MDPI

Article

Drug-Induced Liver Injury: Role of Circulating Liver-Specific microRNAs and Keratin-18

Romilda Cardin †, Debora Bizzaro †, Francesco Paolo Russo, Francesca D'Arcangelo, Francesco Ideo, Filippo Pelizzaro, Chiara Carlotto, Milena Minotto, Fabio Farinati, Patrizia Burra * and Giacomo Germani

Gastroenterology and Multivisceral Transplant Unit, Department of Surgery, Oncology and Gastroenterology, Padua University Hospital, 35128 Padua, Italy; romilda.cardin@unipd.it (R.C.); debora.bizzaro@gmail.com (D.B.); francescopaolo.russo@unipd.it (F.P.R.); francesca.darcangelo02@gmail.com (F.D.); francesco.ideo@studenti.unipd.it (F.I.); filippo.pelizzaro@unipd.it (F.P.); chiara.carlotto@unipd.it (C.C.); milena.minotto@unipd.it (M.M.); fabio.farinati@unipd.it (F.F.); giacomo.germani@unipd.it (G.G.)

* Correspondence: burra@unipd.it

† These authors contributed equally to this work.

Abstract: Background and Objective: Drug-induced liver injury (DILI) is increasingly becoming a cause of acute hepatitis. The study evaluated the role of liver-specific microRNAs (miRNAs) and keratin-18 (K-18) markers M30 (apoptosis) and M65 (necrosis) as biomarkers of acute hepatitis. **Methods**: Sixty-eight patients were sub-grouped as DILI, HBV- and alcohol-related acute hepatitis. Five healthy controls were included. The expression of plasma miR-21-5p, miR-34a-5p and miR-122-5p was evaluated by RT-qPCR analysis using healthy volunteers as reference. M30 and M65 were determined with ELISA kits. **Results**: All markers were significantly higher in the acute liver disease patients compared to controls. In DILI, miRNA levels positively correlated with M30, M65 and ALT. miR-122-5p had the highest AUC of 0.73, sensitivity of 76.2 and specificity of 72.2 in identifying DILI from other groups. Patients with hepatocellular-pattern DILI showed higher miR-122-5p and miR-21-5p compared to patients with cholestatic or mixed pattern. A new score to discriminate DILI versus other causes of acute hepatitis was developed using the identified risk factors as follows: $0.012 \times$ miR-34a-5p + $0.012 \times$ miR-122-5p $- 0.001 \times$ M30 + 2.642×1 (if mixed pattern) + 0.014×1 (if hepatocellular pattern) + 1.887. The AUC of the score was 0.86, with a sensitivity and specificity of 81%, better than the values of the single markers. **Conclusions**: Liver-specific miRNAs and K-18 could be promising serum biomarkers of DILI, especially when used in combination.

Keywords: drug-induced liver injury; microRNA; keratin-18; acute liver injury

Citation: Cardin, R.; Bizzaro, D.; Russo, F.P.; D'Arcangelo, F.; Ideo, F.; Pelizzaro, F.; Carlotto, C.; Minotto, M.; Farinati, F.; Burra, P.; et al. Drug-Induced Liver Injury: Role of Circulating Liver-Specific microRNAs and Keratin-18. *Gastroenterol. Insights* **2024**, *15*, 1093–1105. https://doi.org/10.3390/gastroent15040075

Academic Editor: David A. Gerber

Received: 5 July 2024
Revised: 9 December 2024
Accepted: 17 December 2024
Published: 19 December 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Drug-induced liver injury (DILI) is an increasing cause of acute liver injury. According to the 2011 consensus criteria, DILI should be suspected in patients with a significant elevation of liver function tests occurring after drug exposure, alanine aminotransferase (ALT) ≥ 5 times the upper limit of normal (ULN), alkaline phosphatase (ALP) $\geq 2 \times$ ULN or ALT $\geq 3 \times$ ULN and total bilirubin (TBIL) $> 2 \times$ ULN [1]. However, ALT and aspartate aminotransferase (AST) elevation can also be induced by other conditions apart from liver damage, such as muscle injury [2]; alkaline phosphatase (ALP) elevation also occurs due to unrelated liver pathologies, such as bone pathologies, renal dysfunction, acute inflammatory diseases or malignancies. Moreover, none of the liver parameters mentioned above are specific to a certain type of liver injury [3].

Several other candidate biomarkers have been described in research studies, including glutamate dehydrogenase that has been shown to correlate well with ALT in DILI, alpha-fetoprotein, arginase-1, osteopontin, sorbitol dehydrogenase, fatty acid binding protein and cadherin-5 [4].

Another DILI candidate biomarker is keratin-18 (K-18), an epithelial cytoskeleton protein, both in its complete and caspase-cleaved form. The latter is associated with cellular apoptosis, while the former is released from the cell during necrosis. K-18 is the principal cytoskeletal protein of the hepatocytes and is very important in the maintenance of cellular integrity. The cleavage of K-18 at two distinct sites during cell death results in the release of two different peptides in the systemic circulation: M30 and M65. The advantage of utilizing these markers in DILI is that they can diagnose early stage DILI. Identifying the type and mechanism of hepatic injury allows the severity of DILI to be evaluated. Numerous studies utilized these markers to identify DILI and the severity of injury. In DILI patients, their levels have been found to be elevated earlier and to a higher degree compared to ALT [5,6].

Recently, it has been shown that changes in circulating microRNAs (miRNAs) may occur earlier than changes seen with traditional liver tests, arousing progressive interest in circulating miRNAs as promising diagnostic and prognostic biomarkers.

Changes in miRNAs expression can occur following stress conditions, such as toxicity or infection, and both acute and chronic damage lead to miRNAs alterations.

One particular miRNA, miR-122-5p, the most abundant liver-specific miRNA present in hepatocytes and released into circulation during hepatocyte damage, has been extensively studied and associated with DILI [7]. Nevertheless, not all authors agree on recognizing the role of this particular miRNA in DILI, especially when single-miRNA expression is determined to differentiate acute liver injury types.

Conversely, it was demonstrated that a combined model that comprises K-18, miR-122-5p and glutamate dehydrogenase or high-mobility group box 1 was able to detect paracetamol-induced liver injury better than ALT alone and differentiate DILI from healthy subjects and patients with other acute disease [8,9].

To explore the clinical applicability of miRNAs as non-invasive circulating biomarkers of acute hepatitis, we evaluated two other miRNAs (miR-21-5p, miR-34a-5p), previously demonstrated to be involved in the pathogenesis of liver disease [10], beyond the miR-122. These biomarkers were also evaluated in relation to K-18 epitope M30 as a marker for apoptosis and M65 as a marker for necrosis and apoptosis.

For this purpose, the results obtained in patients with DILI were compared to those obtained in two groups of patients with acute hepatitis of different etiology: HBV and alcoholic, respectively.

