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iTRAQ reveals candidate pancreatic cancer serum biomarkers: influence of obstructive jaundice on their performance

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Background: The aims of our study were to identify serum biomarkers that distinguish pancreatic cancer (pancreatic ductal adenocarcinoma, PDAC) patients from benign pancreatic disease patients and healthy subjects, and to assess the effects of jaundice on biomarker performance.

Methods: Isobaric tags for relative and absolute quantification were used to compare pooled serum and pancreatic juice samples from a test set of 59 and 25 subjects, respectively. Validation was undertaken in 113 independent subjects.

Results: Candidate proteins Complement C5, inter-α-trypsin inhibitor heavy chain H3, α1-β glycoprotein and polymeric immunoglobulin receptor were elevated in cancer, as were the reference markers CA19-9 and Reg3A. Biliary obstruction had a significant effect on the performance of the markers, in particular within the PDAC group where the presence of jaundice was associated with a significant increase in the levels of all six proteins ($P<0.01$). Consequently, in the absence of jaundice, proteins showed reduced sensitivity for PDAC patients over benign subjects and healthy controls (HCs). Similarly, in the presence of jaundice, markers showed reduced specificity for PDAC patients over benign subjects with jaundice. Combining markers enabled improved sensitivity for non-jaundiced PDAC patients over HCs and improved specificity for jaundiced PDAC patients over jaundiced benign disease subjects.

Conclusions: The presence–absence of jaundice in the clinical scenario severely impacts the performance of biomarkers for PDAC diagnosis and has implications for their clinical translation.

Despite progress in our understanding of pancreatic ductal adenocarcinoma (PDAC; Hidalgo, 2010; Costello and Neoptolemos, 2011; Tuveson and Neoptolemos, 2012), it remains very difficult to detect when at a curable stage. Early warning signs are generally nonspecific, while overt symptoms such as obstructive jaundice, weight loss or pain often manifest when the disease is advanced. Late-stage presentation diminishes treatment options (Stathis and Moore, 2010) and contributes to an especially poor prognosis (Siegel *et al*, 2012; Van Laethem *et al*, 2012).

The diagnostic demands in PDAC are unique given the complex pathophysiology of this cancer and the other neoplastic and inflammatory processes affecting the pancreas from which PDAC needs to be separated. The clinical presentation of these diverse conditions is often similar. In addition, around three quarters of PDAC patients have tumours involving the pancreatic head, associated with obstructive jaundice (Sener *et al*, 1999). Currently, no effective non-invasive methods exist, which discriminate PDAC, located within the head of the pancreas, from inflammatory

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states of the biliary tract and the pancreas. This suggests differing demands on the diagnostic biomarker platform depending on the pre-symptomatic or symptomatic scenario, the presence or absence of a distinct mass in the head of the pancreas and the presence or absence of jaundice. Furthermore, it necessitates appropriate comparator groups in order to test and validate the biomarker platform (Costello *et al*, 2012), as the diagnostic algorithm will be determined by the clinical imperative.

Biliary obstruction whether due to benign disease, such as choledocholithiasis or chronic pancreatitis (CP), or due to cancer in the head of the pancreas will lead to impaired hepatic function resulting in reduced metabolism, such as conjugation of molecules targeted for biliary excretion and the physical obstruction of bile excretion itself. The pharmacological consequence is an alteration in serum levels of proteins, including biomarkers. CA19-9 is an epitope of sialylated Lewis blood group antigen and although not expressed in 5% of the population who are non-secretors (Lewis a-b-), it is currently the only blood-borne biomarker routinely used in the management of pancreatic cancer (Locker *et al*, 2006; Wong *et al*, 2008). Although it is recognised that CA19-9 may also be elevated in pancreatic inflammation and benign as well as malignant cases of biliary obstruction (Locker *et al*, 2006), biomarker studies have not systematically used benign biliary obstruction within the set of control groups either in comparing CA-19-9 *per se* or in the evaluation of newer biomarkers (Yue *et al*, 2011; Costello *et al*, 2012). The performance of the standard CA19-9 assay has a sensitivity of around 80% and a specificity of 75–80% prompting investigations to improve the methodology of CA19-9 detection (Locker *et al*, 2006) as well as searching for newer markers either to augment the accuracy of CA19-9 or as a separate signature panel (Faca *et al*, 2008; Brand *et al*, 2011; Pan *et al*, 2011; Bauer *et al*, 2012).

Labelling with isobaric tags for relative and absolute quantification (iTRAQ; Ross *et al*, 2004) allows simultaneous comparison of protein profiles in several samples. Following the digestion of samples with trypsin, peptides are labelled with iTRAQ reagents (4-Plex or 8-Plex), fractionated and analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Here we report the first iTRAQ-based analysis of blood-borne proteins from pancreatic cancer patients and controls, including patients with CP and benign biliary obstruction. In parallel, we applied LC-MS/MS, with and without iTRAQ labelling, for the analysis of pancreatic juice proteins. Analysis of pancreatic juice has a number of potential advantages as it will contain proteins and other molecules from the whole of the exocrine pancreas in relatively high concentrations compared with blood, making it a rich source of potential biomarkers. The disadvantages of using pancreatic juice are that collection is invasive and it contains many proteases and other enzymes in high concentration that are readily activated. Potentially, pancreas juice is an excellent source for target identification but not for routine clinical application. Therefore, proteins differentially expressed in pancreatic juice were selected for validation in serum. The contribution of biliary obstruction to the circulating levels of candidate biomarkers was assessed by comparing the biomarker levels in PDAC patients with and without biliary obstruction, and in patients with benign biliary obstruction.

