Short Gel, Long Gradient Liquid Chromatography Tandem Mass Spectrometry to Discover Urinary Biomarkers of Chronic Pancreatitis

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Abstract

Background—Chronic pancreatitis (CP) is currently diagnosed using invasive endoscopic as well as radiation and non-radiation-based imaging techniques. However, urine can be safely and non-invasively collected and as such may offer a superior alternative to current techniques of CP diagnosis. We use mass spectrometry-based methods to discover proteins which are exclusive to or differentially abundant in urine of chronic pancreatitis patients.

Methods—We have performed a comparative quantitative proteomic analysis of urine collected from 5 healthy controls and 5 severe CP patients. Proteins from urine were fractionated briefly on SDS-PAGE and subsequently digested in-gel with trypsin. The resulting peptides were fractionated for 3 hours by reversed-phase liquid chromatography in-line with a mass spectrometer. ProteinPilot software and the QSPEC algorithm identified proteins and determined statistically significant differences between cohorts. In addition, we used a third cohort of non-CP disease patients to filter out those proteins which may be indicative of an ailment other than CP.

Results—We identified over 600 proteins from urine, of which several hundred were either exclusive to or differ quantitatively between healthy controls and severe CP patients. Members of the cathepsin protein family were of significantly higher abundance in the severe CP cohort. In addition, we have identified a core set of 50 proteins in all 15 samples, 25 of which showed no significant difference among the cohorts.

Conclusions—Proteomic analysis identified differentially abundant proteins in healthy controls and severe CP patients. Such proteins represent an initial set of targets for directed proteomics experiments for further validation studies. However, larger cohorts will be required to determine if these differences have statistically significant diagnostic potential.

Keywords

Background

Chronic pancreatitis (CP) is characterized by chronic inflammation and progressive fibrosis, clinically manifested as intense pain, and pancreatic exocrine and endocrine insufficiency. In the United States, exocrine pancreatic disorders affect over one million persons and cost approximately $3 billion annually. During the past decade, diseases of the exocrine pancreas have resulted in 277,000 hospitalizations and 475,000 ambulatory care visits per year [1]. Clinical diagnosis of chronic pancreatitis is primarily based on morphological and functional findings. Complications, such as bleeding and fistulae formation, preclude pancreatic biopsy for histologic diagnosis. The non-histological "surrogate" gold standard is pancreas function testing [2], which can diagnose only moderate to late stage chronic pancreatitis in which tissue damage and fibrosis are irreversible [3]. Radiologic imaging is also limited in diagnosing early disease as objective morphologic changes are only associated with moderate to advanced disease. Identification of biomarkers of early chronic pancreatitis would revolutionize diagnosis and potentially lead to novel therapies designed to retard, or modify disease progression, before irreversible organ damage and dysfunction become apparent.

We have previously investigated biomarker candidates of chronic pancreatitis in pancreatic fluid. Using the secretin-stimulated endoscopic pancreatic function test (ePFT), mass spectrometry analysis, and bioinformatic data processing, we showed that pathologic changes in the pancreas may be reflected in the pancreatic fluid proteome [4–9]. The secretin-stimulated ePFT can safely collect pancreatic fluid from the duodenum without cannulation of the pancreatic duct [10–12]. Pancreatic fluid is the proximal body fluid of the pancreas and is a reservoir of locally secreted biomolecules that are likely to include specific markers of disease. ePFT, however, is a relatively invasive procedure requiring endoscopy, sedation and/or anesthesia, and is performed at limited specialized centers. A chronic pancreatitis diagnostic biomarker panel, based on a non-invasively collected fluid, such as urine, would thus be preferred.

The analyses of proximal and systemic fluids have inherent advantages in medical testing. A proximal fluid directly represents a particular organ or region and is concentrated with secretions from surrounding cells. In contrast, systemic fluids, such as urine, represent the entire body and thus provide a snapshot of the whole organism under a given set of systemic conditions. The majority of the proteins detected in a systemic fluid are most likely not directly related to the disease or organ of interest [13]; analysis of systemic fluids may be confounded by proteins representing the normal or abnormal physiology of other organs and/or their pathophysiologic response to the disease of interest. These characteristics limit the utility of systemic fluids in investigating pathogenic and pathophysiologic mechanisms. However, systemic fluid analysis would be appropriate, clinically, for targeted biomarker assays for diagnosis and staging. Compared to other body fluids, urine, in particular, is readily available in large volumes, non-invasively collected, and the same individual can be sampled repeatedly and safely.

Mass spectrometry-based proteomic techniques can identify diagnostic urinary biomarkers to complement established diagnostic methods and improve early diagnosis of chronic pancreatitis [7]. Traditionally, pre-fractionation of proteins and/or peptides is performed prior to LC-MS/MS analysis. Greater sample complexity requires a greater degree of fractionation to achieve maximum proteome coverage. However, compared to whole cell lysates, the proteome of urine is suspected to be less complex [14–17]. As such, we have used a modified strategy to minimize sample processing by altering two parameters: 1) time of SDS-PAGE fractionation and 2) length of column and gradient during liquid chromatography. First, during a typical mini-gel SDS-PAGE fractionation, proteins migrate 7–8 cm and the gel lane is divided into 10–24 fractions, each to be processed separately via GeLC-MS/MS. However, we performed SDS-PAGE fractionation on the TCA-extracted protein sample until the proteins migrated only 1 cm into the gel, after which a single 1 cm gel slice is processed per lane. Second, following peptide extraction from gel slices, traditional LC-MS/MS methods then analyze the eluent from a 12–15 cm long reversed phase capillary column over a 1 hr period, however, we used a 45 cm-long column and eluted over a 3 hr period. This strategy simplifies sample processing while allowing efficient peptide fractionation upstream of mass spectrometric analysis.

