Published in final edited form as:

J Immunol Methods. 2011 June 30; 369(1-2): 98–107. doi:10.1016/j.jim.2011.04.012.

Cytokine Profiling of Pancreatic Fluid using the ePFT Collection Method in Tandem with a Multiplexed Microarray Assay

Joao A. Paulo, PhD,

Department of Pathology, Children's Hospital Boston, Boston, MA Proteomics Center at Children's Hospital Boston, Boston, MA Center for Pancreatic Disease, Division of Gastroenterology, Hepatology and Endoscopy, Brigham and Women's Hospital and Department of Medicine, Harvard Medical School, Boston, MA

Linda S. Lee, MD,

Center for Pancreatic Disease, Division of Gastroenterology, Hepatology and Endoscopy, Brigham and Women's Hospital and Department of Medicine, Harvard Medical School, Boston, MA

Bechien Wu, MD, MPH,

Center for Pancreatic Disease, Division of Gastroenterology, Hepatology and Endoscopy, Brigham and Women's Hospital and Department of Medicine, Harvard Medical School, Boston, MA

Peter A. Banks, MD,

Center for Pancreatic Disease, Division of Gastroenterology, Hepatology and Endoscopy, Brigham and Women's Hospital and Department of Medicine, Harvard Medical School, Boston, MA

Hanno Steen, PhD[#], and

Department of Pathology, Children's Hospital Boston and Harvard Medical School, Boston Proteomics Center at Children's Hospital Boston, Boston, MA

Darwin L. Conwell, MD, MS[#]

Center for Pancreatic Disease, Division of Gastroenterology, Hepatology and Endoscopy, Brigham and Women's Hospital and Department of Medicine, Harvard Medical School, Boston, MA

Abstract

Cytokines are secreted immunomodulating proteins involved in pancreatic stellate cell activation and propagation of fibrosis in chronic pancreatitis. We aim to show that cytokines can be

© 2011 Elsevier B.V. All rights reserved.

corresponding author: Darwin L. Conwell Center for Pancreatic Disease Division of Gastroenterology, Hepatology and Endoscopy 75 Francis St. Boston, MA 02115 dconwell@partners.org phone:(617) 525-8818 fax: (617) 566-0338.

AUTHOR CONTRIBUTIONS

JP carried out the experiments. JP and DC conceived of the study and drafted the original manuscript. JP, HS, and DC participated in its design and coordination. DC, and LL collected the specimens and assisted in experimental design. All authors helped to draft the manuscript and approved the final manuscript.

[#]These authors contributed equally to this work

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

COMPETING INTERESTS

The authors declare no competing interests.

identified from pancreatic fluid by (1) collecting pancreatic fluid with the ePFT method, (2) processing the fluid for cytokine-targeted microarray analysis, and (3) comparing cytokine profiles in pancreatic fluid of chronic pancreatitis (CP) patients and of chronic abdominal pain (CAP) controls. We endoscopically collected pancreatic fluid from patients with CP and those with CAP using the ePFT method. This fluid was subjected directly to a multiplexed cytokine protein microarray assay. Six patients (3 CP, 3 CAP) underwent a secretin-stimulated ePFT. The mean peak bicarbonate concentrations [meq/L] of the CP and CAP patients were 43 and 97, respectively. Statistically significant decreases in the cytokine concentrations of EGF, IP-10, eotaxin, IL-3, MIP-1a, IL-15, PDGF-AB/BB, and IL-1a were observed in the CP specimens ($p < 0.05$). We have successfully identified differences in the abundance of cytokines in ePFT-collected pancreatic fluid with a multiplexed microarray assay comparing CP and CAP controls. Further targeted investigation of cytokines in ePFT-collected fluid will broaden our knowledge of pancreatic immune response and pathogenesis in chronic pancreatitis.

Keywords

ePFT; pancreas; biomarker; pancreatitis; pancreatic juice; pancreas fluid

1. Introduction

Immunomodulating proteins, such as cytokines, are involved in pancreatic stellate cell activation and pathogenesis of chronic pancreatitis. Cytokines are regulatory proteins that act as intercellular signaling molecules and are produced by various cell types. These low-molecular weight proteins interact with specific cell-surface receptors upon which the ensuing cascades modulate inflammatory and immune responses. Cytokines are generally released in picomolar amounts; however, their concentration can increase over 1000-fold under physiological stress, such as trauma or infection (Cannon, 2000). Cytokines are critical to the development and function of the immune response cascade, as they are often secreted by cells that have undergone cellular stress. This class of proteins includes interleukins (IL), cell signal molecules, such as growth factors (GF), tumor necrosis factor (TNF), and interferons (INF), which trigger inflammation and respond to infections.

In addition, chemokines are a superfamily of small cytokines (8-10 kDa) that act as chemoattractants which guide the migration of cells via corresponding chemokine receptors (Fernandez and Lolis, 2002). These proteins are known to attract leukocytes, neutrophils, monocytes, and other effector cells from the blood to sites of infection or tissue damage (Rottman, 1999). Specifically, chemokines interact with G protein-linked transmembrane receptors, which are selectively found on the surfaces of their target cells. As with cytokines, in general, many chemokines are considered pro-inflammatory. Such proteins can be induced during an immune response to transport cells of the immune system to a site of infection, while others are considered homeostatic and have roles in controlling the migration of cells during normal processes of tissue maintenance or development (Fernandez and Lolis, 2002). Chemokines include eotaxin, fractalkine, growth-regulated oncogene (GRO), interferon-inducible protein (IP), monocyte chemoattractant protein (MCP), myeloma cell metalloproteinase (MDC), macrophage inflammatory protein (MIP), and Rantes.

We have developed and described an endoscopic pancreatic function test (ePFT) that collects pancreatic fluid without cannulation of the pancreatic duct and without the risk of procedure-related injury (Conwell et al., 2003a; Conwell et al., 2003b; Wu and Conwell, 2009). As determined from its electrolyte composition and enzyme activity, the ePFT-collected fluid reproduces the classic acinar and duct cell secretory profiles following

hormonal stimulation (Conwell et al., 2002; Conwell et al., 2003a; Conwell et al., 2003b; Stevens et al., 2004a; Stevens et al., 2004b). The ePFT is now considered an acceptable alternative method for the assessment of pancreas secretory physiology (Pollack and Grendell, 2006; Forsmark, 2008). Furthermore, pancreatic fluid collected by the ePFT method is fully amenable to proteomic analysis (Paulo et al., 2011). Thus far, a systematic and comprehensive analysis of immunomodulating proteins, such as cytokines, in pancreatic fluid has not been performed. As the ePFT collection method is a valuable alternative tool to acquire pancreatic fluid even from subjects without pancreas-related disease, we aim to establish this technique, coupled with protein microarray analysis, as a viable strategy to investigate the cytokine profile of pancreatic fluid.

