Review

Circulating miRNAs in nontumoral liver diseases

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Abstract

In recent years, there has been increasing interest in finding new biomarkers for diagnosis and prognostication of liver diseases. MicroRNAs (miRNAs) are small noncoding RNA molecules involved in the regulation of gene expression and have been studied in relation to several conditions, including liver disease. Mature miRNAs can reach the bloodstream by passive release or by incorporation into lipoprotein complexes or microvesicles, and have stable and reproducible concentrations among individuals. In this review, we summarize studies involving circulating miRNAs sourced from the serum or plasma of patients with nontumoral liver diseases in attempt to bring insights in the use of miRNAs as biomarkers for diagnosis, as well as for prognosis of such diseases. In addition, we present pre-analytical aspects involving miRNA analysis and strategies for normalization of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) data related to the studies evaluated.

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Abbreviations: Ago, Argonaute; AFP, α-fetoprotein; AH, alcoholic hepatitis; AIH, autoimmune hepatitis; ALD, alcoholic liver disease; ALL, acute liver injury; ALT, alanine aminotransferase; AMA, antimitochondrial antibody; AP, alkaline phosphatase; APAP, acetaminophen; ASH, alcoholic steatohepatitis; AST, aspartate aminotransferase; AUROC, area under the receiver operating characteristic (curve); CHB, chronic hepatitis B; GC, gastric cancer; GGT, γ-glutamyl transpeptidase; HAI, histologic activity index; HBV, hepatitis B virus; HBV-ACLF, hepatitis B-related acute-on-chronic liver failure; HBV-ASC, chronic asymptomatic HBV carriers; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSC, hepatic stellate cells; INR, international normalized ratio; MELD, Model for End-Stage Liver Disease; MeSH, Medical Subject Headings; MV, microvesicle; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; PBC, primary biliary cholangitis; pri-miRNA, primary microRNA; RISC, RNA-induced silencing complex; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SS, simple steatosis; SVR, sustained virological response; TGF-β, transforming growth factor-beta; UTR, untranslated region.

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1. Introduction

The main causes of liver disease are alcohol abuse, viral hepatitis, nonalcoholic fatty liver disease (NAFLD), hemochromatosis, autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), and primary sclerosing cholangitis. Alcohol and the hepatitis B and C viruses (HBV and HCV) are the most important causes of liver cirrhosis and hepatocellular carcinoma (HCC) [1–4]. Mortality related to liver disease is growing, with more than one million deaths from liver cirrhosis worldwide in 2010 [5]. When deaths from liver cirrhosis are combined with deaths from liver cancer and acute hepatitis, the annual number of fatalities due to liver disease can exceed two million [6]. In Europe, liver cirrhosis is responsible for around 170,000 deaths per year, and more than 5500 liver transplants are performed each year [1]. Chronically infected HBV and HCV patients are at high risk of developing cirrhosis (~20% of patients) [7–9] and HCC (~25% and 7% for HBV and HCV, respectively) [8,10]. HBV and HCV infections are responsible for a mortality rate of approximately 3 and 11 deaths, respectively, per 100,000 inhabitants per year in Spain [11]. Although alcohol control policies, vaccination, and prevention strategies have contributed to decreased incidence of chronic liver diseases, these diseases remain public health problems because of their associated complications. In addition, there is a high cost related to antiviral therapy for HBV and HCV chronic infection [12,13].

The health, social, and economic impacts associated with liver diseases could be reduced by improving diagnosis and prognostication. New biomarkers have been investigated for this purpose, including circulating microRNAs (miRNAs). MicroRNAs are small RNAs about 22 nucleotides (nt) long that regulate gene expression by binding to complementary regions of messenger RNA (mRNA) [14]. Circulating miRNAs can be detected in human serum and plasma, and have stable and reproducible concentrations among individuals [15,16]. The purpose of this review is to highlight recent advances in the study of circulating miRNAs as markers of nontumoral liver damage, and to provide an overview of the biological properties of these nucleic acids and analytical challenges associated with their quantitation.

1.1. Overview of miRNA biogenesis

MicroRNAs are endogenous, noncoding RNAs about 22 nt long that regulate gene expression post-transcriptionally by binding to complementary regions of mRNA, resulting in repression of translation or mRNA degradation [14]. Fig. 1 gives a brief overview of miRNA biogenesis. Initially, (1) DNA is transcribed by RNA polymerase II (Pol II), yielding a transcript called primary RNA (pri-miRNA) [17,18], which usually has a stem-loop structure and a length over 1 kb. After that, (2) the RNase III Drosha cleaves the hairpin ~11 base pairs (bp) from the basal junction and ~22 bp from the apical junction connected to the terminal loop [19,20], forming a hairpin RNA ~65 nt long, termed pre-miRNA [21]. Then, (3) pre-miRNA forms a transport complex with the protein exportin-5 and the Ranguanosine-5′-triphosphate complex (RanGTP), allowing export into the cytoplasm. Once in the cytoplasm, (4) pre-miRNA is cleaved by the RNase III Dicer near the terminal loop, releasing

Fig. 1. Schematic representation of miRNA biogenesis (see text for details). This figure as well as the graphical abstract were designed according to the “Guidelines for preparing color figures for everyone including the colorblind” described by Roskoski [42].
a small RNA duplex [22–26], which is loaded into an Argonaute (Ago) protein, forming the pre-RNA-induced silencing complex (pre-RISC) [27–29]. Next, the duplex is unwound, which usually causes degradation of the passenger strand (miRNA+) and the formation of mature miRNA [30].

Once integrated into the RISC, mature miRNA is functional and able to regulate gene expression. This regulation may occur by: (I) inhibition of translation, the most common mechanism in humans, which is dependent on the Ago proteins Ago1, Ago3, or Ago4, and involves imperfect base pairing between the miRNA and the 3′-untranslated region (UTR) of mRNA [31]; (II) cleavage of miRNA, an Ago2-dependent mechanism, which involves perfect base pairing between the miRNA and the 3′-UTR of mRNA; and (III) translation activation, which involves interaction of miRNA with the 5′-UTR of mRNA [32–37]. The mature miRNA can reach the bloodstream by (A) active release (by incorporation into lipoprotein complexes, microvesicles, or exosomes) or (B) passive release (caused by cell death; by incorporation into apoptotic bodies) [38–40]. In associated form, circulating miRNAs are resistant to nuclease activity, making them a promising source of diagnostic or prognostic biomarkers [41].

Although miRNAs were discovered in 1993 by Lee et al. [42] while studying the development of the free-living nematode Caenorhabditis elegans, the term “miRNA” started to be used in mid-2001 [28,44–46]. Since the early 2000s, when a correlation between miRNA levels and human disease was demonstrated [47–49], the number of studies on the applications of miRNAs as biomarkers and therapeutics has progressively increased (Fig. 2). In 2015, there were 11,216 new citations related to miRNA in PubMed (Fig. 2A), of which 978 (9%) related to both miRNA and the liver (Fig. 2B). In 2016, although the number of new citations related to miRNA was lower than in 2015 (9022 citations), the proportion of these citations that also related to the liver was similar (685/9022, 8%). Considering all 53,503 citations related to miRNA up to 2016, 3883 (7%) related to both miRNA and the liver, that is, approximately one-fourteen of citations identified using the Medical Subject Headings (MeSH) search terms “microRNA” and “miRNA” were also annotated with the MeSH term “liver.”

