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# Role of microRNA in non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH): a comprehensive review

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## Abstract

Non-alcoholic fatty liver disease (NAFLD) is a prevalent liver condition that affects people who do not overconsume alcohol. Uncertainties exist over how microRNAs (miRNAs) in the blood and liver relate to NAFLD. The aim of this narrative review was to investigate the role of miRNAs in the onset and progression of non-alcoholic steatohepatitis (NASH) from NAFLD, and explore their potential as diagnostic tools and treatment targets for NAFLD patients. Liver miRNA-34a levels were found to accurately represent the degree of liver damage, with lower levels suggesting more damage. In patients with NAFLD and severe liver fibrosis, higher levels of miRNA-193a-5p and miRNA-378d were found. Moreover, miRNA-34a, miRNA-122, and miRNA-192 levels might aid in differentiating NASH from NAFLD. Similar to this, miRNA-21 and miRNA-27 levels in rats were able to distinguish between steatosis and steatohepatitis. High-fat diets enhanced the expression of 15 distinct miRNAs in rats, and there were substantial differences in the miRNA

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expression patterns between obese and lean people. The results from the present review imply that miRNA microarrays and sequencing may be helpful diagnostic tools, and miRNAs may be a possible treatment target for patients with NAFLD.

**Keywords**
Non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, microRNA, diagnostic tool, disease progression, therapeutic target

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## Introduction

Non-alcoholic fatty liver disease (NAFLD) has become a significant cause of chronic liver disease worldwide.[1–4] NAFLD is defined as the presence of more than 5% steatosis in the liver in the absence of other causal factors for fatty liver, such as drug or alcohol misuse, autoimmune disorders, or viral hepatitis.[1,3,5,6] Due to its multiple extrahepatic associations, including obesity, hypertension, and insulin resistance, it is classified as a metabolic syndrome.[1,4,5] NAFLD is a benign condition; however, it may progress toward more serious diseases, including non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and ultimately, hepatocellular carcinoma (HCC) and cardiovascular disease mortality.[4,7–9] The condition is thought to affect 25% of adults worldwide.[4,9,10]

MicroRNAs (miRNAs) are small, non-coding RNAs comprising 18–25 nucleotides that alter gene expression by interfering at the post-transcriptional level.[9,11] The miRNAs have been highlighted as critical epigenetic factors in the development of NAFLD.[9,10,12] Dysregulated levels of miRNAs involved in lipid metabolism, inflammation activation, fibrosis, insulin resistance, oxidative stress, and apoptosis, are observed in patients with NAFLD, suggesting their roles in development and progression of the disease.[9,13]

The pathophysiology of NAFLD remains poorly understood, which impedes the development of minimally invasive techniques for its diagnosis and a definite treatment plan for its management. Understanding the role of miRNAs in NAFLD may potentially lead to a better understanding of its pathogenesis, aiding the development of minimally invasive diagnostic tools and effective pharmacotherapy. Accordingly, using relevant articles published in the PubMed (NCBI) and Embase (Ovid) databases between their inception and November 2019, the current narrative review aims to discuss the abovementioned roles of miRNAs and their uses in improving treatment regimens for patients with NAFLD.

## Histology and pathophysiology

Many years ago, it was hypothesized that the initiation of NASH transpires as a 'two-hit process'.[14] This theory suggests that in the setting of steatosis alone (i.e, NAFLD), a second 'hit' from other factors (for example, oxidant stress) was required to develop NASH. The first hit includes deposition and accumulation of fatty acids or triacylglycerols in liver cells, leading to fatty acid metabolism dysregulation and steatosis. This also sensitizes the hepatocytes for the second hit, which causes the mitochondrial oxidation of free fatty acids mobilized from

peripheral adipose tissues; when it exceeds metabolic capacity, the excess triacylglycerols accumulate, leading to hepatic inflammation and necrosis, which may lead to fibrosis and cirrhosis in some patients. Increased lipid peroxide levels have also been evidenced, hinting at the contribution of oxidative stress, particularly in the second hit involving lipid peroxidation in steatotic hepatocytes.[15] However, this view is now considered outdated. Many molecular pathways contribute to the development of NASH, and it remains uncertain whether NASH is always preceded by NAFLD. Moreover, pathogenic drivers are unlikely to be identical among all patients. Thus, both the mechanisms leading to disease and their clinical manifestations are highly heterogeneous.

