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miRNA-Seq identifies a serum miRNA panel, which combined with APRI can detect and monitor liver disease in paediatric Cystic Fibrosis

by

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List of abbreviations:

ΔΔCT	Comparative CT method
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	1-way analysis of variance
APRI	Aspartate aminotransferase to platelet ratio index
AST	Aspartate aminotransferase
AUC	Area under the ROC curve
AUROC	Area under the ROC curve
CF	Cystic fibrosis
CFLD	Cystic fibrosis liver disease
CFnoLD	Cystic fibrosis with no liver disease
CFTR	Cystic fibrosis transmembrane regulator gene
CI	Confidence interval
C-statistic	Concordance statistic
FC	Fold change
FIB-4	Fibrosis-4
HCC	Hepatocellular carcinoma
HSC	Hepatic stellate cells
IQR	Interquartile range
miRNA	microRNA
NAFLD	Non-alcoholic fatty liver disease
NGS	Next-generation sequencing
qRT-PCR	Reverse transcription-quantitative polymerase chain reaction
ROC	Receiver operating characteristics
r_s	Spearman's correlation coefficient
RT	Reverse transcription

SD	Standard deviation
SEM	Standard error of mean
TGFβRI	TGFβ-receptor 1
TIMP-4	Tissue inhibitor of matrix metalloproteinase 4
WHO	World Health Organization
GGT	γ-glutamyl transpeptidase

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ABSTRACT

Cystic fibrosis (CF)-associated liver disease (CFLD) is a hepatobiliary complication of CF. Current diagnostic modalities rely on non-specific assessments, while liver biopsy is the gold standard to assess severity of fibrosis. MicroRNAs (miRNAs) regulate liver disease pathogenesis and are proposed as diagnostic biomarkers. We investigated the combined use of serum miRNAs and aspartate aminotransferase to platelet ratio (APRI) to diagnose and assess CFLD severity. This was a cross-sectional cohort study of the circulatory miRNA signature of 124 children grouped by clinical, biochemical and imaging assessments as follows: CFLD (n=44), CF patients with no evidence of liver disease (CFnoLD, n=40) and healthy controls (n=40). Serum miRNAs were analysed using miRNA-sequencing. Selected differentially expressed serum miRNA candidates were further validated by qRT-PCR and statistical analysis performed to evaluate utility to predict CFLD and fibrosis severity validated by liver biopsy, alone or in combination with APRI. Serum miR-122-5p, miR-365a-3p and miR-34a-5p levels were elevated in CFLD compared to CFnoLD, while miR-142-3p and let-7g-5p were downregulated in CFLD compared to CFnoLD. Logistic regression analysis combining miR-365a-3p, miR-142-3p and let-7g-5p with APRI showed 21 times greater odds of accurately predicting liver disease in CF with an AUROC=0.91 (sensitivity=83%, specificity=92%; P<0.0001). Expression levels of serum miR-18a-5p were correlated with increasing hepatic fibrosis stage in CFLD (r_s=0.56, P<0.0001), showing good diagnostic accuracy for distinguishing severe (F3-4) from mild/moderate fibrosis (F0-2). A unit increase of miR-18a-5p showed a 7-fold increased odds of having severe fibrosis with an AUROC=0.82 (sensitivity=93%, specificity=73%; P=0.004), indicating its potential to predict fibrosis severity. ***Conclusion:*** We identified a distinct circulatory miRNA profile in pediatric CFLD with potential to accurately discriminate liver disease and fibrosis severity in children with CF.

Cystic fibrosis-associated liver disease (CFLD) is the leading cause of non-respiratory mortality and morbidity associated with cystic fibrosis (CF). Abnormal cystic fibrosis transmembrane conductance regulator (CFTR) is proposed to lead to the inspissation of viscid bile in the biliary tree, followed by biliary obstruction, hepatocyte and cholangiocyte injury and periductal inflammation resulting in fibrosis and cirrhosis (1). During the recent decades, the median life span of patients with CF has dramatically improved as a result of improved respiratory and microbial management and is expected to exceed 50 years of age in patients born after the year 2000 (2). Further, with the advent of CFTR modulators and anticipated life expectancy of 70 years of age by some estimates (3), vigilant monitoring of non-pulmonary complications such as CFLD is paramount.

According to the World Health Organization (WHO), CF affects 1 out of 2,500 newborns (4), 15.6% of whom are eventually diagnosed with clinically significant liver abnormalities and, prior to the advent of transplantation, was responsible for the death of up to 5% due to end stage liver disease (5). The true prevalence of CFLD, however, could be as high as 70% based on autopsy studies (6). The true impact of CFLD on mortality has been underestimated as demonstrated by Lewindon *et al* and Rowland *et al* who report increased all cause, childhood mortality in those with CFLD (7, 8). The diagnosis of CFLD prior to the advancement of cirrhosis and portal hypertension is challenging. Liver biopsy is not widely popular as it is invasive and management pathways are not well elucidated. Alone, it may be unreliable given sampling limitations and the known focal distribution of CFLD lesions; however, it is considered the gold standard to assess the severity of liver damage in CFLD (7).

