

Decreased serum thrombospondin-1 levels in pancreatic cancer patients up to 24 months prior to clinical diagnosis: association with diabetes mellitus

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Translational Relevance

The majority of patients with pancreatic ductal adenocarcinoma (PDAC) are diagnosed with advanced stage disease and survive less than 12 months. Biomarkers enabling earlier diagnosis are sorely needed. Serum biomarker discovery studies tend to use samples from diagnosed patients, meaning current research efforts are potentially missing critical changes occurring in the months and years prior to diagnosis. Additionally a high proportion of PDAC patients are either hyperglycaemic or diabetic. The impact of diabetes on circulating biomarkers is poorly understood. Here we demonstrate, in serum taken up to 4 years prior to a PDAC diagnosis, that circulating TSP-1 levels are significantly reduced up to 24 months prior to diagnosis and low serum TSP-1 levels in PDAC patients are significantly associated with diabetes mellitus. Early detection strategies could benefit from including TSP-1. Future studies investigating biomarkers for pancreatic cancer detection should take into account the influence of diabetes mellitus on biomarker behaviour.

Abstract

Purpose: Identification of serum biomarkers enabling earlier diagnosis of pancreatic ductal adenocarcinoma (PDAC) could improve outcome. Serum protein profiles in patients with pre-clinical disease and at diagnosis were investigated.

Experimental Design: Serum from cases up to 4 years prior to PDAC diagnosis and controls (UKCTOCS, n=174) were studied, alongside samples from patients diagnosed with PDAC, chronic pancreatitis, benign biliary disease, type 2 diabetes mellitus and healthy subjects (n=298). iTRAQ enabled comparisons of pooled serum from a test set (n=150). Validation was undertaken using MRM and/or western blotting in all 472 human samples and samples from a KPC mouse model.

Results: iTRAQ identified thrombospondin-1 (TSP-1) as reduced preclinically and in diagnosed samples. MRM confirmed significant reduction in levels of TSP-1 up to 24 months prior to diagnosis. A combination of TSP-1 and CA19-9 gave an AUC of 0.86, significantly outperforming both markers alone (0.69 & 0.77 respectively; $P < 0.01$). TSP-1 was also decreased in PDAC patients compared to healthy controls ($P < 0.05$) and patients with benign biliary obstruction ($P < 0.01$). Low levels of TSP-1 correlated with poorer survival, pre-clinically ($P < 0.05$) and at clinical diagnosis ($P < 0.02$). In PDAC patients, reduced TSP-1 levels were more frequently observed in those with confirmed diabetes mellitus ($P < 0.01$). Significantly lower levels were also observed in PDAC patients with diabetes compared to individuals with type 2 DM ($P = 0.01$).

Conclusions: Circulating TSP-1 levels decrease up to 24 months prior to diagnosis of PDAC and significantly enhance the diagnostic performance of CA19-9. The influence of diabetes mellitus on biomarker behaviour should be considered in future studies.

Introduction

For the majority of patients, pancreatic ductal adenocarcinoma (PDAC) goes undetected until it is at an advanced stage. Symptoms, such as obstructive jaundice, weight loss or pain often manifest late in the course of the disease when effective treatment options are limited. Consequently, overall survival is poor (1). CA19-9, the only biomarker in routine use for the management of pancreatic cancer (2, 3) has a number of limitations including lack of expression in ~5% of the population, and elevation in related diseases including chronic pancreatitis and obstructive jaundice (2, 4). Alternative biomarkers that can facilitate earlier diagnosis are actively sought (5, 6).

To date, PDAC serum biomarker discovery work has almost exclusively used samples taken following diagnosis. Serum protein levels in these samples may not accurately reflect changes occurring in the months or years prior to diagnosis. To explore alterations in serum proteins that occur pre-clinically, we performed biomarker discovery using samples collected as part of the prospective cohort 'United Kingdom Collaborative Trial of Ovarian Cancer Screening' (UKCTOCS; www.ukctocs.org.uk/ (7, 8)). The nested cohort of samples analysed here were from women who were subsequently diagnosed with pancreatic cancer. We included samples taken up to 48 months prior to diagnosis of pancreatic cancer, along with control samples, matched for date of donation and the centre at which samples were donated.

For biomarker discovery we used isobaric tags for relative and absolute quantification (iTRAQ), which allows for the simultaneous accurate, precise and reproducible quantification of proteins across several samples (9, 10). For validation we used multiple reaction monitoring (MRM), a mass spectrometry (MS)-based approach, which provides an alternative to immune-based protein quantification. MRM enables the detection and precise quantification of predetermined proteins in complex mixtures (11) and is capable of accurately discriminating between protein isoforms.

We discovered significantly reduced levels of serum Thrombospondin-1 levels pre-clinically, during a period of 24 months prior to diagnosis with PDAC, and at diagnosis of PDAC. Importantly, we demonstrated a significant relationship between TSP-1 levels and diabetes mellitus in PDAC patients, suggesting that TSP-1 merits investigation as a marker for PDAC-associated diabetes.

METHODS

Patient Groups

Blood was obtained, with ethically approved informed written consent from two independent sources; UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS; ref.05/Q0505/57) and National Institute for Health Research Liverpool Pancreas Biomedical Research Unit (PBRU; ref11/NM/0083 and 08/H1005/1). The UKCTOCS study set comprised serum from women recruited to UKCTOCS between 2001 and 2005(12) who went on to develop pancreatic cancer and time matched controls. The samples were subcategorised as follows; 0-6 months pre-diagnosis (n=30 cases, 30 controls), >6-12 months (n=17 cases, 17 controls), >12-24 months (n=17 cases, 17 controls), >24-36 months (n=11 cases, 11 controls) and >36-48 months (n=12 cases, 12 controls). In total, 174 UKCTOCS case samples from 76 individuals were used in the study. This included serial samples from 26 individuals. Two independent PBRU sets were analysed. The first PBRU set (n=199) consisted of 98 patients with histologically confirmed PDAC, 39 patients with chronic pancreatitis (CP), 20 with jaundice due to gall stones (benign biliary obstruction, BBO) and 42 healthy control individuals (HC). PDAC patients were further subcategorised into those with low bilirubin levels (49 patients, <20 µmol/L; upper level of normal for our Centre) and high bilirubin levels (49 patients, >20 µmol/L). To determine levels of TSP-1 in individuals with type 2 diabetes mellitus (DM), a second independent PBRU cohort (n=99) was analysed. This included 54 patients with histologically confirmed PDAC, 18 patients with chronic pancreatitis (CP), 14 healthy control individuals (HC) and 13 patients with long-term (for 5 or more years) type 2 DM. Of the PDAC and CP patients, 26 and 9 respectively had confirmed diabetes. The clinical characteristics of the study populations are provided in Table 1A & B.

