Research article

JHEP|Reports

Profiling circulating microRNAs in patients with cirrhosis and acute-on-chronic liver failure

Delia Blaya,[1,2] Elisa Pose,[1,2,3] Mar Coll,[1,2,3] Juan José Lozano,[2] Isabel Graupera,[1,2,3] Robert Schierwagen,[4] Christian Jansen,[8] Pedro Castro,[9] Sara Fernandez,[9] Julia Sidorova,[10] Mariuca Vasa-Nicotera,[4] Elsa Solà,[1,2,3] Joan Caballería,[1,2,3] Jonel Trebicka,[4,5,6,7,**] Pere Ginès,[1,2,3] Pau Sancho-Bru[1,2,*]

[1]*Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain;* [2]*Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Barcelona, Spain;* [3]*Liver Unit, Hospital Clinic of Barcelona, Barcelona, Spain;* [4]*Goethe University Clinic, Frankfurt, Germany;* [5]*European Foundation for the Study of Chronic Liver Failure, Barcelona, Spain;* [6]*Institute for Bioengineering of Catalonia, Barcelona, Spain;* [7]*Faculty of Health Sciences, University of Southern Denmark, Odense, Denmark;* [8]*University Clinic Bonn, Bonn, Germany;* [9]*Medical Intensive Care Unit, Hospital Clinic of Barcelona, IDIBAPS, University of Barcelona, Barcelona, Spain;* [10]*Instituto de Tecnología del Conocimiento (ITC). Campus de Somosaguas, Universidad Complutense de Madrid (UCM), Spain*

JHEP Reports **2021.** https://doi.org/10.1016/j.jhepr.2021.100233

Background & Aims: MicroRNAs (miRNAs) circulate in several body fluids and can be useful biomarkers. The aim of this study was to identify blood-circulating miRNAs associated with cirrhosis progression and acute-on-chronic liver failure (ACLF).
Methods: Using high-throughput screening of 754 miRNAs, serum samples from 45 patients with compensated cirrhosis, decompensated cirrhosis, or ACLF were compared with those from healthy individuals (n = 15). miRNA levels were correlated with clinical parameters, organ failure, and disease progression and outcome. Dysregulated miRNAs were evaluated in portal and hepatic vein samples (n = 33), liver tissues (n = 17), and peripheral blood mononuclear cells (PBMCs) (n = 16).
Results: miRNA screening analysis revealed that circulating miRNAs are dysregulated in cirrhosis progression, with 51 miRNAs being differentially expressed among all groups of patients. Unsupervised clustering and principal component analysis indicated that the main differences in miRNA expression occurred at decompensation, showing similar levels in patients with decompensated cirrhosis and those with ACLF. Of 43 selected miRNAs examined for differences among groups, 10 were differentially expressed according to disease progression. Moreover, 20 circulating miRNAs were correlated with model for end-stage liver disease and Child-Pugh scores. Notably, 11 dysregulated miRNAs were associated with kidney or liver failure, encephalopathy, bacterial infection, and poor outcomes. The most severely dysregulated miRNAs (*i.e.* miR-146a-5p, miR-26a-5p, and miR-191-5p) were further evaluated in portal and hepatic vein blood and liver tissue, but showed no differences. However, PBMCs from patients with cirrhosis showed significant downregulation of miR-26 and miR-146a, suggesting a extrahepatic origin of some circulating miRNAs.
Conclusions: This study is a repository of circulating miRNA data following cirrhosis progression and ACLF. Circulating miRNAs were profoundly dysregulated during the progression of chronic liver disease, were associated with failure of several organs and could have prognostic utility.
Lay summary: Circulating miRNAs are small molecules in the blood that can be used to identify or predict a clinical condition. Our study aimed to identify miRNAs for use as biomarkers in patients with cirrhosis or acute-on-chronic liver failure. Several miRNAs were found to be dysregulated during the progression of disease, and some were also related to organ failure and disease-related outcomes.

Introduction

Acute-on-chronic liver failure (ACLF) is a clinical syndrome characterised by acute decompensation of cirrhosis, development of hepatic and/or extrahepatic organ dysfunction, and impaired systemic inflammation.[1–4] It is associated with high short-term mortality (28 days), which increases with the number of failed organs, with a rate ranging from 20% to 25% with 1 organ failure (ACLF-1) to 70–80% with 3 or more organ failures (ACLF-3).[5]

Great effort has been made to identify biomarkers with prognostic value for acute decompensation of cirrhosis and ACLF. So far, several biomarkers correlating with poor patient outcome

Keywords: Liver decompensation; Non-coding RNAs; Biomarkers; Chronic liver disease.
Received 23 April 2020; received in revised form 2 December 2020; accepted 25 December 2020; available online 19 January 2021
* Corresponding authors. Addresses: Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), C/ Rosselló, 149-153, third floor, 08036, Barcelona, Spain. Tel.: +34 93 227 54 00 ext. 3371. (P. Sancho-Bru), or Department of Internal Medicine I, University Clinic Frankfurt, Theodor-Stern-Kai 7, 60590, Frankfurt, Germany. Tel.: +34 93 227 14 11; Fax: +34 93 227 14 19 (J. Trebicka).
E-mail addresses: jonel.trebicka@kgu.de (J. Trebicka), psancho@clinic.cat (P. Sancho-Bru).

have been identified in ACLF, including neutrophil gelatinase-associated lipocalin,[6] systemic circulatory dysfunction,[7] and C-X-C motif chemokine ligand 10 (CXCL10),[8] among others.[4,9,10] However, recent data show that no single marker alone can define ACLF or predict its development; instead, the profile of several inflammatory markers could be the key to evaluating this heterogenous disease.[11]

MicroRNAs (miRNAs) are small RNA molecules that regulate gene expression at the post-transcriptional level by promoting mRNA degradation or inhibiting mRNA translation.[12] miRNAs are dysregulated in the liver and are described as having an important role in the pathophysiology of liver diseases.[13–16] Moreover, they can also be used as biomarkers.[17–19]

Circulating miRNAs have been proposed as biomarkers in cholangiocarcinoma,[20] hepatocellular carcinoma,[21] non-alcoholic steatohepatitis (NASH),[22,23] and cirrhosis,[24–26] among others. Based on previous studies, we hypothesised that circulating miRNAs could be useful biomarkers to both stratify patients with advanced chronic liver disease and predict patient outcomes.

The aim of this study was to determine circulating miRNAs dysregulated during the progression of chronic liver disease that might be useful as biomarkers. Specifically, the study aimed to profile circulating miRNAs specifically dysregulated in patients with decompensated cirrhosis or ACLF compared with patients with compensated cirrhosis. To achieve these aims, high-throughput analysis of circulating miRNAs and a subsequent more directed analysis of 43 miRNAs were performed in patients with different stages of chronic liver disease. The results showed that there was a dysregulation of circulating miRNAs with the progression of cirrhosis, mainly associated with decompensation of cirrhosis. Moreover, the data showed no important differences between patients with decompensated cirrhosis or ACLF.

This study provides a comprehensive report of circulating miRNAs associated with the progression of cirrhosis, including patients with compensated or decompensated cirrhotic, or ACLF, as well as valuable information for future studies in the field of circulating miRNAs in liver cirrhosis and ACLF.

Materials and methods

Patients

For the analysis of circulating miRNAs, serum samples were collected from 30 patients from the CANONIC study of the European Foundation for the Study of Chronic Liver Failure (EF CLIF) consortium: 15 patients with decompensated cirrhosis and 15 patients with ACLF. All patients with ACLF were grade ACLF-2 or ACLF-3, with 12 patients having 2 organ failures (80% ACLF-2) and 3 patients presenting with 3 or more organ failures (20% ACLF-3). A group of 15 patients with compensated cirrhosis admitted to the Liver Unit of the Hospital Clinic of Barcelona and a group of 15 healthy individuals were included. The clinical characteristics of the patients are described in Table 1.

Informed consent was obtained from all patients and the study was approved by the Ethics Committee of the Hospital Clinic of Barcelona. The study protocol was performed according to the ethical guidelines of the 1975 Declaration of Helsinki.

The clinical characteristics of other patient cohorts analyzed in this study are described in supplementary material and Tables S1 and S2.

