ORIGINAL ARTICLE

The Proteome of Mesenteric Lymph During Acute Pancreatitis and Implications for Treatment

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ABSTRACT

Context The protein fraction of mesenteric lymph during acute pancreatitis and other critical illness is thought to contain toxic factors. However, we do not have a complete description of the mesenteric lymph proteome during acute pancreatitis. **Objective** The aim of this study was to define the proteomic changes in mesenteric lymph during acute pancreatitis. **Setting** Animal Laboratory, University of Auckland, New Zealand. **Design** Mesenteric lymph was collected from sixteen male Wistar rats randomised to Group 1 (n=8) with taurocholate induced acute pancreatitis and Group 2 (n=8) sham control. The lymph was subjected to proteomic analysis using iTRAQ™ (Applied Biosystems, Foster City, CA, USA) and liquid chromatography-tandem mass spectrometry. **Results** Two hundred and forty-five proteins including 35 hypothetical proteins were identified in mesenteric lymph. Eight of the 245 proteins had a significant increase in their relative abundance in acute pancreatitis conditioned mesenteric lymph, and 7 of these were pancreatic catabolic enzymes (pancreatic amylase 2, pancreatic lipase, carboxypeptidase A2, chymotrypsinogen B, carboxypeptidase B1, cationic trypsinogen, ribonuclease 1). **Conclusions** This is the first comprehensive description of the proteome of mesenteric lymph during acute pancreatitis and has demonstrated a significantly increased relative abundance of 7 secreted pancreatic catabolic enzymes in acute pancreatitis conditioned mesenteric lymph. This study provides a clear rationale for further research to investigate the efficacy of enteral protease inhibitors in the treatment of acute pancreatitis.

INTRODUCTION

Acute pancreatitis is a common inflammatory disease that remains a significant clinical challenge. For the third of patients who develop severe acute pancreatitis, the risk of mortality remains high at 20-30% [1, 2] despite improvements in resuscitation and intensive care support [3, 4]. The mortality is due to multiple organ failure, and this has a bimodal time course distribution. Early deaths, during the first week, are due to a fulminant cytokine mediated systemic inflammatory response syndrome and multiple organ dysfunction (MODS), without an overt septic focus [5].

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Abbreviations IPI: International Protein Index; LC-MS/MS: liquid chromatography-tandem mass spectrometry; MODS: multiple organ dysfunction syndrome; MS: mass spectrometry; NBF: neutral buffered formalin; XO: xanthine oxidase

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Later deaths, after 2 or more weeks, are due to MODS associated with infection of necrotic pancreas [6]. Many pathophysiological processes in acute pancreatitis have been described, but the critical factors that drive the MODS have yet to be fully elucidated [1].

There is a body of experimental work, largely derived from rodent studies, suggesting that mesenteric lymph, collected during critical illness, contains toxic factors [7, 8, 9, 10, 11] that contribute to the development of MODS and might be more important than translocated bacteria [12, 13]. Disease conditioned mesenteric lymph is reported to be toxic and associated with neutrophil dysfunction [14, 15], bone marrow suppression [16], and damage to pulmonary epithelial and endothelial cells [17, 18]. Indeed, Magnotti *et al.* reported that it was disease conditioned mesenteric lymph and not portal venous blood that caused increased endothelial cell permeability and lung injury [19]. This should not be a surprise because the anatomical route of the mesenteric lymph to the subclavian vein via the thoracic duct bypasses the liver. Therefore, unlike portal blood, mesenteric lymph is able to avoid any hepatic first-pass modification or detoxification, and potentially 'toxic' factors are instead delivered directly to distant organs (especially

the heart and lungs). We have recently reported an increase in the histological severity of experimental acute pancreatitis with the peripheral administration of mesenteric lymph conditioned by mild intestinal ischaemia and reperfusion [1]. Other studies have demonstrated a protection against MODS in a model of hypovolaemic shock when mesenteric lymph is excluded by division [20] or ligation [21] of the main rodent mesenteric lymph duct or by diversion of the thoracic duct [16]. The toxic factors in mesenteric lymph that are responsible for these effects have yet to be identified, although some recent work has suggested the toxic factors are largely carried in the aqueous or protein fraction of mesenteric lymph [8] and that pancreatic enzymes may contribute to the toxicity of disease conditioned mesenteric lymph [10, 22, 23, 24, 25, 26].

We recently published the first comprehensive description of normal rodent mesenteric lymph in the fasted and fed states using the advanced proteomic techniques of isobaric tags (iTRAQ™ Reagent Multi-Plex Kit, Applied Biosystems, Foster City, CA, USA) for relative protein quantitation together with LC-MS/MS (liquid chromatography-tandem mass spectrometry) for the identification of the component proteins [27]. The aim of this current study was to use these state-of-the-art proteomic techniques to provide the first comprehensive description of the mesenteric lymph rodent proteome associated with acute pancreatitis, and to determine whether there were any significant increases in the relative abundance of detected proteins compared with sham control mesenteric lymph.

METHODS

Animals

Sixteen inbred male Wistar rats (466±2.9 g; mean±SEM) fed a standard 18% plant protein derived rodent diet (Harlan Teklad 2018, Madison, WI, USA), were randomised to two groups. Group 1 (acute pancreatitis, n=8) had 90 minutes of acute pancreatitis followed by collection of mesenteric lymph for a further 60 minutes. Group 2 (sham control, n=8) had matched interventions and lymph collection to the pancreatitis group but without the induction of acute pancreatitis. In each case the surgery commenced at the same time each day (09:00) and animals were fed *ad libitum*.

Acute Pancreatitis Model

We used an established model of acute pancreatitis [28, 29, 30, 31]. General anaesthesia was induced by isoflurane (2-5%; 2 L/min O_2 via nasal cone). A tracheostomy was inserted (modified 14g angiocath) and connected to a small animal ventilator (Kent Scientific Corporation, Torrington, CT, USA). Balanced general anaesthesia was maintained with isoflurane (2-3.5%) and buprenorphine (0.05 µg/kg, s.c., Temgesic®, Reckitt and Coleman, Hull, England). The fraction of inspired oxygen/air was 40%; the respiratory rate was 50-80 breaths per minute; and the peak inspiratory pressures 11-15 cmH_2O kept the expired CO_2 at 35-45 mL/L as measured by a capnograph (Pryon Corporation, Menomonee Falls, WI, USA). Body temperature was maintained between 36-38°C by use of a warming plate. Maintenance fluid (0.9% sodium chloride, NaCl) was infused at 1-2 mL/h for the duration of the experiment via a femoral intravenous line. Mean arterial pressure was maintained between 80 and 100 mmHg with the use of intravenous NaCl and monitored using a solid-state 2F pressure transducer (Millar Instruments Inc., Houston, TX, USA) placed in the right femoral artery.

The common pancreatic duct was cannulated with a 24g angiocath passed transduodenally into the pancreato-biliary duct through a 1.5 cm abdominal midline incision. The rostral part of the animal was raised 60° to the horizontal for 5 min to allow the biliary tree to drain (about 0.1 mL). During the last 2 min of this procedure, the common hepatic bile duct was occluded at the hilum of the liver (Biemer atraumatic vascular clip, AESCULAP, Center Valley, PA, USA).

Sodium taurocholate (4% w/v in 0.9% NaCl; 0.1 mL/100 g BW; Sigma Aldrich Pty Ltd., Castle Hill, New South Wales, Australia) was infused at 0.1 mL/min by a controlled infusion pump (Genie Precision Pump, Kent Scientific, Torrington, CT, USA). The Biemer clip and angiocath were removed upon completion of the infusion, and the common pancreatic duct was ligated to prevent reflux of taurocholate into the duodenum.

Severe acute pancreatitis was allowed to develop over a 90-minute period. We chose this relatively early time point and careful control of the blood pressure because we wanted to minimise the risk of hypotension and reflex splanchnic vasoconstriction resulting in an intestinal ischaemia-reperfusion injury that is known to occur during the course of severe acute pancreatitis [32, 33]. An intestinal ischaemia-reperfusion injury could potentially have altered the mesenteric lymph composition and confounded our results.

