





## NAFLD and vitamin D: Evidence for intersection of microRNA-regulated pathways

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### Abstract

Non-alcoholic fatty liver disease (NAFLD) is now the most common cause of chronic liver disease, worldwide. The molecular pathogenesis of NAFLD is complex, involving numerous signalling molecules, including microRNAs (miRNAs). Dysregulation of miRNA expression is associated with hepatic inflammation, fibrosis and hepatocellular carcinoma. Although miRNAs are also critical to the cellular response to vitamin D, mediating regulation of the vitamin D receptor and vitamin D's anti-cancer effects, the role of vitamin-D-regulated miRNAs in NAFLD pathogenesis has been relatively unexplored. Therefore, this review aims to critically assess the evidence for a potential subset of miRNAs that are both dysregulated in NAFLD and modulated by vitamin D. Comprehensive review of eighty-nine human studies identified twenty-five miRNAs found dysregulated in more than one NAFLD study. In contrast, only seventeen studies, including a protocol for a trial in NAFLD, had examined miRNAs in relation to vitamin D status, response to supplementation, or vitamin D in the context of the liver. This paper summarises these data and reviews the biological roles of six miRNAs (miR-21, miR-30, miR-34, miR-122, miR-146, miR-200) found dysregulated in multiple independent NAFLD studies. While modulation of miRNAs by vitamin D has been understudied, integration of the data suggests seven vitamin-D-modulated miRNAs (miR-27, miR-125, miR-155, miR-192, miR-223, miR-375, miR-378) potentially relevant to NAFLD pathogenesis. Our summary tables provide a significant resource to underpin future hypothesis-driven research, and we conclude that the measurement of serum and hepatic miRNAs in response to vitamin D supplementation in larger trials is warranted.

**Keywords:** NAFLD; MAFLD; vitamin D; miRNA; obesity; type 2 diabetes

(Received 17 March 2021; revised 23 November 2021; accepted 2 December 2021; accepted manuscript published online 9 December 2021)

### Introduction

Non-alcoholic fatty liver disease (NAFLD) is now the most common cause of chronic liver disease and a significant public health problem worldwide. Closely associated with obesity, the global prevalence of NAFLD is estimated to be 24%, placing a significant clinical and economic burden on many countries, including the United Kingdom<sup>(1,2)</sup>. Defined physiologically by excess accumulation of lipids in the liver, NAFLD is an umbrella term encompassing a range of histopathology from hepatic steatosis (non-alcoholic fatty liver, NAFL), to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis. There is tremendous inter-individual variation both in disease phenotype and its progression, determined by dynamic interactions between genetic, metabolic and environmental factors that are not completely understood<sup>(3,4)</sup>. Although several genetic variants have been identified that influence NAFLD susceptibility, dietary and lifestyle factors strongly influence disease progression, and weight loss is the mainstay of current clinical management guidelines<sup>(5–7)</sup>.

The integral relationship between metabolic dysfunction and NAFLD, along with its complex and variable pathogenesis between individuals, prompted a consensus-driven proposal

in April 2020 for a name change to 'metabolic associated fatty liver disease' (MAFLD)<sup>(4,8)</sup>. More than a name change, this would change the diagnostic criteria from being exclusionary (absence of excess alcohol intake and other chronic liver diseases) to a positive diagnosis of: hepatic steatosis alongside either overweight/obesity, type 2 diabetes, or the presence of two metabolic risk factors in normal-weight individuals<sup>(8)</sup>. Importantly, the recognition of the heterogeneity of NAFLD presentation and the disease as a continuum rather than a dichotomous stratification between NASH and non-NASH would permit the recognition of the coexistence of metabolic and other chronic liver diseases (including alcohol-related fatty liver), and potentially present opportunities for improved clinical trial designs<sup>(4,8)</sup>. Multiple positive endorsements for the proposal have been published in recent months, from patients<sup>(9)</sup>, nurses<sup>(10)</sup>, and professional associations<sup>(11–13)</sup>. Nonetheless, time is required to update International Classification of Diseases (ICD) diagnostic codes. At the time of writing, the most recent codes (ICD-11, 09/2020) refer only to NAFLD and NASH; therefore, we use these terms here.

Individual dietary nutrients have been implicated in NAFLD pathogenesis and may differentially affect disease development

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and/or progression. For example, separate from their provision of energy, dietary macronutrients (saturated and *n*-3 fatty acids, fructose) appear to differentially effect lipid accumulation in the liver through multiple molecular and cellular mechanisms<sup>(14,15)</sup>. Similarly, micronutrient deficiencies have been associated with NAFLD, and a mechanistic basis exists for their therapeutic targeting<sup>(16)</sup>. However, to date, only a limited number of intervention trials have examined dietary supplements in patients with NAFLD, with mixed results<sup>(3,16)</sup>. Vitamin D is of interest in part because it has anti-proliferative, anti-inflammatory and anti-fibrotic properties that have been shown to attenuate NAFLD progression in pre-clinical models<sup>(17,18)</sup>. Moreover, low levels of dietary vitamin D intakes and poor vitamin D status are widespread and are notably observed in paediatric NAFLD<sup>(19–21)</sup>. However, intervention trials of vitamin D supplementation in patients with NAFLD have been heterogeneous in their duration, dosing and outcome measurements, and questions remain about optimal regimens for efficacy in chronic liver disease<sup>(22)</sup>.

The molecular pathogenesis of NAFLD is complex, involving numerous signalling molecules involved in hepatic metabolism, oxidative, inflammatory and fibrotic processes<sup>(3)</sup>. These include microRNAs (miRNAs) that play an essential role in gene expression and regulatory networks involved in lipid and carbohydrate metabolism and cellular stress response pathways<sup>(23)</sup>. Dysregulation of miRNA expression in the liver is associated with hepatic inflammation, fibrosis and the development and progression of multiple liver diseases, including hepatocellular carcinoma (HCC)<sup>(24,25)</sup>. Separately, a body of evidence exists for miRNAs in mediating the cellular response to vitamin D, including the post-transcriptional regulation of the vitamin D receptor (VDR) and vitamin D's anti-cancer properties<sup>(26)</sup>. Interestingly, while the anti-cancer effects of vitamin D have been observed in liver<sup>(27,28)</sup>, the potential role of vitamin-D-regulated miRNAs in NAFLD remains unexplored.

Therefore, the aims of the present paper were to first comprehensively review the data from human studies for involvement of miRNA in NAFLD pathogenesis. Secondly, we aimed to review serum miRNA profiling studies that have examined vitamin D status or response to supplementation, along with the limited research that has investigated miRNAs and vitamin D in the context of liver pathology. Finally, integrating these data, this paper aimed to critically assess the evidence for a potential subset of miRNAs that are both dysregulated in NAFLD and modulated by vitamin D.

### MicroRNAs in health and disease

Typically only twenty-two nucleotides long<sup>(29)</sup>, miRNAs are small, non-coding RNAs that are critical for development, regulate a variety of normal physiological processes, and are found dysregulated in cancer<sup>(30)</sup>, liver<sup>(24)</sup> and metabolic diseases<sup>(31)</sup>. They play a key role in the post-transcriptional regulation of gene expression, binding complementary sequences within mRNA transcripts and typically suppressing gene expression through mRNA degradation and translation repression. Recent estimates suggest there are approximately 2300 human miRNAs<sup>(32)</sup>. However, miRNAs can have numerous mRNA

targets, and individual mRNAs can be targeted by several miRNAs. Indeed, miRNAs regulate an estimated 60% of human protein coding genes impacting almost all biological processes<sup>(33,34)</sup>.

Primary miRNAs (pri-miRNAs) are transcribed and processed in the nucleus into pre-miRNA molecules that contain a characteristic hairpin structure. After export into the cytoplasm, pre-miRNAs are processed into miRNA duplexes by the Dicer enzyme. During the subsequent multistep assembly of the RNA-induced silencing complex, only one of the miRNA strands (the guide strand) of these duplexes will be retained, becoming the mature miRNA targeting complementary mRNA for repression<sup>(35)</sup>. By current nomenclature conventions, mature miRNAs are given the prefix 'miR' and a number indicating identification order; family members with nearly identical sequences expressed from different precursors or genomic loci have lettered suffixes (e.g. miR-30b and miR-30c)<sup>(36)</sup>. Mature miRNAs derived from the 5' and 3' arms of the pre-miRNA hairpin structure are annotated with the suffixes *-5p* and *-3p*, respectively. Although both the *-5p* and *-3p* forms may be functional depending on context, experimental data suggest the *5p* form is more frequently found as mature miRNAs<sup>(32,37)</sup>.

Similar to mRNA, the expression of miRNAs varies by cell and tissue type; and while some miRNAs are ubiquitously expressed, others are tissue specific<sup>(38)</sup>. For example, miR-122 is specifically and highly expressed in the liver, representing a remarkable 70% of liver miRNA content<sup>(39)</sup>. Present in blood, urine and other body fluids<sup>(40)</sup>, miRNA levels have been shown to be altered in multiple diseases as a result of genomic events or alterations in miRNA biogenesis, prompting significant interest in miRNAs as clinical biomarkers and therapeutic targets<sup>(24,31,41,42)</sup>. In the context of cancer in particular, functional and mechanistic studies have established that miRNA dysregulation can be causal, with miRNAs acting as tumour suppressors or oncogenes (oncomiRs)<sup>(30,41)</sup>. Although still at an early stage of development, both miRNA mimics and inhibitors of miRNAs (anti-miRs) have been investigated as therapeutics for cancer, liver and other diseases in early phase clinical trials<sup>(43,44)</sup>. Interestingly, miR-122 binds a region of the hepatitis C virus (HCV) genome promoting its accumulation<sup>(39)</sup>, and Miravirsin, an anti-miR-122 oligonucleotide, was the first miRNA-targeting drug administered in humans. Although found to be safe and specific in phase 2a studies in HCV patients, its use was superseded by effective direct-acting anti-viral drugs<sup>(45,46)</sup>.

A limited number of miRNA-based diagnostic assays have already been brought to market<sup>(43)</sup>, and considerable research has focused on profiling circulating miRNAs as biomarkers of obesity and metabolic disease, including NAFLD<sup>(23,31,42)</sup>. However, the identification of miRNAs as clinical biomarkers or therapeutics is complicated by both technical and confounding factors related to miRNA biology<sup>(47,48)</sup>. While a growing body of evidence suggests miRNAs secreted in extracellular vesicles are stable in circulation and act as paracrine and endocrine factors in metabolic diseases<sup>(31,42,49)</sup>, this complicates the interpretation of miRNA expression and activity in any given cell, tissue or pathology. Age, sex and disease state all influence circulating miRNA profiles<sup>(50)</sup>. Profiles vary between plasma and serum, and further variables include how long the sample was

stored, whether the donor was fed or fasted and what the donor's activity levels are<sup>(48,51,52)</sup>. Importantly, expression and regulatory mechanisms observed in experimental models are not always conserved in humans<sup>(53,54)</sup>. Historically authors have not consistently specified miRNA  $-5p$  and  $-3p$  forms or family member suffixes confounding data interpretation<sup>(55)</sup>, and quantitative polymerase chain reaction (qPCR) primer sequences are also often omitted from methods sections, potentially contributing to reproducibility issues. Given the missing detailed information of mature miRNAs in most studies, we do not use this notation throughout the text, but do detail the reported family member names and mature form suffixes from the studies reviewed and summarised in our tables.

