Increased serum miR-193a-5p during non-alcoholic fatty liver disease progression: Diagnostic and mechanistic relevance

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Graphical abstract

Serum miRNA was sequenced in 183 NAFLD cases of varying severity and 10 population controls.

Plasma levels of miR-193a-5p were significantly increased in patients with advanced fibrosis, high NAS scores, or high SAF scores.

Other miRNAs including miR378d and miR378e were also significantly increased in certain comparisons.

The findings for miR-193a-5p were replicated in a cohort of 372 additional NAFLD cases.

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Background & Aims: Serum microRNA (miRNA) levels are known to change in non-alcoholic fatty liver disease (NAFLD) and may serve as useful biomarkers. This study aimed to profile miRNAs comprehensively at all NAFLD stages.

Methods: We profiled 2,083 serum miRNAs in a discovery cohort (183 cases with NAFLD representing the complete NAFLD spectrum and 10 population controls). miRNA libraries generated by HTG EdgeSeq were sequenced by Illumina NextSeq. Selected serum miRNAs were profiled in 372 additional cases with NAFLD and 15 population controls by quantitative reverse transcriptase PCR.

Results: Levels of 275 miRNAs differed between cases and population controls. Fewer differences were seen within individual NAFLD stages, but miR-193a-5p consistently showed increased levels in all comparisons. Relative to NAFL/non-alcoholic steatohepatitis (NASH) with mild fibrosis (stage 0/1), 3 miRNAs (miR-193a-5p, miR-378d, and miR378d) were increased in cases with NASH and clinically significant fibrosis (stages 2–4), 7 (miR193a-5p, miR-378d, miR-378e, miR-320b, miR-320c, miR-320d, and miR-320e) increased in cases with NAFLD activity score (NAS) 5–8 compared with lower NAS, and 3 (miR-193a-5p, miR-378d, and miR-378e) increased but 1 (miR-19b-3p) decreased in steatosis, activity, and fibrosis (SAF) activity score 2–4 compared with lower SAF activity. The significant findings for miR-193a-5p were replicated in the additional cohort with NAFLD. Studies in Hep G2 cells showed that following palmitic acid treatment, miR-193a-5p expression decreased significantly. Gene targets for miR-193a-5p were investigated in liver RNAseq data for a case subgroup (n = 80); liver GPX8 levels correlated positively with serum miR-193a-5p.

Conclusions: Serum miR-193a-5p levels correlate strongly with NAFLD activity grade and fibrosis stage. MiR-193a-5p may have a role in the hepatic response to oxidative stress and is a potential clinically tractable circulating biomarker for progressive NAFLD.

Keywords: MicroRNA; Non-alcoholic fatty liver disease; Biomarker; Sequencing.

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EASL The Home of Hepatology
Introduction

Non-alcoholic fatty liver disease (NAFLD) affects approximately one-quarter of the adult general population and is the most common cause of liver disease in the Western world. The complex, multisystem disease is characterised by intrahepatic triglyceride accumulation in the absence of excess alcohol intake. In a significant minority of patients, NAFLD can progress from steatosis (non-alcoholic fatty liver [NAFL]) to its inflammatory form (non-alcoholic steatohepatitis [NASH]) with hepatocyte injury and progressive fibrosis and may lead to cirrhosis, hepatocellular carcinoma, and end-stage liver disease.

NAFLD pathogenesis is multifactorial, and so, in addition to metabolic comorbidities and environmental influences, genetic and epigenetic factors confer an increased risk of NAFLD-associated end-stage liver disease. Inflammatory disease severity may be assessed histologically using one of 2 widely adopted semiquantitative scoring systems: the NASH Clinical Research Network NAFLD activity score (NAS), which confabulates the degree of steatosis with steatohepatitic activity grade, and the fatty liver inhibition of progression (FLIP) steatosis–activity–fibrosis (SAF) score, which preserves the distinction between steatosis and activity. Histological scoring of a liver biopsy remains the current reference standard for grading and staging NAFLD for clinical trials, even though sampling error as well as inter- and intra-observer discrepancies are well recognised to increase variability and result in misclassification. Consequently, circulating biomarkers have been sought to circumvent the need for invasive biopsies.

MicroRNAs (miRNAs) are small (~22 nucleotides in length) non-coding RNA molecules that can post-transcriptionally regulate gene expression. Mature miRNAs, complexed with facilitative proteins (i.e. the Argonaute protein family), exert their effects by binding mRNA molecules and inducing mRNA degradation or inhibiting translation. Currently, over 2,000 known human miRNAs are recorded on the miRBase database, some of which have been characterised as diagnostic biomarkers for diseases such as cardiovascular disease and cancer. Despite several studies postulating the association of various miRNAs with NAFLD, very few have been unequivocally replicated and validated. The lack of consensus may in part be caused by differences in study design, relatively small numbers of cases being studied, technical approaches, and variances within the chosen techniques. The most abundantly expressed miRNA known in adult liver, miR-122-5p, is possibly the strongest candidate NAFLD biomarker, having been consistently corroborated in independent studies. More recently, levels of another miRNA, miR-34a-5p, have been shown to increase in serum as NAFLD progresses, and miR-34a-5p has been incorporated in an in vitro diagnostic test to assist in the identification of patients with fibrosing steatohepatitis. Our aim was to identify additional serum miRNAs biologically relevant to NAFLD pathophysiology and progression with the capacity to serve as biomarkers. We report a comprehensive and unbiased profile of over 2,000 miRNAs in a large international cohort of patients with histologically characterised NAFLD, with separate discovery and replication performed using diverse techniques in independent patient cohorts.

