Molecular Profiling of Decompensated Cirrhosis by a Novel MicroRNA Signature

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Noninvasive staging of decompensated cirrhosis is an unmet clinical need. The aims of this study were to characterize and validate a novel microRNA (miRNA) signature to stage decompensated cirrhosis and predict the portal pressure and systolic cardiac response to nonselective beta-blockers (NSBBs). Serum samples from patients with decompensated cirrhosis (n = 36) and healthy controls (n = 36) were tested for a novel signature of five miRNAs (miR-452-5p, miR-429, miR-885-5p, miR-181b-5p, and miR-122-5p) identified in the secretome of primary human hepatocytes and for three miRNAs (miR-192-5p, miR-34a-5p, and miR-29a-5p) previously discovered as biomarkers of chronic liver disease. All patients had ascites, which was refractory in 18 (50%), and were placed on NSBBs for variceal bleeding prophylaxis. In all patients, serum miRNAs, hepatic venous pressure gradient, and an echocardiogram study were performed before and 1 month after NSBBs. Patients with cirrhosis had lower serum levels of miR-429, miR-885-5p, miR-181b-5p, miR-122-5p, miR-192-5p, and miR-29a-5p ($P < 0.05$). Baseline serum miR-452-5p and miR-429 levels were lower in NSBB responders ($P = 0.006$). miR-181b-5p levels were greater in refractory ascites than in diuretic-sensitive ascites ($P = 0.008$) and correlated with serum creatinine. miR-452-5p and miR-885-5p were inversely correlated with baseline systemic vascular resistance ($\rho = -0.46$, $P = 0.007$; and $\rho = -0.41$, $P = 0.01$, respectively) and with diminished systolic contractility ($\rho = -0.55$, $P = 0.02$; and $\rho = -0.55$, $P = 0.02$, respectively) in patients with refractory ascites after NSBBs. *Conclusion:* Analysis of a miRNA signature in serum discriminates between patients with decompensated cirrhosis who show more severe systemic circulatory dysfunction and compromised systolic function after beta-blockade and those more likely to benefit from NSBBs. (*Hepatology Communications* 2021;5:309-322).

MicroRNAs (miRNAs) are small noncoding RNA molecules (18-25 nucleotides) involved in the posttranscriptional regulation of protein-coding genes controlling many cellular processes.[1] In serum, miRNAs may be bound to circulating proteins or, more frequently, contained within extracellular vesicles (EVs).[2] EVs are nanometric double-membrane structures that envelop miRNAs and shuttle this biological information from one cell type to another. Because their cargo dynamically

changes depending on different physiopathological stimuli, it has been hypothesized that EVs themselves and/or EV-housed miRNAs may serve as noninvasive biomarkers to assess disease course, progression, and therapeutic response.[3]

Cirrhosis is a progressive disease that develops over two consecutive stages, compensated and decompensated, each with different clinical characteristics and prognoses.[4] Within the decompensated stage, further decompensation leads to refractory ascites, a stage of cirrhosis characterized by severe peripheral vasodilation, reduced cardiac reserve, and a dismal prognosis.[5-7] For patients with cirrhosis, nonselective beta-blockers (NSBBs) are the cornerstone treatment to prevent first and recurrent variceal bleeding.[8] The response to NSBBs, as defined by a reduction in the hepatic venous pressure gradient (HVPG) $\geq$ 10%, has been associated with reduced variceal bleeding and ascites development and increased survival.[9-12] However, the beneficial effects of NSBBs may not extend to the whole population of patients with decompensated cirrhosis. In fact, NSBBs may be harmful in patients with refractory ascites as they may compromise their cardiac compensatory reserve with the consequences of insufficient cardiac output and renal failure.[7,13] Clinicians, therefore, need to be cautious about prescribing NSBBs to these patients with refractory ascites. There is a lack of noninvasive biomarkers to identify patients with decompensated cirrhosis more prone to develop complications. Personalized molecular profiling could be useful to identify those subjects with severe circulatory dysfunction in which NSBBs may be risky.

This study was designed to examine the correlation of a novel serum miRNA signature with several outcomes in decompensated cirrhosis. This signature was identified according to its differential expression in EVs in the secretome of hepatocytes from livers of patients with cirrhosis. Our working hypothesis was that the proposed miRNA signature would likely reflect disease stage and therefore serve as a valuable biomarker in patients with decompensated cirrhosis. This signature was assessed in terms of its capacity to 1) discriminate between patients with diuretic-sensitive and refractory ascites; 2) anticipate the development of cardiac dysfunction in response to NSBBs; and 3) predict the HVPG response to NSBBs.

Materials and Methods

STUDY DESIGN

Our study consisted of three steps: 1) identification of a novel miRNA signature in the secretome of cultures of human hepatocytes; 2) analysis of the miRNA signature in serum samples from a well-characterized cohort of patients with decompensated cirrhosis and from age- and sex-matched healthy controls; and 3) correlation of serum levels of these miRNAs with clinical outcomes in patients with decompensated cirrhosis (Fig. 1).

