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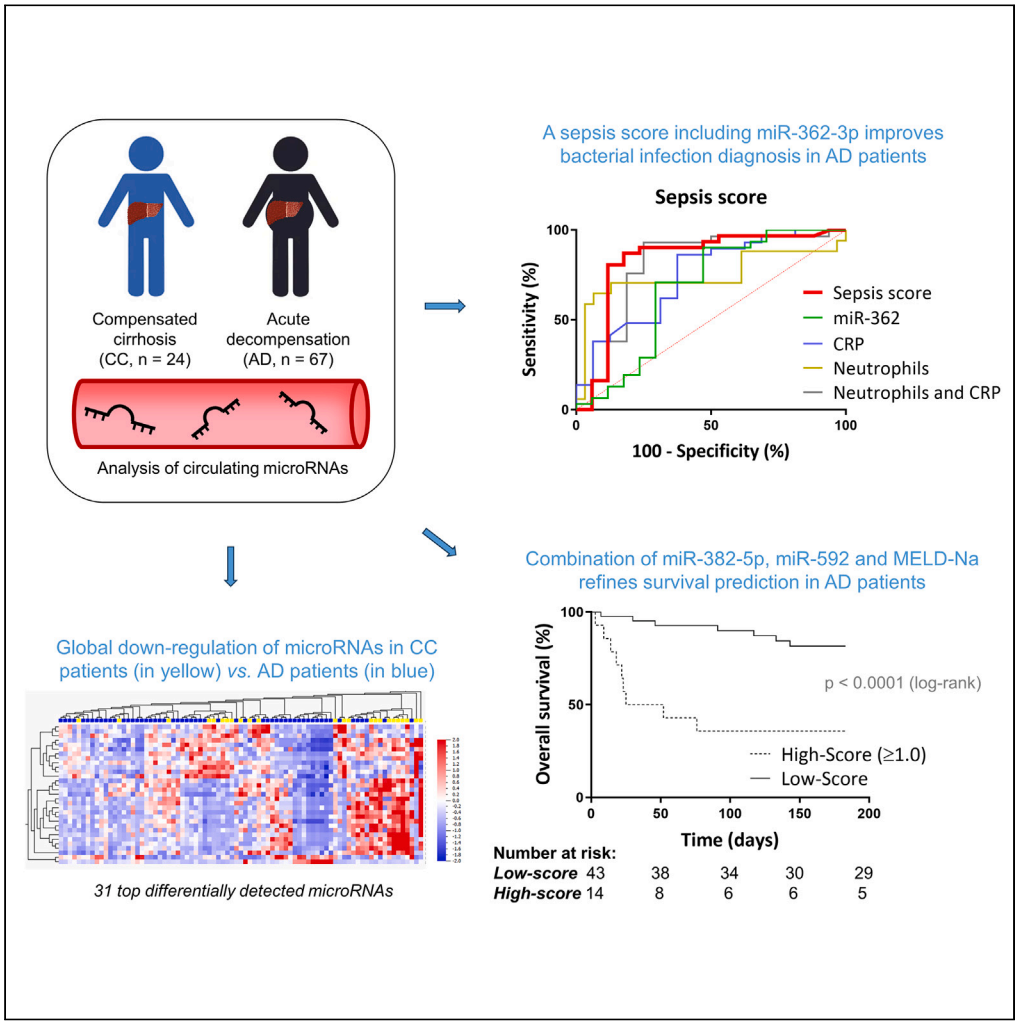

Yasmina Chouik, Fanny Lebossé, Marie-Laure Plissonnier, ..., Pierre-Emmanuel Rautou, Fabien Zoulim, Massimo Levrero

yasmina.chouik@inserm.fr (Y.C.)
massimo.levrero@inserm.fr (M.L.)

Highlights

Plasma miRNAs are down-regulated in AD of cirrhosis

miR-362-3p improves bacterial infection diagnosis in AD patients

miR-382-5p and miR-592 refine MELD-Na survival prediction in AD patients

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Circulating microRNAs improve bacterial infection diagnosis and overall survival prediction in acute decompensation of liver cirrhosis

Yasmina Chouik,[1,2,3,10,*] Fanny Lebossé,[1,2,3] Marie-Laure Plissonnier,[1] Jean-Christophe Lega,[4] Pierre Pradat,[5] Teresa Antonini,[2] Miroslava Subic,[3] Kerstin Hartig-Lavie,[2] Domitille Erard,[2] François Villeret,[2,3] Céline Guichon,[6] Audrey Payancé,[7] Sylvie Radenne,[2] Pierre-Emmanuel Rautou,[7,8] Fabien Zoulim,[1,2,3] and Massimo Levrero[1,2,3,9,*]

SUMMARY

Bacterial infections are the most frequent precipitating event in patients with acute decompensation of cirrhosis (AD) and are associated with high mortality. Early diagnosis is challenging due to cirrhosis-related systemic inflammation. Here we investigated the potential of circulating microRNAs to diagnose bacterial infections and predict survival in cirrhotic patients with AD. High throughput profiling of circulating microRNAs was performed using the Nanostring technology in 57 AD patients and 24 patients with compensated cirrhosis (CC). Circulating miRs profiling showed that: (a) miRs differentially detected in AD vs. CC were mostly down-regulated; (b) a composite score including absolute neutrophil count, C reactive protein and miR-362-3p could diagnose bacterial infection with an excellent performance (AUC of 0.825 [95% CI = 0.671–0.980; p < 0.001]); (c) a composite score including miR-382-5p, miR-592 and MELD-Na improved 6-month survival prediction. Circulating miRs are strongly dysregulated in patients with AD and may help to improve bacterial infection diagnosis and survival prediction.

INTRODUCTION

Acute decompensation (AD) of liver cirrhosis is defined by the rapid development of overt ascites, hepatic encephalopathy, variceal bleeding, or any combination of them.[1] The occurrence of AD is associated with a high short-term mortality, particularly in patients with bacterial or fungal infection.[2,3] Indeed, bacterial infections are identified in 38%–50% of the patients admitted for decompensation of cirrhosis[4,5] with 1-month mortality reaching 30%.[2,5,6] The multi-factorial immune dysfunction associated with cirrhosis seems to play a key role in the increased risk of both bacterial infections and mortality in AD.[7] Diagnosis of bacterial infection in liver cirrhosis is challenging, due to the basal systemic inflammation present in advanced liver diseases and to hepatic insufficiency, skewing usual biomarkers as C reactive protein.[8] Since delayed diagnosis and treatment have a negative impact on prognosis,[3,9] identifying new biomarkers for diagnosis of bacterial infection in cirrhosis is key.

