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Novel Plasma Biomarkers Associated With Liver Disease Severity in Adults With Nonalcoholic Fatty Liver Disease

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Despite the high prevalence of nonalcoholic fatty liver disease (NAFLD), therapeutic options and noninvasive markers of disease activity and severity remain limited. We investigated the association between plasma biomarkers and liver histology in order to identify markers of disease activity and severity in patients with biopsy-proven NAFLD. Thirty-two plasma biomarkers chosen *a priori* as possible discriminators of NAFLD were measured in participants enrolled in the Nonalcoholic Steatohepatitis (NASH) Clinical Research Network. Dichotomized histologic outcomes were evaluated using centrally read biopsies. Biomarkers with statistically significant associations with NAFLD histology were evaluated in multivariable models adjusted for clinical factors. Of 648 participants (74.4% white, 61.7% female, mean age 47.7 years), 58.0% had definite NASH, 55.5% had mild/no fibrosis (stage 0-1), and 44.4% had significant fibrosis (stage 2-4). Increased activated plasminogen activator inhibitor 1 had a strong association with definite NASH compared to not NASH or borderline NASH in multivariable analysis (odds ratio = 1.20, 95% confidence interval 1.08-1.34, $P < 0.001$). Biomarkers associated with significant fibrosis (versus mild/no fibrosis) in multivariable analysis included higher levels of interleukin-8, monocyte chemoattractant protein-1, resistin, soluble interleukin-1 receptor I, soluble interleukin-2 receptor alpha, and tumor necrosis factor alpha and lower levels of insulin-like growth factor 2. *Conclusions:* Specific plasma biomarkers are significantly associated with disease activity and severity of fibrosis in NAFLD and are potentially valuable tools for noninvasive stratification of patients with NAFLD and identification of targets for therapeutic intervention. (HEPATOLOGY 2016; 00:000-000).

Approximately one-third of the US adult population is estimated to have nonalcoholic fatty liver disease (NAFLD).[1] NAFLD is characterized by a spectrum of histologically defined stages from steatosis to steatohepatitis, advanced fibrosis, cirrhosis, and hepatocellular carcinoma.[2] However, only a subset of patients progress through each stage. An improved understanding of risk factors associated with disease severity could help in risk stratification of patients with NAFLD.

The diagnosis and staging of NAFLD rely on histologic evaluation of a liver biopsy, an invasive test that is associated with some risk of complications and inconvenient as a repeat measure of disease severity.

Abbreviations: ALT, alanine aminotransferase; aPAI1, activated PAI1; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; FDR, false discovery rate; HOMA-IR, homeostasis model assessment of insulin resistance; IGFII, insulin-like growth factor 2; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NASH CRN, NASH Clinical Research Network; OR, odds ratio; PAI1, plasminogen activator inhibitor 1; PIVENS, Pioglitazone or Vitamin E for Nonalcoholic Steatohepatitis; sIL-1R1, soluble IL-1 receptor 1; TNFα, tumor necrosis factor alpha; tPAI1, total PAI1.

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Moreover, a liver biopsy is susceptible to significant sampling variability.[3] A growing appreciation of the relationship of mediators of inflammation with the histologic features of NAFLD coupled with the ability to detect many mediators of inflammation in the peripheral blood has resulted in a growing interest in novel biomarkers that could improve the utility of or even replace liver biopsy.[4]

Plasma levels of several candidate biomarkers that correlate with steatosis, steatohepatitis, or fibrosis have been identified in small clinical studies of patients with NAFLD. Interleukin (IL)-6 levels were higher in patients with NAFLD than controls; however, the authors did not discriminate between those with steatosis and those with steatohepatitis.[5] IL-8 levels were higher in patients with nonalcoholic steatohepatitis (NASH) than steatosis; however, other studies showed no difference.[6,7] Elevated monocyte chemoattractant protein 1 (MCP-1) levels and increased *MCP1* gene expression were associated with NAFLD and NASH.[5,8] Reduced adiponectin levels were independently associated with NASH, and increasing tumor necrosis factor alpha (TNF-α) levels correlated with insulin resistance, steatohepatitis, and fibrosis.[9,10] Multiple other biomarkers (resistin, leptin, retinol binding protein-4, hyaluronic acid, procollagen type 3 N-terminal peptide, tissue inhibitor of metalloproteinases 1, transforming growth factor beta 1) have also been associated with NAFLD severity and fibrosis, but all of these studies were limited by a small to modest sample size or examination of only a limited number of biomarkers.[11-14]

The purpose of this study was to evaluate a broad set of candidate biomarkers of inflammation, fibrosis, angiogenesis, and insulin and glucose metabolism in a large, well-characterized cohort of adults with NAFLD to determine their association with the histologic features of NAFLD.

Participants and Methods

STUDY DESIGN AND PARTICIPANTS

This was a cross-sectional study of adult participants recruited into the NASH Clinical Research Network (NASH CRN), a multicenter network sponsored by the National Institute of Diabetes and Digestive and Kidney Diseases. Participants were drawn from two groups, at baseline, within the NASH CRN: (1) the NAFLD Database study, a prospective observational cohort which enrolled participants beginning in 2004, and (2) the Pioglitazone or Vitamin E for Nonalcoholic Steatohepatitis (PIVENS) trial. Both studies have institutional review board approval at each of the eight clinical centers participating in the NASH CRN (Supporting Information). Informed consent was obtained at entry into the study.