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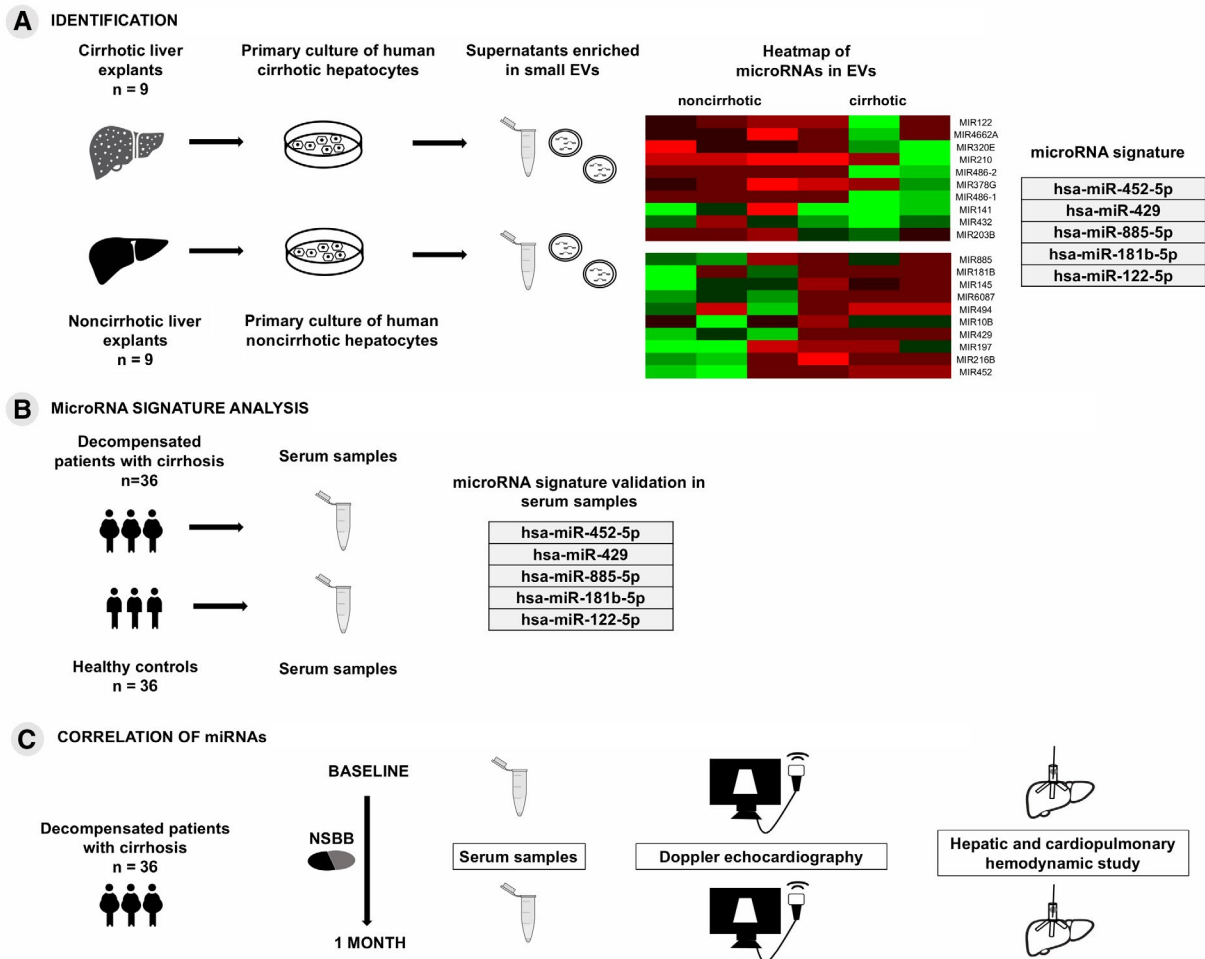
FIG. 1. Study design. The study was developed in three steps: A) Identification of a novel miRNA signature in the secretome of cultures of human hepatocytes from cirrhotic and noncirrhotic livers. The heatmap represents the 20 most deregulated miRNAs (the top 10 up-regulated and the top 10 down-regulated) detected after sequencing small EVs from cirrhotic and noncirrhotic hepatocyte secretome. The five miRNAs integrating the novel signature are those that presented a greater fold change and statistically significant difference. B) miRNA signature analysis in serum samples of patients with decompensated cirrhosis and age- and sex-matched healthy controls. The novel signature of five miRNAs identified in the previous step was measured in serum samples of patients with cirrhosis. C) Correlation of miRNAs in serum and clinical outcomes in patients with decompensated cirrhosis. miRNAs were measured in patients' serum at baseline and 1 month after treatment with NSBBs. miRNAs levels were correlated with disease stage (diuretic-sensitive vs. refractory ascites), the cardiac systolic function measured by echocardiography, and the portal pressure response to NSBBs.

This was a collaborative translational project involving human samples. The study protocol adhered to the principles of the Declaration of Helsinki. The study´s first stage in which the miRNA signature was identified was conducted at the August Pi i Sunyer Biomedical Research Institute-Hospital Clinic of Barcelona, and its protocol was approved by this center´s Ethics Committee (HCB/2018/0028). The protocol for subsequent stages received approval from the Ethics Committee for Clinical Research of the Hospital Universitario Ramon y Cajal (institutional review board number 362/19; approval date April 1, 2019). All included subjects provided written informed consent authorizing the storage and research use of their biological samples.

IDENTIFICATION OF A NOVEL miRNA SIGNATURE IN THE SECRETOME OF CULTURES OF HUMAN HEPATOCYTES

Hepatocytes were isolated from explants of livers from patients with decompensated cirrhosis (n = 9, all of alcoholic etiology) and livers from patients undergoing partial liver resection for hepatic metastases of

colorectal cancer (n = 9) who had not received chemotherapy. The viability of samples from liver metastases was assessed by the Pathology Department to ensure that the isolated hepatocytes were not affected by their proximity to tumor tissue. For the isolation and culture of hepatocytes, we used validated protocols.[14] Cells were seeded on collagen-coated 150-cm^2 Petri dishes at a density of 2×10^7 cells per dish and cultured (37°C, 5% CO_2) in Dulbecco's modified Eagle's medium F-12 Ham supplemented with penicillin-streptomycin solution (1% volume [vol]/vol), L-glutamine (1% vol/vol), and exosome-depleted fetal bovine serum (2% vol/vol). After 24 hours of culture, the supernatant was collected, centrifuged at 2,000g for 10 minutes at room temperature, and filtered with 0.22-μm filters to remove apoptotic bodies. Subfractions enriched in small EVs generated by the hepatocytes, mainly exosomes, were obtained by subsequent ultracentrifugations.[15,16] Small EV subfractions were routinely tested for purity and quality by nanoparticle tracking analysis (NanoSight). The Bradford assay was used to determine protein concentration, and the EVs were stored at –80°C for further analysis. EV samples were pooled in groups of three for proper RNA isolation and analysis. Total RNA was isolated (miRVANA Paris Kit; Ambion/Thermo Fisher Scientific) from 50 μg of EV preparation and 2 μL of 1 nM. Each sample was spiked with *Caenorhabditis elegans* miR-39-5p. The quantity and profiles of the obtained RNAs were examined using Agilent RNA 6000 Pico Chips (#5067-1513; Agilent Technologies). Sequencing libraries were prepared using NEXTflex Small RNA-Seq Kit v3 (#5132-05; Bioo Scientific Corporation) following the protocol for NEXTflex Small RNA-Seq Kit v3 V16.06. Briefly, RNA samples were incubated for 2 minutes at 70°C, then 3′ 4N adenylated adapter and ligase enzyme were added; ligation was conducted overnight at 20°C. After removal of excess 3′-adapter, 5′-adapter and ligase enzyme were added and the mix incubated at 20°C for 1 hour. The ligation product was used for reverse transcription with Moloney murine leukemia virus reverse transcriptase in a thermocycler for 30 minutes at 42°C and 10 minutes at 90°C. Next, complementary DNA (cDNA) was enriched by polymerase chain reaction (PCR) cycling as follows: 2 minutes at 95°C; 22-25 cycles of 20 seconds at 95°C, 30 seconds at 60°C, and 15 seconds at 72°C; final elongation for 2 minutes at 72°C; and the reaction stopped at 4°C. PCR products were resolved on 6% Novex Tris/borate/ethylene diamine tetraacetic acid polyacrylamide gel electrophoresis gels (#EC6265BOX; Thermo

