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3 **miR-122-regulated metabolic circuits:**

4 **micro-management of lipid metabolism in the human liver**

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25 **Abbreviations:** FFA: free fatty acids, HCC: hepatocellular carcinoma, HCV: hepatitis
26 C virus, HFD: high fat diet, LA: lauric acid; miRNA: microRNA, NAFLD: nonalcoholic
27 fatty liver disease, OA: oleic acid, PA: palmitic acid, PPAR: peroxisome proliferator-
28 activated receptor, ROR: retinoic-related orphan receptor; TG: triglycerides
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The liver plays a key role in protein, carbohydrate and lipid metabolism. Alteration of liver metabolic functions, e.g. by excessive alcohol intake, obesity, diabetes mellitus or dyslipidemia can lead to accumulation of triglycerides (TG) in the liver, referred to as fatty liver or hepatic steatosis. Hepatic steatosis can be accompanied by inflammation, a condition called steatohepatitis that may progress to liver cirrhosis and liver cancer (hepatocellular carcinoma (HCC)). Due to the increase of obesity, nonalcoholic fatty liver disease (NAFLD) is one of the most important causes of liver disease worldwide with a global prevalence estimated at 24% (1). With more than 100 million individuals affected in the US and Europe, it is expected that NAFLD-related HCC will dramatically increase within the next decades (1, 2). However, to date no effective pharmacological strategies are available to prevent progression of liver cirrhosis and HCC development in the context of NAFLD.

Liver metabolic functions are tightly regulated by microRNA (miRNA) (reviewed in (3)) and various forms of liver disease have been associated with altered intrahepatic miRNA profiles. As the most abundant miRNA in the adult liver, miR-122 plays an important role in the regulation of lipid metabolism and acts as a tumor suppressor in the liver (reviewed in (4)). Moreover, since the hepatitis C virus (HCV) uses miR-122 to establish its infection (reviewed in (4)), miR-122 also represents an antiviral target and anti-miR-122 strategies have been evaluated in clinical trials (5, 6). Of note, while the administration of antisense miR-122 to chronic hepatitis C patients did not trigger any dose-limiting adverse event in clinical trials, a sustained and reversible decrease in serum cholesterol levels was observed in treated patients (5, 6) in line with the important role of miR-122 in the regulation of hepatic cholesterol and fatty acid metabolism (7-9). However, the molecular mechanism underlying the interplay between miR-122 and lipid homeostasis has not yet been completely deciphered.

Interplay between miR-122 and lipid metabolism in the human liver, white adipose tissue and muscle

While different studies have characterized the role of miR-122 in the mouse liver using transgenic mice and anti-miR-122 strategies (8, 10, 11), the cellular pathways regulated by miR-122 in the human liver are less well known. The study of tumoral and nontumoral liver tissues from HCC patients revealed that liver miR-122 expression inversely correlates with patient survival and metastasis (12). Indeed, miR-122 appears to be specifically repressed in poor prognosis HCC subsets exhibiting gene expression profiles linked to the suppression of hepatic phenotype and the acquisition of invasive properties (12). Most recently, it has been shown that miR-122 levels were decreased in liver samples from patients with alcoholic liver disease (13) suggesting that miR-122 networks may be deregulated in the course of different forms of liver disease. In their recent study, Chai and colleagues have shed new light on the lipid metabolic circuit involving miR-122 in the human liver (14) and have provided another piece to the puzzle of the cellular pathways involving human miR-122. They showed that some lipids can modulate miR-122 expression and secretion from liver-derived cells, circulating miR-122 in turn being able to act remotely on non-liver derived tissues.

In contrast to other free fatty acids (FFA), lauric acid (LA), palmitic acid (PA) and oleic acid (OA) enhanced the miR-122 promoter activity in human hepatoma Huh7 cells. Deciphering the underlying molecular mechanism using mutation of the predicted ROR α binding site within the miR-122 promoter, a retinoic-related orphan receptor alpha (ROR α) agonist and chromatin immunoprecipitation (ChIP) experiments, the authors showed that binding of ROR α was involved in this process. These findings were corroborated by mouse experiments where TG hydrolysis into FFA was induced *in vivo* by the administration of the β 3-adrenergic receptor agonist CL316243. These

mice showed an increase in plasma FFA levels as well as hepatic TG, pre-miR-122 and miR-122 levels.