### The role of miRNAs in NAFLD pathogenesis

NAFLD pathogenesis is now recognised to involve 'multiple hits' and crosstalk between multiple organs and the intestinal microbiome<sup>(3,56)</sup>. Although 'simple' fatty liver (NAFL) is typically benign, NASH, defined by steatosis with inflammation, hepatocyte injury (ballooning) and possibly fibrosis, more frequently develops into advanced liver disease<sup>(57)</sup>. In the liver at a cellular level, exacerbated by insulin resistance, lipid accumulation in the hepatocytes is driven by: increased lipid influx (from diet or adipose tissue lipolysis) and increased lipid synthesis (*de novo* lipogenesis), as well as impaired lipid oxidation and export. Notably, some of the miRNAs identified as altered in NAFLD play critical roles in hepatic lipid and carbohydrate metabolism and lipogenesis, regulating multiple transcription factors such as sterol regulatory element-binding protein 1 (SREBP-1), carbohydrate response element binding protein (ChREBP) and the peroxisome proliferator-activated receptors (PPARs)<sup>(23)</sup>.

Progression from NAFL to NASH is a result of lipotoxicity and the over-production of reactive oxygen species (ROS), which leads to mitochondrial dysfunction, endoplasmic reticulum stress and the recruitment and signalling of immune cells. Adipokines from peripheral adipose tissue dysfunction and signalling molecules derived from the gut further drive this pro-inflammatory state systemically, as well as the resulting wound healing response and fibrosis locally in the liver<sup>(58)</sup>. Although progression typically occurs on the order of decades, prognosis is dependent on severity of fibrosis on index biopsy<sup>(59)</sup>, and NASH significantly increases risk of HCC and liver-specific mortality<sup>(57)</sup>. At each stage of the progression of NAFLD, from inflammation to NASH-related HCC, miRNA-regulated pathways are also implicated, and the potential utility of miRNAs as either diagnostic biomarkers or therapeutic targets is of significant interest<sup>(23,24,60)</sup>.

In light of the aforementioned variables that may confound the reproducibility of miRNA research, we comprehensively surveyed the literature and examined the data from serum profiling or mechanistic studies involving liver tissues to identify miRNAs with good evidence for altered levels in humans with NAFLD. Specifically, we defined 'good evidence' as experimentally replicated beyond array or RNAseq profiling, and identified in at least two independent studies. From a PubMed search using the terms NAFLD, NASH, MAFLD, MASH and miRNA, we found eighty-

nine papers that examined miRNA levels in serum or liver in humans with NAFLD. Close data review identified seventy-four unique miRNAs (fifty-one in liver, forty-three in serum and twenty in both) validated beyond array or RNAseq profiling (Fig. 1A). Focusing on miRNAs found dysregulated in subjects with NAFLD in at least two independent studies, identified twelve miRNAs in liver and nineteen miRNAs in serum. Notably, six miRNAs (miR-21, miR-30, miR-34, miR-122, miR-146 and miR-200; Table 1) were found dysregulated in both liver and serum in at least four independent studies (Fig. 1B). An additional six miRNAs were identified as altered in livers (miR-33, miR-141, miR-155, miR-199, miR-223 and miR-378; Supplementary Table 1), and a further thirteen (miR-16, miR-20, miR-22, miR-27, miR-29, miR-99, miR-125, miR-181, miR-192, miR-197, miR-375, miR-379 and miR-451; Supplementary Table 2) were found altered in serum from participants with NAFLD in more than one study.

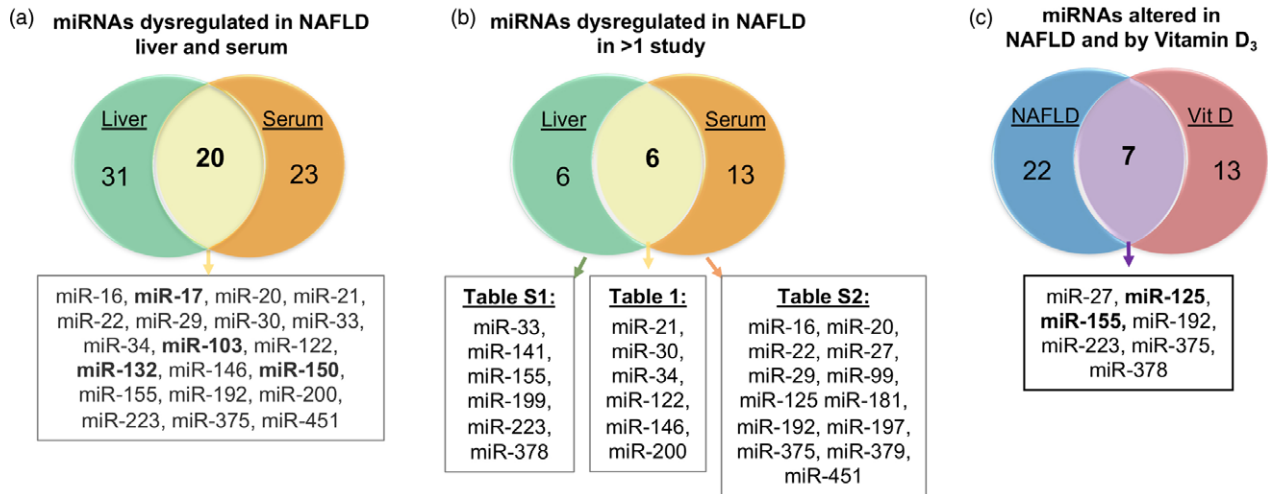
Human NAFLD studies requiring a liver biopsy are typically done in single centres and are often limited in size, similar to miRNA biomarker discovery studies more generally<sup>(48)</sup>. For this reason, the number of participants in each study group was noted in addition to summarising the direction of expression of the miRNAs and the stages of NAFLD examined. Unsurprisingly, liver sample sizes ( $n$  ranged from three to fifty-eight per group; Table 1 and Supplementary Table 1) were smaller than the number of observations in serum ( $n$  ranged from 8 to 392 per group Table 2 and Supplementary Table 2). While serum samples typically came from NAFLD case series, liver samples were often from bariatric surgery patients or tissue banks, and in one case<sup>(61)</sup>, a post-mortem series. In spite of heterogeneous study designs, there was reasonably good concordance between studies in the (generally increased) direction of the miRNAs found dysregulated.

Given the diagnostic and therapeutic potential of miRNAs in NAFLD, we briefly outline relevant regulatory functions of the six miRNAs found altered in both liver and serum (Table 1) below. The limited data concerning functional and pathophysiological effects of their dysregulation arising from these studies, specifically in humans with NAFLD, are summarised in Table 2.

#### MiR-21

Originally described as an oncomiR dysregulated in multiple cancers, miR-21 is now recognised to play a role in numerous inflammatory and fibrotic diseases, including multiple liver diseases<sup>(62)</sup>. In the context of NAFLD, miR-21 is up-regulated in both liver<sup>(63-65)</sup> and serum<sup>(65-68)</sup>, and circulating miR-21 levels have been positively correlated with serum ALT<sup>(67,69)</sup>, steatosis, lobular inflammation<sup>(67)</sup> and hepatic activity<sup>(68)</sup> (Table 1). A single study found decreased hepatic miR-21 levels, but this was in post-mortem samples from people who had died of sudden cardiac death from severe coronary artery disease<sup>(61)</sup>. The single study reporting decreased serum hepatic miR-21 levels had relatively fewer participants (twenty-five NAFLD versus twelve healthy controls (HCs)), and the diagnosis of NAFLD was not specified<sup>(70)</sup>.

Experimental knockdown<sup>(71)</sup> or deletion of miR-21<sup>(72)</sup> in mice markedly reduces lipogenesis and hepatic steatosis in response



**Fig. 1.** Evidence for intersection of miRNA pathways between NAFLD and vitamin D. **(A)** A PubMed search using the terms NAFLD, NASH, MAFLD, MASH and miRNA found eighty-nine papers that examined miRNA levels in serum or liver in humans with NAFLD. Close data review identified seventy-four miRNAs (fifty-one in liver, forty-three in serum and twenty in both) validated beyond array or RNAseq profiling. Of the twenty miRNAs found in both liver and serum, sixteen were found in three or more studies, while four (bold) were identified in only one serum and one liver study. **(B)** Focusing on miRNAs found dysregulated in subjects with NAFLD in at least two independent studies led to the identification of twelve miRNAs in liver and nineteen miRNAs in serum. Six miRNAs were found dysregulated in both liver and serum in at least four independent studies, which are detailed in Table 1. **(C)** A PubMed search using the terms calcitriol, vitamin D and miRNA found twenty vitamin-D-associated miRNAs. Venn analysis of these twenty with the twenty-nine NAFLD-dysregulated miRNAs (the four bold from panel A plus the twenty-five in panel B) suggested seven vitamin-D-modulated miRNAs potentially relevant to NAFLD pathogenesis. Only two (bold) miRNAs were found in more than one vitamin D study.

to high-fat feeding. Among the numerous targets of miR-21 are multiple metabolic and signalling pathways implicated in NAFLD pathogenesis<sup>(73,74)</sup>. These include the genes for several master transcriptional regulators involved in glucose and lipid metabolism, including the hepatocyte nuclear factor 4 alpha (HNF4 $\alpha$ ), forkhead box protein O1 (FOXO1) peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) and sterol regulatory element-binding transcription protein 1c;(SREBP-1c)<sup>(71,72)</sup>, as well as 3-hydroxy-3-methylglutaryl-co-enzyme A reductase (HMGCR)<sup>(70)</sup>. In addition, miR-21 is pro-fibrotic and, as the most up-regulated miRNA during hepatic stellate cell (HSC) activation<sup>(75)</sup>, serves to amplify multiple genes involved in inflammation, fibrosis and NAFLD progression<sup>(73,74)</sup>. While the therapeutic targeting of miR-21 is considered to have potential for multiple fibrotic diseases<sup>(76)</sup>, somewhat surprisingly the deletion of miR-21 did not inhibit fibrosis in mouse models of toxic and biliary liver injury<sup>(75)</sup>. More research is required to resolve the likely differences in miR-21 targets between humans and mice, and the complexity of miR-21 actions in the context of NAFLD, as well as other chronic liver diseases.

### MiR-30

Of the miRNAs profiled in Table 1, relatively fewer studies suggest a role for miR-30 in NAFLD. One challenge in interpreting the data is that the miR-30 family has five members (miR30a–e). Beyond the aforementioned issues of inconsistent specification of family member and –5p and –3p forms in the literature, the miR-30 family members have overlapping sequences that have been found to interfere with expression profiling, and it has been suggested that only high-throughput sequencing can clearly differentiate between miR-30 family members<sup>(77)</sup>. While two studies described decreased hepatic miR-30b<sup>(78,79)</sup> expression in

NAFLD, along with decreased hepatic miR-30c in one of these reports<sup>(79)</sup>, these data appear to be from the same bariatric surgery patients. Separately, two studies have reported a decrease in circulating miR-30c in NAFLD<sup>(69,80)</sup>, with one of these reporting decreased levels and negative correlations between miR-30c and multiple measures of disease severity<sup>(69)</sup>. On the other hand, a single study has reported increased serum levels of miR-30a in a smaller number of participants (eleven NAFLD versus twelve HCs)<sup>(81)</sup>.

Nonetheless, there are some experimental data to suggest a role for miR-30 in NAFLD pathogenesis. As recently reviewed<sup>(82)</sup>, miR-30 has anti-fibrotic properties and has been found down-regulated in the context of HSC activation, hepatic fibrosis and cirrhosis in multiple models of liver injury, as well as human cirrhotic liver. Indeed, overexpression or restoration of miR-30a has been shown both *in vitro* and *in vivo* to suppress HSC activation by inhibiting epithelial–mesenchymal transition, reducing cell proliferation and migration, in line with its reported tumour suppressor activity<sup>(83)</sup>. Separately, limited *in vitro* data suggest increased miR-30a-3p expression prompts triacylglycerol accumulation in hepatocytes via targeting and decreasing PPAR $\alpha$  expression<sup>(81)</sup>. Conversely, in separate experiments also in immortalised hepatocytes, fatty acid deposition triggered by both AMP-activated protein kinase (AMPK) disruption and Dicer knockdown was attenuated by overexpression of miR-30b and miR-30c associated with increased PPAR $\alpha$  expression<sup>(79)</sup>. These limited data nevertheless suggest that a biological role for miR-30 family members cannot be ruled out.

