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Published on: 01 Jul 2019 - bioRxiv (Cold Spring Harbor Laboratory)

Topics: Fatty liver, Steatohepatitis and Biomarker (medicine)

Serum miR-379 expression is related to the development and progression of hypercholesterolemia in non-alcoholic fatty liver disease

Short title: Serum miR-379 relates hypercholesterolemia in NAFLD

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Abstract

Introduction: Non-alcoholic fatty liver disease (NAFLD) has a wide spectrum, eventually leading to cirrhosis and hepatic carcinogenesis. We previously reported that a series of microRNAs (miRNAs) mapped in the 14q32.2 maternally imprinted gene region (Dlk1-Dio3 mat) are related to NAFLD development and progression in a mouse model. We examined the suitability of miR-379, a circulating Dlk1-Dio3 mat miRNA, as a human NAFLD biomarker.

Methods: Eighty NAFLD patients were recruited for this study. miR-379 was selected from the putative Dlk1-Dio3 mat miRNA cluster because it exhibited the greatest expression difference between NAFLD and non-alcoholic steatohepatitis in our preliminary study. Real-time PCR was used to examine the expression levels of miR-379 and miR-16 as an internal control.

Results: Compared to normal controls, serum miR-379 expression was significantly up-regulated in NAFLD patients. Receiver operating characteristic curve analysis suggested that miR-379 is a suitable marker for discriminating NAFLD patients from controls, with an area under the curve value of 0.72. Serum miR-379 exhibited positive correlations with alkaline phosphatase, total cholesterol, and low-density-lipoprotein cholesterol levels in patients with early stage NAFLD (Brunt fibrosis stage 0 to 1). The correlation between serum miR-379 and cholesterol levels was lost in early stage NAFLD patients treated with statins. Software-based predictions indicated that various energy metabolism–related genes, including insulin-like growth factor-1 (IGF-1) and IGF-1 receptor, are potential targets of miR-379.

Conclusions: Serum miR-379 exhibits high potential as a biomarker for NAFLD. miR-379 appears to increase cholesterol lipotoxicity, leading to the development and

progression of NAFLD, via interference with the expression of target genes, including those related to the IGF-1 signaling pathway. Our results could facilitate future research into the pathogenesis, diagnosis, and treatment of NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is an important cause of chronic liver injury, with an increasing incidence worldwide [1]. NAFLD, regarded as a hepatic manifestation of metabolic syndrome, is defined by significant lipid deposition in hepatocytes (excessive numbers of fat-laden hepatocytes are observed by light microscopy), unrelated to excessive alcohol consumption [2]. The prevalence of NAFLD is almost 25% worldwide and expected to increase with increasing incidence of obesity and metabolic diseases such as type 2 diabetes mellitus (T2DM) and hyperlipidemia [3].

The mechanism underlying the development of NAFLD has not been fully elucidated. Currently, the multiple parallel hit theory is the most widely accepted mechanism for the progression of NAFLD [4]. This theory suggests that the disease process begins with the development of insulin resistance resulting from excessive energy intake [5]. Insulin resistance in turn leads to hyperinsulinemia, resulting in upregulated hepatic *de novo* lipogenesis and adipose tissue lipolysis. These "primary hits" increase the susceptibility of hepatocytes to multiple pathogenetic factors, such as upregulated expression of pro-inflammatory cytokines and eicosanoids, Fas ligand, and Toll-like receptor ligands; increased reactive oxygen species (ROS) generation; and altered production of adipokines [6]. Whole-body organs such as adipose tissue, the gut, and gut microbiota are also involved in the pathologic process [7, 8]. Collectively, these factors promote hepatocyte apoptosis through mitochondrial dysfunction [9] and an endoplasmic reticulum stress reaction [10]. Such continuous liver tissue injury ultimately leads to fibrosis [11].

The clinical status of NAFLD patients is generally classified broadly into one of

just two categories: non-alcoholic fatty liver (NAFL) or non-alcoholic steatohepatitis (NASH) [12]. NAFL encompasses most of the NAFLD spectrum and is a benign condition. NASH, on the other hand, is defined as the combination of steatosis with lobular inflammation and hepatocyte ballooning; it can progress to liver fibrosis and result in cirrhosis and cancerous malignancies [12]. In contrast to NAFL, NASH is a life-threatening disease. Indeed, a cohort study showed that 35% of NASH patients die during the 7.6-year average follow-up period, whereas no NAFL patients followed in that study died during the same period [13].

Considering the wide disease spectrum of NAFLD, which can result in significant differences in prognosis, it is likely that mechanisms that regulate one or more of these multiple-hit factors exist. Some risk factors for the development of liver fibrosis in NAFLD include age over 50 years, severe obesity, complications associated with T2DM, increased ferritin levels, and patatin-like phospholipase domain–containing 3 gene polymorphisms [14, 15]. However, more-sensitive and -reliable biomarkers are urgently needed to predict outcome in NAFLD patients and enable treatment to begin in the early stage.

MicroRNAs (miRNAs) are a class of endogenous, noncoding, small RNAs that regulate gene expression [16]. Mature miRNAs are introduced into RNA-induced silencing complexes (RISCs) [17]. A RISC bearing a miRNA binds to a partially complementary mRNA sequence and represses the translation of that mRNA. Because miRNAs cause incomplete base-pair matching with mRNAs, a single miRNA can inhibit the translation of hundreds to thousands of target genes [18]. As such, miRNAs play an important role in many cellular processes, including metabolism, inflammation, and fibrosis [19]. Accumulating evidence from both animal model and human patients

102 indicates that miRNAs contribute to the pathogenesis and progression of NAFLD. For
103 example, the expression levels of miR-29c, miR-34a, miR-155, and miR-200b in mouse
104 model liver and miR-122 and miR-34a in human liver are thought to be involved in the
105 development of NASH [20-22]. Our previous study showed that a series of miRNAs
106 mapped in the 14q32.2 maternally imprinted gene cluster region delineated by the
107 *delta-like homolog 1* and *type III iodothyronine deiodinase* genes (Dlk1-Dio3 mat) are
108 related to NAFLD development and progression in a NAFL/NASH mouse model (fatty
109 liver Shionogi [FLS] and mutated leptin gene transferred FLS *ob/ob*) [23]. Seven
110 miRNAs in the Dlk1-Dio3 mat (miR-127, -136, -376c, -379, -409-3p, -411, and -495)
111 are strongly upregulated in both FLS and FLS *ob/ob* liver tissues. In contrast to
112 previously reported NAFLD-related miRNAs, the expression of these seven miRNAs
113 was higher in NAFL model mice than NASH model mice.

114 Recent studies have clearly indicated that miRNAs are secreted into circulating
115 body fluids from various tissues [24]. A considerable amount of secreted miRNAs are
116 protected from enzymatic and physical degradation by binding to proteins or
117 lipoproteins that are then stored in exosomes [25]. These observations suggest that
118 serum miRNAs are potential biomarkers for NAFLD, as they could reflect various
119 pathologic changes in miRNA expression in the liver. Indeed, our preliminary study in
120 human NAFLD patients indicated that serum levels of the respective human homologs
121 of the candidate Dlk1-Dio3 mat miRNAs are related to NAFLD progression [23]. The
122 aim of the present study was to examine the suitability of circulating 14q32.2 mat
123 miRNA as a human NAFLD biomarker.

Materials and Methods

Ethics statement

This study was approved by the committee for ethics in medical experiments on human subjects of the medical faculty of Tottori University (protocol no. 2374) and all collaborative medical institutes: Hiroshima University Hospital, JA Hiroshima General Hospital, Kawasaki University Hospital, and Shimane University Hospital. The study was conducted in accordance with the declaration of Helsinki. Written informed consent was obtained from each patient before blood was collected.

Patient population and collection of blood samples

Ninety patients were enrolled in this study. The patients were divided into three groups, as follows: 10 patients with asymptomatic gallbladder stones as disease controls, 9 NAFL patients, and 71 NASH patients. In another analysis, NAFLD patients were divided into early stage (n = 53) and advanced-stage (n = 26) groups. Early stage was defined as Brunt fibrosis stage 0 or 1, and the advanced stage was defined as Brunt fibrosis stage 2 to 4. Patients with asymptomatic gallbladder stones without liver function abnormalities and fatty liver changes by ultrasound imaging were selected as controls. The clinicopathologic features of each patient group are shown in Table 1. All participants were Japanese and underwent continuous clinical follow-up at the Tottori University Hospital or collaborative institutes. Exclusion criteria included chronic hepatitis B or C virus infection, habitual alcohol consumption over 20 g/day, administration of liver steatotic drugs (such as glucocorticoids, tamoxifen, amiodarone, methotrexate, or valproate), primary biliary cirrhosis, or autoimmune liver disease. All patients except controls underwent liver biopsy to confirm the diagnoses of NAFLD,

148 and the histologic grade and NAFLD stage was determined according to the Brunt
149 system [26]. NAFL and NASH were defined by >5% fat-laden hepatocytes in biopsy
150 samples and at least 6 months of continuous blood test results in which alanine
151 aminotransferase (ALT) and aspartate aminotransferase (AST) remained at <2-fold of
152 the normal range or in excess, respectively. Blood sample collection for serum miRNA
153 isolation and clinical blood tests were performed at the same time and within 1 month of
154 liver biopsy. Blood samples were collected in the fasted state. For each sample, blood
155 serum was isolated by refrigerated centrifugation at 4°C and 1500 × *g* for 10 min and
156 then stored at −80°C until use.
157
158 Table 1. Clinicopathologic features of NAFLD patients and controls.