A normal functioning liver maintains a balance between lipogenesis and $\beta$-oxidation. In NAFLD, this homeostasis is altered, and the presence of macrovesicular lipid droplets characterizes hepatic steatosis without evidence of hepatic inflammation.[16] This is reversible, but its persistence may cause progression to NASH, which is a necro-inflammatory subtype, characterized by the presence of macro-and-micro-vesicular steatosis, hepatocellular injury with ballooning of hepatocytes, and Mallory bodies near the nucleus containing hyperphosphorylated and misfolded cytokeratin filaments, inflammatory cell infiltration and apoptotic hepatocytes.[17] With continued injury, the native Kupffer cells/macrophages are activated and release pro-inflammatory cytokines, feeding into the inflammatory cycle.[18] The stellate cells are also activated, leading to fibrosis and end-stage cirrhosis. However, a linear relationship between steatosis and fibrosis is not always observed and can be variable.

Non-alcoholic fatty liver disease occurs in the background of metabolic dysfunction, and is considered to be the hepatic manifestation of metabolic syndrome. Hence, NAFLD is associated with insulin resistance, visceral adiposity, diabetes mellitus, hyperlipidemia, and hypertension.[19] Ultimately, these conditions are also associated with lifestyle choices, such as a high-fat diet and sedentarism, and obesity.

The main immunopathogenesis behind these associations can be explained by the link between NAFLD and type 2 diabetes mellitus (T2DM). Under normal conditions, there is a balance between lipid uptake, i.e., free fatty acids or de novo lipogenesis, and lipid removal, i.e., metabolism via $\beta$-oxidation and elimination as very low-density lipoprotein (VLDL).[20] In NAFLD, lipogenesis exceeds the capacity for VLDL clearance, resulting in VLDL buildup in the liver. The hormone insulin also affects this fat accumulation, and influences triglyceride storage in peripheral adipose tissues and inhibition of lipolysis. Hepatic insulin metabolism is dysregulated in patients with T2DM, leading to hyperinsulinemia and insulin resistance, defined as a suboptimal cellular response to physiological insulin levels, causing a steady increase in blood glucose levels.[20] This, coupled with high caloric intake, further feeds this cycle, leading to a pro-inflammatory environment in the liver.

## Hereditary component of NAFLD

Polymorphisms in several genes have been linked to NAFLD, proving the existence of a genetic or hereditary component in the pathogenesis and progression of this disease.[21] Genes described to date include the following:

- Patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), which encodes an enzyme with lipase activity.

- Transmembrane 6 superfamily member 2 (*TM6SF2*), which encodes a regulator in hepatic VLDL secretion.
- Hydroxysteroid 17-beta dehydrogenase 13 (*HSD17B13*), which is expressed in the human liver and encodes a protein that exhibits enzymatic activity against several lipid species.
- Cholesteryl Ester transfer protein (*CETP*), which encodes a protein with an imperative role in cholesterol transport.
- Sterol regulatory element-binding factor 1 (*SREBF1*), which encodes a transcription factor that regulates genes involved in cellular cholesterol synthesis and homeostasis.
- Glucokinase regulator (*GCKR*), encoding glucokinase regulatory protein that inhibits the glucokinase enzyme and, in return, de novo lipogenesis.[21]

## Liver specific microRNAs

MicroRNA-122 is among the early few examples of a tissue-specific miRNA,[22] and makes up 70% of the total miRNA pool of the liver.[22–25] MiRNA-122 is associated with cholesterol metabolism and HCC, and promotes hepatitis C virus replication.[22,26] Here, the role of miRNA-122 and miRNA-34a in the liver is reviewed.