### MiR-34

In contrast, many more studies have been in complete agreement in finding increased expression of miR-34 in liver (seven

**Table 1.** miRNAs dysregulated in both liver and serum from NAFLD patients

miRNA	Sample	Summary
<b>miR-21</b>	Liver	<p><b>Up</b> (<i>miR-21</i>) in NASH (<math>n = 3</math>) versus controls (<math>n = 3</math>) [NIH liver tissue repository]<sup>(63)</sup></p> <p><b>Up</b> (<i>miR-21</i>) in NASH (<math>n = 11</math>) versus steatosis (<math>n = 8</math>) and HCs (<math>n = 6</math>) [pathology database]<sup>(64)</sup></p> <p><b>Up</b> (<i>miR-21</i>) in NASH versus steatosis [<math>N = 28</math>, bariatric surgery patients]<sup>(65)</sup></p> <p><b>Down</b> (<i>miR-21-5p</i>) in NAFLD (<math>n = 12</math>) versus non-NAFLD (<math>n = 15</math>) [postmortem samples, NCSD]<sup>(61)</sup></p>
	Serum	<p><b>Up</b> (<i>miR-21</i>) in NAFLD (<math>n = 48</math>) versus HCs (<math>n = 90</math>) [in males but not females (<math>n = 44</math> NAFLD, <math>n = 221</math> HC)]<sup>(66)</sup></p> <p><b>Up</b> (<i>miR-21</i>) in NASH (<math>n = 87</math>) versus NAFL (<math>n = 50</math>) and HCs (<math>n = 61</math>); positive correlation with ALT, steatosis and lobular inflammation<sup>(67)</sup></p> <p><b>Up</b> (<i>miR-21</i>) in NASH (<math>n = 31</math>) versus HCs (<math>n = 37</math>); positive correlation with hepatic activity<sup>(68)</sup></p> <p><b>Up</b> (<i>miR-21</i>) in NASH versus steatosis [<math>N = 24</math>, bariatric surgery patients]<sup>(65)</sup></p> <p><b>Up</b> (<i>miR-21-5p</i>) in <math>F &gt; 2</math> (<math>n = 29</math>) versus <math>F \leq 2</math> (<math>n = 46</math>); positive correlation with ALT, AST, APRI (NAFL <math>n = 25</math>, NASH <math>n = 50</math>, HCs <math>n = 17</math>)<sup>(69)</sup></p> <p><b>Down</b> (<i>miR-21</i>) in NAFLD (<math>n = 25</math>) versus HCs (<math>n = 12</math>)<sup>(70)</sup></p>
<b>miR-30</b>	Liver	<p><b>Down</b> (<i>miR-30b-5p</i>) in NAFLD (<math>n = 17</math>) and borderline NAFLD (<math>n = 24</math>) versus controls (<math>n = 19</math>); negative correlation with NAFLD [bariatric surgery patients]<sup>(78)</sup></p> <p><b>Down</b> (<i>miR-30b/c</i>) in <math>&gt;5\%</math> steatosis (<math>n = 41</math>) versus <math>&lt;5\%</math> steatosis (<math>n = 19</math>) [bariatric surgery patients]<sup>(79)</sup></p>
	Serum	<p><b>Up</b> (<i>miR-30a-3p</i>) in NAFLD (<math>n = 11</math>) versus HCs (<math>n = 10</math>)<sup>(81)</sup></p> <p><b>Down</b> (<i>miR-30c</i>) in NAFLD (<math>n = 18</math>) versus HCs (<math>n = 62</math>)<sup>(80)</sup></p> <p><b>Down</b> (<i>miR-30c-5p</i>) in <math>SAF \geq 2</math> (<math>n = 50</math>) versus <math>SAF &lt; 2</math> (<math>n = 25</math>), <b>down</b> in <math>NAS \geq 5</math> (<math>n = 38</math>) versus <math>NAS &lt; 5</math> (<math>n = 37</math>), <b>down</b> in <math>F &gt; 2</math> (<math>n = 29</math>) versus <math>F \leq 2</math> (<math>n = 46</math>); negative correlation with FIB4, BARD, NAFLD_FS (NAFL <math>n = 25</math>, NASH <math>n = 50</math>, HCs <math>n = 17</math>)<sup>(69)</sup></p>
<b>miR-34</b>	Liver	<p><b>Up</b> (<i>miR-34a</i>) in NASH (<math>n = 25</math>) versus normal histology (<math>n = 25</math>) [participants with metabolic syndrome]<sup>(114)</sup></p> <p><b>Up</b> (<i>miR-34a</i>) in NASH (<math>n = 13</math>) versus steatosis (<math>n = 15</math>) [bariatric surgery patients]<sup>(96)</sup></p> <p><b>Up</b> (<i>miR34a</i>) in NASH (<math>n = 8</math>) versus normal histology (<math>n = 8</math>) [liver tissue bank]<sup>(180)</sup></p> <p><b>Up</b> (<i>miR-34a-5p</i>) in NASH (<math>n = 42</math>) versus steatosis (<math>n = 16</math>) and non-NAFLD (<math>n = 37</math>) [postmortem samples, CSD and NCSD]<sup>(61)</sup></p> <p><b>Up</b> (<i>miR-34a-5p</i>) in NASH (<math>n = 11</math>) versus controls (<math>n = 10</math>) and NAFL (<math>n = 12</math>) [bariatric surgery patients]<sup>(181)</sup></p> <p><b>Up</b> (<i>miR-34a</i>) in NAFLD (<math>n = 5</math>) versus non-steatosis (<math>n = 3</math> CHB and PBC patients) [liver biopsy patients]<sup>(182)</sup></p> <p><b>Up</b> (<i>miR-34a</i>) in steatosis (<math>n = 4</math>) versus non-steatosis (<math>n = 4</math>) [liver tissue bank]<sup>(120)</sup></p>
	Serum	<p><b>Up</b> (<i>miR-34a</i>) in NAFLD (<math>n = 34</math>) versus HCs (<math>n = 19</math>), <b>up</b> in NASH (<math>NAS &gt; 5</math>) versus steatosis, <b>up</b> in steatosis versus HCs discriminated NASH from steatosis (AUROC = 0.764)<sup>(84)</sup></p> <p><b>Up</b> (<i>miR-34a</i>) in NAFLD (<math>n = 44</math>) versus HCs (<math>n = 221</math>) [adult females], <b>up</b> in NAFLD (<math>n = 48</math>) versus HCs (<math>n = 90</math>) [adult males]<sup>(66)</sup></p> <p><b>Up</b> (<i>miR-34a</i>) in NAFLD (<math>n = 28</math>) versus HCs (<math>n = 36</math>); discriminated NAFLD from HCs (AUROC = 0.781)<sup>(88)</sup></p> <p><b>Up</b> (<i>miR-34a</i>) in NAFLD (<math>n = 18</math>) versus HCs (<math>n = 62</math>)<sup>(80)</sup></p> <p><b>Up</b> (<i>miR-34a</i>) in NASH (<math>n = 31</math>) versus NAFL (<math>n = 17</math>) and HCs (<math>n = 37</math>); positive correlation with histological severity but not fibrosis; discriminated NASH from non-NASH (AUROC = 0.811)<sup>(68)</sup></p> <p><b>Up</b> (<i>miR-34a-5p</i>) in <math>SAF \geq 2</math> (<math>n = 50</math>) versus <math>SAF &lt; 2</math> (<math>n = 25</math>), <b>up</b> in <math>NAS \geq 5</math> (<math>n = 38</math>) versus <math>NAS &lt; 5</math> (<math>n = 37</math>); positive correlation with ALT, AST, ferritin, APRI, FIB4 and total bilirubin (NAFL <math>n = 25</math>, NASH <math>n = 50</math> and HCs <math>n = 17</math>); discriminated <math>SAF \geq 2</math> from <math>SAF &lt; 2</math> (AUROC = 0.76)<sup>(69)</sup></p> <p><b>Up</b> (<i>miR-34a</i>) with inflammation versus non-inflammation (<math>N = 116</math>, post-transplant protocol biopsy in liver transplant recipients)<sup>(86)</sup></p> <p><b>Up</b> (<i>miR-34a</i>) in NAFLD (<math>n = 34</math>) versus HCs (<math>n = 90</math>), <b>up</b> in NASH (<math>n = 86</math>) versus steatosis (<math>n = 124</math>); positive correlation with ALT, AST and histological severity; discriminated NAFLD from HCs (AUROC = 0.77) and NASH from steatosis (AUROC = 0.84)<sup>(85)</sup></p> <p><b>Up</b> (<i>miR-34a-5p</i>) with increasing fibrosis severity [<math>N = 132</math>, NAFLD patients]; in multivariate analyses, positive correlation with steatosis, fibrosis, the PNPLA3 I148M and TM6SF2 E167K variants; discriminated fibrosis from no fibrosis (AUROC = 0.75, 0.73, 0.75 and 0.76 for stages 1, 2, 3 and 4)<sup>(87)</sup></p>
<b>miR-122</b>	Liver	<p><b>Up</b> (<i>miR-122</i>) in <math>&gt;33\%</math> steatosis versus <math>&lt;33\%</math> steatosis, <b>down</b> in severe fibrosis versus no or mild fibrosis [<math>N = 52</math> biopsied NAFLD patients]; negative correlation with fibrosis, positive correlation with serum miR-122 levels<sup>(110)</sup></p> <p><b>Up</b> (<i>miR-122-5p</i>) in steatosis (<math>n = 20</math>) versus NNL (<math>n = 14</math>) and NASH (<math>n = 31</math>); negative correlation with AST [liver biopsy patients]<sup>(111)</sup></p> <p><b>Up</b> (<i>miR-122-5p</i>) in NAFLD (<math>n = 13</math>) versus controls (<math>n = 3</math>) [female bariatric surgery patients]<sup>(116)</sup></p> <p><b>Down</b> (<i>miR-122</i>) in NASH (<math>n = 25</math>) versus normal histology (<math>n = 25</math>) [participants with metabolic syndrome]<sup>(114)</sup></p> <p><b>Down</b> (<i>miR-122</i>) in the more severe NASH (<math>n = 8</math>) versus less severe NASH (<math>n = 5</math>) and steatosis (<math>n = 15</math>) [bariatric surgery patients]<sup>(96)</sup></p> <p><b>Down</b> (<i>miR-122</i>) in steatosis versus non-steatosis [<math>N = 14</math> non-tumour tissue from non-hepatitis B/C HCC resections]<sup>(115)</sup></p> <p><b>Down</b> (<i>miR-122-5p</i>) in NASH (<math>n = 42</math>) versus non-NAFLD (<math>n = 37</math>) [postmortem samples, CSD and NCSD], positive correlation with NAFLD scoring<sup>(61)</sup></p> <p><b>Down</b> (<i>miR-122-5p</i>) in NAFLD (<math>n = 17</math>) and borderline NAFLD (<math>n = 24</math>) versus controls (<math>n = 19</math>); negative correlation with NAFLD [bariatric surgery patients]<sup>(78)</sup></p>
	Serum	<p><b>Up</b> (<i>miR-122</i>) in NAFLD (<math>n = 34</math>) versus HCs (<math>n = 19</math>), <b>up</b> in NASH versus steatosis, <b>up</b> in steatosis versus HCs; discriminated steatosis from HCs (AUROC = 0.927) and NASH from steatosis (AUROC = 0.698)<sup>(84)</sup></p> <p><b>Up</b> (<i>miR-122</i>) in NASH (<math>n = 20</math>) versus HCs (<math>n = 24</math>)<sup>(183)</sup></p>



