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NAFLD Phosphoproteomics: A Functional Piece of the Precision Puzzle

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Abstract

Molecular signaling events associated with the necroinflammatory changes in nonalcoholic steatohepatitis are not well understood. To understand the molecular basis of NASH, we evaluated reversible phosphorylation events in hepatic tissue derived from Class III obese subjects by phosphoproteomic means with the aim of highlighting key regulatory pathways that distinguish NASH from NAFLD. Class III obese subjects undergoing bariatric surgery underwent liver biopsy (8 NOR, 8 simple steatosis and 8 NASH). Our strategy was unbiased, comparing global differences in liver protein reversible phosphorylation events across the 24 subjects.

Of the 3,078 phosphorylation sites assigned (2,465 phosphoserine, 445 phosphothreonine, 165 phosphotyrosine), 53 were altered by a factor of 2 among cohorts, and of those, 12 were significantly increased or decreased by ANOVA ($P < 0.05$). Statistical analyses of canonical signaling pathways identified carbohydrate metabolism and RNA post-transcriptional modification among the most over-represented networks. Collectively these results raise the possibility of abnormalities in carbohydrate metabolism as an important trigger for the development of NASH, in parallel with already established abnormalities in lipid metabolism.

Keywords

NASH; nonalcoholic steatohepatitis; NAFLD; nonalcoholic fatty liver disease; phosphoproteomics; pathway analysis; phosphorylation

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) links chronic aberrant metabolism to structural changes in the liver, varying from increased fat deposition (steatosis) to potential cirrhosis. While insulin resistance is the main driver of NASH, the inflammatory subtype of NAFLD, several other factors also have been implicated as contributory. These include genetic predisposition¹, hepatic ATP content^{2, 3}, redox state^{4, 5}, gut-derived endotoxins^{6, 7}, hormone exposure^{8, 9}, innate immunogenic response^{10, 11} and lipid content, composition and zonation¹². Given the presence of steatosis in NAFLD, much attention has been paid to lipid handling and the development of lipotoxic species as contributors to the development of NASH. A more thorough understanding of the collective metabolic changes associated with NAFLD progression is needed to understand the mechanism of development of NASH.

Quantitative proteomics profiling has been used to differentiate protein expression within the disease spectrum of NAFLD^{13–18}. Serum proteomic profiling studies have reported remarkably few protein expression differences between different liver histologies ranging from no differences¹⁶, to increased hemoglobin expression with NASH^{15, 17}, to alterations in pathways mediating immune responses, coagulation, cellular and extracellular matrix structure and function¹³. Miller *et al.* utilized a labeled quantitative proteomics strategy and identified significantly increased serum apolipoprotein E, catalase, CD5L and lymphocyte cytosolic protein1 (LCPI) expression and decreased vitamin D binding protein with NASH relative to SS¹⁹. Proteomic profiling applied to NAFLD human liver thus far has identified only a few proteins differentially expressed including lumican (LUM), fatty acid binding protein-1 (FABP-1), carbamoyl phosphate synthase 1 (CPS1), glucose-regulated protein (GRP78), and 17 β -hydroxysteroid dehydrogenase-13 (17 β -HSD13) in NASH relative to controls^{18, 20}. 17 β -HSD13 was recently identified as a lipid droplet-associated protein up-regulated in NAFLD, and found to be pathogenic in the development of NAFLD when overexpressed in diet-induced obese mice²¹.

Protein post-translational modifications (PTMs)²², such as phosphorylation, oxidation, ubiquitination, acetylation, acylation, sulfation, and glycosylation elicit different functional consequences that determine protein stability, degradation, and protein-protein interactions²³. Phosphorylation, mediated through the addition and removal of phosphate groups by kinases and phosphatases, respectively, is among the most prevalent and dynamic of PTM and is most frequently found on hydroxyl-containing amino acids such as serine (S), threonine (T) and tyrosine (Y) and is central to metabolic pathway signaling.

Phosphoproteomics, or the large-scale determination of protein phosphorylation status, can be used to characterize and compare a variety of disease states, but has been applied to NAFLD in only a handful of studies. Younossi *et al.*²⁴ described protein phosphorylation differences in visceral adipose tissue to be involved in the pathogenesis of NASH but thus far there have been few studies examining large-scale differences in protein phosphorylation in human liver.

The purpose of this study was to discern the differences in global phosphorylation profiles²⁵ differentiating SS from NASH. Our findings indicate that the progression from SS to NASH involves multiple phosphoproteomic pathways primarily involving carbohydrate

metabolism, gastrointestinal diseases, and RNA post-transcriptional modifications. When applying stringent criteria for inclusion we identified 12 phosphorylation sites that differ significantly among the NAFLD cohorts.

