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MicroRNA 122 Reflects Liver Injury in Children with Intestinal Failure–Associated Liver Disease Treated with Intravenous Fish Oil

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ABSTRACT

Background: There is evidence that microRNA (MIR) 122 is a biomarker for various liver diseases in adults and children. To date, MIR122 has not been explored in children with intestinal failure–associated liver disease (IFALD, or hyperbilirubinemia associated with prolonged parenteral nutrition).

Objectives: This study's purpose was to investigate changes in plasma *miR-122*, correlate *miR-122* with serum liver function tests and enzymes, and investigate changes in whole blood transcripts including *miR-122* targets in a group of children with IFALD who received pure intravenous fish oil (FO) as a treatment for cholestasis.

Methods: This was a prospective, observational study that enrolled children with IFALD who received intravenous FO (1 g/kg/d) and whose cholestasis resolved with FO. Plasma *miR-122* was measured using reverse transcription-quantitative real-time PCR, and whole blood *miR-122* targets were quantified using RNA sequencing.

Results: Fourteen subjects with median age 6 mo (IQR: 3–65 mo) were enrolled. RNA sequence data were available for 4 subjects. When compared with the start of FO, median *miR-122* concentrations at 6 mo of FO therapy decreased [1.0 (IQR: 1.0–1.0) compared with 0.04 (IQR: 0.01–0.6), $P = 0.009$]. At the start of FO, *miR-122* correlated with conjugated bilirubin ($r = 0.56$; $P = 0.038$). At ~3 mo of FO, *miR-122* correlated with conjugated bilirubin ($r = 0.56$; $P = 0.045$). Reactive oxygen species, heme metabolism, coagulation, adipogenesis, IL-6-Janus kinase–signal transducer and activator of transcription (JAK-STAT) 3, IL-2-STAT5, transforming growth factor-β, TNF-α, inflammatory response, mammalian target of rapamycin gene families (normalized enrichment scores < -1.4), and *miR-122* target genes were significantly downregulated with FO.

Conclusions: In this small cohort of young children with IFALD, *miR-122* decreased with FO therapy and correlated with conjugated bilirubin. Key pathways involving oxidation, inflammation, cellular differentiation, and nutrient regulation were downregulated. Data from this study provide information about IFALD and FO. This trial was registered at www.clinicaltrials.gov as NCT00969332. *J Nutr* 2020;150:1144–1150.

Introduction

In children, prolonged parenteral nutrition (PN) is associated with intestinal failure–associated liver disease (IFALD) (1, 2). On liver biopsy, cholestasis and periportal inflammation are prominent features with steatosis being less common (3). IFALD can progress to cirrhosis and irreversible liver damage (1–3). Although serum conjugated hyperbilirubinemia indicates cholestasis, it does not provide information about inflammation, steatosis, or fibrosis. Liver function tests (i.e., alanine aminotransferase and aspartate aminotransferase) are not liver specific and are confounded by age, sex, and other diseases (4). The gold standard for staging liver disease is a biopsy; however, biopsies are costly, invasive, and can be diagnostically inadequate (5).

MicroRNA (miR) 122 makes up >70% of hepatic miRNAs and predicts cholestasis, steatosis, and fibrosis in animals and humans (6–10). When hepatocytes are injured, they release *miR-122* into the circulation. To date, there are no studies examining *miR-122* in patients with IFALD. Because *miR-122* is the most abundant noncoding RNA synthesized by hepatocytes and is well studied in adults with other liver diseases, we sought to explore its role in children with IFALD.

We and others have demonstrated that when intravenous soybean oil is replaced with low-dose intravenous fish oil (FO), IFALD resolves in ~75% of children (2, 11, 12). Soybean oil is a standard intravenous lipid emulsion prescribed with PN as a source of fatty acids and nonprotein calories and postulated to play a critical role in IFALD (13, 14). Compared with FO, soybean oil contains substantially higher concentrations of phytosterols and linoleic acid, a proinflammatory n–6 fatty acid, and a small amount of the antioxidant vitamin E. In contrast, FO contains negligible amounts of phytosterols, higher concentrations of docosahexaenoic and eicosapentaenoic acids,

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TABLE 1 Composition of intravenous fish oil compared with soybean oil

Constituent	Fish oil	Soybean oil
Fatty acids, % by weight		
Linoleic acid (18:2n–6)	1.5	44–62
α-Linolenic acid (18:3n–3)	1.1	4–11
Arachidonic acid (20:4n–6)	0.2–2	0
Docosahexaenoic acid (22:6n–3)	14–27	0
Eicosapentaenoic acid (20:5n–3)	13–26	0
n–6:n–3 ratio	1:8	7:1
Phytosterols,[1] μg/mL		
β-Sitosterol	ND	243.26 ± 4.10
Stigmasterol	1.37 ± 0.35	49.57 ± 0.62
Campesterol	0.95 ± 0.08	37.19 ± 0.54
α-Tocopherol, mg/L	150–300	38

[1] Values for phytosterols are represented as mean +/- standard deviation. ND, not determined.