We present the first comparative mass spectrometry-based proteomic analysis of urine in the study of chronic pancreatitis. The aim of this study is to determine the feasibility of

identifying diagnostic protein biomarker candidates of chronic pancreatitis in urine using a new variation of well-established GeLC-MS/MS strategy employing minimal pre-fractionation.

Results

Several hundred proteins were identified in each specimen

SDS-PAGE analysis (Figure 2) showed that, as expected, simple visualization of gels in not sufficient to determine proteomic differences between cohorts. Our subsequent mass spectrometry-based analysis of the gel lanes identified a total of 609 unique proteins in 15 samples (Supplemental Table 1). Figure 3A depicts the redundancy of each protein over all samples, i.e. proteins were grouped according to the number of samples in which they were identified. Approximately 25% of the proteins were identified in 10 or more samples and greater than 45% of the proteins were identified in 5 or more samples.

When examining each cohort individually (Figure 3B), the protein redundancy distribution within each cohort was similar to that of the whole sample set; approximately 30 – 45% of the identified proteins were identified in 5 or more samples and the largest proportion of proteins (25 – 40%) were identified in only one sample.

Differentially-expressed proteins were identified in severe chronic pancreatitis (sCP) versus healthy controls (HC)

We identified a total of 430 unique proteins in healthy control subjects and 474 unique proteins in subjects with sCP. Comparing proteins identified in both cohorts, 127 proteins (Supplemental Table 2) were exclusive to the sCP cohort and 82 (Supplemental Table 3) were exclusive to the HC cohort (Figure 4A). The majority of the proteins exclusive to a specific cohort were identified in only 1 or 2 individual samples, making it difficult to assess the significance of these proteins as markers of pancreatic disease. However, we did identify several proteins in 3 or more of the 5 samples. In the sCP cohort, Ig lambda-7 chain C region, carbonic anhydrase 1 and neutrophil gelatinase-associated lipocalin were identified in 3 of the 5 samples. In the HC cohort, 6-phosphogluconolactonase appeared in all 5 sCP samples; ephrin type-B receptor, phosphatidylcholine -sterol acyltransferase, and N(G),N(G)-dimethylarginine dimethylaminohydrolase appeared in 4 samples; and Poliovirus receptor-related protein 4 and CD276 appeared in 3 of the 5 samples. These exclusive proteins, particularly those appearing in 4 or 5 samples, represent promising biomarker candidates and targets for further investigation.

Additionally, a total of 348 proteins were found to be common to both cohorts. Using the QSPEC algorithm, we compared the spectral counts of these common proteins to identify proteins present in statistically significant different abundance between the two cohorts. We identified 49 proteins (Supplemental Table 4) significantly more abundant in the sCP cohort and 52 proteins (Supplemental Table 5) significantly more abundant in the HC cohort (Figure 4B). Combining these two sets of proteins (those exclusive to and those statistically more abundant in a particular cohort) we determined that 176 (i.e., 127+49) and 134 (i.e.,

82+52) proteins were differentially abundant in the sCP and HC cohorts, respectively, which we refer to as sCP′ and HC′.

A third (mixed diagnosis, MD) cohort was analyzed to control for pathologic changes in the urine proteome that may not be directly due to chronic pancreatitis

A cohort composed of patients with chronic abdominal pain, acute pancreatitis, and a participant with "borderline" chronic pancreatitis was included in our analysis as these individuals represent the intended use population, i.e., clinical patients commonly presenting to a tertiary care pancreas center for evaluation of abdominal pain.

We identified a total of 386 unique proteins in this mixed diagnosis (MD) cohort. We compared the sCP′ protein subset to the proteins identified from MD cohort, as well as the HC′ protein subset to the proteins identified from MD cohort. In each of these comparisons, overlapping proteins (not exclusive to either the sCP′ or the HC′ cohort) will be eliminated from our list of biomarker candidates. Compared to the MD cohort, 104 (Supplemental Table 6) of the 176 proteins in the sCP′ subset remained exclusive to the sCP cohort, while 67 (Supplemental Table 7) of the 135 proteins in the HC′ subset continued to be exclusive to the HC cohort (Figure 5A). As such, 72 proteins were identified in both the sCP′ subset of proteins and the MD proteome, while 67 proteins were identified in both the HC′ subset and the MD proteome.

We also investigated if differences existed quantitatively in the proteins determined to be in common between the sCP′ and MD cohorts, as well as the HC′ and MD cohorts. Using spectral counting and QSPEC, we compared the MD cohort to the HC′ and sCP′ for significant differences in the abundance of common proteins (Figure 5B). Those proteins found with similar abundance likely represent confounding pathophysiological processes, and not chronic pancreatitis. Consequently these proteins were eliminated as potential diagnostic biomarkers of chronic pancreatitis. First, we examined the comparison of the HC′ and MD cohorts (Figure 5B *left*). This analysis showed that of the 72 proteins common to both sCP′ and MD, 1 was of greater abundance in the MD cohort, 42 were of similar abundance. More importantly, 29 proteins (Supplemental Table 8) were of significantly greater abundance in the sCP′ subset, representing the diagnostic biomarker candidates of chronic pancreatitis. Second, we examined the comparison of the HC′ and MD cohorts (Figure 5B **right**). Of the 67 proteins common to both HC′ and MD, 18 proteins (Supplemental Table 9) were of significantly higher abundance in HC′ subset and thus remained potentially useful targets for diagnostic testing. Additionally, 1 protein was more abundant in the MD cohort and 48 did not differ in abundance. Thus, a total of 49 proteins were eliminated as potential biomarker candidates of a normal pancreas. Therefore, our filtering strategy reduced potential confounding by eliminated many proteomic changes representing a disease state, but not necessarily chronic pancreatitis.

