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## Observational Study

**Circulating microRNA expression and nonalcoholic fatty liver disease in adolescents with severe obesity**

Yi-Jie Li, Brittney O Baumert, Nikos Stratakis, Jesse A Goodrich, Hao-Tian Wu, Jing-Xuan He, Yin-Qi Zhao, Max T Aung, Hong-Xu Wang, Sandrah P Eckel, Douglas I Walker, Damaskini Valvi, Michele A La Merrill, Justin R Ryder, Thomas H Inge, Todd Jenkins, Stephanie Sisley, Rohit Kohli, Stavra A Xanthakos, Andrea A Baccarelli, Rob McConnell, David V Conti, Lida Chatzi

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## Abstract

### BACKGROUND

Nonalcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases in children and adolescents. NAFLD ranges in severity from isolated hepatic steatosis to nonalcoholic steatohepatitis (NASH), wherein hepatocellular inflammation and/or fibrosis coexist with steatosis. Circulating microRNA (miRNA) levels have been suggested to be altered in NAFLD, but the extent to which miRNA are related to NAFLD features remains unknown. This analysis tested the hypothesis that plasma miRNAs are significantly associated with histological features of NAFLD in adolescents.

### AIM

To investigate the relationship between plasma miRNA expression and NAFLD features among adolescents with NAFLD.

### METHODS

This study included 81 adolescents diagnosed with NAFLD and 54 adolescents without NAFLD from the Teen-Longitudinal Assessment of Bariatric Surgery study. Intra-operative core liver biopsies were collected from participants and used to characterize histological features of NAFLD. Plasma samples were collected during surgery for miRNA profiling. A total of 843 plasma miRNAs were profiled using the HTG EdgeSeq platform. We examined associations of plasma miRNAs and NAFLD features using logistic regression after adjusting for age, sex, race, and other key covariates. Ingenuity Pathways Analysis was used to identify biological functions of miRNAs that were associated with multiple histological features of NAFLD.

### RESULTS

We identified 16 upregulated plasma miRNAs, including miR-193a-5p and miR-193b-5p, and 22 downregulated plasma miRNAs, including miR-1282 and miR-6734-5p, in adolescents with NAFLD. Moreover, 52, 16, 15, and 9 plasma miRNAs were associated with NASH, fibrosis, ballooning degeneration, and lobular inflammation, respectively. Collectively, 16 miRNAs were associated with two or more histological features of NAFLD. Among those miRNAs, miR-411-5p was downregulated in NASH, ballooning, and fibrosis, while miR-122-5p, miR-1343-5p, miR-193a-5p, miR-193b-5p, and miR-7845-5p were consistently and positively associated with all histological features of NAFLD. Pathway analysis revealed that most common pathways of miRNAs associated with multiple NAFLD features have been associated with tumor progression, while we also identified linkages between miR-122-5p and hepatitis C virus and between miR-199b-5p and chronic hepatitis B.

### CONCLUSION

Plasma miRNAs were associated with NAFLD features in adolescent with severe obesity. Larger studies with more heterogeneous NAFLD phenotypes are needed to evaluate miRNAs as potential biomarkers of NAFLD.

**Key Words:** MicroRNA; Nonalcoholic fatty liver disease; Non-alcoholic steatohepatitis; Liver fibrosis; Lobular inflammation; Ballooning degeneration

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**Core Tip:** Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the world, and its prevalence in adolescents is increasing. Studies suggest plasma microRNAs (miRNAs) are dysregulated in NAFLD, but relevant observational studies are scarce. In this study, we analyzed the expression of plasma miRNA in adolescents diagnosed with NAFLD by liver biopsy. We identified associations between histological features of NAFLD and plasma miRNA expression. Further, we found consistent expression of miRNA across different features of NAFLD. Although these results need further testing and validation, our findings suggest these miRNAs could be diagnostic and prognostic biomarkers of NAFLD.

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## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is defined by excessive fat accumulation in the liver and the presence of steatosis without heavy alcohol use[1,2]. NAFLD is comprised of two conditions: Nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). Although both NAFL and NASH include accumulation of fat in hepatocytes, the histopathological abnormality in NASH involves further hepatocellular ballooning, fibrosis, and lobular inflammation[3-6]. In the United States, the overall estimated prevalence of NAFL and NASH are approximately 30% and 5%, respectively[7]. The prevalence of NAFLD in adolescents is 18.5%[8] and has more than doubled over the past 20 years to affect approximately one-half of adolescents with obesity[9,10]. Studies suggest that histopathological features of pediatric NAFLD are different from adult NAFLD[11,12], and children with NAFLD may experience increased risk of severe liver disease and higher liver-related mortality in adulthood[13].

Thus, early diagnosis and prevention of NAFLD among adolescents are crucial. Liver biopsy is the gold standard to diagnose NAFLD[14] yet is invasive and costly[15]. Alternately, noninvasive assessments for NAFLD such as blood tests of aspartate aminotransferase and alanine aminotransferase are commonly used[15,16], but are less predictive of more advanced NAFLD features such as NASH and fibrosis[17,18]. Nonetheless, more robust, noninvasive diagnostic biomarkers of the full spectrum of NAFLD disease severity, are needed.

