Supplementary Material

Patients

Three groups of patients with other etiologies of liver disease were also included in the study. Patients with ALD-induced cirrhosis (ALD-CH) without any features of AH in liver biopsy, patients with genotype 1 HCV-induced cirrhosis (HCV-CH) who had not taken any previous antiviral therapy and patients with NASH (with or without cirrhosis). NASH was diagnosed by liver biopsy considering the Brunt criteria[1] and/or the Kleiner scoring system[2]. As controls we included a group of normal livers obtained from optimal cadaveric liver donors or resection of liver metastasis. All controls had normal serum aminotransferase levels and normal histology, as previously described [3].

MicroRNA Array Analysis

RNA samples were labelled using The FlashTag Biotin HSR kit (Genisphere).

The process begins with a brief tailing reaction followed by ligation of the biotinylated signal molecule to the target RNA sample. Afterwards, the biotin-labelled RNA was hybridized onto Genechip miRNA Arrays (Affymetrix) for 16 hours at 48ºC, scanned and detected the following day with Streptavidin-PE.

The Expression Console software (Affymetrix, Inc.) was used for data summarization, normalization, and quality control. For the detection of differentially expressed miRNAs, a linear model was fitted only to the human microarray data, and empirical Bayes moderated statistics were calculated using the limma package[4]. Adjustment of p-values was done by the determination of false discovery rates (FDR) using the Benjamini-Hochberg procedure. Heat Map representation of miRNA results shows the intensity of each color representing the normalized ratio between each value and the average expression of each miRNA across all samples. miRNAs representing a change of 1.5-fold or greater and moderated $p < 0.05$ were considered as differentially expressed.

RNA Isolation and miRNA or mRNA Expression Analysis

Total RNA containing total mRNA including miRNA was extracted from liver tissues using Trizol following the manufacture's instructions (Life Technologies, Carlsbad, CA). Total RNA extracted from liver biopsies underwent quality control by Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). MiRNA quantification was performed using the commercial kit Mir-X miRNA First-Strand Synthesis Kit according to the manufacture's instructions (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). Data were normalized using U6 snoRNA as the endogenous control. The sequences of miRNA primers, either human or mice, were obtained from the miRbase database. Custom primers were used (Integrated DNA Technologies, BVBA, Leuven, Belgium). mRNA quantification was accomplish using Taqman gene expression assay probe and primers. Both miRNA and mRNA levels were measured by quantitative real-time PCR (qPCR) with an ABI 7900 HT cycler (Life Technologies). Expression values were calculated based on the $\Delta\Delta$Ct method. The results were expressed as $2^{-\Delta\Delta Ct}$.

Primer Sequences

hsa-miR-182: TTTGGCAATGGTAGAACTCACACT

mmu-miR-182: TTTGGCAATGGTAGAACTCACACCG

hsa-miR-422a: ACTGGACUUAGGGUCAGAAGGC

hsa-miR-21: TAGCTTATCAGACTGATGTTGA

hsa- miR-214: TGCCTGTCTACACTTGCTGTGC

hsa-miR-432: TCTTGGAGTAGGTCATTGGGTGG

Animal Models

In order to investigate the expression of miR-182 in fibrosis a model of chronic liver injury was used (CCl_4) [3]. Male C57/BL6 mice were treated with CCl_4 intraperitoneally

(n=5) during 2 weeks (7 injections in total). Control mice (n=3) were injected with vehicle (corn oil).

To determine the hepatic expression of miR-182 in a model of acute and acute-on-chronic ethanol-induced liver injury we used a mouse model previously performed in our group [3]. Briefly, the acute model was performed in Balb/c mice by administering a single dose of 5mg/kg body weight of ethanol 50% (n=3) or water (n=3) by gavage, and the acute-on-chronic (n=4) was done by administering CCl_4 (diluted 1:4 in corn oil) intraperitoneally 4 injections in total at a dose of 0,5 mg/kg body weight and a final gavage of 5g/kg of ethanol. Control animals were injected with vehicle (corn oil).

To determine hepatic expression of miR-182 in a model of sclerosing cholangitis and biliary fibrosis [5], a 3,5-diethoxycarbonyl-1,4-dihydro-collidin (DDC) diet was used. Male C57/BL6 mice (n=5) were fed with 0,1% of DDC diet during 3 or 4 weeks as previously described [6]. Control animals were fed with standard rodent chow diet.