MicroRNA-122 plays a role in maintaining tissue identity and regulates differentiation in adult liver cells.[23,26,27] The high activity of the promoter region pri-miRNA-122 in hepatic cell lines suggests that it depends on liver-specific transcription factors.[23,28] MiRNA-122 regulates the pathway of cholesterol and fatty acid synthesis and oxidation.[25,26,28] Serum miRNA-122 levels may be helpful as a marker in diagnosing NAFLD.[29] Similarly, the regulation of miRNA-34a by tumor protein 53 suggests its role in hepatic cell behavior.[30–33] Expression of miRNA-34a during liver regeneration helps in hepatic cell survival and regeneration.[30,34–36] Also, miRNA-34a increases the expression of transforming growth factor beta receptor 1 (*TGFBR1*), transforming growth factor beta 1 (*TGF-β1*), and levels of phosphorylated-smad2/3, thus promoting liver fibrosis in cases of liver injury.[37,38] MiRNA-34a plays a role in liver fibrosis by regulating apoptosis and autophagy in epithelial and endothelial cells in the liver,[33,38] and also inhibits tumor growth in the liver from distant metastasis.[35,39–41]

## Current diagnostic modalities of NAFLD

An ideal screening test identifies a disease rapidly and with high accuracy. Similarly, an excellent diagnostic test should be able to exclude those who do not have the disease in question. When determining whether or not to use a specific diagnostic test, crucial factors such as cost-effectiveness, ease of administration, and reproducibility must be considered.[42]

To date, ultrasound is considered the first-line screening tool for defining steatosis in a selected population. However, to diagnose NAFLD, other chronic liver disease etiologies or other steatosis-causing conditions must be excluded.[43] Liver biopsy remains the current gold standard for diagnosing NAFLD, despite limitations regarding sampling variability, invasive nature, and high cost.[43,44] This necessitates the development of more non-invasive approaches for diagnosing NASH, the progressive form of NAFLD. Several novel biomarkers have been proposed for diagnosing NASH, but their clinical value has yet to be been determined. Some non-invasive tests currently employed are the liver function parameters aspartate aminotransferase (AST) and alanine transaminase (ALT), which can generally show hepatocyte damage, though this depends on the severity of NASH. For

instance, the AST and ALT values may be normal in some NASH patients.[45]

Cytokeratin 18 may be used as an index for diagnosing NASH because it predicts the percentage of hepatocytes undergoing necrosis and apoptosis. Despite having a good specificity, the sensitivity of cytokeratin 18 is not as high. To elevate the sensitivity of this biomarker, it needs to be coupled with other indexes.[46] Inflammation indexes, such as interleukin (IL)-6 and tumor necrosis factor-α, have shown low specificity for detecting NASH.[47]

Comprehensive scoring systems, such as NashTest and ActiTest, are reported to be costly, with moderate diagnostic accuracy for NASH, and involve excessive indices.[45]

## Use of miRNA as a biomarker and diagnostic modality

Although some biomarkers have been employed to diagnose NAFLD, several new ones have also been proposed. These biomarkers help to identify the specific pathways that cause disease progression, including hepatocyte death or apoptosis, oxidative stress, and inflammation.[48–52] Because of its keen sensitivity, serum miRNA may be more effective in helping clinicians diagnose patients earlier than other methods. MiRNA-34a, miRNA-122, and miRNA-192 have been identified as potential diagnostic markers to distinguish NAFLD from NASH. Both miRNA-122 and miRNA-34a have demonstrated moderate accuracy in differentiating NAFLD from healthy controls and NASH from NAFLD, respectively.[53]

