Serum Proteomics and Biomarker Discovery Across the Spectrum of Nonalcoholic Fatty Liver Disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD), ranging from relatively benign simple steatosis to progressive nonalcoholic steatohepatitis (NASH) and fibrosis, is an increasingly common chronic liver disease. Liver biopsy is currently the only reliable tool for staging the subtypes of NAFLD; therefore, noninvasive serum biomarkers for evaluation of liver disease and fibrosis are urgently needed. We performed this study to describe changes in the serum proteome and identify biomarker candidates in serum samples from 69 patients with varying stages of NAFLD (simple steatosis, NASH, and NASH with advanced bridging [F3/F4] fibrosis) and 16 obese controls. Using a label-free mass spectrometry-based approach we identified over 1,700 serum proteins with a peptide identification (ID) confidence level of >75%, 605 of which changed significantly between any two patient groups (false discovery rate <5%). Importantly, expression levels of 55 and 15 proteins changed significantly between the simple steatosis and NASH F3/F4 group and the NASH and NASH F3/F4 group, respectively. Classification of proteins with significant changes showed involvement in immune system regulation and inflammation, coagulation, cellular and extracellular matrix structure and function, and roles as carrier proteins in the blood. Further, many of these proteins are synthesized exclusively by the liver and could potentially serve as diagnostic biomarkers for identifying and staging NAFLD.

Conclusion—This proteomic analysis reveals important information regarding the pathogenesis/progression of NAFLD and NASH and demonstrates key changes in serum protein expression levels between control subjects and patients with different stages of fatty liver. Future validation of these potential biomarkers is needed such that these proteins may be used in place of liver biopsy to facilitate diagnosis and treatment of patients with NAFLD.

The incidence of nonalcoholic fatty liver disease (NAFLD) continues to increase, and prevalence estimates for NAFLD range from 17%–33% in the general population of Western countries.1 Fatty liver encompasses an entire pathological spectrum of disease, from relatively benign accumulation of lipid (simple steatosis) to progressive nonalcoholic...
steatohepatitis (NASH) associated with fibrosis, necrosis, and inflammation.\textsuperscript{2–4} It has been estimated that \textasciitilde 20\% of NAFLD patients exhibit histological characteristics of NASH,\textsuperscript{5} but it remains unclear why some patients develop steatohepatitis and cirrhosis. An important limitation in the clinical management of NAFLD and NASH is the requirement for liver biopsy in order to definitively diagnose and stage the disease.\textsuperscript{6} Noninvasive methods for diagnosis of NAFLD and NASH have been developed, albeit with important limitations and the need for large validation studies. For example, several imaging techniques can be used to detect steatosis but are unable to stage liver fibrosis.\textsuperscript{7–9} Several individual proteins (hyaluronic acid and endothelin-1) and diagnostic biomarker panels (the NAFLD fibrosis score and the European Liver Fibrosis Panel) for identifying and staging NAFLD and NASH have been identified but not validated in prospective clinical studies with large sample sizes.\textsuperscript{10–13}

To address the urgent need for both increased understanding of NASH and identification of novel diagnostic biomarkers to facilitate diagnosis and treatment of liver disease, we applied a label-free quantitative proteomics approach (LFQP) to profile the global protein expression of serum samples from patients with varying stages of NAFLD and obese controls. LFQP is a rapid, sensitive approach for quantification of many proteins in complex biological samples, including tissue, blood, or urine.\textsuperscript{14} The objectives of this study were to (1) identify differentially expressed serum proteins among different patient groups (control, simple steatosis, NASH, and NASH with advanced [F3/F4] fibrosis), and (2) use this information to discover biomarker candidates to diagnose and stage NAFLD.