Putative biomarkers of CFLD have focused on biological and physical properties to indirectly assess the degree of fibrosis in the liver. Studies have shown increased expression of tissue inhibitor metalloproteinase 4 (TIMP-4) and endoglin in serum of adult CFLD patients (9), while the aspartate aminotransferase (AST) to platelet ratio index (APRI) and the fibrosis-4 (FIB-4) index have shown value as biomarkers for the diagnosis and severity of CFLD in children (10). Other non-invasive alternatives to liver biopsy such as imaging-based transient elastography have been used to indirectly assess liver fibrosis through the measurement of liver stiffness which increases with the progression of fibrosis and cirrhosis. However, the value of elastography in the detection/prediction and monitoring of liver disease and fibrosis in CF has not been fully established, primarily due to the lack of liver biopsy-validated CFLD patients in these studies (11-13).

miRNAs are short interfering RNAs which silence gene expression at a post-transcriptional level. Altered miRNA expression has been associated with a broader fibrogenic response or liver damage (14). Furthermore, miRNAs are accessible in body fluids, are disease- and tissue-specific, and are stable in the circulation, suggesting their potential utility as biomarkers of disease (15). Despite the increasing number of studies investigating the use of miRNAs as biomarkers for chronic liver disease, only a few have been conducted in paediatric liver diseases (reviewed elsewhere (16)), and only one study, using a limited miRNA PCR array, has focused on CFLD (17). We hypothesized that there is an alteration in the circulating miRNA signature of children with CFLD that can be used to diagnose liver disease and assess the progression of fibrosis severity among the CF population. We have used next-generation sequencing (NGS) to identify differentially expressed serum miRNAs in children with liver biopsy-validated CFLD vs. children with CF without evidence of liver disease (CFnoLD) and healthy

controls. Selected miRNA candidates were then validated by reverse transcription-quantitative polymerase chain reaction (qRT-PCR) in an extended patient cohort.

METHODS

Patients

This study was approved by the human research ethics committees of the QIMR Berghofer Medical Research Institute (Brisbane, Australia) and the Lady Cilento's Children Hospital (Brisbane, Australia). Enrollment of patients and sample collection were conducted between 1997 and 2016. Parental informed consent was obtained for every patient prior to the start of the study. Sera from 124 well characterized children were selected and allocated in one of three study cohorts as described: children with CFLD (n=44) were selected by the presence of at least two of the following signs or symptoms: hepatomegaly ± splenomegaly; persistent elevation (> six months) of serum alanine aminotransferase (ALT >1x upper limit of normal) and abnormal ultrasound scan (showing abnormal echogenicity or nodularity suggesting cirrhosis), as has previously been described (6, 7, 18). All patients had percutaneous dual-pass liver biopsies to assess CFLD and stage liver fibrosis, as previously described (7). Liver histology was staged for hepatic fibrosis by two independent and blinded pathologists based on the METAVIR scoring system. Severity of fibrosis was assessed as follows: F0: no fibrosis; F1: portal fibrosis only (minimal scaring); F2: portal fibrosis with rare fibrous septa; F3: portal fibrosis with numerous fibrous septa and F4: cirrhosis. The higher fibrosis stage was used if fibrosis stages between the two biopsies were not the same. From the 44 biopsy-proven CFLD children, fibrosis staging was as follows: F0, n=14; F1, n=8; F2, n=8; F3, n=8; F4, n=6. Age- and sex-matched children with CF but no signs of liver disease by clinical, biochemical or ultrasonographic assessment at enrolment and over the two year duration prior to enrollment/sampling were selected as CF no liver disease (CFnoLD, n=40). No patients with CF were on CFTR modulators at the time of enrolment in this study. A group of non-CF and non-liver disease age- and sex-matched paediatric control patients were enrolled as the healthy control group (n=40), while attending the hospital for minor routine procedures such as: orthopaedic, plastic, otolaryngology and burns consultations. APRI and FIB-4 scores were calculated as described previously (10) on CF patients with available liver biochemistries and platelet counts (CFLD, n=36 and CFnoLD, n=36).

RNA extraction

Total free circulating RNA was extracted from 200µL serum using the Plasma/Serum RNA Purification Mini Kit (Norgen Biotek Corporation, Thorold, Canada) following manufacturers' instructions with minor modifications. RNA extraction is described in detail in the supplementary Methods.

Small RNA library construction and Next-Generation Sequencing (NGS)

Small RNA libraries were constructed from an initial subset of 90 serum samples corresponding to the three study cohorts: controls (n=30), CFnoLD (n=30) and CFLD (n=30). Following RNA extraction, ten RNA samples were pooled together to create three small RNA libraries (*i.e.,* 10 patients/library) per study cohort. This strategy, adapted from previous studies (19, 20), maintained the precision and statistical power obtained from the sample size calculation (see Statistical analysis of the Methods section). Libraries were prepared using the Ion Total RNA-Seq Kit v2 protocol (Thermo Fisher, Revision E) and sequenced on the Ion Proton System, using the Ion PI Chip v3 following the Ion PI Hi-Q Sequencing 200 Kit protocol (Thermo Fisher, Revision C). Small RNA library construction and NGS are described in detail in supplementary Methods.

