SUPPLEMENTARY MATERIAL:

Liver MicroRNA-21 is Overexpressed in Non Alcoholic Steatohepatitis and Contributes to the Disease in Experimental Models by Inhibiting PPARα Expression

Authors:

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Supplementary Methods

Bright-field in Situ Hybridization

Briefly, 3 µm-thick paraffin embedded liver sections were post-fixed in 4% paraformaldehyde for 10 min, and then acetylated for 10 min. After washes with PBS, sections were incubated with protein kinase K (Sigma-Aldrich) at 37°C for 5 min. After washes with PBS, sections were incubated with hybridization buffer for 5 hours at room temperature. MicroRNA probes (miR-21, 5'-3'–Digoxigenin-labeled Locked Nucleic Acid probe, Exiqon reference #38102-15, 20 nmol/L; U6snRNA, 3'-Digoxigenin labeled Locked Nucleic Acid probe, 10 nmol/L, Exiqon reference #99002-15, scramble probe Exiqon, reference# 99004-05) were mixed with denaturation buffer and then incubated with the sections overnight at 56 °C. Then, the sections were washed with decreasing concentrations of saline-sodium citrate (SSC) buffer (Invitrogen) (5 min at 56°C once in 5X SSC buffer, then twice in 1X SSC buffer, then 3 times in 0.2X SSC buffer) and then washed in PBS. After incubation for 1 hour in a blocking solution (Tris + 3% fetal calf serum + 0.1% Tween-20), sections were incubated with an anti-Digoxigenin antibody conjugated with alkaline phosphatase (Roche; 1:2000) overnight at 4°C. Sections were then incubated with NitroBlueTetrazolium/5-bromo-4-choloro-3-indolyl-phosphate (Promega) in NTMT (NaCl, Tris, MgCl2, Tween) buffer with levamisole (0.2 mmol/L; Sigma-Aldrich) for 48 hours in the dark at room temperature. NitroBlueTetrazolium/5-bromo-4-choloro-3-indolyl-phosphate solution was changed every 12 hours. Afterwards, slides were fixed in 4% paraformaldehyde for 10 min and mounted with Fluoprep (Biomerieux).

Human Fluorescence *in Situ* Hybridization

After fixation, acetylation and incubation with proteinase K, sections were incubated twice in freshly prepared 3% H_2O_2 for 3 min to inactive endogenous peroxidases. Slides were then

rinsed 3 times in PBS and sections were incubated with hybridization buffer for 1 hour at 37°C. MicroRNA probes (miR-21, 5'-3'–Digoxigenin-labeled Locked Nucleic Acid probe, Exiqon, 40 nmol/L; U6snRNA, 3'-Digoxigenin labeled Locked Nucleic Acid probe, 2 nmol/L) were mixed with denaturation buffer and then incubated with the sections overnight at 56°C. Liver sections were washed with decreasing concentrations of SSC as described for bright-field *in situ* hybridization. Then, sections were again incubated twice in freshly prepared 3% H_2O_2 for 5 min and washed three times in PBS. Liver sections were incubated with blocking solution (Tris + 3% fetal calf serum + 1% bovine serum albumin) for 1 hour at room temperature, and then with anti-DIG-FAB peroxidase (POD) (Roche) diluted 1:400 in blocking solution for 1 hour at room temperature. After washes with PBS, TSA Plus Cy3 system working solution was applied onto the sections for 10 min at room temperature in the dark according to the manufacturer's protocol (PerkinElmer Life sciences). The slides were washed 3 times in PBS.

To identify cells that expressed miR-21 in the liver, sections were processed for double fluorescence staining to visualize simultaneously miR-21 and the T-cell -specific protein CD3, or the known biliary cells marker cytokeratin 19. Sections were incubated in demasking citrate buffer for 20 min at 95°C. A solution containing a primary anti-CD3 antibody (1:200; Dako, #A0452) or a primary anti-CK19 antibody (1:200; Dako M0888) was then applied overnight at 4°C. The next day, slides were twice immerged in PBS and incubated with a secondary AlexaFluor488 conjugated antibody (Invitrogen) for 1 hour at room temperature. Nuclei were stained with DAPI and sections were mounted using a drop of Fluorescent mounting medium (Dako).

