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Differential expression of miRNAs in the visceral adipose tissue of patients with non-alcoholic fatty liver disease

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SUMMARY

Background
Progression of non-alcoholic fatty liver disease (NAFLD) can be facilitated by soluble molecules secreted by visceral adipose tissue (VAT). MicroRNAs (miRNAs) are likely to regulate some of these molecular pathways involved in pathogenesis of NAFLD.

Aim
To profile miRNA expression in the visceral adipose tissue of patients with NAFLD.

Methods
Visceral adipose tissue samples were collected from NAFLD patients and frozen. Patients with biopsy-proven NAFLD were divided into non-alcoholic steatohepatitis (NASH) (n = 12) and non-NASH (n = 12) cohorts controlled for clinical and demographic characteristics. Extracted total RNA was profiled using TaqMan Human MicroRNA arrays. Univariate Mann–Whitney comparisons and multivariate regression analysis were performed to compare miRNA profiles.

Results
A total of 113 miRNA differentially expressed between NASH patients and non-NASH patients ($P < 0.05$). Of these, seven remained significant after multiple test correction (hsa-miR-132, hsa-miR-150, hsa-miR-433, hsa-miR-28-3p, hsa-miR-511, hsa-miR-517a, hsa-miR-671). Predicted target genes for these miRNAs include insulin receptor pathway components (IGF1, IGFR13), cytokines (CCL3, IL6), ghrelin/obestatin gene, and inflammation-related genes (NFKB1, RELB, FAS). In addition, two miRNA species, hsa-miR-197 and hsa-miR-99, were significantly associated with pericellular fibrosis in NASH patients ($P < 0.05$). Levels of IL-6 in the serum negatively correlated with the expression levels of all seven miRNAs capable of down regulating IL-6 encoding gene.

Conclusions
miRNA expression from VAT may contribute to the pathogenesis of NAFLD – a finding which may distinguish relatively simple steatosis from NASH. This could help identify potential targets for pharmacological treatment regimens and candidate biomarkers for NASH.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) comprises a range of chronic liver conditions that span from simple steatosis to non-alcoholic steatohepatitis (NASH). Steatosis occurs when, due to an inability by the liver to metabolize an excess of free fatty acids, triglycerides accumulate in hepatocytes. The prevalence of NAFLD in general population is about 25–30%, but in morbidly obese, it can reach up to 80–90%.[1–5] As many as 25–30% of NAFLD patients may subsequently develop NASH and are at higher risk for hepatic fibrosis, cirrhosis and hepatocellular carcinoma.[4]

Several chronic liver diseases, including NAFLD and NASH, have a strong association with visceral obesity.[6] Progression to NASH can be due, in part, to the changes in the levels of soluble signalling molecules such as cytokines and adipokines secreted by white adipose tissue (WAT). The list of these molecules includes adiponectin, leptin, resistin, visfatin, IL-1β, IL-6, IL-8, IL-18, C3, TNF-α and others.[6, 7] Adipokines and cytokines from white adipose tissue (WAT) are closely tied to insulin resistance and pathogenesis of NASH and NASH-associated fibrosis.[3] A number of therapeutic strategies (including weight loss) have been proposed to target these pathways and potentially improve patients' outcomes.[8]

MicroRNAs (miRNAs) are 21–23nt regulatory RNA molecules that suppress gene expression through either transcriptional degradation or translational repression. Over 30% of human genes are predicted to be regulated by miRNA.[9] MicroRNAs provide means for coordinated tissue-wide gene regulation worthy of investigation in patients with NAFLD. It is especially important to assess the role of miRNA in the WAT, as this tissue is most likely involved in the generation of the secreted milieu strongly associated with development of NASH.

This study investigates expression of all known human miRNAs in the visceral WAT of patients with NAFLD using qPCR arrays.

MATERIALS AND METHODS

Patient population

Tissue and serum samples were obtained from patients with biopsy-proven NAFLD. These samples were collected from 24 obese patients undergoing bariatric surgery for weight management. All patients had their serum obtained just prior to surgery and immediately frozen at −80. In addition, all patients had liver and omental fat biopsies performed at the beginning of their surgery. All liver biopsies were read by a single pathologist. Furthermore, extensive clinical and demographic information for all patients was collected at the same time. Thorough histories were obtained including drug and alcohol use, personal and family histories of diabetes mellitus, hypertension, or hyperlipidaemia (all were defined by clinical diagnosis requiring medical therapy). Data from presurgery physical examination, including height, weight, hip and waist measurements were collected. Laboratory tests included fasting glucose, serum aminotransferases (ALT and AST) and lipid panel. All the specimens and clinical data were collected from each patient after obtaining informed consent. Patients with evidence of excessive alcohol use (≥10 g/day), other causes of liver disease (e.g. hepatitis B, hepatitis C, autoimmune liver disease, iron overload) and those receiving treatment with PPAR-γ agonists were not included in this analysis. As previously noted, each patient's liver biopsy was read by a single hepatopathologist (ZG) using a standardized pathological approach (see below). All patients' liver biopsies showed NAFLD.

