

Serum apolipoprotein C-II is prognostic for survival after pancreatic resection for adenocarcinoma

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BACKGROUND: Pancreaticoduodenectomy remains a major undertaking. A preoperative blood test, which could confidently predict the benefits of surgery would improve the selection of pancreatic cancer patients for surgery. This study aimed to identify protein biomarkers prognostic for long-term survival and to validate them with clinico-pathological information.

METHODS: Serum from 40 preoperative patients was used to train for predictive biomarkers using surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI), and the results were verified on 21 independent samples. Two predictive proteins were identified by tryptic peptide mass fingerprinting and sequencing, and validated on serum from another 57 patients by enzyme-linked immunosorbent assay (ELISA). The influence of these proteins on growth and invasion of two cancer cell lines was tested *in-vitro*.

RESULTS: The SELDI panel of *m/z* 3700, 8222 and 11 522 peaks predicted <12 months' survival (ROC AUC: 0.79, 0.64–0.90; $P < 0.039$). When CA19-9 was added, the ROC AUC increased to 0.95 (0.84–0.99; $P < 0.0001$). The six subjects in the verification group who died within 12 months were correctly classified. The *m/z* 8222 and 11 522 proteins were identified as Serum ApoC-II and SAA-I, respectively. In the validation samples, ELISA results confirmed that ApoC-II was predictive of survival (Kaplan–Meier $P < 0.009$), but not SAA-I. ApoC-II, CA19-9 and major-vessel involvement independently predicted survival. ApoC-II and SAA-I increased cell growth and invasion of both cancer cell lines.

CONCLUSION: Serum ApoC-II, CA19-9 and major-vessel invasion independently predict survival and improves selection of patients for pancreaticoduodenectomy.

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Surgical resection and adjuvant chemotherapy for pancreatic cancer provide the best chance of long-term survival (Castellanos *et al*, 2011). The outcome appears to be independent of pylorus preservation (Akizuki *et al*, 2008), but may be improved by more extensive resection of mesopancreatic tissue (Samra *et al*, 2006) and vascular resection (Tang *et al*, 2011). Despite the introduction of these improvements over the last decade, one-fifth of patients do not survive 12 months; an unsatisfactory result, given that the survival time includes prolonged recovery from major surgery and that palliative treatments may have achieved the same outcome (Distler *et al*, 2010). When faced with the decision to undergo surgery, it is important for patients to be given an accurate prognosis, particularly regarding the chance of long-term survival.

Prognosis generally relies on established pathological classification such as the TNM staging of malignant tumours (TNM) (American Joint Committee on Cancer, 2009). To further improve the prognosis, the expression of a range of tissue proteins has been reported to have prognostic value in cohorts of patients undergoing pancreatic resection, but none has been studied in a prospective randomised manner (Jamieson *et al*, 2011), and their usefulness in the clinic remains unproven. Plasma biomarkers are

more available in the preoperative setting, with Carbohydrate Antigen 19-9 (CA 19-9) being the most commonly used for diagnosis and for prognosis in clinical practice. However, although reviews have supported the prognostic use of CA 19-9 (Mehta *et al*, 2010), others have reported it to be unreliable (Kondo *et al*, 2010). An elevated preoperative value of CA 19-9 was found not to be prognostic of survival, while its continual elevation in the postoperative setting was a significant indicator of poor outcome (Hata *et al*, 2012). Carbohydrate Antigen 19-9 is frequently elevated in benign conditions and more so in the jaundiced patient (Molina *et al*, 2012). Other serum markers of the host's response to cancer such as human C-reactive protein (CRP) (Pine *et al*, 2009), serum amyloid A-1 (SAA-1) (Firpo *et al*, 2009) and the neutrophil to lymphocyte ratio (Bhatti *et al*, 2010) are also reported to predict survival.

Such studies justify an exploratory examination of serum in an attempt to improve prediction of outcome, particularly in the era of extensive surgery and neoadjuvant therapy. One potential source of such biomarkers is the pool of low-abundance small proteins in serum. Surface-Enhanced Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (SELDI) was used to discover diagnostic protein biomarkers, including ApoA-II and ApoC-I (Xue *et al*, 2010), which were not previously recognised. Further, the SELDI technique has also revealed biomarkers of pancreatic cancer-related outcomes such as invasiveness (Valkovskaya *et al*,

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2007), responsiveness to gemcitabine (Cao *et al*, 2010) and cachexia (Felix *et al*, 2011). It is therefore possible that SELDI could identify prognostic markers of long-term survival in the serum.

This study uses the SELDI platform to discover markers of prognosis in a training set of serum samples, and verifies the markers by SELDI in an independent set of serum samples. The prognostic capability of these proteins is then assessed using ELISA, along with other predictors of survival, in a further independent validation set of serum samples.

MATERIALS AND METHODS

Patients and sera

The study was approved by the Northern Sydney Health Human Research Ethics Committee, Sydney, Australia (HREC 909 228M) and the University of Verona, Ethics Committee, Verona, Italy.

The training set Sera from 40 consecutive patients about to undergo pancreaticoduodenectomy for pancreatic ductal carcinoma (PDCA) at the University of Verona (UniVer) from 2006–2007 were prospectively collected and stored at -80°C as the 'training set' until analysed by SELDI.

The verification set Sera from 21 consecutive patients (the 'verification set'), collected before pancreatic resection at Royal North Shore Hospital (RNSH) in 2007 were analysed by SELDI.

The validation set Further 82 serum samples from subjects at RNSH from 2009 to 2010, consisting of sera from 57 PDCA patients before pancreatic resection, 13 with unresectable (advanced) pancreatic cancer (APDCA) and 12 healthy volunteers (HV), which were controls for the analysis technique were collected prospectively as a 'validation set'. Clinical and pathological data for the validation set were prospectively recorded in a Microsoft Access database. The analysis of the data was delayed until 12 months after surgery. Two 'validation set' patients were lost to follow-up at 4 and 10 months, and their data were censored at the last review day, when they were alive. Tumours were synoptically classified according to defined pathological prognostic criteria by one of the authors, AG, as previously described (Gill *et al*, 2009). Carbohydrate Antigen 19-9 and CRP were measured by enzyme-linked immunosorbent assay (ELISA) and the neutrophil-lymphocyte ratio was recorded for the validation-set patients.

