

## ORIGINAL ARTICLE

# Serum microRNAs explain discordance of non-alcoholic fatty liver disease in monozygotic and dizygotic twins: a prospective study

Amir Zarrinpar,<sup>1,2</sup> Shakti Gupta,<sup>3</sup> Mano R Maurya,<sup>3</sup> Shankar Subramaniam,<sup>3,4</sup> Rohit Loomba<sup>1,2,5</sup>

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For numbered affiliations see end of article.

## Correspondence to

Professor Rohit Loomba, Division of Gastroenterology and Epidemiology, University of California at San Diego School of Medicine, 9500 Gilman Drive, La Jolla, CA 92093-0063, USA; [roloomba@ucsd.edu](mailto:roloomba@ucsd.edu) and Dr Shankar Subramaniam, Department of Bioengineering, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0412, USA; [shankar@ucsd.edu](mailto:shankar@ucsd.edu)

AZ and SG are contributed equally.

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## ABSTRACT

**Objective** In the setting where two individuals are genetically similar, epigenetic mechanisms could account for discordance in the presence or absence of non-alcoholic fatty liver disease (NAFLD). This study investigated if serum microRNAs (miRs) could explain discordance in NAFLD.

**Design** This is a cross-sectional analysis of a prospective cohort study of 40 (n=80) twin-pairs residing in Southern California. All participants underwent a standardised research visit, liver MRI using proton-density fat fraction to quantify fat content and miR profiling of their serum.

**Results** Among the 40 twin-pairs, there were 6 concordant for NAFLD, 28 were concordant for non-NAFLD and 6 were discordant for NAFLD. The prevalence of NAFLD was 22.5% (18/80). Within the six discordant twins, a panel of 10 miRs differentiated the twin with NAFLD from the one without. Two of these miRs, miR-331-3p and miR-30c, were also among the 21 miRs that were different between NAFLD and non-NAFLD groups (for miR-331-3p:  $7.644 \pm 0.091$  vs  $8.057 \pm 0.071$ , respectively,  $p=0.004$ ; for miR-30c:  $10.013 \pm 0.126$  vs  $10.418 \pm 0.086$ , respectively,  $p=0.008$ ). Both miRs were highly heritable (35.9% and 10.7%, respectively) and highly correlated with each other ( $R=0.90$ ,  $p=2.2 \times 10^{-16}$ ) suggesting involvement in a common mechanistic pathway. An interactome analysis of these two miRs showed seven common target genes.

**Conclusions** Using a novel human twin-study design, we demonstrate that discordancy in liver fat content between the twins can be explained by miRs, and that they are heritable.

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is common with worldwide prevalence ranging from 6.3% to 33%.<sup>1</sup> Because of the increasing prevalence of obesity, NAFLD is the most common form of liver disease in the developed world.<sup>2</sup> It is projected to become the leading cause of end-stage liver disease, liver transplantation and hepatocellular carcinoma (HCC) by 2025.<sup>3,4</sup> The understanding of the pathophysiology of NAFLD is poor and treatments are limited. Although the pathogenesis is thought to be multi-genetic, studies on various pedigrees have estimated that the heritability of NAFLD ranges from 20% to 100%.<sup>5,6</sup> Furthermore, it is unclear whether epigenetic

## Significance of this study

### What is already known on this subject?

- MicroRNAs (miRs) are small non-coding RNAs that regulate multiple physiological processes by controlling transcription and translation of mRNA.
- Recent studies suggest epigenetic mechanisms, such as miRs, could account for some of the genetic variability in patients with non-alcoholic fatty liver disease (NAFLD).
- Discordant twins have been used for decades to estimate the importance of genetic and environmental factors on complex diseases, but have been rarely used to study epigenetic mechanisms, such as miRs.

### What are the new findings?

- Using a twin-study design, we demonstrate that serum miRs explain discordancy between twins with and without NAFLD. In particular, miR-331-3p and miR-30c not only explained NAFLD discordancy between twins but also were significantly different between participants with and without NAFLD.
- Using a novel twin study, we show that the serum levels of miRs are heritable traits.
- Interactome analysis of miR-331-3p and miR-30c demonstrates their functional relevance in influencing pathophysiological mechanism.

### How might it impact on clinical practice in the foreseeable future?

- MiRs can serve as biomarkers for non-invasive assessment of NAFLD.
- MiRs may also be considered as therapeutic targets in NAFLD.

mechanisms such as those from microRNAs (miRs) are heritable and could account for its pathophysiology.

MiRs are small non-coding RNAs that regulate multiple physiological processes by controlling transcription and translation of mRNA.<sup>7,8</sup> The human genome encodes approximately 1000 miRs, which can regulate about one-third of transcripts.<sup>9</sup> They are frequently dysregulated in many



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pathological conditions.<sup>8</sup> Recently, there has been a great interest in the role that miRs play in NAFLD and whether they can serve as novel markers for the diagnosis and monitoring of liver diseases.<sup>7 10–13</sup> However, previous studies have been limited by testing the association of very few and specific miRs. Furthermore, although several miRs have been correlated with disease activity in NAFLD, their role in the heritability of the condition or the pathophysiology of disease has not yet been described.

A wide array of miRs has been suggested to assess liver disease, mostly HCC, but including viral hepatitis and fatty liver disease. Early studies investigating the role of miRs in NAFLD found significantly elevated serum levels of miR-122, miR-34a and miR-16 compared with controls.<sup>14</sup> In particular, miR-122 and miR-34a were positively correlated with disease severity (ie, steatosis to steatohepatitis), serum lipids and serum levels of liver enzymes and pathological assessment of inflammation activity. A subsequent study showed serum levels of miR-181d, miR-197 and miR146b were lower in patients with NAFLD; however, this study found no significant differences in miR-122 and miR-34a.<sup>15</sup> A more recent study which analysed a larger set of circulating miRs found miR-122 was markedly different in NAFLD and non-alcoholic steatohepatitis (NASH).<sup>16</sup> However, these previous studies only tested for a limited number of miRs. It is plausible that different miRs may play distinct roles at different steps along the spectrum of NAFLD. Furthermore, data regarding the relationship of any of these miRs and the pathophysiology of early NAFLD or its heritability are limited.