MicroRNAs have been implicated in the regulation of many important biological processes, such as cell growth and differentiation, development, apoptosis, and modulation of the host response to viral infection [50]. For example, miR-122 plays an important role in liver physiology, since it is involved in acquisition and maintenance of the hepato-specific phenotype, and participates in the regulation of cholesterol and fatty acids in hepatic metabolism [51,52]. Moreover, the propagation of HCV RNA occurs after binding of miR-122 to the 5′-noncoding region of the HCV genome [51,53,54]. The following section describes other circulating miRNAs, beyond miR-122, that have been described in patients with liver disease.

The names of miRNAs are composed of a three-letter prefix that identifies the source organism, the prefix “miR,” which denotes mature miRNA, and a unique identification number, which is simply sequential. The 5′ and 3′ arms are denoted by the suffixes 5p and 3p, respectively. For example, “hsa-miR-122-5p” corresponds to the 5′ arm of mature miR-122 in Homo sapiens (hsa) [55,56] (www.mirbase.org). Since this work focuses on human miRNA, the prefix specifying the organism will be omitted.

Table 1 summarizes the content covered in the subsequent sections. In the present review, impact factor (IF) was not used as a criterion for the selection of research articles because it does not necessarily reflect the quality of studies [57]. In addition, only 9 studies (listed in Table 1) developed independent validation cohort: liver cirrhosis [58,59], CHB cirrhosis [60], HBV–HCC [61], HCV [62–64], NAFLD [65], and PBC [66]. For this reason, we sought to include, in this review, studies involving miRNA expression in serum or plasma samples from humans with non-tumoral liver diseases, even though independent validation cohort studies were not available in majority of the cases. Although the focus of the review is circulating miRNAs (serum or plasma) in nontumoral liver diseases, we also address some studies involving HCC, which is associated with several chronic liver diseases.

2. Circulating miRNA profile in patients with liver disease

2.1. Liver cirrhosis

MicroRNAs are fundamental in the pathogenesis of various diseases, including cancer and other chronic liver diseases. In addition, miRNAs detected in patients' serum may serve as biomarkers and represent a novel approach for diagnostic blood screening [92]. The expression of various miRNAs is altered in the serum of patients with liver disease; for example, miR-146a, miR-215, miR-224, miR-574-3p, and miR-885-5p were shown to be upregulated in the serum of patients with HCC and liver cirrhosis. In the same study, the expression of these miRNAs was also evaluated in the serum of patients with chronic hepatitis B (CHB) or gastric cancer (GC); among these miRNAs, in the independent validation cohort, miR-885-5p presented higher expression in patients with HCC (n = 46), liver cirrhosis (n = 26), and CHB (n = 23), compared with GC patients (n = 17) and healthy controls (n = 24). In addition, the area under the receiver operating characteristic (AUROC) curve for miR-885-5p was 0.904, with sensitivity and specificity of 90.5% and 79.2%, respectively, in distinguishing patients with liver disease from the control group. However, miR-885-5p did not correlate with the hepatic function markers α-fetoprotein (AFP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), or γ-glutamyl

Table 1
Circulating miRNAs in nontumoral liver diseases (serum or plasma).

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Expression</th>
<th>Reference group</th>
<th>Disease</th>
<th>Variables correlated</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-885-5p</td>
<td>up</td>
<td>Healthy controls</td>
<td>LC, CHB, HCC</td>
<td>AFP, ALT, AST, GGT NC</td>
<td>[58]</td>
</tr>
<tr>
<td>miR-122</td>
<td>down</td>
<td>Compensated LC</td>
<td>Decompensated LC</td>
<td>-- Creatinine, INR, MELD</td>
<td>[59]</td>
</tr>
<tr>
<td>miR-181b</td>
<td>up</td>
<td>Healthy controls</td>
<td>LC</td>
<td>AFP, ALT, AST, GGT NC</td>
<td>[59]</td>
</tr>
<tr>
<td>miR-571</td>
<td>up</td>
<td>Healthy controls</td>
<td>LC</td>
<td>-- Albumin, bilirubin, total protein</td>
<td>[60]</td>
</tr>
<tr>
<td>miR-106b</td>
<td>down</td>
<td>Healthy controls</td>
<td>CHB cirrhosis</td>
<td>AFP, ALT, PT</td>
<td>[61]</td>
</tr>
<tr>
<td>miR-181b</td>
<td>up</td>
<td>Healthy controls</td>
<td>CHB, HCC</td>
<td>-- HBV DNA</td>
<td>[62]</td>
</tr>
<tr>
<td>16 miRNAs</td>
<td>up</td>
<td>Healthy children</td>
<td>CHB, HCC</td>
<td>-- HBV DNA</td>
<td>[63]</td>
</tr>
<tr>
<td>miR-29</td>
<td>down</td>
<td>Healthy controls</td>
<td>CHB, HCC</td>
<td>AFP, ALT, PT</td>
<td>[64]</td>
</tr>
<tr>
<td>miR-122</td>
<td>up</td>
<td>Healthy controls</td>
<td>HBV-HCC</td>
<td>AFP, ALT, PT</td>
<td>[65]</td>
</tr>
<tr>
<td>miR-15b</td>
<td>up</td>
<td>Healthy controls</td>
<td>HBV-HCC</td>
<td>AFP, ALT, PT</td>
<td>[66]</td>
</tr>
<tr>
<td>miR-130b</td>
<td>up</td>
<td>Healthy controls</td>
<td>HBV-HCC</td>
<td>AFP, ALT, PT</td>
<td>[67]</td>
</tr>
<tr>
<td>miR-15b</td>
<td>down</td>
<td>Healthy controls</td>
<td>HBV-HCC</td>
<td>AFP, ALT, PT</td>
<td>[68]</td>
</tr>
<tr>
<td>let-7b</td>
<td>up</td>
<td>Healthy controls</td>
<td>HBV-ASC, CHB,</td>
<td>AFP, ALT, PT</td>
<td>[69]</td>
</tr>
<tr>
<td>miR-16</td>
<td>up</td>
<td>Healthy controls</td>
<td>HBV-ACLF</td>
<td>AFP, ALT, PT</td>
<td>[70]</td>
</tr>
<tr>
<td>miR-106a</td>
<td>up</td>
<td>Healthy controls</td>
<td>HBV-ACLF</td>
<td>AFP, ALT, PT</td>
<td>[71]</td>
</tr>
<tr>
<td>miR-19b</td>
<td>up</td>
<td>Healthy controls</td>
<td>HBV-ACLF</td>
<td>AFP, ALT, PT</td>
<td>[72]</td>
</tr>
<tr>
<td>miR-2223</td>
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<td>Healthy controls</td>
<td>HBV-ACLF</td>
<td>AFP, ALT, PT</td>
<td>[73]</td>
</tr>
<tr>
<td>miR-210</td>
<td>up</td>
<td>Healthy controls</td>
<td>HBV-ACLF</td>
<td>AFP, ALT, PT</td>
<td>[74]</td>
</tr>
<tr>
<td>miR-21</td>
<td>up</td>
<td>Healthy controls</td>
<td>HBV-ACLF</td>
<td>AFP, ALT, PT</td>
<td>[75]</td>
</tr>
<tr>
<td>miR-122</td>
<td>up</td>
<td>Healthy controls</td>
<td>HCV</td>
<td>AFP, ALT, AST, HAI NC</td>
<td>[76]</td>
</tr>
<tr>
<td>miR-19c</td>
<td>up</td>
<td>Healthy controls</td>
<td>HCV</td>
<td>AFP, ALT, AST, HAI NC</td>
<td>[77]</td>
</tr>
<tr>
<td>miR-21</td>
<td>up</td>
<td>Healthy controls</td>
<td>HCV</td>
<td>AFP, ALT, AST, HAI NC</td>
<td>[78]</td>
</tr>
<tr>
<td>miR-122</td>
<td>up</td>
<td>Healthy controls</td>
<td>HCV</td>
<td>AFP, ALT, AST, HAI NC</td>
<td>[79]</td>
</tr>
<tr>
<td>miR-212</td>
<td>up</td>
<td>Healthy controls</td>
<td>HCV</td>
<td>AFP, ALT, AST, HAI NC</td>
<td>[80]</td>
</tr>
<tr>
<td>miR-122</td>
<td>up</td>
<td>Healthy controls</td>
<td>HCV</td>
<td>AFP, ALT, AST, HAI NC</td>
<td>[81]</td>
</tr>
<tr>
<td>miR-122</td>
<td>up</td>
<td>Healthy controls</td>
<td>HCV</td>
<td>AFP, ALT, AST, HAI NC</td>
<td>[82]</td>
</tr>
<tr>
<td>miR-122</td>
<td>up</td>
<td>Baseline</td>
<td>Cholestyramine</td>
<td>AFP, ALT, AST, HAI NC</td>
<td>[83]</td>
</tr>
<tr>
<td>miR-16</td>
<td>up</td>
<td>Healthy controls</td>
<td>Paraquat poisoning</td>
<td>AFP, ALT, AST, HAI NC</td>
<td>[84]</td>
</tr>
<tr>
<td>miR-34a</td>
<td>up</td>
<td>Healthy controls</td>
<td>NAFLD, HCV</td>
<td>AFP, ALT, AST, HAI NC</td>
<td>[85]</td>
</tr>
<tr>
<td>miR-122</td>
<td>up</td>
<td>Healthy controls</td>
<td>NAFLD, HCV</td>
<td>AFP, ALT, AST, HAI NC</td>
<td>[86]</td>
</tr>
</tbody>
</table>
transpeptidase (GGT). Despite this, miR-885-5p exhibited better diagnostic performance than ALT (AUROC of 0.742) [58].