Determination of circulating miRNA expression

Isolated miRNAs from serum samples underwent retro-transcription and pre-amplification reactions using the Human miRNA Megaplex (Life Technologies) following the manufacturer's instructions. Amplification of 754 miRNAs was performed by OpenArray Technology, which is a high-throughput quantitative (q)PCR system. OpenArray was performed by the Molecular Genomics Veterinary Service of the Universitat Autonoma of Barcelona. Data were normalised by global means. After normalising miRNA expression, several samples were excluded because of poor amplification, and further analyses were performed with samples from 14 healthy individuals, 11 patients with compensated cirrhosis, 14 patients with decompensated cirrhosis, and 12 patients with ACLF, with a total of 51 samples.

Further description of the material and methods used can be found in the supplementary material.

Table 1. Demographic, clinical, and laboratory characteristics of patients included in the circulating miRNA analysis.

Patient characteristics	Patients with compensated cirrhosis (n = 15)	Patients with decompensated cirrhosis (n = 15)	Patients with acute-on-chronic liver failure (n = 15)	*p* value
Age (year)	67 (58-73)	56 (51-65)	55 (47-57)	0.39
Male sex, n (%)	11 (73)	9 (60)	11 (73)	0.71
Alcoholic cirrhosis, n (%)	7 (47)	7 (47)	8 (53)	0.41
Presence of ascites, n (%) 0		11 (73)	14 (93)	0.16
Presence of encephalopathy, n (%) 0		3 (20)	10 (67)	0.13
Presence of gastrointestinal bleeding, n (%) 0		2 (13)	2 (13)	0.7
Serum bilirubin (mg/dl)	1.3 (0.9–2.8)	3.0 (2.0–5.0)	16.0 (5.0–44.0)	0.000
Serum albumin (g/L)	37 (34–45)	30 (26–34)	24 (20–30)	0.000
INR	1.1 (1.0–1.2)	1.5 (1.4–1.7)	1.9 (1.6–2.9)	0.000
Platelet count (×10^9/ml)	145 (99–175)	94 (63–139)	57 (42–89)	0.001
Serum creatinine (mg/dl)	0.7 (0.7–0.9)	0.9 (0.7–1.1)	1.9 (0.9–3.1)	0.002
Serum sodium (mEq/L)	141 (140–144)	137 (131–139)	129 (127–139)	0.002
Leukocyte count (×10^9/ml)	5.6 (3.8–8.2)	6.0 (4.0–9.7)	7.5 (4.1–12.5)	0.547
C-reactive protein (mg/dl)	0.7 (0.2–1.5)	1.8 (0.5–4.9)	1.8 (1.1–6.5)	0.083
MELD score	8 (7–10)	16 (12–19)	32 (28–37)	0.000

INR, international normalised ratio; MELD, model for end-stage liver disease.
Comparison between groups were made by ANOVA test.

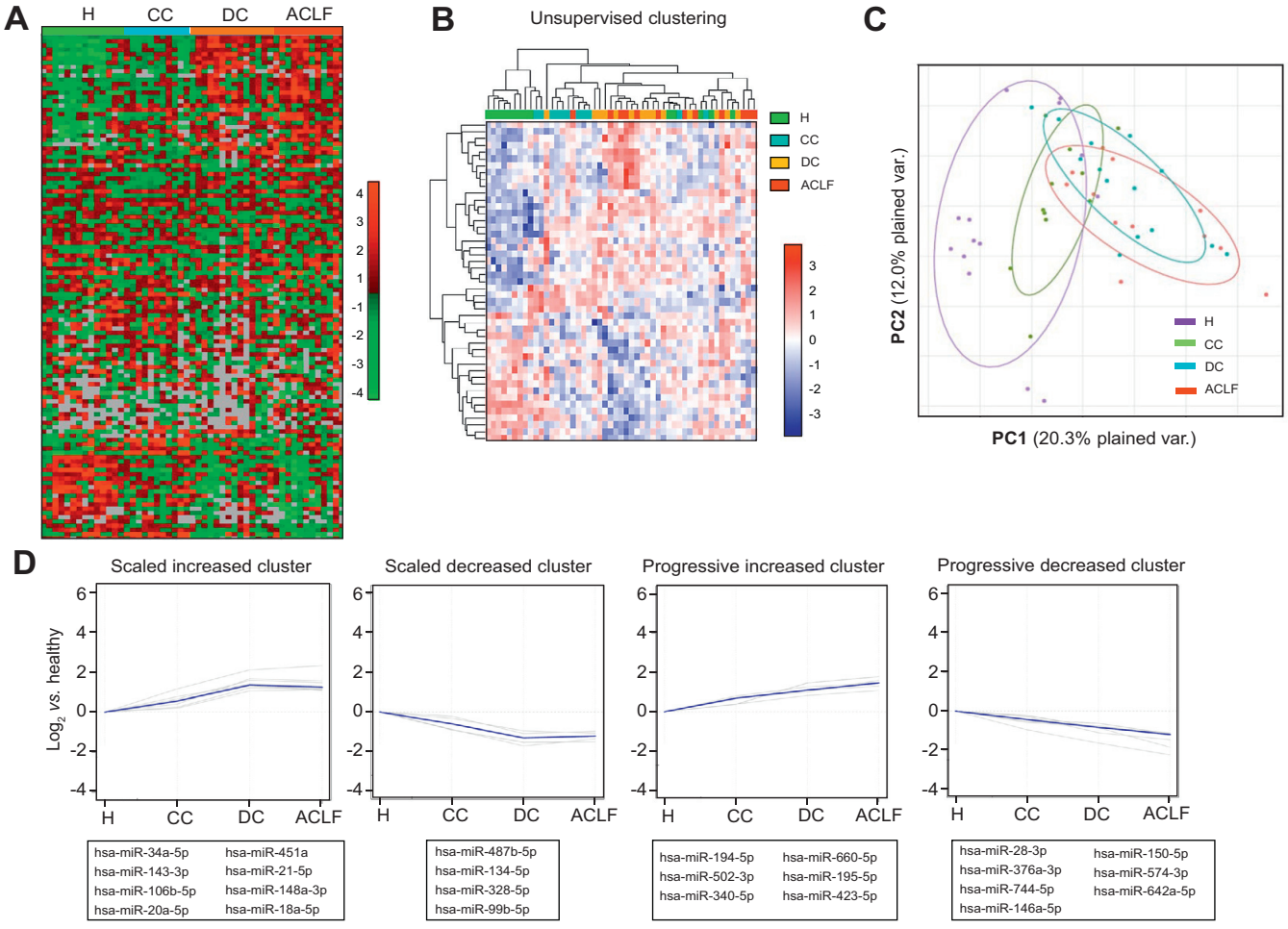

Fig. 1. Profile of circulating miRNAs in patients with cirrhosis and ACLF. (A) Heatmap of circulating miRNAs detected in serum samples of healthy individuals (H; n = 14), patients with compensated cirrhosis (CC; n = 11), patients with decompensated cirrhosis (DC; n = 14), and patients with ACLF (n = 12). Red pixels represent an increase in miRNA level in the indicated sample, whereas green pixels represent a decrease in miRNA abundance. (B) Unsupervised clustering heatmap. Samples are clustered according to similarities in those circulating miRNAs with a reliable level of expression. Red pixels represent an increase in miRNA level in the indicated sample, whereas blue pixels represent a decrease in miRNA abundance. (C) Principal component analysis where the 51 samples are represented according to expression of the 2 variables that best represent the variability of miRNA levels. (D) Graphs showing distinct significant expression profiles clustered by the STEM algorithm. H are in purple, CC in green, DC in light blue and ACLF in red. ACLF, acute-on-chronic liver failure.

Results

High-throughput analysis of serum circulating miRNAs

To analyse the presence of dysregulated miRNAs in the serum of patients with chronic liver disease and ACLF, high-throughput quantitative PCR technology was used to analyse serum from 15 healthy individuals, 15 patients with compensated cirrhosis, 15 patients with decompensated cirrhosis, and 15 patients with ACLF. After normalisation of miRNA expression, some samples were excluded from the analysis and only samples with reliable amplification were included. Thus, samples from 14 healthy individuals, 11 patients with compensated cirrhosis, 14 patients with decompensated cirrhosis, and 12 patients with ACLF were analysed.