Twins offer a unique opportunity to research the aetiology of disease development, especially when their underlying biology is diverse. These studies, specifically those that compare discordant twins, have been used for decades to estimate the importance of genetic and environmental factors on complex diseases.<sup>17</sup> However, discordant twin studies that have analysed epigenetic mechanisms (eg, miRs) of any disease are rare. The role of various epigenetic mechanisms in the pathophysiology of NAFLD has been only recently investigated and their heritability and role in disease progression remain unknown.<sup>18 19</sup>

In this study, we present the results of serum miR analysis from the Twin and Family Study. A total of 818 unique miRs were assessed in our cohort. We first compared the serum miRs between six pairs of discordant twins. In the setting where two individuals are genetically similar, it is plausible that epigenetic mechanisms such as miRs could account for discordance in the presence or absence of NAFLD. The heritability of miRs that were most different between these two groups was assessed. An interactome analysis of implicated miRs was also performed suggesting specific pathophysiological pathways in NAFLD. Although certain miRs are associated with NAFLD, their role in the causality of the disease is unknown. This twin cohort study identifies potential miRs that are likely to be important for causality and attempts to deduce their functional role.

## EXPERIMENTAL PROCEDURES

Please see online supplementary material for extended experimental procedures.

### Design

This is a cross-sectional analysis of a prospective cohort study of the patients from the Twin and Family Study (ClinicalTrials.gov: NCT01643512). The study was designed to examine the genetics of NAFLD and all participants underwent a standardised research visit including a complete history and physical exam, biochemical phenotyping and testing to rule out other causes of

chronic liver disease, alcohol quantification. In addition, we quantified liver fat content using MRI estimated proton-density fat fraction (MRI-PDFF) and performed miR profiling of the participants' serum samples which were collected on the same day of research clinic visit. Patients also attended a research clinic visit and underwent standardised history, physical exam, anthropometric exam and biochemical/serum testing at the University of California San Diego NAFLD Translational Research Unit. Written informed consent was obtained from every participant.

### Participants

Participants were recruited from newspaper advertisements and twin-birth registry. They were from urban Southern California (mainly the San Diego area). The inclusion criteria for this study included age of at least 18 years, willing to give informed consent and complete all procedures in the protocol. The exclusion criteria included women who are pregnant or nursing; if the participant has any contraindication to MRI (eg, metal in body, extreme claustrophobia, extreme obesity); use of steatogenic medications (eg, amiodarone, glucocorticoids, methotrexate) for at least 3 months in the last 6 months; chronic diseases other than NAFLD that could induce hepatic steatosis (eg, viral hepatitis, HIV, coeliac disease, cystic fibrosis); significant alcohol use (eg, >10 g/day in women and >20 g/day in men) for more than three consecutive months in the last 12 months; prior bariatric surgery; metabolic and/or genetic liver disease (eg, Wilson's disease, haemochromatosis, polycystic liver disease, dysbetalipoproteinaemia); clinical or laboratory evidence of systemic infection or any other clinical evidence of liver disease.

### Twin cohort

This study included 80 twin participants (40 twin-pairs), including 29 pairs of monozygotic twins and 11 pairs of dizygotic twins. In addition, there were two twin-pairs where either one or both individuals did not have MRI-PDFF (three individuals) due to contraindications to MRI but who received miR profiling. Although the NAFLD status of these patients was unknown, their data were included in the heritability analysis as well as the correlation analyses. For each participant, a detailed assessment of twin-ship status (ie, monozygotic (MZ) or dizygotic (DZ)) was obtained. The majority of twin-pairs (34) were diagnosed by their physician as either MZ or DZ by genetic testing. Furthermore, twin-ship status was confirmed by using a previously published questionnaire.<sup>20</sup>

### Sibling–sibling and parent–offspring (family) cohort

This cross-sectional analysis of a prospective study also included a cohort of participants who were either siblings or parent–offspring pairs. These participants also underwent the same rigorous evaluation as our twin cohort. They consisted of 57 individuals. Eight of these individuals had a contraindication for an MRI, and one individual had an MRI-PDFF which was borderline. Although the NAFLD status of these nine individuals was unknown, these patients received miR profiling and their data were included in the correlation analyses.

### MRI protocol

It would be unethical to perform a liver biopsy in someone who has no clinical indication for one. Since the vast majority of participants in the study were normal and had no evidence of liver disease, a non-invasive and a quantitative method was used to estimate liver fat. Previous studies from our group have shown that MRI-PDFF is a precise, accurate and reproducible measure

of liver fat.<sup>21–25</sup> Liver fat measurements using this technique have a <1% variability and correlate robustly with MR spectroscopy ( $r^2=0.99$ ).<sup>21</sup> It routinely outperforms CT and ultrasound in accurately quantifying liver steatosis.<sup>26</sup> The MRI procedure took place on the same day as the research clinic visit for both members of each twin-pair.

To assess NAFLD, hepatic steatosis was quantified using the previously described MRI-PDFF protocol.<sup>27</sup> Briefly, this method was performed on a 3T research scanner. The goal is to minimise T1 bias and collect multiple echoes at echo times at which fat and water signals are in phase or out of phase with each other. By processing the data through a non-linear least-squares fitting algorithm, a corrected T2\* effect is produced which estimates fat and water proton densities from which the fat content is calculated. Using custom analysis software developed at the UCSD Liver Imaging Group, the mathematical model is applied pixel by pixel on the source images to generate parametric PDFF maps that depict quantity and distribution of fat throughout the entire liver.

#### Definition of NAFLD and characterisation of twin-pairs

NAFLD was defined as a MRI-PDFF  $\geq 5\%$  without any secondary causes of steatosis. All the twins in our cohort were stratified into two groups: concordant for NAFLD (both members had MRI-PDFF  $\geq 5\%$ ), discordant for NAFLD (one twin had MRI-PDFF  $\geq 5\%$  and the other twin did not) and concordant for the absence of NAFLD (both members had MRI-PDFF  $< 5\%$ ).

