Clinical Fellowship (095898/Z/11/Z).

REFERENCES

- Banerjee, R., Pavlides, M., Tunnicliffe, E.M., Piechnik, S.K., Sarania, N., Philips, R., Collier, J.D., Booth, J.C., Schneider, J.E., Wang, L.M. et al. (2014).** Multiparametric magnetic resonance for the non-invasive diagnosis of liver disease. *J Hepatol.* **60**(1), 69-77.
- Bergmeyer, H. U., Scheibe, P., Wahlefeld, A. W. (1978).** Optimization of methods for aspartate aminotransferase and alanine aminotransferase. *Clin Chem.* **24**(1), 58-73.
- Bonny, J. M., Zanca, M., Boire, J. Y., Veyre, A. (1996).** T2 maximum likelihood estimation from multiple spin-echo magnitude images. *Magn Reson Med.* **36**(2), 287-93.
- Britton, L. J., Subramaniam, V. N., Crawford, D. H. (2016).** Iron and non-alcoholic fatty liver disease. *World J Gastroenterol.* **22**(36), 8112-22.
- Bydder, M., Yokoo, T., Hamilton, G., Middleton, M.S., Chavez, A.D., Schwimmer, J.B., Lavine, J.E., Sirlin, C.B. (2008).** Relaxation effects in the quantification of fat using gradient echo imaging. *Magnetic resonance imaging.* **26**(3), 347-59.
- Clapper, J.R., Hendricks, M.D., Gu, G., Wittmer, C., Dolman, C.S., Herich, J., Athanacio, J., Villescaz, C., Ghosh, S.S., Heilig, J.S. et al. (2013).** 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.* **305**(7), G483-95.
- Deichmann, R. and Haase, A. (1992).** Quantification of T1 Values by Snapshot-Flash Nmr Imaging. *J Magn Reson.* **96**(3), 608-12.
- Fallowfield, J.A., Hayden, A.L., Snowdon, V.K., Aucott, R.L., Stutchfield, B.M., Mole, D.J., Pellicoro, A., Gordon-Walker, T.T., Henke, A., Schrader, J. et al. (2014).** Relaxin modulates human and rat hepatic myofibroblast function and ameliorates portal hypertension in vivo. *Hepatology.* **59**(4), 1492-504.
- Fujita, K., Nozaki, Y., Wada, K., Yoneda, M., Endo, H., Takahashi, H., Iwasaki, T., Inamori, M., Abe, Y., Kobayashi, N. et al. (2008).** Effectiveness of antiplatelet drugs against experimental non-alcoholic fatty liver disease. *Gut.* **57**(11), 1583-91.
- Glover, G. H., Schneider, E. (1991).** Three-point Dixon technique for true water/fat decomposition with B0 inhomogeneity correction. *Magn Reson Med.* **18**(2), 371-83.

