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A serum-induced gene signature in hepatocytes is associated with pediatric nonalcoholic fatty liver disease

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Abstract

Objective: Pediatric nonalcoholic fatty liver disease (NAFLD) is a growing problem, but its underlying mechanisms are poorly understood. We used transcriptomic reporter cell assays to investigate differences in transcriptional signatures induced in hepatocyte reporter cells by the sera of children with and without NAFLD.

Methods: We studied serum samples from 45 children with NAFLD and 28 children without NAFLD. The sera were used to induce gene expression in cultured HepaRG cells and RNA-sequencing was used to determine gene expression. Computational techniques were used to compare gene expression patterns.

Results: Sera from children with NAFLD induced the expression of 195 genes that were significantly differentially expressed in hepatocytes compared to controls with obesity. NAFLD was associated with increased expression of genes promoting inflammation, collagen synthesis, and extracellular matrix remodeling. Additionally, there was lower expression of genes involved in endobiotic and xenobiotic metabolism, and downregulation of peroxisome function, oxidative phosphorylation, and xenobiotic, bile acid, and fatty acid metabolism. A 13-gene signature, including upregulation of *TREM1* and *MMP1* and downregulation of *CYP2C9*, was consistently associated with all diagnostic categories of pediatric NAFLD.

Conclusion: The extracellular milieu of sera from children with NAFLD induced specific gene profiles distinguishable by a hepatocyte reporter system. Circulating

[Correction added on 4 April 2024, after first online publication: Study dates were added to the Methods section.]

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factors may contribute to inflammation and extracellular matrix remodeling and impair xenobiotic and endobiotic metabolism in pediatric NAFLD.

KEYWORDS

patient serum, pediatric NAFLD, RNA-sequencing, transcriptomic reporter cell assay

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1 | INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent liver disorder among children in the United States, and the incidence has been increasing rapidly.[1–3] In addition to hepatic disease, children with NAFLD have an increased risk for comorbid conditions including cardiometabolic disease, diabetes, kidney disease, obstructive sleep apnea, anxiety, and depression.[4] The trend toward earlier onset of NAFLD has implications for a higher lifetime risk for NAFLD-associated morbidity and mortality. However, mechanisms underlying the pathogenesis of NAFLD in children are not fully elucidated.

Liver biopsy is the current standard for diagnosing NAFLD and monitoring disease progression. Liver biopsy is an invasive procedure that carries risks and cannot be performed in all patients.[5] Moreover, liver biopsy is subject to sampling error due to the nonuniform distribution of disease throughout the liver in relation to the small sample obtained during the procedure.[6] Thus, there is an urgent need to explore new, noninvasive approaches to understanding pediatric NAFLD.

To overcome limited access to affected organs, cellular reporter systems have been used as biosensors that transcriptionally respond to circulating factors. Transcriptomic reporter cell assays which use patient plasma or serum to induce gene expression in a reporter cell population, have been utilized as noninvasive tools to predict and understand several systemic diseases such as sepsis, diabetes, arthritis, and inflammatory bowel disease.[7–10] A cellular reporter system may be a valuable, noninvasive system that could improve our understanding of pediatric NAFLD and/or assist with predicting children who are at risk for developing the disease. In this study, we aimed to investigate differences in gene expression in hepatocytes induced by the sera of children with and without histologically confirmed NAFLD to gain insights into the pathogenesis of pediatric NAFLD.

What is Known

- Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease in children in the United States.
- Children with NAFLD are at risk for end-stage liver disease, liver failure and comorbid conditions.
- By responding to circulating factors, transcriptomic reporter cell assays have improved the understanding of different diseases.

What is New

- Compared to control children with obesity, sera from children with NAFLD induces transcriptional changes in cultured hepatocytes that favor inflammation and extracellular matrix remodeling, and inhibition of endobiotic and xenobiotic metabolism.
- A 13-gene signature is consistently associated with pediatric NAFLD and contributes to prediction of histological features of disease.

2 | METHODS

2.1 | Study participants

This was an ancillary study to the NASH Clinical Research Network (NASH CRN) and was conducted between October 2016 and November 2022. Children 8–17 years-old with NAFLD (cases) and without NAFLD (controls) were evaluated at the Altman Clinical and Translational Research Institute at the University of California, San Diego Medical Center as previously described.[11] Cases were enrolled in the NASH CRN Pediatric Database 2 study (NCT01061684) and underwent detailed phenotyping including demographic, clinical, and histological evaluation. Liver biopsy was evaluated in consensus conference by the NASH CRN Pathology Committee who were unaware of clinical or demographic details. Controls were recruited from primary care clinics with normal weight or obesity without any evidence of liver disease. The absence of liver disease was confirmed by clinical history, laboratory studies, and liver magnetic resonance spectroscopy (MRS).

Biopsies were evaluated for individual and composite features of NAFLD per published criteria.[12] The diagnosis of NAFLD without NASH, borderline NASH type 1a, borderline NASH type 1b, and definite NASH was based on the combined presence and degree of distinct features. Definite NASH generally requires presence of steatosis, inflammation, ballooning degeneration and/or fibrosis. Borderline NASH may have fewer features and

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are further classified by the predominant location of histologic findings in the portal/periportal zone (type 1b) or centrilobular zone (type 1a).