MATERIALS AND METHODS

Patient populations. Blood was obtained, with ethically approved informed consent, from 172 subjects comprising a test set of 59 subjects and a validation set of 113 subjects. There were 126 subjects recruited from the Royal Liverpool University Hospital, UK and 46 recruited from the Rechts der Isar Hospital, Munich, Germany (for clinical characteristics, see Table 1). This included 64 patients with histologically confirmed PDAC, who underwent surgical resection (for staging information, see Table 1; fasting blood samples collected; none of the patients have previously received chemotherapy or radiation), 48 patients with histologically confirmed CP, 31 patients with benign jaundice (benign biliary obstruction, BBO), including 23 with gallstones and 8 with liver disease, and 29 healthy control (HC) individuals.

Preoperative total serum bilirubin (μmol l^{-1}; Roche Modular SWA, Pleasanton, CA, USA.) and CA19-9 levels were measured in hospital Clinical Biochemistry Departments, or by CA19-9 enzyme-linked immunoabsorbent assay (ELISA; Human Pancreatic and GI Cancer ELISA Kit, Alpha Diagnostics International, San Antonio, TX, USA). Blood was 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. Pancreatic juice was obtained at the Royal Liverpool University Hospital, either during endoscopic retrograde cholangiopancreatography (without secretin stimulation) or during surgery, both using direct cannulation of the main pancreatic duct and aspiration.

iTRAQ analysis of serum and pancreatic juice. iTRAQ experiments using pooled serum from male individuals (to minimise variation because of gender) was analysed as described (Tonack *et al*, 2009). For the 4-Plex experiment, samples were from PDAC ($n=19$), CP ($n=20$), BBO ($n=8$) or HC ($n=14$) and sample set 1 (Table 1) was used for the 8-Plex experiment. Following lipid and high abundance protein depletion using Sigma Top 20 Depletion column (Sigma Aldrich, Gillingham, UK), samples were concentrated, buffer exchanged and 50 μg from each disease group (4-Plex experiment) or 2 × 50 μg protein from each disease group (8-Plex experiment) was digested with trypsin and iTRAQ labelled (AB Sciex, Warrington, UK). The labelled peptides were combined, fractionated by strong cation exchange chromatography, and the fractions analysed using nano-LC-ESI-MS/MS, on a QSTAR-Pulsar i Hybrid Mass Spectrometer (AB Sciex) as described previously (Tonack *et al*, 2009). Data analysis was performed using ProteinPilot software (version 3, AB Sciex).

For iTRAQ analysis of pancreatic juice, proteins were extracted and pooled from patients with CP ($n=$ 8; 13 μg per sample), PDAC ($n=$ 13; 8 μg per sample) or BBO controls ($n=$ 4; 30 μg per sample), precipitated using cold (−20 °C) acetone, washed with 1 : 4 water:acetone and dissolved in the iTRAQ Kit Dissolution buffer. Protein (60 μg) was digested, labelled with iTRAQ tags, labelled peptides combined, and fractionation, mass spectrometry and data analyses were performed as for serum.

LC-MS-MS analysis of pancreatic juice. For single sample LC-MS/MS analysis ($n=3$ per group), samples were processed essentially as described (Tonack *et al*, 2010). Pancreatic juice samples were mixed with nine volumes of ice-cold methanol, centrifuged at 13 000 **g** for 30 min and proteins resuspended in 50 mM ammonium bicarbonate buffer. Protein (3 μg) was diluted in 12.5 μl of buffer (50 mM ammonium bicarbonate, 0.5 μl 2% SDS) and 1 μl 50 mM TCEP was added, mixed and incubated at 60 °C for 1 h. Subsequently, 0.5 μl of 84 mM iodoacetamide was added and incubation continued at room temperature for 30 min in the dark. Proteins were precipitated using methanol (135 μl) and recovered by centrifugation at 13 000 **g** for 30 min, resuspended in 5 μl of water containing 100 ng of trypsin and incubated overnight at 37 °C. The resulting peptides were analysed by nano-LC-MS and data analysis performed using ProteinPilot software.