Sample collection

UKCTOCS blood samples were collected and processed throughout the trial according to a standardised SOP (8, 13). Blood was taken in Greiner gel tubes (8 mL separation tubes; Greiner Bio-one 455071, Stonehouse UK) at one of 13 trial centres, transported overnight at ambient temperature to a central laboratory, centrifuged at 4,000 rpm for 10 minutes and serum aliquoted and stored in liquid nitrogen. All UKCTOCS blood samples used in this study were processed within 20 hours of venepuncture. PBRU blood samples were collected in Sarstedt Monovette tubes (Sarstedt Ltd, Leicester, UK), placed at 4 °C for 15 min and centrifuged at 800 ×g for 10 min at 4°C. Serum was

stored in aliquots at -80°C . Preoperative total serum bilirubin ($\mu\text{mol/L}$) (Roche Modular SWA) and CA19-9 levels were measured in hospital Clinical Biochemistry Departments, or by CA19-9 ELISA (Human Pancreatic & GI Cancer ELISA Kit, Alpha Diagnostics International, San Antonio, Texas, USA).

Murine sample collection

Animals were treated in accordance with European and institutional guidelines (Legislative Order No. 116/92). 129SvJae/B6 H-2Db mice carrying mutated $Kras^{G12D}$ and $Trp53^{R172H}$ under the endogenous promoter, and flanked by Lox-STOP-Lox cassettes ($LSL-Kras^{G12D/+}$ and $LSL-Trp53^{R172H/+}$). Serum samples were collected from 10 $LSL-Kras^{G12D/+};LSL-Trp53^{R172H/+};Pdx-1-Cre$ (KPC) mice and 9 age-matched control ($LSL-Trp53^{R172H/+};Pdx-1-Cre$) mice via cardiac bleed under isoflurane gaseous anaesthesia. Blood was collected at 6 weeks, 2 months and 3-6 months and centrifuged at 1000x g for 20 minutes at room temperature. Serum was collected, snap frozen (in liquid nitrogen) and stored at -80°C in 60ul aliquots. Mice were surgically and pathologically examined to confirm the presence of pancreatic tumours and metastases.

iTRAQ analysis

An iTRAQ 8-plex experiment using pooled, delipidated and high abundance protein-depleted serum from a discovery subset of pre-diagnosis UKCTOCS samples; 0-6 months cases & controls (n=18 per group), >6-12 months cases & controls (n=17 per group) and PBRU samples; PDAC non obstructed (n=20), PDAC obstructed (n=20), CP (n=20) and HC (n=20) (total n= 150), was analysed as described previously(10, 14) on a QSTAR-Pulsar i Hybrid Mass Spectrometer (AB Sciex, Framingham, USA). Two replicate analyses were also performed on a Triple TOF-5600 (AB Sciex) as described(15). Data were analysed using ProteinPilot software (Version 4.0, AB Sciex). The clinical characteristics of the study subset populations are provided in Supplementary Table S1.

Ingenuity Pathway Analysis

A protein list of significantly altered proteins generated from our iTRAQ data was uploaded into Ingenuity Pathway Analysis (IPA) software server (<http://www.ingenuity.com>). The iTRAQ dataset was converted from iTRAQ ratio to fold change and a significance cut-off of $P\text{-value} < 0.05$ was utilised. Both a Core Analysis and Biomarker Filter were performed.

MRM analysis

Multiple reaction monitoring was carried out on all 150 samples (UKCTOCS n=70; PBRU n=80) used in iTRAQ discovery alongside 196 additional validation samples (UKCTOCS n=104; PBRU n=92). MRM analysis on single serum samples was performed on a 5500 QTRAP mass spectrometer (ABSciex, Framingham, USA) coupled with an Ultimate 3000 HPLC (Dionex-ThermoScientific, UK). Two target peptides were chosen for TSP-1 and three optimum transitions for each peptide were determined empirically using synthetic peptides (Peptide Protein Research Limited, Fareham, UK; See Supplementary Table S2). Corresponding stable-isotope labelled versions of each peptide (C^{13} , N^{15} labelled leucine), were used as internal standards with three transitions selected for each. Standard curves (7 point) were prepared with each peptide from 0.125 fmol to 10 fmol (on-column) in a peptide digest from human serum (diluted to $\sim 0.25 \mu\text{g/mL}$ with 2% acetonitrile + 0.1% formic acid), with subsequent regression analysis showing acceptable linearity ($r^2 \geq 0.95$). Serum samples (1 μL), digested overnight with trypsin, were diluted 1 in 6 with a solution containing 2% acetonitrile, 0.1% formic acid, spiked with the internal standard peptides (to give a final concentration of 50 fmol) and ionized using a spray voltage of 5500 V and a source temperature of 475 $^{\circ}\text{C}$. Analyzer parameters were optimized for each peptide/transition pair to ensure maximum selectivity, dwell time was 50 ms. Peptide separation was achieved with a Hypersil Gold 50 x 1 mm, 1.9 μm , 175 \AA column, using a 20 min gradient, at a flow rate of 100 $\mu\text{L min}^{-1}$, with buffer A (0.1% formic acid) and Buffer B (95% acetonitrile + 0.1% formic acid). The LC gradient comprised the following: 2% buffer B for 2 min, ramped to 10% buffer B in 0.1 min, 40% buffer B in 10 min, 80% buffer B in 0.1 min held for 3 min, and 2% buffer B in 0.1 min held for 5 min. Prior to analysing each batch of serum samples, chromatographic performance and mass spectrometric stability were evaluated using a tryptic peptide mixture of beta-galactosidase (Sigma Aldrich, Dorset, UK). Three aliquots of each serum sample was analysed, each in duplicate with 2 MRM transitions measured for each of 2 peptides (6 readings for each of 4 MRM transitions), generating a total of 24 MRM readings per sample. The acquired MRM .wiff files were analysed using MultiQuant™ software (Version 2.1), where peak-area was determined for each peptide transition and calculated concentrations determined using the software-generated standard curves. Percentage coefficients of variance (%CV) for each of the 4 MRM transitions were calculated and those over 25% were excluded. The average CV for the UKCTOCS samples was 17.7% between the 4 MRM transitions and 11.4% for the PBRU samples.