On the other hand, other authors have found a positive correlation between miR-122 and ALT, AST, GGT, and alkaline phosphatase (ALP) in patients with liver cirrhosis. In this study, serum miR-122 levels were evaluated in patients with compensated and decompensated liver cirrhosis, which included 107 patients in a test cohort and 143 patients in a validation cohort. Patients with decompensated liver cirrhosis presented lower miR-122 levels than those with compensated liver cirrhosis both in the cohort test and in the independent validation cohort. Furthermore, lower miR-122 levels were associated with shorter survival in the Cox regression analysis, even after adjusting for the Model for End-Stage Liver Disease (MELD) score [59].

In addition to the miRNAs described above, the expression of miR-181a and miR-181b in serum from 22 patients with liver cirrhosis has been evaluated by Wang et al. [67]. While miR-181b showed no significant difference compared with the control group (n = 17), miR-181b was significantly higher in patients with liver cirrhosis. In addition, the authors demonstrated, in vitro, that transforming growth factor-beta 1 (TGF-β1) induced expression of miR-181b in hepatic stellate cells-T6 (HSC-T6). In turn, miR-181b promoted HSC-T6 proliferation by targeting the cell cycle regulator p27. Thus, TGF-β1 can induce the expression of miR-181b and promote the proliferation of hepatic stellate cells, which are important mediators of liver fibrosis. Therefore, miR-181b is a potential diagnostic biomarker for liver cirrhosis [67]. In another study, Roderburg et al. demonstrated that isolated liver cells (hepatocytes and hepatic stellate cells) increased the expression of miR-571 in response to treatment with TGF-β [68]. These authors identified changes in three miRNAs (miR-513-3p, miR-571, and miR-652) in 67 patients with liver cirrhosis induced by alcohol or hepatitis C, compared with the control group (n = 17). These three miRNAs showed altered serum levels, miR-513-3p and miR-571 being upregulated and miR-652 downregulated. The AUROC for miR-571 was 0.91, but when miR-571 was combined with the other two miRNAs (miR-513-3p and miR-652) the AUROC was approximately 0.97, showing even greater power to discriminate patients with chronic liver disease and liver cirrhosis from healthy controls. However, among these three miRNAs, only miR-571 was associated with disease stage, which was classified according to the Child-Pugh classification; miR-571 was significantly elevated in patients with Child C cirrhosis compared with those with Child A cirrhosis [68].

### 2.2 Hepatitis B

In a three-phase study (exploration, training, and validation), Chen et al. demonstrated that plasma miR-106b and miR-181b were downregulated and upregulated, respectively, in CHB-related cirrhosis (training phase, n = 90), compared with healthy controls (n = 60) [60]. The authors used miR-106b and miR-181b to construct a miRNA panel with an AUROC of 0.882 (sensitivity: 0.86; specificity: 0.75) that could discriminate CHB cirrhosis patients from healthy individuals better than miR-106b (AUROC: 0.715) or miR-181b (AUROC: 0.833) alone. In addition, they demonstrated that there was no significant difference in miR-106b and miR-181b levels when comparing patients with CHB-related cirrhosis and non-CHB-related cirrhosis (such as alcoholic, schistosomiasis, autoimmune, and biliary), suggesting that variations in the plasma levels of these miRNAs are independent of etiology [60].

