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Proteomic Analysis (GeLC-MS/MS) of ePFT-Collected Pancreatic Fluid in Chronic Pancreatitis

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Abstract

Chronic pancreatitis is characterized by inflammation, fibrosis, pain, and loss of exocrine function of the pancreas. We aimed to identify differentially-expressed proteins in the ePFT-collected pancreatic fluid from individuals with chronic pancreatitis (CP; n=9) and controls with chronic abdominal pain not associated with the pancreas (NP; n=9). Using GeLC-MS/MS techniques, we identified a total of 1391 different proteins in 18 pancreatic fluid samples. Of these proteins, 257

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and 413 were identified exclusively in the control and chronic pancreatitis cohorts, respectively, and 721 were identified in both cohorts. Spectral counting and statistical analysis thereof revealed an additional 38 and 77 proteins that were up- or down-regulated, respectively, in the pancreatic fluid from individuals with chronic pancreatitis. As expected, gene ontology analysis illustrated that the largest percentage of differentially-regulated proteins was secreted/extracellular in origin. In addition, proteins that were down-regulated with statistical significance in the chronic pancreatitis cohort were determined to have biological function of proteases, corresponding to the canonical pancreatic insufficiency associated with chronic pancreatitis. Proteins enriched in pancreatic fluid from chronic pancreatitis patients had roles in fibrosis, inflammation, and pain, whereas digestive enzymes were significantly less abundant. Our workflow provided a mass spectrometry-based approach for the further study of the pancreatic fluid proteome which may lead to the discovery potential biomarkers of chronic pancreatitis.

Keywords

pancreas; pancreas juice; pancreatic function test; biomarkers; label-free quantification

1. Introduction

Chronic pancreatitis (CP) is a disease manifested by severe inflammation, progressive fibrosis, intense pain, and eventually loss of exocrine (i.e., fat and protein malabsorption) and endocrine (i.e., diabetes) function of the pancreas. Disorders of the exocrine pancreas affect over 1 million persons in the United States and cost nearly \$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. Nearly 25 % of these hospitalizations and outpatient visits are due to chronic pancreatitis. In addition, patients with chronic pancreatitis are at greater risk of developing pancreatic cancer, which results in more than 35,000 deaths annually and is the 4th leading cause of cancer death in the United States¹.

Clinical diagnosis of chronic pancreatitis is based currently on identifying advanced functional, morphological, and histological features. However, the tissue damage and fibrosis are often irreversible by the time that objective diagnostic features appear. Pancreatic biopsy for histologic diagnosis is not recommended due to potential complications, such as bleeding and fistulae formation, as well as the possibility of sampling error. Currently, pancreas function testing is the non-histological “surrogate” gold standard that is used to diagnose moderate to late stage chronic pancreatitis². The degree of pancreatic dysfunction is determined by measuring specific concentrations of cellular secretory components, typically bicarbonate, in pancreatic fluid following hormone stimulation³. The ability to diagnose chronic pancreatitis prior to irreversible organ dysfunction would revolutionize treatment and potentially lead to therapies designed to retard or modify disease progression before irreversible damages to the pancreas become apparent.

Pancreatic fluid is an excellent source of potential protein biomarkers for the diagnosis of early chronic pancreatitis. As a proximal body fluid, pancreatic juice is rich in proteins that are shed directly from the pancreas during the cell necrosis which is prevalent in the course of chronic pancreatitis. Changes in the protein expression patterns specific to pancreatic fluid collected from diseased patients can be identified using high-throughput proteomic technologies, such as mass spectrometry. Using mass spectrometric techniques, qualitative and quantitative changes in pancreatic fluid protein profiles can be determined. Proteomic alterations may reflect the underlying mechanisms of disease that have yet to progress to

fibrosis or pancreatic insufficiency and may offer a means for amelioration or retardation of the disease.

To date, several mass spectrometry-based proteomic investigations of pancreatic fluid have been performed using specimens collected surgically or via endoscopic retrograde cholangiopancreatography (ERCP)^{4–12}. In contrast, we use the well-established secretin-stimulated ePFT (endoscopic pancreatic function test) collection method, which is less invasive compared to ERCP and surgery. ePFT permits the safe collection of 10-fold larger volumes of pancreatic fluid without cannulation of the pancreatic duct, thus reducing the risk of post-procedure acute pancreatitis. Secretin stimulates bicarbonate secretion from pancreatic duct cells^{13, 14} and essentially flushes out the proteins, and potentially protein plugs, present in the pancreatic acini and duct. These advantages support ePFT as an exceptional fluid collection method for the comprehensive analysis of the pancreatic fluid proteome^{14–19}.

We present a comparative mass spectrometry-based proteomic analysis of pancreatic fluid from patients with advanced chronic pancreatitis and non-pancreatitis controls. Using our previously established protocol^{20, 21}, we 1) collected pancreatic fluid from the two cohorts via the ePFT method, 2) inactivated proteases and extracted proteins via trichloroacetic acid (TCA) precipitation, 3) fractionated proteins via one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 4) tryptically digested proteins in-gel, 5) analyzed resulting peptides via liquid chromatography-tandem mass spectrometry (LC-MS/MS), and 6) performed bioinformatic data processing which included: a) database searching, b) label-free quantification (spectral counting and statistical analysis thereof) and c) gene ontology and pathway analysis. We have compiled a list of statistically-significant differentially-expressed proteins between our two cohorts, which can be validated further by orthogonal methodologies, such as western blotting, ELISA and protein microarrays.

2. Materials and Methods

Study Population

This protocol was approved by the Institutional Review Board at Brigham and Women's Hospital (BWH) (IRB # 2007-P-002480/1). The study population included adult patients evaluated for abdominal pain in the Center for Pancreatic Disease at BWH. Subjects were referred to BWH to eliminate a pancreatic etiology for their gastrointestinal symptoms. All subjects underwent the following: 1) comprehensive review of history and physical examination, 2) review of radiologic and endoscopic data, and 3) upper endoscopy with ePFT followed by a gastric and duodenal mucosal biopsy.

Materials

ChiRhoStim® synthetic human secretin was from ChiRhoClin (Burtonsville MD). 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). Other reagents and solvents were from Sigma-Aldrich and Burdick & Jackson, respectively.

Experimental Workflow

Figure 1 illustrates the general workflow for the overall analysis as follows: 1) ePFT sample collection, 2) protein precipitation, 3) SDS-PAGE, 4) in-gel tryptic digestion, 5) LC-MS/MS peptide mass determination, and 6) bioinformatic data processing.

