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Association Between Cytokines and Liver Histology in Children with Nonalcoholic Fatty Liver Disease

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Reliable noninvasive markers to characterize inflammation, hepatocellular ballooning, and fibrosis in nonalcoholic fatty liver disease (NAFLD) are lacking. We investigated the relationship between plasma cytokine levels and features of NAFLD histology to gain insight into cellular pathways driving nonalcoholic steatohepatitis (NASH) and to identify potential noninvasive discriminators of NAFLD severity and pattern. Cytokines were measured from plasma obtained at enrollment in pediatric participants in NASH Clinical Research Network studies with liver biopsy-proven NAFLD. Cytokines were chosen *a priori* as possible discriminators of NASH and its components. Minimization of Akaike information criterion was used to determine cytokines retained in multivariable models. Of 235 subjects, 31% had "Definite NASH" on liver histology, 43% had "Borderline NASH," and 25% had NAFLD but not NASH. Total plasminogen activator inhibitor 1 (PAI1) and activated PAI1 levels were higher in pediatric participants with Definite NASH and with lobular inflammation. Interleukin-8 (IL-8) was higher in those with stage 3-4 fibrosis and lobular inflammation. Soluble IL-2 receptor alpha was higher in children with stage 3-4 fibrosis and portal inflammation. In multivariable analysis, PAI1 variables were discriminators of Borderline/Definite NASH, Definite NASH, lobular inflammation, and ballooning. IL-8 increased with steatosis and fibrosis severity; soluble IL-2 receptor alpha increased with fibrosis severity and portal inflammation. IL-7 decreased with portal inflammation and fibrosis severity. *Conclusion*: Plasma cytokines associated with histology varied considerably among NASH features, suggesting promising avenues for investigation. More targeted analysis is needed to identify the role of these markers in NAFLD and to evaluate their potential as noninvasive discriminators of disease severity. (*Hepatology Communications* 2017;1:609–622)

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common pediatric liver disease in the United States. A subset of children with NAFLD have nonalcoholic steatohepatitis (NASH), which can progress to cirrhosis[(1)] and increases the risk for morbidities, including diabetes, cardiovascular disease, and hepatocellular carcinoma.[(2,3)] Routine imaging and blood tests are helpful to identify children and adolescents with NAFLD, but liver biopsy is currently

Abbreviations: AIC, Akaike information criterion; ALT, alanine aminotransferase; aPAI1, activated plasminogen activator inhibitor 1; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; hpf, high-powered field; IGF-II, insulin-like growth factor 2; IL, interleukin; MMP-9, matrix metallopeptidase 9; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NASH CRN, NASH Clinical Research Network; OR, odds ratio; OSA, obstructive sleep apnea; PAI1, plasminogen activator inhibitor 1; sIL-1RI, soluble IL-1 receptor 1; TGF, transforming growth factor; TNF, tumor necrosis factor; TONIC, treatment of nonalcoholic fatty liver disease in children; tPAI1, total plasminogen activator inhibitor 1; VEGF, vascular endothelial growth factor.

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required for diagnosis of NASH and for staging NASH severity.

Immune system pathways that drive hepatic steatosis, inflammation, apoptosis, and fibrosis in NASH are insufficiently understood. In addition, diagnosing NASH severity and histologic pattern requires a liver biopsy, an invasive procedure that relies on a limited liver sample. We lack reliable noninvasive markers for inflammation severity, hepatocellular ballooning, fibrosis, and the age-specific pattern of NASH histology. To address these two gaps, we have recently reported on assocations between NASH histologic severity and cytokine levels in adult NASH Clinical Research Network (NASH CRN) participants.[4] NASH histologic patterns stereotypically differ between children and adults, although overlap is observed. This parallel investigation of cytokine associations with histology in pediatric NASH CRN participants provides novel insight into differences and similarities. By examining a panel of cytokines in a large pediatric cohort, we provide direction for more targeted investigations of promising biomarkers.

Children can have the "adult-type" zone 3 NASH, with pericentral/sinusoidal fibrosis and primarily lobular inflammation, with or without ballooned hepatocytes. However, they can also have a "pediatric" NASH, called zone 1 NASH, characterized by periportal (i.e., zone 1) or panacinar steatosis, portal fibrosis, and primarily portal inflammation. It is not yet clear whether zone 1 NASH is a precursor to or distinct entity from zone 3 NASH, nor how their natural histories differ, although advanced fibrosis and cirrhosis can be an end stage for either.[5]

Previous studies of biomarkers for NASH in children examined a limited number of biomarkers in small cohorts.[6-14] Most studies did not evaluate NASH histology, instead relying on surrogate markers or imaging as a substitute for diagnosis.[8,11-14] Plasminogen activator inhibitor 1 (PAI1) has been associated with NAFLD in three studies in children[9,10,15] as well as adult studies; but other markers, including tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-10, and resistin have been inconsistent predictors of NAFLD in smaller cohorts of adults and children (n = 40-65).[7,9,10,16-18]

This study provides a unique examination of a set of plasma biomarkers in a large cohort of children with biopsy-proven NAFLD. Cytokines and other analytes were chosen *a priori* as possible NASH predictors based on their known role in steatosis, inflammation, fibrosis, angiogenesis, or glucose metabolism. Our aims were to gain insight into the cellular pathways driving NASH and to refine the search for potential noninvasive biomarkers of NASH severity and subtype. We also evaluated associations in children with borderline zone 3 and borderline zone 1 NASH to investigate whether processes driving NASH in these two groups appear to be similar or distinct.

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Patients and Methods

STUDY DESIGN

This was a cross-sectional study using enrollment data from pediatric participants in the NASH CRN, a multicenter network sponsored by the National Institute of Diabetes, Digestive and Kidney Diseases since 2002.[19] Study participants were drawn from two groups within the NASH CRN: (1) Treatment of NAFLD in Children (TONIC) trial (ClinicalTrials.gov Identifier:NCT00063635) and (2) NAFLD Database study, an observational cohort. Both studies have Institutional Review Board approval at all clinical centers participating in the NASH CRN. Written permission was obtained prior to participation from a parent/guardian; written assent was obtained from children ≥8 years old. NASH CRN Pediatric and Resource centers are listed in the Acknowledgment section.