**Patients and Methods**

Blood samples used for proteomics studies were collected from NAFLD patients on the morning of their scheduled liver biopsy and control subjects in the fasting state. Blood was collected, centrifuged, aliquoted, and stored in plastic vials (NUNC, Rochester, NY) at \textasciitilde 80\degree C until use. Sixty-nine subjects with suspected NAFLD who underwent a liver biopsy were enrolled in this study. The diagnosis of NAFLD was based on standard clinical, imaging, and histological criteria. Patients in the NAFLD group lacked significant alcohol consumption, viral hepatitis, autoimmune liver disease, and hemochromatosis. Histological diagnosis of NASH and extent of fibrosis were assessed by an experienced hepatopathologist. Based on liver histology, patients were classified into three groups: simple steatosis, NASH without advanced fibrosis (NASH, as defined by steatosis with lobular and/or portal inflammation and fibrosis stages 0–2), and NASH with advanced (F3/ F4) fibrosis (defined the same as the NASH group but with fibrosis stages 3–4). Serum samples from the 16 obese control subjects originated from a previous set of studies where underlying liver disease was excluded by liver ultrasound and biochemical parameters.\textsuperscript{15–17} As reported, these obese controls had normal liver biochemistries, no physical examination evidence of liver disease, and lacked significant insulin resistance and metabolic syndrome.\textsuperscript{16} Blood samples from both NAFLD patients and obese controls were obtained as part of two clinical research studies (one ongoing and one completed) that were reviewed and approved by the Institutional Review Board at Indiana University School of Medicine. All of the volunteers gave written informed consent prior to their participation in these studies.

**Sample Preparation**

As described,\textsuperscript{14} proteins were extracted from <100 \(\mu\)L serum in lysis buffer containing 8 M urea and 10 mM dithiothreitol (DTT). Highly abundant proteins were removed by SepproTip columns and protein concentrations were determined by Bradford assay.\textsuperscript{18} The resulting protein extracts were reduced and alkylated with DTT, iodoacetamide, triethylphosphine, and iodoethanol.\textsuperscript{19} Protein mixtures were digested with trypsin and filtered through 0.45-\(\mu\)m spin filters before being applied to the high-performance liquid
chromatography (HPLC) system. To assess the stability of the HPLC system and mass spectrometry (MS) instrument, chicken lysozyme was spiked into each sample at a constant amount as an internal reference for assessment of technical variations before tryptic digestion.

Liquid Chromatography-Tandem Mass Spectrometry (LC/MS-MS)

Tryptic peptides (<20 μg) were injected onto an Agilent 1100 nano-HPLC system (Agilent Technologies, Santa Clara, CA) with a C18 capillary column in random order. Peptides were eluted with a linear gradient from 5%–45% acetonitrile developed over 120 minutes at a flow rate of 500 nL/min and the effluent was electro-sprayed into the LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Data were collected in the “Triple Play” (MS scan, Zoom scan, and MS/MS scan) mode. The acquired data were filtered and analyzed by a proprietary algorithm.20 Database searches against the International Protein Index (IPI) human database (European Bioinformatics Institute, 2005) and the nonredundant Homo sapiens database (National Center for Biotechnology Information, 2005) were carried out using the X!Tandem21 and SEQUEST22 algorithms.

Protein Identification and Quantification

Proteins were classified from priority 1 to 4 according to identification (ID) quality. Confidence in protein ID is greater with an increasing number of distinct amino acid sequences identified and increasing peptide ID confidence. Priority 1 proteins have the greatest likelihood of correct ID (multiple unique sequences identified) and priority 4 proteins have the least likelihood of correct ID. The “peptide ID confidence” (the ID quality of the amino acid sequence(s)) of the “best peptide” (the peptide with the highest peptide ID confidence) was used to assign the protein to a “high” (between 90%–100% confidence), “moderate” (between 75%–89% confidence), or “low” (<75% confidence) identification category. All “low” category proteins were discarded. The X!Tandem21 and SEQUEST22 algorithms were used for amino acid sequence ID as described.23

Quantification of proteins was carried out as described.20 Briefly, when raw files were acquired from the LTQ mass spectrometer, all extracted ion chromatograms (XICs) were aligned by retention time. After alignment, area under the curve (AUC) for each individually aligned peak from each sample was measured, normalized, and compared for relative abundance.

Biostatistical Analysis

The current study was an exploratory “discovery” proteomics study; therefore, our study sample was not based on a formal sample size calculation. However, our sample size is generally consistent with other discovery proteomics analyses. ANOVA (analysis of variance) was used to detect significant changes in protein expression among patient groups. To eliminate technical bias, randomization of order of measurement and “quantile normalization” was used.24 Normalization was done on a log2 scale (one unit difference on this log scale is equivalent to a 2-fold change).

From the ANOVA model a P-value was obtained. The P-value is an estimate of the FPR (false positive rate). The P-value was transformed to a q-value, a number that estimates the FDR (false discovery rate). The P-value threshold was fixed to control the FDR at 5% (<0.05). A protein with a “significant change” or “differential expression” was defined as a difference in protein expression between any two patient groups with a q-value < 0.05.