The study was approved by the Institutional Review Boards at each participating center. Written informed consent was obtained from parents/guardians and written assent was obtained from children. Serum samples used for clinical laboratories and used in this study were obtained by phlebotomy after a 12-h overnight fast and stored at −80°C until use.

2.2 | HepaRG cell culture and serum exposure

Terminally differentiated HepaRG cells were cultured per manufacturer guidelines. Briefly, cryopreserved HepaRG cells (BioPredic International) were resuspended and plated on to 48 well polypropylene tissue culture plates at 1.06×10^6 cells/mL with HepaRG Thaw, Plate, and General Purpose medium (Invitrogen). Cells were cultured at 37°C in 5% CO_2 and fed with HepaRG Maintenance/Metabolism medium (Invitrogen) on Days 1, 4, and 7. After culture, terminally differentiated HepaRG cells were exposed to media supplemented with 40% patient serum. After 24 h of incubation at 37°C in 5% CO_2, cells were harvested with Trizol (Life Technologies) and stored at −80°C until use.

2.3 | RNA preparation

Total mRNA was isolated from serum-exposed cells using the RNeasy Mini Kit with on-column DNase treatment (Qiagen) as per manufacturer instructions. RNA integrity number (RIN) was determined using the Agilent Technologies 2100 Bioanalyzer and all samples with RIN > 9 underwent RNA-sequencing (RNA-seq).

2.4 | Library preparation and RNA-seq

Sequencing libraries of RNA from serum-exposed HepaRG cells were prepared by The Jackson Laboratory using the TruSeq RNA library prep kits (Illumina) and sequenced on an Illumina HiSeq. 4000 to generate an average of 23,029,334 2×150 bp paired-end reads per sample.

2.5 | Computational analysis

FastQC (version 0.11) was used to validate raw sequence data quality. Reads were aligned to the Human reference genome (GRCh38) using STAR alignment tool (version 2.6).[13] Absolute mRNA abundance was quantified with FeatureCounts (version 1.6)[14] that counts mapped reads for genomic features and the whole pipeline was run using cgat-flow (https://github.com/cgat-developers/cgat-flow). Differential expression analysis of RNA-seq data was done using DESeq2 package (version 1.16.1)[15] in R (version 3.4.2) and the Benjamini-Hochberg approach to estimate false discovery rate and adjust p-values for multiple testing. All gene expression data were used as an input for gene set/pathway analysis with Gene Set Enrichment Analysis software (version 4.0.3) (GSEA, http://software.broadinstitute.org/gsea/index.jsp),[16] using the Molecular Signatures Database hallmark gene set collection generated by computational methodology to include genes from well-defined biological processes.[17] Genesis (version 1.8.1)[18] was used to generate heatmaps of GSEA enriched genes. Default parameters were used, with exception of the data being adjusted on "Mean Center Genes" and the color parameter was set to "Maximum Positive Expression."

2.6 | Analysis of publicly available data

A study of adults with and without NAFLD (GSE135251)[19] that utilized RNA-seq to examine the transcriptome of liver biopsy samples was compared with the current study to check for similarities and differences with adult NAFLD. The study included 10 adult controls with obesity and 206 adults with NALFD (51 with steatosis or nonalcoholic fatty liver (NAFL) and 153 with NASH with different stages of fibrosis stages F0, F1, F2, F3, and F4). Raw gene counts were downloaded from the NCBI Gene Expression Omnibus (GEO) database. Ensembl gene IDs were mapped to the gene symbol and differential expression analysis was applied to the gene count data comparing all adult NAFLD subcategories combined and individual adult NAFLD subcategories to adult controls with obesity using DESeq2 package in R.

2.7 | Statistical analysis

Patient characteristics were compared among NAFLD categories using Kruskal-Wallis test for continuous variables or Fisher's exact test for categorical variables. Unsupervised principal component analysis (PCA) was performed using all genes as well as differentially abundant genes using R package FactoMineR.[20] Random Forest analysis, for predictive modeling of pediatric NAFLD disease categories by select genes, was performed using the Salford Predictive Modeler® (SPM) software suite Random Forests® (© 2019 Minitab, LLC). Statistical analysis was performed in SAS 9.4 (SAS Institute). $p < 0.05$ was considered statistically significant.

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3 | RESULTS

3.1 | Participants

We included 75 children in this study. Of these, 29 children without NAFLD were controls (15 had normal weight and 14 had obesity). The remaining participants included the following diagnostic categories: 15 NAFLD without NASH (NAFLD), 9 borderline NASH 1a, 11 borderline NASH 1b, and 11 definite NASH. Data from hepatocyte reporter cells exposed to one sample from control children with normal weight and one sample from children with NAFLD were excluded from the analyses due to failed cDNA library preparation.

The overall median age of children in our study was 13 years (IQR 11–15 years). Patient characteristics for each group are shown in Table 1. The study population had similar racial composition across the groups. However, there was a significant difference in age and gender among groups. Notably, all children in the NASH 1b group were Hispanic males. In pairwise comparisons, children in the obesity control group had similar weight and body mass index as children in each of the diagnostic groups (Supporting Information: Tables 1 and 2, Supporting Information: Digital Content 1 and 2, respectively).