A suspension microarray uses capture antibodies that are coupled to color-coded microspheres and allows the simultaneous analysis of numerous cytokines in a single experiment. Such a multiplexed immunoassay has the advantages of specificity, small sample volumes, and cost-effectiveness. For these reasons, the cytokine protein microarray is both sensitive to the low concentration of cytokines and amenable to high-throughput analysis (Fitzgerald et al., 2008). Although in clinical settings such technology is primarily used for the analysis of urine and blood, proximal body fluids, such as pancreatic fluid, may also benefit from such microarray-based approaches.

The primary objectives of our exploratory investigation are as follows:

1. collect pancreatic fluid with the ePFT method after secretin stimulation,
2. process the pancreatic fluid for cytokine-targeted microarray analysis, and
3. compare cytokine profiles in the pancreatic fluid of chronic pancreatitis and of non-pancreatitis/chronic abdominal pain controls.

The methodology established herein may allow targeted investigations of the cytokines in the pancreatic fluid secretome and broaden our knowledge of pancreatic immune-response and disease pathogenesis.

2 Materials and Methods

2.1 Study population

The study design was a cytokine analysis of endoscopically collected pancreatic fluid in the setting of an academic center. This protocol was approved by the Institutional Review Board at Brigham and Women's Hospital and Children's Hospital Boston (IRB # 2007-P-002480/1). The study population (**Table 1**) included adult patients seen in the Center for Pancreatic Diseases at Brigham and Women's Hospital to where they were referred for pancreatic function testing. All subjects underwent the following: 1) informed consent, 2) comprehensive history and physical examination, 3) review of radiologic and endoscopic data, and 4) upper endoscopy with ePFT followed by mucosal biopsy. Chronic pancreatitis was classified as definitive based on the MANNHEIM diagnostic criteria (Schneider et al., 2007). Conversely, the M-ANNHEIM criteria for chronic pancreatitis were not met by any of the chronic abdominal pain patients.

The M-ANNHEIM (Multiple risk factors, Alcohol, Nicotine, Nutrition, Hereditary factors, Efferent duct factors, Immunological factors, and Miscellaneous and metabolic factors) classification is a standardized system which has been developed for the classification of chronic pancreatitis according to etiology, clinical staging, and severity of the disease (Schneider et al., 2007). This system considers clinical imaging data resulting from a wide array of laboratory test results, including ultrasound (US), endoscopic ultrasound (EUS), magnetic resonance imaging (MRI), computed tomography (CT) as well as risk factors, such

as alcohol consumption and diabetes, in addition to hereditary factors, such as SPINK1 and PRSS1 (Schneider et al., 2007; Diaconu et al., 2009). The amalgamation of these factors has established a single classification system for the different stages of chronic pancreatitis.

2.2 Material

ChiRhoStim® synthetic human secretin was from ChiRhoClin (Burtonsville, MD).

2.3 Experimental workflow

The overall analysis was as follows: 1) ePFT sample collection on ice, 2) particulate removal via centrifugation, and 3) division of the sample for biochemical and microarray assays. For microarray assays, the samples were centrifuged to remove particulates, cytokine multiplex microarray analysis was performed, and the resulting cytokine profiles were statistically analyzed, as illustrated in **Figure 1**.

2.4 Pancreatic fluid collection (ePFT method)

The ePFT procedure included the following: 1) pre-procedural assessment, 2) endoscopic procedure and 3) post-procedural assessment/recovery.

2.4.1 Pre-procedural assessment—Prior to upper endoscopy, all study subjects underwent a history and physical examination including list of allergies, medications, substance use/abuse, and vital signs. Pre-procedural sedation review included airway assessment based on Mallampati airway scale and American Society of Anesthesiologists Physical Status Classification (ASA Class). All study subjects in this protocol had a Mallampati score of B, Class 2 and ASA Class II or better. In addition, the study subjects were required to fast for at least 12 hrs.

2.4.2 Procedure—Endoscopic collection is performed in a stepwise manner as follows: 1) The patient was placed in 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 given 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 (2 to 5 minutes). 7) Gastroduodenal fluid was aspirated (approximately 1 minute) as completely as possible through the gastroscope. 8) A standard weight-based intravenous bolus (0.2 µg/kg) given over 1 minute of synthetic human secretin (ChiRhoStim®) was administered. 9) Pancreatic fluid was aspirated from the descending duodenum at specific timed intervals following hormonal stimulation and temporarily stored on ice.

The duodenal aspirates were collected at 0, 5, 10, 15, 20, 30, 45 and 60 minutes after stimulation. Sample volumes ranged from 1 mL to 5 mL for each time point collection. Only the 30-minute time point for each patient was used for the ensuing cytokine analysis. Samples were divided and aliquots were sent to the Brigham and Women's Hospital Biochemistry Laboratory for measurement of electrolyte profiles and the Proteomics Center at Children's Hospital Boston for proteomic analysis. Biopsies of the stomach and duodenum were obtained to rule out microscopic gastrointestinal disease, such as *Helicobacter pylori* or celiac sprue, as a cause of dyspepsia.

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

2.5 Pancreatic fluid biochemical analysis

All biochemical measurements were conducted within 2-4 hours of sample collection. A 1 mL aliquot was 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 ions were measured by indirect ion-selective electrodes, and total bicarbonate was measured by the two-step phosphoenolpyruvate carboxylase-malate dehydrogenase enzymatic-photometric method (Forrester et al., 1976). Samples with results greater than the upper assay limit were diluted into the linear range. Based on previously published studies, the expected mean peak bicarbonate concentration in secretin-stimulated pancreatic fluid from non-pancreatitis controls was 103 ± 11 meq/L (Stevens et al., 2004a). A cut-off point of 80 meq/L was two standard deviations below the mean and considered abnormal (Stevens et al., 2004b; Wu and Conwell, 2009).

2.6 Pancreatic fluid cytokine microarray analysis

In brief, pancreatic fluid samples were collected on ice, centrifuged at 4°C/14,000 rpm to remove cellular debris, and divided 500 µL aliquots prior to storage at -80°C. Protein concentrations were determined using the Pierce BCA (bicinchoninic acid) Protein Assay (Waltham, MA) to ensure that the total protein concentration of each sample was within the 0.8-1.4 mg/mL range, as previously reported (Paulo et al., 2010c).

Levels of cytokines (**Table 2**) in pancreatic fluid were determined using microsphere-based suspension microarray technology (AssayGate, Ijamsville, MD) (Opalka et al., 2003). The methodology used for the microarray analysis was previously published (Carson and Vignali, 1999; Vignali, 2000; Sachdeva and Asthana, 2007). In brief, multiple analytes in a single aliquot (75 µL) of pancreatic fluid were determined quantitatively and simultaneously with Bio-Plex 200 Bead Reader System (Bio-Rad, Hercules, CA). Microparticles were conjugated to differing concentrations of two fluorophores to generate distinct bead sets. Each bead set was coated with capture antibody specific for one analyte. Captured analyte was detected using a biotinylated detection antibody and streptavidin-phycoerythrin (S-PE). The bead analyzer was a dual laser, flow-based, sorting and detection platform. One laser was bead-specific and determined which analyte was being detected. The second laser determined the magnitude of PE-derived signal, which was in direct proportion to the amount of analyte bound. At most, 75 µL of pancreatic fluid was used for each assay and each sample was tested in duplicate.