Preparation of serum and proteinchip arrays for SELDI-TOF MS

Serum was separated into aliquots 30 min after collection and stored at -80°C . From each sample, 20 μl was diluted 1:1 with denaturing buffer (8M urea per 1% CHAPS), then centrifuged at 12 000 rpm for 5 min. Protein levels were measured by Micro BCH protein assay kits (Thermo Scientific, Chicago, IL, USA), and the amount of buffer added to the samples adjusted to provide 2 μg per spot. The supernatant was diluted to 1:25 with 10% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA). Treated samples were applied to hydrophobic (H50) proteinchip arrays and processed, as previously described (Xue *et al*, 2010). The proteinchip arrays were analysed using the Bio-Rad Enterprise SELDI System (Bio-Rad, Hercules, CA, USA).

SELDI-TOF MS Analysis

Duplicate spectra were obtained in the mass/charge (m/z) range 2500–75 000 with a laser intensity set at 220 (arbitrary units), detector sensitivity at 8 and a signal to noise ratio of 5%. The m/z value for each peak was determined using external calibration with

standards (Sigma–Aldrich, St Louis, MO, USA) which are the following: bovine insulin (5734.51 + 1H), equine cytochrome *c* (12 361.96 + 1H), equine apomyoglobin (16 952.27 + 1H) and rabbit muscle aldolase (39 212.28 + 1H). Spectra were analysed using ProteinChip Software, Version 3.1 (Bio-Rad).

Protein peak identification

Serum samples were separated into six fractions eluted at pH 9, 7, 5, 4, 3 and the organic phase using the ProteinChip Serum Fractionation Kit (Bio-Rad). The fractions of interest were immediately applied to a C18 reverse-phase HPLC column eluted with a gradient of 15–60% ACN in 0.1% TFA. Eluted fractions containing the protein peaks of interest were lyophilised and then subjected to 1D SDS-PAGE, staining with Coomassie Blue to visualise the proteins. The bands containing the putative proteins of interest were cut and sent to the Australian Proteome Analysis Facility (APAF, Macquarie University, Sydney, Australia) who used nano-LC-ESI MS/MS (nano-liquid chromatography electro-spray ionisation tandem MS) for protein identification by MS sequencing. Peak lists were generated using MASCOT script in Analyst 2.0 (AB Sciex, Framingham, MA, USA). The MS/MS spectra with >10 peaks were identified using a centroid identification protocol, de-isotoped, and the peak areas >1% maximum reported. The search parameters used by MASCOT were: database: SwissProt 55.2 (362782 sequences; 130497792 residues); taxonomy: *homo sapiens* (human) (19117 sequences); type of search: MS/MS ion search; enzyme: trypsin; mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: ± 300 ppm; fragment mass tolerance: ± 0.6 Da; maximum missed cleavages: 1; criteria for acceptance: MudPIT scoring, significant threshold $P < 0.05$, peptide ion score >25 and bold red only.

Protein identities were confirmed by a SELDI immunoadsorption approach. An ApoC-II antibody (AF-4497, R&D System, McKinley, Minneapolis, MN, USA) and a SAA-1 antibody (sc-59679, Santa Cruz Biotechnology, Santa Cruz, CA, USA), both diluted 1:2000, were separately conjugated to RS100 proteinchips and immunoreactive proteins were allowed to bind, then the chips were washed and analysed by SELDI.

Measurement of serum levels of CA19-9, SAA-1 and ApoC-II by ELISA

The levels of CA 19-9, ApoC-II and SAA-1 in serum were measured using a CA 19-9 ELISA Kit (Alpha Diagnostic International, San Antonio, TX, USA), AssayMax ApoC-II ELISA kit (AssayPro, St Charles, MO, USA) and Human SAA Assay Kit (Invitrogen, Carlsbad, CA, USA), respectively. Sample dilution was fivefold for CA 19-9 and 200-fold for ApoC-II and SAA-1. The absorbance values for the three analytes were measured at 450 nm using a Microplate Reader (Tecan, Salzburg, Austria) within 10 min. Duplicate samples were measured blind in a random order with controls and the average coefficient of variation was 3.7%.

Pancreatic cancer cell lines and cell proliferation assay

Two human pancreatic cancer cell lines, CFPAC-1 (ATCC CRL-1918; ATCC, Manassas, VA, USA) and PANC-1 (ATCC CRL-1469) were kindly provided by Professor Barry Allen (St George Hospital, Sydney, Australia). CFPAC-1 cells were cultured in RPMI 1640 containing 10% foetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA). PANC-1 cells were cultured in Dubecco's Modified Eagle's Medium (DMEM) with 10% FBS. Cells were grown in a humidified (37°C , 5% CO_2) incubator and passaged upon reaching 80% confluence at a ratio of 1:3. The effect of the candidate prognostic markers on cell growth was determined in CFPAC-1 and PANC-1 cells by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT,