Patient sets for validation of iTRAQ- and LC-MS-MS-derived data. Validation of serum candidates (Figure 1) was performed initially using individual samples from the 8-Plex iTRAQ discovery experiment (Set 1; Table 1). Markers taken forward for further

Table 1. Clinical characteristics of the study population (serum sets 1 and 2)

Discovery set (iTRAQ 8-Plex) (set 1)

PDAC				Healthy control			Benign disease control			
Source	Age median (IQR)	Gender	Stage	Source	Age range	Gender	Source	Age median (IQR)	Gender	Diagnosis
Liverpool ($n=15$)	66 (55.2–73)	Male (100%)	T2, N0 = 2; T2, N1 = 1; T3, N0 = 2; T3, N1 = 2; Bypass = 2 U = 6	Liverpool ($n=14$)	All >50	Male (100%)	Liverpool ($n=15$)	46 (42.2–58.2)	Male (100%)	CP
							Liverpool ($n=15$)	72 (61.7–76.7)	Male (100%)	BBO (gall stones)

Validation set (set 2)

PDAC				Healthy control			Benign disease control			
Source	Age median (IQR)	Gender	Stage	Source	Age median (IQR)	Gender	Source	Age median (IQR)	Gender	Diagnosis
Liverpool ($n=19$)	68 (63.2–70.7)	11F, 8M	T1, N0 = 1; T2, N0 = 1; T2, N1 = 1; T3, N0 = 1; T3, N1 = 12; Bypass = 3	Liverpool ($n=15$)	45 (40.8–64.5)	8F, 7M	Liverpool ($n=17$)	53 (44.2–61)	8F, 9M	CP
Munich ($n=30$)	63.5 (55–68)	15F, 15M	T2, N0 = 1; T3, N0 = 7; T3, N1 = 16; U = 6				Munich ($n=16$)	57 (50–65)	8F, 8M	CP
							UoL ($n=15$)	50 (33.5–63)	9F, 7M	BBO (8 gall stones; 8 alcoholic liver disease)

Abbreviations: BBO = benign biliary obstruction; Bypass = palliative biliary-enteric bypass surgery to relieve blockages in unresectable patients; CP = chronic pancreatitis; F = female; IQR = interquartile range; M = male; PDAC = pancreatic ductal adenocarcinoma; U = unknown.

validation were analysed in an independent sample set (set 2; Table 1). Candidates derived from pancreatic juice underwent preliminary evaluation in individual pancreatic juice samples from PDAC ($n=$ 10 or 8), CP ($n=$ 8) and control ($n=$ 4), and were subsequently analysed in individual serum samples from validation sets 1 and 2 (Table 1).

Western blotting and ELISA. The primary antibodies used in this study were: goat anti-inter-α-trypsin inhibitor heavy chain H3 (ITIH3; 1 : 400), mouse anti-C5 alpha chain antibody (1 : 500; Abcam, Cambridge, UK); goat anti-polymeric immunoglobulin receptor (PIGR; 1 : 800; R&D Systems, Abingdon, UK); mouse anti-alpha-1B-glycoprotein (1 : 10 000; Abnova GmbH, Heidelberg, Germany). The secondary antibodies, goat anti-mouse HRP conjugated (1 : 3000) and rabbit anti-goat HRP conjugated (1 : 3000) were purchased from Dako (Ely, UK). For western blotting, individual serum samples were diluted 1 : 10 and 3–7 μl sample analysed, depending on antibody sensitivity. A standard comprising 14 HC samples, pooled together, used at three different dilutions per gel, allowed comparison and quantification across blots. For pancreatic juice, 8 μg protein, plus internal standards were analysed. Protein was separated on 6–15% SDS–polyacrylamide gels, transferred onto nitrocellulose membranes and blocked for 1 h in 5% milk/PBS Tween (PBST). Primary antibodies were 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 either X-ray film or scanned directly using a Kodak Imaging station (Carestream Health, Hemel Hempstead, UK). Densitometry was performed (Kodak MI SE software, Carestream Health), and protein quantities recorded relative to internal standards. All samples were analysed at least in duplicate. ELISA measurement for Reg3A (Pancrepap, Dynabio, Marseille, France) was performed using serum diluted 1 : 100 or 50 ng pancreatic juice protein in a volume of 100 μl.

Data analysis. Statview V.5.01 (SAS Institute Inc., Cary, NC, USA) and Medcalc software (version 11, Mariakerke, Belgium) were used. iTRAQ data were compared using the Mann–Whitney *U*-test. Validation data were analysed using the two-tailed Mann–Whitney *U*-test and diagnostic accuracy compared by receiver operating characteristic analyses.