2. Materials and Methods

2.1. Patients

In this study, 68 consecutive patients, prospectively evaluated between June 2019 and December 2023 from the Multivisceral Transplant Unit and Gastroenterology Unit of Padua University Hospital (Italy), were enrolled and sub-grouped as DILI, HBV-related acute hepatitis (HBV) and acute alcohol-related hepatitis (AAH). Five healthy controls (controls) with no known history of liver disease, normal levels of ALT, AST, ALP and TBIL and no serologic evidence of active hepatitis were also included.

Each subject provided written informed consent to participate in the study, which was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Padova University Hospital (Protocol n° 46093).

For each patient, demographic, clinical and bioumoral data were collected at the time of diagnosis. Data collection was carried out by consulting the hospital computer system and medical records. The demographic variables considered were gender (male/female), age at diagnosis, the presence of comorbidities (cardiovascular, autoimmune, renal, gastrointestinal, oncological, dyslipidemia, diabetes mellitus) and the presence of underlying liver disease.

Regarding bioumoral data, they included parameters for the assessment of liver damage such as AST, ALT, gamma-glutamyl transferase (GGT), ALP, TBIL, and parameters for the evaluation of hepatic synthesis, such as albumin levels and international normalized

ratio (INR). The severity of liver disease was assessed by calculating the Model for End-stage Liver Disease (MELD) score.

Diagnosis of DILI was reached by a thorough examination of the medical history and after exclusion of other causes of acute hepatitis (i.e., viral, alcohol-related, autoimmune, metabolic, etc.). Radiological investigations were also performed to evaluate the liver morphology, the possible presence of focal lesions, and in order to exclude mechanical causes of cholestasis. Liver biopsy was performed in the case of uncertain diagnosis or overlapping liver disease. Drug classes responsible for the liver damage were evaluated and the pattern of liver damage was determined using the "R" value, defined as the ratio between ALT/ULN (upper limit of normal) and ALP/ULN. By convention, liver damage is defined as hepatocellular if "R" is greater than 5, cholestatic if "R" is less than 2, and mixed if "R" is between 2 and 5 [11]. It has been demonstrated that a higher R value, associated with hepatocellular injury, often suggests a more severe form of liver damage. In these cases, the prognosis can vary depending on the extent of liver dysfunction and the drug involved, but high R values are generally seen in cases where the liver injury is more acute and severe, with a higher risk of progression to acute liver failure if not identified and managed promptly. Conversely, a lower R value, which is indicative of cholestatic injury, tends to have a more favorable prognosis in most cases of DILI. Cholestatic injury usually presents with milder liver enzyme elevations, and while it can be associated with chronic liver damage in some instances, the risk of acute liver failure is lower compared to hepatocellular injury [12].

Patients with HBV-related liver failure were screened and enrolled in this study according to the following criteria: serum hepatitis B surface antigen and/or HBV-DNA positivity for a least 6 months; pre-existing chronic liver disease, compensated and decompensated cirrhosis and total bilirubin levels >85.5 Umol/L and INR > 1.5. The exclusion criteria were as follows: aged younger than 18 or older than 80 years old; pregnancy and hepatocellular carcinoma or extrahepatic malignancy [13].

Acute alcohol-related hepatitis was clinically diagnosed according to previously published criteria [14].

2.2. RNA Isolation and miRNAs Analysis

Blood samples (10 mL) from healthy donors and patients were collected and used for serum and plasma separation within two hours of blood withdrawal. Blood was first centrifuged at 3000 rpm for 15 min to obtain the serum, followed by a second centrifugation of the supernatant of plasma at 2700 rpm for 10 min to remove any cellular debris. The plasma supernatant was stored at −80 °C until the assays of biochemical markers were performed.

MiRNAs analysis was performed on the plasma of patients and controls. Total RNA was extracted from 200 µL of plasma samples using the commercially available miRNeasy Serum/Plasma Advanced Kit (Qiagen-GmbH, Hilden, Germany), following the manufacturer's protocol, and finally dissolved in 20 uL of RNase-free water. Extraction efficiency was checked by adding synthetic oligonucleotides (UniSp2, UniSp4, UniSp5) at recommended concentrations.

According to the manufacturer's instructions, reverse transcription for cDNA synthesis was performed using the miRCURY LNA RT kit (Qiagen, Hilden, Germany).

The reverse transcription reaction system contained 4 µL of template RNA, 4 µL of miRCURY RT Reaction Buffer, 2 µL miRCURY RT Enzyme Mix and 9 µL RNase-free water; samples were incubated for 60 min at 42 °C, successively for 5 min at 95 °C to inactivate the reverse transcriptase enzyme and, finally, were stored at −20 °C.

Reverse transcription efficiency was checked by adding synthetic oligonucleotides (UniSp6).

A miRCURY LNA miRNA PCR Assays and PCR Panels (Qiagen, Hilden, Germany) were used for relative quantification of miRNAs by RT-PCR analysis. RT-PCR was performed on a PRISM 7900HT system (Applied Biosystems—Foster City, CA, USA).

The reaction system contained 5 uL of SYBER Green Master Mix, 1 µL of PCR primer mix, 3 µL of cDNA template (60 × diluted) and 1 µL of RNase-free water.

The cycling conditions for RT-PCR were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s and 56 °C for 60 s and melting curve analysis from 60 to 95 °C.

The relative expression of each miRNA was calculated using the comparative cycle threshold (CT) method and $2^{-\Delta\Delta Ct}$ (fold change-fc) method, with miR93-5p, miR103a-3p, miR425-5p as internal controls for normalization. The expression levels of miRNAs were calibrated using the healthy volunteers (controls) as a reference control group.

2.3. Enzyme-Linked Immunosorbent Assay

The M30 Apoptosense ELISA (Peviva, Bromma, Sweden) measures the concentration of caspase-cleaved K-18 in plasma. The assay is based on the unique M30 antibody, which recognizes a neo-epitope of K-18 formed after caspase cleavage. The assay can be combined with the M65 ELISA (Peviva, Bromma, Sweden) which measures soluble K-18 released from dying cells due to apoptosis and necrosis.

All assays were conducted according to the manufacturer's instructions. Both assays were calibrated against the identical reference and the absorbance was determined at 450 nm. The concentration of the antigen was expressed as units per liter (U/L).

2.4. Statistical Analysis

Descriptive statistics, including means and SD, were calculated. For interval data, Student's *t*-test or analysis of variance (ANOVA) was used to compare groups. If the data were not normally distributed, the Wilcoxon rank sum test or the Kruskal–Wallis test was employed. Descriptive statistics for liver injury markers were expressed as median and interquartile ranges. Spearman's correlations were calculated to assess monotonic relationships between variables measured on an interval scale. All tests were conducted as two tailed and $p = 0.05$ was considered statistically significant. IBM SPSS Statistics (Version 25.0. Armonk, NY, USA: IBM Corp.) and GraphPad Prism version 8.3.1 (GraphPad Software, La Jolla, CA, USA) were used for all the calculations in this study.