For each protein a separate ANOVA model was fit using PROC MIXED in SAS software (SAS Institute, Cary, NC):

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where \( \log_2(\text{Intensity}) \) is the protein intensity based on the weighted average of the quantile normalized \( \log_2 \) peptide intensities, Group Effect is the fixed effects (not random) caused by the experimental conditions or treatments that are being compared, and Sample Effect (nested within group) is the random effects from individual biological samples and sample preparation.

Positive fold changes (FC), when mean treated group \( \geq \) mean control group, were computed from the means on the AUC scale (antilog): \( \text{FC} = \frac{\text{mean treated group}}{\text{mean control group}} \).

Negative FCs, when mean control group > mean treated group, were computed from the means on the AUC scale (antilog): \( \text{FC} = \frac{-\text{mean treated group}}{\text{mean control group}} \).

Absolute (positive) values of the FCs were computed. The median percent coefficient of variation (%CV) for each priority level was determined by dividing the standard deviation (SD) by the mean on the AUC scale and is given on a percent scale.

**Development of Biomarker Candidates**

Only priority 1 proteins with a significant change \( (q < 0.05) \) between any two patient groups were considered for further analyses (72 proteins). However, the maximum observed change in the mean \( \log_2 \) intensity for the internal standard (chicken lysozyme) between groups was 14% (1.14-fold change); therefore, only priority 1 proteins with a significant change >14% \( (q < 0.05) \) were considered for characterization of biological function (56 proteins).

In order to further evaluate priority 1 proteins as biomarker candidates, more stringent criteria were applied to discriminate between groups. For these analyses, only priority 1 proteins with a significant change >30% between any two groups \( (q < 0.05) \) were considered (27 proteins). Hierarchical clustering (Ward’s method using mean \( \log_2 \) intensities) was used to describe changes in protein expression patterns among patient groups and linear discriminant analysis (LDA) combined with the area under the receiver operating curve (AUROC) was used to determine the diagnostic accuracy of potential biomarker panels. ID confidence of protein biomarker candidates was verified by quantitating the percent sequence coverage (percent of the complete protein amino acid sequence where matching peptides for protein ID were found). Statistical analyses were performed using JMP software (SAS Institute Inc., Cary, NC).

**Results**

**Patient Demographics**

A total of 85 subjects were included in this study, of which 69 were in the varying spectrum of NAFLD. The NAFLD cohort had several common features of metabolic syndrome including diabetes mellitus, dyslipidemia, hypertension, and obesity. As shown in Table 1, patient clinical characteristics were consistent with what would be expected in the presence or absence of NAFLD. There were five patients in the NAFLD group with methotrexate use, three in the simple steatosis and two in the NASH group.

**Proteomic Profiling**

Findings from the global serum protein analysis are summarized in Table 2. Of the 1,738 proteins that were identified, 183 had multiple unique amino acid sequences identified and high peptide ID confidence (priority 1), and there was a significant change observed \( (q < \)
0.05) in the protein expression level between any two patient groups for 72 of these proteins (Table 2). The significant changes observed between groups are further described in a pairwise fashion in Table 3. Of the priority 1 proteins identified, there were 21 significant changes in protein levels observed between simple steatosis and NASH F3/F4 groups and 9 significant changes between NASH and NASH F3/F4 groups. It is important to note that no serum proteins had significant differential expression when the simple steatosis and NASH groups were compared.

Characterization of Priority 1 Proteins

A comprehensive list of all 56 priority 1 proteins with a significant change >14% (1.14-fold) among any two patient groups (\(q < 0.05\)), sorted by fold change, is shown in Supporting Table 1. Protein identification numbers, mean protein intensity (log\(_2\)) for each patient group, and a description of protein function is listed. Biological functions of these proteins are summarized in Table 4. The function of two proteins, 13 kDa protein and 11 kDa protein, is unknown.

Identification of Diagnostic Biomarkers

Of the 72 proteins with significant differential expression (\(q < 0.05\)) identified as priority 1, 27 had expression levels that differed by at least 30% (1.30-fold change). Of these 27 proteins, six patterns of protein expression changes among the four patient groups were noted: (1) decreased in all stages of NAFLD; (2) increased in all stages of NAFLD; (3) decreased in NASH F3/F4 only; (4) increased in NASH F3/F4 only; (5) decreased with progression of NAFLD; and (6) increased with progression of NAFLD. A protein from each of these patterns was chosen as an example and the mean protein intensity (log\(_2\)) and standard error for these six representative proteins are shown in Fig. 1A–F. In a separate analysis, hierarchical clustering was used to group all priority 1 proteins with a significant change of at least 30% (\(q < 0.05\)) by similarities in their expression patterns (mean log\(_2\) intensities) among patient groups. A heatmap showing the differential expression of these proteins is shown in Supporting Fig. 1.