We identified a core set of proteins common to all 15 samples

Of the 609 proteins identified, 50 were present in all 15 samples (Table 2). These 50 proteins are readily identified using our minimal sample processing GeLC-MS/MS workflow. An ideal biomarker would be detected without the need for excessive enrichment

or sample processing. Differentially expressed proteins readily identified with mass spectrometry are advantageous over those requiring large-scale preparation, as long as sensitivity and specificity are not compromised. Paired comparisons of the cohorts using QSPEC analysis identified 25 proteins common to all cohorts and present in statistically similar abundance. These proteins include beta-2-glycoprotein, fibronectin, insulin-like growth factor-binding protein 7, osteopontin, prothrombin, and serotransferrin. If validated in larger cohorts, several of these proteins may serve as candidates for normalization controls, which may be integrated into future, directed mass spectrometry workflows.

Gene ontology analysis revealed different classes of proteins which were identified exclusively or in higher abundance in the sCP and HC cohorts

Using the DAVID interface, we categorized the potential biomarker candidate proteins according to their functional class (Figure 6). We compared the proteins in the sCP′ subset (which includes the 104 proteins that are exclusive to the sCP cohort, and the 29 proteins that were of higher abundance after filtering out those which overlap with the mixed diagnosis (MD) cohort) and those in the HC′ subset (which includes the 67 proteins that are exclusive to the HC cohort and the 18 proteins that were of higher abundance, after filtering out those which overlap with the mixed diagnosis (MD) cohort). The sCP cohort proteins correspond to those listed in Supplementary Tables 6 and 8 and the HC cohort proteins correspond to those listed in Supplementary Tables 7 and 9. A wide range of protein classes were identified; of note, proteases and other enzyme classes (hydrolase, oxidoreductase, and transferase) were more commonly identified in the sCP cohort. Proteins in this class included both secreted and lysosomal proteins: lysosomal protective protein, dipeptidyl peptidase 4, plasma glutamate carboxypeptidase, dipeptidyl-peptidase 1, pepsin, and members of the cathepsin family (B, D, and Z). The HC cohort urine was rich in defense/ immunity-related proteins, cell adhesion molecules, receptors, and extracellular matrix (ECM) proteins. Of note are the defense/immunity-related proteins which included nectin 3, neogenin, and several immunogloblins which appear to be more highly abundant in the HC cohort. The investigation of these protein classes will allow us to target, not only single proteins, but classes, with protein microarrays ELISA, western blotting and/or targeted mass spectrometry experiments.

Discussion

We used a modified GeLC-MS/MS strategy for a chronic pancreatitis biomarker discovery assay to identify potential protein targets in urine for subsequent validation. Using this strategy, we identified a total of 609 proteins in the urine of 15 individuals. We qualitatively and quantitatively compared the proteins identified in severe chronic pancreatitis (sCP) and healthy control (HC) urine. We established subsets of differentially abundant proteins (i.e., exclusive to and of statistically greater abundance): 176 proteins in the sCP cohort and 134 proteins in the HC cohort. To isolate chronic pancreatitis as the cause for the enrichment of certain proteins, we eliminated proteins which were also present in a mixed diagnosis (MD) cohort. The MD cohort represents the typical clinical presentation of referred patients: individuals with chronic abdominal pain, acute pancreatic, and probable, but not severe, chronic pancreatitis. This strategy removed approximately one third of the proteins in each

subset. Furthermore, gene ontology analysis identified certain classifications of proteins, such as proteases (particularly serine proteases) and defense/immune proteins as potential targets for directed analysis. In addition, of the 609 proteins identified, 50 were identified in all 15 samples, and as such may be useful for normalization in downstream assays.

Of particular interest for further investigation may be the cathepsin family of proteins. Three cathepsin subtypes (cathepsin B, D, and Z) were found to be more abundant in sCP compared to the HC and MD cohorts. This family of proteins is involved in mammalian cellular turnover, including bone absorption [18]. In a prior study, low-trauma bone fracture appeared to have a higher prevalence in chronic pancreatitis [19]. However, cathepsins have a wide range of cellular functions, including amyloid plaque breakdown in strokes [20], cancer [21], and arthritis [22] but have not been thoroughly studied in pancreatic disease. The role of these particular members of the cathepsin family in bone turnover and its relationship to chronic pancreatitis merits further investigation.

Relative to recent studies, in which thousands of proteins have been identified in urine, 609 proteins is a small number to be identified via mass spectrometry-based proteomics [14–17]. However, our goal was not to obtain a comprehensive proteomic profile of urine, but rather to use minimal sample processing to discover potential protein targets which could be utilized in efficient directed mass spectrometry experiments on larger cohorts. By limiting fractionation, we will likely identify the most abundant proteins. As such, for translational studies seeking an ELISA or dip-stick test as a diagnostic, enrichment and fractionation is a deterrent to general acceptance and the characterization of abundant proteins is a more practical strategy.

Our previous biomarker-driven studies focused on the proteomic analysis of pancreatic fluid [4, 7–9]. While pancreatic fluid is the proximal exocrine secretion of the pancreas, its collection is invasive, requires sedation and is performed only at a limited number of specialized centers. Biomarkers found in systemic fluids may be less specific than those obtained from proximal organ fluids, as the origin of the suspected biomarkers may be difficult to attain. Moreover, the complexity of systemic fluids poses a considerable challenge to investigating the mechanisms of disease. However, our aim is to pursue a non-invasively-collected fluid (i.e., urine) as an alternative for use in biomarker discovery due to the relative ease of collection and analysis, a favored characteristic of a diagnostic clinical test.

A major advantage of the methodology described herein is the minimal sample processing necessary for proteomic analysis. Apart from TCA precipitation, to concentrate and extract proteins from the non-protein constituents of urine, no sample enrichment is performed. Although SDS-PAGE was used to visualize proteins prior to tryptic digestion, gel-free in-solution digestion may be an equivalent alternative. We do caution that for protease-rich fluids, such as pancreatic fluid, in-solution digestion may promote undesired proteolysis by endogenous enzymes producing non-tryptic peptides, which will complicate database searching and protein identification. Regarding pancreatic fluid, we postulate that the denaturing conditions of SDS-PAGE and subsequent fixing of the protein in the polyacrylamide matrix prevents refolding of protease active sites, limiting non-tryptic

cleavage of proteins. However, in urine such spurious protease activity may not be as prevalent, but caution may be warranted.