Table 1 Demographic and pathological details of the study groups

Sex (M:F)	Patient groups					
	Training set 23:17		Verification set 12:9		Validation set 33:24	
	Mean	Range	Mean	Range	Mean	Range
Age (years)	60.5	57.7–70.2	70.9	28–80	67	61–75
Bilirubin ($\mu\text{mol l}^{-1}$)	66	16–243	60	9–189	24**	9–41
ALP (IU l^{-1})	194	93–361.5	193	69–433	170	66–524
AST (IU l^{-1})	82	30–135	66	43–133	63	12–400
ALT (IU l^{-1})	110	25–248	87	35–218	76	8–622
Albumin (g l^{-1})			36.5	33–41	31	23–38
CA 19-9 (U ml^{-1})	128	28–703	130	147–229	96	31–266
Number of Whipples procedures	32		21		57	
Total pancreatectomy	8					
Portal vein resection			3		22	
Added arterial resection					4	
Adjuvant chemotherapy	29		4		42	
R0	24		7		25	
R1	11		10		31	
R2	5		4		1	
Stage I–II:III	25:15		13:8		41:16	
Grade I–II:III	25:15		16:5		39:18	
T 1, 2, 3, 4	4, 21, 11, 4		5, 10, 6, 1		5, 6, 40, 6	
N 1, 2, 3	24, 12, 4		10, 11		19, 10, 28	
Median survival (<i>m</i>)	15	11–22	17.3	11–23	14	10–18

Abbreviations: ALP = serum alkaline phosphatase; ALT = alanine amino transferase; AST = aspartate amino transferase; CA 19-9 = Carbohydrate Antigen 19-9. Geometric mean values with the inner quartile range; Bilirubin (serum bilirubin), ALP, AST, ALT, CA 19-9. Resection clearance R0, R1, R2 indicate clear margins > 1 mm, cancer within 1 mm of the margin and macroscopic tumour at to the margins. Otherwise values are number of cases. Stage I and II indicate the tumour is confined to the pancreas with or without early nodal spread. Stage III indicates the tumour has reached the margin of resection or that there is more extensive nodal spread. Grade I, II and III indicates well, moderate and poor differentiation. ** indicates geometric mean was different from both training and verification sets values by analysis of variance with LSD *post hoc* analysis, $P < 0.005$.

Sigma–Aldrich) assay method described previously (Xue et al, 2009).

Invasion assay

Cell invasion was studied using a modification of the Cytoselect 24-well Cell Invasion Assay (Cell Biolabs, San Diego, CA, USA). Briefly, $0.5\text{--}1.0 \times 10^6$ cells ml^{-1} were seeded onto the insert chamber of the plate in medium containing 1% FBS, while medium containing 10% FBS was added to the lower chamber of the invasion plate. The plate was incubated at room temperature for 1 h. Purified human plasma-derived ApoC-II (Sigma–Aldrich) or recombinant human SAA-1 (PeproTech, Rocky Hill, NJ, USA) were added to the inner chamber. The plate was incubated for 24–48 h at 37°C in 5% CO_2 . The non-invading cells were removed and invading cells stained using the cell-stain solution and counted with a light microscope under $\times 10$ magnification, with at least three individual fields counted per insert.

Statistics and bioinformatics

For SELDI analysis, spectra were normalised to the total ion current between 2500 and 75 000 m/z and peaks were detected using the biomarker wizard utility (Bio-Rad). In the training set, univariate analysis by Mann–Whitney test at $P < 0.05$ was used to distinguish patients who survived more or < 12 m. Six-fold crossover validation and logistic regression were used to predict > 12-m survival after surgery. The discriminatory power for each marker was characterised by receiver operating characteristics (ROC) area under the curve (AUC) analysis, and the AUCs were compared using the DeLong test (MedCalc Version 11.6.1.0, Miriakerke, Belgium).

ELISA measurements of protein concentrations were normalised by logarithmic transformation for analysis by analysis of variance

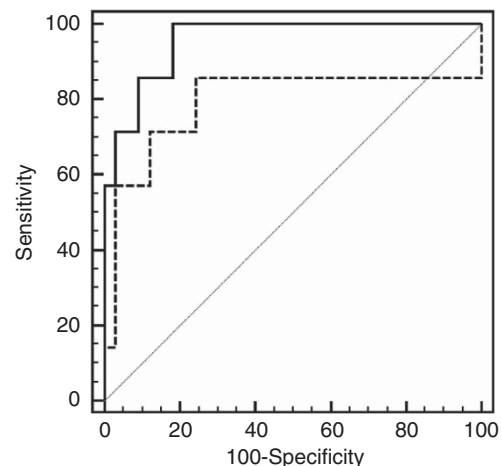


Figure 1 ROC curves for training samples. Dashed line indicates the three-member panel of m/z 3700, 8222 and 11522 with an AUC of 0.79 (s.e., 0.13). Solid line indicates the panel, including CA 19-9 with AUC of 0.97 (s.e., 0.03). These lines were not significantly different ($P = 0.24$, DeLong test).

(ANOVA) and survival analysis and the geometric means presented. *Post hoc* analysis was by the Fishers least-significance difference (LSD) method. To determine the influence of ApoC-II and SAA-1 on survival in Kaplan–Meier analysis of the validation-set samples, the cutoff values, which demonstrated the most significant difference between groups were selected. Co-correlation of protein indices with clinical and pathological indices was assessed by Spearman’s test. Univariate and multivariate effects on

survival were tested using univariate Cox proportional hazard regression analysis and the five most significant independent variables were analysed by backward multivariate regression.

RESULTS

Characteristics of patients in the three sets

The three patient sets were similar with respect to age, sex, serum and liver enzyme levels with the exception of serum bilirubin,

which was lower in the validation set, probably because of the wider use of stenting in these patients (Table 1). In the 40 training-set patients, the surgical procedures undertaken were the following: pyloric preservation resection in 28, total pancreatectomy in eight and standard pancreaticoduodenectomy in four. Twenty-nine received adjuvant chemotherapy, six received chemoradiotherapy and five had no adjuvant therapy. Eight patients died from cancer progression within 12 months of surgery. Median survival was 15 months (IQR 11–22 months).