Pancreatic Fluid Collection (ePFT method)

The ePFT procedure was performed in three steps:

1. *Pre-procedural assessment.* Prior to upper endoscopy, all study subjects underwent a history and physical examination recording allergies, medications, substance use/abuse, and vital signs. Pre-procedural sedation review included airway assessment based on the Mallampati airway scale and the American Society of Anesthesiologists Physical Status Classification (ASA Class). All study subjects in this protocol had a Mallampati score of B, Class 2, or better, and ASA Class II, or better.
2. *Endoscopic procedure.* Endoscopic collection was performed in a stepwise manner as follows: 1) The patient was placed in the left lateral decubitus position with slight head elevation. 2) The posterior pharynx was sprayed with topical cetacaine spray. 3) A sedation and analgesia bolus was administered. 4) Further sedation doses were administered, if necessary, for patient comfort. 5) After the sedation bolus, a bite-block was placed. 6) Esophagogastroduodenoscopy (EGD) was performed using a standard (10 mm) gastroscope for visualization of the esophagus, stomach, and duodenum. 7) Gastroduodenal fluid was aspirated as completely as possible through the gastroscope. 8) A test dose of synthetic human hormone secretin was administered and patients were monitored for anaphylaxis or adverse reaction. Subsequently a standard weight-based intravenous bolus (0.2 µg/kg) was given over 1 min. 9) Pancreatic fluid was aspirated from the descending duodenum at specific timed intervals following hormonal stimulation and stored on ice.

The duodenal aspirates were collected at 0, 5, 10, 15, 20, 30, 45, and 60 min after stimulation. Based on previously published pancreatic output patterns, only the 30-min time point was used for the ensuing analysis²¹. Biopsies of the stomach and duodenum were obtained to eliminate microscopic gastrointestinal disease, such as *Helicobacter pylori* or celiac sprue as a cause of dyspepsia.

3. *Post-procedural Assessment / Recovery.* Study participants were discharged from the endoscopy unit based on hospital procedural sedation guidelines assessing levels of consciousness, vital signs, oxygen saturation, alertness, gag reflex, degree of nausea, and ability to ambulate.

Pancreatic fluid biochemical analysis

All samples underwent biochemical analysis within 24 hrs. Samples were passed through a serum filter (ML0550, MarketLab, Caledonia, MI) to remove particulates and fibrin microthrombi prior to analysis. All measurements were conducted in the CLIA-certified Brigham and Women's Hospital Clinical Chemistry Laboratory, under the standard operating procedures on an AU640 (Olympus America, Center Valley, PA) automated chemistry analyzer. Sodium, potassium, and chloride were measured by indirect ion-selective electrodes, and total bicarbonate was measured by the two-step phosphoenolpyruvate carboxylase-malate dehydrogenase enzymatic-photometric method²². Samples with results greater than the upper assay limit were diluted into the linear range. The mean peak bicarbonate concentration from previously published studies in secretin-stimulated pancreatic fluid was 103 ± 11 meq/L¹⁵. A threshold limit of 80 meq/l was two standard deviations below the mean and considered abnormal^{14, 19}. Excess pancreatic fluid samples were frozen at -80°C and stored until proteomic analysis.

Pancreatic Fluid Proteomic Analysis

Pancreatic fluid sample preparation—As described above, aliquots of pancreatic fluid samples for proteomic analysis were collected on ice, centrifuged at 4°C at 20,000 × g for 15 min to remove cellular debris, and aliquoted (500 µL) prior to storage at −80°C. Protein concentration was determined using the BioRAD protein assay according to the manufacturer's instructions. In preparation for SDS-PAGE analysis, the proteins from pancreatic fluid specimens were isolated by precipitation with the addition of 12.5% trichloroacetic acid (TCA), as described previously^{20, 21, 23}. This process i) limited protein degradation by instantaneously deactivating enzymes and ii) removed salts that will interfere with the subsequent electrophoretic mobility-based fractionation by SDS-PAGE. The precipitated protein pellets were re-dissolved in 50 µL of reducing Laemmli buffer²⁴ (with 10 mM DTT) for 1 hr at 56°C and alkylated with 1% acrylamide at room temperature for 30 min for subsequent GeLC-MS/MS analysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) prefractionation and liquid chromatography-tandem mass spectrometry (GeLC-MS/MS) of pancreatic fluid specimens—The proteins were fractionated using 4–12% NuPAGE pre-cast SDS-PAGE gels at 175V for 45 min using MES-SDS running buffer. Gels were incubated with SimplyBlue Coomassie stain for 3 hrs and afterward destained in water overnight. Subsequently, entire gel lanes were divided into 15 sections. Proteins in each gel section were digested, in-gel, with trypsin^{25, 26}. The extracted peptides from each gel section were subjected to peptide fractionation using reversed-phase high performance liquid chromatography (HPLC; Thermo Scientific, Waltham, MA) and the gradient-eluted peptides were analyzed by a hyphenated linear trap quadrupole-Fourier Transform ion cyclotron resonance (LTQ-FTICR) mass spectrometer (Thermo Scientific, Waltham, MA). The liquid chromatography columns (15 cm × 100 µm ID) were packed in-house (Magic C18, 5 µm, 100Å beads, Michrom BioResources) into PicoTips (New Objective, Woburn, MA). Samples were analyzed with a 90 minute linear gradient (0–35% acetonitrile with 0.2% formic acid) and data were acquired in a data dependent manner, in which MS/MS fragmentation was performed using the 6 most intense peaks of every full MS scan.

Bioinformatics and Data Analysis

Database searching—All data generated from the gel sections were searched against the IPI-human database (v3.56) using the Mascot search engine (v.2.204; Matrix Science). One miscleavage per peptide was allowed and mass tolerances of ± 10 ppm for precursor and ± 0.8 Da for fragment ions were used. Amino acid modifications: fixed: propionamide (Cys); variable: deamidation (Asn/Gln), pyro-glutamate (N-terminal Glu/Gln), and oxidation (Met). Mascot search results were combined using in-house-developed software. In compliance with recommendations^{27–29} proposed by the major proteomic journals, we presented the following protein identification validation method that minimizes false positives and reports only high confidence identifications. Our false discovery rate (FDR) was 1% at the protein level as determined by searching the same dataset against the target database and a decoy database; the latter featured the reversed amino acid sequences of all the entries in the IPI-human database (v3.56)^{30, 31}.

Spectral counting—Relative protein quantification was accomplished using a label-free technique, spectral counting, which compared the number of identified tandem mass spectra 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 previously outlined³². Specifically, spectral counts of each protein were first divided by the total spectral counts of all proteins from the same sample, and then multiplied by the total

spectral counts of the sample with the maximum total 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 counting data from different cohorts³³. This algorithm used the Bayes Factor in lieu of the p-value, as a measure of evidential strength^{34, 35}. 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 threshold³⁶.

Gene ontology analysis—Gene ontology analysis³⁷ was performed, using the GoFact online tool^{38, 39}, for those proteins common to, exclusive to, or significantly (statistically) different between the cohorts. GoSlim Annotation categories were chosen so as to avoid overlapping categories as follows: *cellular component*: cytoskeleton, cytosol, endoplasmic reticulum (ER), endosome, extracellular matrix, extracellular region, Golgi apparatus, lysosomes, membrane, mitochondrion, nucleus, peroxisome, ribonucleoprotein (RNP) complex, and vacuole; *molecular functions*: enzyme regulator activity, ion binding, kinase activity, lipid binding, nucleic acid binding, nucleotide binding, oxygen binding, peptidase, protein binding, signal transducer, structural molecule, transcription regulator, translation regulator, and transporter.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis—KEGG pathway analysis integrates knowledge about molecular interaction networks, including that generated by genome mapping projects, with information about biochemical compounds and reactions. This allows the classification of a protein, or a group of proteins to specific biological pathways^{40–42}. We used the DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Database (<http://david.abcc.ncifcrf.gov/>) interface^{43, 44} to analyze our protein lists with KEGG pathway analysis.