MicroRNAs (miRs) are small non-coding RNA molecules, varying from 18 to 24 nucleotides in length, that control protein homeostasis by binding to the 3′ untranslated region (UTR) of a target mRNA, thereby causing its degradation or inhibition of translation.[10] miRs participate in the post-transcriptional regulation of many physiological and pathological processes, including cell death and proliferation, inflammation, fibrosis, and epithelial-mesenchymal transition. Each miR targets about a hundred genes, and several miRs can regulate the same target gene. miRs can exercise their action in the cell where they are synthetized, but they are also released in the extracellular milieu and can act remotely, thus behaving as hormones.[11]

miRs are quite stable in body fluids and circulating miRs have been widely investigated as candidate biomarkers in various conditions, including in liver diseases.[12] Circulating miRs are dysregulated in cirrhotic patients as they progress to decompensation and acute-on-chronic liver failure (ACLF), defined by AD associated with one or more other organ failure.[13] Specific miRs have been correlated with MELD and Child-Pugh scores, hepatic encephalopathy, renal failure[13] and with early survival in ACLF.[14] Other

[1]Cancer Research Center of Lyon (CRCL), INSERM U1052, CNRS UMR5286, Lyon, France

[2]Department of Hepatology, Hôpital Croix-Rousse, Hospices Civils de Lyon, Lyon, France

[3]University of Lyon Claude Bernard 1 (UCLB1), Lyon, France

[4]Department of Internal Medicine, Hôpital Lyon Sud, Hospices Civils de Lyon, Lyon, France

[5]Clinical Research Center, GHN, Hospices Civils de Lyon, Lyon, France

[6]Department of Anesthesiology and Intensive Care, Hôpital Croix-Rousse, Hospices Civils de Lyon, Lyon, France

[7]Université Paris-Cité, Inserm, Centre de recherche sur l'inflammation, UMR 1149, Paris, France

[8]Service d'Hépatologie, AP-HP, Hôpital Beaujon, DMU DIGEST, Centre de Référence des Maladies Vasculaires du Foie, FILFOIE, ERN RARE-LIVER, Clichy, France

[9]Department of Medicine SCIAC and the Italian Institute of Technology (IIT) Center for Life Nanosciences (CLNS), University of Rome La Sapienza, Rome, Italy

[10]Lead contact

*Correspondence: yasmina.chouik@inserm.fr (Y.C.), massimo.levrero@inserm.fr (M.L.)

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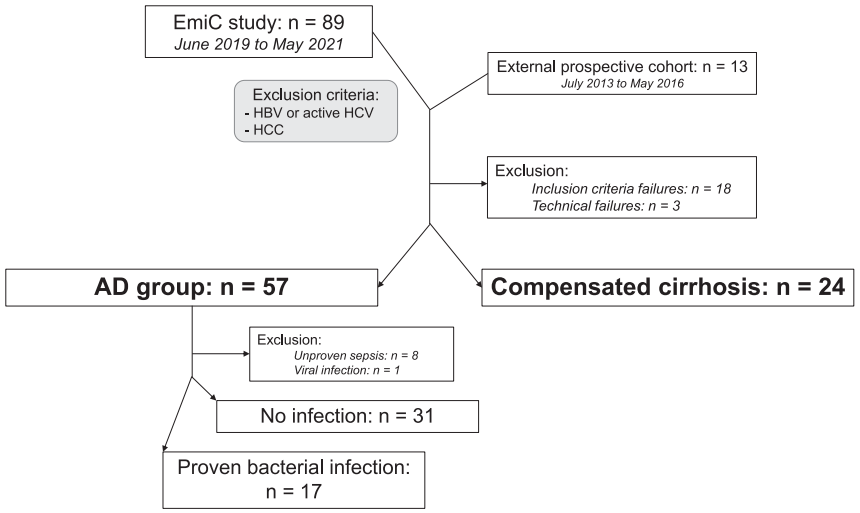

Figure 1. Flow-chart of the study population disposition

Eighty-nine consecutive patients (61 AD and 28 CC) of the prospective observational EMIC cohort were included from June 2019 to May 2021. During the COVID-19 pandemic, 13 additional consecutive patients (8 AD and 5 CC patients) were recruited, with the same inclusion and exclusion criteria, in an external prospective cohort at the Beaujon University Hospital from July 2013 to May 2016, were also included. 18 out of the 102 patients were excluded from the miR profiling study because of inclusion criteria failures [COVID-19 infection (n = 2), HCC diagnosis during the hospitalization (n = 2), incorrect definition of AD or CC (n = 4), absence of plasma sample (n = 10)]. Two additional patients were excluded due to samples hemolysis. miR analysis was performed on 57 patients with and 25 patients with CC. In 12 patients a second run was required due to poor ligation, and 1 CC patient was finally excluded due to persistent poor ligation after 3 runs. miR profiling results are presented for 57 AD and 24 CC patients. In the AD group, 17 had a proven bacterial infection at the hospital admission while 31 patients had no suspected or proven sepsis. In 8 cases sepsis remained unproved and 1 patient had a viral infection.

circulating miRs have been shown to predict ascites onset in patients with compensated cirrhosis and refractoriness to treatment of ascites[15] or to be associated with systemic circulatory and systolic dysfunctions after beta-blockade.[16] Outside the liver disease field, several studies showed that miRs, due to their rapid induction following pathogens recognition and inflammation, can be useful diagnostic biomarkers of bacterial infections.[17,18] However, miRs performance in AD of cirrhosis as biomarkers for early diagnosis of bacterial infection and their ability to improve prediction of survival have not been assessed.

We thus performed a high-throughput analysis of circulating miRs in a prospective cohort of cirrhotic patients with AD with the aim:(i) to identify a specific miR signature for the diagnosis of bacterial infection, and (ii) to evaluate the value of miRs as prognostic biomarkers in patients hospitalized for AD of cirrhosis (secondary infection and 6-month mortality).

RESULTS

Clinical characteristics of the study population

Clinical, demographic, and laboratory data of the study population are shown in Table S1. Median age was 59.3 years old (IQR, 51.2–65.5), with male predominance (69.1%). The most common underlying liver disease was alcohol-related liver disease (ALD) (60.5%) followed by NASH (22.2%). No difference was observed between AD and CC groups for age or sex. Active alcohol consumption was more frequent in AD group whereas NASH showed a higher prevalence in the CC group. Median MELD score was respectively 8 (IQR, 7–9) and 22 (IQR, 16–25) in CC and AD patients. Among the 57 AD patients, 18 fulfilled the criteria for ACLF (9 grade 1, 9 grade $\geq$2). Median time from hospital admission to sample collection was 1 day (IQR, 1–2). Median SOFA and CLIF-OF scores were respectively 4 (IQR, 3–5) and 7 (IQR, 6–8).

Global downregulation of circulating miRs in AD of cirrhosis

Out of the 798 miRs present in the Nanostring Human v3b panel, 208 were not detected in any sample. To assess if the 590 detected miRs could stratify the patients according to their disease stage, we performed a

PCA. As shown in Figure S1A, CC and AD patients did not cluster separately. Selection of the 96 miRs differentially detected between the 2 groups ($p < 0.05$), allowed to separate AD from CC patients whereas AD patients with ACLF were not different from AD patients with no ACLF (Figures 2A and S1B). Figure 2B shows the heatmap for the unsupervised two-way hierarchical clustering analysis of the 31 top-differentially detected miRs. These 31 miRs were globally down-regulated in AD patients as compared with CC patients (Figure 2C), except for the upregulated miR-371a-5p and miR-493-3p. Gene Set Enrichment Analysis (GSEA) of putative target gene for the 31 miRs revealed a strong overrepresentation of pathways involved in cancer such as the Hippo, Ras, TGF-beta, FoxO, PI3K-Akt and Wnt signaling pathways (Table S2). Additional pathways significantly enriched by dysregulated miRs included circadian rhythm, and platelet activation pathways (Table S2).