This NAFLD cohort was divided into those with histologically proven NASH (N = 12) and those with non-NASH type of NAFLD (N = 12). Cohorts were matched for age, race, gender, body mass index (BMI) and presence of diabetes mellitus (Table 1). In a subgroup analysis, NASH patients with pericellular fibrosis were compared with non-NASH type of NAFLD without pericellular fibrosis (Table 2).

For each patient, omental fat or visceral adipose tissue samples were collected and snap-frozen with liquid nitrogen. Samples were stored at −80 before miRNA expression assays. The study was approved by the Inova Health System Institutional Review Board.

Histopathology

Each liver biopsy specimen was fixed in formalin, routinely processed for histology, sectioned and stained with haematoxylin-eosin and Masson trichrome. The liver biopsies were read by a single experienced hepatopathologist (ZG) who was blinded to all clinical and laboratory data. The slides were reviewed according to a predetermined histological grading system. Haematoxylin and eosin stained slides were used to determine the extent of steatosis. The degree of steatosis was assessed in haematoxylin-eosin-stained sections and graded as an estimate of the percentage of tissue occupied by fat vacuoles as follows: 0 = none; 1 ≤ 5%; 2 = 6–33%; 3 = 34–66%; 4 ≥ 66%. Other histological features evaluated in haematoxylin-eosin sections included portal inflammation, lymphoplasmacytic lobular inflammation, polymorpho-

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Table 1 | Clinico-demographic and laboratory data for cohorts of NASH and Non-NASH patients. N.S., not significant ($P > 0.05$)

	NASH ($N = 12$)	Non-NASH NAFLD ($N = 12$)	*P*-value
Age, years (mean ± s.d.)	42.9 ± 13.6	49 ± 7	N.S.
Female, %	75% (9)	75% (9)	N.S.
Caucasian, %	83.3% (10)	83.3% (10)	N.S.
Body mass index (BMI) (mean ± s.d.)	48.4 ± 7.3	45.4 ± 7.8	N.S.
AST level, IU/L (mean ± s.d.)	25.2 ± 9.7	22.2 ± 8.2	N.S.
ALT level, IU/L (mean ± s.d.)	33.3 ± 14.0	30.8 ± 12.1	N.S.
AST/ALT (mean ± s.d.)	0.79 ± 0.18	0.75 ± 0.20	N.S.
Type 2 DM, %	50% (6)	50% (6)	N.S.
Fasting serum triglyceride, mg/dL (mean ± s.d.)	178.0 ± 112.2	161.8 ± 56.8	N.S.
Fasting serum cholesterol, mg/dL (mean ± s.d.)	185.3 ± 70.4	195.4 ± 23.0	N.S.
Fasting serum glucose, mg/dL	113.6 ± 52.0	111.8 ± 26.9	N.S.

Table 2 | Clinico-demographic and laboratory data for cohorts of NASH patients with pericellular fibrosis and patients with non-NASH. N.S., not significant ($P > 0.05$)

	NASH with pericellular fibrosis ($N = 6$)	Non-NASH NAFLD ($N = 6$)	*P*-value
Age, years (mean ± s.d.)	52 ± 6.4	44.2 ± 12.1	N.S.
Female, %	66.7% (4)	100% (6)	N.S.
Caucasian, %	83.3% (5)	83.3% (5)	N.S.
Body mass index (BMI) (mean ± s.d.)	46.3 ± 4.8	47.7 ± 10.0	N.S.
AST level, IU/L (mean ± s.d.)	26.7 ± 11.5	19.8 ± 9.5	N.S.
ALT level, IU/L (mean ± s.d.)	37.2 ± 18.4	29.2 ± 13.0	N.S.
AST/ALT (mean ± s.d.)	0.76 ± 0.15	0.70 ± 0.21	N.S.
Type 2 DM, %	50 (3)	66.67 (4)	N.S.
Fasting serum triglyceride, mg/dL (mean ± s.d.)	227.7 ± 129.9	163.8 ± 80.4	N.S.
Fasting serum cholesterol, mg/dL (mean ± s.d.)	223.0 ± 46.1	196.0 ± 12.5	N.S.
Fasting serum glucose, mg/dL	130.8 ± 49.5	110.4 ± 10.8	N.S.

nuclear lobular inflammation, Kupffer cell hypertrophy, apoptotic bodies, focal parenchymal necrosis, glycogen nuclei, hepatocellular ballooning, and Mallory-Denk bodies. NASH was defined as steatosis, lobular inflammation and ballooning degeneration with or without Mallory-Denk bodies and/or peri-cellular fibrosis with no history of alcohol abuse.