Western Blot Analysis

For western blotting, individual serum samples were diluted 1:10 and 4 μ L of each sample analysed using anti-TSP-1 A6.1 mouse monoclonal antibody (1:400; ThermoScientific, Hemel Hempstead, UK). A standard comprising 20 pooled HC samples was used at three different dilutions per gel, allowing comparison and quantification across blots. Protein was separated on Any kD™ Mini-PROTEAN® TGX™ Precast gels (Biorad, Hemel Hempstead, UK), transferred onto PVDF membranes and blocked for 1 h in 5% milk/PBS Tween (PBST). Primary antibody was incubated overnight at 4 °C in 5% milk/PBST. Membranes were washed with PBST and incubated with HRP-conjugated secondary antibodies diluted in 5% milk/PBST. Bands were visualised with enhanced chemiluminescence developed with X-ray film. Densitometry was performed (Kodak MI SE software, Carestream Health), and protein quantities recorded relative to HC standards. All samples were analysed at least in triplicate.

Immunohistochemistry

Formalin fixed paraffin embedded (FFPE) tissue from 49 PDAC patients was used to construct a tissue microarray (TMA). Sections were deparaffinised and antigen retrieval was performed using a PT Link (Dako) with Target Retrieval Solution, High pH (Dako). Anti-TSP-1 A6.1 mouse monoclonal antibody (1:100; ThermoScientific) was incubated at room temperature for one hour. Positively stained tumour cells and tumour-associated stroma were identified by a specialist histopathologist.

Data analysis

Statview V.5.01 (SAS Institute Inc., Cary, North Carolina) and Medcalc software (Version 13, Mariakerke, Belgium) were used. iTRAQ data were compared using the Mann Whitney U test. MRM and western data were analyzed using the two-tailed Mann Whitney U test and diagnostic accuracy compared by Receiver Operating Characteristic (ROC) Analyses. Patient immunohistochemistry data were compared with clinicopathological parameters using Fisher's exact, Pearson's chi-squared and Mann Whitney U tests as appropriate.

RESULTS

Study Characteristics

No differences in time to centrifugation were observed between case and control samples in any of the UKCTOCS time to diagnosis groupings. A significant difference was noted in median age of the 36-48 month groups ($P=0.01$), however a Spearman's rank correlation for comparison of age and TSP-1 levels showed no significant correlation (Spearman's rank correlation coefficient $\rho=0.003$, $P=0.674$). Likewise in the PBRU samples a significant difference was seen in age between cancer patients and controls ($P=0.002$) but no significant correlation was seen when Spearman's rank was performed (Spearman's rank correlation coefficient $\rho=-0.07$, $P=0.328$). No gender differences in TSP-1 were found in the PBRU cohort.

Serum iTRAQ analysis

Pooled sera from pre-diagnosis UKCTOCS samples; 0-6 months cases & controls, >6-12 months cases & controls and PBRU samples; PDAC non-obstructed, PDAC obstructed, CP and HC, were compared in an 8-plex iTRAQ experiment. To increase the sensitivity of protein detection, high abundance proteins were depleted from pooled samples prior to labelling with iTRAQ tags. Since the presence of jaundice can alter serum protein levels (14, 16), PDAC patients were split into those with and those without obstructive jaundice. The experiment was performed three times, yielding a total of 225 proteins identified using at least two peptides at 95% confidence. Protein expression differences between groups were assessed by comparing the relative intensity of reporter ions released from each labelled peptide and calculating protein ratios. A representative dataset is provided in Supplementary Table S3.

TSP-1 levels are decreased in pooled pre-diagnosis and diagnosed PDAC samples

We sought markers that were altered in the lead into pancreatic cancer diagnosis and in diagnosed pancreatic cancer patients irrespective of jaundice. Using IPA-Biomarker we compared iTRAQ data from pre-diagnostic sample pools versus their respective control pools, along with non-obstructed PDAC patient pools versus HC pools and CP pools, to generate a shortlist of candidates. The high abundance proteins targeted in our depletion protocols were excluded, along with proteins previously characterised by our group (10, 17). TSP-1 emerged as a candidate biomarker as its levels were

reduced with significant fold change differences in case samples in the 0-6 month and 6-12 month time groups prior to diagnosis as well as in PDAC *versus* HC PBRU samples taken at diagnosis (Table 2).

Validation of TSP-1 in individual pre-diagnosis and diagnosed PDAC samples

For the accurate quantification of serum TSP-1 in individual serum samples (n=346), we used a LC-MS/MS system, operated in MRM mode with stable isotope labelled internal standards. The two peptides chosen were unique for TSP-1 (Supplementary Figure S1A) allowing unambiguous discrimination from other thrombospondin family members. The lower limit of detection of the assay was 0.1 fmol/ μ L and where TSP-1 fell below detection, the level was arbitrarily assigned a value of zero. When all 87 pre-diagnostic cases, covering up to 48 months prior to diagnosis, were compared with their time-matched controls, TSP-1 levels were significantly lower in cases *versus* ($P<0.001$; Figure 1 A). Separating samples into their individual time to diagnosis groups revealed TSP-1 levels were significantly reduced compared to time matched controls in the 0-6 month, ($P=0.04$), 6-12 month ($P=0.004$) and 12-24 month ($P=0.03$) cases (Figure 1 B). Significantly lower levels of TSP-1 were observed in PDAC patients in the presence ($P=0.02$) and in the absence ($P=0.03$) of jaundice compared to healthy subjects and compared to patients experiencing biliary obstruction due to gallstones (BBO: $P=0.01$ and $P=0.05$ respectively) (Figure 1 C). By contrast, no significant difference in TSP-1 level was established between patients with CP (Figure 1 C). Furthermore, we could not establish any difference between levels of TSP-1 in resectable PDAC cases *versus* non resectable cases (Supplementary Figure S2).

Western blotting using a mouse monoclonal (A6.1) antibody for TSP-1 revealed a single band in human foreskin fibroblast (HFF) cells, which was not detected following treatment with TSP-1-targeting siRNA (Supplementary Figure S1B). The banding pattern obtained in pre-diagnosis and diagnosed serum samples with this antibody is shown in Supplementary Figure S1B. Weak, although significant correlations for TSP-1 in MRM and western measures were observed in pre-diagnosis samples (n=128; Spearman's rank correlation coefficient $\rho=0.29$, $P=0.005$) and diagnosed samples (n=199; Spearman's rank correlation coefficient $\rho=0.48$, $P=0.0001$). Semi-quantitative western analysis confirmed a significant decrease in the 6-12 month pre-diagnosis samples (n=17) *versus*

controls (n=17, $p=0.03$) and in diagnosed PDAC patients in the presence (n=48; $p=0.001$) and absence (n=49; $p=0.0007$) of jaundice compared to healthy subjects (n=42).