MicroRNAs (miRNAs) are non-coding RNA that regulate gene expression[19]. In addition to intracellular activities, miRNA can be encapsulated in circulating extracellular vesicles that can convey biological information to recipient cells [20]. Hence, miRNAs play crucial roles in various aspects of metabolism and are frequently dysregulated in the context of diseases[21]. Evidence suggests that dysregulation of miRNA is associated with NAFLD pathogenesis[22-26], *via* multiple pathways, including lipid metabolism, insulin signaling, hepatocyte apoptosis, hepatic inflammation, and liver fibrosis [22,23]. To date, NAFLD-miRNA association studies in humans are scarce and have presented inconsistent results[25-39], and only two studies included adolescents[38,39]. Moreover, only one study measured associations between lobular inflammation and ballooning degeneration with miRNA expression[32], and it focused solely on the expression of miR-34a, miR-122, miR-191, miR-192, and miR-200a. Therefore, it is critical to further investigate the relationship between NAFLD and miRNA expression in adolescents.

The objectives of this study were to: (1) Examine the associations between circulating miRNA levels and histological characteristics of NAFLD in adolescents with obesity; and (2) investigate the pathways of identified NAFLD-related miRNA.

## MATERIALS AND METHODS

### Study population and design

This study was based on data from the Teen-Longitudinal Assessment of Bariatric Surgery (Teen-LABS study, ClinicalTrials.gov NCT00465829), a prospective, multicenter, observational study of adolescents ( $\leq 19$  years of age) with severe obesity who underwent bariatric surgery in 2007-2012 and enrolled at participating clinical centers in the United States: Cincinnati Children's Hospital Medical Center (Cincinnati, Ohio), Nationwide Children's Hospital (Columbus, Ohio), University of Pittsburgh Medical Center (Pittsburgh, Pennsylvania), Texas Children's Hospital (Houston, Texas), and Children's Hospital of Alabama (Birmingham, Alabama)[10,40-42]. The protocol, assent/consent forms, and monitoring plans for data and safety were approved by the institutional review boards of each institution, the independent data and safety monitoring board prior to study initiation, and the University of Southern California review board[10,40-42]. Detailed cohort information is described in previous studies[10,40-42].

### Outcome measurement

Liver biopsies were obtained by a laparoscopically controlled, transabdominal core needle biopsy technique after induction of anesthesia and before performing the bariatric procedure[10,40-42]. Biopsies were evaluated by an experienced hepatopathologist using the NASH Clinical Research Network scoring system[43]. NAFLD features were categorized as definite NASH, borderline NASH, NAFLD not NASH (NAFL), and no NAFLD[10,44]. Other histological features of NAFLD were also categorized, including ballooning, lobular inflammation, and fibrosis[10].

### MiRNA profile

Plasma samples were collected at baseline, typically within 30 days of bariatric surgery and stored at  $-70^{\circ}\text{C}$ . Analyses were performed at HTG Molecular Diagnostics, Inc. (Tucson, AZ) for HTG EdgeSeq miRNA sequencing. HTG EdgeSeq

uses EdgeSeq miRNA Whole Transcriptome Assay and Illumina HiSeq 4000 to quantify 2083 mature miRNA. For quality control, triplicate internal human brain tissue controls were sequenced. HTG EdgeSeq Parser software (version 5.0.535.3181) was used for alignment to a priori defined target sequences. The following quality control measures were implemented: (1) Percentage of bases with a quality score  $30 \geq 87\%$ ; (2) percentage of clusters passing filter  $\geq 75\%$ ; (3) cluster density of 180-290 k/mm<sup>2</sup>; and (4) all samples passing HTG-defined criteria, including  $> 500000$  reads,  $< 14\%$  reads aligned to positive control probes, and  $> 0.08$  relative standard deviation of reads allocated to each probe with each sample. To correct technical batch effects, we used the ComBat\_seq function of the *sva* package in R[45]. Sequencing reads were normalized within and across plates using relative log expression[46]. The reliability of each probe was determined by calculating the coefficient of variation for each miRNA across human brain tissue control samples. Analysis included a total of 843 miRNAs with coefficient of variation  $\leq 0.25$  in replicate control samples. All final counts were converted to counts per million and log<sub>2</sub>-transformed prior to analysis.

### Confounders and covariate data

Standardized methods for Teen-LABS data collection have been described previously[10,40-42]. We included participant characteristics as important confounders, including: Age[47-49], body mass index (BMI)[49-51], sex[49,52,53], weight loss prior to surgery[54,55], and covariates, such as race[56,57], parents' income[58,59] and clinical site of surgery. Data were collected within 30 days of bariatric surgery at in-person visits with trained study personnel. Detailed descriptions of methods, comorbidities, data definitions, medical record data, and laboratory testing can be found in previous publications[10,40-42].

### Statistical analysis

Due to the low frequency of borderline NASH ( $n = 22$ , 16.3%) and definite NASH ( $n = 8$ , 5.9%) in the study population, these two categories were combined and referred to as general NASH ( $n = 30$ , 22%). Similarly, we grouped the two ballooning degeneration conditions, which were prominent ( $n = 5$ , 3.7%) and less characteristics ( $n = 16$ , 11.9%), to create a general ballooning group ( $n = 21$ , 15.6%). These groupings ensured an adequate sample size for meaningful analysis and interpretation of results. To investigate associations between histological features of NAFLD and miRNA, we used multivariate logistic regression to investigate miRNA expression in participants with NAFLD (NAFL and NASH). Additional comparisons between each histological grouping, including NASH (NASH *vs.* NAFL), fibrosis, lobular inflammation, and ballooning, were performed using independent logistic regression models for each comparison. Coefficient estimates of miRNA expression change (log odds ratio), standard errors, and *P* value for each miRNA relationship were calculated. To account for multiple comparisons, we applied the false discovery rate (FDR) approach with a threshold of 0.05 to adjust *P* values from each regression analysis. All models were adjusted by covariates. All statistical analysis was conducted in RStudio version 1.0.143 (RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, United States, <http://www.rstudio.com/>).