Collection of Mesenteric Lymph

After 90 minutes elapsed from the induction of pancreatitis, the duodenum and intestines were reflected to the left thus exposing the base of the mesentery. The mesenteric lymph duct was then cleared of surface peritoneum and fat. Silastic tubing (0.96 mm internal diameter, pre-soaked in 70% (v/v) ethanol, rinsed Milli-Q™ (Millipore, Billerica, MA, USA) water, 18 MΩ) was drawn through the right posterolateral abdominal wall using a 14g angiocatheter. The mesenteric lymph duct was cannulated with the silastic tube and secured in place with a drop of cyanoacrylate tissue glue (Aesculap Inc., Center Valley, PA, USA). The intestines were then returned to their original position and the abdomen closed. Mesenteric lymph was collected for the following 60 minutes. Collection was performed

directly into sterile ice-cold siliconised Eppendorff tubes pre-loaded with protease inhibitors (final: 16.7 µM bestatin, 8.3 µM pepstatin and 5 mM EGTA; Sigma Aldrich Pty Ltd, Castle Hill, New South Wales, Australia). We chose to use Eppendorf tubes pre-loaded with protease inhibitors to prevent any *ex-vivo* protein modification of the mesenteric lymph samples. At the end of the experiment the mesenteric lymph was centrifuged (1,700 g, 4°C, 10 min) to remove any cellular material then immediately stored at -80ºC until analysis.

Histology and Assays

At the end of the mesenteric lymph collection (150 minutes from the start of the experimental protocol), animals were euthanised for collection of organs and blood. A 1 cm^3 piece of the pancreatic tail was fixed (10% neutral buffered formalin, (NBF)), and histological severity scoring was performed by a blinded consultant histopathologist on 5 µm thick longitudinal paraffin sections using haematoxylin and eosin stain. Pancreatic histology was assessed using a published 5 point scale (from 0=normal to 4=severe) for each of the following criteria: leukocyte infiltration, pancreatic oedema, haemorrhage, fat necrosis, and acinar necrosis for a total score out of 20 [34].

A 5 cm length of small intestine, 20 cm from the caecum, was fixed (10% NBF) and histological severity scoring was performed by a blinded consultant histopathologist on 5 µm thick longitudinal paraffin sections using haematoxylin and eosin stain. The small intestine histology was assessed on a published 6 point scale (from 0, normal to 5, severe) for mucosal injury, inflammation and haemorrhage respectively for a total score out of 15 [35].

Biochemical assays were performed on rodent serum using a Roche/Hitachi MODULAR® analytical system (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the manufacture's methods.

Depletion of the Major Proteins in Mesenteric Lymph

IgY immunoaffinity columns were used to deplete the most abundant proteins and enhance the detection of lower abundance proteins [36]. In this study, the expected major abundant proteins of mesenteric lymph (albumin, IgG, fibrinogen, transferrin, alpha1-antitrypsin, and haptoglobin) were depleted using ProteomeLab IgY-R7 affinity spin columns (Beckman Coulter, Fullerton, CA, USA). Each of the samples from the 16 rats was individually depleted. The protein concentration of the mesenteric lymph samples was determined using the EZQ® protein assay (Molecular Probes, Eugene, OR, USA). The depleted samples were concentrated by ultrafiltration using Vivaspin 4 concentrators with a 5 kDa polyethersulfone filter (Sartorius AG, Goettingen, Germany).

LC-MS/MS Based Proteomics

The mesenteric lymph samples underwent LC-MS/MS based proteomics both with and without immunodepletion of the top 6 most abundant proteins. Each sample underwent reduction (incubation of 100 µg protein with 10 mM DTT at 56°C for one hour) and alkylation (incubation with 20 mM iodoacetamide at pH 8.0 in the dark for one hour). Protein was then digested by incubation with 1 µL trypsin (Promega, Madison, WI, USA) at 1 mg/mL and incubated at 37°C overnight. The peptides were then desalted on 10 mg Oasis SPE cartridges (Waters Corporation, Taunton, MA, USA), eluted with 70% acetonitrile and completely dried using a speed vacuum concentrator (Thermo Savant, Holbrook, NY, USA).

iTRAQ™ has previously been evaluated and validated against SDS-PAGE and western blotting as a method of tracking relative concentrations of proteins in four different samples [37, 38]. The dried protein digests were reconstituted with 30 µL of dissolution buffer from the iTRAQ™ and labelled with iTRAQ™ reagents according to the manufacturer's instructions. Labelled material was then combined, acidified by addition of 10% (v/v) formic acid, concentrated to approximately 200 µL, and then diluted to 2 mL with 0.1% formic acid. This sample was desalted as above, the eluate then concentrated to 100 µL, and finally diluted to 270 µL with 0.1% (v/v) formic acid.

Samples were then fractionated on-line on a BioSCX II 0.3x35 mm column (Agilent Technologies, Santa Clara, CA, USA). A 20 salt-step protocol was performed using 10 µL injections of 10, 20, 40, 60, 70, 80, 90, 100, 110, 120, 130, 140, 160, 180, 200, 220, 240, 260, 400 and 500 mM KCl. Peptides were captured on a 0.3x5 mm PepMap cartridge (LC Packings, Dionex Corporation, Sunnyvale, CA, USA) before being separated on a C18 300SB 0.3x100 mm Zorbax column (Agilent Technologies, Santa Clara, CA, USA). The HPLC gradient between Buffer A (0.1% formic acid in water) and Buffer B (0.1% formic acid in acetonitrile) was formed at 6 µL/min as follows: 10% B for the first 3 min, increasing to 35% B by 80 min, increasing to 95% B by 83 min, held at 95% until 91 min, back to 10% B at 91.5 min and held there until 100 min. The liquid chromatography effluent was directed into the ion spray source of a QSTAR XL hybrid mass spectrometer (Applied Biosystems, Foster City, CA, USA) scanning from 300-1,600 m/z. The three most abundant, multiply-charged peptides were selected for MS/MS analysis (80-1,600 m/z). The mass spectrometer and HPLC system were under the control of the Analyst QS software package (Applied Biosystems, Foster City, CA, USA).

Sequence Database Searches

ProteinPilot (version 1.0, Applied Biosystems, Foster City, CA, USA) [39] was used to search the MS/MS data against the International Protein Index (IPI) Rat database v3.27 (http://www.ebi.ac.uk/IPI/IPIhelp.html) with the following search parameters: Cys alkylation - Iodoacetamide; Digestion - Trypsin; Instrument - QSTAR ESI; Search Effort - Rapid. The data were also searched against the above database using Mascot 2.0.5

software (Matrix Science, London, UK), and a similar set of protein hits obtained (data not shown). Proteins that were identified as potentially hypothetical by the ProteinPilot IPI Rat database v3.27 search were then subjected to a NCBI Basic Local Alignment Search Tool search against the 'UniProt Clusters 100%' database (BLAST; http://www.ncbi.nlm.nih.gov/blast/).

Validation of Protein Identifications

A search of the IPI Rat database v3.27 with the reversed amino acid sequence of each entry was carried out to determine the minimum required ProteinPilot score for the proteins that would yield an overall confidence greater than 97%. Protein matches were considered valid if their ProteinPilot scores were equal to or above the minimum required score for each run.

Validation of Protein Changes

After immunodepletion and LC-MS/MS, only small volumes (10-20 µL per animal) of the original mesenteric lymph samples were available for cross-validation of protein changes reported by iTRAQ™ LC-MS/MS. Despite the small sample volumes, we were able to measure albumin, pancreatic amylase, and lipase in mesenteric lymph using commercial reagents (Pointe Scientific, Canton, MI, USA) on a COBAS MIRA analyser (Roche, Basel, Switzerland) in accordance with the manufacturer's instructions.

STATISTICS

Protein abundance was calculated from the peptide summary data generated by ProteinPilot. Strict criteria were applied when calculating the protein abundance from peptide data - peptides identified as belonging to more than one protein were eliminated, and any spectra below the confidence threshold set by ProteinPilot were also eliminated. The remaining peak areas were log-transformed and, for each sample, average log peak areas were calculated from the spectra within each reporter region for every identified protein. Differences in relative abundance were calculated as differences in log peak areas (acute pancreatitis - sham) and reported as fold differences between the two.

