RESEARCH ARTICLE

A Novel Diagnostic Biomarker Panel for Obesity-related Nonalcoholic Steatohepatitis (NASH)

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Abstract

Background Within the spectrum of nonalcoholic fatty liver disease (NAFLD), only patients with nonalcoholic steatohepatitis (NASH) show convincing evidence for progression. To date, liver biopsy remains the gold standard for the diagnosis of NASH; however, liver biopsy is expensive and associated with a small risk, emphasizing the urgent need for noninvasive diagnostic biomarkers. Recent findings suggest a role for apoptosis and adipocytokines in the pathogenesis of NASH. The aim of this study was to develop a noninvasive diagnostic biomarker for NASH.

Methods The study included 101 patients with liver biopsies who were tested with enzyme-linked immunosorbent assay (ELISA)-based assays. Of these, 69 were included in the biomarker development set and 32 were included in the biomarker validation set. Clinical data and serum samples were collected at the time of biopsy. Fasting serum samples were assayed for adiponectin, resistin, insulin, glucose, TNF-alpha, IL-6, IL-8, cytokeratin CK-18 (M65 antigen), and caspase-cleaved CK-18 (M30 antigen).

Results Data analysis revealed that the levels of M30 antigen (cleaved CK-18) predicted histological NASH with 70% sensitivity and 83.7% specificity and area under the curve (AUC)=0.711, $p<10^{-4}$, whereas the predictive value of the levels of intact CK-18 (M65) was higher (63.6% sensitivity and 89.4% specificity and AUC=0.814, $p<10^{-4}$). Histological NASH could be predicted by a combination of Cleaved CK-18, a product of the subtraction of Cleaved CK-18 level from intact CK-18 level, serum adiponectin, and serum resistin with a sensitivity of 95.45% sensitivity, specificity of 70.21%, and AUC of 0.908 ($p<10^{-4}$). Blinded validation of this model confirmed its reliability for separating NASH from simple steatosis.

Conclusions Four ELISA-based tests were combined to form a simple diagnostic biomarker for NASH.

Keywords Obesity · NASH · Biomarkers · NASH diagnostics

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome. Over 90% of patients with morbid obesity who are undergoing bariatric surgery have NAFLD [1]. A subtype of NAFLD, nonalcoholic steatohepatitis (NASH), can follow a progressive course. In fact, the progression is associated with age, impaired glucose metabolism, and insulin resistance [1–3]. The cost

associated with managing patients with NASH can be substantial, emphasizing the importance of early diagnosis [4]. To date, liver biopsy remains the only accurate way to diagnose NASH, but liver biopsy is expensive and associated with a small but definite risk [5]. Additionally, liver biopsy can be flawed by sampling errors [6]. Despite their widespread use in clinical practice, noninvasive radiological modalities such as ultrasound, computerized tomography, and magnetic resonance imaging can accurately detect hepatic steatosis but are unable to distinguish NASH or the stage of fibrosis [7]. Although several serum markers of fibrosis or other noninvasive measures of liver elasticity have been developed [8, 9], their role in the management of NASH remains unproven. This presents an urgent need for additional, serum-based, noninvasive diagnostic biomarkers for NASH.

Various components of the adipocytokine pathway and the pathways involved in hepatocyte apoptosis and necrosis may be involved in the pathogenesis of NASH [10–13]. Recently, two markers of apoptosis and necrosis were suggested as potential markers of NASH: cleaved and uncleaved forms of cytokeratin 18 [14, 15]. These biomarkers have previously been proposed as surrogate end-points reflecting rates of the cell death in cancer patients. Furthermore, they have been extensively tested in the clinical trials of anticancer drugs [16, 17]. Additionally, adipocytokines secreted by white adipose tissue of patients with visceral obesity have been shown to play an important role in the pathogenesis of NASH. Given the important role of adipocytokines and apoptosis in the pathogenesis of NASH, a set of biomarkers focusing on both pathways may provide a valuable diagnostic marker for NASH. This study investigates whether enzyme-linked immunosorbent assay (ELISA)-based assays, which measure components of apoptosis and necrosis markers, as well as adipocytokines, to be used as a potential set of biomarkers for NASH.

