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Non-Alcoholic Fatty Liver Disease Proteomics

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is an important cause of chronic liver injury that has gained concern in clinical hepatology. The principal aim of this study was to find differences in protein expression between patients with NAFLD and healthy controls. Changes in protein expression of liver samples from each of the three groups of subjects, controls, non-alcoholic steatosis, and non-alcoholic steatohepatitis (NASH), were analyzed by two dimensional differential in gel electrophoresis (DIGE) combined with MALDI TOF/TOF analysis, a proteomic approach that allows to compare hundreds of proteins simultaneously. Forty-three proteins exhibiting significant changes (ratio ≥ 1.5 , $p < 0.05$) were characterized, twenty-two comparing steatosis samples versus control samples and twenty-one comparing NASH versus control samples. Ten of these proteins were further analyzed by Western blot in tissue samples to confirm the observed changes of protein expression using DIGE. The proteins validated were further tested in serum samples of different cohorts of patients. Following this approach we identified two candidate markers, CPS1 and GRP78, differentially expressed between control and NASH. This proteomics approach demonstrates that DIGE combined with MALDI TOF/TOF and Western blot analysis of tissue and serum samples is a useful approach to identify candidate markers associated with NAFLD, resulting in proteins whose level of expression can be correlated to a disease state.

Keywords

Proteomics; DIGE; non-alcoholic fatty liver disease (NAFLD); steatosis; non-alcoholic steatohepatitis (NASH)

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Clinical relevance

Non-alcoholic fatty liver disease (NAFLD) is an important cause of chronic liver injury that has gained concern in clinical hepatology. The principal aim of this study was to find differences in protein expression between patients with NAFLD and healthy controls.

We hypothesized that by analyzing liver samples from NAFLD patients and healthy controls using DIGE combined with MALDI TOF/TOF analysis, a set of differentially expressed proteins may be identified and validated in serum as potential markers for the diagnostic of NAFLD. To our knowledge this is the first time that a comprehensive comparative study has been developed between steatosis and early stages of NASH in humans using DIGE technology.

Following this approach, we identified two serum proteins, CSP1 and GRP78, which differentiate between healthy controls and NAFLD. Hence, and although the diagnosis of NAFLD can be done in a histological manner, the availability of novel specific non-invasive serum biomarkers of steatosis and NASH may be helpful for the grading and staging of NAFLD and therefore, be useful insights for understanding the mechanism of NAFLD pathogenesis and its progression.

1.- Introduction

Non-alcoholic fatty liver disease (NAFLD) is a clinic pathological term that encompasses a spectrum of abnormalities that range from simple triglyceride accumulation in the hepatocytes (hepatic steatosis) to hepatic steatosis with inflammation (NASH), which may lead to hepatic fibrosis and cirrhosis, resulting in increased morbidity and mortality [1–4]. Obesity, type 2 diabetes and hyperlipidemia (the features of the metabolic syndrome) are all associated with NAFLD [1–4]. With the increasing prevalence of these metabolic conditions in the general population NAFLD is now recognized as the most common chronic liver disease in Western world [5,6]. Numerous studies have been carried out to help to elucidate the complex molecular mechanism of NAFLD and its progression to more severe stages of the disease including hepatic cellular carcinoma (HCC) [7–9]. In fact, the poor survival of patients with HCC is related to the lack of reliable tools for early diagnosis. Hence, a combination of these molecular insights and complex algorithms has been used to design a predictive test of fibrosis [10–12] and even more, tests which may distinguish cirrhotic patients with HCC from those without [13].

Proteomics is a powerful tool in the study of changes in protein expression of proteomes of different populations of patients. The identification of these proteins may have a huge impact by increasing the availability of molecular markers for early diagnosis and therapy. Two-dimensional electrophoresis (2-DE) is a widespread method in proteomics useful to compare changes in protein expression [14,15]. Hence, 2-DE has been used to look for differences in protein expression in NAFLD through the study of tissue, cell lines, human serum or animal models [16–23]. A proteomic investigation of drug-induced steatosis in rat liver has led to the identification of protein markers [24]. HCC related biomarkers have also been identified in some previous studies [25–28]; and markers of oxidative stress, inflammation, apoptosis and fibrosis are being tested as potential novel biomarkers for NASH diagnosis [29–32].

Two-dimensional differential in gel electrophoresis (DIGE) overcomes the problems of reproducibility of 2-DE since allows the co-separation of different samples in the same gel incorporating a pooled internal standard [33,34]. The aim of this study was to identify proteins that may be used as serum markers for the diagnostic of NAFLD. We hypothesized

that by analyzing liver samples from NAFLD patients and healthy controls using DIGE combined with MALDI TOF/TOF analysis, a set of differentially expressed proteins may be identified and validated in serum as potential markers for the diagnostic of NAFLD. Following this approach, we identified two serum proteins (CSP-1 and GRP78) that differentiate between healthy controls and NAFLD. To our knowledge this is the first time that a comprehensive comparative study has been developed between steatosis and early stages of NASH in humans using DIGE technology.