The NAFLD Database is a prospective observational study of participants at least 18 years of age with either a histologic diagnosis of NAFLD or cryptogenic cirrhosis, suspected NAFLD based on imaging studies, or clinical evidence of cryptogenic cirrhosis. Exclusion criteria included clinical evidence of alcoholic liver disease or alcohol consumption during the 2 years before entry of more than 20 g daily for men and 10 g daily for women and evidence of other forms of chronic liver disease, including suspected or confirmed hepatocellular carcinoma and known human immunodeficiency virus positivity. Additional details of the study have been published.[15] The PIVENS trial was conducted from 2005 to 2008 and included nondiabetic adults without cirrhosis but with definite or possible steatohepatitis. The details of the study protocol and the main results of the trial have been published.[16,17] This study included all adult participants at the time of study selection on April 1, 2010,

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with available plasma samples drawn within 6 months of a centrally read liver biopsy at the baseline visit. Of the 1,048 adult participants of the Database study, 1,020 had sufficient plasma available for this study; of these, 850 participants had a centrally reviewed enrollment liver biopsy, 414 of these being within 6 months of the plasma blood draw. The final number of participants with assay data on at least one analyte was 411. Of the 247 adults enrolled in the PIVENS trial, 237 had available baseline serum and were included in this study, bringing the total study population to 648.

CLINICAL AND LABORATORY ASSESSMENT

Demographic data and self-reported doctor-diagnosed comorbidities were obtained by structured interview. Height, weight, waist, and hip measurements were taken in duplicate while standing and wearing light clothing and averaged for analyses. Body mass index (BMI) was calculated as weight (kilograms) divided by height (meters) squared.

Fasting whole blood samples were obtained by 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 white blood cell count (10^3 cells per microliter), hematocrit (percentage), platelet count (10^3 cells per microliter), total bilirubin (milligrams per deciliter), alanine aminotransferase (ALT, units per liter), aspartate aminotransferase (AST, units per liter), alkaline phosphatase (units per liter), gamma-glutamyltransferase (units per liter), albumin (grams per deciliter), fasting lipids including triglycerides and cholesterol fractions (milligrams per deciliter), fasting glucose (milligrams per deciliter), and fasting insulin (milliunits per milliliter). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the equation (fasting glucose [millimoles per liter] × fasting insulin [milliunits per liter])/22.5.

HISTOLOGIC EVALUATION

Biopsy specimens were evaluated centrally by the NASH CRN Pathology Committee for the following histologic features, according to the validated histologic scoring system (NAFLD Activity Score) by Kleiner et al.[18]: steatosis (grade 0 [<5% macrovesicular fat], grade 1 [5%-33%], grade 2 [34%-66%], and grade 3 [>66%]), portal inflammation (grade 0 [none], grade 1 [mild], and grade 2 [>mild]), lobular inflammation (grade 0 [none per ×20 field], grade 1 [<2 foci per ×20 field], grade 2 [2-4 foci per ×20 field], and grade 3 [>4 foci per ×20 field]), ballooning degeneration (grade 0 [none], grade 1 [few], and grade 2 [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]). For analysis, steatosis was dichotomized into grade 1 compared to grade 2 or 3. Lobular inflammation was dichotomized as grades 0-1 versus grades 2-3. Ballooning degeneration was dichotomized as grade 0 versus grades 1-2. Significant fibrosis was defined as stages 2-4 and compared to mild or no fibrosis (stages 0-1). Diagnosis of NASH was classified as definite NASH, NAFLD not NASH, or suspicious for NASH ("borderline" NASH) based upon central pathology reading, as defined.[18] These categories were assigned prior to conducting statistical analyses.

PLASMA BIOMARKER MULTIPLEX ASSAY

Thirty-two plasma biomarkers were chosen *a priori* as possible predictors of NASH and its histologic components (i.e., steatosis, inflammation, fibrosis). Plasma samples were stored at -80°C until testing. Plasma samples were measured in duplicate using the Luminex Multiplex platform (Millipore, St. Louis, MO) and processed according to standard protocol. Cytokine detection by Luminex is comparable to that with enzyme-linked immunosorbent assay,[19,20] 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 <20%, a criterion met for all 32 biomarkers (Supporting Information). Four biomarkers that were unable to be measured in at least 90% of the samples were excluded (glucagon-like peptide 1, soluble vascular endothelial growth factor receptor 1, IL1α, transforming growth factor beta 3).

STATISTICAL ANALYSIS

This exploratory analysis evaluated associations between plasma biomarkers, clinical factors, and specific histologic outcomes: NASH diagnosis (primary outcome of interest), steatosis grade, lobular inflammation, hepatocyte ballooning, and fibrosis stage. Histologic outcomes with more than two categories were

TABLE 1. Clinical and Demographic Characteristics by NASH Diagnosis

	Total (n = 648)	Borderline/Not NASH (n = 272)	Definite NASH (n = 376)
Age at biopsy (years)	47.7 ± 12.2	47.5 ± 12.0	47.9 ± 12.3
Male sex	248 (38.3)	122 (44.9)	126 (33.5)
Race/ethnicity			
White	482 (74.4)	205 (75.4)	277 (73.7)
Black	14 (2.2)	7 (2.6)	7 (1.9)
Hispanic	88 (13.6)	34 (12.5)	54 (14.4)
Other	64 (9.9)	26 (9.6)	38 (10.1)
BMI (kg/m^2)	34.6 ± 6.4	34.7 ± 6.7	34.5 ± 6.1
Diabetes	143 (22.1)	48 (17.7)	95 (25.3)
ALT (U/L)	78.9 ± 53.0	62.9 ± 37.2	90.4 ± 59.4
AST (U/L)	56.1 ± 37.7	41.5 ± 19.9	66.7 ± 43.6
Total bilirubin (mg/dL)	0.76 ± 0.41	0.83 ± 0.47	0.71 ± 0.35
Platelet count (1,000/μL)	244 ± 70	248 ± 71	242 ± 69
Triglycerides (mg/dL)	182 ± 133	167 ± 142	194 ± 125
HDL-C (mg/dL)	43.5 ± 11.6	44.7 ± 11.8	42.6 ± 11.4
HOMA-IR	5.9 ± 5.7	4.9 ± 4.2	6.7 ± 6.5

Values are n (%) or means ± standard deviation unless otherwise specified.
Abbreviation: HDL-C, high-density lipoprotein cholesterol.