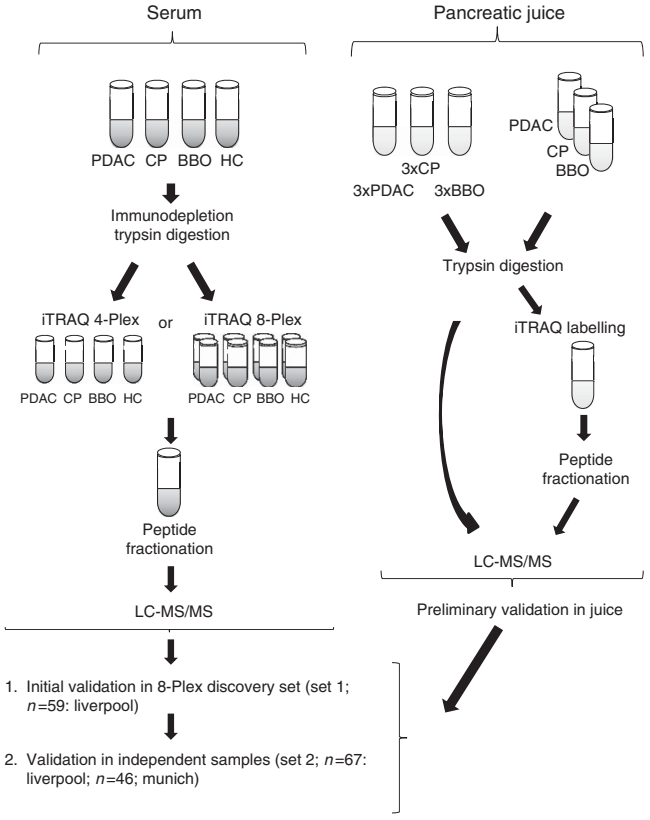

Figure 1. Workflow of serum and pancreatic juice proteomic analysis. Pools of serum for the 4-Plex experiment from PDAC ($n=19$), CP ($n=20$), BBO ($n=8$) or HC ($n=14$), and for the 8-Plex experiment from PDAC ($n=15$), CP ($n=15$), BBO ($n=15$) or HC ($n=14$), were processed similarly and combined after differential isotopic (iTRAQ) labelling. Subsequent protein fractionation involved strong cation-exchange and reversed-phase chromatography. Individual fractions were analysed by LC-MS/MS. Pancreatic juice samples were digested and analysed by LC-MS/MS without iTRAQ labelling (three individual samples per group) or with iTRAQ labelling (pooled samples, PDAC ($n=13$), CP ($n=8$) and BBO ($n=4$)). Selected pancreatic juice proteins underwent preliminary validation in juice. All proteins selected from serum and juice then underwent validation in serum validation sample sets.

RESULTS

Serum iTRAQ analysis: identification of candidate biomarkers. Pooled sera from patients with PDAC, CP, BBO and HCs were compared in a 4-Plex iTRAQ experiment (Figure 1). To maximise the likelihood of identifying proteins in the tissue leakage range, high abundance proteins were depleted before analysis. Comparative protein expression differences between groups were measured by mass spectrometry, relative intensity of reporter ions released from each labelled peptide compared and protein ratios, and *P*-values for proteins calculated. To ensure high quality data, only proteins identified using at least three peptides at 95% confidence were included. Ninety proteins were identified and quantified with a confidence level of 95% and false discovery rate (FDR of 1%). iTRAQ was repeated using an 8-Plex format, which allowed each of the four sample groups to be analysed in duplicate. This led to the identification and quantification of 154 proteins, 88 of which overlapped with the 4-Plex experiment (FDR of 1%; Supplementary Table 1).

Inter-α-trypsin inhibitor heavy chain H3, α1-β glycoprotein (A1BG) and complement C5 alpha chain (C5) were selected for validation. The rationale for this selection included differential expression based on the analysis of iTRAQ data; published literature indicating a promising association with pancreatic cancer (A1BG; Tian *et al*, 2008; Li *et al*, 2009) or lack of published literature, suggesting novel potential candidate biomarkers of pancreatic cancer (ITIH3 and C5).

Validation of candidates in individual serum samples. Initial validation in individual patient sera from the discovery iTRAQ 8-Plex experiment (set 1) revealed that A1BG, C5 and ITIH3 (see Figure 2A for representative western blots) were significantly elevated in PDAC compared with HC ($P<0.001$) and in CP compared with HC ($P<0.01$). Each protein was elevated in patients with BBO (data not shown). Further evaluation in independent individual patient samples (set 2, Figure 2) confirmed the significant elevation of A1BG, C5 and ITIH3 in PDAC, compared with HC (Figure 2B; $P<0.004$) and also showed significant increases in CP compared with HC for C5 and ITIH3 (Figure 2B). Significant elevation in BBO compared with HC was also confirmed ($P<0.001$ A1BG; $P<0.002$ ITIH3; $P<0.04$ C5). The reference marker CA19-9 was significantly elevated in PDAC, CP and BBO compared with HC (Figure 2B).

Pancreatic juice LC-MS-MS and iTRAQ analysis: identification of candidate biomarkers. LC-MS/MS analysis on individual cancer, CP and control (negative for disease following investigation) samples ($n=3$ per group; Supplementary Table 2), led to the identification of a total of 99 proteins with a 1% FDR (average of 28 proteins identified in control; 54 in CP and 41 in PDAC). For quantitative data on proteins in pancreatic juice, iTRAQ was subsequently performed, identifying 108 proteins (Supplementary Table 3), 58 of which had been detected using LC-MS/MS, leaving 50 and 49 detected uniquely by iTRAQ and LC-MS/MS, respectively. Polymeric immunoglobulin receptor and Reg3A were elevated in cancer juice, and were selected for further analysis in both pancreatic juice and serum. Reg3A was included as a reference, since it has been well-characterised in both pancreatic juice and serum in the past (Okamoto, 1999; Rosty *et al*, 2002; Faca *et al*, 2008).