#### miR extraction and profiling

Total RNA was isolated from the serum using the miRNeasy RNA protocol (QIAGEN). The RNA extracted was used as sample input for miR profiling on the OpenArray Real-Time PCR System using the manufacturer's instructions (Life Technologies). The TaqMan OpenArray Human MicroRNA Panel was used for the quantitative real time polymerase chain reaction (q-PCR) qPCR step. Using this method, we obtained profiling for 818 different miRs for each patient (see online supplementary table S1). miRs that were not measurable in >70% of participants (total of 605) were excluded from further analysis.

#### Statistical analysis

For patient characteristics, a Student's unpaired t test (two sample, unequal variance) was used to compare continuous variables (eg, age, weight, body mass index, blood pressure) and Fisher's exact test was used to compare categorical variables (eg, gender). For the discordant twin analysis, a Student's paired t test was used to determine differences in miR expression between patients with NAFLD and without NAFLD. However, when comparing the entire NAFLD and non-NAFLD cohorts, a Student's unpaired t test (two sample, unequal variance) was used to determine the differences.

## RESULTS

### Cohort characteristics

This cross-sectional analysis of a prospective study included 80 twin participants (40 twin-pairs), including 29 pairs of monozygotic twins and 11 pairs of dizygotic twins. Participants with NAFLD were characterised using an accurate and previously validated quantitative imaging-based biomarker, the MRI-estimated proton-density fat fraction (MRI-PDFF)  $\geq 5\%$ .<sup>27</sup> The prevalence of NAFLD was 22.5% (18/80), and the rest were classified as non-NAFLD 77.5% (62/80). The participants without an MRI-PDFF were excluded from analysis in the NAFLD versus non-NAFLD comparisons, but were included in

the correlation and heritability analysis of miRs. In all, this yielded 40 twin-pairs, 6 were concordant for NAFLD, 28 were concordant for non-NAFLD and 6 were discordant for NAFLD (see online supplementary table S2). The demographics for the NAFLD and non-NAFLD, including physical, anthropometric and serum biomarkers, are described in detail in [table 1](#).

### MiRs distinguish between discordant twins

A total of 818 unique miRs were identified; 203 were present in over 30% of participants. First, we aimed to determine whether the epigenetic mechanisms driven by miRs could account for NAFLD discordance in two individuals with genetic and environmental similarities. By comparing the serum miRs between the six pairs of discordant twins, we identified 10 miRs that could differentiate the discordance for NAFLD between twins (see [figure 1](#), online supplementary figure S1 and [table 2](#)). Three miRs were more prominently different: miR-331-3p ( $7.71 \pm 0.19$ ; in NAFLD vs  $8.18 \pm 0.21$  in non-NAFLD,  $p=0.004$ ; [figure 1A](#)), miR-30c ( $10.15 \pm 0.22$ ; in NAFLD vs  $10.87 \pm 0.24$  in non-NAFLD,  $p=0.008$ ; [figure 1B](#)) and the miR-precursor let7c ( $0.00 \pm 0.00$ ; in NAFLD and  $2.78 \pm 0.60$  in non-NAFLD,  $p=0.006$ ; [figure 1C](#)). These 10 miRs were able to cluster the discordant twins efficiently ([figure 1D](#)). Reanalysis of the data with just the monozygotic twins changed the results only slightly, with only six miRs identified: miR-30b, miR-140, miR-30c, miR-324-3p and miR-331-3p (see online supplementary figure S2 and [table S3](#)). Previously described miRs thought to be involved in NAFLD, including miR-122, miR-34a, miR-181d, miR-197, miR146b and miR-16, were not significantly different between discordant twins.

### MiRs distinguish those with NAFLD

Some of the observed miRs were significantly different between the discordant twins, but did not appear so in the concordant NAFLD and concordant non-NAFLD twins (eg, miR-let7; [figure 1C](#)). Hence, the levels of all of the serum miRs were compared between the NAFLD and non-NAFLD cohorts for the entire twin and family cohort (see online supplementary table S4). We found 21 miRs that were significantly different between the two populations (see online supplementary figure S3 and [table 2](#)). The miR that was most discriminative between the two populations was miR-331-3p ( $7.64 \pm 0.09$ ; in NAFLD vs  $8.06 \pm 0.07$ ; non-NAFLD;  $p=0.0007$ ; [figure 2A](#)), which had been previously identified in the discordant twin analysis. In addition, miR-30c was also significantly different when comparing all the NAFLD and non-NAFLD cohorts ( $10.01 \pm 0.13$ ; in NAFLD vs  $10.42 \pm 0.09$ ; non-NAFLD;  $p=0.011$ ; [figure 2B](#)). None of the other miRs identified in the discordant twin analysis were significantly different between the NAFLD and non-NAFLD cohorts.

MiRs that were previously described to be associated with NAFLD were also analysed. Among the 21 miRs that were significantly different between the NAFLD and non-NAFLD cohorts, miR-122 ( $8.23 \pm 0.27$  vs  $7.03 \pm 0.26$ , respectively;  $p=0.002$ ; [figure 2C](#)) and miR-34a\* ( $1.57 \pm 0.23$  vs  $1.04 \pm 0.09$ , respectively;  $p=0.04$ ; [figure 2D](#)) were significantly different (see online supplementary table S5 and [figure S3](#)).