Fisher Scientific), and the band between 150 base pairs (bp) and 300 bp was cut out from the gel. The products were extracted from the polyacrylamide gel using an adapted protocol in which cDNA from gel slices was diluted in double-distilled H_2O. Next, the libraries were analyzed quantitatively and qualitatively in an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit (#5067-4626; Agilent Technologies) and Qubit double-stranded DNA HS DNA Kit (#Q32854; Thermo Fisher Scientific). The libraries were single-read sequenced for 51 nucleotides in a HiSeq2500 System (Illumina) at the CIC bioGUNE genome analysis platform. After sequencing, the raw data were processed to identify miRNAs differentially expressed among the established groups by univariate analysis according to the criteria that the fold change was at least 1.5 and $P <$ 0.05 (all microRNAs identified after sequencing are indicated in Supporting Fig. S1). The differential expression of selected miRNAs was validated by specific TaqMan individual quantitative reverse-transcription (qRT)-PCR assays. All five miRNAs comprising the newly identified signature (miR-452-5p, miR-429, miR-885-5p, miR-181b-5p, and miR-122-5p) were registered at the miRBase database (www.mirbase.org). RNA sequencing data are deposited and stored in Gene Expression Omnibus under accession number GSE150867.

ANALYSIS OF THE miRNA SIGNATURE IN SERUM SAMPLES FROM PATIENTS WITH DECOMPENSATED CIRRHOSIS AND HEALTHY CONTROLS

The novel miRNA signature along with three miRNAs (miR-192-5p, miR-34a-5p, and miR-29a-5p) identified previously as biomarkers of chronic liver disease[17-19] were measured in serum samples from patients with decompensated cirrhosis and healthy individuals.

Serum samples from patients with decompensated cirrhosis were from a registered collection (C.0005352, Collections Section of the Biobank Registry of Instituto de Salud Carlos III, Madrid, Spain) of a cohort of 36 patients with cirrhosis and ascites treated with an NSBB (propranolol) for primary prophylaxis of variceal bleeding. Serum samples were collected before and 4 weeks after starting treatment with NSBBs between April 2016 and December 2018, and miRNA assays were performed in April 2019. Serum samples were centrifuged at

2,000g for 15 minutes at 22°C, and the supernatants aliquoted and stored at –80°C until miRNA analysis.

Healthy individuals were those matched for sex and age with no history of liver disease. Serum samples (n = 36) from the local blood transfusion center were provided by the Hospital Universitario Ramon y Cajal-Instituto Ramon y Cajal de Investigacion Biosanitaria (IRYCIS) (Registry of Biobanks of the Instituto de Salud Carlos III No. B.0000678) within the National Network of Biobanks (PT17/0015/0010). All samples were obtained with the approval of the corresponding ethics and scientific committees and processed following standard procedures. Serum was obtained by centrifugation at 1,100g for 10 minutes at 4°C, then aliquoted and immediately stored at –80°C.

For serum miRNA analysis, we first isolated total RNA from 200 μL of serum with the Plasma/Serum RNA Purification Mini Kit (#55000; Norgen Biotek, Thorold, Canada) according to the manufacturer´s protocol. Next, the expression of miRNAs was analyzed through amplification in individual qRT-PCR reactions using commercially available locked nucleic acid probes specific for each miRNA of interest (miRNA primer sets from Exiqon A/S, Vedbæk, Denmark). All reactions were performed in triplicate with Light Cycler 480 equipment (Roche) following the supplier´s guidelines. The relative expression of each miRNA was calculated using miR-103a-3p as the housekeeping gene to minimize analytic variability and obtain a reliable and reproducible result. This housekeeping gene showed the least variability among the tested normalizers recommended for serum by the supplier (Qiagen) (miR-103a-3p, miR-191-5p, miR-30a, and miR-124-3p). miR-103a-3p was chosen for data normalization as it showed the lower SD between all samples tested. In fact, miR-103a-3p and miR-191-5p have been reported to be stable endogenous reference genes in serum for data normalization.[20] miRNA levels are expressed according to the comparative cycle threshold (Ct) method ΔCt, where ΔCt = average $Ct_{\text{tested miRNA}}$ – average $Ct_{\text{miR-103a-3p}}$ (average Ct is the mean of the technical triplicate), which inversely correlates with the amount of miRNA in the serum.

CLINICAL CHARACTERIZATION AND STUDY OF PATIENTS WITH DECOMPENSATED CIRRHOSIS

A cohort of 36 patients with decompensated cirrhosis, 18 of whom had refractory ascites defined according to the International Club of Ascites,[21] was examined before and 4 weeks after starting treatment with an NSBB (propranolol) as part of a prospective protocol (ClinicalTrials.gov NCT02163512).[7] Examinations included a comprehensive Doppler echocardiography study, hepatic and cardiopulmonary hemodynamic assessment, and blood tests. In brief, heart rate and mean arterial pressure were measured with an automatic sphygmomanometer; free and wedged hepatic venous pressures were obtained by placing a balloon catheter in the hepatic vein; cardiac output was measured by thermodilution using a Swan-Ganz catheter; and color Doppler M-mode images from the five-chamber view were obtained by echocardiography. The HVPG was calculated as the difference between mean wedged and free hepatic pressures. Systemic vascular resistance was estimated according to the formula (mean arterial pressure – right atrial pressure) × 80 / cardiac output. Hemodynamic response to NSBBs was defined as a decrease in HVPG by ≥10% because this has been proposed as the best target to define a response in primary prophylaxis.[9,10] Cardiac systolic function was estimated by Doppler echocardiography using custom software through the ejection intraventricular pressure difference (EIVPD). This index accounts for the difference in pressure between the apex and the left ventricle outflow tract in the normal heart due to blood flow acceleration during early systole. The EIVPD is a robust loading-independent index closely related to the force of contractility of the left ventricle that has been proved highly reliable in decompensated cirrhosis.[7,22] Detailed information on the methods used for clinical measurements is provided as online Supporting Material.

BIOINFORMATICS STUDIES

The potential functional significance and interactions of the miRNAs were assessed through bioinformatics data mining. Functional enrichment analysis was performed on predicted target genes of the selected miRNAs using cluster Profiler software.[23] To assess interactions, we used the miRGate database and accepted those predictions with three or more computational validations or one experimental validation.[24] Bipartite networks were built using the selected miRNAs as source and regulated genes as targets.

