Serum Interleukin-8, Osteopontin, and Monocyte Chemoattractant Protein 1 Are Associated With Hepatic Fibrosis in Patients With Nonalcoholic Fatty Liver Disease

Oliver Glass,[1] Ricardo Henao,[2,3] Keyur Patel,[4] Cynthia D. Guy,[5] Hans J. Gruss,[6] Wing-Kin Syn,[7,8] Cynthia A. Moylan,[9] Robert Streilein,[10] Russell Hall,[10] Anna Mae Diehl,[9] and Manal F. Abdelmalek[9]

The severity of hepatic fibrosis is the primary predictor of liver-related morbidity and mortality in patients with nonalcoholic fatty liver disease (NAFLD). Unfortunately, noninvasive serum biomarkers for NAFLD-associated fibrosis are limited. We analyzed baseline serum samples for 24 cytokines of 97 patients with biopsy-proven NAFLD. These patients were prospectively enrolled in a clinical study (ClinicalTrials.gov NCT00794716) to identify cytokines associated with liver fibrosis in patients with nonalcoholic steatohepatitis. Patients were stratified according to severity of hepatic fibrosis (mild, stage 0-1, n = 37; moderate, stage 2, n = 40; and advanced, stage 3-4, n = 20) while controlling for age, race, sex, body mass index, and diabetes mellitus. Interleukin-8 (IL-8), osteopontin (OPN), and monocyte chemoattractant protein 1 (MCP1) were associated with liver fibrosis ($P < 0.001$, $P = 0.005$, $P = 0.016$, respectively). After controlling for steatosis, lobular inflammation, hepatocyte ballooning, age, sex, body mass index, diabetes mellitus, hypertension, and metabolic syndrome status, IL-8 remained strongly associated with fibrosis ($P = 0.001$). Furthermore, IL-8 was also a strong predictor of increased fibrotic liver injury compared to established markers of hepatic fibrosis. Hepatic gene expression from 72 patients with NAFLD (n = 40 mild fibrosis; n = 32 advanced fibrosis) from the Duke University Health System NAFLD Clinical Database and Biorepository revealed IL-8, MCP1, and OPN gene expression to be increased and differentially expressed in patients with advanced hepatic fibrosis. Thus, serum IL-8, MCP1, and OPN may reflect up-regulated gene expression during liver fibrosis in NAFLD. *Conclusion:* Serum IL-8, MCP1, and OPN may serve as a test for advanced hepatic fibrosis in NAFLD and thus reveal novel targets for antifibrotic therapies. The increased serum IL-8, MCP1, and OPN that correspond with associated hepatic gene expression lend strength to such analytes as ideal surrogate serum biomarkers for severity of hepatic fibrosis. (*Hepatology Communications* 2018;2:1344-1355)

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease worldwide. Due to the increasing prevalence of diabetes and obesity, NAFLD now affects 20%-30% of the Western general population.[1] The histologic spectrum of NAFLD includes simple steatosis

and nonalcoholic steatohepatitis (NASH) with or without hepatic fibrosis. NASH is characterized histopathologically by lipid accumulation in the liver with hepatocellular injury characterized by necroinflammation, ballooned hepatocytes, and/or hepatic fibrosis. Of those with NASH, 30% are at risk for fibrosis progression and complications of cirrhosis, including portal hypertension, diminished liver synthetic function, and hepatocellular carcinoma.[2-4]

The mechanisms underlying the pathophysiologic progression of NAFLD remain poorly understood. Diagnosis and treatment prior to development of advanced-stage disease is difficult without the ideal "gold standard" by which to stratify the severity of hepatic fibrosis. While a liver biopsy remains the current gold standard for the grading and staging of NASH, the procedure is associated with discomfort, increased cost, use of health care resources, potential risk, and is impractical for population-based screening.[5] Thus, there is a strong need and vibrant ongoing investigations in the quest for serum biomarkers to accurately stratify those patients at risk for fibrosis, the primary predictor of morbidity and mortality in patients with NASH.[6,7] For several reasons, cytokine measures are attractive biomarkers in the diagnostic landscape of NASH.

Circulating inflammatory cytokines have been implicated in the pathogenesis of NASH and fibrosis.[8] Compared to simple steatosis, patients with NASH have elevated serum cytokine levels of tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6), inflammatory mediators of NASH progression.[9] Moreover, TNF-α has been shown to be independently associated with liver fibrosis, emphasizing a critical role of inflammatory cytokines in liver fibrogenesis

and progression.[10] However, it is unclear whether serum cytokine levels predict severity of liver fibrosis in NASH and whether other cytokines besides TNF-α are associated with fibrosis. Therefore, the aim of the present study was to evaluate whether serum cytokines associate with severity of liver fibrosis in patients with biopsy-proven NAFLD.

Patients and Methods

HUMAN SUBJECTS

We performed a cross-sectional study using prospectively collected data from subjects with NAFLD who enrolled in a prospective, single-center, open-label study that was designed to assess the pharmacokinetics of cholyl-lysl-fluorescein (NRL972) in patients with clinical evidence for NAFLD (Clinical Trials. gov NCT00794716). The study was approved by the Duke University Health System Institutional Review Board. Patients were enrolled at the time of a standard of care percutaneous liver biopsy at which time a serum sample was obtained and analyzed for serum cytokines. Homeostasis model assessment of insulin resistance was calculated in all subjects irrespective of severity of underlying liver disease and/or use of medical treatment for diabetes mellitus. Fasting lipids were obtained at the time of liver biopsy on all participants.

