The Connection between MicroRNAs from Visceral Adipose Tissue and Non-Alcoholic Fatty Liver Disease

Veronika Zubáňová[1],*, Zuzana Červinková[2], Otto Kučera[2], Vladimír Palička[1]

ABSTRACT

Non-Alcoholic Fatty Liver Disease (NAFLD) is one of the most important causes of liver disease worldwide leading the foreground cause of liver transplantation. Recently miRNAs, small non-coding molecules were identified as an important player in the negative translational regulation of many protein-coding genes involved in hepatic metabolism. Visceral adipose tissue was found to take part in lipid and glucose metabolism and to release many inflammatory mediators that may contribute to progression of NAFLD from simple steatosis to Non-Alcoholic SteatoHepatitis. Since visceral adipose tissue enlargement and dysregulated levels of miRNAs were observed in patients with NAFLD, the aim of this paper is to reflect the current knowledge of the role of miRNAs released from visceral adipose tissue and NAFLD.

AUTHOR AFFILIATIONS

[1] Department of Clinical Biochemistry and Diagnostics, Charles University, Faculty of Medicine in Hradec Králové and University Hospital Hradec Králové, Czech Republic
[2] Department of Physiology, Charles University, Faculty of Medicine in Hradec Králové, Czech Republic
* Corresponding author: Department of Clinical Biochemistry and Diagnostics, Faculty Hospital Hradec Králové, Sokolská 581, 500 05 Hradec Králové, Czech Republic; e-mail: zubanovv@lfhk.cuni.cz

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INTRODUCTION

Non-Alcoholic Fatty Liver Disease (NAFLD) also known as Metabolic Associated Fatty Liver Disease (MAFLD) (1) is the most common chronic liver disease, which afflicts approximately a quarter of the worldwide population (2, 3). However, there also exist some variances in prevalence according to interindividual differences, such as ethnicity and gender. The highest prevalence has been reported in industrialized countries (Western Europe, USA and Middle East) and the lowest number of affected people is in Africa (3, 4). NAFLD is defined by liver fat accumulation in the absence of any other known cause of hepatic steatosis (extensive alcohol intake, chronic viral hepatitis, drug intake or genetic disorders etc.) (5). After hepatitis C, NAFLD is the second leading cause for liver transplantation in developed countries such as the USA. This is the result of asymptomatic follow-up in most of NAFLD patients, which may lead to late detection of the disease at an advanced stage (3).

MicroRNAs (miRNAs) are approximately 20 nucleotides long naturally occurring non-coding RNAs, which at post transcriptional level negatively regulate a large number of genes playing the role in many physiological and pathophysiological processes in the liver and in the human body in general (6–9). Until now, few thousand miRNAs were identified in human genome. miRNAs can be classified according to their characteristics and well defined role in organ development, and may be used as a very powerful, specific and sensitive biomarker in NAFLD diagnosis and follow-up as it was previously seen in many human diseases (7, 10). It has been suggested that microRNAs may influence almost 60% of human protein-coding genes (11). To regulate gene expression, they bind to 3′ untranslated region (UTR) of complementary mRNA (messenger RNA) target and inhibit its translation to protein or cause mRNA degradation. According to this effect, they are involved in the regulation of many biological processes, such as glucose, cholesterol or iron homeostasis, immune response or cellular proliferation, differentiation and cell death as it was reviewed in many articles (8, 9, 12–14). Since one miRNA can target multiple genes (miRNA multi-functionality) and multiple miRNAs may influence a single gene (redundancy), it is difficult to identify direct connection between miRNA and the target gene (9).