Of 754 miRNAs analysed, 481 were not detected in any of the samples, whereas 277 miRNAs (36%) were detected in 1 or more samples. To select miRNAs with a reliable level of expression among all groups of samples, miRNAs were selected that were amplified in at least 6 samples from each group of patients, yielding a set of 118 miRNAs that were selected for further analysis (Fig. 1A and Table S3). To assess whether the set of 118 miRNAs was useful to stratify patients according to disease stage, an unsupervised clustering analysis was performed. The results showed that healthy individuals, and patients with compensated cirrhosis, decompensated cirrhotic or ACLF did not cluster independently (Fig. 1B). Instead, two main clusters were observed, one formed of healthy individuals and patients with compensated cirrhosis, and the second by patients with decompensated cirrhosis and ACLF. These data suggest that the decompensation event determines major changes in circulating miRNAs levels.

Principal component analysis (PCA) was performed to further evaluate whether dysregulation of circulating miRNAs could differentiate the different groups of samples. The miRNAs

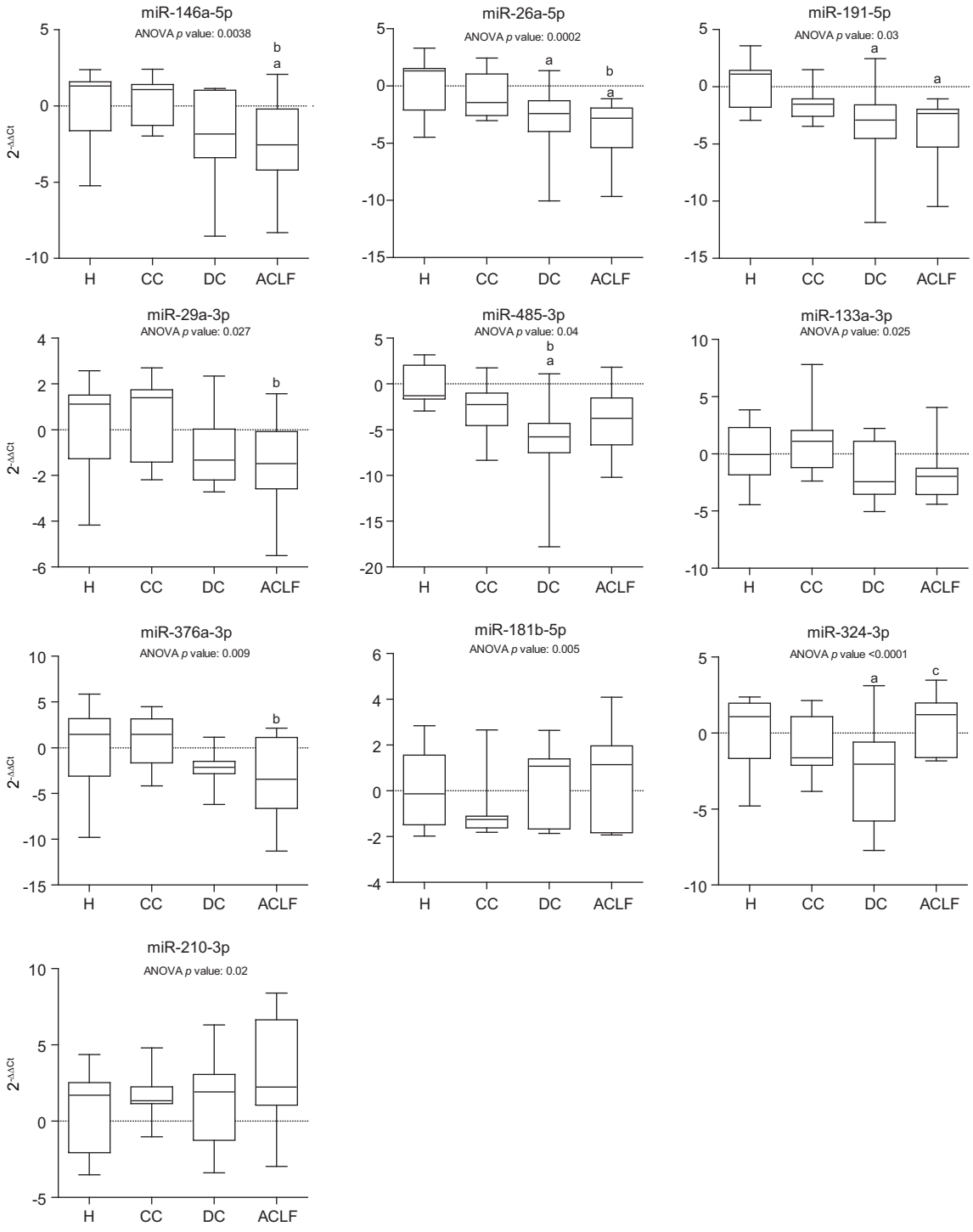

Fig. 2. Expression of 10 circulating miRNAs significantly differentiated during the progression of liver disease. Boxplots graphs representing the fold change (expressed as $2^{-\Delta\Delta Ct}$) between healthy individuals (H; n = 14) and patients with compensated cirrhosis (CC; n = 15), patients with decompensated cirrhosis (DC; n = 14), and patients with ACLF (n = 13). All miRNAs were significant (p <0.05) for the ANOVA test. Subsequent comparisons between groups were made with the Bonferroni test: (a) p <0.05 *vs.* healthy individuals; (b) p <0.05 *vs.* CC; (c) p <0.05 *vs.* DC. ACLF, acute-on-chronic liver failure.

identified were not able to stratify patients according to disease progression (Fig. 1C). However, whereas patients with decompensated cirrhosis displayed a complete overlap with patients with ACLF, healthy individuals and patients with compensated cirrhosis clustered separately. This suggests that circulating miRNAs are altered by decompensation of liver disease, but do not appear to be further affected by the development of ACLF.

Next, the STEM algorithm,[27] which looks for expression profiles in short data series, was used to evaluate the presence of groups of miRNAs with the same expression pattern during the progression of chronic liver disease. The algorithm revealed 4