METHODS

Subjects

Class III obese women (n=24; ages: 26–59 years) were recruited at Vanderbilt. Eight subjects with an established histologic diagnosis of SS, 8 with NASH and 8 with NOR liver histology were selected. Exclusion criteria included presence of viral hepatitis, autoimmune hepatitis, hemochromatosis, significant alcohol use, concurrent infections, active cancer diagnosis within 5 years, glycosylated hemoglobin A1C >7.0, and use of anti-diabetic drugs. The study protocol was approved by the institutional review board (#090647) and registered at [ClinicalTrials.gov](https://clinicaltrials.gov) (NCT00983463). Wedge liver biopsies of the left lateral lobe were collected at the time of laparoscopic RYGB. The presence of steatosis, ballooning, fibrosis, and inflammation were determined by a hepatopathologist from two independent histology specimens using the NAFLD activity score (NAS).²⁶ The definition of simple steatosis (SS) is histologic evidence of greater than or equal to 5% lipid deposition within the liver parenchyma without features of steatohepatitis (STH). The definition of steatohepatitis includes steatosis as defined above, but with features of inflammation, ballooning and/or fibrosis in accordance with Kleiner et al. (²⁶)

Protein isolation and In-Solution Trypsin Digestion

Portions of frozen human liver were homogenized and solubilized as previously described.²⁵ Briefly, One hundred mg portions of frozen human liver were homogenized using a polytron homogenizer on maximum speed for 30 s on ice in MPER reagent (Pierce, Rockford, IL) containing (final concentrations) 2 mM Na₃VO₄; 10 mM NaF; 1 mM sodium pyrophosphate; 1 mM ammonium molybdate; 250 μM PMSF; 10 μg/mL leupeptin; and 10 μg/mL aprotinin. Homogenates were allowed to sit for 30 min prior to centrifugation at 12,000 × g for 15 min at 4°C to precipitate insoluble material. One milligram of soluble protein (concentration determined by the Bio-Rad DC Protein Assay, Hercules, CA) was used for phosphopeptide enrichment. To prepare tryptic peptides for enrichment, aliquots of soluble protein from each sample homogenate were diluted up to 100 μL with 1M Tris-HCl, pH 8.0 prior to addition of 100 μL trifluoroethanol (TFE). The samples were then reduced by addition of 2 μL of 0.5M TCEP for 1h at room temperature, and alkylated with 4 μL of 500mM iodoacetamide for 30 min in the dark at room temperature. Samples were diluted 10-fold with 100mM Tris-HCl, pH 8.0 to reduce the solution to 5% TFE, and digested overnight at 37°C with proteomics-grade trypsin (Sigma Chemical Co., St. Louis, MO) at a ratio of 1:25 enzyme to protein. The resulting peptides were then desalted by solid-phase extraction (Sep-pak Light C18 cartridges, WAT023501 Waters Corporation, Milford, MA). Digested samples were first acidified with TFA, diluted 2-fold with 0.1% TFA, and loaded via syringe onto the Sep-pak SPE material. After sample loading, the cartridges were washed with 0.1% TFA, and eluted with 60% acetonitrile with 0.1% TFA. Three sequential 0.5 mL elutions were performed, and eluates were reduced to dryness via vacuum centrifugation.

TiO₂ Enrichment of Phosphopeptides

Phosphopeptides were enriched as previously described, but with minor modifications²⁷. Purified tryptic peptides were resuspended in Buffer A (0.5% heptafluorobutyric acid/2% ACN) containing 300 mg/ml lactic acid (Buffer B). Thirty mg of Titanosphere TiO₂ 5 µm beads (GL Sciences, Japan) were placed in a 1.5 ml Eppendorf tube, where they were washed twice with Buffer C (0.05% HFBA/80% ACN), and then added to the resuspended tryptic peptides. The mixtures were rotated at room temperature for 30 min at room temperature, centrifuged for 30 sec at 5,000 × g, and the supernatant was discarded. The phosphopeptide-bound beads were washed with 500 µl of Buffer D (Buffer C containing 300 mg/ml lactic acid) then twice with 500 µl of Buffer C. Phosphopeptides were eluted from the TiO₂ beads using 500 µl of 0.5M ammonia and then twice with 500 µl of 5M ammonia prior to drying by vacuum centrifugation. Each eluate was reconstituted in 0.1% formic acid to pH 3.

LC-ESI/MS/MS Analysis

Peptides were loaded onto a self-packed biphasic C18/SCX MudPIT column using a helium-pressurized cell (pressure bomb). The MudPIT column consisted of 360 × 150 µm i.d. fused silica, which was fritted with a filter-end fitting (IDEX Health & Science) and packed with 6cm of Luna SCX material (5µm, 100Å) and 4cm of Jupiter C18 material (5µm, 300Å, Phenomenex). Once the sample was loaded, the MudPIT column was connected using an M-520 microfilter union (IDEX Health & Science) to an analytical column (360µm × 100µm i.d.), equipped with a laser-pulled emitter tip and packed with 20cm of C18 reverse phase material (Jupiter, 3µm beads, 300Å, Phenomenex). Using an Eksigent NanoLC Ultra HPLC and Autosampler, MudPIT analysis was performed with an 11-step salt pulse gradient (25, 50, 75, 100, 150, 200, 250, 500, 750, and 1000 mM ammonium acetate). Peptides were eluted from the analytical column after each salt pulse with a 105-min reverse-phase solvent gradient (2–45% acetonitrile containing 0.1% formic acid) for the first ten salt pulses, and a 105-min gradient of 2–95% acetonitrile containing 0.1% formic acid for the last salt pulse. Gradient-eluted peptides were introduced via nanoelectrospray into a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific). The data were collected using a 6-scan event data-dependent method. Full scan (m/z 400–2000) spectra were acquired with the Orbitrap as the mass analyzer (resolution 60,000), and the five most abundant ions in each MS scan were selected for fragmentation in the LTQ. An isolation width of 2 m/z, activation time of 30 ms, and 35% normalized collision energy were used to generate MS/MS spectra. The MSn AGC target value was set to 1e⁴, and the maximum injection time was set to 100 ms. Dynamic exclusion was enabled, using a repeat count of 1 and an exclusion duration time of 60 ms.