2 anti-inflammatory n–3 fatty acids, and a relatively high concentration of vitamin E (Table 1). In animal models, intravenous phytosterols (plant-derived analogs of cholesterol) and cytokines act synergistically to cause IFALD by inhibiting farsenoid X receptor (FXR) and liver X receptor (LXR) (14). When hepatic FXR and LXR are inhibited, expression of bile acid, bilirubin, and sterol transporters is downregulated and genes involved in lipogenesis are upregulated. As a result, bile acids, bilirubin, and lipids accumulate and liver damage ensues. For these reasons, a common treatment for IFALD is a reduction of the dose of soybean oil or substitution of soybean oil with pure FO or an FO-containing lipid emulsion (2, 11, 12, 15).

In this study, in a group of children with IFALD whose cholestasis resolved with FO treatment, we aimed to: *1*) investigate changes in plasma *miR-122*; *2*) correlate plasma *miR-122* with serum conjugated bilirubin (CB) and liver enzymes; and *3*) examine changes in molecular pathways known to be altered by FO and *miR-122* targets (8, 16–21). We hypothesized that FO would decrease circulating *miR-122*, correlate with CB, and alter *miR-122* pathways and targets involved in inflammation, oxidation stress, antioxidant protection, and lipid metabolism.

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Sequence data can be accessed at https://www.ncbi.nlm.gov/SRA/PRJNA533311 (Sequence Read Archive Database accession no. PRJNA533311).
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Supplemental Table 1 and Supplemental Figures 1 and 2 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.
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Abbreviations used: CB, conjugated bilirubin; ELOVL, elongation of very long chain fatty acids; FC, fold change; FDR, false discovery rate; FO, fish oil; FXR, farsenoid X receptor; IFALD, intestinal failure–associated liver disease; JAK-STAT, Janus kinase–signal transducer and activator of transcription; LXR, liver X receptor; miRNA, microRNA; *miR-122*, microRNA 122; mTOR, mammalian target of rapamycin; p, phosphorylated; PI3K-Akt, phosphatidylinositol-3-kinase-Akt; mTORC1, mammalian target of rapamycin complex 1; PN, parenteral nutrition; ROS, reactive oxygen species; SREBP, sterol regulatory binding protein; UCSC, University of California Santa Cruz.

Methods

Our original study that investigated the efficacy and safety of FO is registered at www.clinicaltrials.gov (NCT00969332). The main study's primary outcome was time to resolution of cholestasis, defined as a CB <2 mg/dL on 2 consecutive measurements separated by 1 wk (11).

In this article, we report the results of a prospective observational study whose primary outcome was changes in plasma *miR-122* concentrations (end of FO compared with start of FO) in children whose cholestasis resolved with FO. Secondary outcomes included correlations between *miR-122* and serum CB and liver enzymes, and changes in targeted and nontargeted transcripts and their involvement in specific molecular pathways.

Subjects were recruited from University of California Los Angeles Mattel Children's Hospital. Inclusion criteria for this study included FO treatment for IFALD, resolution of cholestasis, and 2 plasma samples (1 sample at baseline and a further sample at 2 wk, 3 mo, or 6 mo of FO) (11). Eligibility criteria for the original treatment trial have been previously described and included >2 wk of age, <18 y of age, an acquired or congenital gastrointestinal disorder, and IFALD (11). IFALD was defined as a serum CB ≥2 mg/dL on 2 consecutive measurements separated by ≥1 wk, anticipated PN course >30 d, and >60% of calories from PN (11). Subjects with a primary liver disease, inborn error of metabolism, seafood, egg, or fish allergy, hemorrhagic disorder, hemodynamic instability or shock, comatose state, stroke, pulmonary embolism, myocardial infarction, diabetes, or fatal chromosomal disorder, or receiving extracorporeal membrane oxygenation were excluded (11).

After obtaining parental consent and child assent, if applicable, the subject's intravenous soybean oil (Intralipid; Fresenius Kabi) was replaced with FO (Omegaven; Fresenius Kabi) as the primary parenteral lipid source (Table 1). Soybean oil was dosed according to the clinical team's discretion and generally restricted at a dose of 1 g/kg/d intravenously. FO was dosed at 0.5 g/kg/d intravenously for the first 2 d, then 1 g/kg/d intravenously over 8–24 h for 6 mo. FO was discontinued before 6 mo if the subject no longer required PN, underwent liver and/or multivisceral transplantation, developed a serious adverse complication attributed to FO, or was withdrawn from the study (11).