STUDY POPULATION

The NAFLD Database was an observational study of participants 2 years and older with NAFLD. Eligibility for the NAFLD Database study included having either a histologic diagnosis of NAFLD or cryptogenic cirrhosis with suspected NAFLD based on imaging studies. The TONIC trial was a phase IIb, double-masked, randomized, placebo-controlled trial of metformin or vitamin E versus placebo in children 8-17 years of age with NAFLD. Participants in TONIC were required to have a baseline alanine aminotransferase (ALT) value of 60 U/L or greater and biopsy-proven NAFLD.[20] Exclusion criteria for both studies included no or restricted alcohol intake, other chronic liver diseases, history of parenteral nutrition, bariatric or hepatobiliary surgery, human immunodeficiency virus infection, inborn errors of metabolism, or short-bowel syndrome. Additional exclusion criteria for TONIC included age less than 8 years, diagnosis of diabetes, cirrhosis, use of drugs associated with NAFLD, antidiabetic or anti-NAFLD drugs, metabolic acidosis, and renal dysfunction. Enrollment began in September 2004 for the NAFLD Database and in August 2005 for TONIC.[21] Data collection for this analysis was completed by April 2010. Both studies have been described.[20,22] Participants from both studies age 17 years and younger (total TONIC, n = 173; NAFLD Database, n = 116) were eligible for inclusion in the current study if they had a fasting plasma sample available within 6 months of liver biopsy reviewed by the central Pathology Committee (TONIC, n = 173; NAFLD Database, n = 79) and had at least two plasma aliquots available for analysis (TONIC, n = 162; NAFLD Database, n = 73).

CLINICAL AND LABORATORY ASSESSMENT

Demographic data and self-reported doctor-diagnosed comorbidities were obtained via structured interview and questionnaires. Height, weight, and waist/hip measurements were taken in duplicate while standing, wearing light clothing, and averaged for analyses. Height and weight were measured without shoes to the nearest 0.1 cm and 0.1 kg, respectively. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. The BMI z-score was determined according to age, sex, height, and weight based on data from the Centers for Disease Control and Prevention 2000 reference population.[23]

Fasting whole blood samples were obtained via venipuncture after an overnight fast of 8 hours or more and processed for plasma and serum within 2 hours. Laboratory assays were performed at individual clinical centers and included platelet count (10^9 cells/L), bilirubin (mg/dL), ALT (U/L), aspartate aminotransferase (AST; U/L), triglycerides and cholesterol fractions (mg/dL), glucose (mg/dL), and insulin (mU/mL). Homeostasis model assessment of insulin resistance was calculated from fasting insulin and glucose values.[24]

HISTOLOGIC EVALUATION

Biopsy specimens were evaluated centrally by the NASH CRN Pathology Committee according to the validated histologic scoring system by Kleiner et al.[25] for steatosis (grade 0 [<5% macrovesicular fat], grade 1 [5%-33%], grade 2 [34%-66%], and grade 3 [>66%]), portal inflammation (0-2 [none, mild, more than mild]), lobular inflammation (0-3 [none, <2 foci per high-powered field [hpf], 2-4 foci, >4 foci per 20× field]), ballooning degeneration (0-2 [none, few, many]), and fibrosis (stage 0, stage 1a [mild perisinusoidal], stage 1b [moderate perisinusoidal], stage 1c [portal/periportal fibrosis only], stage 2 [zone 3 and periportal], stage 3 [bridging fibrosis], and stage 4 [cirrhosis]).

A diagnostic categorization was determined for each case: NAFLD, Not NASH, Borderline Zone 3, Borderline Zone 1, or Definite NASH.[22,26] Borderline Zone 1 NASH presents mainly in children and is

TABLE 1. CLINICAL AND DEMOGRAPHIC CHARACTERISTICS OF THE COHORT BY NASH DIAGNOSIS

	Total (n = 235) %	Not NASH (n = 62) %	Borderline Zone 1 NASH (n = 53) %	Borderline Zone 3 NASH (n = 47) %	Definite NASH (n = 73) %
Female	22.4	26.7	13.2	26.1	23.3
Race					
Hispanic	59.5	61.2	64.2	58.7	54.7
White, non-Hispanic	33.6	31.2	32.1	32.6	36.9
Black, non-Hispanic	1.7	1.7	0	4.4	1.3
Other, non-Hispanic	5.2	5.0	3.8	4.4	6.8
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Age at biopsy (years)	13.1 ± 2.6	13.6 ± 2.9	11.8 ± 2.0	13.6 ± 2.8	13.5 ± 2.3
BMI z-score	2.3 ± 0.4	2.3 ± 0.7	2.3 ± 0.3	2.3 ± 0.4	2.3 ± 0.3
ALT (U/L)	107.5 ± 74.6	76.4 ± 42.9	104.6 ± 53.4	97.5 ± 61.9	143.5 ± 99.7
AST (U/L)	62.7 ± 40.2	45.8 ± 25.3	61.3 ± 31.7	54.1 ± 26.6	83.6 ± 52.9
Total bilirubin (mg/dL)	0.7 ± 0.3	0.7 ± 0.4	0.7 ± 0.3	0.7 ± 0.3	0.6 ± 0.3
Triglycerides (mg/dL)	147.7 ± 94.6	126.9 ± 59.0	137.3 ± 109.7	151.0 ± 108.5	170.4 ± 94.5
HDL cholesterol (mg/dL)	37.9 ± 9	37.3 ± 7.7	41.6 ± 10.8	38.0 ± 10.4	35.6 ± 6.8
HOMA-IR	8.3 ± 10.7	7.5 ± 9.4	6.9 ± 6.1	7.2 ± 5.6	10.5 ± 15.1

Abbreviations: HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance.

characterized by periportal fibrosis with steatosis involving zone 1 and minimal or no zone 3 injury.[26] Definite NASH unequivocally fulfilled previously defined criteria for steatohepatitis,[25] whereas the category NAFLD, Not NASH encompasses cases of NAFLD in which diagnostic NASH features are absent.

PLASMA BIOMARKER MULTIPLEX ASSAY

Thirty-two plasma biomarkers were chosen *a priori* as possible predictors of NASH or its components (steatosis, inflammation, fibrosis), energy homeostasis, angiogenesis, or metabolism of lipids, glucose, and insulin. Biomarkers were measured from an enrollment sample for all children, which was prior to beginning medications for those in the TONIC trial. Blood samples were obtained by venipuncture and plasma collected by centrifugation and stored at –80°C. Plasma samples were measured in duplicate using the Luminex Multiplex platform (Millipore, St. Louis, MO) and processed according to standard protocol. Detection by Luminex is comparable to that with enzyme-linked immunosorbent assay, but the required input plasma volume is drastically reduced. Quality control procedures were employed to ensure high-quality data for downstream analyses.

The coefficient of variation for plasma biomarkers was required to be less than 20%, a criterion met for all biomarkers included in this analysis (Supporting Table S1). Biomarkers available in less than 90% of the sample were excluded (glucagon-like peptide 1, soluble vascular endothelial growth factor receptor 1, IL-1α, IL-12/40 kDa subunit, IL-13, transforming growth factor beta 3 [TGF-β3]).