Table 1. (Continued)

miRNA	Sample	Summary
		<b>Up</b> ( <i>miR-122</i> ) in NAFLD ( <i>n</i> = 44) versus HCs ( <i>n</i> = 221) [adults females], <b>up</b> in NAFLD ( <i>n</i> = 48) versus HCs ( <i>n</i> = 90) [adult males] positive correlation with steatosis severity <sup>(66)</sup>
		<b>Up</b> ( <i>miR-122</i> ) in >33% steatosis versus <33% steatosis, <b>down</b> in severe fibrosis versus no or mild fibrosis [ <i>N</i> = 67 NAFLD patients]; negative correlation with fibrosis, positive correlation with hepatic miR-122 levels; discriminated fibrosis (AUROC = 0.82) <sup>(110)</sup>
		<b>Up</b> ( <i>miR-122-5p</i> ) in NAFLD ( <i>n</i> = 103) versus HCs ( <i>n</i> = 80); discriminated NAFLD from HCs (AUROC = 0.759) <sup>(108)</sup>
		<b>Up</b> ( <i>miR-122-5p</i> ) in NASH ( <i>n</i> = 47) versus steatosis ( <i>n</i> = 30) and HCs ( <i>n</i> = 19), <b>up</b> with histological severity; positive correlation with ALT, AST, GGT and serum CK-18 levels; discriminated histological severity (AUROC range 0.61–0.71) <sup>(111)</sup>
		<b>Up</b> ( <i>miR-122</i> ) in NASH ( <i>n</i> = 87) versus NAFL ( <i>n</i> = 50) and HCs ( <i>n</i> = 61), <b>up</b> in NAFL versus HCs; positive correlation with ALT, steatosis, lobular inflammation and serum CK18-Asp396 <sup>(67)</sup>
		<b>Up</b> ( <i>miR-122</i> ) with histological severity except fibrosis stage 4; positive correlation with ALT, AST, GGT and ferritin [ <i>N</i> = 305 NAFLD] <sup>(106)</sup>
		<b>Up</b> ( <i>miR-122</i> ) in NAFLD ( <i>n</i> = 28) versus HCs ( <i>n</i> = 36); discriminated NAFLD from HCs (AUROC = 0.858) <sup>(88)</sup>
		<b>Up</b> ( <i>miR-122</i> ) in NAFLD ( <i>n</i> = 18) versus HCs ( <i>n</i> = 62) <sup>(80)</sup>
		<b>Up</b> ( <i>miR-122</i> ) in NAFLD ( <i>n</i> = 40) versus controls ( <i>n</i> = 22), <b>up</b> in NASH ( <i>n</i> = 22) versus steatosis ( <i>n</i> = 18) [MO women]; discriminated NAFLD from controls (AUROC = 0.82) and histological severity from mild disease (AUROC = 0.76) <sup>(112)</sup>
		<b>Up</b> ( <i>miR-122</i> ) in circulating exosomes in advanced stage NAFLD ( <i>n</i> = 3) versus early stage NAFLD ( <i>n</i> = 3) <sup>(184)</sup>
		<b>Up</b> ( <i>miR-122</i> ) in severe NAFLD ( <i>n</i> = 14) and mild NAFLD ( <i>n</i> = 36) versus HCs ( <i>n</i> = 61), [independent European cohorts of children with obesity]; positive correlation with ALT, AST and serum CK18 <sup>(185)</sup>
		<b>Up</b> ( <i>miR-122</i> ) in NAFLD ( <i>n</i> = 58) versus HCs ( <i>n</i> = 34), <b>up</b> in NAS ≥ 4 ( <i>n</i> = 32) versus NAS < 4 ( <i>n</i> = 24) and HC ( <i>n</i> = 34), <b>up</b> in NAS < 4 ( <i>n</i> = 24) versus HC ( <i>n</i> = 34); discriminated NAFLD from HCs (AUROC = 0.831) <sup>(186)</sup>
		<b>Up</b> ( <i>miR-122-5p</i> ) in NAFLD ( <i>n</i> = 52) versus controls ( <i>n</i> = 48); discriminated NAFLD from controls (AUROC = 0.774) [adults with T2DM] <sup>(109)</sup>
		<b>Up</b> ( <i>miR-122</i> ) in NAFLD ( <i>n</i> = 210) versus HCs ( <i>n</i> = 90) and <b>up</b> in NASH ( <i>n</i> = 86) versus steatosis ( <i>n</i> = 124); positive correlation with ALT, AST and histological severity; discriminated NAFLD versus HCs (AUROC = 0.92) and NASH (AUROC = 0.81) <sup>(85)</sup>
		<b>Up</b> ( <i>miR-122</i> ) in NASH ( <i>n</i> = 31) versus NAFL ( <i>n</i> = 17) and HCs ( <i>n</i> = 37), <b>up</b> in NAFL ( <i>n</i> = 17) versus HCs ( <i>n</i> = 37); positive correlation with histological severity but not fibrosis <sup>(68)</sup>
		<b>Up</b> ( <i>miR-122-5p</i> ) in SAF ≥ 2 ( <i>n</i> = 50) versus SAF < 2 ( <i>n</i> = 25), <b>up</b> in NAS ≥ 5 ( <i>n</i> = 38) versus NAS < 5 ( <i>n</i> = 37); positive correlation with ALT, AST, ferritin, APRI and BARD (NAFL <i>n</i> = 25, NASH <i>n</i> = 50 and NL <i>n</i> = 17) <sup>(69)</sup>
		<b>Up</b> ( <i>miR-122</i> ) with inflammation versus non-inflammation and ballooning versus non-ballooning ( <i>N</i> = 116, post-transplant protocol biopsy in liver transplant recipients) <sup>(86)</sup>
		<b>Up</b> ( <i>miR-122-5p</i> ) with increasing fibrosis severity ( <i>N</i> = 132 NAFLD patients); in multivariate analyses, positive correlation with steatosis, fibrosis, the PNPLA3 I148M and TM6SF2 E167K variants <sup>(87)</sup>
		<b>Up</b> ( <i>miR-122</i> ) in steatosis ( <i>n</i> = 120) versus controls ( <i>n</i> = 60) and fibrosis ( <i>n</i> = 120) versus controls ( <i>n</i> = 60); positive correlation with ALT, AST and GGT [obese patients] <sup>(187)</sup>
		<b>Down</b> ( <i>miR-122</i> ) with improved histopathological features; positive correlations between serum miR-122 ratio (ratio of level at second biopsy to that at first biopsy) and changes in histological scores as well as ALT, AST, GGT and ferritin [ <i>N</i> = 36 NAFLD patients with repeat biopsies] <sup>(105)</sup>
		<b>Down</b> ( <i>miR-122</i> ) associated with risk of mortality (HR 4.35, <i>P</i> = 0.025) in multivariate analyses, and poor cumulative mortality rates over 10 years [ <i>N</i> = 392 biopsy confirmed NAFLD patients with median 4.7 years follow-up] <sup>(107)</sup>
<b>miR-146</b>	Liver	<b>Up</b> ( <i>miR-146b</i> ) in NASH ( <i>n</i> = 25) versus normal histology ( <i>n</i> = 25) [participants with metabolic syndrome] <sup>(114)</sup>
		<b>Up</b> ( <i>miR-146b-5p</i> ) in NAFLD ( <i>n</i> = 17) versus controls ( <i>n</i> = 19) and borderline NAFLD ( <i>n</i> = 24); positive correlation with NAFLD [bariatric surgery patients] <sup>(78)</sup>
		<b>Up</b> ( <i>miR-146</i> ) in steatosis ( <i>n</i> = 4) versus non-steatosis ( <i>n</i> = 4) [liver tissue bank] <sup>(120)</sup>
	Serum	<b>Down</b> ( <i>miR-146b</i> ) in NAFLD ( <i>n</i> = 20) versus HCs ( <i>n</i> = 20); discriminated NAFLD from HCs (AUROC = 0.75) <sup>(121)</sup>
		<b>Up</b> ( <i>miR-146b</i> ) in NASH ( <i>n</i> = 31) versus HCs ( <i>n</i> = 37) <sup>(68)</sup>
<b>miR-200</b>	Liver	<b>Up</b> ( <i>miR-200c</i> ) in NASH fatty liver ( <i>n</i> = 20) versus NASH non-fatty liver ( <i>n</i> = 15) and normal histology ( <i>n</i> = 10) [liver tissue bank] <sup>(131)</sup>
		<b>Up</b> ( <i>miR-200a/b/c</i> ) in steatosis ( <i>n</i> = 4) versus non-steatosis ( <i>n</i> = 4) [liver tissue bank] <sup>(120)</sup>
		<b>Down</b> ( <i>miR-200b/c</i> ) in NAFLD ( <i>n</i> = 11) versus HCs ( <i>n</i> = 11) [liver biopsy patients] <sup>(132)</sup>
	Serum	<b>Up</b> ( <i>miR-200a</i> ) with increasing fibrosis severity ( <i>N</i> = 132 NAFLD patients); in multivariate analyses, positive correlation with fibrosis and TM6SF2 E167K variants <sup>(87)</sup>
		<b>Up</b> ( <i>miR-200</i> ) in NAFLD ( <i>n</i> = 57) versus HCs ( <i>n</i> = 30) <sup>(133)</sup>

ALT, alanine aminotransferase; AST, aspartate transaminase; APTI, AST-to-platelet ratio index (fibrosis score); AUROC, the area under the receiver operating characteristic; CHB, chronic hepatitis B; CK18, cytokeratin-18; CSD, cardiac sudden death; CVD, cardiovascular disease; eLP-IR, enhanced lipo-protein insulin-resistance index; F, fibrosis stage; FIB4, fibrosis 4; GGT, gamma-glutamyl transpeptidase; HC, healthy control; HCC, hepatocellular carcinoma; HR, hazard ratio; LFTs, liver function tests; MO, morbidly obese; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; NCSD, non-cardiac sudden death; NNL, near-normal liver; PBC, primary biliary cirrhosis; PNPLA3, patatin-like phospholipase domain containing protein 3; SAF, steatosis, activity, and fibrosis score; T2DM, type 2 diabetes mellitus; TM6SF2, transmembrane 6 superfamily member 2.

† Activity is the sum of the score of lobular inflammation and hepatocellular ballooning.