Blood samples for research purposes were collected to measure plasma *miR-122* concentrations and whole blood *miR-122* targets before starting FO (baseline), and at ∼2 wk, and 3 and 6 mo of FO. All 6-mo samples were collected prior to FO termination. Blood was collected from central venous catheters. Intravenous lipids were suspended for ≥2 h prior to research sample collections. Clinical data were collected prospectively from the electronic medical record. Data on liver function tests and enzymes were collected at baseline, and ∼2 wk, and at 3 and 6 mo of FO. Nutritional data were collected at baseline (on intravenous soybean oil and prior to FO) and at 6 mo of FO.

The institutional review board at the University of California Los Angeles approved the study. This study followed the ethical standards of the institutional review board and complied with the Helsinki Declaration as revised in 1983.

Plasma RNA and miRNA extraction

Total cell-free RNA enriched with miRNA was isolated from 200 μL of human plasma using the miRNeasy serum/plasma kit (QIAGEN Sciences) following the manufacturer's instructions. RNA concentrations and quality were measured using the Nano Drop 2000c spectrophotometer (ThermoFisher Scientific).

miRNA quantification: reverse transcription-quantitative real-time PCR

Experiments were performed in triplicate. Reverse transcription was carried out using the Taqman Advanced miRNA Assay cDNA synthesis kit (Applied Biosystems) according to the protocol provided by the manufacturer. The extracted RNA (in 2 μL) was reverse transcribed for each sample (n-7–11/time point), the resultant cDNA template (5 μL) was diluted and subjected to qPCR. Each sample was run in triplicate using the TaqMan Advanced miRNA Assay (Applied Biosystems) to evaluate mature *miR-122* expression (Assay ID#

477,855_mir). MiR-24, another miRNA expressed by the liver, was used as the endogenous control (Mir24, Assay ID# 477,992_mir; Applied Biosystems). Quantification was carried out using the ABI 7900HT system (Applied Biosystems). The -fold increase relative to baseline samples was determined by the comparative Ct method of calculation (22).

Detection of miR-122 transcriptomic targets

Total RNA was purified from whole blood samples collected from 4 subjects who were arbitrarily selected. These blood samples were collected into PAXgene blood RNA tubes at baseline and 6 mo of FO according to the protocol provided by the PAXgene Blood miRNA kit (763,134; Qiagen). Before library construction, each RNA sample was quantified using a Qubit Fluorometer (Invitrogen Q32850 for dsRNA BR assay kit) and RNA quality was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies). The library in preparation for RNA sequencing was constructed using a TruSeq Stranded Total RNA Sample Preparation kit (RS-122–2201; Illumina) according to the manufacturer's instructions. The final library preparation was diluted to achieve a 10-nM concentration using Qiagen EB Buffer with 0.1% Tween 20. RNA sequencing was conducted in 50-bp reads for single-end sequencing using Illumina's Hi-Seq 2500 sequencing system.

Statistical methods

To demonstrate reliability, CVs were computed for *miR-122* at each time point (baseline, 2 wk, and 3 and 6 mo). *miR-122*, CB, and liver function tests during the study were compared with baseline using Wilcoxon matched-pairs signed rank test. Pearson correlation coefficients were used to investigate correlations of *miR-122* with CB and liver function tests at the same time point after log transformation. To assess the association between changes in *miR-122* and CB, Pearson correlation coefficients were calculated for the differences between each subject's log-transformed baseline and 2-wk, baseline and 3-mo, and baseline and 6-mo values.

Because not all liver enzymes and tests were measured exactly at 2 wk and 3 mo, local linear interpolation was used. For missing liver function tests and enzymes at 6 mo, values measured in the preceding 4 wk were used. P values < 0.05 were considered statistically significant. Analyses were performed using Prism 7 software (GraphPad). Because this was an exploratory study in a group of children with a rare disease, a power calculation was not performed.

For the RNA sequencing analysis, raw sequencing reads were demultiplexed to make fastq files. The quality of the Raw-seq data was reviewed using Fastqc. Single-end reads from individual libraries were aligned with the human reference genome (hg38) using STAR aligner (23). Uniquely aligned reads were used to obtain raw counts for all the *Homo sapiens* genes [hg38; University of California Santa Cruz (UCSC) Gene Browser] with the featureCounts tools from the Subread package (24). Further analysis and data visualization were performed using various R packages (version number 3.5). DESeq2 (25) was used to calculate the size factors and normalize the library size. Differential expression was calculated between samples at baseline and 6 mo of FO using a negative binomial generalized linear model, and correction for multiple testing with false discovery rates (FDRs) was estimated using the Benjamini–Hochberg method (26). FDRs <0.05 were considered statistically significant. The DEseq2 comparison table was used for pathway analysis. A ranked list of genes was generated as mentioned by Plaisier et al. (27). Gene set enrichment analysis was performed against MSigDB using the clusterProfiler tool (28). Predicted biological mRNA targets of *miR-122*-5p were identified by Target scan software (version 7.0), searching for the presence of conserved sites that match the seed region of *miR-122* (29), or sites with mismatches in the seed region that are compensated by conserved 3′ pairing (30), or centered sites. Predictions were ranked based on the predicted efficacy and cumulative weighted context scores of the sites (31). Predictions were also ranked by their probability of conserved targeting (30), and consideration to matches with the human 3′-untranslated region and their orthologues was given, as defined by UCSC whole-genome alignments. These identified *miR-122* targets (~4000) were next compared with the differentially expressed genes obtained from the RNA-sequencing analysis to detect the predicted *miR-122* targets that displayed differential expression between the baseline and 6 mo of FO. Based on the observed -fold change (>2) and FDR (<0.05), 150 differentially expressed *miR-122* target genes were selected.