A miR-based composite score for bacterial infection diagnosis in AD patients

Next, we sought to determine whether circulating miRs could identify AD patients with bacterial infection. To this end, we compared circulating miR profiles between AD patients with proven bacterial infection at the time of sample collection (n = 17) vs. AD patients without suspected or proven bacterial infection, as a control group (n = 31) (Table 1). Patients with unproven infection (n = 8) or viral infection (n = 1) were thus not included in this analysis. The causes of bacterial infections in our study population were distributed as follows: 5 upper urinary tract infections (UTI) (including 4 bacteremic UTI), 4 spontaneous bacteriemia, 3 spontaneous bacterial peritonitis, 3 pulmonary infections, 1 intra-abdominal abscess and 1 ileocolitis with bacteriemia. Gram-negative and positive bacteria were equally detected (n = 7 and n = 7 patients). Thirty-four miRs were differentially detected between the 2 groups (Table S3). The PCA of these 34 miRs in infected and non-infected AD patients is shown in Figure 3A. According to miRs targets bioinformatic analysis, these 34 dysregulated miRs were notably involved in NF-κB/TNFα signaling and IFNγ responses (Table S4).

A multiple logistic regression model was used to develop a miR-based bacterial infection composite diagnostic score. Three biological parameters significantly higher in AD patients with bacterial infection at the univariate analysis were incorporated (C reactive protein [CRP], absolute neutrophil count [ANC], and level of IL-6) (Figure 3B). From the 34 miRs, miR-362-3p, miR-612, and miR-3144-3p were selected as the most significantly differentially detected miRs based on univariate logistic regression. After focused PCA (Figure 3C), 3 significant variables carrying non-redundant information were chosen due to the limited number of events. The most performant 3-variables model to diagnose bacterial infection combined CRP, ANC, and the levels of miR-362-3p, the most dysregulated miR (Table S5).

Based on the coefficients assessed by multivariate Cox analysis, the *sepsis score* formula was established as follows: 1/(1+EXP-(0,019xmiR362 + 0,021xCRP +0,217xANC - 3.3)). Optimal threshold value was 0.408 (sensitivity 81.3%, specificity 86.2%). The model discriminated bacterial infection diagnosis with an AUC of 0.825 (95% CI 0.671–0.980; $p < 0.001$) (Figure 3D), higher than the AUCs of each of its components taken separately and the combination of CRP, ANC ± IL-6 (Table S5).

Prediction of secondary infection by miRs in AD

Then, we wondered whether circulating miRs could predict the occurrence of nosocomial infections in AD patients. We compared patients developing a secondary infection within 7 days of hospital admission (n = 8) and patients with no proven or suspected infection during hospitalization and who were not receiving any antibiotic treatment at the time of the sample collection (n = 18). PCA showed a clear separation between the 2 subgroups, thus suggesting that circulating miRs could help identify patients who will develop secondary infection during the hospitalization (Figure S2A). 127 miRs were differentially detected between the 2 groups. Among the 25 top-differentially detected miRs, 14 were downregulated (Figure S2B), including miR-362-3p. miRs target bioinformatic analysis for these downregulated miRs identified several immune-related genes, such as IL6, IL1A, TNFRSF10B, TNFAIP3, TGFBR2, TGFBR3, TGFB2, STAT3, and FOXO3 (Figure S2C), with an enrichment of inflammation, apoptosis, and epithelial-mesenchymal transition pathways.

Circulating miRs predict survival in AD patients

One-month and 6-month mortality rates were 15.8% and 28.1%, respectively, in our AD patients. Median time to death was 0.9 months (IQR, 0.6–2.6). Seven patients underwent OLT in a median time of 2.0 months (IQR, 0.9–2.5). Patients with poor outcome at 6 months were admitted more frequently to the hospital in the

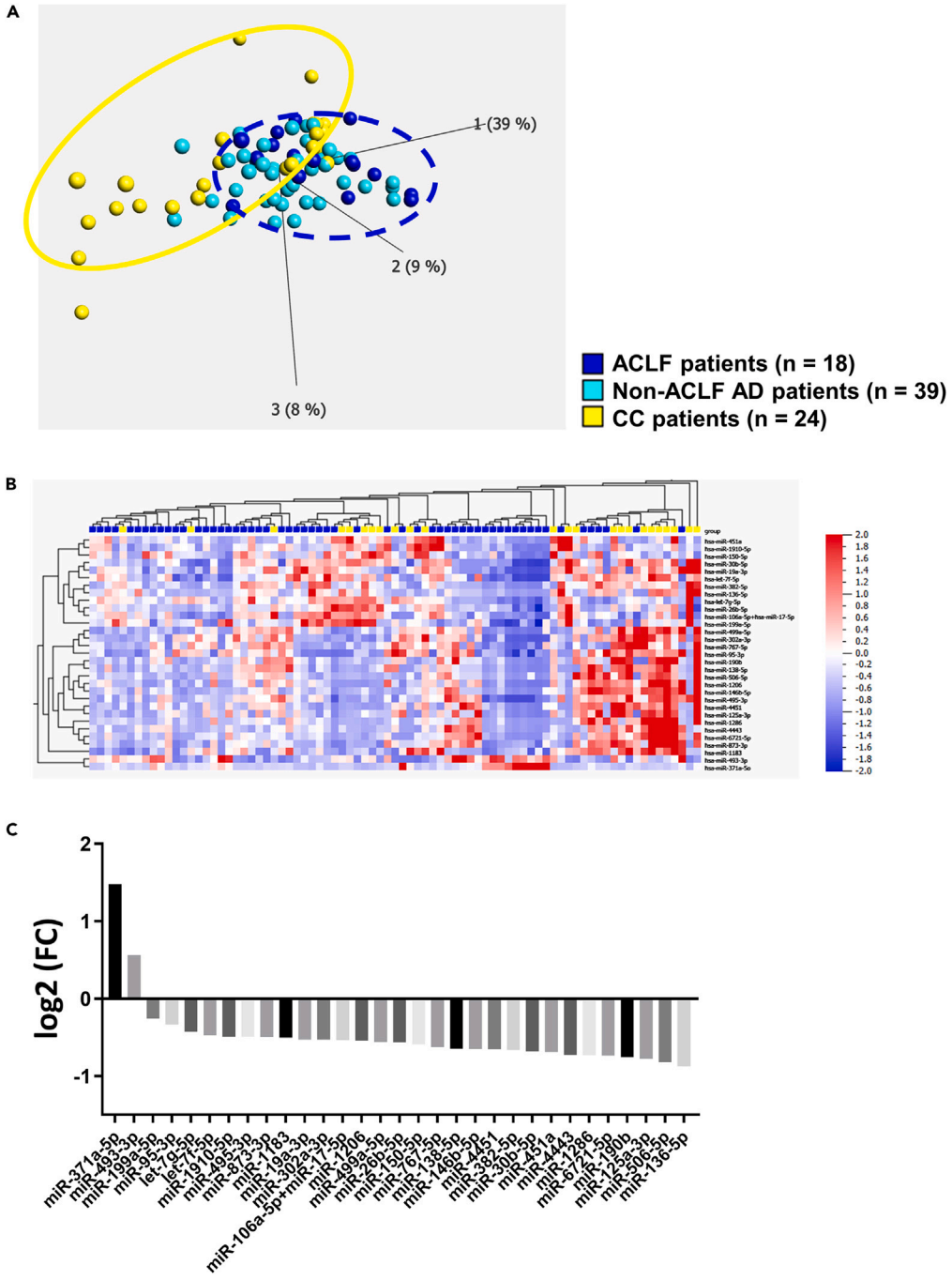

Figure 2. Dysregulation of circulating miRs in AD of liver cirrhosis

(A) PCA of the 96 differentially detected miRs ($p < 0.05$) between all AD patients (light and dark blue; n = 57) and CC patients (yellow; n = 24). AD patients with ACLF (dark blue, n = 18) and non-ACLF AD patients (light blue, n = 39) clustered together.