### Pathway analysis

We investigated pathways of NAFLD-related miRNA in both miRbase and the Kyoto Encyclopedia for Genes and Genomes from Ingenuity Pathway Analysis (IPA) (Qiagen Inc., <https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis>)[60-62]. Given that an individual miRNA can participate in numerous pathways, we used the miRNA Target Filter tool from IPA and selected experimentally observed diseases and functions of NAFLD-related miRNA in humans.

## RESULTS

### Characteristics of the study population

The analysis consisted of 135 study participants with complete data. The mean age was 16.9 years (SD = 1.5), mean BMI was 53.8 kg/m<sup>2</sup> (SD = 9.8), and 73.3% were female. Because more than half of study participants were recruited from a single clinical site, we re-categorized study site as a binary variable for subsequent regression models. Study population characteristics are summarized in Table 1.

### Prevalence of histological features of NAFLD

By histological analysis, 40% of participants did not have NAFLD, while 37.8% were diagnosed with NAFL, 16.3% with borderline NASH, and 5.9% with definite NASH at the time of surgery. Notably, a high proportion of participants from the Teen-LABS cohort exhibited progressive histopathological features associated with NAFLD-19.3% were diagnosed with fibrosis, and 71.9% were diagnosed with lobular inflammation. Furthermore, 3.7% of participants were diagnosed with prominent ballooning degeneration, while 11.9% exhibited ballooning with fewer characteristics. Distribution of NAFLD features is summarized in Table 1.

### Associations of plasma miRNA expression with histological features of NAFLD

The distribution of NAFLD-miRNA associations is shown in Supplementary Table 1, Figure 1A. We found 38 associations between NAFLD and plasma miRNA expression levels. A subset of 16 miRNA displayed upregulation, while a subset of 22 miRNAs demonstrated downregulation. There was dysregulation of 17 downregulated miRNAs and 35 upregulated miRNAs in patients with NASH relative to those with NAFL (Supplementary Table 2, Figure 1B). However,

**Table 1** Baseline characteristics of teen-longitudinal assessment of bariatric surgery participants, *n* = 135

Characteristics	Mean (SD)/ <i>n</i> (%)
Age (yr)	16.86 (1.53)
BMI (kg/m <sup>2</sup> )	53.80 (9.81)
Weight loss prior to surgery (kg)	0.69 (8.36)
Sex	
Female	99 (73.33)
Male	36 (26.67)
Race (binary)	
White or Caucasian	93 (68.89)
Others	42 (31.11)
Parents' income	
< \$25000	53 (39.26)
\$25000-\$74999	57 (42.22)
≥ \$75000	25 (18.52)
NAFLD	
NAFL	51 (37.78)
Borderline NASH	22 (16.30)
Definite NASH	8 (5.93)
No NAFLD	54 (40.00)
Fibrosis	
Presence	26 (19.26)
None	109 (80.74)
Ballooning degeneration	
Many, prominent	5 (3.70)
Less characteristics	16 (11.86)
None	114 (84.44)
Lobular inflammation	
Presence	97 (71.85)
None	38 (28.15)