STATISTICAL ANALYSIS

This analysis considered clinical and plasma biomarker predictors of histologic outcomes. Histologic outcomes were collapsed into binary categories as follows: NASH diagnosis (Not/Borderline NASH versus Definite NASH, Not versus Definite NASH), steatosis severity (steatosis $\leq$33% versus steatosis $>$33%), inflammatory pattern and severity (lobular, $<$2 foci per hpf versus $\geq$2 per hpf; portal, none/mild versus more than mild), hepatocellular ballooning (none versus few/many), and fibrosis severity. For fibrosis severity, two categorizations were considered: (1) no fibrosis (stage 0) versus any fibrosis (stage 1-4) and (2) no/minimal fibrosis (stage 0-2) versus significant fibrosis (stage 3-4).

Descriptive statistics and frequency distributions were generated on the sample demographic and clinical characteristics and plasma biomarker measurements. The Student t test accounting for unequal variances (Tables 1 and 2; Supporting Tables S2-S4) and logistic regression (Tables 3 and 4) were used for bivariate analysis of associations between continuous clinical characteristics or biomarker discriminators and histologic outcomes, and Pearson's chi-square test was used for categorical clinical characteristics (Table 1). All odds ratios (ORs) for cytokines in univariable and

TABLE 2. PLASMA BIOMARKER LEVELS AND NASH DIAGNOSIS

	n	Total Mean ± SD	Not/Borderline NASH (n = 162) Mean ± SD	Definite NASH (n = 73) Mean ± SD	*P*
Adiponectin (μg/mL)	233	13.1 ± 7.48	13.20 ± 7.26	13.00 ± 7.98	0.92
αPAI1 (ng/mL)	233	54.97 ± 43.96	50.46 ± 45.09	64.87 ± 39.93	0.02
FGF2 (pg/mL)	232	98.6 ± 141.3	93.8 ± 146	109.1 ± 130.9	0.42
Fibrinogen (mg/mL)	234	4.39 ± 2.11	4.44 ± 1.69	4.31 ± 2.84	0.72
Haptoglobin (mg/mL)	235	2.47 ± 1.27	2.48 ± 1.27	2.44 ± 1.28	0.81
IFN-γ (pg/mL)	218	8.7 ± 21.3	8.3 ± 21.3	9.6 ± 21.5	0.69
IGF-II (mg/mL)	234	1.83 ± 0.99	1.79 ± 0.99	1.89 ± 1.02	0.48
IL-1b (pg/mL)	215	0.5 ± 0.8	0.5 ± 0.8	0.6 ± 0.6	0.78
IL-2 (pg/mL)	226	3.7 ± 8.8	3.6 ± 9.8	4.0 ± 5.8	0.65
IL-4 (pg/mL)	226	24.8 ± 31.9	24.5 ± 32.5	25.4 ± 30.7	0.84
IL-5 (pg/mL)	230	1.0 ± 1.8	0.9 ± 1.9	1.1 ± 1.6	0.52
IL-6 (pg/mL)	235	15.2 ± 91.1	16.7 ± 108.6	12.0 ± 24.0	0.6
IL-7 (pg/mL)	226	5.0 ± 6.4	5.2 ± 7.4	4.7 ± 3.5	0.47
IL-8 (pg/mL)	235	3.1 ± 2.0	2.9 ± 2.2	3.4 ± 1.5	0.07
IL-10 (pg/mL)	234	24.9 ± 99.4	26.1 ± 118.2	22.2 ± 27.5	0.69
MCP-1 (pg/mL)	235	246.5 ± 100.6	246.9 ± 105.3	245.7 ± 90	0.93
MMP-9 (ng/mL)	235	63.9 ± 33.3	64.1 ± 35.7	63.4 ± 27.6	0.88
Resistin (ng/mL)	234	14.89 ± 6.32	14.91 ± 6.49	14.87 ± 5.97	0.97
sFasL (pg/mL)	227	107.6 ± 71.4	110.2 ± 78.8	102.1 ± 51.1	0.36
sIL-1RI (pg/mL)	235	33.8 ± 23.0	32.2 ± 23.8	37.2 ± 32.4	0.11
sIL-2Rα (ng/mL)	235	0.79 ± 0.36	0.79 ± 0.34	0.79 ± 0.41	0.91
sIL-6R (ng/mL)	235	21.24 ± 5.39	21.33 ± 5.37	21.04 ± 5.49	0.71
TGF-β1 (ng/mL)	235	6.86 ± 7.61	7.11 ± 8.63	6.28 ± 4.59	0.34
TGF-β2 (pg/mL)	223	359.6 ± 343.1	377.9 ± 401.1	321.2 ± 161.2	0.13
TNF-α (pg/mL)	235	8.4 ± 8.8	7.6 ± 5.0	10.1 ± 13.9	0.13
tPAI1 (ng/mL)	235	45.8 ± 24.8	43.2 ± 24.3	51.4 ± 25.1	0.02
VEGF (pg/mL)	235	551.3 ± 1083.4	473.9 ± 981.7	723 ± 1271.3	0.14

Abbreviations: FGF2, fibroblast growth factor 2; IFN-γ, interferon gamma; MCP-1, monocyte chemoattractant protein-1; sFasL, soluble Fas ligand.

multivariable analysis are reported per 0.5 SD change in the cytokine based on SDs for the total group as reported in Table 2. Significance using the Benjamini-Hochberg correction with a false discovery rate of 10% also provided for univariate logistic regression of cytokine changes with outcomes.[27]

Given the number of predictors considered and the exploratory aim of this analysis, predictors were chosen for the final multivariable models to minimize the Akaike information criterion (AIC) (Table 5). AIC minimization allows choice of the model that best fits the data with penalization for the number of predictors included; it is an empiric method for optimizing model fit without overfitting. The AIC statistic is calculated using a model's log-likelihood, the number of parameters included in the model, and the sample size. AIC for candidate models are compared, and the model with the lowest AIC is retained. AIC comparisons are automated, as described below, such that all variable combinations can be tested. The order of variable inclusion/exclusion does not impact results, thus avoiding incorrect selection that can be caused by stepwise selection methods in data sets with many predictors.

First, all cytokines were competed in a logistic regression model. The model with the lowest AIC, i.e., the best data fit with penalization for the number of predictors included, was retained for each outcome. That limited groups of cytokines competed in a second logistic regression model with clinical predictors chosen *a priori* for their association with NASH diagnosis and severity (age, sex, BMI z-score, AST, ALT, triglycerides, high-density lipoprotein, and homeostasis model assessment of insulin resistance). Cytokine and clinical predictors retained in the AIC-minimized model were re-entered into a final logistic regression to obtain the results reported in Table 5. Thus, inclusion in this final model was based on AIC values, not on *P* values. Sensitivity analysis was done excluding those children with Borderline Zone 1 NASH.

