# MicroRNA-26b protects against MASH development and can be efficiently targeted with lipid nanoparticles.

**Short title:** MicroRNA-26b protects against MASH

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## Abbreviations

| | |
|---|---|
| ABCA1 | ATP binding cassette subfamily A member 1 |
| ACAT2 | Acetyl-CoA acetyltransferase 2 |
| ACTA2 | Actin alpha 2 |
| BSA | Bovine serum albumin |
| CCL2 | CC-chemokine ligand 2 |
| CD36 | Platelet glycoprotein 4 |
| CXCL1 | C-X-C motif chemokine ligand 1 |
| eLNPs | Empty LNPs |
| ERK | Extracellular-signal, regulated kinase |
| IL-6 | Interleukin-6 |
| JNK | c-Jun-N-terminal kinase |
| LNP | Lipid nanoparticle |
| MAPK | Mitogen-activated protein kinase |
| MASH | Metabolic dysfunction-associated steatohepatitis |
| MASLD | Metabolic dysfunction-associated steatotic liver disease |
| mLNPs | Mimic LNPs |
| MMP13 | Matrix metalloproteinase 13 |
| NAFLD | Non-alcohol fatty liver disease |
| NF-κB | Nuclear factor-kappa B |
| miR-26b | MicroRNA-26b |
| Ox-LDL | Oxidized low-density lipoprotein |
| PCA | Principal component analysis |
| PDGFR-β | Platelet-derived growth factor receptor-beta |
| SEM | Standard error of the mean |
| Sra | Scavenger receptor a |
| STK | Serine/Threonine kinase |
| TAK1 | TGF-activated kinase-1 |
| Tgfb | transforming growth factor β |
| WTD | Western-type diet |

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## Disclosures

JJ is cofounder of AMICARE GmbH. The other authors declare no conflict of interest.

## Synopsis

Our study highlights the protective function of microRNA-26b in MASH by lowering lipid levels, inflammation, and fibrosis. Using microRNA-26b-containing lipid nanoparticles MASH severity was reduced in mice, providing a novel therapeutic approach that was validated in human precision-cut liver slices.

**Abstract**

**Background & Aims.** The prevalence of metabolic dysfunction-associated steatohepatitis (MASH) is increasing, urging more research into the underlying mechanisms. MicroRNA-26b (miR-26b) might play a role in several MASH-related pathways. Therefore, we aimed to determine the role of miR-26b in MASH and its therapeutic potential using miR-26b mimic-loaded lipid nanoparticles (LNPs).

**Methods.** *Apoe*$^{-/-}$*Mir26b*$^{-/-}$, *Apoe*$^{-/-}$*LysM*$^{cre}$*Mir26b*$^{fl/fl}$ mice, and respective controls were fed a western-type diet to induce MASH. Plasma and liver samples were characterized regarding lipid metabolism, hepatic inflammation, and fibrosis. Additionally, miR-26b mimic-loaded LNPs were injected in *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice to rescue the phenotype and key results were validated in human precision-cut liver slices. Finally, kinase profiling was used to elucidate underlying mechanisms.

**Results.** *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice showed increased hepatic lipid levels, coinciding with increased expression of scavenger receptor a and platelet glycoprotein 4. Similar effects were found in mice lacking myeloid-specific *miR-26b*. Additionally, hepatic TNF and IL-6 levels and amount of infiltrated macrophages were increased in *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice. Moreover, *Tgfb* expression was increased by the *miR-26b* deficiency, leading to more hepatic fibrosis. A murine treatment model with miR-26b mimic-loaded LNPs reduced hepatic lipids, rescuing the observed phenotype. Kinase profiling identified increased inflammatory signaling upon *miR-26b* deficiency, which was rescued by LNP treatment. Finally, miR-26b mimic-loaded LNPs also reduced inflammation in human precision-cut liver slices.

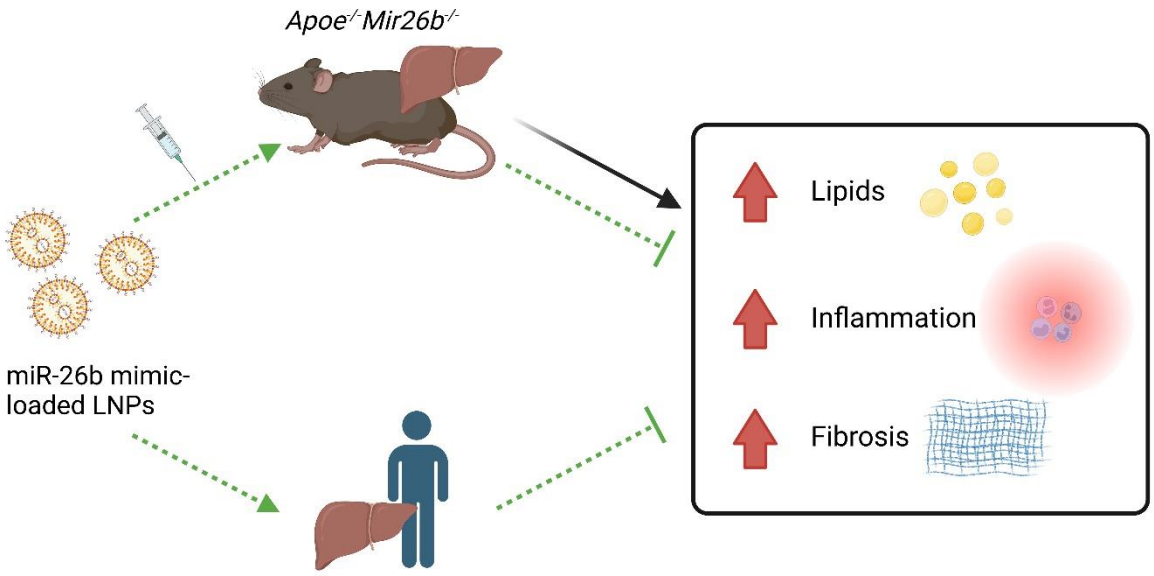
**Conclusions.** Overall, our study demonstrates that the detrimental effects of *miR-26b* deficiency in MASH can be rescued by LNP treatment. This novel discovery leads to

more insight into MASH development, opening doors to potential new treatment options using LNP technology.

**Keywords**

Metabolic dysfunction-associated steatohepatitis; hepatic inflammation; microRNAs; lipid nanoparticles

**Graphical abstract**



## 1. INTRODUCTION

‘Metabolic dysfunction-associated steatotic liver disease’ (MASLD), formerly known as non-alcohol fatty liver disease (NAFLD) [1], is the most common form of fatty liver disease. It accounts for roughly 25% of liver-disease cases globally and is defined by fat accumulation in the liver in the absence of heavy alcohol consumption [1]. MASLD refers to a group of disorders that include simple metabolic dysfunction-associated steatotic liver (MASL) and metabolic dysfunction-associated steatohepatitis (MASH) [1]. An estimated 20% to 25% of MASL patients will acquire MASH, and both MASL and MASH prevalence are predicted to rise even higher over the next decade due to increased frequency of risk factors such as obesity and insulin resistance [2]. While MASL is still considered reversible and generally benign, its progression into MASH is thought to be harmful in most cases since it precedes the development of liver fibrosis, cirrhosis, and cancer [2]. Moreover, the underlying mechanisms that initiate inflammation and cause MASL to progress into MASH are still poorly understood, which restricts the range of available treatments.

One of the main players that may be involved in these processes is microRNAs (miRs). MiRs are short non-coding RNAs that regulate gene expression post-transcriptionally by limiting messenger RNA (mRNA) translation [3]. Because miRs are involved in the control of numerous genes and processes, multiple miRs have been demonstrated to play a role in fundamental aspects of MASH, including lipid metabolism, hepatic inflammation, and fibrosis [3, 4].

Although many miRs have been examined in MASLD, the possible pathogenic involvement of numerous others, remains unknown. One of the miRs that has been researched in a number of pathologies, including cancer, cardiovascular illnesses, and

neurological disorders is miR-26b [5]. However, so far very little is known about the function of miR-26b in the liver and MASH. Nonetheless, some studies have already revealed that miR-26b may play a role in several MASH-related pathways. For example, miR-26b appears to suppress the nuclear factor-kappa B (NF-κB) pathway in human hepatocellular carcinoma cell lines by inhibiting the expression of TGF-activated kinase-1 (TAK1) and TAK1-binding protein-3, both of which are positive regulators of the NF-κB system [6]. Furthermore, miR-26b has been shown to downregulate the gene expression of platelet-derived growth factor receptor-beta (PDGFR-β), hence reducing hepatic fibrogenesis [7].

The findings above show that miR-26b may play a role in the pathogenesis of MASH, although no study has determined its exact causal involvement in MASH development. In this investigation, we used previously described miR-26b knockout mice [5] and myeloid cell-specific miR-26b knockout mice to clarify the role of miR-26b in hepatic lipid metabolism, inflammation, and fibrogenesis. Furthermore, lipid nanoparticles (LNPs), which act as clinically and therapeutically relevant vehicles [8], loaded with miR-26b mimics were used to restore miR-26b levels in mice as well as in human precision-cut liver slices to investigate its therapeutic potential.