The presence of NAFLD was defined histologically according to the criteria established by the NASH Clinical Research Network.[11] For the present study, NAFLD was defined as: (1) presence of >5% hepatic steatosis on liver biopsy; (2) absence of histologic and serologic evidence for other chronic liver disease in a patient with risk factors for the metabolic

ARTICLE INFORMATION:

From the [1]Division of General Internal Medicine, Duke University, Durham, NC; [2]Department of Biostatistics and Bioinformatics, Duke University, Durham, NC; [3]Duke Clinical Research Institute, Duke University, Durham, NC; [4]University Health Network, University of Toronto, Toronto, Canada; [5]Department of Pathology, Duke University, Durham, NC; [6]Norgine, Ltd., Uxbridge, United Kingdom; [7]Division of Gastroenterology and Hepatology, Medical University of South Carolina, Charleston, SC; [8]Section of Gastroenterology, Ralph H Johnson Veterans Affairs Medical Center, Charleston, SC; [9]Division of Gastroenterology and Hepatology; and [10]Department of Dermatology, Duke University, Durham, NC.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Manal F. Abdelmalek, M.D., M.P.H.
Duke University Medical Center, Division of Gastroenterology
P.O. Box 3913

Durham, NC 27710
E-mail: manal.abdelmalek@duke.edu
Tel.: +1-919-684-3262

syndrome. Three groups of NAFLD across the spectrum of fibrosis defined our study cohort: 1) "low-risk" NAFLD, defined as fibrosis stages 0 or 1 (n = 37), the group with little probability of developing clinically significant liver disease over the next 1-2 decades; 2) "high-risk" NAFLD, defined as fibrosis stage 3 or 4 (n = 20), the group with significant likelihood of developing liver-related morbidity and mortality over the same time period (i.e., poor NAFLD outcomes); and 3) "indeterminate-risk" NAFLD, defined as fibrosis stage 2 (n = 40), the group for which clinical risk and outcomes are not well defined. Exclusion criteria included: i) current alcohol consumption of ≥14 servings per week (for men) and ≥7 servings per week (for women), ii) serologic evidence of alternative forms of liver disease (e.g., chronic viral hepatitis, primarily biliary cirrhosis autoimmune hepatitis, hemochromatosis, Wilson's disease, alpha-1-antitrypsin deficiency), or iii) histologic features suggesting co-existing liver diseases. Further, patients with liver impairment due to space-occupying processes (e.g., carcinoma); liver transplant recipients; end-stage renal, pulmonary, or cardiac disease; and pregnant woman were excluded from participation in the parent clinical study (ClinicalTrials.gov NCT00794716). The parent study also excluded patients with any changes in treatment regimen or initiation of new medications within 72 hours of liver biopsy and sample collection or those who received any investigational drug or treatment of NAFLD/NASH within 30 days of study participation. All data were extracted through a systematic chart review to include demographics, body mass index (BMI), medical history, and concomitant medications at time of biopsy.

For our analysis, diabetes mellitus was defined as the presence of an existing medical diagnosis in the medical record, glycosylated hemoglobin (hemoglobin A1c) >6.5%, and/or use of any medication (oral insulin-sensitizing agents or insulin therapy) used to treat diabetes mellitus. Hypercholesterolemia was defined as an existing diagnosis, total cholesterol >200 mg/dL, and/or use of cholesterol-lowering medication. Hyperlipidemia was defined as an existing diagnosis in the medical record, low-density lipoprotein >110 mg/dL, triglycerides >200 mg/dL, and/or use of lipid-lowering therapy at the time of liver biopsy. Hypertriglyceridemia was defined as an existing diagnosis in the medical record and/or isolated triglycerides >200 mg/dL. Hypertension was defined as an existing medical diagnosis and/or use of antihypertensive medication at the time of liver biopsy. Groups were matched for sex, age (±5 years), and BMI (kg/m^2) (±3 points). Demographic data (i.e., height, weight, BMI, age, sex, race, ethnicity, smoking status, and comorbid illnesses) and laboratory studies (i.e., lipids, liver aminotransferases, and measures of liver synthetic function) were obtained within 6 months of liver biopsy for all patients.

Liver Histology

All liver biopsy specimens were stained with hematoxylin and eosin and Masson trichrome stains and reviewed and scored according to the published NASH Clinical Research Network grading and scoring system.[12] For our analysis, fibrosis stages 1a, 1b, and 1c were combined and treated as stage 1. NASH was defined as a nonalcoholic steatohepatitis activity score (NAS) ≥4 points. Portal inflammation was also evaluated as present (grade 1) or absent (grade 0). The grade of steatosis, inflammation and ballooning, stage of fibrosis, and NAS were evaluated as covariates.

Cytokine Assay

Human cytokines, resistin, plasminogen activator inhibitor 1, visfatin, and leptin were measured using a Bio-Rad 24-plex Bio-Plex assay (171-AL002M; Bio-Rad, Hercules, CA). A monoclonal antibody specific for each cytokine of interest was coupled onto a particular set of beads of known internal fluorescence; because several combinations of cytokine antibody-coated beads could be included, multiple cytokines were measured simultaneously. Assays were performed in the Duke Immunology Laboratory according to the manufacturer's instructions.

Acoustic Radiation Force Imaging

Shear wave data acquisition and processing were performed as reported[13] using a customized Siemens SONOLINE Antares scanner and a CH41 transducer (Siemens Healthcare, Mountain View, CA). Shear

stiffness was characterized in three different locations of the liver: 1) superior intercostal (i.e., intercostal space between ribs 9 and 10, coinciding most often with the location of the liver biopsy needle insertion), 2) inferior intercostal (i.e., intercostal space between ribs 10 and 11, typically one to two rib spaces inferior to the superior location), and 3) lateral subcostal. Three replicate shear stiffness data acquisitions were performed at each imaging location for a total of nine data acquisitions per patient.