### Heritability of miRs associated with fatty liver disease

Since NAFLD is a complex disease with both heritable and environmental risk factors, the heritability of the miRs that were identified in the previous two analyses was assessed. The heritability of miR-331-3p was high with an  $H^2$  of 0.36 ([figure 3A, B](#)), suggesting that 36% of the serum level of this miR is attributable to genetics. Serum levels of miR-30c were also heritable with a

**Table 1** Baseline characteristics of the twin cohort

	Subjects with NAFLD (MRI-PDFF>5%)	Subjects without NAFLD (MRI-PDFF<5%)	p Value
Number	18	62	
Demographics			
Age (years)	59.1 (±3.47)	45.4 (±2.63)	0.0109
Sex (% male)	38.9	17.7	0.2648
Race (% White/Black/Hispanic/Asian)	66.67/0/22.22/0	80.65/0/16.13/3.23	NA
Physical			
Weight (kg)	85.82 (±4.81)	65.98 (±1.45)	8.7×10 <sup>-7</sup>
Body mass index (kg/m <sup>2</sup> )	30.14 (±1.39)	23.25 (±0.55)	5.5×10 <sup>-7</sup>
Systolic blood pressure (mm Hg)	139.33 (±3.81)	123.68 (±2.61)	0.00395
Diastolic blood pressure (mm Hg)	84.22 (±3.3)	77.45 (±1.44)	0.038
Waist circumference (cm)	98.44 (±2.63)	85.61 (±1.29)	1.5×10 <sup>-5</sup>
Laboratory data			
Glucose (mg/dL)	110.72 (±8.3)	87.24 (±1.06)	5.8×10 <sup>-6</sup>
Hgb A1c (%)	14.09 (±0.33)	13.69 (±0.16)	0.234
HOMA-IR	12.47 (±1.3)	6.73 (±0.44)	9.9×10 <sup>-7</sup>
AST (U/L)	24.28 (±1.47)	22.98 (±0.85)	0.468
ALT (U/L)	26.78 (±3.14)	20.19 (±1.44)	0.0406
Alk Phos (U/L)	66.28 (±2.8)	67.42 (±2.79)	0.833
Total bilirubin (mg/dL)	0.45 (±0.05)	0.45 (±0.03)	0.935
Albumin (g/dL)	4.49 (±0.05)	4.6 (±0.05)	0.226
GGT (U/L)	32.72 (±7.47)	18.98 (±1.25)	0.00374
Total cholesterol (mg/dL)	197.94 (±10.01)	192.54 (±5.72)	0.65
HDL-cholesterol (mg/dL)	52.28 (±3.18)	70.1 (±2.85)	0.00189
LDL-cholesterol (mg/dL)	115.67 (±8.39)	107.15 (±4.96)	0.407
Triglycerides (mg/dL)	149.83 (±18.01)	76.41 (±5.2)	7.25×10 <sup>-7</sup>
INR	1.1 (±0.1)	1.05 (±0.03)	0.547
Ferritin (ng/mL)	137.22 (±40.29)	90.44 (±10.14)	0.105
Imaging data			
MRI-PDFF (%)	10.71 (±0.94)	2.25 (±0.09)	1.7×10 <sup>-26</sup>
MR spectroscopy (%)	16 (±2.48)	1.98 (±0.27)	4.8×10 <sup>-11</sup>
MR elastography (stiffness)	2.99 (±0.3)	2.07 (±0.05)	8.1×10 <sup>-6</sup>

All numbers are means (±SEM). A Student's unpaired t test was used for all comparisons, except those of categorical variables (ie, gender) where a Fisher's exact test was used. Alk Phos, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; GGT,  $\gamma$ -glutamyl transferase; HDL, high-density lipoprotein; Hgb A1c, glycosylated haemoglobin; HOMA-IR, homeostatic model assessment—insulin resistance; INR, international normalised ratio; LDL, low-density lipoprotein; MRI-PDFF, MRI-estimated proton-density fat fraction; NA, not applicable; NAFLD, non-alcoholic fatty liver disease.

significant  $H^2$  of 0.11 (figure 3A, C). Many of the other miRs identified in our study were also highly heritable, particularly miR-223\*, miR-191, miR-127, miR-193a-5p, miR-411 and miR30b. However, both miR-122 and miR-34a\* had an  $H^2$  that was less than 0.1 (figure 3A).

#### Correlation among miRs identified in cohorts with NAFLD

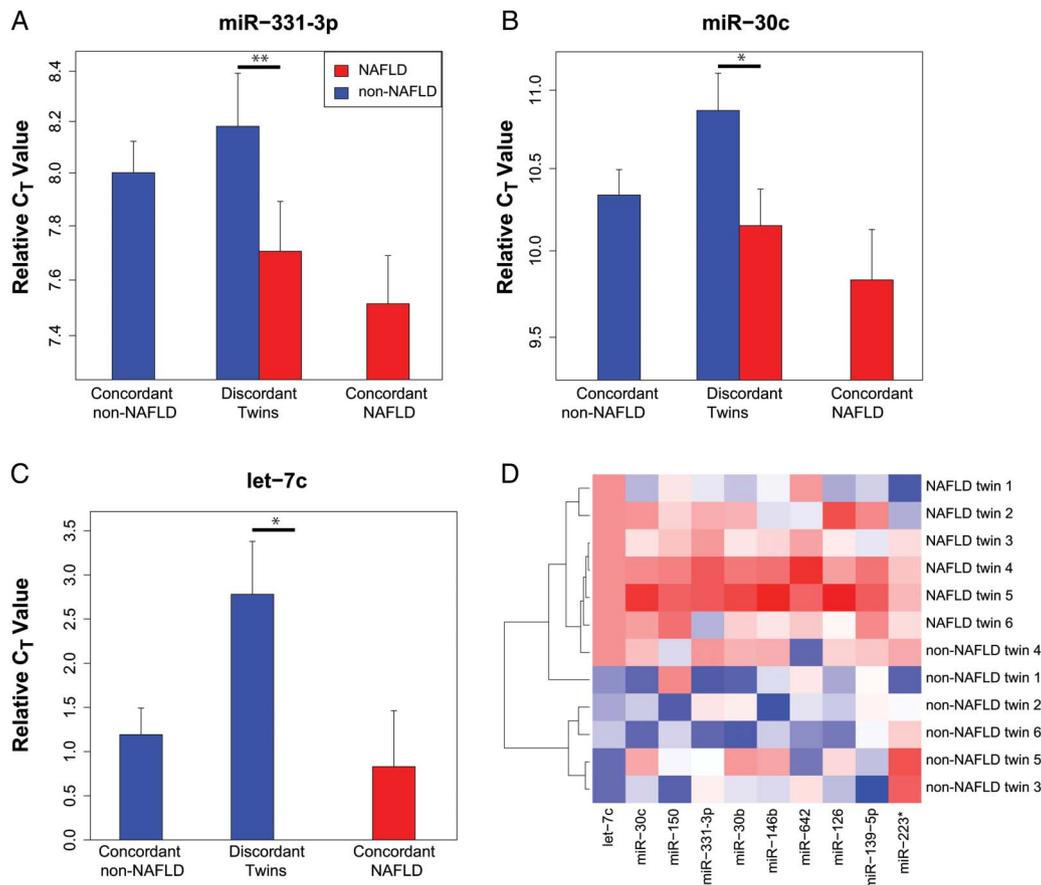
Since many miRs appeared to be significantly different between the NAFLD and the non-NAFLD groups, a correlation analysis was performed to assess whether they could be part of the same physiological processes (figure 4). Many of the miRs identified were highly correlated with each other. In particular, we noted that the correlation between miR-331-3p and miR-30c was the highest ( $r=0.90$ ,  $p=2.2\times 10^{-16}$ ; figure 4). Neither miR-331-3p nor miR-30c was highly correlated with miR-122 ( $r=0.43$  and  $r=0.47$ , respectively) or miR-34a ( $r=0.29$  and  $r=0.25$ , respectively; figure 4) nor with any serum biomarkers (see online supplementary figure S4).