Reverse transcription (RT) and qRT-PCR Validation

Due to low yields of miRNA in serum and the limits of detection of traditional RNA quantification methods, a fixed RNA volume input of 4µL for every sample was used in each reaction, a common strategy when working with circulatory miRNAs (17, 21). Reverse transcription was performed using the miScript II RT Kit (Qiagen, Venio, The Netherlands) following manufacturer's instructions. qRT-PCR was performed on 124 samples using the miScript SYBR Green PCR Kit (Qiagen) as suggested by the manufacturer with 2µL of diluted cDNA added in triplicate for each sample. miRNA primers were designed using the miR primer algorithm (22). RT and qRT-PCR validation are described in detail in Supplementary Methods. miRNA primers sequences are found in **(Supp. Table 1)**.

Sequence data acquisition and analysis

Sequencing data was analysed using the Torrent Suite v5.0.4 (Thermo Fisher) and mirPRo software pipeline (23). Differential expression analysis was performed in R v3.3.2 (24) using the DESeq2

v1.15.28 package (25). NGS sequence data acquisition and analyses are described in detail in Supplementary Methods.

qRT-PCR data analysis and Validation of reference miRNAs

Quantification cycle (Cq) values were determined using the CFX Manager Software v3.1 (Biorad) with the linear regression setting. Relative quantities of miRNA expression were calculated using the comparative CT method. NormFinder and geNorm algorithms were used in a subset of 30 independent samples to find the most stable expressed endogenous miRNAs. miR-93-5p, miR-20a-5p and let-7i-5p were used as endogenous reference miRNAs. Validation of endogenous miRNA is described in detail in Supplementary Methods.

Statistical analysis

All statistical analyses were performed in GraphPad Prism v7 (GraphPad Software, San Diego, USA) and STATA v15 (Stata Corp LLC, Texas, USA), with P values <0.05 considered statistically significant. Sample size calculation was based on a previous study (17) using a method developed by Hart *et al.* (26), showing that a minimum of 10 samples per group are required to detect a fold change of two or greater, reading depth of 20X, 80% of power and 5% error.

Normalised qRT-PCR data was transformed to a log2 base scale and analysed using One-way analysis of variance (ANOVA) and Tukey's post-hoc test. Correlation analyses were performed using Spearman rank correlations. Receiver operating characteristics (ROC) analysis was used to assess the performance of selected miRNAs to distinguish between CFnoLD and CFLD children. miRNA panel calculations to differentiate study groups were based on stepwise logistic regression using the lowest Bayesian information criterion for model selection and Liu's method to determine the optimal cut-point. Performance of the miRNA panels were evaluated using 5-fold cross validation statistical evaluation. During the 5-fold cross validation the observations are randomly divided into five groups of equal sizes, using one of the groups as validation set while the others are fitted into the model. Considering that the METAVIR scoring system consists of five categories, concordance statistic (c-statistic) and Obuchowski measures were used as predictive discriminators of fibrosis staging in CFLD children; in contrast to ROC curve analysis, c-statistic and Obuchowski measure do not dichotomise the observations (more detail is described in Supplementary Methods).

Data deposition

Raw NGS data used in this study are available from the Gene Expression Omnibus (GEO) under accession number GSE111754.

RESULTS

Patient characteristics

For this study, sera from 124 children were collected. The number of subjects for each of the study groups included 40 controls, 40 CFnoLD and 44 CFLD. There was no significant difference in age or sex between the CFLD and CFnoLD cohorts. The CFLD group had significantly higher serum aspartate aminotransferase (AST, $p<0.0001$), alanine aminotransferase (ALT, $p<0.0001$), γ-glutamyl transpeptidase (GGT, $p<0.0001$) and alkaline phosphatase (ALP, $p=0.0003$) vs. CFnoLD. APRI was significantly higher in CFLD compared to CFnoLD ($p<0.0001$). While FIB-4 was increased in CFLD, this did not reach statistical significance ($p=0.6709$). Patient characteristics are shown in **Table 1.**

Next-generation sequencing (NGS) revealed a circulatory miRNA signature in CFLD children

In order to identify differentially expressed circulatory miRNAs, nine libraries were sequenced using the Ion Proton System producing 64,658,545 total reads, with average read lengths between 17-23 nucleotides **(Supp. Table 2)**, the expected size for miRNAs. During processing of the sequencing data, we noticed significant low counts mapped from the CFnoLD library 3. Following principal component analysis this library was confirmed to be an outlier and excluded from the analysis, however this did not decrease statistical power based on our sample size calculation. Analysis of the remaining libraries identified 659 miRNAs with a minimum average count of 10. miRNAs were then selected for further validation by qRT-PCR based on the NGS differential analysis and previous literature reporting the functions of these miRNAs related to liver function or fibrosis. Similarly, stable expressed miRNAs were selected to identify endogenous miRNAs necessary for qRT-PCR normalization. Based on miRNA-seq and differential expression analysis miR-122-5p; miR-25-3p; miR-199a-3p; miR-365a-3p; miR-18a-5p; miR-126-5p; miR-142-3p; let-7g-5p; miR-103a-3p; miR-34a-5p and miR-484 were selected as potential biomarkers of CFLD, while, miR-19b-3p; miR-93-5p; miR-20a-5p; let-7i-5p; let-7b-5p; let-7d-5p; miR-27a-3p and miR-146a-5p were selected as potential endogenous reference miRNAs.