The use of lengthy reversed-phase columns and extended liquid chromatography gradients provide fractionation that is directly in-line with the mass spectrometer. The typical column length for a nanospray mass spectrometry experiment is 12–18 cm, however, we used 45 cm for the present study, along with an extension of the gradient from 1 to 3 hr. Coinciding with the advent of ultra-high pressure liquid chromatography (UHPLC) systems, longer columns, typically packed with reversed-phase C18 particles of smaller diameter, can be packed and show promise for the in-line fractionation of complex digests [23, 24]. Additional pre-fractionation, at the subcellular level and/or via full 24 fractions on SDS-PAGE, strong cation exchange chromatography, isoelectric focusing, or OFFGEL (Agilent), fractionation would increase the proteome coverage. However, such procedures would concomitantly increase sample processing times and may result potentially in sample loss due to the additional processing procedures. Future studies may further lengthen the column to provide greater protein loading capacity and elongate the gradient for greater fractionation.

To increase further the proteome coverage, depletion of highly abundant proteins may be justified, although caveats persist. The depletion of relatively high abundance proteins, such as albumin and immunoglobulins found in our core proteome subset, may be advantageous for biomarker discovery. Albumin can be depleted using dye-based columns, often with Cibachrome Blue dye [25], or monoclonal antibodies that improve specificity [26]. Additional abundant proteins are depleted often to improve identification of low abundance proteins. For example, multiple affinity removal columns capable of removing up to 14 of the most abundant proteins, simultaneously, are commercially available (MARS - Multiple Affinity Removal System, Agilent) [27] [26]. However, depletion of certain proteins, such as albumin (that functions as carrier proteins), may result in the loss or co-depletion of other significant proteins [28]. Furthermore, each additional step in the workflow may result in greater sample loss and variability.

Protein biomarkers of chronic pancreatitis in urine could potentially offer an innovative and non-invasive modality for diagnosis, staging and prognostication of the disease. In addition to urine, blood can also be collected non-invasively and used in protein-based chronic pancreatitis biomarker discovery. The human blood proteome is a reflection of the specific physiological state at a given point in time, rich in potential biomarkers from a variety of organ systems. Consistently detected alterations in the proteome can potentially be used for disease diagnosis, staging and prognosis [29]. Blood (both serum and plasma) has one of the highest dynamic ranges of any body fluid, with specific protein concentrations spanning over 10 orders of magnitude [30]. Although the dynamic range of the urine proteome still spans several orders of magnitude, its simplicity compared to serum makes it an attractive alternative in the search for diagnostic biomarkers of chronic pancreatitis [31]. For example, during urine production, renal blood flow undergoes glomerular ultrafiltration, greatly reducing urine protein complexity, thereby simplifying analysis and preparation methods [32–34]. In addition, up to 30% of proteins in urine are not of urinary tract origin, but are from distant sites [35]. Although urine was chosen over blood derivatives for analysis because of this decreased complexity and narrower dynamic range, the methods described

herein are also relevant to proteomic biomarker discovery in blood, as well as other body fluids [36].

Conclusions

The diagnosis of early chronic pancreatitis remains elusive. Early detection is vital to initiate treatment within the therapeutic window before irreversible pancreatic scarring and dysfunction, and before symptoms become debilitating. Here we have presented a mass spectrometry-based strategy for chronic pancreatitis biomarker discovery in urine. Our data show both qualitative and quantitative differences between the urine proteomes of healthy subjects and that of subjects with severe chronic pancreatitis. To control for potential confounding diagnoses, we also compared a mixed disease cohort representing typical patients who are commonly referred to a pancreas center, whose protein profile may reflect a diseased state, but not necessarily that of chronic pancreatitis. Based on these investigations, we have assembled a subset of potential diagnostic biomarkers for chronic pancreatitis. Several of these proteins can be categorized as proteins having roles in defense and immunity and proteases. As such, future studies may develop assays to target these particular classes of proteins. The specific proteins may also serve as targets for further studies using directed mass spectrometry approaches or classical biochemistry techniques, such as ELISA or western blotting.

Methods

Study Population

This protocol was approved by the Institutional Review Board at Brigham and Women's Hospital (BWH) (IRB # 2007-P-002480/1 for severe chronic pancreatitis (sCP) and mixed disease (MD) cohorts, and 2011P-001391 for healthy control volunteers). The experimental cohorts were assembled from adult patients referred to the Center for Pancreatic Disease (BWH) for evaluation of abdominal pain, and the healthy control (HC) adult volunteers were recruited from the general population. Patient characteristics are listed in Table 1.

Materials

SeeBluePlus2 Pre-Stained standard (LC5925), LDS (lithium dodecyl sulfate) sample buffer (NP0008), NuPAGE 4–12% Bis-Tris polyacrylamide gels (NP0335), SimplyBlue Coomassie stain (LC0665), and MES-SDS (2-(N-morpholino)ethanesulfonic acid-sodium dodecyl sulfate) electrophoresis buffer (NP002) were from Invitrogen (Carlsbad, CA). Sequencing-grade modified trypsin (V5111) was obtained from Promega (Madison, WI). Other reagents and solvents were from Sigma-Aldrich and Burdick & Jackson, respectively.