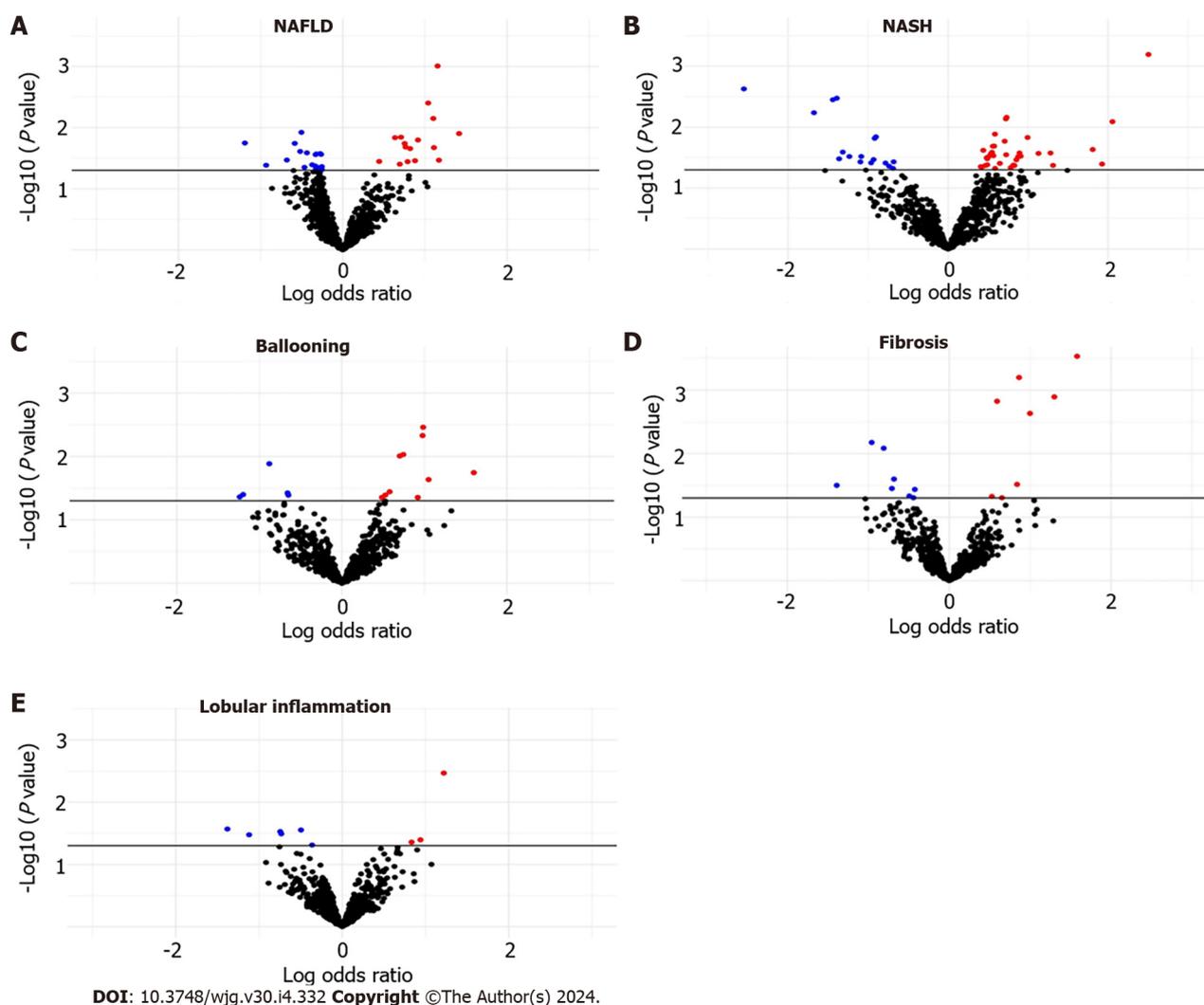
BMI: Body mass index; NAFL: Nonalcoholic fatty liver; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

these findings did not retain significance after applying multiple comparison adjustments (FDR > 0.05).

Within the group of participants with fibrosis (*n* = 26), we observed downregulation of 8 miRNAs and upregulation of 8 miRNAs compared to participants without fibrosis (*n* = 109). Additionally, in participants with ballooning (*n* = 21), we identified 15 altered miRNAs, including downregulation of miR-1224-5p, miR-369-5p, miR-411-5p, and miR-500b-5p. Among participants diagnosed with lobular inflammation (*n* = 97), we identified downregulation of 6 miRNAs and upregulation of miR-1244, miR-125b-2-3p, and miR-365b-5p compared to individuals without lobular inflammation. Associations among ballooning, fibrosis, and lobular inflammation with plasma miRNA levels are depicted in [Supplementary Tables 3-5](#) and [Figure 1C-E](#). However, no associations met statistical significance after multiple comparison adjustment (FDR > 0.05).

### Integration of miRNA profiles associated with multiple histological features of NAFLD

A total of 16 miRNAs exhibited differences in expression across two or more histological features of NAFLD ([Figure 2](#)). MiR-193a-5p was consistently upregulated in NASH, ballooning and fibrosis; miR-193b-5p was consistently upregulated in NAFLD, NASH, and fibrosis; expression of miR-411-5p was downregulated in NASH, ballooning, and fibrosis. Additionally, we observed inconsistent expression patterns of miR-1301-5p and miR-1296-5p between NAFLD and NASH-miR-1301-5p and miR-1296-5p were upregulated in NAFLD yet downregulated in NASH. Additionally, we



**Figure 1** Volcano plots of associations between histological features of nonalcoholic fatty liver disease and microRNA expression. A: MicroRNAs (miRNAs) associated with nonalcoholic fatty liver disease (NAFLD); B: MiRNAs associated with nonalcoholic steatohepatitis (NASH) relative to nonalcoholic fatty liver; C: MiRNAs associated with ballooning; D: MiRNAs associated with fibrosis; E: MiRNAs associated with lobular inflammation. Solid horizontal line represents  $P = 0.05$ , and any dots above the line indicate miRNAs with significant associations. Negative associations are in blue; positive associations are in red; black dots below the solid line represent insignificant miRNAs; higher absolute x-value of a dot indicates greater magnitude of change in miRNA expression in patients with histological progression of NAFLD, either increased ( $x > 0$ ) or decreased ( $x < 0$ ); higher y-value of a dot indicates smaller  $P$  value of associations. NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

observed downregulation of miR-7150 in NAFLD, and this miRNA was conversely upregulated in individuals with ballooning.

The 16 miRNAs associated with two or more histological features of NAFLD were subsequently subjected to scaling and grouped into two distinct clusters using the k-means clustering algorithm and the elbow method[63-66]. Cluster 1 comprised 6 miRNAs, the majority of which were upregulated in individuals with NAFLD. However, these miRNAs were mostly downregulated in patients with NASH, fibrosis, lobular inflammation, and ballooning. Cluster 2 encompassed 10 miRNAs, most of which were upregulated in NASH, fibrosis, and ballooning. In addition to overall inconsistency between the clusters, we noted consistent upregulation of miR-122-5p, miR-1343-5p, miR-193a-5p, miR-193b-5p, and miR-7845-5p across histological features of NAFLD. Figure 2 shows a graphical representation of associations between multiple histological features of NAFLD and miRNA expression.