Validation of the potential markers detected in pancreatic juice. Preliminary evaluation in individual pancreatic juice samples showed that PIGR levels were elevated in PDAC compared with controls ($P=0.03$), whereas Reg3A levels were not significantly higher in pancreatic juice from cancer patients compared with benign controls (Supplementary Figures 1A and B).

The levels of PIGR and Reg3A were then analysed in individual patient sera using samples from validation sets 1 and 2 (Figure 2C for box plots; Figure 2A for representative blot for PIGR). The levels of both PIGR and Reg3A were significantly elevated in PDAC, CP and BBO compared with HC ($P<0.001$). The levels of PIGR in the BBO group were significantly higher than those in the PDAC and CP group respectively ($P=0.02$ both cases, Mann–Whitney *U*-test).

Assessment of the influence of biliary obstruction on marker levels in cancer and CP. As PIGR, ITIH3, C5 and A1BG were raised in patients with BBO, we sought to determine the contribution of biliary obstruction to the circulating levels of markers in PDAC patients. For the purposes of this analysis, PDAC patients were divided into those with bilirubin levels $>$ or $\leqslant 20\,\mu mol\,l^{-1}$ (the upper limit of normal at the participating centres). The levels of CA19-9, Reg3A, PIGR, ITIH3, C5 and A1BG were significantly higher in cancer patients with biliary obstruction compared with patients with lower bilirubin levels (Figure 3). The mean levels of all markers in different patient groups are summarised in Table 2.

Elevated marker levels in the presence of jaundice favoured the detection of PDAC over HCs when jaundice was present (compare