Database searching and phosphopeptide scoring

Tandem mass spectra were analyzed using a suite of custom-developed bioinformatics tools. MS/MS spectra were converted into DTA files and searched on a 2500 node Linux cluster supercomputer using a custom version of the Sequest algorithm²⁸ and compared to the Uniprot human database (v155, December 31, 2009) containing 83,946 protein sequences. Search parameters included a 2.5 Da precursor mass tolerance, digestion with trypsin (K/R), and variable modifications of carbamidomethylation of cysteine, oxidation of methionine

residues, and phosphorylation of serine (Ser), threonine (Thr), and tyrosine (Tyr) residues. All search results were assembled through the use of Scaffold (v3.3.3, Proteome Software, Portland, OR) where protein and peptide probabilities were calculated. Ascore values for phosphosite assignments were calculated in Scaffold PTM (v1.1.3). A detailed list of all spectra, phosphopeptides, and phosphoproteins identified is provided (Supplemental Information 1).

Determination of differential phosphosite abundance

Counts of tandem mass spectrometry (MS/MS) spectra assigned to a given phosphosite, inclusive of miscleaved phosphopeptides, were normalized to the length of the protein (number of amino acids) then again against the sum of spectra in the sample, resulting in a Normalized phosphosite spectral abundance factor, or NPSAF. NPSAFs observed in at least 5 of 8 subjects in at least 2 cohorts were used for statistical testing. P-values were calculated using a Kruskal-Wallis test using Graphpad Prism (v 5.04, La Jolla, CA). Significance (*) = $P < 0.05$.

Mapping detected phosphopeptides to intracellular signaling pathways

We queried our data against the Phosphosite database, a curated bioinformatics resource housing information regarding 156,143 non-redundant phosphorylation sites as of June 2014 (<http://www.phosphosite.org>). We determined proteins displaying differentially abundant phosphorylation sites using Ingenuity Pathway Analysis (IPA; Mountain View, CA) for identification of highly interconnected proteins.²⁹ The coverage of various biological pathways associated with the complete (NOR, SS and NASH) phosphoprotein dataset was scored using Fisher's Exact Test and the highest scoring pathways were ranked by P-value. Kinase motif analysis was then performed.³⁰ We then quantified phosphopeptide abundance and calculated normalized phosphopeptide spectral abundance factors (NPSAFs).²⁵ NPSAFs observed in at least 5 subjects in a single cohort were used for statistical testing. P-values were calculated using a Kruskal-Wallis test using Graphpad Prism (v 5.04, La Jolla, CA). Finally, NPSAFs were subjected to Pearson hierarchical clustering using average linkage clustering with MultiExperiment Viewer (version 4.8.0; <http://www.tm4.org>)

RESULTS

Anthropomorphic Measurements

The presence of steatosis, ballooning, inflammation and fibrosis was determined by a hepatopathologist using two independent histologic specimens for classification and subsequent scoring by NAS.²⁶ Eight obese subjects from each cohort were selected. NAFLD subjects were similar in terms of gender, ethnicity, BMI and fasting blood glucose. Subjects with NASH were slightly older ($P < 0.001$) than the NOR cohort (Table 1).

Plasma AST, ALT, ALT/AST ratio, total bilirubin, albumin and platelets were not significantly different among the three groups, however plasma levels of alkaline phosphatase were elevated ($P < 0.01$) by nearly two-fold in both SS and NASH vs. NOR. Estimates of fibrosis were made by trichrome stain. Normal specimens were almost uniformly void of steatosis, inflammation and fibrosis resulting in an overall NAFLD

activity score (NAS) of 0.4 ± 0.2 while SS and NASH specimens had a mean NAS of 2.2 ± 0.1 and 5.0 ± 0.4 respectively ($P < 0.001$). Details of individual patient biopsy NAFLD Activity Scores can be found in Table 2.

Identification of Human Liver Phosphoproteins

An overview of the workflow for phosphopeptide and phosphosite analysis is shown in Figure 1. Using RP-nanoLC-ESI-MS/MS we identified 3,532 unique phosphopeptides, corresponding to 3,078 non-redundant sites on 1,434 phosphoproteins. Over 70% of the identified phosphosites were present in the phosphosite database indicating the reliability of our approach. A variable number of phosphopeptides and phosphorylation sites were identified in each of the 24 subjects, averaging 585 phosphopeptides and 445 sites per sample. Multiply phosphorylated peptides were frequently observed, consistent with reported enrichment strategies using TiO_2 .³¹ While the total number of phosphopeptide spectra identified was similar across the three cohorts (Figure 2a), phosphopeptides containing phosphoserine (pSer) were most abundant, followed by phosphothreonine (pThr) and phosphotyrosine (pTyr) (Figure 2b). Specimens in each histologic category generated similar numbers of total non-redundant phosphopeptides indicating our enrichment strategy was not biased with regards to any particular sample group (1,975 in SS and 2,040 sites in NASH vs. 2,080 in obese normal).