BioBase ExPlain 3.0 for comparison of proteomics data with gene expression data for pancreatic disease—ExPlain integrates genomic information with biological knowledge databases and computational analysis methods^{45–47}. This database was manually compiled from microarray, miRNA and ChIP-chip/Seq experiments. ExPlain applied an upstream analysis approach, based on the implementation of machine learning and graph topological analysis algorithms, to identify causality key-nodes in the network of pancreatic disease. We used ExPlain 3.0 to compare our proteomics data to that of previously identified biomarkers of pancreatic disease, including pancreatitis and pancreatic neoplasms.

Statistical analysis—Wilcoxon rank-sum non-parametric tests were performed using SAS 9.2 (Cary, NC). P-value < 0.05 was considered statistically significant.

3. Results

Sample collection

Patient medical histories and case report forms were extensively reviewed prior to initiation of our proteomics study. Table 1 summarizes the two patient cohorts in terms of their demographic, radiologic, endoscopic, histologic, and function test data. The chronic pancreatitis cohort had a mean age of 53.7±10.0 years and 5 of 9 individuals were female. The non-pancreatitis control cohort had a mean age of 45.3±13.6 years and 4 of 9 were female. The mean secretin-stimulated pancreatic fluid peak bicarbonate concentration was 93.4±13.6, meq/L in control subjects, consistent with normal pancreatic function, and 37.7±13.3 meq/L in the chronic pancreatitis patients, consistent with severe pancreatic

insufficiency. The control subjects had no history of alcohol abuse, acute recurrent pancreatitis or therapeutic endoscopic pancreaticobiliary procedures involving instrumentation of the pancreas or pancreatic duct. In addition, all imaging studies, including computed tomography (CT), magnetic resonance imaging (MRI), and endoscopic ultrasound (EUS) were not indicative of acute or chronic pancreatic disease. Endoscopic biopsies of the stomach and duodenum were normal in all patients and without evidence of *Helicobacter pylori* infection or celiac sprue. Careful review of all clinical, laboratory, and radiologic data eliminated pancreatic disease as a cause of chronic abdominal pain in the control cohort.

GeLC-MS/MS identified differentially expressed proteins

A total of 3 to 5 mL of pancreatic fluid with a protein concentration ranging from 1–2 mg/mL was collected at the 30 minute time point and used for analysis. Pancreatic fluid was cleared via centrifugation and protein was extracted by TCA precipitation -using our previously published optimized protocol^{20, 21}- in preparation for SDS-PAGE analysis. SDS-PAGE revealed relatively similar, yet distinct, protein patterns within and between cohorts (Figure 2). We performed gel densitometry, using the ImageJ software⁴⁸. Upon performing the Wilcoxon rank sum test on the densitometry measurements between the two cohorts, there is no statistically significant difference between the cohorts (p-value = 0.0811). Although there is patient-to-patient variability within cohorts, there is no overall difference in protein concentration between cohorts. Each gel lane was sectioned into 15 slices, which were individually in-gel tryptically digested, after which peptides were analyzed using liquid chromatography coupled to tandem mass spectrometry (GeLC-MS/MS).

In total, 1391 non-redundant proteins were identified from the 18 samples. “Non-redundant” implies that the same protein is not counted more than once if it appears in multiple samples in a specified comparison. An average of 367 proteins was identified from individual control samples and an average of 433 proteins from individual chronic pancreatitis samples (Figure 3A). These proteins corresponded to an average of 2324 and 2808 peptides identified per control and chronic pancreatitis sample, respectively (Figure 3B). Wilcoxon rank sum tests revealed no statistically significant difference (95% confidence interval) between the controls and the chronic pancreatitis patients in terms of either the number of proteins (p-value = 0.2029) or peptides (p-value=0.2092), agreeing with the statistical analysis of protein concentrations as determined by densitometry of the SDS-PAGE results, as described above.

Examination of the total non-redundant proteins in each of the two cohorts identified 978 proteins in the control cohort and 1134 proteins in the chronic pancreatitis cohort. Seven hundred and twenty-one (721) proteins were common to both cohorts, 257 (Supplemental Table 1) proteins were unique to the control cohort and 413 (Supplemental Table 2) proteins were unique to the chronic pancreatitis cohort (Figure 4). Of the total 1391 non-redundant proteins, 69 proteins appeared in all 18 samples (Table 2), representing a core set of proteins identified from ePFT-collected pancreatic fluid. These proteins included various isoforms of pancreatic enzymes amylase, aminopeptidase, carboxypeptidase, chymotrypsin, elastase, lipase, and trypsin, as well as several protease inhibitors of the serpin family. When examining these common proteins, it was noted that several proteins did not show significant difference in abundance in the chronic pancreatitis cohort when compared to controls. These proteins include alpha-2-macroglobulin, alpha-1-acid glycoprotein 1, Xaa-Pro dipeptidase, enteropeptidase, as well as several serpins (A1, A3, C1, and G1). As such, one or more of these proteins may be used as normalization standards for proteomic analysis of pancreatic fluid; in a manner analogous to how actin or glyceraldehyde-3-phosphate dehydrogenase is generally used for various types of cell culture. In addition, we have included, in the supplementary material, the core proteomes for the chronic pancreatitis (Supplementary Table 3) and control cohorts (Supplementary Table 4) which are comprised

of proteins identified in all 9 samples of each cohort. Further studies with much larger cohorts and targeted assays will be needed to assess the utility of these proteins for sample normalization.

Spectral counting analysis revealed statistically significant differences in the relative abundance of certain proteins between the two cohorts

Spectral counting analysis was performed using the QSPEC statistical tool⁴⁹. We applied a strict filtering strategy, in which the threshold for statistically significant differences was determined by a Bayes factor greater than 10. Utilizing this threshold, we identified 77 proteins significantly up-regulated (Supplemental Table 5) and 38 significantly down-regulated (Supplemental Table 6) in the chronic pancreatitis cohort. The remaining 606 proteins were not significantly different in abundance in chronic pancreatitis compared to control samples (Supplemental Table 7).

Gene ontology analysis revealed differences in protein function between the two cohorts

We performed gene ontology (GO) analysis of subcellular localization (Figure 5A) and biological function (Figure 5B) using the GOfact online interface (<http://61.50.138.118/gofact>) for proteins which we identified to be exclusive to a particular cohort, differentially expressed (with statistical significance), as well as common to both cohorts. We divided the analysis into 5 groups of proteins that were a) down-regulated in CP, b) absent in CP (i.e., exclusive to controls), c) exclusive to CP, d) up-regulated in CP and e) common to both NP and CP. GO analysis of subcellular localization of proteins in these distinct groups revealed that the highest percentage of identified proteins in 3 of the 5 comparison groups originated from the secreted category (e.g., 47% and 76% of proteins up- and down-regulated in chronic pancreatitis, respectively). As for biological function, the highest percentage of proteins in 4 of the 5 groups were of the protein binding category, with the exception of proteins up-regulated in chronic pancreatitis, was classified as having peptidase activity.