2.5. Biomarkers of DILI Diagnosis and Prognosis

Receiver operating characteristic (ROC) analysis was used to evaluate the performance of candidate biomarkers for diagnosis of DILI patients, taking as threshold the value with maximal sensitivity and specificity (Youden J test), followed by multivariate logistic regression analysis.

Accurate outcome assessments were available for all patients. ROC curve analysis was used to evaluate which biomarkers determined in DILI, HBV and AHH patients could significantly predict death or the requirement for liver transplantation (LT).

3. Results

From June 2015 to April 2020, 68 patients were included in the study, of whom, 32 (47.1%) patients had suspected DILI, 15 (22.1%) patients had HBV-related acute hepatitis, and 21 (30.9%) had AAH. Overall, 50% of patients were male and the median (IQR) age was 45 years (37–65 years). Thirty (44.1%) patients presented an underlying liver disease and 57.3% reported other comorbidities.

When patients were stratified according to etiology of acute liver disease, no differences were found between the three groups in terms of gender, median age and presence of other comorbidities, whereas patients with AAH presented a significantly higher percentage of underlying liver disease (Table 1).

Table 1. Baseline demographics and median values of liver injury markers in the 68 patients included in the study.

	DILI *n* **= 32 (%)**	**HBV** *n* **= 15 (%)**	**AAH** *n* **= 21 (%)**	***p*-Value**
Gender, male	12 (37.5)	10 (66.7)	12 (57.1)	0.1
Age, years, median (IQR)	50 (34.5–58.3)	46 (39–56)	44 (36–56)	0.09
Underlying liver disease, *yes*	9 (28.1)	3 (20)	18 (85.7)	<0.001
Comorbidities, *yes*	19 (59.4)	6 (40)	14 (70)	0.2
INR, median (IQR)	1.2 (1.1–2.7)	1.4 (1.3–3.2)	2.1 (1.7–2.9)	<0.001
Creatinine, mg/dL, median (IQR)	0.7 (0.6–1.8)	0.8 (0.7–1.2)	1 (0.7–2.7)	0.05
Sodium, median (IQR)	139 (136–142)	136 (135–141)	132 (129–140)	<0.001
AST, U/L, median (IQR)	321 (134.8–1765)	1259 (592–2739)	107 (91–167)	<0.001
ALT, U/L, median (IQR)	470 (242.8–2449)	2171 (1442–4337)	39 (29–96)	<0.001
Bilirubin, mg/dL, median (IQR)	11.7 (1.8–37.6)	15.5 (6.8–23)	23.9 (16.8–36.5)	0.003
GGT, U/L, median (IQR)	85 (58.5–559	99 (67–473)	200 (100–392)	0.004
ALP, U/L, median (IQR)	139 (107.3–665)	144 (110–203)	141 (108–285)	0.8
Albumin, median (IQR)	35.5 (33–41)	35 (31–38.8)	26 (24–42)	0.002
MELD score, median (IQR)	19.5 (16–28)	20 (17–30.8)	30 (23–38)	<0.001
MELD-Na score, median (IQR)	21 (16.5–24.8)	22 (19–27)	32 (27–35)	<0.001

Among the DILI patients, the most frequent therapeutic classes considered responsible for the liver damage were herbs and supplements ($n = 12$, 37.5%), antimicrobials ($n = 6$, 18.8%), non-steroidal anti-inflammatory drugs (NSAIDs) ($n = 4$, 12.5%), recreational drugs ($n = 4$, 12.5%) and other agents ($n = 6$, 18.8%). When single agents were analyzed, the most commonly involved drug was amoxicillin-clavulanic acid ($n = 5$). As regards the type of liver damage, calculated using the "R" value, most patients presented hepatocellular liver damage ($n = 21$, 65.6%), while it was cholestatic in six (18.8%) patients and mixed in five (15.6%). In 3/32 (9.4%) patients, a liver biopsy was performed, the histological picture of which was concordant with the type of liver damage predicted by calculating the "R" value.

Only two DILI patients required an urgent liver transplantation and two died after admission on the waiting list for LT within 6 months (Table 2).

Table 2. Outcome data in the three patient groups.

	DILI *n* **= 32 (%)**	**HBV** *n* **= 15 (%)**	**AAH** *n* **= 21 (%)**
Normalization	28 (87.5)	13 (86.7)	0
Chronicity	0	0	7 (33.3)
Liver transplantation	2 (6.3)	1 (6.7)	6 (28.6)
Death	2 (6.3)	1 (6.7)	8 (38.1)

In the HBV group, one patient died and one underwent liver transplantation, whereas in the AAH group, eight patients died, six underwent liver transplantation and seven developed chronic liver disease.

3.1. Levels of Circulating Biomarkers

3.1.1. Plasma miRNA Expression

All three patient groups had a median level of miR-21-5p significantly higher compared to controls (DILI 2.670 fc [1.728–4.458]; HBV (2.860 fc [1.890–3.940]; AAH (2.810 fc [1.380–4.110] vs. controls 1.020 fc [0.8950–1.105]; $p = 0.0003$, $p = 0.0001$ and $p = 0.003$, respectively).

MiR-34a-5p expression was significantly higher in the three patient groups with respect to controls with the higher level in DILI (DILI 16.93 fc [3.423–37.65]; HBV (13.61 fc [8.110–20.61]; EAA (4.090 fc [2.000–12.85] vs. controls 1.040 fc [0.5000–2.015]; $p = 0.0002$, $p = 0.0001$ and $p = 0.009$, respectively).

A statistically significant drop in miR-34a-5p was observed from DILI to AAH ($p = 0.03$).

Lastly, miR-122-5p was significantly higher in DILI and HBV with respect to controls and AAH (DILI 14.84 fc [4.538–41.46]; HBV 12.70 fc [4.560–53.59]; AAH 2.390 fc [1.065–5.375]; controls 1.300 fc [0.4050–2.280]. DILI vs. controls $p = 0.0002$; HBV vs. controls $p = 0.0003$ and DILI vs. AAH $p = 0.0001$ (Figure 1).