Materials and Methods

Patient Population

Serum samples were obtained from our ongoing epidemiology of nonalcoholic fatty liver disease (EPI-NAFLD) database and specimen repository. The EPI-NAFLD database-specimen repository was created by enrolling histologically proven patients with NAFLD from Inova Fairfax Hospital (October 2001 to the present). The EPI-NAFLD database contains the extensive clinical and laboratory data routinely collected for each patient after obtaining informed consent. Patients with evidence of excessive alcohol use

($\geq$10 g/day), other causes of liver disease (e.g., hepatitis B, hepatitis C, autoimmune liver disease), and those receiving treatment with PPAR-γ agonists were excluded. In addition to extensive clinical and laboratory data, each patient included in this study had a liver biopsy, which was read by a single hepatopathologist (ZG) with a standardized approach. For each patient, fasting serum specimens had been obtained at the time of biopsy and stored at −80°C.

This study included a total of 101 patients. Of these, 69 patients were part of the "biomarker development set" and 32 were part of the "biomarker validation set." The biomarker development set included three groups of patients: (1) biopsy-proven NASH (NASH, $N=22$), (2) biopsy-proven simple steatosis (SS, $N=15$), and (3) age- and body mass index (BMI)-matched controls whose liver biopsy did not show NAFLD (controls, $N=32$). Additionally, the biomarker validation cohort included patients with biopsy-proven NASH or biopsy-proven SS ($N=32$) who were randomly selected from the same database. Histology and the clinical characteristics of the validation cohort were blinded for all the investigators performing the validation assays and subsequent analysis. After the completion of biomarker validation, the characteristics of the validation set were unblinded. Of these patients ($N=32$), 21 had histological NASH and 11 had histological SS. The protocol was approved by our Institutional Review Boards.

Histopathology

Each liver biopsy specimen was fixed in formalin, routinely processed for histology, sectioned, and stained with hematoxylin–eosin and Masson trichrome. All biopsies were evaluated by a single hepatopathologist (ZG). The degree of steatosis was assessed in hematoxylin–eosin-stained sections and graded as an estimate of the percentage of tissue occupied by fat vacuoles as follows: 0=none, 1=<5%, 2=6–33%, 3=34–66%, 4=>66%. Other histological features evaluated in hematoxylin–eosin sections included portal inflammation, lymphoplasmacytic lobular inflammation, polymorphonuclear lobular inflammation, Kupffer cell hypertrophy, apoptotic bodies, focal parenchymal necrosis, glycogen nuclei, hepatocellular ballooning, and Mallory bodies.

These histological features were graded as follows: 0=none, 1=mild or few, 2=moderate, and 3=marked or many. Fibrosis was assessed with the Masson trichrome stain. Portal fibrosis and interlobular pericellular fibrosis were graded as follows: 0=none, 1=mild, 2=moderate, and 3=marked. When present, bridging fibrosis was noted as few or many bridges, and cirrhosis was identified by parenchymal nodules surrounded by fibrous tissue. Cirrhosis was further categorized as incomplete or established, depending

on the degree of loss of acinar architecture. Each liver biopsy was assigned to one of four diagnostic categories: (1) no fatty liver disease present (controls), (2) SS, (3) steatosis with nonspecific inflammation (excluded), or (4) NASH. Patients were defined as having SS if they had any degree of hepatocellular fat accumulation as their sole pathology. Patients with steatosis and nonspecific inflammation had, in addition to fat, spotty hepatocellular dropout with focal inflammation or Kupffer cell hypertrophy. These patients were excluded from this analysis. NASH was identified when, in addition to steatosis, the pathologist identified one of the following features: (1) prominent hepatocellular ballooning with associated lobular inflammation, (2) Mallory bodies, or (3) perisinusoidal fibrosis.

The Homeostasis Model Assessment

Glucose levels were measured by glucose oxidase-based kits (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. Insulin levels in serum samples were quantified by sandwich ELISA (LINCO Research, St Charles, MO, USA). Homeostasis model assessment (HOMA) scores were obtained with HOMA calculator software, version 2.2 (http://www.dtu.ox.ac.uk/). The analyses were subdivided into three groups of patients: high HOMA (>3.0), low HOMA (<1.8), and midrange HOMA (1.8–3.0).