	Control (CON)	NAFL	NASH	p value			NAFLD early stage	NAFLD advanced stage	p value		
				NAFL and CON	NASH and CON	NAFL and NASH			Early stage and CON	Advanced stage and CON	Early stage and advanced stage
Age	59.3 ± 16.6	44 ± 10	50 ± 16	0.080	0.162	0.533	45.4 ± 14.7	55.2 ± 14.9	0.023*	0.742	0.021*
Gender M/F	4 / 6	7 / 2	47 / 24	0.170	0.161	0.710	38 / 15	16 / 11	0.071	0.460	0.261
BMI	21.9 ± 5.2	26.4 ± 2.2	29.8 ± 6.3	0.270	0.002*	0.259	29.8 ± 5.5	28.4 ± 7.2	0.003*	0.024*	0.628
Brunt Stage	-	0.89 ± 0.33	1.58 ± 0.87	-	-	0.041*	-	-	-	-	-
Brunt Grade	-	1.0 ± 0	1.58 ± 0.67	-	-	0.021*	1.3 ± 0.6	1.9 ± 0.6	-	-	0.001*

T-Bil.	0.8 ± 0.3	0.9 ± 0.3	1.0 ± 0.4	0.927	0.479	0.805	0.9 ± 0.4	1.2 ± 0.3	0.908	0.071	0.014
Alb	4.3 ± 0.4	4.6 ± 0.4	4.4 ± 0.4	0.123	0.175	0.701	4.5 ± 0.4	4.4 ± 0.4	0.378	0.880	0.470
PT (%)	96.7 ± 9.5	107.9 ± 12.5	99.2 ± 13.2	0.415	0.187	0.938	104.0 ± 12.6	92.4 ± 11.7	0.578	0.837	0.001*
AST (U/L)	27.8 ± 18.8	40 ± 19	49 ± 19	0.360	0.005*	0.404	45.5 ± 16.4	53.3 ± 23.1	0.021*	0.002*	0.198
ALT (U/L)	25.5 ± 15.1	72 ± 41	77 ± 40	0.028*	0.001*	0.923	78.3 ± 39.1	74.5 ± 41.6	0.001*	0.002*	0.910
ALP (U/L)	276.5 ± 91.7	259 ± 67	237 ± 84	0.886	0.350	0.752	240.5 ± 73 .4	238.6 ± 100.2	0.434	0.451	0.995
GGT (U/L)	47.3 ± 45.6	65 ± 45	62 ± 45	0.667	0.598	0.980	63.7 ± 46.1	61.4 ± 41.6	0.542	0.676	0.976
LDH (U/L)	158.3 ± 45.6	215 ± 84	209 ± 47	0.244	0.226	0.958	216.3 ± 58.4	199.5 ± 32.6	0.144	0.391	0.362
Ch-E (U/L)	348.3 ± 66.2	351 ± 85	379 ± 82	0.997	0.511	0.634	388.9 ± 79.7	352.8 ± 84.8	0.310	0.988	0.150
BUN (mg/dL)	11.0 ± 2.4	13.8 ± 2.5	13.1 ± 2.4	0.216	0.301	0.766	13.1 ± 2.5	13.3 ± 1.9	0.296	0.276	0.955
Cr (mg/dL)	0.56 ± 0.17	0.79 ± 0.13	0.75 ± 0.15	0.054	0.092	0.638	0.76 ± 0.14	0.74 ± 0.16	0.068	0.109	0.920
UA (mg/dL)	5.7 ± 1.2	6.0 ± 1.1	6.3 ± 1.4	0.973	0.792	0.883	6.3 ± 1.4	6.2 ± 1.4	0.805	0.867	0.985
Ferritin	42.4 ± 33.0	142.1 ± 74.0	210.6 ± 174.5	0.723	0.338	0.477	190.6 ± 158.6	229.1 ± 186.6	0.439	0.287	0.614
FBS (mg/dL)	93.7 ± 9.7	104.0 ± 11.5	117.6 ± 45.6	0.849	0.204	0.621	117.7 ± 47.8	113.9 ± 33.8	0.220	0.394	0.923
HgbA1c %	6.3 ± 1.0	5.9 ± 0.6	6.3 ± 1.5	0.911	0.996	0.658	6.3 ± 1.5	6.2 ± 1.4	0.995	0.999	0.938

IRI (μU/mL)		17.1 ± 19.6	18.3 ± 13.5	-	-	0.820	18.8 ± 15.0	17.2 ± 12.8	-	-	0,897
HOMA-IR		4.6 ± 5.7	5.3 ± 6.7	-	-	0.767 8	5.5 ± 7.4	4.9 ± 4.5	-	-	0.921
T-Chol (mg/dL)	202 ± 44	199 ± 47	204 ± 35	0.978	0.988	0.913	206.6 ± 36.7	197.5 ± 36.3	0.936	0.940	0.936
LDL-C (mg/dL)	134.1 ± 37.4	130. 3 ± 43.9	131.3 ± 33.2	0.974	0.978	0.996	135.1 ± 33.9	122.5 ± 34.9	0.997	0.709	0.288
HDL-C (mg/dL)	67.2 ± 34.3	50.9 ± 6.9	49.4 ± 9.0	0.033 *	0.004 *	0.930	49.1 ± 7.9	50.6 ± 10.6	0.003 *	0.012*	0.853
TG (mg/dL)	104.3 ± 64.8	112. 1 ± 50.9	149.9 ± 69.0	0.967	0.139	0.255	154.5 ± 70.6	128.4 ± 60.7	0.104	0.629	0.255

Early stage NAFLD was defined as Brunt fibrosis stage 0 or 1, and advanced stage was defined as Brunt fibrosis stage 2 to 4. *: $p < 0.05$ in analysis of variance (ANOVA). T-Bil: total bilirubin, Alb: albumin, AST: alanine aminotransferase, ALT: aspartate aminotransferase, ALP: alkaline phosphatase, GGT: gamma-glutamyl transferase, LDH: lactate dehydrogenase, Ch-E: choline esterase, BUN: blood urea nitrogen, Cr: creatinine, UA: uric acid, T-Chol: total cholesterol, LDL-C: low-density-lipoprotein cholesterol, HDL-C: high-density-lipoprotein cholesterol, TG: triacylglycerol, FBS: fasting blood sugar, HgbA1c: hemoglobin A1c, IRI: immunoreactive insulin, HOMA IR: homeostasis model assessment of insulin resistance.

miRNA expression analysis with human serum

miR-379 was selected from the putative Dlk1-Dio3 mat miRNA cluster because it exhibited the greatest difference in expression between NAFL and NASH in our preliminary study [23]. We selected miR-16 as an endogenous control. miR-16 is one of

the most commonly used reference miRNAs in serum miRNA expression analyses [27, 28] . To the best of our knowledge, no previous reports have indicated a relationship between liver disease and miR-16. A miRNeasy serum/plasma kit (Qiagen, Venlo, Nederland) was used to extract miRNAs from each 200-μL serum sample according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was used to examine the expression levels of miR-379 and miR-16, and data were analyzed using the ΔΔCT method of relative quantification. Applied Biosystems TaqMan® MicroRNA Assays (Applied Biosystems, Waltham, MA, USA) and an ABI7900HT system (Applied Biosystems) were used for quantitative RT-PCR amplification of serum miRNAs. The hsa-miR-379 and hsa-miR-16 primer sequences were UGGUAGACUAUGGAACGUA and UAGCAGCACGUAAAUAUUGGCG, respectively.

Predicting miRNA targets

Putative miR-379 targets were predicted using the web-driven software DIANA microT-CDS 5.0 (http://diana.cslab.ece.ntua.gr/). The threshold for the target prediction score in DIANA microT-CDS was set to 0.7. Database for Annotation, Visualization, and Integrated Discovery (DAVID) 6.8 (http://david.abcc.ncifcrf.gov/) was used for gene ontology (GO) annotation, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) was used for pathway enrichment analysis.

Statistical analysis

Statistical analysis was performed using JMP 11.2.1 software (SAS Institute Inc., Cary, NC, USA). Value data are expressed as the mean ± standard deviation. The

197 statistical significance of differences between groups was determined using the
198 Student's *t* test or ANOVA, followed by Dunnett's test for multiple comparisons.
199 Receiver operating characteristic (ROC) curve analysis was performed to assess
200 NAFLD, NAFL, and NASH diagnostic accuracy. Linear regression analysis was used to
201 examine correlations between miRNA levels and clinicopathologic parameters. Fisher's
202 exact test and the chi-square test were selected depending on the sample size and used
203 to determine distribution differences of categorical variable. Differences were
204 considered statistically significant at a p value < 0.05.
205

Results

Serum miR-379 expression was up-regulated in NAFLD patients

One NASH patient was excluded from this study due to low RT-PCR signal, even after 60 PCR cycles. Compared to controls, serum miR-379 expression was significantly up-regulated in NAFLD patients (Fig. 1). In a subgroup analysis of NAFL and NASH patients, serum miR-379 expression was significantly higher in NAFL patients than normal controls (Fig. 1). We also compared early stage NAFLD (Brunt fibrosis stage 0 to 1) and advanced-stage NAFLD (Brunt fibrosis stage 2 to 4) patients with controls. Patients with early stage NAFLD exhibited significantly higher miR-379 expression than controls (Fig. 1). Expression of miR-379 in NASH patients was also higher than in controls, but the difference was not significant ($p = 0.061$) (Fig. 1). There was no significant difference in miR-379 expression between NAFL and NASH patients or between those with early or advanced-stage NAFLD.