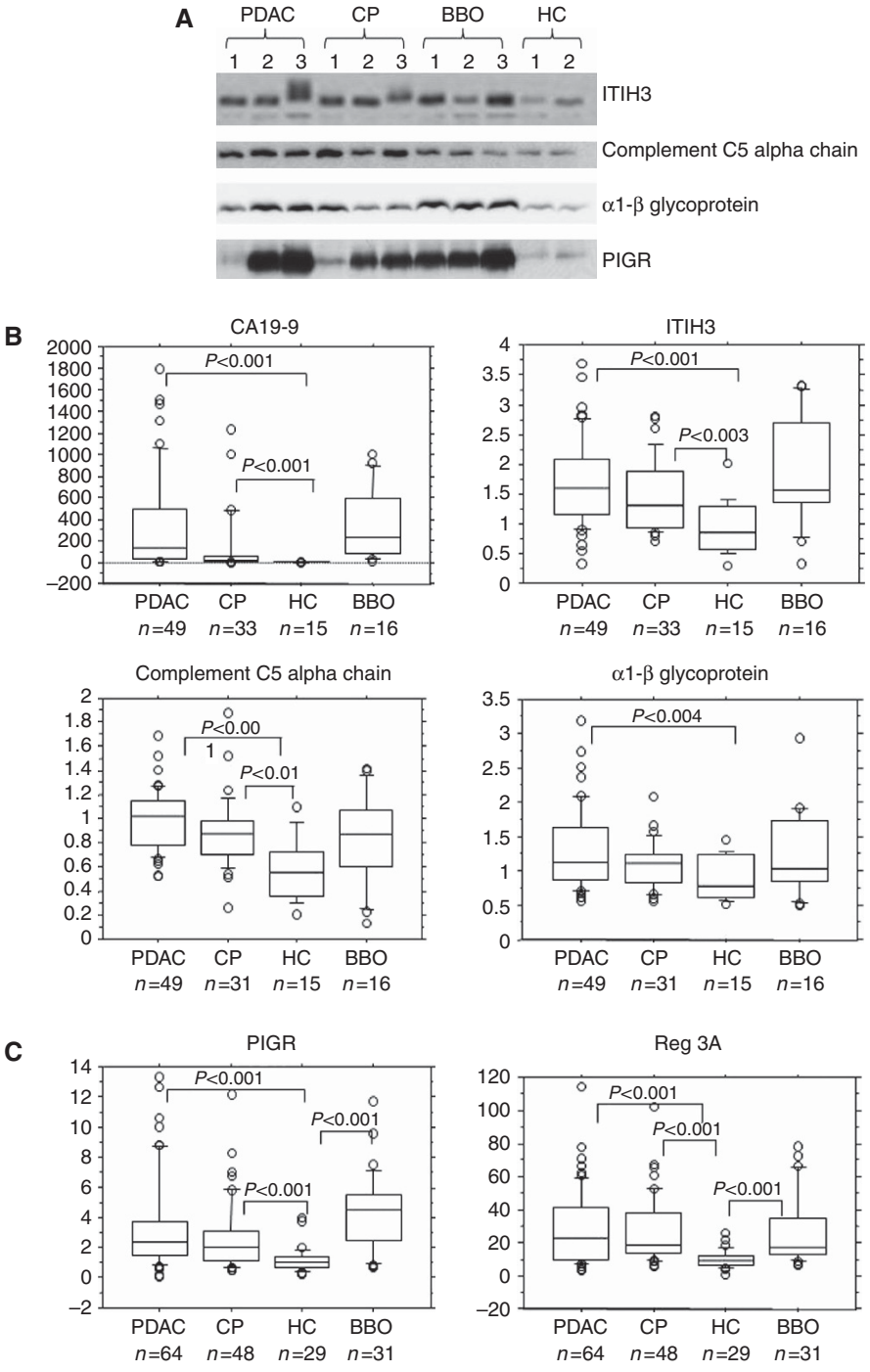

Figure 2. Analysis of the indicated candidates in individual serum samples. (**A**) Representative western blots for proteins validated in serum. Validation was performed by western blotting (A1BG, C5, ITIH3 and PIGR) or ELISA (Reg3A), apart from CA19-9 that was derived from clinical laboratory data. (**B**) *P*-values are shown for comparison (using the Mann–Whitney *U*-test) of the median levels of candidates in indicated groups for the proteins identified in serum iTRAQ or (**C**) pancreatic juice iTRAQ. BBO = benign biliary obstruction; CP = chronic pancreatitis; HC = healthy control; PDAC = pancreatic ductal adenocarcinoma.

Figure 4A with Figure 4B). The sensitivities of individual markers for PDAC over HCs ranged from 74% to 100% when jaundice was present compared with 32% to 82% when it was absent. Combining CA19-9, C5 and A1BG improved the sensitivity for PDAC in the absence of jaundice (SN/SP 86/90%; AUC 0.92; Figure 4B) compared with CA19-9 alone (SN/SP 73/97%; AUC 0.88; Figure 4B).

The consequence of marker elevation in benign disease subjects with jaundice was a reduction in marker specificity for PDAC (specificities of markers ranged from 33% to 73%), compared with specificities for jaundiced PDAC patients over HCs (ranged from 76% to 100%) or over non-jaundiced benign disease subjects (ranged from 49% to 92%). When jaundice was present in both PDAC and benign disease subjects, combining C5, CA19-9 and A1BG significantly improved the specificity for PDAC patients (SN/SP 76/73%; AUC 0.77, Figure 4C) compared with CA19-9 alone (SN/SP 76/51%; AUC 0.66, Figure 4C).

Finally, distinguishing PDAC from the benign condition, CP is vitally important. We compared the performance of markers to

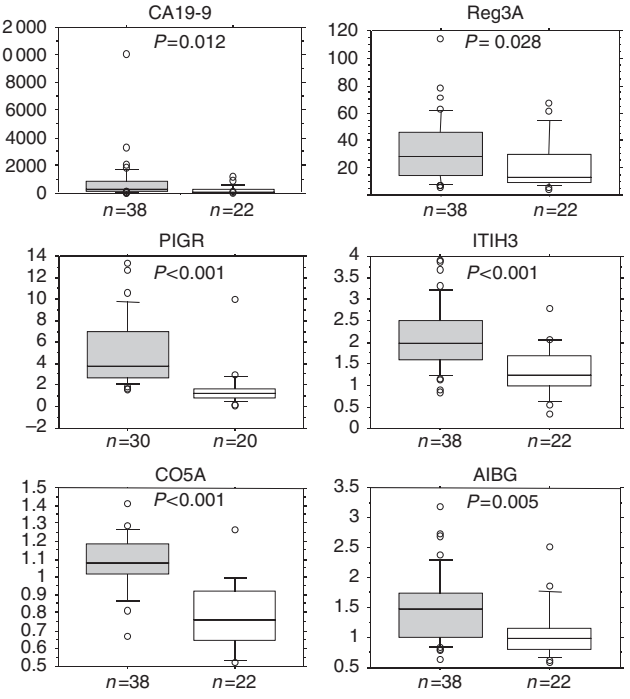

Figure 3. Influence of biliary obstruction on candidate markers. The levels of indicated candidate markers in PDAC patients were separated for patients with bilirubin levels $>20\,\mu mol\,l^{-1}$ (grey box) or $<20\,\mu mol\,l^{-1}$ (white box).

distinguish PDAC in the presence or absence of jaundice, over CP in the absence of jaundice (eight CP patients with bilirubin levels of $>20\,\mu mol\,l^{-1}$ were excluded from this analysis). Markers were better at distinguishing PDAC from CP when PDAC patients had jaundice (compare Figures 4D and E). Markers had higher sensitivity for PDAC in the presence of jaundice (e.g., SN/SP of 76%/90% for CA19-9) than in the absence of jaundice (SN/SP of 50%/90% for CA19-9). Combining CA19-9, Reg3A and PIGR improved the sensitivity for non-jaundiced PDAC patients over non-jaundiced CP patients compared with CA19-9 (50% to 63%), however, a loss in specificity resulted (90% to 74%).

DISCUSSION

In this study, we found that iTRAQ provided significant leads on potential biomarkers for pancreatic cancer detection, including the novel candidates C5 and ITIH3. Moreover, we demonstrated remarkable variability of biomarker performance depending on the presence or absence of biliary obstruction.