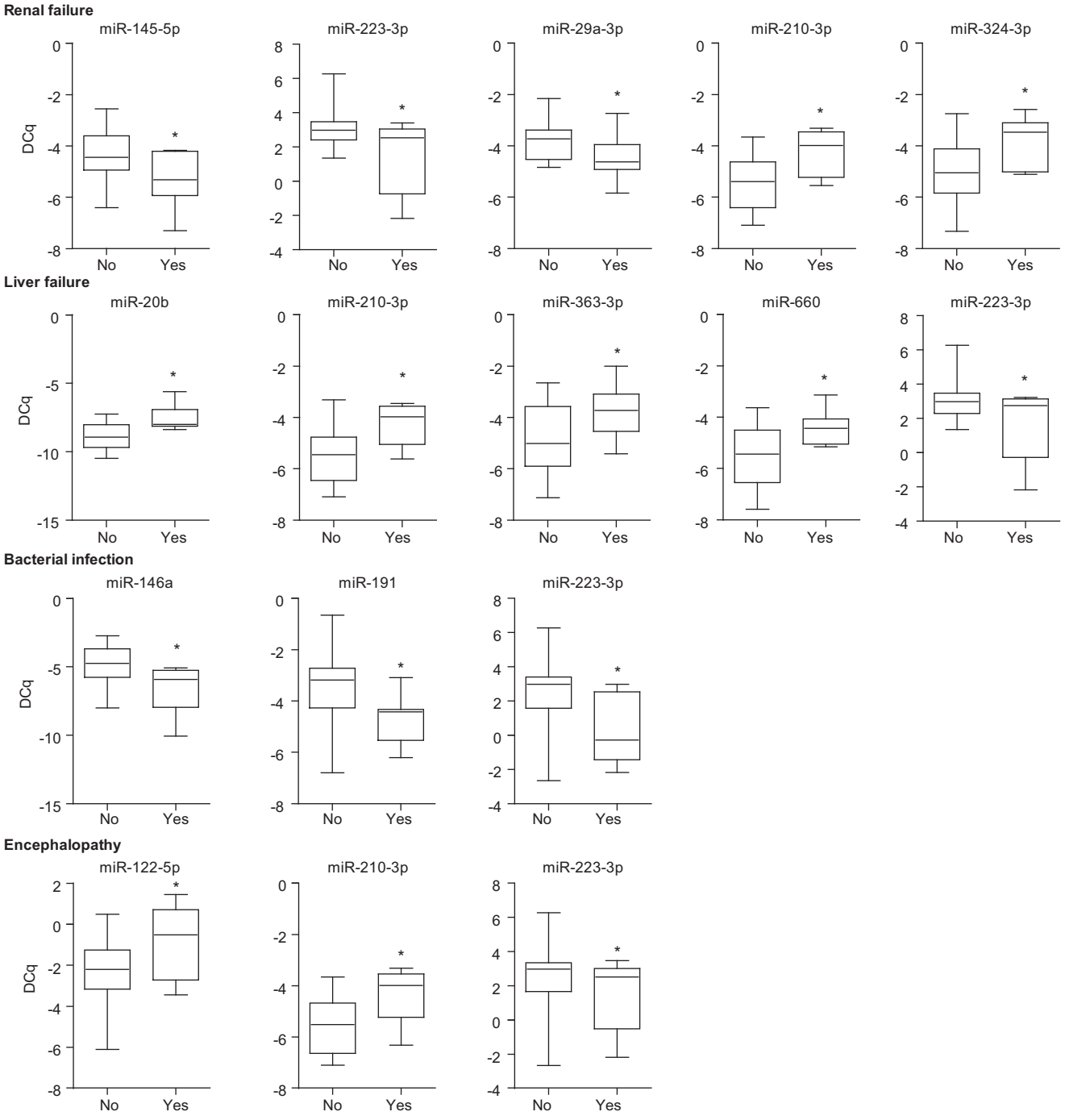

Fig. 3. Circulating miRNA with significant changes in expression between patients with or without a decompensation event. Evaluation of circulating miRNAs in patients with decompensated cirrhosis (n = 30) divided according to the presence or absence of: renal failure, liver failure, encephalopathy, and bacterial infection. Comparison between groups were made with t test, $^{*}p$ >0.05.

different miRNAs clusters (Fig. 1D). Two clusters were identified with miRNAs increasing from healthy samples to patients with ACLF. The first cluster contained 8 miRNAs with a scaled increase in patients with decompensated cirrhosis or ACLF compared with healthy controls and patients with compensated cirrhosis. Functional analysis of this cluster indicated that the miRNAs identified were involved in the transforming growth factor (TGF)-β, forkhead box O (FoxO), or mitogen-activated protein kinase (MAPK) signalling pathways and were associated with liver disease (Table S4). The second cluster contained 6 miRNAs showing a constant increase in miRNA serum content with disease progression ('progressive increase cluster'), and showed an association with the phosphatidylinositol-3-kinase (PI3K)-Akt, Wnt, and Hippo pathways (Fig. 1D and Table S4).

By contrast, 2 clusters of miRNAs were identified with a serum content decreasing with disease progression. The 'scaled decreased cluster' comprised 4 miRNAs that decreased with disease progression but showed no changes between patients

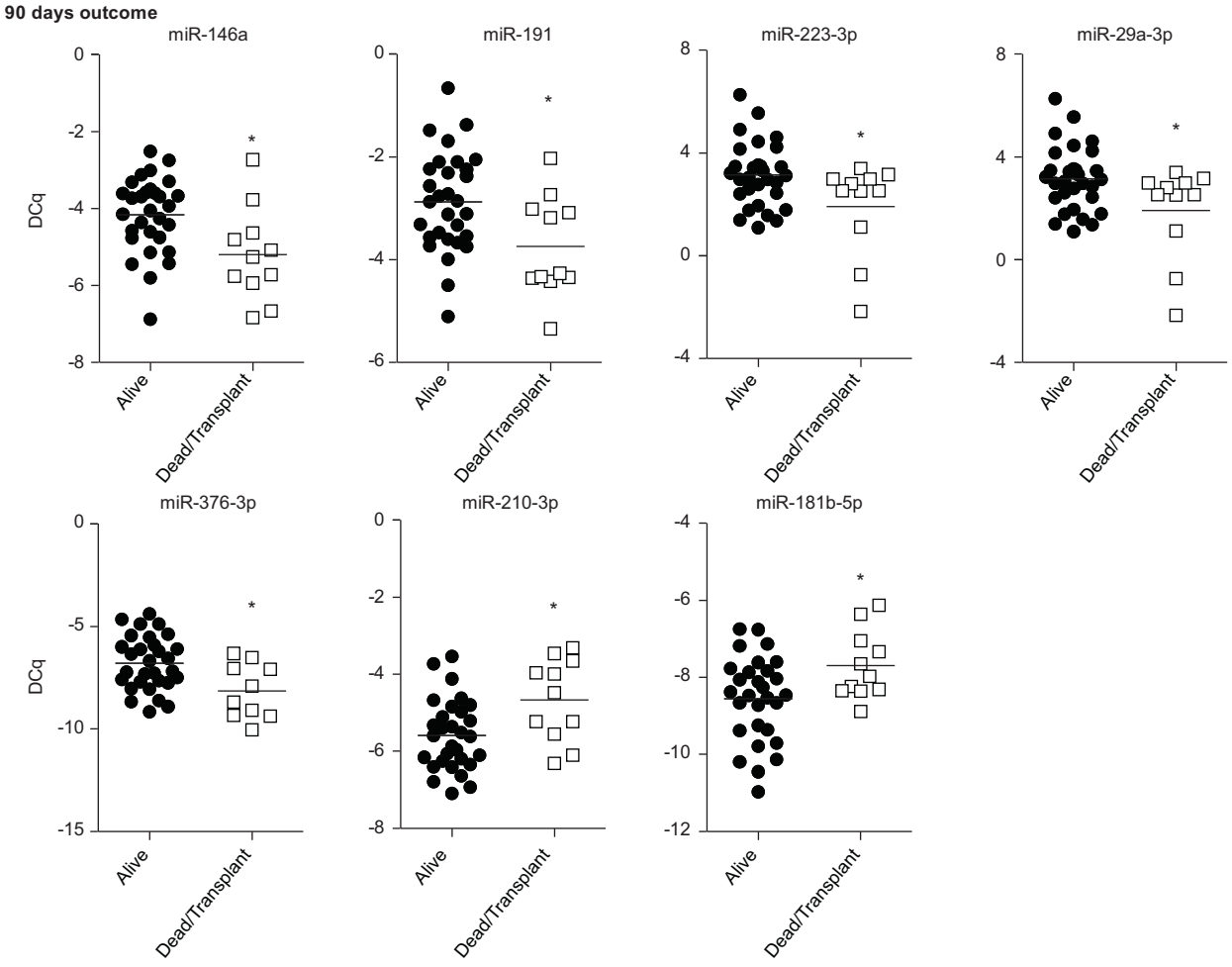

Fig. 4. Circulating miRNAs with significant changes in expression in patients with poor outcomes. Circulating miRNAs expression was evaluated in patients (n = 45) with different outcomes 90 days after hospital admission. Comparison between groups were made with t test, *p >0.05.

with decompensated cirrhosis and ACLF. The functional analysis of this cluster showed an association with liver damage and liver inflammation (Fig. 1D and Table S4). Finally, the 'progressive decrease cluster' contained 7 miRNAs potentially involved with renal dysfunction, endocytosis, and the ErbB signalling pathway (Fig. 1D and Table S4).

Circulating miRNAs differentially expressed with disease progression

Next, we sought to identify miRNAs differentially expressed with cirrhosis progression. Of the 118 miRNAs, 51 miRNAs were significantly differentially expressed among the 4 groups (ANOVA p ≤0.05) (Fig. S1A), representing the miRNAs most dysregulated during disease progression.

To depict the distribution of the 118 miRNAs along cirrhosis progression, we represented them in a XY plot according to their fold change *vs.* healthy individuals (Fig. S1B). The XY plot presents the fold change of every miRNA (Y axis) in each group of patients compared with healthy values (X axis). As shown in the XY plot, miRNA expression in patients with ACLF diverged most from the middle axis, showing the highest fold change from levels in healthy individuals. ACLF values were closer to patients with decompensated cirrhosis (blue dots) and more distant from patients with compensated cirrhosis (yellow dots).

Furthermore, we investigated whether the number of dysregulated miRNAs increased with disease progression. In total, 21 dysregulated miRNAs were found in patients with compensated cirrhosis *vs.* healthy individuals, whereas there were 37 dysregulated miRNAs in patients with decompensated cirrhosis *vs.* healthy individuals; finally, 47 dysregulated miRNAs were found in patients with ACLF compared with healthy individuals (Fig. S2). These data confirmed that dysregulation of circulating miRNAs increased with the progression of chronic liver disease.