TABLE 2 Characteristics of the children with IFALD who responded to intravenous fish oil treatment and who were included in the study ($n = 14$)[1]

Characteristic	Value
Age, mo	6 (3–65)
Male gender	71 (10)
White race	71 (10)
Hispanic ethnicity	50 (7)
Gestational age, wk	36 (33–39)
Weight, kg	5.2 (4.4–17.4)
BMI, kg/m^2	16 (14.7–17.2)
Primary gastrointestinal diagnosis	
Gastroschisis	21.4 (3)
Necrotizing enterocolitis	21.4 (3)
Volvulus	28.5 (4)
Intestinal atresia	7.2 (1)
Other	21.4 (3)
Small bowel length, cm	15 (15–28)

[1]Data are presented as median (IQR) or % (*n*). IFALD; intestinal failure–associated liver disease.

Results

Subjects

Twenty-five subjects were enrolled in our FO treatment trial between July 2013 and July 2016. Subjects were excluded because parents/legal guardians refused blood collection for research purposes ($n = 2$), insufficient blood for experiments ($n = 2$), a second sample was not available for comparison ($n = 4$), anemia and/or small body weight ($n = 1$), and failure to respond to FO ($n = 1$). One subject was excluded from the analysis because they required hemodialysis, which is known to increase *miR-122* concentrations (32). From this cohort, 14 subjects were included in the *miR-122* analysis (**Supplemental Figure 1**). For the *miR-122* analysis, 14 samples were collected at baseline, 9 at 2 wk, 13 at 3 mo, and 12 at 6 mo. Samples at baseline and 6 mo of FO were collected from 4 subjects for RNA sequencing.

At the start of the study, the median age for the 14 subjects was 6 mo (IQR: 3–65 mo); 71% ($n = 10$) were male. Median body weight at the start of the study was 5.2 kg (IQR: 4.4–17.4 kg). Most children were diagnosed with volvulus, gastroschisis, or necrotizing enterocolitis and had short bowel syndrome (Table 2). Cholestasis resolved (serum CB <2 mg/dL on 2 consecutive measurements) in all 14 subjects. None of these subjects achieved enteral autonomy, received a transplant, or died during the study. Median PN requirements were less at the end of FO compared with the start of FO [80 kcal/kg/d (IQR: 66, 90) compared with 63 kcal/kg/d (IQR: 51, 76); $P = 0.02$]. The median dose of intravenous fat was similar at the start of the study compared with the end of the study [1 g/kg/d (IQR: 0.9, 1.5) of soybean oil compared with median 1 g/kg/d (IQR: 1, 1) of FO; $P = 0.14$].

TABLE 3 Laboratory values in children with IFALD who responded to intravenous fish oil treatment[1]

	Baseline	2 Weeks	3 Months	6 Months
miR-122	1.0 (1.0–1.0)	0.7 (0.1–2.0)	0.5 (0.1–0.9)	0.04 (0.01–0.6)**
CB, mg/dL	3.8 (2.6–5.1)	3.6 (2.8–5.1)	0.3 (0.2–1.9)**	0.2 (0.2–0.3)**
AST, IU/L	106 (81–146)	133 (104–229)	63 (46–102)	50 (40–104)
ALT, IU/L	91 (63–126)	134 (71–168)	53 (40–100)	45 (33–100)
GGT, IU/L	184 (140–238)	161 (120–205)	73 (42–209)	65 (60–117)

[1] Data are presented as median (IQR). $^{**}P < 0.01$ compared with baseline. $n = 14$ except for the following: *miR-122* at 2 wk, $n = 9$; at 3 mo, $n = 13$; and at 6 mo, $n = 12$; CB at 6 mo, $n = 11$; AST and ALT at 6 mo, $n = 12$; and GGT at baseline, $n = 8$; at 2 wk, $n = 9$; at 3 mo, $n = 9$; and at 6 mo, $n = 5$. Analysis done using Wilcoxon matched-pairs signed rank test. *miR-122* was measured in the plasma, and liver function tests and enzymes were measured in the serum. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CB, conjugated bilirubin; GGT, γ-glutamyl transferase; IFALD; intestinal failure–associated liver disease; *miR-122*, microRNA 122 is expressed as a ratio relative to an internal control.