RNA extraction

Frozen visceral adipose tissue samples (~150 mg) were ground in 1 mL of the homogenization buffer from a mirVana miRNA Isolation Kit (Applied Biosystems, Carlsbad, CA, USA) using a Powergen 125 Homogenizer (Fisher Scientific, Hampton, NH, USA). Homogenate was then subjected to the mirVana miRNA Isolation Kit protocol for total RNA isolation according to manufacturers' instruction.

miRNA Reverse Transcription

Total RNAs, including miRNA from each patient sample, were assayed using two independent pulsed megaplex reverse-transcription (RT) reactions. Each reaction was

performed using 350–500 ng of total RNA combined with the components of TaqMan miRNA Reverse Transcription Kit (Applied Biosystems) and v2.0 Human Megaplex Reverse Transcription Primer Pool Set A and B (Applied Biosystems). While maintaining supplier recommended concentrations for each component, a final volume of 22.5 μL was used for each RT reaction. The RT reaction concentrations were as follows: (10×) RT Megaplex Primer Mix, (10×) RT Buffer, Multiscribe Reverse Transcriptase (10 U/μL), dNTPs with dTTP (0.5 mM each), $MgCl_2$ (3 mM) and Applied Biosystems RNase inhibitor (0.25 U/μL). Megaplex Primer pool A consists of 377 stem-loop RT primers, which are specific for mature miRNAs, as well as four endogenous controls. The Megaplex Primer pool B consists of 290 stem-loop RT primers specific for mature miRNAs, as well as four endogenous controls. A pulsed RT thermal cycling protocol was used as per the manufacturer's protocol (40 cycles of 16 °C for 2 min, 42 °C for 1 min and 50 °C for 1 s, with a final reverse transcriptase inactivation at 85 °C for 5 min).

Real-Time qPCR

Mature miRNAs were detected and quantified using the TaqMan miRNA Array v 2.0 (Applied Biosystems). Twelve microlitres of the Megaplex RT product was combined with the TaqMan Universal Master Mix NO UNG (Applied Biosystems) and loaded on the individual TaqMan miRNA Arrays as per the manufacturer's protocol. The arrays were run on the Applied Biosystems 7900 HT Real Time PCR instrument using the following thermal cycling conditions: 50 °C for 2 min followed by 94.5 °C for 10 min and 40 cycles of 97 °C for 30 s and 59.7 °C for 1 min. Raw cycle threshold (Ct) values were generated using the Applied Biosystems SDS 2.3 and RQ Manager 1.2 software. An automatic baseline combined with a global threshold of 0.2 (delta Reporter signal normalized or ΔRn) was used throughout the analysis. Target miRNA Ct values were normalized to the mammalian U6 snRNA endogenous control.

Serum cytokines

Serum samples were profiled with The Bio-Plex Human Cytokine 17-Plex panel was used with the Bio-Plex Suspension Array System (Bio-Rad, Hercules, CA, USA) to profile expression of 17 inflammatory mediators, including IL-6 and TNF-α. The assay was performed according to the manufacturer's instructions. Briefly, serum samples were thawed and then centrifuged at 2000 g for 3 min at 4-C. Serum samples were then incubated with microbeads labelled with specific antibodies to one of the aforementioned cytokines for 30 min. After a wash step, the beads were incubated with the detection antibody cocktail, each antibody specific to a single cytokine. After another wash step, the beads were incubated with streptavidinphycoerythrin for 10 min and washed, and the concentrations of each cytokine were determined using the array reader.

Analysis

Nonparametric groupwise Mann–Whitney comparisons and multivariate analysis with logistic regression were performed to compare subjects with NASH with Non-NASH NAFLD, or NASH patients with significant fibrosis with NASH patients without significant fibrosis. An analysis of correlations of serum cytokine levels with expression levels of individual miRNAs was also performed using Pearson's formula.

Functional analysis of differentially expressed miRNA and predicted target genes was performed using Ingenuity Pathway Analysis 7.5 (IPA) (Ingenuity Systems, Redwood City, CA, USA). Predicted miRNA target genes were obtained from miRBase (Sanger Institute) using the miRanda target prediction algorithm. In IPA analysis, expression values for predicted target genes were artificially set as equal to the number of differentially expressed miRNA species targeting given gene.

RESULTS

Patients' clinical and demographic data are summarized in Tables 1 and 2.

Differentially expressed miRNA in the adipose tissue of patients with NASH

In this study, we profiled a total of 664 human miRNA species. Interestingly, the global landscape of the statistically significant changes in miRNA expression in the visceral adipose tissue of NASH patients (Fold Change > −1.7, $P < 0.05$) was characterized by overall downregulation. A total of 113 species of miRNA were differentially expressed in the visceral adipose tissue of NASH patients compared with those with non-NASH type of NAFLD (|Fold Change| > 1.7, $P < 0.05$) (Table 3). Of these, seven differentially expressed miRNA species passed stringent multiple test correction, including hsa-miR-132 (Fold Change = −1.74, $P < 0.05$), hsa-miR-150 (Fold Change = −1.86, $P < 0.01$), hsa-miR-28-3p (Fold Change = −1.71, $P < 0.05$), hsa-miR-433 (Fold Change =