Supplementary Tables

Supplemental Table 1. Oligonucleotide Primer Sequences Used

Symbole	Name	Oligonucleotide	Sequence
TNFα	*Mmu-Tnfalpha*	Upper primer	5'-GAT GGG GGG CTT CCA GAA CT-3'
		Lower primer	5'-CTG GGG CTA CAG GCT TGT CAC-3'
MCP-1	*Mmu-Mcp-1*	Upper primer	5'- ATG CTT CTG GGC CTG CTG CTG TTC A -3'
		Lower primer	5'- GAG TGG GGC GTT AAC TGC ATC TG -3'
TGFβ	*Mmu-Tgf-beta*	Upper primer	5'-CGG AGA GCC CTG GAT ACC AAC TA-3'
		Lower primer	5'-GCC GCA CAC AGC AGT TCT TCT CT-3'
GAPDH	*Mmu-Gapdh*	Upper primer	5'-CGT CCC GTA GAC AAA ATG GTG AA-3'
		Lower primer	5'-GCC GTG AGT GGA GTC ATA CTG GAA CA-3'
Col1a2	*Mmu-Collagen1a2*	Upper primer	5'-GCT GAG GGC AAC AGC AGG TTC ACC TA-3'
		Lower primer	5'-GGA ACG GCA GGC GAG ATG GCT TAT T-3'
PPARα	*Mmu-PPARα*	Upper primer	5'-AAC CTG AGG AAG CCG TTC TGT GAC AT-3'
		Lower primer	5'-GAC CAG CTG CCG AAG GTC CAC CAT-3'
HPRT	*Mmu-HPRT*	Upper primer	
		Lower primer	
PPIA	*Mmu-PPIA*	Upper primer	5'-CAC CGT GTT CTT CGA CAT CA-3'
		Lower primer	5'-CAG TGC TCA GAG CTC GAA AGT-3'
ACOX1	*Mmu-Acox1*	Upper primer	5'-CGC GCC TGC ACC TTC GAG GGG GAG A-3'
		Lower primer	5'-GCT GGA TAC GCT GGC TCG GCA GGT CA-3'
CPT-1	*Mmu-CPT1*	Upper primer	5'-GGT TGC TGA TGA CGG CTA TGG TGT-3'
		Lower primer	5'-GCG GTG AGG CCA AAC AAG GTG ATA-3'

Supplementary Table 2. Metabolic parameters of *Ldlr*$^{-/-}$ mice upon HFD

	WT mice, chow diet (n = 7)	*Ldlr*$^{-/-}$ mice fed a high fat diet and treated with:		
		PBS (n = 11 to 12)	Antagomir control (n = 10 to 12)	Antagomir-21 (n = 10 to 11)
Fasting serum glucose (mmol/L)	NA	5.5 (4.1-8.9)	5.5 (4.5-9.0)	6.1 (3.7-8.9)
Serum triglyceride (mmol/L)	0.7 (0.6-1.0)	4.2 (2.3-5.2)	3.8 (2.6-4.2)	3.2 (2.9-3.3)
Serum total cholesterol (mmol/L)	1.9 (1.7-2.0)	39.8 (33.7-45.4)	34.5 (31.4-37.2)	31.7 (30.2-35.2)
Serum HDL cholesterol (mmol/L)	1.2 (1.1-1.3)	5.9 (5.4-6.3)	5.8 (4.9-6.4)	5.2 (4.2-6.1)

Data are given as median (interquartile range).

WT mice fed a chow diet had significantly lower serum triglyceride, total and HDL cholesterol than Ldlr$^{-/-}$ *mice fed a high fat diet and treated with PBS, antagomir control or antagomir-21 ($p < 0.001$ for each comparison).*

There was no significant difference between Ldlr$^{-/-}$ *mice treated with antagomir control and antagomir-21, and between Ldlr*$^{-/-}$ *mice treated with antagomir control and PBS in any of these parameters. Antagomir-21 reduced serum total cholesterol as compared with PBS ($p = 0.003$), but did not change other variables.*

Abbreviations: NA, not available; WT, wild type.