For multivariable models, missing values for cytokine and clinical data were imputed using a multiple imputations technique with progressive mean matching and iterative chained equations. To allow for automated AIC minimization, the mean of 20 imputed values was retained and used for predictor selection in the same process described above. All statistical

TABLE 3. UNADJUSTED OR (95% CI) FOR CHANGE IN CYTOKINE LEVEL BY 0.5 SD BY HISTOLOGIC OUTCOME

	Definite NASH (vs. Not/Borderline)		Any Fibrosis (stage 1-4)		Significant Fibrosis (stage 3-4)		Steatosis (>33%)	
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Adiponectin (μg/mL)	0.99	0.86-1.14	0.91	0.80-1.04	1.01	0.84-1.21	0.95	0.82-1.09
aPAI1 (ng/mL)	1.18*	1.01-1.38	1.09	0.93-1.28	1.15	0.99-1.35	1.13	0.95-1.35
FGF-2 (pg/mL)	1.05	0.92-1.20	0.92	0.80-1.06	1.05	0.91-1.22	0.97	0.85-1.10
Fibrinogen (mg/mL)	0.97	0.84-1.12	1.03	0.89-1.19	1.09	0.94-1.27	0.92	0.80-1.05
Haptoglobin (mg/mL)	0.98	0.82-1.18	0.95	0.83-1.10	0.98	0.82-1.18	0.92	0.80-1.06
IFN-γ (pg/mL)	1.03	0.90-1.18	0.99	0.86-1.13	0.86	0.57-1.29	0.94	0.82-1.07
IGF-II (mg/mL)	1.05	0.92-1.21	0.98	0.85-1.12	0.90	0.73-1.09	1.18*	1.00-1.38
IL-1b (pg/mL)	1.02	0.88-1.18	0.96	0.83-1.11	0.87	0.65-1.17	0.90	0.77-1.04
IL-2 (pg/mL)	1.03	0.90-1.17	0.90	0.76-1.05	0.77	0.44-1.35	0.93	0.81-1.07
IL-4 (pg/mL)	1.01	0.88-1.16	1.02	0.88-1.18	0.89	0.70-1.15	0.98	0.85-1.13
IL-5 (pg/mL)	1.04	0.91-1.19	0.98	0.86-1.12	0.92	0.69-1.22	1.03	0.87-1.20
IL-6 (pg/mL)	0.97	0.81-1.16	0.89	0.69-1.15	0.98	0.78-1.23	1.05	0.84-1.31
IL-7 (pg/mL)	0.96	0.82-1.12	0.89	0.77-1.03	0.77	0.55-1.09	0.98	0.85-1.12
IL-8 (pg/mL)	1.11	0.97-1.27	1.13	0.97-1.33	1.20*	1.03-1.39	1.12	0.95-1.32
IL-10 (pg/mL)	0.98	0.83-1.15	0.93	0.79-1.09	1.01	0.86-1.19	1.06	0.83-1.35
MCP-1 (pg/mL)	0.99	0.87-1.14	1.01	0.88-1.16	1.13	0.97-1.32	1.03	0.89-1.19
MMP-9 (ng/mL)	0.99	0.86-1.14	1.13	0.96-1.32	0.98	0.82-1.18	0.88	0.77-1.01
Resistin (ng/mL)	1.00	0.87-1.15	1.08	0.93-1.25	1.16	0.99-1.36	1.08	0.93-1.26
sFasL (pg/mL)	0.94	0.81-1.09	1.02	0.88-1.17	0.98	0.81-1.18	1.14	0.96-1.36
sIL-1RI (pg/mL)	1.11	0.97-1.27	1.00	0.87-1.14	1.05	0.89-1.24	1.11	0.93-1.32
sIL-2Rα (ng/mL)	0.99	0.86-1.14	1.13	0.97-1.31	1.39†	1.18-1.64	0.98	0.85-1.12
sIL-6R (ng/mL)	0.97	0.85-1.12	0.99	0.86-1.13	1.20	0.99-1.44	1.00	0.86-1.15
TGF-β1 (ng/mL)	0.94	0.81-1.10	1.05	0.91-1.21	0.89	0.70-1.12	1.00	0.87-1.16
TGF-β2 (pg/mL)	0.90	0.75-1.08	1.04	0.89-1.22	0.96	0.77-1.18	0.99	0.85-1.14
TNF-α (pg/mL)	1.18	0.97-1.44	1.10	0.89-1.326	0.87	0.62-1.23	1.06	0.88-1.29
tPAI1 (ng/mL)	1.17*	1.02-1.34	1.07	0.92-1.23	1.09	0.92-1.29	1.08	0.93-1.25
VEGF (pg/mL)	1.11	0.97-1.27	1.04	0.89-1.20	1.02	0.86-1.21	1.01	0.87-1.16

*$P \leq 0.05$ before correction for multiple comparisons.
†$P \leq 0.05$ before correction and significance retained with Benjamini-Hochberg correction.
Abbreviations: FGF2, fibroblast growth factor 2; IFN-γ, interferon gamma; MCP-1, monocyte chemoattractant protein-1; sFasL, soluble Fas ligand.

analyses were performed using STATA (StataCorp LP, College Station, TX). Multivariable model results without multiple imputations are reported in Supporting Table S5.

Results

CHARACTERISTICS OF THE STUDY POPULATION

Included in this study were 235 subjects, 73 from the NAFLD Database and 162 from the TONIC trial. Children from the NAFLD Database and TONIC were grouped together because there were no differences in their participation or care prior to collection of baseline data. Of the 235 children and adolescents included, 4 had no steatosis, 58 were classified as NAFLD, Not NASH (25%), and 73 (31%) were categorized as Definite NASH. The remaining 100 had Borderline NASH, with 47 of those (20% of total) having a Borderline Zone 3 NASH pattern and 53 (23% of total) a Borderline Zone 1 pattern. Clinical characteristics associated with NASH are noted in Table 1 and have been described in additional detail in previous NASH CRN analyses.[20,22]

Children classified as Borderline Zone 1 NASH were younger than those with Borderline/Definite Zone 3 NASH (11.8 $\pm$ 2.0 years versus 13.5 $\pm$ 2.5, $P < 0.0005$), but there were no differences by sex, race, ethnicity, BMI z-score, transaminases, glucose, or insulin.