The statistical analysis was carried out using the LIMMA package v2.9.17 [40] in the R software v2.6.1 (R Development Core Team, 2007) [41]. The analysis for differential expression was performed on a protein-by-protein basis using a linear model that included run, label and treatment effects. A moderated t-statistic, in which the standard errors were moderated across proteins using a Bayesian model, was used for the significance analysis [42]. P values were adjusted for multiple testing using Benjamini and Hochberg's false discovery rate setting the expected proportion of false discoveries to 5% [43]. Changes in protein abundance with an adjusted P value less than 0.05 were considered significant.

For non-proteomic data, such as histology scores and biochemical parameters, the non-parametric Mann-Whitney U test was used to derive statistical significance and two-tailed P value less than 0.05 was considered significant.

Bioinformatics

Proteins that were found to have a statistically significant difference in abundance between sham and acute pancreatitis conditioned mesenteric lymph were then further analysed for functional and biological relevance. With the help of Gaggle [44], an open-source Java software environment, and Gene Ontology (GO) provided free by the Gene Ontology Consortium (http://www.geneontology.org/index.shtml) [45], these proteins were classified by their molecular function and cellular location.

ETHICS

This study was approved by the University of Auckland Animal Ethics Committee. All animals received humane care in keeping with the "Guide for Care and Use of Laboratory Animals (1996)" prepared by the National Academy of Sciences.

Table 1. Histological and serum biochemical parameters in the two experimental groups.

	Acute pancreatitis (n=8)	Sham (n=8)	P value
Pancreas histology score [34]	7.20±0.57	1.20±0.20	<0.001
Intestinal histology score [35]	0.47±0.16	0.31±0.15	0.541
Sodium (mmol/L)	141.9±0.35	139.7±0.58	0.012
Potassium (mmol/L)	6.31±0.22	6.22±0.17	0.515
Chloride (mmol/L)	111.6±0.93	108.8±0.98	0.099
Glucose (mmol/L)	14.85±1.61	14.8±0.96	0.959
Urea (mmol/L)	10.2±0.39	10.5±0.41	0.460
Creatinine (µmol/L)	42.13±6.64	42.4±2.75	0.274
Calcium (mmol/L)	2.2±0.06	2.4±0.34	0.027
Amylase (U/L)	3,829±302	2,053±81	<0.001
Lipase (U/L)	4,377±1,088	310±61	<0.001
Bilirubin (µmol/L)	<2.0	<2.0	-
GGT (U/L)	<2.0	<2.0	-
ALP (U/L)	47±3	56±4	0.101
AST (U/L)	592±267	101±15	0.019
ALT (U/L)	120±34	46±5	0.009

Values listed are mean±SEM. P values derived using Mann-Whitney U test. Bilirubin and GGT had values below the lower limits of the assays.

RESULTS

Acute Pancreatitis Model

The taurocholate model produced acute pancreatitis with the expected elevation in serum amylase (3,829±302 U/L *vs*. 2,053±81 U/L; sham *vs*. acute pancreatitis, respectively; P<0.001) and serum lipase (4,377±1,088 U/L vs. 310±61 U/L; sham *vs*. acute pancreatitis, respectively; P<0.001) (Table 1). The histology of the pancreas confirmed severe acute pancreatitis (Table 1). There was no significant difference between the two groups for histology of the intestine.

Mesenteric Lymph Proteomics

There were 245 proteins identified in mesenteric lymph from all the mass spectrometry runs (with and without depletion) that met the validity criteria. All of the proteins were identified in both experimental groups, and there were no proteins unique to either experimental group. A non-redundant list of these proteins is provided (Supplementary Table 1).

Forty-seven of the 245 identified proteins (19.2%) were listed as potentially hypothetical proteins according to the International Protein Index (Rat database v3.27). These proteins were then subjected to a NCBI BLASTP analysis. Thirty-five proteins (14.3%) were confirmed as hypothetical but 12 proteins were not, being identical to other rat proteins (Supplementary Table 2). Nine of these 35 proteins were previously identified in a recent proteomic study of normal mesenteric lymph [27] but continue to be listed as hypothetical according to the International Protein Index (Rat database v3.27) (Supplementary Table 2).

Prior to the immunoaffinity depletion, the 6 proteins removed by this process (albumin, fibrinogen, transferrin, alpha1-antitrypsin, IgG and haptoglobin) were investigated using mass spectrometry data and there were no statistically significant differences between the two experimental groups (Supplementary Table 1).

There was a statistically significant increase in the relative abundance of 8 proteins in the mesenteric lymph of the acute pancreatitis experimental group after immunoaffinity depletion (Table 2). An additional 2 proteins (hemoglobin beta chain complex and phosphoglycerate mutase 1) had changes in their relative abundance that approached significance (P<0.05 and an adjusted P<0.10).

The 8 proteins that were significantly increased in acute pancreatitis conditioned mesenteric lymph were then classified by their cellular location and molecular function using the Gene Ontology classification system. Seven of the proteins were extracellular pancreatic enzymes and one was cytosolic (Table 2). In regards to their molecular function, all 8 were catabolic enzymes and 4 were also ion binding (Table 2). Of the 7 extracellular pancreatic catabolic enzymes, four had peptidase activity (carboxypeptidase B1, chymotrypsinogen B, carboxypeptidase A2 and cationic trypsinogen), one had ester hydrolase activity (pancreatic lipase), one had endoribonuclease activity (ribonuclease), and one acted on glycosyl bonds (pancreatic amylase 2).

The results of the specific biochemical assays performed for albumin, pancreatic amylase and lipase in mesenteric lymph are consistent with the LC-MS/MS findings. Albumin was not different between the two groups (mean±SEM; 14.7±2.8 g/L *vs*. 14.0±1.8 g/L; acute pancreatitis *vs*. sham, respectively; P=0.867) while both pancreatic amylase (7,024±2,079 U/L *vs*. 901±196 U/L; acute pancreatitis *vs*. sham, respectively; P<0.001) and lipase (1,424±367 U/L *vs*. 272±132 U/L acute pancreatitis *vs*. sham, respectively; P=0.036) were significantly increased.

DISCUSSION

This study provides the first comprehensive description of the changes that occur in the proteome of mesenteric

Table 2. List of the 8 proteins that had an adjusted P value less than 0.05 in their relative abundance between the pancreatitis and sham experimental groups.

Name	Gene symbol	Protein identifier	Location	Molecular function	No. of animals [a]		Fold change [b]	P value	Adjusted P value
					AP [c]	Sham			
Amylase 2, pancreatic	Amy2	IPI00211904	Extracellular	Catabolic Ion binding	7	6	46.96	<0.001	<0.001
Pancreatic lipase	Pnlip	IPI00198916	Extracellular	Catabolic	6	5	38.09	<0.001	<0.001
Carboxypeptidase A2 (pancreatic)	Cpa2_predicted	IPI00193391	Extracellular	Catabolic Ion binding	6	5	34.64	<0.001	<0.001
Chymotrypsinogen B	Ctrb	IPI00206309	Extracellular	Catabolic	6	5	18.22	<0.001	<0.001
Carboxypeptidase B1 (tissue)	Cpb1	IPI00193393	Extracellular	Catabolic Ion binding	6	5	22.89	<0.001	<0.001
Cationic trypsinogen	LOC286911	IPI00211212	Extracellular	Catabolic Ion binding	6	5	29.64	<0.001	<0.001
Glutathione S-transferase, mu 2	Gstm2	IPI00411230	Intracellular	Catabolic	8	8	3.52	<0.001	0.012
Ribonuclease, RNase A family, 1 (pancreatic)	Rnase1	IPI00211902	Extracellular	Catabolic	3	2	40.50	<0.001	0.024

[a] Number of animals in each group where the protein was identified (maximum n=8 for each group)
[b] Fold change: acute pancreatitis *vs*. sham group
[c] Taurocholate induced acute pancreatitis group

lymph during acute pancreatitis. A total of 245 proteins were identified in mesenteric lymph using strict acceptance criteria and with greater than 97% confidence. All identified proteins were present in both the acute pancreatitis and sham control groups. There were 8 proteins that were significantly more abundant in acute pancreatitis conditioned mesenteric lymph. All 8 of these proteins were catabolic enzymes with 7 being secreted pancreatic catabolic enzymes. Also identified in the mesenteric lymph of both groups were 35 hypothetical proteins.