Additionally, when juvenile CHB patients were compared with healthy children (n = 60), 16 plasma miRNAs were found to be upregulated: miR-99a, miR-100, miR-122, miR-122*, miR-125b,
miR-192, miR-192*, miR-193b, miR-194, miR-215, miR-365, miR-455-5p, miR-455-3p, miR-483-3p, miR-885-5p, and miR-1247 [69]. All 16 miRNAs presented higher expression in Hepatitis B Antigen (HBeAg)-positive children (n = 34) than in HBeAg-negative children (n = 26). In addition, the authors found a strong positive correlation between all miRNAs and viral load (HBV DNA) suggesting that circulating miRNAs might play a role in the establishment and maintenance of CHB in children [69]. Jinato et al. evaluated miRNA expression profile in liver biopsies collected from patients with hepatitis B virus genotype C (HBeAg positive) both before and after treatment with pegylated-interferon alpha-2a for 48 weeks (n = 10). After the treatment, patients were sorted as responders with HBsAg clearance (n = 3), responders without HBsAg clearance (n = 3), and non-responders (n = 4). Out of these three groups, only patients classified as “responders with HBsAg clearance” presented downregulated miR-185-5p and miR-186-5p levels suggesting that these miRNAs might indicate the response to treatment [93].

Xing et al. recently evaluated serum miR-29 and miR-122 levels in HCC (n = 20), liver cirrhosis (n = 20), and CHB patients (n = 29), and HBV inactive carriers (n = 20) [70]. The authors demonstrated that serum miR-122 levels were upregulated in HCC and CHB patients compared with healthy controls (n = 20). On the other hand, miR-29 was downregulated in liver cirrhosis patients and upregulated in CHB patients compared with healthy controls. In addition, a positive correlation was found between serum miR-29 and miR-122 levels and viral load (HBV DNA) in CHB patients. However, the increased miR-122 expression was not associated with ALT in CHB patients. The authors suggest that downregulation of miR-29 expression may be related to hepatic fibrosis [70].

In patients with HBV-related HCC (HBV-HCC), Liu et al. found that serum miR-15b and miR-130b levels were upregulated [61]. This study was divided into three phases: exploration, selection, and validation. The validation phase included 30 healthy individuals, 29 CHB carriers, and 57 HCC patients. In this phase, the authors used miR-15b and miR-130b to construct a miRNA panel to distinguish between HCC patients and non-cancerous controls (healthy individuals and CHB carriers), which had an AUROC of 0.981 (sensitivity: 98.3%; specificity: 91.5%). In addition, they demonstrated that this miRNA panel was useful for diagnosing HCC in subjects with low serum AFP level (lower than 20 ng/ml) and that it could detect cases of HCC in the early stages of development [61].

Interestingly, Li et al. studied plasma microvesicles (MV) miRNAs in patients with CHB (n = 354) and HBV-HCC (n = 265) [71]. The authors found 219 miRNAs expressed only in CHB MVs, 189 miRNAs expressed only in HCC MVs, and 53 miRNAs coexpressed in these two groups. The following miRNAs that target genes related to the inflammatory response were expressed in both groups: miR-1, miR-17, miR-20a, miR-23a, miR-30b, miR-128, miR-188-5p, miR-194, miR-199a, miR-401, miR-432, miR-494, miR-936, and miR-939. In addition, miR-15b and miR-130b were downregulated in CHB MVs, but upregulated in HCC MVs [71], which is in accordance with the result of Liu et al. described previously [61], miR-125b, which acts as a tumor suppressor, was upregulated in CHB MVs and downregulated in HCC MVs. Thus, these results indicate that miRNAs may serve as biomarkers for HCC screening and risk stratification in CHB [71].

Ji et al. compared the expression of serum-circulating miRNAs in chronic asymptomatic HBV carriers (HBV-ASC) (n = 9), CHB (n = 21), HBV-related acute-on-chronic liver failure (HBV-ACLF) (n = 21), and healthy controls (12) [72]. Using an array of 377 miRNAs, a total of 37 miRNAs were detected in the control samples, while 77, 101, and 135 miRNAs were detected in the HBV-ASC, CHB, and HBV-ACLF samples, respectively. Interestingly, the amount of miRNA detected increased according to disease severity. Moreover, expression of miR-122, -194, -223, -16, -92a, -19b, -20a, -106a, let-7b was confirmed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). These miRNAs were upregulated in most of the HBV patients, compared with healthy controls. Expression levels of miR-223 and miR-19b were similar between the HBV-ASC and control groups but increased significantly in the CHB and HBV-ACLF groups. Furthermore, expression of miR-122 was significantly higher in the HBV-ASC and CHB groups, but not the HBV-ACLF group, compared with the control group [72]. Conversely, Zheng et al. found that miR-122 expression was significantly higher in patients with HBV-ACLF (n = 39) than in healthy controls (n = 20), but did not observe a significant difference in miR-194 expression [73]. Increased levels of miR-21, -486-5p, -130a, -192, -148a, -143, and -200a were also observed in HBV-ACLF patients (P < 0.05). Moreover, expression of these miRNAs was evaluated in patients who had recovered (n = 20) and not recovered (n = 19) from HBV-ACLF: low levels of miR-130a and miR-486-5p were observed in the serum of the patients who had not recovered from the disease. Thus, miR-130a and miR-486-5p were suggested to be related to prognosis [73]. To investigate markers of disease progression, Song et al. evaluated miR-210 expression in the serum of CHB subjects [74]: this miRNA was chosen because its expression had already been described in inflammation and hypoxia. In this study, 30 mild CHB patients, 30 severe CHB patients, 30 HBV-ACLF patients, and 30 healthy controls were included. Serum miR-210 expression was shown to increase significantly with the severity of HBV-related chronic liver disease. Serum miR-210 levels in the three groups were higher than in the control group (P < 0.01). Severe CHB and HBV-ACLF serum samples showed higher expression of miR-210 than mild CHB serum samples (P < 0.01), and miR-210 levels were higher in the HBV-ACLF group than in the severe CHB group (P < 0.05). Serum miR-210 was found to be positively correlated with ALT, AST, and total bilirubin, and negatively correlated with prothrombin activity. The authors suggested that increased serum miR-210 expression may be related to the severity of hepatitis, whereas increased liver miR-210 may be a response of hepatic cells to hypoxia during liver inflammation [74].

In summary, serum miRNA expression profiles in HBV patients varied across studies. Expression of some miRNAs varied depending on the severity of the disease and the presence of HCC, suggesting that these miRNAs might have potential as biomarkers for disease progression and cancer screening. Further studies are necessary to establish a panel of specific miRNAs for each disease stage, allowing clinical use of miRNAs with high specificity and sensitivity.

2.3. Hepatitis C

miR-122 has also been studied in patients with chronic hepatitis C virus (HCV) infection. Bihrer et al. demonstrated that these patients (n = 68) had higher serum miR-122 levels than healthy subjects (n = 19) [75]. Although no correlation was observed between miR-122 and international normalized ratio (INR), liver fibrosis, or viral load (serum HCV RNA), miR-122 was correlated with ALT, AST, histologic activity index (HAI), and necroinflammatory activity. In addition, the authors demonstrated that miR-122 levels did not differ between healthy subjects and patients with normal ALT. Therefore, increased concentrations of circulating miR-122 may reflect inflammatory activity in patients with chronic HCV infection [75]. In another study, Bihrer et al. found that serum miR-21 was upregulated in chronic HCV (n = 62) and in HCV–associated HCC (n = 29) patients compared with healthy controls (n = 19) [62]. However, miR-21 was not able to differentiate between HCV patients with and without HCC, because its levels were similar in both groups. On the other hand, miR-21 correlated with HAI, ALT, AST, bilirubin, GGT, and INR. In order to evaluate the correlation between miR-21 and necroinflammatory activity, a validation study was performed using a validation cohort of patients with chronic HCV (n = 47), and it was demonstrated that miR-21 was able to differ-

entiate between minimal and mild to severe necroinflammation (AUROC: 0.76; sensitivity: 53.3%; specificity: 95.2%) [62].