collapsed into binary outcomes based on clinical significance. Descriptive statistics and frequency distributions were generated on the sample demographic and clinical characteristics as well as plasma biomarker measurements (Table 1). Logistic regression was used for univariable analysis of the association between biomarkers and histologic outcomes, with the biomarkers scaled per 0.5 standard deviation change based on standard deviations for the total group as reported in Table 2. To account for multiple comparisons, the Benjamini-Hochberg false discovery rate (FDR) adjusted threshold for statistical significance was calculated for the association between plasma biomarkers and histologic outcomes. Multiple imputation was performed with progressive mean matching and iterative-chained equations with the mean of 20 imputed values retained and used for multivariable models. Univariable linear regression of clinical factors chosen *a priori* for their known association with NASH diagnosis and severity (age, sex, BMI, AST, ALT, triglycerides, high-density lipoprotein, and HOMA-IR) on statistically significant biomarkers was used for analysis of the association between biomarkers and clinical factors. Pearson's correlation was used to evaluate the correlation among statistically significant biomarkers. Finally, biomarkers meeting the threshold for statistical significance in univariable analysis ($P < 0.05$) were evaluated in a multivariable model with the aforementioned clinical factors. In this analysis, the biomarkers meeting the threshold for statistical significance but not meeting the FDR adjusted threshold on univariable analysis were highlighted (with an asterisk). All statistical analyses were performed using STATA (StataCorp LP, College Station, TX).

Results

CHARACTERISTICS OF THE STUDY POPULATION

Six hundred and forty-eight patients with NAFLD from the NASH CRN were included in the analysis. Participants had a mean age of 47.7 years and were predominantly female (61.7%), white (74.4%), and obese (mean BMI = 34.6 kg/m^2). Diabetes (22.1%), hypertension (46.1%), and hyperlipidemia (55.7%) were common comorbidities. Of the 648 adult participants, 376 were classified as definite NASH (58.0%), 143 as not NASH (22.1%), and 129 as "borderline" NASH (19.9%).

BIOMARKERS ASSOCIATED WITH THE DIAGNOSIS OF DEFINITE NASH

In univariable logistic regression analysis, participants with definite NASH had significantly higher levels of total plasminogen activator inhibitor 1 (tPAI1), activated plasminogen activator inhibitor 1 (aPAI1), IL-8, and soluble IL-1 receptor 1 (sIL-1R1) than those with no or borderline NASH (Tables 2 and 3). In multivariable analysis adjusting for clinical factors,

TABLE 2. Plasma Biomarkers Levels by NASH Diagnosis

Biomarker	n	Total	Borderline/Not NASH	Definite NASH
Adiponectin (μg/mL)	642	12.8 ± 8.3	13.5 ± 8.6	12.3 ± 8.0
aPAI1 (ng/mL)	641	43.0 ± 33.5	35.5 ± 25.5	48.5 ± 37.4
FGF-2 (pg/mL)	618	86.9 ± 87.7	83.3 ± 93.6	89.5 ± 83.2
Fibrinogen (mg/mL)	645	4.5 ± 1.7	4.4 ± 1.7	4.5 ± 1.8
Haptoglobin (mg/mL)	647	2.9 ± 1.9	2.9 ± 1.8	2.9 ± 1.9
IFNγ (pg/mL)	585	7.8 ± 13.5	8.0 ± 12.6	7.7 ± 14.1
IGFII (ng/mL)	648	1.4 ± 1.1	1.5 ± 1.1	1.3 ± 1.1
IL-1β (pg/mL)	582	0.6 ± 1.9	0.6 ± 0.6	0.7 ± 2.4
IL-2 (pg/mL)	594	5.3 ± 17.2	5.1 ± 17.3	5.5 ± 17.1
IL-4 (pg/mL)	622	21.8 ± 26.0	23.1 ± 27.3	20.9 ± 25.1
IL-5 (pg/mL)	629	0.7 ± 0.8	0.8 ± 0.8	0.7 ± 0.8
IL-6 (pg/mL)	645	10.5 ± 38.7	11.8 ± 54.9	9.5 ± 20.6
IL-7 (pg/mL)	612	4.3 ± 4.3	4.5 ± 4.5	4.2 ± 4.2
IL-8 (pg/mL)	648	4.3 ± 4.7	3.7 ± 5.1	4.7 ± 4.3
IL-10 (pg/mL)	647	16.8 ± 23.3	15.9 ± 19.2	17.3 ± 25.8
IL-12/p40 (pg/mL)	501	80.6 ± 246.7	77.4 ± 222.0	83.0 ± 263.5
MCP-1 (pg/mL)	648	251.5 ± 90.8	247.3 ± 79.8	254.4 ± 98.0
MMP-9 (ng/mL)	648	63.3 ± 37.8	61.0 ± 33.7	64.9 ± 40.5
Resistin (ng/mL)	644	16.2 ± 7.5	16.0 ± 6.6	16.3 ± 8.1
sFasl (pg/mL)	609	71.3 ± 38.5	73.8 ± 39.1	69.5 ± 38.0
sIL-1R1 (pg/mL)	644	32.9 ± 26.2	28.6 ± 20.2	36.0 ± 29.3
sIL-2Rα (ng/mL)	644	0.7 ± 0.4	0.6 ± 0.3	0.7 ± 0.4
sIL-6R (ng/mL)	644	19.9 ± 5.5	20.0 ± 5.5	19.8 ± 5.4
TGFβ1 (ng/mL)	648	5.2 ± 5.3	5.2 ± 4.7	5.3 ± 5.7
TGFβ2 (pg/mL)	598	299.3 ± 247.3	285.7 ± 230.1	309.1 ± 258.8
TNFα (pg/mL)	648	7.4 ± 7.5	6.7 ± 4.0	7.8 ± 9.2
tPAI1 (ng/mL)	648	43.2 ± 21.8	40.5 ± 18.1	45.2 ± 23.9
VEGF (pg/mL)	623	413.0 ± 711.4	410.4 ± 790.2	415.0 ± 648.9

Abbreviations: FGF-2, fibroblast growth factor 2; IFNγ, interferon gamma; MMP-9, matrix metallopeptidase 9; sFasl, soluble Fas ligand; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

only increased aPAI1 was independently associated with definite NASH (odds ratio [OR] = 1.20, 95% confidence interval (CI) 1.08-1.34, $P = 0.001$) (Table 4). aPAI1 was associated with BMI, HOMA-IR, high-density lipoprotein cholesterol, and triglycerides (Table 5); and levels strongly correlated with tPAI1, $\rho = 0.65$ (Supporting Table S3). In sensitivity analysis excluding patients with borderline NASH from the comparison group, only aPAI1 remained associated with definite NASH after multivariable adjustment (Supporting Table S4).