Measurements of Adipokines and Cytokines

Serum levels of adipokines and cytokines were measured with enzyme immunoassays according to the manufacturers' instructions. Each measurement was performed in duplicate. TNF-α, IL-6, and IL-8 were quantified by using Compact ELISA kits from the RDI Division of Fitzgerald Industries (Concord, MA, USA). Resistin levels were assessed with kits provided by BioVendor Laboratory Medicine, (Candler, NC, USA). Adiponectin and visfatin serum levels were measured with competitive ELISA assays from Phoenix Pharmaceuticals (Belmont, CA, USA) according to the manufacturers' manual. The absorbance values were measured with the ELISA Reader at 450 nm. The calibration curves were constructed by plotting the net average absorbances of the standards on Y axis and the concentrations on X axis using logit-log function to linearize and draw the best-fitting curve. Concentrations of the adipocytokines in each sample were calculated from the calibration curve with Sigma Plot software v.7. The correlation coefficients were linear in a concentration range between 1 and 700 pg/mL for TNF-α ($R=0.973$), 2 and 300 pg/mL for IL-8 ($R=0.989$), 1.5 and 400 pg/ml for IL-6 ($R=0.968$), 1.82 and 49 ng/ml for visfatin ($R=0.977$), 1.5 and 50 ng/ml for resistin ($R=0.969$), 0.3 and 100 μg/ml for adiponectin ($R=0.975$), and 2 and 200 μU/ml for insulin ($R=0.982$). The samples with higher concentrations of analytes were quantified after dilutions.

Measurements of Apoptosis and Necrosis

Cytokeratin CK-18 (M65 antigen, a measurement of overall cell death due to both apoptosis and necrosis) and caspase-cleaved CK-18 (M30 antigen, a specific measurement of apoptosis) [18, 19] were profiled by M65 and M30 (Apoptosense) ELISA Kits (AXXORA, San Diego, CA, USA), respectively. Each measurement was performed in duplicate. Concentrations of the antigens in each sample were calculated from the calibration curve as described above.

Statistical Analyses

Variables are presented as means +/− standard deviation or percentages. Between groups of patients, pair-wise comparisons of the serum concentrations of insulin, glucose, visfatin, TNF-α, resistin, adiponectin, IL-8, IL-6, and M30 and M65 antigens were performed by nonparametric Mann–Whitney tests. One-way ANOVA test or the Kruskal–Wallis test was used to compare three or more groups. Linear regression analysis was performed with S-Plus v. 7.0. The sensitivity, specificity, positive predictive values, negative predictive values, and confidence intervals (CIs) were assessed by using the receiver operating characteristic curve analysis provided by MedCalc demo (http://www.medcalc.be/).

Associations between the concentration levels for pairs of adipokines and cytokines of interest were tested with the use of Pearson correlation coefficients after appropriate log-normalizations of concentration values. Additionally, multivariate linear regressions with stepwise variable selection were used to test for significant relations in continuous data with adjustment for possible confounders [20]. Unless otherwise noted, we used two-tailed hypothesis tests and p values <0.05 were considered significant.

Results

Clinical, demographic, and adipocytokine data for the biomarker development set are summarized in Table 1. The three groups were well matched in terms of age, gender, ethnic background, and measures of obesity (BMI and hip/waist ratio). Additionally, fasting serum insulin (8.9±6.4, 10.7±6.7, and 10.3±15.0, respectively) and HOMA score (2.1±2.2, 3.4±2.3, and 3.2±7.0, respectively)

Table 1 Clinicodemographic and laboratory data of training and validation patient cohorts (mean±standard deviation or %)

Clinical parameter	SS (training) (N=15)	NASH (training) (N=22)	Matched controls (training) (N=32)	Validation set (N=32)
Age, years	37.4±8.3	42.5±10.4	39.3±9.8	41.6±10.6
Female, %	93.3% (14)	59.1% (13)	90.6% (19)	70.9%
Caucasian, %	73.3% (11)	72.7% (16)	78.% (25)	75%
Hip-to waist ratio	1.06±0.11	1.01±0.1	1.07±0.13	1.07±0.13
Body mass index	45.7±4.8	48.2±8.7	47.0±9.1	52.30±13.08
AST level, IU/L (*)	19.9±6.8	35.9±27.1	18.0±3.7	32.9±30.3
ALT level, IU/L (*)	22.1±12.2	47.9±32.1	21.9±8.1	42.4±34.3
Serum triglyceride, mg/dL	154.6±88.8	177.8±78.3	134.9±73.4	177.1±87.6
Serum cholesterol, mg/dL	192.2±42.7	193.4±32.7	178.6±27.9	205.7±29.6
Serum glucose, mg/dL (*)	109.6±24.1	128.45±46.5	103.6±24.4	102.8±16.9
TNF-α, pg/ml	2.8±0.9	9.1±23.6	1.9±0.2	NA
IL-8, pg/ml	26.9±56.5	24.0±29.4	7.3±3.1	NA
IL-6, pg/ml (*)	51.1±122.5	8.0±2.9	7.2±5.3	NA
Resistin, ng/ml	7.9±3.4	5.9±3.0	7.9±4.1	5.43±3.27
Adiponectin, μg/ml (*)	12.1±8.4	6.1±5.2	9.3±6.3	9.48±4.48
Visfatin, pg/ml (*)	52.5±67.0	16.7±6.3	25.8±18.0	NA

