Decreased Serum Thrombospondin-1 Levels in Pancreatic Cancer Patients Up to 24 Months Prior to Clinical Diagnosis: Association with Diabetes Mellitus

Claire Jenkinson1,2, Victoria L. Elliott1,2, Anthony Evans1,2, Lucy Oldfield1,2, Rosalind E. Jenkins3, Darragh P. O’Brien4, Sophia Apostolidou4, Aleksandra Gentry-Maharaj4, Evangelia-O Fourkala4, Ian J. Jacobs4,5, Usha Menon4, Trevor Cox1, Fiona Campbell6, Stephen P. Pereira7, David A. Tuveson8, B. Kevin Park3, William Greenhalph1,2, Robert Sutton1,2, John F. Timms4, John P. Neoptolemos1,2, and Eithne Costello1,2

Abstract

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

Experimental Design: Serum from cases up to 4 years prior to PDAC diagnosis and controls (URCTOCS, 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). Isobaric tags for relative and absolute quantification (iTRAQ) enabled comparisons of pooled serum from a test set (n = 150). Validation was undertaken using multiple reaction monitoring (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 and 0.77, respectively; P < 0.01). TSP-1 was also decreased in PDAC patients compared with healthy controls (P < 0.05) and patients with benign biliary obstruction [P < 0.01]. Low levels of TSP-1 correlated with poorer survival, preclinically (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 with individuals with type 2 diabetes mellitus (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 behavior should be considered in future studies. Clin Cancer Res; 22(7); 1734–43. ©2015 AACR.

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 approximately 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 preclinically, we performed biomarker discovery using samples collected as part of the prospective cohort “United Kingdom...
Reduced Serum TSP-1 Prior to Clinical Diagnosis of PDAC

Translational Relevance

A 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. In addition, a high proportion of PDAC patients are either hyperglycemic 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 behavior.

Collaborative Trial of Ovarian Cancer Screening” (UKCTOCS; www.ukctos.org.uk); ref.s 7, 8). The nested cohort of samples analyzed 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 (Traq), 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 TSP-1 levels preclinically, 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.

Table 1. Patient characteristics of UKCTOCS prediagnostic cases and controls for whole study set

<table>
<thead>
<tr>
<th>Patient groups</th>
<th>Cases</th>
<th>Controls</th>
<th>Cases</th>
<th>Controls</th>
<th>Cases</th>
<th>Controls</th>
<th>Cases</th>
<th>Controls</th>
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<tbody>
<tr>
<td>Mean time from sample collection to diagnosis (days)</td>
<td>IQR)</td>
<td>IQR)</td>
<td>IQR)</td>
<td>IQR)</td>
<td>IQR)</td>
<td>IQR)</td>
<td>IQR)</td>
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<tr>
<td>Age (years)</td>
<td>Median</td>
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<td>64.7</td>
<td>68.3</td>
<td>67.4</td>
<td>62.9</td>
<td>60.8</td>
<td>62.4</td>
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<tr>
<td>(IQR)</td>
<td>(60.9-70.8)</td>
<td>(58.2-69.2)</td>
<td>(59.2-70.6)</td>
<td>(60.3-71.7)</td>
<td>(59.1-68.5)</td>
<td>(55.1-67.1)</td>
<td>(59-69.9)</td>
<td>(52.9-67.8)</td>
</tr>
<tr>
<td>Mean time to spin (hours)</td>
<td>IQR)</td>
<td>IQR)</td>
<td>IQR)</td>
<td>IQR)</td>
<td>IQR)</td>
<td>IQR)</td>
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<td>IQR)</td>
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<tr>
<td>(IQR)</td>
<td>(19.3-23.1)</td>
<td>(19.2-23.3)</td>
<td>(19.8-22.5)</td>
<td>(19.7-23.1)</td>
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<td>(20.3-23.6)</td>
<td>(20.6-24.6)</td>
<td>(20.4-24.5)</td>
</tr>
</tbody>
</table>

Abbreviation: IQR, interquartile range.

Methods

Patient groups

Blood was obtained, with ethically approved written informed 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 (PBRI; ref.1/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 subcategorized as follows: 0–6 months prediagnosis (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 PBRI sets were analyzed. The first PBRI set (n = 199) consisted of 98 patients with histologically confirmed PDAC, 39 patients with chronic pancreatitis, 20 with jaundice due to gall stones (benign biliary obstruction, BBO), and 42 healthy control individuals. PDAC patients were further subcategorized 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, a second independent PBRI cohort (n = 99) was analyzed. This included 54 patients with histologically confirmed PDAC, 18 patients with chronic pancreatitis, 14 healthy control individuals, and 13 patients with long-term (for 5 or more years) type 2 diabetes mellitus. Of the PDAC and chronic pancreatitis patients, 26 and 9, respectively, had confirmed diabetes. The clinical characteristics of the study populations are provided in Tables 1 and 2.