Materials and methods

Patient recruitment and sample collection

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Cases were derived from the European NAFLD Registry (NCT04442334) with the collection and use of NAFLD patient data and samples approved by the relevant ethical committees in the participating centres and all patients having provided informed consent. The collection of blood samples from ‘healthy’ controls (no reported comorbidities or evidence of NAFLD) was approved by the County Durham and Tees Valley Research Ethics Committee (12/NE/012), and informed consent was given by all donors. Disease stage and grade of NAFLD samples were histologically proven by liver biopsy, with the serum samples taken within 6 months of said biopsy. Liver biopsy specimens were scored centrally by 2 expert liver pathologists (DT and PB) to ensure consistency in the analysis and interpretation of histology data. NAFLD was defined by the presence of steatosis in >5% of hepatocytes. Cases were divided into those with NAFL, if steatosis only or steatosis with lobular inflammation was present, and those with NASH, which is characterised by the presence of both lobular inflammation and hepatocellular ballooning in addition to steatosis. NAS was calculated as the unweighted sum of steatosis, ballooning, and lobular inflammation, whereas SAF activity was calculated as the unweighted sum of hepatocyte ballooning and lobular inflammation. NAFLD activity was graded as low (NAS 1–4 or SAF activity 0–2) and high (NAS 5–8 or SAF activity 3–4), and fibrosis staged as none/minimal fibrosis stage (F0–F1) and clinically significant (F2–F4).

Serum sample RNA extraction, library preparation, sequencing, and quality control

Serum samples (15 μl) were processed through the HTG EdgeSeq platform (HTG Molecular Diagnostics, Inc., Tucson, AZ, USA) using the EdgeSeq miRNA Whole Transcriptome Assay according to the manufacturer’s instructions. Briefly, the samples were lysed with a 1:1 ratio of HTG biofluids lysis buffer to extract RNA, and the lysates input into nuclease protection assays run on the HTG EdgeSeq processor. Sequence adapters were added to the samples in a 16-cycle PCR. PCR products were purified using AMPure XP beads (Beckman Coulter, High Wycombe, UK) and quantified using a KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA, USA). The barcoded libraries were pooled and 2,083 miRNAs (plus 5 negative controls, 1 positive control and 13 housekeeping genes) sequenced at the Genomics Core Facility, Newcastle University, UK, using an Illumina NextSeq 500 System (Illumina Inc., Cambridge, UK) according to the manufacturer’s instructions (single end run, 50 bp). FASTQ files were processed by the pre-installed HTG EdgeSeq parser software to align the probe sequences to the results, giving an output of raw count data. Quality control was conducted using the raw counts according to HTG guidelines. Delta mean values within 2 standard deviations (calculated by the manufacturer as 4.8 for serum samples) were considered acceptable.

miRNA sequencing data analysis

Data for the 2,083 sequenced miRNAs were analysed using RStudio version 1.0.143 (RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, USA, http://www.rstudio.com/). Raw
Development for R. RStudio, Inc., Boston, MA, USA, http://www.rstudio.com/). The NucleoSpin® miRNA Plasma Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany; distributed by Fisher Scientific UK, Loughborough, UK) according to the manufacturer’s instructions. Total RNA was eluted in 30 μl nuclease-free water and stored at -80°C.

miRNA analysis in the replication cohort

Total RNA extraction for analysis by quantitative reverse transcriptase PCR

Total RNA was extracted from serum using the MACHEREY-NAGEL NucleoSpin® miRNA Plasma Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany; distributed by Fisher Scientific UK, Loughborough, UK) according to the manufacturer’s instructions with only slight modifications to the protocol. Serum (130 μl) was vortexed with 90 μl Buffer MLP and incubated for 3 min at room temperature. As processing controls, 100 fmol of a non-phosphorylated exogenous synthetic cel-miR-39-3p and 100 fmol of a phosphorylated exogenous synthetic cel-miR-39-3p were spiked-in (5’-UCACC GGUGUA AAUCAGCUUG-3’; Integrated DNA Technologies, Inc., Coralville, IA, USA). Total RNA was eluted in 30 μl nuclease-free water and stored at -80°C.

Complementary DNA synthesis and quantitative reverse transcriptase PCR

Using the TaqMan™ Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, Paisley, UK), complementary DNA (cDNA) was synthesised from 3 μl total RNA by reverse transcription according to the manufacturer’s instructions. The miRNA reverse transcription product was diluted 1:10 in nuclease-free water and stored at -20°C. The expressions of 3 miRNAs, including the phosphorylated spike-in, were quantified using pre-formulated TaqMan™ Advanced miRNA Assays (Thermo Fisher Scientific, Armonk, NY, USA), according to the manufacturer’s instructions. Sample data were transformed into counts per million (CPM), and the miRNAs with a CPM ≤100 were filtered out of the raw count dataset. DESeq2 version 1.18.1 (https://bioconductor.org/packages/release/bioc/html/DESeq2.html) was used to generate a dataset – corrected for batch, age, and centre – from which principal component analysis (PCA) plots were constructed. Outliers were defined by visualisation of the PCA plots and removed from the dataset. Comparisons between each of the histological groupings (control, NAFL, NASH-F0/F1, NASH-F2, NASH-F3, and NASH-F4) and various subgroups were performed using limma version 3.34.9 (https://bioconductor.org/packages/release/bioc/html/limma.html). Statistical significance was determined using a Benjamini–Hochberg-adjusted p value ≤0.05; no log2 fold change (logFC) threshold was set. Area under the receiver operating characteristic (AUROC) curves was assigned based on the DeLong, DeLong, and Clarke-Pearson method in RStudio version 1.0.143 (RStudio: Integrated Development for R, RStudio Inc., Boston, MA, USA, http://www.rstudio.com/).