## 2. METHODS

### 2.1 Animals

*Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice were generated as described in [5]. *Apoe*$^{-/-}$ mice were used as control and all mice were on C57BL/6J background for more than 10 generations. Both male and female mice were used for this study. Furthermore, myeloid lineage-specific *Mir26b*$^{fl/fl}$ mice on an *Apoe*$^{-/-}$ background were generated by crossing *Apoe*$^{-/-}$*Mir26b*$^{fl/fl}$ with *Lysm*$^{Cre+}$ transgenic mice to form *Apoe*$^{-/-}$*Mir26b*$^{fl/fl}$*Lysm*$^{Cre+}$ mice. *Apoe*$^{-/-}$ *Mir26b*$^{fl/fl}$*Lysm*$^{Cre-}$ mice were used as control. Starting at 8 weeks of age, the mice were fed a Western-type diet (WTD) containing 21% fat and 0.20% cholesterol (Sniff TD88137) for 4 or 12 weeks after which the mice were euthanized by intraperitoneal injection of ketamine (0.1 mg/g body weight) and xylazine (0.02 mg/g body weight). The blood was collected, and livers were harvested and snapfrozen using liquid nitrogen. The livers were embedded in Tissue-Tek O.C.T. compound (Sakura) and cryosectioned in 5µm cuts and mounted on glass slides. For metabolic measurements and further analysis, 100µm tissue sections were collected in 2ml reaction tubes. All animal studies performed were approved by the local ethical committee (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Germany, approval number 81-02.04.2019.A363 and Regierung von Oberbayern, approval number 55.2-1-54-253245-2015).

### 2.2 Lipid Nanoparticle Production

The ionizable cationic lipid DLin-MC3-DMA (Hycultec GmbH, Beutelsbach, Germany) was combined with DSPC (1,2-distearoyl-sn-glycero-3-phosphorylcholine, Avanti), Cholesterol (Sigma) and DMG-PEG 2000 (Avanti) in absolute ethanol at molar ratios of 50:10:38.5:1.5 and a final lipid concentration of 50 mM. The aqueous phase was

prepared by dissolving a combination (1:1) of miRNA-26b-3P and -5P in sterile 100 mM acetate buffer (pH 4), to achieve a 0.17 µg/µL solution. For LNP formulation, the ethanol and aqueous phase were injected into a microfluidic herringbone mixer (Microfluidic ChipShop, Jena, Germany) via syringe pumps at a flow rate ratio of 1:3, respectively, with a total flow rate of 4 mL/min to obtain an N/P ratio of 4. Generated lipid nanoparticles were diluted with PBS to a concentration of ethanol below 2 %, followed by concentrating via ultra-centrifugation (3000 *xg*) using 10 kDa Amicon Ultrafilitration units (Sigma), and were finally dialyzed against PBS using a dialysis membrane with 30 kDa MWCO.

To characterize the particle size and surface charge, samples were diluted with PBS and equilibrated for 15 min at 20°C before analysis. Particle sizes and polydispersity were determined by dynamic light scattering and Zetapotential measurements were carried out both, using a Zetasizer Nano ZS (Malvern Instruments Ltd).

The encapsulation efficiency of RNA of prepared lipid nanoparticles was determined using a Quant-iT RiboGreen assay according to the instructions of the manufacturer (Thermo Fisher Scientific). Samples were analyzed by fluorescence quantification on a microplate reader (Cytation 3, BioTek Instruments Inc.). The encapsulation efficiency was calculated as the difference between the total RNA and the non-encapsulated RNA, divided by the total RNA (x100%). The dosing of RNA-LNPs was based on the Ribogreen assay result.

### 2.3 LNP injections

LNPs were produced as described above LNPs containing 2mg/kg RNA were administered via intraperitoneal injection every 3 days. Empty LNPs (eLNPs) and LNPs loaded with murine miR-26b-3p (Duplex Sequences: 5'-

/5Phos/rCrCrUrGrUrUrCrUrCrCrArUrUrArCrUrUrGrGmCmUrC-3' and 5'-mGmCrCmArAmGrUmArAmUrGmGrAmGrAmArCmArGG-3') and miR-26b-5p (Duplex Sequences: 5'-/5Phos/rUrUrCrArArGrUrArArUrUrCrArGrGrArUrAmGmGrU-3' and 5'-mCmUrAmUrCmCrUmGrAmArUmUrAmCrUmUrGmAA-3') mimics (IDT) were injected into *Apoe*$^{-/-}$*miRNA-26b*$^{-/-}$ mice to determine potential therapeutic effects.

**2.4 RNA isolation and quantitative polymerase chain reaction**

One frozen liver piece of 25mg per mouse was homogenized in a closed tube with glass beads and 1mL Qiazol by using the Tissue Lyser (Qiagen) for 5min at 50Hz. RNA was isolated using the miRNeasy mini Kit according to the manufacturer's protocol (Qiagen). Following RNA isolation, cDNA was made from 500ng total RNA by adding 1µl Oligo (dT)-Primer (Eurofins Genomics). Secondary RNA structure was denaturalized at 70˚C for 5min after which the samples were briefly cooled on ice to allow primer annealing. Subsequently, M-MLV Reverse Transcriptase, M-MLV RT 5x Buffer, and dNTP Mix (Promega) were added and cDNA synthesis was completed by incubation for 1h at 37˚C. The relative gene expression levels were determined by quantitative polymerase chain reaction (qPCR) using primer sequences listed in **Table 1**. For the qPCR reaction, 10ng of cDNA template was used to which 1X PowerUp™ SYBR™ Green Master Mix (ThermoFisher) and primers (Eurofins Genomics) were added. PCR cycling was performed on QuantStudio 3 Real-Time PCR system (ThermoFisher) with the following conditions: 50˚C for 2min for 1 cycle (UDG activation); 95˚C for 2min for 1 cycle (Dual-Lock DNA polymerase); and 95˚C for 15sec (Denature), 58˚C for 15sec (Anneal) and 72˚C for 1min (Extend) for 40 cycles. The reference gene *Cyclophilin* was used for normalization.

**Table 1. Primer sequences for genes measured with qPCR.**

| | **Sequence in 5'-3'- direction** | |
|---|---|---|
| **Primer** | **Forward** | **Reverse** |
| *Abca1* | CCCAGAGCAAAAAGCGACTC | GGTCATCATCACTTTGGTCCTTG |
| *Acat2* | ACCAATTCCAGCCATAAAGCA | GGTTTAATCCAAGTTCTTTAGCTATTGC |
| *Acta2* | ACGAACGCTTCCGCTGC | GATGCCCGCTGACTCCAT |
| *Cd36* | GCCAAGCTATTGCGACATGA | AAAAGAATCTCAATGTCCGAGACTTT |
| *Cyclophilin* | TTCCTCCTTTCACAGAATTATTCCA | CCGCCAGTGCCATTATGG |
| *Mmp13* | ACAAAGATTATCCCCGCCTCATA | CACAATGCGATTACTCCAGATACTG |
| *Sr-A* | CATACAGAAACACTGCATGTCAGAGT | TTCTGCTGATACTTTGTACACACGTT |
| *Tgf-β* | GCCCTTCCTGCTCCTCATG | CCGCACACAGCAGTTCTTCTC |

## 2.5 Protein isolation

One frozen liver piece of 25mg per mouse was homogenized in 0.5ml SET buffer (sucrose 250mmol/L, EDTA 2mmol/L, TRIS 10mmol/L) by vortexing briefly. Cell destruction was completed by 2 freeze-thaw cycles with liquid nitrogen and subsequently passing the sample through a 27-gauge needle for 3 times. After one last freeze-thaw cycle the total protein content was measured on the NanoDrop One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific).

## 2.6 Lipid analysis

Cholesterol and triglyceride levels were quantified in liver protein lysates and EDTA-plasma using enzymatic colorimetric assays (c.f.a.s. cobas, Roche Diagnostics) according to the manufacturer’s protocol. Absorbance was measured at 510nm with the microplate reader infinite M200 (Tecan).

### 2.7 Enzyme-linked Immunosorbent Assay

Mouse TNF, interleukin-6 (IL-6), CC-chemokine ligand 2 (CCL2), and C-X-C Motif Chemokine Ligand 1 (CXCL1) levels were measured in liver protein lysates and EDTA-plasma by enzyme-linked immunosorbent assay (ELISA) (ThermoFisher) according to the manufacturer's instructions. Absorbance was measured at 450nm with wavelength subtraction at 570nm using the microplate reader infinite M200 (Tecan).

Human TNF, IL-6, CCL2, and CXCL1 levels were measured in the supernatant of human precision-cut liver slices using ELISA (ThermoFisher) according to the manufacturer's instructions. Absorbance was measured at 450nm with wavelength subtraction at 570nm using the microplate reader infinite M200 (Tecan).