Sample collection

UKCTOCS blood samples were collected and processed throughout the trial according to a standardized SOP (8, 13). Blood was taken in Greiner gel tubes (8 mL separation tubes; Greiner Bio-one 455071) at one of 13 trial centers, 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 venipuncture. PBRI blood samples were collected in Sarstedt Monovette tubes (Sarstedt Ltd), placed at 4°C for 15 minutes and centrifuged at 800 × g for 10 minutes at 4°C. Serum was stored in aliquots at −80°C.
Table 2. Patient characteristics of PBRU diagnosed PDAC samples and controls for whole study set

<table>
<thead>
<tr>
<th>PBRU cohort 1</th>
<th>PBRU cohort 2</th>
</tr>
</thead>
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<tr>
<td><strong>PDAC</strong></td>
<td><strong>PDAC</strong></td>
</tr>
<tr>
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<td>obstructive</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>49</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td><strong>F/M</strong></td>
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<tr>
<td><strong>Resection margin</strong></td>
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<tr>
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<tr>
<td><strong>F/M</strong></td>
<td>14</td>
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</tbody>
</table>

abbreviations: BBO, benign biliary obstruction; CP, chronic pancreatitis; F, female; HC, healthy control; I, inoperable; IQR, interquartile range; M, male; U, unknown.

Preoperative total serum bilirubin (μmol/L; Roche Modular SWA) and CA19-9 levels were measured in hospital Clinical Biochemistry Departments, or by CA19-9 ELISA (Human Pancreatic and GI Cancer ELISA kit, Alpha Diagnostics International).

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 KrasG12D and Trp53R172H under the endogenous promoter, and flanked by Lox-STOP-Lox cassettes (LSL-KrasG12D/Cre; LSL-Trp53R172H/Cre). Serum samples were collected from 10 LSL-KrasG12D/Cre; LSL-Trp53R172H/Cre; Pdx-1-Cre (KPC) mice and 9 age-matched control (LSL-Trp53R172H/Cre; Pdx-1-Cre) mice via cardiac bleed under isoﬂurane gaseous anesthesia. Blood was collected at 6 weeks, 2 months, and 3 to 6 months and centrifuged at 1,000 × g for 20 minutes at room temperature. Serum was collected, snap frozen (in liquid nitrogen), and stored at −80°C in 60 μL aliquots. Mice were surgically and pathologically examined to confirm the presence of pancreatic tumors and metastases.

iTRAQ analysis

An iTRAQ 8-plex experiment using pooled, delipidated, and high abundance protein-depleted serum from a discovery subset of prediagnosis UKCTOCS samples [0–6 months cases and controls (n = 18/group), >6–12 months cases and controls (n = 17/group) and PBRU samples; PDAC nonobstructed (n = 20), PDAC obstructed (n = 20), chronic pancreatitis (n = 20), and healthy control individuals (n = 20; total n = 150)] was analyzed as described previously (10, 14) on a QSTAR-Pulsar Hybrid Mass Spectrometer (AB Sciex). Two replicate analyses were also performed on a Triple TOF-5600 (AB Sciex) as described (15). Data were analyzed using ProteinPilot software (Version 4.0, AB Sciex). The initial 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 value <0.05 was utilized. Both a Core Analysis and Biomarker Filter were performed.