The presence of jaundice is known to elevate serum levels of CA19-9 (Locker *et al*, 2006; Duraker *et al*, 2007; Smith *et al*, 2008; Marrelli *et al*, 2009; Singh *et al*, 2011) and this study has demonstrated a similar effect on a range of potential biomarkers with differing biochemical characteristics, including C5, ITIH3, A1BG, PIGR and Reg3A. Candidate biomarkers had highest sensitivity for PDAC over HCs or CP when jaundice was present in PDAC patients. On the other hand, when jaundice was present in benign disease subjects, the specificity of markers for PDAC was adversely affected. Our findings, therefore, have genuine significance for the translation of biomarkers into clinical use, and highlight the importance of calibrating markers according to the requirements of individual clinical scenarios. Currently, several experimental approaches are being applied to the derivation of markers or marker panels for pancreatic cancer but without consideration of the potential confounding factor of the presence

Table 2. Marker levels in combined validation sets 1 and 2

	Disease group								Mann–Whitney *U*-test *P*-value							
	PDAC low bil ($n=22$)		PDAC high bil ($n=38$)		BBO ($n=31$)		CP ($n=48$)		HC ($n=29$)		HC		CP		BBO	
Protein	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	*vs* PDAC low bil	*vs* PDAC high bil	*vs* PDAC low bil	*vs* PDAC high bil	*vs* PDAC low bil	*vs* PDAChigh bil
CA19-9	201.60	289.54	819.45	1699.22	271.22	361.02	96.36	238.71	6.21	4.84	<0.001	<0.001	0.03	<0.001	0.38	0.04
A1BG	1.08	0.46	1.46	0.59	1.17	0.55	1.11	0.34	0.79	0.29	0.008	<0.001	0.36	0.004	0.38	0.04
C5A	0.86	0.22	1.10	0.21	0.89	0.32	0.91	0.27	0.67	0.26	0.004	<0.001	0.57	<0.001	0.57	0.002
ITIH3	1.34	0.59	2.11	0.76	2.04	0.96	1.61	0.68	1.00	0.51	0.018	<0.001	0.18	0.002	0.008	0.68
PIGR	1.72	1.99	4.43	3.12	4.32	2.58	2.64	2.31	1.16	0.86	0.10	<0.001	0.013	<0.001	<0.001	0.67
Reg3A	21.45	18.81	33.47	23.34	28.08	21.96	27.15	20.12	9.96	5.43	0.014	<0.001	0.07	0.19	0.09	0.27

Abbreviations: A1BG = α1-β glycoprotein; BBO = benign biliary obstruction; bil = bilirubin; CP = chronic pancreatitis; HC = healthy control; ITIH3 = inter-α-trypsin inhibitor heavy chain H3; PDAC = pancreatic ductal adenocarcinoma; PIGR = polymeric immunoglobulin receptor.