Corresponding CA19-9 data for the pre-diagnosis and diagnosed PDAC samples are presented in Supplementary Figures S2 A and B respectively. CA19-9 levels were significantly up-regulated in the 0-6 month pre-diagnosis cases compared to controls ($p=0.001$), in the 6-12 month pre-diagnosis cases compared to controls ($p=0.04$), in patients with PDAC in the presence and absence ($P=0.0001$) of biliary obstruction compared to HC ($P=0.001$ and $P=0.0001$, respectively) and CP (both $P=0.0001$), consistent with our previous observations (12).

Validation of TSP-1 in murine samples

Low TSP-1 serum levels were observed in LSL-Kras^{G12D/+};LSL-Trp53^{R172H/+};Pdx-1-Cre (KPC) mice with cancer, compared to KPC mice with high or low grade PanIN and compared to age-matched control (LSL-Trp53^{R172H/+};Pdx-1-Cre) mice (Figures 1 D and E).

Low TSP-1 levels correlate with poor outcome

Next we examined the relationship between circulating TSP-1 levels and survival. For this analysis, TSP-1 levels equal to the 25th percentile (0.00 fmol/ μ L) were classified as low with the remaining values classified as high. The preclinical levels of TSP-1, up to 24 months prior to diagnosis were associated with a significantly reduced survival time from diagnosis (Figure 2 A; logrank $\chi^2_1=3.7$ $p=0.05$). Only diagnosed patients with resectable disease (n=75) were included and the median survival of these patients with low circulating TSP-1 was significantly lower than those with high TSP-1 (Figure 2 B; logrank $\chi^2_1=4.27$ $P=0.04$).

Ability to distinguish pre-diagnosis cases from controls

While our work was in progress, Nie *et al* (18) reported that TSP-1 provided AUCs of 0.78 and 0.83 for the discrimination of clinically diagnosed PDAC from HC and CP respectively. In samples up to 24 months prior to diagnosis (Figure 2 C), we found that TSP-1 distinguished cases from controls with an AUC of 0.69. During this time period, CA19-9 yielded an AUC of 0.77. The combination of both markers achieved a significantly higher AUC of 0.85 ($p=0.02$; Figure 2 C).

TSP-1 levels do not relate to platelet count

TSP-1 was identified as a molecule released from platelets in response to thrombin treatment (19). Concerned that fluctuations in circulating TSP-1 may reflect changes in blood platelet levels, we correlated PDAC patient platelet count (n=96 PDAC patients) with TSP-1 levels. The median platelet count was $293 \times 10^9/L$, but was significantly elevated in patients with advanced cancer (n=20; count $344 \times 10^9/L$), compared to resectable cancer (n=76; count $277 \times 10^9/L$; $p=0.02$). No significant correlation was detected between TSP-1 levels and platelet count in either resectable (Spearman Rank rho= 0.01, $p=0.88$) or in advanced patients (rho= -0.13, $p=0.57$).

TSP-1 serum levels are associated with diabetes mellitus

Although TSP-1 levels were significantly lower in cancer patients than controls (Figure 1 C), not all cancer patients had reduced levels. We therefore hypothesised that the marker may be regulated in a sub-set of patients only and examined for associations between circulating TSP-1 levels and clinicopathologic parameters, separating PDAC patients into low or high for TSP-1 based on circulating TSP-1 values $<$ or \geq the median (0.267 fmol/ μ L). Associations could not be established between gender, age at surgery, presence of obstructive jaundice, operable *versus* advanced, resection margin status, T stage or nodal status (Supplementary Table S4). However, the presence of diabetes in PDAC patients was significantly associated with TSP-1 (Chi-squared test, $P=0.02$, Supplementary Table S4). Examining this further, of 27 patients with confirmed diabetes, 19 (70.3%) had less than the median level of TSP-1 (14 of those patients had undetectable TSP-1). By contrast, of the remaining 71 patients without confirmed diabetes, only 30 (42.2%) had less than the median serum TSP-1 level (Chi-squared test, $P=0.01$). Upon separating our clinically diagnosed PDAC patients into those with- and without-diabetes, a significant reduction in TSP-1 levels were observed for the PDAC patients with diabetes compared to all other control groups (Figure 3A). Finally, given the correlation between diabetes and TSP-1, we examined whether diabetes was associated with outcome in this cohort, but no association was observed (Figure 3 B; logrank $\chi^2_1=1.53$ $P=0.22$).

PDAC related diabetes often goes undiagnosed (20); it is therefore unsurprising that only 10% of UKCTOCS cases were recorded as having diabetes. Since a substantial number of UKCTOCS cases probably had occult diabetes or glucose intolerance, no analysis was undertaken for this cohort.

TSP-1 serum levels are lower in PDAC patients with diabetes compared to individuals with long-term type 2 diabetes mellitus

Measurement of TSP-1 levels by western blot in an independent cohort confirmed our previous observation that significantly lower levels of TSP-1 are present in PDAC patients with diabetes compared to PDAC patients without diabetes ($P=0.002$), healthy subjects ($P<0.0001$) and CP patients ($P=0.05$); Figure 4). Significantly lower levels were also observed in PDAC patients with diabetes compared to individuals with long term type 2 DM ($P=0.01$).

TSP-1 tumour expression is not associated with diabetes mellitus

To determine whether tumour levels of TSP-1 were associated with diabetes mellitus, a PDAC TMA was stained for TSP-1 (Figure 3 C). Tumour cell staining was observed in 7/49 cases (14%) while 8 patients (16%) expressed TSP-1 in desmoplastic stroma. Two patients (4%) were positive for both tumour and stromal staining. Thirty-two (65%) patients lacked detectable tissue TSP-1 expression. Associations could not be established between TSP-1 expression and other clinicopathological parameters, including diabetes (Supplementary Table S5). This remained true when expression was categorised as either tumoural or stromal.