Recently, Kumar et al. evaluated miR-122 expression in serum from 25 patients with HCV genotype 3 and viral load greater than 10,000 IU/ml [76]. These patients exhibited increased miR-122 expression compared with the control group (n = 25). In addition, miR-122 correlated positively with ALT, AST, and viral load. For this group of patients, the AUROC was 0.929 with sensitivity and specificity of 92.0% and 84.0%, respectively, for distinguishing HCV patients from healthy controls [76]. In another study, Su et al. evaluated the potential of miR-122 in predicting virological response to pegylated interferon and ribavirin, retrospectively in 126 patients and prospectively in another 51 patients (this group corresponded to an independent validation cohort) with chronic HCV infection by quantifying circulating miR-122 both before and during treatment [64]. miR-122 was positively correlated with hepatic necroinflammation. Patients who achieved sustained virological response (SVR), especially in genotype 2 infection, had higher miR-122 levels in the pre-treatment phase than those with non-SVR. In addition, a strong correlation was found between serum and hepatic miR-122 expression [64]. Murakami et al. demonstrated in the liver biopsies of patients with HCV genotype 1b (n = 99) that the expression level of 9 miRNAs (miR-18a, miR-27b, miR-34b*, miR-122, miR-143, miR-145, miR-378, miR-422b*, miR-652) was significantly different in the SVR (n = 46) and non-responder (NR) (n = 25) groups [94]. Waidmann et al. evaluated miR-122 expression in the serum of patients with chronic hepatitis C virus genotype 1 infection treated with a combination of pegylated interferon-α and ribavirin (n = 52). There was no significant difference in miR-122 expression in the serum from patients who showed sustained virological response (n = 28) in comparison to that from the non-response group (n = 24), suggesting that this miRNA may not be a suitable marker for response prediction of the treatment [95]. However, Butt et al. evaluated the expression of liver and serum miR-122 in patients with CHC genotype 3 (n = 123). The authors found liver miR-122 downregulated and serum miR-122 upregulated in those patients in relation to healthy individuals (n = 60), demonstrating a significant inverse correlation. In addition, the expression of serum miR-122 before the pre-treatment with pegylated-interferon plus ribavirin was upregulated in patients with SVR (n = 70) compared to that in the non-responders or relapsers (n = 53), and it remained independently associated with treatment responses as obtained from multivariate analysis, hence suggesting that serum miR-122 has the potential for predicting response to treatment [96].

Waring et al. evaluated the expression of miR-122 in serum samples from patients with HCV (n = 86, 45 being genotype 1; 20, genotype 2; and 21, genotype 3) before (baseline), during, and 8 weeks after the end of treatment with paritaprevir plus ritonavir combined with dasabuvir or ombitasvir, which can be associated or not with ribavirin. In patients with genotype 1 who achieved SVR (n = 33) miR-122 expression decreased over the treatment period and remained below the baseline level 8 weeks after the end of the treatment. On the other hand, in non-responders (n = 7) the expression of miR-122 initially decreased but returned to the baseline after the second week of the treatment. Regarding relapsers (n = 5), although they had decreased miR-122 expression throughout the treatment, the level returned to the baseline 8 weeks after the end of the treatment. The miR-122 expression profile was similar for all patients with genotype 2 [SVR (n = 14), relapsers (n = 2), non-responders (n = 2), and lost to follow-up (n = 2)]. In all the patients with genotype 3, miR-122 expression decreased with treatment and did not return to the baseline even 8 weeks after the end of the treatment [SVR (n = 6), relapsers (n = 3), non-responders (n = 11), and lost to follow-up (n = 1)]. In addition, the authors demonstrated that miR-122 expression in patients with SVR 8 weeks after the treatment completion was greater in relation to healthy subjects (n = 30) [97].

Wang et al. evaluated serum miR-122 levels in 105 patients with chronic HCV infection and 11 patients with acute hepatitis (six with hepatitis E, four with hepatitis B, and one with hepatitis A) [77]. They found that miR-122 levels were significantly higher in chronic HCV and acute hepatitis patients than in healthy individuals (n = 33). A strong correlation was found between ALT activity and hepatic inflammation, which was graded using the Ishak scoring system, but there was no correlation between miR-122 and ALT serum levels or liver inflammatory activity in chronic HCV patients [77]. Additionally, Zhang et al. reported that HCV-infected patients (HCV, n = 34; HCV-HCC, n = 10) had higher miR-155 expression in hepatic tissue than healthy controls (n = 7) and nonalcoholic steatohepatitis (NASH) patients (n = 12) [78]. They also demonstrated that, before interferon plus ribavirin therapy, circulating miR-155 levels were positively correlated with the levels of plasma interferon-gamma-inducible protein of size 10 kDa (IP-10), which is a chemokine with increased expression in inflammatory diseases. Patients who achieved SVR had lower expression of circulating miR-155, indicating that this miRNA has potential as a response predictor [78]. In a different study, Zhang et al. compared expression of serum miR-143 and miR-215 in 118 chronic HCV and 95 HCC patients with expression in healthy controls (n = 127) [79]. These miRNAs showed increased expression in the case of both chronic HCV and HCC. The AUROC was 0.617 (sensitivity: 78.0%; specificity: 64.0%) for miR-143 and 0.802 (sensitivity: 78.0%; specificity: 89.0%) for miR-215 in distinguishing HCV patients from healthy controls. For distinguishing between the HCC group and the control group, the AUROC was 0.795 (sensitivity: 73.0%; specificity: 83.0%) for miR-143 and 0.816 (sensitivity: 80.0%; specificity: 91.0%) for miR-215 [79].