Of the verification-set patients, twenty-one underwent a pancreaticoduodenectomy. Six died from cancer progression

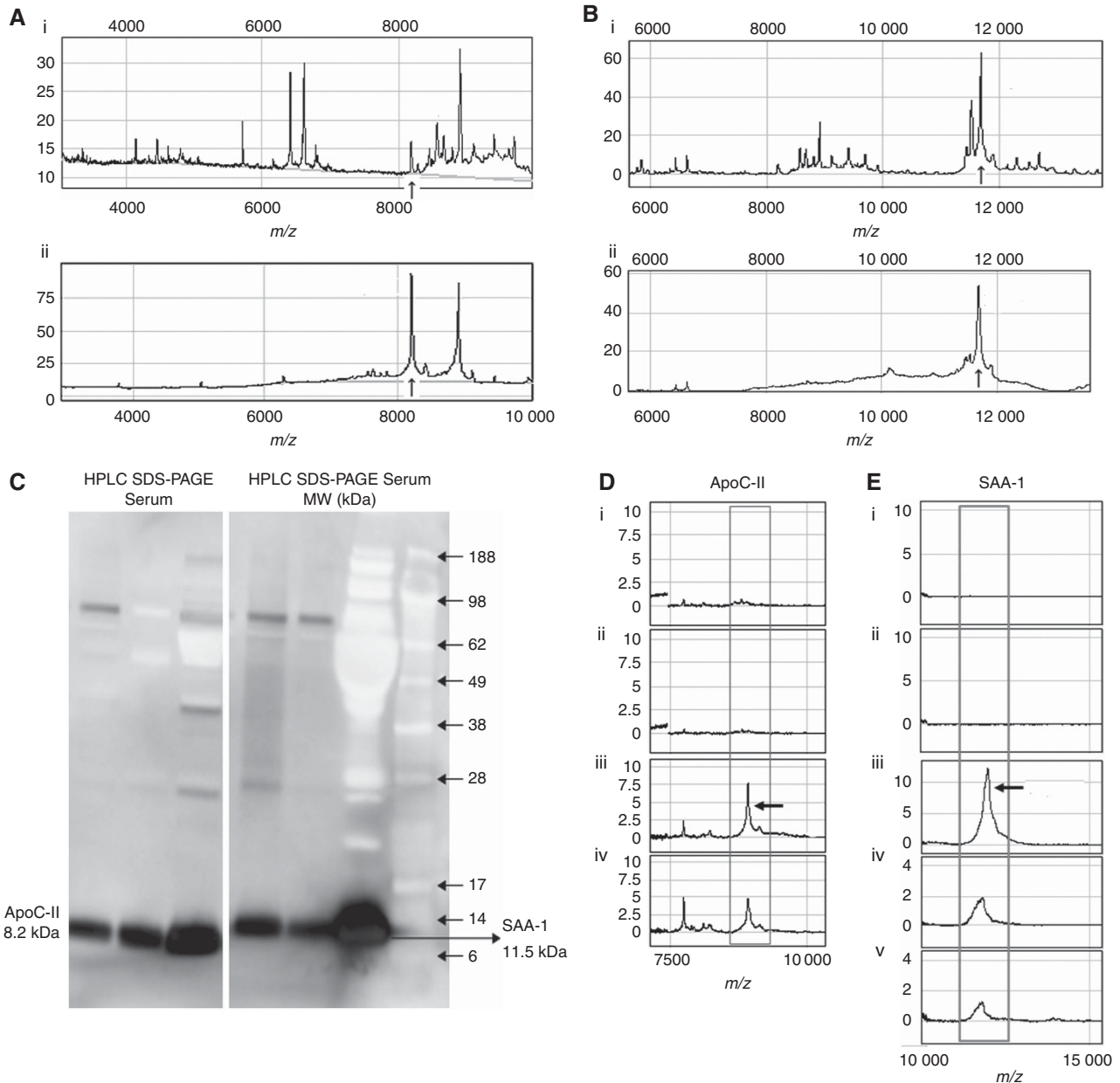


Figure 2 Purification and identification of the proteins corresponding to m/z 8222 and 11522 peaks. **(Ai)** Initial SELDI profile from serum; **(Aii)** semi-purified peak after HPLC fractionation showing the m/z 8222 peak (arrowed) selected for sequencing. **(Bi)** Initial SELDI for serum; **(Bii)** semi-purified sample for the m/z 11522 peak (arrowed). **(C)** Western-blot lanes are labeled: serum (crude serum), HPLC (the HPLC fraction containing the peaks of interest), SDS-PAGE (the protein extracted from ID-SDS-PAGE gel bands, which were subjected to protein sequencing and molecular weight (MW) (the protein standards, which are presented in kDa) for ApoC-II in the panel on the left and SAA-1 in the panel on the right). **(D)** Confirmation of m/z 8222 peak identity using SELDI immuno-adsorption on RS100 chips containing ApoC-II antibody. **(Di)** Affinity-purified IgG negative control; **(Dii)** unprocessed serum without antibody; **(Diii)** unprocessed serum with antibody; **(Div)** antibody with semi-purified ApoC-II from patient serum. **(E)** Confirmation of m/z 11522 peak identity using SAA-1 antibody. **(Ei)** Affinity-purified IgG (negative control); **(Eii)** unprocessed serum without antibody; **(Eiii)** recombinant human SAA-1 (positive control); **(Eiv)** unprocessed serum with antibody; and **(Ev)** semi-purified SAA-1 from patient serum after HPLC purification with antibody.

within 12 months of surgery, whereas the median survival was 17.3 months (IQR 11–23 months).

Of the validation-set patients, fifty-seven underwent a pancreaticoduodenectomy with 22 requiring vascular resection, indicating that they had more advanced tumours. Twelve died within 12 months of surgery because of recurrence, with a median of 14.3 months (IQR 10.3–17.6 months).