Histological characteristics

| Fibrosis grade | Sequencing cohort | Replication cohort | p value |
|----------------|------------------|--------------------|---------|
| F0             | 59 (32%)         | 43 (12%)           | <0.0001 |
| F1             | 21 (11%)         | 127 (34%)          |        |
| F2             | 47 (26%)         | 66 (18%)           |        |
| F3             | 38 (21%)         | 92 (25%)           |        |
| F4             | 18 (10%)         | 44 (12%)           |        |

Histological characteristics

| NAS score >4 | Sequencing cohort | Replication cohort | p value |
|--------------|------------------|--------------------|---------|
| Yes          | 128 (70%)        | 315 (85%)          | <0.0001 |
| No           | 55 (30%)         | 57 (15%)           |        |

Histological characteristics

| SAF activity score >2 | Sequencing cohort | Replication cohort | p value |
|-----------------------|------------------|--------------------|---------|
| Yes                   | 67 (37%)         | 147 (40%)          | 0.52    |
| No                    | 116 (63%)        | 225 (60%)          |        |

Table 1. Clinical data for the NAFLD serum samples used in sequencing and quantitative PCR replication.

| Patient demographic and clinical characteristics | Sequencing cohort | Replication cohort | p value |
|--------------------------------------------------|------------------|--------------------|---------|
| Sex (% female)                                   | 40%              | 41%                | 0.79    |
| Age in years (median [IQR])                      | 54 (44–60)       | 56 (47–64)         | 0.007   |
| BMI (median [IQR])                               | 31.6 (27.7–36.1) | 31.5 (28.4–36.5)   | 0.46    |
| AST in IU/L (median [IQR])                       | 37 (27–52)       | 42 (30–60)         | 0.014   |
| ALT in IU/L (median [IQR])                       | 53 (36–79)       | 57 (38–84)         | ns      |

Histological characteristics

| Fibrosis grade | Sequencing cohort | Replication cohort | p value |
|----------------|------------------|--------------------|---------|
| F0             | 59 (32%)         | 43 (12%)           | <0.0001 |
| F1             | 21 (11%)         | 127 (34%)          |        |
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Histological characteristics

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| SAF activity score >2 | Sequencing cohort | Replication cohort | p value |
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| Yes                   | 67 (37%)         | 147 (40%)          | 0.52    |
| No                    | 116 (63%)        | 225 (60%)          |        |

Counts were first transformed into counts per million (CPM), and the miRNAs with a CPM ≤100 were filtered out of the raw count dataset. DESeq2 version 1.18.1 (https://bioconductor.org/packages/release/bioc/html/DESeq2.html) was used to generate a dataset – corrected for batch, age, and centre – from which principal component analysis (PCA) plots were constructed. Outliers were defined by visualisation of the PCA plots and removed from the dataset. Comparisons between each of the histological groupings (control, NAFL, NASH-F0/F1, NASH-F2, NASH-F3, and NASH-F4) and various subgroups were performed using limma version 3.34.9 (https://bioconductor.org/packages/release/bioc/html/limma.html). Statistical significance was determined using a Benjamini–Hochberg-adjusted p value ≤0.05; no log2 fold change (logFC) threshold was set. Area under the receiver operating characteristic (AUROC) curves was assigned based on the DeLong, DeLong, and Clarke-Pearson method in RStudio version 1.0.143 (RStudio: Integrated Development for R, RStudio Inc., Boston, MA, USA, http://www.rstudio.com/).
Table 2. Differences in miRNA levels detected in the NAFLD-only sequencing analysis.

| miRNA     | logFC | logFC SE | Adj. p val.* |
|-----------|-------|----------|--------------|
| NASH grouped relative to NAFL |       |          |              |
| miR-193a-5p | 0.49  | 0.11     | 3.70E-03     |
| miR-378d   | 0.37  | 0.09     | 4.97E-03     |
| miR-378e   | 0.35  | 0.09     | 1.01E-02     |
| miR-4484   | 0.35  | 0.09     | 2.05E-02     |
| NASH with significant fibrosis (F2–F4) relative to NAFL/NASH with minimal fibrosis (F0/F1) |       |          |              |
| miR-193a-5p | 0.43  | 0.10     | 1.79E-03     |
| miR-378e   | 0.34  | 0.07     | 1.79E-03     |
| miR-378d   | 0.33  | 0.07     | 1.85E-03     |
| High NAS (NAS 5–8) relative to low NAS (NAS 1–4) |       |          |              |
| miR-193a-5p | 0.44  | 0.09     | 2.61E-04     |
| miR-378d   | 0.29  | 0.07     | 7.14E-03     |
| miR-320d   | 0.32  | 0.09     | 2.39E-02     |
| miR-320b   | 0.26  | 0.07     | 2.39E-02     |
| miR-320c   | 0.32  | 0.09     | 2.39E-02     |
| miR-320e   | 0.29  | 0.08     | 2.39E-02     |
| miR-320e   | 0.34  | 0.09     | 2.70E-02     |
| miR-378d   | 0.34  | 0.09     | 2.70E-02     |
| miR-19b-3p | -0.74 | 0.20     | 2.73E-02     |
| High SAF activity (SAF activity 3–4) relative to low SAF activity (SAF activity 0–2) |       |          |              |
| miR-193a-5p | 0.43  | 0.11     | 2.70E-02     |
| miR-378d   | 0.34  | 0.09     | 2.70E-02     |
| miR-320d   | 0.34  | 0.09     | 2.70E-02     |
| miR-378e   | 0.34  | 0.09     | 2.70E-02     |
| miR-320e   | 0.34  | 0.09     | 2.70E-02     |
| miR-19b-3p | -0.74 | 0.20     | 2.73E-02     |

Adj. p val., adjusted p value; logFC, log2 fold change; miRNA, microRNA; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; SAF, steatosis–activity–fibrosis.