By contrast, comparison of the different stages of liver cirrhosis revealed 20 differentially expressed miRNAs in patients with compensated cirrhosis compared with decompensated cirrhosis, and 30 dysregulated miRNAs compared with patients with ACLF. Interestingly, only 5 miRNAs were differentially expressed in patients with ACLF *vs.* decompensated cirrhosis (let-7c, miR-324-3p, miR-99, miR-26a, and miR-192). Altogether, these data suggest that, whereas miRNAs in patients with

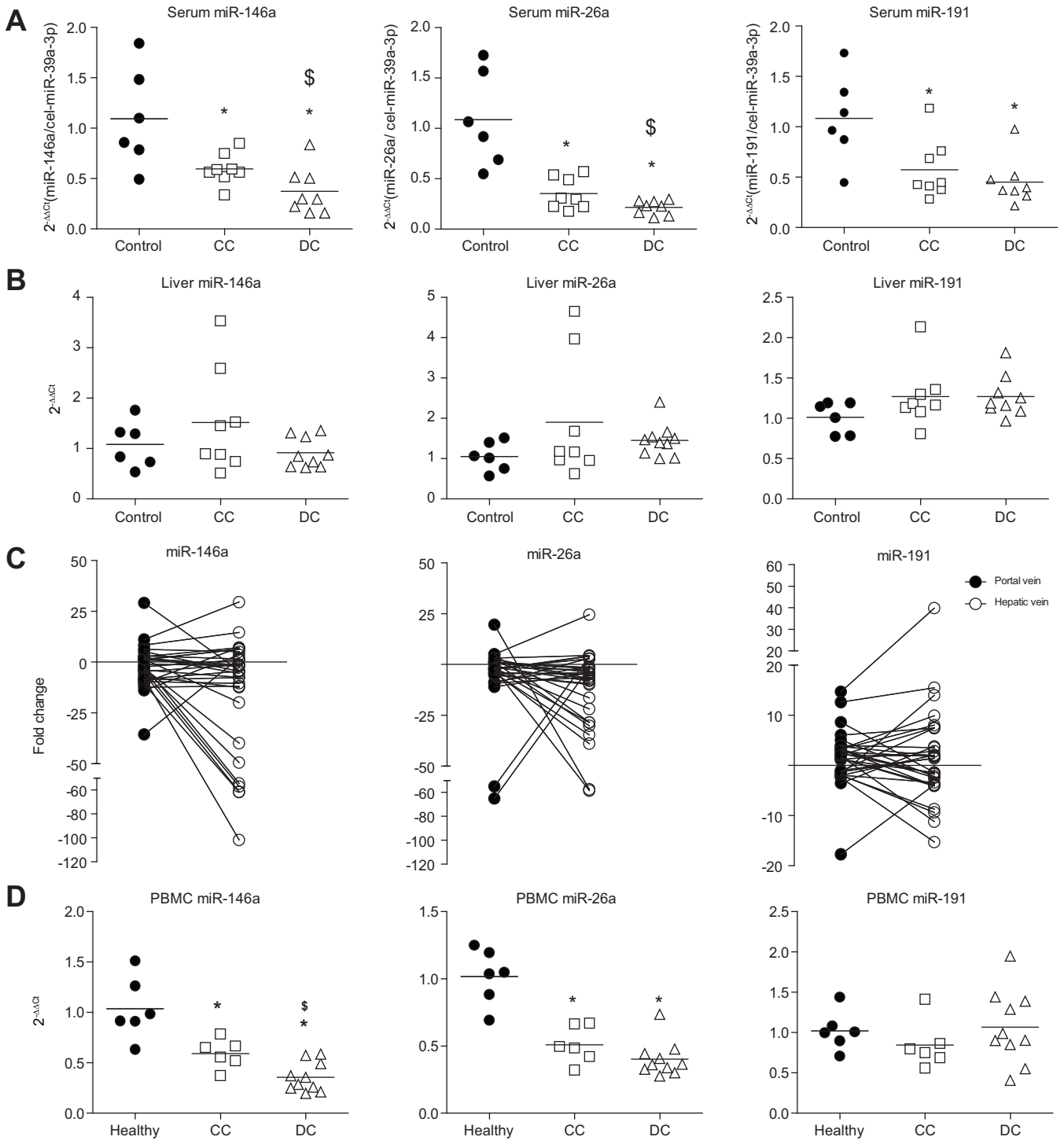

Fig. 5. Validation of miR-146a, miR-26a, and miR-191. (A) miRNAs were validated in serum samples of control individuals (n = 6), patients with compensated cirrhosis (CC; n = 8), and patients with decompensated cirrhosis (DC; n = 8). $^{*}p$ <0.05 *vs.* control individuals; $^{\$}p$ <0.05 *vs.* CC. (B) miRNAs were validated in liver tissue from CC (n = 8), DC (n = 9), and control liver tissue (n = 6). (C) Expression of miRNAs was validated in 33 CC in paired samples from the portal and hepatic veins before TIPS procedure. (D) miRNAs were validated in PBMCs of CC (n = 6), DC (n = 10), and healthy individuals (n = 6). Comparison between groups were made with *t* test, $^{*}p$ <0.05 *vs.* healthy individuals, $^{\$}p$ <0.05 *vs.* CC. PBMC, peripheral blood mononuclear cell; TIPS, transjugular intrahepatic portosystemic shunt.

decompensated cirrhosis or ACLF do not show major changes, decompensation determines an important change in circulating miRNA patterns with cirrhosis progression.

Targeted analysis of 43 dysregulated miRNAs

To validate the results obtained with the OpenArray technology, a smaller analysis was performed evaluating 43 miRNAs in the same set of samples. This included the initial 60 samples with

correct amplification in 14 healthy individuals, 15 patients with compensated cirrhosis, 14 patients with decompensated cirrhosis, and 13 patients with ACLF. These 43 miRNAs were selected out of the 51 miRNAs differentially expressed among the 4 groups. In agreement with the results of the high-throughput analysis, 10 miRNAs (miR-146a-5p, miR-26a-5p, miR-191-5p, miR-29a-3p, miR-133a-3p, miR-376-3p, miR-181b-5p, miR-210-3p, miR-324-3p, and miR-485-3p) had a significant ANOVA *p* value (Fig. 2). Out of the 10 significantly dysregulated miRNAs, 7 were downregulated with disease progression. Importantly, no changes in miRNA expression were detected among patients with ACLF and decompensated cirrhosis or between patients with compensated cirrhosis and healthy controls, thus supporting the previous results that major circulating miRNAs changes occur during decompensation of liver disease. miR-146a-5p, miR-26a-5p, and miR-191 were consistently dysregulated among patients with ACLF, compensated cirrhosis, or healthy individuals (fold change of ALCF *vs.* compensated cirrhosis: -2.8 ± 3.3, -2.8 ± 3.2, and -2.08 ± 2.13, respectively; fold change of ACLF *vs.* healthy patients: -2.5 ± 3.2, -3.5 ± 2.7, and -3.7 ± 2.6, respectively), but did not show any differences from patients with decompensated cirrhosis.

We further evaluated whether a group of miRNAs was able to determine the probability of a patient having ACLF. Using the data from the OpenArray analysis as well as the Exiqon algorithm described in the Materials & methods, joint expression of miR-21, miR-26a, and miR-376a identified a patient with ACLF with high sensitivity and specificity. In both data sets, this group of 3 miRNAs was mainly dysregulated in patients with ACLF with an AUC of 0.938 in the OpenArray data set and of 0.848 in the Exiqon data set (Fig. S3).

Correlation of miRNA levels with clinical data and disease severity scores

Given that the main changes in circulating miRNAs were associated with cirrhosis decompensation and disease severity, we evaluated changes in miRNAs in patients with renal failure, liver failure, bacterial infection, or encephalopathy. The miRNAs differentially expressed in each of the conditions are shown in Fig. 3. To determine whether the downregulation was caused by bacterial infection or chronic liver damage, the miR-223-3p, miR-146a, and miR-191 levels were analysed in serum samples from patients with sepsis. miR-146a and miR-223-3p were downregulated in patients with bacterial infection compared to healthy individuals (Fig. S4), suggesting that bacterial infection is the main event driving the dysregulation of these miRNAs. By contrast, miR-191 levels were unchanged in patients with sepsis (Fig. S4), suggesting that the main driver of miR-191 downregulation is the underlying chronic liver injury, which is further affected by bacterial infection (Fig. 4).