BIOMARKERS ASSOCIATED WITH MODERATE TO SEVERE STEATOSIS, HEPATOCYTE BALLOONING, AND LOBULAR INFLAMMATION

Twenty-five percent of participants had >66% steatosis (grade 3), 34% had 34%-66% (grade 2), 37% had 5%-33% (grade 1), and 5% had <5% steatosis (grade 0). Participants with moderate to severe steatosis (>33%) had lower levels of adiponectin and sIL-2Rα as well as elevated tPAI1, aPAI1, and insulin-like growth factor 2 (IGFII) (Table 3; Supporting Table S1) compared to those with no or mild steatosis ($\leq$33%) in univariable logistic regression. sIL-2Rα did not meet the FDR adjusted threshold for statistical significance. In a multivariable analysis of biomarkers associated with steatosis >33% compared to steatosis $\leq$33%, decreased adiponectin (OR = 0.91, 95% CI 0.83-0.99, $P = 0.029$) and increased aPAI1 (OR = 1.20, 95% CI 1.09-1.32, $P < 0.001$), tPAI1 (OR = 1.31, 95% CI 1.18-1.45, $P < 0.001$) and IGFII (OR = 1.16, 95% CI 1.05-1.28, $P = 0.005$) were associated with higher grades of steatosis (Table 4). Adiponectin was associated with female sex and increased age and high-density lipoprotein cholesterol and inversely associated with ALT, HOMA-IR, and triglycerides (Table 5). IGFII was associated with male sex and increased ALT and triglycerides and inversely associated with age and BMI. tPAI1 was associated with increased age, BMI, AST, HOMA-IR, and triglycerides. IGFII was inversely correlated with IL-8 and sIL-2Rα ρ = -0.20 and ρ = -0.22, respectively (Supporting Table S3). Adiponectin was not strongly correlated with any

TABLE 3. Univariable ORs* for Plasma Biomarkers by Histologic Dichotomies

	NASH Diagnosis (Definite Versus Borderline/Not) OR (95% CI) *P*	Fibrosis (Stages 2-4 Versus 0-1) OR (95% CI) *P*	Steatosis (Grade 2-3 Versus 0-1) OR (95% CI) *P*	Ballooning (Any Versus None) OR (95% CI) *P*	Lobular Inflammation (Grade 2-3 Versus 0-1) OR (95% CI) *P*
Adiponectin (μg/mL)	0.93 (0.86-1.01) 0.090	1.05 (0.97-1.13) 0.251	**0.88 (0.81-0.95) 0.001**	0.97 (0.89-1.05) 0.482	0.95 (0.88-1.03) 0.226
aPAI1 (ng/mL)	**1.25 (1.14-1.38) <0.001**	1.04 (0.96-1.12) 0.358	**1.20 (1.10-1.31) <0.001**	**1.16 (1.06-1.27) 0.002**	1.06 (0.98-1.14) 0.170
FGF-2 (pg/mL)	1.04 (0.95-1.13) 0.386	1.01 (0.94-1.10) 0.730	0.93 (0.86-1.01) 0.100	1.06 (0.96-1.16) 0.249	1.01 (0.93-1.09) 0.838
Fibrinogen (mg/mL)	1.02 (0.95-1.11) 0.550	1.07 (0.99-1.15) 0.089	0.99 (0.92-1.07) 0.833	0.98 (0.90-1.06) 0.569	0.98 (0.91-1.06) 0.657
Haptoglobin (mg/mL)	1.00 (0.92-1.08) 0.957	**0.92 (0.84-0.99) 0.035**†	1.07 (0.99-1.17) 0.086	0.97 (0.89-1.05) 0.436	0.98 (0.91-1.06) 0.622
IFNγ (pg/mL)	0.99 (0.91-1.07) 0.774	1.01 (0.93-1.09) 0.897	0.96 (0.89-1.05) 0.374	1.00 (0.92-1.09) 0.990	1.03 (0.95-1.12) 0.499
IGFII (ng/mL)	0.95 (0.87-1.02) 0.160	**0.71 (0.63-0.78) <0.001**	**1.21 (1.10-1.32) <0.001**	**0.92 (0.84-0.99) 0.033**	1.05 (0.97-1.13) 0.239
IL-1β (pg/mL)	1.03 (0.92 -1.15) 0.568	1.15 (0.91-1.45) 0.256	0.94 (0.81-1.08) 0.361	1.04 (0.90-1.21) 0.572	1.10 (0.90-1.36) 0.351
IL-2 (pg/mL)	1.01 (0.93-1.10) 0.772	1.07 (0.97-1.17) 0.162	0.92 (0.83-1.01) 0.072	1.03 (0.94-1.14) 0.543	1.02 (0.94-1.11) 0.592
IL-4 (pg/mL)	0.96 (0.89-1.04) 0.313	1.04 (0.96-1.12) 0.373	1.02 (0.94-1.11) 0.581	0.99 (0.92-1.08) 0.904	0.98 (0.90-1.06) 0.593
IL-5 (pg/mL)	0.97 (0.89-1.05) 0.405	1.00 (0.93-1.09) 0.940	0.96 (0.89-1.05) 0.382	1.01 (0.92-1.10) 0.902	0.98 (0.91-1.07) 0.705
IL-6 (pg/mL)	0.97 (0.89-1.06) 0.490	1.18 (0.96-1.45) 0.107	0.99 (0.92-1.07) 0.874	0.96 (0.88-1.05) 0.325	0.98 (0.91-1.07) 0.687
IL-7 (pg/mL)	0.97 (0.90-1.05) 0.513	1.07 (0.99-1.16) 0.096	1.03 (0.95-1.11) 0.538	1.01 (0.93-1.11) 0.757	1.00 (0.92-1.08) 0.927
IL-8 (pg/mL)	**1.21 (1.06-1.37) 0.004**	**2.35 (1.92-2.88) <0.001**	0.98 (0.91-1.06) 0.638	**1.62 (1.34-1.97) <0.001**	1.02 (0.94-1.10) 0.608
IL-10 (pg/mL)	1.00 (0.99-1.02) 0.452	1.01 (1.00-1.02) 0.065	1.00 (0.99-1.02) 0.426	1.01 (0.99-1.02) 0.232	1.01 (0.99-1.02) 0.305
IL-12/p40 (pg/mL)	1.01 (0.92-1.11) 0.803	1.02 (0.93-1.11) 0.692	0.92 (0.83-1.02) 0.121	1.00 (0.91-1.10) 0.974	1.02 (0.93-1.11) 0.702
MCP-1 (pg/mL)	1.04 (0.96-1.13) 0.326	**1.20 (1.10-1.31) <0.001**	0.96 (0.89-1.04) 0.361	**1.09 (1.00-1.20) 0.046**†	**0.92 (0.85-0.99) 0.031**†
MMP-9 (ng/mL)	1.06 (0.97-1.15) 0.201	1.05 (0.97-1.14) 0.236	1.05 (0.96-1.14) 0.280	1.07 (0.97-1.18) 0.154	1.00 (0.92-1.08) 0.938
Resistin (ng/mL)	1.03 (0.95-1.11) 0.519	**1.12 (1.03-1.22) 0.006**	0.96 (0.89-1.04) 0.295	1.02 (0.94-1.12) 0.568	0.93 (0.86-1.01) 0.080
sFasl (pg/mL)	0.95 (0.87-1.02) 0.178	0.97 (0.89-1.05) 0.436	0.94 (0.87-1.02) 0.160	0.97 (0.89-1.06) 0.499	0.95 (0.87-1.02) 0.171
sIL-1R1 (pg/mL)	**1.22 (1.09-1.37) 0.001**	**1.22 (1.10-1.35) <0.001**	1.05 (0.96-1.14) 0.296	**1.18 (1.05-1.33) 0.006**	1.05 (0.97-1.14) 0.219
sIL-2Rα (ng/mL)	1.09 (1.00-1.20) 0.058	**1.44 (1.29-1.61) <0.001**	**0.91 (0.83-0.99) 0.029**†	1.09 (0.99-1.21) 0.094	1.05 (0.96-1.15) 0.259
sIL-6R (ng/mL)	0.98 (0.91-1.07) 0.707	1.00 (0.92 -1.08) 0.989	0.96 (0.89-1.04) 0.360	0.98 (0.91-1.07) 0.718	0.97 (0.89-1.04) 0.373