#### Pathway analysis of miRNA associated with multiple histological features of NAFLD

We conducted pathway analysis on the 16 miRNAs associated with two or more histological features of NAFLD. Analysis revealed 16 experimentally confirmed pathways predominantly involving 6 overlapping NAFLD-related miRNAs in humans (Table 2). Specifically, miR-122-5p, miR-193b-5p, miR-199b-5p, and miR-323-3p were associated with apoptosis of tumor cell lines, while miR-122-5p, miR-193a-5p, and miR-199b-5p were associated with cell migration. Notably, miR-122-5p and miR-199b-5p were associated with multiple pathways in humans. For example, miR-122-5p was associated with production of hepatitis C virus, RNA decay, metastatic hepatocellular carcinoma, replication of viral replicon, and invasion of hepatoma cell lines. Similarly, miR-199b-5p was associated with congenital adrenal hyperplasia, chronic hepatitis B, early-stage invasive cervical squamous cell carcinoma, and proliferation of myeloma cell lines.

**Table 2** Pathway analysis for miRNA associated with multiple histological features of non-alcoholic fatty liver disease in teen-longitudinal assessment of bariatric surgery participants

Disease and functions	miRNA
Apoptosis of tumor cell lines	miR-122-5p, miR-193b-5p, miR-199b-5p, miR-323-3p
Migration of cells	miR-122-5p, miR-193a-5p, miR-199b-5p
Apoptosis of myeloma cell lines	miR-122-5p, miR-193a-5p
Dedifferentiated liposarcoma	miR-193a-5p, miR-199b-5p
Production of hepatitis C virus	miR-122-5p
Decay of RNA	miR-122-5p
Metastatic hepatocellular carcinoma	miR-122-5p
Replication of viral replicon	miR-122-5p
Invasion of hepatoma cell lines	miR-122-5p
Chemosensitivity of squamous cell carcinoma cell lines	miR-193a-5p
Epithelial-mesenchymal transition of adenocarcinoma cell lines	miR-193a-5p
Migration of adenocarcinoma cell lines	miR-193a-5p
Congenital adrenal hyperplasia	miR-199b-5p
Chronic hepatitis B	miR-199b-5p
Early-stage invasive cervical squamous cell carcinoma	miR-199b-5p
Proliferation of myeloma cell lines	miR-199b-5p

A total of 16 miRNA were included as input for independent practice association. To ensure reliability and relevance of results, we specifically extracted pathways that were experimentally confirmed in human studies, considering only those with  $P < 0.05$ .

## DISCUSSION

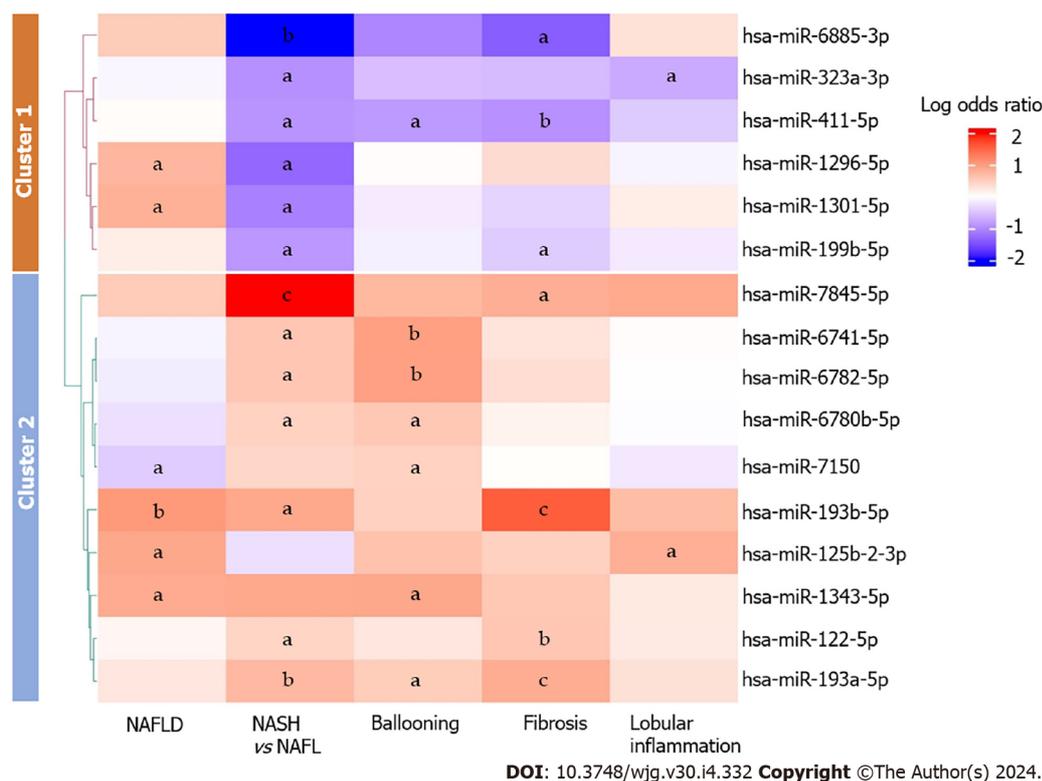
Our study is the first to show associations between histological features of NAFLD and expression of plasma miRNA in adolescents with severe obesity. The IPA results revealed that miRNAs associated with multiple NAFLD features were linked to cancer, hepatitis B and hepatitis C. Our findings have several important implications. First, our findings were consistent with previous epidemiological studies[26]. Additionally, we identified novel NAFLD-miRNA associations. Moreover, our findings revealed consistent patterns of miRNA expression across various histological features of NAFLD, diagnosed using gold standard methods. The consistency of miRNA expression trends across different NAFLD features strengthen their potential utility as valuable diagnostic and prognostic markers for NAFLD.