### 2.8 Oil-red-O staining

Liver sections were prepared as described above. Following 30min drying to the air, the cryo sections were fixed with 3.5% formaldehyde for 30min at room temperature. Then the liver sections were stained with Oil-red-O (Sigma-Aldrich) for 1 hour and counterstained with Mayer's heamlum solution (Merck) for 30sec. After mounting the slides with glycerin jelly, images were acquired with an automated upright microscope (Leica microsystems), and the lipid content in the livers was quantified using ImageJ Fiji software (Laboratory for Optical and Computational Instrumentation, University of Wisconsin-Madison, Madison, Wisconsin, United States). All analyses were performed in a blinded manner.

### 2.9 Immunofluorescent stainings

For the visualization of inflammation and leukocyte infiltration, several immunofluorescent stainings were performed. The cryo-sectioned livers were first air-

dried for 5min at room temperature and subsequently fixed with ice-cold acetone for 10min. Tissue sections were blocked with 1% bovine serum albumin (BSA) and 0.03% normal horse serum blocking solution (Vector) in 1X PBS for 1h. Neutrophils, resident macrophages, infiltrating neutrophils and macrophages, and T-cells were visualized by staining with anti-mouse Ly6G (Biolegend, dilution 1:100), anti-mouse CD68 (Biolegend, dilution 1:250), anti-mouse Mac-1 (R&D Systems, dilution 1:100) or anti-mouse CD3 (Abcam, dilution 1:100), respectively, overnight at 4˚C. Liver sections were incubated with secondary antibodies Cy3-conjugated donkey anti-rat IgG (Jackson ImmunoResearch, dilution 1:300) or Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, dilution 1:300) for 30min at room temperature after which nuclei were stained with Hoechst (ThermoFisher, dilution 1:10,000) for 10min at room temperature. Following the staining, the sections were mounted with Immuno-Mount (ThermoFisher), and images were acquired using an inverted microscope Dmi8 (Leica microsystems). The number of Ly6-G, CD68, Mac-1, and CD3 positive immune cells was counted with the ImageJ Fiji software. All analyses were performed in a blinded manner.

### 2.10 Picrosirius red staining

Liver fibrosis was visualized by a Picrosirius red staining. First, the liver sections were fixed for 2h in 10% formalin. This was followed by a 90min incubation with 0,1% Sirius Red (Polysciences) in 1% saturated picric acid solution (Applichem). Slides were subsequently incubated in 0.01N HCl for 2min and dehydrated using an ethanol range. After incubation in xylol, the slides were mounted with Vitro-Clud (R.Langenbrinck). Images were acquired using an automated upright microscope (Leica microsystems),

after which the sirius red positive area was analyzed and calculated in each liver section by using ImageJ Fiji. All analyses were performed in a blinded manner.

### 2.11 Kinase activity profiling

Serine-Threonine kinase profiles were determined using the PamChip® Ser/Thr Kinase assay (STK; PamGene International, ´s-Hertogenbosch, The Netherlands). Each STK-PamChip® array contains 144 individual phospho-site(s) that are peptide sequences derived from substrates for Ser/Thr kinases. One 100µm liver section was lysed for 15 min on ice using M-PER Mammalian Extraction Buffer containing Halt Phosphatase Inhibitor and EDTA-free Halt Protease Inhibitor Cocktail (1:100 each; Thermo Fischer Scientific). Lysates were centrifuged for 15min at 16.000g at +4°C in a pre-cooled centrifuge. Protein quantification was performed with a NanoDrop One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific).

For the STK assay, 2.0µg of protein and 400µM ATP were applied per array (n=4 per condition) together with an antibody mix to detect the phosphorylated Ser/Thr. After incubation for an hour (30$^{o}$C) where the sample is pumped back and forth through the porous material to maximize binding kinetics and minimize assay time, a 2nd FITC-conjugated antibody is used to detect the phosphorylation signal. Imaging was done using an LED imaging system and the spot intensity at each time point was quantified (and corrected for local background) using the BioNavigator software version 6.3 (PamGene International, 's-Hertogenbosch, The Netherlands). Upstream Kinase Analysis (UKA), a functional scoring method (PamGene) was used to rank kinases based on combined specificity scores (based on peptides linked to a kinase, derived from 6 databases) and sensitivity scores (based on treatment-control differences).

For the peptides, a principal component analysis (PCA) was performed with the help of the R package stats V4.3.1 depicting the singular peptide decomposition examining

the covariances / correlations between the samples. The R package gglot2 v3.4.2 was used to visualize the results. The distribution of the phosphorylated peptides is shown in the volcano plots created with the R package EnhancedVolcano v1.18.0. Blue dots depict peptides with an adjusted P value <0.05.

For kinases, the median final score of the kinase with a score > 1.2 and with an adjusted P value for multiple comparisons by the false discovery rate (FDR) of <0.05 are depicted in a heatmap. The R package disease ontology semantic and enrichment analysis (DOSE) [9] was utilized to analyze the enriched pathways depicting the biological complexities in which these kinases correlate with multiple annotation categories, which was visualized in a network plot with the help of the R package Reactome Pathway Analysis (ReactomePA) v1.44.0 [10].

### 2.12 Precision-cut liver slices

Small human liver wedges of an equivalent size of approximately 10g were collected from 3 human donors following partial resection or when livers were unsuitable for transplantation. The study was approved by the Medical Ethical Committee of the University Medical Centre Groningen (UMCG), according to Dutch legislation and the Code of Conduct for dealing responsibly with human tissue in the context of health research, refraining the need for written consent for 'further use' of coded-anonymous human tissue. The procedures were carried out in accordance with the experimental protocols approved by the Medical Ethical Committee of the UMCG. Liver tissue was stored in University of Wisconsin preservation solution (UW, 4°C). The total cold ischemic time was between 3 and 29h. Slice viability for each donor liver was tested after one hour of culture by checking ATP production as previously described [11]. Slices were cultured in GFIPO medium [12] (36mM Glucose, 5mM Fructose, 1nM

Insulin, 480 µM Oleic acid, 240µM Palmitic acid) and cultured for 24 h and 48 h in the presence of empty LNPs (eLNPs) or LNPs loaded with murine miR-26b-3p (Duplex Sequences: 5'-/5Phos/rCrCrUrGrUrUrCrUrCrCrArUrUrArCrUrUrGrGmCmUrC-3' and 5'-mGmCrCmArAmGrUmArAmUrGmGrAmGrAmArCmArGG-3') and miR-26b-5p (Duplex Sequences: 5'-/5Phos/rUrUrCrArArGrUrArArUrUrCrArGrGrArUrAmGmGrU-3' and 5'-mCmUrAmUrCmCrUmGrAmArUmUrAmCrUmUrGmAA-3') mimics (IDT). The medium was refreshed every 24 hours. The slices were collected to check the viability of the slices by measuring ATP levels and supernatant was collected for further analysis.

**2.13 MiR-26b expression in patient cohort**

All patients were prospectively recruited in the Department of Medicine II (Saarland University Medical Center, Homburg, Germany) between December 2021 and March 2023. Included patients were adults and had either type 1 or type 2 diabetes. Alcohol consumption above the National Institute on Alcohol Abuse and Alcoholism's (NIAAA) definition of chronic alcohol use (four drinks or more on any day or 14 drinks per week for men or three drinks or more on any day or 7 drinks per week for women) was regarded as exclusion criterium. Serum and EDTA blood samples were collected from fasted patients. Genomic DNA was isolated from EDTA anticoagulated blood according to the membrane-based QIAamp DNA extraction protocol (Qiagen, Hilden, Germany). The common genetic variants involved known to modulate the risk of fatty liver, namely *PNPLA3*, *TM6SF2*, *MBOAT7*, *SERPINA*, *HSD17B13*, and *MTARC1*, were genotyped using a solution-phase hybridization reaction with 5'-nuclease and fluorescence detection. Transient elastography (TE) and controlled attenuation parameter (CAP) were performed to non-invasively quantify liver fibrosis and steatosis,

respectively. Cirrhosis was defined by TE greater or equal 15 kPa, and fibrosis F0 was defined by TE below 6,5 kPa [13, 14].

RNA was isolated from 100µl serum, using the miRNeasy serum/plasma kit (Qiagen), according to the manufacturer's instructions. C. elegans miR-39 miRNA mimic was added as spike-in control. Subsequently, cDNA was generated using the TaqMan MicroRNA Reverse Transcription Kit (ThermoFisher) according to the manufacturer's instructions using 10ng of total RNA. The relative gene expression levels were determined by quantitative polymerase chain reaction (qPCR) using TaqMan MicroRNA-assays (ThermoFisher) for miR-39 (Assay-ID: 000200), miR-26b-3p (Assay-ID: 000407) or miR-26b-5p (Assay-ID: 002444). For the qPCR reaction, 0.5ng of cDNA template was used to which TaqMan gene expression master mix (ThermoFisher) and above-described primers were added. PCR cycling was performed on QuantStudio 3 Real-Time PCR system (ThermoFisher) with the following conditions: 50˚C for 2min for 1 cycle (UDG activation); 95˚C for 10min for 1 cycle (Dual-Lock DNA polymerase); and 95˚C for 15sec (Denature), 60˚C for 60sec (Anneal/Extend) for 40 cycles. Expression of miR-39 as spike-in control was used for normalization.