**Table 2.** Functional and pathophysiological effects of dysregulated miRNA in NAFLD

MicroRNA	Sample type	Summary
<b>miR-21</b>	Liver	<i>P47phox</i> mRNA, 3-nitrotyrosine immunoreactivity, NF- $\kappa$ B activation and the immunoreactivity of TGF- $\beta$ , CTGF, EDAFN, $\alpha$ -SMA and Col1 $\alpha$ , and SMAD2/3 and SMAD7 nuclear colocalisations: <b>up</b> in NASH ( $n = 3$ ) versus HCs ( $n = 3$ ); SMAD7 protein: <b>down</b> in NASH versus HCs <sup>(63)</sup>
		Hepatic PPAR $\alpha$ protein: <b>down</b> in NASH versus steatosis [ $N = 28$ , bariatric surgery patients] <sup>(65)</sup>
	Serum	Serum <i>HMGCR</i> mRNA and protein: <b>up</b> NAFLD ( $n = 25$ ) versus HCs ( $n = 12$ ) <sup>(70)</sup>
<b>miR-30</b>	Liver	Hepatic <i>ACSL1</i> , <i>Dicer</i> and <i>AMPK</i> mRNA: <b>down</b> in >33% steatosis ( $n = 16$ ) versus <5% steatosis ( $n = 19$ ) <sup>(79)</sup>
	Serum	Not investigated
<b>miR-34</b>	Liver	Hepatic SIRT1 protein: <b>down</b> in NASH ( $n = 13$ ) versus steatosis ( $n = 15$ ); p53 acetylation: <b>up</b> in NASH versus steatosis; TUNEL-positive cells: <b>up</b> in more severe NASH ( $n = 8$ ) versus less severe NASH ( $n = 5$ ) and steatosis [obese patients] <sup>(96)</sup>
		<i>HNF4<math>\alpha</math></i> mRNA: <b>down</b> in NASH versus HCs [HCs or NASH $n = 8$ ] <sup>(180)</sup>
		Hepatic circRNA_0046367: <b>down</b> in NAFLD ( $n = 5$ ) versus non-steatosis ( $n = 3$ ); hepatic <i>PPAR<math>\alpha</math></i> mRNA and protein, PPAR $\alpha$ / $\beta$ -actin ratio: <b>down</b> in NAFLD versus non-steatosis <sup>(182)</sup>
	Serum	Not investigated
<b>miR-122</b>	Liver	Hepatic <i>CTDNEP1</i> mRNA, lipin-1 mRNA and protein: <b>up</b> in NAFLD ( $n = 13$ ) versus controls ( $n = 3$ ) [female obese adults] <sup>(116)</sup>
	Serum	Not investigated
<b>miR-146</b>	Liver	Not investigated
	Serum	Not investigated
<b>miR-200</b>	Liver	Hepatic SREBP1 and FAS: <b>up</b> in NAFLD ( $n = 11$ ) versus HCs ( $n = 11$ ) [liver biopsy patients] <sup>(132)</sup>
	Serum	Not investigated

ACSL1, acyl-CoA synthetase long-chain family member 1; Col1 $\alpha$ , collagen  $\alpha$ 1; CTGF, connective tissue growth factor; CTDNEP1, contactin-associated protein 1; EDAFN, extra domain A-fibronectin; HMGCR, 3-hydroxy-3-methylglutaryl-co-enzyme A reductase; HNF4 $\alpha$ , hepatocyte nuclear factor 4 $\alpha$ ; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NNL, near-normal liver; NS, not specified; PPAR $\alpha$ , peroxisome proliferator-activated receptor  $\alpha$ ; PRKAA1, AMP-activated protein kinase; SIRT1, sirtuin 1;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; SMAD7, mothers against decapentaplegic homologue 7; SREBP1, sterol regulatory element-binding protein 1; TGF- $\beta$ , transforming growth factor- $\beta$ ; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

studies) and increased serum levels (nine studies) in patients with NAFLD (Table 1). Higher serum miR-34 levels have been found in more advanced stages of NAFLD<sup>(66,68,69,84–87)</sup>, and found to positively correlate with multiple markers of disease severity<sup>(69,85)</sup>, including histological severity<sup>(68,85,87)</sup> and the PNPLA3 I148M and TM6SF2 E167K variants<sup>(87)</sup>. Moderate diagnostic accuracy has been found in multiple studies for miR-34 in discriminating NAFLD<sup>(85,88)</sup>, NASH<sup>(68,69,84,85)</sup> and fibrosis<sup>(69,87)</sup>, with area under the receiver operating characteristics (AUROCs) ranging from 0.73 to 0.84.

Notably, a recent meta-analysis published in 2020 that examined the utility of miRNAs as non-invasive biomarkers of NAFLD concluded that miR-34 had moderate diagnostic accuracy for total NAFLD with the lowest heterogeneity (sensitivity  $P^2 = 5.73\%$ , specificity  $P^2 = 33.16\%$ , AUROC = 0.85) of the three miRNAs found most commonly studied (miR-34a, miR-99a, miR-122)<sup>(89)</sup>. An earlier meta-analysis from 2018 had concluded (from three studies at the time) miR-34a to have the best diagnostic accuracy (AUROC = 0.7783) for discriminating NASH versus NAFLD, whereas, (from three studies) miR-122, discussed in detail below, was best at discriminating NAFLD from healthy controls (AUROC = 0.8174)<sup>(90)</sup>. In addition, a recent systematic review has concluded that both miR-34a and miR-122 may be useful biomarkers in children with obesity and NAFLD<sup>(91)</sup>.

As direct targets of the tumour suppressor gene p53, induction of miR-34 family members is associated with apoptosis and cell cycle arrest, and inactivation of the miR-34a and miR-34b/c genes is typically observed in cancer<sup>(92)</sup>. Among multiple cell cycle and apoptosis-related direct gene targets of miR-34 is sirtuin 1 (SIRT1), which, in a fascinating regulatory loop, links cholesterol, lipid and energy homeostasis to inflammation and p53-dependent apoptosis<sup>(92,93)</sup>. An NAD-dependent deacetylase, SIRT1 directly deacetylates multiple metabolic regulators

of relevance to NAFLD pathogenesis, including SREBP-1c, the PPARs (alpha and gamma), the farnesoid X receptor (FXR) and liver X receptors (LXR, alpha and beta), as well as nuclear factor- $\kappa$ B (NF- $\kappa$ B) and p53 itself<sup>(93–95)</sup>. Data from experimental models suggest possible benefit from targeting and inhibiting the miR-34/p53 pathway<sup>(96–98)</sup>, although some conflicting data exist around effects in HSCs<sup>(98,99)</sup>. Interestingly, resveratrol, a naturally occurring dietary polyphenol that activates SIRT1, has been tested in a number of small clinical trials for benefit in NAFLD and other metabolic diseases<sup>(95,100)</sup>. A recent meta-analysis synthesising data from six randomised controlled trials (RCTs) found that, although resveratrol significantly lowered tumour necrosis factor alpha (TNF- $\alpha$ ) and high-sensitivity C-reactive protein (hs-CRP) levels, there were no changes in numerous other cardiometabolic risk markers<sup>(101)</sup>. More potent, synthetic SIRT1 agonists are under development, and some are in early phase clinical trials<sup>(100)</sup>.

### miR-122

In addition to its aforementioned role in HCV promotion, miR-122 is essential for lipid metabolism and has anti-inflammatory and anti-carcinogenic effects in the liver<sup>(102,103)</sup>. The role and therapeutic targeting of miR-122 in liver disease has long been of considerable interest<sup>(39,104)</sup>, and unsurprisingly, miR-122 has been the most explored miRNA in NAFLD. We identified twenty-three studies that measured miR-122 levels in serum, and eight studies that examined hepatic miR-122 expression in NAFLD (Table 1). Of the twenty-three serum studies, twenty-one found increased levels of miR-122 positively correlated with markers of NAFLD severity. Consistent with these findings, one study in a small number of patients in Japan reported decreased serum miR-122 levels with histological improvement on second

biopsy<sup>(105)</sup>, although, other data from the same group suggest that at stage 4 fibrosis miR-122 levels decrease<sup>(106)</sup> and decreased levels of miR-122 (expressed relative to the median of the cohort) may be associated with risk of mortality<sup>(107)</sup>.

Multiple studies found miR-122 to have moderate diagnostic accuracy in discriminating either NAFLD<sup>(84,88,108,109)</sup>, NASH<sup>(84,85)</sup> or histological severity<sup>(86,110–112)</sup>. Although a diagnostic assay for miR-122 is in pre-clinical development and has been tested in the context of drug-induced liver injury<sup>(113)</sup>, given that miR-122 is altered in multiple liver diseases, any potential utility as a biomarker for NAFLD will most likely be in combination with other miRNAs or biochemical markers<sup>(67)</sup>. Notably, the limited number of currently available miRNA-based diagnostics for other diseases are panels of ten or more miRNAs<sup>(45)</sup>.

On the other hand, the studies of miR-122 hepatic expression in aggregate were more inconclusive. Sample sizes were typically low, and participants and/or liver samples were heterogeneous in origin and variable in disease stage of NAFLD. Of the five studies reporting that miR-122 decreased in liver biopsies, three were staged as NASH<sup>(61,96,114)</sup>, one as NAFLD<sup>(78)</sup> and one involved a small number of non-tumour HCC resected liver samples with steatosis<sup>(115)</sup>. Of the three studies reporting increased expression of hepatic miR-122 in steatosis, two found decreased expression in more advanced disease, such as NASH<sup>(111)</sup> and fibrosis<sup>(110)</sup>, and the third specifically excluded NASH samples, only examining steatosis in bariatric surgery patients<sup>(116)</sup>.

The hepatic data perhaps suggest decreased expression of miR-122 in advanced disease, and the possibility of increased expression in steatosis. This fits the hypothesis of an early defensive response (in steatosis) and later causal factor in NASH progression<sup>(24)</sup>, and reconciles with several lines of experimental data highlighting the complexity of the dynamics of miR-122 expression and secretion in the regulation of lipid metabolism. While antisense oligonucleotide inhibition of miR-122 *in vivo* had beneficial effects on plasma cholesterol and hepatic steatosis in high-fat-fed (60% lard, for 19 weeks) mice<sup>(102)</sup>, genetic deletion of miR-122 causes steatohepatitis and tumourigenesis<sup>(103,117)</sup>. In addition, NEFAs have been demonstrated to increase the expression and secretion of miR-122 inhibiting triacylglycerol synthesis in both liver and muscle<sup>(118)</sup>. This mechanism would account for the increased serum levels of miR-122, but underscores that circulating miRNAs do not always reflect tissue expression or activity<sup>(23)</sup>. The question of whether humans with NAFLD might benefit from therapeutic targeting of miR-122 through either antagomirs (anti-miRs) or miRNA mimics will require considerable more research and development and larger trials with careful staging of NAFLD.

### MiR-146

Along with miR-155, miR-146 is recognised for multiple roles in the innate and adaptive immune responses and is considered an oncomiR<sup>(119)</sup>. However, knowledge regarding a role for miR-146 during NAFLD pathogenesis is limited. Three studies identified an increase in hepatic miR-146 in biopsies from participants with metabolic syndrome and NASH<sup>(114)</sup>, bariatric surgery patients with NAFLD<sup>(78)</sup>, and steatotic tissue bank biopsies<sup>(120)</sup>. Two studies detecting circulating miR-146 levels were conflicting, possibly

relating to clinical stage of NAFLD or ethnic differences. Whereas one European cohort comparing histologically proven NAFLD patients with healthy age-matched participants ( $n = 20$  per group) found miR-146b decreased in NAFLD<sup>(121)</sup>, a separate Chinese cohort found miR-146b increased in serum from NASH patients (biopsy diagnosed,  $n = 31$ ) versus healthy controls ( $n = 37$ )<sup>(68)</sup>.

In experimental models, decreased expression of miR-146 has been detected in dietary-induced NAFLD models<sup>(122,123)</sup>, and *in vitro* data show that miR-146 mimics can suppress lipid accumulation and inflammatory cytokines, such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6)<sup>(123,124)</sup>. In addition, miR-146 is pro-fibrotic, and modulates fibrosis signalling pathways in HSCs<sup>(82)</sup>. In transforming growth factor-beta (TGF- $\beta$ )-stimulated cellular models, overexpression of miR-146 inhibited the proliferation and apoptosis of HSCs and the expression of pro-fibrogenic markers<sup>(125,126)</sup>. Furthermore, in a hepatic fibrosis rat model induced by CCl<sub>4</sub>, vein injection of miR-146a-expressing adenovirus increased the level of miR-146 and served to alleviate fibrogenesis<sup>(127)</sup>. Collectively, the data suggest that roles of the miR-146 family in NAFLD pathogenesis should be further explored, paying attention to the different isoforms and their unique regulatory functions as previously recommended<sup>(55)</sup>.