Severe acute pancreatitis is associated with hypotension and reflex splanchnic vasoconstriction resulting an intestinal ischaemia-reperfusion injury [32, 33]. It has previously been hypothesized that pancreatic enzymes which are normally present in the intestinal lumen in high concentration may be able to pass through a compromised intestinal barrier and cause remote organ injury [22, 46]. In the current study, we controlled the mean arterial pressure to help prevent ischaemic injury of the intestine, and this was confirmed by the normal intestinal histology scores in the acute pancreatitis group. Thus, we show for the first time that high levels of several pancreatic catabolic enzymes are present in acute pancreatitis conditioned mesenteric lymph early in disease process in the presence of normal intestinal histology.

A strength of this study is the use of state-of-the-art iTRAQ™ LC-MS/MS techniques used to define the proteome of acute pancreatitis conditioned mesenteric lymph. Previous studies have used enzyme specific biochemical methods to identify amylase, lipase and trypsin in the thoracic duct lymph of animals [47] and humans [7, 26] with acute pancreatitis. In addition to confirming the presence of these three catabolic enzymes, we report for the first time the presence of ribonuclease 1, carboxypeptidase B1, chymotrypsinogen B and carboxypeptidase A2 in acute pancreatitis conditioned mesenteric lymph. The most abundant protein class identified in normal mesenteric lymph was previously reported to be protease inhibitors [27]. It is striking that despite a substantial increase in the relative abundance of proteases in acute pancreatitis conditioned mesenteric lymph identified here, there was no concomitant rise in relative abundance of protease inhibitors.

A study published in 2008 by Mole *et al*. used two different proteomic techniques to investigate acute pancreatitis conditioned mesenteric lymph [9]. The SELDI-TOF (surface-enhanced laser desorption - ionization time-of-flight mass spectrometry) technique generated spectra that differentiated acute pancreatitis conditioned mesenteric lymph from sham mesenteric lymph, but this could not perform identification of individual proteins [48]. In a separate experiment they used 2D-PAGE (two-dimensional gel electrophoresis) to identify just 4 proteins (transferrin, haptoglobin, alpha1-protease inhibitor and apolipoprotein A1) that showed a relative increase or decrease in acute pancreatitis conditioned mesenteric lymph. Using these techniques it was not possible for Mole *et al*. to demonstrate any numerical fold change data or statistical measures of significance. If immunodepletion had been used to deplete the major abundant proteins prior to 2D-PAGE it might have been possible to achieve a higher level of resolution and identification of additional protein changes. Another limitation of the study by Mole *et al*. [9] is that mean arterial pressure was not controlled. This raises the possibility that the reported proteomic changes in acute pancreatitis conditioned mesenteric lymph might have been due, at least in part, to concomitant hypotension and intestinal ischaemia.

Pancreatic enzymes contribute to the development of distant organ injury and MODS by the proteolytic cleavage of cellular membranes and extracellular proteins, and by activating leucocytes to generate reactive oxygen species (ROS) [49, 50, 51, 52, 53]. Pancreatic proteases are also thought to contribute to the generation of ROS by the limited proteolytic conversion of the enzyme xanthine dehydrogenase to xanthine oxidase (XO) [54]. In the oxidase form, this enzyme produces the superoxide anion and thus generates ROS [55]. It is recognized that pancreatic proteases are not the only factor responsible for the development of MODS in acute pancreatitis. Pancreatic amylase has also been implicated as a potentially toxic factor. It is now thought that high levels of pancreatic amylase disrupt the binding of tissue XO by hydrolyzing the internal alpha1-4 linkages of some of the glycoproteins present in the extracellular space. Once mobilized, XO is able to concentrate in distant organs with low intrinsic XO activity and produce ROS contributing to organ dysfunction [56].

There is evidence that pancreatic enzymes contribute to the toxicity of mesenteric lymph in acute pancreatitis and other critical illnesses. In a human study of severe acute pancreatitis, diversion of trypsin rich thoracic duct lymph reduced lung injury [26]. In the setting of haemorrhage it was found in animal models that shock conditioned mesenteric lymph caused neutrophil dysfunction [14, 15], bone marrow suppression [16], and damage to endothelial cells of the pulmonary microvasculature [17, 18]. These effects were prevented by either ligation of the pancreatic duct prior to the induction of shock [10, 22] or by the intraintestinal inhibition of pancreatic serine proteases [23, 24, 25, 50] thereby implicating pancreatic proteases as toxic factors in mesenteric lymph.

The findings of this study support the proposal that pancreatic catabolic enzymes in mesenteric lymph during acute pancreatitis could be therapeutic targets. The history of intravenous anti-protease treatment in acute pancreatitis, using gabexate and nafamostat, is disappointing [57]. After more than 70 clinical trials and several meta-analyses, there is no convincing evidence to recommend the use of intravenous protease inhibition in acute pancreatitis [58]. Of the 16 recently published clinical guidelines there are only two, from Japan and China, that recommend the use of

intravenous protease inhibitors [58, 59], and not on the basis of high level evidence. The findings of the present study would suggest that protease inhibition might be more effective if given by the enteral rather than the intravenous route, especially if it were lymphotropic and concentrated in mesenteric lymph. To our knowledge, there is only one clinical trial that investigated the use of oral protease inhibition (FOY 305) in acute pancreatitis [60] and showed significant improvement in abdominal pain scores and urinary amylase in the treatment arm. Further studies to evaluate the efficacy of protease inhibition delivered by the enteral route appear to be justified in this context. There have been previous reports of enteral protease inhibitor treatment improving haemodynamic parameters, reducing intestinal injury and leukocyte activation in models of intestinal ischaemia-reperfusion injury [24, 25] and septic shock [61].

One of the challenges of proteomics is that high abundance proteins mask low abundance proteins when compositional analysis is attempted. This problem was addressed in the present study by the immunodepletion of the highly abundant proteins using a validated method [36, 62, 63, 64, 65]. Unfortunately, unintentional protein loss inevitably occurs during immunodepletion because of non-specific binding to the column, specific binding to immunoglobulin with structural homology to the proteins being depleted, and/or binding to the proteins that are being depleted [36, 62]. Given the modest amount of lymph that can be collected from a rat during the experimental protocol (750-1,000 μL), only 10-20 μL of the original sample is left after immunodepletion and LC-MS/MS for further evaluation thus prohibiting further analyses by complementary gel-based proteomic methods. The protein fraction of mesenteric lymph is unlikely to contain all of the factors responsible for the toxicity found in acute pancreatitis and other critical illnesses, although it has been found to be more toxic than the lipid fraction [8]. Delineating the composition of the lipid fraction of acute pancreatitis conditioned mesenteric lymph is the focus of further studies.

CONCLUSION

This is the first comprehensive description of the proteome of mesenteric lymph conditioned by acute pancreatitis. It has demonstrated a significant increase in the relative abundance of 8 proteins amongst the 245 proteins identified using state-of-the-art iTRAQ^{TM} based LC-MS/MS techniques, 7 of which are secreted pancreatic catabolic enzymes. This study provides a clear rationale for further research to investigate the efficacy of enteral protease inhibitors in the treatment of acute pancreatitis.

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Supplementary Table 1. Non-redundant list of 245 proteins identified in the mesenteric lymph from the pancreatitis and sham experimental groups.