An independent subset of 30 samples (n=10/patient cohort) were used to measure the expression of potential endogenous reference miRNAs. We used geNorm and NormFinder algorithms and determined an optimal panel of miR-93-5p; miR-20a-5p and let-7i-5p to be used as endogenous reference miRNA for data normalization.

Circulatory miRNA signature confirmation by qRT-PCR

The expression of differentially expressed miRNA candidates was then confirmed in the entire cohort of 124 patients **(Supp. Table 3)**. Serum levels of miR-122-5p were significantly elevated in CFLD compared to both CFnoLD (fold change [FC] =3.1, P=0.0015) and Controls (FC=7.5, P<0.0001), and also in CFnoLD compared to Controls (FC=2.4, P=0.022) **(Figure 1A)**. Expression levels of serum miR-34a-5p were also elevated in CFLD compared to both CFnoLD and Controls (FC=2.6, P=0.0042 and FC=2.9, P=0.0011, respectively) **(Figure 1B)**. Similarly, circulatory miR-365a-3p was overexpressed in CFLD when compared to both CFnoLD and Controls (FC=2.2, P=0.0002 and FC=2.2, P=0.0001, respectively) **(Figure 1C)**. In contrast, expression levels of let-7g-5p were downregulated in CFLD compared to both CFnoLD and Controls (FC=-1.8, P=0.0034 and FC=-2.1, P=0.0003, respectively) **(Figure 1D)**, while expression levels of miR-142-3p were elevated in CFnoLD compared to CFLD (FC=1.6, P=0.0047) **(Figure 1E)**. miR-126-5p was significantly downregulated in CFnoLD compared to Controls (FC=-1.7, P=0.042) and increased in CFLD compared to CFnoLD however this did not reach statistical significance (FC=1.7, P=0.0549).

Serum miR-18a-5p levels were upregulated in both CFnoLD and CFLD compared to controls (FC=1.7, P<0.0001 and FC=1.7, P<0.0001, respectively) **(Figure 1F).**

Circulatory miRNA expression in CFLD F0 fibrosis stage

Fourteen children within the CFLD cohort had F0 fibrosis on dual-pass liver biopsy. Despite the absence of overt histological collagen deposition these patients were classified as having liver disease based on clinical assessment. A sub-analysis was performed on these patients with F0 fibrosis to determine whether they were distinct from children classified as CFnoLD. CFLD subjects with F0 fibrosis showed significantly higher serum levels of miR-122-5p (FC=4.9, P=0.001), miR-365a-3p (FC=2.2, P=0.009) and APRI (FC=2.0, P<0.0003) compared to CFnoLD children **(Figure 2A-C)**, while miR-142-3p (FC=-2.95, P0.0006) showed significantly decreased expression in F0 compared to CFnoLD **(Figure 2D)**. Moreover, ROC curve analysis revealed the ability of miR-365a-3p (area under

the curve [AUC]=0.74, P=0.007), miR-142-3p (AUC=0.75, P=0.006) and APRI (AUC=0.86, P=0.0006) to discriminate F0 from CFnoLD children.

Validated miRNA candidates can discriminate liver disease in children with CF

miR-365a-3p demonstrated the best diagnostic accuracy for liver disease (AUC=0.74; sensitivity=70%; specificity=67%; P=0.0001), while miR-122-5p (AUC=0.70; sensitivity=70%; specificity=63%; P=0.0001), miR-34a-5p (AUC=0.71; sensitivity=71%; specificity=64.%; P=0.0014) and let-7g-5p (AUC=0.71; sensitivity=70%; specificity=65%; P=0.0010) showed similar differential diagnostic performance **(Figure 3A)**.

Next, to derive a panel of miRNAs that could diagnose liver disease in CF children, stepwise logistic regression analysis was performed using subsets of up to four miRNAs (794 models in total). Model selection, based on Bayesian information criterion, resulted in a model including miR-365a-3p, miR-142-3p and let-7g-5p yielding an AUC=0.87 (P<0.0001) **(Figure 3B).** In order to assess the performance of this selected miRNA panel to diagnose liver disease in CF children, the statistical model evaluation method 5-fold cross validation was performed within the same cohort of CFLD and CFnoLD children. The result of the cross-validation showed AUC=0.79 with a sensitivity of 63% and specificity of 77% across the different validation sets.

Addition of APRI to miRNA panel for differential diagnosis of CFLD

We have previously shown the utility of APRI to detect liver disease in children with CF (10). In our present cohort of CFLD vs. CFnoLD children, APRI demonstrated an AUC=0.80 (P<0.0001) with a sensitivity of 79% and specificity of 80%, recapitulating the results obtained in our previous report **(Figure 3A)** (10). Logistic regression analysis combining APRI with our three miRNA panel demonstrated a markedly improved diagnostic accuracy with an AUC=0.91. A cut-point of -5.81 was optimal for liver disease discrimination obtaining a sensitivity of 83% and specificity of 92% **(see Supp. Table 4 for logistic regression equation) (Figure 3C)**. Performance evaluation of the new proposed CFLD panel using 5-fold cross validation resulted in an AUC=0.86 with a sensitivity of 73% and specificity of 76%. Using our three miRNA + APRI panel with a cut-point of -5.81 predicted a 21 times greater odds of children with CF having CFLD, compared to the ~6 times greater odds when APRI is used alone, as previously described (10).