3.2 | NAFLD vs controls with obesity

We first examined whether patient sera induced gene expression in hepatocyte reporter cells in sham experiments. We found that hepatocytes exposed to media supplemented with patient sera showed distinct differences in gene expression compared to those exposed to media alone (Figure, Supporting Information: Digital Content 3). To investigate whether hepatocyte reporter cells respond differently to sera from similar children with and without NAFLD, we then compared gene transcripts of hepatocytes exposed to sera from children with NAFLD and those exposed to sera from controls with obesity. PCA was performed to provide a global comparison of all RNA-seq data. This analysis showed overlap between samples associated with NAFLD and controls with obesity (Figure, Supporting Information: Digital Content 4).

To focus the analysis, we examined the most substantial transcriptome differences induced by sera from children with NAFLD and controls with obesity. Differentially expressed genes (DEG) were defined as those with $|\text{Log2FC}| > 0.5$ (at least a 40% difference in expression) and p-value < .05. A total of 195 DEG showed distinct clustering based on the presence of disease (Figure 1), with 73 (37%) upregulated and 122 (63%) downregulated genes associated with NAFLD compared to controls with obesity. (Supporting Information: Tables 3 and 4, Supporting Information: Digital Content 5 and 6, respectively). Among the most significantly DEG, sera from children with NAFLD were associated with higher expression of genes involved in inflammation (*TREM1, CCL7*), collagen synthesis (*COL13A1, COL6A3*), and remodeling of extracellular matrix (*MMP1, MMP9*). Sera from children with NAFLD were also associated with lower expression of genes involved in bile acid metabolism (*SLC10A1, ABCG8, SULT2A*), fatty acid metabolism (*ACOX2*), xenobiotic metabolism (*NR1I3 [CAR]*, and cytochrome P450 enzymes [CYPs] including *CYP2A6, CYP3A4, CYP2C9)*. These finding suggest that genes promoting inflammation, collagen synthesis, and remodeling of extracellular matrix are increased, while those involved in metabolism of endogenous compounds (endobiotics) and xenobiotics are inhibited by the extracellular milieu of sera from children with NAFLD compared to control children with obesity.

To examine potential pathways impacted by disease, we performed untargeted analysis of the RNA-seq data set to determine directional enrichment of gene sets differentially expressed in hepatocytes exposed to sera from children with NAFLD vs control children with obesity. Gene Set Enrichment Analysis identified eight gene sets significantly enriched by sera from controls with obesity compared to NAFLD (adjusted FDR < 0.25), whereas no gene sets were significantly enriched by sera from children with NAFLD compared to controls with obesity (Supporting Information: Table 5, Supporting Information: Digital Content 7). Compared to controls with obesity, exposure to NAFLD sera was associated with downregulation of peroxisome function, oxidative phosphorylation, and xenobiotic, bile acid, and fatty acid metabolism; indicating that transcriptomic changes related to inhibition of energy production and metabolism of compounds occur in hepatocytes in response to sera from children with NAFLD.

3.3 | A core set of 13 DEGs is consistently associated with pediatric NAFLD

For insights into potential mechanisms of disease, we performed pairwise analyses between individual diagnostic categories and controls with obesity. As noted previously, we identified 195 significant DEG in hepatocytes exposed to sera from children with NAFLD compared to controls with obesity; whereas 147, 371, and 149 DEG were identified in hepatocytes exposed to sera from children with NASH 1a, NASH 1b, and definite NASH, respectively, compared to sera from controls with obesity.

To identify a core set of genes associated with pediatric NAFLD, we focused on shared DEG between the different pairwise comparisons. The intersection of comparisons of all diagnostic categories with controls

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TABLE 1 Patient characteristics.