Table 3 | Differentially expressed miRNA species between NASH and non-NASH NAFLD

Name	*P* value	Fold Change
hsa-let-7a	<0.001	−4.93
hsa-let-7b	<0.01	−4.2
hsa-let-7c	<0.05	−3.37
hsa-let-7d	<0.05	−3.48
hsa-let-7f-2	<0.01	−3.58
hsa-miR-100	<0.05	−3.75
hsa-miR-107	<0.05	−3.92
hsa-miR-10a	<0.05	−1.94
hsa-miR-10b	<0.05	−2.49
hsa-miR-125a-3p	<0.01	−3.79
hsa-miR-125b	<0.05	−2.15
hsa-miR-132*	<0.05	−1.74
hsa-miR-133b	<0.05	−4.05
hsa-miR-134	<0.01	−3.02
hsa-miR-135a	<0.01	−4.98
hsa-miR-139-3p	<0.01	−3.03
hsa-miR-145	<0.05	−2.47
hsa-miR-150*	<0.01	−1.86
hsa-miR-16-1	<0.05	−3.5
hsa-miR-181a	<0.05	−2.41
hsa-miR-181a-2	<0.05	−2.77
hsa-miR-181c	<0.01	−4.27
hsa-miR-188-5p	<0.01	−4.67
hsa-miR-190b	<0.01	−250
hsa-miR-192	<0.01	−3.94
hsa-miR-193a-5p	<0.001	−3.12
hsa-miR-193b	<0.01	−4.65
hsa-miR-197	<0.01	−2.04
hsa-miR-19b-1	<0.05	−3.37
hsa-miR-200c	<0.05	−2.67
hsa-miR-206	<0.01	−5.38
hsa-miR-21	<0.05	−2.89
hsa-miR-210	<0.05	−2.22
hsa-miR-214	<0.01	−2.51
hsa-miR-23a	<0.01	−3.62
hsa-miR-26a-1	<0.01	−4.13
hsa-miR-26a-2	<0.01	−3.97
hsa-miR-27a	<0.05	−3.05
hsa-miR-27b	<0.05	−3.34
hsa-miR-28-3p*	<0.05	−1.71
hsa-miR-296-5p	<0.01	−2.16
hsa-miR-299-5p	<0.05	−2.29
hsa-miR-30a	<0.05	−1.83
hsa-miR-320	<0.05	−2.05
hsa-miR-323-3p	<0.05	−3.38
hsa-miR-324-3p	<0.05	−1.7
hsa-miR-324-5p	<0.05	−2.2
hsa-miR-328	<0.01	−2.35
hsa-miR-330-3p	<0.01	−3.79
hsa-miR-331-3p	<0.01	−1.82
hsa-miR-337-3p	<0.01	−4.17
hsa-miR-337-5p	<0.05	−4.9
hsa-miR-339-3p	<0.05	−2.22
hsa-miR-339-5p	<0.05	−2.3
hsa-miR-33a	<0.05	−3.03
hsa-miR-345	<0.05	−1.87
hsa-miR-361-5p	<0.05	−2.43
hsa-miR-374a	<0.01	−4.44
hsa-miR-378	<0.05	−2.48
hsa-miR-423-5p	<0.01	−6.45
hsa-miR-432	<0.01	−4.31
hsa-miR-433*	<0.05	−2.45
hsa-miR-483-5p	<0.05	−3.98
hsa-miR-485-3p	<0.05	−2.7
hsa-miR-487a	<0.01	−4.9
hsa-miR-502-3p	<0.05	−3.82
hsa-miR-502-5p	<0.05	−3.31
hsa-miR-505	<0.05	−2.46
hsa-miR-511*	<0.05	−2.11
hsa-miR-516a-3p	<0.05	−3.05
hsa-miR-517a*	<0.05	−3.82
hsa-miR-517c	<0.05	−3.6
hsa-miR-518e	<0.01	−3.92
hsa-miR-518f	<0.05	−4.22
hsa-miR-519b-3p	<0.01	−4.18
hsa-miR-519d	<0.05	−2.93
hsa-miR-522	<0.05	−3.07
hsa-miR-532-3p	<0.05	−1.73
hsa-miR-543	<0.001	−4.69

Table 3 | (Continued)