To assess the diagnostic utility of these proteins, we established three different classification groupings to distinguish: (1) all four patient groups (control, simple steatosis, NASH, and NASH F3/F4); (2) patients with NAFLD (simple steatosis and NASH) from those with advanced disease (NASH F3/F4); and (3) control subjects from patients with all forms of NAFLD (simple steatosis, NASH, and NASH F3/F4). LDA was used to explore diagnostic utility for all 27 proteins, both in an individualized manner (Supporting Table 2) and in a grouped fashion to identify biomarker panels. Overall, we identified 10 biomarker candidate proteins with a high percent ID confidence (Table 5) that are able to differentiate between groups, as depicted in Fig. 2A–C.

A panel of six proteins (fibrinogen \(\beta\) chain, retinol binding protein 4, serum amyloid P component, lumican, transgelin 2, and CD5 antigen-like) differentiates all four patient groups with an overall success rate of 76% (AUROC for control group = 1.0, simple steatosis = 0.83, NASH = 0.86, and NASH F3/F4 = 0.91) and the correct classification percentage for each individual group is shown in Fig. 2A. A group of three proteins (complement component C7, insulin-like growth factor acid labile subunit, and transgelin 2), as shown in Fig. 2B, overall correctly categorizes 90% of patients as having NAFLD (simple steatosis and NASH) or NASH F3/F4 (AUROC = 0.91). Finally, two proteins (prothrombin fragment and paraoxonase 1) are able to accurately differentiate between control subjects and patients with all forms of NAFLD 100% of the time with an AUROC = 1.0 (Fig. 2C).
**Diagnostic Comparison of Potential Biomarker Panels to Alanine Aminotransferase (ALT) Levels**

LDA was also performed to differentiate patient groups by ALT levels (Fig. 2A,B). Control subjects were classified based on normal ALT levels and were not included in this analysis. When discriminating between the three liver disease groups, ALT correctly classified 53% of the steatosis patients (AUROC = 0.68), 15% of the NASH patients (AUROC = 0.59), and 40% of the NASH F3/F4 group (AUROC = 0.69). Differentiation of NAFLD patients with simple steatosis and NASH from those with advanced fibrosis (NASH F3/F4) was performed with an overall success rate of 55% (AUROC = 0.53) by ALT levels.

**Discussion**

In this proteomics study, we identified protein expression patterns in the blood that differ significantly between control subjects without fatty liver disease and patients with various forms of NAFLD (simple steatosis, NASH, and NASH F3/F4) and developed potential biomarker panels to aid in the diagnosis of these common liver diseases. Proteomics is a promising area of research that has recently provided important insight into the pathogenesis of human NAFLD and NASH. There are two previously published proteomics studies using serum or liver tissue from patients across the spectrum of NAFLD. In the first and only other proteomics study using serum samples, Younossi et al. identified 12 protein peaks with significant differential expression when patient groups and controls were compared. In a recent proteomics study utilizing liver tissue from patients with NAFLD and controls, Charlton et al. identified nine proteins with differential expression between study groups, and all proteins exhibited functions previously implicated in the pathogenesis of NAFLD and NASH, including biological response to increased hepatic lipid content, inflammation, mediation of fibrosis, and fatty acid transport. Our study utilized an ion-intensity based, label-free quantitative proteomics approach (LFQP) that has gained popularity in recent years as the performance of mass spectrometers has improved. Using this LFQP approach, we detected 1,738 proteins in serum samples obtained from control subjects and NAFLD patients. Of these proteins, expression of 605 proteins differed significantly (q < 0.05) between any two patient groups. Further analysis revealed that expression of 229 proteins differed significantly when control subjects were compared with any of the three NAFLD patient groups. There were no significant differences observed between the simple steatosis and NASH groups, suggesting that systemic markers of fatty liver and NASH may not be present in serum from patients with mild disease. However, there were 55 proteins that were different between the simple steatosis and NASH F3/F4 group and 15 proteins that differed between the NASH and NASH F3/F4 patients. These proteins may be particularly helpful in identifying biomarkers to diagnose and stage NAFLD and NASH.