Experimental workflow

The general experimental workflow (Figure 1) was as follows: 1) Urine samples were collected (clean-catch); 2) proteins were extracted from the sample by trichloroacetic acid (TCA) precipitation; 3) proteins were reduced with dithiotreitol (DTT) and alkylated with acrylamide; 4) proteins were electrophoresed into an SDS-PAGE gel for a length of 1 cm; 5) proteins from each sample were in-gel tryptically digested in a single gel slice; 6) peptides

were fractionated using a 45 cm capillary reversed-phase (C18) column with a 3 hr linear gradient, in-line with an LTQ-FT Ultra mass spectrometer; 7) bioinformatics processing using ProteinPilot for database searching, QSPEC [37] to determine statistically significant differences in spectral counts between cohorts, and DAVID [38, 39] to perform gene ontology analysis.

Sample preparation

Urine collection and storage—Urine from the subjects' second void of the day was collected using the clean-catch method. The urine was promptly aliquoted and stored at −80°C until analysis. The urinary protein concentration was determined using the bicinchoninic acid (BCA) colorimetric assay.

TCA precipitation of urine—A total of 125 μL of ice-cold 100% TCA was added to 1 mL of each urine sample. The samples were vortexed for 5 seconds and incubated at 4°C for 2 hours. The samples were then centrifuged at 20,000×g at 4°C for 30 minutes and the supernatant carefully aspirated and discarded. One milliliter of 100% ice-cold acetone was added to the pellets, which were then briefly vortexed and incubated at −20°C for 1 hour. The samples were centrifuged again at 20,000×g at 4°C for 30 minutes, and the pellets washed twice with 100% ice-cold acetone. The final pellets were allowed to air dry at room temperature.

SDS-PAGE analysis

LDS sample buffer, with 50mM DTT, was added to each sample to achieve a 1X concentration. The samples were incubated at 56°C for 30 min and allowed to cool. The samples were then alkylated with 1% acrylamide and incubated for 30 min at 23°C. Proteins (approximately 100 μg) were fractionated by SDS-PAGE at 150 volts in MES buffer for a total migration length of 1 cm. Gels were rinsed in deionized water for 10 min, fixed in 45% methanol/45% water/10% acetic acid for 30 min, stained with SimplyBlue Coomassie for 1 hour, and destained overnight in deionized water.

GeLC-MS/MS analysis

Proteins in the entire 1 cm gel section underwent standard in-gel tryptic digestion using established techniques [40, 41]. Peptides were extracted from each gel section and fractionated by a nanoflow reversed-phase ultra-high pressure liquid chromatography system (nanoLC, Eksigent) in-line with a linear trap quadrupole-Fourier transform ion cyclotron mass spectrometer (LTQ-FT Ultra, Thermo Scientific). The reversed-phase liquid chromatography columns (45 cm long × 150 μm ID, New Objective, Woburn, MA) were packed in-house (Magic C18, 5 μm, 100 Å, Michrom BioResources). Samples were analyzed with a 3-hr linear gradient (5–29% acetonitrile with 0.2% formic acid), and data were acquired in a data-dependent manner using 6 MS/MS scans for every full scan spectrum.

Bioinformatics and Data Analysis

Mascot generic files ("mgf") were generated using MSconvert software [42]. All MS data generated from the gel sections were searched against the UniProt database (downloaded November 11, 2011) using the Paragon Algorithm [43] integrated into the ProteinPilot search engine (v. 4; ABSciex). Search parameters were set as follows: sample type, identification; Cys alkylation, acrylamide; Instrument, Orbitrap/FT (1–3 ppm), LTQ MS/MS; special factors, gel-based ID; ID focus, Biological Modifications; database, UniProt database; detection protein threshold, 95.0%; and search effort, thorough ID. Species specificity was *Homo sapiens*. We used Proteomics System Performance Evaluation Pipeline (PSPEP) to determine the cutoff that would be result in a 1% global false discover rate at the protein level.

Venn diagrams

The VENNY on-line Venn diagram plotter was used to obtain lists of unique and common proteins among the cohorts investigated [44].

QSPEC spectral counting analysis

Relative protein quantification was performed using a label-free technique, spectral counting, which compared the number of identified peptide-spectra matches for the same protein across multiple data sets. To search for differences in the protein profile among data sets, spectral counts were normalized based on the total spectral counts, as outlined previously [45]. Briefly, spectral counts of each protein were first divided by the total spectral counts of all proteins from the same sample, and then scaled by multiplying the total spectral counts of each sample by the number of spectral counts of the sample with the highest number of spectral counts. Significance analysis of our normalized spectral count data was performed using QSPEC, a recently published algorithm for determining the statistical significance of differences in spectral count data from two cohorts [37]. This algorithm used the Bayes Factor, in lieu of the p-value, as a measure of evidential strength [46, 47]. By convention, a Bayes factor greater than 10 suggested strong evidence that a particular protein was differentially expressed between the two cohorts; thus a value of 10 was used as our significance threshold [48].

DAVID Functional Annotation Bioinformatics Microarray Analysis

We used the DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Database (http://david.abcc.ncifcrf.gov/) interface [38, 39] to classify proteins according to the Panther Protein Class [49].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

CP	chronic pancreatitis
GeLC-MS/MS	in-gel tryptic digestion followed by liquid chromatography-tandem mass spectrometry
HC	healthy controls
LTQ-FTICR	linear trap quadrupole- Fourier transform ion cyclotron resonance mass spectrometry
MD	mixed diagnosis
sCP	severe chronic pancreatitis
TCA	trichloroacetic acid