#### Target interactome of highly correlated miRs associated with fatty liver disease

In order to understand the functional contribution of miR-331-3p and miR-30c to the pathophysiology of NAFLD, an interactome analysis was performed. Using Targetscan and a

probability of preferentially conserved targeting ( $P_{CT}$ ) score cut-off of at least 50% and miRWalk ( $p$  value cut-off 0.05)<sup>28</sup> (ie, the intersection of targets predicted by both), we found 70 gene targets for miR-331-3p and 480 targets for miR-30c. Since miR-331-3p and miR-30c are associated with each other based on their high correlation, they may work in similar pathways in the pathophysiology of NAFLD. We found that the two miRs shared seven gene targets: adaptor-related protein complex 2, alpha 1 subunit (AP2A1), histone deacetylase 5 (HDAC5), kinase suppressor of Ras 1 (KSR1), adhesion G protein-coupled receptor L3 (ADGRL3; LPHN3), MAX network transcriptional repressor (MNT), suppressor of cytokine signalling 1 (SOCS1) and signal sequence receptor, gamma (SSR3).

In order to investigate a pathway that is affected by both miR-331-3p and miR-30c, a targeted interactome analysis was performed to determine areas of functional overlap between the two miRs (figure 5). The combined list of targets, which included 543 genes, was used. Since many of the miRs identified in our analysis have altered levels in patients with HCC, our initial targeted interactome involved the Kyoto Encyclopedia of Genes and Genomes (KEGG) cancer pathway. Eight of the 543 genes are from the KEGG Pathways in Cancer. The immediate neighbouring proteins of these eight targets were identified in the protein–protein interaction (PPI) and such connections were



**Figure 1** MicroRNAs (MiRs) that are significantly different between discordant twins. Ten miRs were identified as being significantly different between discordant twins. The three most significantly different were (A) miR-331-3p, (B) miR-30c and (C) let-7c. (D) Heatmap of the 10 miRs in discordant twins. \* $p < 0.05$ ; \*\* $p < 0.005$ . NAFLD, non-alcoholic fatty liver disease.

used to build the PPI subnetwork. The overall subnetwork included 303 nodes and 332 interactions and was visualised using cytoscape (figure 5A). The miR targets appear as hubs since they interact with many other proteins. Hence, multiple cancer pathway genes are affected by the genes modulated by miR-30c and miR-331-3p.

Metabolic pathways are not enriched among the targets of miR-30c and miR-331-3p. However, since lipid metabolism is thought to play a significant role in NAFLD pathophysiology, a targeted interactome using the KEGG lipid metabolism pathway

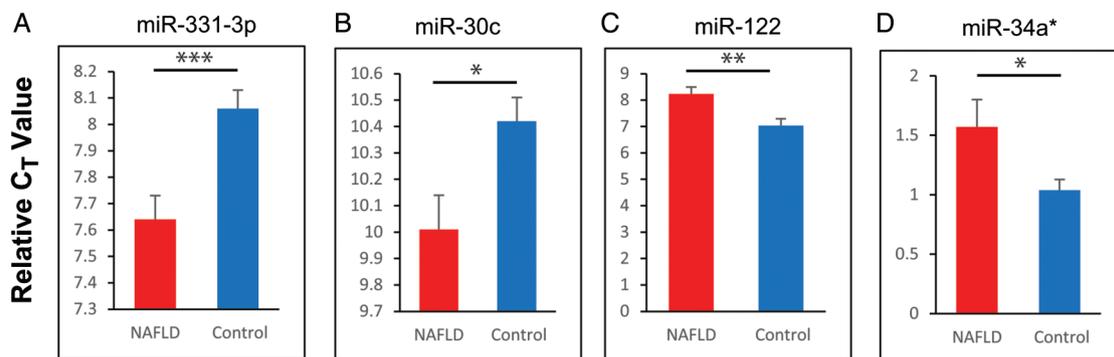
was also created. Targets of either miR-331-3p or miR-30c in this pathway included 22 genes from the lipid and some energy/metabolism-related pathways. All the immediate neighbouring proteins of these 22 targets were identified in the PPI and such connections were used to build the PPI subnetwork (figure 5B). The overall subnetwork included 356 nodes and 428 interactions. Some of the miR targets appear as hubs since they interact with many other proteins. Important kinases such as diacylglycerol kinase delta (DGKD) and zeta (DGKZ) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PIK3CD) are