### 2.14 Statistics

Statistical analysis was performed using GraphPad Prism version 9.1.1 (GraphPad Software, Inc., San Diego, CA, USA). Outliers were identified using the ROUT = 1 method after which normality was tested via the D'Agostino-Pearson and Shapiro-Wilk normality test. Significance was tested using either Welch's t-test for normally distributed data or Mann-Whitney U test for non-normally distributed data. All data are

expressed as mean ± standard error of the mean (SEM) and results of <0.05 for the *p*-value were considered statistically significant.

All authors had access to the study data and have reviewed and approved the final manuscript.

## 3. RESULTS

### 3.1 Mice deficient in *miR-26b* show increased hepatic lipid levels and an increased expression of hepatic lipid uptake receptors.

To determine the role of miR-26b in hepatic lipid metabolism and MASH development, hepatic cholesterol and triglyceride levels were measured in *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice that were fed a 4-week WTD (**Figure 1A**). Total cholesterol and triglyceride levels were significantly increased in the livers of *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice compared to control mice (**Figure 1B-C**). This hepatic lipid effect was also confirmed by Oil-red-O staining, showing increased lipid accumulation in the livers of *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice (**Figure 1D-E**). Moreover, pathological scoring of the Oil-red-O staining unveiled that the *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice showed a clear tendency towards increased steatosis compared to controls, especially of macrovesicular steatosis (**Figure 1F**).

To elucidate possible mechanisms underlying the increased hepatic lipid levels, gene expression levels of key proteins involved in lipid metabolism were measured in liver tissues of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice. Notably, a knockout of miR-26b did not affect the expression levels of 'ATP binding cassette subfamily A member 1' (*Abca1*) or 'acetyl-CoA acetyltransferase 2' (*Acat2*) (**Figure 1G-H**). However, livers of *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice showed a clearly increased expression of scavenger receptor *Cd36* (**Figure 1I**) and a striking 2-fold increase of the expression of scavenger receptor A (*Sra*) (**Figure 1J**). Since these scavenger receptors are highly expressed on macrophages, we have evaluated the contribution of myeloid miR-26b to the observed hepatic lipid effects. Interestingly, mice that have a myeloid-specific lack of *miR-26b* also show increased hepatic cholesterol levels and lipid accumulation demonstrated

by Oil-red-O staining, coinciding with an increased hepatic *Cd36* expression (**Figure 2**), demonstrating that myeloid miR-26b plays a major role in the observed steatosis.

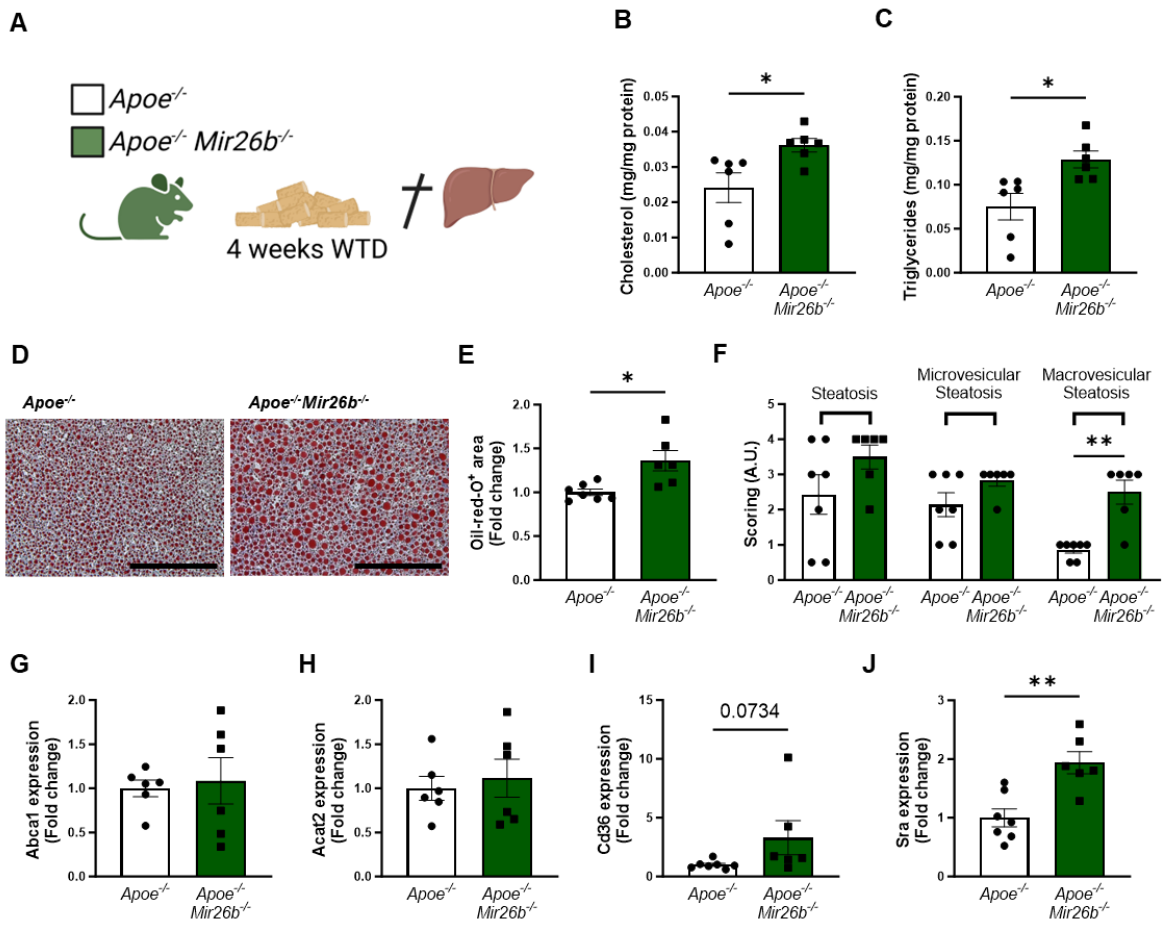



**Figure 1. Hepatic lipid levels and the expression of lipid uptake receptors are increased by a whole-body knockout of miR-26b in mice.**

(**A**) Schematic overview of the experimental approach. (**B-C**) Hepatic total cholesterol (**B**) and triglyceride (**C**) measurements normalized against total protein. (**D**) Representative pictures of Oil-red-O staining of liver sections. Scale bar=200µm. (**E**) Quantification of the Oil-red-O staining. (**F**) Pathological scoring of the Oil-red-O staining. (**G-J**) Gene expression analysis of (**G**) *Abca1*, (**H**) *Acat2*, (**I**) *Cd36*, and (**J**) *Sra*. Fold change is corrected for sex. *$p$<0.05; **$p$<0.01. $n$=6-7 animals per group.

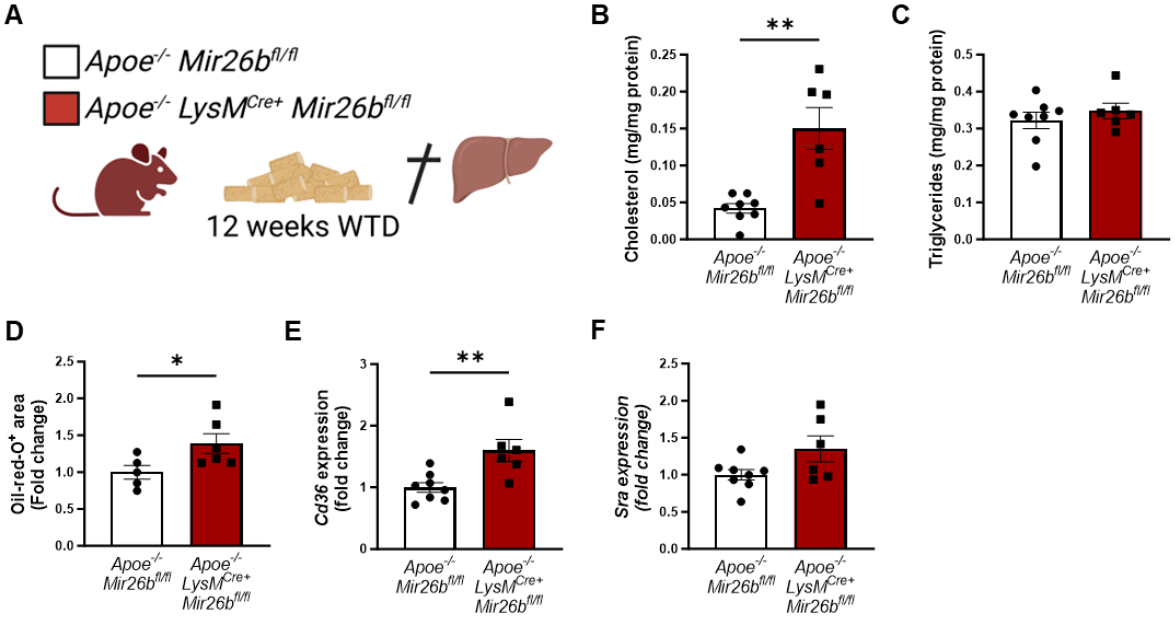



**Figure 2. Hepatic lipid levels and the expression of lipid uptake receptors are increased by a myeloid-specific *miR-26b* deficiency in mice.**

(**A**) Schematic overview of the experimental approach. (**B-C**) Hepatic total cholesterol (**B**) and triglyceride (**C**) measurements normalized against total protein. (**D**) Quantification of the Oil-red-O staining. (**E-F**) Gene expression analysis of (**E**) *Cd36* and (**F**) *Sra*. Fold change is corrected for sex. *$p$<0.05; **$p$<0.01. $n$=6-8 animals per group.