(B) Heatmap of the top differentially detected miRs generated by hierarchical clustering. Color scale is blue to red with high values getting the red color.

(C) Histogram of the top 31 miRs (log 2 old-change values). Only 2 miRs (miR-371a-5p and miR-493-3p) are significantly upregulated in AD patients.

Table 1. Clinical and biological characteristics of AD patients according to the presence of proven bacterial infection

	Bacterial infection (n = 17)	No infection (n = 31)	p value
Age (y)	55.8 (48.8–59.2)	61.4 (53.0–66.4)	p = ns
Sex (% male)	52.9% (9/17)	83.9% (26/31)	p = 0.050
Active alcohol consumption	52.9% (9/17)	54.8% (17/31)	p = ns
Diabetes	23.5% (4/17)	32.3% (10/31)	p = ns
Cardiovascular diseases	38.6% (22/57)	37.5% (9/24)	p = ns
Child-Pugh score	C12 (C10-C13)	C10 (B9-C13)	p = ns
MELD score	23 (20–28)	23 (16–25)	p = ns
SOFA score	5 (4–5)	5 (3–6)	p = ns
CLIF-OF score	7 (6–9)	7 (7–8)	p = ns
Acute-on-chronic liver failure	35.3% (6/17)	25.8% (8/31)	p = ns
Alcoholic hepatitis	35.3% (6/17)	16.1% (5/31)	p = ns
Antibiotics at the time of sample collection	82.4% (14/17)	38.7% (12/31)	p = 0.006
Biological parameters at the admission			
WBC (G/L)	11.8 (4.0–13.3)	5.7 (3.5–8.3)	p = 0.011
Neutrophils (G/L)	9.1 (2.7–9.8)	3.4 (2.0–5.6)	p = 0.004
Neutrophils-to-lymphocyte ratio	5.7 (5.4–9.7)	3.7 (2.5–5.3)	p = 0.002
Platelet count (G/L)	80 (60–125)	91 (50–114)	p = ns
INR	1.8 (1.6–2.6)	1.8 (1.5–2.2)	p = ns
Creatinine (mg/dL)	0.68 (0.55–0.89)	0.84 (0.61–1.16)	p = ns
Albumin (g/L)	24.8 (24.0–30.6)	28.6 (26.2–32.6)	p = ns
Total bilirubin (mg/dL)	8.6 (2.6–18.4)	3.8 (1.6–7.8)	p = 0.041
C reactive protein (mg/L)	38.6 (14.2–58.8)	15.0 (7.4–25.2)	p = 0.006
IL-6 (pg/mL)	59.4 (22.3–104.0)	31.6 (20.1–43.7)	p = 0.020
IL-8 (pg/mL)	38.0 (27.2–221.0)	34.1 (23.9–63.0)	p = ns
TNFα (pg/mL)	11.7 (8.8–12.8)	11.1 (9.0–13.4)	p = ns
Persistent ACLF at D7	29.4% (5/17)	16.1% (5/31)	p = ns
Lenght of stay (days)	20 (10–26)	10 (7–27)	p = ns
1-month mortality	29.4% (5/17)	16.1% (5/31)	p = ns
6-month mortality	41.2% (7/17)	22.6% (7/31)	p = ns

Data shown as either n (%) or median (interquartile range) for continuous variables. Categorical variables were compared with the Fisher's exact test and quantitative variables were compared using the Mann-Whitney test.

setting of a proven bacterial infection (43.8% vs. 26.8%, p = *ns*) and developed more frequently a secondary infection (37.5% vs. 12.2%, p = 0.023). Main cause of death in our AD patients was sepsis (n = 8). Two other patients died of upper GI bleeding and subdural hematoma, respectively, and cause of death was unknown in 6 patients.

Regarding 6-month survival, 17 miRs were differentially detected in survivors as compared to non-survivors, including 7 miRs in common with the 127 miRs signature associated with secondary infection (Figure 4A). All 17 miRs were validated as significant predictors of 6-month overall survival in competing risk analysis (Table S6). MELD (HR = 1.20; 95% CI = 1.01–1.43; p = 0.037) and MELD-Na (HR = 1.20; 95% CI = 1.02–1.41; p = 0.025) were also significantly associated with 6-month mortality (Table 2). After adjusting for MELD-Na, which performed better than the MELD alone, 7 of the 17 miRs (miR-382-5p, miR-30d-5p, miR-148b-3p, miR-493-3p, miR-592, miR-22-3p, and miR-21-5p) remained as independent predictive factors (Table S6). Three of them were associated with survival, including miR-493-3p, one of the 2 miRs we showed to be upregulated in AD patients compared to CC (Figures 2B and 2C). Four could predict death, including miR-382-5p, which is significantly down-regulated in AD patients