Table 3. miRNAs showing significantly different levels in NASH grouped by fibrosis stage.

| F2 | F3 | F4 |
|----|----|----|
| miR-193a-5p | 0.41 | 0.22 |
| miR-378d | -0.79 | 0.48 |
| miR-378e | 0.35 | 0.43 |
| miR-378e | 0.35 | 0.45 |
| miR-4534 | -0.46 | -0.46 |
| miR-649 | -0.59 | -0.59 |
| miR-670a-3p | 0.37 | 0.22 |
| miR-6769a-5p | 0.22 | 0.27 |
| miR-25-3p | -0.61 | -0.51 |
| miR-330-3p | 0.25 | 0.25 |
| miR-5489-5p | -0.51 | -0.51 |
| miR-548d-5p | 0.35 | 0.27 |

miRNA levels were assessed by sequencing and are shown relative to NAFL. Adjusted p value threshold ≤0.05. F0/F1 showed no significant differences to NAFL. Adj. p val., adjusted p value; logFC, log2 fold change; miRNA, microRNA; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis.

Paisley, UK): 478293_mir (for the spiked-in cel-miR-39-3p), 477954_mir (hsa-miR-193a-5p), and 477855_mir (hsa-miR-122-5p). Each quantitative PCR (qPCR) mixture contained 5 μl diluted cDNA, 1× TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific, Paisley, UK), and 1× TaqMan™ Advanced miRNA Assay, and was made up to 15 μl with nuclease-free water.

TaqMan™ MicroRNA Reverse Transcription Kits (Thermo Fisher Scientific, Paisley, UK) were used to synthesise cDNA from 5 μl total RNA according to the manufacturer’s instructions for miRNAs where analysis with material generated via the Advanced miRNA Kit was unsuccessful owing to low levels. The reverse transcription reaction products were diluted 1:10 in nuclease-free water and stored at -20°C. The expressions of 3 miRNAs, including the non-phosphorylated spike-in, were quantified using pre-formulated standard TaqMan™ MicroRNA Assays (Thermo Fisher Scientific, Paisley, UK): 000200 (cel-miR-39-3p), 464645_mat (hsa-miR-3687) and 000426 (hsa-miR-34a-5p). Each qPCR reaction mixture contained 2.5 μl diluted cDNA, 1× TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific, Paisley, UK), and 1× TaqMan™ Small RNA Assay (Thermo Fisher Scientific, Paisley, UK) and was made up to 20 μl with nuclease-free water.
All qPCRs were run in triplicate in MicroAmp™ Fast Optical 96-Well Reaction Plates (Thermo Fisher Scientific, Paisley, UK) on a QuantStudio™ 5 Real-Time PCR machine (Thermo Fisher Scientific, Paisley, UK). PCR cycling conditions were 50°C for 2 min and 95°C for 20 s, followed by 40 cycles of 95°C for 1 s and 60°C for 20 s. A reference control, made of pooled cDNA, for each assay was run in triplicate on every plate, meaning the raw cycle threshold (Ct) values could be corrected for batch effects. The batch corrected data were analysed per miRNA by the 2-\(\Delta\Delta C_t\) method. Data were plotted using GraphPad Prism version 8.3.1 (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Statistical analyses were performed using the Mann–Whitney U and Kruskal–Wallis 1-way ANOVA tests.

**Target gene predictions and bioinformatic searches**

Two online databases were searched for predicted gene targets of miR-193a-5p, namely, TargetScan and miRDB, and the overlapping genes between the 2 were identified. Common genes were identified between the resulting predicted genes and the differentially expressed genes in NAFLD liver as described by Govaere et al. Normalised and transformed counts of miR-193a-5p, GPX8, and COL1A1 were correlated for the 80 overlapping samples for which miRNA-seq and RNA-seq data were available; a linear model was performed to obtain the slopes and p values of the correlations. Single-cell RNA-seq data for GPX8 and COL1A1 were extracted from the Liver Cell Atlas gene browser on 13 August 2020. Data on gene expression were obtained from the Gene Page of the Genotype-Tissue Expression (GTEx) Portal (GTEx Analysis Release V8; dbGaP Accession phs000424.v8.p2).

**Hep G2 cell culture and treatment**

The human hepatoblastoma Hep G2 cell line (ATCC® HB-8065™, Middlesex, UK), was cultured as described previously. To assess effects on miRNA expression, cells were treated for 24 h with the following: (i) 1% bovine serum albumin-conjugated palmitic acid (250 μM), (ii) 1% bovine serum albumin-conjugated oleic acid (500 μM), (iii) 1% bovine serum albumin-conjugated palmitic and oleic acid (250/500 μM) combined solution, and (iv) 1% bovine serum albumin as a lipid-loading control. All treatments were performed in