KEGG pathway analysis revealed differences in biological pathways between the two cohorts

Using the DAVID interface^{43, 44}, we surveyed the biomolecular pathways of proteins that we determined to be differentially expressed in the pancreatic fluid of the chronic pancreatitis cohort when compared to controls. Tables 3 and 4 list the pathways, the number of proteins, and the percentage of differentially expressed proteins which were present in that particular pathway. Among the pathways identified for proteins unique to or up-regulated in chronic pancreatitis (Table 3) were the regulation of the cytoskeleton, complement and coagulation cascades, and type 1 diabetes mellitus pathophysiological pathways, as well as other disease-specific pathways. For those proteins that were not identified or were down-regulated in chronic pancreatitis (Table 4), most of the pathways identified were specific to metabolism (e.g., glycolysis, TCA cycle, fatty acid, and glycerolipid metabolism). The identification of these metabolic pathways may be expected, as cellular processes are generally diminished in diseased pancreata.

4. Discussion

We have identified differentially-expressed proteins in ePFT-collected pancreatic fluid of chronic pancreatitis patients, using a GeLC-MS/MS strategy. In total, 1391 non-redundant proteins are determined to be present among 18 samples. Of these, 257 and 413 proteins are identified exclusively in the controls and chronic pancreatitis samples, respectively. In addition, label-free quantification followed by statistical evaluation determined that of the 606 common proteins, 77 are up-regulated and 38 are down-regulated in chronic pancreatitis

when compared to controls. Moreover, GO analysis of these differentially expressed proteins from each cohort reveals that most of these proteins are extracellular. In terms of biological function classification, proteins with peptidase activity comprised the largest proportion (43%) of the proteins that were down-regulated in chronic pancreatitis. This result is expected as we would anticipate that a healthy pancreas would secrete digestive enzymes into the duodenum upon stimulation, however this function would be diminished in the case of a diseased fibrotic pancreas.

We classified further the identified proteins according to the pathways in which they have been previously identified. The KEGG pathway analysis of our differentially expressed proteins reveals that those up-regulated in chronic pancreatitis are identified in disease-related pathways, such as Alzheimer's disease (11 proteins), and prion disease (6 proteins). The identification of pathways indicative of diseased states unrelated to the pancreas may be a result of analogous deregulation at the biomolecular / subcellular level. Specifically, diseases for which given pathways are better understood, compared to chronic pancreatitis, have more entries in the KEGG database. In addition, those proteins that are down-regulated in chronic pancreatitis are involved in cellular homeostasis, and as such may be indicative of cellular dysfunction. Therefore, our bioinformatics analysis agrees with expected findings for chronic pancreatitis: abrogated cellular function in a diseased state, resulting in down-regulation of pancreatic proteases (enzyme insufficiency).

To expand on the KEGG pathway results, we used BioBase to search for proteins which are involved in the four main manifestations of chronic pancreatitis related to exocrine function: fibrosis, inflammation, pain, and exocrine insufficiency. The proteins found in these processes merit further investigation, and may have roles in the pathogenesis and pathophysiology of the disease. Below we highlight several proteins from each of the four categories. Understanding the function of these proteins in relation to chronic pancreatitis may further the knowledge of the molecular mechanisms resulting in the development and progression of the disease.

Several proteins involved in fibrosis-related pathways were determined to be exclusive to or up-regulated in chronic pancreatitis (Table 5). Proteins in this category include alpha-smooth muscle actin, haptoglobin, and serpin A5. Alpha-smooth muscle actin (α -SMA, actin alpha 2) is an isoform of the highly conserved actin family of proteins that is involved in cell motility, structure and integrity. The alpha actin subfamily is a major constituent of the contractile apparatus, and has been shown to be highly expressed in activated pancreatic stellate cells^{50, 51} and may be the direct link to the development of chronic pancreatitis^{51, 52}. Therefore up-regulation of this protein in chronic pancreatitis patients is consistent with pancreatic stellate cell activation.

Haptoglobin (HP protein) is identified in all 9 chronic pancreatitis patients and only 6 controls (fold change: 3.45 higher in chronic pancreatitis with a Bayes Factor of 45.66). Haptoglobin is commonly associated with liver fibrosis⁵³ and may also play a role in the pancreatic disease, as altered post-translational modifications of this protein are involved in pathways related to pancreatic cancer⁵⁴. In fact, a mass spectrometry-based assay, which analyzes the fucosylation of this protein, has been developed for pancreatic cancer. The role of this protein in chronic pancreatitis has not been determined.

Serpin A5 is identified in 8 of the 9 chronic pancreatitis specimens and none of the control specimens. Serpin A5 inhibits activated protein C^{55,56} and plasminogen activators⁵⁷ and has been shown to interact with prostate specific antigen⁵⁸, and PLAU (urokinase-type plasminogen activator)⁵⁹. In chronic pancreatitis, specific changes in protein C expression in the stroma of the pancreas have been shown to modulate the intracellular signaling pathways

that control homeostatic mechanisms, however the specific role of Serpin A5 has not been investigated⁶⁰.

In addition to these fibrosis-related proteins, several proteins involved in inflammation-related pathways were determined to be exclusive to or up-regulated in chronic pancreatitis (Table 6). Proteins in this category include: annexin A5, defensin 5, and neprilysin. Annexin A5 was identified in 5 of the 9 chronic pancreatitis specimens and none of the control specimens. Annexin A5 is involved in the blood coagulation cascade and inhibits the activity of protein kinase C^{61, 62}. A previous study has identified annexin A5 in the pancreatic fluid of pancreatic cancer patients⁹. Interestingly, annexin A5 also inhibits the secretion of phospholipase^{63, 64}, which is one of several pancreatic enzymes that our data shows to have a lower abundance in chronic pancreatitis versus control pancreatic fluid.

Defensin 5 is identified in 7 of the 9 chronic pancreatitis specimens and only 2 of the 9 control specimens (fold change: 5.24 higher in chronic pancreatitis with a Bayes Factor of 149.78). In addition, pro-defensin 5 has been shown to be processed to mature defensin 5 in the human intestinal lumen by trypsin, in a complex in which chymotrypsinogen is also cleaved and activated. The increase in defensin 5 may be due to persistence of this complex resulting from decreased levels of pancreatic enzymes or increased levels of protease inhibitors, such as alpha1-antitrypsin⁶⁵. However, further studies are needed to clarify the roles of both proteins in possible mechanisms regulating the development and progression of chronic pancreatitis.

Neprilysin (MME) is identified in 8 of the 9 chronic pancreatitis specimens and only 2 of the 9 control specimens (fold change: 5.15 higher in chronic pancreatitis with a Bayes Factor of 1176.29). MME - also known as neutral endopeptidase (NEP), CD10, and common acute lymphoblastic leukemia antigen (CALLA) - (is a zinc-dependent metalloprotease enzyme that degrades a number of small secreted polypeptides of up to 30 amino acids⁶⁶. In addition, MME cleaves various isoforms of angiotensin and is involved in the degradation of atrial natriuretic factor^{67, 68} which has been shown to stimulate pancreatic exocrine secretion by interacting with hormones that regulate pancreatic function^{69, 70}. As such, the increase in MME is associated with an decrease in atrial natriuretic factor which in turn decreases the pancreatic exocrine secretion of digestive enzymes. In agreement with our data, the overexpression of neprilysin has been linked previously to pancreatitis and pancreatic cancer⁷¹⁻⁷³.