NAFLD PATHOGENESIS AND PROGRESSION

The pathogenesis of NAFLD is multifactorial and is triggered by environmental factors such as hypernutrition (excessive caloric intake, high intake of saturated fats, cholesterol, fructose, etc.) and physical inactivity in the context of genetic predisposition (15). Elevated incidence of NAFLD is related to the worldwide rise in metabolic syndrome. NAFLD is associated with increased risk of cardiovascular disease, metabolic syndrome and cancer development (16, 17). Metabolic syndrome (MS) is defined as a complex disorder including many interconnected factors (dyslipidemia, abdominal obesity, dysregulated glucose metabolism, etc.) that together contribute to higher risk of cardiovascular disease and type 2 diabetes mellitus

(18). Above 90% of patients with NAFLD have more than one component of MS and about 60% of patients with liver disease have the complete diagnosis of MS (minimal 3 of 5 signs) (19). Insulin resistance (IR) was identified as a central point in pathogenesis of NAFLD, thus considering NAFLD to be the liver manifestation of metabolic syndrome. IR leads to the retention of triacylglycerols (TAG) by the hepatocyte together with impaired carbohydrate metabolism (20). In general, insulin decreases the production of VLDL (very low-density lipoprotein) by inhibition of adipose tissue lipolysis and hepatic VLDL production. Nevertheless, in patients with NAFLD (or MS) insulin fails to inhibit lipolysis and hepatic production, leading to an increase in serum triacylglycerols and their accumulation in the liver (21). In this case, the global prevalence of NAFLD among patients with type 2 diabetes mellitus (T2DM) was estimated to be almost 60% (22).

Several dysfunctions may participate in the pathogenesis of NAFLD such as alterations in metabolic pathways, imbalance in lipid import, *de novo* formation, oxidation and export, and thus leading to lipid accumulation in the liver (steatosis is defined as hepatic TAG content > 5% of liver weight) and to further progression – inflammation state (steatohepatitis) complicated with fibrosis, cirrhosis and carcinoma formation (Figure 1) (23). Non-Alcoholic SteatoHepatitis (NASH), an advanced stage of fatty liver disease, develops in 10–30% of patients with simple steatosis and in 40% of T2DM patients where is associated with a worse long-term prognosis (22). NASH is accompanied by hepatocellular damage characterized by hepatocellular ballooning, apoptosis, lobular inflammation and with or without hepatic fibrosis (23). Contrary to simple steatosis, NASH may progress to cirrhosis and, in some number of patients to hepatocellular carcinoma (23, 24). Unique miRNA profiles were found to be changed in obesity and different stages of NAFLD. Sharma et al. also found correlation of differentially expressed miRNA with various variables such as body weight, body mass index, fasting glycemia, TAG concentrations, adipocyte differentiation or adipose tissue inflammation (25). In addition to previously mentioned study, Klöting et al. found higher miR-17-5p, miR-132 and miR-134 expression and opposite pattern of miR-181a in omental fat comparing the group of patients with T2DM and normal glucose tolerance (26). Some miRNAs were identified to be dysregulated in omental and subcutaneous fat (subcutaneous adipose tissue – SAT) that significantly correlate with glucose and lipid metabolism parameters, adipose tissue morphology (macrophage infiltration, adipocyte volume) and visceral fat area, suggesting the role of miRNAs in obesity associated disorders (26). As an example, serum free fatty acid levels negatively correlate with adipose tissue level of miR-99a in human. Additionally, an expression of miR-155 was significantly related to macrophage infiltration and the strongest correlation was found between the mean omental adipocyte diameter and miR-198. These findings point out a role of miRNAs in immune cells attraction to adipose tissue and thus its proinflammatory role in NAFLD development (26). Furthermore, it has been demonstrated that visceral adipose tissue (VAT) and SAT exhibit specific gene expression patterns and in case of

that regulate different physiological functions (27). Up to now, an exact role of miRNAs is difficult to estimate, and *in silico* analyses are widely used. For instance, Capobianco et al. studied miRNA and protein expression profiles of visceral adipose tissue. Based on the transcriptomic and proteomic profiles, they found a functional association between dysregulated miRNA and corresponding protein. Two pairs were identified as contributors to altered gene expression in obesity, namely, miR-141/YWHAG (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide) and miR-520e/RAB11A (Ras-related protein). Decreased expression of miRNA was associated with up-regulation of their pairing proteins and this hypothesis was also validated by functional analysis with luciferase assay confirming binding site of these miRNAs on 3'UTR region of corresponding transcripts (28). However, further detailed research on miRNA functional studies is needed.