Among the well-studied serum miRNAs, miRNA-34a is the most available diagnostic index for NAFLD.[54] Peroxisome proliferator-activated receptor (PPAR)-α is a crucial transcription factor in regulating hepatic lipid metabolism and is downregulated in NASH. Levels of miRNA-34a have been shown to be inversely associated with the severity of liver injury,[55] and it also plays a role in liver inflammation by activating Kupffer cells.[55] In terms of apoptosis, miRNA-34a represses sirtuin-1, which leads to increased p53 acetylation and transcription. This results in the induction of pro-apoptotic genes, such as BCL2 binding component 3 (also known as PUMA), eventually leading to apoptosis.[56] MiRNA-34a moderates the pathogenesis of NAFLD by abnormal lipid metabolism and inflammation. Since miRNA-34a is present throughout NAFLD, including its onset and progression, it may be more reliable for diagnosing the disease than other miRNAs.[57]

Circulating miRNAs, especially miRNA-122, might be promising diagnostic biomarkers with high accuracy for NAFLD; however, more large-scale studies are required to back up these findings.[53,57] In a previously published systematic review and meta-analysis, 17 studies comprising a total of 1408 patients with NAFLD, and 926 healthy subjects from six other studies, were analyzed to assess the probability of circulating miRNAs serving as new diagnostic biomarkers in patients with NAFLD. The analysis revealed that miRNA-122 had high diagnostic accuracy, with a pooled sensitivity, specificity, and area under the curve of 0.88, 0.66, and 0.86, respectively.[53]

Compared with NAFLD patients, or NASH patients with only mild fibrosis (stage 0/1), those with NASH and more significant fibrosis (stages 2–4) have been shown to exhibit increased levels of three miRNAs (miRNA-193a-5p, miRNA-378d, and miRNA-378d). In addition, increased levels of seven miRNAs (miRNA-193a-5p, miRNA-378d, miRNA-378e, miRNA-320b, miRNA-320c, miRNA-320d and miRNA-320e) has been shown in cases with NAFLD activity scores between 5 and 8. Amongst the four microRNAs, three increased while only one decreased in those with high steatosis, activity, and

fibrosis (SAF) activity scores (scores of 2, 3, or 4) compared with lower SAF activity.[53,58]

The correlation between serum miRNA-193a-5p levels and NAFLD activity grade suggests that this microRNA may mediate the body's response to oxidative stress. Its ability to serve as a circulating biomarker for progressive liver diseases makes it a promising target for future clinical research.[54]

## Use of mRNA as therapeutics

Despite NAFLD being a leading liver disease, no definite line of treatment has been proposed to date.[59–62] Although the pathogenesis of NAFLD is not entirely understood, it is believed to respond to treatments other than lifestyle modifications when the latter is not useful,[63] and miRNAs have proven to be an essential regulator of inflammation in NAFLD.[64,65]

With technologies such as miRNA microarrays and miRNA sequencing, the circulating levels of unregulated miRNAs can be detected in body fluids and tissues that are thought to have prognostic significance.[66,67] Currently, miRNA sensors and decoy libraries are being established to assess miRNA activities and inhibit their physiological effects.[66–68] Other techniques, such as luciferase reporter assays, pull-down assays, and software, such as miRDB and miRGator, are also being employed to identify miRNA/mRNA interaction to identify targets for their potential use in therapeutics.[66]

Depletion of miRNA-122 expression in hepatic cells has been shown to lead to the progressive development of different stages of NASH, leading to HCC in both humans and in STAM mice, consequently establishing miRNA-122 as a tumor suppressor.[69–71] Sorafenib, an anti-neoplastic agent used in HCC, is proven to induce apoptosis more effectively in cells retaining a certain degree of miRNA-122 expression, leading to a proposal of combination therapy of miRNA-122 analog and sorafenib as a therapeutic regimen for HCC.[72] MiRNA-34a is another important miRNA involved in the pathogenesis of NAFLD.[61,63,65] Its inhibitors may be used as a therapeutic agent to improve the degree of steatosis by increasing the expression of *PPAR-α* and *SIRT1* genes.[54,61]

Ursodeoxycholic acid, used in treating primary biliary cholangitis, exerts its effects by lowering miRNA-122 levels and is being considered as a potential therapy for NAFLD.[73] Prescribing patients with palmitic acid is suggested to decrease levels of miRNA-193a-5p, which is increased in NASH with mild and significant fibrosis [stage 0–4].[58] The findings of such clinical trials raise the possibility of incorporating miRNA-based pharmacological therapies for NAFLD in the future.[66,74]