Fig. 1. Relative expression of serum miR-379 in NAFLD patients.
Quantitative real-time PCR (qRT-PCR) was used to examine miRNA levels. All qRT-PCR data were normalized to that for serum miR-16, and fold-change was calculated relative to data from normal controls. *$p < 0.05$.

Serum miR-379 is a potential NAFLD diagnostic marker

ROC curve analysis revealed that miR-379 is a potential marker for discriminating NAFLD patients from controls (area under the ROC curve [AUROC]: 0.72) (Fig. 2). AUROC values for discriminating NAFL, NASH, and early and

advanced-stage NAFLD patients from controls were 0.76, 0.72, 0.74, and 0.67, respectively (Fig. 2).

Fig. 2. Receiver operating characteristic (ROC) curve analysis.

Positive correlations were observed between serum miR-379 and alkaline phosphatase (ALP) or cholesterol levels in patients with NAFL or early stage NAFLD

We analyzed the correlations between clinicopathologic parameters and serum miR-379 levels in NAFLD patients. No significant correlation was identified between serum miR-379 expression in NAFLD patients and any of the parameters examined (Supplemental Fig. 1). However, positive correlations were observed between serum miR-379 expression and ALP, total cholesterol, and low-density-lipoprotein cholesterol (LDL-C) levels in patients with early stage NAFLD (Fig. 3). In contrast, there was no correlation between these parameters and serum miR-379 levels in controls or patients with advanced-stage NAFLD (Fig. 3, Supplemental Fig. 3).

Fig. 3. Correlation between miR-379 and ALP, T-Chol, and LDL-C levels.
Left, middle, and right columns present the results for the normal, early stage NAFLD, and advanced-stage NAFLD groups, respectively. *$p < 0.05$.

Statin treatment weakened the correlation between miR-379 and cholesterol level

Nine of 51 patients with early stage NAFLD were undergoing treatment for hypercholesterolemia with hydroxymethyl glutaryl coenzyme A reductase (HMG

CoA-reductase) inhibitors; commonly called statins. Among statin-treated and non-treated patients with early stage NAFLD, serum levels of total cholesterol, LDL-C, and triglycerides were similar (Fig. 4). miR-379 expression levels were higher in the statin-treated group than the non-treated group, but the difference was not significant (5.1 ± 4.4 and 3.2 ± 4.8 log2 folds, respectively. $p = 0.29$). Linear regression analysis showed the non-treated group exhibited a significant positive correlation between total cholesterol and serum miR-379 expression. This trend was also observed in the statin-treated group, but the correlation was not significant ($p = 0.10$) (Fig. 4).

Fig. 4. Statin treatment and serum miR-379 expression, and correlation with cholesterol levels.

*$p < 0.05$.

Software-based predictions of miR-379 target genes

We predicted potential target genes of miR-379 using web-based software. Based on the selection criteria, 1423 human genes were identified as candidates. The candidate genes were classified according to GO annotation in *Homo sapiens* (Fig. 5), and 12 GO terms were significantly enriched (Table 2).

Fig. 5. Simple aggregation of Gene Ontology (GO) terms among putative miR-379 target genes.

The predicted miR-379 target gene dataset were fed into DAVID, version 6.8. Pie chart slices represent the number of genes associated with each GO term.

278 Table 2. GO-term enrichment analysis of predicted miR-379 target genes.

Go Term	Gene Count	%	Fold enrichment	p value
Positive regulation of macromolecule biosynthetic process	176	12.4	1.5	> 0.001*
Positive regulation of RNA metabolic process	156	11.0	1.5	> 0.001*
Positive regulation of gene expression	178	12.5	1.4	0.001*
Positive regulation of nucleobase-containing compound metabolic process	175	12.3	1.4	0.001*
Positive regulation of cellular biosynthetic process	181	12.7	1.4	0.002*
Positive regulation of transcription, DNA-templated	148	10.4	1.5	0.002*
Regulation of cellular macromolecule biosynthetic process	365	25.7	1.3	0.002*
Positive regulation of RNA biosynthetic process	149	10.5	1.5	0.002*
Regulation of macromolecule biosynthetic process	370	26.0	1.2	0.006*
Regulation of gene expression	387	27.2	1.2	0.010*

Cellular protein modification process	342	24.0	1.2	0.034*
Protein modification process	342	24.0	1.2	0.034*

Percentages indicate the number of predicted target genes associated with a GO term category compared to all predicted genes examined in the GO-term analysis. Fold-enrichment shows the abundance ratios of predicted miR-379 target genes and DAVID pre-built human genome backgrounds among GO terms. Only statistically significant results ($p < 0.05$) are displayed.

Next, we explored the KEGG pathway database to determine specific gene functions. Ontology annotation via KEGG pathway mapping showed that biological functions have been identified for 32.8% of the candidate genes (467 of 1423 genes). Function-labeled miR-379 candidate target genes were primarily enriched in clusters associated with nutrition and energy regulation (FOXO and mTOR signaling pathways), cancer (melanoma, prostate cancer, p53 signaling, Hippo signaling, and transcriptional misregulation in cancer), and multi-functional cellular mechanisms or signaling pathways (cGMP-PKG signaling, focal adhesion, Hippo signaling pathway, pluripotency regulation in stem cells, TGF-beta signaling, and ubiquitin-mediated proteolysis) (Table 3).

Table 3. Enriched KEGG pathways among putative miR-379 target genes.

KEGG pathway	Gene count	%	Fold enrichment	p value
FOXO signaling pathway	21	1.5	2.3	> 0.001*

TGF-beta signaling pathway	15	1.1	2.6	0.001*
Ubiquitin mediated proteolysis	20	1.4	2.2	0.002*
Hippo signaling pathway	19	1.3	1.8	0.013*
Prostate cancer	13	0.9	2.2	0.015*
Transcriptional misregulation in cancer	20	1.4	1.9	0.018*
Signaling pathways regulating pluripotency of stem cells	17	1.2	2.3	0.027*
p53 signaling pathway	10	0.7	1.8	0.036*
cGMP-PKG signaling pathway	18	1.3	1.6	0.038*
Focal adhesion	21	1.5	2.1	0.038*
mTOR signaling pathway	9	0.6	1.7	0.040*
Melanoma	10	0.7	2.2	0.048*

297 Percentages indicate the number of predicted miR-379 target genes associated with a
298 KEGG pathway compared to all predicted genes explored in the KEGG pathway
299 analysis. Fold-enrichment shows the abundance ratios of predicted miR-379 target
300 genes and DAVID pre-built human genome backgrounds among GO terms. Only
301 statistically significant results ($p < 0.05$) are displayed.

302

303 Finally, to identify probable miR-379 target genes related to the pathology of
304 NAFLD, we conducted a keyword search of the U.S. National Library of Medicine
305 database PubMed (https://www.ncbi.nlm.nih.gov/pubmed) using the terms “KEGG
306 annotated putative target gene” and “NAFLD” or “NASH”. A total of 27 predicted
307 genes were associated with NAFLD development or progression, including

308 multi-functional cellular mechanisms or signaling pathways (*HDAC2*), fibrosis and
309 inflammation (*CAT*, *CTGF*, *IL10*, *PDGFA*, *PDGFRA*, *SMAD4*, *TGFBR1*, and *THBS1*),
310 cell survival and proliferation (*Bcl2*, *CCNB1*, *HGF*, *PMAIP1*, *PTEN*, and *YAP1*), and
311 energy management, including gluconeogenesis and lipogenesis (*CREB1*, *EIF4E*,
312 *FOXO1*, *INSR*, *IGF1*, *IGF1R*, *ITPR2*, *PRKAA1* and *2*, *RICTOR*, *SOCS1*, and *TCF7L2*)
313 (Table 4) [29-54].
314
315 Table 4. Keyword search of the U.S. National Library of Medicine database PubMed to
316 identify KEGG annotated miR-379 putative target genes associated with NAFLD or
317 NASH.