THE ROLE OF VISCERAL ADIPOSE TISSUE IN NAFLD

Adipose tissue plays a central role in health and disease. Its primary function is to store energy in the form of triacylglycerols and release them in the usable form during fasting and starvation, when energy is needed (29). In general, obesity is characterised by an excess of white adipose tissue. Central obesity, i.e., fat accumulated in VAT, is stated to be the major risk factor for metabolic-syndrome related disorders. Although, there is considerable physiological difference in genders, whereas in men VAT represents almost 20% of total fat and in women 8% (16,

29). The abdominal adipose tissue has been characterised as an endocrine tissue, releasing many hormonal signals that may affect the hypothalamic-pituitary-gonadal axis (30, 31). However, few epidemiological studies revealed the fact, that NAFLD occurred also in group of non-obese patients without metabolic syndrome (32, 33). In the group of non-obese Chinese adults, hepatic fat accumulation showed the relation to abdominal fat examined by CT (34).

A) VAT inflammation

In obesity, adipocytes enlarge their size due to higher accumulation of TAGs. However, it is hypothesised that this expansion may be limited and fat overload of adipocytes may create a hypoxic environment (35), the condition leading to adipocyte death (36). Monocytes are then attracted and infiltrate adipose tissue partially due to monocyte chemo-attractant protein-1 (MCP-1), chemokine released from damaged adipocytes (37). Knockout or pharmacological blockage of MCP-1 receptor gene reduced macrophage infiltration of adipose tissue and insulin resistance in mouse on high-fat diet (38). In addition, the anatomy of the liver enables the portal and arterial circulation interaction together with hepatocytes and liver immune-system cells. In comparison to subcutaneous adipose tissue, VAT has a greater lipolytic potential and free fatty acids (FFAs) released from visceral fat are directly transported to the liver via portal circulation. These FFAs, together with exogenous fat supply and *de novo* lipogenesis are the main source of hepatic TAGs in NAFLD (39).
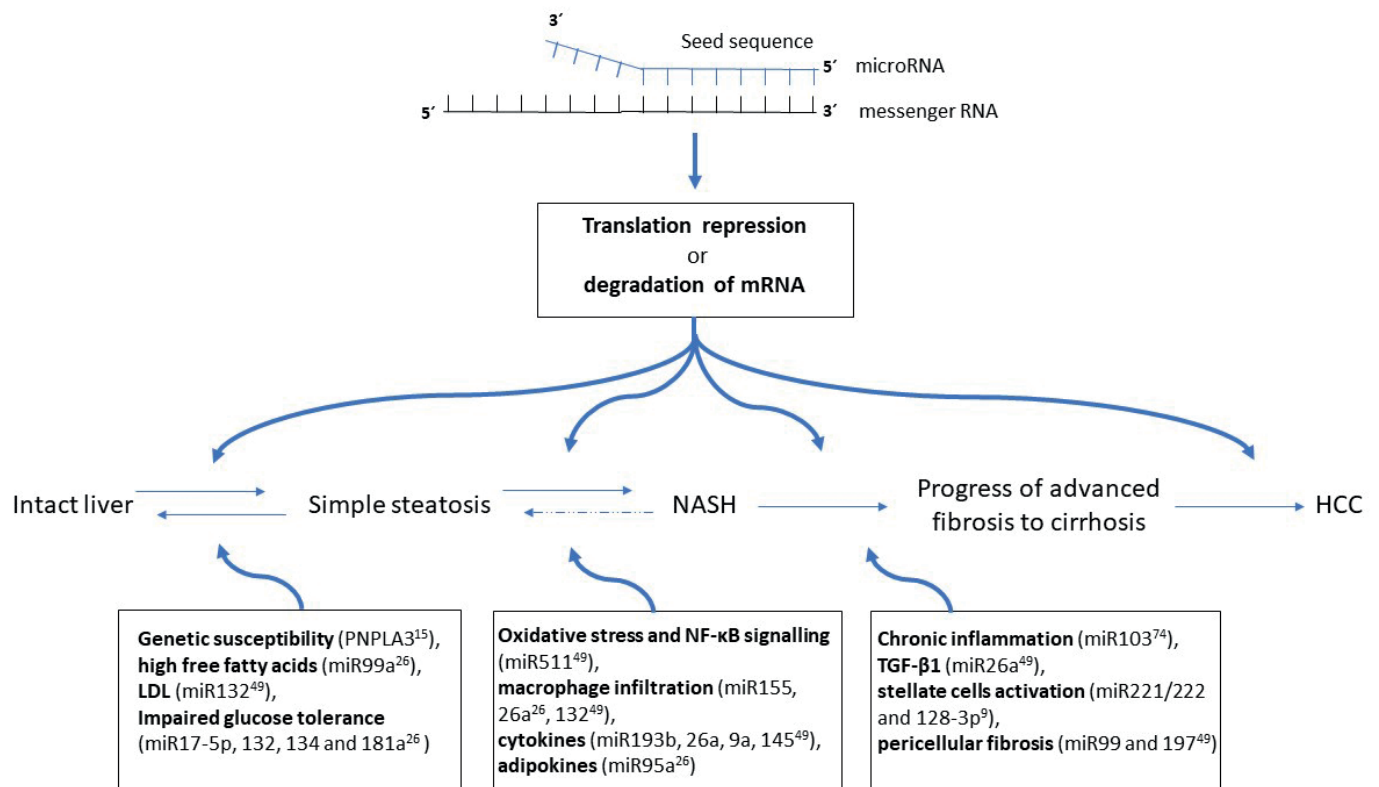