SELDI-TOF MS derivation of a prognostic panel

Analysis of the training set of serum samples by SELDI-TOF identified 59 protein peaks with intensities at least 10-fold greater than baseline. Logistic regression and sixfold crossover validation analysis demonstrated that 18 peaks ($m/z = 3208, 3432, 3476, 3700, 3879, 4398, 4461, 5051, 5367, 5785, 7333, 8222, 9422, 11522, 12862, 13889, 16963, 17367$) along with CA 19-9 were possibly predictive of survival of <1 year. Seven of these peaks and CA19-9 were chosen on two or more occasions in the sixfold cross-validation analysis. Three of these peaks, $m/z 3700, 8222, 11522$, were independent of each other by Spearman's correlation, and AUC for the combination of these peaks to predict 1-year survival was 0.79 (CI, 0.64–0.90). The mean value was greater in those surviving <1 year, but this was only significant for the $m/z 8222$ peak ($P = 0.034$). When CA 19-9 was included in the panel ($m/z 3700, 8222, 11522 + CA 19-9$), survival of <1 year was predicted by ROC analysis with AUC = 0.957 (CI, 0.84–0.996; Figure 1). Two patients who were predicted to live >12 months died at 336 and 364 days after surgery. One patient predicted to live <12 months lived for 972 days. The sensitivity of this panel was 0.86 and specificity was 0.82 at the strongest criterion value. When this equation was tested on the SELDI results from the verification set, the six patients predicted to have a poor prognosis died within the first 12 months after surgery, whereas 10 of 15 patients were correctly predicted to live >12 months. The other five died at 21, 26, 45, 50 and 51 weeks after surgery. Thus, although the sensitivity was only 0.55, the specificity was 1.0 and the post-test probability of outcome, given a positive test, was also 1.0.

Identification of the $m/z 8222$ protein as ApoC-II and the $m/z 11522$ protein as SAA-1

Figure 2 illustrates the steps involved in identification of the two proteins $m/z 8222$ and 11522 after the ion-exchange fractionation step. The data obtained by LC-ESI/MS/MS showed five major tryptic fragments from the protein peak at $m/z 8222$, two of them overlapping. MASCOT analysis revealed that the biomarker at $8222 m/z$ corresponded to ApoC-II with sequence coverage of 82.6%. Two major tryptic fragments were obtained from the protein peak at $m/z 11522$, which was identified as SAA-1 with sequence coverage of 47.2%. The intensity of the protein peak at $m/z 3700$ was too low for identification.

SELDI immuno-adsorption and western-blotting validation

Western-blotting analysis was performed for ApoC-II ($m/z 8222$ peak) comparing crude serum samples, HPLC-purified serum and the excised SDS-PAGE band. A similar comparison was performed for SAA-1 ($m/z 11522$ peak). The results showed that the anti-ApoC-II and anti-SAA-1 antibodies recognised the respective target proteins in purification fractions and SDS-PAGE gel-bands excised for MS/MS analysis. The peaks migrated in a similar manner to the originally targeted proteins (Figure 2C). Other less intense peaks were also seen in the 26 and 90 kDa region, but these were considered to be owing to the polyclonal nature of the antibodies used, although they were purified to be >90% specific for the protein of interest.

Further immunological confirmation that the protein peaks were ApoC-II and SAA-1 was undertaken with SELDI immuno-adsorption using commercial ApoC-II and SAA-1 antibodies covalently bound to RS100 chips (Figure 2D and E). Here the $m/z 8222$ and 11522 proteins bound to ApoC-II and SAA-1 antibodies, respectively, with minimal non-specific binding to IgG control. Further, ApoC-II antibody binds to the $m/z 8222$ peak in unprocessed serum (Figure 2D(iii)) and HPLC-purified serum (Figure 2D(iv)). The SAA-1 antibody bound to the $m/z 11522$ peak with a recombinant human SAA-1 sample (Figure 2E(iii)) (positive control), with unprocessed serum (Figure 2E(iv)) and with semi-purified SAA-1 (Figure 2E(v)).

ELISA study on validation-set samples

ELISA serum measurements (Table 2) demonstrated that ApoC-II (analysed as geometric means) were lower in resectable cases than in advanced cases (APDCa) ($P < 0.001$) with about half of the values being $< 10 \mu\text{g ml}^{-1}$, the lowest value for HV cases (Figure 3). Serum SAA-1 levels were higher in PDCa serum than APDCa ($P = 0.028$) or HV samples ($P = 0.003$) but, CRP was not different among the groups. Kaplan–Meier survival analysis demonstrated that ApoC-II at the cut-off values of $10 \mu\text{g ml}^{-1}$ significantly influenced survival (Figure 4, A, $P = 0.009$), whereas SAA-1 and CRP were not predictive of survival (data not shown). CA 19-9 at a cut-off of 200 IU l^{-1} (Figure 4B, $P = 0.009$) and the pathological finding of large-vessel invasion (Figure 4C, $P = 0.025$)

Table 2 Measurement of SAA, ApoC-II, CRP and CA19.9 by ELISA in validation set

	N	SAA (ng ml^{-1})		ApoC-II ($\mu\text{g ml}^{-1}$)		CRP (ng ml^{-1})		CA19.9 U l^{-1}	
		Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
PDCa	57	104.0	3.9	6.2	2.5	3.7	4.1	105	4.1
APCA	13	32.9	3.8	15.3	3.7	3.4	4.3	267	2.1
HV	12	9.9	2.0	13.7	1.6	2.4	4.9	—	—
<i>P</i> by ANOVA		0.000		0.001		n.s.		n.s.	
<i>P</i> for PDC vs APCA		0.013		0.007					
<i>P</i> for PDC vs HV		0.000		0.027					

Abbreviations: ANOVA = analysis of variance; APCA = advanced pancreatic carcinoma; HV = healthy volunteers; PDCa = pancreatic ductal adenocarcinoma. Values are geometric means \pm s.d.