2.- Materials and Methods

2.1.- Patients

The clinical characteristics of patients are summarized in Table 1. We obtained six liver samples from each of the following three groups of subjects: (1) six cholecystectomy controls with normal liver function and histology; (2) six morbidly obese patients diagnosed of non-alcoholic hepatic steatosis. A diagnostic of hepatic steatosis in the absence of other (viral, alcohol, metabolic) causes of liver disease was established histologically; and (3) six morbidly obese patients diagnosed of early-stage NASH. A diagnostic of steatohepatitis grade 1 was established histologically (macrovesicular steatosis, lobular and portal inflammation) in the absence of other (viral, alcohol, metabolic) causes of steatohepatitis [35,36]. Liver samples were obtained during open cholecystectomy (1) or open bariatric surgery (groups 2 and 3) using the same procedure and treated in an identical fashion. No pre-operation regimen was used before surgery. Biopsies were obtained at the beginning of the procedure. A single pathologist who was unaware of the clinical data of patients made the histological diagnostic. Liver samples were divided into two groups, one group was processed for routine histology and the other group was immediately frozen in liquid nitrogen and stored at -80°C . Serum was prepared by incubating patient venous blood in serum separator tubes for 30 minutes before centrifugation (2500 g, 15 min). Aliquot supernatants were placed into micro tubes and stored at -80°C until required. Fifteen serum samples of each cohort of patients were used for this study.

Patients with other forms of liver disease, including hemochromatosis, Wilson's disease, celiac disease and drug-induced liver disease were excluded from the study. None of the patients included in this study was treated with insulin. The Human Research Review Committees of the participating hospitals approved the study. The tissue samples were collected at Hospital Clinic (Barcelona) and Hospital Príncipe de Asturias (Alcalá de Henares, Madrid) and the serum samples were from BIOEF BioBank (www.bioef.org, Basque Country). Informed consent was obtained from each patient included in the experimental section and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

2.2.- Sample preparation for 2D-PAGE analysis

Liver samples were homogenized in 1 ml of lysis buffer containing 7M urea, 2M thiourea, 4% (v/v) CHAPS, 1% (v/v) DTT and 0.5% (v/v) Bio-Lyte 3–7 ampholytes (Amersham Biosciences). Protein concentration of the supernatant was measured with the Bradford assay kit (Biorad) using bovine serum albumin (Sigma) as standard protein. Samples were desalted and concentrated with the Clean up kit (Amersham Biosciences) following manufacturer's recommendations and resuspended in 30mM Tris, 7M urea, 2M thiourea and 4% (v/v) CHAPS.

2.3.- Two-dimensional differential in gel electrophoresis

Steatosis and NASH experiments were run separately but in identical fashion. The DIGE analysis has been performed on individual samples. In one hand, 50 μg of steatosis samples

and 50 µg of control counterparts were labeled alternatively with Cy-3 or Cy-5 in six different gels. A pool of both samples (25 µg each) was labeled with Cy-2 for normalization, using 400 pmol of fluorochrome per 50 µg of protein. In parallel, NASH samples were compared against control samples.

Twenty-four cm, 3–7 IPG strips (Biorad) were actively re-hydrated with 460 µl of re-hydration buffer (7M urea, 2M thiourea, 4% (v/v) CHAPS, 1% (v/v) DTT and 1% (v/v) Biolyte 3–7 ampholytes) at 20°C for at least 12 hours using a Protean isoelectric focusing Cell (Biorad). After the re-hydration, 50 µl of sample was added by cup-loading and the IEF was carried out at 300 V (1h), 300–600V (1h), 600V (3h), 600–2000V (7h), 2000V (3h), 2000–3500V (7h) at 20°C, with a maximum current setting 50µA/strip in a Protean IEF Cell. The IPG strips were equilibrated in equilibration buffer (50mM Tris/HCl pH 7.5, 6M urea, 30% (v/v) glycerol, 2% (v/v) SDS) and further reduced with 2% (v/v) DTT for 15 min at room temperature, followed by alkylation with 2.5% (v/v) iodoacetamide in equilibration buffer for another 15 minutes at room temperature in the dark. The IPG strips were then loaded at the top of a 12.5% SDS-PAGE. The temperature was maintained at 20°C throughout the run by use of an external cooler (Multitemp III, Amersham Pharmacia). The gels were run over night using a constant current of 2 mA per gel. For Sypro Ruby staining, the gels were stained following manufacturer's instructions.

2.4.- Image analysis

DIGE gels were visualized using the Typhoon TRIO (Amersham Pharmacia) with excitation at 553 nm (Cy3), 648 nm (Cy5), 491 nm (Cy2) and emission at 572 nm (Cy3), 669 nm (Cy5) and 506 nm (Cy2). The Typhoon imager allows adjust sensitivity by tuning the voltage setting of the photomultiplier tube (PMT) that captures the fluorescent image. The starting point for the PMT was around 600 V. All gels were scanned at 100 µm resolution. Images were cropped to remove areas extraneous to the gel image using Image Quant (Amersham Pharmacia) prior to analysis. Gel images were stored as .gel file.

Using the DeCyder software (v6.5, Amersham Biosciences), Cy-2 images (pooled samples) were compared with Cy-3 or Cy-5 images that were alternatively steatosis and control for the first analysis and NASH and control for the second analysis. Images from separate gels were matched with the BVA (Biological Variance Analysis) module of the DeCyder software using the Cy-2 labeled image on each gel for normalization. Quantitative differences were only accepted when at least a 1.5-fold change was confirmed in five out of six independent experiments. The statistical significance of the differences was calculated using Student's t-test and accepted when the value was $p < 0.05$.

2.5.- In-gel digestion and protein identification

Proteins were identified by peptide mass fingerprinting from spots digested from preparative gels stained with Sypro Ruby. Protein spots were excised from the 2D gel using the Spot Picker (GE-Healthcare).