Immediately prior to the microarray analysis, the concentration of each cytokine in each sample was determined by 5-parameter logistic regression algorithm with analysis of the median fluorescence intensity readings of an 8-point protein standard curve. This procedure ensures that the reading is within the linear range of the assay. Once a regression equation was derived, the fluorescence intensity values of the standards were treated as unknowns and the concentration of each standard was calculated. A ratio of the calculated value to the expected value of this standard was determined. A ratio between 70 and 130% for each of standards indicated a good fit. If fluorescence intensity values of samples reached a plateau or were outside the range of standard curves, a re-test with diluted samples was performed to ensure the fluorescence intensity measurement of unknown samples fall inside the linear range of standard curves. The results were expressed in picograms per milliliter of pancreatic fluid and analyzed by the Wilcoxon rank-sum non-parametric test using SAS 9.2 (Cary, NC), with which boxplots were constructed. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Demographics

Table 1 displays the characteristics of the 6 subjects in the study cohort. Pancreatic fluid was safely collected via secretin-stimulated ePFT from three controls (CAP) and three patients with definite chronic pancreatitis (CP) based on the M-ANNHEIM classification. Conversely, the MANNHEIM criteria for chronic pancreatitis were not met by any of the three individuals with CAP. The mean peak bicarbonate concentrations of the CP and CAP subjects were 43 (abnormal) and 97 (normal) meq/L, respectively.

3.2 Protein microarray assay detected cytokines directly from pancreatic fluid

The pancreatic fluid samples from the six individuals were directly assayed using a suspension microarray assay to quantitatively determine the presence of 42 cytokines (**Table 2**). Unlike mass spectrometry-based proteomic assays of fluids collected by ePFT (Paulo et al., 2010b; Paulo et al., 2010a; Paulo et al., 2010c), minimal sample preparation was necessary for this assay, as the only prerequisite was the removal of particulates via centrifugation prior to performing the assay. From our initial analysis, we noted that the detectable levels of cytokines that were identified in pancreatic fluid ranged in concentration from 25 to 250 pg/mL. **Table 3** lists the cytokine concentrations (pg/mL) in ePFT-collected pancreatic fluid for all six individual samples ordered from the highest to the lowest concentration.

3.3 Different abundances of cytokines were present in pancreatic fluid of individuals with chronic pancreatitis and chronic abdominal pain

We examined the differences in cytokine concentration between the cohort of individuals with chronic pancreatitis (abnormal secretory function) and the chronic abdominal pain (normal secretory function) cohort. As was listed in **Table 3**, and as was illustrated in **Figure 2**, cytokines IL-1a, IL-15, and IL-3, as well as the chemokines MIP-1a, eotaxin and IP-10, and growth factors PDGF-AB/BB and EGF were lower in concentration with statistical significance in the CP patients when compared to individuals with CAP (Wilcoxon rank-sum test, $p < 0.05$). Moreover, EGF, IP-10, eotaxin, and IL-3 were among the cytokines with the highest concentrations that we detected in pancreatic fluid.

4. Discussion

We have identified cytokines in secretin stimulated, ePFT-collected pancreatic fluid using a microsphere-based suspension protein microarray assay. To our knowledge, this is the first published report profiling cytokines in ePFT-collected pancreatic fluid. We have compared the levels of 42 different cytokines between chronic pancreatitis (CP) and chronic abdominal pain (CAP) control patients. Our analysis has revealed differences in the cytokine profile of pancreatic fluid between these two cohorts. We have identified eight cytokines, EGF, IP-10, eotaxin, IL-3, MIP-1a, IL-15, PDGF-AB/BB, and IL-1a which are decreased in chronic pancreatitis patients with statistical significance. This targeted method of directly investigating pancreatic fluid has the advantages of specificity, small sample volumes, cost-effectiveness, and is complementary to other detection methods, such as mass spectrometry and western blotting (Pollard et al., 2007).

Our data show that four cytokines (EGF, IP-10, eotaxin, and IL-3) have a greater than 15-fold difference in median concentration in pancreatic fluid when comparing CP and CAP individuals. **EGF** (epidermal growth factor) has a 29-fold decrease in concentration in CP versus CAP individuals. EGF is a known inducer of differentiation in IL-3 dependent cell lines and is also involved in macrophage-mediated cellular proliferation and is mitogenic for

fibroblasts and smooth muscle cells (Wang et al., 1989). Moreover, EGF plays an important role in the regulation of cell growth, proliferation, and differentiation by binding to its receptor EGFR (Carpenter and Cohen, 1990; Herbst, 2004). In addition, growth factors also showed statistically significant decreases when comparing pancreatic fluid from our two cohorts. Likewise, **IP-10** has nearly a 17-fold decrease in concentration in the pancreatic fluid of CP individuals compared to CAP individuals. **IP-10** is a chemokine that is a chemotactic for monocytes and T-lymphocytes, which binds to the CXCR3 G protein-coupled receptor (Dufour et al., 2002). In support of our finding, a previously published study examining pancreatic tissue showed that expression of IP-10 is high in healthy pancreatic tissue but low in tissue of individuals with chronic pancreatitis (Walz et al., 1997). Similarly, **eotaxin** has a 25-fold increase in concentration when comparing CP to CAP individuals. Eotaxin is a chemokine that directly promotes the accumulation of eosinophils, a prominent feature of allergic inflammatory reactions (Kampen et al., 2000; Palikhe et al., 2009). Another differentially abundant cytokine, **IL-3** has a 19-fold decrease in the median concentration when comparing CP and CAP individuals. IL-3 acts in hematopoiesis by controlling the production, differentiation, and function of granulocytes and the monocyte-macrophages (Ihle et al., 1981; Yang et al., 1986). IL-3 is expressed by T-lymphocytes, that are activated by IL-15 which is also found to be differentially abundant in our experiments (Giri et al., 1994).