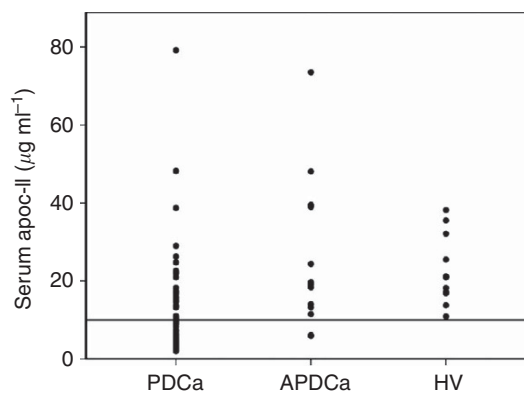


Figure 3 Plot of serum concentrations of ApoC-II for the different groups in the Validation study. The horizontal line indicates the cut-off value used for prognosis ($10 \mu\text{g ml}^{-1}$).

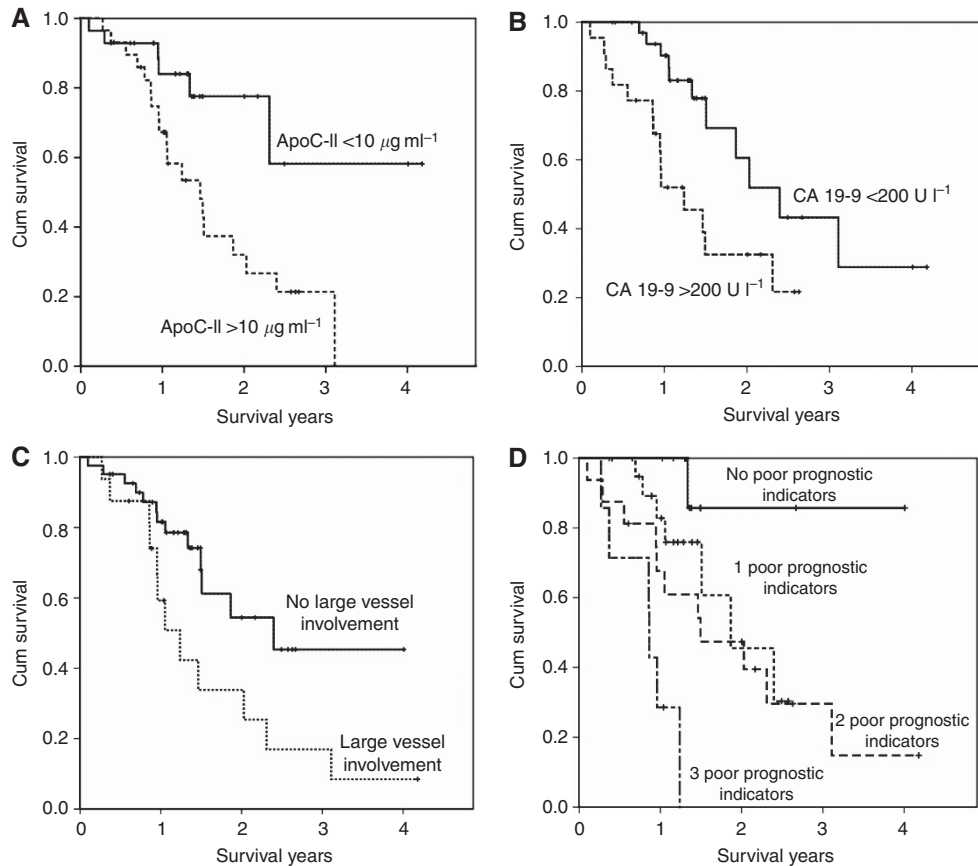


Figure 4 Kaplan–Meier survival curves for factors influencing survival after resection for PDCa in validation-2 samples. **(A)** The survival was significantly reduced if ApoC-II was $> 10 \mu\text{g ml}^{-1}$ (solid line, $n = 28$) compared with $< 10 \mu\text{g ml}^{-1}$ (dotted line, $n = 29$), log rank $P = 0.009$. **(B)** The survival curves for CA 19-9 at the cutoff of 200 U l^{-1} where dotted line represents patients with $\text{CA 19-9} > 200 \text{ U l}^{-1}$ ($n = 24$) and solid line represents patients with values were $< 200 \text{ U l}^{-1}$ ($n = 33$), $P = 0.009$. **(C)** The influence of pathologically determined large-vessel invasion (dotted line, $n = 16$) vs rest of the cases (solid line, $n = 41$) $P = 0.025$. **(D)** Combination of the three measures with the solid line representing 15 subjects with three low-risk measures, dotted line indicates survival of 8 subjects with 3 abnormal values, the dashed line indicates survival of 25 subjects with 1 abnormal measure and the dashed-dotted line indicated survival of 9 subjects with 2 abnormal values. Note that three patients with large-vessel involvement had low values of ApoC-II and CA19-9. The curves were different, $\chi^2 = 20.4$, $\nu = 3$, $P < 0.001$.

also demonstrated influence on survival. The combination of these three measures indicated that survival was excellent for the 15 subjects (26%) with all three values in the low-risk range but patients were unlikely to survive 12 months when the three measures were in high-risk range (8 subjects, 14%). The remaining patients with one or two high-risk measures had a median survival of 2 years (Figure 4D).

Cox proportional hazard assessment of prognostic factors

Multiple potential prognostic factors were assessed by univariate Cox proportional hazard analysis (Supplementary Table S1). This allowed comparison of the proteins of interest, SAA-1 and ApoC-II, with CRP, CA 19-9, serum bilirubin and the ratio of total neutrophil to lymphocytes in the preoperative blood film (Neu/LCC), along with pathological indices. Spearman's correlation indicated that ApoC-II was independent of other measures, whereas SAA-1 had a weak but non-significant correlation with the Neu/LCC ratio ($P = 0.07$), and CA19-9 only correlated with the need for vascular reconstruction. Large-vessel invasion correlated with T-stage ($P = 0.03$), retroperitoneal nerve invasion ($P = 0.02$), perineural invasion ($P = 0.006$) and small-vessel invasion ($P = 0.008$). Lymph node involvement correlated with T-stage and retroperitoneal extension. Therefore to avoid interference by co-correlation, the following indices were entered into the Cox

proportional hazards model using backward regression: $\log_{10}\text{ApoC-II}$, $\log_{10}\text{SAA-1}$, $\log_{10}\text{CA19-9}$, lymph node involvement and large-vessel involvement. The final equation included $\log_{10}\text{ApoC-II}$, $\log_{10}\text{CA19-9}$ and lymph node involvement for predicting survival ($\nu = 3$, $\chi^2 = 12.1$ and $P = 0.007$).