The spots were in gel digested with trypsin in a Proteineer DP automated digester (Bruker) following Bruker's standard in gel digestion protocol (reduction phase with DTT (10mM) followed by alkylation with IAA (55mM)). After alkylation, gel spots were dried with ACN and rewetted with Trypsin (0.1 µg/ul) during four hours. Following digestion, peptides were extracted with two phases of ACN/TFA 0.1% dry/rewet and finally dried in a SpeedVac (Christ) and stored until preparation at -20°C. The resulting peptides were desalted with home made microcolumns using GELoader tips (Eppendorf) containing 1 mm chromatographic bed consisting of Poros R2 (Applied Biosystems). Peptides were eluted with HCCA matrix over a MALDI target and let dried at room temperature. MS and MS/MS

analysis was performed with an Ultraflex TOF/TOF (Bruker) spectrometer equipped with a LIFT ion selector and a Reflectron ion reflector. The resulting spots were analyzed manually. Typically, 1000 spectra for peptide mass fingerprint (PMF) and 1500 spectra for peptide fragment fingerprinting (PFF) were acquired.

Protein identities were obtained by using Mascot searching engine (Matrix Science) against SwissProt (51.3) non-redundant databases selected for human taxonomy. A precursor mass tolerance of 30 ppm for MS and 0.7 Da for MS/MS was allowed. Up to two missed cleavages were allowed; oxidation of methionine was set as variable modification and carboxyamidomethylation of cysteine as fixed modification.

2.6.- Western blot

Equal amounts of protein (20µg) extracted from liver tissue as described previously were resolved in 12% SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes. Western blot analysis were performed using the following primary antibodies: Alpha 1 acid glycoprotein (Abcam); Beta actin (Abcam); Calumenin (Santa Cruz Biotechnology, Inc.); Calreticulin (Abcam); Carbamoyl phosphate synthase 1 (CPS1) (Proteintech Group, Inc.); 14-3-3 epsilon protein (IBL); BiP/GRP78 (BD Biosciences); Grp94/Endoplasmic (Abcam); Heat shock protein 90 (Hsp 90) (BD Biosciences); Peroxiredoxin 4 (Abcam). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam) was used as control loading. The protein concentration was measured in serum by Bradford assay. Equal amounts of protein (80 µg) were resolved in 12% SDS-PAGE. Western Blot analysis was performed using the same set of antibodies used for successful validation of the potential markers in tissue.

3.- Results

3.1.- Differentially expressed proteins

The identification of potential markers differentially expressed in steatosis and early stages of NASH was performed by differential proteomics comparing 2-DE protein patterns of tissue from steatosis and NASH versus control patients. The 2-DE images obtained from liver protein extracts showing the location of the differentially expressed spots in steatosis versus control and NASH versus control are shown in Figure 1. Proteins were resolved in a pH range between 3 and 7 and approximately six hundred spots were detected using image analysis software DeCyder.

Only spots with a fold change equal or above 1.5 (in absolute value) and a *p* value <0.05 were considered as differentially expressed spots. The mean and standard deviation values of the normalized spot volumes are shown in supplementary table 1 (steatosis) and supplementary table 2 (NASH). In total, fifty-six protein spots were identified comprising forty-three different proteins that were differentially expressed between the pathological stages and the control cases (Tables 2 and 3). The selected protein spots were excised and subjected to in-gel tryptic digestion. The extracted peptides were analyzed by MALDI TOF and, when feasible, by MALDI TOF/TOF.

3.2.- Identification of differentially expressed proteins: steatosis versus control

Twenty-five spots characterized as twenty-two different proteins were significantly different between steatosis and control (Table 2). The majority of these spots, twenty, were up-regulated (ranging from 1.6- to 3.4-fold) and five of them were down-regulated (ranging from 0.4- to 0.5-fold). Some differentially expressed protein spots are products of the same gene but not all the spots corresponding to the same protein show the same expression behavior. A study comparing tumor and non tumor tissues from patients with HCC showed

that GRP78 exhibited cleavage in HCC. It was not observed variation in the intensity of the full-length protein however microarray data suggests that GRP78 is cleaved in HCC. In our study, spots 2 and 3 (Figure 1, gel A), two proteins spots with an apparent molecular weight near 30 kDa, up-regulated in steatosis, were identified by mass spectrometry as GRP78 while the full length GRP78 protein was identified as protein spots 2, 3 and 4 (Figure 1, gel B), down-regulated in NASH. Likewise, three spots were identified as calreticulin but while two of them were down-regulated in steatosis versus control samples (spot 11 and 12, Figure 1, gel A) the third spot was up-regulated (spot 10, Figure 1, gel A). Four spots were identified as endoplasmin, showing two of them up-regulation in steatosis versus control samples (spot 14 and 15, Figure 1, gel A) and the other two down-regulation (spot 16 and 17, Figure 1, gel A). Hence, there is a need to identify the PTMs underlying these specific isoelectric point and molecular weight isoforms of the same protein because these PTMs are the specific signature to relate these proteins with a specific stage of the pathology. Other studies have reported that different protein isoforms are linked to different pathological situations such as HCC [17,28,37], suggesting that the origin of these disease markers relates with posttranslational events and it is independent of changes in the total expression profile of the protein. These findings suggest that specific isoforms in general and cleavage in particular should be further evaluated as possible markers arising from protein-processing deregulation specific of NAFLD.

3.3.- Identification of differentially expressed proteins: NASH versus control

Thirty-one spots representing twenty-one different proteins were differentially expressed in NASH versus control, of which twenty were down-regulated (ranging from 0.3- to 0.5-fold) whilst the remainder three were increased (ranging from 1.6- to 3.9-fold) (Table 3). Again, several differentially expressed protein spots were found to be products of the same gene. For instance, three spots were identified as GRP78, four as CPS1, and five as short/branched chain specific mitochondrial acyl-CoA dehydrogenase; all of them were down-regulated while three spots corresponding to the protein serum albumin were up-regulated. Serum albumin precursor has also been shown to be up-regulated in a study that characterizes the effects of clorofibrate on protein expression in rat liver [38]. To observe the same tendency in the ratio of several spots corresponding to products of the same gene emphasizes the importance of the variation of the protein. These proteins may be considered markers of the disease independently of the isoform and could be easily tested as immunological markers.