Supplementary Table 3. Metabolic parameters miR-21-/- mice upon MCD diet

	WT mice, chow diet (n=10)	**WT mice, MCD diet (n=9)**	***miR-21*$^{-/-}$ mice , MCD diet (n=10)**
Fasting serum glucose (mmol/L)	NA	6.3 (5.7-7.2)	6.2 (5.6-6.8)
Serum triglyceride (mmol/L)	1.1 (0.5-1.5)	0.3 (0.3-0.3)	0.4 (0.3-0.5)
Serum total cholesterol (mmol/L)	2.3 (2.0-2.4)	0.4 (0.3-0.5)	0.5 (0.4-0.6)
Serum HDL cholesterol (mmol/L)	1.7 (1.5-1.8)	0.2 (0.1-0.3)	0.2 (0.2-0.3)

Data are given as median (interquartile range).

WT mice fed a chow diet had significantly higher serum triglyceride, total and HDL cholesterol than mice fed with MCD diet ($p < 0.001$ for each comparison).

There was no significant difference between WT and miR-21$^{-/-}$ mice upon MCD diet in any of these parameters.

Abbreviations: NA, not available; MCD, methionine-choline- deficient; WT, wild type.

Supplementary Table 4. Metabolic parameters of Wild type (WT) and Pparα-/- mice upon MCD diet

Diet	**Chow diet**	**MCD Diet**				
Genotype	**WT**	**WT**	**WT**	**WT**	***Pparα*$^{-/-}$**	***Pparα*$^{-/-}$**
AntagomiR	**-**	**-**	**Anti-Ctrl**	**Anti-21**	**Anti-Ctrl**	**Anti-21**
N=	**9**	**8**	**10**	**10**	**10**	**11**
Fasting serum glucose (mmol/L)	NA	2.7 (2.3-3.0)	2.5 (2.2-2.7)	2.2 (1.8-2.6)	1.7 (1.4-2.0)	1.7 (1.3-2.2)
Serum triglyceride (mmol/L)	0.8 (0.6-1.0)	0.5 (0.4-0.6)	0.6 (0.4-0.6)	0.5 (0.4-0.6)	0.5 (0.4-0.6)	0.5 (0.4-0.6)
Serum total cholesterol (mmol/L)	2.1 (2.0-2.3)	0.9 (0.7-1.0)	0.9 (0.6-1.2)	0.8 (0.5-0.9)	0.8 (0.6-1.0)	0.8 (0.6-1.0)
Serum HDL cholesterol (mmol/L)	1.6 (1.5-1.8)	0.7 (0.6-0.8)	0.8 (0.6-0.9)	0.6 (0.4-0.7)	0.5 (0.4-0.6)	0.5 (0.4-0.6)

Data are given as median (interquartile range).

WT mice fed a chow diet had significantly higher serum triglyceride, total and HDL cholesterol than mice fed with MCD diet ($p < 0.001$ for each comparison). Ppar$\alpha^{-/-}$ mice had lower serum HDL cholesterol and serum glucose levels than WT mice fed MCD diet. As compared to antagomir control, antagomir-21 did not change any of these parameters in WT or in Ppar$\alpha^{-/-}$ mice.

Abbreviations: Ctrl, control; NA, not available; MCD, methionine-choline- deficient diet; WT, wild type.

Supplementary Table 5. Patients' Main Features.

Characteristics	Controls with no or mild abnormalities at liver histological examination (n = 6)	Bland steatosis patients (n = 8)	NASH patients (n = 11)
Age (yrs)	51 (31-54)	55 (41-65)	55 (42-62)
Male gender – N (%)	2 (33)	5 (63)	6 (55)
Body mass index ($kg.m^{-2}$)	23.6 (20.0-24.5)	25.9 (23.7-27.4)*	31.7 (27.4-34.8)** ##
Cardio-vascular risks factors			
Hypertension – N (%)	0	3 (38)	2 (18)
Smoking – N (%)	1 (17)	2 (29)	1 (9)
Diabetes – N (%)	1 (17)	1 (13)	3 (27)
Dyslipidemia – N (%)	1 (17)	4 (50)	5 (45)
Serum levels			
Platelet count (x 10^9/L)	255 (212-304)	192 (187-226)	197 (152-279)
Prothrombin index (%)	99 (94-101)	97 (92-105)	93 (84-100)
AST (x ULN)	1.0 (0.8-1.4)	1.0 (0.9-1.2)	1.6 (1.3-2.0) *#
ALT (xULN)	1.5 (1.3-2.9)	1.5 (1.1-2.9)	2.2 (1.7-2.6)
γ-GT (x ULN)	3.2 (2.6-4.8)	1.4 (0.8-11.9)	3.0 (1.1-4.4)
Bilirubin (µmol/L)	13 (9-18)	12 (8-14)	11 (10-15)
Pathological findings			
Steatosis: S0, S1, S2, S3	6-0-0-0	0-5-2-1 **	0-5-2-4 ***
Ballooning: 0, 1, 2	6-0-0	3-4-1 *	0-3-8 *** ##
Lobular inflammation: 0, 1, 2	6-0-0	7-1-0	0-7-4 *** ###
Fibrosis: F0, F1, F2, F3, F4	5-1-0-0-0	3-4-1-0-0	0-5-3-2-1 *** #