Discussion

Previous serum biomarker studies have carried out discovery work on samples taken from patients after a diagnosis of PDAC has been made (11, 29, 30, 31, 32), thereby potentially missing critical changes to the serum disease profile that occur months and years prior to diagnosis. Our study is virtually unique in that we have simultaneously subjected pre-clinical and clinical samples to a proteomic biomarker discovery protocol and identified a protein of interest, TSP-1, that appeared altered before and after diagnosis, highlighting its potential as an early biomarker. We used MRM in this study to verify these changes in individual serum samples as it afforded unequivocal identification and accurate quantification of TSP-1 by selecting peptides unique to the TSP-1 protein. We saw similar trends in reduction of TSP-1 levels using an antibody-based approach. However cross-reaction between the antibody used in our study and TSP-2 cannot be ruled out (21) and might explain differences between MRM and western data. TSP-1 is a large secreted multimeric extracellular matrix protein (22), whose role in cancer is controversial. TSP-1 has been described as anti-

carcinogenic due to its potent anti-angiogenic properties, mediated in large part through binding to CD36 (fatty acid translocase; FAT) and CD47 (integrin-associated protein; IAP) and its role in activation of TGF-beta (23). Serum levels of TSP-1 are decreased in patients with prostate cancer (24) and lung cancer (25)(26), while plasma levels of TSP-1 are elevated in breast cancer patients (27). In pancreatic cancer, serum TSP-1 levels were found to be significantly increased in patients with unresectable cancer (28), although we could not establish any difference between levels in unresectable *versus* resectable pancreatic cancer patients. Our analysis of TSP-1 expression in pancreatic cancer tissue did not help explain the reduction in the circulating levels of TSP-1 in PDAC patients. Currently, we cannot propose a mechanism for our observations.

As we have previously reported, the presence of jaundice, a late symptom in PDAC patients, can influence biomarker levels (14, 16). Potential markers that are not affected by jaundice are more likely to be indicative of early disease. We allowed for this here by separately analysing PDAC samples taken from diagnosed patients in the presence *versus* absence of jaundice. The reduction of TSP-1 levels in patients' serum, regardless of the presence of jaundice, distinguished it from previous candidates evaluated by our group (10) and made it an attractive candidate for early diagnosis. Consistent with our findings, others have reported lower circulating TSP-1 levels in PDAC patients compared to healthy controls (18, 28). Here we go further by showing significant reduction of TSP-1 levels in patients up to 24 months prior to diagnosis of pancreatic cancer. We recently showed that CA19-9 discriminated preclinical PDAC cases from controls (12). Combining TSP-1 and CA19-9 offered a significant improvement over either marker alone. Our analysis of serum from KPC mice provided evidence for a decrease in circulating TSP-1 in mice with PDAC, but not in mice with PanIN lesions. This raises the question as to whether the decreases in TSP-1 observed in pre-diagnostic UKCTOCS cases are occurring in a background of already formed PDAC. Further study is required to unravel this question.

Upon separating our PDAC patients into those with and without diabetes we found that levels of TSP-1 were significantly reduced in clinically diagnosed PDAC patients with diabetes compared to all control groups, including individuals with long-term type 2 DM. Our findings therefore suggest a link between reduced serum TSP-1 levels and PDAC-associated diabetes (type III C). Pannala *et al.* reported that 85% of PDAC patients have hyperglycaemia or diabetes with some 47% having

diabetes (20). In our study, only 27% of diagnosed PDAC patients had confirmed diabetes, which is likely to be an underestimate. TSP-1 has been shown to be an adipokine, associated with insulin resistance (29). Moreover, TSP-1-null mice are markedly glucose intolerant and have decreased glucose-stimulated insulin release and capacity for (pro)insulin biosynthesis, although they possess an increased beta-cell mass (30). This phenotype was attributed to the lack of activation of islet TGF β -1 by endothelial-derived TSP-1. Failure of glucose regulation may occur before a rise in CA19-9, perhaps explaining why TSP-1 adds to CA19-9 in discriminating early disease. More work is needed to uncover the true extent of the relationship between TSP-1 and diabetes in PDAC patients, and to determine whether TSP-1 might serve as a PDAC screening tool in individuals newly diagnosed with type 2 DM. Aggarwall *et al.* (31) reported elevated plasma levels of adrenomedullin in PDAC patients with new-onset DM compared to PDAC patients with normal fasting glucose and compared to non-cancer subjects with new-onset type DM.

Limitations of this study include using pooled samples for iTRAQ profiling as this is sensitive to outliers and can lead to false positives. Extensive validation was therefore undertaken using both individual samples of the iTRAQ set and also independent samples. It is possible that the delay of processing in UKCTOCS samples may have led to the loss of some proteins. In addition, it was not possible to evaluate longitudinal preclinical alterations in TSP-1 as the number of UKCTOCS cases with available longitudinal samples was insufficient to adequately assess this. Finally, as mentioned above, the number of individuals with diabetes in our PBRU cancer cohort is likely to be underestimated, and accurate diabetes data were only available for a small number of UKCTOCS cases, preventing sensible evaluation of TSP-1 levels and diabetes in the preclinical setting.

In conclusion, our study allowed a valuable appraisal of TSP-1 levels during the lead into pancreatic cancer diagnosis, demonstrating that its inclusion in diagnostic panels could have potential in early detection strategies. We have also highlighted the effect diabetes can have on the performance of potential biomarkers and have shown its influence should be controlled for in future biomarker studies.

TABLE AND FIGURE LEGENDS:

Table 1A). Patients Characteristics of UKCTOCS pre-diagnostic cases and controls for whole study set and **1B)** Patients Characteristics of PBRU diagnosed PDAC samples and controls for whole study set.

Table 2. IPA Biomarker Filter Analysis from iTRAQ data set.

Figure 1. Detection of thrombospondin 1 (TSP-1) by MRM in (A) all pre-diagnostic samples versus controls, (B) in individual time to diagnosis groups, (C) in diagnosed samples and controls (PDAC obs = bilirubin $>20\mu\text{mol/L}$, presence of jaundice; PDAC non-obs = bilirubin $<20\mu\text{mol/L}$, absence of jaundice; CP = chronic pancreatitis, BBO = benign biliary obstruction, HC = healthy control) and (D) genetically engineered mice (PanIN = pancreatic intraepithelial neoplasia (pre-neoplastic lesion). Low grade = PanIN I or II, High grade = PanIN III).

Figure 2. Survival curves for TSP-1 in (A) pre-diagnosis and (B) diagnosed patients (low levels = $0.00\text{ fmol}/\mu\text{L}$), (C) Performance of TSP-1, based on MRM measurements, to discriminate 0-24 month pre-diagnostic patients ($n=64$) from time matched controls ($n=64$).

Figure 3. TSP-1 is associated with diabetes in diagnosed samples compared to all other groups (A), Diabetes mellitus does not relate to outcome (B), TSP-1 staining in PDAC tissue samples (C).

Figure 4. Detection of thrombospondin 1 (TSP-1) by western analysis in an independent cohort of diagnosed samples and controls (CP = chronic pancreatitis, HC = healthy control).