In addition, there are several other cytokines (MIP-1a, IL-15, IL-1a, and PDGF-AB/BB) that show statistically significant, but less than 10-fold decrease in median concentration when comparing individuals with CP and CAP. **MIP-1a**, is produced by macrophages, activated granulocytes and also induces the synthesis of IL-1, IL-6, and TNF- α from fibroblasts and macrophages (Menten et al., 2002), including those in the pancreas (Andoh et al., 2000). **IL-15** has been shown also to enhance the anti-tumor immunity of CD8⁺ T cells in pre-clinical models, in addition to stimulating the activation and proliferation of T-lymphocytes, (Klebanoff et al., 2004). Another interleukin, **IL-1a** is a cytokine that is generally produced by activated macrophages. IL-1a can induce IL-2 release, B-cell maturation and proliferation, and fibroblast growth factor activity. IL-1a is also involved in the inflammatory response and has been shown to stimulate the release of prostaglandin and collagenase (Dinarello, 1994; Barland et al., 2004). Additionally, our data show that **PDGF-AB/BB** is also of decreased abundance in the CP specimens. PDGF isotypes bind the receptor tyrosine kinase and have downstream effects which include regulation of gene expression and the cell cycle (Fredriksson et al., 2004; Tallquist and Kazlauskas, 2004). In late stage pancreatitis, myofibroblast activity decreases, which may result in the decreased concentration of PDGF-AB/BB, as is seen in our results (Detlefsen et al., 2006). The cytokines mentioned above offer promising targets for future studies aimed at discovering biomarkers of chronic pancreatitis or discerning immunological mechanisms of the disease.

Cytokines in pancreatic fluid have been previously identified using other molecular biology techniques. In a work by Noh, et al (Noh et al., 2006), modified enzyme-linked immunosorbent assays (ELISA) were used to compare the levels of interleukin-8 (IL-8), interleukin-6 (IL-6), and transforming growth factor- β (TNF- β) in pancreatic fluid. Similar to our work, Noh, et al do not detect differences in the amount of IL-6 and TNF- β using their method. However, using IL-8, this group is able to discriminate between pancreatic fluid from normal and chronic pancreatitis patients. With our cytokine microarray assay, we are unable to reproduce this result, as there is no significant difference in IL-8 concentration between the two cohorts. Several confounding factors may result in disparities between these assays, including variations in sample preparation and reagents, particularly different antibodies used in each of the assays. A more systematic comparison between the two methods must be performed before conclusions can be drawn as to the reasons for this disparity.

In terms of the development and progression of chronic pancreatitis, it has been hypothesized that cytokine production may be closely linked to pancreatic stellate cell function (Apte and Wilson, 2004; Ellenrieder et al., 2004; Patel and Fine, 2005; Shimizu, 2008). In response to pancreatic injury, pancreatic stellate cells express several growth factors and cytokines, which are known to participate in inflammatory and fibrotic processes (Apte et al., 1999; Mews et al., 2002; Vonlaufen et al., 2007; Aust et al., 2008; Farrow et al., 2008; Shimizu, 2008; Masamune et al., 2009), and which potentially may be identified in ePFT-collected pancreatic fluid. Pancreatic stellate cells are often activated by cytokines (IL-1, IL-6, IL-8, and TNF- α) and growth factors (PDGF and TGF- β 1) and in turn, can express cytokines, including IL-1 and IL-6, and growth factors, such as PDGF and TGF- β 1 (Omary et al., 2007). Factors controlling the cellular functions of pancreatic stellate cells, including these and other cytokines, could provide potential targets for the treatment or diagnosis of pancreatic fibrosis or inflammation, which may be a prelude to the development of chronic pancreatitis (Masamune and Shimosegawa, 2009). Further experiments based on the work discussed herein may also focus on the investigation of stellate cells in culture; however, this is beyond the scope of the present work.

Although we have noted statistically significant decreases in the concentration of certain cytokines, it is possible that the peak concentration of certain cytokines in pancreatic fluid may not be at its highest point in severe chronic pancreatitis, which we studied herein, but rather during the early development of chronic pancreatitis. Markers for disease are generally classified as early, mid-, or late stage, and it is possible that the concentrations of certain cytokines are elevated during the early to mid- stages of pancreatitis and dissipate in later disease stages, as a result of cellular dysfunction. Comparing cytokine levels during early- and mid- stages of chronic pancreatitis with those cytokine levels in healthy individuals, as well as advanced chronic pancreatitis, would enhance future studies. With the current study design, it is possible that at the stage of advanced chronic pancreatitis, cytokine production was decreased due to pancreatic dysfunction and such phenomena may be analogous to decreases in pancreatic enzyme concentrations exhibited by advanced chronic pancreatitis patients. Although the mechanisms of cytokines are well studied, their role as markers of the pathogenesis or progression of chronic pancreatitis is yet to be completely determined.

A potential limitation of our strategy for pancreatic fluid analysis is the possible impact of stimulation with secretin on the cytokine levels. Secretin stimulates pancreatic secretion mainly by promoting the secretion of water and bicarbonate from the ductal cells of the pancreas (Catalano et al., 1998; Bali et al., 2007). However, there is a potential for cytokine cascade activation via indirect pathways upon this stimulation. To address such issues, one may propose future experiments which collect pancreatic fluid with the alternative cholecystokinin (CCK)-stimulated ePFT (Conwell et al., 2003a). However, in the current study, the cohorts with and without pancreatic disease are similarly injected with secretin, which controls for the effects of that hormone. Alternatively, non-hormone stimulated ERCP (endoscopic retrograde cholangiopancreatography) collection may also be a viable option, although less safe and resulting in the collection of less fluid (Andriulli et al., 2007). Such approaches may be useful in the investigation of cytokine levels in pancreatic fluid and merit further investigation.

The ePFT-collected fluid is essentially an admixture of biliary, gastric, duodenal, and pancreatic secretions. However, as all specimens are handled in a uniform manner, we inherently control for cytokines which may be a result of these other secretions. Furthermore, histological biopsies of gastric and duodenal mucosa and a review of radiologic imaging minimize the potential contribution of non-pancreatic diseases to cytokine production in both cohorts, which is of particular importance for the chronic

pancreatitis cohort. Because our control patients have chronic abdominal pain, there is potential for confounding ailments, apart from those which were tested, that may be attributed to or are a result of the elevated cytokine levels in these non-pancreatitis diseased patients. Establishing a baseline with healthy patients would be ideal; however, such a cohort group would be difficult to acquire, as there would be no medical benefit for the healthy patient to undergo an endoscopic pancreatic function test. Further investigations of cytokine profiles may necessitate the incorporation of a larger number and a broader range of pancreatic disease samples, such as fluid collected from mild or moderate chronic pancreatitis patients.

5. Conclusion

In conclusion, we have successfully identified cytokines in pancreatic fluid using the ePFT collection method in tandem with cytokine microarray technology. Such a workflow is a valuable tool for deciphering the extracellular signaling cascades, which modulate the inflammatory response of the pancreas and which may lead to insights into pancreatic dysfunction. The application of this approach to the various etiologies and clinical stages of pancreatic dysfunction may lead to major insights into cytokine-mediated mechanisms of inflammation and pathogenesis of chronic pancreatitis.