Name	Gene symbol	Protein identifier	No. of animals [a]		Fold change [b]	P value	Adjusted P value
			AP [c]	Sham			
Ab1-018	Hps5	IPI00382131	8	8	-1.03	0.797	0.98
Ig gamma-2B chain C region	Igh-1a	IPI00655256	3	2	NA	NA	NA
22 kDa protein [d]	-	IPI00204640	8	8	1.07	0.742	0.961
Actin alpha cardiac 1	Actc1	IPI00194087	1	1	NA	NA	NA
Actin, alpha 1, skeletal muscle	Acta1	IPI00189813	3	3	1.80	0.150	0.589
Actin, gamma, cytoplasmic 1	Actg1	IPI00764461	4	4	1.90	0.160	0.589
Adiponectin, C1Q and collagen domain containing [d]	Adipoq	IPI00202515	6	5	1.20	0.380	0.833
Afamin	Afm	IPI00777658	2	2	NA	NA	NA
AHNAK nucleoprotein [d]	Ahnak	IPI00769072	3	5	1.35	0.653	0.930
Albumin	Alb	IPI00191737	8	8	-2.12	0.026	0.242
Alcohol dehydrogenase1	Adh1	IPI00331983	2	3	NA	NA	NA
Alcohol dehydrogenase 4 (class II), pi polypeptide	Adh4	IPI00476212	1	2	NA	NA	NA
Aldolase A	Aldoa	IPI00231734	6	6	1.32	0.269	0.745
Aldolase B	Aldob	IPI00471911	2	3	NA	NA	NA
Alpha 1 microglobulin/bikunin	Ambp	IPI00210900	8	8	1.04	0.778	0.975
Alpha-1-inhibitor III	A1i3	IPI00201262	8	8	-1.07	0.601	0.902
Alpha-2-glycoprotein 1, zinc	Azgp1	IPI00211103	8	8	-1.05	0.790	0.974
Alpha-2-HS-glycoprotein	Ahsg	IPI00327469	8	8	-1.10	0.560	0.875
Alpha-2u globulin PGCL1	LOC259246	IPI00400456	6	6	1.80	0.121	0.589
Amiloride binding protein 1	Abp1	IPI00204571	2	2	NA	NA	NA
Amylase 1, salivary	Amy1	IPI00198466	8	8	2.24	0.010	0.121
Amylase 2, pancreatic	**Amy2**	**IPI00211904**	**7**	**6**	**46.96**	**<0.001**	**<0.001**
Angiotensinogen	Agt	IPI00209744	8	8	1.04	0.816	0.986
Apolipoprotein A-I	Apoa1	IPI00563778	8	8	-1.02	0.905	0.986
Apolipoprotein A-II	Apoa2	IPI00197700	7	6	-1.24	0.466	0.876
Apolipoprotein A-IV	Apoa4	IPI00324272	8	8	-1.03	0.896	0.986
Apolipoprotein B	Apob	IPI00555161	8	8	1.03	0.947	0.986
Apolipoprotein C-I	Apoc1	IPI00200102	8	8	-1.00	0.995	0.999
Apolipoprotein C-II	Apoc2	IPI00194583	8	8	-1.30	0.448	0.873
Apolipoprotein C-III	Apoc3	IPI00206600	8	8	-1.45	0.271	0.745
Apolipoprotein C-IV	Apoc4	IPI00191952	7	7	-1.22	0.521	0.876
Apolipoprotein E	Apoe	IPI00190701	8	8	1.15	0.260	0.745
Apolipoprotein H	Apoh	IPI00778633	8	8	-1.78	0.010	0.121
B-cell CLL/lymphoma 10	Bcl10	IPI00776589	2	1	NA	NA	NA
Beta-2 microglobulin	B2m	IPI00204359	8	8	1.03	0.892	0.986
Haemoglobin beta chain isoform	MGC72973	IPI00207146	8	8	7.66	0.039	0.304
Betaine-homocysteine methyltransferase	Bhmt	IPI00332027	2	3	NA	NA	NA
Biliverdin reductase B (flavin reductase (NADPH)) (predicted)	Blvrb_predicted	IPI00392676	2	1	NA	NA	NA
Cadherin 1	Cdh1	IPI00206662	3	4	-1.20	0.695	0.930
Cadherin 17	Cdh17	IPI00215358	1	2	NA	NA	NA
Calmodulin	Calm1	IPI00231955	5	4	1.33	0.539	0.876
Calreticulin	Calr	IPI00191728	2	1	NA	NA	NA
Carbonic anhydrase 1 [d]	Car1_predicted	IPI00360930	8	8	2.16	0.076	0.467
Carbonic anhydrase 3	Ca3	IPI00230788	8	8	-1.03	0.940	0.986
Carboxypeptidase A1	Cpa1	IPI00327713	2	3	NA	NA	NA
Carboxypeptidase A2 (pancreatic) [d]	**Cpa2_predicted**	**IPI00193391**	**6**	**5**	**34.64**	**<0.001**	**<0.001**

Carboxypeptidase B1 (tissue)	**Cpb1**	**IPI00193393**	**6**	**5**	**22.89**	**<0.001**	**<0.001**
Carboxypeptidase B2 (plasma)	Cpb2	IPI00190501	8	8	-1.53	0.313	0.773
Carboxypeptidase N, polypeptide 1	Cpn1	IPI00190500	7	7	-1.06	0.690	0.927
Cathepsin B	Ctsb	IPI00562653	2	3	NA	NA	NA
Cationic trypsinogen [d]	**LOC286911**	**IPI00211212**	**6**	**5**	**29.64**	**<0.001**	**<0.001**
Gastrotropin	Fabp6	IPI00231649	2	3	NA	NA	NA
Ceruloplasmin	Cp	IPI00476292	8	8	-1.11	0.513	0.876
Chymotrypsinogen B	**Ctrb**	**IPI00206309**	**6**	**5**	**18.22**	**<0.001**	**<0.001**
Clusterin	Clu	IPI00198667	8	8	1.08	0.563	0.876
Coagulation factor II	F2	IPI00189981	8	8	-1.11	0.537	0.876
Coagulation factor IX	F9	IPI00765267	4	4	1.07	0.779	0.975
Coagulation factor X	F10	IPI00206786	8	8	-1.13	0.339	0.852
Coagulation factor XI	F11	IPI00569754	4	2	NA	NA	NA
Coagulation factor XII	F12	IPI00365752	8	8	-1.21	0.178	0.615
Cofilin 1	Cfl1	IPI00327144	3	4	1.72	0.247	0.728
Col6a3_predicted	Col6a3_predicted	IPI00360737	1	1	NA	NA	NA
Complement component 1, q subcomponent, alpha polypeptide	C1qa	IPI00215296	1	2	NA	NA	NA
Complement component 1, r subcomponent [d]	C1r	IPI00361108	5	6	-1.18	0.625	0.913
Complement component 1, s	C1s	IPI00199519	8	8	1.07	0.672	0.927
Complement component 2	C2	IPI00194044	8	8	-1.07	0.619	0.913
Complement component 3	C3	IPI00480639	8	8	-1.68	0.455	0.876
Complement component 4 binding protein, alpha	C4bpa	IPI00209973	8	8	1.03	0.909	0.986
Complement component 4, gene 2	C4-2	IPI00422037	6	7	1.03	0.900	0.986
Complement component 4a	C4a	IPI00213036	8	8	-1.09	0.644	0.927
Complement component 5	C5	IPI00764698	8	8	1.01	0.942	0.986
Complement component 6	C6	IPI00331776	8	8	1.07	0.678	0.927
Complement component 7 [d]	C7	IPI00766303	8	8	1.03	0.876	0.986
Complement component 8, alpha polypeptide [d]	C8a_predicted	IPI00358382	8	8	-1.01	0.928	0.986
Complement component 8, beta polypeptide	C8b	IPI00387929	7	8	-1.04	0.834	0.986
Complement component 8, gamma polypeptide [d]	C8g_predicted	IPI00373395	8	8	1.00	0.999	0.999
Complement component 9	C9	IPI00231423	8	8	1.08	0.594	0.898
Complement component factor H	Cfh	IPI00208659	7	7	-1.02	0.907	0.986
Complement component factor h-like 1	Cfhl1	IPI00554226	7	6	1.12	0.503	0.876
Complement factor B	Cfb	IPI00422011	5	5	-1.10	0.617	0.913
Complement factor D	Cfd	IPI00212480	8	8	1.21	0.336	0.804
Complement factor I	Cfi	IPI00204451	8	8	-1.06	0.680	0.927
C-reactive protein, pentraxin-related	Crp	IPI00188225	8	8	-1.35	0.281	0.745
Creatine kinase, brain	Ckb	IPI00470288	8	8	-1.06	0.862	0.986
Creatine kinase, muscle	Ckm	IPI00211053	8	8	1.50	0.245	0.728
Cystatin C	Cst3	IPI00231801	6	7	1.55	0.192	0.632
Desmoglein 2 [d]	Dsg2_predicted	IPI00358687	1	2	NA	NA	NA
Diazepam binding inhibitor	Dbi	IPI00231069	1	1	NA	NA	NA
Dmx-like 1 [d]	Dmxl1_predicted	IPI00367836	4	2	NA	NA	NA
Enolase 1, alpha	Eno1	IPI00464815	2	3	NA	NA	NA
Enolase 1, alpha pseudogene [d]	LOC688509	IPI00767147	2	3	NA	NA	NA
Enolase 3, beta	Eno3	IPI00231631	8	8	1.59	0.205	0.658
Epidermal growth factor receptor	Egfr	IPI00212694	8	8	-1.12	0.400	0.851
Esterase 2	Es2	IPI00195148	8	5	-1.70	0.124	0.589
Eukaryotic translation initiation factor 5A	Eif5a	IPI00211216	1	2	NA	NA	NA
Expressed in non-metastatic cells 2	Nme2	IPI00325189	4	2	NA	NA	NA
Extracellular link domain-containing 1 (predicted)	Xlkd1_predicted	IPI00359024	1	1	NA	NA	NA
Extracellular matrix protein 1	Ecm1	IPI00231772	6	7	-1.13	0.516	0.880
Fatty acid binding protein 1, liver	Fabp1	IPI00190790	2	3	NA	NA	NA
Fatty acid binding protein 3	Fabp3	IPI00231971	1	1	NA	NA	NA
Fatty acid binding protein 4, adipocytes	Fabp4	IPI00207890	8	7	1.27	0.516	0.876
Fetuin beta	Fetub	IPI00212708	7	6	-1.24	0.353	0.822
Fibrinogen, alpha polypeptide	Fga	IPI00382317	7	7	1.18	0.416	0.858
Fibrinogen, B beta polypeptide	Fgb	IPI00382134	1	3	NA	NA	NA
Fibrinogen, gamma polypeptide	Fgg	IPI00190759	4	4	1.03	0.945	0.986
Fibrinogen-like 2	Fgl2	IPI00324102	3	2	NA	NA	NA
Fibronectin 1	Fn1	IPI00231982	3	5	-2.47	0.077	0.467
Follistatin-like 1	Fstl1	IPI00207063	2	1	NA	NA	NA
Four and a half LIM domains 1 [d]	Fhl1	IPI00780699	3	2	NA	NA	NA
Gelsolin	Gsn	IPI00363974	8	8	-1.03	0.902	0.986
Globin, alpha [d]	LOC287167	IPI00213611	7	6	3.11	0.149	0.589
Glucose phosphate isomerase	Gpi	IPI00364311	8	8	-1.01	0.961	0.986
Glutathione peroxidase 1	Gpx1	IPI00192301	1	2	NA	NA	NA
Glutathione peroxidase 3	Gpx3	IPI00476458	8	8	-1.33	0.133	0.589
Glutathione S-transferase, mu 1	Gstm1	IPI00231639	6	7	4.61	0.007	0.109
Glutathione S-transferase, mu 2	**Gstm2**	**IPI00411230**	**8**	**8**	**3.52**	**<0.001**	**0.013**
Glutathione-S-transferase, alpha type2	Gsta3	IPI00231150	6	4	2.41	0.022	0.221