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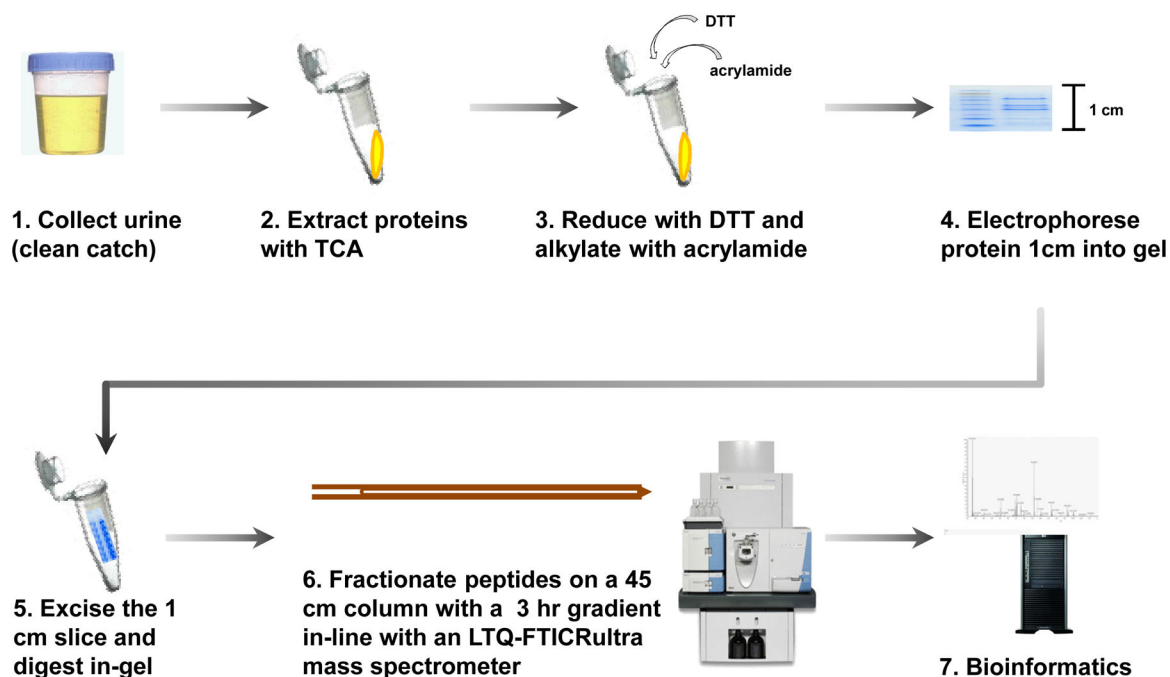
Figure 1. Experimental workflow

The method was as follows: 1) Urine was collected via clean-catch. 2) Proteins were extracted with trichloroacetic acid (TCA) precipitation. 3) Proteins were reduced with dithiotreitol (DTT) and alkylated with acrylamide. 4) Proteins were separated via SDS-PAGE for a length of 1 cm. 5) The single band was excised and in-gel tryptically digested. 6) Peptides were fractionated on a 45 cm column with a 3 hr gradient in-line with an LTQ-FTICR Ultra mass spectrometer. And 7) Bioinformatics tools, such as ProteinPilot, QSPEC, and DAVID were used to identify, quantify, and characterize the collected data.
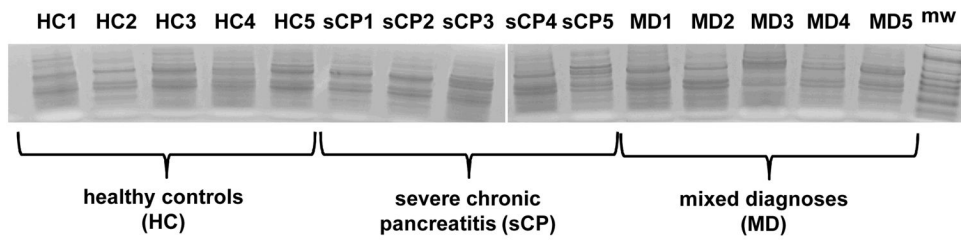
Figure 2. SDS-PAGE 1 cm separation gel image
Fifteen samples - 5 each for healthy controls (HC), severe chronic pancreatitis (sCP), and mixed diagnoses (MD) - were analyzed via SDS-PAGE.
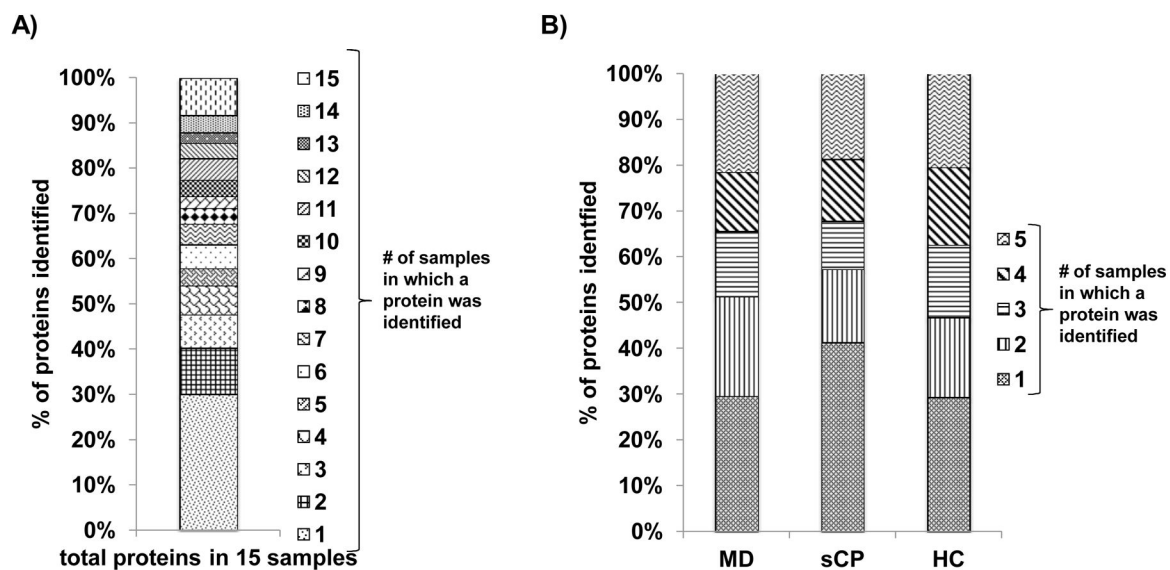
Figure 3. Bar graphs examining redundancy among identified proteins