Impact of SAA-1 and ApoC-II on PDCa cell growth and invasion

In-vitro studies of the influence of ApoC-II and SAA-1 on the growth of CFPAC-1 and PANC-1 pancreatic cancer cell lines demonstrated a dose-dependent response to 0.05 , and $2.5 \mu\text{g ml}^{-1}$ of purified ApoC-II and to $25 \mu\text{g ml}^{-1}$ of SAA-1 (Figure 5A, ANOVA, $P < 0.01$) but not to $50 \mu\text{g ml}^{-1}$ of purified SAA-1 at 24 h. Furthermore, when CFPAC-1 and PANC-1 cells were pre-treated with recombinant ApoC-II or SAA-1 compared with control PBS, before seeding onto Matrigel-coated transwells there was significant increase in cell invasion ($P < 0.001$) in both cell lines but there was no additive effect of combining SAA-1 and ApoC-II (Figure 5B and C).

DISCUSSION

This paper demonstrates that preoperative levels of ApoC-II are prognostic of long-term survival in patients undergoing resection

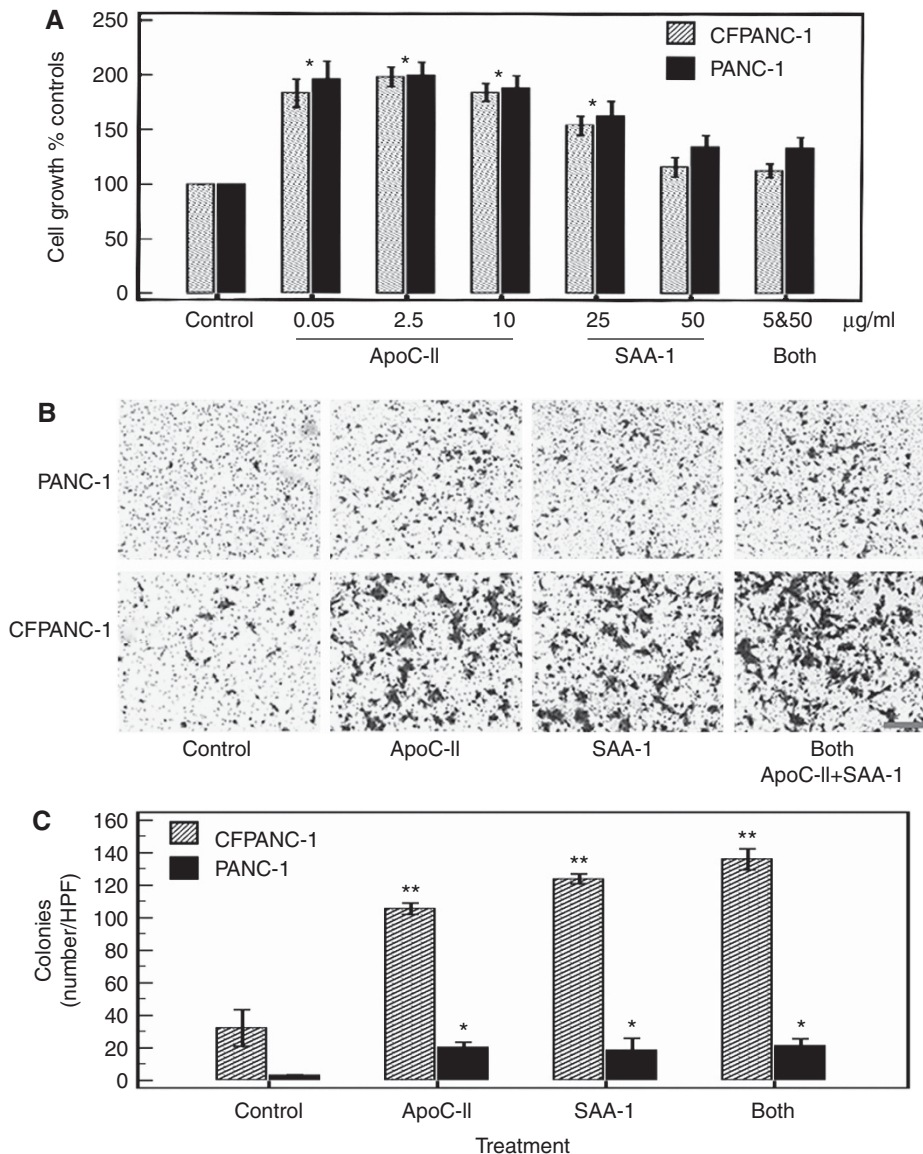


Figure 5 (A) The influence of ApoC-II and SAA-1 on cell growth (mean values \pm s.e.m.), as a percentage of controls after 24 h for CFPAC-1 (shaded bars), and PANC-1 (solid bars). Two-way analysis of variance showed no difference between cell lines. ApoC-II increased cell growth above that of controls ($P < 0.001$ for both cell lines) and greater than the combined treatment with ApoC-II and SAA-1. SAA-1 increased cell growth at the lower dose of $25 \mu\text{g ml}^{-1}$ ($P < 0.001$), but not at the higher dose $50 \mu\text{g ml}^{-1}$, which was less stimulatory than each dose of ApoC-II ($P < 0.001$). ** indicates different from controls, $P < 0.001$. 5 and 50 indicates $5 \mu\text{g ml}^{-1}$ ApoC-II and $50 \mu\text{g ml}^{-1}$ SAA-1. (B) Micrograph of $\times 10$ magnification transwell invasion assay for PANC-1 and CFPAC-1 cells treated with control solutions, ApoC-I, SAA-1 and both treatments. Bar = $200 \mu\text{m}$. (C) Cell counts per high power field at $\times 10$ results for 3–4 fields (mean values \pm s.e.m.). Analysis of variance indicated a significant increase from controls for CFPAC-1 cells (** $P < 0.001$) and for PANC-1 cells (* $P < 0.02$). When treatments were combined, the apparent increase did not reach significance.