Fig. 1 Schematic diagram of possible effect of miRNAs on pathogenesis of NAFLD. HCC: Hepatocellular Carcinoma; NASH: Non-Alcoholic SteatoHepatitis; PNPLA3: Patatin-like Phospholipase Domain-containing Protein 3; TGF-β1: Transforming growth factor β1.

B) VAT mediators

The excessive expansion of VAT results in low-grade inflammation due to the presence of macrophages secreting several bioactive mediators. Dysregulation in soluble signalling molecules such as cytokines and adipokines (leptin, resistin, visfatin, interleukin 6 (IL-6) and 8 (IL-8), tumour necrosis factor alpha (TNF-α), adiponectin) secreted by visceral adipose tissue can partially contribute to proinflammatory state participating on NAFLD progression to NASH, NASH-associated fibrosis and insulin resistance (40, 41). Since the role of adiponectin is to protect the liver from inflammation via direct antagonism of TNF-α, dysregulation of adipocytokines may represent and important link between fat mass in obesity (metabolic syndrome) and NAFLD development (42). Adiponectin exhibits insulin-sensitising effects in the insulin-dependent tissues, such as the liver, skeletal muscle and adipose tissue. Concentrations of adiponectin were found to be downregulated in obesity and T2DM, in which inversely correlated with fat amount (43). Adipose tissue shows selective gene expression patterns, to which miRNAs contribute (26, 29, 44, 45). Enlargement of adipose tissue is associated with a transcriptional alteration of coding and also non-coding RNAs. In that case, miRNA expression from VAT may contribute to the pathogenesis of NAFLD, as it was detected in previous studies revealing their association with NAFLD (41, 46).

In comparison to subcutaneous adipose tissue, visceral adipose tissue contains larger adipocytes resistant to insulin and macrophages releasing pro-inflammatory cytokines such as TNF-α, IL-6 and C-reactive protein (47). Many miRNAs, such as miR-193b, miR-26a, miR-9a, miR-145, were found to regulate the production of MCP-1, TNF-α and adiponectin during visceral adipose tissue inflammatory process (48). Several adipokines have been reported to be related to changes in miRNA profile, and differential expression of miRNAs was found in the liver and also in VAT of patients with NAFLD (49). Estep et al. studied expression of miRNAs in visceral adipose tissue from NASH patients in which they found overall down-regulation of miRNA expression in comparison to non-NASH NAFLD. In addition, the decreased levels of miR-99 and miR-197 were significantly associated with pericellular fibrosis in NASH. Other miRNAs were confirmed to be associated with insulin receptor pathway (IGF1, IGFR13), cytokines (CCL3, IL6), genes connected to inflammation response (NFκB1, RELB, FAS) or ghrelin/obestatin gene. Moreover, four of differentially expressed miRNAs are positioned inside genes connected to metabolic syndrome, liver disease, obesity or T2DM, confirming their possible effect on target genes (49). This down-regulation of miRNAs was also found in the serum of NASH patients, but without any relationship to the stage of fibrosis (50). The study of Cheung et al. also supported these findings when they found eighteen miRNA species to be differentially expressed in the adipose tissue of NASH patient and confirmed their dysregulation in the liver of NASH patients (51).