Name	*P* value	Fold Change
hsa-miR-550	<0.01	−6.75
hsa-miR-565	<0.001	−3.89
hsa-miR-572	<0.05	−3.11
hsa-miR-573	<0.05	−2.65
hsa-miR-574-3p	<0.01	−2
hsa-miR-576-3p	<0.05	−3.6
hsa-miR-582-5p	<0.05	−2.89
hsa-miR-604	<0.01	−4.2
hsa-miR-610	<0.001	−5.21
hsa-miR-618	<0.05	−3.42
hsa-miR-622	<0.001	−5.46
hsa-miR-625	<0.01	−1.85
hsa-miR-629	<0.001	−3.73
hsa-miR-630	<0.001	−5.1
hsa-miR-642	<0.01	−2.55
hsa-miR-643	<0.01	−3.8
hsa-miR-650	<0.05	−4.17
hsa-miR-652	<0.01	−2.59
hsa-miR-656	<0.01	−3.91
hsa-miR-671-3p*	<0.05	−2.65
hsa-miR-7	<0.05	−-3.85
hsa-miR-744	<0.05	−2.82
hsa-miR-768-3p	<0.01	−1.76
hsa-miR-875-5p	<0.001	−5.21
hsa-miR-885-5p	<0.01	−2.87
hsa-miR-886-3p	<0.01	−8.93
hsa-miR-886-5p	<0.05	−6.62
hsa-miR-9	<0.01	−4.2
hsa-miR-923	<0.05	−4.56
hsa-miR-92a	<0.01	−2.4
hsa-miR-941	<0.001	−5.92
hsa-miR-942	<0.01	−4
hsa-miR-99a	<0.05	−2.08
hsa-miR-99b	<0.01	−2.95

* miRNA species that passed multiple test correction.

−2.45, $P < 0.05$), hsa-miR-511 (Fold Change = −2.11, $P < 0.05$), hsa-miR-517a (Fold Change = −3.82, $P < 0.05$), hsa-miR-671-3p (Fold Change = −2.65, $P < 0.05$).

Differentially expressed miRNA in the adipose tissue of NASH patients with pericellular fibrosis

Thirty five species of miRNA were differentially expressed in the visceral adipose tissue of NASH patients with pericellular fibrosis vs. those with non-NASH (|Fold Change| > 1.7, $P < 0.05$) (Table 4). Two miRNA species, hsa-miR-197 (Fold Change = −2.6, $P < 0.01$) and hsa-miR-99b (Fold Change = −3.57, $P < 0.05$) passed multiple test corrections. Additionally, the expression of two miRNA species, hsa-miR-146b-3p (Fold Change = −4.88, $P < 0.05$) and hsa-miR-149 (Fold Change = −2.03, $P < 0.05$) were specifically altered only in the subgroup of patients with both NASH and pericellular fibrosis, but were not changed when entire NASH non-NASH NAFLD cohorts were compared.

Systems biology analysis

As a means to elucidate coordinated systemic changes in the visceral adipose tissue of NAFLD patients and to identify downstream genes likely to be deregulated as an indirect result of miRNA suppression of upstream targets, a knowledge-based approach was employed to analyse the data (IPA 7.5, Ingenuity Systems).

Functional analysis of the seven miRNA species differentially expressed in NASH indicated significant association with pathways involved in the development of the hepatocellular carcinoma and the cardiac diseases (Figure 1).

The same type of analysis performed on the nonstringent precorrection list of 113 miRNA species differentially expressed in the visceral adipose tissue of NASH patients produced similar results. Moreover, an analysis of the 35 miRNA species differentially expressed in the subset of NASH patients with pericellular fibrosis yielded the same two pathways; however, in this case, only hepatocellular carcinoma-related gene list passed cut-off *P*-value of less than 0.05 (Figure 2).

At the time of this analysis (July 2009), IPA 7.5 did not allow performance of direct analysis of the miRNA targets, as it did not include miRanda miRNA target gene knowledge base. To enable this analysis, we uploaded an entire set of miRanda-selected targets (9542) into the Ingenuity software. For each gene, the levels of gene expression were artificially set as equal to the number of miRNA species previously predicted to target that gene. Subsequent functional analysis of this dataset returned a set of pathways involved in a variety of cardiac diseases (Figure S1). It is notable that cardiac diseases have been associated with metabolic syndrome and obesity and more recently, with NAFLD and NASH. In addition, according

Table 4 | Differentially expressed miRNA species between NASH with Pericellular Fibrosis and non-NASH NAFLD

Name	*P* value	Fold Change
hsa-let-7b	<0.01	−5.08
hsa-miR-133b	<0.05	−8.85
hsa-miR-134	<0.05	−3.22
hsa-miR-139-3p	<0.01	−3.14
hsa-miR-146b-3p	<0.05	−4.88
hsa-miR-149	<0.05	−2.03
hsa-miR-150	<0.01	−2.07
hsa-miR-193a-5p	<0.05	−4.13
hsa-miR-193b	<0.05	−7.72
hsa-miR-197*	<0.01	−2.6
hsa-miR-23a	<0.01	−5.24
hsa-miR-296-5p	<0.05	−2.7
hsa-miR-299-5p	<0.05	−3.34
hsa-miR-324-5p	<0.05	−3.76
hsa-miR-328	<0.01	−2.87
hsa-miR-331-3p	<0.05	−1.98
hsa-miR-337-3p	<0.05	−5.95
hsa-miR-374a	<0.05	−6.94
hsa-miR-432	<0.05	−5.49
hsa-miR-532-3p	<0.01	−2.28
hsa-miR-543	<0.05	−6.45
hsa-miR-550	<0.05	−15.63
hsa-miR-565	<0.05	−4.17
hsa-miR-574-3p	<0.05	−2.27
hsa-miR-604	<0.01	−5.92
hsa-miR-610	<0.05	−6.94
hsa-miR-622	<0.05	−5.85
hsa-miR-629	<0.05	−5.13
hsa-miR-630	<0.05	−5.62
hsa-miR-656	<0.05	−4.61
hsa-miR-744	<0.01	−5.92
hsa-miR-875-5p	<0.05	−4.9
hsa-miR-92a	<0.01	−3.25
hsa-miR-941	<0.01	−6.9
hsa-miR-99b*	<0.05	−3.57