We generated Venn diagrams to illustrate shared and unique (shaded areas) phosphopeptides (Figure 2c), and phosphosites for each cohort (Figure 2d). Among the unique phosphosites found only in SS were fibrinogen alpha chain (FGA) Ser364, serine/arginine repetitive matrix2 (SRRM2) Thr1003, and serine/threonine-protein kinase Wnk1 (WNK1) Ser2032. Differentiating NASH were EH domain binding protein1 (EHBP1) Ser1058, filamin1 (FMN1) Ser1031, PEST proteolytic signal-containing nuclear protein (PCNP) Ser119 and plasminogen activator inhibitor1 RNA-binding protein (SERBP1) Ser25. These contrast with the presence of several phosphosites in livers obtained from the NOR cohort; these include cAMP-dependent protein kinase type II-beta regulatory subunit (PRKAR2B Ser114), regulator of cohesion maintenance, homolog B (PDS5B) Ser1394, protein tyrosine phosphatase-like A domain containing1 (PTPLAD1) Ser114 and protein kinase, AMP-activated, gamma 2 non-catalytic subunit (PRKAG2) Ser157.

Relative Quantification of Detected Phosphorylation Sites on Enriched Peptides

Determinations of relative phosphosite abundance were made using spectral counting as described.^{32–34} Reproducibility of the NPSAF applied to TiO_2 -enriched phosphopeptides was facilitated by comparing phosphopeptide profiles from three separate portions of human liver extract from a single individual, independently digested with trypsin, enriched for phosphopeptides, and processed for MuDPIT. Using linear regression, we observed strong agreement among the abundance measurements of 348 phosphopeptides common to all three sample replicates (r^2 of 0.815 for replicate 1 vs 3 and 0.895 for replicate 1 versus 2; Supplemental Information 2). Four examples illustrating the repeatability of phosphopeptide measurements are shown in Supplemental Information 3.

Combining and collapsing the results from all 24 volunteers allowed identification of 1,134 phosphopeptides bearing 967 phosphorylation sites that were common among cohorts (Supplemental Information 1). Phosphosites that were assigned in at least 5 of 8 subjects (majority) in any group totaled 343 (Supplemental Information 4); these were used for relative quantitative analyses and identified a total of 12 that were significantly ($P < 0.05$) different among the three groups using one-way ANOVA (Table 3). We used the Kruskal-Wallis test to analyze the data due to non-homogenous NPSAF variances for over half of the proteins analyzed. Of the 12 phosphorylation sites that significantly differed by ANOVA, 8 differed significantly by Kruskal-Wallis, including DNA-binding phosphoproteins such as serine/arginine repetitive matrix protein1 (SRRM1 Ser616) and Yorkie homolog protein (YAP1 S109) increased with worsening NAFLD severity. Two phosphosite NPSAFs, those for chromobox homolog3 (CBX3 Ser95) and nuclear ubiquitous casein and cyclin-dependent kinases substrate1 (NUCKS1 Ser79), were decreased in subjects with NASH. Amyloid β A4 precursor protein-binding family A1 and putative uncharacterized protein MATR3 Ser188 showed bimodal patterns of NPSAF-determined phosphosite abundance, with values lowest (APBA1 Ser263) or greatest (MATR3 Ser188) in SS specimens.

Differential Phosphoprotein Expression Networks in NAFLD

Specimens were grouped by similarities in NPSAFs. The relatedness of the relative abundance of the shared phosphosites is indicated by the dendrogram in Figure 3. The length and subdivision of the branches display the relatedness of the liver specimens (top) and NPSAF (left). Hierarchical clustering was able to distinguish liver specimens in two groups (first branch divisions on top). The first group was composed primarily of NOR and SS specimens with only 2 NASH specimens. Six of the 8 NASH specimens along with 3 NOR and 2 SS clustered in the second group. Broadly speaking, group 1 was dominated by mostly lower NPSAFs (darker blue/teal colors), while group 2 displayed increased NPSAFs (red/yellow colors). Taken together, these data suggest that a pattern of phosphopeptide activation/deactivation consistent with the development of NASH rather than an isolated aberrant pathway.

In order to predict the differential activity of kinases in NOR, SS and NASH specimens, we grouped phosphosites based on known kinase consensus motifs present in each phosphopeptide primary amino acid sequence. Table 4 shows the percentage of assigned phosphopeptide spectra for NOR, SS and NASH cohorts matching 1 of 30 described kinase motifs. Overall, there were no significant differences in the abundance levels of phosphopeptides.

HSP1 S27 Differential Abundance with Increasing NAFLD Severity

To establish the expression levels of differentially detected phosphorylation sites, we performed immunoblot analyses on a subset of samples for which additional material was available. Commercially available phosphospecific and total antibodies were identified for only one of the detected phosphorylation sites, heat shock 27kD protein1 (HSPB) Ser78. In concordance with our phosphoproteomics profiling results, densitometric analysis of a 27kD band recognized in each liver protein homogenate confirmed the profiling results

(Supplemental Information 5) with the trend of increased phosphorylation of HSPB1 Ser78 persisting in the NASH specimens.