Glutathione-S-transferase P	Gstp1	IPI00231229	4	5	2.54	0.037	0.297
Glycerol-3-phosphate dehydrogenase 1 (soluble)	Gpd1	IPI00231148	2	3	NA	NA	NA
Glycosylphosphatidylinositol specific phospholipase D1	Gpld1	IPI00325157	7	6	1.41	0.121	0.589
Group specific component	Gc	IPI00194097	8	8	-1.13	0.361	0.824
Guanine deaminase	Gda	IPI00325884	2	3	NA	NA	NA
Isoform 1 of Haptoglobin	Hp	IPI00477597	2	4	NA	NA	NA
Heat shock protein 8	Hspa8	IPI00208205	2	2	NA	NA	NA
Hemoglobin alpha 2 chain	LOC360504	IPI00205036	7	6	5.78	0.064	0.434
Hemoglobin beta chain complex	**Hbb**	**IPI00230897**	**8**	**8**	**20.54**	**0.003**	**0.086**
Hemoglobin subunit alpha-1/2	Hba-a1	PI00287835	1	2	NA	NA	NA
Hemopexin	Hpx	IPI00195516	8	8	-1.14	0.410	0.858
Hepatocyte growth factor activator	Hgfac	IPI00364125	5	4	-1.21	0.304	0.772
Histidine-rich glycoprotein	Hrg	IPI00201347	8	8	-1.27	0.149	0.589
Hypothetical protein LOC678701 [d]	LOC678701	IPI00557598	2	3	NA	NA	NA
Igh-1a protein [d]	Igh-1a	IPI00202440	2	3	NA	NA	NA
Igha_mapped protein	LOC366747	IPI00553949	4	5	-9.46	0.134	0.589
Insulin-like growth factor 1	Igf1	IPI00831729	4	3	1.12	0.669	0.927
Insulin-like growth factor binding protein 3	Igfbp3	IPI00209369	2	3	NA	NA	NA
Insulin-like growth factor binding protein, acid labile subunit	Igfals	IPI00202416	8	8	-1.09	0.448	0.872
Inter alpha-trypsin inhibitor, heavy chain 4	Itih4	IPI00188541	4	5	1.21	0.511	0.876
Inter-alpha trypsin inhibitor, heavy chain 1 [d]	Itih1_predicted	IPI00188338	7	7	-1.03	0.832	0.986
Inter-alpha trypsin inhibitor, heavy chain 3	Itih3	IPI00326984	8	8	-1.05	0.748	0.961
Interleukin 1 receptor accessory protein	Il1rap	IPI00211004	4	4	-2.28	0.022	0.220
Kallikrein B, plasma 1	Klkb1	IPI00203384	8	8	-1.01	0.961	0.986
K-kininogen isoform	LOC25087	IPI00187799	8	8	-1.16	0.227	0.720
K-kininogen 1	LOC25087	IPI00515829	8	8	-1.07	0.776	0.975
Lactate dehydrogenase A	Ldha	IPI00197711	2	2	NA	NA	NA
Lactate dehydrogenase B	Ldhb	IPI00231783	1	1	NA	NA	NA
Lecithin cholesterol acyltransferase	Lcat	IPI00191754	1	2	NA	NA	NA
Lectin, galactose binding, soluble 1	Lgals1	IPI00231275	8	8	1.32	0.329	0.804
LOC366772 BWK3 [d]	LOC366772	IPI00368397	4	5	1.93	0.581	0.884
LOC494499 protein	LOC494499	IPI00555178	5	3	2.62	0.156	0.589
LOC498793 protein [d]	LOC498793	IPI00389806	8	8	1.03	0.817	0.986
LOC500180 Ig kappa chain C region, B allele [d]	LOC500180	IPI00388002	4	5	-1.97	0.271	0.745
LRRGT00046	LOC679040	IPI00454264	1	2	NA	NA	NA
Lumican	Lum	IPI00206403	8	8	1.30	0.173	0.615
Lysozyme	Lyz	IPI00421714	5	4	1.27	0.564	0.876
Malate dehydrogenase 1, NAD (soluble)	Mdh1	IPI00198717	7	7	1.50	0.263	0.745
Mannose binding lectin 1, protein A	Mbl1	IPI00325371	7	4	-1.22	0.284	0.745
Murinoglobulin 1 homolog (mouse)	Mug1	IPI00212666	8	8	-1.00	0.992	0.999
Murinoglobulin 2	Mug2	IPI00564327	8	8	-1.11	0.529	0.876
Muscle glycogen phosphorylase	Pygm	IPI00190181	1	1	NA	NA	NA
Myoglobin	Mb	IPI00214517	8	8	1.55	0.293	0.760
Myosin, light polypeptide kinase [d]	Mylk_predicted	IPI00370703	8	8	-1.42	0.450	0.873
Nucleoside phosphorylase [d]	Np	IPI00207725	2	3	NA	NA	NA
Orosomucoid 1	Orm1	IPI00191715	8	8	-1.14	0.533	0.876
Pancreatic lipase	**Pnlip**	**IPI00198916**	**6**	**5**	**38.09**	**<0.001**	**<0.001**
Paraoxonase 1	Pon1	IPI00555299	8	8	1.09	0.657	0.927
Parkinson disease7	Park7	IPI00212523	3	5	1.14	0.716	0.941
Peptidylprolyl isomerase A	Ppia	IPI00387771	8	8	1.42	0.236	0.721
Peroxiredoxin 1	Prdx1	IPI00211779	5	4	1.37	0.384	0.834
Peroxiredoxin 2	Prdx2	IPI00201561	8	6	2.94	0.007	0.109
Peroxiredoxin 5	Prdx5	IPI00205745	4	4	2.10	0.151	0.589
Peroxiredoxin 6	Prdx6	IPI00231260	6	5	2.17	0.005	0.109
Phosphoglucomutase 1	Pgm1	IPI00780332	7	5	-1.00	0.990	0.999
Phosphoglycerate kinase 1	Pgk1	IPI00231426	8	8	1.41	0.279	0.745
Phosphoglycerate mutase 1	**Pgam1**	**IPI00421428**	**5**	**6**	**2.68**	**0.004**	**0.094**
Phosphoglycerate mutase 2	Pgam2	IPI00231506	4	4	2.48	0.032	0.285
Plasma glutamate carboxypeptidase	Pgcp	IPI00388265	4	6	-1.32	0.312	0.773
Plasminogen	Plg	IPI00206780	8	8	-1.06	0.680	0.927
Platelet factor 4	Cxcl4	PI00206634	1	2	NA	NA	NA
Pregnancy-zone protein	Pzp	IPI00326140	8	8	-1.11	0.64	0.92
Profilin 1	Pfn1	IPI00231358	8	6	1.41	0.182	0.618
Prolyl 4-hydroxylase, beta polypeptide	P4hb	IPI00198887	4	3	13.49	0.005	0.104
Properdin factor, complement [d]	Pfc	IPI00365896	8	8	1.04	0.847	0.986
Protein S (alpha)	Pros1	IPI00213693	4	3	-1.02	0.950	0.986
Protein Z, vitamin K-dependent plasma glycoprotein [d]	Proz_predicted	IPI00365336	8	8	-1.08	0.662	0.927
Putative uncharacterized protein (Fragment) [d]	-	IPI00454446	6	6	1.39	0.281	0.747
Pyruvate kinase, muscle	Pkm2	IPI00231929	1	2	NA	NA	NA
Regenerating islet-derived 3 gamma	Reg3g	IPI00200614	5	3	-1.03	0.967	0.986
Ribonuclease, RNase A family, 1 (pancreatic)	**Rnase1**	**IPI00211902**	**3**	**3**	**40.50**	**<0.001**	**0.024**