Acknowledgments

We would like to thank the Burrill family for their generous support through the Burrill Research Grant. In addition, funds were provided by the Harvard Digestive Diseases Center (NIH 5 P30 DK034854-24) and the NIH NIDDK NRSA Fellowship (NIH NIDDK 1 F32 DK085835-01A1). We would like to thank members of the Steen Lab at Children's Hospital Boston and Harvard Medical School, in particular John FK Sauld and Robert Everley for their technical assistance and critical reading of the manuscript.

LIST OF ABBREVIATIONS

CP	chronic pancreatitis
CAP	chronic abdominal pain
ePFT	endoscopic pancreatic function test
GRO	growth-regulated oncogene
IP	interferon-inducible protein
MCP	monocyte chemoattractant protein
MDC	myeloma cell metalloproteinase
M-ANNHEIM	Multiple risk factors, Alcohol, Nicotine, Nutrition, Hereditary factors, Efferent duct factors, Immunological factors, and Miscellaneous and metabolic factors
MIP	macrophage inflammatory protein
IL	interleukins
GF	growth factors
TNF	tumor necrosis factor
INF	interferons

References

- Andoh A, Takaya H, Saotome T, Shimada M, Hata K, Araki Y, Nakamura F, Shintani Y, Fujiyama Y, Bamba T. Cytokine regulation of chemokine (IL-8, MCP-1, and RANTES) gene expression in human pancreatic periacinar myofibroblasts. *Gastroenterology*. 2000; 119:211–9. [PubMed: 10889171]
- Andriulli A, Loperfido S, Napolitano G, Niro G, Valvano MR, Spirito F, Pilotto A, Forlano R. Incidence rates of post-ERCP complications: a systematic survey of prospective studies. *The American journal of gastroenterology*. 2007; 102:1781–8. [PubMed: 17509029]
- Apte MV, Haber PS, Darby SJ, Rodgers SC, McCaughan GW, Korsten MA, Pirola RC, Wilson JS. Pancreatic stellate cells are activated by proinflammatory cytokines: implications for pancreatic fibrogenesis. *Gut*. 1999; 44:534–41. [PubMed: 10075961]
- Apte MV, Wilson JS. Mechanisms of pancreatic fibrosis. *Dig Dis*. 2004; 22:273–9. [PubMed: 15753610]
- Aust S, Jager W, Kirschner H, Klimpfinger M, Thalhammer T. Pancreatic stellate/myofibroblast cells express G-protein-coupled melatonin receptor 1. *Wien Med Wochenschr*. 2008; 158:575–8. [PubMed: 18998076]
- Bali MA, Sontou R, Arvanitakis M, Metens T, Deviere J, Matos C. Evaluation of the stimulating effect of a low dose of secretin compared to the standard dose on the exocrine pancreas with MRCP: preliminary results in normal subjects (MRCP quantification of secretin stimulation). *Abdom Imaging*. 2007
- Barland CO, Zettersten E, Brown BS, Ye J, Elias PM, Ghadially R. Imiquimod-induced interleukin-1 alpha stimulation improves barrier homeostasis in aged murine epidermis. *J Invest Dermatol*. 2004; 122:330–6. [PubMed: 15009713]
- Cannon JG. Inflammatory Cytokines in Nonpathological States. *News Physiol Sci*. 2000; 15:298–303. [PubMed: 11390930]
- Carpenter G, Cohen S. Epidermal growth factor. *J Biol Chem*. 1990; 265:7709–12. [PubMed: 2186024]
- Carson RT, Vignali DA. Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. *J Immunol Methods*. 1999; 227:41–52. [PubMed: 10485253]
- Catalano MF, Lahoti S, Alcocer E, Geenen JE, Hogan WJ. Dynamic imaging of the pancreas using real-time endoscopic ultrasonography with secretin stimulation. *Gastrointestinal endoscopy*. 1998; 48:580–7. [PubMed: 9852447]
- Conwell DL, Zuccaro G Jr, Vargo JJ, Morrow JB, Obuchowski N, Dumot JA, Trolli PA, Burton A, O'Laughlin C, Van Lente F. An endoscopic pancreatic function test with cholecystokinin-octapeptide for the diagnosis of chronic pancreatitis. *Clin Gastroenterol Hepatol*. 2003a; 1:189–94. [PubMed: 15017490]
- Conwell DL, Zuccaro G Jr, Vargo JJ, Trolli PA, Vanlente F, Obuchowski N, Dumot JA, O'Laughlin C. An endoscopic pancreatic function test with synthetic porcine secretin for the evaluation of chronic abdominal pain and suspected chronic pancreatitis. *Gastrointestinal endoscopy*. 2003b; 57:37–40. [PubMed: 12518128]
- Conwell DL, Zuccaro G, Morrow JB, Van Lente F, O'Laughlin C, Vargo JJ, Dumot JA. Analysis of duodenal drainage fluid after cholecystokinin (CCK) stimulation in healthy volunteers. *Pancreas*. 2002; 25:350–4. [PubMed: 12409828]
- Detlefsen S, Sipos B, Feyerabend B, Kloppel G. Fibrogenesis in alcoholic chronic pancreatitis: the role of tissue necrosis, macrophages, myofibroblasts and cytokines. *Mod Pathol*. 2006; 19:1019–26. [PubMed: 16680157]
- Diaconu BL, Ciobanu L, Mocan T, Pfutzer RH, Scafaru MP, Acalovschi M, Singer MV, Schneider A. Investigation of the SPINK1 N34S mutation in Romanian patients with alcoholic chronic pancreatitis. A clinical analysis based on the criteria of the M-ANNHEIM classification. *J Gastrointest Liver Dis*. 2009; 18:143–50. [PubMed: 19565042]
- Dinareello CA. The interleukin-1 family: 10 years of discovery. *FASEB J*. 1994; 8:1314–25. [PubMed: 8001745]

- Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD. IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *J Immunol.* 2002; 168:3195–204. [PubMed: 11907072]
- Ellenrieder V, Schneiderhan W, Bachem M, Adler G. Fibrogenesis in the pancreas. *Rocz Akad Med Bialymst.* 2004; 49:40–6. [PubMed: 15631312]
- Farrow B, Albo D, Berger DH. The role of the tumor microenvironment in the progression of pancreatic cancer. *J Surg Res.* 2008; 149:319–28. [PubMed: 18639248]
- Fernandez EJ, Lolis E. Structure, function, and inhibition of chemokines. *Annu Rev Pharmacol Toxicol.* 2002; 42:469–99. [PubMed: 11807180]
- Fitzgerald SP, McConnell RI, Huxley A. Simultaneous analysis of circulating human cytokines using a high-sensitivity cytokine biochip array. *Journal of proteome research.* 2008; 7:450–5. [PubMed: 18020322]
- Forrester RL, Watiji LJ, Silverman DA, Pierre KJ. Enzymatic method for determination of CO₂ in serum. *Clinical chemistry.* 1976; 22:243–5. [PubMed: 1248128]
- Forsmark CE. The early diagnosis of chronic pancreatitis. *Clin Gastroenterol Hepatol.* 2008; 6:1291–3. [PubMed: 18986847]
- Fredriksson L, Li H, Eriksson U. The PDGF family: four gene products form five dimeric isoforms. *Cytokine Growth Factor Rev.* 2004; 15:197–204. [PubMed: 15207811]
- Giri JG, Ahdieh M, Eisenman J, Shanebeck K, Grabstein K, Kumaki S, Namen A, Park LS, Cosman D, Anderson D. Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J.* 1994; 13:2822–30. [PubMed: 8026467]
- Herbst RS. Review of epidermal growth factor receptor biology. *Int J Radiat Oncol Biol Phys.* 2004; 59:21–6. [PubMed: 15142631]
- Ihle JN, Pepersack L, Rebar L. Regulation of T cell differentiation: in vitro induction of 20 alpha-hydroxysteroid dehydrogenase in splenic lymphocytes from athymic mice by a unique lymphokine. *J Immunol.* 1981; 126:2184–9. [PubMed: 6971890]
- Kampen GT, Stafford S, Adachi T, Jinquan T, Quan S, Grant JA, Skov PS, Poulsen LK, Alam R. Eotaxin induces degranulation and chemotaxis of eosinophils through the activation of ERK2 and p38 mitogen-activated protein kinases. *Blood.* 2000; 95:1911–7. [PubMed: 10706854]
- Klebanoff CA, Finkelstein SE, Surman DR, Lichtman MK, Gattinoni L, Theoret MR, Grewal N, Spiess PJ, Antony PA, Palmer DC, Tagaya Y, Rosenberg SA, Waldmann TA, Restifo NP. IL-15 enhances the in vivo antitumor activity of tumor-reactive CD8⁺ T cells. *Proceedings of the National Academy of Sciences of the United States of America.* 2004; 101:1969–74. [PubMed: 14762166]
- Masamune A, Kikuta K, Watanabe T, Satoh K, Hirota M, Hamada S, Shimosegawa T. Fibrinogen induces cytokine and collagen production in pancreatic stellate cells. *Gut.* 2009; 58:550–9. [PubMed: 19052021]
- Masamune A, Shimosegawa T. Signal transduction in pancreatic stellate cells. *J Gastroenterol.* 2009; 44:249–60. [PubMed: 19271115]
- Menten P, Wuyts A, Van Damme J. Macrophage inflammatory protein-1. *Cytokine Growth Factor Rev.* 2002; 13:455–81. [PubMed: 12401480]
- Mews P, Phillips P, Fahmy R, Korsten M, Pirola R, Wilson J, Apte M. Pancreatic stellate cells respond to inflammatory cytokines: potential role in chronic pancreatitis. *Gut.* 2002; 50:535–41. [PubMed: 11889076]
- Noh KW, Pungpapong S, Wallace MB, Woodward TA, Raimondo M. Do cytokine concentrations in pancreatic juice predict the presence of pancreatic diseases? *Clin Gastroenterol Hepatol.* 2006; 4:782–9. [PubMed: 16713745]
- Omary MB, Lugea A, Lowe AW, Pandol SJ. The pancreatic stellate cell: a star on the rise in pancreatic diseases. *J Clin Invest.* 2007; 117:50–9. [PubMed: 17200706]
- Opalka D, Lachman CE, MacMullen SA, Jansen KU, Smith JF, Chirmule N, Esser MT. Simultaneous quantitation of antibodies to neutralizing epitopes on virus-like particles for human papillomavirus types 6, 11, 16, and 18 by a multiplexed luminex assay. *Clin Diagn Lab Immunol.* 2003; 10:108–15. [PubMed: 12522048]

- Palikhe NS, Kim SH, Cho BY, Ye YM, Choi GS, Park HS. Genetic variability in CRTH2 polymorphism increases eotaxin-2 levels in patients with aspirin exacerbated respiratory disease. *Allergy*. 2009
- Patel M, Fine DR. Fibrogenesis in the pancreas after acinar cell injury. *Scand J Surg*. 2005; 94:108–11. [PubMed: 16111091]
- Paulo JA, Lee LS, Wu B, Banks PA, Steen H, Conwell DL. Mass spectrometry-based proteomics of endoscopically collected pancreatic fluid in chronic pancreatitis research. *Proteomics Clin Appl*. 2011; 5:109–20. [PubMed: 21360826]
- Paulo JA, Lee LS, Wu B, Repas K, Banks PA, Conwell DL, Steen H. Optimized sample preparation of endoscopic collected pancreatic fluid for SDS-PAGE analysis. *Electrophoresis*. 2010a; 31:2377–87. [PubMed: 20589857]
- Paulo JA, Lee LS, Wu B, Repas K, Banks PA, Conwell DL, Steen H. Proteomic analysis of endoscopically (endoscopic pancreatic function test) collected gastroduodenal fluid using in-gel tryptic digestion followed by LC-MS/MS. *Proteomics Clin Appl*. 2010b; 4:715–25. [PubMed: 21137089]
- Paulo JA, Lee LS, Wu B, Repas K, Morteale KJ, Banks PA, Steen H, Conwell DL. Identification of pancreas-specific proteins in endoscopically (endoscopic pancreatic function test) collected pancreatic fluid with liquid chromatography--tandem mass spectrometry. *Pancreas*. 2010c; 39:889–96. [PubMed: 20182389]
- Pollack BJ, Grendell JH. Where have all the dreiling tubes gone? *The American journal of gastroenterology*. 2006; 101:356–9. [PubMed: 16454843]
- Pollard HB, Srivastava M, Eidelman O, Jozwik C, Rothwell SW, Mueller GR, Jacobowitz DM, Darling T, Guggino WB, Wright J, Zeitlin PL, Paweletz CP. Protein microarray platforms for clinical proteomics. *Proteomics Clinical Applications*. 2007; 1:934–952. [PubMed: 21136748]
- Rottman JB. Key role of chemokines and chemokine receptors in inflammation, immunity, neoplasia, and infectious disease. *Vet Pathol*. 1999; 36:357–67. [PubMed: 10490203]
- Sachdeva N, Asthana D. Cytokine quantitation: technologies and applications. *Front Biosci*. 2007; 12:4682–95. [PubMed: 17485405]
- Schneider A, Lohr JM, Singer MV. The M-ANNHEIM classification of chronic pancreatitis: introduction of a unifying classification system based on a review of previous classifications of the disease. *J Gastroenterol*. 2007; 42:101–19. [PubMed: 17351799]
- Shimizu K. Mechanisms of pancreatic fibrosis and applications to the treatment of chronic pancreatitis. *J Gastroenterol*. 2008; 43:823–32. [PubMed: 19012035]
- Stevens T, Conwell D, Zuccaro G, Van Lente F, Khandwala F, Hanaway P, Vargo JJ, Dumot JA. Analysis of pancreatic elastase-1 concentrations in duodenal aspirates from healthy subjects and patients with chronic pancreatitis. *Dig Dis Sci*. 2004a; 49:1405–11. [PubMed: 15481311]
- Stevens T, Conwell DL, Zuccaro G, Van Lente F, Khandwala F, Purich E, Vargo JJ, Fein S, Dumot JA, Trolli P, O'Laughlin C. Electrolyte composition of endoscopically collected duodenal drainage fluid after synthetic porcine secretin stimulation in healthy subjects. *Gastrointestinal endoscopy*. 2004b; 60:351–5. [PubMed: 15332022]
- Tallquist M, Kazlauskas A. PDGF signaling in cells and mice. *Cytokine Growth Factor Rev*. 2004; 15:205–13. [PubMed: 15207812]
- Vignali DA. Multiplexed particle-based flow cytometric assays. *J Immunol Methods*. 2000; 243:243–55. [PubMed: 10986418]
- Vonlaufen A, Apte MV, Imhof BA, Frossard JL. The role of inflammatory and parenchymal cells in acute pancreatitis. *J Pathol*. 2007; 213:239–48. [PubMed: 17893879]
- Walz A, Schmutz P, Mueller C, Schnyder-Candrian S. Regulation and function of the CXC chemokine ENA-78 in monocytes and its role in disease. *J Leukoc Biol*. 1997; 62:604–11. [PubMed: 9365115]
- Wang HM, Collins M, Arai K, Miyajima A. EGF induces differentiation of an IL-3-dependent cell line expressing the EGF receptor. *EMBO J*. 1989; 8:3677–84. [PubMed: 2531081]
- Wu B, Conwell DL. The endoscopic pancreatic function test. *The American journal of gastroenterology*. 2009; 104:2381–3. [PubMed: 19806083]