Gene Code	Protein name	Reference
Bcl2	*B-cell lymphoma 2*	Panasiuk et al. 2006
CAT	*Catalase*	Kumar et al. 2013
CCNB1	*Cyclin B1*	Gentric et al. 2015
CREB1	*cAMP responsive element binding protein 1*	Oh et al. 2013
CTGF	*Connective tissue growth factor*	Colak et al. 2012
EIF4E	*Eukaryotic translation initiation factor 4E*	Wang et al. 2014
FOXO1	*Forkhead box o1*	Pan et al. 2017
HDAC2	*Histone deacetylase 2*	Kolodziejczyk et al. 2019
HGF	*Hepatocyte growth factor*	Kosone et al. 2007

INSR	*Insulin receptor*	Wu et al. 2017
IGF1	*Insulin like growth factor 1*	Adamek et al. 2018
IGF1R	*Insulin like growth factor 1 receptor*	Go et al. 2014
IL10	*Interleukin 10*	Cintra et al. 2008
ITPR2	*Inositol 1, 4, 5-trisphosphate receptor type 2*	Khamphaya et al. 2018
PDGFA	*Platelet derived growth factor subunit A*	Hardy et al. 2017
PDGFRA	*Platelet derived growth factor receptor A*	Abderrahmani et al.
PMAIP1	*Phorbol 12-myristate 13-acetate induced protein 1*	Kung et al. 2016
PRKAA1	*5' AMP-activated protein kinase catalytic subunit alpha 1*	Garcia et al. 2019
PRKAA2	*5' AMP-activated protein kinase catalytic subunit alpha 2*	Garcia et al. 2019
PTEN	*Phosphatase and tensin homolog*	Matsuda et al. 2013
RICTOR	*Rapamycin-insensitive companion of mammalian target of rapamycin*	Sydor et al. 2017
SMAD4	*Small worm phenotype and mothers against decapentaplegic 4*	Qin et al. 2018
SOCS1	*Suppressor of cytokine signaling 1*	Wang et al. 2017
TCF7L2	*Transcription factor 7-like 2*	Musso et al. 2009
TGFBR1	*Transforming growth factor beta receptor 1*	Matsubara et al. 2012
THBS1	*Thrombospondin 1*	Li et al. 2017
YAP1	*yes-associated protein 1*	Chen et al. 2018

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Discussion

The present study revealed significantly higher serum levels of miR-379 in NAFLD patients compared to controls. Our previous study indicated that miR-379 expression in liver tissues of an NAFLD mouse model is strongly upregulated (>4 log2 compared to the normal control group) [23]. miR-379 secretion from liver tissue, probably via exosome particles rich in miR-379, appears to be related, at least in part, to the high circulating level observed in NAFLD patients.

Relatively little is known regarding the mechanism regulating miR-379 expression. miR-379 has been mapped to the miRNA cluster in the Dlk1-Dio3 mat region. Major regulators of Dlk1-Dio3 locus expression include methylated regulatory regions such as the germline-derived intergenic differentially methylated region and somatic MEG3-differentially methylated region [55, 56]. Moreover, CpG islands that are embedded in or near miRNA-coding regions also regulate the expression of Dlk1-Dio3 mat miRNA [57]. Dai et al. reported that miR-379 expression is directly regulated by DNA methylation [58]. In addition, histone acetylation functions synergistically with DNA methylation to regulate the Dlk1-Dio3 locus [57].

With respect to non–DNA methylation regulation, Guia and colleagues reported that the miRNA cluster miR-379/410 is a direct transcriptional target of the glucocorticoid receptor, which promotes insulin resistance and systemic dyslipidemia [59]. Guia et al. also showed that miR-379 is upregulated in liver tissue of obese subjects and that hepatic miR-379 expression in patients with obesity is correlated with both serum cortisol and triacylglycerol (TG) levels [59]. However, in our present study, TG levels in NAFLD patients did not differ significantly from those of controls (Table

1), and serum miR-379 expression was not correlated with TG level ($p = 0.738$, Supplemental Fig. 1). This discrepancy may be related to differences between obese patients and NAFLD patients whose diagnosis was confirmed by liver biopsy. The mechanism of serum miRNA expression may also be related to this discrepancy. For example, sorting and selection occur during incorporation of cytosolic miRNAs into exosomes [60]. Because the level of circulating miRNAs is the sum total of miRNAs secreted from tissues/organs throughout the body, other metabolism-related organs may affect the level of circulating miRNA. Chartoumpekis et al. reported that miR-379 is overexpressed in white adipose tissue in an obese mouse model [61].

ROC curve analyses showed that miR-379 provides fair diagnostic accuracy for NAFLD. The AUROC of serum miR-379 for NAFLD diagnosis was >0.7 and similar to other single serologic markers for non-invasive detection of NAFLD, such as tumor necrosis factor–alpha, interleukin-6, and ferritin [62]. Most non-invasive NAFLD markers exhibit higher values and diagnostic accuracy in patients with liver fibrosis and cirrhosis [63]. In contrast to the majority of NAFLD diagnostic markers, the serum miR-379 level was significantly increased relative to NAFL, but there was no difference between NAFL and NASH. This distinctive feature of serum miR-379 may confer an advantage for detecting NAFLD in the early stage. For instance, serum miR-379 is a candidate factor for use in NAFLD diagnosis algorithms combining multiple biomarkers as a means of increasing sensitivity for early stage diagnosis [64].

Our present study showed that the serum miR-379 level is positively correlated with ALP in early stage NAFLD. Serum ALP is the traditional marker of cholestasis. However, the other cholestasis markers, such as bilirubin and gamma-glutamyl transferase, were not significantly correlated with miR-379 (Supplemental Fig. 2). ALP

368 is a plasma membrane–bound enzyme that catalyzes the hydrolysis of phosphate esters
369 [65]. Though found in most body tissues, ALP is particularly abundant in the liver,
370 bone, kidneys, and intestinal mucosa, with liver and bone serving as the predominate
371 organs supplying ALP to circulating body fluids [65]. Chronic liver diseases, including
372 NAFLD, increase serum ALP levels [66, 67]. Moreover, previous reports indicated that
373 the serum ALP level is an independent marker of NAFLD development and
374 progression. Pantsari et al. showed that a subset of NAFLD patients (elderly females)
375 exhibit isolated elevation in ALP rather than aminotransferases [68]. Kocabay et al.
376 reported that serum levels of ALP, but not gamma-glutamyl transferase, are increased in
377 NAFLD patients with early fibrosis stage (stage 1 to 2) [69]. ALP is richly expressed in
378 the canalicular membrane side of hepatocytes, and previous studies suggested that ALP
379 relates the transport of bile acid, which plays a major role in cholesterol metabolism and
380 excretion [70]. However, details regarding the physiologic functions of ALP are
381 unclear. miR-379 may be related to NAFLD development and progression by directly or
382 indirectly modulating ALP expression.

383 Our present study also showed that the serum miR-379 level is positively
384 correlated with serum cholesterol in early stage NAFLD. The contribution of
385 hypercholesterolemia to the development of NAFLD has not been fully elucidated;
386 however, previous studies showed that hepatic cholesterol synthesis and circulating total
387 cholesterol and LDL are increased in NAFLD [71]. Disruption of hepatic cholesterol
388 homeostasis and free cholesterol (FC) accumulation in liver tissue is related to the
389 pathogenesis of NAFLD [72, 73]. Some studies have shown that hepatic cholesterol
390 synthesis is up-regulated in NAFL and NASH patients due to increased activity of a
391 major regulator of cholesterol synthesis, sterol regulatory element–binding protein 2 and

its downstream effector HMG CoA-reductase, which catalyzes a rate-limiting step in cholesterol synthesis [74-76]. Interestingly, Min et al. also reported that up-regulation of cholesterol synthesis was not observed in control obese subjects [74].

Regarding other cholesterol-related metabolic functions in the liver of NAFLD patients, cholesterol de-esterification is increased, and cholesterol catabolism to bile acid and cholesterol efflux via the bile duct are attenuated [74]. These NAFLD-specific changes in cholesterol metabolism are believed to increase FC levels in liver tissues. FC accumulation in hepatocytes induces mitochondrial dysfunction, which results in increased production of ROS and leads to the unfolded protein response in the endoplasmic reticulum, leading to localized stress and apoptosis [73]. Mari et al. also reported that FC loading (but not that of fatty acids or triglycerides) into hepatocyte mitochondria membranes sensitizes the hepatocyte to pro-inflammatory cytokines (e.g., tumor necrosis factor–alpha and Fas) in mouse models, resulting in steatohepatitis [77]. Moreover, FC accumulation in non-parenchymal cells in liver tissues such as Kupffer cells and stellate cells promotes activation of these cells [78, 79]. The activated Kupffer cells secrete pro-inflammatory cytokines such as interleukin-1β and tumor necrosis factor–alpha, and activated stellate cells differentiate into myofibroblasts, which exhibit a high ability to produce extracellular matrix and fibrogenic cytokines, such as transforming growth factor–β [78, 79]. It has been hypothesized that miR-379 promotes the development and progression of NAFLD as a result of continuous over-nutrition—manifested primarily as obesity—by increasing the lipotoxicity of cholesterol. Cirrhosis and hepatocellular carcinoma are the most common liver-related causes of morbidity associated with NAFLD [80]. However, cardiovascular disease is the most common cause of death in NAFLD patients without cirrhosis [13]. Therefore,

some reviewers have recommended giving priority to the prevention of cardiovascular or renal diseases over liver-specific treatments in patients with non-aggressive NAFLD [81].

miR-379 has also been associated with the risk of cardiovascular disease in early stage NAFLD via up-regulation of the serum cholesterol level, which plays an important role in atherosclerosis development. In the present study, however, no significant correlation between serum miR-379 and cholesterol levels was observed in control subjects and NAFLD patients with advanced fibrosis (Brunt stage 2 to 4). This suggests that such a correlation is pertinent only under limited conditions, such as early stage NAFLD–specific pathophysiologic and nutritional states. The serum miR-379 level in controls was significantly lower than that in patients with early stage NAFLD. Normal levels of miR-379 may be insufficient to affect cholesterol metabolism. With respect to advanced-stage NAFLD, it is known that serum cholesterol levels decline with progression of liver fibrosis, independent of the etiology of chronic liver disease [82]. The effect of miR-379 on cholesterol metabolism may be attenuated by decreased hepatic parenchymal function.