DISCUSSION

In this study we used a quantitative phosphoproteomic technique to analyze human liver samples with a histologic diagnosis of NOR, SS or NASH. We used a NPSAF approach to gauge relative differences in phosphosite abundance among all liver samples and employed bioinformatics approaches to understand the implications of differences in phosphosite abundance at both the single protein and pathway level. Importantly, the patterns of liver protein phosphorylation site abundance we identified were able to discriminate the histopathologic relatedness of 6 out of 8 SS and 6 out of 8 NASH specimens (Figure 3). These patterns were suggestive of defects in canonical pathways of carbohydrate metabolism as well as RNA post-transcriptional modifications, gastrointestinal disease, cell-to-cell signaling and cancer. Several different reports of whole liver phosphoproteomes^{35, 36} and sub-fractions (e.g. mitochondria and cell lines)^{37–40} have been reported in mice but only a handful of studies in human liver^{41–46} almost all from subjects of Asian descent^{42–44, 46}. Our findings identified several phosphorylation differences in both homeostatic and disease-specific processes and highlight the utility of phosphoproteomic profiling leading to the development of novel targeted therapeutic approaches for NASH.^{47, 48}

In order to determine the biologic relevance of our findings, we subjected our phosphoproteins to Ingenuity Pathway Analysis (www.ingenuity.com). Canonical signaling pathways associated with the entire detected liver phosphoprotein dataset was scored using Fisher's Exact Test and the highest scoring (best covered) pathways were ranked by P-value (Figure 4a). Carbohydrate metabolism and gastrointestinal disease (Supporting Information 6) received the highest scores followed by RNA post-transcriptional modification (Supporting Information 7), cell-to-cell signaling (Supporting Information 8), lipid metabolism (Supporting Information 9) and cancer (Supporting Information 10). We selected Carbohydrate Metabolism to explore the relationships among detected phosphoproteins (Figures 4b, c). Thirty-five proteins comprise this pathway with different node shapes (diamonds, ovals or rectangles) denoting functional classification of the proteins. Forty-nine of the phosphosites we measured, mapping to 29 phosphoproteins, were present on this pathway and include binding proteins (CDC42EP1), translational and transcriptional regulators (EEF1D, SRRM1, SRRM2, PEBP1) and enzymes (ALDOA, ALDOC, GAPDH, ERCC5, PDI1, XRN2) (Figure 4b). When comparing expression differences in the NPSAF values of these phosphosites between NOR and SS specimens, 4 phosphosites (shades of red) were increased in abundance while 32 phosphosites (shades of green) were decreased. A different pattern of phosphosite abundance was noted when comparing NASH and SS specimens. There was a modest increase in NPSAF abundance noted for sites such as PDCD5 Ser119, PDLIM2 Ser124, Ser134 and Ser197 and PEBP1 Ser52. Certain phosphosites, such as CDC42EP1 Ser121, increased over 3.8-fold, while five others such as KTN1 Ser75, LSP1 Ser189, SRRM1 Ser616, SRRM2 Ser1083 and TRAFD1 Ser327 increased over 2-fold in NASH versus SS specimens (Figure 4c). This differential activation of signaling networks in NASH and SS versus NOR specimens suggests a central role for these carbohydrate metabolism phosphosites in mediating the disease processes

driving the development of NASH. These are consistent with recent findings examining the role of polymorphisms in patatin-like phospholipase domain containing3 (PNPLA3), strongly associated with NAFLD in humans⁴⁹, which showed that excess carbohydrate relative to fat potentially regulates PNPLA3.⁵⁰ More work is needed to fully understand how aberrant carbohydrate metabolism drives NAFLD progression.

“RNA post-transcriptional modification” is another canonical pathway identified as important in our analyses. These findings are noteworthy given the significant increase of HSPB1 (Hsp27) Ser78 we identified (Supplemental Information 9) and given its known role in the regulatory sequence AU-rich element-mediated mRNA decay.⁵¹ Tian *et al* recently showed in a phosphoproteomic study of hepatocellular carcinoma that proteins in the spliceosome pathway were important for regulating mRNA processing and RNA splicing.⁴⁵ In our study, heterogeneous nuclear ribonucleoproteins (HNRNPs) C, D, K and U, known to bind to pre-mRNA, were among the phosphoproteins mapped to this pathway, and may be associated with the splicing apparatus⁵². As regards comparison with other available phosphorylation sites in NAFLD, Younossi and colleagues recently examined phosphoproteomic biomarkers in visceral adipose tissue to characterize the predictive value of a predetermined panel of phosphorylation sites in diagnosing NASH and related fibrosis.²⁴ Their best performing model relied on levels of the phosphorylation of GSK3B Tyr279 as well as phosphosites on two subunits of c-AMP regulated protein kinase C α (PKC) Thr197 and protein kinase C β (catalytic subunit) Thr638/641. In our study, we did not see such trends, suggesting that protein phosphorylation in visceral adipose and liver tissues may be inherently different in signaling networks involved in NASH.