TABLE 3. *Continued*

	NASH Diagnosis (Definite Versus Borderline/Not) OR (95% CI) *P*	Fibrosis (Stages 2-4 Versus 0-1) OR (95% CI) *P*	Steatosis (Grade 2-3 Versus 0-1) OR (95% CI) *P*	Ballooning (Any Versus None) OR (95% CI) *P*	Lobular Inflammation (Grade 2-3 Versus 0-1) OR (95% CI) *P*
TGFβ1 (ng/mL)	1.01 (0.94-1.10) 0.758	1.04 (0.96-1.13) 0.309	1.05 (0.96-1.14) 0.295	1.00 (0.92-1.09) 0.958	0.95 (0.88-1.03) 0.235
TGFβ2 (pg/mL)	1.05 (0.96-1.15) 0.261	1.08 (0.99-1.18) 0.081	1.05 (0.96-1.15) 0.269	1.04 (0.95-1.15) 0.376	0.96 (0.89-1.05) 0.391
TNFα (pg/mL)	1.14 (0.99-1.32) 0.067	**1.38 (1.17-1.62) <0.001**	0.91 (0.81-1.02) 0.122	**1.21 (1.02-1.43) 0.028**	1.01 (0.94-1.10) 0.735
tPAI1 (ng/mL)	**1.12 (1.03-1.23) 0.007**	1.08 (1.00-1.17) 0.066	**1.25 (1.14-1.38) <0.001**	**1.10 (1.01-1.20) 0.038**[†]	1.04 (0.96-1.12) 0.353
VEGF (pg/mL)	1.00 (0.93-1.09) 0.936	0.99 (0.91-1.07) 0.741	0.97 (0.90-1.05) 0.449	1.04 (0.95-1.15) 0.380	1.00 (0.93-1.08) 0.954

Associations with $P < 0.05$ are bold.
*ORs scaled to one-half standard deviation for each biomarker.
[†]The Benjamini-Hochberg FDR adjusted threshold for statistical significance for the outcomes of NASH, fibrosis, steatosis, ballooning, and lobular inflammation are 0.0071, 0.0125, 0.0071, 0.036, and 0.001, respectively. *P* values below 0.05 that do not meet the FDR adjusted threshold are identified.
Abbreviations: FGF-2, fibroblast growth factor 2; IFNγ, interferon gamma; MMP-9, matrix metallopeptidase 9; sFasl, soluble Fas ligand; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

other biomarker, and tPAI1 only correlated strongly with aPAI1.

Thirty-two percent of participants had no evidence of hepatocyte ballooning (grade 0), 28% had a few ballooned hepatocytes (grade 1), and 39% displayed many ballooned hepatocytes (grade 2). In univariable logistic regression, participants with hepatocellular ballooning had increased aPAI1, tPAI1, IL-8, MCP-1, sIL-1R1, and TNFα and decreased IGFII compared to those without ballooning (Table 3; Supporting Table S1). MCP-1 and tPAI1 did not meet the FDR adjusted threshold for statistical significance. In a multivariable logistic regression analysis, the presence of hepatocyte ballooning was associated with increased IL-8 (OR = 1.27, 95% CI 1.04-1.56, $P = 0.021$) and decreased IGFII (OR = 0.86, 95% CI 0.77-0.96, $P = 0.006$) (Table 4). IL-8 was associated with increased age, AST, and HOMA-IR (Table 5).