To observe the effects of chronic administration of ethanol and the effects of DDC over chronic ethanol, a modified Lieber-de-Carli model was performed. Four groups of 8-12 weeks old mice were established. The first group was administered with increasing amounts of ethanol; every 2 days the ethanol percentage was increase until a total of 4% ethanol (v/v) which remained during 3 more weeks (n=5). The second group received control Lieber-de-Carli (LdC) diet until the fourth week when they received 0.05% DDC LdC diet (n=3). The third group receives the ethanol diet following the same pattern as the first group and in addition 0.05% DDC during the last week (n=6). The last group took only control LdC diet (n=3).

Next, we assessed the expression of miR-182 in the presence of non-alcoholic steatohepatitis features. Liver steatosis mouse model was performed in 4 male C57/BL6 mice fed during 16 weeks with a high fat diet (D09031101, 60% Kcal from fat; Research Diets, New Brunswick, New Jersey, USA). Control mice (n=5) were fed with the standard rodent chow as previously described [7].

To evaluate the effect of a fulminant inflammation of the liver in the hepatic levels of miR-182 we carried out a model of acute hepatitis. Eight to twelve weeks old male Balb/c mice were injected with ConA (Sigma-Aldrich Química, Madrid, Spain) dissolved in saline (NaCl 0,9%)(n=3) or saline alone as controls (n=3). The ConA dose was established at 10mg/kg body weight and it was administered intravenously through the tail vein. The animals were sacrificed 18 hours after the injection.

Isolation of Ductular Reaction Cells

Ductular reaction cells were obtained from Hnf1bCreER^{YFP} transgenic mice, which express a reporter yellow fluorescence protein (YFP) after the Hnf1b promoter. We have previously shown that Hnf1b is expressed in ductular reaction cells allowing the possibility to sort these cells based on the expression of the reporter YFP[6]. The ductular reaction cells were obtained by a liver perfusion method followed by flow cytometry cell sorting from uninjured mice (n=3), and from mice treated with DDC (n=3). Livers were perfused with EGTA solution (NaCl 137 mM, KCl 5.4 mM, NaH2PO4H2O 0.64 mM, Na2HPO4 0.85 mM, HEPES 1mM, NaHCO3 4.17 mM, EGTA 0.5 mM and glucose 5mM, pH= 7.4) at a flow rate of 5ml/min until liver got pale. After that, the liver was perfused with collagenase solution (NaCl 137 mM, KCl 5.4 mM, NaH2PO4H2O 0.64mM, Na2HPO4 0.85 mM , HEPES 1 mM, NaHCO3 4.17 mM and CaCl2·2H2O 3.8 mM, pH=7.4) containing Collagenase A 0.5 g/L (Roche Diagnostics GmbH, Manheim, Germany) at a flow rate of 5 mL/min for 11 minutes. The digested liver was minced in Petri dish with ice cold 1x Hank's Balanced Salt Solution (HBSS) (Biological Industries) and filtered through 70-µm cell strainer (BD Biosciences, Erembodegen, Belgium). The undigested liver was re-digested with collagenase solution containing 0.5 g/L Collagenase A, 0.5 g/L Pronase and 50mg/L DNAse I (Roche Diagnostics GmbH) and stirred for 30 minutes at 37°C. After slow centrifugations at 100g for 2 minutes, the supernatant was collected and centrifuged at 300g for 5 minutes; the pellet containing non-parenchymal cells were sorted on a

FACSAria (BD Biosciences). YFP+ gate was established based on wild type mice. Dead cells were excluded by Live/Dead cell stain kit (Life Technologies, Eugene, Oregon) and YFP+ cells were collected in Qiazol buffer (Qiagen GmbH, Hilden, Germany).

RNA Extraction from Ductural Reaction Cells

Total RNA containing miRNAs were extracted using the commercial kit miReasy Mini Kit (Quiagen) following manufacture's instructions.

Isolation of Hepatocytes.