A recent multicenter study correlates cytokeratin 18 (CK18) with the magnitude of hepatocyte apoptosis and described it as a good indicator of NASH diagnosis. In our study CK18 was found to be up-regulated in steatosis and NASH what reasserts the hypothesis made by Feldstein and co workers which states that the odds of having fibrosis on liver biopsy increased with increasing plasma CK18 fragment levels [39].

3.4.- Protein validation by Western blot

Liver samples from patients corresponding to the three different groups were pooled and we conducted additional Western blot experiments to confirm the observed changes of protein expression on 2D-gels. In total, ten antibodies were tested to validate different expression changes observed by DIGE (alpha 1 acid glycoprotein, beta actin, calumenin, calreticulin, CPS1, 14-3-3 epsilon protein, GRP78, endoplasmin, Hsp 90, and peroxiredoxin 4

We found by DIGE that different isoforms of GRP78 and CPS1 were all decreased in NASH versus control patients (Table 3). These results were confirmed by Western blot analysis (Figure 2). In summary, we were able to identify and validate two candidate markers of NAFLD in biopsies combining DIGE and MALDI TOF/TOF methodology with Western blot analysis.

These two liver-biopsies markers were also validated in serum reasserting that a tissue-based approach followed by a serum validation approach can be successfully used for finding candidate markers in serum (Figure 3). Serum CPS1 levels were found to be similar in steatosis as compared to controls, but significantly reduced in NASH. Serum GRP78 followed the same pattern as CPS1 being slightly reduced in steatosis and markedly decreased in NASH. The validation on individual samples is shown in Supplementary Figure 1.

4.- Discussion

The aim of this work was to identify differences in serum protein expression between patients with NAFLD and healthy controls. The strategy followed was to search for new candidate markers in diseased liver where the marker protein must be at higher concentration than in serum samples, facilitating the detection of such markers and the protein identification by MS. Further validation of two candidate markers in serum samples allows us to point a set of non-invasive markers of relevant importance for prognosis and diagnostic of NAFLD. An inherent risk in the approach is that the very high dynamic range of the serum would interfere with the detection. Besides this, the use of tissue samples gives us a stronger source of proteomic analysis and allow us to avoid the drawback derived of using serum samples where variations in clotting time, storage time and temperatures can cause changes in the different proteomic profiles of the sample thus compromising their predictive value when are analyzed as the same set of samples [40].

A set of twenty-two and twenty-three proteins were found, using DIGE in combination with MALDI TOF/TOF analysis, to be differentially expressed in liver samples from patients with steatosis and NASH respectively. Whereas in the case of patients with steatosis the majority of the proteins identified by DIGE and MALDI TOF/TOF were up-regulated, in the case of NASH patients the majority of the proteins found to be differentially expressed using this technology were down-regulated. Moreover, although five of the proteins identified by DIGE and MALDI TOF/TOF were common to both steatosis and NASH (calreticulin, calumenin, CK18, GRP78 and endoplasmin), the majority of them were specific of a single pathological stage. This argues against the possibility that the observed differences in protein expression is due to differences in BMI between the group of NAFLD patients and the controls, and supports the concept that these proteins may be markers of NAFLD. Five of the forty three deregulated proteins were described among the top fifteen most often identified differentially expressed proteins: Peroxiredoxin 4, Apolipoprotein A1, GRP78, HSP 60 and Calreticulin [41]. Although the validation of the data presented in this manuscript strengthens the deregulation of GRP78 as a characteristic of NAFLD and GRP78 had been previously associated to HCC (28), it is unlikely that this protein is intrinsic associated to NAFLD. GRP78 is highly regulated in different pathological conditions (42, 43) and could be associated to the general inflammation process which the liver suffers in NAFLD.

While some of the proteins identified had been previously associated to NAFLD, like CK18 [39,44] or Apo A1 which is one of the biomarkers used in the Nash Test for the prediction of NASH in patients with NAFLD [45], others, like calreticulin, calumenin, CPS1 and endoplasmin, had not been previously linked to NAFLD. Among the proteins identified there are several heat shock proteins, such as Hsp 90, GRP78, calreticulin and endoplasmin, which are associated with endoplasmic reticulum stress. Endoplasmic reticulum stress is common in NAFLD [45], as well as in obesity and diabetes [46–48], two conditions which are well known to be associated with NAFLD [1–4]. Several key enzymes and proteins implicated in the urea cycle (CPS1), calcium metabolism (calpain small subunit 1 and calreticulin), and fatty acid metabolism (enoyl coA hydratase and short/branched mitochondrial acyl-CoA dehydrogenase) were also found to be differentially expressed in

liver samples from NAFLD patients, which agrees with numerous studies linking abnormal hepatic metabolism with the pathogenesis and progression of NAFLD [1–4].

Of the proteins identified to be associated with NAFLD using DIGE in combination with MALDI TOF/TOF, ten were randomly selected to be validated by Western blot analysis. Of these ten proteins, two, GRP78 and CPS1, were validated using this approach. It is important to note that, even though not all the proteins identified by DIGE in combination with MALDI TOF/TOF were validated by Western blot, this observation does not invalidate the proteomics findings. Protein isoforms with different isoelectric points due to PTMs can be identified by DIGE experiments but not by Western blot analysis. Therefore, these results suggest that the protein markers of NAFLD identified by DIGE in combination with MALDI TOF/TOF which were not validated by Western blot analysis may have different PTMs and post-processing forms whose description is a requisite for the fully comprehension of a candidate marker. CPS1 and GRP78 were also confirmed to be serum candidate markers of NAFLD. CPS1 and GRP78 serum concentration decreased gradually from control subjects to steatosis and NASH patients. Despite the discreet success of this study, further validation of these proteins in a higher number of patients is needed to ensure the strength of these candidate markers.