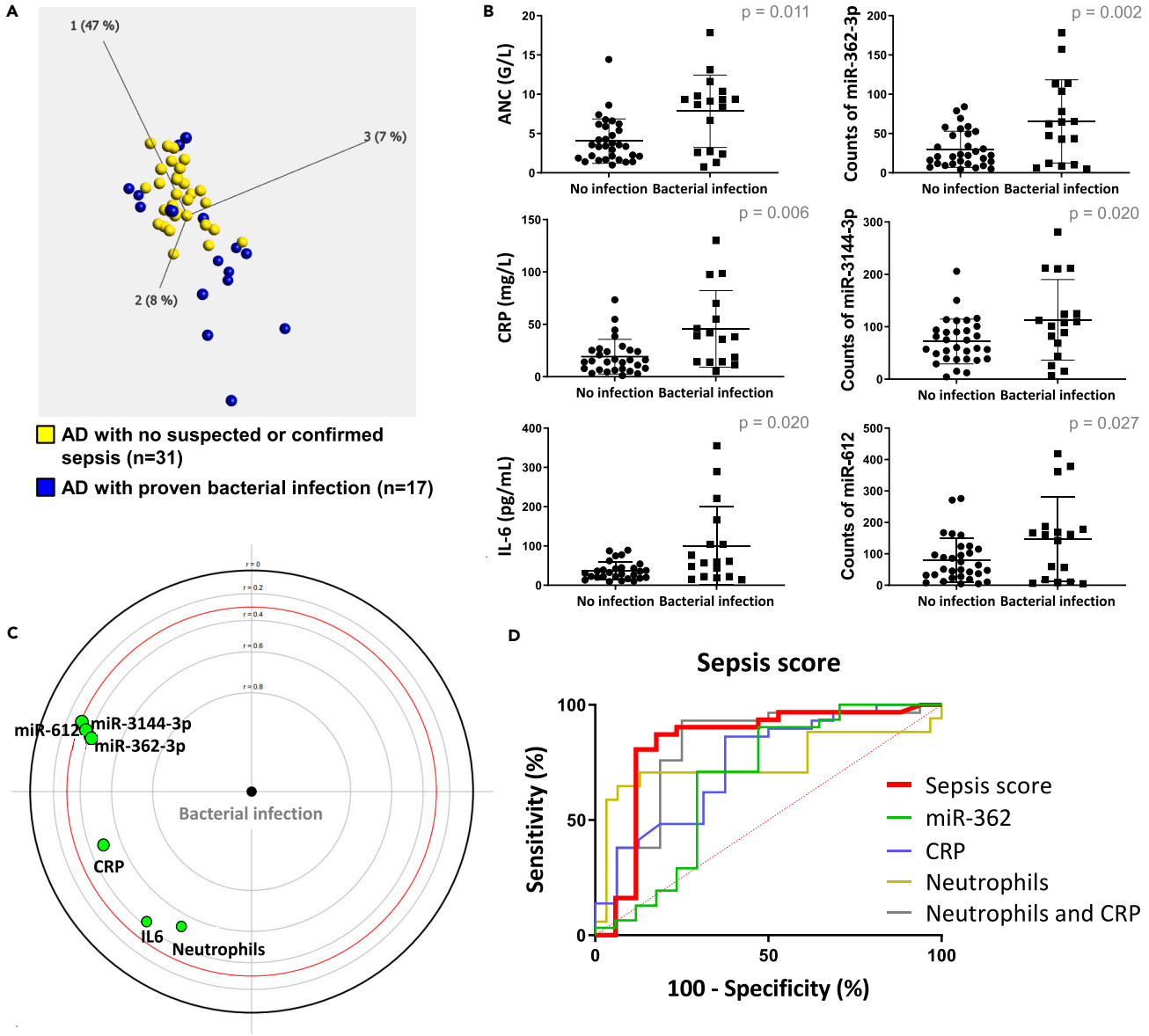

Figure 3. Development of a bacterial infection diagnostic score in AD patients

(A) PCA of the 34 differentially detected miRs between patients with proven bacterial infection (n = 17) and patients without any suspected or confirmed bacterial infection at the time of hospital admission (n = 31).

(B) Median levels of cytokines (IL-6, IL-8, and TNFα), miRs (miR-362-3p, miR-612, and miR-3144-3p), ANC and CRP in infected and non-infected AD patients. ANC, CRP, IL-6 levels were significantly higher in infected patients (respectively p = 0.011, p = 0.006 and p = 0.020), as plasma counts of miR-362-3p (p = 0.002), miR-3144-3p (p = 0.020) and miR-612 (p = 0.027).

(C) Focused PCA of significant biological parameters (ANC, CRP, and IL-6) and the top 3 differentially detected miRs (miR-362-3p, miR-612, and miR-3144-3p), allowing to visualize covariance and correlation strength of the different variables. After adjusting for ANC, IL-6 was not significant anymore. miRs carried redundant information whereas CRP and ANC were independent of each other.

(D) ROC curves of the *sepsis score* (combining miR-362-3p, ANC, and CRP), miR-362-3p, CRP, ANC, and the combination of ANC and CRP in relation to bacterial infection diagnosis. Targeting a negative predictive value of 92.0%, optimal cut-off value was 0.310 (positive predictive value 70%, sensitivity 87.5%, and specificity 79.3%). Targeting a positive predictive value of 88.9%, threshold value was 0.668 (negative predictive value 77.8%, sensitivity 50.0%,and specificity 96.6%). Bacterial infection is unlikely in patients with score <0.310 while patients with score >0.668 are likely infected.

(Figures 2B and 2C). Similar results were obtained after adjusting for the MELD score (data not shown). A multivariate model combining miR-382-3p, miR-592, and MELD-Na score showed better prognostic discrimination compared to the MELD-Na or MELD score (c-index of 0.843 [0.770–0.916] vs. 0.766

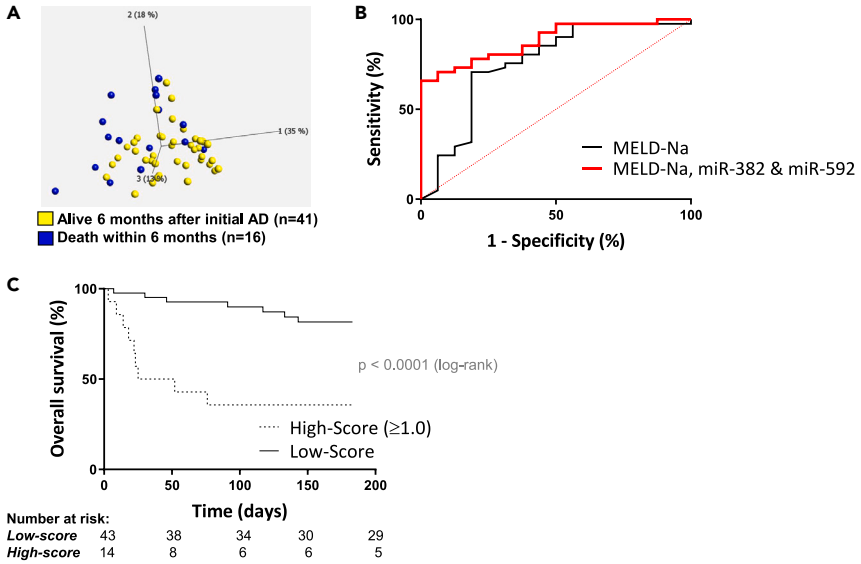

Figure 4. Predictive value of circulating miRs in AD

(A) PCA of the 27 differentially detected miRs between patients alive 6 months after sample collection (n = 41) and patients with poor outcome (n = 16).

(B) ROC analysis of 6-month survival probability relative to MELD-Na score or prognostic score combining MELD-Na, miR-382-5p and miR-592. C-index of MELD-Na was 0.766 (95% CI, 0.633–0.899), while c-index of composite score combining MELD-Na and the 2 miRs was 0.843 (95% CI, 0.770–0.916), representing and improvement in prediction error of 32.9%.

(C) Comparison of Kaplan-Meier curves between patients with a miR-based score >1.0 (corresponding to the 25th percentile) to patients with lower score ($p < 0.0001$ using log rank test). For the establishment of the new prognostic score, variables were mean centered and, using the regression coefficients, the score was calculated as follows: 0.1721x(MELD-Na-23.5) + 0.0398x(miR-382-25) – 0.0901x(miR-592-17).

[0.633–0.899] and 0.762 [0.623–0.901], respectively, representing an improvement in prediction error of 32.9% and 34.0%, respectively) (Figure 4B). Figure 4C shows the Kaplan-Meier curves for mortality during the 6-month follow-up period, according to the new miR-based model (patients at high-risk: score >75th percentile).