We also observed that 7 miRNAs, including miR-146a and miR-191, were significantly changed in patients who died within 90 days after admission (Fig. 4). Overall, these results highlight the association of circulating miRNAs with liver disease severity, clinical decompensation, and patient outcomes.

Next, we assessed whether the expression of the 39 miRNAs correlated with scores of disease progression, such as model for end-stage liver disease (MELD) and Child-Pugh scores, or with clinical parameters. In total, 20 miRNAs were found that correlated with one or more clinical parameters (Fig. S5). Moreover, a group of 6 miRNAs (miR-146a-5p, miR-26a-5p, miR-191-5p, miR-29a-3p, miR-133a-3p, and miR-324-3p) correlated negatively with MELD and Child-Pugh scores and also with bilirubin and the International Normalised Ratio (INR). By contrast, there was a positive correlation with serum albumin, suggesting that a decrease in miRNA levels is associated with a decrease in serum albumin, which also reflects impaired liver function. Altogether these data support the idea that a decrease in these 6 miRNAs is associated with a deterioration in liver function and an increase in the severity of liver disease. Interestingly, no miRNAs correlated with aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH), indicating that the levels of circulating miRNAs are not associated with parenchymal damage.

Assessment of the source of circulating miRNAs

miRNAs are expressed in all cell types of the body and can be released into circulation in response to a variety of stimuli and situations. Therefore, we assessed the possible origin of 3 miRNAs that were significantly downregulated with the progression of the disease and associated with clinical scores: miR-146a, miR-26a, and miR-191. These 3 miRNAs were evaluated in the liver, hepatoportal circulation, and peripheral inflammatory cells. First, it was determined that the circulating levels of these miRNAs in the validation cohort were the same as in the study population. miR-146a, miR-26a, and miR-191 were confirmed to be downregulated with the progression of the disease, with a decreased level in the serum of patients with compensated cirrhosis and being lower in the serum of patients with decompensated cirrhosis (Fig. 5A).

Next, we assessed the liver expression of miR-146a, miR-26a and miR-191(Fig. 5B). The expression of these 3 miRNAs in liver tissue did not differ between patients with compensated cirrhosis or decompensated cirrhosis compared with control liver tissue.

Next, we evaluated the level of these miRNAs in plasma samples obtained from the hepatic and the portal veins of 32 patients before undergoing transjugular intrahepatic portosystemic shunt (TIPS). All the patients included had decompensated cirrhosis (Table S1). Evaluation of miR-146a, miR-26a, and miR-191 in paired blood samples from hepatic and portal veins showed changes in the levels of miRNA expression, with some patients presenting an increase in miRNA levels and others a decrease (Fig. 5C). Considering all patients together, there was no conclusive trend in the changes of plasma levels observed in the hepatic and portal veins. We then stratified patients into 2 categories: patients with either a decrease or an increase in miRNA levels between hepatic and portal veins. No differences were detected regarding the MELD score (miR-146a, $p = 0.36$; miR-26a, $p = 0.72$; miR-191, $p = 0.71$) the Child-Pugh score (miR-146a, $p = 0.60$; miR-26a, $p = 0.51$; miR-191, $p = 0.90$) or serum transaminases, between the two groups of patient.

These data suggest that the dysregulation of these miRNAs in patients with cirrhosis is the result of changes in expression in other tissues or organs. Given the importance of inflammation in patients with hepatic cirrhosis, especially those with decompensated cirrhosis, we next evaluated the expression of miR-146a, miR-26a, and miR-191 in peripheral blood mononuclear cells (PBMCs) from patients with compensated cirrhosis or decompensated cirrhosis, and healthy individuals. The

expression of miR-146a and miR-26a was significantly lower in PBMCs from patients with compensated and decompensated cirrhosis compared with healthy individuals (Fig. 5D). Interestingly, miR-146a was significantly downregulated in patients with decompensated cirrhosis compared with those with compensated cirrhosis.

Altogether, these results suggest that the source of dysregulated circulating miRNAs is not the diseased liver, but rather extrahepatic tissues and organs, the dysfunction and failure of which defines ACLF. Moreover, changes in the expression of miRNAs in circulating inflammatory cells might be partially responsible for the differences observed in circulating miRNAs, such as miR-146a.

Discussion

This study is a comprehensive stepwise analysis of circulating miRNAs in the serum of liver from patients with cirrhosis and ACLF. Using high-throughput qPCR technology, 754 circulating miRNAs were evaluated and several miRNAs were identified as being associated with disease progression and being related to relevant functions in hepatic disease. Moreover, miRNAs were identified that correlated with survival and organ dysfunction. Finally, the source of some miRNAs was determined, indicating that dysregulation of miR-26a and miR-146a might derive from altered PBMC expression. In addition, the results of this study are an important resource of miRNA data that will be useful for future research in the field of miRNAs in patients with liver cirrhosis and ACLF.

Previous studies have evaluated the presence and relevance of circulating miRNAs in liver disease, such as cirrhosis[24,25,28] and hepatocellular carcinoma.[21,29–31] Recently, Cisilotto *et al.*[32] evaluated circulating miRNAs in ACLF and identified miR-25-3p and miR-223-3p as being significantly dysregulated; however, these results were not confirmed in our study. Of note, we focussed our study on the serum profile of miRNA with the progression of cirrhosis and patients with ACLF, whereas Cisilotto *et al.* investigated patients with decompensated cirrhosis with or without ACLF. Thus, we consider these studies to be complementary.

Furthermore, we identified a set of 3 miRNAs (miR-21, miR-26a, and miR-376a) with the power to predict whether a patient has ACLF. Further studies should evaluate this set of miRNAs in patients with decompensated cirrhosis to evaluate their potential to predict ACLF even before its appearance.

The high-throughput approach allowed the identification of an important number of miRNAs potentially dysregulated during cirrhosis progression. Among those identified in the OpenArray and Exiqon studies, only some were validated by focused alternative methods, and future studies could use these data as a resource for identifying new biomarkers. Importantly, the profile of dysregulated miRNAs in patients with decompensated cirrhosis or ACLF patients was similar, and only 5 miRNAs were differentially expressed. By contrast, both patients with decompensated cirrhosis or ACLF showed important differences compared with healthy individuals or those with compensated cirrhosis. These results suggest that cirrhosis decompensation has an important impact regarding the circulating miRNA serum profile, which is not further altered in ACLF.

We reassessed 43 selected miRNAs by qPCR, including 26 miRNAs that were significant in the first screening and 17 extra miRNAs selected based on tendency and/or previous studies.[24,25] This targeted analysis allowed evaluation of their correlation with clinical variables and disease progression. Some of these circulating miRNAs showed a correlation with clinical parameters, liver function scores, cirrhosis decompensation events, and poor outcomes. Some of the miRNAs identified as being correlated with organ failure have been previously described. For instance, miR-210 and miR-145 were described to be related to renal damage,[33–35] whereas miR-122 and miR-223-3p were observed as being dysregulated in studies of brain damage.[36,37] In addition, miR-223-3p and miR-146a were downregulated in patients with cirrhosis and bacterial infection, and also in a cohort of patients with sepsis but without liver disease, which suggests that bacterial infection drives the downregulation of these miRNAs. These results are in agreement with previous data describing the downregulation of these miRNAs in patients with sepsis.[38,39] Larger cohorts of patients are needed to evaluate these miRNAs as biomarkers, and mechanistic studies should be performed.

Variations in circulating miRNAs might reflect changes in miRNA expression in tissues or organs related to the disease. In a previous study, miR-571 and miR-652 were found to be dysregulated in the serum of patients with cirrhosis, and were also altered in liver and cell compartments involved in the pathogenesis of liver cirrhosis.[24] For this reason, we assessed whether dysregulated miRNAs in peripheral blood reflected changes in miRNA expression in liver tissue. First, miR-146a, miR-26a, and miR-191 were evaluated in paired samples of portal and hepatic veins to assess whether the liver was the origin of the dysregulated miRNAs. Although there were changes between the portal and hepatic veins, these alterations were not significant and did not have a correlation with any clinical parameter. Some limitations regarding portal flow circulation should be considered, for instance, patients with cirrhosis could have a reverse portal flow, which could have impacted our results, although 80% of patients with TIPS have correct hepatoportal flow.[40] Further studies with larger cohorts of patients could determine whether these changes have clinical significance.