### Associations between histological features of NAFLD and miRNA expression

Among the 38 NAFLD-related miRNAs identified in our study, several associations are comparable to published epidemiological studies. Our findings demonstrating positive associations between NAFLD and expression of miR-193a-5p, and miR-7150 align with current epidemiological studies[26]. However, it is noteworthy that Soronen *et al*[67] reported increased expression of hepatic miR-584-5p in those with NAFLD, while we found a negative association between NAFLD and plasma miR-584-5p levels. However, this differential expression may be attributed to variation in miRNA measurement sites, as contrasting expression patterns between hepatic miR-122[30] and serum miR-122[36] have been reported in those with NASH.

Additionally, NASH-related miRNA findings from our study align with current research findings. For example, we identified increased expression of miR-2861, miR-3940-5p, miR-6727-5p, miR-6771-5p, miR-6780b-5p, miR-6845-5p, and miR-7114-3p in participants with NASH, consistent with reported positive associations between these miRNAs in the setting of NAFLD and/or NASH[26]. However, it is important to note that this previous study also demonstrated downregulation of miR-6741-5p, miR-6782-5p, and miR-7108-5p in individuals with NAFLD and/or NASH[26], which contrasts with our observation of positive associations between these miRNA and NASH. We also observed a negative association between NASH and miR-182-5p, while mouse studies suggest this miRNA attenuates NASH[68]. In addition, Katsura *et al*[69] reported downregulation of miR-301b in mice with NASH, which is consistent with our findings.

We identified more miRNAs specifically associated with ballooning degeneration, fibrosis, and lobular inflammation, respectively. Among these miRNAs, we identified increased levels of plasma miR-34a-5p in participants with liver fibrosis, while epidemiological studies also show upregulation of miR-34a in NAFLD and NASH patients[29].



**Figure 2 Heatmap of microRNA associated with two or more histological features of nonalcoholic fatty liver disease.** Heatmap for association between microRNA (miRNA) expression and histological features of nonalcoholic fatty liver disease (NAFLD) in Teen-Longitudinal Assessment of Bariatric Surgery participants. Red indicates positive association, and purple indicates negative association. 16 miRNAs were associated with two or more histological features and were assigned into two clusters by k-means clustering. Cluster 1 (red bar on left) includes 6 miRNAs that are mostly upregulated in patients with NAFLD, but downregulated in patients with nonalcoholic steatohepatitis (NASH) (relative to nonalcoholic fatty liver), ballooning, fibrosis, and lobular inflammation. Cluster 2 (green bar on left) includes 10 miRNAs that are mostly upregulated in the presence of NASH, ballooning and fibrosis. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001. NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

### Integration of miRNA expression across NAFLD features

We identified several miRNAs associated with two or more NAFLD features. Interestingly, plasma levels of miR-122-5p, miR-1343-5p, miR-193a-5p, miR-193b-5p, and miR-7845-5p exhibited consistent increases across all histological features of NAFLD. Similarly, previous studies reported that miR-122-5p and miR-193a-5p were upregulated in individuals with NAFLD[26,33]. Moreover, Johnson *et al*[26] found strong associations between increased miR-193a-5p levels and NAFLD activity grade and fibrosis stage. Furthermore, Pirola *et al*[36] reported increased expression of serum miR-125b in individuals with NAFLD. Similarly, we found upregulated plasma miR-125b-2-3p expression with NAFLD and lobular inflammation.

Additionally, our analysis revealed decreased levels of miR-1296-5p, miR-1301-5p, miR-199b-5p, miR-411-5p, and miR-6885-3p in NASH compared to NAFL, while these levels were elevated in NAFLD compared to individuals without NAFLD. These miRNAs also demonstrated predominantly negative associations with fibrosis, lobular inflammation, and ballooning (Figure 2). Notably, the downregulation of miR-411-5p aligned with a recent study by Wan *et al*[70], which reported decreased expression of serum miR-411-5p in persons with NASH. Collectively, the distinct expression patterns observed across various NAFLD features suggest that these miRNAs may serve as potential biomarkers for NAFLD progression.