This study has limitations, the most important of which relates to the small sample size of Class III obese subjects and hence our findings may not apply to Class I or II obese. Additionally, subjects with NASH were older, which we acknowledge may be an independent source of phosphoproteomic differences between NASH and other cohorts. Yet despite these limitations, an identifiable, modified HSP27 Ser78 emerged as a site of interest. Hsp27 serves several diverse functions: in regulating mRNA decay, mRNA stabilization⁵³, inhibition of apoptosis⁵⁴ and modulation of actin polymerization.^{55, 56} Ser78 serves as one of 3 different phosphorylation sites in HSP27 (the others being Ser15 and Ser78) that are phosphorylated by MAPKAPK-2.⁵⁷ Phosphorylation of HSP27 is usually associated with the disassembly of HSP27 complexes⁵⁸ and has been correlated with function such as cell migration⁵⁹ and with stress protection.⁶⁰ Recent reports showed that HSP27 Ser78 phosphorylation was essential to TRAIL-triggered Src-Akt/ERK signaling, possibly serving as a survival signaling mechanism.⁶¹

In summary, we have provided an expansive and as yet undescribed view of human liver phosphorylation in the context of an increasingly prevalent disease. Our data paint a highly complex picture of protein signaling in human liver and suggest a shift in kinase abundance and activity in NASH. Furthermore, our findings delineate site-specific alterations in NAFLD and NASH and provide a previously unavailable catalog of abundant phosphorylated proteins in human liver. Taken together with emerging loci of interest from genomic studies in NAFLD, these data should serve as a basis for generation of novel hypotheses concerning the precise molecular mechanisms discriminating SS from NOR and

NASH. As instrumentation and bioinformatics tools improve^{62–64}, the understanding of these and other phosphorylated protein relationships to specific aspects of NAFLD pathophysiology will increase, thereby providing new therapeutic targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ALT	alanine aminotransferase
AST	aspartate aminotransferase
BMI	body mass index
NAFLD	nonalcoholic fatty liver disease
NAS	NAFLD activity score
NASH	nonalcoholic steatohepatitis
MuDPIT	multidimensional protein identification technology
pS	phosphoserine
pT	phosphothreonine
pY	phosphotyrosine RP-nanoLC, reversed phase nano-flow liquid chromatography
SCX	strong cation exchange
RYGB	Roux-en-Y Gastric Bypass
SS	simple steatosis

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Figure 1.

Workflow of sample preparation and data analysis. (a) Obese subjects were subjected to wedge liver biopsy (100–200 mg) during elective RYGB surgery. Specimens were scored as NOR, SS or NASH (n=8/cohort) using NAS. One milligram of soluble liver protein homogenate was digested with trypsin and the resulting peptide/phosphopeptide mixture subjected to TiO₂ enrichment in the presence of lactate to diminish non-specific binding. (b) MS/MS spectra from 24 subjects were assigned to 3,532 phosphopeptides. Protein FDR, peptide FDR and phosphopeptide FDR (Ascore) filtering criteria as well as orthogonal Sequest scores such as XCorr per charge state were used to generate a final filtered list of 3,078 phosphosites. Phosphosite abundance was determined by NPSAF. Twelve phosphosites were found significantly different by one-way ANOVA among specimens with NOR, SS or NASH.

Figure 2. Inventory and relationships of detected phosphopeptides and phosphorylation sites across NAFLD categories. **(a)** Spectral counts were determined for phosphopeptides meeting the following criteria: +2 charge, XCorr ≥ 2.0 , Ascore ≥ 13 (<5% FDR), +3 charged peptides - Xcorr ≥ 2.5 ; Ascore ≥ 13 . **(b)** Total number of phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) containing phosphopeptides. Venn diagrams illustrating **(c)** phosphopeptides and **(d)** phosphosites shared among and unique to NOR, SS or NASH specimens.

Figure 3. Hierarchical clustering of 24 NAFLD liver specimens by normalized phosphosite spectral abundance factor (NPSAF). Fold change heat map (–2.0 to 2.0-fold) by NPSAF based across histologically NOR, SS and NASH specimens (top). NPSAFs for each sample were log transformed and scaled to the average detected value (defined as 0). Saturated red color indicates at least a two-fold increase in mean levels of phosphopeptide NPSAF; saturated blue color indicates a two-fold decrease in mean levels of phosphosite NPSAF.

Figure 4. Unbiased phosphoproteomic pathway analysis in human NAFLD. **(a)** Based on a pathway analysis of phosphorylation sites differentially regulated in liver specimens of NOR, SS or NASH. The following top six pathways were significantly enriched: Carbohydrate Metabolism, Gastrointestinal Disease, RNA post-transcriptional modification, Cell-to-Cell signaling, Lipid metabolism, Cancer. **(b)** Pathway diagram of proteins involved in Carbohydrate Metabolism. Direct interactions between the genes in the network pathways are indicated by solid lines and indirect relationships are indicated by dashed lines. Diamond shapes represent enzymes, ovals represent transcription regulators, circles represent cytokines and triangles represent kinases. **(c)** Phosphorylation sites on proteins mapping to the Carbohydrate Metabolism canonical pathway. The relative fold change (SS versus NOR, NASH versus SS) in the mean NPSAF for each detected phosphorylation site. The green color indicates lower and the red higher expression levels in relation to the other samples analyzed. $n = 8$ subjects per group.