Hence, and although the diagnosis of NAFLD can be done histologically [35,36], the availability of novel specific non-invasive serum biomarkers of NAFLD may be helpful for the grading and staging of NAFLD and therefore, be useful insights for understanding the mechanism of NAFLD pathogenesis and its progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ApoA1	apolipoprotein A1
CPS1	carbamoyl phosphate synthase 1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GRP78	78 kDa glucose-regulated protein
HCC	hepatocellular carcinoma
CK18	cytokeratin type I, cytoskeletal 18
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis

5.- References

1. Matteoni CA, Younossi ZM, Gramlich T, Boparai N, et al. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology*. 1999; 116:1413–9. [PubMed: 10348825]
2. Reid AE. Nonalcoholic Steatohepatitis. *Gastroenterology*. 2001; 121:710–723. [PubMed: 11522755]
3. Clark JM, Diehl AM. Hepatic steatosis and type 2 diabetes mellitus. *Curr Diab Rep*. 2002; 2:210–5. [PubMed: 12643175]
4. Marchesini G, Bugianesi E, Forlani G, Cerelli F, et al. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology*. 2003; 37:917–23. [PubMed: 12668987]
5. Adams LA, Lindor KD. Nonalcoholic fatty liver disease. *Ann Epidemiol*. 2007; 17:863–9. [PubMed: 17728149]
6. Angulo P. Obesity and nonalcoholic fatty liver disease. *Nutr Rev*. 2007; 65:S57–63. [PubMed: 17605315]
7. Younossi ZM, Baranova A, Ziegler K, Del Giacco L, et al. A genomic and proteomic study of the spectrum of nonalcoholic fatty liver disease. *Hepatology*. 2005; 42:665–674. [PubMed: 16116632]
8. Rubio A, Guruceaga E, Vázquez-Chantada M, Sandoval J, et al. Identification of a gene pathway associated with non-alcoholic steatohepatitis. *J Hepatol*. 2007; 46:708–18. [PubMed: 17275126]
9. Charlton M, Viker K, Krishnan A, Sanderson S, et al. Differential expression of lumican and fatty acid binding protein-1: New insights into the histologic spectrum of nonalcoholic fatty liver disease. *Hepatology*. 2009; 49:1375–1384. [PubMed: 19330863]
10. Poordad FF. FIBROSpect II: a potential noninvasive test to assess hepatic fibrosis. *Expert Rev Mol Diagn*. 2004; 4:593–97. [PubMed: 15347253]
11. Imbert-Bismut F, Ratziu V, Pieroni L, Charlotte F, et al. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. *Lancet*. 2001; 357:1069–1075. [PubMed: 11297957]
12. Sandrin L, Fourquet B, Hasquenoph JM, Yon S, et al. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol*. 2003; 29:1705–13. [PubMed: 14698338]
13. Vanderschaeghe D, Laroy W, Sablon E, Halfon P, et al. GlycoFibroTest is a highly performant liver fibrosis biomarker derived from DNA sequencer-based serum protein glycomics. *Mol Cell Proteomics*. 2009; 8(5):986–94. [PubMed: 19181623]
14. Rabilloud T. Two-dimensional gel electrophoresis in proteomics: old fashioned, but still climbs up mountains. *Proteomics*. 2002; 2:3–10. [PubMed: 11788986]
15. Bengtson S, Krogh M, Szgyarto CA, Uhlen M, et al. Large-Scale proteomics analysis of human ovarian cancer for biomarkers. *J Proteome Res*. 2007; 6:1440–50. [PubMed: 17315909]
16. Douette P, Navet R, Gerkens P, de Puw E, et al. Steatosis-induced proteomic changes in liver mitochondria evidenced by two-dimensional differential in-gel electrophoresis. *J Proteome Res*. 2005; 4:2024–31. [PubMed: 16335947]
17. Zeindl-Eberhart E, Haraida S, Liebmann S, Jungblut PR, et al. Detection and identification of tumor-associated protein variants in human hepatocellular carcinomas. *Hepatology*. 2004; 39:540–49. [PubMed: 14768008]
18. Ding SJ, Li Y, Zhou H, Zeng R, et al. Proteome analysis of hepatocellular carcinoma cell strains, MHCC97-H and MHCC97-L, with different metastasis potentials. *Proteomics*. 2004; 4:982–94. [PubMed: 15048980]
19. Yokoyama Y, Terai S, Ishikawa T, Aoyama K, et al. Proteomic analysis of serum marker proteins in recipient mice with liver cirrhosis after bone marrow cell transplantation. *Proteomics*. 2006; 6:2564–70. [PubMed: 16548057]
20. Sun W, Xing B, Du X, Lu M, et al. Proteome analysis of hepatocellular carcinoma by two-dimensional difference gel electrophoresis. *Mol Cell Proteomics*. 2007; 10:1798–808. [PubMed: 17627933]
21. Meneses-Lorente G, Watt A, Salim K, Gaskell SJ, et al. Identification of early proteomics markers for hepatic steatosis. *Chem Res Toxicol*. 2006; 19:986–98. [PubMed: 16918237]