- Hernando, D., Levin, Y.S., Sirlin, C.B., Reeder, S.B.** (2014). Quantification of liver iron with MRI: State of the art and remaining challenges. *J Magn Reson Imaging*. **40**(5), 1003-1021.
- Kleiner, D.E., Brunt, E.M., Van Natta, M., Behling, C., Contos, M.J., Cummings, O.W., Ferrell, L.D., Liu, Y.C., Torbenson, M.S., Unalp-Arida, A., et al.** (2005). Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. **41**(6), 1313-21.
- Li, W., Griswold, M., Yu, X.** (2010). Rapid T1 mapping of mouse myocardium with saturation recovery Look-Locker method. *Magn Reson Med*. **64**(5), 1296-303.
- Noureddin, M., Lam, J., Peterson, M.R., Middleton, M., Hamilton, G., Le, T.A., Bettencourt, R., Changchien, C., Brenner, D.A., Sirlin, C. et al.** (2013). Utility of magnetic resonance imaging versus histology for quantifying changes in liver fat in nonalcoholic fatty liver disease trials. *Hepatology*. **58**(6), 1930-40.
- O'Regan, D.P., Callaghan, M.F., Wylezinska-Arridge, M., Fitzpatrick, J., Naoumova, R.P., Hajnal, J.V., Schmitz, S.A.** (2008). Liver fat content and T2*: simultaneous measurement by using breath-hold multiecho MR imaging at 3.0 T--feasibility. *Radiology*. **247**(2), 550-7.
- Pavlidis, M., Banerjee, R., Sellwood, J., Kelly, C.J., Robson, M.D., Booth, J.C., Collier, J., Neubauer, S., Barnes, E.** (2016). Multiparametric magnetic resonance imaging predicts clinical outcomes in patients with chronic liver disease. *J Hepatol*. **64**(2), 308-15.
- Pavlidis, M., Banerjee, R., Tunnicliffe, E.M., Kelly, C., Collier, J., Wang, L.M., Fleming, K.A., Cobbold, J.F., Robson, M.D., Neubauer, S. et al.** (2017). Multi-parametric magnetic resonance imaging for the assessment of non-alcoholic fatty liver disease severity. *Liver Int*. **37**(7),1065-1073.
- Pearlman, F. C. and Lee, R. T.** (1974). Detection and measurement of total bilirubin in serum, with use of surfactants as solubilizing agents. *Clin Chem*. **20**(4), 447-53.
- Runge, J.H., Bakker, P.J., Gaemers, I.C., Verheij, J., Hakvoort, T.B., Ottenhoff, R., Nederveen, A.J., Stoker, J. .** (2014). Measuring liver triglyceride content in mice: non-invasive magnetic resonance methods as an alternative to histopathology. *MAGMA*. **27**(4), 317-27.
- Scheuer, P. J., Williams, R., Muir, A. R.** (1962). Hepatic pathology in relatives of patients with haemochromatosis. *J Pathol Bacteriol*. **84**, 53-64.

- Singh, S., Venkatesh, S.K., Wang, Z., Miller, F.H., Motosugi, U., Low, R.N., Hassanein, T., Asbach, P., Godfrey, E.M., Yin, M. et al.** (2015). Diagnostic performance of magnetic resonance elastography in staging liver fibrosis: a systematic review and meta-analysis of individual participant data. *Clin Gastroenterol Hepatol.* **13**(3),440-51 e6.
- Tanner, M. A., He, T., Westwood, M. A., Firmin, D. N., Pennell, D. J., Thalassaemia International Federation Heart T I.** (2006). Multi-center validation of the transferability of the magnetic resonance T2* technique for the quantification of tissue iron. *Haematologica.* **91**(10), 1388-91.
- Torok, N. J., Dranoff, J. A., Schuppan, D. and Friedman, S. L.** (2015). Strategies and endpoints of antifibrotic drug trials: Summary and recommendations from the AASLD Emerging Trends Conference, Chicago, June 2014. *Hepatology.* **62**(2), 627-34.
- Wilman, H.R., Kelly, M., Garratt, S., Matthews, P.M., Milanesi, M., Herlihy, A., Gyngell, M., Neubauer, S., Bell, J.D., Banerjee, R. et al.** (2017). Characterisation of liver fat in the UK Biobank cohort. *PLoS One.* **12**(2), e0172921.
- Younossi, Z. M., Koenig, A. B., Abdelatif, D., Fazel, Y., Henry, L. and Wymer, M.** (2016). Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology.* **64**(1), 73-84.
- Zhu, B., Wei, L., Rotile, N., Day, H., Rietz, T., Farrar, C.T., Lauwers, G.Y., Tanabe, K.K., Rosen, B., Fuchs, B.C. et al.** (2017). Combined magnetic resonance elastography and collagen molecular magnetic resonance imaging accurately stage liver fibrosis in a rat model. *Hepatology.* **65**(3), 1015-25.