Table 1

Clinical characteristics of subjects in the study

	NOR	SS	NASH
n	8	8	8
Mean age (years)	38.9 ± 2.3	32.1 ± 2.1	46.4 ± 2.2 ^a
Gender (F/M)	8/0	8/0	8/0
Caucasian/African American	6/2	6/2	8/0
Weight (kg)	126.5 ± 7.1	121.2 ± 5.9	117.3 ± 4.6
BMI (kg/m ²)	45.7 ± 2.5	44.3 ± 1.3	43.9 ± 0.9
Glucose (mg/dl)	94.9 ± 3.1	96.6 ± 3.4	96.0 ± 6.1
AST (normal range: 0–65 U/I)	21.4 ± 2.2	24.4 ± 6.3	33.6 ± 5.1
ALT (normal range: 0–65 U/I)	23.9 ± 3.4	30.6 ± 9.0	54.0 ± 27.9
AST/ALT ratio	1.0 ± 0.7	0.8 ± 0.1	0.9 ± 0.1
Alkaline phosphatase (U/I)	57.5 ± 7.2	92.4 ± 10.9 ^b	83.4 ± 6.1 ^b
Total bilirubin (mg/dl)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.04
Albumin (g/dl)	4.1 ± 0.1	4.1 ± 0.1	4.2 ± 0.1
Platelets (1000/mm ³)	268.3 ± 30.3	275.6 ± 30.3	287.9 ± 15.1
Fibrosis (0–2)	0.1 ± 0.1	0.2 ± 0.1	0.4 ± 0.2
NAFLD Activity Score (0–8)	0.3 ± 0.3	2.1 ± 0.2	5.3 ± 0.4 ^{ac}

Data presented as mean ± SEM. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; NOR, normal; SS, simple steatosis; NASH, nonalcoholic steatohepatitis; NAFLD, nonalcoholic fatty liver disease.

^aP < 0.001 vs. SS

^bP < 0.05 vs. NOR

^cP < 0.001 vs. NOR (Kruskal-Wallis one-way analysis of variance with Dunn's post-test).

Table 2

Subject information and histological scoring of liver specimens.

ID	Category	Ethnicity	* NAS	Steatosis	Ballooning	Inflammation	Fibrosis	Sex	Age	BMI
S17	Normal	Caucasian	0	0	0	0	0	F	46	44.9
S23	Normal	African Am	1	0	1	0	0	F	40	37.1
S29	Normal	Caucasian	0	0	0	0	0	F	37	47.7
S35	Normal	Caucasian	0	0	0	0	0	F	37	49.1
S40	Normal	Caucasian	0	0	0	0	0	F	51	41.6
S45	Normal	Caucasian	1	1	0	0	0	F	34	59.4
S49	Normal	Caucasian	0	0	0	0	0	F	33	37.9
S52	Normal	African Am	1	0	1	0	0	F	33	47.5
S01	SS	Caucasian	2	1	0	1	1	F	36	44.0
S02	SS	African Am	2	1	1	0	1	F	36	49.7
S27	SS	Caucasian	2	1	0	1	0	F	28	49.5
S32	SS	Caucasian	3	2	1	0	1	F	26	42.4
S36	SS	African Am	2	1	1	0	0	F	38	39.9
S46	SS	Caucasian	2	2	0	0	1	F	24	40.9
S50	SS	Caucasian	2	1	1	0	0	F	39	42.6
S51	SS	Caucasian	3	3	0	0	0	F	30	44.9
S04	NASH	Caucasian	6	3	2	1	0	F	43	49.3
S07	NASH	Caucasian	7	3	2	2	0	F	39	41.0
S14	NASH	Caucasian	6	2	2	2	1	F	55	42.7
S18	NASH	Caucasian	4	1	2	1	0	F	44	42.7
S20	NASH	Caucasian	5	2	1	2	2	F	47	43.6
S21	NASH	Caucasian	6	3	2	1	1	F	47	43.1
S47	NASH	Caucasian	4	2	1	1	0	F	56	45.1
S48	NASH	Caucasian	4	1	1	2	0	F	40	44.0

* NAFLD Activity scores calculate per Kleiner et al., 2005.