Hepatocytes were isolated from livers of mice fed with DDC diet during 6 weeks (n=3). Livers were perfused with EGTA and collagenase A solution, as descried above, with the important difference that the last digestion was performed without pronase that specifically digests hepatocytes. Afterwards, disaggregated livers were centrifuge at 30g during 3 minutes allowing hepatocyte to settle thanks to their higher density and size. Supernatant was discarded and hepatocytes resuspended in Trizol to isolate total RNA.

Hepatocytes for *in vitro* experiment were isolated from healthy C57BL/6J mice (n=4) by a three-step in situ retrograde perfusion procedure using 0.04% collagenase IV through the inferior cava vein, as previously described [7].

Cell culture

The H69 normal cholangiocyte cell line was grown in a 50/50% (v/v) DMEM/Ham's F-12 along with other additives as previously described [9]. The RAW 264.7 cell line was grown with DMEM Glutamax supplemented with 10%FBS. Primary mouse hepatocytes were grown with E-Williams supplemented with 10%FBS.

***In vitro* transfection with miR-182 mimic.**

H69 human cholangiocytes cell line were plated in a 6 well culture plate at a density of $4X10^5$ cells per well. At 70-80% of confluence, 3% fetal bovine serum (FBS) medium was added. After 3 hours cells were transected with 50nM of cel-miR-39-3p or hsa-miR-182, both labeled with Cy3.3 (Exiqon, Vedbaek Denmark). After 24 hours, yield of transfection reached a 60-80%. 48 hours after transfection, cells were harvested with Trizol (Thermo Fisher) to collect RNA. Gene expression was measured by qPCR.

Primary mouse hepatocytes were plated in a 6 well culture plate at a density of $7X10^5$ cells per well. After 4 hours, 1% FBS medium was added overnight. Cells were transfected with 50nM of mmu-miR-182 or cel-miR-39-3p, labeled with Cy3.3 (Exiqon). 24 hours after transfection total RNA was extracted with Rneasy Mini Kit (Quiagen) following manufacturer's instructions.

RAW 264.7 cells were plated in a 6 well culture plate at a density of $2X10^5$ cells per well. At 70-80% of confluence, 2% fetal bovine serum (FBS) medium was added. After 4 hours cells were transected with 50nM of cel-miR-39-3p or mmu-miR-182 labeled with Cy3.3. 48 hours after transfection total RNA was extracted with Rneasy Mini Kit (Quiagen) following manufacturer's instructions.

The transfectant reagent used was jetPRIME (PolyPlus) in all cell types.

Determination of total bile acids

Fragments of liver weights 30 mg were homogenized in PBS 1X. Bile acids were determined with Total Bile Acid Assay Kit (Cell Biolabs, INC.) following manufacturer's instructions.

Tissue Staining and Immunohistochemistry

Mouse paraffin embedded liver sections were deparaffinised and incubated in Target Retrieval Solution, Citrate pH 6 (DAKO, Glostrup, Denmark), heated in a pressure cooker for 20 minutes. Sections were then incubated with primary antibodies overnight at 4ºC. Appropriate secondary antibodies were incubated for 1 hour at room

temperature. Finally, the sections were stained with 3,3'-diaminobenzidine (DAB, Dako) and counterstained with hematoxylin. Hepatic MPO and KRT19 tissue expression and cell localization in mouse samples were assessed by immunohistochemistry using primary antibodies against MPO (Cell Signalling Technology) and against KRT19 (Developmental Studies Hybridoma Bank, University of Iowa). Positive-stained area was quantified using a morphometric system analysis described in detail previously [8].

Statistical Analysis

Continuous variables were described as means (95% confidence interval) or medians (interquartile range). Categorical variables were described by means of counts and percentages. Comparisons between groups were performed using the Student´s *t* test or the Mann-Whitney *U* test when appropriate. Correlations between variables were evaluated using Spearman's *rho* or Pearson's *r*, when appropriate. The Area under the Receiver Operating Characteristic (AUROC) curve analysis was used to determine the best cut-off value and the accuracy (sensitivity and specificity) of continuous variables associated with 90-day mortality. Finally, we performed a comparative risk analysis using the Kaplan-Meier method. Comparisons were performed by the log-rank test. All statistical analyses were performed using SPSS version 14.0 for Windows (SPSS Inc., Chicago, IL).