Lobular inflammation was seen in fewer than two foci per ×20 field (grade 1) in 52% of participants, two to four foci (grade 2) in 37% of participants, and more than four foci (grade 3) in 11% of participants. Compared to participants with grade 0-1 lobular inflammation, those with grade 2-3 lobular inflammation had decreased MCP-1 (Table 3; Supporting Table S2) in univariable logistic regression. MCP-1 did not meet the FDR adjusted threshold for statistical significance. In multivariable logistic regression analysis for the presence of grade 2-3 lobular inflammation compared to grade 0-1 lobular inflammation, decreased MCP-1 (OR = 0.90, 95% CI 0.82-0.99, $P = 0.025$) was associated with increased odds of lobular inflammation (Table 4). In addition, MCP-1 was associated with increased age, BMI, and HOMA-IR. Because advancing fibrosis in NAFLD is associated with a shift from lobular inflammation to more portal-based inflammation[21] and MCP-1 is associated with fibrosis, we performed sensitivity analysis on the association between MCP-1 and lobular inflammation excluding patients with cirrhosis; and the association was no longer statistically significant (OR = 0.92, 95% CI 0.83-1.01, $P = 0.073$).

BIOMARKERS ASSOCIATED WITH FIBROSIS

Twenty-six percent of participants had no fibrosis on liver biopsy, 29% had stage 1, 20% had stage 2, 17% had stage 3, and 8% had stage 4 fibrosis. In univariable logistic regression participants with significant fibrosis (stage 2-4) had lower levels of IGFII and haptoglobin and higher levels of IL-8, resistin, sIL-1R1, sIL-2Rα, MCP-1, and TNFα compared with no or mild fibrosis (Table 3; Supporting Table S2).

TABLE 4. Multivariable* ORs for Individual Plasma Biomarkers by Histologic Dichotomies Adjusted for Clinical Factors

Outcomes	Biomarker (per one-half SD increase)	OR†	95% CI	P
NASH: not/borderline versus definite	aPAI1 (per 16.75 ng/mL increase)	1.20	1.08-1.34	0.001
Steatosis: grade 0-1 versus grade 2-3	Adiponectin (per 4.15 μg/mL increase)	0.91	0.83-0.99	0.029
	aPAI1 (per 16.75 ng/mL increase)	1.20	1.09-1.32	<0.001
	IGFII (per 0.55 ng/mL increase)	1.16	1.05-1.28	0.005
	tPAI1 (per 10.9 ng/mL increase)	1.31	1.18-1.45	<0.001
Ballooning: grade 0 versus grade 1-2	IGFII (per 0.55 ng/mL increase)	0.86	0.77-0.96	0.006
	IL-8 (per 2.35 pg/mL increase)	1.27	1.04-1.56	0.021
Lobular inflammation: grade 0-1 versus grade 2-3	MCP-1 (per 45.4 pg/mL increase)	0.90	0.82-0.99	0.025
Fibrosis: stage 0-1 versus stage 2-4	IGFII (per 0.55 ng/mL increase)	0.75	0.67-0.84	<0.001
	IL-8 (per 2.35 pg/mL increase)	1.81	1.47-2.23	<0.001
	MCP-1 (per 45.4 pg/mL increase)	1.12	1.02-1.22	0.020
	Resistin (per 3.75 ng/mL increase)	1.10	1.00-1.20	0.040
	sIL-1R1 (per 13.1 pg/mL increase)	1.16	1.04-1.29	0.009
	sIL-2Rα (per 0.2 ng/mL increase)	1.27	1.13-1.43	<0.001
	TNFα (per 3.75 pg/mL increase)	1.22	1.04-1.44	0.018

*All ORs adjusted for sex, age, BMI, AST, ALT, high-density lipoprotein cholesterol, HOMA-IR, and triglycerides.
†ORs scaled to one-half standard deviation for each biomarker

Haptoglobin did not meet the FDR adjusted threshold for statistical significance.

In a multivariable analysis of significant fibrosis compared to no or mild fibrosis, increased IL-8 (OR = 1.81, 95% CI 1.47-2.23, $P < 0.001$), MCP-1 (OR = 1.12, 95% CI 1.02-1.22, $P = 0.020$), resistin (OR = 1.10, 95% CI 1.00-1.20, $P = 0.040$), sIL-1R1 (OR = 1.16, 95% CI 1.04-1.29, $P = 0.009$), sIL-2Rα (OR = 1.27, 95% CI 1.13-1.43, $P < 0.001$), and TNFα (OR = 1.22, 95% CI 1.04-1.44, $P = 0.018$) and decreased IGFII (OR = 0.75, 95% CI 0.67-0.84, $P < 0.001$) were associated with significant fibrosis (Table 4). Resistin was associated with female sex and increased HOMA-IR and BMI and inversely associated with ALT. sIL-1R1 was associated with female sex and increased BMI, ALT, AST, and HOMA-IR. sIL-2Rα was associated with female sex and increased age, BMI, and AST and inversely associated with triglycerides. TNFα was associated with increased age (Table 5). sIL-2Rα correlated with IL-8, MCP-1, resistin, and sIL-1R1. IL-8 correlated with MCP-1 and TNFα as well. IGFII was inversely correlated with IL-8 and sIL-2Rα (Supporting Table S3).

Discussion

In this study, using a large population with well-characterized histological NAFLD, we explored the relationship between putative biomarkers and the histologic disease spectrum associated with NAFLD. We identified biomarkers that remain strongly and independently associated with specific NAFLD phenotypes after adjustment for clinical factors. We found that higher aPAI1 levels were associated with NASH (versus non-NASH or borderline NASH). Higher aPAI1, tPAI1, and IGFII and lower adiponectin were associated with significant steatosis; and increased IL-8 levels and decreased IGFII were associated with hepatocyte ballooning. In addition, among seven biomarkers associated with the presence of significant fibrosis, increased IL-8 and sIL-2Rα and decreased IGFII demonstrated the strongest associations.