Serum miRNAs reflect hepatic fibrosis severity in CFLD

The expression of serum miRNA candidates was analysed to assess the potential association with hepatic fibrosis severity in CFLD **(supp. Table 5)**. Expression levels of miR-19b-3p (FC=1.6, P=0.0465), miR-18a-5p (FC=1.8, P=0.0011) and miR-142-3p (FC=2.6, P=0.0244) were all increased in CFLD patients with severe fibrosis/cirrhosis (F3/F4) compared to CFLD children with no fibrosis (F0). Expression of miR-142-3p was increased in CFLD with mild/moderate fibrosis (F1/F2) compared to F0 fibrosis (FC=2.5, P=0.026), whereas expression of miR25-3p was significantly lower (FC=-1.9, P=0.023) in F1/F2 vs. F0 fibrosis. We observed a significant positive correlation between miR-19b-3p (r_s=0.35, P=0.021) and miR-18a-5p (r_s=0.56, P<0.0001) and disease progression. miRNA expression levels and correlations with hepatic fibrosis stage are depicted in **Figure 4.**

Association between serum miR-18a-5p levels and hepatic fibrosis stage in CFLD

From the validated differentially expressed miRNAs between fibrosis stages only miR-18a-5p demonstrated potential for discriminating the severity of liver disease in CF children **(supp. Table 5).** ROC curve analysis to determine the ability of miR-18a-5p to distinguish liver disease severity within CFLD yielded the following: F0 vs. any fibrosis/cirrhosis (F1-4), exhibited an AUC=0.78(P=0.003; sensitivity=76%; specificity=64%), while F0-F1 vs. F2-F4 showed an AUC=0.76(P=0.003; sensitivity=86%; specificity=59%) and F0-F2 vs. F3-4 demonstrated an AUC=0.82(P=0.004; sensitivity=92%; specificity=73%)**(Figure 5)**. In our model, miR-18a-5p demonstrated a c-statistic = 0.74, while APRI had a c-statistic = 0.64; in addition, miR-18a-5p had an Obuchowski measure = 0.74, whereas APRI had an Obuchowski measure = 0.59 **(Table 2)**.

Stepwise ordinal logistic regression analysis was performed to evaluate the performance of selected miRNAs + APRI in predicting CFLD severity, however, only miR-18a-5p demonstrated a statistically significant result (P=0.001). In our model, one unit of increased log2 relative expression is associated with 7-fold increased odds of having advanced fibrosis. When APRI was added to miR-18a-5p as a predictor of CFLD severity in combined logistic regression, there was no improvement in its performance with an Obuchowski measure of 0.71 for both miR-18a-5p alone and combined with APRI.

Serum miRNA expression levels correlate with liver enzymes

In CFnoLD children, AST and GGT showed significant positive correlation with miR-365a-3p and miR-34a-5p respectively, while significant negative correlations were observed between GGT with miR-19b-3p, miR-103a-3p and miR-484; and ALP with miR-34a-5p (**supp. Table 6)**. In CFLD children, significant positive correlations were observed for AST with miR-34a-5p; ALT with miR-122-5p, miR-34a-5p and miR-19b-3p; and ALP with miR-19b-3p (**supp. Table 6)**. Similar correlations were observed between APRI and these miRNAs which is expected as APRI is based on AST and platelet count. In fact, when compared to APRI as a biomarker for liver disease progression, miR-365a-3p showed a positive correlation in CFnoLD children, whereas, miR-34a-5p was positively correlated in CFLD patients (**supp. Table 6).**

DISCUSSION

The prevalence of liver disease in children with CF ranges between 20-40% (27, 28) depending on definitions and tools employed. The reasons why only select CF patients develop clinically significant liver damage remain unclear. The lack of a uniform clinical definition hinders the diagnosis of CFLD in children as it relies on non-specific clinical, biochemical and imaging assessments. We have previously demonstrated the utility of a variety of different serum biomarkers as non-invasive alternatives to distinguish between CFnoLD and CFLD in children (10, 29), although the relative diagnostic accuracy of these modalities are less than ideal. We have also demonstrated the potential of circulatory miRNA for discriminating liver disease in CF children and disease severity using a limited miRNA array screen (17). In the present study, we used serum miRNA-Seq with PCR-validation to assess novel and known miRNAs in children with liver biopsy-validated CFLD to demonstrate differential miRNA expression compared to children with CFnoLD and healthy Controls, as well as to assess utility in predicting hepatic fibrosis severity.