Data are median (interquartile range) or frequency (%).

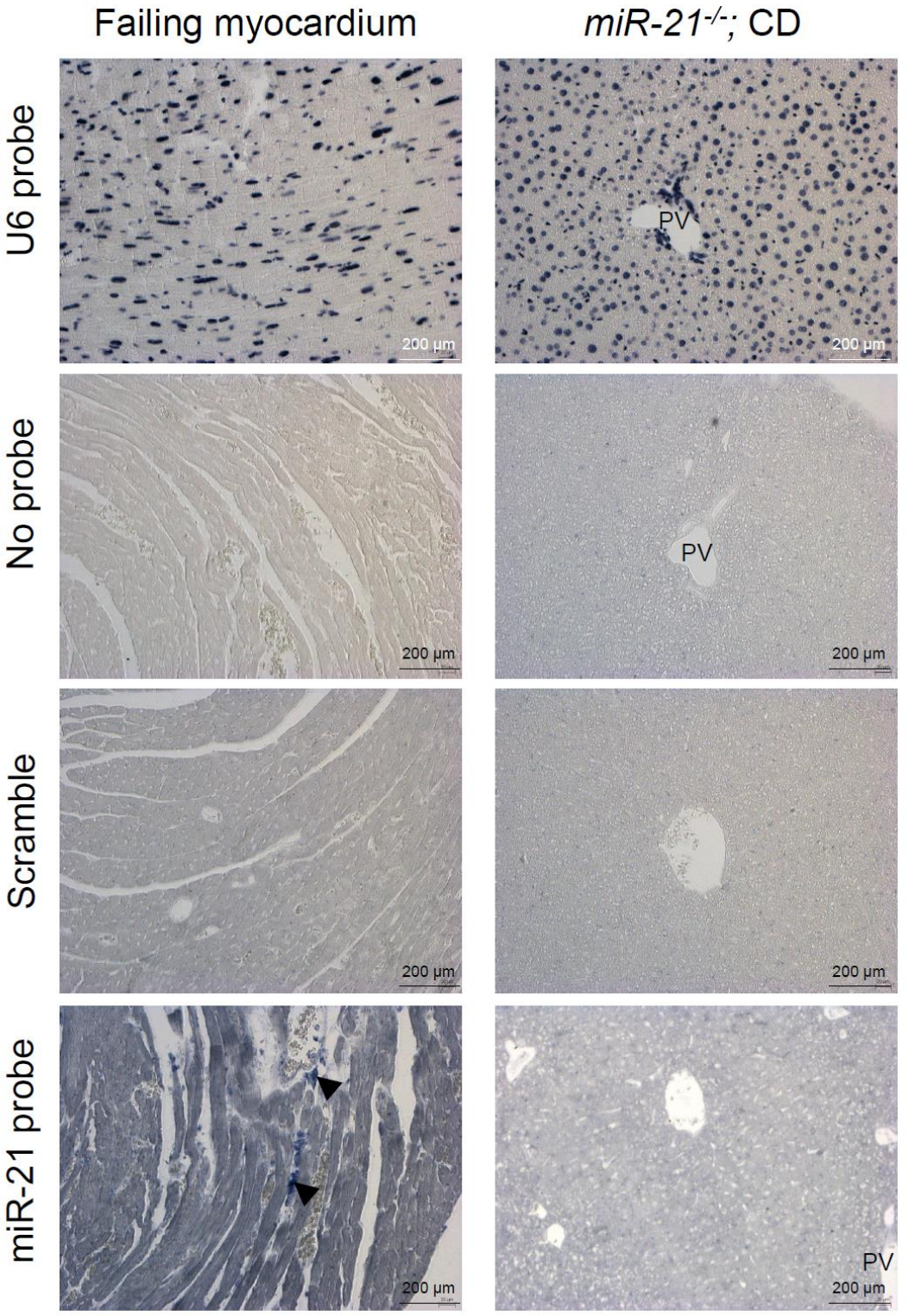
Abbreviations: NASH, non alcoholic steatohepatitis; ULN, upper limit of normal.