MRM analysis

MRM was carried out on all 150 samples (UKCTOCS n = 70; PBRU n = 80) using 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) coupled with an Ultimate 3000 HPLC (Dionex, Thermo Scientific). 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; See Supplementary Table S2). Corresponding stable isotope–labeled versions of each peptide (C13-, N15-labeled 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 ∼0.25 μg/mL with 2% acetonitrile + 0.1% formic acid), with subsequent regression analysis showing acceptable linearity (r2 > 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 5,500 V and a source temperature of 475°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 × 1 mm, 1.9 μm, 175 Å column, using a 20-minute gradient, at a flow rate of 100 μL/min, with buffer A (0.1% formic acid) and buffer B (95% acetonitrile + 0.1% formic acid). The liquid chromatography (LC) gradient comprised the following: 2% buffer B for 2 minutes, ramped to 10% buffer B in 0.1 minute, 40% buffer B in 10 minutes, 80% buffer B in 0.1 minute held for 3 minutes, and 2% buffer B in 0.1 minute held for 5 minutes. Prior to analyzing each batch of serum samples, chromatographic performance and mass spectrometric stability were evaluated using a tryptic peptide mixture of...
β-galactosidase (Sigma Aldrich). Three aliquots of each serum sample were analyzed, 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 analyzed 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 µL of each sample analyzed using anti-TSP-1 A6.1 mouse monoclonal antibody (1:400; Thermo Scientific). A standard comprising 20 pooled healthy control samples was used at three different dilutions per gel, allowing comparison and quantification across blots. Protein was separated on Any kD MINIPROTEAN TGX Precast gels (Bio-Rad), transferred onto polyvinylidene difluoride (PVDF) membranes and blocked for 1 hour 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 hors eradish peroxidase–conjugated secondary antibodies diluted in 5% milk/PBST. Bands were visualized with enhanced chemiluminescence developed with X-ray film. Densitometry was performed (Kodak MI SE software), and protein quantities recorded relative to healthy control (HC) standards. All samples were analyzed 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 deparaffinized 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; Thermo Scientific) was incubated at room temperature for one hour. Positively stained tumor cells and tumor-associated stroma were identified by a specialist histopathologist.

**Statistical analysis**

Statview V 5.01 (SAS Institute Inc.) and Medcalc software (Version 13) were used. iTRAQ data were compared using the Mann–Whitney U test. MRM and Western blotting data were analyzed using the two-tailed Mann–Whitney U test and diagnostic accuracy compared by ROC analyses. Patient immunohistochemistry data were compared with clinicopathologic parameters using Fisher exact, Pearson χ², 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- to 48-month groups (P = 0.01); however, a Spearman rank correlation for comparison of age and TSP-1 levels showed no significant correlation (Spearman rank correlation coefficient ρ = 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 rank correlation was performed (Spearman rank correlation coefficient ρ = –0.07; P = 0.328). No gender differences in TSP-1 were found in the PBRU cohort.

**Serum iTRAQ analysis**

Pooled sera from prediagnosis UKCTOCS samples (0–6 months cases and controls, >6–12 months cases and controls and PBRU samples; PDAC nonobstructed, PDAC obstructed, chronic pancreatitis, and healthy control individuals) 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 labeling with iTRAQ tags. As 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 labeled peptide and calculating protein ratios. A representative dataset is provided in Supplementary Table S3.

**TSP-1 levels are decreased in pooled prediagnosis 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 prediagnostic sample pools versus their respective control pools, along with nonobstructed PDAC patient pools versus healthy control pools and chronic pancreatitis pools, to generate a shortlist of candidates. The high abundance proteins targeted in our depletion protocols were excluded, along with proteins previously characterized 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- to 6-month and 6- to 12-month time groups prior to diagnosis as well as in PDAC versus healthy control PBRU samples taken at diagnosis (Table 3).

**Validation of TSP-1 in individual prediagnosis and diagnosed PDAC samples**

For the accurate quantification of serum TSP-1 in individual serum samples (n = 346), we used a liquid chromatography/tandem mass spectrometry system, operated in MRM mode with stable isotope–labeled internal standards. The two peptides chosen were unique for TSP-1 (Supplementary Fig. S1A) allowing unambiguous discrimination from other thrombospondin family members. The lower limit of detection of the assay was 0.1 fmol/µL and where TSP-1 fell below detection, the level was arbitrarily assigned a value of zero. When all 87 prediagnostic 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 controls (P < 0.001; Fig. 1A). Separating samples into their individual time-to-diagnosis groups revealed TSP-1 levels were significantly reduced compared with time-matched controls in the 0- to 6-month (P = 0.04), 6- to 12-month (P = 0.004), and 12- to 24-month (P = 0.03) cases (Fig. 1B). Significantly lower levels of TSP-1 were observed in PDAC patients in the presence (P = 0.02) and in the absence (P = 0.03)
Table 3. IPA biomarker filter analysis from iTRAQ dataset

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<tr>
<th>Dataset</th>
<th>Symbol</th>
<th>Entrez gene name</th>
<th>Fold change</th>
<th>P</th>
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<td>Apolipoprotein E</td>
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<td>ICAM1</td>
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Abbreviations: CP, chronic pancreatitis; HC, healthy controls.