**Table 2** Serum microRNAs (miRs) that are significantly different between discordant twins

	Twins with NAFLD (MRI-PDFF $\geq 5\%$ )	Twins without NAFLD (MRI-PDFF $< 5\%$ )	p Value
N	18 (6 concordant twin-pairs; 6 individuals in discordant twin-pairs)	62 (28 concordant twin-pairs; 6 individuals in discordant twin-pairs)	NA
miR-331-3p	7.71 ( $\pm 0.19$ )	8.18 ( $\pm 0.21$ )	0.0039
let-7c	0 ( $\pm 0$ )	2.78 ( $\pm 0.6$ )	0.0056
miR-30c	10.15 ( $\pm 0.22$ )	10.87 ( $\pm 0.24$ )	0.0080
miR-146b	6.56 ( $\pm 0.17$ )	6.96 ( $\pm 0.19$ )	0.013
miR-30b	10.29 ( $\pm 0.16$ )	10.76 ( $\pm 0.23$ )	0.017
miR-223*	3.22 ( $\pm 0.42$ )	2.6 ( $\pm 0.52$ )	0.022
miR-126	9.57 ( $\pm 0.38$ )	10.49 ( $\pm 0.21$ )	0.023
miR-150	10.47 ( $\pm 0.15$ )	11.45 ( $\pm 0.31$ )	0.031
miR-139-5p	6.16 ( $\pm 0.19$ )	6.79 ( $\pm 0.24$ )	0.038
miR-642	2.06 ( $\pm 0.15$ )	2.78 ( $\pm 0.15$ )	0.040

A paired t test was used for all comparisons.

MRI-PDFF, MRI-estimated proton-density fat fraction; NA, not applicable; NAFLD, non-alcoholic fatty liver disease.

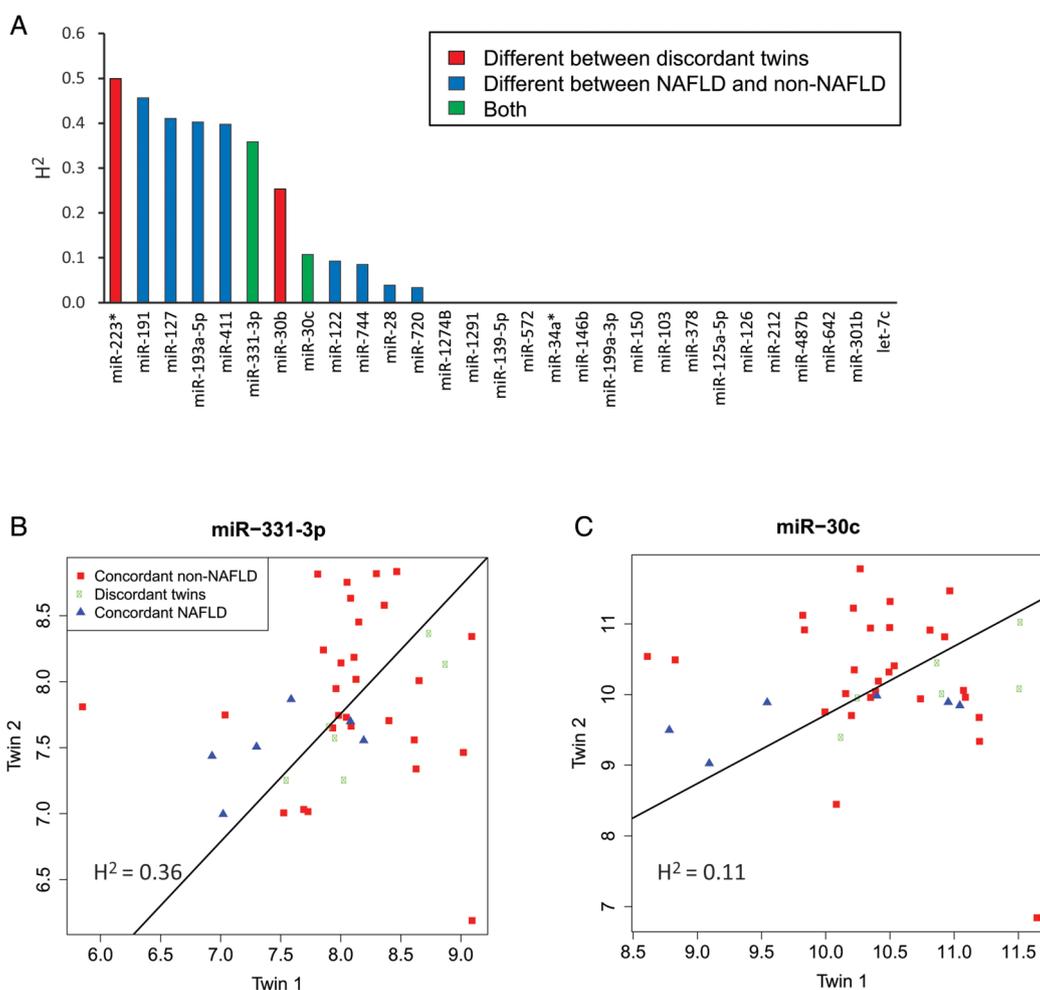


**Figure 2** Select microRNAs (miRs) that are significantly different between non-alcoholic fatty liver disease (NAFLD) and non-NAFLD participants. Of the 10 miRs that were significantly different between discordant twins, only two were significantly different between NAFLD and non-NAFLD groups in general: (A) miR-331-3p and (B) miR-30c. Previously identified miRs, such as (C) miR-122 and (D) miR-34a\*, which were not significantly different between discordant twins, were significantly different in the entire cohort of patients. \* $p < 0.05$ ; \*\* $p < 0.005$ ; \*\*\* $p < 0.001$ .

direct targets of miR-30c. Other important genes associated with NAFLD include leptin receptor (LEPR) and interleukin 1 $\alpha$  (IL1A). The former can affect appetite, while the latter is involved in inflammation.<sup>29</sup> In addition, MMP17, which interacts with tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), can also play an important role in NAFLD.<sup>30</sup>