* indicates significant differences with controls with no or mild abnormalities at liver histological examination

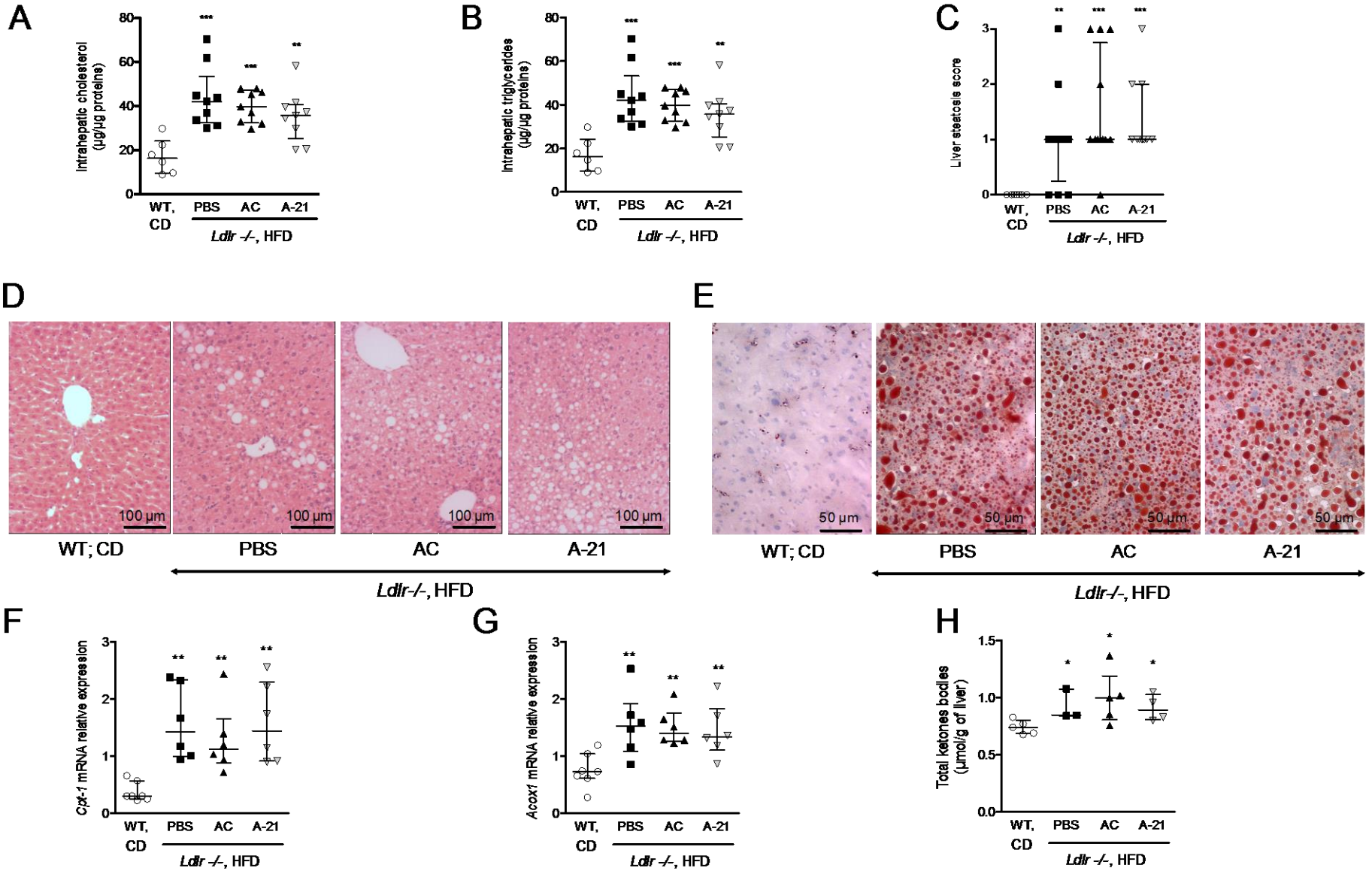
# indicates significant differences with bland steatosis patients