HEPATIC GENE EXPRESSION

Our group previously published results of hepatic gene expression in patients with mild (i.e., fibrosis stage 0-1; n = 40) versus advanced (i.e., fibrosis stage 3-4, n = 32) NAFLD in a subset of patients from the Duke University Health System NAFLD Clinical Database and Biorepository.[14] We reanalyzed our gene expression data for candidate genes, including IL-8, monocyte chemoattractant protein 1 (MCP1/chemokine [C-C motif] ligand 2), and osteopontin (OPN/secreted phosphoprotein 1). Two probes were available for IL-8 and OPN, while one probe was available for MCP1.

STATISTICAL ANALYSIS

Demographic and clinical data were compared between fibrosis stages using analysis of variance or chi-squared tests for continuous and categorical variables, respectively, with $P < 0.05$ considered significant. Cytokine values (concentrations) were log-transformed and tested for normality (Kolmogorov-Smirnov) prior to statistical analyses. Generalized linear regression was used to assess cytokine associations with fibrosis ($P < 0.05$ considered significant). Multiple generalized linear regression analysis was used to assess cytokine association with fibrosis while controlling for age, sex, BMI, and diabetes, ($P \leq 0.05$ considered statistically significant). We evaluated the predictive ability of the 24 cytokines and the 10 noninvasive markers of liver fibrosis (FibroTest score,[15] FIBROSpect II,[16] FibroMeter,[17] HepaScore,[18] NAFLD fibrosis score,[19] enhanced liver fibrosis score,[20] fibrosis-4,[21] BARD,[22] aspartate aminotransferase [AST]-to-platelet ratio index,[23] acoustic radiation force impulse [ARFI] stiffness score[24]) on our cohort of 97 subjects, using leave-one-out cross-validated area under the receiving operating characteristic curve.

We applied this to four different binary groupings of fibrosis, namely stages 0,1 versus 3,4; 0,1,2 versus 3,4; 0,1 versus 2,3,4; and 0 versus 1,2,3,4. Missing values of fibrosis markers were imputed with median observed values prior to calculating area under the curve and receiver operating characteristic values. Imputation of missing values is a limitation of the study; however, we note that missing values accounted for less than 7% of all fibrosis marker observations. Additionally, we considered all cytokines as univariate markers and as multivariate predictors (sparse logistic regression model) consisting of all markers in addition to all cytokines (34 markers total). Statistical analysis of the data was performed using MatLab (MathWorks, Inc., Natick, MA). Generalized linear models were used to assess gene associations with primary outcomes while controlling for age, sex, BMI, race, diabetes mellitus, hyperlipidemia, hypertension, hypercholesterolemia, hypertriglyceridemia and hemoglobin A1c ($P < 0.05$ considered significant). The Benjamini-Hochberg procedure was used to correct for multiple testing with a false discovery rate at 5%. Gene expression statistical analyses were performed using MatLab.

Results

PATIENT CHARACTERISTICS

Demographic and clinical data obtained from 97 study participants are summarized in Table 1. The mean age of patients was 59.4 ± 9.5 years; 31% of patients were male individuals, and 89% were Caucasian. The average BMI of participants was 35.0 ± 7.0 kg/m^2; 56% had diabetes mellitus, 77% had hypertension, and 88% had metabolic syndrome. In univariate analysis, increased fibrosis stage was associated with age, diabetes mellitus, AST, AST/alanine aminotransferase ratio, platelets, high-density lipoproteins, and homeostatic model assessment of insulin resistance ($P = 0.008$, $P < 0.001$, $P = 0.008$, $P = 0.009$, $P = 0.001$, $P = 0.005$, $P < 0.001$, respectively) but not sex, BMI, or metabolic syndrome. Lobular inflammation, portal inflammation, and hepatocyte ballooning were also associated with increased fibrosis stage ($P = 0.002$, $P = 0.011$, $P = 0.005$, respectively), but steatosis grade and NAS were not. Overall, most demographic variables were relatively balanced across fibrosis risk groups, although higher risk groups on average were older than low- to intermediate-risk groups.

TABLE 1. PATIENT CHARACTERISTICS

Number of Samples	Fibrosis Stages				
	Low Risk (n = 37)		Indeterminate Risk (n = 40)	High Risk (n = 20)	
	Stage 0 (n = 20)	Stage 1 (n = 17)	Stage 2 (n = 40)	Stage 3, 4 (n = 20)	P Value*
Age (years)	52.4 ± 13.5	54.1 ± 12.1	54.4 ± 11.2	62.7 ± 7.9	0.0075
Sex (male)	7 (35%)	9 (53%)	16 (40%)	5 (25%)	0.3641
Caucasian	15 (75%)	14 (82%)	38 (95%)	19 (95%)	0.0791
African American	3 (15%)	2 (12%)	2 (5%)	1 (5%)	0.5089
BMI (kg/m^2)	33.8 ± 6.8	36.4 ± 8.5	38.0 ± 7.3	36.6 ± 8.3	0.1869
Diabetes mellitus	4 (20%)	6 (35%)	17 (43%)	17 (85%)	0.0003
Metabolic syndrome	13 (65%)	16 (94%)	34 (85%)	19 (95%)	0.1265
Current smoker	3 (15%)	2 (12%)	3 (8%)	3 (15%)	0.7716
Steatosis grade (0-1)	12 (60%)	8 (47%)	19 (48%)	12 (60%)	0.6811
Steatosis grade (2-3)	8 (40%)	9 (53%)	21 (52%)	8 (40%)	0.6811
Lobular inflammation (0-1)	19 (95%)	13 (76%)	19 (48%)	12 (60%)	0.0023
Lobular inflammation (2-3)	1 (5%)	4 (24%)	21 (52%)	10 (50%)	0.0023
Portal inflammation	10 (50%)	9 (53%)	25 (63%)	19 (95%)	0.0113
Hepatocyte ballooning	11 (55%)	14 (82%)	35 (88%)	19 (95%)	0.0054
NAS (0-3)	11 (55%)	7 (41%)	9 (23%)	5 (25%)	0.0584
NAS (4-7)	9 (45%)	10 (59%)	31 (77%)	15 (75%)	0.0584
AST	35.8 ± 25.0	49.4 ± 35.0	52.5 ± 27.7	60.6 ± 25.6	0.0083
ALT	51.9 ± 48.0	60.8 ± 37.8	68.2 ± 42.2	63.6 ± 30.2	0.2783
AST/ALT ratio	0.8 ± 0.3	0.8 ± 0.2	0.8 ± 0.2	1.1 ± 0.5	0.0088
Total bilirubin	0.7 ± 0.2	1.0 ± 0.4	0.8 ± 0.3	0.8 ± 0.3	0.8014
Platelets	269.4 ± 55.2	219.1 ± 67.9	239.6 ± 77.5	182.1 ± 55.8	0.0014
Total cholesterol	178.7 ± 34.8	187.7 ± 34.7	174.4 ± 42.0	172.1 ± 33.8	0.3599
HDL	41.0 ± 8.6	35.8 ± 8.3	35.4 ± 9.8	32.2 ± 8.2	0.0045
LDL	109.7 ± 30.3	122.1 ± 30.4	107.7 ± 33.2	115.1 ± 26.2	0.9344
Total triglycerides	141.0 ± 58.7	149.2 ± 61.6	150.4 ± 83.7	146.8 ± 95.0	0.8115
Hyperlipidemia	17 (85%)	15 (88%)	38 (95%)	19 (95%)	0.5089
Hypertension	11 (55%)	12 (71%)	30 (75%)	17 (85%)	0.1907
HOMA-IR	2.9 ± 1.8	4.0 ± 2.2	5.2 ± 4.3	8.0 ± 4.0	9.9e-05