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**Fig. 2. Replication by qPCR of miR-193a-5p and miR-3687 associations.** Levels of miR-193a-5p are shown for (A) significant fibrosis (NASH F2–F4) relative to minimal fibrosis (NAFL–NASH F0/F1) (n = 359), (B) advanced NAS (NAS 5–8) relative to mild NAS (NAS 1–4) (n = 359), and (C) advanced SAF activity (SAF activity 3–4) relative to mild SAF activity (SAF activity 0–2) (n = 359). Levels of miR-3687 are shown for (D) significant fibrosis (NAFL–NASH F0/F1) (n = 371), (E) advanced NAS (NAS 5–8) relative to mild NAS (NAS 1–4) (n = 371), and (F) advanced SAF activity (SAF activity 3–4) relative to mild SAF activity (SAF activity 0–2) (n = 371). 2-\(\Delta\Delta C_t\) was calculated relative to controls for all samples. Median values are shown with 95% CIs. Mann–Whitney U tests were performed for all comparisons. NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; qPCR, quantitative PCR; SAF, steatosis–activity–fibrosis.
triplicate. Total RNA was isolated from the cells as described previously, and cDNA was synthesised and qPCR performed as described above using the TaqMan Advanced miRNA cDNA Synthesis Kit and TaqMan Advanced miRNA Assay for miR-193a-5p.

Results

Study cohort description

The current study comprised cases that were histologically characterised and represented the full spectrum of NAFLD severity from NAFL to NASH F4 (cirrhosis). The samples were divided between discovery (n = 183) and replication (n = 372) cohorts. Discovery cases to cover the range of NAFLD phenotypes were selected from those already recruited within the European NAFLD Registry by July 2018 with replication cases selected from the larger cohort available by June 2019. Demographic and clinical data are reported in Table 1. There were some significant differences between the cohorts. In particular, the discovery cohort included a relatively large number of NAFL cases without detectable fibrosis or inflammation to allow detailed comparisons between early and late disease. The replication cohort was significantly older with more severe disease and included a wider spectrum of phenotypes.

Comparison of NAFLD serum miRNA profiles with controls

The serum miRNA profiles of 183 patients across the NAFLD spectrum and 10 population controls were generated using the HTG EdgeSeq and Illumina NextSeq technologies. Complete sequence data are available as GSE185062 on Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/). Initial PCA confirmed that there was no clustering by centre, plate/batch, or age, indicating that batch correction was effective (Fig. S1). PCA showed that the control samples appeared to cluster together, suggesting they had a different miRNA profile to the NAFLD samples, although some overlap was observed (Fig. 1).

Relative to controls, levels of 275 serum miRNAs were different between the cohorts. Further 15 control individuals (9 male, 6 female, mean age 42 years) were included in the analysis with the discovery cohort. A further 15 control individuals (9 male, 6 female, mean age 42 years) were included in the replication cohort analyses.

Fig. 3. Differentially expressed predicted target genes of miR-193a-5p in liver RNA-seq. (A) RNA-seq data from liver tissue were analysed for 3 comparisons: advanced NAS (NAS 5–8) relative to mild NAS (NAS 1–4), advanced SAF activity (SAF activity 3–4) relative to mild SAF activity (SAF activity 0–2), and significant fibrosis (NAS F2–F4) relative to minimal fibrosis (NAFL–NAS F0/F1). The genes that overlapped with those predicted by in silico tools to be targets of miR-193a-5p are shown in the heatmap. Hierarchical clustering is based on levels of fold change in gene expression in the liver tissue. Statistical significance in the RNA-seq data was determined using a Benjamini-Hochberg adjusted p value ≤0.05: ‘p ≤0.05, ”p ≤0.01, and ***p ≤0.001. (B and C) Linear models of 80 overlapping NAFLD samples between the miRNA-seq and RNA-seq datasets for miR-193a-5p and (B) GPX8 and (C) COL1A1. AFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; SAF, steatosis–activity–fibrosis.
Hep G2 cells were treated with fatty acids (oleic acid [500 μM], palmitic acid [250 μM], or a combination of oleic [500 μM] and palmitic acid [250 μM]) for 24 h. All treatments were performed in triplicate, and qCRs of each were performed in triplicate. Data were normalised to the control condition (untreated) using the 2-△△CT method. An unpaired Student’s t-test was performed for all conditions relative to the control conditions (**p ≤ 0.01). Data are presented as the mean with error bars representing the SEM. qPCR, quantitative PCR.

3687 for which decreased levels in NAFLD were seen (fold change [FC] 0.10, p = 4.57×10^{-02}) (Table S2) When cases with NASH and different stages of fibrosis were compared with the population controls, in excess of 200 significant differences in miRNA levels were detected for each fibrosis stage, with the top 200 for each condition listed in Table S2. The most significant alteration was again the decrease seen for miR-3687 but for NASH F0/F1, F2, and F3 only.

Comparison of NAFLD miRNA profiles with NAFL as baseline
We next conducted an analysis amongst NAFLD cases only, excluding population controls. Using NAFL as the baseline, we initially looked for serum miRNA changes in NASH cases generally; 4 miRNAs showed increased levels with no miRNAs decreased (Table 2). The miRNA showing the largest increase was miR-193a-5p (FC 1.34, p = 3.70×10^{-03}). We subsequently characterised the miRNA profiles of NASH cases with particular stages of fibrosis (F0–F4) against NAFL (Table 3). The miRNA profile for NASH-F0/F1 was similar to that for NAFL; thereafter, progressive changes in miRNA levels were seen for cases with NASH-F2, NASH-F3, and NASH-F4 (cirrhosis), suggesting that it was the stage of fibrosis rather than grade of steatohepatitic activity that was driving the association. All changes seen in F3 and F4 were increases in miRNA levels, but for F2, both increases and decreases in levels were detected. MiR-193a-5p showed the most significant change with a progressive increase in levels from F2 (FC 1.32) via F3 (FC 1.65) to F4 (FC 1.84) (Table 3). These increases in miR-193a-5p levels with increasing fibrosis score were also in line with those seen for the NAFLD cases compared with population controls (Table S2).