miR-122 and liver function

The overall CV for *miR-122* was 7%, and ranged from 6% to 8% for each time point. As anticipated, median serum CB concentration was significantly less at the end of the FO when compared with the start of FO [3.8 mg/dL (IQR: 2.6–5.1) compared with 0.2 mg/dL (IQR: 0.2–0.3); $P = 0.001$]. Median plasma *miR-122* concentrations followed a similar trend [1.0 (IQR: 1.0–1.0) compared with 0.04 (IQR: 0.01–0.6); $P = 0.009$] (Table 3). Baseline *miR-122* correlated with baseline CB ($r = 0.56$; $P = 0.038$); 3-mo *miR-122* correlated with 3-mo CB ($r = 0.56$; $P = 0.045$) (Table 4). Correlations for changes in plasma *miR-122* and changes in serum CB were not statistically significant (baseline − 2 wk: $r = 0.34$; 95% CI: −0.4, 0.8; baseline − 3 mo: $r = 0.29$; 95% CI: −0.3, 0.7; and baseline − 6 mo: $r = -0.15$; 95% CI: −0.7, 0.5).

RNA sequencing and pathway analysis

Descriptive data from 4 subjects whose RNA was sequenced is presented in **Supplemental Table 1**. We observed a significant reduction fold change (FC) >2; FDR <0.05) in abundance of 573 protein-coding mRNAs in the blood in response to FO treatment (Figure 1). Gene Set Enrichment Analysis with hallmark pathway gene sets from the Molecular Signatures Database (MySigDB) showed a concordant difference in the enrichment score of various metabolic and immune-associated pathways between baseline and 6-mo FO-treated samples (**Supplemental Figure 2**, Table 5). Further expression analysis of genes associated with adipogenesis, the mammalian target of rapamycin (mTOR) pathway, the reactive oxygen species (ROS) pathway, the transforming growth factor-β signaling pathway, TNFα signaling pathway, IL-6-JAK-STAT3, IL-2-STAT5, and other inflammatory pathways revealed reduced relative abundance after 6 mo of FO (Figure 2A, B).

TABLE 4 Correlation coefficients for *miR-122* and liver function tests and enzymes at the same time in children with IFALD who responded to intravenous fish oil treatment[1]

	CB	AST	ALT
Baseline, $n = 14$	0.56* (0.04, 0.8)	0.36 (−0.2, 0.8)	0.27 (−0.3, 0.7)
2 Weeks, $n = 9$	− 0.14 (−0.7, 0.6)	0.34 (−0.4, 0.8)	0.10 (−0.6, 0.7)
3 Months, $n = 13$	0.56* (0.01, 0.9)	0.42 (−0.2, 0.8)	0.25 (−0.4, 0.7)
6 Months, $n = 11$	0.43 (−0.2, 0.8)	0.40 (−0.3, 0.8)	0.30 (−0.4, 0.8)

[1] Data are presented as *r* value (95% CI); Correlation significant, $^{*}P < 0.05$. Analysis done using Pearson correlation after log transformation. *miR-122* was measured in the plasma, and liver function tests and enzymes were measured in the serum. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CB, conjugated bilirubin; IFALD, intestinal failure–associated liver disease; *miR-122*, microRNA 122.

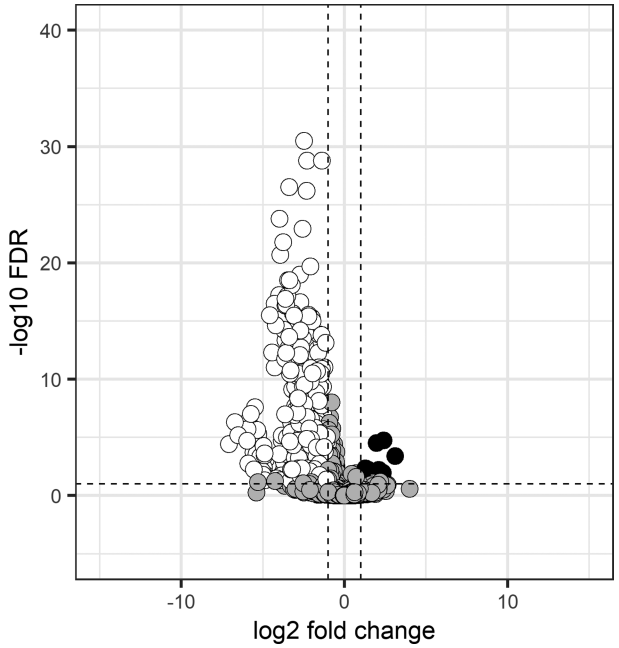

FIGURE 1 Volcano plot showing the distribution of transcripts by comparing the log2-fold change in gene expression between baseline and 6 mo of fish oil treatment in 4 children with intestinal failure–associated liver disease. White and black circles represent significant decrease or increase in expression, respectively. Genes with no change are highlighted in gray. The *x*-axis represents log2-fold change in expression, and the *y*-axis represents negative log10 value of the false discovery rate (FDR). Genes with a log2-fold change value ≥1 and FDR value <0.05 are considered significant.