	Controls with Normal Weight ($n = 14$)	Controls with Obesity ($n = 14$)	NAFLD ($n = 14$)	NASH 1a ($n = 9$)	NASH 1b ($n = 11$)	Definite NASH ($n = 11$)	*p* Value
Gender							0.04*,[a]
Male, *n* (%)	10 (71.4)	8 (57.1)	6 (42.9)	6 (66.7)	11 (100)	9 (81.8)	
Race							0.97[a]
White	5 (35.7)	5 (35.7)	4 (28.6)	4 (44.4)	4 (36.4)	5 (45.5)	
Others	9 (64.3)	9 (64.3)	10 (71.4)	5 (55.6)	7 (63.6)	6 (54.5)	
Ethnicity							0.05[a]
Hispanic, *n* (%)	10 (71.4)	8 (57.1)	13 (92.9)	8 (88.9)	11 (100)	7 (63.6)	
Non-Hispanic, *n* (%)	4 (28.6)	6 (42.9)	1 (7.1)	1 (11.1)	0 (0)	4 (36.4)	
Age	14.5 (12–15)	11.5 (10–14)	12 (10–14)	13 (12–15)	11 (10–11)	13 (11–15)	0.03*,[b]
Growth parameters							
Height (cm)	161.9 (154–169.3)	163.2 (141.7–170.3)	159.35 (149.6–163.8)	163.5 (159.5–164.4)	151.5 (139.3–159.7)	166.5 (154.5–166.9)	0.41b
Weight (lbs)	50.5 (40.9–52.9)	81.6 (51.2–86.4)	68.3 (60.5–90.5)	84.2 (74.0–109.5)	60.6 (47.1–78.3)	93.2 (81.9–102.1)	<0.0001*,[b]
BMI	18.3 (17.6–21)	27.80 (26.12–30.73)	27.65 (26.31–29.36)	32.44 (29.56–36.62)	27.10 (23.76–30.97)	34.45 (30.64–36.83)	<0.0001*,[b]
BMI-for-age percentile	39.01 (21.62–78.01)	98.15 (96.81–99.32)	98.27 (96.38–98.99)	98.85 (97.83–99.42)	98.29 (96.36–99.38)	99.36 (98.92–99.49)	<0.0001*,[b]
BMI z-score	−0.28 (−0.79 to 0.77)	2.09 (1.85–2.47)	2.12 (1.80–2.32)	2.27 (2.02–2.52)	2.12 (1.79–2.50)	2.49 (2.30–2.57)	<0.0001*,[b]
Liver function							
ALT	13 (11–17)	17 (12–24)	38 (35–77)	57 (50–134)	43 (31–63)	97 (71–249)	<0.0001*,[b]
AST	22 (19–25)	21.5 (18–25)	31 (23–58)	43 (27–72)	35 (27–52)	58 (45–129)	<0.0001*,[b]
GGT	13.5 (11–17)	18 (15–21)	27 (22–32)	38 (27–40)	22 (18–36)	47 (28–98)	<0.0001*,[b]
Insulin resistance							
Insulin	10 (8–12)	13 (10–26)	19 (16–31)	36 (25–58)	20 (13–24)	53 (36–89)	<0.0001*,[b]
Glucose	87.5 (82–91)	85 (82–89)	84 (80–88)	82 (78–86)	86 (74–88)	84 (82–97)	0.71[b]
Fat Composition							
MRS Liver Fat	1.60 (1.30–2.90)	2.80 (2.10–4.90)	17.95 (13.34–25.69)	21.95 (19.90–28.64)	18.03 (15.52–23.40)	19.26 (12.51–29.70)	<0.0001*,[b]
Body Fat	22.15 (16.20–24.10)	39.40 (34.50–43.40)	41.50 (38.40–46.00)	46.25 (38.15–48.90)	44.80 (38.40–47.30)	42.40 (41.10–45.40)	<0.0001*,[b]
Serum lipids							

TABLE 1 (Continued)

	Controls with Normal Weight ($n = 14$)	Controls with Obesity ($n = 14$)	NAFLD ($n = 14$)	NASH 1a ($n = 9$)	NASH 1b ($n = 11$)	Definite NASH ($n = 11$)	p Value
Triglycerides	58 (42–66)	112.5 (54–203)	135.5 (115–232)	132 (93–216)	122 (105–206)	157 (94–236)	0.0003*[,b]
Total cholesterol	136 (124–150)	157 (126–183)	171.5 (143–191)	157 (149–170)	169 (146–193)	176 (136–183)	0.12[b]
HDL	51 (44–60)	41 (33–50)	46 (43–50)	42 (41–43)	45 (36–53)	39 (32–42)	0.07[b]
LDL	78 (65–85)	86 (69–103)	80 (72–108)	89 (64–95)	101 (80–121)	93 (79–112)	0.34[b]
Steatosis							
5–33%			6 (42.9)	1 (11.1)	4 (36.4)	2 (18.2)	
34–66%			6 (42.9)	2 (22.2)	5 (45.5)	2 (18.2)	
>66%			2 (14.3)	6 (66.7)	2 (18.2)	7 (63.6)	
Fibrosis							
Stage 0			13 (92.9)	3 (33.3)	0 (0)	0 (0)	
Stage 1a			0 (0)	4 (44.4)	0 (0)	0 (0)	
Stage 1b			0 (0)	0 (0)	0 (0)	1 (9.1)	
Stage 1c			1 (7.1)	0 (0)	9 (81.8)	1 (9.1)	
Stage 2			0 (0)	1 (11.1)	1 (9.1)	6 (54.6)	
Stage 3			0 (0)	1 (11.1)	1 (9.1)	3 (27.3)	

Note: Data are presented as median (interquartile range) or *n* (%).

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; cm, centimeters; GGT, gamma-glutamyl transferase; HDL, high-density lipoprotein; lbs, pounds; LDL, low-density lipoprotein; MRS, magnetic resonance spectroscopy; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

[a]Fisher's Exact test.

[b]Kruskal–Wallis test.

*$p < 0.05$.