* miRNA species that passed multiple test correction.

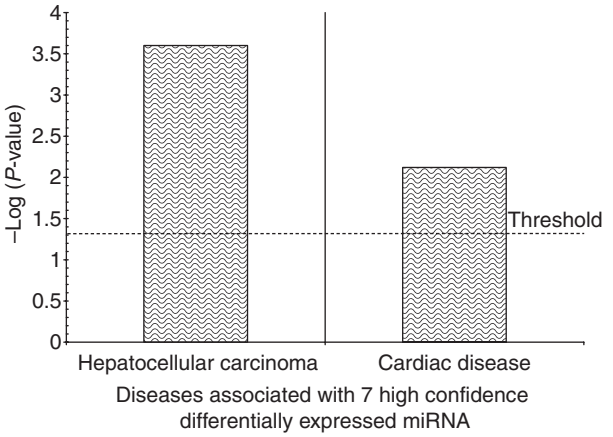

Figure 1 | IPA Toxicity analysis of the seven miRNA species differentially expressed in NASH that passed multiple text correction. The *X* axis is the disease or process most associated with the data set according to the IPA knowledge base, the *Y* axis is the −log of a *P* value generated by a Fisher Exact test (the threshold corresponds to a *P* of 0.05).

to the IPA knowledge base, miRanda-predicted target genes were associated with relevant hepatic diseases (Table 5); once again, of particular interest were the cytokines, especially those participating in inflammation.

Levels of miRNA expression negatively correlate with serum IL-6 levels

A profiling of Il-6 and TNF-α cytokines identified as targets of multiple miRNAs down regulated in NASH has been performed in nine serum samples (non-NASH, $n = 3$, NASH, $n = 6$). Levels of TNF-α were below detection threshold for six of nine samples, thus precluding analysis of correlations. Levels of IL6 negatively correlated with levels of each miRNAs down regulated in NASH (Table 6). For three of seven miRNAs capable of regulating IL-6, correlations reached statistical significance (hsa-miR-149: $R = -0.751$, $P < 0.02$; hsa-miR-574-3p: $R = -0.694$, 0.04; hsa-miR-132: $R = -0.755$, $P < 0.02$).

DISCUSSION

It is widely known that inflammation and insulin resistance-related molecular pathways play an important role in the development of NAFLD and its progressive form, NASH.[10–12] In obese subjects, both pathways are stimulated by the soluble molecules produced by excessive white adipose tissue. Furthermore, many genes differentially expressed in the white adipose tissue of patients

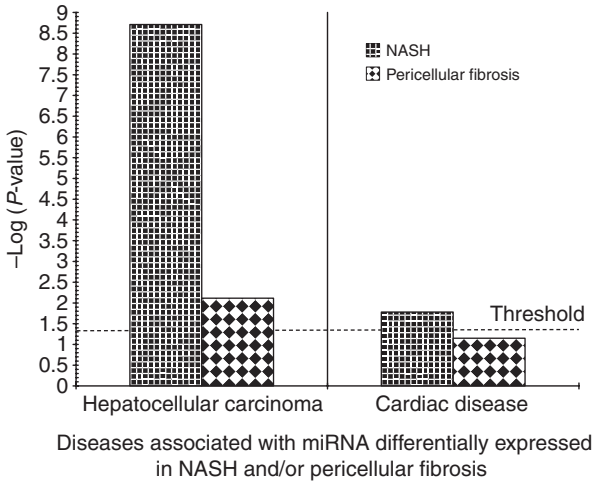

Figure 2 | IPA Toxicity analysis of total differentially expressed miRNA in NASH compared with total differentially expressed miRNA in the subset of patients with pericellular fibrosis.