*P < 0.05 considered significant.

Abbreviations: ALT, alanine aminotransferase; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein.

SERUM CYTOKINES

In univariate analysis of 24 cytokines in 97 participants, serum cytokines IL-8, OPN, and MCP1 were associated with hepatic fibrosis (P < 0.001, P = 0.005, P = 0.016, respectively) (Table 2; Fig. 1A-C). After controlling for age, sex, BMI, diabetes mellitus, hypertension, and metabolic syndrome status, only the association of IL-8 with hepatic fibrosis remained significant (P = 0.001) (Table 3). Moreover, the association of IL-8 with hepatic fibrosis persisted (P = 0.001) after controlling for additional differences in histologic parameters, including steatosis, lobular inflammation, and hepatocyte ballooning status (Table 4).

Predictive Ability of Serum Cytokines and Additional Noninvasive Measures of NAFLD Fibrosis

Additional analyses of the predictive ability of the 24 serum cytokines and 10 existing noninvasive

TABLE 2. UNIVARIATE ASSOCIATION OF CYTOKINES WITH FIBROSIS

Cytokine	Beta	Standard Error	P Value*	FDR
IL-8	0.710	0.145	4.18e-06	1.00e-04[†]
OPN	0.933	0.322	0.005	0.056
MCP1	0.497	0.203	0.016	0.130
IL-17	−0.165	0.086	0.058	0.349
PAI-1	0.337	0.213	0.117	0.496
Leptin	0.175	0.113	0.124	0.496
Visfatin	0.083	0.075	0.275	0.592
IFN-γ	0.055	0.051	0.283	0.592
IL-6	0.097	0.090	0.283	0.592
IL-13	0.150	0.144	0.299	0.592
MIP1β	0.255	0.248	0.306	0.592
IL-1b 39	0.102	0.102	0.322	0.592
Resistin	0.311	0.313	0.323	0.592
IL-2	0.061	0.068	0.369	0.592
IFN-α 2	−0.089	0.103	0.390	0.592
GCSF	0.068	0.080	0.395	0.592
IL-7	0.051	0.070	0.469	0.662
GMCSF	0.024	0.052	0.647	0.809
TNF-α	0.024	0.057	0.669	0.809
IL-18	−0.047	0.112	0.674	0.809
IL-10	0.050	0.146	0.731	0.834
Adiponectin	0.072	0.239	0.764	0.834
IL-5	0.024	0.148	0.874	0.912
IL-12 p70	−0.007	0.084	0.936	0.936

*$P < 0.05$ considered significant; [†]Significant.
Abbreviations: FDR, false discovery rate; GCSF, granulocyte colony-stimulating factor; GMCSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; MIP, macrophage inflammatory protein; PAI, plasminogen activator inhibitor.

measures of hepatic fibrosis, with stratification of fibrosis into different groups (i.e., none [stage 0] versus any fibrosis [stage 1-4]; low risk [stage 0-1] versus moderate-high risk [stage 2-4] or versus high risk [stage 3-4]; none-low-moderate risk [stage 0-2] versus high risk [stage 3-4]) demonstrated that IL-8 remained a strong predictor of increased fibrotic liver injury compared to established noninvasive measures of hepatic fibrosis (Fig. 2, S1-4). IL-8 alone was in the top 11 of fibrosis predictors in all comparisons. We included a prototype measure of assessing liver stiffness (ARFI) in order to compare an imaging-based method of evaluating liver fibrosis to blood-based measures.[13] ARFI was superior to blood-based measures for low risk versus high risk and low-moderate risk versus high risk. Considering IL-8 as a multivariate predictor using a sparse logistic regression model resulted in the best predictive overall model for low

risk versus high risk, although this was not statistically significant ($P > 0.05$).