S-adenosylhomocysteine hydrolase	Ahcy	IPI00476295	1	2	NA	NA	NA
Selenium binding protein 2	Selenbp1	IPI00208026	6	7	1.56	0.164	0.593
Selenoprotein P, plasma, 1	Sepp1	IPI00188060	8	8	1.10	0.719	0.941
Serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 10	Serpina10	IPI00210340	5	5	-1.03	0.889	0.986
Serine (or cysteine) peptidase inhibitor, clade A, member 3K	Serpina3k	IPI00200593	5	5	1.05	0.905	0.986
Serine (or cysteine) peptidase inhibitor, clade A, member 3N	Serpina3n	IPI00211075	8	8	-1.08	0.749	0.961
Serine (or cysteine) peptidase inhibitor, clade C (antithrombin), member 1 [d]	Serpinc1	IPI00372372	8	8	-1.34	0.114	0.589
Serine (or cysteine) peptidase inhibitor, clade D, member 1	Serpind1	IPI00210947	8	8	-1.08	0.566	0.876
Serine (or cysteine) peptidase inhibitor, clade F, member 1	Serpinf1	IPI00777549	2	3	NA	NA	NA
Serine (or cysteine) peptidase inhibitor, clade F, member 2	Serpinf2	IPI00199695	8	8	-1.11	0.537	0.876
Serine (or cysteine) peptidase inhibitor, clade G, member 1	Serping1	IPI00372792	8	8	-1.27	0.133	0.589
Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	Serpina1	IPI00324019	4	4	1.24	0.422	0.856
Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4	Serpina4	IPI00205568	8	8	-1.02	0.889	0.986
Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6	Serpina6	IPI00551705	6	5	-1.42	0.129	0.589
Serine (or cysteine) proteinase inhibitor, clade A, member 3M	Serpina3m	IPI00210091	7	7	-1.15	0.432	0.858
Serine peptidase inhibitor, Kunitz type 1	Spint1	IPI00454389	4	3	-1.03	0.965	0.986
Serine protease inhibitor	LOC299282	IPI00200591	8	8	-1.12	0.478	0.876
SH3-binding domain glutamic acid-rich protein like (predicted)	Sh3bgrl_predicted	IPI00358292	1	1	NA	NA	NA
Signal recognition particle receptor, B subunit	Srprb	IPI00476177	5	6	-2.70	0.008	0.112
Similar to Actin, cytoplasmic 2 (Gamma-actin) [d]	LOC295810	IPI00765011	4	4	1.46	0.461	0.876
Similar to ADP-ribosylation factor-like 1	LOC688311	IPI00766239	4	3	-1.27	0.616	0.913
Similar to alpha-1 major acute phase protein prepeptide [d]	MGC108747	IPI00679245	8	8	-1.18	0.554	0.876
Similar to amylase 2, pancreatic [d]	LOC499694	IPI00563187	1	2	NA	NA	NA
Similar to B7-like protein GL50-B [d]	RGD1562791_predicted	IPI00391338	1	2	NA	NA	NA
Similar to carboxylesterase 5 [d]	LOC679368	IPI00763603	2	1	NA	NA	NA
Similar to Carboxypeptidase N 83 kDa chain (Carboxypeptidase N regulatory subunit) [d]	RGD1305170_predicted	IPI00769284	8	8	-1.14	0.422	0.858
Similar to Cysteine-rich protein 1 [d]	LOC691657	IPI00192188	1	2	NA	NA	NA
Similar to GTPase activating protein testicular GAP1 [d]	RGD1563562_predicted	IPI00367684	2	3	NA	NA	NA
Similar to heat shock protein 8 [d]	LOC689908	IPI00566672	3	3	-1.77	0.160	0.589
Similar to heat shock protein 8 [d]	LOC680121	IPI00764197	1	2	NA	NA	NA
Similar to histidine-rich glycoprotein [d]	LOC681544	IPI00769165	8	8	-1.33	0.141	0.589
Similar to L-lactate dehydrogenase A chain [d]	RGD1562690_predicted	IPI00203823	3	3	2.30	0.154	0.589
Similar to mKIAA0386 protein [d]	RGD1306939	IPI00382226	6	4	1.11	0.906	0.986
Similar to Murinoglobulin 1 homolog [d]	RGD1566313_predicted	IPI00368704	8	8	-1.09	0.526	0.876
Similar to Myh11 protein [d]	RGD1564935_predicted	IPI00765351	2	1	NA	NA	NA
Similar to peptidoglycan recognition protein 2 [d]	LOC687320	IPI00779290	3	2	NA	NA	NA
Similar to RIKEN cDNA 1300017J02 [d]	RGD1310507	IPI00655254	8	8	-1.55	0.128	0.589
Similar to tropomyosin 1, embryonic fibroblast – rat [d]	MGC109519	IPI00187731	6	4	1.39	0.577	0.883
Similar to Vanin-3 (predicted) [d]	RGD1560609_predicted	IPI00212508	5	5	-1.71	0.278	0.745
SPARC-like 1	Sparcl1	IPI00203494	8	8	1.54	0.068	0.433
Superoxide dismutase 3, extracellular	Sod3	IPI00200507	8	8	1.01	0.929	0.986
Thioredoxin 1	Txn1	IPI00231368	7	6	1.70	0.050	0.352
Thymosin, beta 4	Tmsb4x	IPI00230925	1	2	NA	NA	NA
Transaldolase 1	Taldo1	IPI00190377	1	2	NA	NA	NA
Transferrin	Tf	IPI00679202	3	5	-1.06	0.879	0.986
Transforming growth factor, beta induced [d]	Tgfbi	IPI00188622	8	8	1.19	0.506	0.876
Transgelin	Tagln	IPI00231196	8	8	-1.04	0.921	0.986
Transgelin 2	Tagln2	IPI00555171	1	1	NA	NA	NA
Transketolase	Tkt	IPI00231139	2	3	NA	NA	NA
Transthyretin	Ttr	IPI00324380	7	6	-1.01	0.963	0.986
Triosephosphate isomerase 1	Tpi1	IPI00231767	8	8	1.63	0.121	0.589
Tropomyosin 1, alpha	Tpm1	IPI00204206	2	2	NA	NA	NA
Type II keratin Kb1	Kb1	IPI00421857	4	4	1.65	0.179	0.615
14-3-3 epsilon polypeptide	Ywhae	IPI00325135	3	4	3.37	0.042	0.314
14-3-3 gamma polypeptide	Ywhag	IPI00230835	2	2	NA	NA	NA
14-3-3 zeta polypeptide	Ywhaz	IPI00324893	8	8	1.23	0.346	0.814
Vitronectin	Vtn	IPI00210120	8	8	-1.33	0.059	0.405