### MiR-200

Key inhibitors of the epithelial-to-mesenchymal transition, the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) play critical roles in both normal development and cancer metastases. High expression of miR-200 is associated with an epithelial phenotype, and recent bioinformatic analyses suggest potential binding sites for miR-200b in the 3' untranslated regions of sixty different mRNAs involved in the epithelial-to-mesenchymal transition, the majority of which are associated with a mesenchymal phenotype that miR-200 likely inhibits<sup>(128)</sup>. Typically considered tumour suppressors, the miR-200 family have generally been reported down-regulated in multiple cancers, including HCC<sup>(129)</sup>. However, this may depend on tumour stage, with data suggesting the miR-200 family are down-regulated at the primary tumour site permitting intravasation, but up-regulated in distal metastases facilitating colonisation of metastatic breast cancer cells<sup>(130)</sup>.

In the context of NAFLD, to date, there are only limited data about the miR-200 family. While two studies have found miR-200a/b/c increased in steatotic and NASH liver samples from tissue banks<sup>(120,131)</sup>, a separate study using biopsies found miR-200b/c decreased in NAFLD compared with healthy controls<sup>(132)</sup>. An additional two studies have found miR-200 increased in serum from NAFLD patients<sup>(87,133)</sup>. Some evidence from animal models supports the idea that inhibition of miR-200 may attenuate steatosis, inflammation and fibrosis. For example, double deletion of miR-141 and miR-200 in mice with NASH induced from a methionine and choline deficient diet resulted in decreased steatosis and inflammation and alterations in multiple signalling pathways<sup>(131)</sup>. In a similar vein, high-fat-fed (20% lard for 4 weeks) mice transduced with an miR-200 inhibitor also exhibited reduced steatosis and fibrosis<sup>(133)</sup>. However, in a



separate study of high-fat-fed (45% fat for 10 weeks) mice, miR-200b and miR-200c, but not miR-200a, were found markedly decreased<sup>(132)</sup>. These data may reflect the use of different animal models, but could also relate to disease stage. While a rationale can be made for increased expression of miR-200 early in NAFLD, and decreased expression later during disease progression conferring risk of HCC, more data at greater resolution (e.g. from single-cell experiments discriminating between hepatocytes and HSCs) are required to confirm this.

### Intersection of miRNA pathways between NAFLD and vitamin D

Vitamin D is a misnomer for a family of secosteroid hormones with varying degrees of activity<sup>(134)</sup>. The biological effects of the active form of vitamin D (calcitriol, 1,25-dihydroxycholecalciferol) are mediated through the interaction with the vitamin D receptor (VDR), a ligand-activated, nuclear receptor transcription factor<sup>(135)</sup>. A significant amount of experimental research shows vitamin D has anti-proliferative, anti-inflammatory and anti-fibrotic properties that might impact disease progression in chronic liver diseases, including NAFLD<sup>(136,137)</sup>. Polymorphisms in the vitamin D metabolic pathway are associated with histological severity of paediatric NAFLD<sup>(20)</sup>, and inadequate vitamin D status has been commonly observed in association with NAFLD severity in adults<sup>(17)</sup>. While vitamin D has been shown to improve insulin sensitivity and glycaemic control in people with prediabetes and type 2 diabetes<sup>(138,139)</sup>, vitamin D supplementation trials in NAFLD have been typically small (twenty to thirty people per arm<sup>(140)</sup>), single-centre trials and have been heterogeneous in duration, the form (e.g. vitamin D versus calcitriol), dose and mode of delivery of supplement, as well as the liver outcomes measured<sup>(22)</sup>. Only a minority have measured liver biomarkers by magnetic resonance<sup>(141)</sup> or biopsy<sup>(142)</sup>. Multiple questions around dosing regimes, duration of intervention and ideal stage of NAFLD to intervene remain unanswered<sup>(17,22)</sup>, but potential benefits for younger people with earlier stages of NAFLD have been hypothesised<sup>(137)</sup>, and recently demonstrated<sup>(142)</sup>.

Interestingly, miRNAs have multiple essential roles in mediating the cellular response to vitamin D, including the post-transcriptional regulation of VDR<sup>(143)</sup>. Within the 3' untranslated region of the VDR mRNA are binding sites for four miRNAs (miR-27, miR-125, miR-298, miR-346), which have been shown to decrease VDR protein levels<sup>(144–147)</sup>. Indeed, miR-125 inhibitors have been demonstrated to increase VDR expression and decrease proliferation and cell viability *in vitro* in HCC cells, while VDR levels were negatively correlated with miR-125 levels in tumour tissue from patients with HCC<sup>(148)</sup>. Moreover, multiple genes (CYP27B1, CYP24A1, RXR $\alpha$ ) in the vitamin D pathway are regulated by miRNAs<sup>(149–151)</sup>, and VDR directly regulates the transcription of multiple miRNAs<sup>(152)</sup>. While a large body of pre-clinical research suggests the anti-cancer effects of vitamin D are mediated through miRNA regulation, data from human trials are more limited<sup>(26)</sup>. Although anti-cancer effects of vitamin D have been observed in the liver<sup>(27,28)</sup>, potential roles for

vitamin-D-regulated miRNAs in the molecular pathogenesis of NAFLD remain largely unexplored.

With the question 'is there a potential subset of miRNAs that are both dysregulated in NAFLD and modulated by vitamin D?' in mind, we searched PubMed broadly using the terms calcitriol, vitamin D and miRNA. As calcitriol and miRNA are both medical subheadings in PubMed, we searched a large number of related entry terms. In contrast to the number of studies profiling serum miRNAs in NAFLD, this yielded only a very limited number of human studies that had examined miRNAs in relation to either vitamin D status (Table 3; six studies) or response to vitamin D supplementation (Table 4; four studies), or had investigated miRNAs and vitamin D in the context of the liver (Table 5; seven studies). Interestingly, one manuscript identified was a study protocol for an ongoing trial that aims to measure miR-21, miR-34 and miR-122 in response to 12 weeks supplementation of 4000 IU/d vitamin D in NAFLD patients<sup>(153)</sup>. Five studies characterized miRNAs regulated by vitamin D in a variety of diverse pathologies, including chronic hepatitis B (CHB)<sup>(154)</sup>, primary biliary cholangitis (PBC)<sup>(155)</sup>, cirrhosis<sup>(156)</sup> and HCC<sup>(148,157)</sup>. The study involving patients with CHB<sup>(154)</sup> measured miR-378 expression in relation to plasma 25-hydroxycholecalciferol (calcidiol, 25(OH)D) and was therefore placed in both Tables 3 and 5. A single study examined the association between miR-27b expression and its targets VDR and cytochrome P450 3A (CYP3A) in normal liver samples from a tissue bank<sup>(158)</sup>.

From the sixteen non-redundant studies reviewed, we identified twenty miRNAs measured by qPCR. While in some cases this was secondary to microarray analysis or alongside other experimental validation (e.g. luciferase), in some instances there was no within-study experimental validation. Venn analysis of the twenty vitamin-D-associated miRNAs with the twenty-nine NAFLD-dysregulated miRNAs suggested that seven vitamin-D-modulated miRNAs (miR-27, miR-125, miR-155, miR-192, miR-223, miR-375 and miR-378; Fig. 1C) may be relevant to NAFLD pathogenesis. Notably, miR-27b directly targets and regulates VDR<sup>(26)</sup>, and has been found altered in serum from NAFLD patients in three studies<sup>(69,108,159)</sup> summarised in Table S2. Also interesting among the intersecting miRNAs was miR-192, shown to decrease in the serum of adults with prediabetes supplemented with vitamin D (2000 IU [50  $\mu$ g] cholecalciferol) for 4 months in correlation with favourable changes in fasting plasma glucose levels and disposition index (the product of insulin sensitivity and insulin secretion)<sup>(160)</sup>. As eight independent studies have shown miR-192 to be up-regulated in NAFLD patients (plus one outlier showing down-regulation; summarised in Table S2), in light of the data from Nunez Lopez and colleagues<sup>(160)</sup>, it remains tempting to speculate a benefit for vitamin D supplementation in NAFLD patients. Although trials of vitamin D supplementation in adults with NAFLD have been disappointing in terms of liver endpoints, as previously discussed, sufficient questions around trial design preclude completely rejecting vitamin D as having therapeutic benefit<sup>(17,22,137)</sup>, especially given its benefit to people with prediabetes and type 2 diabetes<sup>(138,139)</sup>.

Out of the twenty miRNAs identified as altered by both NAFLD and vitamin D, only two, miR-125 and miR-155, were found in more than one vitamin D study (Fig. 1C). We identified

**Table 3.** Serum miRNA profiling studies examining vitamin D status

Reference	Study design; Group (sample size); 25(OH)D status (nmol/L)	miRNAs related to 25(OH)D status	miRNA-related summary
Enquobahrie <i>et al.</i> , 2011 <sup>(188)</sup>	miRNA expression (microarray) in relation to 25(OH)D status† in early (16 weeks' gestation) pregnancy (~34-year-old women); <b>High 25(OH)D</b> ( <i>n</i> = 6): 98.05±15.3, <b>Low 25(OH)D</b> ( <i>n</i> = 7): 57.10±5.0	Microarray: miR-92b, -93, -138, -196a, -320d, -343-3p, -423-3p, -484, -573, -574-5p, -589, -601	Microarray: <b>Up</b> in high 25(OH)D versus low 25(OH)D: miR-574-5p <b>Down</b> in high 25(OH)D versus low 25(OH)D: miR-92b, -93, -138, -196a, -320d, -343-3p, -423-3p, -484, -573, -589, -601
Lee <i>et al.</i> , 2014 <sup>(189)</sup>	miRNA expression (microarray and qPCR) in relation to 25(OH)D status in AML patients (~60 years old); <b>Normal vitamin D</b> ( <i>n</i> = 34): >80 <b>Insufficient vitamin D</b> ( <i>n</i> = 34): 50–79.8; <b>Deficient vitamin D</b> ( <i>n</i> = 29): <50	Microarray: miR-96, -122, -125b-1, -134, -144, -182, -193b, -329, -451, -486-5p, -511, -595, -663, -886-3p, -1248 qPCR: Not significant	Microarray: <b>Up</b> in <50 ( <i>n</i> = 10) versus >50 ( <i>n</i> = 10): miR-96, -134, -144, -182, -193b, -329, -451, -486-5p, -595, -663, -886-3p <b>Down</b> in <50 versus >50: miR-122, -125b-1, -511, -1248 qPCR ( <i>N</i> = 58): No miRNAs associated with 25(OH)D levels in validation cohort
Beckett <i>et al.</i> , 2014 <sup>(190)</sup>	Circulating level of let-7 (qPCR) in relation to vitamin D intake in elderly cohort (~75 years old); <b>Adequate intake</b> ‡ ( <i>n</i> = 23): ns§; <b>Inadequate intake</b> ( <i>n</i> = 177): ns§	qPCR: let-7b-5p	qPCR: <b>Down</b> in adequate versus inadequate; negative correlation with vitamin D intake
Mohamadkhani <i>et al.</i> , 2015 <sup>(154)</sup>	miR-378 (qPCR) in relation to 25(OH)D status and HBV DNA level in CHB patients (~37 years old); <b>CHB</b> ( <i>n</i> = 173): 55.5±20.7	qPCR: miR-378	qPCR: Positive correlation with plasma 25(OH)D status; negative correlation with viral load
Chen <i>et al.</i> , 2017 <sup>(161)</sup>	miRNA expression (qPCR) in T cells of patients with SLE with 25(OH)D insufficiency (~36 years old); SLE patients ( <i>n</i> = 42): 41.7±12.8 <b>Normal vitamin D</b> ( <i>n</i> = 32): NR <b>Insufficient vitamin D</b> ( <i>n</i> = 10): NR	qPCR: miR-10a, -125a, 342, -374b, -377, -410	qPCR: All miRNAs <b>down</b> in SLE versus controls except miR-377 and -410; all miRNAs were positively correlated with 25(OH)D levels
Ferrero <i>et al.</i> , 2021 <sup>(191)</sup>	Circulating miRNome in healthy volunteers in relation to estimated vitamin D intakes# (~40 years old) <b>25(OH)D status</b> ( <i>n</i> = 120): NR	RNAseq: ~348 miRNAs detected per sample	RNAseq: In GLM, miR-361-3p was positively correlated and let-7a-5p was negatively correlated with estimated vitamin D intake#

AML, acute myeloid leukemia; CHB, chronic hepatitis B; FAS, fatty acid synthase; GLM, generalised linear regression model; ns, not specified; NR, not reported; HBV, hepatitis B virus; 25(OH)D, 25-hydroxycholecalciferol; qPCR, quantitative polymerase chain reaction; SLE, systemic lupus erythematosus.