Figures

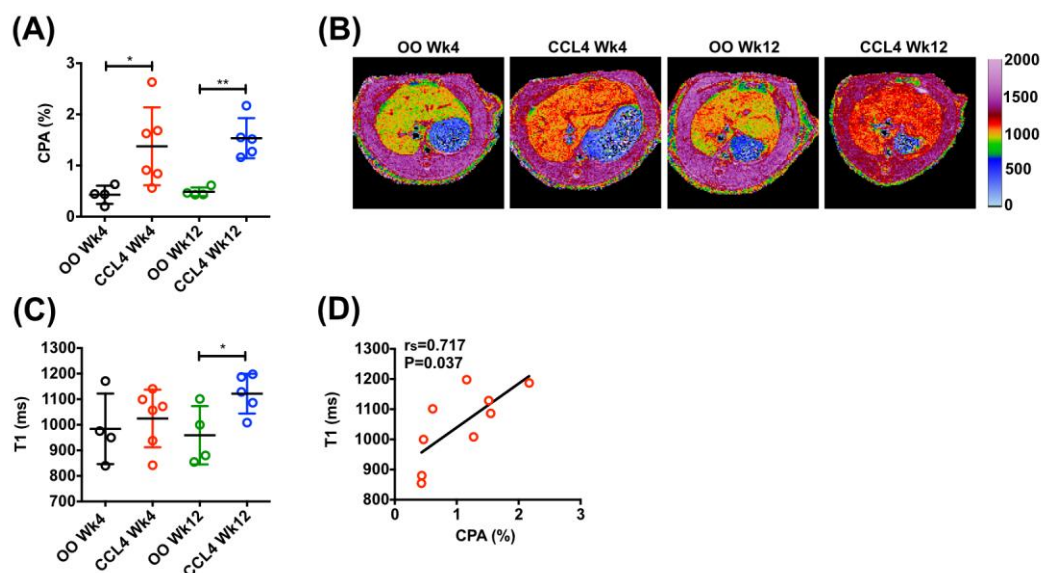


Fig. 1. MRI assessment of hepatic fibro-inflammatory injury in rat CCl₄ model.

A) Quantification of hepatic collagen content (% area of picrosirius red staining). **B)** Representative examples of transverse liver MR T1 relaxation maps. **C)** T1 relaxation quantification in rats receiving CCl₄ or olive oil (OO) for 4 and 12 weeks. **D)** Correlation between hepatic collagen content and T1 in rats receiving CCl₄ and OO for 12 weeks. Data presented as mean±SD, analyzed by unpaired t-test (* $P < 0.05$, ** $P < 0.01$). Spearman (r_s) correlation coefficient was used to examine correlations.