Yang YC, Ciarletta AB, Temple PA, Chung MP, Kovacic S, Witek-Giannotti JS, Leary AC, Kriz R, Donahue RE, Wong GG, et al. Human IL-3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. *Cell*. 1986; 47:3–10. [PubMed: 3489530]

Figure 1. Workflow of pancreatic fluid processing for biochemical assays and cytokine microarray

Pancreatic fluid is collected via ePFT, the sample is divided for biochemical assays (e.g., bicarbonate measurements) and for cytokine microarray analysis following centrifugation.

Figure 2. Box and whisker plots of statistically significant cytokine concentrations between chronic pancreatitis and non-pancreatitis controls

The cytokines included in these plots are those listed in **Table 3** with p-value <0.05. A) Cytokines having concentrations greater than 30 pg/mL. B) Cytokines having concentrations less than 30 pg/mL. The top and bottom edges of the box are located at the sample 75th and 25th percentiles, respectively. The vertical line drawn within the box marks the 50th percentile (median). The whiskers extend from the box as far as the data extend, to a distance of at most 1.5 interquartile ranges. CAP, chronic abdominal pain; CP, chronic pancreatitis.

Demographic and clinical data of study cohort.

Table 1

ID	Reason for referral	Age (years)	Gender	CT Scan	MRI	Pancreatic elastase – I (units/ mg)	EUS Score (0-9)	ePFT Peak Bicarbonate (meq/L)	Endoscopic Biopsy (gastric, duodenum)
CAP1	abdominal pain	28	male	normal	normal	n/a	3	84	normal
CAP2	abdominal pain	53	female	normal	normal	148 (diarrhea)	1	92	gastropathy
CAP3	abdominal pain	54	male	normal	normal	202	n/a	114	normal
CP1	chronic pancreatitis	64	female	atrophy, calcifications, strictured and dilated duct	atrophy, dilated and strictured duct, filling effects, low T1 signal, dilated side branches	221	n/a	37	duodenitis, gastropathy
CP2	chronic pancreatitis	60	female	atrophy, calcifications, strictured and dilated duct	n/a	n/a	8	54	normal
CP3	chronic pancreatitis	52	female	n/a	atrophy, dilated and strictured duct, low T1 signal, dilated side branches	<15	5	38	normal

CT, computed tomography; MRI, magnetic resonance imaging; EUS, endoscopic ultrasound; ePFT, endoscopic pancreas function test; CP, chronic pancreatitis; CAP, chronic abdominal pain; n/a, not available.

Table 2

Cytokines investigated by the microarray panel.

Name	UniProt entry	mol. wt. (Da)
EGF	Q99075	23,067
Eotaxin	P51671	10,732
FGF-basic	P09038	22,623
Flt-3L	P49771	26,416
Fractalkine	P78423	42,203
G-CSF	P09919	22,293
GM-CSF	P01587	16,091
GRO	P09341	11,301
IFN- α 2	P01563	21,550
IFN- γ	P01579	19,348
IL-1 α	P01583	30,607
IL-1 β	P01584	30,748
IL-1 α	P18510	20,055
IL-2	P60568	15,238
IL-2 α	P01589	30,557
IL-3	P08700	17,233
IL-4	P05112	17,492
IL-5	P05113	15,238
IL-6	P05231	23,718
IL-7	P13232	20,187
IL-8	P10145	11,098
IL-9	P15248	15,909
IL-10	P22301	20,517
IL-12 α	P29460	37,169
IL-12 β	P29459	24,874
IL-13	P35225	15,816
IL-15	P40933	18,086
IL-17	Q16552	17,504
IP-10	P02778	10,881
MCP-1	P13500	11,025
MCP-3	P80098	11,200
MDC	O00626	10,581
MIP-1 α	P10147	10,085
MIP-1 β	P13236	10,212
PDGF-AA	P04085	24,043
PDGF-AB/BB	P01127	27,283
Rantes	P13501	9,990
sCD40L	P29965	29,274
TGF- α	P01375	25,644

Name	UniProt entry	mol. wt. (Da)
TNF-a	P01375	25,644
TNF-b	P01374	22,297
VEGF	P15692	27,042

Table 3

Concentrations of cytokines as determined by the microarray assay, ordered by increasing concentration of cytokines in CAP (units: pg/mL).