### Pathway analysis of miRNA associated with histological features of NAFLD

An increasing body of research have investigated the associations between NAFLD and miRNA expression, yet little is known about mechanisms underlying the dysregulation of circulating miRNA in NAFLD patients. We first conducted pathway analysis by IPA, and the results revealed that most overlapping miRNA were associated with tumorigenesis. Analysis also highlighted linkage between miR-122-5p and production of hepatitis C virus and between miR-199b-5p and chronic hepatitis B. Given the high prevalence of NAFLD in those with hepatitis C virus and the reported associations among miR-122, hepatitis C virus, and NAFLD[34], the relationship among miR-122-5p, hepatitis C virus, and NAFLD warrants further attention[71]. Furthermore, meta-analyses suggest an inverse association between hepatitis B virus infection and risk of developing NAFLD[72], offering potential insights into the mechanisms involving miR-199b-5p in NAFLD in the context of hepatitis B virus infection.

Experimental studies provide valuable insights into the molecular mechanisms underlying associations between NAFLD and miRNA expression while minimizing confounding variables intrinsic to human observational studies. Particularly, miR-122, a highly expressed hepatic miRNA in hepatocytes, is associated with NAFLD progression by

**Table 3 Molecular pathways of non-alcoholic fatty liver disease-associated miRNA in teen-longitudinal assessment of bariatric surgery participants**

miRNA	Target	Function
miR-122	SIRT-1[73]; FOXO3[74]	miR-122 downregulates SIRT-1 and induces steatosis and hepatic lipogenesis in NAFLD[73]; miR-122-5p inhibits FOXO3 to attenuate inflammatory response and oxidative stress damage in NAFLD[74]
miR-125b	TNFAIP3[79]	miR-125b targets TNFAIP3 and promotes the NF- $\kappa$ B-mediated inflammatory response in NAFLD [79]
miR-146a	MED1[75]	miR-146a targets MED1 and improves hepatic lipid and glucose metabolism in NAFLD[75]
miR-181a	PPAR $\alpha$ [76]	miR-181a inhibits PPAR $\alpha$ and aggravates lipid accumulation in hepatocytes[76]
miR-22	SIRT-1[78]	miR-22 targets SIRT-1 and inhibits gluconeogenesis[78]
miR-34a	TGF- $\beta$ 1/Smad3[80]	miR-34a-5p targets TGF- $\beta$ 1/Smad3 and inhibits liver fibrosis in hepatic stellate cells[80]
miR-375	RAC1[81]	miR-375 inhibits RAC1 and alleviates liver fibrosis[81]

FOXO3: Forkhead box O 3; SIRT-1: Sirtuin 1; TNFAIP3: Tumor necrosis factor alpha-induced protein 3; MED1: Mediator complex subunit 1; PPAR $\alpha$ : Peroxisome proliferator-activated receptor- $\alpha$ ; TGF- $\beta$ 1: Transforming growth factor- $\beta$ 1; Smad3: Mothers against decapentaplegic family 3; RAC1: Rac family small GTPase 1; NAFLD: Non-alcoholic fatty liver disease.

regulating lipid metabolism[23]. For example, Long *et al*[73] revealed that miR-122 inhibited liver kinase B1/AMP-activated protein kinase signaling pathway, which further induced hepatic lipogenesis and steatosis in NAFLD. Additionally, the inhibition of miR-122-5p may suppress the inflammation and oxidative stress damage in NAFLD[74]. Given the observed upregulation of circulating miR-122 and downregulation of hepatic miR-122 in NASH patients[30, 36], the elevated circulating miR-122-5p across NAFLD features in our study might be released by hepatocytes. Furthermore, we identified downregulation of plasma miR-146a-5p, miR-181a-5p, and miR-22-3p in individuals with NAFLD, which is supported by experimental studies of miRNA. For example, miR-146a targeted complex subunit 1 to attenuate lipid accumulation and alleviate NAFLD progression in mice[75]. Additionally, miR-181a was found to downregulate peroxisome proliferator-activated receptor- $\alpha$  and mediate lipid metabolisms in NAFLD in human liver cells[76]. Moreover, miR-22 is a pivotal regulator of lipid and glucose metabolism, playing a crucial role in mitigating NAFLD progression in mice[77]. For example, miR-22 inhibited sirtuin-1 and regulated gluconeogenesis in NAFLD[78]. We also observed increased expression of miR-125b-2-3p in both NAFLD and lobular inflammation, while studies indicated that miR-125b promoted the nuclear factor kappa-light-chain-enhancer of activated B cells-mediated inflammatory response in NAFLD[79]. Furthermore, we observed positive associations between liver fibrosis and expression of miR-34a-5p and miR-375, while experimental research suggested that both miR-34a-5p and miR-375 could alleviate liver fibrosis[80,81]. Together these experimental data support a plausible biological mechanism of NAFLD-miRNA association (Table 3).

### Strength, limitations, and recommendations for future research

Our study has several strengths. Liver biopsies are considered the gold standard in NAFLD assessment, thus ensuring robust and accurate diagnoses of our study. Besides, the consistency of our NAFLD-miRNA associations with epidemiological studies further reinforced the importance of circulating miRNA in NAFLD, supporting their potential use as diagnostic markers. Additionally, our study revealed NAFLD-miRNA associations that have only been previously recognized in experimental research, enhancing the translational value of our findings. By bridging the gap between experimental research and clinical observations, our study helps unravel the complexities of NAFLD and its potential management strategies. Furthermore, our study provides comprehensive characterization of more severe NAFLD features through liver biopsies. Previous studies only profiled specific miRNAs, namely miR-34a, miR-122, miR-191, miR-192, and miR-200a, in patients with ballooning and lobular inflammation[32], while our study conducted an analysis of 843 miRNAs across various histological features of NAFLD. By integrating miRNA expression across these histological features of NAFLD, we uncovered consistent expression patterns of plasma miRNA that hold promise as potential NAFLD biomarkers. Conversely, miRNAs that exhibit differential expression across histological features also warrant further investigation to understand their specific roles and mechanisms in NAFLD pathogenesis.