† 25(OH)D status defined as high: ≥79.25 nmol/l, or low: <63.75 nmol/l; ‡ The recommended adequate daily intake for vitamin D intake for vitamin D in Australia is 10 µg/d for 51–70 years old and 15 µg/d for those aged over 70 years; § Intake estimated by food frequency questionnaire 0–65.6 g/d; # Estimated from EPIC food frequency questionnaire.

**Table 4.** Serum miRNA profiling studies response to vitamin D supplementation

Reference	Study design; Group (sample size) and vitamin D intake	Serum 25(OH)D status (nmol/l)	miRNAs related to 25(OH)D status§	miRNA-related summary
Jorde <i>et al.</i> , 2012 <sup>(192)</sup>	Obese males (~50 years old) supplemented for 1 year; <b>Vitamin D</b> ( <i>n</i> = 40): 20 000 or 40 000 IU cholecalciferol/wk <b>Placebo</b> ( <i>n</i> = 37)	<b>Vitamin D group:</b> Baseline: 50.2 ± 14.2, 12 months: 101.7 ± 17.8; <b>Placebo group:</b> Baseline: 53.0 ± 19.1, 12 months: 49.6 ± 16.0	miR-211, miR-532-3p	miR-211: <b>Down</b> in 12-month versus baseline [in the placebo group]; <b>up</b> in vitamin D versus placebo; miR-532-3p: Positive correlated with serum 25(OH)D [at baseline]
Yu <i>et al.</i> , 2017 <sup>(193)</sup>	60 allergic rhinitis (AR) patients and 20 healthy controls (HCs) (~35 years old) supplemented for 6 months; <b>Vitamin D</b> ( <i>n</i> = 20): 2000 IU vitamin D <sub>3</sub> /d <b>Placebo</b> ( <i>n</i> = 20)	<b>All groups:</b> <75	miR-19a	miR-19a: <b>Up</b> in AR with vitamin D <sub>3</sub> versus HCs
Nunez Lopez <i>et al.</i> , 2017 <sup>(160)</sup>	Prediabetes adults (~59 years old) supplemented for 4 months; <b>Vitamin D group</b> ( <i>n</i> = 40): 2000 IU cholecalciferol/d <b>Placebo group</b> ( <i>n</i> = 21)	<b>Vitamin D group:</b> Baseline: 62.0 ± 14.8 4 months: 83.8 ± 18.5 <b>Placebo group:</b> Baseline: 66.5 ± 20.0 4 months: 43.3 ± 12.3	miR-7, miR-23b miR-107 miR-152, miR-192-5p	miR-7: <b>Up</b> in vitamin D versus placebo miR-23b: <b>Up</b> in post versus pre [in vitamin D group] miR-107: <b>Up</b> in post versus pre [in vitamin D group] miR-152: <b>Up</b> in vitamin D versus placebo, <b>up</b> in post versus pre [in vitamin D group]; positively correlated with serum 25(OH)D miR-192-5p: <b>Down</b> in vitamin D versus placebo <b>down</b> in post versus pre [in vitamin D group]
Pastuszak-Lewandoska <i>et al.</i> , 2020 <sup>(164)</sup>	20 male ultra-marathon (UM) runners (~38 years old) supplemented for 2 weeks; <b>Vitamin D group</b> ( <i>n</i> = NS): 10 000 IU cholecalciferol/d <b>Placebo group</b> ( <i>n</i> = NS)	NR	miR-155, miR-223	miR-155: <b>Up</b> in both placebo and vitamin D groups [after UM] miR-223: <b>Up</b> in placebo group only [after UM]

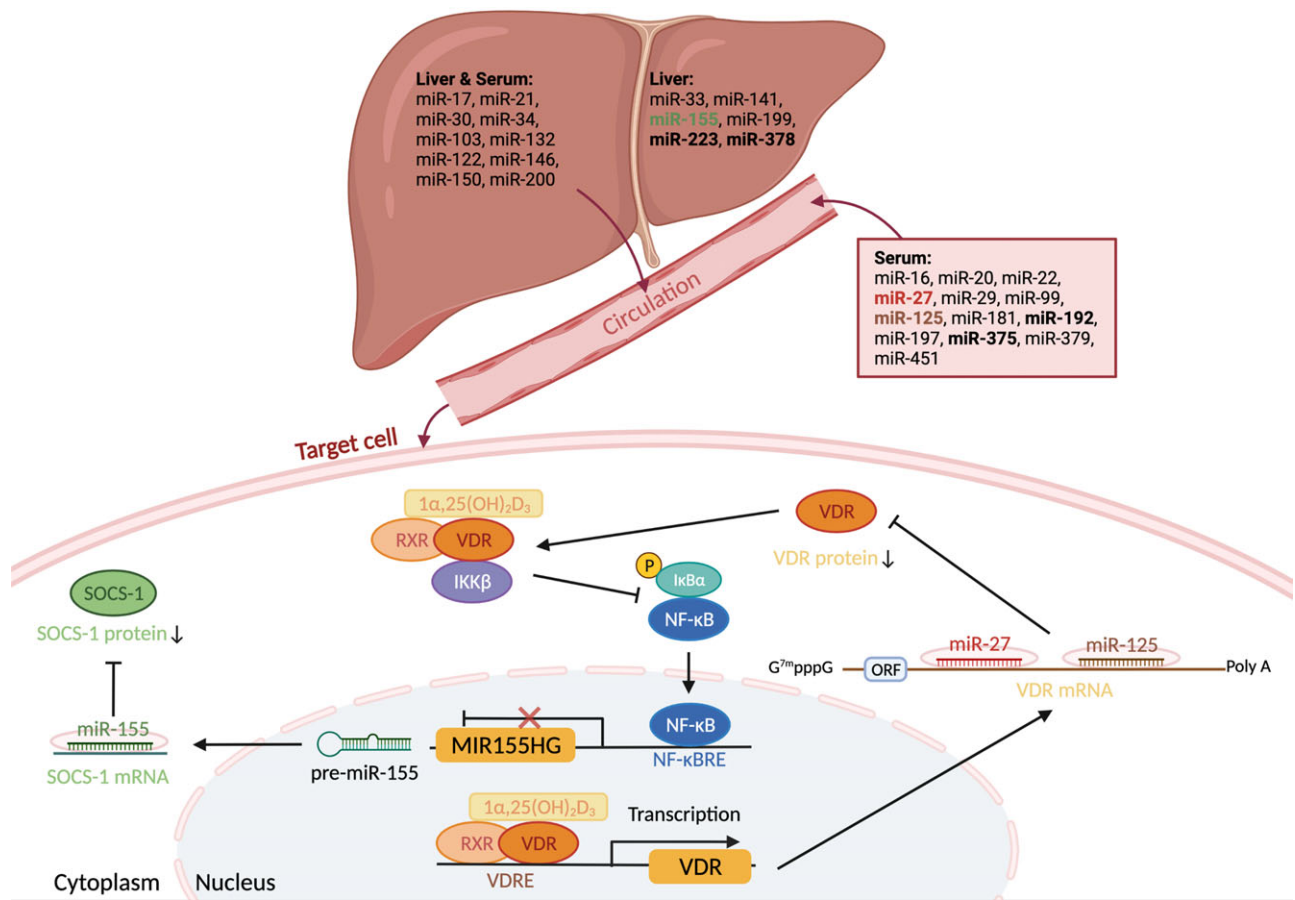
AR, allergic rhinitis; HC, healthy control; NS, not specified; NR, not reported; 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)D, 1,25-dihydroxyvitamin D; PCa, prostate cancer; UM, ultra-marathon. § Jorde<sup>(192)</sup> used microarrays with quantitative polymerase chain reaction (qPCR) for validation; Nunez Lopez<sup>(160)</sup> and Pastuszak-Lewandoska<sup>(164)</sup> used qPCR alone.

**Table 5.** Research studies characterising miRNA regulated by vitamin D involving liver pathology

Reference	Liver pathology; Samples	Vitamin D treatment	Related miRNA	miRNA-related summary
Duan <i>et al.</i> , 2015 <sup>(194)</sup>	HCV; Human cell lines: Huh7.5 HCV Con1b replicon Huh7.5.1 infected with HCV J6/JFH1	1 μM calcitriol for 48 h	miR-130a	qPCR: Calcitriol potentiated miR-130a inhibition of HCV RNA replication, but calcitriol did not affect the expression of miR-130a
Mohamadkhani <i>et al.</i> , 2015 <sup>(154)</sup>	CHB; Serum <i>n</i> = 173;	NR	miR-378	qPCR: Positive correlation with plasma 25(OH)D status;
Ekstrom <i>et al.</i> , 2015 <sup>(158)</sup>	General population; Serum <i>n</i> = 28, Liver <i>n</i> = 20;	NR	miR-27b	qPCR: Negative correlation with CYP3A activity* in both liver and serum; no association with mRNA levels of <i>CYP3A4</i> , <i>VDR</i> and <i>PPARα</i> [liver]
Kemipinska-Podhorodecka <i>et al.</i> , 2017 <sup>(155)</sup>	PBC; Liver: PBC <i>n</i> = 22, PSC <i>n</i> = 13 and Controls <i>n</i> = 23 PBMCs from human: PBC <i>n</i> = 16, PSC <i>n</i> = 10 and Controls <i>n</i> = 11	PBC patients were supplemented with vitamin D/calcium (amount NR) and had normal levels of serum vitamin D	miR155	qPCR: <b>Up</b> in PBC versus PSC and controls in both liver and PBMCs; Positive correlation with hepatic <i>VDR</i> mRNA and SOCS1 protein level [liver]
Xu <i>et al.</i> , 2018 <sup>(148)</sup>	HCC; Liver: HCC <i>n</i> = 31 and NL <i>n</i> = 10; Human cell lines: HepG2 and SMMC-7221	NR	miR-125a-5p	qPCR: <b>Up</b> in HCC versus NL, negative correlation with hepatic <i>VDR</i> mRNA [liver]; Down-regulation of miR-125a-5p increased <i>VDR</i> mRNA and protein expression in HepG2, <b>Target:</b> <i>VDR</i> (luciferase reporter assay) [in cells]
Provisiero <i>et al.</i> , 2019 <sup>(157)</sup>	HCC; Human cell lines: PLC/PRF/5, and JHH-6	With or without 10 <sup>-7</sup> M 1,25(OH) <sub>2</sub> D <sub>3</sub> for 12 h	miR-375	qPCR: <b>Up</b> in vitamin D treated versus untreated; <b>Target:</b> <i>c-MYC</i> (luciferase reporter assay)
He <i>et al.</i> , 2021 <sup>(156)</sup>	Liver cirrhosis Liver: cirrhosis <i>n</i> = 60 NL = 5 Human cell line: 293T	NR	miR-125	qPCR: <b>Up</b> in cirrhosis versus NL [liver]; IHC: <b>Up</b> miR-125 expression with reduced <i>VDR</i> staining [liver]; Positive correlation with liver cirrhosis, negative correlation with hepatic <i>VDR</i> protein [liver] <b>Target:</b> <i>VDR</i> [293T]

CHB, chronic hepatitis B; CYP3A, cytochrome P450 3A; PBC, primary biliary cholangitis; PBMCs, peripheral blood mononuclear cells; PSC, primary sclerosing cholangitis; HC, healthy control; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; NAFLD, non-alcoholic fatty liver disease; NL, normal liver; NR, not reported; qPCR, quantitative polymerase chain reaction; RCT, randomised clinical trial; SOCS1, suppressor of cytokine signalling 1; VDR, vitamin D receptor.