Several proteins involved in pain-related pathways are determined to be exclusive to or up-regulated in chronic pancreatitis (Table 7). Proteins in this category include: complement C3 (fold change: 4.35 higher in chronic pancreatitis with a Bayes Factor of 8.35E7) and complement C5 (fold change: 2.73 higher in chronic pancreatitis with a Bayes Factor of 11.20), both of which are involved in the adaptive immune system^{74, 75}, in addition to neuropathic pain⁷⁶. Complement C3 may be involved in a variety of pathophysiological processes in chronic pancreatitis, including deposition at the basement membrane and inhibition by TGF-beta⁷⁷. Significantly, C5 has been shown to have a role in edema formation in a mouse acute pancreatitis model⁷⁸. In addition to their involvement in the pain pathway, both proteins have roles in fibrosis-related pathways, while complement C5 is also involved in inflammation pathways. However, the immediate function of either protein in chronic pancreatitis has yet to be defined clearly.

While proteins that are involved in fibrosis, inflammation, and pain are identified exclusively in, or determined to be up-regulated in chronic pancreatitis, proteins having a role in digestion are down-regulated in chronic pancreatitis. We have shown that several common pancreatic enzymes, including trypsin, chymotrypsin, lipase, and aminopeptidase

are down-regulated in chronic pancreatitis (Table 8). Such a finding is expected as pancreatic enzyme deficiency, manifested by malabsorption of proteins and fat, is generally associated with late stage chronic pancreatitis^{79–81}. These findings not only support our study, but demonstrate that quantitative analysis of these enzymes from pancreatic fluid collected using the ePFT method, may provide criteria for diagnosing chronic pancreatitis as well as determining disease severity.

To obtain a quantitative estimate of the abundance of identified proteins, we used spectral counting – a frequently-used label-free approach for quantification⁸². This method compares the number of MS/MS spectra for the same protein among several data sets (in the present case, 18 individuals in two cohorts). We chose to analyze our data using spectral counting, as studies have shown a strong linear correlation between relative protein abundance and sequence coverage, with a dynamic range of over two orders of magnitude⁸³. Furthermore, spectral counting quantification has been shown to be more reproducible and have a higher dynamic range than peptide ion chromatogram-based quantification⁸⁴, and is particularly useful if no labeling has been performed *a priori*.

In our study, we investigate proteins in pancreatic fluid collected from patients with advanced chronic pancreatitis for comparison to chronic abdominal pain controls. We did not, however, aim to investigate differences in etiology. Such analysis may reveal proteins that differ significantly in abundance among the various specific causes of chronic pancreatitis. Further comparative proteomic analysis is certainly warranted, as the molecular mechanisms directing disease pathogenesis and progression may be different among etiologies. If early detection of chronic pancreatitis is to be investigated, data for etiology-specific protein differences will be valuable. However, with our current data, we do not have the statistical power to perform such etiology-specific pancreatic fluid proteome analysis. Future investigations will require increased number of patients for both the biomarker discovery and validation phases.

Moreover, a clinically useful diagnostic test must be able to discriminate between individuals with early pancreatic disease and those whose symptoms are of non-pancreatic origin. As such, future proteomic analysis must include a wider spectrum of patients, specifically those with mild and moderate chronic pancreatitis to ensure that identified biomarkers are sensitive and are not solely markers of advanced disease. In addition, longitudinal studies that investigate the progression of chronic pancreatitis in patients at different stages of disease may lend insight into the potential for tracking disease progression, complementing current radiologic and imaging techniques and may have the additional benefit of each patient serving as his/her own control.

Although we have successfully identified a set of pancreatic proteins with statistically significant differences in abundance between cohorts of severe chronic pancreatitis patients and controls, the molecular mechanisms underlying these differences remain unresolved. Protein target-based assays with animal models and/or cell culture must be performed to understand more clearly the mechanisms by which changes in protein expression occur. Such model systems allow the modulation of specific proteins, i.e. by overexpression or transcriptional knockdowns, and the subsequent analysis of cellular and/or physiological changes, in a controlled environment. Analogous experiments would be difficult or impossible to perform with human subjects, thus studies in animal models and cell culture, including that of pancreatic duct and stellate cells, will be necessary to elucidate the molecular mechanisms of chronic pancreatitis and the role of the identified proteins at the cellular level.

In summary, we have identified successfully proteins that are differentially secreted in the ePFT-collected pancreatic fluid of chronic pancreatitis patients compared to non-pancreatitis controls using GeLC-MS/MS. An orthogonal methodology, such as western blotting, ELISA or targeted mass-spectrometry-based assays, may be performed to validate our findings at an individual protein level, using much larger cohorts. The use of the ePFT collection technique coupled with GeLC-MS/MS analysis of proteins extracted from pancreatic fluid has significant potential in the study of the exocrine pancreas. In fact, our study has identified the largest number of proteins from pancreatic fluid to date. Once the molecular mechanisms underlying the differential expression of these proteins have been determined, cell culture systems or animal models, such as mouse, rat, and zebrafish, may be developed to investigate further these proteins of interest in a well-controlled system. In conclusion, we have identified potential biomarkers of chronic pancreatitis, establishing a workflow which may also be applied to related proteomic studies of diseases of the exocrine pancreas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ePFT	endoscopic pancreatic function test
GeLC-MS/MS	in-gel tryptic digestion followed by liquid chromatography-tandem mass spectrometry
LTQ-FTICR	linear trap quadrupole- Fourier transform ion cyclotron resonance mass spectrometry
TCA	trichloroacetic acid

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Figure 1. Experimental workflow. The general workflow for the overall analysis is as follows: 1) ePFT sample collection, 2) protein extraction with trichloroacetic acid (TCA), 3) SDS-PAGE protein fractionation, 4) in-gel tryptic digestion, 5) LC-MS/MS peptide mass determination, and 6) bioinformatic data processing.

Figure 2. SDS-PAGE protein fractionation. Each gel lane represents approximately 100 μ g of ePFT-collected pancreatic fluid that has been TCA precipitated from a particular patient (9 individual patients per gel). A) NP (non-pancreatitis) controls samples. B) CP (chronic pancreatitis) samples.

Figure 3.

The number of unique A) proteins and B) peptides identified in each pancreatic fluid sample analyzed. Grey bars represent NP (non-pancreatitis) controls samples and white bars represent CP (chronic pancreatitis) samples.

Figure 4.

Venn diagrams comparing proteins identified in the NP (non-pancreatitis) and CP (chronic pancreatitis) cohorts. A) Qualitative assessment of proteins in each cohort, depicting those exclusive to either cohort. B) Quantitative assessment of proteins in common between the two cohorts. Bayes factor ≥ 10 indicates a statistically significant difference of the protein between the two cohorts.

Figure 5.

Gene ontology (GO) analysis of subcellular localization and molecular function that were statistically up- or down- regulated in chronic pancreatitis. GO characterization using the categories listed for proteins with statistically significant differences was performed manually with the UniProt⁸⁵ database or using the GoFact online tool^{38, 39}. A) Subcellular localization and B) Molecular function of proteins down-regulated in CP (white bars), absent in CP (light grey), exclusive to CP (medium grey), up-regulated in CP (darker grey bars), and identified in both NP and CP (black bars). CP, chronic pancreatitis; NP, non-pancreatitis controls.