In comparison, some miRNAs were found to be upregulated in adipose tissue. Chartoumpekis et al. found increased levels of miR-146a and miR-146b in obese mice fed by long-term high-fat diet (52). On the other hand, Latorre et al. investigated the decrease of few miRNAs in obese subject with NAFLD. In concordance with the results of human studies, palmitic acid used as an inductor of FA accumulation in hepatocytes *in vitro*, inhibited expression of many miRNAs in cell cultures, increased fatty acid transport, and fat formation, but reduces glucose metabolism and fatty acid oxidation (53). Down-regulation of miRNA miR-146a was also examined in peripheral blood mononuclear cells from T2DM patients when compared to healthy subjects suggesting the role of miR-146a in insulin resistance (54).

THE ROLE OF MICRORNA IN NAFLD DIAGNOSTICS

Until now, it has not been possible to predict NAFLD outcome through the routinely used blood and tissue markers due to their limited sensitivity and tissue specificity. The ultrasonography is a gold standard in NAFLD non-invasive diagnostics, but it has low sensitivity (detects fatty liver when approximately 30% of area is affected by liver steatosis) (55). The best tool for revealing the fine details of histopathological severity is liver biopsy. Nevertheless, this technique is invasive and could be accompanied by some severe complications, thus limiting its indication. Liver biopsy is usually performed at progressed stage of the disease and is not routinely used for mass screening or disease follow-up (56). Due to the limitations of liver biopsy, the use of non-invasive markers has emerged in recent years.

A) Circulating miRNAs

The biggest advantage of miRNAs is their use in non-invasive diagnostic due to their presence in the body fluids such as serum, plasma or urine and correlation between free circulating and tissue levels and thus reflecting the physiological/pathological state of tissue they are released from. Circulating miRNAs can result from a passive release in case of cell death or an active secretion to extracellular space in the form of microvesicles. In that case, they may indicate hepatocellular damage during liver injury or inflammation as described previously (56–58). Due to their presence in microvesicles and exosomes, they show high stability (contrary to long RNAs) in the environment containing RNases (RNA-degrading enzymes) (56, 58). The presence of specific surface proteins on exosomes reflects tissue or cell origin and function in cell-to-cell communications (56, 59). During NASH ballooning degeneration, cell death is enhanced, and many miRNAs are released into the circulation. This feature is thought to mirror molecular event in NAFLD (57). Some miRNAs also exist without cover or are incorporated in and protected by Argonaute 2 and surrounded proteins in high-density lipoproteins (HDL) (56, 58).

RESEARCH MODELS ON THE ROLE OF MICRORNAS IN NAFLD

Murine models play a key role in the deeper understanding of therapeutic effects and provide an opportunity for research using genetic manipulation. Popular models of obesity are genetically and diet-induced animals (mouse)

or, on *in vitro* level, preadipocyte cell-culture differentiated into adipocytes (60).