## DISCUSSION

In this study, approximately 800 miRs, more than 10 times previous reports, were assessed in the serum of a prospectively assessed uniquely phenotyped, community-dwelling, cohort of twins with and without NAFLD as identified by an accurate and previously validated, quantitative imaging-based biomarker



**Figure 3** Heritability of identified microRNAs (miRs). (A)  $H^2$  was calculated for miRs that were significantly different between discordant twins, as well as those that were significantly different between the non-alcoholic fatty liver disease (NAFLD) and non-NAFLD population. MiR-331-3p and miR-30c belong to a small group of miRs that are highly heritable. (B) Correlation of twins illustrates the highly heritable miR-331-3p, which has an  $H^2$  of 0.36. (C) Correlation of twins showing lower heritability of miR-30c, which has an  $H^2$  of 0.11.

	miR-30c	miR-331-3p
miR-28	0.86	0.83
miR-30c	1.00	0.90
miR-103	0.61	0.65
miR-127	0.48	0.56
miR-212	0.13	0.07
miR-331-3p	0.90	1.00
miR-487b	0.49	0.52
miR-411	0.55	0.53
miR-125a-5p	0.57	0.57
miR-122	0.49	0.48
miR-193a-5p	0.10	0.08
miR-191	0.80	0.90
miR-199a-3p	0.66	0.72
miR-744	0.65	0.76
miR-301b	0.50	0.51
miR-572	-0.11	-0.14
miR-378	-0.12	-0.04
miR-378	-0.13	-0.16
miR-34a*	0.27	0.31
miR-1291	0.05	-0.02
miR-1274b	-0.27	-0.13
miR-720	0.14	0.20
let-7c	0.44	0.44
miR-150	0.58	0.45
miR-30b	0.84	0.80
miR-146b	0.72	0.68
miR-642	0.25	0.22
miR-126	0.64	0.62
miR-139-5p	0.73	0.66
miR-223*	0.38	0.51

Color-scale: -0.25 0 0.25 0.5 0.75 1

**Figure 4** Correlation of miR-331-3p and miR-30c with other identified microRNAs (miRs). MiR-331-3p and miR-30c are highly correlated with each with a  $R > 0.9$ . The other miRs are not highly correlated. Intensity of red shading suggests level of correlation.

MRI-PDFF. Among discordant twins, where one had NAFLD and the other didn't have NAFLD by MRI-PDFF, 10 miRs were identified. Furthermore, two of these miRs, miR-331-3p and miR-30c, were significantly different between patients with NAFLD and without NAFLD in this cohort.

The primary risk factors of NAFLD are obesity, type 2 diabetes mellitus and dyslipidemia. Increased sedentary lifestyle and the consumption of foods with high caloric value are the main environmental factors that contribute to NAFLD. Since the participants in our study lived separately as adults, shared environmental factors were limited. Nevertheless, there is also a strong heritable component to NAFLD.<sup>5 6</sup> The serum measurement of these miRs, especially the former, was highly heritable. This is parallel to previous studies where the levels of other serum biomarkers (eg,  $\gamma$ -glutamyl transferase (GGT)) have been observed to be heritable as well.<sup>31</sup> Although previous studies have shown miRs can explain discordancy in autism spectrum disorder<sup>32</sup> or lupus nephritis,<sup>33</sup> a novel finding in this study is that serum levels of miRs are heritable in twins.

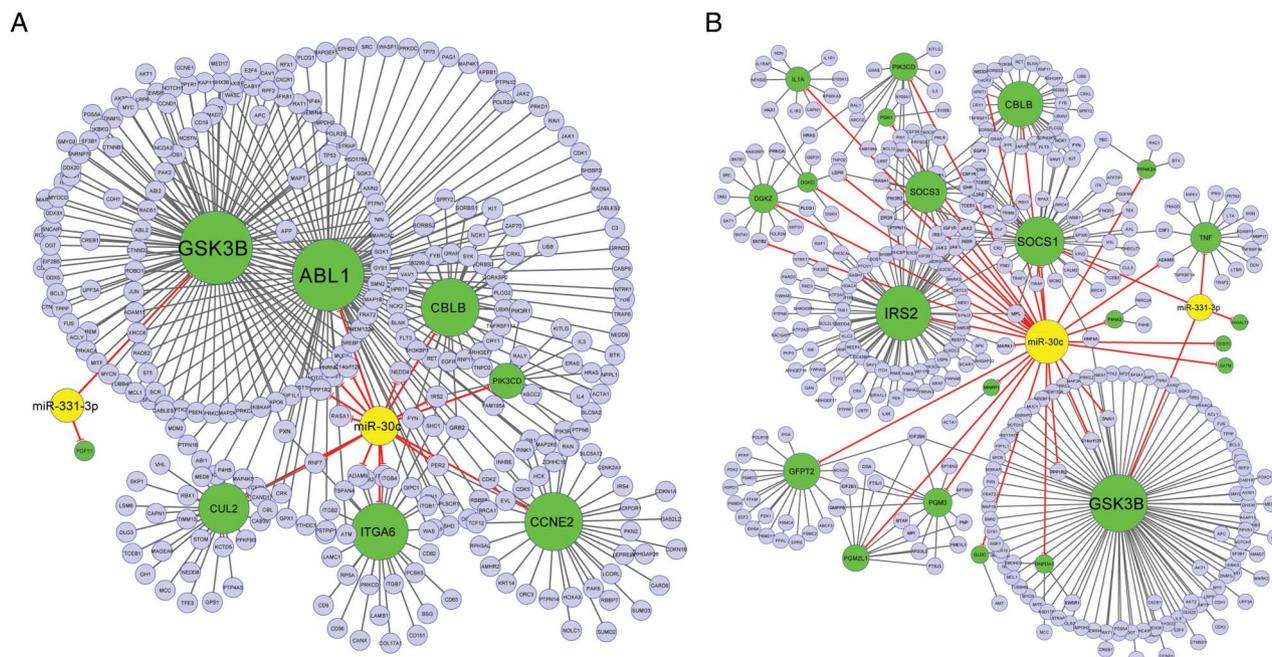
The heritability of epigenetic factors such as miRs is not entirely well understood. Epigenetic factors are cellular modifications that can be heritable, but appear unrelated to DNA sequence changes and can be modified by environmental stimuli.<sup>34 35</sup> Previous twin studies of epigenetics (in this case DNA methylation patterns) showed great amount of heritability with similar epigenetic profiles between twins. However, great differences in epigenetic variability were observed (post hoc) in twins who differed most in lifestyle and age.<sup>36</sup> The fact that 34 of the 40 twin-pairs in our study were concordant suggests that the six discordant pairs perhaps have the greatest differences in lifestyle that contribute to NAFLD, which could be what these two miRs could be detecting.