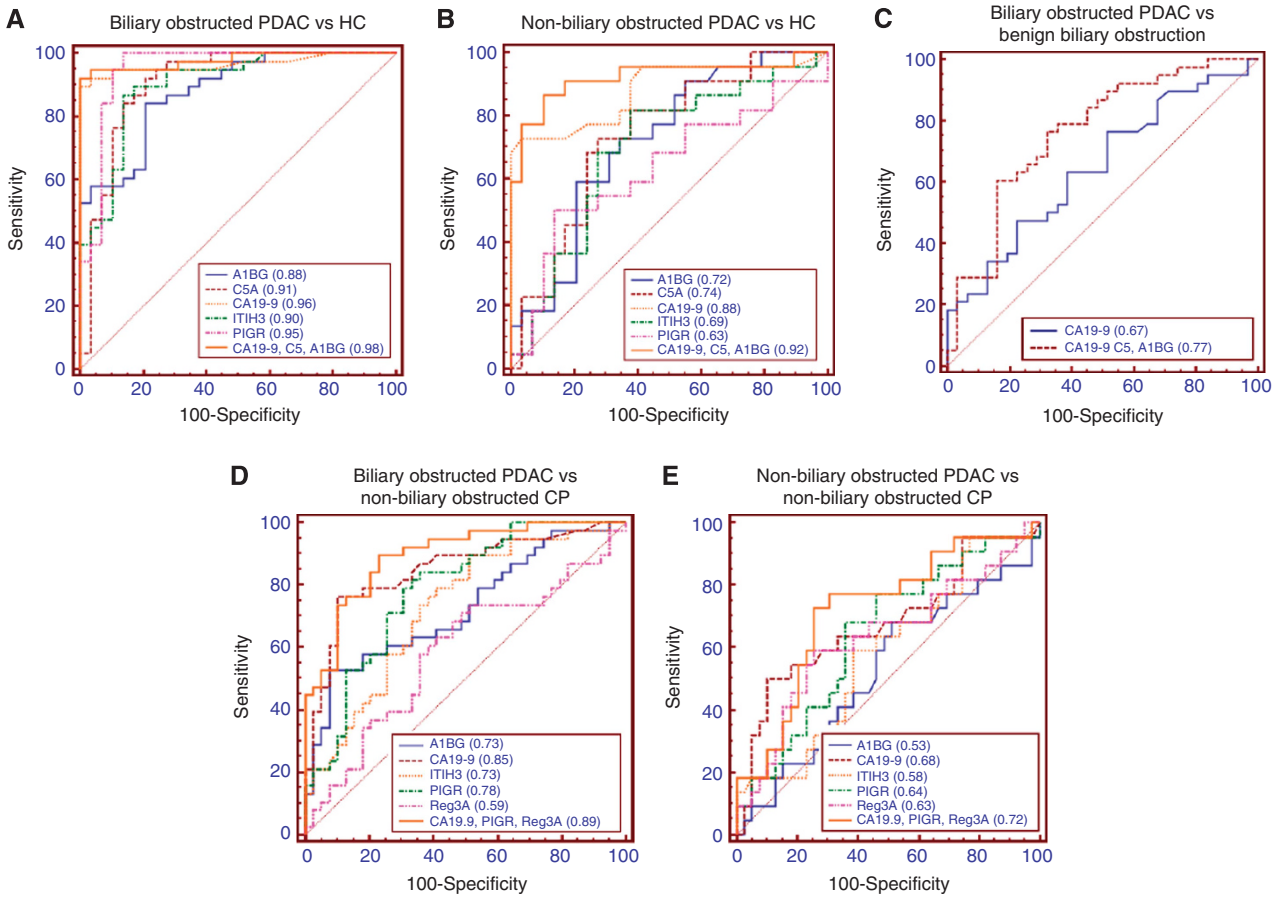

Figure 4. ROC curves comparing markers in biliary obstructed and non-obstructed patient groups. Markers A1BG, C5a, CA19-9, ITIH3, REG3A and PIGR, were examined singly or combined into optimum panels and ROC curves generated to compare the performance of individual markers and panels. (**A**) 44 PDAC, 29HC; AUC for Reg3A = 0.86; (**B**) 22 PDAC, 29 HC; AUC for Reg3A = 0.7; (**C**) 38 PDAC and 37 controls, including 31 BBO and 6 CP; (**D**) 38 PDAC and 39 CP; AUC for C5a = 0.76; (**E**) 22 PDAC and 39 CP; AUC for C5a = 0.52.

of obstructive jaundice (Zhao *et al*, 2007; Faca *et al*, 2008; Brand *et al*, 2011; Pan *et al*, 2011). Given the large proportion of PDAC patients presenting with jaundice, it is necessary to develop and understand the performance of biomarkers in this context. Conversely, studies aimed at the discovery and validation of early PDAC biomarkers may be more likely to find markers relevant to early detection if they focus on samples from patients devoid of jaundice. As obstruction is considered a late event in the disease, markers that are predominantly elevated because of jaundice are not likely to be useful for early disease detection.

In addition to the reference proteins, CA19–9 and Reg3A, our study focused on four serum proteins. Complement C5 has not previously been characterised as a marker of pancreatic cancer. Here, C5 was the best performing marker at distinguishing PDAC in the presence of jaundice from patients with BBO. Inter-α-trypsin inhibitor heavy chain H3 belongs to the inter-α trypsin inhibitor (ITI) family of serine protease inhibitors that occur at relatively high concentrations in blood. ITIH proteins stabilise extracellular matrix by covalently binding to hyaluronic acid, a major component of extracellular matrix (Zhuo *et al*, 2004) and increased plasma levels of ITIH3 have also been reported in gastric cancer (Chong *et al*, 2010). α1-β Glycoprotein was shown previously to be upregulated in pancreatic juice from patients with pancreatic cancer (Tian *et al*, 2008) and its glycosylation pattern altered in serum (Li *et al*, 2009). Polymeric immunoglobulin receptor was recently reported in pancreatic juice, with increased levels in cancer patients compared with HCs (Makawita *et al*, 2011). Our study shows that it has excellent discrimination in the context of patients in the presence of jaundice (AUC 0.95), but much less in patients without biliary obstruction (AUC 0.63).

Combining makers into panels with CA19-9 improved on the discrimination provided by CA19-9 alone in different clinical scenarios: (1) for pancreatic cancer patients without jaundice compared with healthy subjects; and (2) for pancreatic cancer patients with jaundice *vs* benign disease subjects with jaundice. Discriminating pancreatic cancer from CP patients remains a real challenge, whether jaundice is present or not. Although, a panel of CA19-9, Reg3A and PIGR resulted in improved sensitivity for non-jaundiced PDAC patients over non-jaundiced CP, this was at the expense of specificity. All of the marker panels in this study merit validation in independent, larger patient cohorts.

The limitations of this study include the use of pooled samples for discovery and the relatively small sample sizes. Although sample pooling was undertaken for reasons of quantity of sample required and cost, it is not ideal as outlier samples could mask other samples. Nonetheless, all of the markers were validated in individual, independent samples, so the data presented are robust. The samples used in the discovery phase of this project were uniquely from male subjects. Although this minimised potential variation because of gender, it also means that candidate proteins, elevated in females only, will have been overlooked. On validation, we observed no gender differences in protein levels.

Further advances in proteomic technologies, enabling greater proteome coverage, combined with analysis of carefully selected samples, should allow the identification and development of

cancer-specific biomarkers for the improved detection of pancreatic cancer.

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