2.4. Hepatotoxicity

The expression of miRNAs in the serum and plasma of acetaminophen (APAP)-intoxicated patients was evaluated by Starkey Lewis et al. [80]. These authors found that miR-122 and miR-192 levels were upregulated in APAP-induced acute liver injury (APAP-ALI) patients (n = 53) compared with healthy individuals (n = 25) and chronic kidney disease patients (n = 22). Although no significant correlation was found between miR-122 and prothrombin time, total serum bilirubin, or serum creatinine levels, serum miR-122 levels were correlated with ALT in APAP-ALI patients [80]. In another study, Ward et al. evaluated miRNA expression in both plasma and serum of 49 patients with APAP overdose and seven patients with ischemic hepatitis [81]. In this work, more than 100 miRNAs were found to be upregulated at least eight-fold in APAP compared with a control group that did not use APAP in the previous week (n = 12). Among these miRNAs, seven (miR-21-5p, miR-27b-3p, miR-122-5p, miR-125b-5p, miR-193a-5p, miR-194-5p, and miR-1290) were found to be able to differentiate between ischemic hepatitis and APAP-induced liver injury. In this study, the AUROC for miR-122 was 0.897 for distinguishing between APAP-induced and ischemic liver injury. In addition to these miRNAs, miR-23b-5p, miR-28-3p, miR-1247-5p, and miR-452a-3p were shown to compose a miRNA recovery profile capable of differentiating patients with APAP overdose from those with ischemic liver injury during N-acetylcysteine treatment, since these miRNAs normalized after successful N-acetylcysteine treatment [81].

Likewise, Yang et al. studied miRNA expression in serum and urine samples of children hospitalized for APAP overdose [82]. The authors found 147 serum miRNAs that were upregulated in eight children hospitalized for APAP overdose, compared with 10 healthy children (who had not taken APAP for 14 days) and 10 hospitalized children receiving therapeutic doses of APAP. Among these
miRNAs, miR-122 stood out as being strongly correlated with the peak serum level of APAP-protein adducts, which is an indicator of APAP oxidative metabolism. However, no significant correlation was observed between serum miR-122 and ALT. Despite this, it is interesting to note that these miRNAs were able to differentiate between children with APAP overdose and patients receiving therapeutic doses [82].

Recently, Singhal et al. attempted to determine whether new biomarkers that have been proposed for hepatotoxicity, including miR-122 as well as sorbitol dehydrogenase, cytookeratin 18, high-mobility group box-1, and glutamate dehydrogenase, would increase during ALT elevation related to cholestyramine treatment, which is a cholesterol-lowering drug not associated with clinically significant liver damage. In 11 patients out of a total of 67, an ALT elevation greater than three times the upper normal limit was observed and accompanied by an increase in serum miR-122 greater than 20-fold, suggesting a considerable degree of specific liver injury [83].

Ding et al., studying a different kind of poisoning, assessed the expression of miR-122 in the serum of 36 patients with acute hepatotoxicity caused by paraquat (N,N'-dimethyl-4,4'-bipryridinium dichloride), a widely used herbicide [84]. The authors demonstrated that serum miR-122 levels were significantly elevated in this group of patients compared with the control group (n = 19), and a positive correlation was found between miR-122 and ALT greater than 600 U/L. In addition, daily monitoring of miR-122 expression throughout treatment showed that there was a reduction in both miR-122 and ALT with treatment evolution [84].

Although further studies are required to validate the clinical application of miRNAs, in general, miR-122 has stood out among all miRNAs related to hepatotoxicity as a potential marker of acute liver injury. Future studies exploring miR-122 and other miRNAs in non-APAP liver toxicity are needed to discover new diagnostic and prognostic tools in this often-neglected area.

2.5. Nonalcoholic fatty liver disease

Cermelli et al. evaluated the expression of miR-16, miR-21, miR-34a, and miR-122 in chronic HCV infection and nonalcoholic fatty liver disease (NAFLD) [63]. They demonstrated that both in the cohort test and in the independent validation cohort the expression of miR-16, miR-34a, and miR-122 was higher in both HCV (n = 53) and NAFLD (n = 34) patients than in the control group (n = 19), and that miR-21 expression was not altered in either group. In addition, miR-34a and miR-122 correlated positively with ALT, AST, fibrosis stage, and inflammatory activity. In NAFLD patients, miR-122 also correlated with serum lipids (total cholesterol level and low-density lipoprotein). miR-34a and miR-122 could also be used to discriminate between NAFLD patients with NAFLD-simple steatosis (NAFLD-SS) and nonalcoholic steatohepatitis (NASH), which were distinguished using the NAFLD activity score (NAS); for distinguishing between these groups, miR-34a had an AUROC of 0.75 and miR-122 had an AUROC of 0.70. Thus, miR-34a and miR-122 might represent useful diagnostic biomarkers in NAFLD [63].

Likewise, Mijiyaka et al. evaluated miR-122 expression in both liver biopsy (n = 67) and serum samples (52 from 67 samples) from NAFLD patients, and found a significant correlation between serum miR-122 and liver miR-122 [85]. In addition, patients with mild steatosis (less than 33%) had lower serum miR-122 expression than patients with severe steatosis (greater than 33%). There was also lower expression of serum miR-122 in patients with severe fibrosis (greater than F1) than in patients with mild fibrosis (F0 or F1). The authors constructed an ROC curve for serum miR-122 as a predictor of liver fibrosis and found an AUROC of 0.820, which was greater than the AUROCs for hyaluronic acid and type IV collagen, both markers associated with liver fibrosis [85].

Recently, Pirola et al. demonstrated, through an independent validation cohort, that serum miR-122, miR-192, and miR-375 levels were upregulated in patients with NAFLD (both SS and NASH) (n = 65) [65]. These miRNAs correlated with the severity of disease and had greater expression in NASH patients than in SS patients. Despite this, these miRNAs were not related to the severity of fatty liver infiltration and lobular inflammation. On the other hand, miR-122 correlated with advanced fibrosis, AST, ALT, and GGT. The AUROCs for differentiating between the advanced stages of the disease were similar for miR-122, miR-192, and miR-375 (~0.7). In addition, miR-122 has been shown to be able to distinguish a NAS score greater than five to the same extent as hepatic transaminases (AST and ALT) [65].

2.6. Other liver diseases

Serum miR-21 and miR-122 expressions were evaluated by Migita et al. in 46 patients with type 1 autoimmune hepatitis (AIH) [86]. miR-21 and miR-122 were found to be upregulated in AIH patients as compared with chronic HCV patients (n = 40) and healthy controls (n = 13). Both miR-21 and miR-122 were positively correlated with ALT and AST, but there was no significant correlation with type IV collagen. Only miR-21 presented a positive correlation with liver necroinflammation grading, although both miR-21 and miR-122 presented lower expression in patients with advanced fibrosis. In addition, it was demonstrated that these miRNAs are upregulated in untreated AIH patients, but downregulated four weeks after corticosteroid therapy (n = 34, with paired serum samples from the same subjects) [86].

Ninomiya et al. assessed miRNA expression in patients with primary biliary cholangitis (PBC), an autoimmune disease characterized by progressive destruction of bile ducts and positive antimitochondrial antibody (AMA) [87]. In the validation phase of the study, the authors found that miR-197-3p and miR-505-3p were downregulated in PBC patients compared with healthy individuals, CHB patients, and chronic HCV patients (each of the four groups consisted of 10 samples) [87]. In addition, Tan et al. found, in the validation phase of their study, that serum miR-122 and miR-141-3p levels were upregulated, and miR-26b-5p downregulated, in PBC patients (n = 82) compared with healthy individuals (n = 60) [66]. The authors constructed a miRNA panel with these three miRNAs and obtained an AUROC of 0.905 (sensitivity: 80.5%; specificity: 88.3%) for distinguishing between PBC patients and healthy individuals. In addition, this miRNA panel presented better diagnostic performance than ALP and antinuclear antibody, but lower sensitivity and specificity than AMA [66].