Abreviations: ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; γ-GT, γ-glutamyl transferase

Supplementary Figures



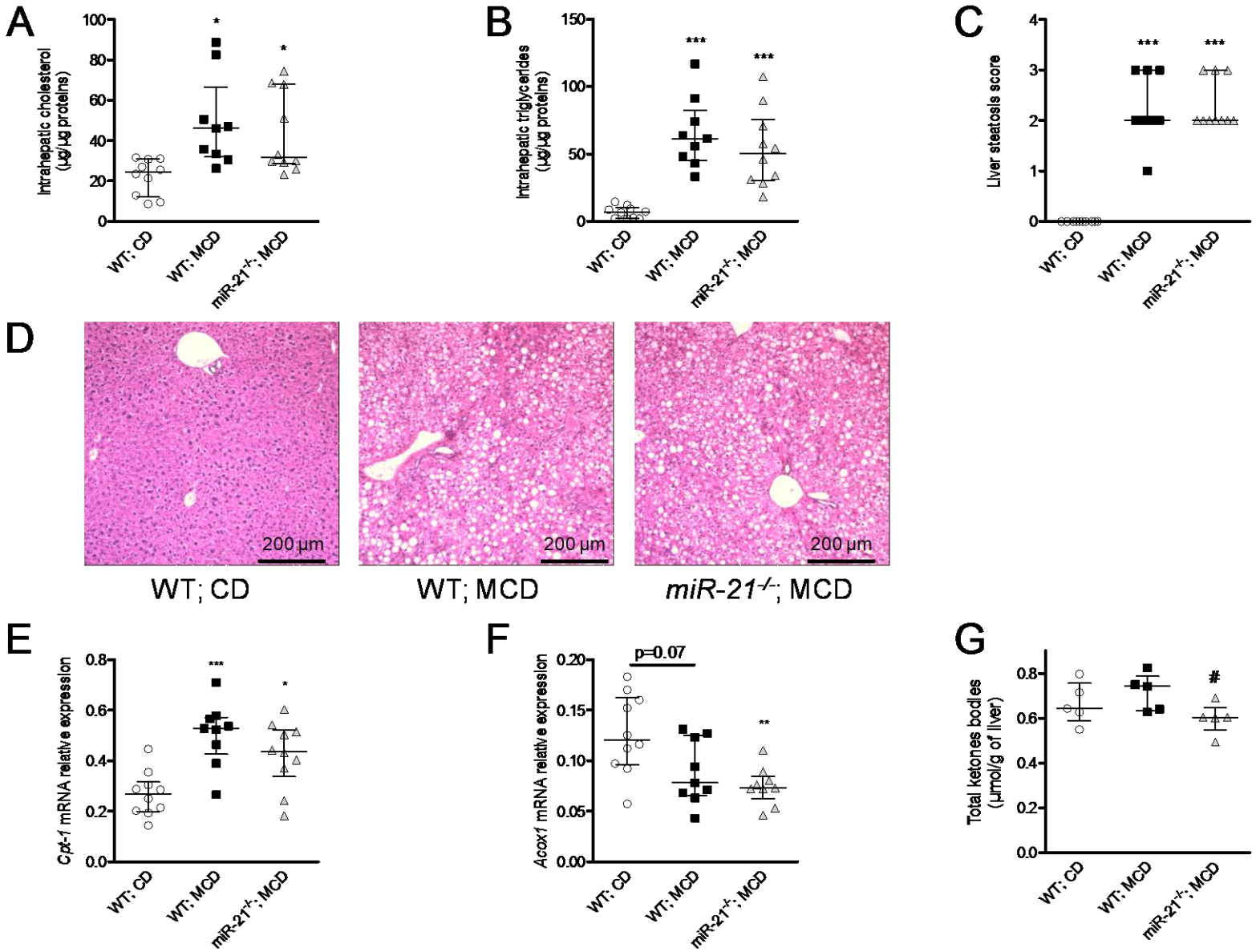
Supplementary figure 1. *In situ* hybridization. Failing myocardium from a wild type mouse having undergone myocardial infarction was used as a positive control. Arrows indicate cardiac fibroblasts expressing miR-21. The liver of a *miR-21*$^{-/-}$ fed a chow diet (CD) was used as a negative control. Original magnification x 100.