STATISTICAL ANALYSIS

The Shapiro-Wilk test and distribution plots were used to establish the normality of the data. Continuous variables provided as the median and interquartile range were compared through univariate analysis using a nonparametric test (Mann-Whitney U test). For paired continuous data, the Wilcoxon signed-rank test was used. Categorical variables are provided as absolute and relative frequencies, and the Fisher´s exact test was used for intergroup comparisons. The expression of miRNAs was correlated with the response to NSBBs by calculating the Spearman correlation coefficient. The area under the curve (AUC) was used to assess the diagnostic performance of the selected miRNAs. Optimal cutoffs of sensitivity and specificity were calculated for each miRNA based on the receiver operating characteristic curves. Statistical analysis was performed using Stata/IC 14.0. All tests were two-tailed. Significance was set at $P < 0.05$.

Results

IDENTIFYING AN miRNA SIGNATURE IN THE SECRETOME OF PRIMARY HUMAN HEPATOCYTES

We first analyzed the secretome of primary human hepatocytes isolated from tissue remnants of liver explants and partial hepatic resections from patients with cirrhosis and liver metastases, respectively. This involved examining the noncodifying RNA contents of small EVs present in the secretome. We identified a signature of five miRNAs (miR-452-5p, miR-429, miR-885-5p, miR-181b-5p, and miR-122-5p) that were differentially expressed in the secretome of hepatocytes from livers with and without cirrhosis.

miRNA SIGNATURE VALIDATION IN SERUM SAMPLES OF PATIENTS WITH CIRRHOSIS AND CONTROLS

Our next step was to validate the identified miRNA signature in the serum of patients with decompensated cirrhosis. As a control, we also checked whether three miRNAs (miR-192-5p, miR-34a-5p, and miR-29a-5p) previously identified as biomarkers of chronic liver disease were differentially expressed in total circulating RNA isolated from patients and healthy controls.

Serum samples from 36 patients with cirrhosis with ascites (50% refractory ascites) and 36 age- and sex-matched healthy controls were tested for the expression of the novel miRNA signature. miRNAs could not be determined in 3 patients with cirrhosis as samples were collected in heparinized tubes causing qRT-PCR inhibition. These 3 patients and their corresponding controls were thus excluded from the study. The baseline characteristics of the study population (33 patients with cirrhosis, 81.8% men, mean age 58 years [range, 54-61], 16 with diuretic-sensitive ascites, and 17 with refractory ascites) are detailed in Table 1.

TABLE 1. BASELINE CHARACTERISTICS OF THE STUDY POPULATION

	Diuretic-Sensitive Ascites n = 16	Refractory Ascites n = 17	P Value
Age, years	55 (52-59)	59 (54-65)	0.4
Male sex	13 (81.2%)	14 (82.3%)	0.64
Etiology of cirrhosis			0.15
Alcohol	13 (81.2%)	10 (58.8%)	
Viral	3 (18.8%)	7 (41.2%)	
Child-Pugh score			0.15
B	13 (81.2%)	10 (58.8%)	
C	3 (18.8%)	7 (41.2%)	
MELD score	12 (10-16)	16 (13-17)	0.12
Baseline laboratory parameters			
Bilirubin (mg/dL)	1.9 (1-3.5)	2.2 (1.3-3.6)	0.63
Albumin (g/dL)	3 (2.5-3.3)	2.9 (2.6-3.3)	0.97
INR	1.3 (1.3-1.5)	1.5 (1.3-1.6)	0.17
Creatinine (mg/dL)	0.7 (0.7-0.8)	0.9 (0.7-1)	0.08
eGFR (mL/minute/1.73 m^2)	103 (84-120)	79 (58-87)	0.01
Cystatin C (mg/dL)	1.2 (1.1-1.2)	1.6 (1.2-2)	0.03
Sodium (mEq/L)	135 (133-137)	132 (131-134)	0.01
Baseline hemodynamic parameters			
Mean arterial pressure (mm Hg)	91 (85-97)	80 (74-92)	0.006
Systemic vascular resistance (dynes.seg/cm^{-5}/m^2)	1,006 (828-1,170)	937 (784-1,179)	0.69
HVPG (mm Hg)	21 (19-25)	23 (19-25)	0.81
EIVPD (mm Hg)	4.1 (3.2-5.4)	4.6 (2.9-5.8)	0.65

Quantitative variables are provided as median and interquartile range. Qualitative variables are provided as absolute values and percentages.
Abbreviations: eGFR, estimated glomerular filtration rate; INR, international normalized ratio; sec, seconds.

Our validation study in serum samples of the miRNA signature confirmed different expression levels in patients versus controls of four of the five miRNAs comprising the signature and two of the three miRNAs assessed as controls. Compared to controls, patients with cirrhosis showed significantly ($P < 0.005$) lower levels of miR-429, miR-885-5p, miR-181b-5p, miR-122-5p, miR-192-5p, and miR-29a-5p ($P < 0.05$) and similar levels of miR-452-5p and miR-34a-5p (Fig. 2). Of these differentially expressed markers, miR-29a-5p showed the highest AUC for the identification of cirrhosis (AUC, 0.88; 95% confidence interval [CI], 0.8-0.95; sensitivity, 78.8%; specificity, 78.8% for cut-off point 1.53), as depicted in Supporting Fig. S2. Of note, miRNA serum levels did not correlate with the classic scores of liver function (Model for End-Stage Liver Disease [MELD], MELD-Na, and Child-Pugh), as detailed in Supporting Table S1.

SERUM miRNA SIGNATURE EXPRESSION AND CLINICAL OUTCOMES IN PATIENTS WITH DECOMPENSATED CIRRHOSIS

Serum miRNA Expression in Refractory Ascites

Baseline levels of miR-181b-5p were significantly higher in patients with refractory ascites than diuretic-sensitive ascites ($P = 0.008$). miR-181b-5p levels showed an AUC of 0.77 (95% CI, 0.59-0.93; sensitivity, 75%; specificity, 70.6% for cut-off point 6.57) to identify patients with refractory ascites, as depicted