### 3.2 Livers of *miR-26b* knockout mice have higher levels of inflammatory cytokines and an increased number of infiltrating macrophages.

Besides lipid accumulation, an increased inflammatory profile is a key characteristic of MASH [2]. Therefore, we aimed to elucidate the role of miR-26b in hepatic inflammation. Hepatic protein levels of the pro-inflammatory cytokines IL-6 and TNF were significantly increased in *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice compared to controls (**Figure 3A-B**), while levels of the chemokines CCL2 and CXCL1 remained unchanged (**Figure 3C-D**). To further investigate the effects of the whole-body knockout on a cellular level, liver sections were stained to identify several key leukocyte subpopulations.

Mac-1-positive cells were significantly increased in livers of mice lacking *miR-26b*, indicating a higher infiltration of macrophages and neutrophils (**Figure 3E**). To identify whether the increase of Mac-1-positive cells is due to macrophage or neutrophil infiltration we also determined the number of Ly6G-positive cells, which remained unchanged, suggesting that the increased number of Mac-1 positive cells was likely the result of an increase in the number of infiltrating macrophages rather than neutrophils (**Figure 3F**). Furthermore, the whole-body knockout of *miR-26b* only affected the number of infiltrating macrophages and not Kupffer cells, which are recognized as CD68-positive cells (**Figure 3G)**. Furthermore, the number of CD3-positive cells did not differ between *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice and controls (**Figure 3H**), suggesting that miR-26b does not affect T-cell counts in the liver.

Collectively, these results indicate that miR-26b plays a protective role in hepatic inflammation by influencing TNF and IL-6 levels and macrophage infiltration in the liver.

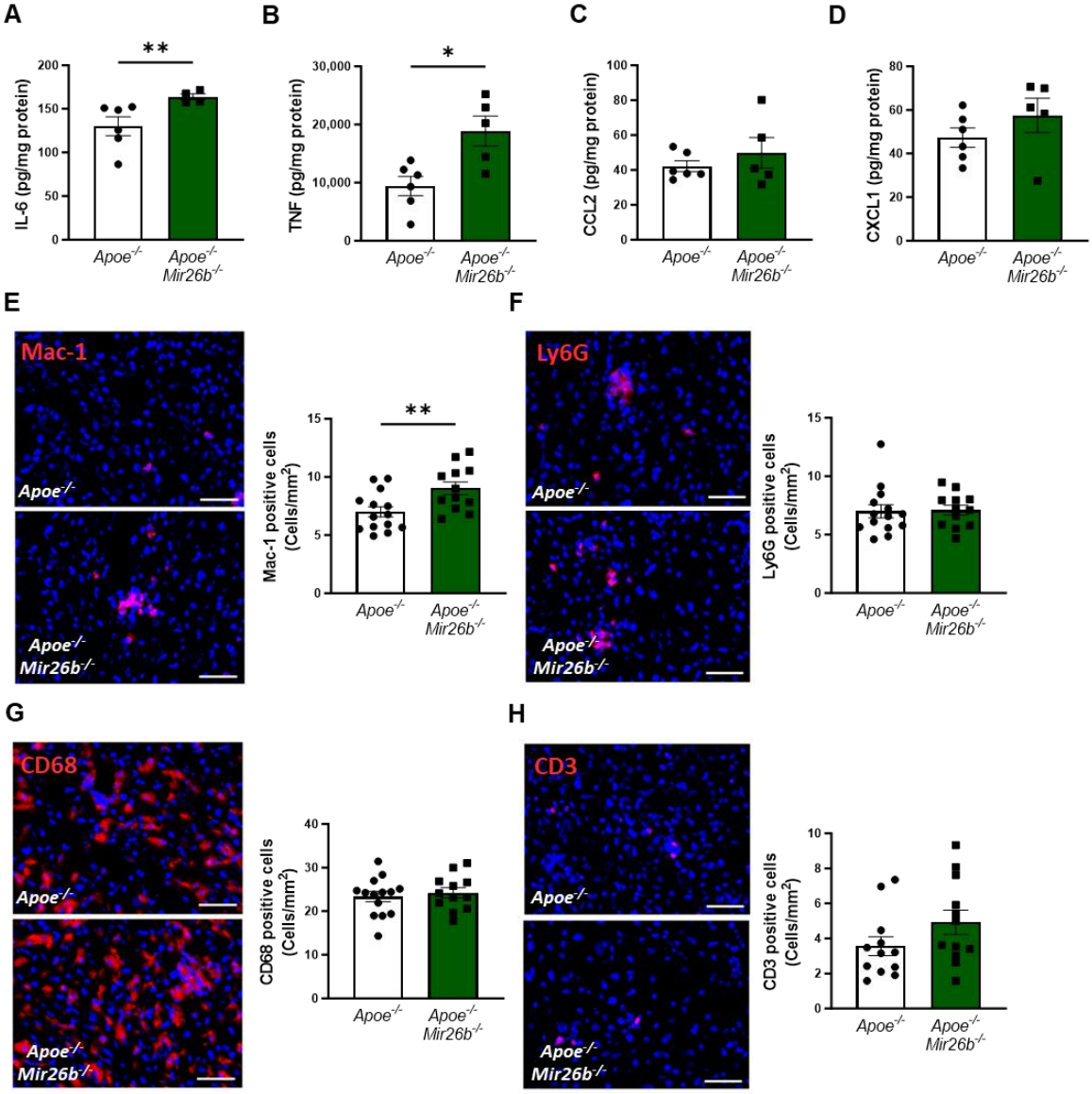



**Figure 3. Livers of *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice show elevated pro-inflammatory cytokine levels and an increased number of Mac-1-positive cells.**

(**A-D**) Cytokine levels of (**A**) IL-6, (**B**) TNF, (**C**) CCL2, and (**D**) CXCL1 were measured in liver protein lysates. (**E-H**) Representative images and quantification of immunofluorescent stainings for (**E**) infiltrating macrophages and neutrophils (Mac-1), (**F**) neutrophils (Ly6G), (**G**) resident monocytes/macrophages (CD68), and (**H**) T-cells (CD3). Scale bar=50µm. *$p$<0.05; **$p$<0.01. $n$=6-7 animals per group.

### 3.3 A knockout of *miR-26b* in mice results in increased hepatic fibrosis, which coincides with an increased expression of *Tgfb*.

Continued hepatic inflammation can cause fibrotic changes in the liver, which is another characteristic of MASH [2]. As such, we also investigated the influence of miR-26b on hepatic fibrosis in mice. Collagen deposition in liver sections was determined by a Sirius-red staining, which showed that a knockout of *miR-26b* significantly exacerbated hepatic fibrosis (**Figure 4A-B**). This was further supported by the increased expression of 'transforming growth factor β' (*Tgfb*) in the livers of *Apoe*$^{-/-}$ *Mir26b*$^{-/-}$ mice compared to controls (**Figure 4C**). Another gene involved in liver fibrosis, i.e. 'actin alpha 2' (*Acta2)*, trended towards an elevated expression in mice lacking miR-26b (**Figure 4D**). Lastly, a whole-body knockout of miR-26b resulted in an increased expression of 'matrix metalloproteinase 13' (*Mmp13)* (**Figure 4E**). Overall, these results imply a protective role of miR-26b in liver fibrosis, which is linked to an altered expression of *Tgfb*.

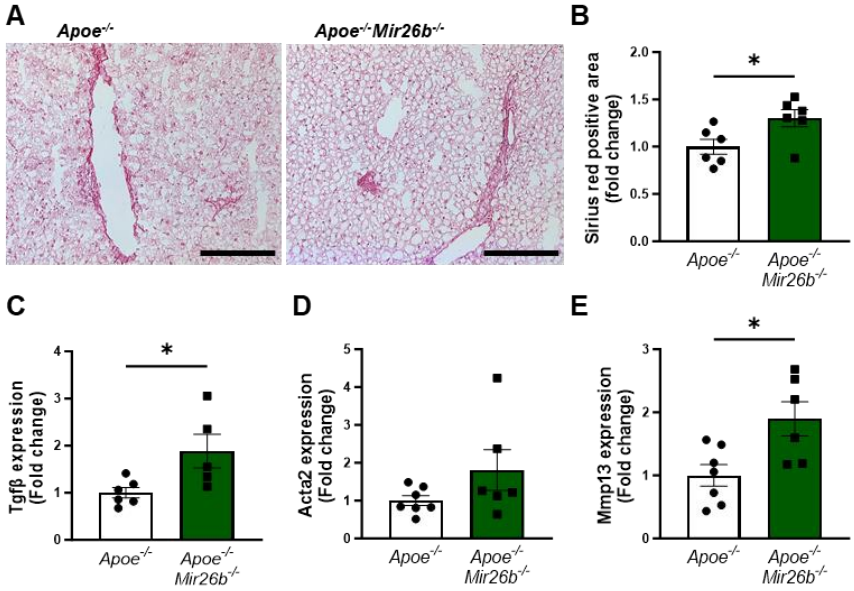



**Figure 4. Hepatic fibrosis is exacerbated by a whole-body knockout of *miR-26b* in mice.**

(**A**) Representative pictures of Sirius-red staining of liver sections. Scale bar=100µm. (**B**) Quantification of the Sirius-red staining. (**C-E**) Gene expression of (**C**) *Tgfb*, (**D**) *Acta2*, and (**E**) *Mmp13*. Fold change is corrected for sex. *$p$<0.05. $n$=6-7 animals per group.