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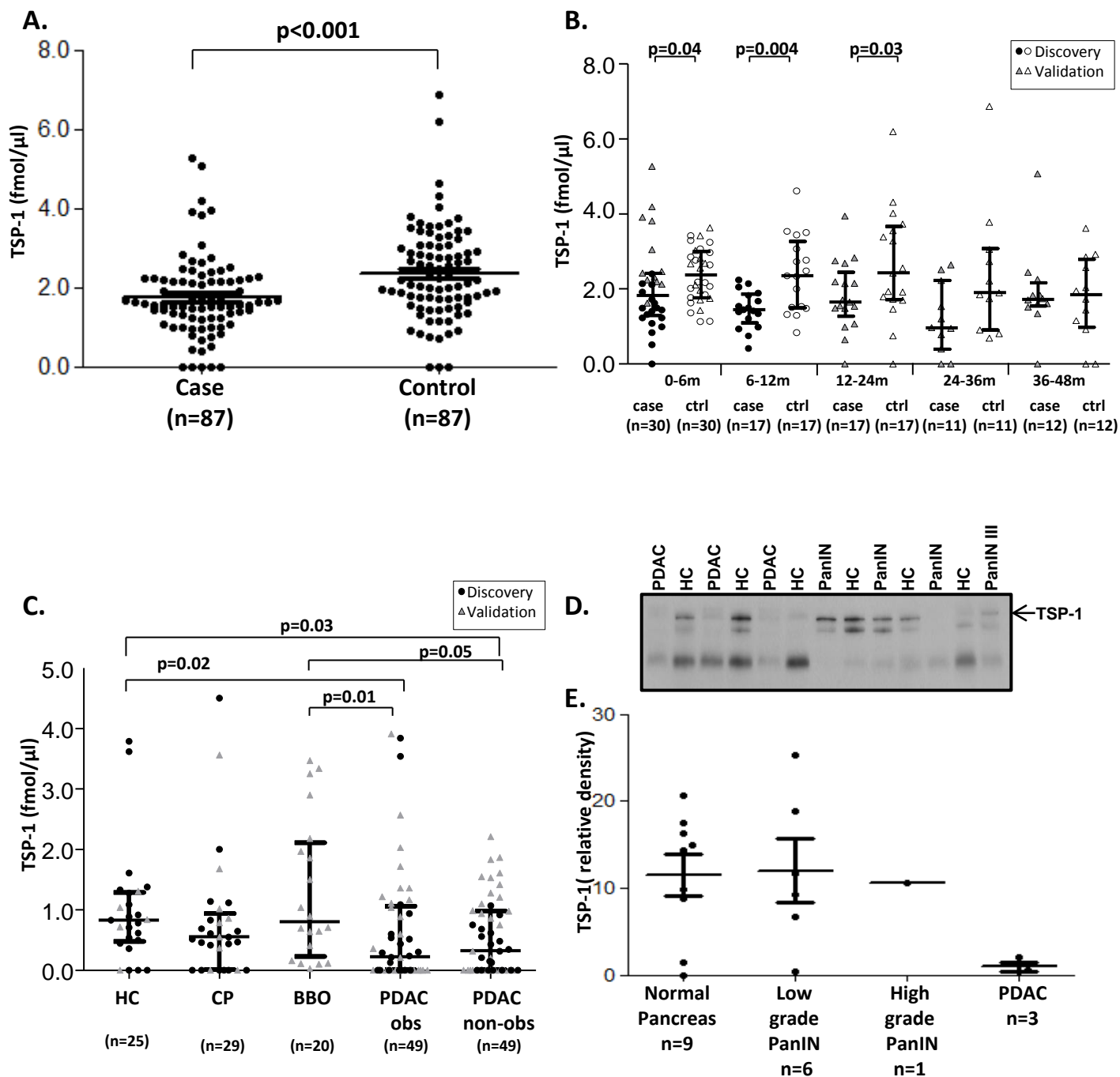


Figure 1

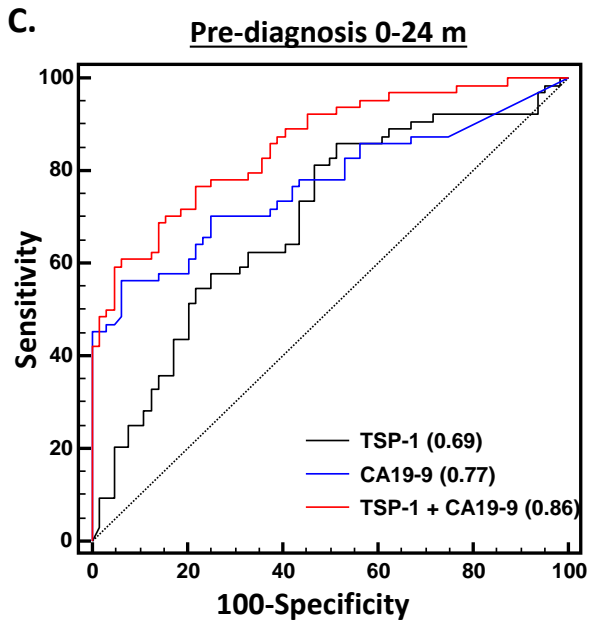
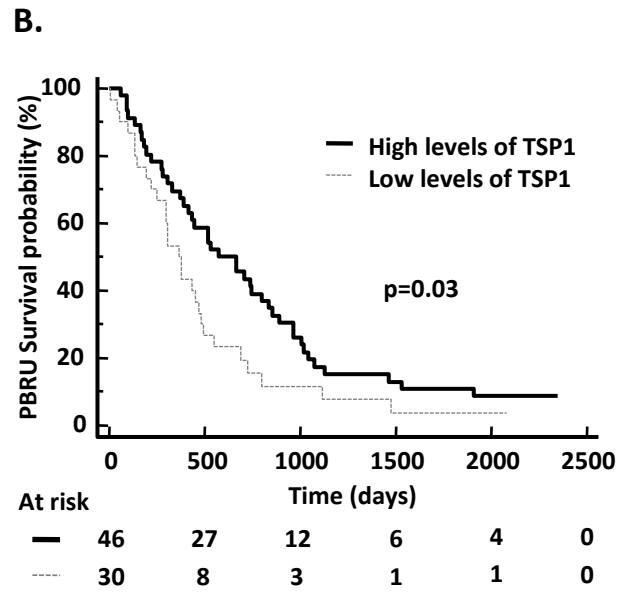
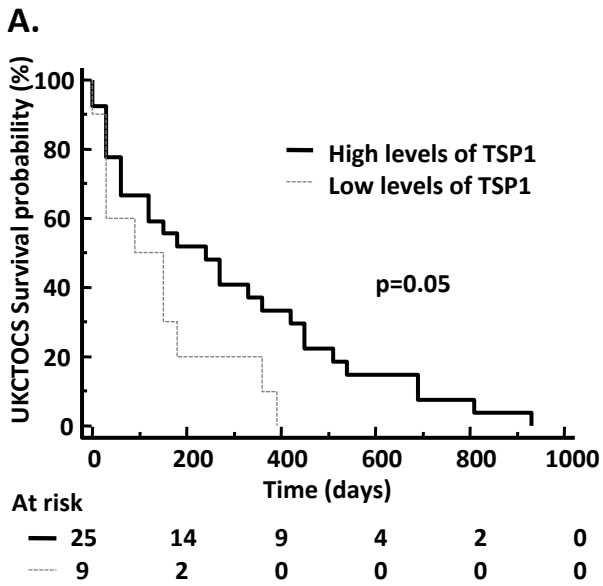