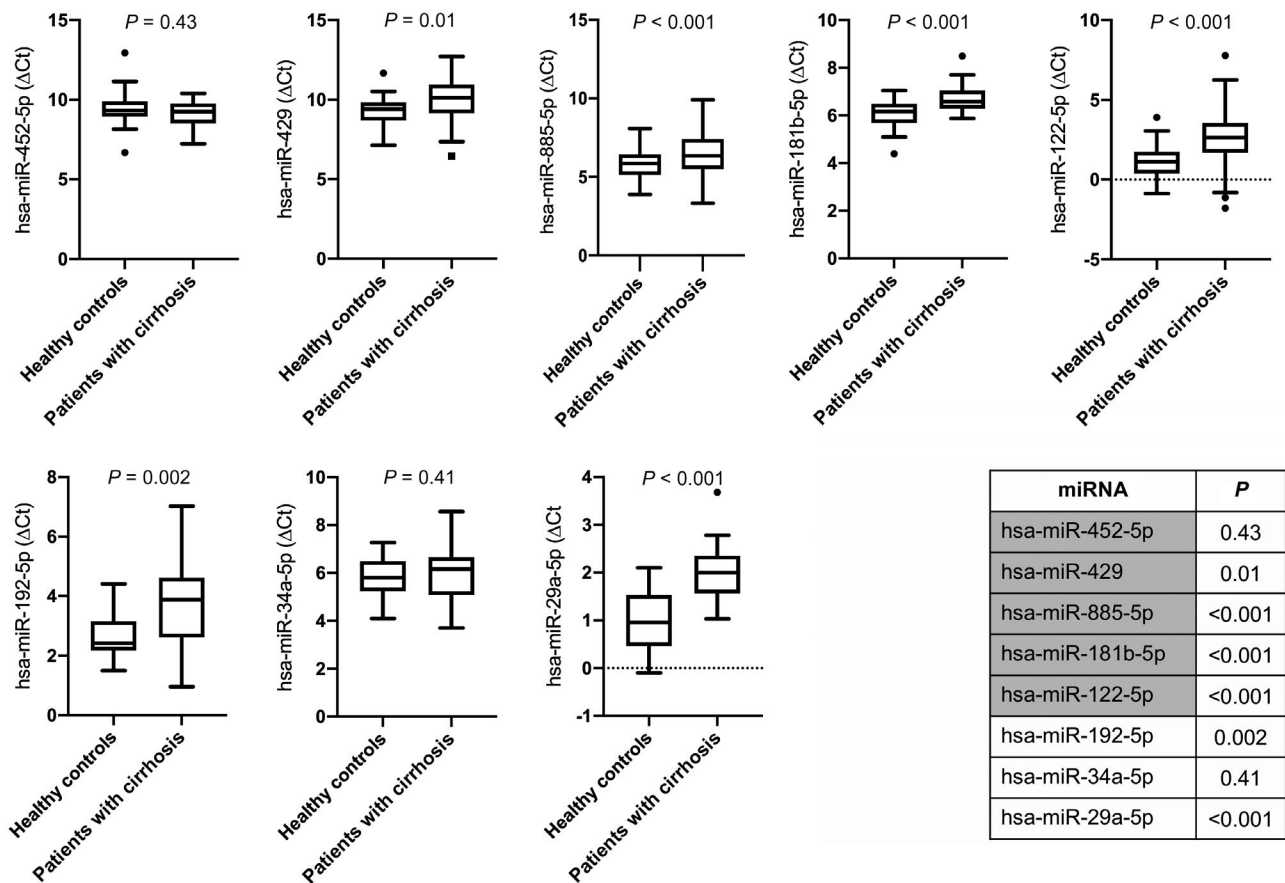| miRNA	P
hsa-miR-452-5p	0.43
hsa-miR-429	0.01
hsa-miR-885-5p	<0.001
hsa-miR-181b-5p	<0.001
hsa-miR-122-5p	<0.001
hsa-miR-192-5p	0.002
hsa-miR-34a-5p	0.41
hsa-miR-29a-5p	<0.001

FIG. 2. miRNA serum expression in patients with cirrhosis and healthy controls. The vertical lines indicate the range, the middle horizontal line is the median, and the horizontal boundaries of the boxes represent the first and third quartile. miRNAs from the novel signature appear highlighted in the table.
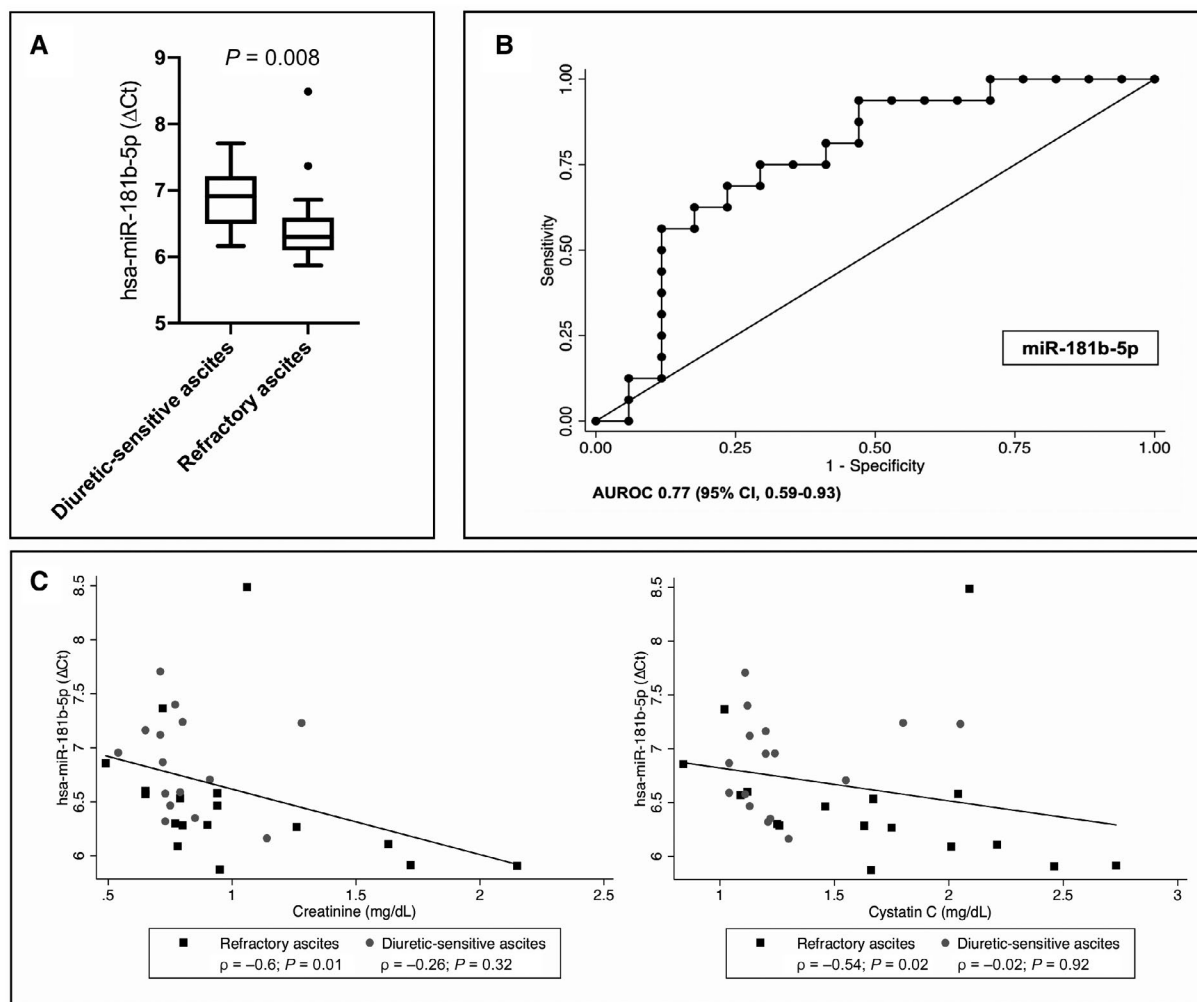
FIG. 3. Diuretic-sensitive versus refractory ascites. (A) miR-181b-5p serum expression in patients with diuretic-sensitive and refractory ascites. The vertical lines indicate the range, the middle horizontal line is the median, and the horizontal boundaries of the boxes represent the first and third quartile. (B) Receiver operator characteristic curve of miR-181b-5p to identify patients with refractory ascites. Each point on the ROC curve represents a different cutoff value for a sensitivity/specificity pair. (C) Correlation between miR-181b-5p and renal function parameters. Points represent individual patients. Abbreviation: AUROC, area under the receiver operator characteristic curve.