### 3.4 Liver of *miR-26b* knockout mice show highly increased kinase activity related to inflammatory pathways.

To elucidate the underlying mechanisms behind the effects of miR-26b on the liver, we have performed a kinase activity profiling, focusing on serine-threonine kinases (STK). In order to evaluate the differentially activated kinases, the degree of phosphorylation of peptides coated on STK arrays is determined. Liver lysates from *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice (KO) showed a strong and very distinct upregulation of peptide phosphorylation compared to liver lysates from *Apoe*$^{-/-}$ mice (WT) (**Figure 5A-B** and **Supplemental Figure 1A**). Remarkably, 84 kinases were significantly more activated in liver lysates from *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice compared to controls (**Figure 5C**), many of which are involved in inflammatory pathways such as c-Jun-N-terminal kinases (JNKs), mitogen-activated protein kinases (MAPKs), and extracellular-signal, regulated kinases (ERKs). This was also further corroborated by pathway analysis (**Figure 5D-E**), showing enrichment in pathways related to inflammation (e.g. MAP kinase activation, TLR signaling) and angiogenesis (e.g. VEGF signaling). Combined, these results demonstrate that the lack of *miR-26b* increases kinase activity in the liver, particularly of kinases related to inflammatory pathways, which can thus be a plausible mechanism behind the hepatic effects observed in *miR-26b* deficient mice.

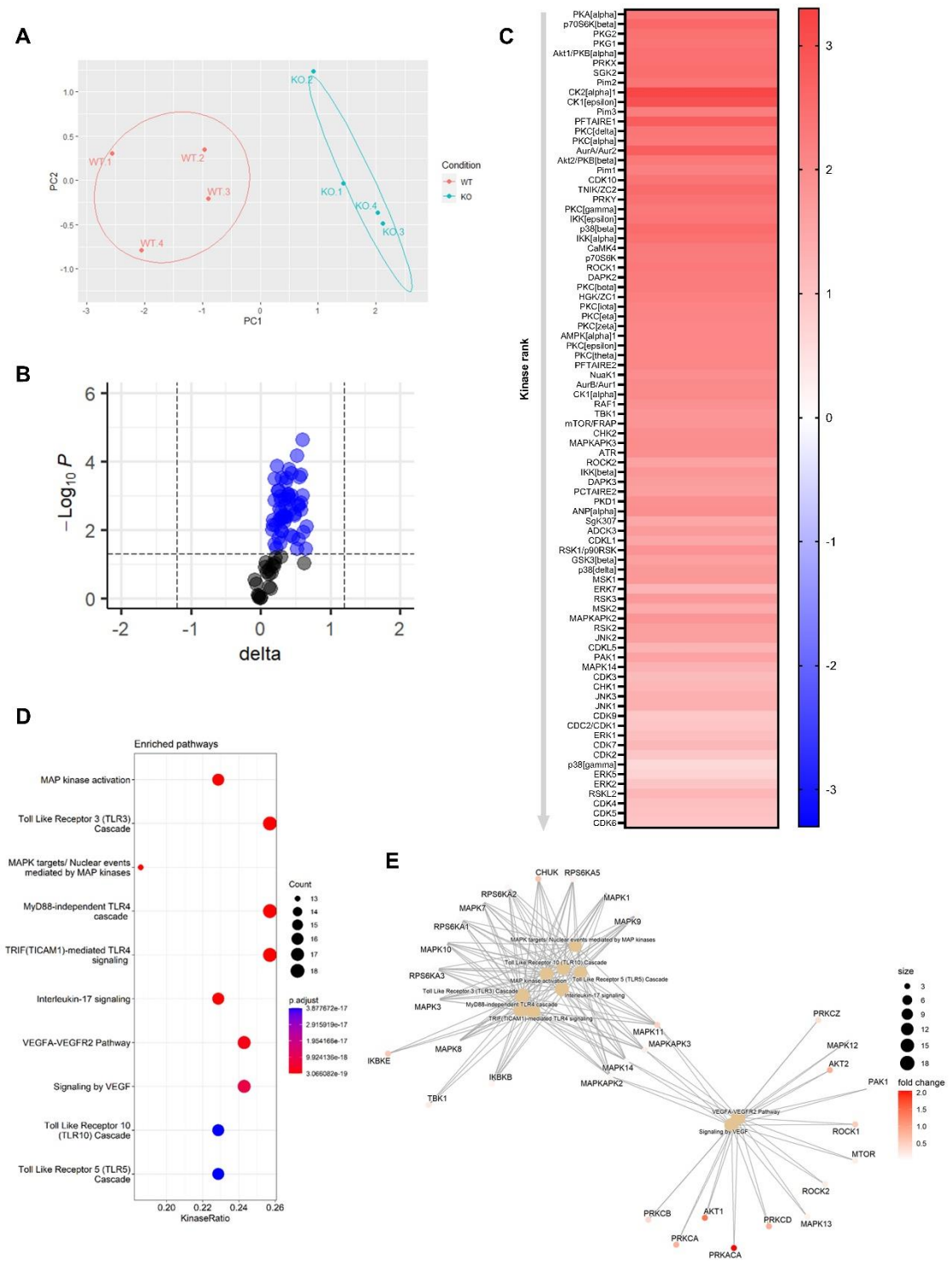



**Figure 5. Knockout of *miR-26b* results in an increased hepatic inflammatory kinase activity.**

(**A**) Principal component analysis (PCA) of phosphorylated peptides from STK array (n=4) of liver lysates from *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice (KO) or *Apoe*$^{-/-}$ (WT) mice**.** (**B**) Volcano plot visualizing fold change and *p*-value for phosphorylated peptides from STK array. Blue dots represent significantly altered phosphopeptides. (**C**) Heatmap of significantly changed kinases are ranked based on Median Final Score (cut-off value of 1.2), STK array performed on liver lysates from *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice (KO)

compared to *Apoe*$^{-/-}$ (WT) mice**.** Color corresponds to the Median Kinase Statistic, which represents effect size and directionality (red = increased activity in KO vs. WT mice). (**D**) Enriched Pathways based on STK array. (**E**) Network diagram of the pathway enrichment analysis.

### 3.5 Lipid nanoparticles loaded with miR-26b mimics can partly rescue the MASH phenotype in whole-body *miR-26b* knockout mice.

Since the whole-body knockout mouse model demonstrated that miR-26b plays a role in MASH, we attempted to rescue the phenotype by injecting *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice on WTD with LNPs, which act as clinically and therapeutically relevant vehicles [8], loaded with miR-26b mimics (mLNPs) and empty lipid nanoparticles (eLNPs) as control for 4 weeks (**Figure 6A**). These mLNPs replenish the miR-26b level in the whole-body deficient mouse, providing insight into the therapeutic potential of miR-26b. Injections with mLNPs lowered hepatic cholesterol levels compared to the vehicle control (**Figure 6B**), whilst triglyceride levels remained unaffected (**Figure 6C**). These findings were further confirmed by demonstrating that treatment with mLNPs significantly reduced the Oil-red-O positive area (**Figure 6D**). While mLNP treatment did not affect *Cd36* expression (**Figure 6E**), it resulted in a 0.67-fold reduction in *Sra* expression compared to mice injected with eLNPs (**Figure 6F**).