Hepatic Gene Expression of IL-8, MCP1, and OPN in NAFLD Fibrosis

After discovering that serum cytokines IL-8, MCP1, and OPN were associated with hepatic fibrosis in our patient cohort, we re-analyzed published gene expression data in the Duke University Health System NAFLD Clinical Database and Biorepository from 72 patients with NAFLD to determine whether IL-8, MCP1, and OPN are up-regulated in liver tissue. Expression of IL-8, MCP1, and OPN from liver tissue is a potential source for elevated levels of these cytokines in serum. Compared to livers of patients with NAFLD with early stage disease (fibrosis stage 0-1, n = 40), livers of patients with NAFLD with advanced fibrosis (stage 3-4, n = 32) had a 2.71-fold and 1.11-fold increase for the OPN/secreted phosphoprotein 1 probes (209875_s_at and 1568574_x_at; $P < 0.001$ and $P = 0.01$, respectively), a 2.22-fold and 1.16-fold increase for the IL-8 probes (216598_s_at and 216598_s_at; $P < 0.001$ and $P = 0.001$, respectively), and a 1.56-fold ($P < 0.001$) higher MCP1 liver gene expression (Fig. 3A-D).

Discussion

Proinflammatory cytokines have been implicated in the pathogenesis and progression of NASH.[25-27] While TNF-α and IL-6 have been shown to be elevated in patients with NASH compared to simple steatosis,[26,28] the association of those and other inflammatory cytokines with fibrosis is less understood. Identifying inflammatory cytokines associated with fibrosis in NAFLD is an urgent clinical need considering that liver fibrosis is the primary determinant of liver-related morbidity and mortality. Currently, there is a lack of noninvasive measures that predict fibrosis severity. Noninvasive measures of liver fibrosis may allow patients to be readily risk stratified for the purpose of guiding future clinical management (i.e., surveillance for hepatocellular carcinoma or screening of esophageal varices) and/or serve as a prescreening tool to identify eligible candidates for investigational therapies. Our study demonstrates that serum IL-8, MCP1,
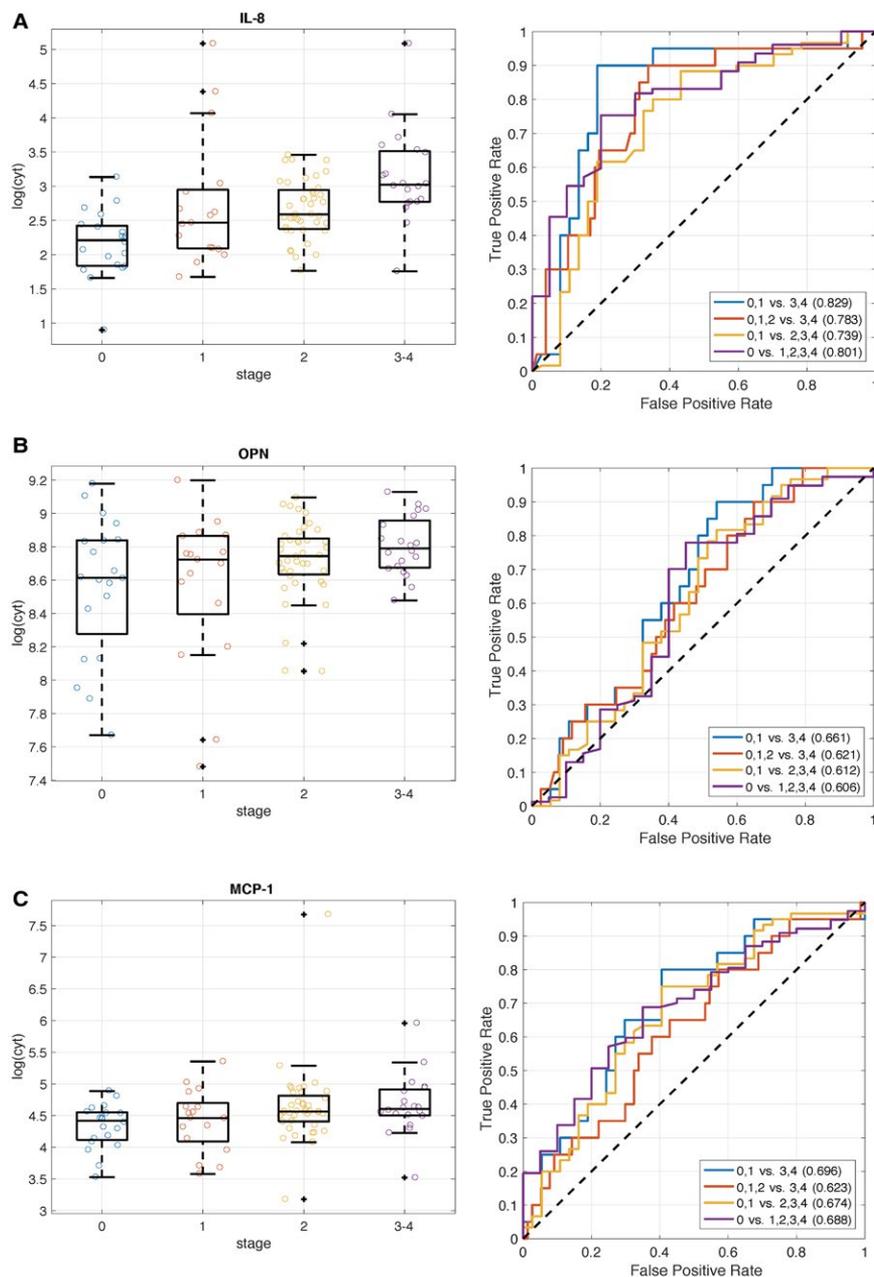
FIG. 1. Serum IL-8, OPN, and MCP1 are associated with hepatic fibrosis. (A) IL-8, (B) OPN, and (C) MCP1. Log-transformed concentrations of serum cytokines (log[cyt]) by fibrosis stage are shown on the left. Receiving operating characteristic curves for four different binary groupings of fibrosis are shown on the right. Legends denote the comparison among stages and their corresponding area under the curve value. Notes: This is a box plot. The middle line of the box is the median, the top of the box is the third quartile and the lower line of the box is the first quartile. The bottom of the box to the top of the box is the interquartile range. The dashed lines are meant to represent the range of the data. The horizontal lines represent the minimum (bottom) and maximum (top) values. Abbreviations: IL-8, interleukin-8; log(cyt), log concentration of cytokine; MCP-1, monocyte chemoattractant protein 1; NAFLD, nonalcoholic fatty liver disease; OPN, osteopontin.

and OPN are important noninvasive biomarkers associated with the severity of liver fibrosis in patients with NAFLD and may serve as important prognostic tools for antifibrotic treatments.