*Validation data set was comprised of 29.1% of SS and 70.9% of NASH. *p* Values: fasting serum glucose ($p<0.03$), AST ($p<0.0011$), ALT ($p<0.0001$), IL-6 ($p<0.04$), adiponectin ($p<0.03$), visfatin ($p<0.008$).
AST = aspartate aminotransferase, ALT = alanine aminotransferase.

were similar among the three groups. Liver biopsies were available for all patients including those from the control group.

As expected, patients with NASH had higher aminotransferase levels (aspartate aminotransferase and alanine aminotransferase), higher fasting glucose, and higher IL-6 levels, but lower adiponectin and visfatin levels. Serum TNF-α, IL-8, and resistin levels were not significantly different between patients with NASH and SS.

Cytokeratin CK-18 and Caspase-cleaved CK-18 Levels in NAFLD

We assessed three interdependent surrogate end-points to determine the relative levels of apoptosis and necrosis in NAFLD: (1) the total level of CK-18 neoepitope (M30 antigen released in the process of the caspase cleavage and reflecting apoptosis), (2) the total released amount of CK-18

(M65 antigen released from all dying cells and reflecting total cell death including both apoptosis and necrosis), and (3) necrosis-reflecting parameter calculated as M65–M30.

Patients with NASH had significantly (p value <0.02) higher levels of M30 antigen than those with SS and controls (NASH 307.0+/−278.2 IU/L, SS 127.3+/−62.2 IU/L, controls 137.4+/−36.8 IU/L) (Fig. 1). Differences in the concentrations of M30 antigen between patients with SS and controls did not reach statistical significance.

The levels of apoptosis in NAFLD patients were positively correlated with HOMA scores ($R=0.5106$, $p=0.0013$). The differences in the levels of apoptosis were highly significant ($p<0.001$) when NAFLD patients were subdivided according to their HOMA scores (high HOMA scores, $N=15$ vs middle and low HOMA scores, $N=22$).

Quantification of the M65 antigen (intact CK-18) that served as a measure of total cell death followed the same

Fig. 1 CK-18 neoepitope (M30 antigen) levels are significantly increased in the serum of NASH patients in comparison to patients with SS and controls. On this scatter plot, each *dot* represents one subject, and the *dashed lines* represent the mean value for each group
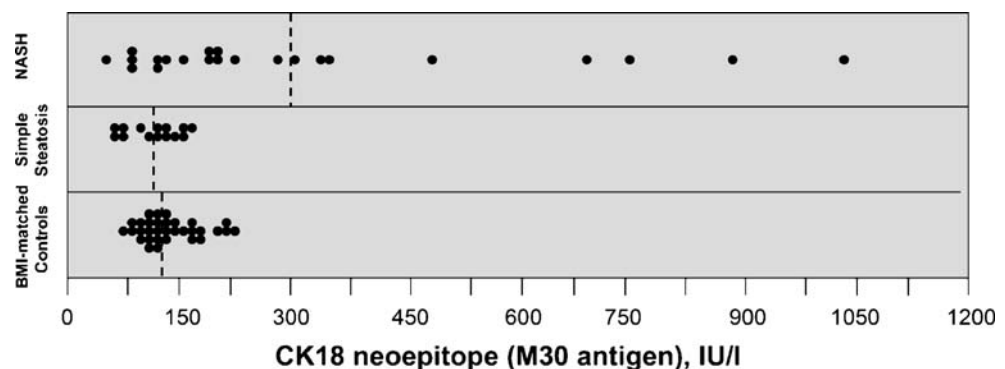
Table 2 Best fitting multiple linear regression model distinguishes NASH patients within the cohort of NAFLD and non-NAFLD controls; regression coefficient β represents slope estimate ± standard error of the estimate