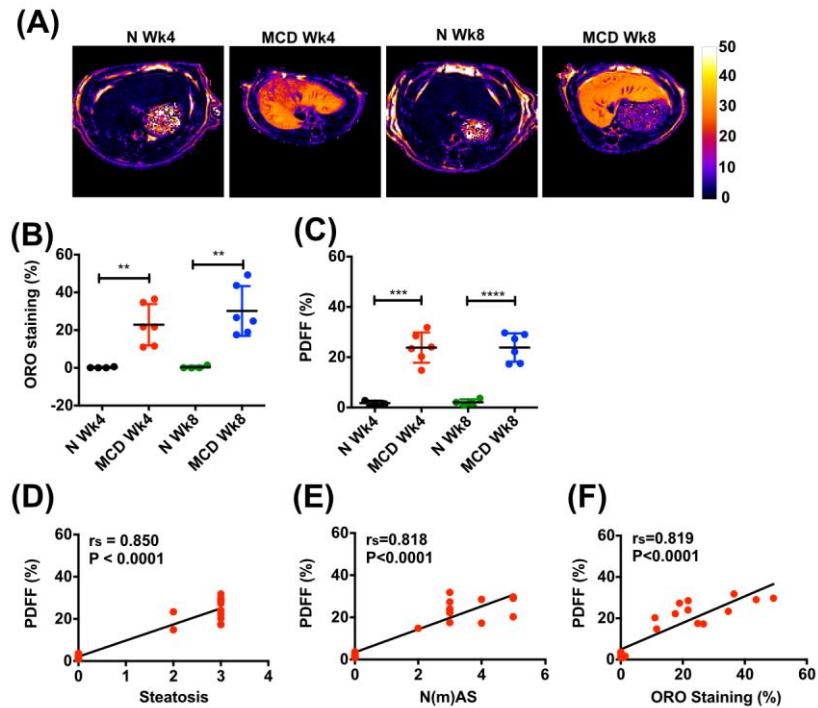


Fig. 2. MRI assessment of hepatic steatosis and steatotic inflammatory injury in rat MCD diet model. **A)** Representative examples of transverse liver MR PDFF maps. **B)** Quantification of Oil red O (ORO) staining of hepatic fat. **C)** Quantification of hepatic proton density fat fraction (PDFF). Correlations between hepatic PDFF and **D)** histologically assessed steatosis, **E)** steatotic inflammatory injury (N(m)AS) score and **F)** ORO staining in rats receiving MCD or control diet (N) for 4 and 8 weeks. Data presented as mean \pm SD, analyzed by unpaired t-test (** $P < 0.01$, *** $P < 0.0001$, **** $P < 0.0001$). Spearman (r_s) correlation coefficient was used to examine correlations.

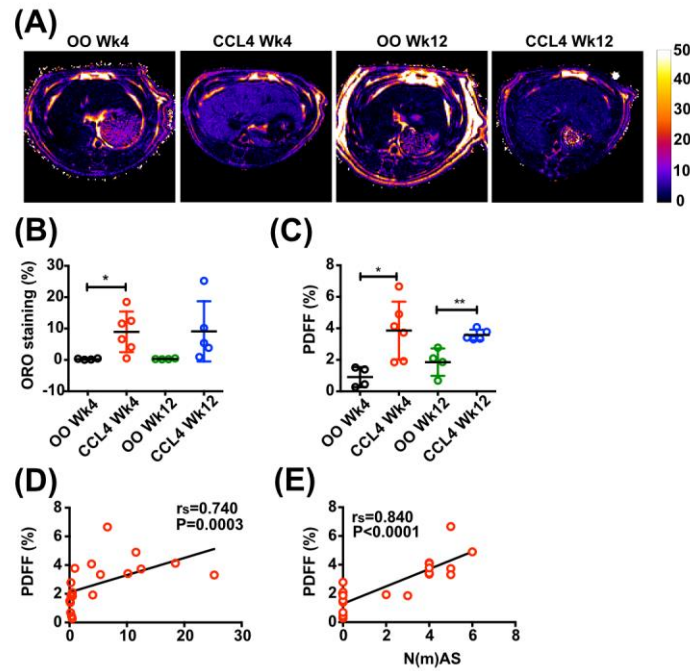


Fig. 3. MRI assessment of hepatic steatosis and steatohepatitis in rat CCl₄ model. **A)** Representative examples of transverse liver MR PDFF maps. **B)** Quantification of Oil red O (ORO) staining of hepatic fat. **C)** Quantification of hepatic proton density fat fraction (PDFF). Correlations between hepatic PDFF and **D)** histologically assessed ORO staining and **E)** steatotic inflammatory injury (N(m)AS) score in rats receiving CCl₄ or olive oil (OO) for 4 and 12 weeks. Data presented as mean±SD, analyzed by unpaired t-test (* $P < 0.05$, ** $P < 0.01$). Spearman (r_s) correlation coefficient was used to examine correlations.