However, this study was not without limitations. The miRNAs were profiled at baseline, limiting the ability to establish a straightforward causal relationship between NAFLD and plasma miRNA expression. Investigations incorporating longitudinal cohort study designs could better elucidate the temporal relationship and causal associations between NAFLD and plasma miRNA expression. Given our specific focus on adolescents with obesity, who are at high risk of developing NAFLD[10,50,51], and the limitations arising from our small sample size, a validation for generalizability is imperative. The limitation in sample size is an inherent consequence of our methodological choice to employ liver biopsy for NAFLD diagnosis. While liver biopsy ensures accurate and definitive diagnosis, its invasiveness and cost present challenges in expanding the participant pool[82]. Studies with larger and more diverse populations would facilitate more robust and conclusive findings regarding NAFLD-miRNA associations.

## CONCLUSION

Our study provides valuable insights into differential miRNA expression in adolescents with NAFLD. In addition to the previously reported miR-122-5p, miR-193a-5p, and miR-34a, our findings reveal the presence of novel NAFLD-associated miRNAs, namely miR-125b-2-3p and miR-193b-5p. Furthermore, our research underscores similar expression trend of specific miRNAs, such as miR-122-5p, miR-1343-5p, miR-193a-5p, miR-193b-5p, and miR-7845-5p, across all histological features of NAFLD, highlighting their potential roles in pathogenesis and promise as diagnostic and prognostic biomarkers for NAFLD. Plasma miRNAs hold potential to distinguish different stages and phenotypes of NAFLD, allowing for more precise clinical disease classification and targeted management strategies.

## ARTICLE HIGHLIGHTS

### Research background

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver diseases in the world, impacting approximately 25% of the population. The gold standard for NAFLD diagnosis is liver biopsy, yet it is invasive and expensive. Therefore, it is essential to provide alternative methods for NAFLD diagnosis. Recent studies propose that plasma microRNAs (miRNAs) are potential biomarkers for NAFLD, though research in this area remains limited.

### Research motivation

This study is motivated by the current gaps of concerning associations between plasma miRNAs and NAFLD. This study aims to identify potential biomarkers for NAFLD diagnosis and NAFLD progression.

### Research objectives

The objective of this study is to investigate associations between histological features of NAFLD and plasma miRNAs in adolescents with severe obesity.

### Research methods

A total of 135 participants from the Teen Longitudinal Assessment of Bariatric Surgery (Teen-LABS) study were included in this study. Within Teen-LABS, the histological features of NAFLD, including NAFLD, nonalcoholic steatohepatitis (NASH), ballooning degeneration, fibrosis, and lobular inflammation, are characterized based on liver biopsy. Multivariate logistic regression was employed to investigate associations between NAFLD features and 843 plasma miRNAs. Pathway analysis was performed for identified NAFLD-associated miRNA by Ingenuity Pathway Analysis (IPA).

### Research results

In the present study, we identified 38, 52, 16, 15, and 9 plasma miRNAs associated with NAFLD, NASH, fibrosis, ballooning, and lobular inflammation, respectively. Among these miRNA, miR-122-5p, miR-1343-5p, miR-193a-5p, miR-193b-5p, and miR-7845-5p were consistently upregulated across NAFLD features. In contrast, miR-1296-5p, miR-1301-5p, miR-199b-5p, miR-411-5p, and miR-6885-3p were positively associated with NAFLD, yet displayed predominant decreasing in NASH, fibrosis, ballooning, and lobular inflammation. IPA results suggested that most of NAFLD-associated miRNAs were related to cancer.

### Research conclusions

Positive and consistent associations were observed between miR-122-5p, miR-1343-5p, miR-193a-5p, miR-193b-5p, and miR-7845-5p and NAFLD features, indicating their potential as biomarkers for NAFLD diagnosis. Additionally, miR-1296-5p, miR-1301-5p, miR-199b-5p, miR-411-5p, and miR-6885-3p showed different patterns of expression in response to NAFLD severity, indicating they had potential for characterizing NAFLD progression.

### Research perspectives

Conducting studies with larger and more diverse populations would contribute to more conclusive findings regarding NAFLD-miRNA associations. Furthermore, experimental research is imperative to understand the underlying molecular mechanisms of NAFLD-miRNA associations.

## FOOTNOTES

**Author contributions:** Li YJ and Chatzi L designed the research study; Li YJ performed the research; Conti DV, Stratakis N, Goodrich JA, Zhao YQ, Wang HX, and He JX contributed new analytic tools; Ryder JR, Inge TH, Jenkins T, Sisley S, Kohli R and Xanthakos SA contributed data collection; Li YJ wrote the manuscript; Baumert BO, Stratakis N, Goodrich JA, Wu HT, Aung MT, Eckel SP, Walker DI, Valvi D, La Merrill MA, Ryder JR, Inge TH, Jenkins T, Sisley S, Kohli R, Xanthakos SA, Baccarelli AA, McConnell R, Conti DV and Chatzi L reviewed and revised manuscript; all authors have read and approve the final manuscript.

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