Figure 2

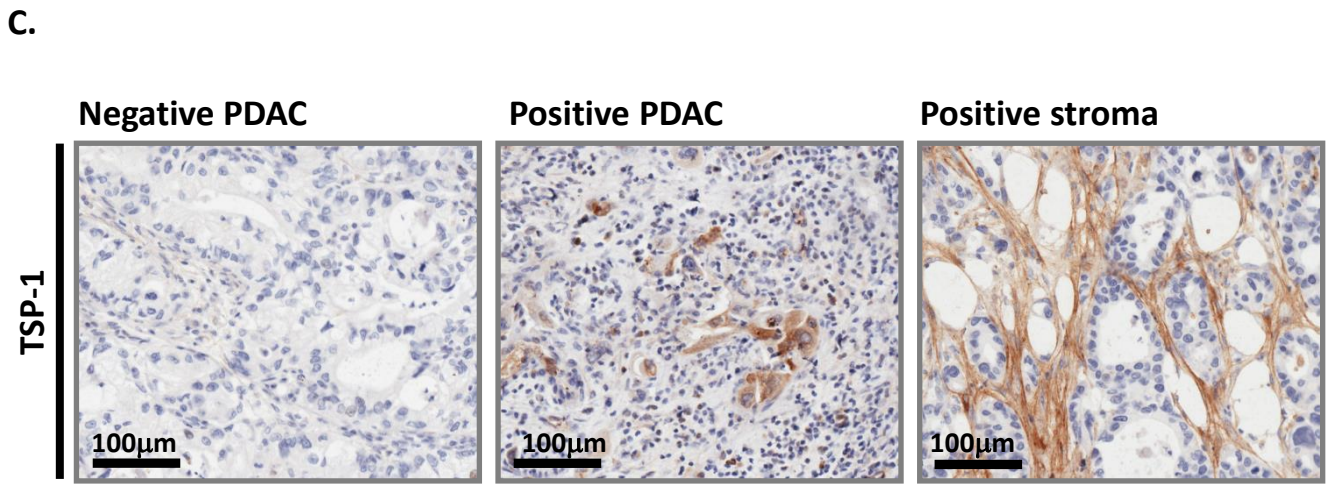
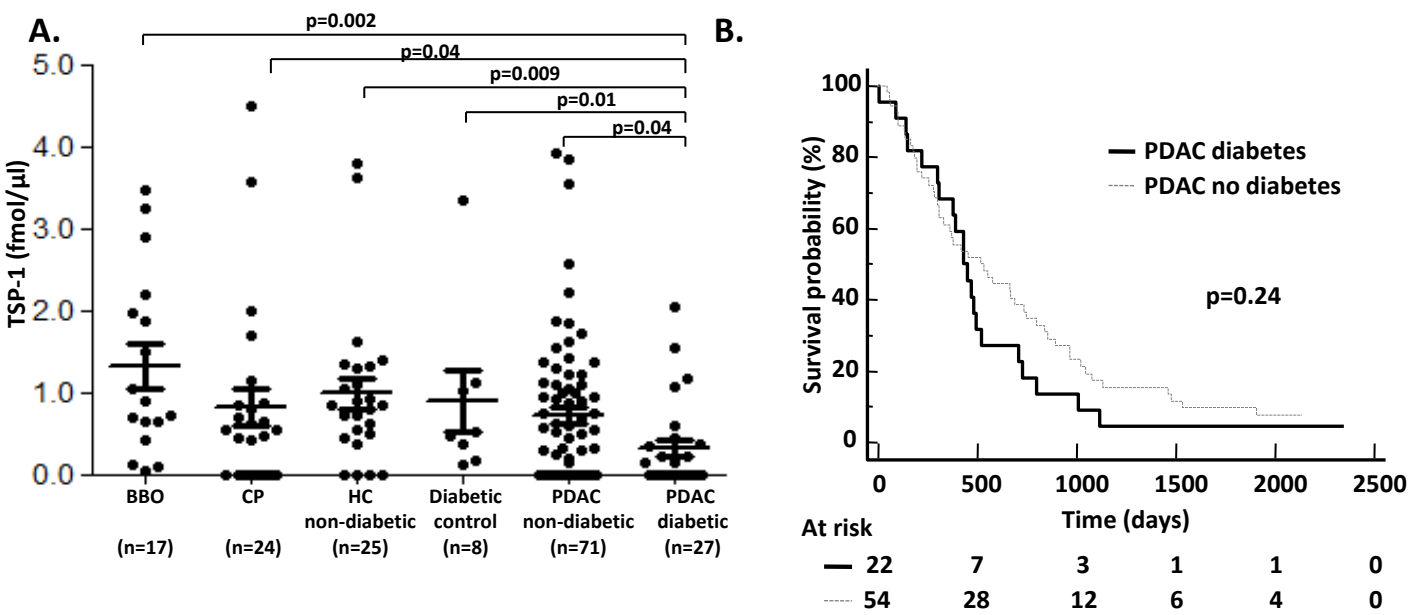