The diagnosis and staging of NASH have long relied on histologic evaluation of liver biopsy, which is invasive, potentially subject to sampling error, and inconvenient as a repeat measure of disease severity over time. Many studies have explored the ability of biomarkers to predict disease activity.[9,11,22-24] These studies have been plagued by small sample sizes, have often lacked liver biopsy for definitive diagnosis, and have measured very few candidate biomarkers. To date, the most promising biomarker for NAFLD has been cytokeratin 18. Wieckowska et al. initially examined the relationship between cytokeratin 18, a protein cleaved during apoptosis, and NASH in 44 consecutive patients with suspected NAFLD. While cytokeratin 18 levels yielded an area under the receiver operating curve of 0.93, only 21 patients had NASH.[25] Moreover, attempts to validate these findings in a larger population of 318 patients yielded unacceptable performance characteristics for the diagnosis of NASH and provided little additional data beyond ALT levels,

TABLE 5. Univariable Association* Between Clinical Factors and Significant Biomarkers

	Adiponectin (μg/mL)	αPAI1 (ng/mL)	Haptoglobin (mg/mL)	IGFII (ng/mL)	IL−8 (pg/mL)	MCP−1 (pg/mL)	Resistin (ng/mL)	sIL−1R1 (pg/mL)	sIL−2Rα (ng/mL)	TNFα (pg/mL)	tPAI1 (ng/mL)
Male (compared to female)	**−3.23** **$P < 0.001$**	1.78 $P = 0.515$	**−1.03** **$P < 0.001$**	**0.19** **$P = 0.029$**	−0.23 $P = 0.550$	−5.68 $P = 0.439$	**−1.86** **$P = 0.002$**	**−9.81** **$P < 0.001$**	**−0.09** **$P = 0.002$**	0.21 $P = 0.727$	−1.44 $P = 0.414$
Age (per 5−year increase)	**0.81** **$P < 0.001$**	−0.88 $P = 0.104$	−0.02 $P = 0.530$	**−0.12** **$P < 0.001$**	**0.18** **$P = 0.016$**	**6.30** **$P < 0.001$**	0.16 $P = 0.176$	−0.03 $P = 0.935$	**0.02** **$P < 0.001$**	**0.30** **$P = 0.013$**	**0.72** **$P = 0.040$**
BMI (per 3 kg/m^2 increase)	0.11 $P = 0.492$	**1.50** **$P = 0.016$**	**0.19** **$P < 0.001$**	**−0.10** **$P < 0.001$**	−0.13 $P = 0.130$	**4.22** **$P = 0.012$**	**0.41** **$P = 0.004$**	**1.37** **$P = 0.005$**	**0.02** **$P = 0.008$**	0.10 $P = 0.479$	**0.98** **$P = 0.015$**
ALT (per 20 U/mL increase)	**−0.38** **$P = 0.002$**	0.63 $P = 0.203$	**−0.06** **$P = 0.028$**	**0.08** **$P < 0.001$**	0.08 $P = 0.279$	−1.33 $P = 0.323$	**−0.26** **$P = 0.018$**	**1.35** **$P < 0.001$**	−0.01 $P = 0.099$	0.14 $P = 0.217$	0.12 $P = 0.718$
AST (per 20 U/mL increase)	−0.05 $P = 0.764$	1.05 $P = 0.132$	**−0.09** **$P = 0.028$**	0.03 $P = 0.245$	**0.60** **$P < 0.001$**	1.75 $P = 0.355$	−0.13 $P = 0.412$	**3.11** **$P < 0.001$**	**0.03** **$P < 0.001$**	0.18 $P = 0.238$	**1.05** **$P = 0.021$**
HOMA-IR (per unit increase)	**−0.17** **$P = 0.004$**	**2.30** **$P < 0.001$**	0.01 $P = 0.505$	0.00 $P = 0.537$	**0.07** **$P = 0.022$**	**1.35** **$P = 0.031$**	**0.12** **$P = 0.022$**	**0.48** **$P = 0.008$**	0.00 $P = 0.058$	0.07 $P = 0.180$	**1.33** **$P < 0.001$**
HDL-C (per 5 mg/dL increase)	**1.06** **$P < 0.001$**	**−1.69** **$P = 0.003$**	0.01 $P = 0.767$	−0.02 $P = 0.207$	−0.04 $P = 0.625$	−2.74 $P = 0.075$	−0.01 $P = 0.955$	−0.12 $P = 0.781$	0.01 $P = 0.358$	−0.23 $P = 0.074$	−0.45 $P = 0.221$
Triglycerides (per 20 mg/dL increase)	**−0.21** **$P < 0.001$**	**1.10** **$P < 0.001$**	0.00 $P = 0.686$	**0.04** **$P < 0.001$**	−0.04 $P = 0.200$	0.26 $P = 0.628$	−0.02 $P = 0.585$	0.18 $P = 0.234$	**−0.01** **$P = 0.011$**	−0.02 $P = 0.699$	**0.38** **$P = 0.003$**

Associations with $P < 0.05$ are bold.
*Reported regression coefficients and P values calculated by linear regression of clinical factor on biomarker.
Abbreviations: HDL-C, high-density lipoprotein cholesterol.

leaving the need for a biomarker(s) to predict disease activity in NAFLD unmet.[26]

In our study, the only biomarker that remained associated with the histological diagnosis of NASH after adjustment for clinical factors was aPAI1. PAI1 is a serine protease inhibitor and a primary regulator of the fibrinolytic system but also has significant effects on cell adhesion, detachment, and migration.[27] PAI1 gene expression in the liver is up-regulated by endotoxin and inflammatory mediators, and levels may reflect TNFα signal dysregulation.[28,29] PAI1 gene expression is up-regulated in livers with NASH, and plasma levels are increased in patients with NASH.[30,31] Previous studies have not evaluated differences in levels of aPAI1 and tPAI1 and their relationship to NAFLD. The active form is secreted by cells but is unstable and spontaneously converts into the latent form within 1-2 hours.[32] The latent form can be converted to the active form, and the increased inflammatory milieu associated with NASH could drive this process. This finding warrants evaluation in further studies.