of jaundice compared with healthy subjects and compared with patients experiencing biliary obstruction due to gallstones (BBO: P = 0.01 and P = 0.05, respectively; Fig. 1C). In contrast, no significant difference in TSP-1 level was established between patients with chronic pancreatitis (Fig. 1C). Furthermore, we could not establish any difference between levels of TSP-1 in resectable PDAC cases versus nonresectable cases (Supplementary Fig. 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 Fig. S1B). The banding pattern obtained in prediagnosis and diagnosed serum samples with this antibody is shown in Supplementary Fig. S1B. Weak, although significant correlations for TSP-1 in MRM and Western blot measures were observed in prediagnosis samples (n = 128; Spearman rank correlation coefficient ρ = 0.29, P = 0.005) and diagnosed samples (n = 199; Spearman rank correlation coefficient ρ = 0.48, P = 0.0001). Semiquantitative Western blot analysis confirmed a significant decrease in the 6–12-month prediagnosis 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 with healthy subjects (n = 42).

Corresponding CA19-9 data for the prediagnosis and diagnosed PDAC samples are presented in Supplementary Fig. S2A and S2B, respectively. CA19-9 levels were significantly upregulated in the 0- to 6-month prediagnosis cases compared with controls (P = 0.001), in the 6- to 12-month prediagnosis cases compared with controls (P = 0.04), and in patients with PDAC in the presence and absence (P = 0.0001) of biliary obstruction compared with healthy control individuals (P = 0.001 and P = 0.0001, respectively) and chronic pancreatitis (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 KPC mice with cancer, compared with KPC mice with high- or low-grade pancreatic intraepithelial neoplasia (PanIN) and compared with age-matched control (LSL-Trp53R172H+/−;Pdx-1-Cre) mice (Fig. 1D 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/μL) were classified as low and 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 (Fig. 2A; log rank χ² = 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 (Fig. 2B; log rank χ² = 4.27, P = 0.04).
Reduced Serum TSP-1 Prior to Clinical Diagnosis of PDAC

Ability to distinguish prediagnosis cases from controls

While our work was in progress, Nie and colleagues (18) reported that TSP-1 provided AUCs of 0.78 and 0.83 for the discrimination of clinically diagnosed PDAC from healthy control individuals and chronic pancreatitis, respectively. In samples up to 24 months prior to diagnosis (Fig. 2C), 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; Fig. 2C).

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 × 10^9/L, but was significantly elevated in patients with advanced cancer (n = 20; count, 344 × 10^9/L), compared with resectable cancer (n = 76; count, 277 × 10^9/L; P = 0.02). No significant correlation was detected between TSP-1 levels and platelet count in either resectable (Spearman rank ρ = 0.01, P = 0.88) or in advanced patients (ρ = −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 (Fig. 1C), not all cancer patients had reduced levels. We therefore hypothesized that the marker may be regulated in a subset 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 ≥ the median (0.267 fmol/μL). 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 (χ^2 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). In contrast, of the remaining 71 patients without confirmed diabetes, only 30 (42.2%) had less than the median serum TSP-1 level (χ^2 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 with all other control groups (Fig. 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 (Fig. 3B; log rank \( \chi^2 \) = 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. As a substantial number of UKCTOCS cases probably had occult diabetes or glucose intolerance, no analysis was undertaken for this cohort.

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Figure 1.
Detection of TSP-1 by MRM in all prediagnostic samples versus controls (A), in individual time to diagnosis groups (B), in diagnosed samples and controls (PDAC obstructive = bilirubin >20 μmol/L, presence of jaundice; PDAC nonobstructive = bilirubin <20 μmol/L, absence of jaundice; CP, chronic pancreatitis; BBO, benign biliary obstruction; HC, healthy control) and genetically engineered mice (PanIN[preneoplastic lesion; D]). Low grade, PanIN I or II; high grade, PanIN III.
TSP-1 serum levels are lower in PDAC patients with diabetes compared with individuals with long-term type 2 diabetes mellitus