To assess the potential for distinguishing between mild and more clinically significant disease, we undertook subgroup comparisons of the miRNA profile in relation to disease activity and fibrosis stage. Three miRNAs showed increased levels in clinically significant fibrosis compared with minimal disease (Table 2); the most statistically significant of these was miR-193a-5p (FC 1.35, p = 1.79×10^{-03}). When the grade of steatohepatitis was considered using NAS and SAF activity, 7 and 4 miRNAs respectively showed altered levels, with all but 1 of these changes an increase with increasing disease severity (Table 2). MiR-193a-5p again showed the most significant increase (NAS FC = 1.36, p = 2.61×10^{-04}; SAF activity FC = 1.35, p = 2.70×10^{-02}).

In light of its association with disease severity, we evaluated miR-193a-5p as a potential circulating biomarker of disease stage and activity by assessing its diagnostic performance in the sequencing dataset using AUROC statistics. AUROCs were also calculated and plotted for miR-122-5p as well as simple composite scores (the aspartate aminotransferase/alanine aminotransferase [AST/ALT] ratio and the fibrosis-4 [FIB-4] score). Relevant curves and values are detailed in Fig. S2. The AUROC for miR-193a-5p was 0.92 for discriminating NAFLD from population controls, whereas the values for differentiating mild disease from significant fibrosis (F0–1 vs. F2–4), high NAS, and high SAF activity were 0.68, 0.73, and 0.71, respectively. MiR-193a-5p significantly outperformed AST/ALT ratio when discriminating between mild vs. significant fibrosis (p = 0.04), high NAS (p = 6.00×10^{-05}), and high SAF activity (p = 0.03). It also had better discriminatory ability than FIB-4 as an indicator of high NAS (p = 3.40×10^{-03}) and exhibited comparable performance with FIB4 for fibrosis. The discriminatory ability of miR-193a-5p was better than that of miR-122-5p for fibrosis (p = 0.019), NAS (p = 0.012), and SAF activity (p = 1.53×10^{-03}).

Replication of miR193a-5 and previously reported miRNAs as predictors of NAFLD progression using qPCR in an independent cohort of samples
We sought to replicate selected findings from our sequencing studies relating to miR-193-5p and miR-3687 and additionally quantify serum levels of 2 miRNAs (miR-122-5p and miR-34a-5p) that have previously been reported as relevant to NAFLD, using the independent replication cohort of 372 NAFLD cases; healthy control samples (n = 15) were also included. Limited volumes of serum and inability to analyse using the Advanced miRNA kit prevented replication of other interesting miRNA signals. Consistent with the results of our sequencing analysis, miR-193a-5p showed significantly higher levels in NAFLD cases with greater disease severity (i.e. more significant fibrosis and high NAS/SAF activity) (Fig. 2). However, the differences observed in miR-3687 levels did not vary significantly with grade or stage of disease in the replication cohort (Fig. 2). Levels of miR-34a-5p were also significantly increased with a higher grade of NAFLD activity (by both NAS and SAF activity) and with more significant fibrosis (Fig. S3). In contrast, miR-122-5p levels did not show significance by fibrosis stage, but levels increased significantly in higher grades of NAFLD activity by both NAS and SAF activity (Fig. S3).

Correlation of miR-193a-5p target gene predictions with differential hepatic gene expression measured by RNA-seq in NAFLD
In view of the significant increase in serum miR-193a-5p levels seen as disease activity increased and its strong discriminatory ability, we sought to investigate the functional significance of miR-193a-5p in disease progression. Bioinformatic analysis using TargetScan27 and miRDB28 predicted that 204 and 327 genes, respectively, were targets of miR-193a-5p, with an overlap of 78 genes between both prediction sets. To better examine the transcriptional correlates of miR-193a-5p, we reanalysed our
recently reported hepatic transcriptomics dataset from 206 histologically characterised NAFLD cases to identify genes differentially expressed between mild and severe steatohepatitis and/or fibrosis.\(^{25}\) We then projected the list of 78 \textit{a priori} predicted miR-193a-5p targets onto these data. The resulting differentially expressed genes for each comparison are detailed in Fig. 3. Nine predicted gene targets of miR-193a-5p were significantly differentially expressed in the liver of patients with greater steatohepatitic activity by NAS (COL1A1, CRYPBC, GPX8, OLA1, RAP2A, SLC7A1, XK, ANK51A, and IGF2) and 3 by SAF activity (COL1A1, GPX8, and IFFO2), and 11 gene targets were differentially expressed in the liver of patients with significant fibrosis (COL1A1, GPX8, IFFO2, NETO2, PCDHA12, SLC7A1, ZNF827, GOLGAG6, ITSNI, KCNH1, and KMT2A). Additionally, the correlation between serum miR-193a-5p and levels in the liver of the 2 genes that were differentially expressed in all 3 comparisons, COL1A1 and GPX8, was investigated in a subgroup of 80 samples for which both serum miRNA-seq and liver RNA-seq data were available (Fig. 3). A significant positive correlation between miR-193a-5p and GPX8 was observed (slope = 0.35, \(p = 0.021\)), and there was a similar trend with COL1A1 (slope = 0.29, \(p = 0.074\)). Moreover, single-cell RNA-seq data extracted from the Liver Cell Atlas gene browser\(^{10}\) showed that both GPX8 and COL1A1 followed a similar pattern of increased expression in cirrhotic compared with uninjured tissue in the mesenchyme (Fig. S4).