We further analyzed the biological significance of all priority 1 proteins with a significant change (q < 0.05) of at least 14% (1.14-fold change) based on the maximum observed change of the internal standard, chicken lysozyme. As described below, several of these biological processes have been previously implicated in the pathogenesis of NAFLD and NASH and many of these identified proteins are only synthesized by the liver. Fifteen of the proteins that changed significantly are involved in immune system regulation and inflammation. One example is retinol binding protein 4 (RBP4), a protein we identified as having significantly decreased expression with increasing NAFLD severity. RBP4 is a cytokine synthesized by both the liver and adipose tissue that carries vitamin A in the blood and is involved in the development of insulin resistance. Although some studies have shown an increase in serum RBP4 levels in patients with NAFLD, a recent study by Nobili et al. reported a stepwise decrease in RBP4 levels with increasing severity of NAFLD and NASH in a pediatric population. Proteins that constitute acute phase response to tissue injury/infection and the complement cascade have also been explored as candidates.
involved in the inflammatory state present in fatty liver disease. In agreement with a previous report, we found that some serum acute phase proteins were significantly elevated in NASH compared to controls, but found no changes in the expression levels of others. The same was observed with several proteins that comprise the complement system, which have been identified in previous proteomic studies as important diagnostic biomarkers for patients with cirrhosis and hepatocellular carcinoma.

Coagulation and development of liver fibrosis are tightly coupled and proteins that contribute to inflammation and immunity, production and remodeling of extracellular matrices, and cell proliferation, motility, and survival are all involved in this process. Serum levels of most proteins involved in platelet aggregation and coagulation were elevated in NAFLD and NASH patients; however, circulating levels of fibrinogen \( \beta \) chain and fibrinogen \( \gamma \) chain were significantly reduced. Interestingly, in the only other proteomics study using serum from NAFLD patients, Younossi et al. provisionally identified fibrinogen \( \gamma \) chain as one of the protein peaks that differed significantly among patient groups and controls. Taken together, these findings highlight the importance of coagulation in the pathogenesis of NAFLD. Structural and extracellular matrix proteins also play a critical role in tissue remodeling and fibrosis in the liver, and we observed significant changes in several of these proteins in NAFLD. Specifically, the expression of lumican, a protein involved in collagen fibril assembly, was significantly elevated in the NASH F3/F4 group. This finding is consistent with the recent proteomics report by Charlton et al. in which they also demonstrated increased lumican messenger RNA (mRNA) and protein expression in liver tissue from patients with NAFLD and progressive NASH.

The liver is the primary site of synthesis for most apolipoproteins and is responsible for the maintenance of lipoproteins and lipid metabolism. Serum apolipoprotein C1 and its precursor have been previously identified as potential biomarkers for patients with hepatitis C virus (HCV)-induced cirrhosis that progresses to HCV-induced hepatocellular carcinoma. Similarly, we observed changes in the serum lipoprotein profile of patients with NAFLD and NASH. These findings may reflect the common observation of hypercholesterolemia and dyslipidemia in fatty liver disease. Finally, we observed a significant reduction in serum levels of proteins known to possess antiinflammatory and antioxidant capabilities, such as the high-density lipoprotein (HDL) particle-associated paraoxonase 1 and several apolipoproteins, in patients with NAFLD and NASH. Paraoxonase 1 inhibits oxidation of low-density lipoprotein (LDL) particles and lipid peroxidation, and levels are reduced in cardiovascular disease, diabetes, and in patients with chronic liver impairment and cirrhosis.