in Fig. 3A,B. The other miRNAs examined did not differ significantly in their levels between refractory and diuretic-sensitive ascites (Supporting Fig. S3). Levels of miR-181b-5p expression showed an inverse correlation with indicators of renal function (serum creatinine, $\rho = -0.5$, $P = 0.002$; and cystatin C, $\rho = -0.44$, $P = 0.01$). This correlation was restricted to patients with refractory ascites (creatinine, $\rho = -0.6$, $P = 0.01$ in refractory ascites vs. $\rho = -0.26$, $P = 0.32$ in diuretic-sensitive ascites; cystatin C, $\rho = -0.54$, $P = 0.02$ in refractory ascites vs. $\rho = -0.02$, $P = 0.92$ in diuretic-sensitive ascites), as detailed in Fig. 3C.

In the whole group of patients with ascites, serum levels of miR-452-5p and miR-885-5p correlated with severity of systemic vasodilation. Lower systemic vascular resistance had lower serum levels of miR-452-5p and miR-885-5p ($\rho = -0.46$, $P = 0.007$; and $\rho = -0.42$, $P = 0.01$, respectively), as represented in Fig. 4.

Serum miRNA Expression and Response to Beta-Blockade

Mean HVPG at baseline was 22 mm Hg,[19-25] with no significant differences between refractory and
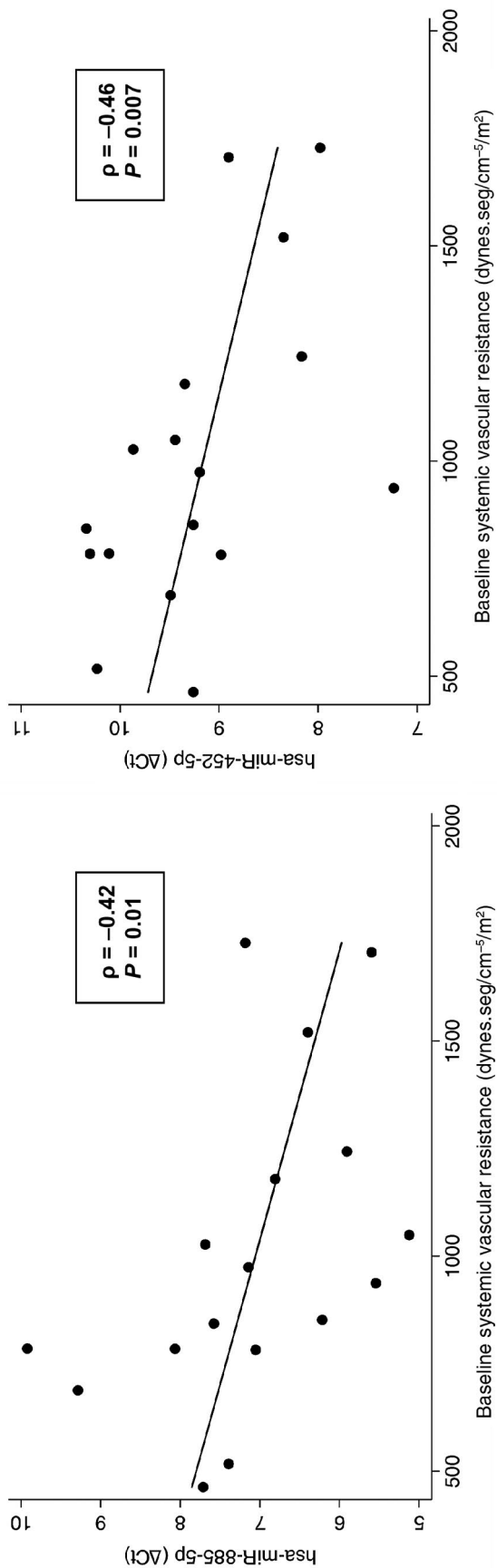
FIG. 4. Correlation between miR-452-5p and miR-885-5p with baseline systemic vascular resistance in patients with refractory ascites. Points represent individual patients. Abbreviation: sec, seconds.

diuretic-sensitive ascites (P = 0.81). Baseline serum miRNAs levels showed no correlation with baseline HVPG (Supporting Table S1). miRNA levels did not significantly change after NSBBs, as represented in Supporting Fig. S4. Seventeen patients (51.5%) showed a hemodynamic response to NSBBs defined as a drop in HVPG ≥ 10%. Baseline serum miR-452-5p and miR-429 levels were significantly lower (P < 0.05) in responders than nonresponders to NSBBs (Fig. 5A). Levels of miR-181b-5p and miR-29a-5p showed a similar tendency, albeit nonsignificant (P = 0.05 and P = 0.06, respectively), while other miRNAs did not differ significantly between responders and nonresponders (Supporting Fig. S5). Their AUC to identify a response to NSBBs was 0.77 (95% CI, 0.59-0.95; sensitivity, 82.4%; specificity, 75% for a 9.26 cutoff) for miR-452-5p and also 0.77 for miR-429 (95% CI, 0.61-0.94; sensitivity, 76.5%; specificity, 81.2% for a 10.2 cutoff), as represented in Fig. 5B.

Treatment with NSBBs resulted in significantly reduced systolic cardiac function as estimated by EIVPD in patients with refractory ascites before NSBBs (mean, 4.6; 2.9-5.8 mm Hg) versus after NSBBs (mean, 3.3; 3-4.1 mmHg; P = 0.005) but not in those with diuretic-sensitive ascites (P = 0.3). Baseline miR-452-5p and miR-885-5p inversely correlated with impaired systolic function produced after NSBBs in patients with refractory ascites (ρ = –0.55, P = 0.02; and ρ = –0.55, P = 0.02, respectively), as shown in Fig. 6.

FUNCTIONAL SIGNIFICANCE OF miRNAs

Our bioinformatics analysis was designed to assess the potential functional implications of the miRNAs examined here. As illustrated in Supporting Fig. S6, these miRNAs and their target genes play essential roles in several biological processes, mostly in cell response to hypoxia.