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with obesity as a baseline shared 13 DEG (Figure 2A and Supporting Information: Table 6, Supporting Information: Digital Content 8). Of these, 7 genes showed increased expression (*CD200R1, MMP1, AC010255.3, PPP2R2C, TREM1, SLAMF8, KLF17*) and 6 genes showed decreased expression (*CCDC129, CASS4, ADH1A, PPP1R1A, CYP2C9*, and *ALPI*) in hepatocytes exposed to sera from children

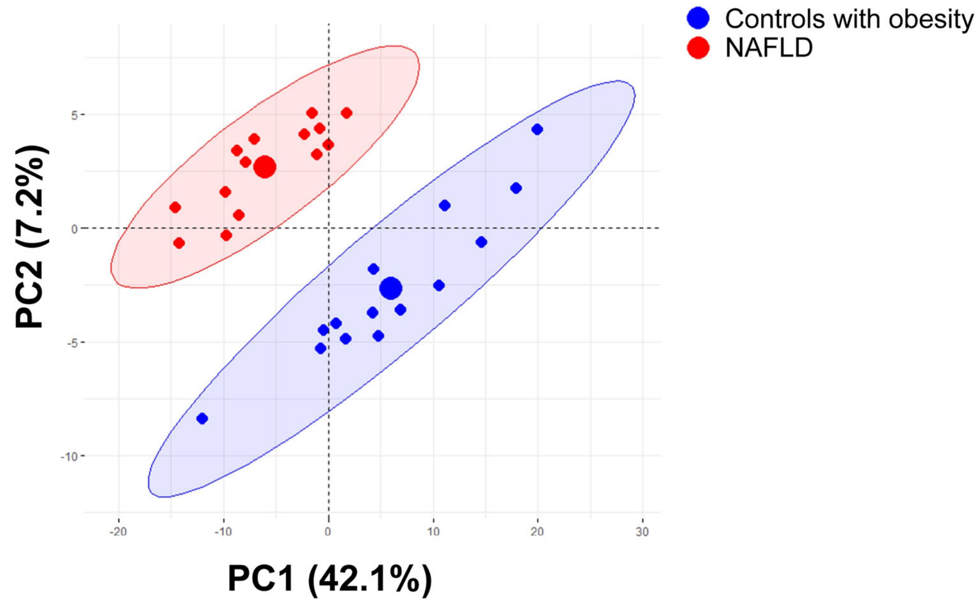
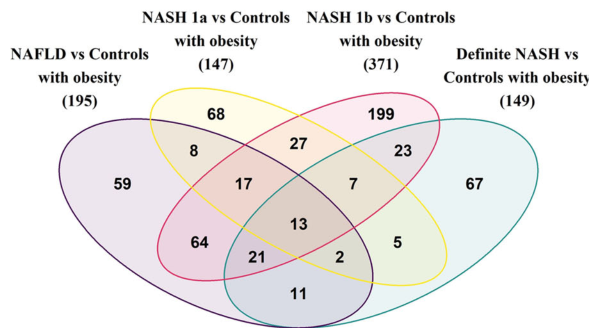


FIGURE 1 Gene expression differences in serum-exposed hepatocyte reporter cells distinguishes children with Nonalcoholic fatty liver disease (NAFLD) and control children with obesity. Principal component analysis (PCA) plot showing the variance in gene expression of 195 differentially expressed genes (DEG) among hepatocyte reporter cells exposed to sera from children with NAFLD ($n = 14$) and controls with obesity ($n = 14$). The variance captured within each coordinate is shown in parenthesis. Smaller symbols represent individual samples, larger symbols represent the centroid (or average of each group), and the ellipses represent the 95th percentile of each group.

(A)



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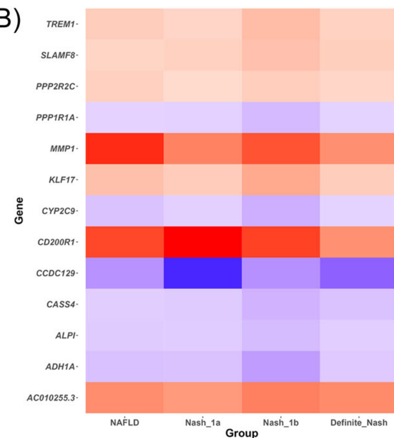

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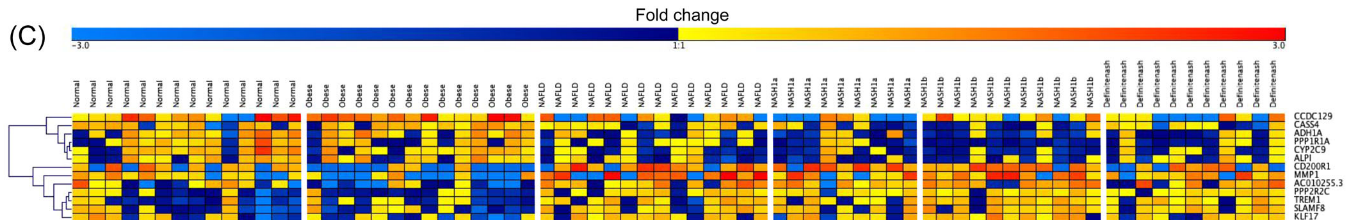

FIGURE 2 Gene signature in serum-exposed hepatocytes associated with pediatric NAFLD. (A) Venn diagram showing the number of differentially expressed genes (DEG) identified by pairwise analyses using controls with obesity as a baseline. (B) Heatmap of the 13 genes associated with NAFLD. Data presented using $\log_2$ fold change (L2FC) in expression compared to controls with obesity. (C) Heatmap showing hierarchical clustering of gene expression profiles induced by sera from controls with normal weight (Normal), controls with obesity (Obese), NAFLD, NASH 1a, NASH 1b, and Definite NASH using the 13 genes associated with NAFLD. Data presented using fold change in expression. NAFLD, Nonalcoholic fatty liver disease; NASH, Nonalcoholic steatohepatitis.