DISCUSSION

We present here the results of a high throughput plasma miRs profiling in a prospective cohort of acutely decompensated cirrhotic patients. Taken together, our findings indicate that circulating miRs are strongly dysregulated in decompensated cirrhosis, and we identified a novel composite score combining ANC and CRP with miR-362-3p, that can diagnose with an excellent performance bacterial infection in patients hospitalized for AD. Furthermore, our results highlight the prognostic role of miRs in the prediction of secondary infections in hospitalized patients and 6-month mortality in AD cirrhotic patients. Notably, mortality and primary or secondary infection rates in our patients align with those reported in the literature.[3,8,19]

Management of bacterial infection is a major issue in cirrhotic patients since it is a frequent and serious event in this population and any delay in diagnosis and treatment has an important negative impact on patient prognosis.[4,9,20] Conversely, increased exposure of cirrhotic patients to antibiotics results in the emergence of multidrug-resistant bacterial infections and fungal infections, which worsens overall prognosis.[21] Moreover, due to combination of chronic systemic inflammation, hepatic insufficiency, and portal hypertension, current biomarkers like CRP and ANC may lack sensitivity and specificity in AD cirrhotic patients.[8] The identification of a miR-based signature associated with bacterial infection may improve the accuracy of its diagnosis in AD cirrhotic patients. Indeed, we have shown that a *sepsis score* that combined miR-362-3p with CRP and ANC diagnoses bacterial infection with an excellent AUC of 0.825, higher than those of each of its components taken separately. Although miR-362-3p has been mainly described as a tumor suppressor in HCC[22] some data have also linked miR-362-3p to the regulation of TLR4 in response to LPS.[23] AH and bacterial infections are the main precipitating events of AD and ACLF.[5] Notably, our patients with AH had, as expected, high ANC and CRP independently from the presence of sepsis (data not shown), whereas

Table 2. Comparison of clinical and biological characteristics of AD patients according to survival at 6 months

	Non-survivors (n = 16)	Survivors (n = 41)	HR (95% CI)	p value
Age (y)	63.1 (52.0–66.9)	59.2 (51.1–63.9)	1.01 (0.97–1.06)	p = ns
Sex (% male)	75.0% (12/16)	73.2% (30/41)	1.08 (0.36–3.30)	p = ns
Active alcohol consumption	56.3% (9/16)	51.2% (21/41)	1.26 (0.48–3.31)	p = ns
Child-Pugh score	C12 (C11-C13)	C11 (B9-C13)	1.16 (0.98–1.38)	p = ns
MELD score	25 (24–30)	22 (16–23)	1.20 (1.01–1.43)	p = 0.037
MELD-Na score	28 (36–32)	23 (18–26)	1.20 (1.02–1.41)	p = 0.025
SOFA score	5 (4–6)	4 (3–5)	1.30 (0.91–1.86)	p = ns
CLIF-OF score	7 (7–8)	7 (6–8)	1.33 (0.88–2.00)	p = ns
Proven initial bacterial infection	43.8% (7/16)	26.8% (11/41)	2.18 (0.82–5.79)	p = ns
Alcoholic hepatitis	37.5% (6/16)	17.1% (7/41)	2.64 (0.94–7.40)	p = ns
Acute-on-chronic liver failure	43.8% (7/16)	26.8% (11/41)	2.27 (0.83–6.21)	p = ns
Biological parameters				
WBC (G/L)	10.3 (5.4–12.1)	7.1 (3.6–9.9)	1.10 (0.98–1.24)	p = ns
Neutrophils (G/L)	6.8 (3.1–12.1)	4.2 (2.1–6.8)	1.16 (1.01–1.33)	p = 0.041
Neutrophils-to-lymphocyte ratio	4.8 (4.2–7.2)	3.8 (2.4–5.9)	1.04 (0.97–1.11)	p = ns
C reactive protein (g/L)	23.1 (14.2–54.9)	15.5 (7.9–31.7)	1.01 (0.99–1.02)	p = ns
IL-6 (pg/mL)	43.1 (24.8–67.8)	32.6 (21.7–55.5)	1.00 (0.99–1.01)	p = ns
IL-8 (pg/mL)	93.8 (33.1–197.5)	30.5 (20.0–68.9)	1.00 (1.00–1.01)	p = 0.034
TNFα (pg/mL)	11.7 (9.4–12.9)	10.6 (8.1–14.5)	1.03 (0.93–1.14)	p = ns
Persistent ACLF at D7	50.0% (8/16)	9.8% (4/41)	7.57 (2.67–21.40)	p < 0.001
Proven secondary infection	37.5% (6/16)	12.2% (5/41)	3.13 (1.17–8.40)	p = 0.023

Data shown as n (%) or median (interquartile range) for continuous variables. Each variable was tested in univariate analysis by competing risk analysis, with OLT considered as a competing event. Absolute neutrophil count was not significant after adjusting on MELD-Na score.

circulating miR-362-3p levels, that are markedly increased in AD patients with bacterial infections, did not significantly differ in AH patients as compared to the rest of the AD cohort (data not shown). Although, due to the small number of patients, no sensitivity analyses were undertaken, these results support the rationale of combining ANR and CRP with miR-362-3p in the design of a *sepsis score* performed across the whole spectrum of AD patients. IL-6 levels also are markedly increased in AD and ACLF[24] and have been proposed for the diagnosis of bacterial infections in cirrhotic patients.[25] In other types of infection, IL-6 was shown to be more sensitive and specific than CRP or procalcitonin for the diagnosis of sepsis.[26] Although IL-6 was significantly raised in our AD patients with bacterial infection, we choose not to include it in the *sepsis score* because it was not independent from ANC in our multivariate analysis. Importantly, IL-6 increase was, like miR-362-3p but differently from ANC and CRP, independent from the presence or absence of AH (data not shown) and its potential to further ameliorate the *sepsis score* warrants to be investigated in larger cohorts of AD patients. Among our 8 patients with *unproven sepsis*, mostly presenting with bacteriuria or CT-suspected pneumonia without clinical symptoms and treated with antibiotics during the hospitalization, only one had an elevated *sepsis score* at 0.719. No bacterial or fungal documentation was obtained for this patient, but he was transferred to ICU for a clinical picture of septic shock requiring vasopressors and intensification of antibiotic and antifungal therapy. The other patients with low *sepsis score* had a median hospitalization time of 7 days, and their 1-month survival was 100%. Altogether these results altogether provide a strong rationale for a prospective validation of our *sepsis score* accuracy to diagnose bacterial infection in AD of cirrhosis.

To identify the patients that will develop an infection during hospitalization for AD of cirrhosis is of great interest since this population is at higher risk of mortality.[27] In our study, these patients had a 62.5% 6-month mortality compared to 12.0% for the patients who did not. We have shown that circulating miRs profiles clearly differentiate AD patients developing a secondary infection as compared to AD patients

without proven or suspected sepsis during hospitalization and without antibiotic therapy at the time of sample collection. Although the small number of patients did not enable us to develop a predictive model, our results suggest that circulating miRs may help to assess the risk of secondary infection and stratify AD patients for antibiotic treatment and avoid their systematic use, potentially associated with the development of multidrug-resistant bacterial or fungal infections. Many of the circulating miRs associated with secondary infection in our study (e.g., miR-223-3p, miR-25-3p, miR-126-3p, or miR-142-3p) regulate immune responses and thus could mirror the underlying immune dysfunction in these patients. Several miRs modulate innate immune responses in the liver and target inflammatory pathways such as TLRs.[28] miR-223-3p has been shown to inhibit IL-6 signaling in liver infiltrating neutrophils in ALD patients.[29]

miR profiling in our AD patients has revealed a strong dysregulation as compared to CC, with a global downregulation of circulating miRs. Cisilotto et al.[14] have reported a global decrease of circulating miRs in ACLF, compared to non-ACLF AD patients and healthy volunteers. The global decrease of circulating miRs in AD patients may reflect either the overall reduction of viable secreting cells or a decrease/change in their secretion in response to various signals. Although hepatocytes are likely to be the major cell compartment affected in AD, immune cells[7] or renal epithelial cells[30] may also contribute. Indeed, Blaya et al.[13] showed that 2 out of 3 miRs dysregulated in AD as compared to CC and healthy volunteers, displayed the same altered profile in PBMCs, while no differences were observed in liver tissue or between hepatic and portal blood, rather suggesting an extra-hepatic origin.