PLASMA BIOMARKERS ASSOCIATED WITH NASH DIAGNOSIS

In bivariate analysis, participants with Definite NASH had significantly higher levels of activated

TABLE 4. UNADJUSTED OR (95% CI) FOR CHANGE IN CYTOKINE LEVEL BY 0.5 SD BY HISTOLOGIC OUTCOME

	Lobular Inflammation (≥2 foci per hpf)		Portal Inflammation (mild/more than mild)		Hepatocellular Ballooning	
	OR	95% CI	OR	95% CI	OR	95% CI
Adiponectin (μg/mL)	1.03	0.91-1.17	1.04	0.82-1.30	1.02	0.90-1.17
aPAI1 (ng/mL)	1.29†	1.09-1.52	1.17	0.86-1.59	1.14	0.98-1.33
FGF-2 (pg/mL)	1.26*	1.01-1.59	1.15	0.76-1.72	1.04	0.91-1.20
Fibrinogen (mg/mL)	0.93	0.81-1.07	1.02	0.81-1.30	0.96	0.84-1.10
Haptoglobin (mg/mL)	1.00	0.88-1.14	0.80*	0.65-0.98	0.99	0.87-1.13
IFN-γ (pg/mL)	1.06	0.92-1.22	1.11	0.73-1.68	0.99	0.87-1.13
IGF-II (mg/mL)	0.99	0.87-1.13	0.98	0.79-1.22	1.01	0.89-1.15
IL-1b (pg/mL)	1.06	0.92-1.23	1.10	0.79-1.53	1.01	0.88-1.16
IL-2 (pg/mL)	1.00	0.88-1.14	0.99	0.80-1.22	1.10	0.92-1.31
IL-4 (pg/mL)	1.06	0.93-1.21	0.97	0.79-1.19	0.97	0.85-1.11
IL-5 (pg/mL)	0.98	0.86-1.12	1.21	0.72-2.05	0.98	0.85-1.11
IL-6 (pg/mL)	1.25	0.66-2.37	1.46	0.30-7.06	1.21	0.67-2.17
IL-7 (pg/mL)	0.96	0.84-1.10	0.95	0.80-1.14	1.01	0.89-1.15
IL-8 (pg/mL)	1.14*	1.00-1.31	1.06	0.83-1.36	1.06	0.93-1.20
IL-10 (pg/mL)	0.93	0.76-1.14	2.47	0.35-17.54	0.94	0.79-1.12
MCP-1 (pg/mL)	1.12	0.98-1.29	0.96	0.79-1.18	1.02	0.90-1.16
MMP-9 (ng/mL)	1.11	0.97-1.27	1.48*	1.02-2.14	1.09	0.95-1.24
Resistin (ng/mL)	1.14	0.99-1.30	0.96	0.78-1.18	0.94	0.83-1.07
sFasL (pg/mL)	0.95	0.83-1.08	1.18	0.88-1.58	0.91	0.79-1.04
sIL-1RI (pg/mL)	0.95	0.83-1.08	1.06	0.82-1.38	1.13	0.98-1.30
sIL-2Rα (ng/mL)	1.03	0.90-1.17	1.48*	1.07-2.06	0.99	0.87-1.12
sIL-6R (ng/mL)	1.17*	1.02-1.33	1.09	0.88-1.37	0.98	0.86-1.11
TGF-β1 (ng/mL)	0.86	0.74-1.00	0.99	0.80-1.23	0.90	0.79-1.04
TGF-β2 (pg/mL)	0.84	0.70-1.00	0.98	0.80-1.21	0.90	0.78-1.05
TNF-α (pg/mL)	0.99	0.87-1.12	1.21	0.75-1.94	1.12	0.93-1.35
tPAI1 (ng/mL)	1.22†	1.06-1.40	1.12	0.87-1.44	1.18*	1.03-1.35
VEGF (pg/mL)	1.11	0.96-1.28	1.43	0.78-2.60	1.14	0.97-1.33

*$P \leq 0.05$ before correction for multiple comparisons.
†$P \leq 0.05$ before correction and significance retained with Benjamini-Hochberg correction.
Abbreviations: FGF2, fibroblast growth factor 2; IFN-γ, interferon gamma; MCP-1, monocyte chemoattractant protein-1; sFasL, soluble Fas ligand.

PAI1 (aPAI1) and total PAI1 (tPAI1) than those with Not/Borderline NASH (Tables 2 and 3). In multivariable regression models, Definite NASH was associated with higher TNF-α levels as well as clinical predictors (Table 5).[22]

Comparison was also made between children with Definite NASH (n = 73) and those with Not NASH (n = 62), excluding those with Borderline NASH. Those with Definite NASH had significantly higher tPAI1 (51.4 ± 25.1 ng/mL versus 38.8 ± 21.9 ng/mL; $P = 0.002$), aPAI1 (64.8 ± 35.9 versus 45.8 ± 35.9 ng/mL; $P = 0.004$), and IL-8 (3.37 ± 1.54 pg/mL versus 2.46 ± 1.89 pg/mL; $P = 0.003$) than those with Not NASH.

In sensitivity analysis excluding participants with Borderline Zone 1 NASH, children with Definite Zone 3 NASH (n = 108) had higher aPAI1 (64.9 ± 39.9 versus 44.8 ± 31.2; $P = 0.0004$), tPAI1 (51.4 ± 21.0 versus 40.7 ± 25.1; $P = 0.003$), IL-8 (3.38 ± 1.54 versus 2.70 ± 1.98; $P = 0.01$), and vascular endothelial growth factor (VEGF; 723.0 ± 1271.3 versus 404.2 ± 609.9; $P = 0.05$) levels than those with Not/Borderline Zone 3 NASH (n = 73). In multivariable analysis excluding those with Borderline Zone 1 NASH, significant discriminators of Definite NASH versus Not/Borderline Zone 3 NASH were aPAI1 (OR, 1.25 per 0.5 SD increase; 95% confidence interval [CI], 1.01-1.56; $P = 0.04$), AST (OR, 1.65 per 20 IU/L increase; 95% CI, 1.30-2.11; $P < 0.005$), and triglycerides (OR, 1.06 per 20 mg/dL increase; 95% CI, 0.98-1.14; $P = 0.14$).

Soluble IL-1 receptor 1 (sIL-1RI) was higher in children with Borderline/Definite Zone 3 NASH (37.06 ± 25.39 pg/mL) than those with Borderline Zone 1 NASH (30.29 ± 16.37 pg/mL; $P = 0.04$). There were no other significant differences in analyte levels between participants with Borderline/Definite Zone 3 and Zone 1 NASH (data not shown).