with NAFLD are linked to the processes of insulin resistance or inflammation.[11, 12] It is possible that many of these targets are directly or indirectly regulated by miRNAs. Indeed, many molecules previously noted as differentially expressed in patients with NASH, such as V-rel reticuloendotheliosis viral oncogene homolog A (RELA) and STEAP family member 4 (STEAP4/STAMP2),[13, 14] have previously been identified in the miRanda database as potential targets of multiple miRNAs. In this study, we extended the list of the potential targets for miRNAs differentially expressed in NASH to include several soluble molecules previously associated with NAFLD, including IL6 and TNFα (Table 6). In an attempt to validate results of the miRNA profiling, we also measured levels of the target cytokines in the serum samples of nine study subjects. For each of seven miRNA down regulated in NASH patients, the serum levels of IL-6 were negatively correlated with the expression levels of individual miRNAs, thus, confirming the hypothesis that the expression of IL-6 encoding gene is under miRNA regulation. Although increases in the levels of IL-6 and many other soluble molecules produced by excessive white adipose tissue suggest the possibility that many of them are regulated by miRNAs, we cannot exclude the possibility that miRNA expression itself is regulated in some way by these inflammatory adipocytokines. To elucidate causative relationships between miRNA and soluble cytokine levels, future studies are warranted.

Most of the miRNAs differentially expressed in adipose tissue of NASH patients were down regulated. This observation was confirmed by careful examination of the levels of the mammalian U6 RNA used for normalization and the fact that several miRNA species were expressed at equal levels in all groups of the patients independently of the comparisons. Interestingly, Xie *et al.* noted an 'inverse regulatory pattern for many miRNAs during adipogenesis and obesity' while measuring the expression of over 370 miRNA species in leptin-deficient ob-/ob- and diet-induced obese mice.[15] Two miRNAs highlighted by this study, hsa-miR-125b and hsa-miR-26a, have been previously implicated in adipogenesis.[16–19] Recent study conducted by Klöting *et al.* showed that although visceral adipose tissue consistently produces more miRNA than subcutaneous adipose tissue, these miRNA levels are lower in the presence of the high levels of IL6, macrophage infiltration, or metabolic conditions such as type II diabetes.[17] Importantly, of 10 miRNA species shown to have a negative correlation with pathogenic factors mentioned above, four were independently found in our study: hsa-miR-132, hsa-miR-134, hsa-miR-181a and hsa-miR-99a. Of particular interest are hsa-miR-132

Table 5 | miRanda-predicted miRNA targets and their functions in the liver as highlighted by an analysis of knowledge base

Pathological conditions	miRanda-predicted target molecules
Chronic hepatitis (any aetiology)	ABCC2, ADORA3, AGTR1, BID, DRD5, ESR2, FAS, FASLG, FRAP1, IFNA2, IFNAR2, IGF1R, IL10, IL22, IL6, IMPDH1, IMPDH2, INSR, MGAM, MPL (includes EG:4352), PDX1 (includes EG:3651), POLA1, POLD1, PPP3CC, PTPRC, RELA (includes EG:5970), SLC6A4, SPP1, TIMP1, TNF, TNFRSF9, TNFSF10
Fibrosis of liver	C5, FGFR1, FGFR2, IL10, IL13RA2, LHX2, PLAU, SOCS1, XYLT2
Hepatic steatosis	ADFP, AMY2B, APOB, CPT1A, DGAT1, FGFR2, GPT (includes EG:2875), HADHA, IKBKG, IL10, LPA, MGAM, MPL (includes EG:4352), MTHFR, PDE11A, PDE3A, PDE4A, PDE4B, PDE4C, PDE4D, PDE7A, SCG5, SOD1, SOD2, SREBF1, THRA (includes EG:7067), TNF, UCN3
Necrosis of hepatocytes	AGER, FAS, FASLG, FGL2, HSPD1, IGF1, IGFBP1, ITGB2, NR1H4

Table 6 | Partial list of notable soluble molecules targeted by a significant number of differentially expressed miRNA

TNFSF14	TNFSF12	TNFSF11	TNF-α	IL15RA	IL6	CD40LG	LEP*
hsa-miR-885-5p	hsa-miR-768-3p	hsa-miR-565	hsa-miR-875-5p	hsa-miR-135a	hsa-miR-643	hsa-miR-582-5p	hsa-miR-875-5p
hsa-miR-629	hsa-miR-573	hsa-miR-543	hsa-miR-519b-3p	hsa-miR-330-3p	hsa-miR-576-3p	hsa-miR-522	
hsa-miR-574-3p	hsa-miR-550	hsa-miR-532-3p	hsa-miR-337-3p	hsa-miR-502-5p	hsa-miR-574-3p	hsa-miR-135a	
hsa-miR-519d	hsa-miR-516a-3p	hsa-miR-485-3p	hsa-miR-149	hsa-miR-516a-3p	hsa-miR-522	hsa-miR-133b	
hsa-miR-502-3p	hsa-miR-502-5p	hsa-miR-337-3p	hsa-miR-125b	hsa-miR-517a	hsa-miR-149		
hsa-miR-339-3p				hsa-miR-517c	hsa-miR-146b-3p		
hsa-miR-331-3p				hsa-miR-622	hsa-miR-132		
hsa-miR-197				hsa-miR-886-3p			
hsa-miR-150				hsa-miR-923			
hsa-miR-125b				hsa-miR-941			
hsa-miR-125a-3p							