22. Santamaría E, Avila MA, Latasa U, Rubio A, et al. Functional proteomics of nonalcoholic steatohepatitis: mitochondrial proteins as targets of S-adenosylmethionine. *Proc Natl Acad Sci USA*. 2003; 100:3065–70. [PubMed: 12631701]
23. Mölleken C, Sitek B, Henkel C, Poschmann G, et al. Detection of novel biomarkers of liver cirrhosis by proteomic analysis. *Hepatology*. 2009; 49:1257–1266. [PubMed: 19177598]
24. Meneses-Lorente G, Guest PC, Lawrence J, Muniappa N, et al. A proteomic investigation of drug-induced steatosis in rat liver. *Chem Res Toxicol*. 2004; 17:605–12. [PubMed: 15144217]
25. Lee IN, Chen CH, Sheu JC, Lee HS, et al. Identification of human hepatocellular carcinoma-related biomarkers by two dimensional difference gel electrophoresis and mass spectrometry. *J Proteome Res*. 2005; 4:2062–69. [PubMed: 16335951]
26. Santamaría E, Muñoz J, Fernández-Irigoyen J, Sesma L, et al. Molecular profiling of hepatocellular carcinoma in mice with a chronic deficiency of hepatic S-adenosylmethionine: relevant in human liver diseases. *J Proteome Res*. 2006; 5:944–53. [PubMed: 16602702]
27. Luk JM, Lam CT, Siu AFM, Lam BY, et al. Proteomic profiling of hepatocellular carcinoma in Chinese cohort reveals heat-shock proteins (Hsp27, HSp70, GRP78) up-regulation and their associated prognostic values. *Proteomics*. 2006; 6:1049–57. [PubMed: 16400691]
28. Chignard N, Shang S, Wang H, Marrero J, et al. Cleavage of endoplasmic reticulum proteins in hepatocellular carcinoma: Detection of generated fragments in patient sera. *Gastroenterology*. 2006; 130(7):2010–22. [PubMed: 16762624]
29. Solga SF, Alkhuraishe A, Cope K, Tabesh A, et al. Breath biomarkers and non-alcoholic fatty liver disease: preliminary observations. *Biomarkers*. 2006; 11:174–83. [PubMed: 16766393]
30. Feldstein AE, Werneburg NW, Canbay A, Guicciardi ME, et al. Free fatty acids promote hepatic lipotoxicity by stimulating TNF- α expression via a lysosomal pathway. *Hepatology*. 2004; 1:185–94. [PubMed: 15239102]
31. Feldstein AE, Canbay A, Guicciardi ME, Higuchi H, et al. Diet associated hepatic steatosis sensitizes to Fas mediated liver injury in mice. *J Hepatol*. 2003; 39:978–83. [PubMed: 14642615]
32. Ratzliff V, Giral P, Charlotte F, Bruckert E, et al. Liver fibrosis in overweight patients. *Gastroenterology*. 2000; 118:1117–23. [PubMed: 10833486]
33. Alban A, David SO, Bjorkesten L, Andresson C, et al. A novel experimental design for comparative two-dimensional gel analysis: Two-dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics*. 2003; 3:36–44. [PubMed: 12548632]
34. Gharbi S, Gaffney P, Yang A, Zvelebil MJ, et al. Evaluation of two-dimensional differential gel electrophoresis for proteomic expression analysis of a model breast cancer cell system. *Mol Cell Proteomics*. 2002; 1:91–98. [PubMed: 12096126]
35. Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, et al. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterology*. 1999; 94:2467–74.
36. Kleiner DE, Brunt EM, Van Natta M, Behling C, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. 2005; 41:1313–21. [PubMed: 15915461]
37. Zeindl-Eberhart E, Klugbaer S, Dimitrijevic N, Junbut PR, et al. Proteome analysis of rat hepatomas: carcinogen-dependent tumor associated protein variants. *Electrophoresis*. 2001; 22:3009–18. [PubMed: 11565795]
38. Léonard JF, Courcol M, Mariet C, Charbonnier A, et al. Proteomic expression of the effects of clorofibrate on protein expression in rat liver. *Proteomics*. 2006; 6:1915–33. [PubMed: 16470657]
39. Feldstein AE, Wieckowska A, Rociolopez A, Liu Y, et al. Cytokeratin-18 fragment levels as noninvasive biomarker for nonalcoholic steatohepatitis: A multicenter validation study. *Hepatology*. 2009 in press. 10.1002/hep.23050
40. West-Nielsen M, Hogdall EV, Marchiori E, Hogdall CK, et al. Sample handling for Mass Spectrometric proteomic investigations of human sera. *Anal Chem*. 2005; 77:5114–5123. [PubMed: 16097747]
41. Petrak J, Ivanek R, Toman O, et al. Déjà vu in proteomics. A hit parade of repeatedly identified differentially expressed proteins. *Proteomics*. 2008; 8(9):1744–9. [PubMed: 18442176]

42. Pyrko P, Schönthal AH, Hofman FM, Chen TC, et al. Glioma in humans is associated with upregulation of human GRP78 [HSPA5] protein in glioma. *Cancer Res.* 2007; 15;67(20):9809–16.
43. Antony JM, Ellestad KK, Hammond R, Imaizumi K, et al. Multiple sclerosis in humans is associated with upregulation of human BIP [HSPA5] protein in brain. *J Immunol.* 2007; 15;179(2):1210–24.
44. Wieckowska A, Zein NN, Yerian LM, Lopez R, et al. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. *Hepatology.* 2006; 44:27–33. [PubMed: 16799979]
45. Poynard T, Ratziu V, Charlotte F, Messous D, et al. Diagnostic value of biochemical biomarkers (Nash Test) for the prediction of non-alcoholic steatohepatitis in patients with non-alcoholic fatty liver disease. *BMC Gastroenterol.* 2006; 6:34. [PubMed: 17096854]
46. Neuschwander-Tetri BA. Fatty liver and the metabolic syndrome. *Curr Opin Gastroenterol.* 2007; 23:193–8. [PubMed: 17268250]
47. Nakatani Y, Kaneto H, Kawamori D, Yoshiuchi K, et al. Involvement of endoplasmic reticulum stress in insulin resistance and diabetes. *J Biol Chem.* 2005; 280:847–51. [PubMed: 15509553]
48. Ozcan U, Cao Q, Yimaz E, Lee AH, et al. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science.* 2004; 306:457–61. [PubMed: 15486293]

Figure 1.