Alcoholic liver disease (ALD) is one of the major causes of cirrhosis and can present histologically as steatosis, steatohepatitis, and cirrhosis. Chen et al. identified a serum miRNA pattern that could be used to distinguish alcoholic steatohepatitis patients (ASH) (n = 3) from healthy subjects (n = 3) [88]. Some of the miRNAs included in this profile were miR-135b, miR-490, miR-761 (all upregulated), miR-203, and miR-214 (both downregulated) [88]. Blaya et al. found that serum miR-182 levels were upregulated in 28 patients with alcoholic hepatitis (AH) compared with the control group (n = 8), but there was no association with disease severity and mortality. In contrast, miR-182 expression in the liver showed association with disease severity and mortality; thus, the authors suggested that serum miR-182 levels might not be a suitable biomarker for AH [89]. Li et al. found that serum miR-223 levels were upregulated in alcoholics (n = 300, including 140 with recent excessive drinking and 160 without recent excessive drinking) compared with healthy individuals (n = 45) [90]. The authors further demonstrated that miR-223 is important for limiting hepatic neutrophil infiltration that occurs in alcoholic liver disease [90].
Interestingly, Hoy et al. evaluated the expression of miRNAs in serum from patients infected with Schistosoma mansoni in the Piída community of Uganda, an area of high endemicity (n = 20), and in the Chiredzi community of Zimbabwe, an area of low endemicity (n = 5) [91]. The control groups were composed of individuals from the same areas whose feces did not present parasite eggs (n = 10 for the Piída community and n = 9 for the Chiredzi community). The authors demonstrated that infected individuals could not be discriminated from uninfected individuals based on serum host-derived miRNAs, but could be discriminated based on parasite-derived miRNAs found in patients’ serum. The three parasite-derived miRNAs found in patient serum were sma-miR-277, sma-miR-3479-3p, and sma-bantam. The authors used these three miRNAs to construct a miRNA panel that presented an AUROC of 0.845 (sensitivity: 90.0%; specificity: 80.0%) for the Piída community and an AUROC of 0.933 (sensitivity: 80.0%; specificity: 89.0%) for the Chiredzi community for distinguishing infected individuals from the control group [91].

2.7. MicroRNA across distinct disorders

Out of the studies presented in Table 1, 19 reported increased expression of circulating miR-122 in patients with HBV [69,70,72,73], HCV [63,64,75–77], HCC [70], NAFLD [63,65,85], AH [86], PBC [66], LC [59], APAP overdose [80–82], cholosteryamine treatment [83], or parquat poisoning [84]. Nine of these studies showed a positive correlation between miR-122 and ALT [59,63–65,75,76,80,81,84], while six others showed not so significant correlation [70,72,77,82,83,85]. Adding to the fact that miR-122 is considered hepatospecific [52], miR-122 could be potent as a marker for liver injury. In addition to miR-122, other miRNAs have also been described as organ-specific, such as miR-208a and miR-208b (heart) [98–100], or miR-9 and miR-124 (brain) [101–103] that may be potential diagnostic markers since they are found in serum or plasma samples after a tissue injury [100,101]. Other miRNAs, such as miR-192 (liver) [80], miR-1 and miR-499 (heart) [80,100], and miR-218 (brain) [80], have been described as organ-enriched, that is, abundant in certain organs but not specific to them; they can also be released into circulation when the organ is injured. This points at the possibility of using both organ-specific and organ-enriched miRNAs to improve the sensitivity and specificity of identification of the specific cause of a liver disease [80,100].

In comparison to the other miRNAs presented in Table 1, there are not enough studies yet to assign their expression to a specific disease or a disease stage. However, it is clearly observed that different circulating miRNAs are altered for the same etiology: miR-21 (upregulated: HBV [73], HCV, HCV-HCC [62], and AH [86]), miR-29a (downregulated: LC [70]; upregulated: CHB [70]), miR-34b (upregulated: NAFLD and HCV [63]), miR-92a (upregulated: HBV [72]), miR-106a (upregulated: HBV [72]), miR-106b (downregulated: CHB cirrhosis [60]), miR-130a (upregulated: HBV [73]), miR-130b (downregulated: CHB [71]; upregulated: HBV-HCC [61,71]), miR-181b (upregulated: CHB cirrhosis [60], and LC [67]), miR-192 (upregulated: HBV [73], APAP overdose [80], and NAFLD [65]), miR-215 (upregulated: CHB [69], HCV, and HCC [79]), miR-223 (upregulated: alcoholics [90], and HBV [72]), miR-885-5p (upregulated: CHB [58,69], LC, and HCC [58]). Table 2 presents some miRNAs, arranged in ascending order of miRNA nomenclature, in order to provide insights into the different etiologies in which a given miRNA might be altered. For example, miR-125b, described as a tumor suppressor by targeting Bcl-2 and stimulating apoptosis [71,104], has been found to be upregulated in microvesicles isolated from plasma samples of patients with CHB, but downregulated in HCC, thereby suggesting that a lower expression of miR-125b might result in loss of tumor suppression, hence causing HCC development [71]. It is important to note that similar independent studies have reported concordant results. For example, circulating miR-122 has been described as upregulated in patients with HCV [63,64,75–77], NAFLD [63,65,85], or APAP overdose [80–82]; the results of these studies have been confirmed by independent cohort validation for HCV [63,64] and NAFLD [63,65]. In this regard, confirmation of these results by independent research groups is critical for validating the potential of circulating miRNAs in prognosis and diagnosis of diseases. Thus, further studies are necessary to evaluate whether an expression profile in a particular liver disease could be correlated to the initial liver injury, inflammation, establishment, and progression of the disease, eventually hinting at the strategies for cure or even to characterize the etiology.

3. Pre-analytical aspects involving miRNA analysis

McDonald et al. evaluated the expression of miR-15b, miR-16, and miR-122 in serum and plasma samples from healthy subjects (n = 10) within 72 h of storage. The authors demonstrated that these miRNAs were stable for up to 72 h if refrigerated (4 °C) or frozen (−20 °C), and up to 24 h if kept at room temperature. In addition, hemolysis (up to 600 mg/dL and 1200 mg/dL hemoglobin) did not alter miR-24 and miR-122, respectively, but 25 mg/dL hemoglobin caused a significant increase in miR-15b and miR-16 concentrations indicating that these miRNAs may be released from erythrocytes [105]. In another study, the expression of miR-15b, miR-16, and miR-24 in plasma samples (n = 2) were found stable when kept at room temperature for up to 24 h or subjected to eight cycles of freeze-thaw. In addition, the authors demonstrated that unlike naked miRNAs that are susceptible to degradation by RNase activity in plasma, the levels of endogenous miRNAs miR-15b, miR-16, and miR-24 were not significantly altered, indicating that circulating miRNAs exist in a form resistant to plasma RNase activity [106].