There was little overlap between the miRs identified in the discordant twins and those identified in the entire NAFLD and non-NAFLD cohorts. This suggests that the NAFLD in discordant twins may be different, and perhaps at a much earlier stage, than those in the general NAFLD population in our study. It is plausible that early in the disease a different group of miRs (such as those identified in the discordant twin set) is involved and as the disease progresses, the miR profile shifts (to something similar to that in the NAFLD cohort). Nine of the 10 miRs identified in discordant twins are decreased in the NAFLD group, whereas only 13 of the 21 miRs identified in the population cohort are decreased. Hence, the miR profile of a patient may change as he/she progresses from NAFLD to NASH, fibrosis and cancer, and a single or group of miR may not be an optimal serum biomarker for all stages of liver diseases. Rather, proportional changes in specific markers may be more important in tracking disease progression. More research is necessary to investigate this further.

Patients with NAFLD were identified with MRI-PDFF. Although previous studies have shown that this methodology is an excellent way to evaluate an individual for NAFLD,<sup>27</sup> one limitation of using this technique is that the wealth of histological information from a liver biopsy (eg, amount of inflammation, pattern) is not available. Correlating disease activity with miR levels may lead to additional information about the levels of these miRs and their role in pathophysiology and warrants further investigation.

Previous studies have associated a number of miRs with NAFLD. In particular, miR-122 has been proposed as a potential marker of NAFLD. MiR-122 is liver specific and its inhibition in mice leads to downregulation of cholesterol and lipid-metabolising enzymes.<sup>37</sup> Furthermore, a recent study found increased levels of circulating miR-122, as well as miR-34a, in patients with NAFLD.<sup>14</sup> Other miRs have been miR-125b, miR-146a and miR-155 which are thought to regulate inflammatory responses to lipopolysaccharide-induced TNF- $\alpha$  in Kupffer cells.<sup>38</sup> Although our study confirmed previous studies by finding differences in miR-122 and miR-34a in the NAFLD versus non-NAFLD cohort, these differences did not exist in the discordant group and these miRs were not heritable. One possible explanation could be that the miR-122 and miR-34a could be the result of environmental perturbations and hence not linked to host genetics, but miR-331-3p and miR-30c could be more affected by genetic processes. This suggests that the genetic underpinnings of NAFLD/NASH appear to be complex.<sup>39</sup>

Many of the miRs found in this study have been associated with HCC or other cancers in previously published studies. These include, but are not limited to, miR-331-3p,<sup>40</sup> miR-34a,<sup>40</sup> let-7c<sup>41</sup> and miR-30c.<sup>42</sup> Furthermore, many of the gene targets from interactome are in cancer pathways. However,



**Figure 5** Targeted interactome of microRNAs (miRs) (miR-30c and miR-331-3p). We performed an interactome analysis using targets from the KEGG (A) cancer pathway and (B) lipid/energy metabolism pathway. Yellow nodes: miRs; green nodes (hubs): targets from the lipid pathways; blue nodes: proteins interacting with the miR targets. miRs to target inhibitory connections are shown in red colour.

there may be a bias in that these miRs have been best studied in the context of cancer and their role in lipid metabolism has not yet been determined. Until done so, it is unclear what the role of miRs will be in developing new therapies for NAFLD/ NASH.<sup>43</sup>

MiR-331-3p and miR-30c were highly correlated with each other. Although this association does not necessarily imply a mutual involvement of a common mechanistic pathway, we investigated whether there are overlapping gene targets. Only seven genes were at the intersection of all the genes the two miRs are known to target. The functional role of these genes in NAFLD is still unknown, but SOCS1 is the only gene which has been associated with hepatic steatosis.<sup>44</sup> HDAC5 and MNT, in addition to SOCS1 have been associated with HCC.<sup>45–49</sup> Targeted PPIs show that the reach of these miR targets is quite broad, particularly in the cancer pathway.

Using a twin-study design, we demonstrate that serum miRs explain discordancy between twins with and without NAFLD. In particular, miR-331-3p and miR-30c not only explained NAFLD discordancy between twins but also were significantly different between participants with and without NAFLD. In addition, we showed that the serum levels of these miRs, as well as others, are heritable traits. Hence, miRs can be used as biomarkers for invasive assessment, or even therapeutic targets, of NAFLD.

#### Author affiliations

<sup>1</sup>NAFLD Translational Research Unit, University of California, San Diego, La Jolla, California, USA

<sup>2</sup>Division of Gastroenterology, Department of Medicine, University of California, San Diego, La Jolla, California, USA

<sup>3</sup>Department of Bioengineering, San Diego Supercomputer Center, University of California, San Diego, La Jolla, California, USA

<sup>4</sup>Departments of Computer Science & Engineering and Cellular & Molecular Medicine, University of California, San Diego, La Jolla, California, USA

<sup>5</sup>Division of Epidemiology, Department of Family Medicine and Public Health, University of California, San Diego, La Jolla, California, USA

**Correction notice** This article has been corrected since it published Online First. Dr Subramaniam has been added as co-corresponding author.

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# Serum microRNAs explain discordance of non-alcoholic fatty liver disease in monozygotic and dizygotic twins: a prospective study

Amir Zarrinpar, Shakti Gupta, Mano R Maurya, Shankar Subramaniam and Rohit Loomba

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