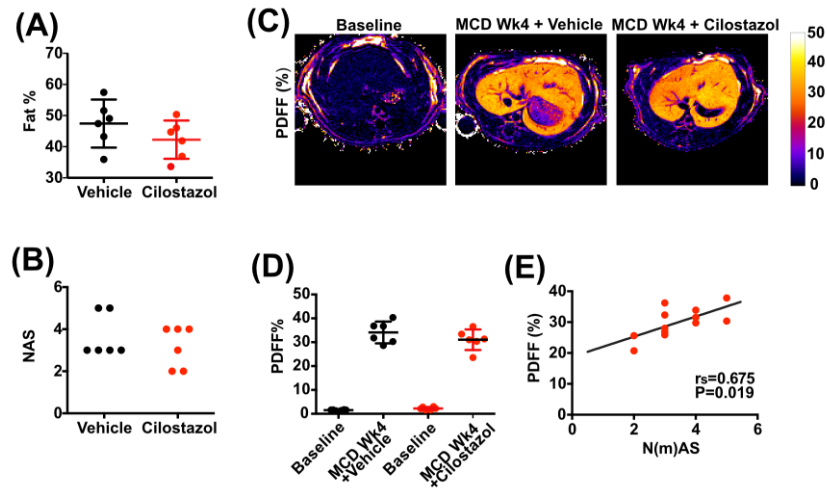


Fig. 4. MRI assessment of hepatic steatosis and steatohepatitis in cilostazol treated rats on MCD diet. Sprague-Dawley rats received MCD diet for 4 weeks plus cilostazol or vehicle for the last 2 weeks of the model. Animals were scanned at baseline and after 4 weeks. **A)** Quantification of hepatic fat by morphometry based on H&E staining. **B)** Steatotic inflammatory injury (N(m)AS) score. **C)** Representative examples of transverse liver MR proton density fat fraction (PDFFF). **D)** Quantification of hepatic PDFFF. **E)** Correlation between PDFFF and steatotic inflammatory injury (N(m)AS) score. Data presented as mean \pm SD, analyzed by unpaired t-test. Spearman (r_s) correlation coefficient was used to examine correlations.

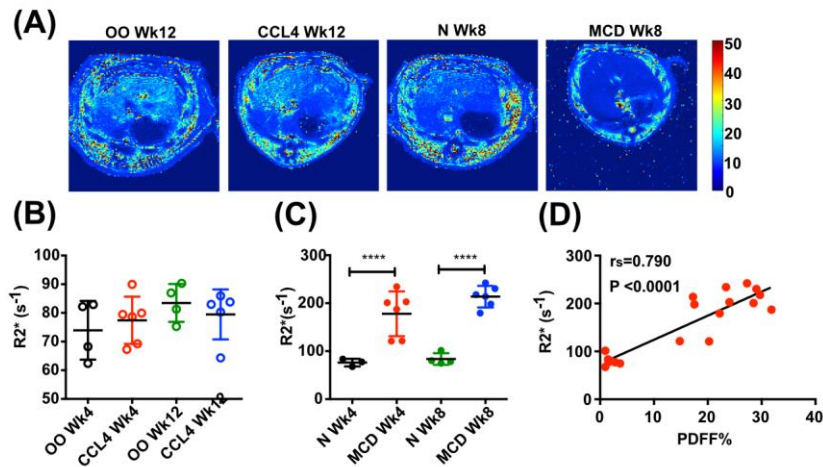


Fig. 5. MRI assessment of T2*/R2* relaxation in preclinical liver injury models.

A) Representative examples of transverse liver MR T2* maps. **B)** Quantification of R2* (1/T2*) in rats receiving CCl₄ or olive oil (OO) for 4 and 12 weeks. **C)** Quantification of R2* (1/T2*) in rats receiving MCD or control diet (N) for 4 and 8 weeks. **D)** Correlation between R2* and PDFF in animals receiving CCl₄ or OO injections and MCD or control diets. Data presented as mean±SD, analyzed by unpaired t-test (*****P*<0.0001). Spearman (*r_s*) correlation coefficient was used to examine correlations.