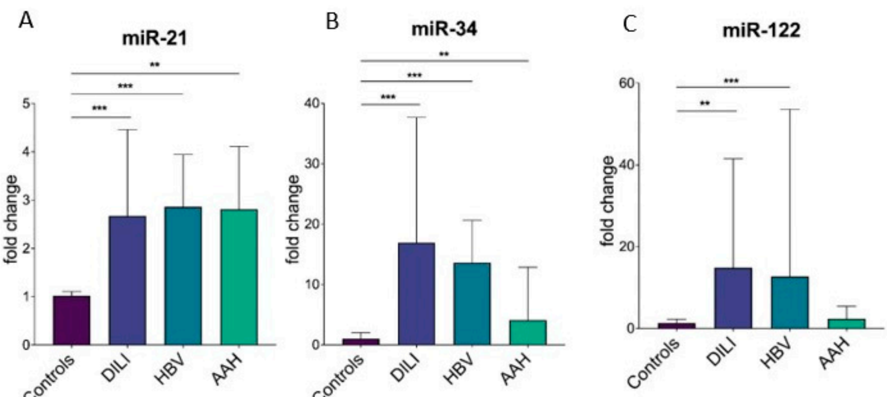

Figure 1. MiRNAs expression levels in plasma of controls, DILI, HBV and AAH patients. (**A**) MiR 21 level expressed as fold change; (**B**) MiR-34a-5p level expressed as fold change; (**C**) MiR-122-5p level expressed as fold change (** $p = 0.01$–0.001, *** $p = 0.001$–0.0001).

Stratifying the DILI patients based on R-value (ratio of ALT/ALP), the patients with a hepatocellular pattern (H) of liver disease showed higher expression of circulating miRNAs compared to patients with cholestatic pattern (C) and mixed pattern (M), considered together. MiR-21-5p: H 3.710 fc [2.320–5.955] vs. M + C 1.930 fc [1.400–2.700] $p = 0.007$. MiR-34a-5p: H 23.33 fc [8.435–65.19] vs. M + C 4.780 fc [3.290–15.35] $p = 0.04$. miR-122-5p: MiR-122-5p: H 24.84 fc [9.215–64.39] vs. M + C 8.690 fc [2.960–49.21] $p = 0.046$ (Figure 2).

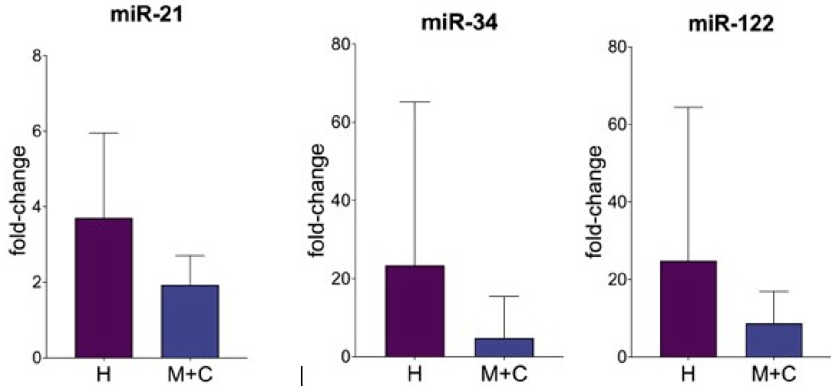

Figure 2. Plasma miRNAs levels stratifying DILI patients based on R-value (ratio of ALT/ALP). Patients with a hepatocellular pattern (H) of liver disease showed higher expression of circulating miRNAs compared to patients with a cholestatic pattern (C) and mixed pattern (M), considered together. MiR-21-5p: $p = 0.007$. MiR-34a-5p: $p = 0.04$. miR-122-5p: MiR-122-5p: $p = 0.046$.

3.1.2. K-18 Levels

The K-18 markers M30 and M65 were significantly elevated in the three patient groups with respect to controls (M30: DILI 1447 U/L [420.5–2383]; HBV (2465 U/L [1817–2934]; AAH 2913 U/L [1636–3322] vs. controls 186 U/L [147.5–202.8]; $p = 0.0002$; DILI vs. AAH $p = 0.003$ and DILI vs. HBV $p = 0.03$.

M65: DILI 1484 U/L [735.5–1962]; HBV 1890 U/L [1620–2559]; AAH (2592 U/L [1466–3364] vs. controls 171.5 U/L [165.3–188.3]; $p = 0.0003$; DILI vs. AAH $p = 0.005$ and DILI vs. HBV $p = 0.03$ (Figure 3).

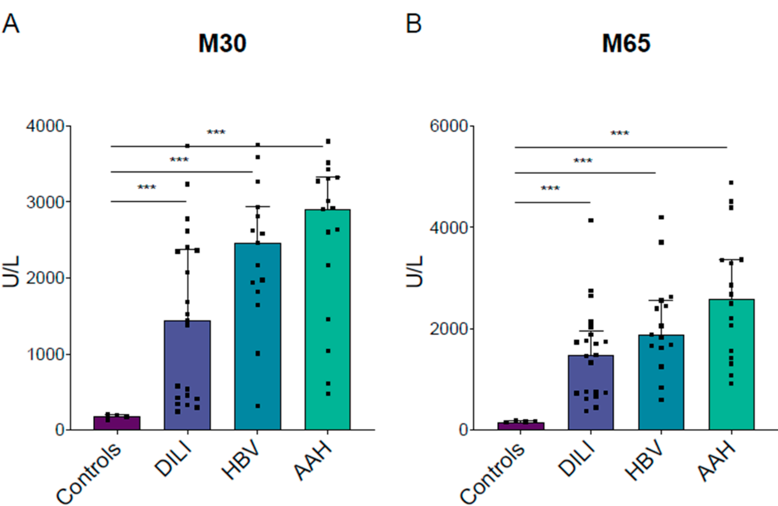

Figure 3. Serum levels of liver injury biomarkers: (**A**) cleaved K-18 (M30) and (**B**) full-length K-18 (M65). Individual dots represent each patient's data The K-18 markers M30 and M65 were significantly elevated in each of the three patient groups with respect to controls (*** p = 0.001–0.0001).

3.1.3. Linear Correlations

No correlations were observed between circulating levels of miR-21-5p, miR-34a-5p and miR-122-5p and any of the characteristics evaluated (sex, age).

In DILI patients, miR-122-5p and miR-21-5p levels positively correlated with M30 (p = 0.04, p = 0.03, respectively) and M65 (p = 0.007, p = 0.005, respectively). Moreover miR-122-5p positively correlated with ALT levels (p = 0.04).

3.1.4. ROC Curves of miRNAs

ROC analysis was performed to evaluate which levels of the three miRNAs could distinguish DILI from other acute hepatitis groups.

The cut-off for each miRNA was selected with the highest Youden's index value. Among the three miRNAs, miR-122-5p had the highest AUC of 0.73 (95% CI 0.6015–0.8642), sensitivity of 76.2 and specificity of 72.2 in separating DILI from the other groups (p = 0.0036). MiR-34a-5p had an AUC of 52.4 (95% CI 0.5486–0.8350), sensitivity of 52.4 and specificity of 83.3 (p = 0.017) (Figure 4).