TABLE 5. PREDICTORS ASSOCIATED WITH NASH AND HISTOLOGIC COMPONENTS IN MULTIVARIABLE ANALYSIS*

NAFLD Group Comparison	Predictor (per unit change†)	Odds Ratio	95% CI	*P*
Definite NASH (vs. Borderline/Not NASH)	Age (1 year)	1.12	0.99-1.26	0.07
	AST (20 U/L)	1.56	1.29-1.88	<0.001
	HDL cholesterol (5 mg/dL)	0.78	0.64-0.95	0.01
	TNF-α (4.4 pg/mL)	1.19	0.97-1.46	0.10
	$\chi^2 = 42.46$, $P < 0.0005$, n = 230			
Definite NASH (vs. Not NASH)	AST (20 U/L)	2.18	1.49-3.19	<0.0001
	Triglycerides (20 mg/dL)	1.15	1.02-1.28	0.02
	VEGF (514.5 pg/mL)	1.30	0.98-1.71	0.07
	$\chi^2 = 45.58$, $P < 0.0005$, n = 134			
Steatosis >33%	Male	1.81	0.88-3.78	0.11
	Age (1 year)	0.87	0.76-0.99	0.03
	BMI z-score (1)	1.96	0.83-4.64	0.13
	ALT (20 U/L)	1.09	0.99-1.22	0.09
	IGF-II (1 pg/mL)	1.28	1.06-1.55	0.01
	MMP-9 (16.5 ng/mL)	0.84	0.72-0.98	0.02
	Resistin (3.15 ng/mL)	1.13	0.95-1.33	0.17
	sFasL (35.5 pg/mL)	1.09	0.92-1.28	0.33
	$\chi^2 = 25.95$, $P = 0.001$, n = 229			
Stage 1-4 fibrosis	AST (20 U/L)	1.30	1.05-1.61	0.02
	IL-5 (0.9 pg/mL)	1.28	0.92-1.78	0.14
	IL-7 (3.2 pg/mL)	0.62	0.46-0.84	0.002
	IL-8 (1 pg/mL)	1.23	0.95-1.59	0.11
	MMP-9 (16.5 ng/mL)	1.20	1.01-1.41	0.03
	$\chi^2 = 26.14$, $P = 0.0001$, n = 233			
Stage 3-4 fibrosis	AST (20 IU/mL)	1.40	1.13-1.74	0.002
	IL-4 (16 pg/mL)	0.63	0.36-1.09	0.10
	IL-7 (3.2 pg/mL)	0.32	0.15-0.66	0.002
	IL-8 (1 pg/mL)	1.77	1.31-2.38	<0.0005
	IL-10 (50 pg/mL)	2.20	1.21-3.98	0.01
	sIL-2rα (0.18 ng/mL)	1.48	1.20-1.84	<0.0005
	$\chi^2 = 60.45$, $P < 0.0005$, n = 234			
Mild/more than mild portal inflammation	Haptoglobin (0.65 mg/mL)	0.80	0.65-1.00	0.05
	sIL-2rα (0.18 ng/mL)	1.45	1.04-2.02	0.03
	MMP-9 (16.5 ng/mL)	1.31	0.93-1.84	0.12
	$\chi^2 = 14.94$, $P = 0.002$, n = 235			
Lobular inflammation (≥2 foci per hpf)	Male	0.47	0.22-0.98	0.04
	AST (per 20 U/L)	1.35	0.89-2.05	0.16
	ALT (per 20 U/L)	1.18	0.95-1.48	0.14
	HDL cholesterol (per 5 mg/dL)	0.86	0.72-1.04	0.13
	Resistin (per 3.15 ng/mL)	1.20	1.02-1.42	0.03
	sIL-2rα (per 0.18 ng/mL)	0.85	0.72-1.00	0.06
	sIL-6R (per 2.7 ng/mL)	1.26	1.07-1.48	0.005
	TGF-β2 (per 171.5 pg/mL)	0.62	0.47-0.82	0.001
	tPAI1 (per 12.4 ng/mL)	1.37	1.13-1.65	0.001
	$\chi^2 = 71.67$, $P < 0.0005$, n = 227			
Hepatocellular ballooning	AST (per 20 U/L)	1.26	1.06-1.50	0.008
	Triglycerides (per 20 mg/dL)	1.09	1.02-1.16	0.02
	TGF-β1 (per 3.8 pg/mL)	0.78	0.65-0.94	0.008
	tPAI1 (per 12.4 ng/mL)	1.23	1.04-1.45	0.02
	$\chi^2 = 30.10$, $P < 0.0005$, n = 233			

*Models include imputation of missing cytokine and clinical values by progressive mean matching and chained iterative equations. See Supporting Table S5 for multivariate models without imputed missing values.
†Per unit change represents 0.5 SD change for cytokine biomarkers and clinically significant change for clinical predictors.
Abbreviations: HDL, high-density lipoprotein; sFasL, soluble Fas ligand

PLASMA BIOMARKERS ASSOCIATED WITH STEATOSIS GRADE

Forty percent of participants had >66% steatosis (grade 3), 32% had 34%-66% (grade 2), 26% had 5%-33% (grade 1), and 2% had <5% steatosis (grade 0). Participants with moderate to severe steatosis (grade 2-3) had higher levels of insulin-like growth factor 2 (IGF-II; $P = 0.03$, Table 3; Supporting Table S3). In multivariable modeling, steatosis >33% was associated with higher IGF-II, resistin, and soluble Fas ligand levels and lower matrix metallopeptidase 9 (MMP-9) levels (Table 4). When subjects with Borderline Zone 1 NASH were excluded for sensitivity analysis, IGF-II remained significantly associated with moderate-severe steatosis (OR, 1.85; 95% CI, 1.16-2.96; $P = 0.01$); IL-1β was also retained in this model (OR, 0.69; 95% CI, 0.41-1.18; $P = 0.18$) with ALT, BMI z-score, and age.

PLASMA BIOMARKERS ASSOCIATED WITH FIBROSIS STAGE

Thirty-two percent of participants had no fibrosis on liver biopsy, 38% had stage 1 (perisinusoidal or periportal only), 15% had stage 2, 14% stage 3, and 1% stage 4 fibrosis. None of the biomarker distributions were significantly different between those with no fibrosis and those with stage 1-4 fibrosis (Table 3). However, IL-8 and sIL-2Rα were significantly higher in those who had stage 3-4 fibrosis compared to stage 0-2, and the latter retained statistical significance after correction for multiple comparisons (Table 3).

In multivariable analysis, IL-7 levels were lower in participants that had any fibrosis (versus none) and stage 3-4 fibrosis (versus 0-1) (Table 4). Stage 3-4 fibrosis was also associated with lower IL-4 and higher IL-8, IL-10, and sIL-2Rα (Table 4).