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

TSP-1 tumor expression is not associated with diabetes mellitus

To determine whether tumor levels of TSP-1 were associated with diabetes mellitus, a PDAC TMA was stained for TSP-1 (Fig. 3C). Tumor cell staining was observed in 7 of 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 clinicopathologic parameters, including diabetes (Supplementary Table S5). This remained true when expression was categorized as either tumoral 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), 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 preclinical 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 blot analysis data. TSP-1 is a large secreted multimeric matrix-cellular protein (22), whose role in cancer is controversial. TSP-1 has been described as anticarcinogenic due to its potent antangiogenic 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 TGFβ (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 analyzing 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 with 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 with all control groups, including individuals with long-term type 2 diabetes mellitus. Our findings therefore suggest a link between reduced serum TSP-1 levels and PDAC-associated diabetes (type III C). Pannala and colleagues 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-stimu-
lated insulin release and capacity for (pro)insulin biosynthesis,
although they possess an increased β-cell mass (30). This phe-
notype was attributed to the lack of activation of islet TGFβ1 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 diabetes mellitus. Aggarwal and colleagues (31) reported
raised plasma levels of adrenomedullin in PDAC patients with new onset diabetes mellitus compared with PDAC patients with normal fasting glucose and compared with non-
cancer subjects with new onset type diabetes mellitus.

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 longitudi-
dinal 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 UKC-
TOCS 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, demon-
strating that its inclusion in diagnostic panels could have poten-
tial in early detection strategies. We have also highlighted the
effect diabetes can have on the performance of potential biomar-
kers and have shown its influence should be controlled for in
future biomarker studies.

Disclosure of Potential Conflicts of Interest

J. Jacobs is an employee of and has ownership interest in Women’s Health Specialists Ltd., is a consultant/advisory board member for Abcodia Ltd, and is listed
as a co-inventor on a patent, which is owned by Massachusetts General
Hospital and Queen Mary London and licensed to Abcodia Ltd., on a risk of
ovarian cancer algorithm for early detection of Ovarian Cancer. U. Menon
reports receiving commercial research grants from and has ownership interest
(including patents) in Abcodia. No potential conflicts of interest were disclosed
by the other authors.

Authors’ Contributions

Conception and design: C. Jenkinson, V. Elliott, A. Evans, D.P. O’Brien,
Development of methodology: C. Jenkinson, V. Elliott, A. Evans, R.E. Jenkins,
D.P. O’Brien, S. Apostolidou, A. Gentry-Maharaj, E.O. Fourkala, U. Menon,
P. Pereira, R. Sutton, J. Neoptolemos

Acquisition of data (provided animals, acquired and managed patients,
provided facilities, etc.): C. Jenkinson, V. Elliott, A. Evans, R.E. Jenkins,
D.P. O’Brien, S. Apostolidou, A. Gentry-Maharaj, E.O. Fourkala, I. Jacobs,
U. Menon, F. Campbell, S.P. Pereira, D.A. Tuveson, K. Park, R. Sutton,
J.F. Timms, J. Neoptolemos, E. Costello

Analysis and interpretation of data (e.g., statistical analysis, biostatistics,
computational analysis): C. Jenkinson, V. Elliott, A. Evans, L. Oldfield,
R.E. Jenkins, D.P. O’Brien, T.F. Cox, F. Campbell, S.P. Pereira, R. Sutton,
J.F. Timms, J. Neoptolemos, E. Costello

Writing, review, and/or revision of the manuscript: C. Jenkinson, V. Elliott,
A. Evans, L. Oldfield, S. Apostolidou, A. Gentry-Maharaj, E.O. Fourkala,
I. Jacobs, U. Menon, F. Campbell, S.P. Pereira, D.A. Tuveson, K. Park, R. Sutton,
J.F. Timms, J. Neoptolemos, E. Costello

Administrative, technical, or material support (i.e., reporting or organizing
data, constructing databases): C. Jenkinson, V. Elliott, A. Evans, L. Oldfield,
E. Costello

Study supervision: W. Greenhalf, E. Costello

Grant Support

This work was supported by Northwest Cancer Research Fund, UK, grant
CR976, The National Institute for Health Research Pancreas Biomedical
Research Unit, Cancer Research UK grant A12790, Pancreatic Cancer UK, and
the European Community’s Seventh Framework Programme (FP7) 2007-2013
grant agreement no. 256974. UKCTOCS was core-funded by the Medical
Research Council, Cancer Research UK, and the Department of Health with
additional support from the Eve Appeal, Special Trustees of Bart’s and the
London, and Special Trustees of UCLH. UKCTOCS researchers were supported
by the National Institute for Health Research University College London
Hospitals Biomedical Research Centre.

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Received April 10, 2015; revised September 3, 2015; accepted September 19,
2015; published OnlineFirst November 16, 2015.

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Claire Jenkinson, Victoria L. Elliott, Anthony Evans, et al.


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