* Although LEP is only targeted by a single differentially expressed miRNA, it is predicted to be only targeted by a total of five miRNA species.

which negatively correlates with macrophage infiltration, visceral area, LDL, and total cholesterol as well as hsa-miR-99a, which negatively correlates with free fatty acid levels and IL6 concentration.[17]

System-wide dysregulation of miRNA may be reflected in the coordinated changes in miRNA levels across a variety of tissues. Interestingly, eighteen of the miRNA species differentially expressed in the adipose tissue of NASH patients were also differentially expressed in the livers of NASH patients.[16] In addition, hsa-miR-26a has been previously shown to be down regulated in another liver disease, primary biliary cirrhosis.[20]

Of the microRNA species differentially expressed in patients with NASH and/or pericellular fibrosis, 21 were mapped to the introns or 5′ UTRs of other protein coding genes. Four of these miRNA species are positioned inside genes previously shown to be associated with obesity, liver disease, metabolic syndrome or type II diabetes, often through a decrease in their expression. For example, the IGF2 gene also encodes hsa-miR-483-5p. Allelic variation studies of IGF2 associate the gene with eating behaviour,[21] foetal growth[22] and obesity,[23] whereas other studies link it to diabetes.[24] An additional example is hsa-miR-642, which is located in the intron seven of the gene encoding gastric inhibitory polypeptide receptor (GIPR), also known as glucose-dependent insulinotropic polypeptide. According to at least one study, GIP/GIPR system is even more influential on eating behaviour than insulin/glucose metabolism.[25] Allelic variants of this gene have also been associated with obesity and metabolic syndrome.[26, 27] Furthermore, low expression of this gene in adipose tissue is associated with diabetes in non-obese women.[28]

Two differentially expressed miRNA species, hsa-miR-378 and hsa-miR-107, are located within genes associated with peroxisome proliferator-activated receptors (PPARs). The miRNA hsa-miR-378 is located within in the PPARγ coactivator gene PPARGC1B that has also been directly implicated in obesity, insulin resistance and type II diabetes.[29–33] The gene PANK1 contains hsa-miR-107; this gene encodes a key enzyme in CoA production and is modulated by PPARα.[34]

A significant number of the differentially expressed miRNA species are located within genes playing direct roles in cell signalling. The miRNAs hsa-miR-582-5P and hsa-miR-139-3p are located within PDE4D and PDE2A loci respectively; both genes participate in cAMP regulation.[35–37] The fifth intron of the mannose receptor MRC1 contains hsa-miR-511; this gene is involved in NFκB signalling.[38] Gene GCP1 contains hsa-miR-149,

one of the two microRNA species that showed a significant association with pericellular fibrosis, and is linked to FGF and TGFβ signal transduction.[39, 40] The gene that contains hsa-miR-26a, CTDSP2, is also associated with the TGFβ cascade as well as androgen signalling.[41, 42] Finally, SVIL and HNRNPK, which contain hsa-miR-604 and hsa-miR-7 respectively, are also important in androgen hormone signalling.[43, 44]

Understanding the differential expression of miRNA in the adipose tissue of obese patients is useful to advance understanding of mechanisms important to the development of NAFLD and NASH. Hopefully, novel insights into NAFLD pathogenesis could facilitate the development of the novel treatment modalities, such as miRNA replacement therapy. Our findings may also help develop novel diagnostic tools to distinguish patients with relatively benign simple steatosis from those patients with NASH, thus, enabling targeted intervention.

Limitations of this study include a relatively small sample size. This was to due to our desire to conduct a study that controlled for as many demographic and clinical factors as possible leading to the decrease in candidates for the study. Although this limitation could influence the *P*-values associated with specific findings and lead to a greater risk of type II error, it is unlikely to affect the hypothesis that miRNA is an important regulatory mechanism in the visceral adipose tissue impacting NAFLD. The limitation of cohort size does inevitably lead to limitations of the types of hypotheses that can be tested; our findings are restricted to miRNA expression as it pertains to NAFLD in a mostly Caucasian, mostly female, morbidly obese population. Therefore, assertions about the pathogenesis of NAFLD in individuals outside of this group cannot be made without additional experimentation. Another unavoidable limitation in our study stems from the lack of experimental data confirming particular interactions of miRNA and its targets. It is probable that the effect of miRNAs' dosage or combination of multiple miRNA species on the expression of a particular human gene could not be computed, but has to be determined experimentally.

In conclusion, this study has shown that miRNAs from visceral adipose tissue is differentially expressed in relation to the subtypes of NAFLD. Furthermore, several adipokines and cytokines that participate in the pathogenesis of NAFLD are either the predicted or proven targets of the miRNA species reported in this study. We believe that our findings offer a novel avenue for the pursuit of diagnostic tools and novel treatments, such as miRNA replacement, for patients with NAFLD.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. IPA implicated functions associated with miRanda predicted target genes.

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