A) Redundancy of proteins identified in all 15 samples indicating the proportion of the total proteins identified in a specified number (1 through 15) of samples. B) Redundancy of proteins categorized by cohort (n=5), indicating the proportion of proteins identified in a specified number (1 through 5) of samples.
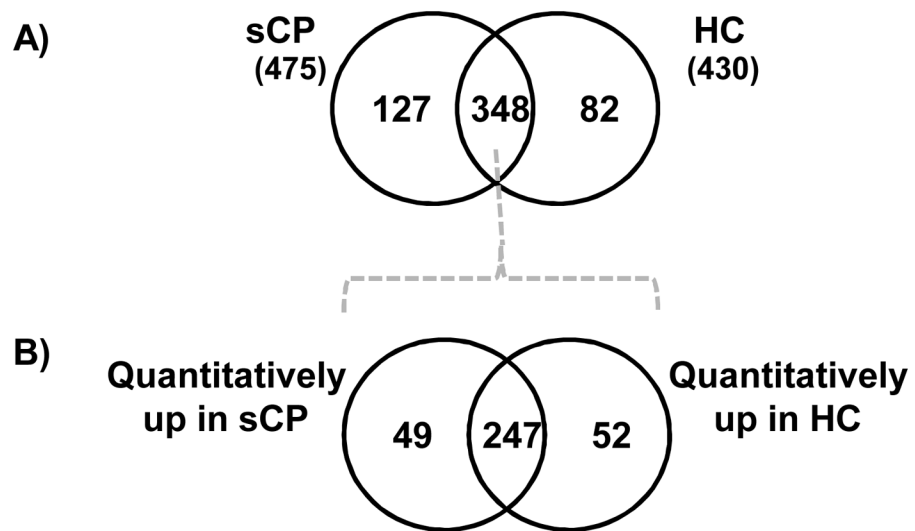
Figure 4. Venn diagrams comparing identified proteins for the sCP and HC cohorts
A) Proteins identified in sCP and HC cohorts were compared qualitatively to identify proteins exclusively to a particular cohort. B) The 348 common proteins were analyzed further using the QSPEC algorithm to identify statistically significant differences between the two cohorts.
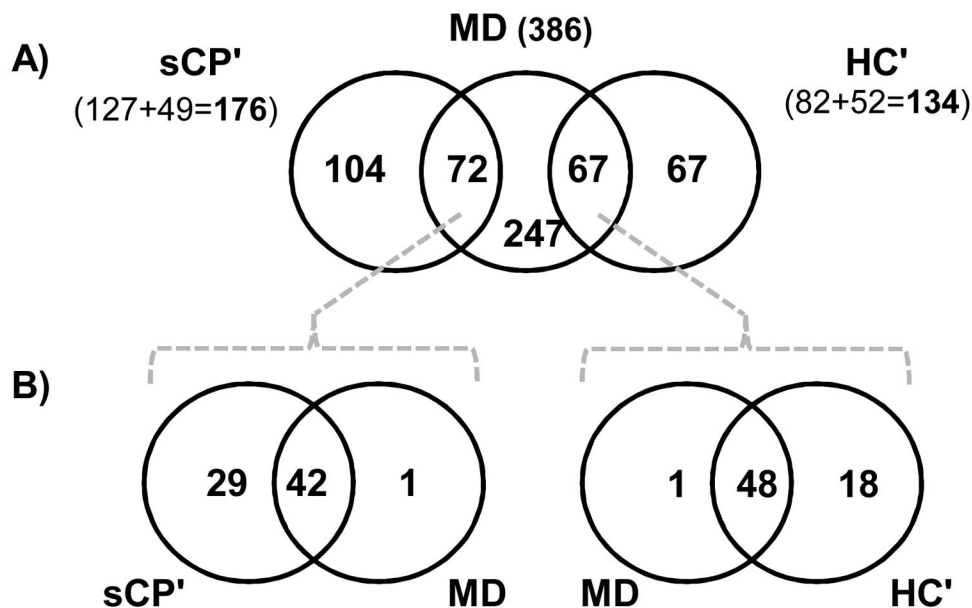
Figure 5. Venn diagrams comparing differentially expressed proteins for the sCP and HC cohorts with those identified in the MD cohort

A) Exclusive and statistically different proteins were combined appropriately into 2 subsets, sCP′ and HC′, which were compared qualitatively with the MD cohort to eliminate proteins that are not specific to the chronic pancreatitis disease state. B) Proteins that were shared between MD and the other cohorts were subjected to QSPEC analysis and proteins with no significant difference between sCP′ and MD, as well as those with no significant difference between HC′ and MD, were removed from the set of potential chronic pancreatitis targets.
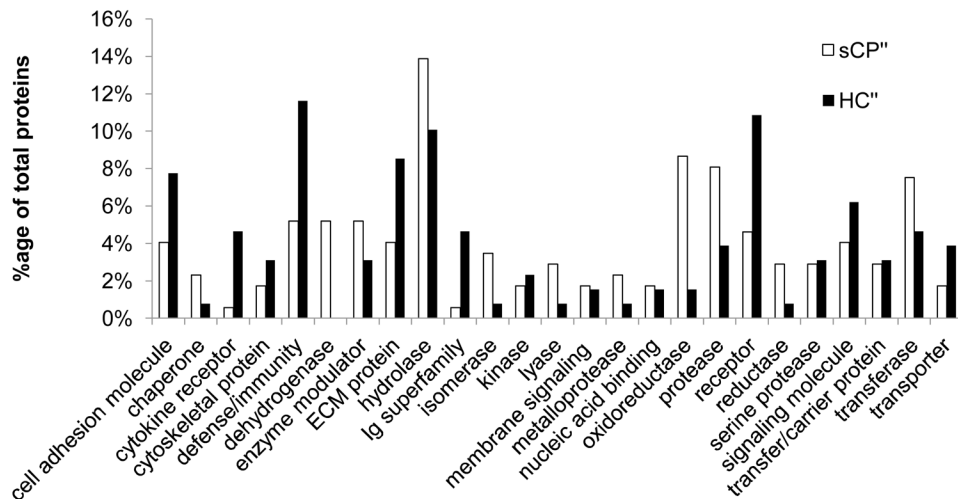
Figure 6. Gene ontology classification of proteins differentially abundant in severe chronic pancreatitis (sCP) and healthy controls (HC)
Functional class categorization of the aforementioned protein subsets according to Panther classification using the DAVID interface.