TABLE 5 Pathway analysis results from gene enrichment analysis from the whole blood of 4 children with IFALD who received intravenous fish oil[1]

	Size	ES	NES	FDR
Heme metabolism	189	− 0.7801	− 4.5862	0
ROS pathway	46	− 0.5318	− 2.3559	0
Protein secretion	92	− 0.4293	− 2.2134	0
Hypoxia	172	− 0.3702	− 2.1681	0.0005
Mitotic spindle	196	− 0.362	− 2.1175	0.0004
Coagulation	101	− 0.3954	− 2.0754	0.0006
Androgen response	92	− 0.3673	− 1.9076	0.0023
Adipogenesis	175	− 0.3303	− 1.8979	0.0027
IL-6-JAK-STAT3 signaling	78	− 0.3763	− 1.8454	0.0042
PI3K-Akt-mTOR signaling	95	− 0.3551	− 1.8446	0.0038
Inflammatory response	167	− 0.32	− 1.8426	0.0034
TNFα signaling via NFκB	188	− 0.2997	− 1.7726	0.0053
mTORC1 signaling	194	− 0.295	− 1.7508	0.0053
Complement	179	− 0.2864	− 1.6686	0.0111
Apoptosis	150	− 0.2985	− 1.6648	0.0107
IL-2-STAT5 signaling	185	− 0.2845	− 1.6399	0.0113
TGFβ signaling	51	− 0.3499	− 1.5770	0.0169

[1] Akt, protein kinase B; EAS, enrichment analysis score; FDR, false discovery rate (q-value); IFALD, intestinal failure–associated liver disease; JAK-STAT, Janus kinase–signal transducer and activator of transcription; mTORC1, mammalian target of rapamycin complex 1; NES, normalized enrichment score; PI3K-Akt-mTOR, phosphatidylinositol-3-kinase-Akt-mammalian target of rapamycin; ROS, reactive oxygen species; TGFβ, transforming growth factor-β. Size refers to number of genes in the pathway. .