Cytokine	Chronic abdominal pain (CAP)							Chronic pancreatitis (CP)							p-value		
	CAP1		CAP2		CAP3		median of all replicates	interquartile range	CP1		CP2		CP3			median of all replicates	interquartile range
	rep1	rep2	rep1	rep2	rep1	rep2			rep1	rep2	rep1	rep2	rep1	rep2			
PDGF-AA	46.2	42.6	157.8	136.8	215.4	175.1	147.3	101.9	201.1	152.8	146.3	162.3	391.3	410.0	181.7	188.6	0.174
EGF	3.2	3.2	203.5	167.0	107.2	82.3	94.8	129.1	3.2	3.2	3.2	3.2	3.2	3.2	3.2	0.0	0.028*
IP-10	103.0	93.2	52.0	44.2	70.4	56.0	63.2	34.5	3.8	3.8	3.8	3.8	29.4	26.6	3.8	17.1	0.004*
Eotaxin	96.3	82.4	56.9	50.8	49.4	46.1	53.9	26.3	2.2	2.2	2.2	2.2	2.2	2.2	2.2	0.0	0.003*
IL-3	32.3	33.0	76.3	73.5	43.5	39.4	41.5	31.4	6.7	5.7	2.1	2.1	2.1	2.1	2.1	2.7	0.004*
GRO	47.2	55.0	33.5	37.0	34.5	36.0	36.5	9.8	24.4	25.9	42.7	46.2	40.7	44.9	41.7	14.8	0.810
IL-12(p40)	95.8	88.2	33.7	29.1	9.8	9.8	31.4	60.0	9.8	9.8	9.8	9.8	9.8	9.8	9.8	0.0	0.051
IL-4	30.0	30.5	72.4	72.1	5.9	5.8	30.3	49.8	4.4	4.2	41.4	38.8	11.9	10.9	11.4	26.1	0.379
FGF-basic	36.7	30.7	27.5	21.5	25.3	30.8	29.1	4.9	4.5	5.2	26.8	29.2	44.3	45.5	28.0	29.9	0.936
MIP-1a	62.4	73.1	22.4	25.7	7.2	8.1	24.1	41.6	6.0	6.0	6.0	6.0	6.0	6.0	6.0	0.0	0.003*
MIP-1b	24.1	22.6	21.5	19.3	15.6	13.4	20.4	5.8	10.2	8.5	15.1	12.0	37.2	28.1	13.6	14.2	0.471
IL-15	12.4	13.6	11.1	11.5	9.9	9.6	11.3	2.0	3.3	3.0	4.9	4.2	9.6	7.7	4.6	3.5	0.006*
IL-2ra	38.6	40.1	8.6	8.4	11.4	10.5	11.0	22.7	2.8	2.4	22.8	18.5	25.7	19.5	19.0	15.3	0.810
IL-13	10.9	10.0	27.2	23.5	0.2	0.2	10.5	17.7	0.2	0.1	13.7	13.2	8.6	7.9	8.3	9.9	0.468
PDGF-AB/BB	10.1	8.3	27.7	23.8	5.0	5.0	9.2	14.6	5.0	5.0	5.0	5.0	5.0	5.0	5.0	0.0	0.028*
sCD40L	11.8	13.4	6.8	7.5	8.3	8.8	8.6	3.4	2.6	2.6	8.6	8.4	17.0	16.0	8.5	10.1	1.000
Flt-3L	7.8	8.3	7.6	8.5	4.7	4.7	7.7	2.8	4.7	4.7	7.2	8.9	7.1	9.0	7.2	3.2	1.000
IFN-γ	10.2	10.9	6.9	7.1	3.0	3.0	7.0	5.5	4.9	4.7	6.3	5.8	2.4	2.1	4.8	2.6	0.128
MCP-3	6.9	6.3	6.9	6.3	6.8	6.4	6.6	0.6	2.4	2.4	6.7	6.5	17.9	18.0	6.6	11.7	1.000
MDC	42.9	34.2	6.4	6.4	6.4	6.4	6.4	20.9	6.4	6.4	6.4	6.4	6.4	6.4	6.4	0.0	0.176
Fractalkine	15.6	17.4	6.0	6.0	6.0	6.0	6.0	7.2	6.0	6.0	6.0	6.0	12.1	10.0	6.0	3.0	0.775
VEGF	5.8	5.8	5.8	5.8	5.8	5.8	5.8	0.0	5.8	5.8	5.8	5.8	5.8	5.8	5.8	0.0	1.000
IFN-α2	7.2	6.9	4.8	4.8	4.8	4.8	4.8	1.6	4.8	4.8	4.8	4.8	4.8	4.8	4.8	0.0	0.176
IL-1a	9.6	9.2	3.3	3.0	3.4	2.9	3.4	4.7	1.1	0.9	1.1	1.1	1.2	1.2	1.1	0.1	0.005*
GM-CSF	5.5	5.0	3.2	2.7	1.0	1.0	3.0	3.1	1.0	1.0	1.0	1.0	1.4	1.3	1.0	0.2	0.104

Cytokine	Chronic abdominal pain (CAP)										Chronic pancreatitis (CP)										p-value												
	CAP1					CAP2					CAP3					CP1						CP2					CP3					interquartile range	median of all replicates
	rep1		rep2		interquartile range	rep1		rep2		interquartile range	rep1		rep2		interquartile range	rep1		rep2		interquartile range		rep1		rep2		interquartile range	median of all replicates						
	rep1	rep2	rep1	rep2		rep1	rep2	rep1	rep2		rep1	rep2	rep1	rep2		rep1	rep2	rep1	rep2			rep1	rep2	rep1	rep2			rep1	rep2	rep1	rep2		
IL-1ra	7.5	8.3	2.8	2.9	1.8	1.8	1.8	1.8	1.8	4.3	1.3	1.2	17.0	14.8	7.8	6.4	7.1	10.5	0.688														
IL-6	2.4	2.3	3.7	3.4	0.3	0.3	0.3	0.3	0.3	2.4	0.3	0.3	6.8	6.7	4.4	4.0	4.2	4.9	0.222														
MCP-1	5.8	4.8	2.3	1.9	2.4	1.9	2.4	1.9	2.4	2.2	0.9	0.9	0.9	0.9	5.8	4.9	0.9	3.0	0.252														
G-CSF	2.8	3.1	1.6	1.8	0.2	0.2	0.2	0.2	1.7	2.0	0.2	0.2	1.7	1.7	2.8	3.2	1.7	2.0	1.000														
TNF-b	4.8	4.9	1.3	1.3	1.4	1.2	1.4	1.2	1.4	2.7	1.2	1.2	1.4	1.1	2.0	1.5	1.3	0.3	0.418														
IL-7	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.0	1.000														
Rantes	2.2	2.6	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.0	0.176														
TGF-a	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.0	0.9	0.9	0.9	0.9	4.8	3.9	0.9	2.3	0.176														
IL-9	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.0	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.0	1.000														
IL-17	1.2	1.4	0.7	0.8	0.7	0.7	0.7	0.7	0.8	0.4	0.7	0.7	0.7	0.7	0.9	0.9	0.7	0.2	0.531														
IL-12(p70)	1.3	1.3	0.5	0.5	0.4	0.4	0.4	0.4	0.5	0.7	0.7	0.7	0.9	0.8	2.7	2.3	0.9	1.2	0.126														
IL-1b	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.0	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.0	1.000														
IL-2	1.4	1.1	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.6	0.3	0.3	1.5	1.5	0.5	0.5	0.5	0.9	0.345														
IL-10	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.0	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.0	1.000														
IL-5	0.2	0.2	0.3	0.3	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.0	0.086														
IL-8	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	1.000														
TNF-a	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	1.000														

* p-value <0.05; CAP, chronic abdominal pain; CP, chronic pancreatitis; rep, replicate.