We found a functional relationship among the validated miRNAs based on shared target prediction, which is represented as a network in Supporting Fig. S7. In this relationship, miR-452-5p, miR-429, miR-181b-5p, miR-122-5p, miR-192-5p, and
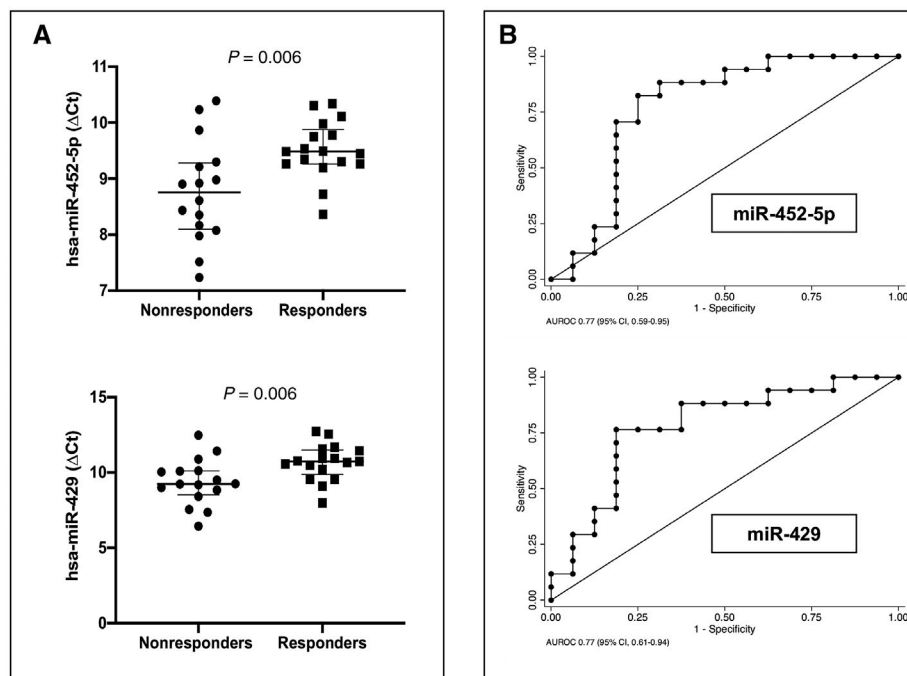
FIG. 5. Hemodynamic response to NSBBs. (A) miR-452-5p and miR-429 serum expression in patients with and without hemodynamic response to NSBBs. The vertical lines indicate the range, and the horizontal lines are the median and first and third quartile. Points represent individual patients. (B) Receiver operator characteristic curves of miR-452-5p and miR-429 to predict hemodynamic response to NSBBs. Each point on the ROC curve represents a different cutoff value for a sensitivity/specificity pair. Abbreviation: AUROC, area under the receiver operator characteristic curve.

FIG. 6. Correlation between miR-452-5p and miR-885-5p with the fall of systolic function after NSBB.

miR-34a-5p control the expression of common targets that might cooperate to promote cell response; miR-429, miR-181b-5p, and miR-34a-5p control the expression of a common target, B-cell lymphoma 2 (BCL2); and miR-429, miR-181b-5p, and miR-192-5p seem to be related through the gene X-linked inhibitor of apoptosis protein (XIAP). Additionally, BCL2 and XIAP are genes involved in the apoptotic process.
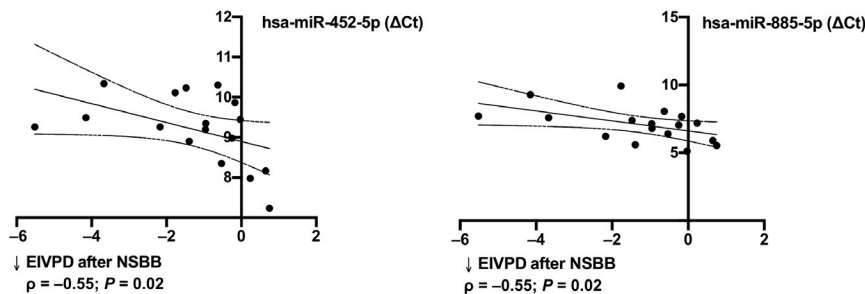
Discussion

To the best of our knowledge, this is the first study to focus on circulating miRNAs as potential biomarkers of disease stage in decompensated cirrhosis. Our findings reveal the differential expression of a novel miRNA signature in the serum of patients with refractory ascites. Our study also shows the capacity of this signature to distinguish patients with decompensated cirrhosis with severe peripheral vasodilation, who are more prone to develop significant cardiac impairment after NSBBs, as well as those more likely to respond to NSBBs in terms of HVPG reduction. In addition to the novel signature, we also examined the behavior of three miRNAs that have been previously related to chronic liver disease. We confirmed that two of these three miRNAs were able to differentiate patients with cirrhosis from healthy controls, but they did not serve to characterize decompensated cirrhosis or to predict the response to beta-blockade.

The translational design of our study allowed for the identification of a signature of circulating miRNAs. We hypothesized that serum miRNAs with potential diagnostic value as biomarkers in decompensated cirrhosis might include, among others, miRNAs previously identified in the secretome of hepatocytes from the cirrhotic livers of patients with decompensated disease. For this purpose, we first identified miRNAs differentially expressed in the EVs of the supernatant of cultures of human hepatocytes from cirrhotic and noncirrhotic livers. We postulated that the miRNA signature characterized *in vitro* could be a marker of clinical outcomes in decompensated cirrhosis. It is conceivable that the miRNA content of EVs is specific of its cell source and reflects the physiological/pathological state of the corresponding tissue.[25] In keeping with this approach and for the sake of methodological simplicity, we opted for the analysis of miRNA in serum instead of isolated serum EVs. Our results confirmed our working hypothesis as the miRNA signature first identified in EVs and thereafter in serum was found to correlate with outcomes in patients with decompensated cirrhosis.

Six of the miRNAs examined (miR-429, miR-885-5p, miR-181b-5p, miR-122-5p, miR-192-5p, and miR-29a-5p) were differentially expressed in our cohort of patients with decompensated cirrhosis compared to controls. The best diagnostic accuracy for cirrhosis was shown by miR-29a-5p, which has been reported to be down-regulated in patients with cirrhosis.[19] The five other miRNAs appearing in significantly lower levels in the serum of patients with cirrhosis were miR-429, which has not been studied in liver disease; and miR-122-5p, miR-885-5p, miR-181b-5p, and miR-192-5p, for which higher serum levels have been detected in patients with chronic liver disease compared to controls. The presence of miRNAs has been explored in several liver diseases, such as viral hepatitis, nonalcoholic fatty liver disease

(NAFLD), or hepatocellular carcinoma.[26-28] In these studies, however, patients with decompensated cirrhosis were barely represented. This could explain the discrepancies (up-regulation vs. down-regulation) in the levels of circulating miRNAs between our study and those of others. In agreement with our findings, reduced serum levels of miR-122, the most abundant miRNA in the liver, and of miR-885-5p have been reported in patients with decompensated compared to those with compensated cirrhosis.[29-31] Such lower serum levels of these miRNAs in patients with more advanced disease may be secondary to their reduced secretion due to a loss of functional hepatocytes rather than to their increased release from injured hepatocytes seen in earlier stages of liver diseases.