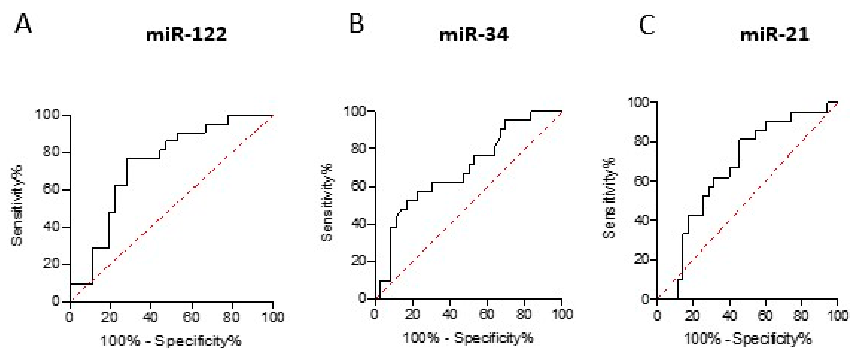

Figure 4. ROC analysis to distinguish DILI from other acute hepatitis groups. (**A**) ROC curve of miR-122-5p, (**B**) ROC curve of miR-34a-5p and (**C**) ROC curve of miR-21-5p.

The cut-off for each miRNA was selected with the highest Youden's index value. miR-122-5p had the highest AUC of 0.73, sensitivity of 76.2 and specificity of 72.2 in separating DILI from the other groups (p = 0.0036).

In contrast, miR-21-5p had an AUC of 0.62 and there were no statistically significant differences between the groups.

From ROC analysis of M30, AUC was 0.6667 (95% CI 0.5222–0.8112), sensitivity 81.0% and specificity 54.3% (p = 0.038). M65 had an AUC of 0.6408 (95% CI 0.4946–0.7870), sensitivity of 81.0% and specificity of 51.4% without, however, reaching statistical significance.

3.1.5. A Model to Predict the Development of DILI

A new score to discriminate DILI versus other causes of acute hepatitis was developed using independent risk factors as follows: 0.012 × miR34 + 0.012 × miR-122-5p − 0.001 × M30 + 2.642 × 1 (if mixed pattern) + 0.014 × 1 (if hepatocellular pattern) + 1887.

ROC analysis was conducted to evaluate the specificity and sensitivity of the score.

The AUC of the score was 0.86 (95% CI 0.7534–0.9578), the sensitivity and specificity 81%, with a better clinical value compared to the single markers (p = 0.0001) (Figure 5).

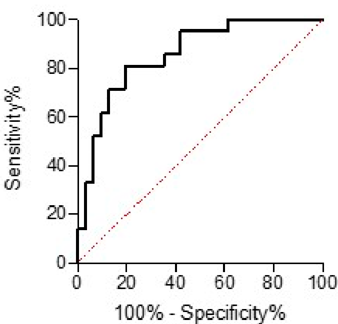

Figure 5. ROC curve of new score to discriminate DILI versus other causes of acute **hepatitis**. ROC analysis was conducted to evaluate specificity and sensitivity of the score: 0.012 × miR34 + 0.012 × miR-122-5p − 0.001 × M30 + 2.642 × 1 (if mixed pattern) + 0.014 × 1 (if hepatocellular pattern) + 1887.

4. Discussion

The inability to differentiate drug-induced liver injury (DILI) from other liver-related pathologies or injury patterns presents significant challenges in clinical practice, leading to delays in diagnosis, inappropriate treatments, and potentially worsening patient outcomes. DILI often shares clinical features with other liver conditions such as viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD) and autoimmune liver diseases, which can make it difficult for clinicians to pinpoint the specific cause of liver dysfunction without clear diagnostic tools. One of the primary challenges is that biomarkers such as elevated liver enzymes (e.g., ALT, AST, ALP) are nonspecific and can be elevated in many different liver conditions. Without clear markers distinguishing DILI from other etiologies, clinicians may mistakenly attribute liver dysfunction to an underlying chronic liver disease or non-drug-related causes. This diagnostic uncertainty can lead to inappropriate management. Moreover, prolonged diagnostic uncertainty can increase the risk of acute liver failure in severe cases, particularly when the hepatocellular injury is progressive.

In this context, several promising biomarkers of DILI have been identified; however, they present poor correlation with the histopathological staging of injury; they lack prognostic capability and struggle to distinguish between liver toxicity mechanisms.

The significant stability in body fluids such as the blood of miRNAs facilitates the use of these regulators of gene expression as translational clinical biomarkers for early diagnosis and prognosis in different liver diseases, including DILI.

MiRNAs can be actively released from the cell at an early stage of cell damage before the damage is detected by conventional markers. MiR-122 is the most abundant hepatic miRNA and for this reason is the best characterized potential circulating liver biomarker in response to drug-related hepatotoxicity. This has been observed for miR-122 in paracetamol-induced liver damage, with an increase in this miRNA before the detection of liver damage symptoms or transaminase elevations [15].

Nevertheless, miR-122 alone may not be an ideal DILI biomarker because it still lacks specificity as it is also altered in other liver pathologies; for example, being elevated in both metabolic diseases and renal cell carcinoma [16,17].

It was recently demonstrated that in combination with glutamate dehydrogenase (GLDH), K-18 and miR-122 were able to accurately detect paracetamol-induced liver damage and differentiate between DILI, healthy controls and patients with non-hepatocellular organ damage [8].

A novel and alternative approach for distinguishing between drug-induced and non-drug-induced phenotypes of liver damage or to differentiate the types of liver damage is to consider different miRNA panels as markers of injury [18].

A combination of different miRNAs or even composite determinations including other types of biomarkers may be more specific to differentiate pathologies of different etiologies [19].

An independent validation study of serum miRNAs for metabolic associated steatotic liver disease (MASLD) found that 5 miRNAs (miR-192, -27b, -22, -197 and 30c) resulted in being specific for MASLD when compared to DILI patients [20] and the combination of clinical parameters and miRNAs showed improved predictivity.

Another pilot study evaluating serum-combined miRNAs for the diagnosis of cirrhosis and hepatocellular carcinoma (HCC) in hepatitis C patients found that miR-122-5p and miR-409-3p were capable of distinguishing cirrhosis from mild disease, and the prediction was improved by adding the aminotransferase-to-platelet ratio [21].

The approach of the present study was to combine the evaluation of miRNAs with tested biomarkers such as K-18 and routine parameters of liver function in order to improve the specificity and ability to diagnose different etiologies of acute liver injury and to assess the biomarkers' performance in DILI.

In this preliminary study, in addition to miR-122-5p, which has demonstrated a superior biomarker performance compared to traditional biomarkers, two other miRNAs, miR-21-5p and miR-34a-5p, were evaluated since they were previously demonstrated to be involved in the pathogenesis of liver injury caused by diverse etiologies.