Model	Independent variable	Regression coefficient β and SE	p Values of independent variables	p Value of the whole model
Prediction of histologic NASH	(Intercept)	0.4909+/−0.1351	<0.0006	$p < 1.232e^{-6}$
	M30, IU/L	0.0011+/−0.0003	<0.0001	
	M65–M30, IU/L	0.0003+/−0.0001	<0.0548	
	Adiponectin, μg/ml	−0.0153+/−0.0069	<0.0316	
	Resistin, ng/ml	−0.0418+/−0.0125	<0.0014	

SE = standard error of the estimate

trend as M30 measures with significantly higher levels in NASH vs SS ($p < 0.003$). In NAFLD patients, the correlation of overall cell death and HOMA scores remained significant ($R=0.51$, $p<=0.002$). When NAFLD patients were subdivided according to their HOMA scores as described above, the differences in the levels of total cell death remained significant ($p < 0.0002$).

The parameter most closely reflecting necrotic cell death was calculated as M65–M30. Patients with SS differed from those with NASH ($p < 0.05$), but not from the controls. The correlation between the necrosis and HOMA scores was not significant. Subdividing the NASH patients according to their necrosis levels discriminated between patients with high HOMA and middle and low HOMA scores ($p < 0.001$).

Relationship Between Levels of CK-18 and Adipocytokines in NAFLD Patients

In the entire cohort (NAFLD and controls), markers of apoptosis (as measured by levels of intact CK-18) correlated with TNF-α levels and IL-8 levels ($R=0.4986$, $p<=1.395e-05$ and $R=0.3052$, $p<=0.0108$, respectively).

On the other hand, in the NAFLD cohort (NASH and SS), cleaved CK-18 levels only correlated with TNF-α ($R=0.4816$, $p<=0.002626$). Similarly, in the entire cohort, measurements of a total cell death positively correlated with both TNF-α and IL-8 ($R=0.3277$, $p<=0.006007$, and $R=0.3095$, $p<=0.009697$, respectively). However, in NAFLD patients, this correlation, although relatively weak, remained significant only for TNF-α ($R=0.3049$, $p<=0.06669$). There were no significant correlations in the levels of CK-18 derived epitopes with any other adipocytokine profiled.

Models Predicting Histologic NASH

Additional analyses revealed that the levels of M30 antigen (cleaved CK-18) predicted histological NASH with 70% sensitivity and 83.7% specificity and area under the curve (AUC)=0.711, $p < 10^{-4}$, whereas the predictive value of the levels of intact CK-18 (M65) was somewhat higher (63.6% sensitivity and 89.4% specificity and AUC=0.814, $p < 10^{-4}$).

On the other hand, the multivariate analysis revealed that histological NASH could be predicted by a combination of Cleaved CK-18 or m30 antigen (apoptosis), a product of

Table 3 Performance of models predicting NASH

Model	Cut-off	Sensitivity (95% CI), %	Specificity (95% CI), %	PPV, %	NPV, %	Negative likelihood ratio	AUC (95% CI)	Model p value
Full model	0.2772	95.45 (77.1–99.2)	70.21 (55.1–82.6)	60.0	97.1	0.065	0.908	$<10^{-5}$
of NASH	0.3499	77.27 (54.6–92.1)	87.23 (74.2–95.1)	73.9	89.1	0.26	(0.814–0.964)	
diagnostics (multivariate)	0.2075	100.00 (84.4–100.0)	46.81 (32.1–61.9)	46.8	100.0	0.00		
Cleaved	174.1	63.64 (40.7–82.8)	87.23 (74.2–95.1)	70.0	83.7	0.42	0.711	$<10^{-4}$
CK-18 or	111.6	81.82 (59.7–94.7)	29.79 (17.4–44.9)	35.3	77.8	0.61	(0.589–0.814)	
M30, U/L	261.35	36.36 (17.2–59.3)	97.87 (88.7–99.6)	88.9	76.7	0.65		
Intact CK-18	384.3	63.64 (40.7–82.8)	89.36 (76.9–96.4)	73.7	84.0	0.41	0.814	$<10^{-4}$
or M65, U/L	242.9	86.36 (65.1–96.9)	65.96 (50.7–79.1)	54.3	91.2	0.21	(0.702–0.897)	
	545	36.36 (17.2–59.3)	95.74 (85.4–99.4)	80.0	76.3	0.66		

Cut-off values correspond to the highest accuracy value (minimal false-positive and false-negative results).
PPV = positive predictive value, NPV = negative predictive value.