We identified novel serum biomarker candidates using only priority 1 proteins with a significant fold change >1.30 (30%) \( (q < 0.05) \). LDA was used to assess the utility of individual and combinations of serum proteins, as well as ALT levels, to correctly classify patients into control or disease groups. Diagnostic utility was determined three ways: (1) the percent of the total number of subjects classified correctly (overall); (2) the percent of the subjects in each individual patient group classified correctly; and (3) the AUROC. Consideration of these three measures together estimates the probability that a subject will be positively identified as belonging to the correct patient group when the expression level of these protein biomarker candidates (or ALT) in a patient serum sample is quantitated. Although serum ALT is generally used as the population-wide screening test to diagnose NAFLD, this measure is not accurate, as patients with advanced NASH and cirrhosis may not exhibit elevated ALT and there is no correlation between ALT levels and the extent of hepatic damage. This is true in the current study, where diagnostic utility of the potential biomarker panels was much greater than ALT levels alone.
Findings from our study confirm that the LFQP approach can be used successfully to identify potential serum biomarkers for NAFLD and NASH. However, limitations of our study require mention. The possibility of mild fatty liver disease that was undiagnosed in our control group exists, and is a possible confounding factor in all studies involving obese subjects. Liver biopsy is the only definitive diagnostic tool, but it would not have been ethical to subject individuals to this invasive procedure. Therefore, all comparisons made with the control group should be interpreted with caution. Inclusion of five NAFLD patients with methotrexate use is a limitation of our study but they constituted a small fraction of the NAFLD group and thus are not likely to alter our results significantly. Another limitation is the fact that our internal standard protein, chicken lysozyme, changed 14% between groups. Therefore, we were limited to analyzing only proteins with a significant change $>1.14$-fold, which eliminated 15 priority 1 proteins from classification of biological function. Finally, because of a relatively limited sample size and using only a “discovery” dataset, we were not able to definitively establish the diagnostic utility of the potential biomarker panels. In the future, serum samples from a prospective “validation” cohort of control subjects and NAFLD patients will be used to perform these confirmatory experiments with the hope that use of such noninvasive biomarkers is incorporated into routine clinical practice.

Supplementary Material
Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

- ALT: alanine aminotransferase
- AUROC: area under the receiver operating curve
- CV: coefficient of variation
- FC: fold change
- FDR: false discovery rate
- FPR: false positive rate
- LDA: linear discriminant analysis
- LFQP: label-free quantitative proteomics
- NAFLD: nonalcoholic fatty liver disease
- NASH: nonalcoholic steatohepatitis

References


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40. Bell AW. Lipid metabolism in liver and selected tissues and in the whole body of ruminant animals. Prog Lipid Res 1979;18:117–164. [PubMed: 396532]


Fig. 1.
Representative proteins displaying significant changes in protein expression among patient groups. All 27 priority 1 proteins with a significant change of at least 30% ($q < 0.05$) were grouped into one of six protein expression patterns and a representative protein from each of these groups was chosen (A–F). The mean log$_2$ intensity ± standard error (y axis) is displayed for each of these proteins (a difference of one unit is equivalent to a two-fold change). Groups are as follows: C = obese controls; F = simple steatosis; N = NASH; R = NASH F3/F4.
Diagnostic utility of biomarker candidate panels and ALT levels for differentiating patient groups. The utility of priority 1 proteins with a significant change of at least 30% ($q < 0.05$) and ALT levels in differentiating between patient groups was assessed by linear discriminant analysis and AUROC. (A, black bars) represents a six-protein panel (fibrinogen β chain, retinol binding protein 4, serum amyloid P component, lumican, transgelin 2, and CD5 antigen-like) able to correctly differentiate between all four patient groups (AUROC for control subjects = 1.0, simple steatosis = 0.83, NASH = 0.86, and NASH F3/F4 = 0.91). The diagnostic utility of ALT levels in differentiating between the three patient groups with liver disease: simple steatosis (AUROC = 0.68), NASH (AUROC = 0.59), and NASH F3/F4
(AUROC = 0.69) is also shown (A; gray bars). Three proteins (complement component C7, insulin-like growth factor acid labile subunit, and transgelin 2) correctly differentiate patients with NAFLD (simple steatosis and NASH) from those with NASH F3/F4 (AUROC = 0.91) (B; black bars). The ability of ALT levels to identify NAFLD patients (simple steatosis and NASH) versus those with advanced disease (NASH F3/F4 group) was also tested (AUROC = 0.53) (B; gray bars). (C) The diagnostic utility of a combination of two proteins (prothrombin fragment and paraoxonase 1) that differentiate control subjects with no liver disease from patients with all forms of NAFLD (simple steatosis, NASH, and NASH F3/F4) is shown (AUROC = 1.0). Normal ALT levels were used to characterize control subjects; therefore, they were not included in the ALT analyses.
Table 1
Clinical Characteristics and Liver Biochemistries of Study Participants