Adipocyte differentiation as a cellular process is regulated by several important regulatory factors, nevertheless the whole mechanism is not fully understood. miRNAs together with insulin secretion have been shown to be involved significantly in stem cell modulation and adipocyte differentiation (61, 62). These results were supported by *in vitro* functional analysis of Esau et al. The reduction of miR-143 by transfecting antisense oligonucleotides inhibited adipocyte differentiation, suggesting that miRNAs may regulate adipose tissue processes (63). Furthermore, the study of Bergenstrate revealed almost 70 miRNAs to be differentially expressed in mesenchymal stem cell-derived adipocytes (terminally differentiated adipocytes) compared to progenitor cells. They also validated the results of *in vivo* studies in mouse and *in vitro* studies in mutant mouse line (64). Moreover, miR-21, miR-27a, miR-103a, and miR-320 have been reported to be involved in adipocyte proliferation and differentiation (65–68). The functional study demonstrated insulin-induced improvement of glucose uptake in insulin-resistant adipocytes after anti-miR-320 oligo addition. Prior to these manipulations, miR-320 expression in insulin-resistant cell line (3T3-L1) was approximately 50 times higher compared to normal cell expression. These results suggest the role of miR-320 in targeting phosphoinositide-3-kinase, regulatory subunit 1 (68). The findings are in the accordance with study of Lin et al., who demonstrated influence of miR-27 overexpression in inhibition of adipocyte formation. Also, adipogenic regulators PPAR-γ (peroxisome proliferator-activated receptor γ) and C/EBPα (CCAAT-enhancer binding protein α) represent targets for this miRNA, which contributes to their close link (69). PPARs are ligand-activated transcription factors from nuclear receptor subfamily that function as lipid and glucose metabolism, energetic, inflammation and atherosclerotic regulators. Few isoforms can be found in different tissues: PPAR-α, PPAR-β/δ and PPAR-γ that is highly expressed in adipose tissue (70, 71). Additionally, data from PPAR-α null mice supported this hypothesis. Activation of this protein was reported to control the metabolic response of adipose tissue to diet income and induce cleavage of intracellular lipids through fatty acid oxidation (72). In adipose tissue, activation of PPAR-γ regulates the change of the macrophages to M2 subtype, that exerts in contrast to M1 type, anti-inflammatory action and may be preventive in NAFLD development (73). In another study, few miRNAs were found to be pro-adipogenic (miR-143, miR-103, miR-146b, miR-148, miR-33b) and serve in the process of preadipocytes accelerated adipogenesis. This process was accompanied by increased expression of transcription factors (PPARγ2), cell cycle regulators, glucose transporters and adipokines (74). However, miR-143 and miR-103 were found to be significantly decreased in adipocytes from obese mice. On the contrary, miR-222 and miR-221 were downregulated during adipogenesis and conversely upregulated in adipocytes from obese subjects. In general, miRNAs that were induced during adipogenesis, were underexpressed in adipocytes from obese mice and *vice versa*, suggesting that these changes are related to chronic local inflammatory environment in adipose tissue (74). Intronic

miRNA – miR-33b and its target gene SREBP-1 (sterol regulatory element-binding protein 1) were also highly upregulated during preadipocyte differentiation and miR-33b inhibition decreases adipose cell differentiation and lipid droplet accretion (75). Moreover, miR-146b expression was increased in high fat (HF) diet-induced obese mice, db/db mice and ob/ob mice its and expression of its target gene SIRT1 (sirtuin 1) was found to be decreased in white adipose tissue representing induced adipogenesis based on SIRT1 pathway. In addition, *in vivo* neutralization of miR-146a revealed increased SIRT1 expression relieving the impact of diet-induced obesity (76).

Similarity of rodent model with human NAFLD or NASH is assessed by liver histology, where degree, phenotype of inflammation and fibrosis are rated (17). For example, HF diet leads to the development of the steatotic phenotype that most characterizes human NAFLD/NASH at early stage. Teufel et al. analysed systemic gene expression profile of liver tissues from patients at different stages of NAFLD compared to various rodent models of NAFLD. Mice model based on high-fat diet seems to be the most similar to human liver affected by NAFLD as documented by resemblance to expression patterns analysed at pathway level (17). Even though the nutritional manipulation-based mouse model is considered the best in terms of correlation to human pathology, no single model is able to reflects the whole process of human disease. However, currently, there are only few models that can mimic the individual aspects of NAFLD (high-fat/high-fructose diet mouse model) (17, 77, 78).

CONCLUSIONS

Genetics, environment and dietetic habits play a key role in NAFLD development and progression. Epigenetic factors influence genetic base of the disease and increase individual susceptibility to NAFLD and may stand for great phenotype variability.

Since visceral adipose tissue secretes significant amount of cytokines and adipokines, adipocytes play an important role in fatty liver development. Genes encoding these inflammatory molecules were found to be regulated by epigenetic factors, thus indicating that understanding of differential expression of miRNAs in the adipose tissue is useful for better understanding of mechanisms participating in the development of NAFLD and its progression to advanced stages. Hopefully, circulating miRNAs secreted from tissues, may become promising tool for non-invasive diagnostic differentiation between NASH and NAFLD.

AUTHOR CONTRIBUTIONS

All authors took part in writing the manuscript. All authors read and approved the final draft.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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