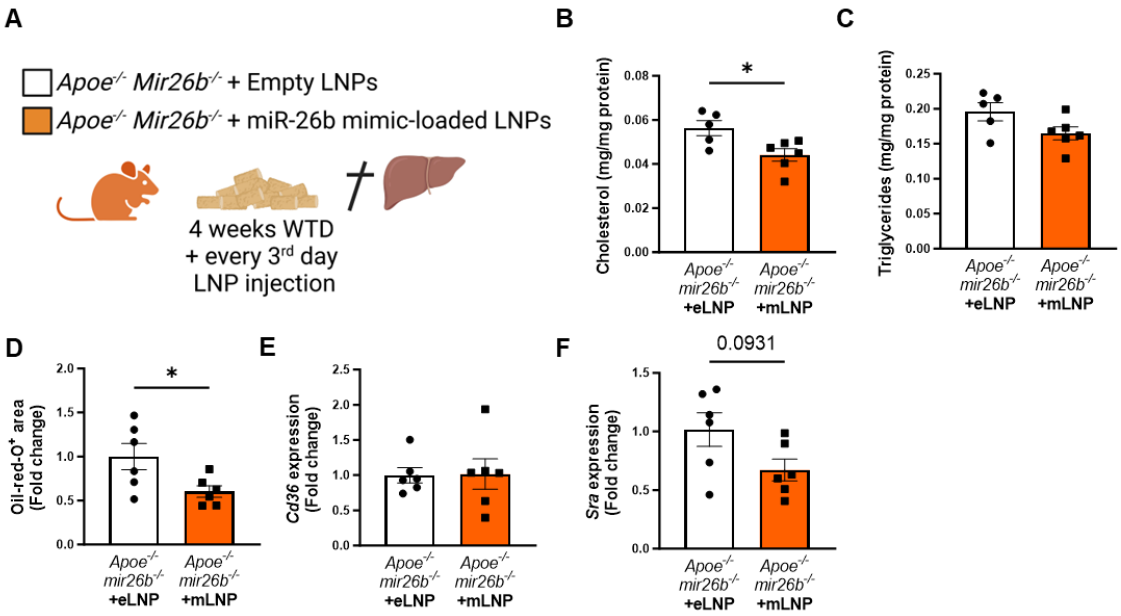



**Figure 6. *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice injected with LNPs containing miR-26b mimics show decreased hepatic lipid levels compared to vehicle control injected mice.**

(**A**) Schematic overview of the experimental approach in which mice on 4-week WTD were simultaneously injected every 3 days with either empty LNPs as vehicle control (eLNP) or LNPs containing miR-26b mimics (mLNP). (**B-C**) Hepatic total cholesterol (**B**) and triglyceride (**C**) measurements normalized against total protein. (**D**) Quantification of the Oil-red-O staining. (**E-F**) Gene expression analysis of (**E**) *Cd36* and (**F**) *Sra*. Fold change is corrected for sex. *$p$<0.05; **$p$<0.01. $n$=6 animals per group.

Furthermore, kinase activity profiling of liver lysates demonstrated a distinct downregulation of peptide phosphorylation upon mLNP treatment of *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ (KO.LNP) mice (**Figure 7A-B** and **Supplemental Figure 1A-B**). Interestingly, principal component analysis (PCA) clearly demonstrated that livers from mLNP-treated *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ (KO.LNP) mice more closely resembled *Apoe*$^{-/-}$ (WT) mice rather than *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ (KO) mice (**Figure 7A**). The kinase activity of 76 kinases in the liver was significantly downregulated upon mLNP treatment of *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice (**Supplemental Figure 1C**). The notion that 60 (79%) of these downregulated kinases were originally upregulated by the *miR-26b* deficiency (**Figure 7C**), furthermore indicates that mLNP treatment rescues the observed effects due to the *miR-26b* deficiency. In line with this, pathway analysis also showed an enrichment of similar pathways as described before, i.e. pathways related to inflammation and angiogenesis (**Figure 7D-E**).

Overall, treatment with mLNPs attenuated MASH development with regard to hepatic lipids and inflammatory kinase activity, highlighting the therapeutic potential of LNPs loaded with miR-26b mimics.

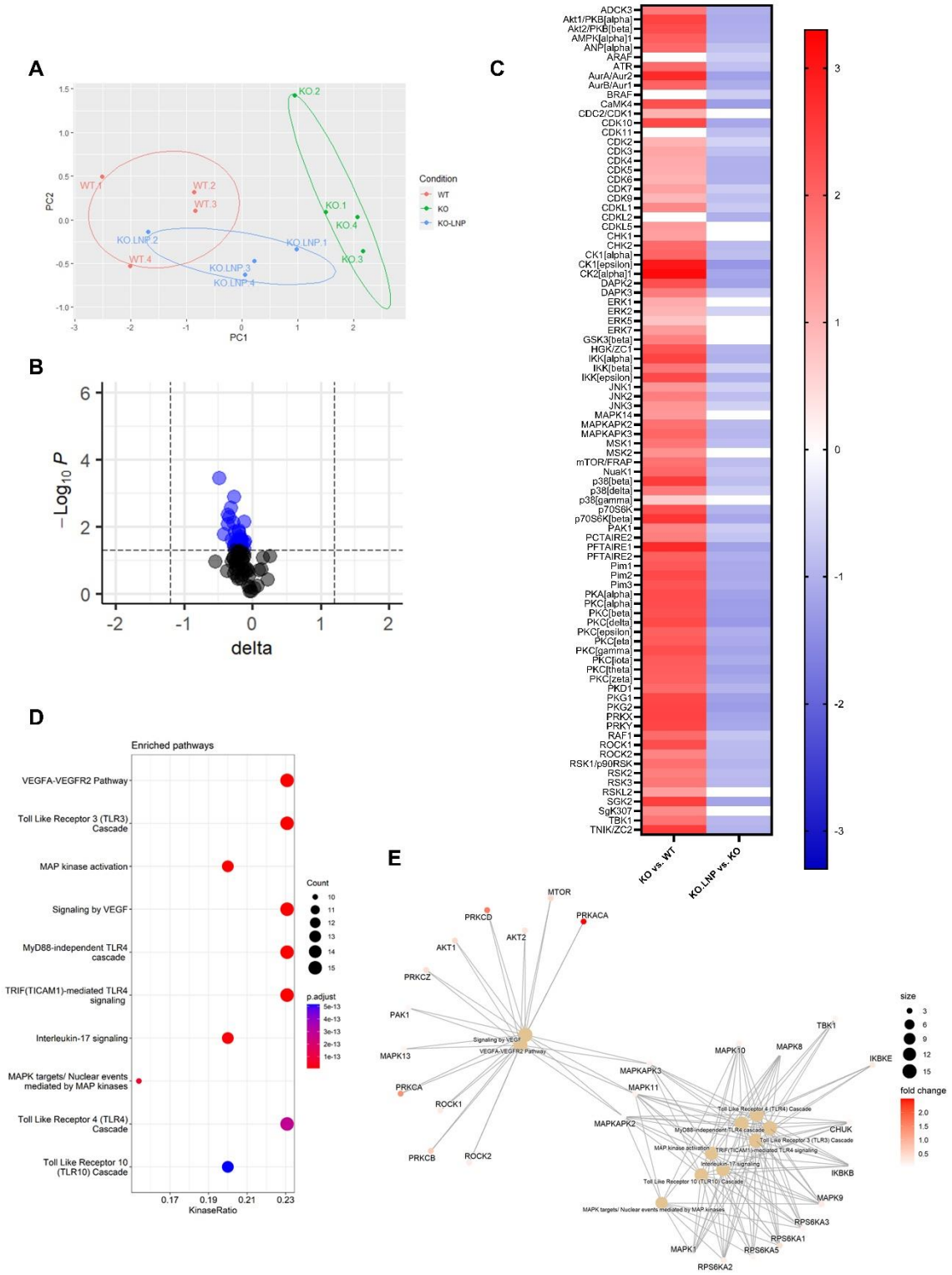



**Figure 7. mLNP treatment of *miR-26b* knockout mice results in a decreased hepatic inflammatory kinase activity.**

(**A**) Principal component analysis (PCA) of phosphorylated peptides from STK array (n=4) of liver lysates from mLNP treated *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice (KO.LNP), *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice (KO) or *Apoe*$^{-/-}$ mice (WT) mice**.** (**B**) Volcano plot visualizing fold change and P value for phosphorylated peptides from STK array.

Blue dots represent significantly altered phosphopeptides. (**C**) The heatmap of significantly changed kinases is ranked based on the Median Final Score (cut-off value of 1.2). Color is corresponding to Median Kinase Statistic, which represents effect size and directionality (red = increased activity in KO vs. WT mice; blue = decreased activity in KO.LNP vs. KO mice) (average of n=4 is shown). (**D**) Enriched Pathways based on STK array. (**E**) Network diagram of the pathway enrichment analysis.

### 3.6 Lipid nanoparticles loaded with miR-26b mimics have anti-inflammatory effects on human livers.

Since the mouse experiments demonstrated a clear therapeutic potential of LNPs loaded with miR-26b mimics, we also set out to explore this potential in a human setting. Human precision-cut liver slices were cultured for 24h or 48h in the presence of mLNPs or eLNPs as control (**Figure 8A**). Although no effects were observed on IL-6 secretion (**Figure 8B**), mLNPs had strong anti-inflammatory effects on these human precision-cut liver slices. The secretion of TNF, CCL2, and CXCL1 was significantly reduced in slices treated with mLNPs compared to eLNP-treated liver slices (**Figure 8C-E**), underlining the clear potential of these miR-26b-loaded LNPs in a human context.

To further evaluate the importance of miR-26b in liver diseases in humans, we have measured the expression levels of miR-26b-3p and miR-26b-5p in the plasma of healthy subjects and patients with liver cirrhosis (**Figure 8F**). Remarkably, both miR-26b-3p and -5p were significantly elevated in the plasma of liver cirrhosis patients (**Figure 8G-H**), suggesting -at least- a strong association between miR-26b and the development of MASH in humans.