<table>
<thead>
<tr>
<th></th>
<th>Controls n=16</th>
<th>Simple Steatosis n=24</th>
<th>NASH n=23</th>
<th>NASH F3/F4 n=22</th>
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<tr>
<td>Age (years)</td>
<td>43 ± 14</td>
<td>50 ± 12</td>
<td>44 ± 12</td>
<td>52 ± 11</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>32.2 ± 3.8</td>
<td>32.6 ± 8.9</td>
<td>33.8 ± 6.2</td>
<td>35.4 ± 8.8</td>
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<td>Male (%)</td>
<td>44</td>
<td>71</td>
<td>39</td>
<td>32</td>
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<tr>
<td>Caucasian (%)</td>
<td>100</td>
<td>83</td>
<td>96</td>
<td>95</td>
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<tr>
<td>Comorbidities</td>
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<tr>
<td>Hypertension (%)</td>
<td>12.5</td>
<td>50</td>
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<td>Diabetes mellitus (%)</td>
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<td>Dyslipidemia (%)</td>
<td>12.5</td>
<td>47</td>
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<td>50</td>
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<tr>
<td>Liver biochemistries</td>
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<tr>
<td>AST (U/L)</td>
<td>22 ± 8</td>
<td>53 ± 37</td>
<td>66 ± 41</td>
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<tr>
<td>ALT (U/L)</td>
<td>25 ± 8</td>
<td>63 ± 29</td>
<td>89 ± 55</td>
<td>70 ± 40</td>
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<tr>
<td>ALP (U/L)</td>
<td>74 ± 17</td>
<td>98 ± 38</td>
<td>99 ± 56</td>
<td>93 ± 49</td>
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<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.4 ± 0.2</td>
<td>0.9 ± 0.6</td>
<td>0.6 ± 0.2</td>
<td>0.8 ± 0.3</td>
</tr>
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</table>

Values are expressed as mean ± SD.

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase.

Five NAFLD patients had methotrexate use, three patients in the simple steatosis group and two patients in the NASH group.
### Table 2

Summary of All Identified Proteins

<table>
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<tr>
<th>Protein Priority</th>
<th>Peptide ID Confidence</th>
<th>Multiple Sequences Quantified</th>
<th>Number of Proteins</th>
<th>Number of Significant Changes*</th>
<th>Maximum Absolute Fold Change</th>
<th>Median % Coefficient of Variation</th>
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<td>1</td>
<td>High</td>
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<td>183</td>
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<td>Overall</td>
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<td>1738</td>
<td>605</td>
<td>14.26</td>
<td>24.73</td>
</tr>
</tbody>
</table>

*False discovery rate (FDR) <5% (q < 0.05).
### Table 3

Pairwise Summary of Significant Changes Among All Four Patient Groups

<table>
<thead>
<tr>
<th>Protein Priority</th>
<th>Number of Proteins</th>
<th>Control Versus Simple Steatosis</th>
<th>Control Versus NASH</th>
<th>Control Versus All NAFLD/NASH</th>
<th>Simple Steatosis Versus NASH F3/F4</th>
<th>NASH Versus NASH F3/F4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>183</td>
<td>30</td>
<td>36</td>
<td>47</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>426</td>
<td>90</td>
<td>107</td>
<td>97</td>
<td>59</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>73</td>
<td>16</td>
<td>21</td>
<td>20</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>1056</td>
<td>187</td>
<td>248</td>
<td>253</td>
<td>138</td>
<td>21</td>
</tr>
<tr>
<td>Overall</td>
<td>1738</td>
<td>323</td>
<td>412</td>
<td>417</td>
<td>229</td>
<td>55</td>
</tr>
</tbody>
</table>

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Table 4
Summary of Biological Processes in Which Differentially Expressed Priority 1 Proteins with a Significant Change >14% (q < 0.05) Are Involved