MiR-21 is considered an oncogenic miRNA detectable at high levels in the tissue [22,23] and serum [24,25] of HCC patients. The miR-21 is reported to induce hepatic inflammation through the promotion of inflammatory gene expression via the STAT3 signaling pathways, leading to liver disease [26]. High levels of miR-21 after liver resection are predictive of disease-progression [23] and poor prognosis [27]. In our previous study, cirrhotic patients had a median level of miR-21-5p, significantly higher compared to healthy volunteers and HCC patients. In HCC, a statistically significant drop in miR-21-5p after drug-eluting bead trans-arterial chemoembolization was observed, returning to levels comparable to those of healthy individuals. miR-21-5p correlated with hypoxia-inducible factor 1-alpha and probably had a role in modulating angiogenesis in HCC [10].

The miRNA-34 family regulates a number of different signaling pathways, including those linked to cancer, the immune system, metabolism and cellular structure [28].

MiR-34a has a significant role in fatty acid oxidation in hepatocytes, leading to altered lipid metabolism in MASLD [29]. MiR-34a inhibits the gene that regulates catabolism increasing lipid synthesis and inhibiting mitochondrial in MASLD. miR-34a inhibits the gene that regulates catabolism [30,31].

Total plasma K-18 can be detected by the epitope M65, present both on the full-length and cleaved form of K-18 (necrosis and apoptosis), whereas the neoepitope M30 is formed on the K-18 fragments during apoptotic cleavage.

In accordance with other studies [6,32], the results of the present study demonstrated that the expression of miR-122-5p, miR-34a-5p, miR-21-5p, M30 and M65 were significantly higher in patients with liver injury compared to healthy controls. Moreover, in patients with DILI, miR-122, -192, -34 and -22 demonstrated a significantly greater induction in those with a hepatocellular pattern [33].

In agreement with previously published data, we found that the expression of the three candidate miRNAs was more elevated in DILI with a hepatocellular pattern compared to a cholestatic and mixed pattern considered together; however, the question remains

whether the altered expression of miRNAs could have a diagnostic value to discriminate these two types of injuries.

The novel aspect of the present study is the development of a score based on the combination of miR-122-5p, miR-34a-5p and M30, which showed a sensitivity and specificity of 81% in diagnosing patients with DILI, higher than miRNA and M30 considered separately. Since the diagnosis of DILI is often based on medical history and exclusion of other causes of acute liver disease, this score could represent a capable instrument to discriminate patients presenting with DILI early in clinical practice.

Moreover, it could help to define patients presenting with a hepatocellular pattern of DILI early, which is usually the pattern more frequently associated with poor outcome and with the potential need for liver transplant. This would allow, especially in rapidly evolving cases, to take decision-making measures in a timely manner. An analysis based on a larger sample of patients and the validation of this score in an external cohort could facilitate a better characterization of the results found in this study.

An increasing number of studies have revealed the roles of miRNAs in viral infection, including HBV infection, and explored their clinical applicability as non-invasive biomarkers of circulating HBV infection. In particular, miR-146a-5p, miR-122-3p and miR-328-3p positively correlate with the severity of liver inflammation in patients with HBV-associated acute-on-chronic liver failure [34].

In a recent study, the miRNA expression profile in peripheral blood mononuclear cells was investigated to explore the potential role of miRNA in the pathogenesis of HBV-related chronic hepatitis. Among thousands of aberrantly expressed miRNAs, 45 upregulated and 62 downregulated miRNAs were found with a fold expression change >1.5 in patients with HBV compared with healthy donors [35].

Bioinformatics analysis revealed that the target genes of these miRNAs were implicated in multiple biological processes and signaling pathways, especially gene expression regulation.

Similarly, our findings demonstrated that miR-122-5p, miR-21-5p and miR-34a-5p were significantly higher with respect to controls and with levels similar to those in DILI.

Several studies also provided important insights into the role of miRNAs and K-18 in alcohol-related disease. The upregulation of miR-155, via NF-kB signaling, might contribute to alcohol liver injury [36], with K-18-M30 and M65 levels > 10 times higher in cirrhotic patients who had consumed alcohol compared to levels reported in healthy individuals.

The data show that K-18 fragments constitute attractive non-invasive diagnostic markers of alcohol disease [37].

The results of our study seem to confirm previously published data, since M30 and M60 were particularly elevated in the patient groups with alcohol-related acute hepatitis, and therefore with recent and elevated alcohol intake.

In summary, the principal findings of our study are: 1. The miRNAs evaluated when considered alone are significantly higher in patients with acute liver diseases compared to controls; 2. Necrosis and apoptosis indexes were significantly higher than in controls and correlated with miR122 and miR21; 3. MiR-122 and M30, as apoptosis indexes, had the highest sensitivity and specificity in separating DILI from other groups; 4. A model comprising different miRNAs (miR122 and miR34), M30 and the hepatocellular pattern (H) of liver disease was potentially able to differentiate DILI from other pathologies.

The principal limitation of this study is represented by the small sample size, which potentially influenced the possibility of performing a statistical analysis in order to identify a prognostic role of miRNAs and K-18 in patients with acute liver injury.

Despite the approach selected in this study for evaluating circulating miRNAs being well established, the technical aspects require more general, common and reproducible standardization in the performance of the assay for miRNA determination across studies, in particular regarding homogenization in the definition of a standard threshold of miRNA levels. Moreover, current options for miRNA detection have positive and negative aspects in terms of range, normalization approach, reproducibility and cost.

5. Conclusions

We concluded that a subset of miRNAs was altered in the plasma of DILI patients, showing specific differences depending on the type of liver injury (hepatocellular vs. cholestatic). Moreover, when a model was created using this subset of miRNAs in association with other markers, it was able to differentiate DILI from other pathologies.

Validating the proposed model in larger and more diverse cohorts of patients with acute liver disease, in particular with DILI, would be crucial for ensuring that it is robust, accurate, and applicable to a broad range of patient populations. Once validated, it would be of utmost importance to include the model in clinical guidelines and protocols. Collaboration with professional societies and medical associations is critical to ensure that the identified biomarkers become part of standard care.

Lastly, the pathway to commercialization involves securing regulatory approval, proving clinical utility, obtaining reimbursement, and integrating the biomarker into clinical practice. Success in these areas depends on strong scientific evidence, collaboration with regulatory bodies, and a clear value proposition to stakeholders across the healthcare ecosystem. Through these efforts, biomarkers can ultimately improve patient care, inform treatment decisions, and support precision medicine approaches.

In conclusion, although the data obtained should be considered still preliminary, they are intriguing and could represent a starting point for future and promising studies, especially with regard to miRNAs. Additional studies are needed to further define the role of miRNAs in each type of acute liver disease and to translate them into clinical settings.

Author Contributions: R.C. and D.B. drafted the manuscript; R.C., C.C. and M.M. performed the laboratory analysis; F.P. performed the data analysis; D.B., F.D. and F.I. performed the sample collection and information recording; G.G., F.P.R. and F.F. performed the clinical data analysis and revision of manuscript and P.B. and G.G. supervised the study. All authors have read and agreed to the published version of the manuscript.

Funding: The APC was funded by the Specialization School in Digestive System Diseases, Padua University.