In this study, following miRNA-Seq, qRT-PCR validation on serum of 124 children confirmed increased expression of miR-122-5p, miR-34a-5p and miR-365a-3p in CFLD vs. CFnoLD while let-7g-5p and miR-142-3p expression was decreased. The most abundant miRNA in the liver is miR-122 which accounts for more than half of the total hepatic miRNA content (31). Multiple studies have reported increased circulatory levels of miR-122 in paediatric chronic liver diseases such as biliary atresia, CFLD, non-alcoholic fatty liver disease (NAFLD) and hepatitis B (17, 32-34). Our study shows increased circulatory miR-122 levels in children with CFLD when compared to both CFnoLD and healthy controls. We also found over expression of miR-122 in CFnoLD compared to healthy

controls. It is possible that altered circulatory miR-122 expression is an early marker of liver injury and thus detected before the existing clinical modalities used in this study to identify children with CFLD. Autopsy studies have reported that up to 70% of CF patients show focal biliary cirrhosis (6), which suggests that some of the CFnoLD children in our group may have liver damage that may manifest as an overexpression of miR-122 before its detection by the traditional diagnostic methods. We also found miR-34a-5p and miR-365a-3p to be upregulated in serum of CFLD compared to CFnoLD children. Increased serum levels of miR-34a have been reported in chronic liver diseases such as hepatitis C and NAFLD (35, 36). miR-34a has been associated with liver regeneration, having a direct repressor effect on hepatocyte proliferation and senescence in alcoholic liver injury and in animal models of partial hepatectomy (37, 38). Moreover, a significant increase of miR-34a expression has been reported in liver disease, including cholestasis (39). Based on our previous studies (40), we have proposed a role for the hydrophobic bile acid taurocholate in the abnormal development of bile ducts, a process known as the ductular reaction, in CFLD (41). Key components of the Notch signalling pathway such as Notch1, Notch2 and Jagged1 are direct targets of miR-34a (42). There is supporting evidence that hepatic stellate cells and macrophages can influence the differentiation of liver progenitor cells into reactive biliary cells and bile ducts via the Notch pathway (43), therefore suggesting a potential role of miR-34a in the manifestation of CFLD. A potential role of miR-365 has been implicated in paediatric chronic liver disease including biliary atresia (44) and increased levels of circulating miR-365a-3p have been found in the plasma of children with hepatitis B, suggesting its potential as a biomarker for disease progression (33, 45). However, further mechanistic studies are required to fully elucidate the role of miR-34a-5p and miR-365a-3p in the pathogenesis of these pediatric liver diseases.

We also found a decreased expression of serum let-7g-5p and miR-142-3p in CFLD compared to CFnoLD children. Both let-7g-5p and miR-142-3p expression are downregulated in hepatocellular carcinoma (HCC) (46, 47) and are involved in suppressing HCC cell migration, motility and invasion (46, 48). Downregulated miR-142-3p has also been found in activated hepatic stellate cells (HSC) controlling cell viability and cell growth by targeting TGFβ-receptor 1 (TGFβRI) (49). Based on the functions that these miRNAs play in HCC and in HSCs, it is possible that their decreased expression in CFLD children may play a role in preventing fibrosis. Functional studies are required to establish the origin and potential role that these differentially expressed miRNAs may play in CFLD and in the development of fibrosis.

We have highlighted serum miR-365a-3p, miR-34a-5p and let-7g-5p which individually demonstrated moderate clinical utility for liver disease detection in CF with AUROCs ranging between 0.71-0.74

(Suppl. Table 3). However, using stepwise logistic regression analysis we identified a miRNA panel consisting of miR-365a-3p, miR-142-3p and let-7g-5p with the ability to discriminate liver disease in CF children with an AUC=0.87. Recently, we reported that APRI can differentiate liver disease in CF children with an AUC=0.75 and severe CFLD (F3-F4) from mild to moderate CFLD (F0-F2) with an AUC=0.81 (10). When APRI was combined with our proposed miRNA panel we observed a marked improvement in discriminating CFLD from CFnoLD with an AUC=0.91. Performance evaluation of the selected miRNA panel using 5-fold cross validation resulted in an AUC=0.79 for our three miRNA panel and an AUC=0.86 when our miRNA panel was combined with APRI, thus validating its significant discriminatory capacity. Our new proposed three miRNA + APRI panel outperforms APRI alone in predicting CFLD even in 5-fold cross validation evaluation. This study represents one of the largest biopsy-proven CFLD cohorts with internal cross-validation methodology that confirms the clinical utility of our combined three miRNA + APRI biomarker panel.

There is a clinical need for the continuous assessment of CFLD to monitor disease progression in order to provide anticipatory guidance or identify patients in need of future liver transplantation. In this study, we found significant differences in the expression of miR-19b-3p, miR-25-3p, miR-18a-5p and miR-142-3p between varying METAVIR fibrosis stages. Moreover, we identified a significant positive correlation between miR-19b-3p and miR-18a-5p with fibrosis advancement. Specifically, miR-18a-5p discriminated severe fibrosis (F3-4) from mild to moderate fibrosis (F0-2) **(Suppl. Table 5)**. miR-18-5p was able to discriminate liver disease severity in CFLD children when comparing F0 vs. F1-4 (AUC=0.78), F0-1 vs. F2-4 (AUC=0.76) and F0-2 vs. F3-4 (AUC=0.82). This data suggests that miR-18a-5p was able to significantly differentiate CFLD severity in all three cases and its diagnostic performance was better than our previously published result for APRI in stratifying disease progression (**Table 2)**. To assess the discriminatory accuracy of predicting CFLD severity, these results were further evaluated using the Obuchowski measure as suggested by Lambert *et al.*(50). In our model, miR-18a-5p produced an Obuchowski measure of 0.74 which was superior to APRI with an Obuchowski measure of 0.59. Lambert *et al.* suggests using penalties proportional to the difference in METAVIR units between stages. Applying this penalty scheme, miR-18a-5p remains superior to APRI in discriminating liver disease severity with an Obuchowski measure of 0.79 and 0.68 respectively **(Suppl. Table 5)**.