A noteworthy finding of our study was the ability of miR-181b-5p to identify patients with refractory ascites. Whereas the role of miRNAs as biomarkers in chronic liver disease has focused on diagnosis and NAFLD stratification,[32] ours is the first study to explore their predictive value specifically in decompensated cirrhosis. Refractory ascites is clinically defined by expert consensus as a stage of further decompensation of cirrhosis characterized by severe circulatory dysfunction and limited cardiac reserve.[7,21] Classical scores of liver function do not reflect the severity of circulatory dysfunction or the labile nature of renal function these patients feature.[7] Indeed, while there were no differences in Child-Pugh and MELD between patients with diuretic-sensitive and refractory ascites in our cohort, remarkably, miR-181b-5p was up-regulated in the serum of patients with refractory ascites and showed an AUC of 0.77 to identify this patient subset. Moreover, serum levels of this miRNA were correlated with the degree of renal impairment. The marked differences in serum miRNA levels between refractory and diuretic-sensitive ascites support the notion of a distinctive pathophysiological background underlying the classical clinical differentiation of the two substages of decompensated cirrhosis, which are accurately identified by the miRNA signature. Further, we have also observed that a greater serum expression of miR-452-5p and miR-885-5p shows a greater severity of peripheral vasodilation, i.e., the ability of these miRNA to identify those patients with the more severe circulatory dysfunction. Interestingly, because the severity of peripheral vasodilation and the reduction in systolic function induced by beta-blockade are closely related, expression levels of miR-452-5p and miR-885-5p were also linked to the declining systolic function after NSBBs.[7] Taken together, our results provide proof-of-concept evidence of the value of miRNA profiling to stratify decompensated cirrhosis.

Our study also explores whether miRNAs are able to predict the HVPG response to NSBBs. NSBBs prevent primary and recurrent variceal bleeding and improve survival in patients with compensated and decompensated cirrhosis.[9-12] The benefits of NSBBs for clinical outcomes are closely linked to the fall in HVPG produced in response to beta-blockade, which is largely variable among patients and can only be addressed by its measurement.[11] Thus, noninvasive biomarkers, i.e., differential expression of miRNAs, could represent an innovative theranostic approach to identify this HVPG response to NSBBs. We found that HVPG responders to NSBBs had lower baseline serum levels of miR-452-5p and miR-429, suggesting their potential clinical utility as prognostic of the portal pressure response to NSBBs. Similarly, other miRNAs, such as miR-19a, miR-101, and let-7e, which regulate β1-adrenergic receptors, and other beta-blocker pharmacodynamics-related genes, have been associated with the antihypertensive response to beta-blockers.[33]

Elucidating the biological functions of individual miRNAs is complex as they may influence several messenger RNAs and more than one miRNA can regulate one messenger RNA. The search of biomarkers is further complicated by the fact that the underlying mechanisms of the clinical processes addressed in this study, i.e., refractory ascites, HVPG response to NSBBs, are multifactorial. Most of the miRNAs shown here to correlate with clinical outcomes in decompensated cirrhosis have also been associated with disorders of renal function and cardiocirculatory dysfunction, all of which are involved in chronic liver disease progression. In this context, miR-181b-5p, which served to identify the most severe stage of cirrhosis (refractory ascites), has been related to proliferation and activation of hepatic stellate cells[34,35] and cardiovascular remodeling.[36-38] Accordingly, miR-181b-5p has been proposed as a marker of fibrosis severity in chronic liver disease,[39] disease progression in chronic hepatitis B,[40] and cardiac dysfunction in diabetic cardiomyopathy.[38] However, it is likely that the role of miRNAs in modifying biological processes involves more their cooperation to regulate gene networks and repress one

or more target genes than individual actions. Our analysis of the functions possibly overrepresented in the set of genes regulated by our novel signature revealed their involvement largely in the response to hypoxia by the control of critical genes involved in the apoptotic process, such as BCL2 or XIAP. Hypoxia plays a pivotal role in the natural history of cirrhosis as it stimulates angiogenesis, inhibits cell proliferation factors, and promotes fibrogenesis, contributing to progressive portal hypertension and hyperdynamic circulation.[41]

We acknowledge certain limitations of our study. First, its exploratory nature and limited sample size means its results need to be validated in larger cohorts. Second, the miRNA signature identified as representative of changes in the secretome of the hepatocytes from cirrhotic livers was identified and sequenced from small EVs, whereas we analyzed individual miRNAs in total serum. We ruled out our initial plan to only isolate small EVs in the serum samples because there is no efficient method to obtain these vesicles from blood, especially in a large number of samples already collected and aliquoted in small volumes. To avoid losing information, we move toward a more reproducible and pragmatic approach that does not require sophisticated equipment to facilitate clinical use. Thus, using a commercial kit, we isolated the whole RNA content of the sample, including that associated with small EVs and exosomes, and then analyzed the individual miRNAs of the signature. It could also be argued that miRNAs are produced by multiple cell types and miRNAs in primary cell cultures may differ from those in circulation. However, our study does not attempt to identify the cell source of the miRNAs but rather their validity as biomarkers in a specific pathological context, namely, for the noninvasive identification of patients in whom NSBBs could be futile or harmful. Finally, validation of the miRNA signature should include cirrhosis etiologies other than alcohol because the latter was the cause of cirrhosis in the livers from which primary human hepatocytes were obtained as well as the most frequent etiology of cirrhosis in our patient series.

In conclusion, this study identifies a novel circulating miRNA signature that allows for the molecular characterization of patients with decompensated cirrhosis. The up-regulation of miRNA 181b-5p was identified as a feature of refractory ascites. In addition, the down-regulation of miRNA 452-5p and 885-5p

was found to reflect the magnitude of hemodynamic impairment that occurs in these patients as these miRNAs identify patients with severe circulatory dysfunction who are more likely to develop systolic cardiac impairment in response to beta-blockade. Finally, down-regulation of miR-452-5p and miR-429 identified patients more likely to have a significant reduction in portal pressure when treated with NSBBs. The present proof-of-concept study indicates that miRNAs are distinctive biomarkers of advanced decompensated cirrhosis and of relevant clinical outcomes, including the patient response to treatment with NSBBs.

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