Compared to children with fibrosis in a zone 1 pattern (periportal only), children with any fibrosis in an adult NASH pattern had higher levels of IL-5 (1.12 $\pm$ 1.65 versus 0.60 $\pm$ 0.45, $P = 0.004$), IL-10 (23.41 $\pm$ 35.79 versus 14.80 $\pm$ 15.00, $P = 0.04$), and IL-1b (0.58 $\pm$ 0.62 versus 0.41 $\pm$ 0.40, $P = 0.05$) with no significant differences in other cytokine levels between the two groups (data not shown).

When participants with Zone 1 NASH fibrosis (periportal only) were excluded, those with any fibrosis in an adult NASH pattern (stage 1a, 1b, or 2-4; n = 98) had higher IL-8 levels (3.41 $\pm$ 2.25 pg/mL) than those with no fibrosis (2.77 $\pm$ 1.96 pg/mL; $P = 0.05$, n = 75). There were no statistically significant differences in other biomarker levels between those with adult NASH pattern fibrosis and those with no fibrosis. Those with significant fibrosis (stage 3-4; n = 138) had higher IL-8 (3.87 $\pm$ 3.05 pg/mL versus 2.95 $\pm$ 1.83 pg/mL in stage 0-2 fibrosis; n = 35), sIL-2rα (1.04 $\pm$ 0.07 ng/mL versus 0.72 $\pm$ 0.03 ng/mL), and sIL-6R (22.83 $\pm$ 4.74 ng/mL versus 20.93 $\pm$ 5.22 ng/mL) levels as well as lower IL-7 levels (3.64 $\pm$ 3.91 pg/mL versus 5.52 $\pm$ 7.40 pg/mL; $P = 0.05$).

In multivariable modeling excluding those with Zone 1 NASH fibrosis (periportal only), any fibrosis (stage 1-4) in an adult NASH pattern was associated with increasing IL-5 (OR, 1.80 per 0.9 pg/mL increase; 95% CI, 1.16-2.79; $P = 0.01$) and IL-8 levels (OR, 1.41 per 1 pg/mL increase; 95% CI, 1.06-1.87; $P = 0.02$) as well as decreasing IL-7 (OR, 0.46 per 3.2 pg/mL increase; 95% CI, 0.30-0.72; $P = 0.001$) and increasing AST (OR, 1.39 per 20 IU/L increase; 95% CI, 1.09-1.78; $P = 0.008$). In the same group, fibrosis stage 3-4 (versus 0-2) remained associated with decreasing IL-7 and increasing AST as well as increasing sIL-2Rα (OR, 1.56 per 0.18 ng/mL increase; 95% CI, 1.22-1.98; $P < 0.0005$) and IL-10 (OR, 2.77 per 50 / mL increase; 95% CI, 1.27-6.04; $P = 0.01$).

PLASMA BIOMARKERS ASSOCIATED WITH INFLAMMATION AND HEPATOCELLULAR BALLOONING

Lobular inflammation was seen in <2 foci per 20× field in 53% of participants, 2-4 foci in 42%, and more than 4 foci in 5% (n = 235). Compared to participants with <2 foci of lobular inflammation, those with ≥2 foci had higher levels of aPAI1 and fibroblast growth factor 2 that remained significant after correction for multiple comparisons. They had higher tPAI1, IL-8, and sIL-6R and lower levels of TGF-β1 and TGF-β2 before correction (Table 4). In multivariable modeling, lobular inflammation was significantly associated with higher resistin, sIL-6R, and tPAI1 levels and lower TGF-β2 and sIL-2Rα levels (Table 4).

Chronic portal inflammation was absent in 9% of children, mild in 81%, and more than mild in 10%. Compared to those with no portal inflammation, those with mild or more than mild portal inflammation had

higher levels of MMP-9 and sIL-2Rα and lower levels of haptoglobin (Table 5; Supporting Table S4). In multivariable models, portal inflammation was associated with higher levels of MMP-9 and sIL-2Rα and lower levels of haptoglobin (Table 4).

Hepatocyte ballooning was seen in 49% of participants. Those with ballooning had higher levels of tPAI1 with no significant differences in other biomarkers (Table 5; Supporting Table S3). The association of ballooning with higher tPAI1, as well as lower TGF-β1, was significant in multivariable analysis (Table 4).

In the sample excluding those with Borderline Zone 1 NASH, participants with ballooning had higher levels of tPAI1 (49.3 ± 24.7 versus 40.0 ± 20.4; $P = 0.006$), aPAI1 (59.4 ± 37.9 versus 45.2 ± 32.8; $P = 0.007$), sIL-1RI (38.1 ± 27.5 versus 30.9 ± 20.0; $P = 0.04$), IL-8 (3.22 ± 1.63 versus 2.68 ± 2.03; $P = 0.05$), and VEGF (667.8 ± 1148.1 versus 373.7 ± 593.1; $P = 0.03$). Only 3% of pediatric participants had Mallory bodies on baseline liver biopsy.

Discussion

This is the first report of an array of plasma biomarkers related to metabolism, inflammation, and fibrosis measured simultaneously with liver biopsy in children and adolescents with NAFLD. The NASH CRN cohort offers a unique opportunity to characterize differences in plasma biomarker levels by histologic pattern and severity. Given the significant number of cytokines evaluated, our aim was to identify promising targets for future research.

In multivariable models, clinical characteristics, such as sex, age, and AST, were associated most consistently with overall NASH diagnosis and particular histologic components. In addition, several analytes emerged repeatedly in bivariate and multivariable analyses as significantly associated with NASH histologic components. These patterns provide targets for future research into the inflammatory, fibrotic, and other pathways that drive NAFLD development and progression.

One of the most consistent findings was the association between NASH features and tPAI1 and aPAI1. In bivariate analysis, increased tPAI1 and aPAI1 were associated with Definite NASH and lobular inflammation. In multivariable analysis, tPAI1 was associated with lobular inflammation and hepatocellular ballooning.

In the parallel analysis of cytokines in the adult NASH CRN cohort, aPAI1 was the only cytokine that discriminated between Definite NASH and Borderline/Not NASH.[4] This association between PAI1 levels and NAFLD has been reported in other cohorts of adults with NASH.[28,29] PAI1 has primarily been studied for its role in atherosclerosis and thrombotic disease.[30] It is not clear if PAI1 is itself mediating inflammation and damage in the NASH liver or if NASH inflammation induces PAI1 release, which then contributes to systemic cardiovascular disease.[29] The mechanisms are deserving of further investigation as NAFLD is emerging as a significant risk factor for cardiovascular events in adults, with severity of liver disease correlating with cardiovascular event risk.[31] PAI1 has also been reported as a predictor of NASH and NAFLD activity score in obese children[9] and as a predictor of steatosis severity in another cohort.[15] The investigators hypothesized that PAI1 might be involved in NASH hepatic inflammation; indeed tPAI1 retained significance as a predictor in multivariable modeling of lobular inflammation and aPAI1 of portal inflammation in our cohort.