Despite the recent advances in successfully modulating miRNA levels to combat miRNA-related diseases, the outcome is invariably associated with severe adverse effects that force its discontinuation in clinical settings.[66,74] Due to this uncertainty in pharmacologic management, advances are being made in an organic treatment approach to halt the progression of NAFLD via targeting microRNAs,[61] such as the inclusion of extra virgin olive oil combined with a low caloric diet,[61,75] curcumin,[61,63,76] Xanthigen, a blend of Wakame (brown seaweed) and pomegranate seed oil,[61,77] and soy milk.[61,78] We advise further investigation into synthetic and natural compounds for the treating NAFLD via targeting miRNAs.

## Animal trials

MicroRNA has been revealed to significantly influence NAFLD development in animals and humans,[79–83] and studies have found that miRNA-21 and miRNA-27 are specific molecules that may help distinguish between steatosis (fatty liver) and

steatohepatitis (fatty liver with inflammation) in rats.[79,82]

Animal model studies have further investigated the relationship between miRNA and NAFLD by placing mice on high-fat and low-fat diets.[2] Their liver progress was closely monitored, and the presence of 15 different miRNAs was documented in the livers of mice on the high-fat diet, leading to various liver diseases.[80,81] The livers of these mice also displayed architectural abnormalities, such as pallor and fibrosis, and a high cellular density due to steatosis.[80]

Most recently, a pivotal study by Tanoglu et al.,[84] in a rat model of high-fructose diet-induced fatty liver, shed light on the correlation between vitamin D supplementation and the expression and circulating levels of microRNAs 200c and 33a. The study's results elucidated that vitamin D does have a protective role against NAFLD, and vitamin D was associated with a significant change in the circulating levels of miRNA-200c and 33a. This further consolidates our belief that levels of circulating miRNAs may act as biomarkers for NAFLD.

These findings demonstrate liver disease progression through NAFLD, NASH, and HCC.[80,85] Additionally, research has found that the downregulation of miRNA-122 in mice and humans and upregulation in rats can contribute to the pathogenesis of NAFLD, suggesting that miRNA may be of therapeutic value in treating NAFLD.[86–88]

## Human trials

The obesity epidemic has contributed to a rise in NAFLD. The correlation between NAFLD and obesity has been extensively explored, revealing that miRNA-378 is elevated in the livers of obese mice and patients with NASH.[89]

MicroRNA-378 plays a crucial role in developing hepatic inflammation and fibrosis by positively regulating the nuclear factor (NF)-$\kappa$B–tumor necrosis factor (TNF)$\alpha$ axis. MiRNA-378 targets protein kinase AMP-activated non-catalytic subunit gamma 2 (*PRKAG2*), which encodes AMP-activated protein kinase (AMPK)$\gamma$2. This reduces sirtuin-1 activity and facilitates an inflammatory pathway involving NF-$\kappa$B–TNF$\alpha$.[90]

In a study investigating the influence of oleoyl ethanolamide (OEA) supplementation and calorie restrictions on different variables, such as inflammation, body composition, and hepatic fibrosis, 76 obese patients with newly diagnosed NAFLD were placed on a weight-loss diet and randomly allocated into OEA or placebo groups. Pre- and post-intervention messenger RNA expression, levels of the transcription factor NF-$\kappa$B, IL-6, and IL-10, body composition, and NAFLD fibrosis score were assessed. A significant reduction in fat mass alongside a remarkable increase in resting metabolic rate was observed in the OEA group compared with the placebo group post-intervention.[91] MiRNA dysregulation in adipose tissue causes inflammation directly linked with obesity,[92] and a difference in the expression of 21 miRNAs has been noted in the epididymal adipose tissue of lean and obese people.[93]

Another study that analyzed subcutaneous adipose tissue in lean and obese individuals had similar findings. Out of 799 miRNAs, 50 exhibited variability in expression between the two groups and 17 of these 50 miRNAs are linked with body mass index.[94]