Figure 3

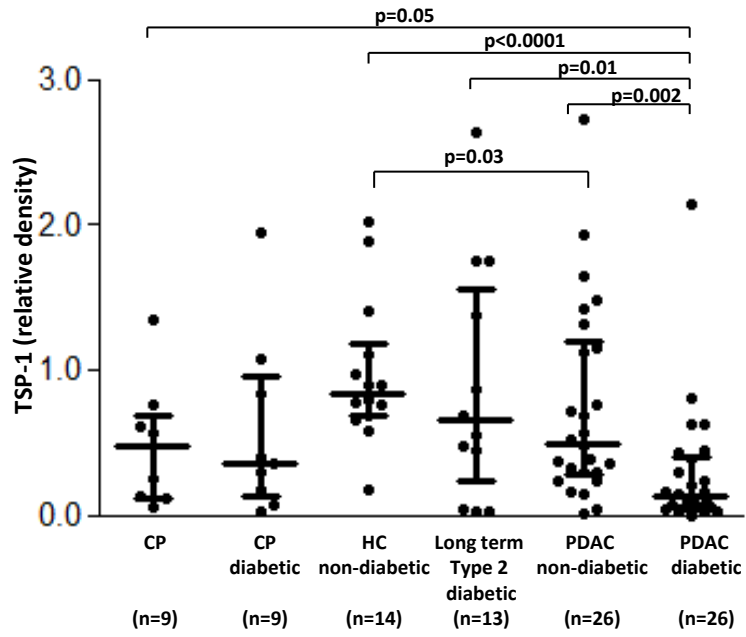


Figure 4

Table 1a) Patients Characteristics of UKCTOCS pre-diagnostic cases and controls for whole study set

	0-6m		6-12m		12-24m		24-36m		36-48m	
	cases	ctrls	cases	ctrls	cases	ctrls	cases	ctrls	cases	ctrls
n	30	30	17	17	17	17	11	11	12	12
Median Age (y)	66.9	64.7	68.3	67.4	62.9	60.8	62.4	60.2	65.8	57.4
(IQR)	(60.9-70.8)	(58.2-69.2)	(59.2-70.6)	(60.3-71.7)	(59.1-68.5)	(55.1-67.1)	(59-69.9)	(52.9-67.8)	(60.5-71)	(55.6-61.4)
Mean time from sample collection to diagnosis (days)	91.5	n/a	282.9	n/a	535.6	n/a	853.4	n/a	1288.9	n/a
(IQR)	(49.5-152.6)		(243.5-314.4)		(476.3-684.5)		(814-944.5)		(1197-1344)	
Mean time to spin (hours)	21.3	21.0	21.0	20.8	22.7	22.7	22.2	21.7	23.6	22.7
(IQR)	(19.3-23.1)	(19.2-23.3)	(19.8-22.5)	(19.7-23.1)	(20.5-23.6)	(20.3-23.6)	(20.6-24.6)	(20.4-24.5)	(20.8-24.9)	(20.6-24.5)

Abbreviations: ctrls - controls; IQR - interquartile range.

Table 1b) Patients Characteristics of PBRU diagnosed PDAC samples and controls for whole study set

		PBRU cohort 1					PBRU cohort 2					
		PDAC non-obs	PDAC obs	HC	CP	BBO	PDAC	PDAC (w/diabetes)	HC	Type II diabetics	CP	CP (w/diabetes)
n		49	49	42	39	20	26	26	14	13	9	9
Median Age (IQR)		67 (60-73)	66 (62.8-72)	30.5 (25-37.8)	50 (43-60)	65.5 (56.8-75.3)	71 (63-73)	70 (65-74)	56 (53-60)	67 (62-75)	46 (44-59)	54 (46-59)
Gender	F/M	28/21	26/23	20/22	20/19	5/15	16/12	9/17	7/7	7/6	3/6	2/7
Diabetes		14	13	n/a	5	3	-	26	-	13	-	9
Resection Margin	R0	10	11	-	-	-	5	6	-	-	-	-
	R1	24	26	-	-	-	16	14	-	-	-	-
	U	3	2	-	-	-	1	-	-	-	-	-
	I	12	10	-	-	-	4	6	-	-	-	-
Staging	IA	1	-	-	-	-	2	1	-	-	-	-
	IB	-	1	-	-	-	-	-	-	-	-	-
	IIA	6	6	-	-	-	3	6	-	-	-	-
	IIB	28	30	-	-	-	16	12	-	-	-	-
	III/IV	12	10	-	-	-	4	7	-	-	-	-
	U	2	2	-	-	-	1	-	-	-	-	-

Abbreviations: BBO - benign biliary obstruction; CP - chronic pancreatitis; F - female; HC – healthy control; I – Inoperable; IQR -interquartile range; M - male; obs – obstruction;

PDAC - pancreatic ductal adenocarcinoma; U - unknown

Table 2. IPA Biomarker Filter Analysis from iTRAQ data set

Data Set	Symbol	Entrez Gene Name	Fold Change	p-value
0-6m case 'v' ctrls	APOE	apolipoprotein E	-6.54	0.017
	HP	haptoglobin	1.42	0.000
	KRT6B	keratin 6B	3.56	0.039
	TSP-1	thrombospondin 1	-2.09	0.010
6-12m case 'v' ctrls	FN1	fibronectin 1	-1.08	0.000
	ICAM1	intercellular adhesion molecule 1	-1.51	0.004
	KRT6B	keratin 6B	-5.55	0.038
	SERPINA1	serpin peptidase inhibitor, clade A member 1	-1.21	0.002
	TSP-1	thrombospondin 1	-4.49	0.042
PDAC non obs 'v' HC	APOE	apolipoprotein E	8.40	0.005
	CRP	C-reactive protein, pentraxin-related	52.96	0.031
	FGA	fibrinogen alpha chain	-10.19	0.042
	FN1	fibronectin 1	-1.14	0.000
	HP	haptoglobin	-1.06	0.002
	ICAM1	intercellular adhesion molecule 1	35.65	0.003
	KRT6B	keratin 6B	-2.63	0.004
	LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein	2.47	0.038
	MCAM	melanoma cell adhesion molecule	-4.29	0.001
	NCAM1	neural cell adhesion molecule 1	-2.13	0.030
	PLG	plasminogen	2.75	0.013
	SERPINA1	serpin peptidase inhibitor, clade A member 1	1.29	0.008
	TSP-1	thrombospondin 1	-4.97	0.032
PDAC non obs 'v' CP	APOA1	apolipoprotein A-I	32.21	0.000
	APOE	apolipoprotein E	32.81	0.000
	FGA	fibrinogen alpha chain	-24.66	0.007
	FN1	fibronectin 1	1.03	0.011
	HP	haptoglobin	-1.82	0.000
	ICAM1	intercellular adhesion molecule 1	42.46	0.003
	KRT6B	keratin 6B	-1.91	0.005
	MCAM	melanoma cell adhesion molecule	-4.29	0.001
	NCAM1	neural cell adhesion molecule 1	-1.26	0.033
	SERPINA1	serpin peptidase inhibitor, clade A member 1	1.20	0.000