Table 4 Results of the blinded validation of NASH Diagnostics™ model predicting NASH

Threshold	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
0.2085	19	8	3	2	90.5	27.3	70.4	60.0
0.2793	19	8	3	2	90.5	27.3	70.4	60.0
0.2805	19	8	3	2	90.5	27.3	70.4	60.0
0.3199	17	6	5	4	81.0	45.5	73.9	55.6
0.3377	16	5	6	5	76.2	54.5	76.2	54.5
0.3692	15	4	7	5	75.0	63.6	78.9	58.3
0.3825	15	3	8	6	71.4	72.7	83.3	57.1
0.5500	11	2	9	10	52.4	81.8	84.6	47.4

AUC of this model is 0.732 with 95% CI of (0.546–0.872).

TP = true positives, FP = false positives, TN = true negatives, FN = false negatives, PPV = positive predictive value, NPV = negative predictive value

the subtraction of Cleaved CK-18 level from Intact CK-18 level (necrosis), serum adiponectin and serum resistin. This combination set, called NASH Diagnostics™, had a sensitivity of 95.45%, specificity of 70.21%, and AUC of 0.908 ($p < 10^{-4}$) (Tables 2 and 3).

Despite an excellent AUC for TNF-α, the overall significance of the model based on the serum TNF-α was much lower than that based on CK-18 measurements. Performance-related characteristics of the models described above are summarized in the Table 3.

Validation of the NASH Diagnostics™ in Predicting Histological NASH

To validate the model described here, a blinded cohort of NAFLD patients ($N = 32$) was subjected to the same measurement. The histological findings of NAFLD, as well as the clinical and biochemical variables, were blinded until completion of the analysis. After the analysis was complete, clinical and demographic data for the biomarker validation set were unblinded. As depicted in Table 1, no differences were observed between the two sets of patients.

The performance of the model in the validation was characterized by an AUC of 0.732 (95% CI, 0.55–0.87). A threshold of 0.3825 for the model was associated with a sensitivity of 71.4%, a specificity of 72.7%, a positive predictive value of 83.3%, and a negative predictive value of 57.1%. A full list of thresholds for the validation set is depicted in Table 4. As noted in Table 4, the model had the best PPV when a threshold of 0.5500 was chosen. The best NPV for the model was obtained at a threshold of 0.2085.

To further assess the performance of this model, the training data set was extended to all 101 patients by inclusion of the subjects whose clinical and pathologic data were uncovered after the completion of the validation phase. This extension resulted in a model with an increase of the optimal threshold to 0.4320, an AUC of 0.854 (CI 95% 0.770 to 0.917, $p < 2.1e^{-7}$), a sensitivity of 72.1%, and a specificity of 91.4%.

Discussion

The role of cell death in the development of NASH has been previously suggested [21–23]. Hepatic gene expression data [21], assessment of apoptosis-related molecules in the liver biopsies [22], and data from animal models [24] have all provided evidence to support the role of cell death in the pathogenesis of NASH. Recently, two cytokeratin 18-derived antigens, M30 and M65, released in the NAFLD patients' serum in the process of the cellular death, have been proposed as possible markers of NASH [14, 15]. These studies have provided further support for the role of apoptosis in the pathogenesis of NASH. Given the complexity of the pathogenesis of NASH, it is likely that other pathways (cytokines and adipokines) also play critical roles in the development of NASH [25]. Therefore, a set of biomarkers that include not only the markers of apoptosis, but also those from adipocytokine pathway, may provide a better means of discriminating NASH from other, less progressive subtypes of NAFLD, e.g., SS. Given that NASH is the only subtype of NAFLD that can potentially progress, an initial diagnosis has important prognostic implications and provides a potential target for future therapeutic options. Also, panel of biomarkers that can reliably establish the diagnosis of NASH can reduce the need for liver biopsy, thus reducing both biopsy-related costs and its associated risks.