Interestingly, the effect on miR-122 secretion from Huh7 cells appeared to be independent on the effect of FFA on miR-122 expression since FFA that demonstrated no effect on the miR-122 promoter (i.e. decanoic and myristic acids) were able to increase the secretion of miR-122 from these cells. Like miR-122 expression, miR-122 secretion is under the control of ROR α since a ROR α agonist and antagonist respectively increased and decreased LA-induced miR-122 secretion from Huh7 cells. This process appears to also take place *in vivo* since increased serum miR-122 levels were observed prior to increased miR-122 expression in CL316243-treated mice as well as in mice following a 24h-starvation period. To further investigate the clinical relevance of these findings, the authors assessed serum miR-122 levels in individuals subjected to plasmapheresis, a procedure that has been reported to increase plasma FFA levels (15), as well as following fasting. Plasmapheresis indeed increased serum FFA and miR-122 levels and a positive correlation was observed between FFA and miR-122 levels in healthy individuals following fasting. Noteworthy, secreted miR-122 was able to be transferred to miR-122-negative cells and to repress miR-122-target genes in these cells as shown by experiments using supernatants from LA-treated Huh7 cells and non-liver-derived recipient cells that do not express miR-122. The underlying molecular mechanism was not studied but miR-122 may be included in exosomes or be associated with lipoproteins (16). Furthermore, the addition of miR-122 mimics to miR-122-negative non-liver-derived cells triggered a decrease of LA-induced lipid droplet formation underscoring that exogenous miR-122 can modulate lipid pathways in non-liver-derived cells.

To corroborate their findings *in vivo*, Chai and colleagues treated mice with antagomiR-122 and/or the β 3-adrenergic receptor agonist CL316243 and monitored TG synthesis pathway gene expression as well as TG and miR-122 levels in the liver, white adipose tissues and skeletal muscle. CL316243 increased miR-122 levels in the serum, liver, white adipose tissue and skeletal muscle while the concomitant administration of antagomiR-122 reduced miR-122 levels in those compartments and lead to an increase of TG levels in the liver and skeletal muscle in line with an increase in the expression of miR-122-target genes involved in TG synthesis. Next-generation RNA-sequencing and pathway analysis revealed that miR-122 is likely involved in several metabolic pathways that regulate TG levels, including circadian rhythm pathways and adipogenesis. Furthermore, antagomiR-122-treated mice exhibited increased food uptake and reduced voluntary activity, behaviors that favor TG accumulation and steatosis. It has to be pointed out that in contrast to the study by Chai and colleagues, a previous study reported a reduction of steatosis following short-term administration of antagomiR-122 to mice fed with high-fat diet (HFD) (8). These different observations may be due to different experimental conditions and analysis timelines since different strains of miR-122 knock-out mice were shown to exhibit extensive lipid accumulation in the liver (10, 11).

Chai and colleagues uncovered novel direct targets of miR-122 in humans, namely *Agpat1* and *Dgat1* that play a major role in TG synthesis. This is in line with previously published data demonstrating that mouse *Agpat1* is a miR-122 target (10, 11). They also validated *Dgat1* as miR-122 target in mice. Based on Ingenuity pathway analysis (IPA) and GeneAnalytics analysis of their elegant *in vivo* gain-of-function and loss-of-function experiments, the authors hypothesized that miR-122 decreases *Foxo1* and *PPAR γ* , transcription factors that have been previously linked to hepatic steatosis