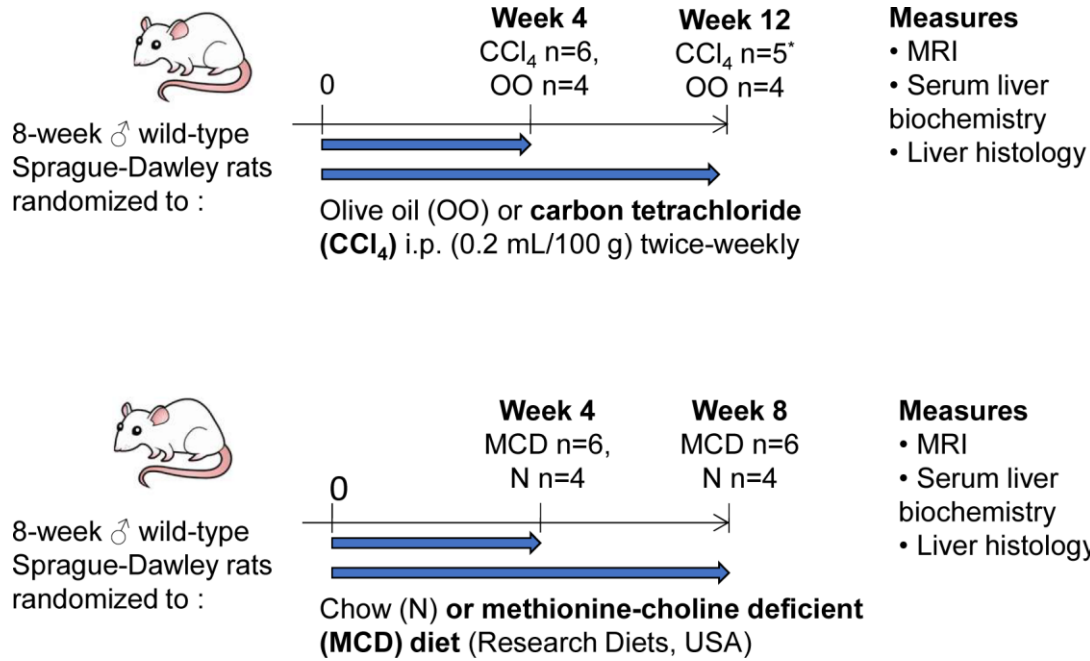


Fig. 6. Rat models of chronic liver disease.