In addition, we analyzed the expression of miR-146a, miR-26a, and miR-191 in liver tissue; however, the level of expression did not change and, therefore, could not explain the changes observed in the circulation. Inflammatory cells can also be a source of changes in circulating miRNAs and, thus, we also evaluated miRNA expression in PBMCs of patients with compensated and decompensated cirrhosis. Interestingly, there was a significant reduction in the expression of miR-26a and miR-146a in PBMCs from patients with cirrhosis compared with healthy individuals. Remarkably, miR-146a was further decreased in patients with decompensated cirrhosis compared with those with compensated cirrhosis.

MiR-146a has been widely studied in inflammation and has been demonstrated to have a role in haematopoiesis.[41,42] Previous studies also reported the downregulation of miR-146a in hepatocellular carcinoma[43,44] and ischaemia/reperfusion damage.[45] Interestingly, a recent study described the downregulation of miR-146a in the serum, liver tissue, and PBMCs of patients with liver cirrhosis,[46] supporting our results. Our results are also in agreement with a previous study in which miR-155 levels in inflammatory cells were shown to be altered in response to liver injury and to regulate inflammatory cell recruitment and liver injury.[47] Taken together, these results suggest that dysregulation of miRNAs in circulating inflammatory cells is responsible for the changes observed in circulatory miRNAs in chronic liver disease and ACLF.

Overall, the current study shows that advanced chronic liver diseases are associated with important changes in circulating miRNAs, particularly after cirrhosis decompensation. However, these findings should be confirmed in a larger cohort of patients, especially patients with ACLF, given that ACLF is a very heterogenous clinical condition.

In this study, we showed that there is important dysregulation of circulating miRNAs with the progression of chronic liver disease. Moreover, we focused on miR-146a, miR-26a, and miR-191, which are significantly downregulated during the progression of liver cirrhosis and show a correlation with clinical parameters. Furthermore, miR-146a dysregulation correlates with patient outcomes. Further analysis elucidated that these miRNAs are not specifically dysregulated in liver tissues. Conversely, miR-146a and miR-26a are significantly downregulated in the inflammatory cell compartment, suggesting an important role for inflammatory cells in the dysregulation of circulating miRNAs in ACLF.

Abbreviations

ACLF, acute-on-chronic liver failure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CXCL10, C-X-C motif chemokine ligand 10; EF CLIF, European Foundation for the Study of Chronic Liver Failure; FoxO, forkhead box O; INR, International Normalised Ratio; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MELD, model for end-stage liver disease; NASH, non-alcoholic steatohepatitis; PBMCs, peripheral blood mononuclear cells; PCA, principal component analysis; qPCR, quantitative PCR; TGF, transforming growth factor; TIPS, transjugular intrahepatic portosystemic shunt.

Financial support

Supported by grants from Fondo de Investigación Sanitaria Carlos III (FIS), co-financed by Fondo Europeo de Desarrollo Regional (FEDER), Unión Europea, 'Una manera de hacer Europa' (FIS PI17/00673, PI20/00765, PI16/00043, FIS PI12/01265 to P.S-B., P.G., and J.C.), from the NIH National Institute on Alcohol Abuse and Alcoholism grant 1U01AA026972-01 to P.S-B. and from the European Foundation for Alcohol Research (ERAB) Grant EA1653. P.S-B is funded by Instituto de Salud Carlos III, Miguel Servet (CP11/00071), and co-financed by Fondo Europeo de Desarrollo Europeo (FEDER), Unión Europea, 'Una manera de hacer Europa'. P.G. is funded by Agencia de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) 2014 SGR 708, Centro de Investigación en Red Enfermedades Hepáticas y Digestivas (CIBERehd), and Institució Catalana de Recerca i Estudis Avançats(ICREA), EU .H2020 Research & Innovation program, No. 731875 (LIVERHOPE). I.G. is funded by Instituto de Salud Carlos III, Rio Hortega grant. E.P. is funded by a PhD4MD grant. J.T. received funding from the Deutsche Forschungsgemeinschaft (SFB TRR57 P18), European Union's Horizon 2020 research and innovation program's GALAXY study (No. 668031), LIVERHOPE (No. 731875) and MICROB-PREDICT(No. 825694) and the Cellex Foundation (PREDICT).

Conflicts of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

D.B. contributed to the design of the study, performed the experiments, collected, and analysed the data, interpreted the data and wrote the manuscript. E.P. enrolled patients, collected human samples, performed analysis and interpretation of human data, and critically reviewed the manuscript. I.G., R.S., C.J., P.C., and S.F. enrolled patients and collected human samples. J.J.L. and J.S. helped with the analysis and interpretation of the data. M.C., M.V.N., E.P., J.C., and P.G. helped with data interpretation and critically reviewed the final version of the manuscript. J.T. and P.S-B. conceived the study design and were involved in the analysis, interpretation, and drafting of the final version of the manuscript.

Acknowledgements

The study was supported by the European Foundation for the Study of Chronic Liver Failure (EF-Clif), a non-profit private organisation. EF-CLIF receives unrestricted donations from Cellex Foundation and Grifols and, furthermore, is a partner in, or contributor to, several EU Horizon 2020 program projects. The funders had no influence on study design, data collection and analysis, decision to publish, or preparation of the manuscript. We are indebted to the HCB-IDIBAPS Biobanc, integrated in the Spanish National Biobanks Network, for the biological human samples and data procurement.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material. Further questions can be addressed to the corresponding authors.

Supplementary data

Supplementary data to this article can be found at https://doi.org/10.1016/j.jhepr.2021.100233.

References

Author names in bold designate shared co-first authorship

[1] Hernaez R, Solà E, Moreau R, Ginès P. Acute-on-chronic liver failure: an update. Gut 2017;66:541–553.

[2] Moreau R, Jalan R, Gines P, Pavesi M, Angeli P, Cordoba J, et al. Acute-on-chronic liver failure is a distinct syndrome that develops in patients with acute decompensation of cirrhosis. Gastroenterology 2013;144:1426–1437.

[3] Arroyo V, Moreau R, Jalan R, Ginès P, EASL-CLIF Consortium CANONIC Study. Acute-on-chronic liver failure: a new syndrome that will re-classify cirrhosis. J Hepatol 2015;62:S131–S143.

[4] Solé C, Solà E, Morales-Ruiz M, Fernàndez G, Huelin P, Graupera I, et al. Characterization of inflammatory response in acute-on-chronic liver failure and relationship with prognosis. Sci Rep 2016;6:32341.

[5] Gustot T, Fernandez J, Garcia E, Morando F, Caraceni P, Alessandria C, et al. Clinical course of acute-on-chronic liver failure syndrome and effects on prognosis. Hepatology 2015;62:243–252.

[6] **Ariza X**, **Graupera I**, Coll M, Solà E, Barreto R, García E, et al. Neutrophil gelatinase-associated lipocalin is a biomarker of acute-on-chronic liver failure and prognosis in cirrhosis. J Hepatol 2016;65:57–65.

[7] **Clària J**, **Stauber RE**, Coenraad MJ, Moreau R, Jalan R, Pavesi M, et al. Systemic inflammation in decompensated cirrhosis: characterization and role in acute-on-chronic liver failure. Hepatology 2016;64:1249–1264.

[8] Lehmann JM, Claus K, Jansen C, Pohlmann A, Schierwagen R, Meyer C, et al. Circulating CXCL10 in cirrhotic portal hypertension might reflect systemic inflammation and predict ACLF and mortality. Liver Int 2018;38:875–884.

[9] Grønbæk H, Rødgaard-Hansen S, Aagaard NK, Arroyo V, Moestrup SK, Garcia E, et al. Macrophage activation markers predict mortality in patients with liver cirrhosis without or with acute-on-chronic liver failure (ACLF). J Hepatol 2016;64:813–822.

[10] Bernsmeier C, Triantafyllou E, Brenig R, Lebosse FJ, Singanayagam A, Patel VC, et al. $CD14^{+}$ $CD15^{-}$ $HLA\text{-}DR^{-}$ myeloid-derived suppressor cells impair antimicrobial responses in patients with acute-on-chronic liver failure. Gut 2018;67:1155–1167.

[11] Trebicka J, Amoros A, Pitarch C, Titos E, Alcaraz-Quiles J, Schierwagen R, et al. Addressing profiles of systemic inflammation across the different clinical phenotypes of acutely decompensated cirrhosis. Front Immunol 2019;10:476.