Our study found an association between increased aPAI1, tPAI1, and IGFII as well as decreased adiponectin and significant steatosis. PAI1 levels correlate more with liver fat content than visceral adipose content.[33] Insulin induces PAI1 expression, and improvement in insulin resistance with weight loss or troglitazone is associated with a reduction in PAI1 levels.[34-36] Multiple studies have shown an association between PAI1 levels and NAFLD, which our study corroborates.[30,37,38] IGFII is part of the family of ligands that mediate growth, development, and differentiation and act primarily through IGF1 receptor.[39] IGFII has significant structural homology with insulin,[40] shares biological actions through the insulin receptor,[41] and thereby could lead to decreased lipolytic activity and increased steatosis, as was seen in our study. Adiponectin is an adipokine with anti-inflammatory[42] and insulin-sensitizing[43] effects. Our study confirms previously documented inverse associations with hepatic steatosis[44] but contrasts with previous studies suggesting an inverse association with NASH severity.[9]

We found that increased IL-8 was associated with the presence of hepatocyte ballooning. IL-8 is a chemokine that serves as a chemoattractant for neutrophils and contributes to acute liver inflammation.[45] Previous studies have shown an association between NASH and increased IL-8 levels; however, ours is the first to document a significant association specifically between IL-8 levels and hepatocyte ballooning.[46,47] Increased lobular inflammation was associated with decreased MCP-1. MCP-1 is a chemoattractant that activates target cells including macrophages and has been associated with hepatic steatosis and NASH; however, prior studies had no or few participants with fibrosis.[5,8,48] Our finding of decreased MCP-1 among those with more lobular inflammation is likely confounded by the presence of cirrhosis. Patients with cirrhosis had higher MCP-1 and less lobular inflammation, and when excluded from the analysis the association between MCP-1 and lobular inflammation was no longer statistically significant. Furthermore, MCP-1 did not meet the FDR adjusted threshold for statistical significance on univariable analysis. Therefore, these results should be interpreted with caution.

Seven biomarkers were strongly associated with significant fibrosis in the multivariable model: IL-8, MCP-1, resistin, sIL-1R1, sIL-2Rα, TNFα, and IGFII. The strongest associations in terms of statistical significance and effect size were IL-8, sIL-2Rα, and IGFII. In addition to its relationship with hepatocyte ballooning, increased IL-8 was associated with significant fibrosis. In hepatitis C virus models of hepatic fibrosis, IL-8 was the strongest inducer of alpha-smooth muscle actin expression in primary hepatic stellate cells.[49] IL-8 serum levels and increased gene expression were associated with fibrosis and advanced cirrhosis in a study of other causes of chronic liver disease,[50] and our study supports these findings in patients with NAFLD. sIL-2Rα is formed by proteolytic cleavage of the IL-2 receptor from the cell surface of multiple immune cells and is proportional to its membrane-bound expression.[51] An association between increased levels and advanced fibrosis has been documented in non-NAFLD liver disease.[52-54] Our study is the first to document this association in patients with NAFLD. Finally, our study found an association between decreased IGFII and significant fibrosis, in addition to the previously highlighted association between increased IGFII and steatosis. Prior studies of IGFII have revealed that levels are lower in patients with cirrhosis.[55] In a small study of pediatric patients with NAFLD, lower IGFII levels correlated with the degree of fibrosis; and our study corroborates this finding in adults.[56] IGFII was found to protect against the effects of caspases 3 and 9 in the liver, thereby decreasing free radical damage and apoptosis in rats; and this may underlie the association of IGFII with fibrosis.[57] Further studies are warranted.

Our study has a number of strengths, including the large number of well-characterized participants and the

number of biomarkers evaluated. All of the biomarkers were chosen based on *a priori* hypotheses regarding their relationship with NAFLD, and our findings are well supported by previous literature. We did evaluate multiple biomarkers and outcomes in this study; however, we have highlighted only those with strong associations in multivariable models in our discussion. We provided FDR adjusted *P* value thresholds in univariable analysis for readers to interpret. By applying the FDR adjusted thresholds to biomarker selection for multivariable models, only the association between MCP-1 and lobular inflammation would have been eliminated from our multivariable models. Furthermore, a corresponding study among pediatric patients in the NASH-CRN corroborates many of our findings including the associations between IGFII and steatosis and between IL-8 and fibrosis. While we have demonstrated significant relationships between plasma levels of biomarkers and the pathological spectrum of NAFLD, we cannot evaluate causal relationships. Therefore, while certain biomarkers may be critical to the pathogenesis of NAFLD, their plasma levels may not correlate with disease activity or the relationship may have been diminished by a clinical factor included in the multivariable model. However, the goal of this study was to explore the most impactful biomarkers in NAFLD and evaluate the relationships between biomarkers and clinical factors. Therefore, we also evaluated the associations between significant biomarkers and clinical factors to provide context for the potential role of the biomarker in NAFLD. Our study was limited by the cross-sectional measurement of our outcome, which does not allow evaluation of the temporal relationship between specific biomarkers and disease activity or prediction of how changes in biomarkers will affect disease progression. However, the findings of this study should inform biomarker selection for future longitudinal studies and clinical trials. Finally, the patients drawn from the NASH CRN represent those with an *a priori* diagnosis of NAFLD. Inclusion of patients from the PIVENS trial decreased the prevalence of diabetic patients in our study sample compared to the NAFLD population at large and may affect generalizability. However, our multivariable models adjusted for HOMA-IR, and strong associations between biomarkers and histology remained present.

In conclusion, our study explored relationships between clinical factors, biomarkers, and histologic severity of disease in NAFLD. We found strong associations, after multivariable adjustment for clinical factors, of specific biomarkers with the histologic manifestations of NAFLD. Measurement of serum biomarkers will advance our ability to stratify disease severity in NAFLD and may identify additional pathways to target for therapeutic intervention.

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