The present study also demonstrated that the use of statins to treat hypercholesterolemia in NAFLD patients weakens the relationship between serum miR-379 and cholesterol levels. Statins target hepatocytes and inhibit HMG-CoA reductase, which catalyzes the rate-limiting step in the cholesterol biosynthesis pathway, known as the mevalonate pathway [83]. HMG-CoA reductase converts HMG-CoA into mevalonic acid, a cholesterol precursor. Stains have a higher binding affinity for HMG-CoA reductase than HMG-CoA and thus block access to the active site by the substrate [83]. Previous studies indicated that statins improve hepatic

steatosis and reduce hepatic inflammation and fibrosis in NAFLD patients [84, 85]. Moreover, long-term observations of NAFLD patients indicated that continuous statin treatment reduces rates of liver-related death and liver transplantation [86]. Statins may attenuate the effect of miR-379 on cholesterol biosynthesis, resulting in reduced cholesterol lipotoxicity in NAFLD.

GO term annotation analyses showed enrichment of cellular biosynthesis and metabolism–related genes among predicted miR-379 targets. Aberrations in biosynthesis and metabolism play important roles in metabolic disorders such as NAFLD. miR-379 appears to affect the development and progression of NAFLD by interfering with these target genes.

KEGG pathway mapping of prospective miR-379 target genes extracted biological functions such as nutrition and energy regulation, the down-regulation of which leads to the development of NAFLD. Searches of PubMed combining keywords with the selected putative target genes identified in the KEGG pathway analysis and NAFLD identified a number of metabolism-, inflammation-, and fibrosis-related genes. Among the selected putative target genes, *IGF1* and *IGF1R* were identified as targets of miR-379 interference in previous studies [87, 88]. IGF-1 is an insulin-like anabolic hormone primarily secreted by hepatocytes, and circulating IGF-1 levels reflect hepatic IGF-1 expression [89]. Previous studies reported that adults with growth hormone deficiency in which hepatic IGF-1 production is impaired exhibit an increased prevalence of NASH; IGF-1 substitution ameliorated NAFLD in a mouse model [90, 91]. In NAFLD patients without growth hormone deficiency, serum IGF-1 levels are also significantly reduced [89, 92].

The mechanism by which IGF-1 and its signaling pathways protect against

NAFLD have been found to involve a variety of biological functions, such as improving insulin sensitivity, decreasing ROS production, and inducing senescence of hepatic stellate cells [93-95]. With respect to lipid metabolism, it has been reported that IGF-1 accelerates lipid oxidation and lipolysis [93]. Moreover, several previous studies revealed that serum IGF-1 is inversely correlated with serum levels of total cholesterol and LDL-C [96]. *IGF1* appears to be one of the most significant miR-379 target genes with regard to promoting the development and progression of NAFLD via the enhancement of cholesterol lipotoxicity. Among other keyword-selected putative target genes, B-cell lymphoma 2 (*BCL2*), catalase (*CAT*), and cAMP responsive element binding protein 1 (*CREB1*) are reportedly down-regulated in the liver in NAFLD [30, 97, 98]. *BCL2* and *CAT* are major anti-apoptosis genes that function by protecting against mitochondrial outer membrane permeabilization and detoxifying ROS, respectively [30, 97]. Down-regulation of *BCL2* and *CAT* expression in liver tissue drives hepatocyte apoptosis, which is an important pathologic event in the development and progression of NAFLD. CREB1 is a transcription factor that regulates energy balance by suppressing hepatic fatty acid generation and accumulation via downregulation of hepatic-specific peroxisome proliferator activated receptor–γ and fatty acid transporter CD36 expression [98]. miR-379 may affect the development and progression of NAFLD by interfering with the expression of these target genes, which is reportedly down-regulated in NAFLD.

A relationship with NAFLD has also been reported for other miR-379 target genes. For example, 5'-AMP–activated protein kinase catalytic subunit alpha 2 (*PRKAA2*) is the catalytic subunit alpha 2 of AMPK, a key sensor of energy status in mammalian cells. In the liver, AMPK phosphorylates and inactivates both

488 acetyl-coenzyme A carboxylase and HMG-CoA reductase [99]. Acetyl-coenzyme A
489 carboxylase regulates the biosynthesis of malonyl-CoA, which is the initial committed
490 intermediate in fatty acid biosynthesis. Malonyl-CoA can inhibit carnitine palmitoyl
491 transferase 1, which controls mitochondrial fatty acid oxidation [100]. Therefore,
492 AMPK downregulation increases fatty acid and cholesterol biosynthesis and inhibits
493 fatty acid oxidation, resulting in hepatic lipid accumulation. Although AMPK appears
494 to be related to NAFLD development, details regarding levels of AMPK in hepatocytes
495 are controversial [101].

496 Previous studies reported the relationship between miR-379 and various
497 diseases. The majority of these studies suggest that miR-379 plays tumor suppressive
498 role in many types of carcinomas, including nasopharyngeal carcinoma, cervical cancer,
499 lung cancer, gastric cancer, hepatocellular carcinoma, bladder cancer, and osteosarcoma
500 [102-107]. With regard to metabolic disorders as described above, de Guia et al.
501 revealed a relationship between miR-379 and lipid homeostasis dysregulation [59].
502 Additionally, patients with a congenital disease known as maternal uniparental disomy
503 for chromosome 14, which causes overexpression of miR-379 of the Dlk1-Dio3 mat
504 miRNA cluster, exhibit characteristic weight gain in early childhood that results in
505 truncal obesity [108].

506 Our study had some limitations associated with sample size and study design.
507 We used software programs to predict target genes of the candidate miRNAs. Although
508 this method is commonly used, it carries a risk of missing some real targets because the
509 software is designed to assess the relative strength of partial sequence complementarity
510 between mRNA and miRNA. Ontology selection was used to select putative targets that
511 might be relevant to cellular functions. However, ontology selection can only identify

proteins for which the function has been identified. Notably, our understanding of the detailed mechanisms that promote the development and progression of NAFLD to NASH is still developing, but new insights are being obtained regularly.

Moreover, we did not confirm whether any NAFLD candidate miRNA actually interfered with any of the predicted target genes in vivo (mouse model liver) or in vitro, such as direct binding experiments. Complex intracellular regulatory networks influence the tissue-specific function of miRNAs [109]. Therefore, further studies are needed to assess whether the predicted targets are actual targets of these miRNAs.

Concerning the correlation between serum ALP and miR-379, we could not definitively conclude that the correlation reflects only liver tissue pathologic changes. Bone is another major ALP-secreting organ, and the serum level of the bone isozyme of ALP is elevated in children, adolescents, and elderly people due to bone tissue turnover [110, 111].

Regarding our study participants, all NAFLD patients and control subjects were adults (age ranging from 20 to 76 years), and there was no significant relationship between serum ALP level and age ($R^2 = 0.0286$; $p = 0.115$). Additionally, no pregnant subjects were included. The number of patients in this study was small, at less than 100. Consequently, the statistical power of the human serum data was relatively limited.

Our findings from NAFLD mouse models could not be confirmed by miRNA expression profiling in human liver tissue. A parallel examination of microarray analyses of human liver samples would have enhanced the confidence of NAFLD candidate miRNAs. However, we could not conduct miRNA expression profiling in human liver tissues, primarily because we could not obtain liver tissue specimens from controls due to ethical considerations. Larger human population-based studies are

needed to confirm and extend our findings.

In conclusion, the serum level of miR-379, a member of Dlk1-Dio3 mat miRNA cluster, exhibits high potential as a biomarker for NAFLD. miR-379 also appears to increase cholesterol lipotoxicity, which promotes the development and progression of NAFLD by interfering with the expression of target genes, including those of the IGF-1 signaling pathway. To confidently identify more associations between highly complex and interactive miRNAs with NAFLD, future longitudinal studies with greater sample sizes will be necessary.

Supporting Information

Supplemental Fig. 1. Linear regression analysis of relationships between serum miR-379 and clinical features of NAFLD patients. Normalized relative to serum miR-16; miR-379 values represent fold-difference relative to the normal control.

Supplemental Fig. 2. Linear regression analysis of the relationships between serum miR-379 and clinical features of early stage NAFLD patients (Brunt fibrosis stage 0 to 1). Normalized relative to serum miR-16; miR-379 values represent fold-difference relative to the normal control.

Supplemental Fig. 3. Linear regression analysis of the relationships between serum miR-379 and clinical features of advanced-stage NAFLD patients (Brunt fibrosis stage 2 to 4). Normalized relative to serum miR-16; miR-379 values represent fold-difference relative to the normal control.