[12] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–297.
[13] Blaya D, Coll M, Rodrigo-Torres D, Vila-Casadesús M, Altamirano J, Llopis M, et al. Integrative microRNA profiling in alcoholic hepatitis reveals a role for microRNA-182 in liver injury and inflammation. Gut 2016;65:1535–1545.
[14] Szabo G, Bala S. MicroRNAs in liver disease. Nat Rev Gastroenterol Hepatol 2013;10:542–552.
[15] Otsuka M, Kishikawa T, Yoshikawa T, Yamagami M, Ohno M, Takata A, et al. MicroRNAs and liver disease. J Hum Genet 2017;62:75–80.
[16] **Schueller F**, **Roy S**, Vucur M, Trautwein C, Luedde T, Roderburg C, et al. The role of miRNAs in the pathophysiology of liver diseases and toxicity. Int J Mol Sci 2018;19:261.
[17] **Gilad S**, **Meiri E**, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N, et al. Serum microRNAs are promising novel biomarkers. PLoS ONE 2008;3:e3148.
[18] **Lutz P**, **Mhaimid M**, Pohlmann A, Lehmann J, Jansen C, Schierwagen R, et al. MicroRNA-155 is upregulated in ascites in patients with spontaneous bacterial peritonitis. Sci Rep 2017;7:40556.
[19] **Mitchell PS**, **Parkin RK**, **Kroh EM**, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci 2008;105:10513–10518.
[20] Loosen SH, Lurje G, Wiltberger G, Vucur M, Koch A, Kather JN, et al. Serum levels of miR-29, miR-122, miR-155 and miR-192 are elevated in patients with cholangiocarcinoma. PLoS ONE 2019;14:e0210944.
[21] Huang Y-H, Liang K-H, Chien R-N, Hu T-H, Lin K-H, Hsu C-W, et al. A circulating microRNA signature capable of assessing the risk of hepatocellular carcinoma in cirrhotic patients. Sci Rep 2017;7:523.
[22] **Liu Y**, **Meyer C**, Xu C, Weng H, Hellerbrand C, Ten Dijke P, et al. Animal models of chronic liver diseases. Am J Physiol Gastrointest Liver Physiol 2012;304:G449–G468.
[23] López-Riera M, Conde I, Quintas G, Pedrola L, Zaragoza Á, Perez-Rojas J, et al. Non-invasive prediction of NAFLD severity: a comprehensive, independent validation of previously postulated serum microRNA biomarkers. Sci Rep 2018;8:10606.
[24] Roderburg C, Mollnow T, Bongaerts B, Elfimova N, Vargas Cardenas D, Berger K, et al. Micro-RNA profiling in human serum reveals compartment-specific roles of miR-571 and miR-652 in liver cirrhosis. PLoS ONE 2012;7:e32999.
[25] **Chen Y-J**, **Zhu J-M**, Wu H, Fan J, Zhou J, Hu J, et al. Circulating microRNAs as a fingerprint for liver cirrhosis. PLoS ONE 2013;8:e66577.
[26] Fernández-Ramos D, Fernández-Tussy P, Lopitz-Otsoa F, Gutiérrez-de-Juan V, Navasa N, Barbier-Torres L, et al. MiR-873-5p acts as an epigenetic regulator in early stages of liver fibrosis and cirrhosis. Cell Death Dis 2018;9:958.
[27] Ernst J, Bar-Joseph Z. STEM: a tool for the analysis of short time series gene expression data. BMC Bioinformatics 2006;7:191.
[28] **Tan Y**, **Pan T**, **Ye Y**, Ge G, Chen L, Wen D, et al. Serum microRNAs as potential biomarkers of primary biliary cirrhosis. PLoS ONE 2014;9:e111424.
[29] Fornari F, Ferracin M, Trerè D, Milazzo M, Marinelli S, Galassi M, et al. Circulating microRNAs, miR-939, miR-595, miR-519d and miR-494, Identify Cirrhotic Patients with HCC. PLoS ONE 2015;10:e0141448.
[30] Weis A, Marquart L, Calvopina D, Genz B, Ramm G, Skoien R. Serum microRNAs as biomarkers in hepatitis C: preliminary evidence of a microRNA panel for the diagnosis of hepatocellular carcinoma. Int J Mol Sci 2019;20:864.
[31] Amaral AE do, Rode MP, Cisilotto J, Silva TE da, Fischer J, Matiollo C, et al. MicroRNA profiles in serum samples from patients with stable cirrhosis and miRNA-21 as a predictor of transplant-free survival. Pharmacol Res 2018;134:179–192.
[32] Cisilotto J, do Amaral AE, Rosolen D, Rode MP, Silva AH, Winter E, et al. MicroRNA profiles in serum samples from acute-on-chronic liver failure patients and miR-25-3p as a potential biomarker for survival prediction. Sci Rep 2020;10:1–11.
[33] Kito N, Endo K, Ikesue M, Weng H, Iwai N. miRNA profiles of tubular cells: diagnosis of kidney injury. Biomed Res Int 2015;2015:1–9.
[34] Watany MM, Hagag RY, Okda HI. Circulating miR-21, miR-210 and miR-146a as potential biomarkers to differentiate acute tubular necrosis from hepatorenal syndrome in patients with liver cirrhosis: a pilot study. Clin Chem Lab Med 2018;56:739–747.
[35] Lorenzen JM, Kielstein JT, Hafer C, Gupta SK, Kümpers P, Faulhaber-Walter R, et al. Circulating miR-210 predicts survival in critically ill patients with acute kidney injury. Clin J Am Soc Nephrol 2011;6:1540–1546.
[36] Morquette B, Juźwik CA, Drake SS, Charabati M, Zhang Y, Lécuyer M-A, et al. MicroRNA-223 protects neurons from degeneration in experimental autoimmune encephalomyelitis. Brain 2019;142:2979–2995.
[37] Li D-B, Liu J-L, Wang W, Luo X-M, Zhou X, Li J-P, et al. Plasma exosomal miRNA-122-5p and miR-300-3p as potential markers for transient ischaemic attack in rats. Front Aging Neurosci 2018;10:24.
[38] **Wang L**, **Wang HC**, Chen C, Zeng J, Wang Q, Zheng L, et al. Differential expression of plasma miR-146a in sepsis patients compared with non-sepsis-SIRS patients. Exp Ther Med 2013;5:1101–1104.
[39] **Wang H**, **Zhang P**, Chen W, Feng D, Jia Y, Xie L. Serum microRNA signatures identified by Solexa sequencing predict sepsis patients' mortality: a prospective observational study. PLoS ONE 2012;7:e38885.
[40] Iranpour P, Lall C, Houshyar R, Helmy M, Yang A, Choi J-I, et al. Altered Doppler flow patterns in cirrhosis patients: an overview. Ultrasonography 2016;35:3–12.
[41] Zhao JL, Starczynowski DT. Role of microRNA-146a in normal and malignant hematopoietic stem cell function. Front Genet 2014;5:219.
[42] Kroesen B-J, Teteloshvili N, Smigielska-Czepiel K, Brouwer E, Boots AMH, van den Berg A, et al. Immuno-miRs: critical regulators of T-cell development, function and ageing. Immunology 2015;144:1–10.
[43] **Zhang Z**, **Zhang Y**, **Sun X-X**, Ma X, Chen Z-N. microRNA-146a inhibits cancer metastasis by downregulating VEGF through dual pathways in hepatocellular carcinoma. Mol Cancer 2015;14:5.
[44] Rong M, He R, Dang Y, Chen G. Expression and clinicopathological significance of miR-146a in hepatocellular carcinoma tissues. Ups J Med Sci 2014;119:19–24.
[45] Chen Q, Kong L, Xu X, Geng Q, Tang W, Jiang W. Down-regulation of microrna-146a in the early stage of liver ischemia-reperfusion injury. Transpl Proc 2013;45:492–496.
[46] Yang Z, Peng Y, Yang S. MicroRNA-146a regulates the transformation from liver fibrosis to cirrhosis in patients with hepatitis B via interleukin-6. Exp Ther Med 2019;17:4670–4676.
[47] Blaya D, Aguilar-Bravo B, Hao F, Casacuberta-Serra S, Coll M, Perea L, et al. Expression of microRNA-155 in inflammatory cells modulates liver injury. Hepatology 2018;68:691–706.