This study compares the predictive values of several previously established markers of apoptosis, necrosis, and adipocytokines in patients with NASH, SS, and matched controls [12, 26–28]. To this end, we profiled adiponectin, resistin, insulin, glucose, TNF-α, IL-6, and IL-8, as well as M30 and M65 antigens representing cleaved and uncleaved

forms of cytokeratin 18, and reflecting apoptosis and the overall level of the cellular death, respectively. Among the three measures reflecting the levels of cell death, M30 (apoptosis), M65 (overall cell death), and M65–M30 (necrosis), it was the cleaved CK-18 fragment M30 was able to predict histological NASH with a sensitivity of 63.6% and specificity of 87.2% (AUC=0.710). This finding confirms previously published observations [14, 15]. Additionally, our analysis revealed that the levels of this apoptosis-related marker are closely correlated with levels of proinflammatory cytokines (TNF-α and IL-8), but not with levels of any of the adipokines.

A predictive model based on concentrations of the M30 antigen outperformed the model based only on the level of TNF-α. Interestingly, multivariate logistic regression analyses of the complete set of noninvasive tests demonstrated that the additional measure of adipokine levels significantly improved predictive power of the model for NASH, improving both its sensitivity and AUC, while adding overall confidence in the model performance ($p<1.232e^{-6}$). It is important to note that having cleaved CK-18 fragment or M30 as a major component of the best performing model allowed us to eliminate the need for quantifying TNF-α and IL-8 in the patient's serum, thus reducing the number of measurements. Apoptosis levels were also closely correlated with HOMA scores, confirming the importance of insulin resistance in the pathogenesis and progression of NAFLD [29].

Data reflecting the total amount of cell death (M65) followed the same trend as the results of the apoptosis quantification (M30). This is not surprising, as the M30 antigen represents a fragment that is located within the larger molecule of cytokeratin 18 that can be released both in apoptotic and necrotic processes. According to our data, the levels of necrosis markers (M65–M30) alone were not predictions of histological NASH, but served as a valuable component in the full multiparametric model. One possible limitation of the use of CK-18-based antigens for the prediction of NASH is its intrinsic inability to discriminate between NASH and other chronic diseases that involve apoptosis, for example, cholangitis and cholestasis [30], chronic viral infection C [31], and various malignancies [16, 17]. In addition, these M30 levels may be influenced by acute conditions such as trauma [32].

This study shows that the best performing model (NASH Diagnostics™) for predicting the diagnosis of NASH included a combination of Cleaved and Intact CK-18, serum adiponectin, and serum resistin measurements. An attempt to validate the performance of this model in a blinded set of patients with NAFLD confirmed its diagnostic value in a circumstance in which the model was tested under very rigorous conditions. If this model were used to minimize the use of liver biopsies at the threshold of 0.2085, 19 out of 24 patients with NAFLD (79.2%) could avoid a liver biopsy. Depending on the particular NAFLD cohort, the use of this noninvasive NASH biomarker could lead to the significant reduction in the liver biopsies needed to establish the diagnosis of NASH. Given that this threshold (0.2085) has a lower positive predictive value, patients diagnosed with NASH using this biomarker may not necessarily have NASH, potentially leading to an "overdiagnosis." Nevertheless, establishing that patients do not have NASH has important prognostic implications. In fact, one could argue that these patients would not be harmed by the false positive diagnosis and should be encouraged to undergo management of their risk factors, such as exercise and weight loss, as well as treatment of hyperlipidemia and insulin resistance. These risk factors are not only associated with NAFLD, but also with coronary artery disease.

One way to improve the performance of this model is to adjust its threshold after testing additional subjects. When the training data set was extended to include all patients profiled ($N=101$), the optimal threshold was reset to 0.4320 and AUC of the model improved to 0.854 with sensitivity of 72.1% and specificity of 91.4%. Studies extending the training data set and subsequent independent validation experiments are under way.

In summary, this study attempted to predict the presence of NASH in a cohort of patients by using a panel consisting of two adipokines and two biomarkers of cellular death. This panel of biomarkers can predict NASH, potentially decreasing the number of required biopsies. NASH Diagnostics™ needs to be externally validated in various cohorts of patients with NAFLD. After careful validation, this panel of biomarkers may become very useful in the clinical management of these patients.

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