The most appropriate case definition of CFLD remains a topic of ongoing discussion in pediatric hepatology. Our study used the definitions and guidelines recommended by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) to categorise CFLD. These guidelines state that CFLD should be considered when at least two of the following conditions are

present: abnormal physical examination (hepatomegaly ± splenomegaly), persistent abnormalities of liver function tests and ultrasonographic evidence of liver involvement (including increased and/or heterogeneous echogenicity, nodularity) or portal hypertension (18). By using these criteria we decreased the risk of ignoring important aspects of the disease as well as over interpreting others such as elevation of serum transaminases, which are relatively common among the CF population. Others have proposed that CFLD should be defined as the presence of cirrhosis/portal hypertension, or intermittent elevation of liver transaminases, steatosis, fibrosis, cholangiopathy or ultrasound abnormalities (30). Both definitions use non-specific diagnostic criteria and prioritise advanced liver disease/cirrhosis against early manifestations of the disease or fibrogenic processes that could potentially lead to misclassification. The case definition in our cohort for CFLD should be considered when assessing the clinical utility of our findings. However, we believe our observations are robust for important reasons. Firstly we performed dual-pass liver biopsy to stage hepatic fibrosis in all children classified as CFLD, as previously reported (7). Second, fibrosis staging revealed 14 children without histological evidence of fibrosis (F0 fibrosis) but with a serum miRNA profile distinct from CFnoLD. In a sub-analysis, we showed that serum miR-122-5p, miR-365a-3p and APRI were all significantly increased, while miR-142-3p was significantly decreased in CFLD F0 fibrosis when compared to CFnoLD, which mirrored the observations for the collective CFLD cohort. Thus, these findings suggest that subjects classified as CFLD were correctly classified, even in the absence of overt histological fibrosis.

In summary, novel serum miRNA biomarkers, when used in combination with the free and readily available APRI index, are able to predict CFLD and assess disease severity with an excellent diagnostic accuracy compared to the current gold standard of liver biopsy. Here, we report that a combination of circulatory levels of miR-365a-3p, miR-142-3p and let-7g-5p, together with APRI, is capable of early diagnosis of CFLD. Furthermore, the expression of miR-18a-5p was able to stratify disease severity in children with CFLD. Although our proposed panel requires further validation, it may provide vital information to assess prognosis and disease severity. In conclusion, our proposed three miRNA+APRI panel has the potential to identify and stratify CFLD especially at an early stage where timely intervention is critical to improve patient outcomes. Furthermore, functional studies on the role that these miRNAs play in the development of CFLD have the potential to reveal future therapeutic strategies and may offer further insights into CFLD pathogenesis.

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FIGURES LEGENDS

Figure 1. Relative serum miRNA expression levels in CFLD, CFnoLD and Control groups. Log2 relative expression of (A) miR-122-5p, (B) miR-34a-5p, (C) miR-365a-3p, (D) let-7g-5p, (E) miR-142-3p and (F) miR-18a-5p. Lines represent mean ± SEM. * *P*<.05, ***P*<.01 and ****P*<.001 by ANOVA with Tukey post-hoc test. miR = microRNA; SEM = standard error of mean; CFnoLD = cystic fibrosis no liver disease; CFLD = cystic fibrosis-associated liver disease.

Figure 2. Relative serum miRNA expression and APRI levels in CFLD F0 fibrosis vs. CFnoLD. Relative expression of (A) log2 miR-122-5p, (B) log2 miR-365a-3p, (C) APRI and (D) log2 miR-142-3p. Lines represent mean ± SEM for miRNAs, or geometric mean and geometric standard deviation for APRI. * *P*<.05, ***P*<.01 and ****P*<.001 by two-tailed T test for miRNAs and Mann-Whitney test for APRI. miR = microRNA; SEM = standard error of mean; CFnoLD = cystic fibrosis no liver disease; F0= no fibrosis based on METAVIR score.

Figure 3. ROC curves for the discrimination of CFLD vs CFnoLD. (A) ROC curves of APRI (AUC = 0.80,n=75), miR-122-5p (AUC = 0.70, n=84), let-7g-5p (AUC = 0.71, n=84), miR-34a-5p (AUC = 0.71, n=84) and miR-365-3p (AUC = 0.74, n=84) (B) Logistic regression model based on the combination of miR-365a-3p, miR-142-3p and let-7g-5p with the addition of log(APRI) (AUC = 0.91, n=75) and without the addition of log(APRI) (AUC = 0.87, n=84) to detect liver disease in CF (C) miRNA panel cut point of 1.41*miR-365a-3p-0.86*miR-142-3p-0.79*let-7g-5p+5.21*log10(APRI) = -5.81 based on Liu's method discriminates CFLD from CFnoLD.