Patient charact en sties

Table 1

	NP1	NP2	NP3	NP4	NP5	NP6	NP7	NP8	NP9	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8	CP9
Gender	male	male	male	female	female	female	male	female	male	male	female	male	female	male	male	female	female	female
Age (years)	55	53	48	30	32	30	23	55	56	42	44	41	49	58	68	64	61	53
Race	white	white	white	white	white	white	white	white	Hispanic	white	white	Hispanic	white	white	white	white	white	white
Smoker	–	+	+	–	–	–	–	–	–	–	+	+	+	+	+	+	–	+
Alcohol	+	–	+	–	–	–	–	–	–	–	–	+	+	–	+	+	–	+
TIGAR-O	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	G	G	T	T	I	T	T	I	T
Symptoms	pain	pain	pain, weight loss	Pain, Diarrhea	pain	pain	pain	diarrhea	pain	pain, recurrent pancreat it is	pain, recurrent pancreat it is	pain	pain	pain	pain	none	weight loss, pain	pain
CT Scan Findings	none	none	none	none	none	none	none	none	none	calcifications	calcifications, dilated main duct	calcifications	n/a	calcifications	calcifications, dilated main duct, atrophy	dilated duct, calcifications, atrophy	calcifications, dilated duct, dilated	calcifications, cyst
CT Grade	normal	n/a	normal	n/a	normal	n/a	normal	normal	normal	definite	definite	definite	n/a	definite	definite	definite	definite	definite
EUS Score	1	4	2	n/a	4	2	3	1	n/a	5	5	n/a	7	n/a	5	n/a	8	6
ERCP findings	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	dilated ducts & side branch, stones	n/a	n/a	n/a	n/a	n/a	n/a
ERCP grade	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	IV	n/a	n/a	n/a	n/a	n/a	n/a
MRI grade	n/a	0	n/a	0	0	0	0	0	0	IV	IV	IV	III	IV	IV	IV	IV	IV
CP based on imaging	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
Peak HCO ₃ [–]	80	84	101	115	81	90	84	92	114	60	26	22	41	22	39	37	54	38
Secretory dysfunction	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
Classification	A	A	A	A	A	A	A	A	A	D	D	D	D	D	D	D	D	D

TIGAR-O: T, Toxic-metabolic; I, Idiopathic; G, Genetic; A, Autoimmune; R, Recurrent and severe acute pancreatitis; O, Obstructive. EUS scores: 0–2 is normal 3–4 is equivocal, >5 is definite CP. ERCP Grade (Cambridge classification): normal=0, equivocal=I, mild=II, moderate=III, severe=IV. MRI/MRCP Grade (Cambridge classification: normal=0, equivocal=I, mild=II, moderate=III, severe=IV). Secretory dysfunction: yes (+) <80, no (–) ≥80. Classification of patient: A=healthy, B=Equivocal (Cambridge II, EUS 0–2), C=moderate (Cambridge III, EUS 3–4),D=severe (Cambridge IV, EUS ≥5), E=acute recurrent. n/a: not available; CP, chronic pancreatitis; NP, non-pancreatitis controls.

Table 2

Proteins identified in all 18 samples.

Protein	IPI #	spectral counts				Bayes Factor	Fold Change	Statistically significant difference
		total NP	total CP	avg. NP	avg. CP			
A2M Alpha-2-macroglobulin	IP[00478003.1	2548	3075	283.11	341.67	0.00	1.18	no
ALB Isoform 1 of Serum albumin	IP[00745872.2	10452	13709	1161.33	1523.22	3.88	1.62	no
AMBP Protein AMBP	IP[00022426.1	70	194	7.78	21.56	468.36	2.48	
AMY1C;AMY1B;AMY1A;AMY2A Alpha-amylase 1	IP[00300786.1	5353	2529	594.78	281.00	284.76	2.07	
AMY1C;AMY1B;AMY1A;AMY2A Pancreatic alpha-amylase	IP[00025476.1	9488	4003	1054.22	444.78	2.67E+06	2.33	
AMY2B Alpha-amylase 2B	IP[00021447.1	8215	3424	912.78	380.44	6231.48	2.44	
ANPEP Aminopeptidase N	IP[00221224.6	834	1417	92.67	157.44	6.58	2.17	no
Anti-(ED-B)scFV	IP[00915411.4	239	461	26.56	51.22	25.34	1.88	
C3 Complement C3	IP[00783987.2	1339	2757	148.78	306.33	8.35E+07	4.34	
cDNA FLJ78387	IP[00876888.1	1252	1542	139.11	171.33	0.38	1.35	no
CEL carboxyl ester lipase precursor	IP[00099670.2	1898	1202	210.89	133.56	2237.61	2.38	
CLCA1 Calcium-activated chloride channel regulator 1	IP[00014625.3	438	664	48.67	73.78	12.92	1.66	
CPA1 Carboxypeptidase A1	IP[00009823.3	8474	3610	941.56	401.11	6.63E+07	2.28	
CPB1 Carboxypeptidase B	IP[00009826.2	5599	2537	622.11	281.89	9.38E+05	2.37	
CTRB1 Chymotrypsinogen B	IP[00015133.1	2323	1149	258.11	127.67	3.01E+04	2.33	
CTRB2 chymotrypsinogen B2	IP[00515087.2	4055	1781	450.56	197.89	1.09E+05	2.14	
CTRC Chymotrypsin-C	IP[00018553.1	2049	793	227.67	88.11	2.45E+04	2.71	
DCD Dermcidin	IP[00027547.2	120	158	13.33	17.56	1.67	1.30	no
DMBT1 Isoform 4 of Deleted in malignant brain tumors 1 protein	IP[00418512.5	412	175	45.78	19.44	26.37	1.89	
ELA2A Elastase-2A	IP[00027722.1	5087	2780	565.22	308.89	87.38	1.94	
ELA3A Elastase-3A	IP[00295663.1	3142	1328	349.11	147.56	624.24	2.52	
ELA3B Elastase-3B	IP[00307485.3	1699	637	188.78	70.78	1987.17	3.13	
FCGBP IgGfc-binding protein	IP[00242956.5	986	1323	109.56	147.00	8.72	1.45	no
GP2 Isoform Alpha of Pancreatic secretory granule membrane major	IP[00914943.1	1678	438	186.44	48.67	5.74E+09	5.98	
HBA1;HBA2 Hemoglobin subunit alpha	IP[00410714.5	597	480	66.33	53.33	0.72	1.31	no
HBB Hemoglobin subunit beta	IP[00654755.3	898	904	99.78	100.44	4.59	1.39	no