Supporting Information

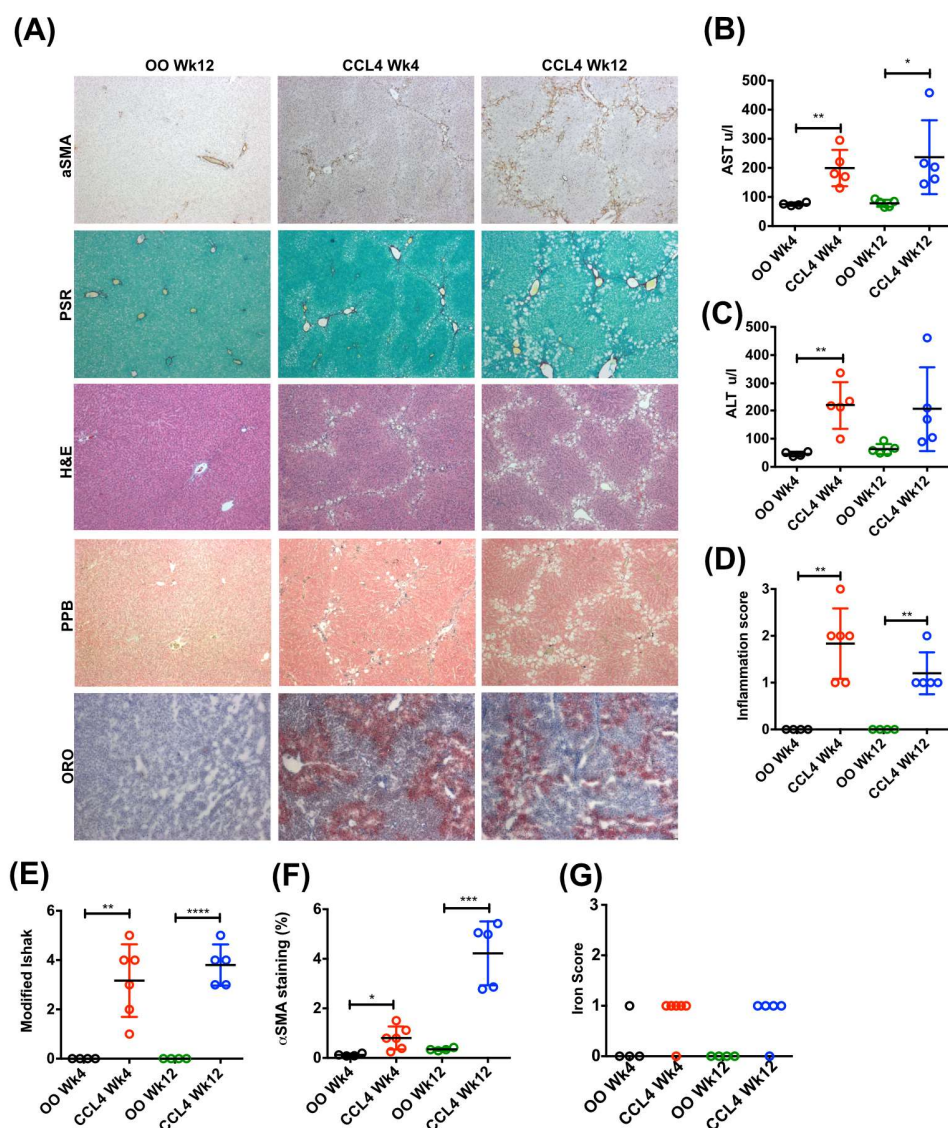


Fig. S1. Characterization of rat CCl₄ model. Liver histology and serum markers were assessed in male Sprague-Dawley rats receiving CCl₄ or olive oil (OO) injections for 4 or 12 weeks. **A)** Representative liver staining images of: alpha-smooth muscle actin (α -SMA) staining of activated hepatic stellate cells, picrosirius red (PSR) staining of fibrillar collagen, hematoxylin and eosin (H&E), Perls' Prussian Blue (PPB) staining of iron, Oil

Red O (ORO) fat staining. **B**) Serum alanine aminotransferase (ALT) levels. **C**) Serum aspartate aminotransferase (AST) levels. **D**) Iron score based on PPB staining. Data presented as mean \pm SD, analyzed by unpaired t-test (* P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001).

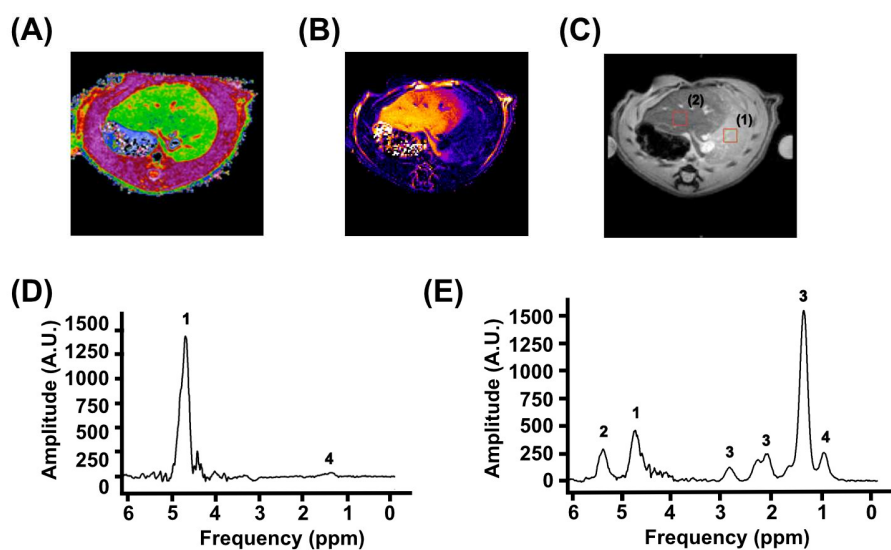


Fig. S2. Influence of fat on hepatic T1. **A**) Transverse liver MR T1 relaxation map. **B**) Transverse liver MR proton density fat fraction (PDFF) map. **C**) Structural T2-weighted MRI scan with marked voxels. **D**) Magnetic resonance spectroscopy (MRS) spectra acquired from voxel 1, and **E**) MRS spectra acquired from voxel 2. Peak assignment is as follows, 1: H₂O, 2: Olefinic protons (CH=CH-), 3: Methylene protons (-CH=CHCH₂-), 4: Methylene protons (-CH₂-), 5: Methyl protons (-CH₃).