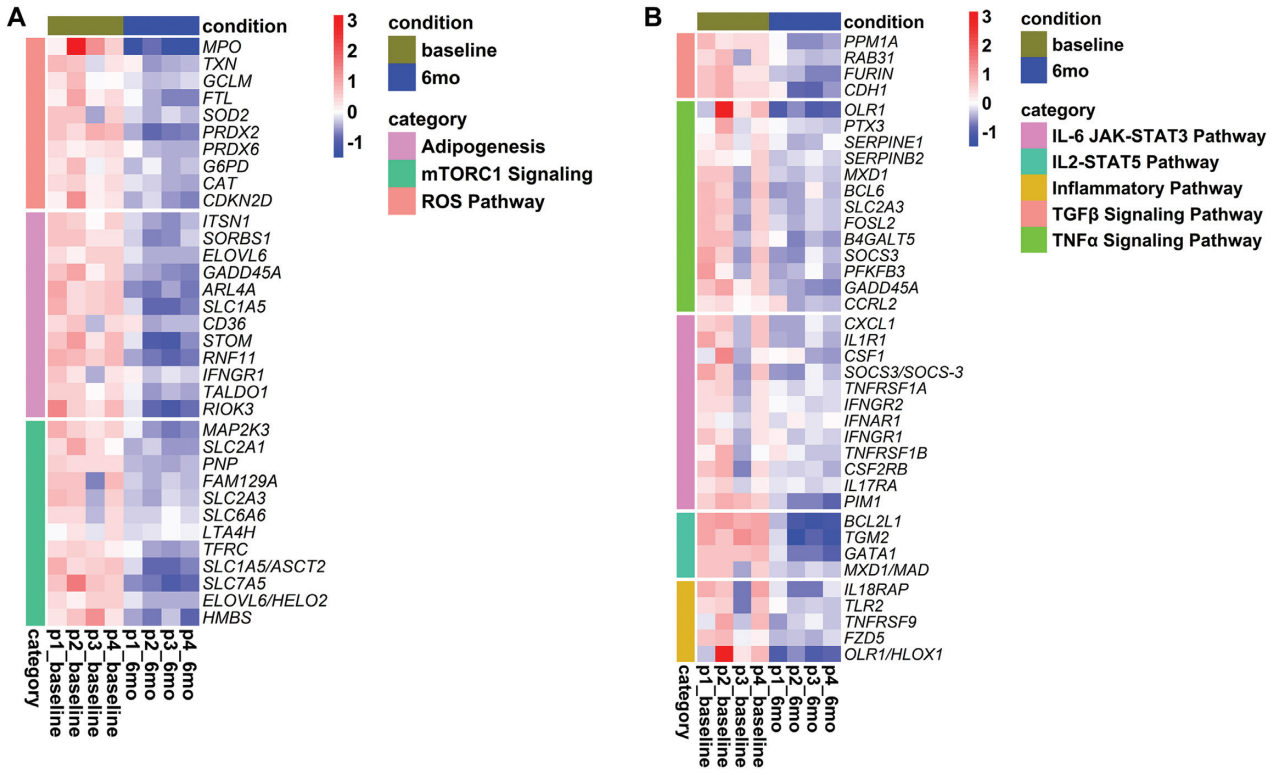

FIGURE 2 Heat maps for 4 children (refered to as p1 to p4) with intestinal failure–associated liver disease before and after 6 mo of treatment with fish oil. Heatmaps show the abundance of circulating blood mRNA/transcript associated with: (A) adipogenesis, mammalian target of rapamycin complex (mTORC)-1 signaling, and reactive oxygen species (ROS) pathways, and (B) IL-6-Janus kinase–signal transducer and activator of transcription (JAK-STAT) 3 pathway, IL2-STAT5 pathway, inflammatory pathway, and transforming growth factor (TGF)-β signaling and TNFα pathways. p refers to patient. Scale bar represents the mean-centered log2-normalized counts (row *z*-score) and is represented by the red to blue vertical bar scaled 3 to -1.

miR-122 targets

Finally, our analyses revealed that multiple predicted targets of *miR-122* were also differentially expressed between these 2 time points in 4 subjects (p1-p4). Examples include downregulation with FO therapy of *IL1RN* (interleukin 1-receptor, a gene that encodes the interleukin 1 cytokine family), *HMGCR* (3-hydroxy-3-methyl-glutaryl coenzyme A, a gene that encodes the enzyme β-hydroxy-β-methylglutaryl coenzyme A, the rate-limiting enzyme involved in cholesterol synthesis), and *FKBP5* (FK binding protein 5, a gene involved in the FXR pathway). Of note, our analysis revealed an altered expression of several *miR-122* targets that regulate apoptosis, insulin, glucose, and amino acid metabolism (**Figure 3**).

Discussion

In a small cohort of children with IFALD, we demonstrated that *miR-122* decreases with FO treatment and correlates with serum CB. miRNAs are well-characterized, short, single-stranded, highly conserved noncoding RNAs that regulate gene expression at the posttranscriptional level. Because miRNAs are tissue-specific, stable in plasma, and can be easily detected in bodily fluids at low cost, they appear to be ideal biomarkers. In this study, we observed that FO treatment induced downregulation in pathways that regulate oxidative stress, adipogenesis, inflammation, and nutrient and insulin signaling. Our observations suggest that plasma *miR-122* could serve as a noninvasive marker of liver disease in children with IFALD.

IFALD is a multifactorial disease. Prematurity, sepsis, and altered intestinal permeability are risk factors for IFALD (1, 33). Currently, effective treatments for children with IFALD include a multidisciplinary approach to intestinal failure, FO and/or FO-containing lipid emulsions, and transplant (12, 13, 15, 33). We hypothesize that long-term intravenous soybean oil in combination with repeated bouts of sepsis and small bowel bacterial overgrowth promote hepatic inflammation, oxidative stress, and phytosterol and lipid accumulation in the liver (12–14, 34–37). Our targeted pathway analysis revealed significant changes in transcripts encoding ROS (i.e., myeloperoxidase, superoxide dismutase, catalase) and inflammatory pathways (i.e., IL-6-JAK-STAT3, TNFα signaling via NF-κB, and IL2-STAT5). Using a targeted approach, we noted a decrease in *IL1R1* (encodes IL-1 receptor 1, a mediator of NF-κB- and cytokine-induced inflammation) and *ALOX5AP* (encodes arachidonate 5-lipoxygenase activating protein, a gene that regulates production of the 4 series of leukotrienes, which are metabolites of arachidonic acid and regulate inflammation). In PN-exposed children, we and others have noted an increase in anti-inflammatory mediators and a decrease in markers of oxidative stress and inflammation with FO and FO-containing emulsions (13, 19, 37). In fact, in PN-infused mice with intestinal injury, LPS induces inflammation, activating liver macrophages, which release IL-1β, thus activating NF-κB and downregulating FXR and LXR expression resulting in cholestasis (38).