A
Intrahepatic cholesterol (µg/µg proteins)
WT, CD
PBS
AC
A-21
Ldlr -/-, HFD
B
Intrahepatic triglycerides (µg/µg proteins)
C
Liver steatosis score
D
100 µm
WT; CD
PBS
AC
A-21
Ldlr-/-, HFD
E
50 µm
F
Cpt-1 mRNA relative expression
G
Acox1 mRNA relative expression
H
Total ketones bodies (µmol/g of liver)

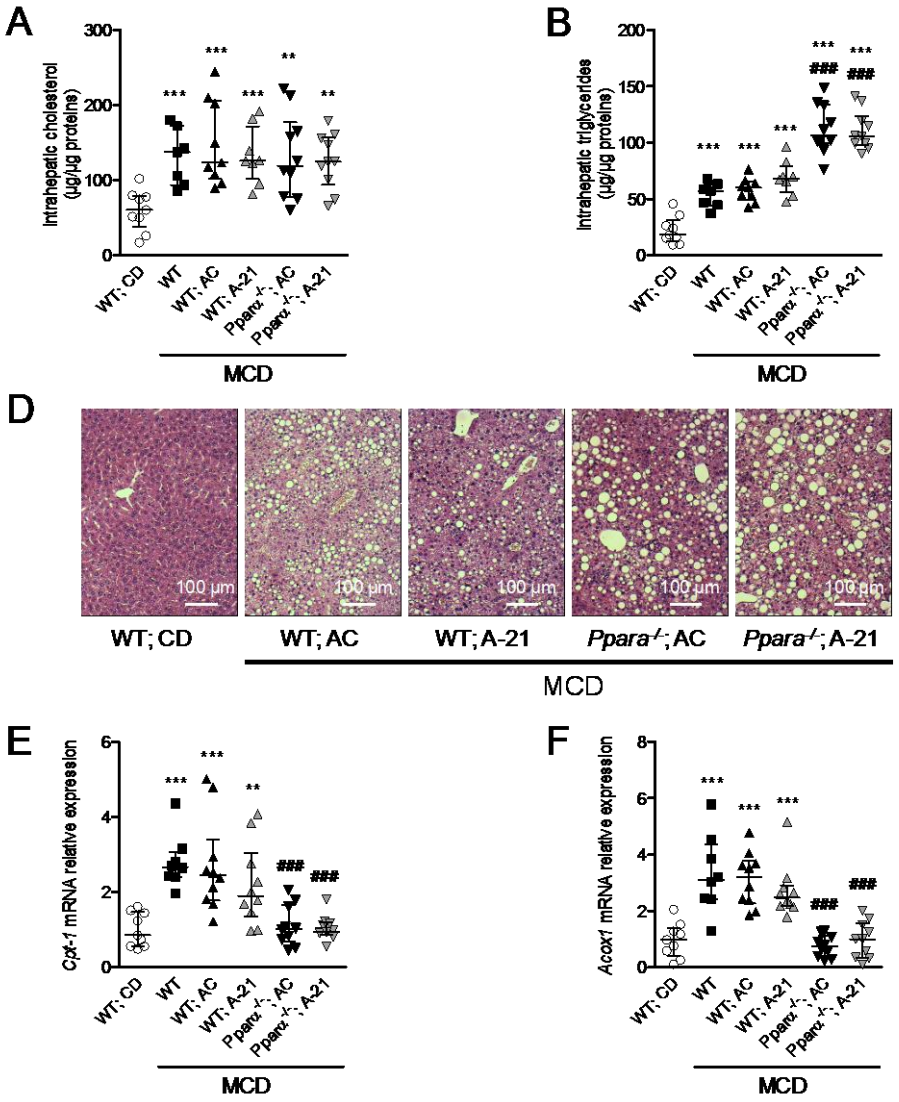
Supplementary figure 2. Antagomir-21 (A-21) did not change liver lipid accumulation or liver fatty acid ß-oxidation in *Ldlr*$^{-/-}$ mice fed a high fat diet (HFD). (A, B) Liver total cholesterol and triglycerides levels. (C) Quantification of liver steatosis by hematoxylin-eosin staining. (D, E) Representative images of hematoxylin-eosin (D; original magnification x 200) and Oil Red O staining (E; original magnification x 400). (F, G) Liver normalized *Cpt-1* and *Acox* mRNA expression. (H) Liver total ketone bodies.