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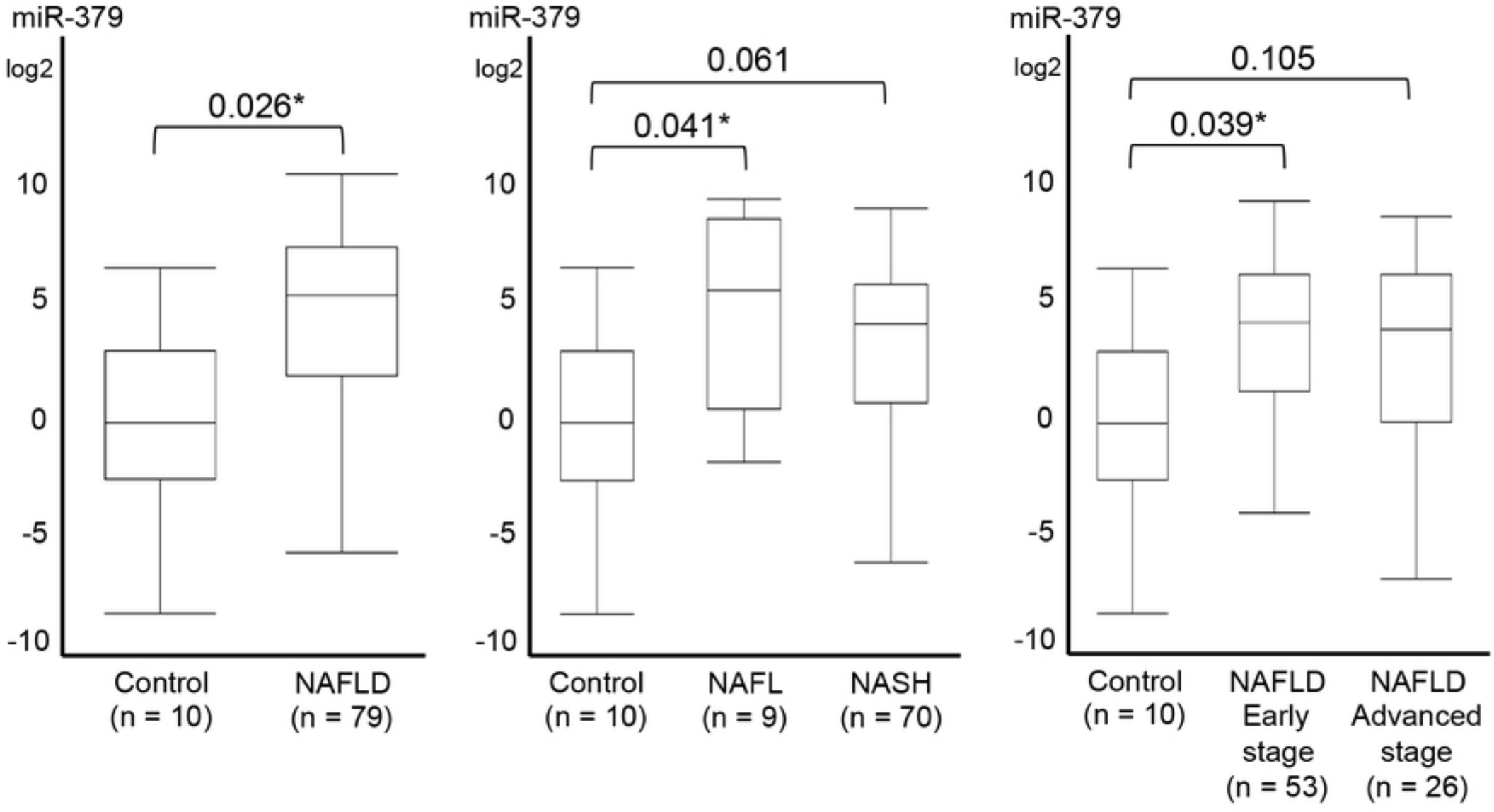
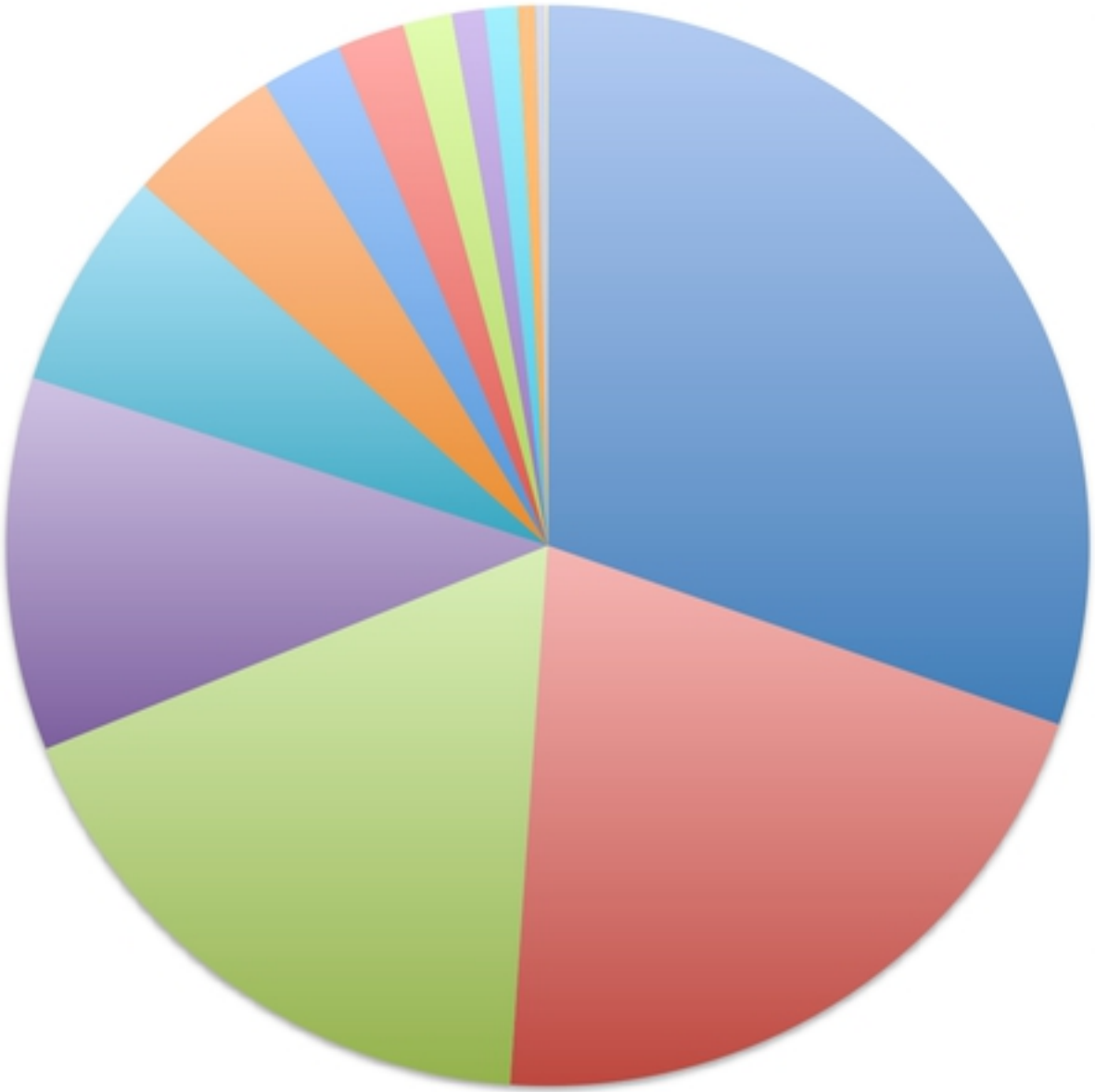


	Control (n = 10)	NAFLD (n = 79)	NAFLD subgroup		NAFLD Brunt fibrosis stage	
			NAFL (n = 9)	NASH (n = 70)	Early (n = 53)	Advanced (n = 26)
miR-379	0 ± 4.55	3.49 ± 4.58	4.87 ± 4.50	3.32 ± 4.60	3.65 ± 4.73	3.17 ± 4.35
p-value versus control		0.026*	0.041*	0.061	0.039*	0.105