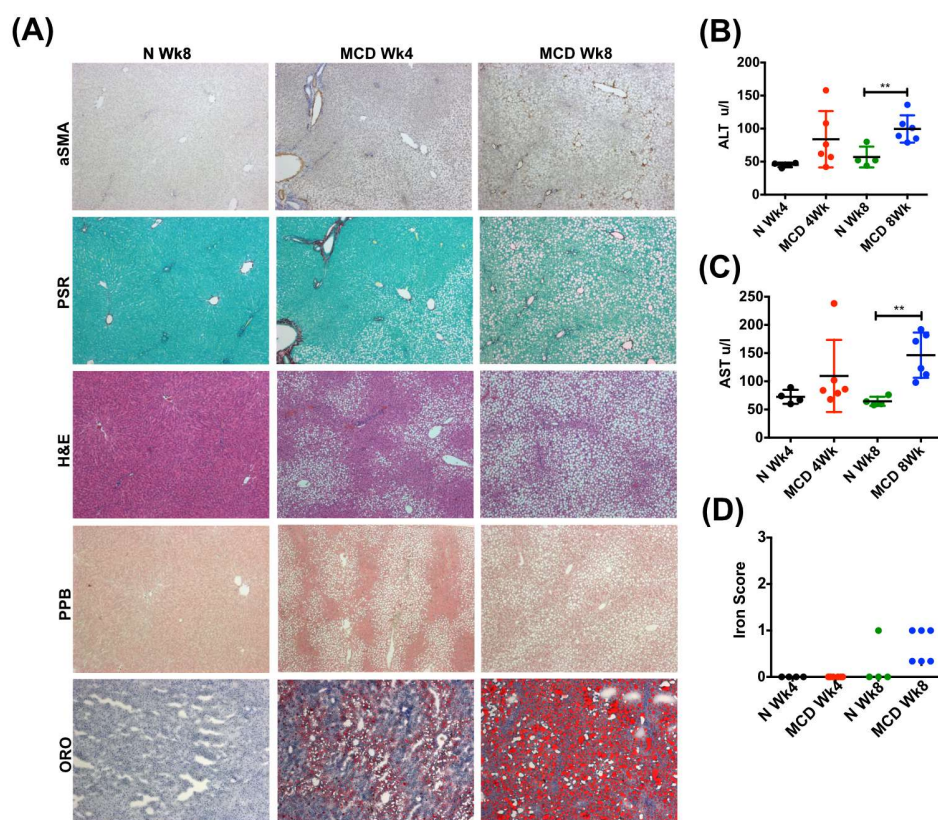


Fig. S3. Characterization of MCD diet model. Liver histology and serum markers were assessed in male Sprague-Dawley rats receiving MCD or control diet (N) for 4 or 8 weeks. **A)** Representative liver staining images of: alpha-smooth muscle actin (α -SMA) staining of activated hepatic stellate cells; picosirius red (PSR) staining of fibrillar collagen; hematoxylin and eosin (H&E), Perls' Prussian Blue (PPB) staining of iron; Oil Red O (ORO) fat staining. **B)** Serum aspartate aminotransferase (AST) levels. **C)** Serum alanine aminotransferase (ALT) levels. **D)** Inflammation score based on H&E staining. **E)** Modified Ishak fibrosis score. **F)** Quantification of α -SMA staining by morphometry. **G)** Iron score based on PPB staining. Data presented as mean \pm SD, analyzed by unpaired t-test (** $P < 0.01$).

Score	Description	Fibrosis Severity
0	No fibrosis	None
1	Zonal fibrosis involving a minority of portal tracts and/or zone 3 areas	Mild
2	Zonal fibrosis involving a majority of portal tracts and/or zone 3 areas	Mild
3	Bridging fibrosis - occasional foci	Moderate
4	Bridging fibrosis – widespread	Moderate
5	Bridging fibrosis – widespread, with occasional nodules (incomplete/early cirrhosis)	Severe
6	Cirrhosis, probable or definite	Severe

Table S1. Modified Ishak score for hepatic fibrosis