### Quantification of miR-193a-5p expression in a hepatoma cell line

We utilised data from the GTEx Portal to visualise the tissue-specific expression of miR-193a-5p, confirming that – in addition to the renal medulla, omentum and subcutaneous adipose tissue, mammary tissue, and cultured fibroblasts – miR-193a-5p was expressed in the liver (Fig. S5). Considering this evidence, we characterised the expression of miR-193a-5p \textit{in vitro} in a model system of human hepatocytes in the context of lipid accumulation as seen in NAFLD (Fig. 4). Hep G2 cells were loaded with fatty acids, and miR-193a-5p expression was quantified by qPCR. After a 24-h treatment with lipotopic palmitic acid, miR-193a-5p expression was decreased significantly relative to the control condition (\(p = 0.009\)). A less lipotopic but more steatoenic lipid, oleic acid,\(^{32}\) had a negligible effect relative to the control condition, and, also in line with a previous report,\(^{32}\) there was no effect when the 2 lipids were combined.

### Discussion

An unbiased profile of circulating miRNAs in NAFLD patients and controls has been generated using high-throughput sequencing techniques. Although other studies have identified some changes in serum miRNA levels in NAFLD,\(^{17,33,34}\) our study is the largest of its kind – in terms of sample number and number of miRNAs analysed – spanning the full spectrum of the disease including cirrhosis. Although targeting only known miRNAs and unable to detect novel miRNAs, within these limitations we conducted a comprehensive and unbiased profile of miRNAs in NAFLD. Our sequencing approach revealed hundreds of miRNAs showing different levels at every NAFLD fibrosis stage relative to the controls, suggesting that these levels might correlate with, and therefore potentially predict, disease progression. We also saw numerous significant differences in miRNA levels within the entire NAFLD group compared with controls. Altered serum miRNA levels could be a useful diagnostic tool for NAFLD generally, but identifying such changes was not an aim of this study.

By combining the NAFLD sequencing data with an independent qPCR replication, we have shown consistently that serum levels of miR-193a-5p can distinguish between mild and advanced disease activity and fibrosis. Our finding for miR-193a-5p is in line with a recent report that performed miRNA sequencing of plasma samples from NAFLD cases but focussed on predominantly mild steatosis and fibrosis.\(^{34}\) We also saw increased levels of miR-122-5p, which was generally in line with previous reports,\(^{16,17,35}\) apart from a failure to detect significant differences with increasing fibrosis severity. This suggests that miR-193a-5p is a more sensitive biomarker of NAFLD progression than miR-122-5p. In addition to miR-193a-5p and miR-122-5p, we detected several other miRNAs that show differences in levels as NAFLD progresses, although the effect was not as significant as that for miR-193a-5p and overall levels of these miRNAs were lower. These additional significant miRNAs included miR-378d and miR-378e, which both increased in NASH and advanced fibrosis. This finding is in line with a recent sequencing study by Zhang et al.,\(^{32}\) who, while reporting that these 2 miRNAs were present at higher levels in plasma from NAFLD cases compared with controls and showed a significant relationship with extent of fibrosis, also found lower levels in plasma compared with miR-193a-5p, in both NAFLD cases and controls. We also saw significantly raised levels of miR-4484 in NASH compared with NAFLD. The previous sequencing study reported levels of this miRNA increased in severe steatosis but did not include data on inflammation.\(^{34}\) Our sequencing analysis did not detect changes in levels of miR-34a, another previously reported miRNA that increased in NAFLD,\(^{39}\) but we were able to confirm previous data for this relatively low-abundance miRNA as a significant marker for NAFLD progression by use of a highly sensitive qPCR assay. Indicating its potential usefulness as a biomarker, serum miR-193a-5p achieved high AUROC values in our study. There is increasing evidence that at least a proportion of circulating miRNAs is encapsulated within extracellular vesicles in NAFLD.\(^{10}\) The sequencing approach we used detects all miRNAs within serum without distinguishing between those present in vesicles and those circulating outside vesicles that are possibly protein-bound.

There is evidence from other studies that levels of miR-193a-5p are decreased in liver tissue in fibrosis\(^{37}\) and in hepatocellular carcinoma\(^{38}\) and that hepatic miR-193a-5p levels exhibit an inverse correlation with serum levels\(^{37,39}\) similar to that reported for other miRNAs including miR-122-5p.\(^{17,37}\) This would also be broadly consistent with our observations in Hep G2 cells where proapoptotic palmitic acid\(^{32}\) decreases miR-193a levels but steatogenic oleic acid, either alone or in combination with palmitic acid, has no significant effect. The miR-193a target genes, found to show altered expression during NAFLD in our transcriptomic analysis, all increased in expression, which is typical of genes regulated by miRNA with a decreased miRNA level facilitating increased mRNA levels. In a previous study, miR-193a-5p has been shown to act within a network of miRNAs to modify the transforming growth factor-
beta (TGF-β)-dependent regulation of extracellular matrix-related genes in hepatic fibrosis. It is likely, therefore, that miR-193a-5p in NAFLD will exert its effects on target genes as part of a large interaction network over a longer time course rather than, for example, as an immediate response to an acute stress. One of the other miRNAs that showed increased serum levels in severe fibrosis, miR-378d, has also been demonstrated to show decreased expression during fibrosis in an animal model.