Serum IL-8 has been shown to be elevated in patients with NASH[29] as well as in patients with chronic liver disease, suggesting that IL-8 is a likely contributor of hepatic inflammation.[30] Additionally, serum IL-8 has

TABLE 3. UNIVARIATE ASSOCIATION OF CYTOKINES WITH FIBROSIS CONTROLLING FOR AGE, SEX, BMI, DIABETES MELLITUS, HYPERTENSION, AND METABOLIC SYNDROME

Cytokine	Beta	Standard Error	P Value*	FDR
IL-8	0.555	0.159	0.001	0.018[†]
OPN	0.612	0.326	0.064	0.767
PAI-1	0.326	0.204	0.113	0.846
Visfatin	0.106	0.073	0.151	0.846
IL-17	−0.109	0.084	0.195	0.846
Leptin	0.243	0.194	0.213	0.846
MCP1	0.242	0.207	0.247	0.846
IL-7	0.073	0.067	0.284	0.852
IL-13	0.112	0.145	0.441	0.890
IL-2	0.047	0.064	0.470	0.890
IFN-α 2	−0.063	0.096	0.514	0.890
Resistin	0.182	0.307	0.553	0.890
IFN-γ	0.027	0.049	0.580	0.890
IL-6	0.046	0.084	0.587	0.890
GMCSF	0.021	0.048	0.671	0.890
Adiponectin	0.094	0.227	0.679	0.890
TNF-α	0.022	0.053	0.680	0.890
GCSF	0.028	0.077	0.722	0.890
IL-10	−0.044	0.136	0.747	0.890
IL-5	−0.042	0.139	0.763	0.890
MIP1β	0.062	0.242	0.799	0.890
IL-12 p70	−0.019	0.080	0.816	0.890
IL-18	−0.017	0.104	0.872	0.910
IL-1b 39	0.010	0.099	0.918	0.918

*P < 0.05 considered significant; [†]Significant.
Abbreviations: FDR, false discovery rate; GCSF, granulocyte colony-stimulating factor; GMCSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; MIP, macrophage inflammatory protein; PAI, plasminogen activator inhibitor.

TABLE 4. UNIVARIATE ASSOCIATION OF CYTOKINES WITH FIBROSIS CONTROLLING FOR AGE, SEX, BMI, DIABETES MELLITUS, HYPERTENSION, METABOLIC SYNDROME, STEATOSIS, LOBULAR INFLAMMATION, AND HEPATOCYTE BALLOONING

Cytokine	Beta	Standard Error	P Value*	FDR
IL-8	0.509	0.153	0.001	0.030[†]
OPN	0.565	0.313	0.074	0.632
IL-7	0.107	0.064	0.099	0.632
Visfatin	0.113	0.069	0.105	0.632
IL-2	0.078	0.060	0.202	0.921
MCP1	0.236	0.196	0.230	0.921
IL-13	0.138	0.139	0.325	0.969
PAI-1	0.200	0.203	0.328	0.969
IFN-α 2	−0.060	0.091	0.509	0.969
Leptin	0.124	0.188	0.514	0.969
IL-6	0.048	0.080	0.553	0.969
IFN-γ	0.027	0.046	0.554	0.969
GMCSF	0.026	0.045	0.569	0.969
TNF-α	0.028	0.051	0.576	0.969
IL-12 p70	0.035	0.077	0.653	0.969
Resistin	0.094	0.295	0.750	0.969
IL-5	−0.042	0.136	0.756	0.969
IL-18	−0.028	0.098	0.776	0.969
GCSF	−0.019	0.074	0.792	0.969
MIP1β	−0.038	0.233	0.869	0.969
IL-10	−0.019	0.128	0.880	0.969
Adiponectin	0.023	0.219	0.917	0.969
IL-1b 39	−0.009	0.096	0.928	0.969
IL-17	0.002	0.085	0.983	0.983

*P < 0.05 considered significant; [†]Significant.
Abbreviations: FDR, false discovery rate; GCSF, granulocyte colony-stimulating factor; GMCSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; MIP, macrophage inflammatory protein; PAI, plasminogen activator inhibitor.

been shown to be associated with progression to fibrosis in patients with chronic liver disease.[31] Here, we extend prior work by showing that serum IL-8 is independently associated with liver fibrosis in patients with NASH. The independent association of IL-8 remained after controlling for known confounders, including age, sex, BMI, diabetes mellitus, hypertension, and metabolic syndrome. This finding underscores the importance of IL-8 as a noninvasive biomarker of liver fibrosis in NAFLD. Furthermore, IL-8 outperformed other more established predictive markers of liver fibrosis. We also showed that IL-8 hepatic gene expression was up-regulated as much as 2.2-fold in patients with clinically occult (but advanced) disease, suggesting that IL-8 protein detected in serum may reflect changes in liver gene expression before liver fibrosis becomes overt. Our results validate the utility of profiling liver gene

expression to identify biologically plausible serum biomarkers that reflect the severity of NAFLD-related liver fibrosis and thus support ongoing efforts by the NAFLD field to apply this approach more widely. More specifically, our results identify IL-8 as a useful biomarker for stratifying patients with fibrosis in NAFLD, and this insight is immediately applicable to prioritize patients in most need of interventional therapies.