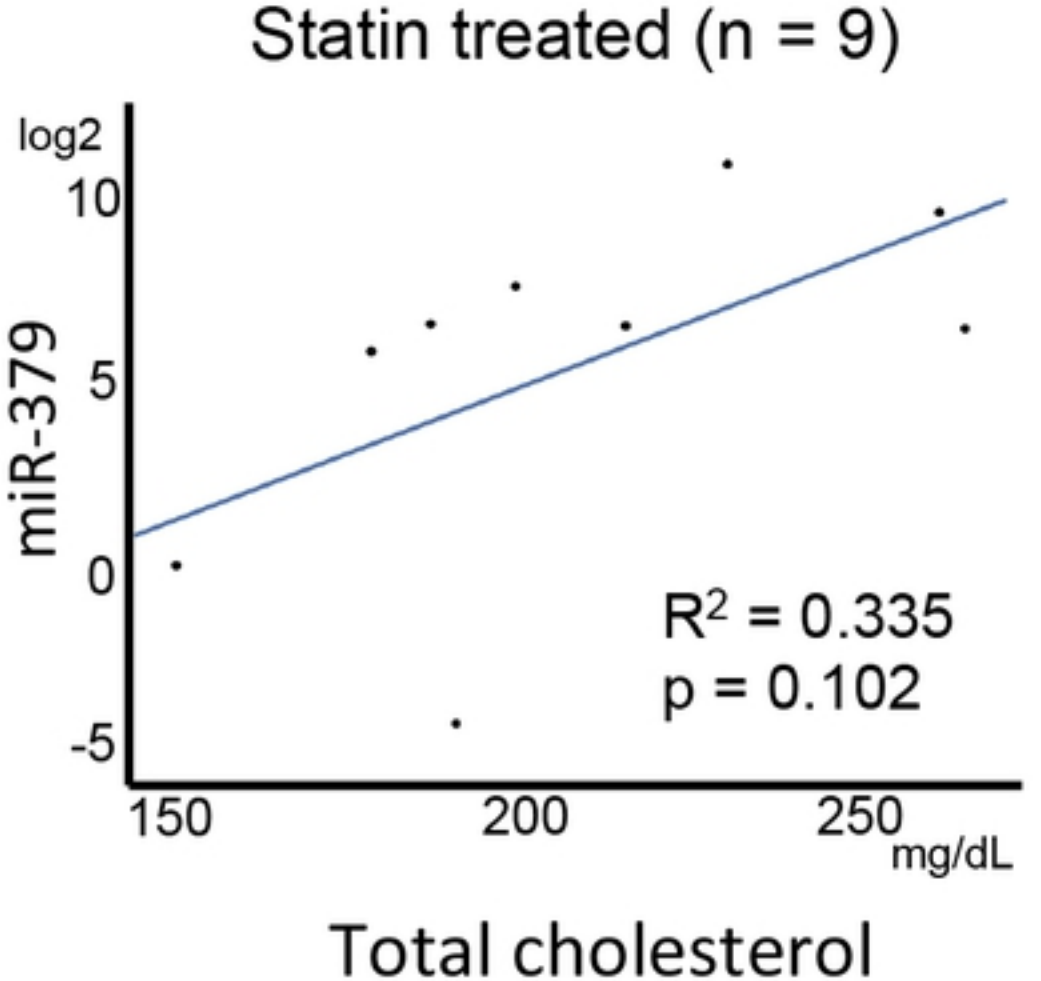
figure

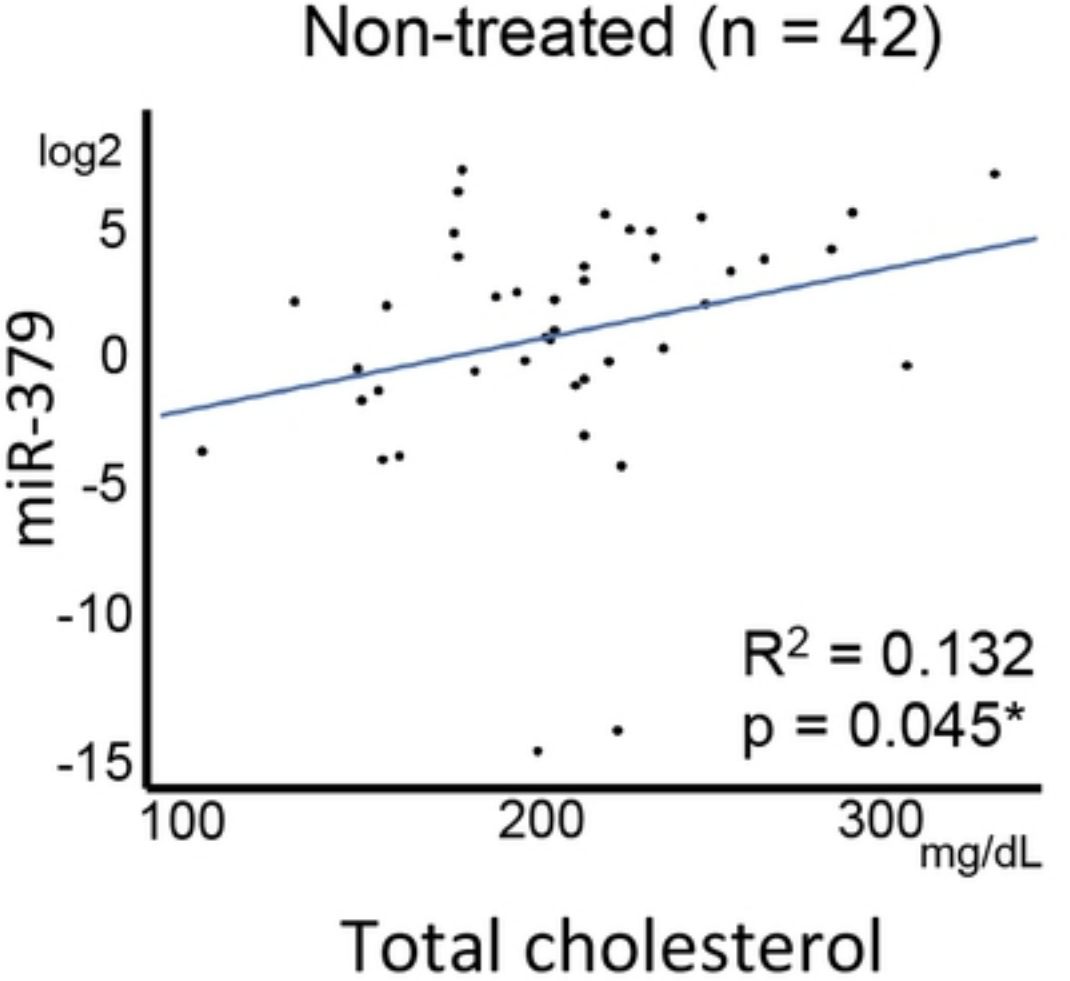


GO term	GO ID	Gene Count	%
Cellular process	0009987	438	31.5
Metabolic process	0008152	299	21.5
Biological regulation	0065007	256	18.4
Localization	0051179	161	11.6
Multicellular organismal process	0032501	95	6.8
Response to stimulus	0050896	67	4.8
Developmental process	0032502	35	2.5
Biological adhesion	0022610	29	2.1
Immune system process	0002376	21	1.5
Cellular component organization or biogenesis	0071840	14	1
Reproduction	0000003	14	1
Cell proliferation	0008283	8	0.6
Rhythmic process	0048511	3	0.2
Biological phase	0044848	1	0.1
Pigmentation	0043473	1	0.1

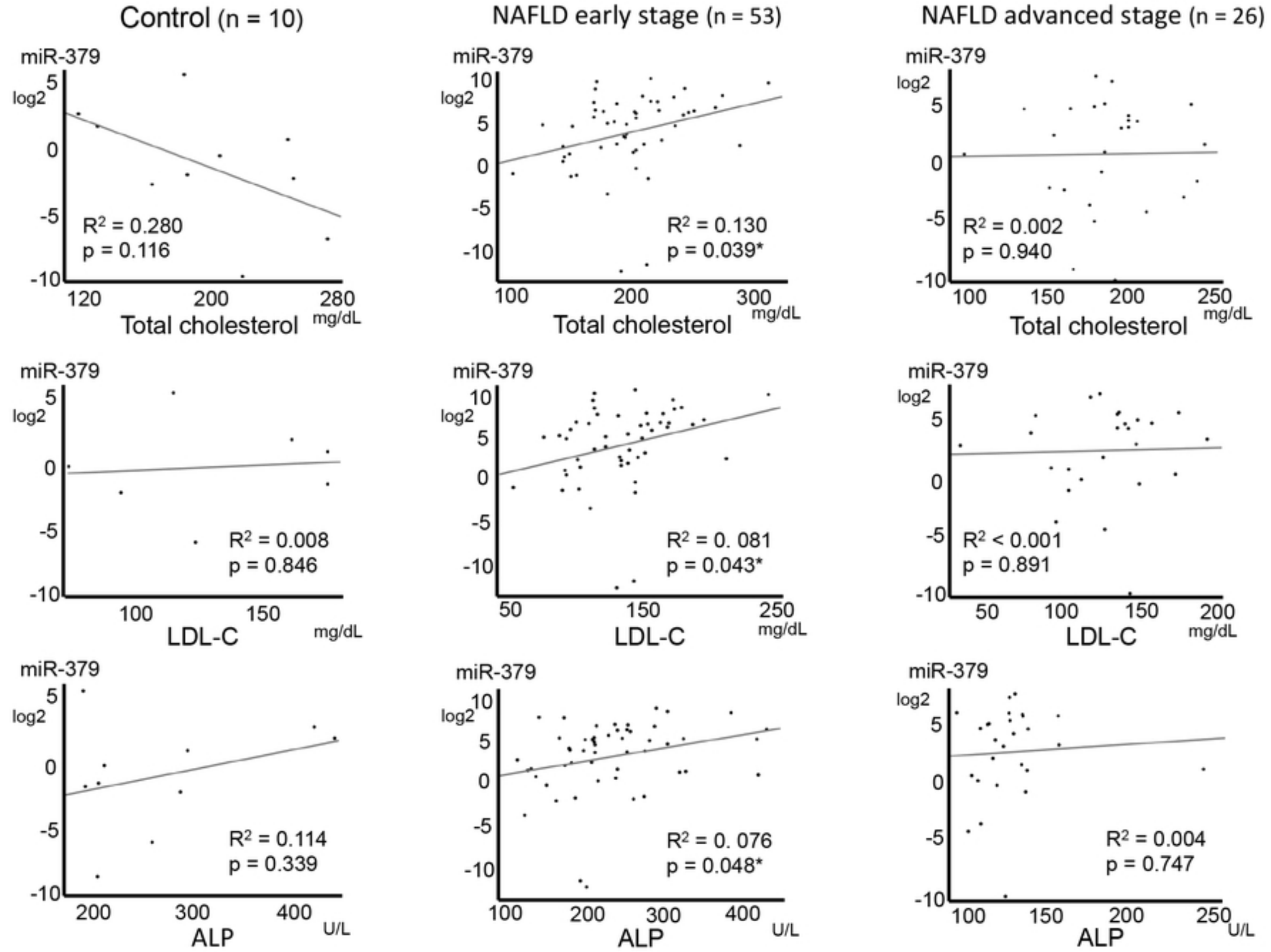
figure

	NAFLD early stage		
	Statin treated (n = 9)	Non-treated (n = 42)	p-value
T-Chol (mg/dL)	205.4 ± 30.9	209.9 ± 38.1	0.916
LDL-C (mg/dL)	134.4 ± 32.1	135.3 ± 34.6	0.945
HDL-C (mg/dL)	50.2 ± 7.3	48.8 ± 8.1	0.631
TG (mg/dL)	152.1 ± 57.0	154.9 ± 73.6	0.914
miR-379 (log2 fold)	5.1 ± 4.4	3.2 ± 4.8	0.293