Institutional Review Board Statement: The study was conducted in accordance to the Declaration of Helsinki and was approved by the Ethics Committee of the Padova University Hospital (Protocol n° 46093). Each subject provided written informed consent to participate to the study.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data that support the findings of this study are available on reasonable request from the corresponding author [PB].

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Aithal, G.P.; Watkins, P.B.; Andrade, R.J.; Larrey, D.; Molokhia, M.; Takikawa, H.; Hunt, C.M.; Wilke, R.A.; Avigan, M.; Kaplowitz, N.; et al. Case definition and phenotype standardization in drug-induced liver injury. *Clin. Pharmacol. Ther.* **2011**, *89*, 806–815. [CrossRef]
2. Pettersson, J.; Hindorf, U.; Persson, P.; Bengtsson, T.; Malmqvist, U.; Werkström, V.; Ekelund, M. Muscular exercise can cause highly pathological liver function tests in healthy men. *Br. J. Clin. Pharmacol.* **2008**, *65*, 253–259. [CrossRef] [PubMed]
3. Weber, S.; Gerbes, A.L. Challenges and future of drug-induced liver injury research-laboratory tests. *Int. J. Mol. Sci.* **2022**, *23*, 6049. [CrossRef] [PubMed]
4. Church, R.J.; Kullak-Ublick, G.A.; Aubrecht, J.; Bonkovsky, H.L.; Chalasani, N.; Fontana, R.J.; Goepfert, J.C.; Hackman, F.; King, N.M.P.; Kirby, S.; et al. Candidate biomarkers for the diagnosis and prognosis of drug-induced liver injury: An international collaborative effort. *Hepatology* **2019**, *69*, 760–773. [CrossRef] [PubMed]
5. Korver, S.; Bowen, J.; Pearson, K.; Gonzalez, R.J.; French, N.; Park, K.; Jenkins, R.; Goldring, C. The application of cytokeratin-18 as a biomarker for drug-induced liver injury. *Arch. Toxicol.* **2021**, *95*, 3435–3448. [CrossRef] [PubMed]
6. Cueto-Sánchez, A.; Niu, H.; Álvarez-Álvarez, I.; López-Longarela, B.; Del Campo-Herrera, E.; Ortega-Alonso, A.; García-Cortés, M.; Pinazo-Bandera, J.; Sanabria-Cabrera, J.; Díaz-Mochón, J.J.; et al. Evaluation of diagnostic and prognostic candidate biomarkers in drug-induced liver injury vs. other forms of acute liver damage. *Br. J. Clin. Pharmacol.* **2023**, *89*, 2497–2507. [CrossRef] [PubMed]

7. Cione, E.; Abrego Guandique, D.M.; Caroleo, M.C.; Luciani, F.; Colosimo, M.; Cannataro, R. Liver damage and microRNAs: An update. *Curr. Issues Mol. Biol.* **2022**, *45*, 78–91. [CrossRef] [PubMed]
8. Llewellyn, H.P.; Vaidya, V.S.; Wang, Z.; Peng, Q.; Hyde, C.; Potter, D.; Wang, J.; Zong, Q.; Arat, S.; Martin, M.; et al. Evaluating the sensitivity and specificity of promising circulating biomarkers to diagnose liver injury in humans. *Toxicol. Sci.* **2021**, *181*, 23–34. [CrossRef] [PubMed]
9. Dear, J.W.; Clarke, J.I.; Francis, B.; Allen, L.; Wraight, J.; Shen, J.; Dargan, P.I.; Wood, D.; Cooper, J.; Thomas, S.H.L.; et al. Risk stratification after paracetamol overdose using mechanistic biomarkers: Results from two prospective cohort studies. *Lancet Gastroenterol. Hepatol.* **2018**, *3*, 104–113. [CrossRef] [PubMed]
10. Pelizzaro, F.; Cardin, R.; Sartori, A.; Imondi, A.; Penzo, B.; Aliberti, C.; Ponzoni, A.; Vitale, A.; Cillo, U.; Farinati, F. Circulating MicroRNA-21 and MicroRNA-122 as prognostic biomarkers in hepatocellular carcinoma patients treated with transarterial chemoembolization. *Biomedicines* **2021**, *9*, 890. [CrossRef] [PubMed]
11. Robles-Diaz, M.; Garcia-Cortes, M.; Medina-Caliz, I.; Gonzalez-Jimenez, A.; Gonzalez-Grande, R.; Navarro, J.M.; Castiella, A.; Zapata, E.M.; Romero-Gomez, M.; Blanco, S.; et al. The value of serum aspartate aminotransferase and gamma-glutamyl transpetidase as biomarkers in hepatotoxicity. *Liver Int.* **2015**, *35*, 2474–2482. [CrossRef] [PubMed]
12. Chalasani, N.; Bonkovsky, H.L.; Fontana, R.; Lee, W.; Stolz, A.; Talwalkar, J.; Reddy, K.R.; Watkins, P.B.; Navarro, V.; Barnhart, H.; et al. Features and outcomes of 899 patients with drug-induced liver injury: The DILIN prospective study. *Gastroenterology* **2015**, *148*, 1340–1352.e1347. [CrossRef]
13. European Association for the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J. Hepatol.* **2017**, *67*, 370–398. [CrossRef]
14. Crabb, D.W.; Bataller, R.; Chalasani, N.P.; Kamath, P.S.; Lucey, M.; Mathurin, P.; McClain, C.; McCullough, A.; Mitchell, M.C.; Morgan, T.R.; et al. Standard definitions and common data elements for clinical trials in patients with alcoholic hepatitis: Recommendation from the NIAAA Alcoholic Hepatitis Consortia. *Gastroenterology* **2016**, *150*, 785–790. [CrossRef] [PubMed]
15. Antoine, D.J.; Dear, J.W.; Lewis, P.S.; Platt, V.; Coyle, J.; Masson, M.; Thanacoody, R.H.; Gray, A.J.; Webb, D.J.; Moggs, J.G.; et al. Mechanistic biomarkers provide early and sensitive detection of acetaminophen-induced acute liver injury at first presentation to hospital. *Hepatology* **2013**, *58*, 777–787. [CrossRef]
16. Jafri, I. MiRNA a new insight in metabolic and human diseases: A review. *Cell. Mol. Biol.* **2023**, *69*, 102–110. [CrossRef]
17. Fan, Y.; Ma, X.; Li, H.; Gao, Y.; Huang, Q.; Zhang, Y.; Bao, X.; Du, Q.; Luo, G.; Liu, K.; et al. miR-122 promotes metastasis of clear-cell renal cell carcinoma by downregulating Dicer. *Int. J. Cancer* **2018**, *142*, 547–560. [CrossRef] [PubMed]
18. Yamaura, Y.; Tatsumi, N.; Takagi, S.; Tokumitsu, S.; Fukami, T.; Tajiri, K.; Minemura, M.; Yokoi, T.; Nakajima, M. Serum microRNA profiles in patients with chronic hepatitis B, chronic hepatitis C, primary biliary cirrhosis, autoimmune hepatitis, nonalcoholic steatohepatitis, or drug-induced liver injury. *Clin. Biochem.* **2017**, *50*, 1034–1039. [CrossRef]
19. Martinelli, V.; Dalla Costa, G.; Messina, M.J.; Di Maggio, G.; Sangalli, F.; Moiola, L.; Rodegher, M.; Colombo, B.; Furlan, R.; Leocani, L.; et al. Multiple biomarkers improve the prediction of multiple sclerosis in clinically isolated syndromes. *Acta Neurol. Scand.* **2017**, *136*, 454–461. [CrossRef] [PubMed]
20. López-Riera, M.; Conde, I.; Castell, J.V.; Jover, R. A novel MicroRNA signature for cholestatic drugs in human hepatocytes and its translation into novel circulating biomarkers for drug-Induced liver injury patients. *Toxicol. Sci.* **2020**, *173*, 229–243. [CrossRef] [PubMed]
21. Weis, A.; Marquart, L.; Calvopina, D.A.; Genz, B.; Ramm, G.A.; 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. [CrossRef] [PubMed]
22. Zeng, W.; van den Berg, A.; Huitema, S.; Gouw, A.S.; Molema, G.; de Jong, K.P. Correlation of microRNA-16, microRNA-21 and microRNA-101 expression with cyclooxygenase-2 expression and angiogenic factors in cirrhotic and noncirrhotic human hepatocellular carcinoma. *PLoS ONE* **2014**, *9*, e95826. [CrossRef]
23. Yoon, J.S.; Kim, G.; Lee, Y.R.; Park, S.Y.; Tak, W.Y.; Kweon, Y.O.; Park, J.G.; Lee, H.W.; Han, Y.S.; Ha, H.T.; et al. Clinical significance of microRNA-21 expression in disease progression of patients with hepatocellular carcinoma. *Biomark. Med.* **2018**, *12*, 1105–1114. [CrossRef] [PubMed]
24. Bharali, D.; Banerjee, B.D.; Bharadwaj, M.; Husain, S.A.; Kar, P. Expression analysis of MicroRNA-21 and MicroRNA-122 in hepatocellular carcinoma. *J. Clin. Exp. Hepatol.* **2019**, *9*, 294–301. [CrossRef]
25. Pu, C.; Huang, H.; Wang, Z.; Zou, W.; Lv, Y.; Zhou, Z.; Zhang, Q.; Qiao, L.; Wu, F.; Shao, S. Extracellular vesicle-associated mir-21 and mir-144 are markedly elevated in serum of patients with hepatocellular carcinoma. *Front. Physiol.* **2018**, *9*, 930. [CrossRef] [PubMed]
26. Zhang, T.; Yang, Z.; Kusumanchi, P.; Han, S.; Liangpunsakul, S. Critical role of microRNA-21 in the pathogenesis of liver diseases. *Front. Med.* **2020**, *7*, 7. [CrossRef]
27. Huang, C.S.; Yu, W.; Cui, H.; Wang, Y.J.; Zhang, L.; Han, F.; Huang, T. Increased expression of miR-21 predicts poor prognosis in patients with hepatocellular carcinoma. *Int. J. Clin. Exp. Pathol.* **2015**, *8*, 7234–7238. [PubMed]
28. Mm Yahya, S.; Elsayed, G.H. The role of MiRNA-34 family in different signaling pathways and its therapeutic options. *Gene* **2024**, *931*, 148829. [CrossRef] [PubMed]
29. Salvoza, N.C.; Klinzing, D.C.; Gopez-Cervantes, J.; Baclig, M.O. Association of circulating serum miR-34a and miR-122 with dyslipidemia among patients with non-alcoholic fatty liver disease. *PLoS ONE* **2016**, *11*, e0153497. [CrossRef] [PubMed]