Many of the circulating miRs we found decreased in AD patients regulate cancer pathways, which could be in relation to the increasing risk of HCC in patients with more advanced liver disease. Computational pathway analysis also showed an over-representation of these miRs in neurological processes, thus suggesting a possible involvement in hepatic encephalopathy modulation. miR profiles also differed depending on the presence of clinically significant portal hypertension (CSPH)[1] in CC patients. miR-122, the most abundant liver-specific miR,[31] was significantly decreased in the presence of CSPH and was negatively correlated with the MELD score in our CC patients (data not shown). Additional studies are needed to decipher the origin and the pathophysiological role of the miRs dysregulated in AD patients.

Finally, we showed that circulating miRs can help predict 6-month survival in AD patients. The use of circulating miRs as survival biomarkers has been reported in acetaminophen-induced acute liver failure[32] as well as in sepsis.[33] We identified 7 miRs (miR-382-5p, miR-30d-5p, miR-148b-3p, miR-493-3p, miR-592, miR-22-3p, and miR-21-5p) that were associated with 6-month mortality in AD patients and were independent from MELD-Na score. Combination of miR-382-3p, miR-592, and MELD-Na decreased MELD and MELD-Na prediction error by 34.0% of 32.9%, respectively. The most significant miR, miR-382-5p, has been reported to promote cell invasion in HCC by targeting PTEN[34] and, notably, also to predict acetaminophen liver injury, independently from kidney injury.[35] miR-592 inhibits aerobic glycolysis in HCC cells through the WSB1/HIF-1α axis[36] and low levels of miR-592 contribute to a 5 miRs necroptosis-related prognostic signature in HCC patients.[37] Considering the other 5 independent prognostic miRs identified in our study, they have been involved in apoptosis and cell-fate regulation, the modulation of immune responses or have been proposed as survival biomarkers in other diseases. Notably, miR-21-5p was shown to predict survival independently from MELD score in patients with non-viral decompensated cirrhosis.[38]

Our observation that combining circulating miR levels with MELD or MELD-Na scores improves the predictive power of these scores in patients with AD of cirrhosis raises the question of whether the incorporation of circulating miRs in prognostic scores might help to identify patients in need of rapid access to OLT, an important challenge in the current context of organ shortage. Indeed, by reflecting pathogenetic mechanisms, like apoptosis and immune dysfunction, which are not assessed in the current scores based upon biochemical parameters, miRs could provide new prognostic value.

In conclusion, our data indicate that circulating miRs are strongly dysregulated in AD of cirrhosis as compared to CC and are useful to identify subgroups of patients with different clinical courses and prognoses. The combination of miRs and biological parameters outperforms conventional biomarkers for the diagnosis of bacterial infection diagnosis and refines survival prediction in patients hospitalized for AD of cirrhosis. Altogether, our results provide a strong rationale and the knowledge to design studies aimed to validate miR signatures and miR-based scores in patients with acute or non-acute decompensation of liver cirrhosis. More studies are also needed to decipher the exact cell/organ origin of circulating miRs

in AD patients and to understand the mechanistic basis of their contribution to liver carcinogenesis, hepatic encephalopathy, and cirrhosis-associated immune dysfunction.

LIMITATIONS OF THE STUDY

Our study has some limitations, notably the lack of internal and external validation, and the small size of the study cohort.

- All platforms for miR quantification have specific advantages and drawbacks that need to be taken into consideration, particularly when aiming for development and clinical application of extracellular miRs as biomarkers. The hybridization and digital laser detection technology used in our study is slightly less sensitive for low miRs input than some other PCR or sequencing platforms, but it is less prone to false positives results, not affected by CG content, not subjected to the potential bias associated with target amplification and provides absolute quantification. Importantly, it also has the shortest turnaround time, an interesting feature in the perspective of clinical use.
- Our study has been designed to have sufficient power, but the presence of false discoveries cannot be excluded. To mitigate this risk, the *sepsis score* and the *miR-MELD score* were confirmed by multiple analytical approaches. Thus, our study provides key signals about the interest in circulating miRs as biomarkers in AD of cirrhosis, but other studies are warranted before concluding on their applicability in clinical practice.
- Finally, we have chosen to exclude patients with history of HCC and active chronic viral hepatitis, due to previously reported circulating miRs alterations in these populations. Therefore, the scope of our findings is mainly restricted to patients with ALD and NASH.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107427.

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AUTHOR CONTRIBUTIONS

Y.C., F.L., and M.L. designed the study. Y.C., F.L., M.S., T.A., D.E., S.R., F.V., C.G., and K.H.L. included patients in the EMIC study. Y.C. collected biological and clinical data. P.E.R. and A.P. provided patients data and biological samples from the Beaujon Hospital. Y.C. and M.L.P. performed miR analyses. Y.C.

performed bioinformatic analysis. Statistical analyses were made by Y.C., P.P. and J.C.L. Y.C. wrote the original draft. M.L. edited the manuscript. F.L., F.Z., and P.E.R. critically reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Simple Plex™ Automated Immunoassay (custom panel for IL-6, IL-8 and TNF-α)	ProteinSimple, San Jose, CA	Not available (custom panel)
Biological samples		
Human plasma samples	Clinical trial "Emic"	N/A
Critical commercial assays		
miRNeasy Serum/Plasma Advanced Kit	Qiagen	Cat#217204
Human v3b miRNA panel	Nanostring	Cat#150629
Oligonucleotides		
cel-miR-254 UGC-AAA-UCU-UUC-GCG-ACU-GUA-GG	Eurogentec	custom oligos synthesized
osa-miR-414 UCA-UCC-UCA-UCA-UCG-UCC	Eurogentec	custom oligos synthesized
Software and algorithms		
nSolver™ Analysis Software 4.0	Nanostring	https://nanostring.com/products/analysis-solutions/
Qlucore Omics Explorer software 3.9	Qlucore	https://qlucore.com

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Yasmina Chouik (yasmina.chouik@inserm.fr).

Materials availability

This study did not generate new unique reagents.

There are restrictions to the availability of plasma samples due to ethical restrictions.

Data and code availability

- Data reported in this paper are available from the lead contact upon request.
- This paper does not report any original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The study population comprises patients with an acute decompensation (AD) of cirrhosis[1] and a control group including patients with compensated cirrhosis (CC) (Child-Pugh score A). Cirrhosis was diagnosed on histology (n = 22) or on a combination of clinical, biochemical, ultrasonographic findings and liver elastometry (n = 59). Diagnosis of bacterial infection was made following Bajaj et al.,[39] and defined as the presence of one or more of the following features: a) spontaneous bacteremia: positive blood cultures without a source of infection, (b) spontaneous bacterial peritonitis: ascitic fluid polymorphonuclear cells >250/μL, (c) lower respiratory tract infections: new pulmonary infiltrate in the presence of at least one respiratory symptom with at least one finding on auscultation or one sign of sepsis, (d) bacterial entero-colitis: diarrhea or dysentery with a positive stool culture for *Salmonella, Shigella, Yersinia, Campylobacter, E. coli* or *Clostridium difficile*, (e) skin infection: fever with cellulitis, (f) urinary tract infection (UTI): urine WBC >15/high power field with either positive urine gram stain or culture in a symptomatic patient or (g) intra-abdominal abscess.