**, $p < 0.01$. ***, $p < 0.001$ *vs.* wild type (WT) mice fed a chow diet (CD). Data are given as median (horizontal bar) and interquartile range (error bar).

Abbreviations: AC, antagomir control; HFD, high fat diet.


A
Intrahepatic cholesterol (µg/µg proteins)
100
80
60
40
20
0
*
*
WT; CD
WT; MCD
miR-21-/-; MCD
B
Intrahepatic triglycerides (µg/µg proteins)
150
100
50
0
***
***
WT; CD
WT; MCD
miR-21-/-; MCD
C
Liver steatosis score
4
3
2
1
0
***
***
WT; CD
WT; MCD
miR-21-/-; MCD
D
200 µm
200 µm
200 µm
WT; CD
WT; MCD
miR-21-/-; MCD
E
Cpt-1 mRNA relative expression
0.8
0.6
0.4
0.2
0.0
***
*
WT; CD
WT; MCD
miR-21-/-; MCD
F
Acox1 mRNA relative expression
0.20
0.15
0.10
0.05
0.00
p=0.07
**
WT; CD
WT; MCD
miR-21-/-; MCD
G
Total ketones bodies (µmol/g of liver)
0.8
0.6
0.4
0.2
0.0
#
WT; CD
WT; MCD
miR-21-/-; MCD

Supplementary figure 3. *MiR-21*$^{-/-}$ mice fed a methionine-choline-deficient (MCD) diet had no change liver lipid accumulation. (A, B) Liver total cholesterol and triglycerides levels. (C) Quantification of liver steatosis by hematoxylin-eosin staining. (D) Representative images of hematoxylin-eosin (original magnification x 100). (E, F) Liver normalized *Cpt-1* and *Acox* mRNA expression. (G) Liver total ketone bodies.
*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ *vs.* wild type (WT) mice fed a chow diet (CD*);* #, $p < 0.05$ *vs.* WT mice fed a MCD diet
Data are given as median (horizontal bar) and interquartile range (error bar).


A
Intrahepatic cholesterol (µg/µg proteins)
300
200
100
0
WT; CD
WT
WT; AC
WT; A-21
Ppara-/-; AC
Ppara-/-; A-21
MCD
B
Intrahepatic triglycerides (µg/µg proteins)
200
150
100
50
0
C
Liver steatosis score
3
2
1
0
D
100 µm
WT; CD
WT; AC
WT; A-21
Ppara-/-; AC
Ppara-/-; A-21
MCD
E
Cpt-1 mRNA relative expression
6
4
2
0
F
Acox1 mRNA relative expression
8
6
4
2
0

Supplementary figure 4. Antagomir-21 (A-21) did not change liver lipid accumulation or liver fatty acid ß-oxidation in *Pparα*$^{-/-}$ mice fed a methionine-choline-deficient (MCD) diet. (A, B) Liver total cholesterol and triglycerides levels. (C) Quantification of liver steatosis by hematoxylin-eosin staining. (D) Representative images of hematoxylin-eosin (D; original magnification x 200). (E, F) Liver normalized *Cpt-1* and *Acox* mRNA expression.

*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ *vs.* WT mice fed a chow diet (CD*)*

###, $p < 0.001$ *vs.* WT mice fed a MCD diet

Data are given as median (horizontal bar) and interquartile range (error bar).