Table 3

Phosphorylation sites differing significantly among NAFLD cohorts

Protein name	Gene	Site	NPSAF				P_{ANOVA}	P_{K-W}
			NOR	SS	NASH			
Serum albumin	ALB	S82	112.55 ± 12.33	131.79 ± 21.73	280.23 ± 48.66 [*]		0.011 [‡]	0.513 [‡]
Amyloid beta A4 precursor protein-binding family A1	APBA1	S263	88.34 ± 17.73	58.47 ± 0.68	158 ± 23.04 [§]		0.019 [‡]	0.034
Chromobox homolog3	CBX3	S95	154.07 ± 2.08	76.51 ± 11.57	51 ± 10.06		0.047	0.031 [‡]
Catenin alpha-1	CTNNA1	S655	96.86 ± 10.64	101.05 ± 12.69	183.68 ± 35.75		0.035 [‡]	0.027
Cortactin isoform a variant	CTTN	T401	121.39 ± 18.31	232.53 ± 43.42	336.9 ± 22.27 ^{‡§}		0.003 [‡]	0.002 [‡]
Heat shock 27kDa protein1	HSPB1	S78	64.59 ± 15.01	142.44 ± 32.24	325.33 ± 87.33 [*]		0.036 [‡]	0.053
Putative uncharacterized protein	MATR3	S188	175.06 ± 23.59	325.4 ± 38.00 [*]	177.55 ± 23.14		0.027	0.247 [‡]
Protein LYRIC	MTDH	S426	444.74 ± 45.47	635.82 ± 34.00	731.7 ± 43.88 [*]		0.012	0.010 [‡]
Nuclear ubiquitous casein and cyclin-dependent kinases substrate	NUCKS1	S79	600.42 ± 50.84	215.51 ± 86.64	248.96 ± 71.51		0.038	0.165
Serine/arginine repetitive matrix 1	SRRM1	S616	87.83 ± 17.71	74.42 ± 10.3 [*]	222.06 ± 46.21 [§]		0.011	0.026 [‡]
TRAF-type zinc finger domain containing 1	TRAFD1	S327	114.21 ± 22.05	119.37 ± 22.3	242.48 ± 34.92		0.019	0.271
Yes-associated protein1	YAP1	S109	47.31 ± 3.36	72.49 ± 9.37	175.74 ± 37.85		0.036	0.009

Data are for NPSAF values × 1,000 and are means ± SE. P value listed is for overall ANOVA (P ANOVA) with Bonferroni post-test and Kruskal-Wallis test (P K-W) among groups. Total spectra = sum of spectra across subjects in each group. Individual group a posteriori comparisons:

^{*}P 0.05,

[‡]P 0.01 vs. NOR, by a posteriori tests after ANOVA.

[§]P 0.05,

[§]P 0.01 vs. SS subjects, by a posteriori tests after ANOVA.

[‡]Variances not homogeneous.

Table 4

Relative Kinase Action in NAFLD

Kinase	Sequence Preference	NOR	SS	NASH
PKA	R-X-s/t	938 (5.1%)	992 (4.8%)	810 (4.5%)
	R-R/K-X-s/t	29 (0.2%)	26 (0.1%)	26 (0.1%)
CK1	S-X-X-s/t	1762 (9.6%)	2161 (10.4%)	1838 (10.3%)
	S/T-X-X-X-s	1196 (6.5%)	1248 (6.0%)	1182 (6.6%)
CK2	s/t-X-X-E	6332 (34.5%)	6926 (33.4%)	5892 (32.9%)
GSK3	s-X-X-X-S	884 (4.8%)	1000 (4.8%)	934 (5.2%)
CDK2	s/t-P-X-K/R	454 (2.5%)	553 (2.7%)	487 (2.7%)
CAMK2	R-X-X-s/t	923 (5.0%)	997 (4.8%)	809 (4.5%)
	R-X-X-s/t-V	10 (0.1%)	5 (0.0%)	7 (0.0%)
ERK/MAPK	P-X-s/t-P	234 (1.3%)	173 (0.8%)	131 (0.7%)
	V-X-s/t-P	159 (0.9%)	176 (0.8%)	139 (0.8%)
	P-E-s/t-P	662 (3.6%)	814 (3.9%)	620 (3.5%)
PKB/AKT	R-R/S/T-X-s/t-X-S/T	20 (0.1%)	30 (0.1%)	14 (0.1%)
	R-X-R-X-X-s/t	3 (0.0%)	10 (0.0%)	5 (0.0%)
PKC	R-X-X-s/t-X-R	3 (0.0%)	5 (0.0%)	3 (0.0%)
PKD	L/V/I-X-R/K-X-X-s/t	156 (0.8%)	185 (0.9%)	186 (1.0%)
SRC	E/D-X-X-y-X-X-D/E/A/G/S/T	11 (0.1%)	14 (0.1%)	6 (0.0%)
ALK	y-X-X-I/L/V/M	84 (0.5%)	97 (0.5%)	83 (0.5%)
EGFR	D/P/S/A/E/N-X-y-V/L/D/E/I/N/P	18 (0.1%)	20 (0.1%)	24 (0.2%)
CDK1	s/t-P-X-K/R	454 (2.5%)	553 (2.7%)	487 (2.7%)
	s/t-P-K/R	1149 (6.3%)	1399 (6.7%)	1144 (6.4%)
AURORA	R/K-X-s/t-I/L/V	822 (4.5%)	796 (3.8%)	671 (3.7%)
AURORA-A	R/K/N-R-X-s/t-M/L/V/I	13 (0.1%)	13 (0.1%)	3 (0.0%)
PLK	D/E-X-s/t-V/I/L/M-X-D/E	31 (0.2%)	32 (0.2%)	44 (0.2%)
PLK1	E/D-X-s/t-F/L/I/Y/W/V/M	190 (1.0%)	186 (0.9%)	201 (1.1%)
NEK6	L-X-X-s/t	1264 (6.9%)	1280 (6.2%)	1110 (6.2%)
CHK1/2	L-X-R-X-X-s/t	24 (0.1%)	53 (0.3%)	49 (0.3%)
CHK1	M/I/L/V-X-R/K-X-X-s/t	160 (0.9%)	198 (1.0%)	196 (1.1%)