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with disease compared to controls with obesity. The direction of regulation of all 13 genes was consistent throughout all disease categories (Figure 2B) and similar when all stages of NASH were combined (Figure, Supporting Information: Digital Content 9). Notably, the serum of control children with normal weight induced expression profiles that clustered with those of controls with obesity when using the 13 genes, further associating the gene signature with pediatric NAFLD (Figure 2C). Collectively, these findings demonstrate that the extracellular milieu of sera from children with NAFLD induces disease-specific gene profiles that can be discriminated by a hepatocyte reporter system.

Although most children with NAFLD have obesity, there are some children with NAFLD who have normal weight. To examine whether the 13 DEG that were associated with pediatric NAFLD compared to control children with obesity were also associated with pediatric NAFLD compared to control children with normal weight, we performed pairwise analyses between individual diagnostic categories and controls with normal weight as a baseline. We found 130 DEG that were shared between the different pairwise comparisons (Figure, Supporting Information: Digital Content 10A). Of these, 5 genes were among the 13 DEG of NAFLD compared to controls with obesity (*TREM1, PPP1R1A, CYP2C9*, *ALPI*, and *ADH1A*). The direction of regulation of all 5 genes was consistent throughout all disease categories and similar to the direction of regulation in their comparison to controls with obesity (Figure, Supporting Information: Digital Content 10B). Interestingly, none of the five genes were significantly differentially expressed in the comparison of controls with obesity versus controls with normal weight. These findings suggest that serum-induced expression of these five genes in hepatocyte reporter cells may be useful in discriminating pediatric NAFLD in children with normal weight.

To examine whether there was a serum-induced signature in hepatocytes that was associated with disease progression in pediatric NAFLD, we combined all stages of NASH and determined the overlap of genes between NAFLD compared to controls with obesity and all NASH combined compared to NAFLD. There were no overlapping genes among these two comparisons and suggests that while there is a serum-induced 13-gene signature in hepatocytes that associates with the disease spectrum of pediatric NAFLD compared to controls with obesity, there are no genes in serum-exposed hepatocytes that could detect disease progression.

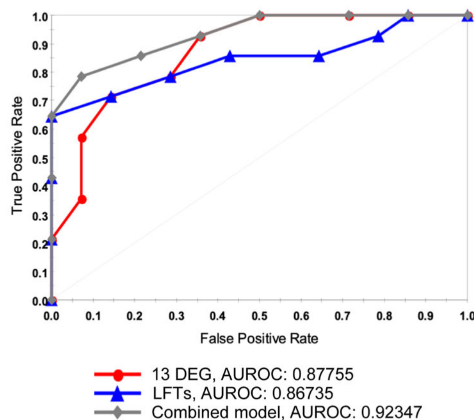
3.4 | Similarities and differences in the 13 DEG in adult NAFLD

Due to the lack of publicly available RNA-seq data from liver samples of children with NAFLD, we were unable to directly evaluate the 13-gene signature in pediatric liver biopsy specimens. To examine whether any of the 13 signature genes showed consistent associations in adult NAFLD, published RNA-seq transcriptomic data from liver biopsy specimens from adults with and without NAFLD[19] were downloaded and computationally analyzed in a similar manner as described in our methods. Pairwise comparisons were performed between all adult NAFLD subcategories combined and individual adult NAFLD subcategories to adult controls with obesity. Among the 13 DEG associated with pediatric NAFLD in our study, *PPP1R1A*, *MMP1*, and *CD200R1* were significantly differentially expressed in adult NAFLD liver. Consistent with the decreased expression of *PPP1R1A* in our pediatric NAFLD comparisons to control children with obesity, the expression *PPP1R1A* was also lower in the liver of all adult NAFLD subcategories combined and decreased with worsening disease in adult NASH F2-4 compared to healthy adults with obesity (Supporting Information: Table S7, Supporting Information: Digital Content 11). *PPP1R1A* encodes the protein phosphatase 1 inhibitor protein 1A and reduced levels of have been associated with impaired beta-cell function in type 2 diabetes,[21] and its decreased expression may reflect impaired insulin sensitivity in NAFLD. In contrast to the increased expression of *MMP1* that we found in pairwise comparisons of all subcategories of pediatric NAFLD to control children with obesity, the expression of *MMP1* was lower in all adult NAFLD subcategories combined and in advancing adult disease. The differential expression of *CD200R1*, involved in limiting inflammation by inhibiting the expression of proinflammatory molecules, was only seen in NASH F4 compared to heathy adults with obesity. Differences in the expression of the 13 signature genes in adult NAFLD may be due to the presence of multiple cell types in liver biopsy specimens versus only hepatocytes in our reporter cell assay and/or differences in the pathogenesis of adult and pediatric NAFLD.