<table>
<thead>
<tr>
<th>Biological Process</th>
<th>Number of Proteins</th>
<th>Protein List</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune system regulation and inflammation</td>
<td>15</td>
<td>α-1-acid glycoprotein 2, complement component C7, α-1-acid glycoprotein 1, serum amyloid P component, α-2-macroglobulin, CD5 antigen-like, C-reactive protein (isoform 1), complement C4A, complement component 4B (preprotein), complement component 4A, N-acetylglucosamidase-L-alanine amidase (isoform 1), complement comp 1q subcomp (β chain precursor), GUGU (γ form), fetuin-B, complement C1q (subcomponent)</td>
</tr>
<tr>
<td>Coagulation cascade</td>
<td>14</td>
<td>Platelet basic protein, fibrinogen β chain, fibrinogen γ chain (isoform γB), SERPIN1, antithrombin II variant, platelet factor 4, platelet factor 1 variant, prothrombin (fragment), α-2-macroglobulin, histidine-rich glycprotein, von Willebrand factor, SERPINF2, fibrinogen α chain (isoform 1), plasminogen</td>
</tr>
<tr>
<td>Structural and extracellular matrix proteins</td>
<td>9</td>
<td>Transgelin 2, actin (cytoplasmic), serum amyloid P component, lumican, proteoglycan 4 (isoform A), transhyretin, extracellular matrix protein 1, α-2-macroglobulin 1 (zinc), gelsolin (isoform 1)</td>
</tr>
<tr>
<td>Blood carrier proteins</td>
<td>7</td>
<td>Retinol binding protein 4, insulin-like growth factor acid labile subunit, transhyretin, insulin-like growth factor binding protein 2, insulin-like growth factor binding protein 3, afamin, fetuin-B</td>
</tr>
<tr>
<td>Cholesterol and triglyceride balance (lipoprotein components)</td>
<td>7</td>
<td>Apolipoprotein C1, apolipoprotein A2, apo lipoprotein A4, apolipoprotein A4 precursor, apolipoprotein B100, apolipoprotein C3, apolipoprotein L1 (isoform 2)</td>
</tr>
<tr>
<td>Cell growth/survival/proliferation</td>
<td>6</td>
<td>Insulin-like growth factor acid labile subunit, insulin-like growth factor binding protein 2, insulin-like growth factor binding protein 3, sterile α motif domain-containing protein 9 (isoform 1), α-2-glycoprotein 1 (zinc), gelsolin (isoform 1)</td>
</tr>
<tr>
<td>Antiinflammatory and anti-oxidant</td>
<td>5</td>
<td>Paraoxonase 1, retinol binding protein 4, apolipoprotein A2, afamin, apolipoprotein L1 (isoform 2)</td>
</tr>
<tr>
<td>Gene/protein processing and expression</td>
<td>3</td>
<td>Poly (A) RNA polymerase mitochondrial (isoform 1), replication initiation-like protein (isoform 1), superhydrol oxidase 1 (isoform 1)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
<td>13 kDa protein, 11 kDa protein</td>
</tr>
<tr>
<td>Blood pressure regulation (renin-angiotensin system)</td>
<td>1</td>
<td>Angiotensinogen</td>
</tr>
<tr>
<td>Insulin action</td>
<td>1</td>
<td>Retinol binding protein 4</td>
</tr>
<tr>
<td>Pro-oxidant</td>
<td>1</td>
<td>Sulphhydrol oxidase 1 (isoform 1)</td>
</tr>
</tbody>
</table>
Table 5
Protein Identification Confidence of Potential Biomarker Candidates

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Annotation</th>
<th>Minimum q-value</th>
<th>Number of Amino Acid Sequences ID'd</th>
<th>Protein ID Confidence (%)</th>
<th>Sequence Coverage* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPI00298497.3</td>
<td>Fibrinogen β-chain</td>
<td>0.000000</td>
<td>17</td>
<td>100</td>
<td>52</td>
</tr>
<tr>
<td>IPI00218732.3</td>
<td>Paraoxonase 1</td>
<td>0.000000</td>
<td>11</td>
<td>100</td>
<td>55</td>
</tr>
<tr>
<td>IPI00019568.1</td>
<td>Prothrombin (fragment)</td>
<td>0.000000</td>
<td>23</td>
<td>100</td>
<td>53</td>
</tr>
<tr>
<td>IPI00022420.3</td>
<td>Retinol binding protein 4</td>
<td>0.000001</td>
<td>9</td>
<td>99.99</td>
<td>43</td>
</tr>
<tr>
<td>IPI00550363.3</td>
<td>Transgelin 2</td>
<td>0.000000</td>
<td>2</td>
<td>99.99</td>
<td>18</td>
</tr>
<tr>
<td>IPI00296608.6</td>
<td>Complement component C7</td>
<td>0.000007</td>
<td>22</td>
<td>100</td>
<td>42</td>
</tr>
<tr>
<td>IPI00022391.1</td>
<td>Serum amyloid P component</td>
<td>0.000000</td>
<td>12</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>IPI00020986.2</td>
<td>Lumican</td>
<td>0.000028</td>
<td>10</td>
<td>99.99</td>
<td>41</td>
</tr>
<tr>
<td>IPI00020996.3</td>
<td>Insulin-like growth factor acid labile subunit</td>
<td>0.000000</td>
<td>6</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td>IPI00025204.1</td>
<td>CD5 antigen-like</td>
<td>0.006677</td>
<td>3</td>
<td>99.99</td>
<td>14</td>
</tr>
</tbody>
</table>

* Percent of the complete protein amino acid sequence where matching peptides for protein identification were found.