Metabolic health and *miR-122* have been previously studied (6, 21, 39). When compared with healthy controls, overweight children and children with nonalcoholic fatty liver disease and

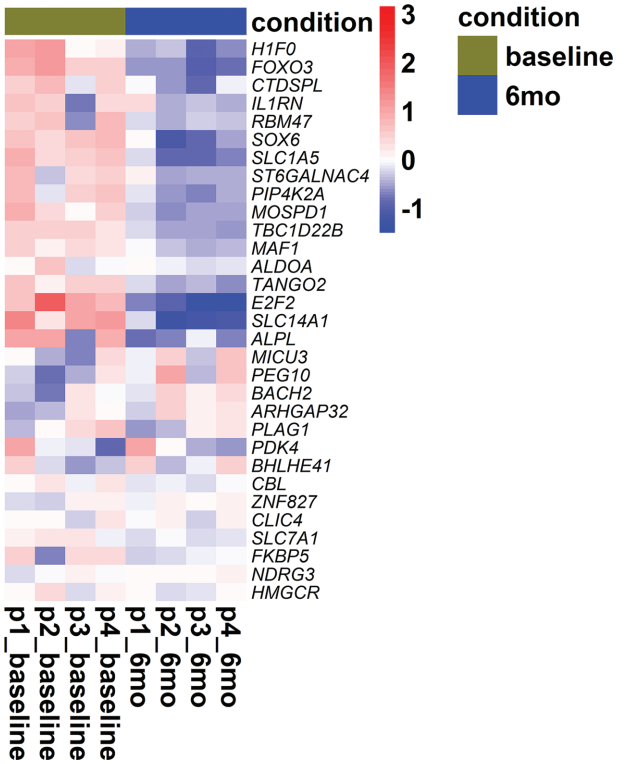

Figure 3 Heat maps in four children (p1, p2, p3, p4) with intestinal failure associated liver disease before and after 6 mo of fish oil (FO) treatment. Heatmaps show the abundance of *miR-122* targets in the whole blood. Scale bar represents the mean-centered log2-normalized counts (row Z score) and is represented by the red to blue vertical bar 1.5 to -1.

insulin resistance have increased circulating *miR-122* concentrations (6). In the Framingham study, *miR-122* correlated with type 2 diabetes mellitus, adiposity, and dyslipidemia in adults and adolescents. Pathway analysis revealed targets such as 5′-AMP-activated protein kinase (*AMPK*), mTOR, and mitogen-activated protein kinase (21). Likewise, our pathway analysis noted a downregulation of the phosphatidylinositol-3-kinase-Akt-mammalian target of rapamycin complex 1 (PI3K-Akt-mTORC1) signaling pathway. mTOR is an important nutrient sensor and essential for cellular growth. Studies demonstrate that n–3 fatty acids regulate mTOR and improve insulin sensitivity (40).

In our present study, we observed a downregulation of genes involved in adipogenesis. Although we hypothesized that FO would alter pathways involved in lipid and cholesterol metabolism, this was not the case. These results might be because our experiments were in blood and not liver tissue. FO has been shown to decrease hepatic β-oxidation via peroxisome proliferator-activated receptor-α (PPAR-α), which is directly activated by n–3 fatty acids, and decrease hepatic fatty acid synthetase via activation of sterol regulatory binding protein-1 (SREBP-1) (41). In our study, we did observe a downregulation in the elongation of very long-chain fatty acids 6 (ELOVL6), a target of SREBP-1 and a microsomal enzyme that regulates the elongation of C12–16 saturated and monosaturated fatty acids. In studies, mice deficient in ELOVL6 are protected against dyslipidemia, insulin resistance, atherosclerosis, and hepatic fibrosis (42, 43).

With respect to *miR-122* and FXR, in a hepatocellular carcinoma cell line, *miR-122* was shown to target FXR indicating that FXR might regulate *miR-122* (8). We speculate that IFALD secondary to prolonged PN with intravenous soybean oil could cause *miR-122* to move from the hepatic extracellular space into the circulation. Increased *miR-122* in the liver could suppress FXR resulting in bile acid and lipid retention. FO could reduce hepatic *miR-122*, and this could facilitate FXR's escape from posttranscriptional inhibition by *miR-122*. *miR-122*/FXR could play an essential role in the pathogenesis of IFALD.

We recognize our study's limitations. Although changes in gene expression occurred with FO, validation is required. The sample size is small and ideally we would have examined *miR-122* in a group of children who failed to respond to FO. Because ~75% of children respond to FO, and some of these children wean off PN during FO treatment, we lacked access to a reasonable sample size of subjects who failed to respond to FO (2). We also lacked healthy controls or children with IFALD who received intravenous soybean oil. We only examined *miR-122* and not multiple miRNAs. Last, before recommending *miR-122* as a biomarker of IFALD, larger clinical trials are required, and results should be correlated with liver biopsy.

In summary, in this study, we report that plasma *miR-122* and its target genes are dysregulated in pediatric IFALD and recover with FO treatment. These findings support that circulating *miR-122* concentrations can be a biomarker for pediatric IFALD. Further study is warranted.

Acknowledgments

The authors' contributions were as follows—SUD: conceived the research, designed the experiments, and provided study oversight; KLC, ST, YD, SG: conducted the research; KLC, SG: analyzed the data; KLC, SG, SUD: wrote parts of the manuscript and edited the manuscript; SUD: had primary responsibility for the final content; and all authors: read and approved the final manuscript.

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