30. Ding, J.; Li, M.; Wan, X.; Jin, X.; Chen, S.; Yu, C.; Li, Y. Effect of miR-34a in regulating steatosis by targeting PPARα expression in nonalcoholic fatty liver disease. *Sci. Rep.* **2015**, *5*, 13729. [CrossRef] [PubMed]
31. Xu, Y.; Zhu, Y.; Hu, S.; Pan, X.; Bawa, F.C.; Wang, H.H.; Wang, D.Q.; Yin, L.; Zhang, Y. Hepatocyte miR-34a is a key regulator in the development and progression of non-alcoholic fatty liver disease. *Mol. Metab.* **2021**, *51*, 101244. [CrossRef] [PubMed]
32. Thulin, P.; Nordahl, G.; Gry, M.; Yimer, G.; Aklillu, E.; Makonnen, E.; Aderaye, G.; Lindquist, L.; Mattsson, C.M.; Ekblom, B.; et al. Keratin-18 and microRNA-122 complement alanine aminotransferase as novel safety biomarkers for drug-induced liver injury in two human cohorts. *Liver Int.* **2014**, *34*, 367–378. [CrossRef] [PubMed]
33. López-Riera, M.; Conde, I.; Quintas, G.; Pedrola, L.; Zaragoza, Á.; Perez-Rojas, J.; Salcedo, M.; Benlloch, S.; Castell, J.V.; Jover, R. Non-invasive prediction of NAFLD severity: A comprehensive, independent validation of previously postulated serum microRNA biomarkers. *Sci. Rep.* **2018**, *8*, 10606. [CrossRef]
34. Wen, Y.; Peng, S.F.; Fu, L.; Fu, X.Y.; Wu, D.X.; Liu, B.J.; Tan, D.M.; Ouyang, Y. Serum levels of miRNA in patients with hepatitis B virus-associated acute-on-chronic liver failure. *Hepatobiliary Pancreat. Dis. Int.* **2018**, *17*, 126–132. [CrossRef] [PubMed]
35. Yu, K.; Li, Q.; Li, N. MicroRNA profile in peripheral blood mononuclear cells from hepatitis B virus infected patients. *Ann. Hepatol.* **2018**, *17*, 1012–1020. [CrossRef]
36. Bala, S.; Szabo, G. MicroRNA signature in alcoholic liver disease. *Int. J. Hepatol.* **2012**, *2012*, 498232. [CrossRef] [PubMed]
37. Atkinson, S.R.; Grove, J.I.; Liebig, S.; Astbury, S.; Vergis, N.; Goldin, R.; Quaglia, A.; Bantel, H.; Guha, I.N.; Thursz, M.R.; et al. In severe alcoholic hepatitis, serum keratin-18 fragments are diagnostic, prognostic, and theragnostic biomarkers. *Am. J. Gastroenterol.* **2020**, *115*, 1857–1868. [CrossRef] [PubMed]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.