Patients with hepatocellular carcinoma (HCC) (active or treated), other active cancers, chronic viral hepatitis (active or eradicated hepatitis C virus infection < 6 months, HBsAg positivity) or HIV infection were excluded due to the specific changes of miR profiles described in these populations that might potentially bias the interpretation of the results.

The prospective observational EMIC study (Study of microRNAs in Decompensated Cirrhosis; *ClinicalTrials.gov* ID number NCT03905746) was launched at the University Hospital of Lyon (France) to investigate the alterations of circulating miRs in patients with AD of liver cirrhosis, and notably to investigate their potential role as diagnostic biomarkers of bacterial infection. EMIC sample size was calculated based on an alternative hypothesis of AUC = 0.80 for the new diagnostic test in discriminating subjects with and without bacterial infection. We used a two-sided test:H1: AUC = 0.8, vs. H0: AUC = 0.5, power = 90%, $\alpha = 0.025$.

The ratio between infected and non-infected AD patients was set at 1:2 in agreement with previous published data.[2,40] We estimated a final sample size of 48 subjects (16 with bacterial infection and 32 without). Due to the suspension of inclusions during the COVID-19 pandemic and the consequent decrease in the expected number of patients included in the EMIC cohort and to meet the calculated sample size in all categories of patients, we included all consecutive patients with the same inclusion and exclusion criteria and available plasma samples from an external prospective cohort launched at the Beaujon University Hospital (Clichy, France) from June 2013 to June 2016 (Microspy study).[41,42] Eighty-nine consecutive patients (61 AD and 28 CC patients) have been included in Lyon from June 2019 to May 2021 and 13 patients (8 AD and 5 CC patients) in Clichy (*Institutional Review Boards of Paris North Hospitals, Paris 7 University, Agreement AP-HP N° 11-112*). A flow-chart of the study population is shown in Figure 1. miR profiles were analyzed in 57 AD and 24 CC patients, after exclusion of technical and clinical failures (Figure 1).

Demographics, liver disease-related events, biochemical data and biological samples were prospectively collected within the 48 hours of the hospital admission for AD patients and at the time of inclusion for CC patients. Clinical and biochemical data were also collected at day 2, day 7 and 6 months after inclusion.

ACLF, defined by AD of chronic liver disease associated with one or more organ failures (renal, coagulation, brain and/or pulmonary), and associated with a high 1-month mortality compared to AD without ACLF, was graded following Moreau at al.[43] Secondary infection was defined as an infection acquired during hospitalization and that was not present at the time of admission. Unproven sepsis was defined as clinical suspicion of infection (fever or leukocytosis) without meeting the established diagnostic criteria described above.[44] The Sepsis-related Organ Failure Assessment (SOFA) score[45] and the Chronic Liver Failure-Organ Failure Assessment (CLIF-OF) score[46] were calculated for AD patients. All alcoholic hepatitis (AH) were confirmed by liver biopsy.

The study protocol adhered to the ethical guidelines of the Declaration of Helsinki and was approved by the Ethics Committee for Clinical Research (Lyon: *Comité de Protection des Personnes du Sud-Ouest et Outre-mer IV*, Agreement n. 2019-A00266-51; Clichy: *Institutional Review Boards of Paris North Hospitals, Paris 7 University, Agreement AP-HP N° 11-112*). All participants granted written informed consent authorizing the storage and research use of their samples.

METHOD DETAILS

miR profiling

Blood collection and RNA extraction

Plasma was collected from peripheral blood and stored at −80°C within 4h from blood drawing. Total RNA was extracted from 200 µL of plasma using an automated QIAcube™ system using the Qiagen miRNeasy Serum/Plasma Advanced Kit. Two exogenous spike-in controls (*cel*-miR-254 and *osa*-miR-414) were added to each sample to assess the quality of RNA extraction and further steps.

miR quantification

miR quantification was performed on a Nanostring nCounter™ Flex platform using the Human v3b miRNA panel which allows the simultaneous detection and quantification of 798 miRs. Briefly, direct target hybridization (without reverse transcription step) with specific probes was followed by a purification step and digital quantitative laser counting. Quality of runs was assessed with the nSolver™ Analysis Software. A second

run was performed for samples with low ligation and/or low spike-in controls. Data normalization was made, after exclusion of samples failing ligation, on the top 100 detected miRs. Background subtraction was done by subtracting the geometric mean of internal negative controls.

Cytokine quantification

Plasma concentrations of IL-6, IL-8 and TNF-α were measured using Simple Plex™ immunoassays on the microfluidic ELLA analyzer (ProteinSimple®, San Jose, CA), according to manufacturer's instructions. Cytokine measurement was performed in one batch.

QUANTIFICATION AND STATISTICAL ANALYSIS

Bioinformatic analysis

The Qlucore Omics Explorer software was used to identify differentially detected miRs, to build heat maps and to perform Principal Component Analysis (PCAs). miRs with > 20 normalized counts in at least 1 patient were considered as detected.

The analysis of biological pathways enrichment was performed using miRPath v3 (http://snf-515788.vm.okeanos.grnet.gr/). The target genes of miR of interest were predicted using miRTargetLink 2.0. (https://ccb-compute.cs.uni-saarland.de/mirtargetlink2). Target genes defined as *strongly validated* by miRTargetLink 2.0 were then analyzed using Enrichr (https://maayanlab.cloud/Enrichr/) for functional enrichment analysis including cellular component, molecular function, biological process and Kyoto Encyclopedia of Genes and Genomes (KEGG).

Statistical analysis

Statistical analyses were performed using SPSS version 23, GraphPad Prism version 7.05 and R software version 3.5.3.

Demographic, clinical and laboratory continuous variables were expressed as median with interquartile (IQR) range and compared using the Mann-Whitney test, while categorical variables were expressed as counts and percentages and compared using the Chi-square test or Fisher's exact test, as appropriate. For all analyses, a two-tailed p value ≤ 0.05 was considered statistically significant. Fold change was defined as the ratio of the final value divided by the initial value.

For the diagnosis of bacterial infection, miRs of interest and relevant biochemical parameters were tested in univariate logistic regression. Only variables with a p value ≤ 0.05 were integrated in the multiple logistic regression. Receiver Operating Characteristic (ROC) curves analysis were reported as area under the ROC curve (AUC) (95% confidence interval (CI)). The optimal threshold value was calculated using Youden's index.[47] Focused PCA was used to represent correlation strength between bacterial infection and explanatory variables as well as covariance of the different explanatory variables.[48]

For survival prediction, patients undergoing orthotopic liver transplantation (OLT) were censored at the day of OLT (n = 7) and OLT was considered as a competing event with death (R "cmprsk" package). The primary endpoint was 6-month survival calculated from the day of plasma collection. From the miRs identified as significant predictors ($p \leq 0.05$) by the Fine & Gray regression, a Cox regression model combining miRs and MELD or MELD-Na score was used to create an alternative prediction model. Discriminatory performance of the newly developed score in comparison to MELD and MELD-Na scores was assessed using Harrell's C concordance index (c-index) using R survival package.