Supplementary Figure Legends

Supplementary Figure 1. Heat map of the 51 miRNA differentially expressed in AH versus ALD-CH and normal livers. 38 were found up-regulated and 13 were found down-regulated ($p < 0.05$).

Supplementary Figure 2. **(A)** Correlation between miR-182 hepatic expression and mortality at 90-days in patients with AH (alive n=26, deceased n=9); *, p=0.019; **(B)** Hepatic expression of miR-182 correlates with the ABIC score (p>0.01).

Supplementary Figure 3. Correlation between miR-182 serum levels and **(A)** miR-182 hepatic expression, **(B)** ABIC score, **(C)** MELD score, **(D)** baseline total serum bilirubin (mg/dl), **(E)** Hepatic venous pressure gradient (HVPG) (p>0.01) and **(F)** mortality at 90-days in patients with AH (alive n=21, deceased n=6). **(G)** MiR-182 serum levels were measured in paired samples of AH patients (n=8) before corticosteroid treatment, and 7 days after treatment.

Supplementary References

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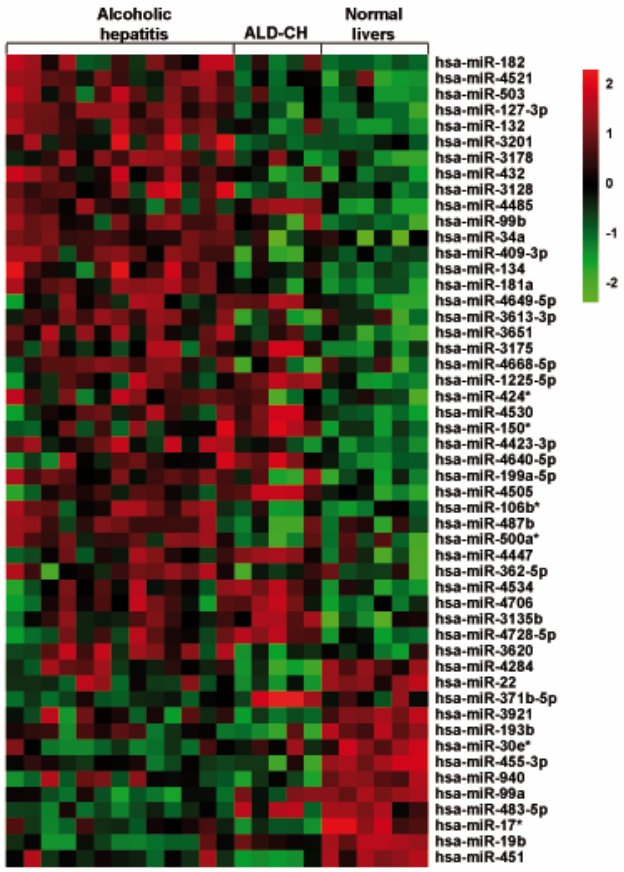
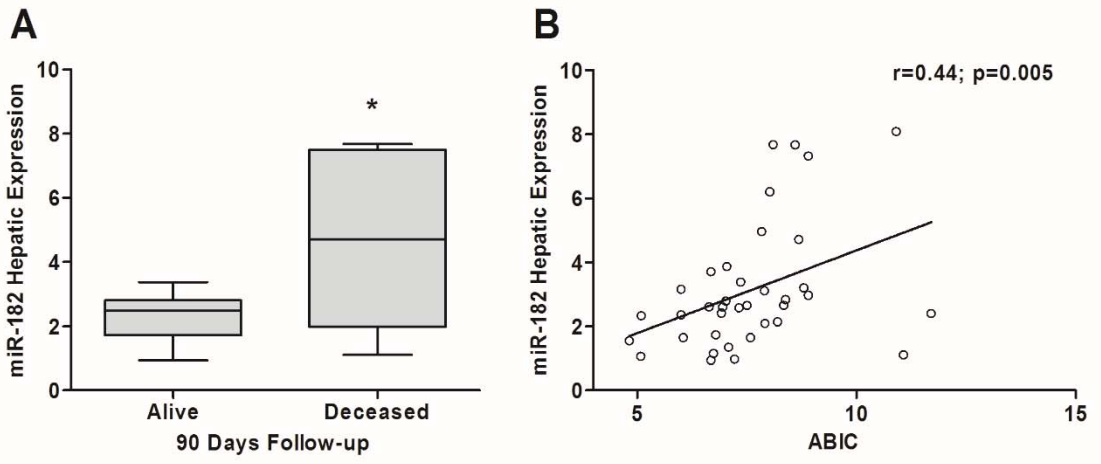
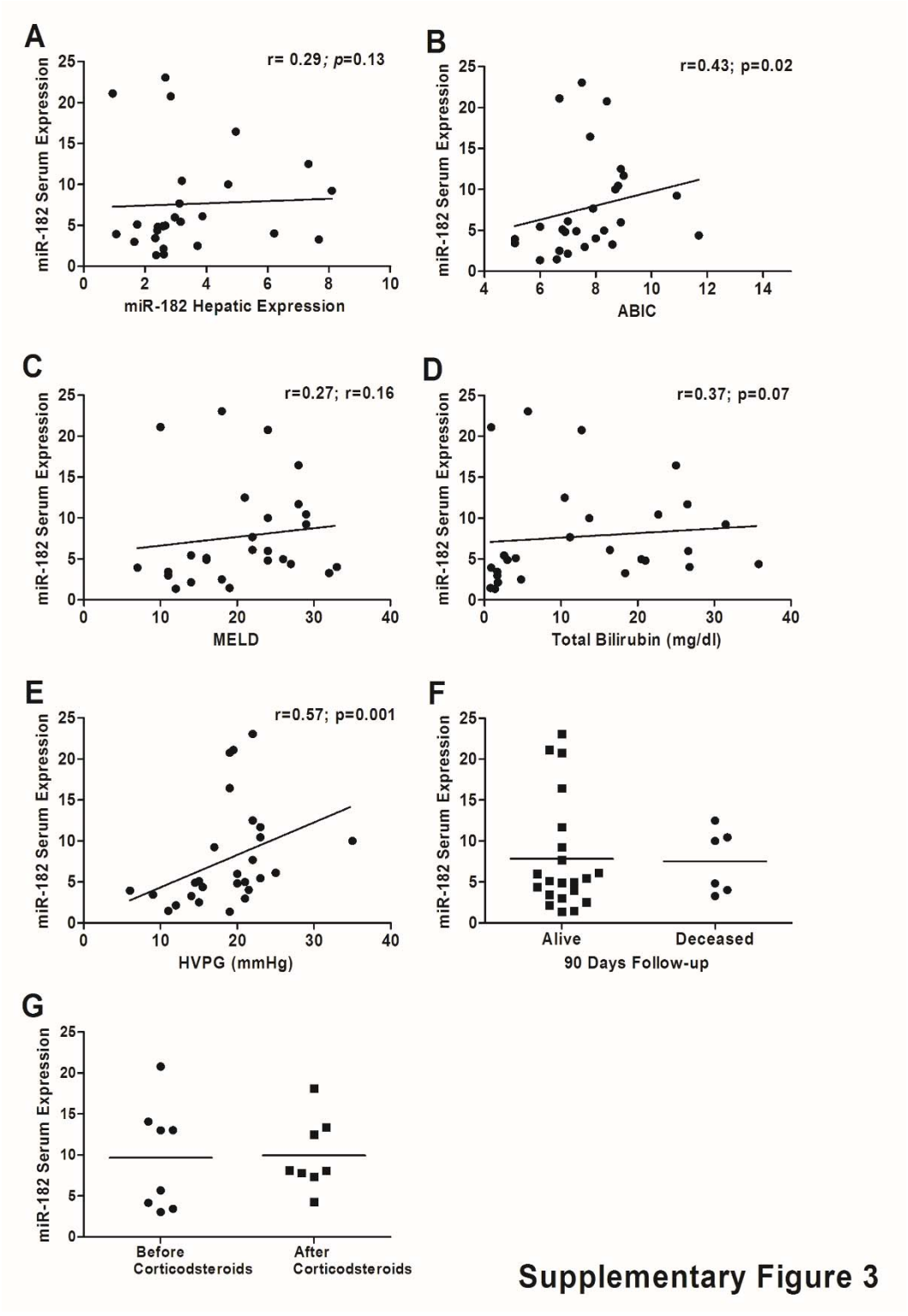


Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3