and adipogenesis in mice (17-19). Furthermore, they also provided novel evidence for the regulation of miR-122 expression in human liver-derived cells. In line with its liver-enriched expression profile, miR-122 expression is known to be driven by liver-enriched transcription factors. The miR-122 promoter can be stimulated by hepatocyte nuclear factor (HNF) 1 α , 4 α and 6 as well as FoxA2 and CCAAT/enhancer binding protein α (20, 21). Chai and colleagues now provided evidence that ROR α plays a role in FFA-mediated miR-122 expression. Interestingly, ROR α knock-out mice have been reported to exhibit a dysregulation of PPAR γ signaling and increased hepatic glucose and lipid metabolism rendering them more susceptible to the development of hepatic steatosis, obesity and insulin-resistance upon feeding with HFD than control mice (19, 22). These data indicate the existence of a complex network involving FFA, miR-122 and several transcription factors including ROR α and PPAR γ that regulates lipid metabolism in the liver with remote effects on TG levels in peripheral tissues.

Taken together, this study increases our understanding about the role of miR-122 in hepatic lipid metabolism and highlights its role as a secreted signaling molecule to regulate lipid storage in adipose tissue and skeletal muscle.

Perspectives of increasing hepatic miR-122 levels for prevention and treatment of liver disease

Several therapeutic strategies targeting miRNAs are in development (for review see (23)) including for metabolic diseases. The safety, tolerability and pharmacokinetics of anti-miR-103/107 will be evaluated in diabetic patients with NAFLD (ClinicalTrials.gov Identifiers: NCT02826525). Due to the lack of strategies to slow down/reverse progression of liver disease and to prevent HCC in cirrhotic patients in the context of NAFLD as well as the key role of miR-122 as a tumor suppressor and a regulator of

lipid metabolism in the liver, novel preventive and therapeutic strategies aiming to increase miR-122 levels may provide perspectives for patients with advanced liver disease. In line with this, a recent study showed that restoration of miR-122 levels reduced ethanol-induced liver injury in mice (13).

Therapeutic approaches using antisense miR-122 strategies were shown to be well tolerated in chronic hepatitis C patients (5, 6) but so far miR-122 mimics have not been used in the clinic. Several miRNA mimics (miR-16, miR-29 and miR-34) have reached phase I clinical trials (ClinicalTrials.gov Identifiers: NCT02369198, NCT02603224 and NCT01829971) and miR-34 mimics (MRX34) were evaluated in cancer patients including patients with liver cancer. However, this study was terminated due to immune related serious adverse events as announced by Mirna Therapeutics on September 20, 2016 and indicated on ClinicalTrials.gov. It will thus be very important to carefully evaluate the potential risks associated with the modulation of miR-122 levels in patients with liver disease.

Another approach to increase miR-122 levels in metabolic disorders without using miRNA mimics or viral vector gene therapy could be through activation of ROR α as suggested by Chai and colleagues (14). This appears to be an interesting strategy since small molecule drugs could be used (24). However, it has to be kept in mind that ROR α does not only regulate glucose and lipid metabolism but also T helper 17 (Th17) cell development that plays an essential role in many autoimmune disorders; moreover, ROR α is involved in the stabilization of p53 /apoptosis and has been suggested as an anti-cancer target (reviewed in (24)). Further preclinical and clinical studies are needed to define and carefully characterize novel therapeutic targets in order to improve the management of patients with liver disease.

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Conflict of interest

The authors declare no conflict of interest.

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Figure legend

Figure 1. Impact of miR-122 on the triglyceride biosynthesis pathway. An increase of free fatty acids (FFA) in the bloodstream due to triglyceride (TG) hydrolysis, e.g. following starvation or treatment with a β 3-adrenergic receptor agonist, enhances the promoter activity of miR-122 through retinoic-related orphan receptor alpha (ROR α) in hepatocytes. In turn, miR-122 regulates the TG biosynthesis in the liver by inhibiting *Agpat1* and *Dgat1*, two genes involved in the production of TG. FFA also increase the secretion of miR-122 from hepatocytes into the bloodstream. Secreted miR-122 remotely decreases the TG levels both in adipose tissues and muscle cells as well as the formation of lipid droplets in muscle cells (14). (Images adapted from SMART (Servier Medical Art))

β 3-adrenergic receptor agonist CL316243
starvation