Blondal et al. evaluated the expression of 119 miRNAs most commonly found in serum and plasma samples, including let-7b, miR-21, miR-23a, miR-29a, miR-92a, miR-106a, miR-130a, miR-192, miR-215, miR-223, miR-451 among others [107]. The serum and plasma samples were grouped as hemolyzed and non-hemolyzed, each group consisting of 381 samples, resulting in an n > 1500. The authors suggested two methods for hemolysis detection: absorbance and delta Cq (miR-23a – miR-451). In the first method, the hemolyzed samples showed an absorbance peak at a wavelength of 414 nm corresponding to oxyhemoglobin, while the non-hemolyzed samples did not. Since as miR-23a expression was stable in serum and plasma samples and remained unaffected by hemolysis, and miR-451 is known to be enriched in erythrocytes, in the second method, the authors found that delta Cq (miR-23a – miR-451) > 5 could be an indicator of possible erythrocyte miRNA contamination, and the delta Cq (miR-23a – miR-451) > 7 indicated a high risk of hemolysis. The authors carefully draw attention to the fact that many miRNAs found in serum and plasma samples remain unaffected by hemolysis and therefore may remain associated as disease biomarkers, but hemolysis may introduce changes in the miRNA expression profile causing systematic error in the data analysis [107].

Grasedieck et al. evaluated the stability and amount of miRNA (pg/μL) using Bioanalyzer Small RNA kit (Agilent Technologies, Santa Clara, CA) and identified individual miRNA (miR-24, miR-93, miR-223, and miR-451) by RT-qPCR in serum samples from healthy subjects (n = 3) and patients with multiple myeloma (n = 3) for short- (10 days), intermediate- (<20 months), and long-term storages (<10 years) at −80 °C and −20 °C. Short-term storage samples of serum from healthy subjects were also evaluated at room temperature. The authors demonstrated that there was no differ-
ence between short-term storage at −80 °C and −20 °C, but storage at room temperature and repeated freeze-thaw cycles did lead to a significant reduction in miRNA levels compared to the continuous storage samples at −80 °C, although expression of miR-24, miR-93, miR-223, and miR-451 remained detectable by RT-qPCR. The authors observed no difference in the miRNA concentration in samples stored for intermediate-term at −80 °C and −20 °C, except for miR-93 that showed a significant decrease, hence suggesting differences in the stability of the individual serum miRNAs. In long-term storage conditions, the authors reported a small reduction in the amount of miRNA within 4 years and a significant reduction after 6 years; these results were confirmed by RT-qPCR for all of miR-24, miR-93, miR-223, and miR-451. Thus, the authors emphasize the importance of consistency of storage conditions for the samples and the stability of miRNAs for use as biomarkers even under conditions considered unsuitable for messenger RNA (mRNA) [108]. Likewise, Balzano et al. demonstrated that the expression of miR-21-5p, miR-125b-5p, miR-126-3p, miR-200b-5p, miR-200c-3p, miR-212-3p, miR-425-5p, and miR-579-3p were stable in plasma samples from healthy subjects (n = 5) even 12 months of storage at −80 °C, compared to that of the same samples before freezing. In addition, the expression of these miRNAs were evaluated in another set of 24 plasma samples from healthy subjects stored at −80 °C for up to 14 years demonstrating that all miRNAs remained detectable, although only miR-212-3p expression did not significantly reduce. The authors suggest that the presence of AU nucleotide sequences may contribute to the reduced miRNA stability. Overall, none of the miRNAs showed any change in stability over a period of 4 years of storage [109].

Köberle et al. evaluated the expression of miR-1, miR-16, miR-21, miR-122, and miR-142-3p in serum samples from healthy subjects (n ≥ 3) incubated at room temperature for up to 24 h; miR-1 and miR-122 levels decreased significantly after 3 h, whereas miR-16, miR-21, and miR-142-3p levels were reduced only slightly after 5 h, suggesting that endogenous miRNAs exhibit different stabilities. In addition, they demonstrated that RNase A, B, and C inhibitor (0.3 U/μL) minimized the loss of miRNAs in serum and whole blood samples (n = 3). The authors also showed that the expression of miR-122, which is not expressed in blood cells, in hemolyzed serum samples were more stable compared to that in non-hemolyzed samples (n = 3) suggesting that hemolysis may contribute to the stability of circulating miRNAs due to the release of RNase inhibitors from blood cells, and that vesicle-associated miRNAs exhibited increased resistance to RNase A activity in relation to non-vesicle-associated miRNAs [110].

Although it has been demonstrated that miRNAs have adequate stability to be used as biomarkers, collection, preparation, handling, and storage of the samples should be standardized to avoid confounding factors that might influence the results [111,112].

### 4. miRNAs as biomarkers: methodological considerations for clinical use

In addition to other considerations, clinical use of miRNAs as biomarkers should account for differences in sample types and methods for normalization of RT-qPCR data. While some research has shown that there is a strong correlation between serum and plasma miRNA profiles [81,106], other studies have reported that some miRNAs may occur at different levels in these sample types [105,113]. Therefore, caution should be exercised when comparing results from different studies. Another important technical consideration is the normalization of RT-qPCR data to reduce variation in the quantification of gene expression among samples [114]. Various methods have been proposed for the normalization of miRNA expression data, including the use of a spike-in (an exogenous synthetic control not present in humans) [68,74,106], RNU6B (U6) [58,67,70,71], miR-16 [59,60,62,75], and a combination of three or more miRNAs that exhibit stable expression between analyzed samples [69,115,116]. It may be most appropriate to use a combination of three or more miRNAs, because this method may reduce
variation between technical replicates and minimize the number of false-positive downregulated miRNAs being possible the detection of the real changes in patient samples profiles in function of the disease evolution. In contrast, the use of spike-in controls should be limited to controlling for the efficiency of RNA extraction and reverse transcription, because this method does not account for all types of experimental variability [116].

5. Conclusions

Liver diseases are common and remain an important public health problem, with increasing mortality and healthcare costs. New biomarkers to improve the diagnosis and prognostication of liver diseases could lead to a better management of these challenging conditions, reducing health, social, and economic impacts associated with them. Over the last decade, the amount of published data on circulating miRNAs as biomarkers for liver disease has increased substantially. Some miRNAs, such as miR-122, have demonstrated potential as diagnostic and prognostic tools for multiple liver diseases. Other miRNAs, such as miR-34a, miR-192, and miR-885-5p, appear to be disease-specific, and the potential of these miRNAs to be used as biomarkers needs further investigation with adequate methodology and sample size. In addition, we highlight the fact that suitable methodological and independent studies with sufficient sample size, preferably involving independent validation cohort, are still required to evaluate the usefulness of miRNAs as biomarkers in clinical practice.

Conflict of interest

None.

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