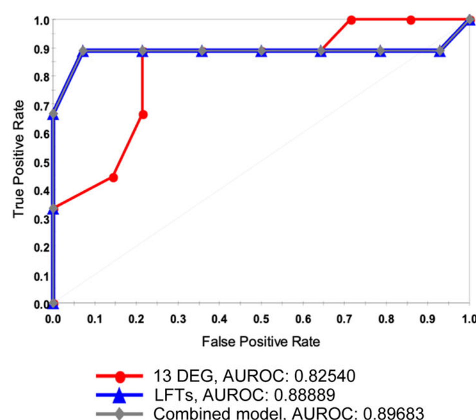
3.5 | A 13-gene signature contributes to prediction of histological features of pediatric NAFLD

We next performed random forest analysis using the 13-gene signature and serum transferases, (AST, ALT, and GGT), to examine their relationship with histological features of pediatric NAFLD (Figure 3). The combination of gene signature with clinical labs resulted in improvement in distinguishing NAFLD and NASH 1b from controls with obesity compared to clinical labs alone. The gene signature alone was better at predicting NASH 1b than clinical labs alone (AUROC 0.922 vs. 0.805). While ALT was the most important variable for distinguishing NAFLD, NASH 1a, and

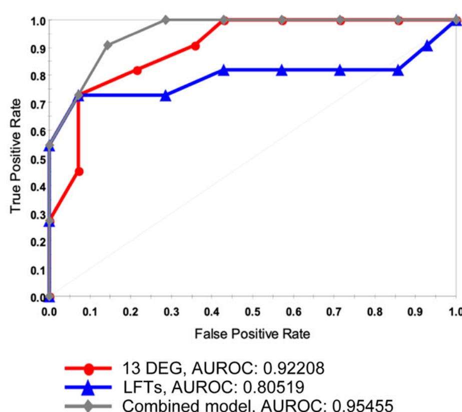
(A) **NAFLD vs Controls with obesity**



(B) **NASH 1a vs Controls with obesity**



(C) **NASH 1b vs Controls with obesity**



(D) **Definite NASH vs Controls with obesity**

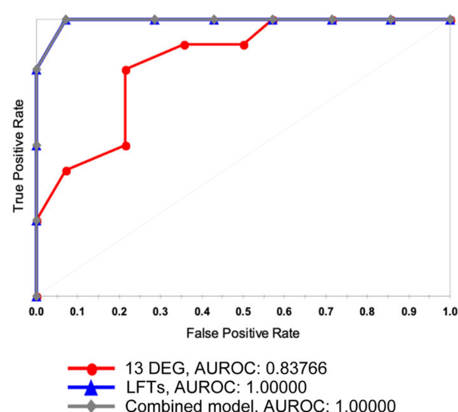


FIGURE 3 Predictive modeling of disease categories versus controls with obesity using the 13-gene signature associated with pediatric NAFLD and liver function tests. Area under the receiver operating characteristic (AUROC) curves showing the random forest predictive models using the 13-gene signature associated with NALFD, liver function tests (LFTs), and the combined model of 13-genes plus LFTs for (A) NAFLD, (B) NASH 1a, (C) NASH 1b, and (D) Definite NASH. NAFLD, Nonalcoholic fatty liver disease; NASH, Nonalcoholic steatohepatitis.

definite NASH from controls with obesity; serum-induced expression of *TREM1*, involved in amplifying neutrophil and monocyte-mediated inflammatory responses triggered by bacterial and fungal infections, emerged as the most important variable in predicting NASH 1b (Figure, Supporting Information: Digital Content 12). These findings suggest that a serum-induced 13-gene signature in hepatocyte reporter cells contributes to prediction models of histological features of pediatric NAFLD with high accuracy.

4 | DISCUSSION

In this study, we have shown the potential of a transcriptomic hepatocyte reporter system in pediatric NAFLD. Our RNA-seq analysis revealed that distinct disease gene signatures can be identified by cultured hepatocytes exposed to sera from children with and without NAFLD. Compared to control children with obesity, the extracellular milieu of sera from children with NAFLD induces transcriptional changes that favor inflammation, collagen synthesis, and extracellular matrix remodeling, while inhibiting energy production and bile acid, fatty acid, and xenobiotic metabolism. We identified 13 DEG that were consistently associated with all diagnostic categories of NAFLD relative to control children with obesity. Importantly, our findings demonstrate that this 13-gene signature contributed to prediction models of histological features of pediatric NAFLD.

This study represents the first attempt to explore the use of reporter cells in the context of pediatric NAFLD. Transcriptomic analysis of reporter cells has been shown to generate informative, disease-specific transcriptional profiles in response to bioactive molecules present in the systemic circulation, without the need for tissue samples. The use of serum- and plasma-exposed cells have provided valuable insights into the pathogenesis of sepsis, type I diabetes, Crohn's disease, and ulcerative colitis[7,8,10] and has led to novel therapeutic interventions for juvenile idiopathic arthritis.[9,22,23]