In children, circulating miRNAs have been proposed as biomarkers of obesity and its comorbidities. One study identified four miRNAs overexpressed in obesity (miRNA-222, miRNA-142–3, miRNA-140-5p, and miRNA-143) and two miRNAs (miRNA-122 and miRNA-34a) overexpressed in children with obesity and NAFLD and insulin resistance.[95] Two miRNAs, miRNA-199a-5p and miRNA-122, were also significantly overexpressed in children with NAFLD compared with healthy controls.[96]

Decreased expression of miRNA-139-5p, miRNA-30b-5p, miRNA-122-5p, and miRNA-422a, and increased miRNA-146b-5p has been observed in obese patients with NAFLD compared with sex, age, and weight-matched controls.[97] Activation of AMPK in hepatocytes stimulates fatty acid uptake and oxidation, while it switches off anabolic pathways, such as the synthesis of glucose, glycogen, and lipids.[98] The saturated fatty acid, palmitate, blocks AMPK phosphorylation and, thus, increases fatty acid biosynthesis independently of insulin.[99]

Decreased miRNA-139-5p and miRNA-122a-5p confirm the involvement of some miRNAs in NAFLD and changes in liver gene expression patterns, modifying fatty acid biosynthesis and glucose metabolism.[100]

Another pathway linking NAFLD to obesity is the liver X receptor (LXR)–sterol regulatory element-binding protein 1c (SREBP1c) pathway. Hepatic lipogenesis is aberrantly induced in NAFLD via activation of the LXR–SREBP1c pathway.[101] Hepatic expression of the long noncoding (lnc)RNA Blnc1 is strongly elevated in mouse models of obesity and NAFLD.[100] Inactivation of the Blnc1 gene in mice liver cells prevents high-fat diet-induced hepatic steatosis and insulin resistance, subsequently protecting mice from diet-induced non-alcoholic steatohepatitis.[100]

These studies and trials have all demonstrated that miRNA dysregulation and obesity are linked to NAFLD and its complications. Further research is needed to elucidate the role of miRNAs in modulating gene expression, metabolism, and other pathways associated with NAFLD, as well as a deeper understanding of how miRNAs can be used for diagnostic and therapeutic purposes.

## Future prospects

In accordance with the present review, we can conclude that considerable deficit exists in the management of NAFLD, particularly in its diagnosis. A patient's good prognosis depends on the timely and accurate diagnosis of NAFLD. The gold standard for diagnosing the complications of NAFLD i.e., NASH, fibrosis, and cirrhosis, is liver biopsy. Due to its invasiveness, biopsy comes with many risks, adding to the patient's anxiety and hesitancy, so there is a dire need for better biochemical and imaging diagnostic tests, to monitor treatment response or disease progression, than those currently in use, as they have limited diagnostic capacity.[102]

Although many underlying causes and main drivers contributing to the progression of NAFLD have been recognized, the key mediators remain unknown. To better understand the pathophysiology of NAFLD, and develop accurate diagnostic tools, research directed towards identifying these key mediators and epigenetic modifications is required.[103]

## Conclusion

Despite its wide prevalence, there are many gaps in the literature regarding NAFLD. Due to insufficient research on this patient demographic, few advances have been made in revising NAFLD treatment protocols. We prompt the identification of various biomarkers and the development of diagnostic tools for this vulnerable population. The present review highlights the contribution and potential therapeutic significance of miRNAs in NAFLD, NASH, and obesity, which have been overlooked in clinical practice. Our review further highlights the deficits in managing this complex ailment, while also elucidating recent trends and interest shown by researchers and clinicians alike for this disease, which, to the best of our knowledge, has not been presented before. With the alarming rise of NAFLD in adults and children, we strongly urge extensive clinical trials and observational studies, which may potentially factor into

improving and redefining treatment plans and prevention strategies for this patient subset.[91]

## Author contributions

All authors contributed equally to this work.

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