MCP1 is a chemokine responsible for the migration and attraction of macrophages and monocytes into the liver following inflammation.[32] Preclinical studies indicate that MCP1 does not contribute to steatosis or inflammation but independently contributes to fibrosis.[33] However, there have been few human studies investigating the role of MCP1 in NAFLD. Of the available studies, MCP1 was shown to be elevated in
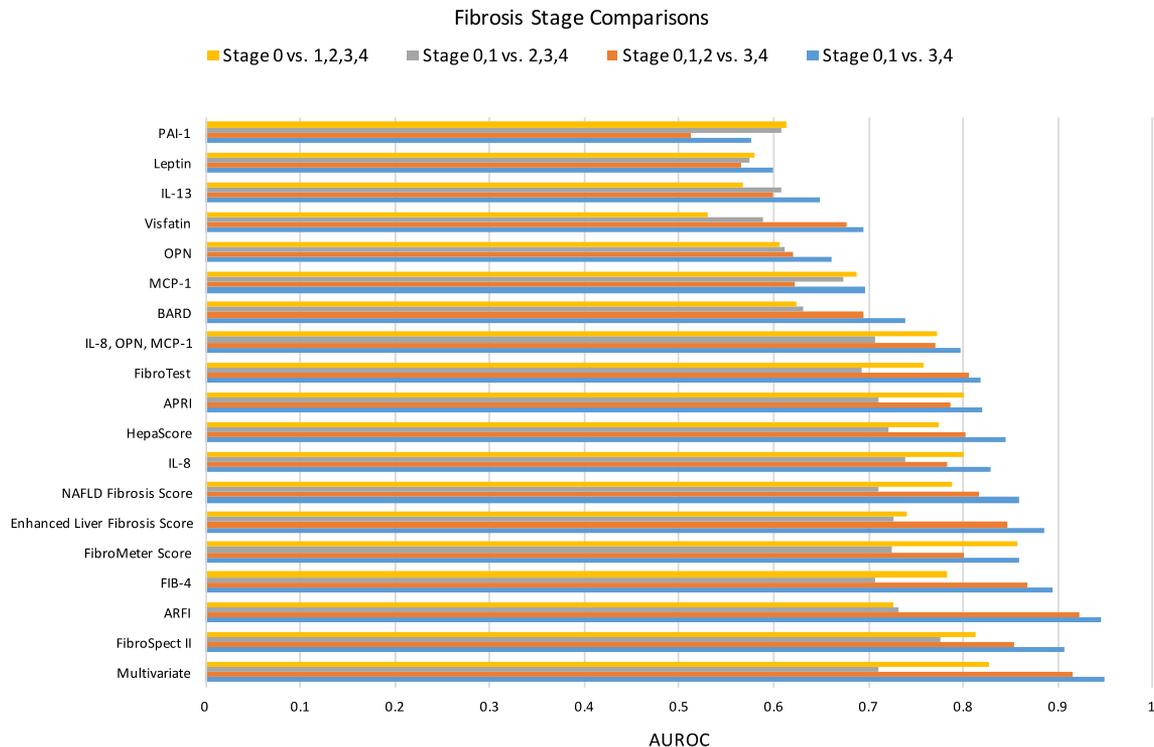
FIG. 2. Serum IL-8 is a strong predictor of liver fibrosis in patients with biopsy-proven NASH compared to established markers. We used leave-one-out cross-validated area under the receiver operating characteristic for 10 noninvasive measures of liver fibrosis, 24 cytokines, IL-8, OPN and MCP1 combined, and a multivariate model, on four different binary groupings of fibrosis: stage 0,1 versus 3,4; stage 0,1,2 versus 3,4; stage 0,1 versus 2,3,4; and stage 0 versus 1,2,3,4. IL-8 was the eighth best overall marker, and the combination of IL-8, OPN, and MCP1 was the twelfth best overall marker. The top 20 noninvasive predictors of liver fibrosis are shown. The multivariate model, consisting of IL-8 in addition to all measures, was the best performer for low risk (stage 0,1) versus high risk (stage 3,4), $P > 0.05$. Abbreviations: APRI, aspartate aminotransferase-to-platelet ratio index; AUROC, area under the receiver operating characteristic; FIB-4, fibrosis-4; PAI-1, plasminogen activator inhibitor 1.

patients with both NAFLD and NASH[34] compared to healthy controls and patients with NAFLD with simple steatosis.[9,35] Our results indicate that elevated MCP1 is associated with fibrosis in NAFLD. The fact that the association did not remain after significant controlling for known confounders that influence the severity of NASH does not undermine the importance of MCP1 in NASH pathogenesis.

OPN is an extracellular matrix protein that assists in the recruitment of macrophages during liver injury,[36] has been shown to be elevated in patients with NAFLD, and independently predicts portal inflammation.[37] We have previously shown that liver OPN expression is significantly elevated in NASH compared to normal livers and correlates with fibrosis stage.[38] We extend our prior work on hepatic gene expression by demonstrating that serum OPN levels may reflect both hepatic expression of OPN and fibrosis severity in patients with NASH. Hence, similar to IL-8, serum

OPN levels in NASH may mirror hepatic production of this profibrogenic factor and as such provide a noninvasive marker to measure liver fibrosis.

Our study has several limitations. First, a small number of subjects with stage 4 fibrosis were included in this study, and this may reflect the presence of other prognoses associated with advanced liver disease in the exclusion criteria. Second, we imputed missing values in our statistical analysis; however, missing values accounted for less than 7% of all fibrosis marker observations and thus are less likely to significantly alter our results. Third, the analysis is a cross-sectional assessment of correlation between cytokine and histologic features of NASH. The study design does not lend us the ability to predict disease progression (or regression) or assessment of clinical outcomes based on cytokine profile.