\* CYP3A activity in serum measured by its endogenous marker 4β-hydroxycholesterol; CYP3A activity in liver measured by dextromethorphan *N*-demethylation.



**Fig. 2.** Overview of miRNAs altered by NAFLD and vitamin D. Twenty-nine miRNAs were identified dysregulated in NAFLD in more than one study. Seven (bold) were also found in separate studies as vitamin D modulated. Two of these miRNAs, miR-27 and miR-125, target vitamin D receptor (VDR) mRNA and decrease translation. The transcription of a third miRNA, miR-155, is inhibited by VDR, which directly interacts with IκB kinase (IKKβ), preventing nuclear factor κB (NFκB) activation and transrepression of the MIR155 host gene. Relevant to NAFLD, in the context of low vitamin D/VDR signalling, miR-155 lowers expression of the suppressor of cytokine signalling 1 (SOCS-1), increasing expression of pro-inflammatory cytokines.

three studies that reported dysregulation of miR-125 in the context of vitamin D<sup>(148,156,161)</sup>, plus two studies that examined miR-125 in the serum of NAFLD patients (Table S2)<sup>(162,163)</sup>. Separately, miR-155 was found in independent studies in a vitamin D context<sup>(155,164)</sup>, as well as in two studies involving NAFLD liver samples (Table S1)<sup>(120,165)</sup>. Both miR-125 and miR-155 are expanded on briefly below.

### miR-125

The miR-125 family (miR-125a, miR-125b-1 and miR-125b-2) play essential roles in haematopoiesis and the normal function of immune cells and, perhaps unsurprisingly, have also been linked to a variety of cancers<sup>(166)</sup>. Their effects in cancer are dependent on cell type, and they have been shown to have both oncogenic and tumour suppressive activities. Along with the previously discussed miR-27, miR-125 is of note because it also targets and inhibits VDR translation<sup>(26)</sup>. We identified two studies where miR-125a was examined in relation to VDR in patients with HCC<sup>(148)</sup> and liver cirrhosis<sup>(156)</sup> (Table 5). In HCC tissues ( $n = 31$ ), miR-125a was found to be negatively correlated with VDR expression, and was expressed at much higher levels than in

non-tumour controls ( $n = 11$ )<sup>(148)</sup>. Similarly, in cirrhotic liver biopsies ( $n = 60$ ), miR-125a expression increased with severity of liver fibrosis in association with a corresponding decrease in VDR expression<sup>(156)</sup>.

To date, only a single observational study has evaluated miR-125 in relation to vitamin D status. Chen and colleagues reported a positive correlation between miR-125 expression in T cells and serum 25(OH)D levels in patients with systemic lupus erythematosus<sup>(161)</sup> (Table 3). Separately, two studies have examined miR-125 in serum from NAFLD patients, with conflicting results<sup>(162,163)</sup>. Whereas Cai and colleagues found miR-125 decreased in serum from patients with ultrasound-diagnosed NAFLD ( $n = 34$ ) compared with non-NAFLD ( $n = 20$ )<sup>(162)</sup>, a separate study reported the opposite, finding miR-125 increased in NAFLD ( $n = 29$ ) compared with healthy volunteers ( $n = 24$ )<sup>(163)</sup>. Differences in NAFLD phenotype and/or qPCR methodologies employed may explain these contradictory findings. Notably, in the latter study, the diagnostic modality for NAFLD was unspecified and SYBR green staining was used for qPCR<sup>(163)</sup>. However, the associated experimental work of Cai and colleagues<sup>(162)</sup>, in combination with previous experimental work demonstrating that miR-125 targets fatty acid synthase<sup>(167)</sup>,



suggests miR-125 up-regulation is likely to be protective for NAFLD and liver fibrosis, and that miR-125 in relation to NAFLD is worthy of further investigation.

### MiR-155

A notorious oncomiR, increased expression of miR-155 has been found in a host of different cancers, including HCC<sup>(168,169)</sup>. Transcription of the MIR155 host gene (*MIRHG155*), historically termed B-cell integration cluster, is regulated by numerous transcription factors involved in the inflammatory response, including nuclear factor-kappa B (NF- $\kappa$ B), interferon regulatory factors, TGF- $\beta$  and hypoxia inducible factor 1 alpha, among others<sup>(170)</sup>. Therefore, the aberrant expression of miR-155 plays a vital role in multiple inflammatory molecule and signalling pathways. Critical to both innate and adaptive immune responses, miR-155 influences the immune inflammatory response in part through directly targeting suppressors of cytokine signalling 1 (SOCS1)<sup>(171)</sup>.

Interestingly, miR-155 is inhibited by VDR, which directly interacts with  $\kappa$ B kinase (IKK $\beta$ ), preventing nuclear factor  $\kappa$ B (NF $\kappa$ B) activation and transrepression of *MIRHG155*<sup>(172)</sup>. Calcitriol decreases miR-155 expression in human macrophages<sup>(173,174)</sup> and adipocytes<sup>(175)</sup>. In mice, vitamin D supplementation ameliorated the increase in miR-155 in adipose tissue in response to high-fat feeding, in further support of an anti-inflammatory role of vitamin D in obesity<sup>(175)</sup>. Moreover, miR-155 has been observed to decrease in response to both dietary weight loss and bariatric surgery, and has been proposed as a biomarker of weight loss<sup>(176,177)</sup>. In the context of NAFLD, hepatic miR-155 expression was shown to be increased alongside miR-34a and miR-200a-c and other miRNAs in a small number ( $n = 4$  per group) of tissue bank biopsies from patients with and without steatosis<sup>(120)</sup>. Hepatic expression of miR-155 has also been found elevated in cholestatic liver disease, and was related to decreased levels of VDR and SOCS1 protein in the peripheral blood mononuclear cells of patients<sup>(155)</sup>. The authors point out that the decreased VDR expression was observed in spite of patients being supplemented with vitamin D and having normal vitamin D status.

Perhaps counterintuitively, in 2016, Wang and colleagues<sup>(165)</sup> reported significantly decreased circulating levels of miR-155 in fifty participants with NAFLD compared with fifty healthy controls, as well as decreased hepatic miR-155 levels in eleven biopsy samples from NAFLD patients compared with eleven control biopsies. However, in accompanying experimental work, they showed miR-155 directly targets LXR $\alpha$ , which targets SREBP-1c and fatty acid synthase (FAS) influencing lipid accumulation. In addition, high-fat-fed mice transfected with miR-155 mimics had significantly reduced hepatic steatosis, as well as decreased expression of LXR $\alpha$ , SREBP-1c and FAS<sup>(165)</sup>. Apart from the aforementioned study in cholestatic liver disease, we identified only one other study examining miR-155 response to vitamin D supplementation. Unusually, it involved very-high-dose vitamin D supplementation (10 000 IU/250  $\mu$ g cholecalciferol) for 2 weeks prior to a 100 km ultra-marathon. In this small

study done in a unique population, miR-155 levels increased in both groups after the ultra-marathon, but there was no difference between groups<sup>(164)</sup>.

Genome-wide analyses have demonstrated miR-155 has many hundreds of gene targets, and furthermore miR-155 binding and miR-155-dependent repression are regulated in a cell-context-dependent fashion<sup>(178,179)</sup>, which may explain these somewhat disparate results. However, the pre-clinical data and data from weight loss intervention studies suggest that the potential interactions between miR-155, vitamin D, and hepatic lipid metabolism and inflammation in the molecular pathogenesis of NAFLD, are worth pursuing.

### Conclusions

This review critically assessed the evidence for a potential subset of miRNAs that are both dysregulated in NAFLD and modulated by vitamin D. Comprehensive review of the literature found numerous studies examining dysregulation of miRNA levels in humans with NAFLD. We identify twenty-nine miRNAs found dysregulated in more than one NAFLD study, including six (miR-21, miR-30, miR-34, miR-122, miR-146 and miR-200) found dysregulated in multiple independent NAFLD studies. On the other hand, a paucity of human studies were identified that had investigated miRNAs in relation to vitamin D status, response to supplementation, or vitamin D in the context of the liver. This is a notable gap in the evidence base, given that VDR mediates its cellular response in part by directly targeting miRNAs that regulate transcription factors involved in NAFLD pathogenesis, and considering that VDR expression is directly regulated by miRNAs likely disrupted in NAFLD.

Our critical review found evidence from human studies for seven vitamin-D-modulated miRNAs (miR-27, miR-125, miR-155, miR-192, miR-223, miR-375 and miR-378) potentially relevant to NAFLD pathogenesis (overall summary in Fig. 2). While we await the results of the ongoing trial of Ebrahimpour-Koujan and colleagues<sup>(153)</sup> with interest, we believe that the measurement of serum and hepatic miRNAs in response to vitamin D supplementation in larger trials or biobanked samples is warranted. While miRNA analyses of liver tissue are unlikely to add diagnostic value to already informative, but invasive, liver biopsies, they may be key to further understanding pathobiology. On the other hand, the measurement of serum miRNAs is non-invasive. Given that current genetic risk factors for NAFLD are non-specific and predict severity of multiple liver diseases, a fascinating, unanswered question worthy of deliberate inquiry is whether serum miRNA signatures might yield diagnostic specificity for either chronic liver disease stage or aetiology. Although individual miRNAs alone seem unlikely to provide such specificity, for the earlier stages of NAFLD in particular, panels of diet-responsive miRNAs may be particularly intriguing. The summary tables within this review provide a significant resource to underpin future hypothesis-driven research to tackle such questions, including gene expression meta-analysis studies. We conclude that the modulation of miRNAs by

vitamin D has been understudied and that, based on of the evidence to date, a therapeutic benefit for vitamin D supplementation in NAFLD can not be ruled out.

### Author contributions

Z.Z., R.M., J.L.T. and J.B.M contributed to review concept and design. Z.Z and J.B.M extracted data. R.M. and Z.Z. contributed to manuscript drafts. J.B.M. wrote the final manuscript. All authors critically reviewed the manuscript for intellectual content and approved the final version of the manuscript. Fig. 2 was created with BioRender.com.

### Financial support

This research received no specific grant from any funding agency, commercial or not-for-profit sectors.

### Conflicts of interest

All authors declare no conflicts of interest.

### Supplementary material

To view supplementary material for this article, please visit <https://doi.org/10.1017/S095442242100038X>.

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