Interestingly, PAI1 levels are also increased in patients with obstructive sleep apnea (OSA).[32] In children with NAFLD, OSA has recently been associated with fibrosis severity, and their hypoxemia severity correlated with inflammation severity and increasing NAS.[33]

IL-8 was also a significant discriminator of NASH severity across multiple histologic components. It was associated with fibrosis in overall multivariable analysis, with NASH diagnosis in multivariable analysis excluding children with pediatric (Borderline Zone 1) NASH, and with lobular inflammation in bivariate analysis. IL-8 was also associated with fibrosis in the adult NASH CRN cohort.[4] IL-8 is a proinflammatory cytokine released from macrophages, endothelium, and other cells and is primarily responsible for recruiting neutrophils to sites of injury. Elevated IL-8 levels have been associated with both NASH[34] and alcoholic liver disease.[35] Lipid accumulation in hepatocytes may induce production of IL-8.[36] IL-8 levels were higher in children with hepatic steatosis measured by magnetic resonance imaging in one study.[8]

IL-7 decreased with increasing fibrosis stage. IL-7 is generally considered a proinflammatory cytokine secreted by adipose tissue as well as other cell types. In high-fat-fed mice, IL-7 deficiency has been associated with glucose intolerance.[37] An association of lower IL-7 levels with higher fibrosis stage has been reported in an adult NASH cohort[38] but does not seem to have been investigated in pediatric NASH.

Similarly, increased sIL-2Rα was associated with fibrosis stage and with portal and lobular inflammation. It also emerged as a significant predictor of fibrosis in the adult NASH CRN cohort.[4] sIL-2Rα is a marker of T-cell activation in the plasma. It is produced by T cells and then shed from their surface, although its role as a mediator of inflammation is not well characterized. Elevated sIL-2Rα levels have been reported in patients with chronic hepatitis C[39] and hepatocellular carcinoma.[40] In children, sIL-2Rα was recently identified as a marker of poor prognosis in pediatric acute liver failure, with levels significantly higher in those children who died or required liver transplantation.[41]

MMP-9 levels increased with portal inflammation and any fibrosis but decreased with steatosis severity. Elevated plasma MMP-9 has been reported in adults with NASH compared to healthy controls[42] and with viral hepatitis[43] but has not been studied in pediatric subjects with NAFLD to our knowledge. MMP-9 levels decreased in both obese children and adults with OSA who were successfully treated, again suggesting that hypoxia may play a role in NASH.[32] The pattern of MMP-9 increase with inflammation and fibrosis but decrease with steatosis severity is intriguing. In light of theories that isolating free fatty acids in triglyceride droplets, thereby increasing steatosis, may be protective against NASH, the pattern we found suggests that MMP-9 may be involved in NASH-mediated damage.

Additional analytes had associations with only one histologic component, suggesting that they may play a more targeted role in NASH. For example, IGF-II and IL-1β emerged only in association with steatosis severity. IGF-II is secreted primarily by hepatocytes and is known to be involved in fetal pancreatic β-cell development, but its role in children and adults is not well characterized. Possibly related to its homology to insulin and weak affinity for the insulin receptor, it may play a role in glucose and fat metabolism.[44] Elevations have been reported in obese individuals.[45] Overexpression of IGF-II in mice leads to an increase in pancreatic β-cell mass, insulin hypersecretion, insulin resistance, and hepatic steatosis,[46,47] but an association with hepatic steatosis in humans has not been reported. Lower levels of IGF-II in children with NAFLD fibrosis was recently observed but was not associated with steatosis in a smaller cohort of obese children[48] but was not observed in our study. Higher levels of IGF-II with increased steatosis and lower levels of IGF-II with advanced fibrosis and ballooning were also seen in the adult NASH CRN cohort.[4]

A decrease in IL-1β was seen with increasing steatosis in our cohort and in adult NASH in other cohorts.[34,49] IL-1β is a proinflammatory cytokine that appears to impact liver insulin sensitivity, with lower levels associated with insulin resistance; higher levels are profibrotic. IL-1β deficiency in mice protected against inflammation in mice with steatosis.[50]

One limitation of this study is that the cohort did not include control subjects. Ideally, a control group would include overweight pediatric patients with liver biopsy available but without NAFLD or other liver disease. Unfortunately, liver biopsies on this group of children are rare for clinical purposes, and it is difficult to justify the risk for research purposes. A control group of nonoverweight children with liver biopsies would not be directly applicable to NASH associations because increased cytokine levels could reflect the low-grade inflammation associated with obesity and insulin resistance instead of just NASH.

Our cohort does explore the utility of plasma biomarkers in differentiating severity of NAFLD and its components among those with biopsy-proven fatty liver disease. As a cross-sectional study, it identifies correlations between analyte levels and NASH histology. We relied on serum levels of analytes, so our analyses do not identify the tissue driving cytokine levels, i.e., adipose, muscle, liver, or other, or actual hepatic levels. Additional longitudinal research of cytokines in serum and liver or other tissues is warranted to investigate the role of these analytes in differentiating between those with and without NAFLD and in NASH progression.

Another limitation of this study is that we examined multiple plasma biomarkers and outcomes in these analyses, increasing the risk of false-positive associations between plasma biomarkers and NASH. However, our analyte choices were hypothesis driven and selected *a priori* based on their known role in processes related to NASH. We have reported actual levels in Supporting Tables S2-S4 so that the reader may put the associations in context. Statistical analysis was designed to minimize spurious associations by using AIC minimization techniques to select predictors for our multivariable models and competing the biomarkers against established clinical discriminators of NASH severity. Using AIC for model selection allowed us to account for the large number of cytokines evaluated while minimizing information lost in the whittling of the model. It does not rely on statistical significance from univariable analysis to choose predictors for consideration in the multivariable models or on

nested models, allowing more flexibility in considering the contribution of each cytokine relative to the others. However, AIC minimization is a relatively liberal technique as concerns predictor retention in final models; our study intended to provide direction to future work and not to definitively identify biomarkers ready for clinical application.

Future investigations are needed to validate and understand associations between the plasma biomarkers and NASH histology identified in this analysis. Of particular interest will be to study whether changes in these biomarker levels correspond to changes in NASH histology over time and to further study their etiologic roles in NASH progression or improvement and to assess whether any might prove useful as noninvasive biomarkers. Correlation of baseline biomarker levels with NASH progression may potentially allow for prognostic testing at initial diagnosis. A central puzzle in pediatric NASH remains the accurate prediction of which patients will progress to cirrhosis and end-stage liver disease in adulthood; this study highlights cytokines deserving of further exploration as biomarkers.

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