DIGE 2-DE images of human liver samples. Spots differentially expressed are labeled with numbers. Samples were run in a 3–7 isoelectric point (pI) gradient. A: Steatosis versus control differentially expressed spots. B: NASH versus control differentially expressed spots. NASH and steatosis were differentially labeled with a Cy3 and Cy5. An internal standard composed of disease sample and control sample labeled with Cy2 was added as normalization standard. Gels were further stained with Sypro Ruby for spot picking.

Figure 2.

GRP78 and CPS1 were validated by Western blot analysis. GAPDH was used as loading control. A three dimensional view (3D view) of the spots corresponding to the selected proteins is displayed. 20 μ g of liver lysate of each sample was incubated against each antibody in order to validate the differences in expression observed by DIGE. GRP78 and CPS1 were down-regulated in NASH samples.

Figure 3.

Validation in human serum of two of the NAFLD associated proteins identified in liver samples by 2-DE. Changes in protein expression of CPS1 and GRP78 were validated using 15 serum samples of each of the three groups of subjects, (control, steatosis, and NASH). (A). CPS1 and GRP78 were down-regulated in NASH samples. Quantification of the signal has been obtained by densitometric scanning; data obtained were plotted as intensity values. The horizontal bar represents the media of the intensity values. Three replicates of each Western were performed. Student t-test of the densitometric values were calculated in order to assess the significance of the results. The p values obtained for each antibody are as follows: CPS1 (steatosis/control $p=4\times 10^{-2}$; NASH/control $p=7.3\times 10^{-13}$); GRP78 (steatosis/control $p=1\times 10^{-3}$; NASH/control $p=1.4\times 10^{-7}$). (B) Representative immunoblot analysis for the evaluation of six representative patients: C1, C2 (controls) S1, S2 (steatosis), N1, N2 (NASH). The expression levels of CPS1 and GRP78 were analyzed using the antibodies described in Materials and Methods. For evaluation of these proteins, 80 μ g of protein was loaded onto a 12% SDS-PAGE. As control loading the PVDF membranes were stained with Ponceau staining (data not shown).

Table 1

Demographic and laboratory data.

Liver samples	Control (n=6)	Steatosis (n=6)	NASH (n=6)
Age (years)	57.6	43.3	41.1
Sex (female/male)	3/3	4/2	4/2
BMI (Kg/m ²)	24.6	48.2	44.4
Triglycerides (mg/dL)	85.8	108.6	178.5
Glucose (mg/dL)	104.7	119.6	140.3
Cholesterol (mg/dL)	170.6	203	175
AST/ALT (U/L)	0.98	0.71	0.62
ALT (U/L)	26.2	51.5	62.7

Serum samples	Control (n=15)	Steatosis (n=15)	NASH (n=15)
Age (years)	70.6	46.2	70.6
Sex (female/male)	7/8	7/8	8/7
BMI (Kg/m ²)	23.1	42.06	28.5

Data are the mean of the patient values. AST: aspartate aminotransferase; ALT: alanine aminotransferase; BMI: body mass index.

Table 2
Identification of proteins which show altered expression in steatosis versus control

Protein spots were analyzed by MALDI TOF peptide mass fingerprinting and MALDI TOF/TOF peptide fragment fingerprinting as described in experimental section. Spot number indicates the number labeling the spots in Figure 1. For each protein, entry name and protein name are annotated as in SwissProt database. The fold change, the estimated MW and pI, the percentage of the sequence coverage observed (SC%), the number of peptides identified per protein (NP) and the Mascot score are also indicated. Only protein spots that were present on every gel and vary with a significance of $p < 0.05$ were considered.

Spot number	Entry name	Protein Name	Fold change	MW/pI	S.C.(%)	NP	Mascot score
1	I433E_HUMAN	14-3-3 protein epsilon	3.4	44/4.	30	11	116
2	GRP78_HUMAN	78 kDa glucose regulated protein precursor	2.4	45/4.5	18	11	159
3	GRP78 + CPNS1_HUMAN	78 kDa glucose regulated protein precursor + Calpain small subunit 1	1.7	45/4	11+15	6+4	80/94
4	ABHEB_HUMAN	Abhydrolase domain containing protein 14 b	1.6	40/5.5	27	5	200
5	NUDT5_HUMAN	ADP sugar pyrophosphatase	1.7	48/4	38	8	124
6	NUDT5 + K1C16_HUMAN	ADP sugar pyrophosphatase + keratine type 1 cytoskeletal 16	2.8	47/4.2	38+20	8+8	160/99
7	ALDH2 + AL1B1_HUMAN	Aldheyde dehydrogenase 2 + Aldheyde dehydrogenase X	0.5	60/6	28+24	12+12	112/97
8	APOA1	Apolipoprotein A1 precursor	1.7	45/5	47	22	267
9	ACTB_HUMAN	Beta actin	1.7	50/4	23	11	110
10	CALR_HUMAN	Calreticulin precursor	2.3	52/3.5	15	6	103
11	CALR_HUMAN	Calreticulin precursor	0.5	64/3.2	66	30	404
12	CALR_HUMAN	Calreticulin precursor	0.5	62/3.2	24	13	187
13	CALU_HUMAN	Calumenin precursor	2.2	55/3.2	17	7	155
14	ENPL_HUMAN	Endoplasmin precursor	1.7	42/3.5	8	7	114
15	ENPL_HUMAN	Endoplasmin precursor	2.2	40/3.6	5	5	109
16	ENPL_HUMAN + PDIA1_HUMAN	Endoplasmin precursor + Protein disulfide isomerase precursor	0.4	62/3.8	19+12	20+6	134/133
17	ENPL_HUMAN	Endoplasmin precursor	0.4	50/3.5	16	17	166
18	FRIL_HUMAN	Ferritin Light chain	2.9	35/5	17	6	187
19	HPT_HUMAN	Haptoglobin precursor	2.6	25/3.5	10	6	139
20	HS90A_HUMAN	Hsp 90 alpha	1.8	60/4.2	10	14	140
21	HS90B_HUMAN	Hsp 90 beta	2.4	45/4	6	5	101
22	K1C18_HUMAN	Keratina type I; cytoskeletal 18	2.1	57/5	62	33	438
23	ACSL1_HUMAN	Long chain fatty acid CoA ligase I	2	40/6	9	10	137
24	NADC_HUMAN	Nicotinate nucleotide pyrophosphorylase	1.7	55/5.5	21	7	147