Figure 4. Association between relative serum miRNA expression and hepatic fibrosis staging in CFLD. Scatter plots show log2 relative expression of (A) miR-19b-3p, (B) miR-18a-5p, (C) miR-142-3p, (D) miR-25-3p vs. hepatic fibrosis severity. Correlation between log2 miRNA expression levels and hepatic fibrosis stage for (E) miR-19b-3p and (F) miR-18a-5p. Lines represent mean ± SEM. * *P*<.05, ***P*<.01 and ****P*<.001 by ANOVA with Tukey post-hoc test. miR = microRNA; SEM = standard error of mean; rs = Spearman's correlation coefficient; CFnoLD = cystic fibrosis no liver disease; CFLD = cystic fibrosis-associated liver disease; F0 = no fibrosis; F1-2 = mild/moderate fibrosis; F3-4 = severe fibrosis/cirrhosis (fibrosis staging based on METAVIR score).

Figure 5. ROC curves for assessment of CFLD severity. (A) ROC curves of miR-18a-5p (n=44) to distinguish between F0 vs. F1-4 (AUC = 0.78); F0-1 vs. F2-4 (AUC = 0.76); F0-2 vs. F3-4 (AUC = 0.82). (B) ROC curves of APRI (n=36) to distinguish between F0 vs. F1-4 (AUC = 0.53); F0-1 vs. F2-4 (AUC = 0.69); F0-2 vs. F3-4 (AUC = 0.77).

Table 1. Patient characteristics.

	Controls (n=40)	CFnoLD (n=40)	CFLD (n=44)	CFLD vs CFnoLD P-value
Sex				
Male, n (%)	24 (60)	19 (47.5)	21 (47.7)	>0.9999[a]
Female, n (%)	16 (40)	21 (52.5)	23 (52.3)	
Age (years)				
Mean ± SD	9.03 ± 3.01	11.23 ± 3.61	9.66 ± 4.07	0.0666[b]
IQR	7 - 11	8 - 14	6 – 13	
Aspartate aminotransferase, AST (U/L)				
Mean ± SD	NA	25.55 ± 9.53	44.38 ± 17.91	<0.0001[b]
IQR		18 - 32	29 - 56	
Alanine aminotransferase, ALT (U/L)				
Mean ± SD	NA	22.25 ± 10.53	49.05 ± 27.24	<0.0001[b]
IQR		15 - 28	28 - 68	
γ-Glutamyl transpeptidase, γGT (U/L)				
Mean ± SD	NA	12.00 ± 4.58	35.54 ± 33	<0.0001[b]
IQR		8 - 15	13 - 44	
Alkaline phosphatase, ALP (U/L)				
Mean ± SD	NA	212.7 ± 80.15	295 ± 109.2	0.0003[b]
IQR		156 - 266	218 - 365	
AST to platelet ratio index , APRI (arbitrary units)				
Geometric mean ± geometric SD	NA	0.20 ± 1.48	0.40 ± 2.05	<0.0001[c]
IQR		0.16 – 0.25	0.25 – 0.52	
Fibrosis-4 index, FIB-4 (arbitrary units)				
Geometric mean ± geometric SD	NA	0.18 ± 1.56	0.22 ± 2.26	0.6709[c]
IQR		0.13 – 0.26	0.14 – 0.28	
Diagnostic criteria, n (%)				
Hepatomegaly ± splenomegaly				
• Present	NA	0 (0)	27 (61.3)	
Persistent elevation of ALT				
• Present	NA	12 (30)	32 (72.7)	
Abnormal ultrasound				
• Present	NA	6 (15)	35 (79.5)	

CFnoLD= cystic fibrosis no liver disease; CFLD= cystic fibrosis-associated liver disease; NA= data not available; SD= standard deviation; a= Fisher's exact test; b= Student t test; c= Mann-Whitney Test; IQR=interquartile range.

Table 2. Diagnostic test accuracy.

Biomarkers performance to predict disease severity in CFLD			
AUC (Pvalue)			
	F0 vs F1-4	**F0-1 vs F2-4**	**F0-2 vs F3-4**
miR-18a-5p (n=44)	0.78 (0.003)	0.76 (0.003)	0.82 (0.004)
APRI (n=36)	0.53 (0.804)	0.69 (0.054)	0.77 (0.002)
Concordance statistic (c-statistic)			
miR-18a-5p (n=44)	0.74		
APRI (n=36)	0.64		
Obuchowski measure			
miR-18a-5p (n=44)	0.74		
APRI (n=36)	0.59		

AUC, c-statistic and Obuchowski measure for miR-18a-5p (n=44) and APRI (n=36) to assess their performance in discriminating fibrosis stages in CFLD. Fibrosis stages based on METAVIR scoring system. AUC=area under the curve; F0= no fibrosis; F1-4= any fibrosis/cirrhosis; F0-1= no fibrosis/mild fibrosis; F2-4= moderate fibrosis/cirrhosis; F0-2= not severe fibrosis; F3-4= severe fibrosis/cirrhosis.