Protein	IPI #	spectral counts				Bayes Factor	Fold Change	Statistically significant difference
		total NP	total CP	avg. NP	avg. CP			
Ig kappa chain V-J region DEE	IP[00387025.1	73	104	8.11	11.56	3.83	1.41	no
IGHA1;IGHV3OR16-13 IGHA1 protein	IP[00061977.1	1559	2264	171.00	251.56	0.96	1.39	no
IGHA2 IGHA2 protein	IP[00783993.1	881	1698	97.89	188.67	41855.83	2.76	no
IGHG1 IGHG1 protein	IP[004448925.3	1371	1700	152.33	188.89	3.01	1.44	no
IGHG2 IGHG2 protein	IP[00784807.1	447	496	49.67	55.11	4.47	1.22	no
IGHG2 Putative uncharacterized protein DKFZp686I04196	IP[00399007.5	894	896	99.33	99.56	0.23	1.18	no
IGHM IGHM protein	IP[00472610.2	1801	2102	200.11	233.56	2.76	1.25	no
IGHV4-31 Putative uncharacterized protein DKFZp686G11190	IP[00784842.1	699	1148	77.67	127.56	22.10	1.65	no
IGJ immunoglobulin J chain	IP[00178926.2	236	344	26.22	38.22	10.86	1.44	no
IGKC IGKC protein	IP[00430847.1	902	1677	100.22	186.33	2.14	1.60	no
IGKV1-5 IGKV1-5 protein	IP[00430820.1	290	1038	32.22	115.33	378.57	2.32	no
IGKV2-24 IGKV2-24 protein	IP[004440577.3	1365	1956	151.67	217.33	114.60	1.54	no
IGKV4-1 Similar to Ig kappa chain V-IV region precursor	IP[00026197.7	81	93	9.00	10.33	1.08	1.16	no
Immunoglobulin heavy chain variable region	IP[00783287.1	164	194	18.22	21.56	0.58	1.30	no
JUP cDNA FLJ60424, highly similar to Junction plakoglobin	IP[00789324.3	212	260	23.56	28.89	1.54	1.18	no
LOC100126583 cDNA FLJ41981 fis, clone SMINT2011888	IP[00784830.1	1650	2420	183.33	268.89	384.12	1.56	no
LOC100133739 Putative uncharacterized protein DKFZp686C15213	IP[00426051.3	428	843	47.56	93.67	498.98	1.93	no
LOC401847 similar to hCG1793095	IP[00888191.1	250	304	27.78	33.78	0.62	1.31	no
LOC642131 similar to hCG1812074	IP[00887113.1	55	80	6.11	8.89	2.31	1.36	no
Myosin-reactive immunoglobulin heavy chain variable region	IP[00783024.1	200	322	22.22	35.78	93.95	1.63	no
ORM1 Alpha-1-acid glycoprotein 1	IP[00022429.3	405	476	45.00	52.89	0.85	1.19	no
ORM2 Alpha-1-acid glycoprotein 2	IP[00020091.1	184	231	20.44	25.67	0.88	1.37	no
PEPD Xaa-Pro dipeptidase	IP[00257882.7	140	176	15.56	19.56	1.02	1.06	no
PIGR Polymeric immunoglobulin receptor	IP[00004573.2	1807	2364	200.78	262.67	1.96	1.24	no
PNLIP Pancreatic triacylglycerol lipase	IP[00027720.1	5785	2729	642.78	303.22	1.44E+09	2.25	no
PRSS1 Trypsin-1	IP[00011694.1	2464	1533	273.78	170.33	20.90	1.77	no
PRSS2 Protease serine 2 isoform B	IP[00011695.8	1300	566	144.44	62.89	54.10	1.79	no
PRSS3 Isoform A of Trypsin-3	IP[00015614.4	1336	804	148.44	89.33	2175.75	2.15	no
PRSS7 Enteropeptidase	IP[00023788.1	422	585	46.89	65.00	2.94	1.33	no

Protein	IPI #	spectral counts				Bayes Factor	Fold Change	Statistically significant difference
		total NP	total CP	avg. NP	avg. CP			
Putative uncharacterized protein	IP100807428.1	1289	1230	143.22	136.67	0.77	1.04	no
REG1A Lithostathine-1-alpha	IP100009027.1	645	284	71.67	31.56	58.20	2.03	
Rheumatoid factor C6 light chain	IP100829956.1	77	100	8.56	11.11	1.40	1.29	no
SCFV Single-chain Fv	IP100748998.1	331	464	36.78	51.56	3.95	1.34	no
SERPINA1 Isoform 1 of Alpha-1-antitrypsin	IP100553177.1	3075	3021	341.67	335.67	4.51	1.00	no
SERPINA3 cDNA FLJ35730 fis	IP100550991.3	544	479	60.44	53.22	1.46	1.01	no
SERPINC1 Antithrombin III variant	IP100032179.2	290	257	32.22	28.56	0.63	1.04	no
SERPING1 Plasma protease C1 inhibitor	IP100291866.5	260	366	28.89	40.67	1.72	1.43	no
SI Sucrase-isomaltase, intestinal	IP100221101.3	671	849	74.56	94.33	15.02	1.30	
Similar to Elastase-3A precursor	IP100921065.1	1609	665	178.78	73.89	2997.68	2.98	
Single-chain Fv	IP100470652.1	361	401	40.11	44.56	0.91	1.16	no
TF Serotransferrin	IP100022463.1	2806	3503	311.78	389.22	214.72	1.73	
TTR Transthyretin	IP100022432.1	284	354	31.56	39.33	0.70	1.02	no
UGa8H	IP100828099.1	275	282	30.56	31.33	0.37	1.08	no

IPI #, international protein index number; avg., average; CP, chronic pancreatitis; NP, non-pancreatitis controls.

Table 3

KEGG pathways analysis of proteins that were exclusive to or up-regulated in chronic pancreatitis.

Pathway	# of proteins	% of total up-regulated proteins
Regulation of actin cytoskeleton	12	3.65
Complement and coagulation cascades	11	3.34
Alzheimer's disease	11	3.34
Cell adhesion molecules (CAMs)	10	3.04
Tight junction	10	3.04
Viral myocarditis	9	2.74
Lysosome	9	2.74
Pathogenic Escherichia coli infection	8	2.43
Leukocyte transendothelial migration	8	2.43
Starch and sucrose metabolism	7	2.13
Glycolysis / Gluconeogenesis	7	2.13
Antigen processing and presentation	7	2.13
Hematopoietic cell lineage	7	2.13
Prion diseases	6	1.82
PPAR signaling pathway	6	1.82
Allograft rejection	5	1.52
Graft-versus-host disease	5	1.52
Type I diabetes mellitus	5	1.52
Autoimmune thyroid disease	5	1.52
Renin-angiotensin system	4	1.22
Nitrogen metabolism	4	1.22

Table 4

KEGG pathways analysis of proteins that were not identified in or down-regulated in chronic pancreatitis.

Pathway	# of proteins	% of total down-regulated proteins
Glycolysis / Gluconeogenesis	11	5.47
Metabolism of xenobiotics by cytochrome P450	7	3.48
Leukocyte transendothelial migration	7	3.48
Citrate cycle (TCA cycle)	6	2.99
Fatty acid metabolism	5	2.49
Proteasome	5	2.49
Drug metabolism	5	2.49
Complement and coagulation cascades	5	2.49
Propanoate metabolism	4	1.99
Pyruvate metabolism	4	1.99
Amino sugar and nucleotide sugar metabolism	4	1.99
Valine, leucine and isoleucine degradation	4	1.99
Glycerolipid metabolism	4	1.99
Glutathione metabolism	4	1.99
Glyoxylate and dicarboxylate metabolism	3	1.49
Pentose phosphate pathway	3	1.49

Table 5

Proteins exclusive to or up-regulated in chronic pancreatitis that are involved in fibrosis.