Spot number	Entry name	Protein Name	Fold change	MW/pI	S.C.(%)	NP	Mascot score
25	PRDX4_HUMAN	Peroxisredoxin 4	2.9	42/5	49	13	197

Table 3
Identification of proteins which show altered expression in NASH versus control

Protein spots were analyzed by MALDI TOF peptide mass fingerprinting and MALDI TOF/TOF peptide fragment fingerprinting as described in experimental section. Spot number indicates the number labeling the spots in Figure 1. For each protein, entry name and protein name are annotated as in SwissProt database. The fold change, the estimated MW and pI, the percentage of the sequence coverage observed (SC%), the number of peptides identified per protein (NP) and the Mascot score are also indicated. Only protein spots that were present on every gel and vary with a significance of $p < 0.05$ were considered.

Spot number	Entry name	Protein Name	Fold change	MW/pI	S.C.(%)	M.P.	Mascot score
1	CH60_HUMAN	60kDa HSP	0.6	65/4.5	41	25	250
2	GRP78_HUMAN	78 kDa glucose regulated protein precursor	0.4	70/4.2	9	6	97
3	GRP78_HUMAN	78 kDa glucose regulated protein precursor	0.5	70/4.2	39	28	279
4	GRP78_HUMAN	78 kDa glucose regulated protein precursor	0.6	70/4.2	53	35	358
5	A1AG1_HUMAN	Alpha1-acid glycoprotein precursor	1.8	55/5	18	4	90
6	CPNS1_HUMAN	Calpain small subunit 1	2.5	45/4	18	6	130
7	CARL_HUMAN	Calreticulin precursor	2.3	65/3.5	27	17	264
8	CALU_HUMAN	Calumenin precursor	2.6	67/3.5	17	7	155
9	CPS1_HUMAN	Carbamoyl phosphate synthase 1	0.5	75/5.7	21	34	278
10	CPS1_HUMAN	Carbamoyl phosphate synthase 1	0.5	75/5.8	26	45	378
11	CPS1_HUMAN	Carbamoyl phosphate synthase 1	0.4	75/6	20	32	230
12	CPS1_HUMAN	Carbamoyl phosphate synthase 1	0.4	75/6	15	23	213
13	CYB5_HUMAN	Cytochrome b5	0.6	25/3.8	49	5	184
14	ENPL_HUMAN	Endoplasmic precursor	0.3	52/3.8	21	31	262
15	ECHM_HUMAN	Enoyl CoA hydratase	0.3	45/5.5	41	14	311
16	GAMT_HUMAN	Guanidinoacetate N methyltransferase	0.6	45/5.5	41	11	130
17	K2C8_HUMAN	Keratin type II; cytoskeletal 8	1.6	62/5	21	11	135
18	K1C18_HUMAN	Keratin type I; cytoskeletal 18	1.9	60/5	24	14	175
19	RCN1_HUMAN	Reticulocalbin 1 precursor	0.6	60/5	30	16	157
20	NQO2_HUMAN	Ribosyl-dihydronicotinamide dehydrogenase	2	45/4.8	25	7	188
21	ALBU_HUMAN	Serum albumin precursor	3.9	70/5.8	18	12	158
22	ALBU_HUMAN	Serum albumin precursor	2.1	70/6	45	33	366
23	ALBU_HUMAN	Serum albumin precursor	1.8	60/6.5	24	15	177
24	ACDSB + ALBU_HUMAN	Short branched chain specific acyl CoA dehydrogenase + Serum albumin precursor	0.6	57/5	28+24	12+14	135/126

Spot number	Entry name	Protein Name	Fold change	MW/pI	S.C.(%)	M.P.	Mascot score
25	ACDSB_HUMAN	Short branched chain specific acyl CoA dehydrogenase	0.6	58/5.2	19	8	128
26	ACDSB + ALDH2_HUMAN	Short branched chain specific acyl CoA dehydrogenase+ aldehyde dehydrogenase	0.6	57/5.2	17+11	7+7	138/112
27	ACDSB_HUMAN	Short branched chain specific acyl CoA dehydrogenase	0.6	57/5.2	51	27	387
28	ACDSB_HUMAN	Short branched chain specific acyl CoA dehydrogenase	0.6	57/5.8	29	13	189
29	TERA_HUMAN	Transitional endoplasmic reticulum ATPase	0.6	50/5	13	9	117
30	TPM4_HUMAN	Tropomyosin alpha 4 chain	1.6	45/3.8	64	26	311
31	TBA1C_HUMAN	Tubulin alpha 1 chain	0.6	55/5	18	7	115