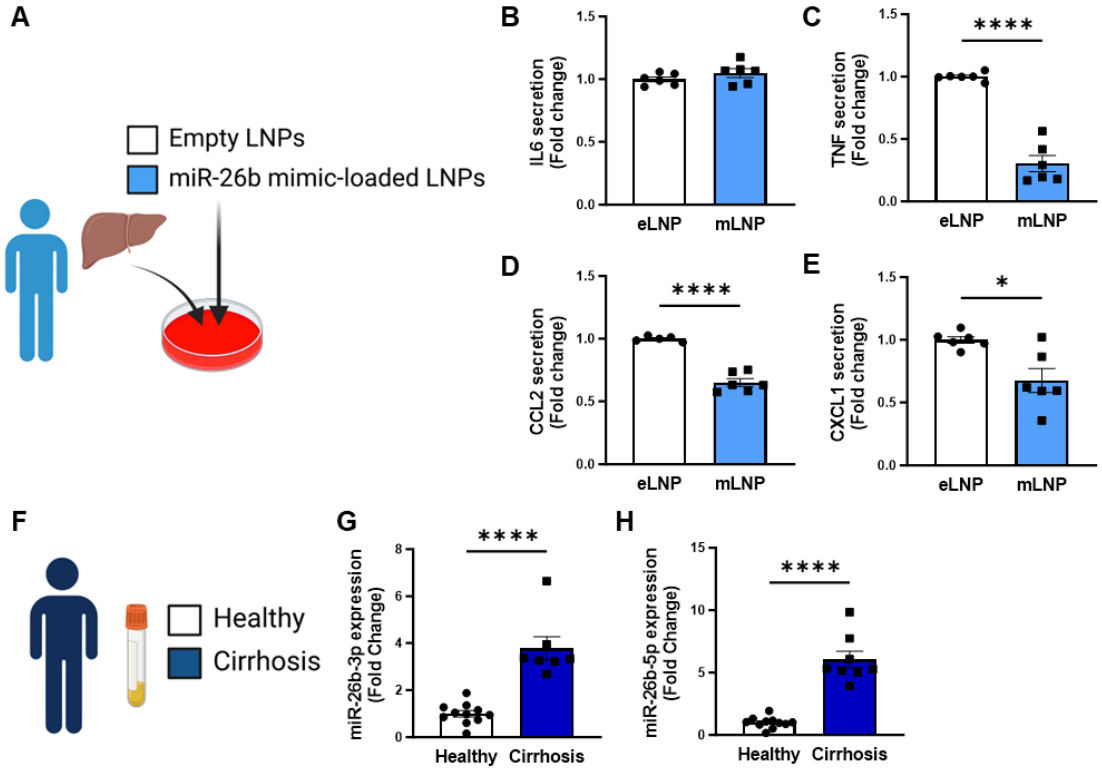



**Figure 8. MiR-26b-loaded LNPs have anti-inflammatory effects on human liver slices and miR-26b plasma levels are significantly increased in patients with liver cirrhosis.**

(**A**) Schematic overview of the experimental approach. (**B-E**) Cytokine levels of (**B**) IL-6, (**C**) TNF, (**D**) CCL2, and (**E**) CXCL1 measured in the supernatant of human precision-cut liver slices after 24h (for IL-6/TNF) or 48h (for CCL2/CXCL1) incubation with mLNPs or eLNPs (n=3). (**F-G**) Plasma was isolated from patients with liver cirrhosis or healthy volunteers (**F**) and miR-26b-3p (**G**) and miR-26b-5p (**H**) plasma levels were measured. *$p$<0.05; ****$p$<0.0001. n=8-11 patients per group.

## 4. DISCUSSION

MiRs have been indicated to play a critical role in the development of several pathologies, including MASH. While the role of miR-26b has already been investigated in various cardio-metabolic diseases [5], its role in MASH remained unknown so far. Therefore, this study focused on the role of miR-26b in MASH showing that mice deficient in miR-26b presented with a higher hepatic lipid content, higher levels of pro-inflammatory cytokines, and an increased number of infiltrated macrophages in the liver and exacerbated hepatic fibrosis. This coincided with an increased activity of kinases that are involved in inflammatory pathways. Finally, when attempting to rescue this phenotype by injecting LNPs loaded with miR-26b mimics, we managed to decrease hepatic cholesterol, overall hepatic lipid levels, gene expression of *Sra*, and the activity of inflammatory kinases. These anti-inflammatory effects of miR-26b loaded LNPs were also confirmed in human precision-cut liver slices. Taken together, these results suggest that miR-26b plays a protective role in MASH development and shows the therapeutic potential of miR-26b loaded LNPs.

In this study, *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice showed elevated hepatic cholesterol and triglyceride levels, which coincided with increased expression of *Sra* and *Cd36*. Generally, SR-A and CD36 play a critical role in lipid metabolism, as 75% to 90% of oxidized low-density lipoprotein (ox-LDL) is taken up by macrophages via these receptors [15]. In line with our observations, previous studies already highlighted that CD36 and SR-A play a key role in MASH development. For example, a hematopoietic deficiency of these receptors resulted in reduced hepatic inflammation [16]. Furthermore, a recent study demonstrated that mice lacking SR-A show decreased hepatic lipid levels and inflammation [17]. Interestingly, they also demonstrated that SR-A induced JNK

signaling [17], which is also in line with our kinase activity results. Moreover, it could be demonstrated that *CD36* expression is upregulated in the livers of MASLD patients, leading to hepatic triglyceride accumulation and consequently an exacerbation of hepatic steatosis [18]. Overall, this strongly indicates that an increased hepatic expression of *Cd36* and *Sra* in both mice and humans causes hepatic cholesterol and triglyceride accumulation as well as inflammation, adding fuel to the notion of CD36 and SR-A as a possible underlying mechanism through which miR-26b exerts its effects on MASH development.

Elevated hepatic lipid levels lead to the release of pro-inflammatory cytokines, consequently mediating liver injury and inflammation in MASH [19], which could also be observed in our study. The elevated levels of pro-inflammatory cytokines in the liver led to the increased number of Mac-1 positive cells, representing infiltrated macrophages. The production of IL-6 and TNF by macrophages in turn led to further local inflammation, also highlighted by the increased inflammatory kinase activity in the liver, thereby causing a vicious cycle of cytokine release and myeloid cell infiltration[20]. Interestingly, TNF has been shown to promote steatosis by altering lipid metabolism and inducing fatty acid uptake in the liver [21, 22], further showing the critical link between inflammation and hyperlipidemia. Additionally, infiltrated macrophages usually form clusters, especially in areas of macrovesicular steatosis [23], which was in line with our current study. Overall, these findings indicate a crosstalk between hepatic lipid levels, inflammation, and macrophage infiltration and show that miR-26b might play a protective role in these processes.

The third characteristic of MASH that was investigated during this study was hepatic fibrogenesis. Hepatic fibrosis is mainly caused by the activation of the fibrogenic factor TGF-β [24], which is also confirmed in the current study as miR-26b knockout mice

displayed a higher amount of Sirius red positive area and an increased expression of *Tgfb*. Additionally, *Mmp13*, a protein responsible for extracellular matrix degradation, was upregulated in *Apoe*$^{-/-}$*Mir26b*$^{-/-}$ mice, which is probably a secondary compensatory response to the increase in fibrosis. Besides this, MMP13 has been shown to play a less straightforward role in liver fibrosis [25]. Uchinami *et al.* demonstrated that levels of TNF and TGF-β were suppressed in MMP13-deficient mice in an early phase of liver fibrosis, suggesting that MMP13 possibly accelerates hepatic fibrogenesis by mediating inflammation. Overall, this indicates that miR-26b plays a protective role in hepatic fibrogenesis, possibly due to modulating TGF-β and MMP13 levels.

Finally, to study the therapeutic potential of miR-26b, we injected whole-body knockout mice with miR-26b mimic-loaded LNPs. LNP-based therapies have emerged over the last few years and have been extensively studied and even already used in clinical settings [26], underlining the translatability and potential of our study. Interestingly, the LNP treatment not only showed significant results on hepatic lipid metabolism but also reversed the effects on inflammatory kinase activity, particularly of pathways mediated by key regulators like JNKs, MAPKs and ERKs. However, a note of caution is the fact that we injected the LNPs simultaneously with the diet, providing a more preventative approach as opposed to a curative therapy. Furthermore, we demonstrated that miR-26b loaded LNPs have anti-inflammatory effects on human precision-cut liver slices, which, combined with the suppressed levels of cholesterol and hepatic lipids in our mouse model, underline its potential as an exciting therapeutic option.

Collectively, these results show that miR-26b plays a protective role in the development of MASH by exerting effects on hepatic lipid metabolism, inflammation, and fibrosis. Future research should focus on the further clinical translation of our results by evaluating the effects of miR-26b loaded LNPs on various human tissues. Overall, we

show here thought-provoking results on the role of miR-26b in MASH development and highlight this miR as a promising therapeutic target, providing a solid base for exciting future research.

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Figures are made with Biorender.

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