High-throughput detection of novel circulating miRNA biomarkers of non-alcoholic fatty liver disease

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BACKGROUND

- Non-alcoholic fatty liver disease (NAFLD) is characterised by fatty liver in the absence of excess alcohol intake (Figure 1)
- Disease activity is graded using NAFLD activity score (NAS), calculated as sum of Kleiner inflammation, ballooning and steatosis histological scores
- Circulating biomarkers are sought to circumvent invasive liver biopsies

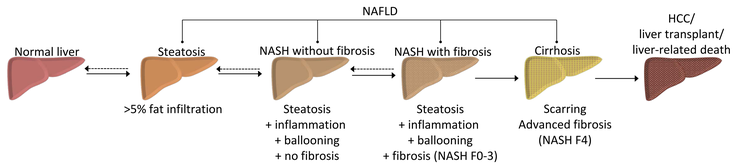

Figure 1. Progression of NAFLD. The disease is diagnosed and staged based on histological features. NAFLD can progress from simple fatty liver to NASH, fibrosis, cirrhosis and HCC. Cirrhosis and HCC are irreversible.

- MicroRNAs (miRNAs) are small (~22 nt) non-coding, single-stranded RNA molecules that post-transcriptionally regulate gene expression (Figure 2)
- Liver miRNAs could function in NAFLD before release into circulation
- Previous studies postulate the association of many miRNAs with NAFLD, including miR-122,[1] miR-34a,[2] miR-21,[3] miR-192[3] and miR-375[4]

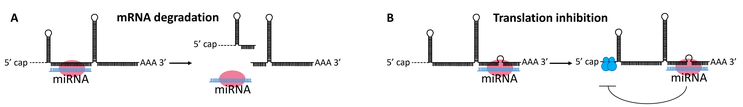

Figure 2. Post-transcriptional miRNA regulation of gene expression. The mechanism of miRNA action depends on seed region (bases 2-7) complementarity with the 3′ UTR of target mRNA. **(A)** Complete complementarity leads to major mode of action, mRNA degradation. **(B)** Partial complementarity is associated with translation inhibition.

AIMS

- To characterise the serum miRNA profiles of a large cohort of histologically characterised NAFLD patients across the full spectrum of disease
- To identify miRNAs that correlate with histological disease severity and progression relative to healthy controls

METHODS

- Serum samples taken from 199 NAFLD patients and 10 healthy controls
- Next-generation sequencing libraries prepared and 2,083 miRNAs sequenced using HTG EdgeSeq and Illumina NextSeq, respectively (Figure 3)
- Data analysed using limma in R software environment
- MiRNAs with a mean CPM ≥100, a log2 fold-change (logFC) of ≥0.3 and an adjusted *p* value ≤0.05 classified as differentially expressed
- Quantitative PCR (qPCR) used to replicate findings in subset of sequenced samples; signals currently being validated in independent NAFLD cohort

Figure 3. Preparation and sequencing of miRNA libraries. Fifteen microlitres of serum from each patient were processed through HTG EdgeSeq. The libraries were sequenced using Illumina NextSeq. Data were parsed through HTG EdgeSeq before count data were assessed for quality and analysed using R.

RESULTS 1 – SEQUENCING QUALITY CONTROL (QC)

- Phred sequencing quality scores all very good (Figure 4A)
- QC performed according to HTG guidelines; all but two samples passed
- 14 outliers removed based on PCA plot (Figure 4B)

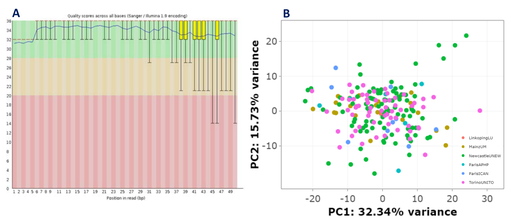

Figure 4. Quality control of sequencing data. (A) Representative example FASTQC plot showing high quality reads. QC performed according to HTG guidelines; two samples removed. **(B)** PCA plot of the data with 19 outliers removed. No clustering observed based on sex, centre or batch or disease stage.

- 183 NAFLD samples and 10 healthy controls carried forward
- 514 miRNAs retained after filtering data to remove background noise

RESULTS 2 – DIFFERENTIAL miRNA EXPRESSION

- 121 significantly differentially expressed miRNAs in NAFLD combined compared to healthy controls (Figure 5A)
- Increase of miR-122 in NAFLD relative to healthy controls is in agreement with previous reports;[1] logFC = 1.07, adj. $p = 2.17 \times 10^{-02}$
- MiR-193a is most significantly differentially expressed miRNA, upregulated in:
  - NAS 5-8 vs NAS 1-4; logFC = 0.68, adj. $p = 3.04 \times 10^{-07}$, AUC 0.71 (Figure 5B)
  - Individual NAFLD stages vs controls (Figure 5C)
  - NAFLD grouped vs controls; logFC = 1.52, adj. $p = 3.47 \times 10^{-10}$, AUC 0.94

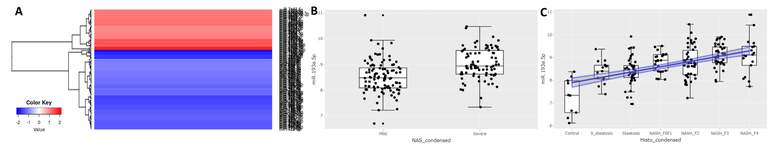

Figure 5. NAFLD miRNA signature. (A) 121 miRNAs were differentially expressed in NAFLD relative to controls. MiR-193a expression was increased in **(B)** NAS 5-8 relative to NAS 1-4, and **(C)** NAFLD stages relative to controls.

RESULTS 3 – REPLICATION BY qPCR

- Eight miRNAs selected for replication by qPCR
- qPCR confirms significantly increased expression of miR-193a in NAS 5-8 relative to NAS 1-4, and in NAFLD (grouped and individual stages) relative to healthy controls (Figure 6)
- Significant increase of miR-122 in NAFLD relative to controls was confirmed

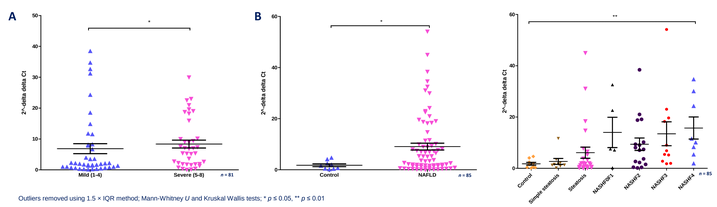

Figure 6. Replication of miR-193a expression using qPCR. The expression of miR-193a was increased in **(A)** severe NAS (5-8) relative to mild NAS (1-4), and **(B)** NAFLD grouped and **(C)** NAFLD stages relative to controls in a replication cohort of samples that were previously sequenced.

CONCLUSIONS

- 121 significantly differentially expressed miRNAs in NAFLD relative to controls
- Eight significant miRNA signals selected for replication and validation
- MiR-193a differentiates controls from NAFLD; expression increases with disease activity and so may serve as a potential biomarker for NASH

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