Protein	IPI	#NP	#CP	Bayes Factor	Fold Change higher in CP
ACTA2 Actin, smooth muscle	IPI00008603.1	0	4	--	--
APOA2 Apolipoprotein A-II	IPI00021854.1	0	2	--	--
C5 Complement C5	IPI00032291.2	2	6	11.20	2.73
HP HP protein	IPI00431645.1	6	9	45.66	3.45
HPX Hemopexin	IPI00022488.1	6	8	6871.66	3.23
SERPINA5 Plasma serine protease inhibitor	IPI00007221.1	0	8	--	--
TF Serotransferrin	IPI00022463.1	9	9	214.72	1.73

IPI, international protein index; #NP, number of the non-pancreatitis cohort samples in which the specified protein was present (maximum = 9); #CP, number of chronic pancreatitis cohort samples in which specified protein was present (maximum = 9).

Table 6

Proteins exclusive to or up-regulated in chronic pancreatitis that are involved in inflammation.

Protein	IPI	#NP	#CP	Bayes Factor	Fold Change higher in CP
AHSG cDNA FLJ55606, similar to Alpha-2-HS-glycoprotein	IP100022431.2	0	2	--	--
AMBP Protein AMBP	IP100022426.1	9	9	468.36	2.48
ANXA5 Annexin A5	IP100329801.12	0	5	--	--
APOA2 Apolipoprotein A-II	IP100021854.1	0	2	--	--
C3 Complement C3	IP100783987.2	9	9	8.4E+07	4.34
C4B complement component 4B preproprotein	IP100418163.3	3	5	4.64	1.95
C5 Complement C5	IP100032291.2	2	6	11.20	2.73
C7 protein	IP100642632.1	5	6	0.07	1.58
CFHR1 Complement factor H-related protein 1	IP100011264.2	0	3	--	--
DEFA5 Defensin-5	IP100008298.1	2	7	149.78	5.24
DMBT1 Putative uncharacterized protein DMBT1	IP100412044.4	8	8	0.33	1.66
DNASE1 Deoxyribonuclease-1	IP100031065.1	3	8	58.91	4.31
F11R Junctional adhesion molecule A	IP100001754.1	2	6	52.35	3.67
F2 36 kDa protein	IP100877967.1	0	3	--	--
GC Vitamin D-binding protein	IP100555812.4	3	7	71.05	4.45
HLA-B HLA class I histocompatibility antigen, B-73 alpha	IP100472943.1	0	2	--	--
HLA-DRA HLA class II histocompatibility antigen, DR alpha	IP100005171.1	0	3	--	--
HP HP protein	IP100431645.1	6	9	45.66	3.45
HPX Hemopexin	IP100022488.1	6	8	6871.66	3.23
ICAM1 Intercellular adhesion molecule 1	IP100008494.4	0	2	--	--
LTF Growth-inhibiting protein 12	IP100298860.5	4	7	20.80	2.28
MME Nephrysin	IP100247063.3	3	9	1178.29	5.18
MUC1 Isoform 1 of Mucin-1	IP100013955.1	0	2	--	--
NT5E 5'-nucleotidase	IP100009456.1	0	3	--	--
S100A9 Protein S100-A9	IP100027462.1	4	9	52.74	4.09
SCGB1A1 Uteroglobin	IP100006705.1	0	2	--	--
SERPINF3 Isoform 1 of Serpin B3	IP100022204.2	1	4	20.58	3.91
SIRPA signal-regulatory protein alpha precursor	IP100332887.5	0	2	--	--
TF Sero transferrin	IP100022463.1	9	9	214.72	1.73

Protein	IPI	#NP	#CP	Bayes Factor	Fold Change higher in CP
TXN Thioredoxin	IP100216298.6	2	6	13.30	3.23
VTN Vitronectin	IP100298971.1	4	7	13.38	2.49

IPI, international protein index; #NP, number of the non-pancreatitis cohort samples in which the specified protein was present (maximum = 9); #CP, number of chronic pancreatitis cohort samples in which specified protein was present (maximum = 9).

Table 7

Proteins exclusive to or up-regulated in chronic pancreatitis that are involved in pain.

Protein	IPI	#NP	#CP	Bayes Factor	Fold Change higher in CP
C3 Complement C3	IP100783987.2	9	9	8.35E+07	4.34
C5 Complement C5	IP100032291.2	2	6	11.20	2.73
CAMP Cathelicidin antimicrobial peptide	IP100292532.6	0	1	--	--
ICAM1 Intercellular adhesion molecule 1	IP100008494.4	0	2	--	--
LTF Growth-inhibiting protein 12	IP100298860.5	4	7	20.80	2.28
SIRPA signal-regulatory protein alpha precursor	IP100333287.5	0	2	--	--
TXN Thioredoxin	IP100216298.6	2	6	13.30	3.23

IPI, international protein index; #NP, number of the non-pancreatitis cohort samples in which the specified protein was present (maximum = 9); #CP, number of chronic pancreatitis cohort samples in which specified protein was present (maximum = 9).

Table 8

Common pancreatic enzymes that are down-regulated in chronic pancreatitis.

Protein	IPI	#NP	#CP	Bayes Factor	Fold Change higher in NP
AMY1C;AMY1B;AMY1A;AMY2A Alpha-amylase 1	IP100300786.1	9	9	284.76	2.07
AMY1C;AMY1B;AMY1A;AMY2A Pancreatic alpha-amylase	IP100025476.1	9	9	2.67E+06	2.33
CTRB1 Chymotrypsinogen B	IP100015133.1	9	9	3.01E+04	2.33
CTRB2 chymotrypsinogen B2	IP100515087.2	9	9	1.09E+05	2.14
CTRC Chymotrypsin-C	IP100018553.1	9	9	2.45E+04	2.71
ELA2A Elastase-2A	IP100027722.1	9	9	87.38	1.94
ELA3A Elastase-3A	IP100295663.1	9	9	624.24	2.52
ELA3B Elastase-3B	IP100307485.3	9	9	1987.17	3.13
PLA2G1B Phospholipase A2	IP100021792.1	9	8	7961.39	3.19
PNLIP Pancreatic triacylglycerol lipase	IP100027720.1	9	9	1.44E+09	2.25
PNLIPRP2 pancreatic lipase-related protein 2	IP100005924.4	9	8	39.47	3.43
PRSS1 Putative trypsin-6	IP100169276.2	9	8	3690.34	2.12
PRSS2 Protease serine 2 isoform B	IP100011695.8	9	9	54.10	1.79
PRSS3 Isoform A of Trypsin-3	IP100015614.4	9	9	2175.75	2.15
RP11-265F14.2 Elastase-2B	IP100027723.2	9	8	34.73	2.00
RP11-265F14.2 Pancreatic elastase IIB	IP100746692.3	5	0	--	--
Similar to Elastase-3A precursor	IP100921065.1	9	9	2997.68	2.98

IPI, international protein index; #NP, number of the non-pancreatitis cohort samples in which the specified protein was present (maximum = 9); #CP, number of chronic pancreatitis cohort samples in which specified protein was present (maximum = 9).