Meta-analyses of randomized controlled trials indicate that antifibrotic therapies in NASH may provide efficacy.[39] It is possible that suppression of
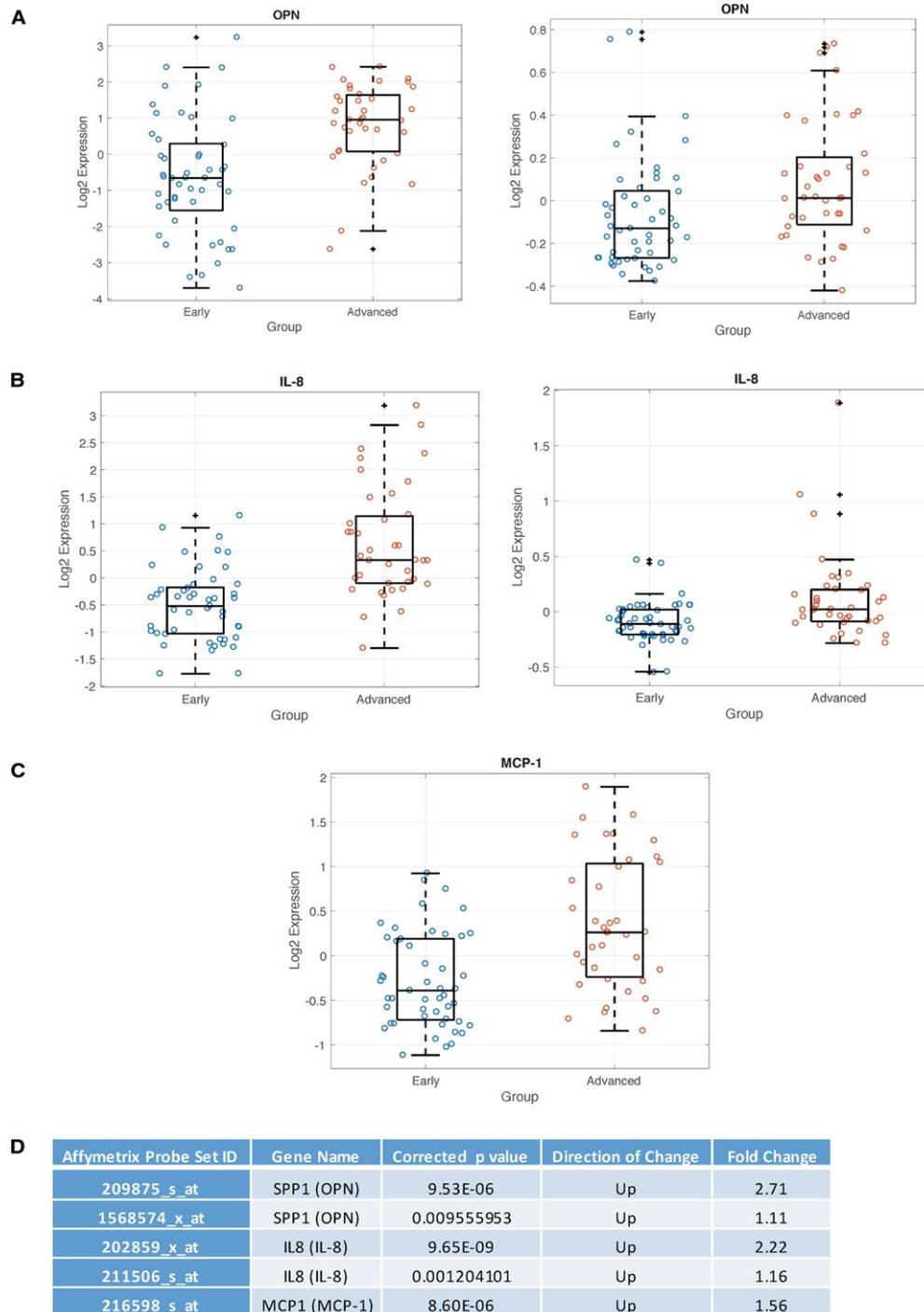
FIG. 3. Liver gene expression of OPN, IL-8, and MCP1 are elevated in patients with NAFLD with advanced versus mild fibrosis. Gene expression analysis from 72 patients with NAFLD with mild- (fibrosis stage 0-1; n = 40) and advanced- (fibrosis stage 3-4, n = 32) stage disease indicate that liver gene expression of OPN, IL-8, and MCP1 are significantly elevated in patients with advanced disease. (A) Log2-fold change of OPN Affymetrix probes, (B) IL-8 Affymetric probes, and (C) MCP1 Affymetrix probe, by mild versus advanced disease groups. (D) *P* values and log2-fold change of OPN, IL-8, and MCP1 Affymetrix probes. Only a single probe was available for analysis of MCP1, while multiple probes for OPN and IL-8 were available for analyses. Notes: This is a box plot. The middle line of the box is the median, the top of the box is the third quartile and the lower line of the box is the first quartile. The bottom of the box to the top of the box is the interquartile range. The dashed lines are meant to represent the range of the data. The horizontal lines represent the minimum (bottom) and maximum (top) values. Abbreviations: IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein 1; NAFLD, nonalcoholic fatty liver disease; OPN, osteopontin.

inflammatory mediators is one potential strategy to improve fibrosis in patients with NASH. Our results in serum from patients with NAFLD suggest that inflammatory cytokines IL-8, MCP1, and OPN may provide novel targets to improve fibrosis. Further, these inflammatory cytokines may optimize the quest for a highly sensitive and specific serum biomarker for patients with NAFLD/NASH and advanced hepatic fibrosis.

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Author names in bold designate shared co-first authorship.