Table 1

Cohort characteristics

	Healthy controls (HC)					Severe chronic pancreatitis (sCP)					Mixed diagnoses (MD)				
	HC1	HC2	HC3	HC4	HC5	sCP1	sCP2	sCP3	sCP4	sCP5	MD1	MD2	MD3	MD4	MD5
Gender	M	M	M	M	M	F	M	M	M	M	M	F	M	F	M
Race	W	B	W	W	W	W	W	W	W	W	W	H	W	W	W
Age (years)	n/a	n/a	n/a	n/a	n/a	53	44	78	68	33	74	42	69	39	54
MANNHEIM diagnosis	n/a	n/a	n/a	n/a	n/a	Def CP	Def CP	Def CP	Def CP	Def CP	AP	AP	Prob CP	CAP	CAP
Atlanta AP severity	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	Severe	Mild	n/a	n/a	n/a
[HCO$_3$]	n/a	n/a	n/a	n/a	n/a	38	50	36	39	60	n/a	n/a	0	0	100
Smoking	n/a	n/a	n/a	n/a	n/a	2	0	1	2	1	0	0	0	0	2
Alcohol	n/a	n/a	n/a	n/a	n/a	1	2	1	2	1	2	0	0	2	2

AP, acute pancreatitis; n/a, available; M, male; F, female; W, white; H, Hispanic; B, black; CP, chronic pancreatitis; Def CP, definite chronic pancreatitis; Prob CP, probable chronic pancreatitis; CAP, chronic abdominal pain. Smoking and Alcohol: 0, never smoking; 1, past; 2, current.

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Table 2

Core set of proteins identified in all 15 samples.

Protein names	UniProt entry	spectral counts			significant difference (p<0.05)
		ΣHC	ΣsCP	ΣMD	
Alpha-1-antichymotrypsin	P01011	556	392	598	no
Alpha-1-antitrypsin	P01009	1527	606	1264	
Alpha-2-HS-glycoprotein	P02765	485	383	397	no
Apolipoprotein D	P05090	2000	1512	1375	
Basement membrane-specific heparan sulfate protein	P98160	2261	2535	1981	
Beta-2-glycoprotein 1	P02749	330	322	364	no
Cathepsin D	P07339	355	760	270	
CD44 antigen	P16070	261	298	352	no
CD59 glycoprotein	P13987	148	283	211	
Clusterin	P10909	612	550	435	no
Collagen alpha-1	P12109	1215	658	693	
Cubilin	O60494	1348	1075	1122	no
EGF-containing fibulin-like extracellular matrix protein 1	Q12805	318	479	295	no
Fibronectin	P02751	587	673	775	no
Galectin-3-binding protein	Q08380	513	504	461	no
Gelsolin	P06396	868	1287	664	no
Hemopexin	P02790	496	353	534	no
Ig alpha-1 chain C region	P01876	821	982	886	no
Ig gamma-1 chain C region	P01857	1949	1350	1794	
Ig gamma-2 chain C region	P01859	2268	1483	1942	no
Ig heavy chain V-III region BRO	P01766	225	264	292	no
Ig kappa chain C region	P01834	3456	8024	3137	
Ig kappa chain V-III region VG	P04433	141	246	117	
Insulin-like growth factor-binding protein 7	Q16270	437	407	386	no
Inter-alpha-trypsin inhibitor heavy chain H4	Q14624	1455	1376	1036	
Keratin, type I cytoskeletal 10	P13645	937	1014	1399	no
Keratin, type I cytoskeletal 9	P35527	861	787	1378	

Protein names	UniProt entry	spectral counts			significant difference (p<0.05)
		ΣHC	ΣsCP	ΣMD	
Keratin, type II cytoskeletal 1	P04264	1409	1543	2209	
Kininogen-1	P01042	2920	1983	2549	
Lysosomal alpha-glucosidase	P10253	885	762	1046	no
Monocyte differentiation antigen CD14	P08571	590	568	452	no
Non-secretory ribonuclease	P10153	374	607	591	
Osteopontin	P10451	790	812	895	no
Pancreatic alpha-amylase	P04746	2035	868	4547	
Peptidoglycan recognition protein 1	O75594	343	244	264	no
Plasma serine protease inhibitor	P05154	737	370	492	no
Polymeric immunoglobulin receptor	P01833	1393	1569	1300	no
Pro-epidermal growth factor	P01133	1730	587	892	
Prostaglandin-H2 D-isomerase	P41222	1395	3798	1617	
Protein AMBP	P02760	2019	6600	3094	
Prothrombin	P00734	406	483	332	no
Retinol-binding protein 4	P02753	337	483	315	
Secreted and transmembrane protein 1	Q8WVN6	242	311	133	no
Serotransferrin	P02787	1933	1449	2179	no
Serum albumin	P02768	16657	13767	20708	
Uromodulin	P07911	8539	2502	5943	
Vasorin	Q6EMK4	689	442	495	
Vesicular integral-membrane protein VIP36	Q12907	847	460	503	
Vitamin D-binding protein	P02774	407	208	425	
Zinc-alpha-2-glycoprotein	P25311	872	1885	1107	

HC, healthy controls; sCP, severe chronic pancreatitis; MD, mixed diagnosis samples.