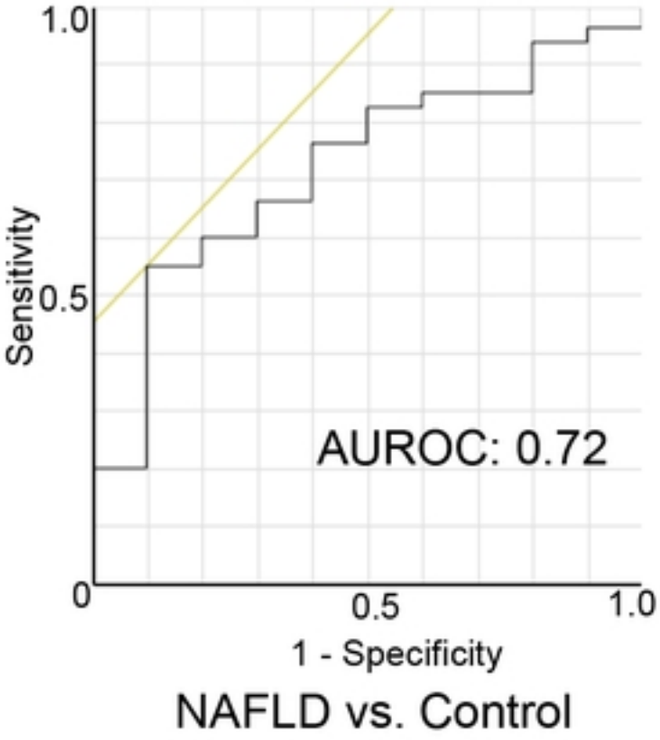




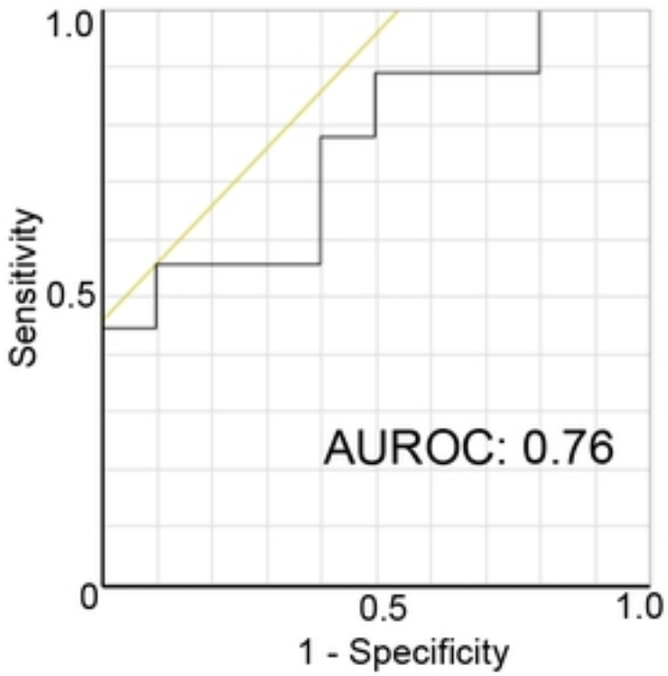
figure


Control (n = 10)
NAFLD early stage (n = 53)
NAFLD advanced stage (n = 26)
miR-379
log2
Total cholesterol
mg/dL
R² = 0.280
p = 0.116
R² = 0.130
p = 0.039*
R² = 0.002
p = 0.940
LDL-C
R² = 0.008
p = 0.846
R² = 0. 081
p = 0.043*
R² < 0.001
p = 0.891
ALP
U/L
R² = 0.114
p = 0.339
R² = 0. 076
p = 0.048*
R² = 0.004
p = 0.747

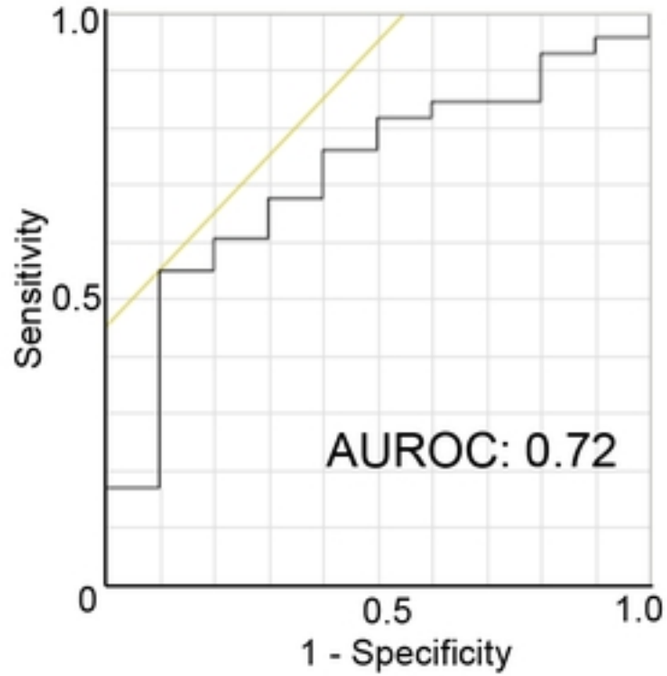
figure



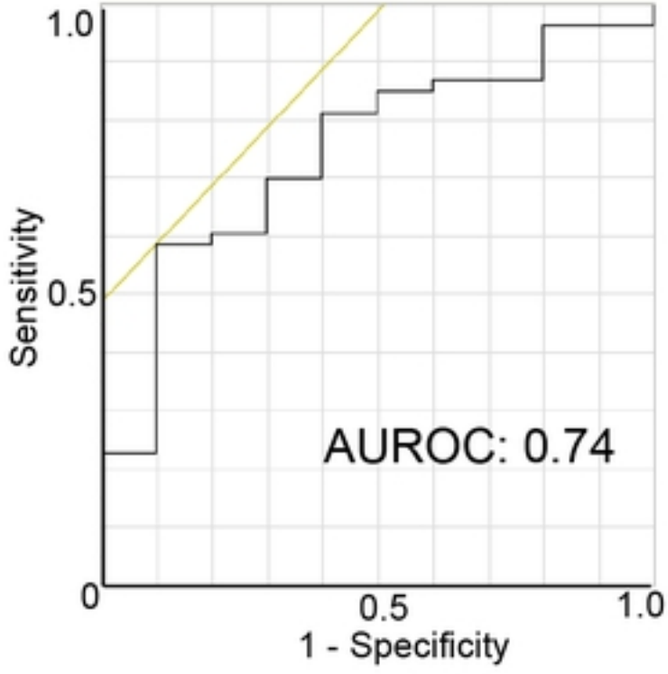
NAFLD vs. Control



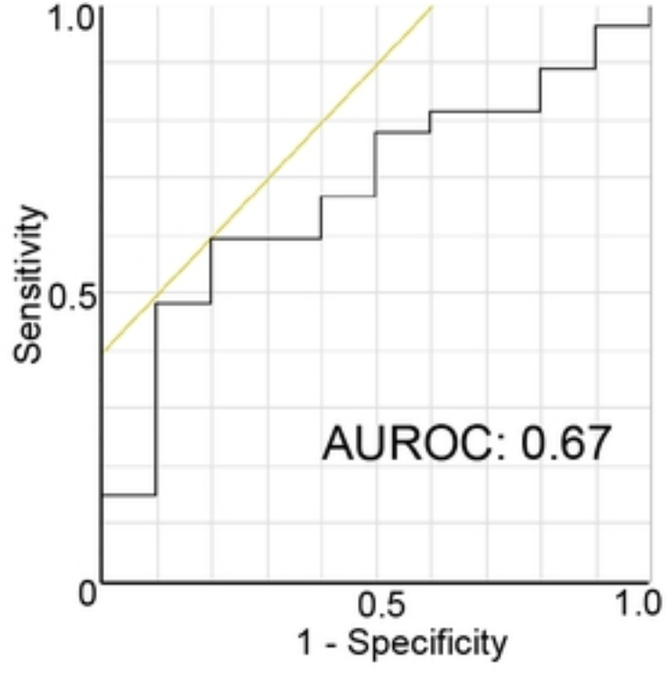
NAFL vs. Control



NASH vs. Control



Early NAFLD vs. Control



Advanced NAFLD vs. Control

figure