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Using RNA-seq for a comprehensive transcriptomic analysis, we demonstrated that hepatocytes exhibit a unique transcriptional response to circulating factors in children with NAFLD compared to children without liver disease. We found that the extracellular milieu of sera from children with NAFLD induces transcriptional changes in genes and pathways known to be involved in the disease. Specifically, the increased hepatocyte expression of *TREM1, CCL7, COL13A1, COL6A3, MMP1*, and *MMP9* in response to sera from children with NAFLD are consistent with the current understanding that inflammation, tissue regeneration, and fibrogenesis are critical in NAFLD.[24] *TREM1* encodes a receptor that stimulates the release of proinflammatory chemokines and cytokines that amplify inflammatory responses to foreign antigens and toxic substances.[25,26] Using an animal model of NAFLD and hepatocyte cell lines, Rao et al showed that *TREM1* regulated key P13K/AKT and NFKB signaling genes that promoted lipid accumulation and secretion of proinflammatory cytokines from hepatocytes.[27] *CCL7* encodes a secreted chemokine that attracts immune cells during injury and inflammation,[28] and CCL7 secreted by hepatocytes damaged by lipopolysaccharide or palmitate was shown to promote macrophage infiltration and inflammation.[29] Collagen proteins and matrix metalloproteinases are important in remodeling the extracellular matrix, and *COL6A3* and *MMP1* were shown to enhance hepatic fibrosis in NAFLD.[30,31] Our findings suggest that hepatocytes may be augmenting inflammation and fibrosis in response to components in the sera of children with NAFLD.

We also found that exposure to sera from children with NAFLD resulted in lower hepatocyte expression of important genes and pathways involved in endobiotic and xenobiotic metabolism, when compared to controls with obesity. This is consistent with current evidence of altered lipid, bile acid, and drug metabolism in patients with NAFLD,[32,33] and in animal models of NAFLD.[34,35] Specifically, we observed that exposure to sera from children with NAFLD was associated with reduced expression of the nuclear receptor *NR1I3* (*CAR*, constitutive androstane receptor), which regulates the transcription of many genes encoding phase I and phase II enzymes involved in drug metabolism and transport.[32] This suggests that the altered metabolism of endobiotics and xenobiotics in children with NAFLD may be related to inhibition of *CAR*.

Through analysis of the response of hepatocyte reporter cells to sera from children with different categories of NAFLD, we were able to identify transcriptome changes specific to pediatric NAFLD. A core set of 13 genes were consistently differentially expressed and similarly regulated in hepatocytes after exposure to sera from children of all NAFLD categories compared to controls with obesity, suggesting their contribution to the pathogenesis of pediatric NAFLD. This NAFLD disease-specific gene signature included upregulation of *TREM1* and *MMP1* and downregulation of *CYP2C9*, suggesting that circulating factors may induce inflammation and extracellular matrix breakdown and impair xenobiotic and endobiotic metabolism in hepatocytes.

We also found that the 13-gene signature contributed to good prediction models of histological features of pediatric NAFLD. Interestingly, our gene signature was more effective at predicting NASH 1b than liver chemistry alone, with *TREM1* being the most important variable in the prediction model for NASH 1b verses controls with obesity. While children with NASH 1a have steatosis, ballooning degeneration, and perisinusoidal fibrosis more typical of adult NASH, children with NASH 1b have findings characterized by steatosis, portal inflammation, and portal fibrosis in the absence of ballooning degeneration and perisinusoidal fibrosis.[36] The location of portal inflammation and fibrosis of NASH 1b is notable, as blood from the gastrointestinal tract reaches the liver via the portal vein. The microbiome has been shown to be related to NAFLD in adults and children.[11,37] Given that TREM1 augments the inflammatory response to bacteria, our finding that the 13-gene signature better predicts NASH 1b and that *TREM1* had the highest variable importance score in distinguishing NASH 1b from controls with obesity suggests that hepatocytes exposed to microbes and/or microbially derived products may contribute to *TREM1*-driven portal inflammation in NASH 1b.

From a clinical perspective, there is an important need for noninvasive approaches for screening and diagnosing pediatric NAFLD given the risks and limitations of liver biopsy. Patient sera are more easily obtained, and while studies have previously measured systemic serum markers, examining the transcriptomic response of hepatocytes to circulating factors may provide more liver-specific information regarding pediatric NAFLD. Detecting differences in serum-induced gene signatures in hepatocyte reporter cells has the potential to improve screening, diagnosis, and enable early interventions. In this study, we used a novel approach of analyzing the response of hepatocyte reporter cells to sera from children with different categories of pediatric NAFLD. This approach allowed us to identify a 13-gene signature that can distinguish the presence and absence of disease in different cohorts. However, the small sample size and heterogeneity of the disease may have limited our ability to detect differences among the different categories of NAFLD. Therefore, our findings need to be validated in larger and more diverse cohorts. Despite these limitations, our findings suggest that circulating factors may play a role in inducing changes in gene expression in hepatocytes, which could be relevant to the

pathophysiology of pediatric NAFLD. However, due to the limited volume of serum samples, we were unable to test for proteins encoded by the genes in our gene signature, cytokines, and other potential mediators. Future studies utilizing metabolomics, lipidomics, and/or inflammatory assays are needed to further examine the mechanistic links between circulating factors and hepatocyte gene expression.

5 | CONCLUSION

In conclusion, our study provides novel insights into pediatric NAFLD. Our findings suggest that circulating factors may alter endobiotic and xenobiotic metabolism, inflammation, and extracellular matrix remodeling in pediatric NAFLD. Moreover, we identified a serum-induced 13-gene signature in hepatocyte reporter cells that may serve as a useful tool for predicting histological features of the disease.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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