Significantly differentially expressed predicted gene targets of miR-193a-5p in our transcriptomics data include RAP2A, IGF2, and SLC7A1, which have all been associated with hepatic function, liver disease, and/or steatosis. Most notably, we found COL1A1 and GPX8 showed increased expression by all 3 comparisons. A separate comparison using single-cell RNA-seq data also showed increased expression in cirrhotic cells compared with uninjured mesenchyme cells. COL1A1 encodes an extracellular matrix component that is extensively deposited in the development of scarring and fibrosis. Accordingly, it is a highly biologically plausible and relevant target of miR-193a-5p and has previously been identified as part of a transcriptomic gene signature associated with advanced NAFLD. Mir-193a-5p has also been shown to interact with COL1A1 in colorectal cancer cells and is known to be involved in extracellular matrix deposition. It is therefore plausible that secretion of miR-193a-5p by hepatocytes permits an increase in COL1A1 expression in stellate cells and thus contributes to hepatic fibrosis as proposed by others.

GPX8 is an endoplasmic reticulum (ER)-resident member of the glutathione peroxidase family that functions to protect cells from oxidative damage; oxidative damage may in turn lead to ER stress and is associated with metabolically driven NAFLD pathologies. Roy et al. have previously described the expression of miR-193a-5p in the liver and its role as a member of a network of miRNAs modifying the TGF-β-dependent regulation of extracellular matrix-related genes expression in hepatic stellate cells to modify the balance between deposition and resorption of liver fibrosis. Recently, it has been demonstrated that GPX8 expression increases during epithelial-to-mesenchymal transition in several cell lines with evidence that increased GPX8 affects IL-6/JAK-STAT3 signalling via the soluble IL6 receptor. Given that hepatic miR-193a-5p expression is known to exhibit an inverse correlation with serum levels, we therefore postulate that the increase in serum miR-193a-5p we observed in patients with advanced NAFLD is indicative of increased hepatocyte export. This could induce increased GPX8 expression in other hepatic cells as they respond to oxidative stress and undergo further transitions during NAFLD progression. Indeed, we observed a significant positive correlation between serum miR-193a-5p and hepatic GPX8. Nonetheless, further investigations are required to identify the specific cells in which GPX8 acts, as low hepatocyte expression in the single-cell RNA-seq dataset suggests that it may exert its effects elsewhere in the liver.

Published reports on miRNAs in NAFLD are seldom in complete agreement, and as such, one should be mindful of pertinent limitations. Discordance between studies could be caused by sampling and interpretation errors, and so the historical classification of patients’ disease might be inconsistent from the outset. Despite being a component of an in vitro diagnostic test for NASH, our sequencing analysis filtered out miR-34a-5p because of low levels, yet subsequent qPCR was in fact able to detect it. Conversely, the strongest signal in NAFLD relative to controls, miR-3687, was not confirmed by qPCR. These examples highlight that sequencing should always be replicated independently using a different methodology to test the strength of the original dataset. This approach combined with use of a replication cohort enabled us to confirm the novel miR-193a-5p signal. However, the qPCR approach also has limitations, and we were unable to replicate all the associations found by sequencing owing to limited serum availability. A higher throughput approach for replication, possibly using nanoString or a custom microarray would be helpful if a further replication cohort was available. Further studies on the functional significance of miR-193a-5p, especially direct confirmation of effects on specific target genes, and the identification of cells and possible extrahepatic tissues in which these interactions and downstream effects occur are still needed.

Overall, we have obtained an unbiased global profile of circulating miRNAs in NAFLD, and our study has identified a number of miRNAs that display changes in levels during disease progression. In particular, the increase in circulating miR-193a-5p was replicated independently, and we showed that several functionally relevant gene targets of this miRNA were differentially expressed in liver tissue. We have also further investigated the reported association of miR-122-5p and miR-34a-5p with NAFLD, finding that miR-122-5p was not a significant predictor of fibrosis progression but confirming that miR-34a-5p is a sensitive, although difficult to detect, marker. MiR-193a-5p had a superior ability to discriminate between mild and advanced NASH compared with AST/ALT and FIB-4 and could have value as an additional biomarker for progression, possibly in combination with other biologically relevant indices. The current study demonstrates how global miRNA profiling may provide both new insights into disease pathophysiology and identify promising new liver disease biomarkers.

Abbreviations
ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUROC, area under the receiver operating characteristic; cDNA, complementary DNA; CPM, counts per million; Ct, cycle threshold; ER, endoplasmic reticulum; FC, fold change; FIB-4, fibrosis-4; FLIP, fatty liver inhibition of progression; GTeX, Genotype-Tissue Expression; logFC, log2 fold change; miRNA, microRNA; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; PCA, principal component analysis; qPCR, quantitative PCR; SAF, steatosis–activity–fibrosis; TGF-β, transforming growth factor-beta.

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Conflicts of interest
The authors have no potential conflicts (financial, professional, or personal) directly relevant to the manuscript. Please refer to the accompanying ICMJE disclosure forms for further details.

Authors’ contributions
Conceptualisation: KJ, OG, QMA, AKD. Methodology: KJ, PJL, OG. Software: PJL, SJC. Validation: KJ. Formal analysis: KJ, PJL. Investigation: KJ, PJL, OG, MJ, SHC, DT, MZ, PB. Resources: JC, ME, GA, KC, JMS, JB, VR, EB, QMA, AKD. Data curation: KJ, PJL. Writing – original draft: KJ, QMA, AKD. Writing – review and editing: KJ, PJL, OG, MJ, SHC, SJC, DT, PB, MJ, JFC, ME, GA, KC, JMS, JB, VR, EB, QMA, AKD. Project supervision: QMA, AKD. Funding acquisition: QMA, AKD

Data availability statement
The complete miRNA sequence data are available on Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) (GSE185062). Other data from the study are available from the corresponding authors upon reasonable request.

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