Highlighted in bold are proteins with an individual P value less than 0.05 and an adjusted P value less than 0.1 for differences in their abundance between the pancreatitis and sham experimental groups.

NA: not available (when proteins were found in only one or two animals in one of the groups, the P value is not reported as the sample size was too small to gain statistical confidence.)

[a] Number of animals in each group where the protein was identified (maximum n=8 for each group)

[b] Fold change: acute pancreatitis *vs*. sham group

[c] Taurocholate induced acute pancreatitis group

[d] Potentially hypothetical proteins as per International Protein Index (Rat database v3.27)

Supplementary Table 2. Non-redundant list of potentially hypothetical proteins identifications and NCBI BLASTP analysis results (n=47). Proteins listed as hypothetical are as per IPI Rat database v3.27. NCBI BLASTP search against the 'UniProt Clusters 100%' database.

Name	Gene symbol	Protein identifier	Highest scoring protein from BLAST	BLAST species	Similarity
22 kDa protein	-	IPI00204640	C-type lectin domain family 3, member b	Rat	100%
Adiponectin, C1Q and collagen domain containing	Adipoq	IPI00202515	30 kDa adipocyte complement-related protein	Rat	100%
Cationic trypsinogen	LOC286911	IPI00211212	Cationic trypsin-3	Rat	100%
Carboxypeptidase A2 (pancreatic)	Cpa2_predicted	IPI00193391	Carboxypeptidase A2 (pancreatic)	Rat	100%
Four and a half LIM domains 1	Fhl1	IPI00780699	Four and a half LIM domains 1	Rat	100%
Similar to B7-like protein GL50-B	RGD1562791 _predicted	IPI00391338	Icos ligand	Rat	100%
Igh-1a protein	Igh-1a	IPI00202440	Ig gamma-2B chain C region	Rat	100%
Protein S (alpha)	Pros1	IPI00213693	Vitamin K-dependent protein S	Rat	100%
Similar to Cysteine-rich protein 1	LOC691657	IPI00192188	Cysteine-rich protein 1	Rat	100%
Similar to alpha-1 major acute phase protein prepeptide	MGC108747	IPI00679245	T-kininogen 2	Rat	100%
Similar to histidine-rich glycoprotein	LOC681544	IPI00769165	Histidine-rich glycoprotein 2	Rat	100%
Similar to mKIAA0386 protein	RGD1306939	IPI00382226	Ab2-162	Rat	100%
Similar to Actin, cytoplasmic 2 (Gamma-actin)	LOC295810	IPI00765011	Actin, gamma, cytoplasmic 1	Mouse	100%
Similar to tropomyosin 1, embryonic fibroblast	MGC109519	IPI00187731	Tpm2 protein	Mouse	100%
Complement component 1, r subcomponent	C1r	IPI00361108	C1r protein	Rat	99%
Complement component 8, alpha polypeptide [a]	C8a_predicted	IPI00358382	Complement component 8, alpha polypeptide	Rat	99%
Enolase 1, alpha pseudogene	LOC688509	IPI00767147	Enolase-alpha	Rat	99%
Myosin, light polypeptide kinase	Mylk_predicted	IPI00370703	Myosin, light polypeptide kinase	Mouse	99%
Similar to amylase 2, pancreatic	LOC499694	IPI00563187	Pancreatic alpha-amylase	Rat	99%
Similar to heat shock protein 8	LOC680121	IPI00764197	Heat shock cognate 71 kDa protein	Mouse	99%
Similar to L-lactate dehydrogenase A chain	RGD1562690 _predicted	IPI00203823	L-lactate dehydrogenase A chain	Rat	98%
Similar to heat shock protein 8	LOC689908	IPI00566672	Heat shock cognate 71 kDa protein	Mouse	98%
Similar to Myh11 protein	RGD1564935 _predicted	IPI00765351	Myh11 protein	Mouse	97%
LOC498793 protein [a]	LOC498793	IPI00389806	Inter-alpha-trypsin inhibitor heavy chain H2	Rat	96%
Dmx-like 1	Dmxl1_predicted	IPI00367836	Dmx-like 1	Mouse	95%
Similar to Complement component 7	C7	IPI00766303	Complement component 7	Rat	95%
Carbonic anhydrase 1 [a]	Car1_predicted	IPI00360930	Carbonic anhydrase 1	Mouse	94%
Putative uncharacterized protein (Fragment)	-	IPI00454446	Superoxide dismutase	Rat	94%
Serine (or cysteine) peptidase inhibitor, clade C (antithrombin), member 1	Serpinc1	IPI00372372	Antithrombin-III precursor	Mouse	94%
Inter-alpha trypsin inhibitor, heavy chain 1 [a]	Itih1_predicted	IPI00188338	Inter-alpha trypsin inhibitor, heavy chain 1	Mouse	93%
Similar to Vanin-3 (predicted) [a]	RGD1560609 _predicted	IPI00212508	Vanin 3	Mouse	93%
Transforming growth factor, beta induced	Tgfbi	IPI00188622	Transforming growth factor-beta-induced	Mouse	93%
Similar to Murinoglobulin 1 homolog [a]	RGD1566313 _predicted	IPI00368704	Murinoglobulin-1	Rat	91%
Similar to Desmoglein 2	Dsg2_predicted	IPI00358687	Desmoglein 2	Mouse	89%
Nucleoside phosphorylase	Np	IPI00207725	Purine nucleoside phosphorylase	Mouse	89%
Properdin factor, complement	Pfc	IPI00365896	Properdin	Mouse	89%
Similar to carboxylesterase 5	LOC679368	IPI00763603	Adult male colon cDNA, RIKEN	Mouse	89%
Similar to RIKEN cDNA 1300017J02 [a]	RGD1310507	IPI00655254	Adult male liver cDNA, RIKEN	Mouse	89%
Similar to AHNAK nucleoprotein	Ahnak	IPI00769072	Ahnak 1	Rat	86%
Similar to peptidoglycan recognition protein 2	LOC687320	IPI00779290	TagL-alpha	Mouse	84%
Similar to Carboxypeptidase N 83 kDa chain (Carboxypeptidase N regulatory subunit)	RGD1305170 _predicted	IPI00769284	Carboxypeptidase N subunit 2	Mouse	83%
Protein Z, vitamin K-dependent plasma glycoprotein [a]	Proz_predicted	IPI00365336	Vitamin K-dependent protein Z	Mouse	82%
LOC366772 BWK3 [a]	LOC366772	IPI00368397	Igh protein	Mouse	80%
Globin, alpha	LOC287167	IPI00213611	Hemoglobin subunit alpha	Hamster	79%
Hypothetical protein LOC678701	LOC678701	IPI00557598	BWK3	Rat	78%
LOC500180 Ig kappa chain C region, B allele	LOC500180	IPI00388002	Anti-colorectal carcinoma light chain	Mouse	69%
Similar to GTPase activating protein testicular GAP1	RGD1563562 _predicted	IPI00367684	GTPase activating protein testicular GAP1	Rat	61%

[a] Previously identified in a proteomic study of normal mesenteric lymph (Mittal *et al*. 2008, [27])