for PDCa. The discovery of this protein was undertaken using the high through-put technique of SELDI-TOF MS and logistic regression analysis with sixfold cross-validation. These findings were verified by SELDI in a separate sample set from a different hospital and, were validated by ELISA in a prospective patient group where the patients with a good outcome had depressed levels of ApoC-II. Survival curve analysis by Cox regression of the validation-set samples confirmed that ApoC-II, CA 19-9 and lymph node involvement independently predict survival. Major-vessel involvement, which may be more easily assessed preoperatively with CT scanning (Manak *et al*, 2009) than lymph node involvement, was shown by Kaplan–Meier analysis to allow for a clear separation of patient outcomes when combined with ApoC-II and CA19-9. The influence of SAA-1, also identified on SELDI

analysis, was not found to be significant in the validation set. Of interest, SAA-1 and CRP have previously been shown to be inflammatory markers with prognostic utility (Raynes and Cooper, 1983).

ApoC-II is mainly synthesised by the liver (Kardassis *et al*, 2003), and is an important component of LDL, where it helps to solubilise lipid and deliver it to peripheral tissues by its ability to activate lipoprotein lipase (LPL). After the lipid is released, LDL shrinks and ApoC-II splits off either to pass back into the circulation or be utilised by the cancer. Interestingly ApoC-II has been shown to be a putative substrate for the metalloproteinases MMP-14 and MMP-7 (Kim *et al*, 2006). ApoC-II is efficiently cleaved by MMP-7, which may account for the depressed levels of ApoC-II in our cases with better outcomes (Giannopoulos *et al*,

2008). Further, pancreatic cancer utilises lipid in preference to carbohydrate (Wang *et al*, 2009) for energy, which may add to the turnover of ApoC-II. Our finding that ApoC-II increases the growth and spread of pancreatic cancer cells *in vitro* may indicate a further role of ApoC-II where high levels predicted poorer survival. Thus, although the prognostic importance of ApoC-II has not been previously reported, there are plausible cellular mechanisms involving ApoC-II in the progress of pancreatic cancer. It is interesting that elevated levels of ApoC-II have been considered to be of diagnostic importance (Chen *et al*, 2007) and associated with pancreatic cancer-associated cachexia (Felix *et al*, 2011).

SAA-1, the second protein selected by SELDI was previously shown to be prognostic of outcome in a number of malignancies related to an induced systemic acute-phase reaction (Weinstein *et al*, 1984), but, when measured by ELISA in our validation set, it failed as a prognostic marker. This is an unexpected result given that a number of these patients required vascular resection. The cachexia of pancreatic cancer is associated with an increase in inflammatory cytokines and would be expected to increase SAA-1 (Argiles *et al*, 2009), but few patients with resectable pancreatic cancer have established cachexia (Aslani *et al*, 2010). Of interest it is a lipoprotein, which is associated with HDL (van der Hilst, 2011) and has a complex, highly conserved, role in acute inflammatory conditions (Manley *et al*, 2006), which accounts for its elevation in our preoperative patients who had recently had stents for obstructive jaundice (Waghlikar *et al*, 2003). Recently SAA-1 was included in a diagnostic panel for pancreatic cancer (Bunger *et al*, 2011). Similarly, the ratio of neutrophils to lymphocytes has intriguingly been associated with poor prognosis (Bhatti *et al*, 2010), particularly in patients undergoing chemotherapy (Chua *et al*, 2011), implying an effect on the body's inflammatory response, although, again we were not able to confirm this.

Carbohydrate Antigen 19-9 level of $>200 \text{ U ml}^{-1}$ and lymph node involvement were independently prognostic of poor survival after pancreatic resection (Berger *et al*, 2004), as confirmed by this study. Although the prognostic value of CA 19-9 has been debated both for (Mehta *et al*, 2010) and against (Kondo *et al*, 2010), it does appear to indicate benefit for selection of patients for adjuvant therapy (Hallemeier *et al*, 2011). Multiple influences on survival include pathological criteria and nutritional and performance status (Schnelldorfer *et al*, 2008), but CA 19-9 may have further independent predictive value (Tsavaris *et al*, 2009). However, CA 19-9 results may be confusing because it can be elevated in the presence of biliary inflammation (Dogan *et al*, 2011) and is not produced in Lewis antigen-negative subjects (Vestergaard *et al*, 1999).

Surgical technique to achieve an R0 resection has an important role in achieving long-term survival (Waraya *et al*, 2009) where

en-bloc resection allows for clearance of involved vessels (Schnelldorfer *et al*, 2008). This approach has been used in the current study (Samra *et al*, 2006). However, margin status continues to be a debated topic and there is a wide belief that the aggressive nature of PDCA over-rides the importance of margin status (Butturini *et al*, 2008). Major-vessel involvement seen on multi-slice computer tomography is predictive of survival (Manak *et al*, 2009) and may be used in the preoperative assessment of prognosis along with levels of ApoC-II and CA 19-9.

Our results led us to analyse whether ApoC-II and SAA-1 were involved in cancer cell proliferation and invasion. ApoC-II and to a lesser extent SAA-1 stimulated cell proliferation and invasion in two pancreatic cancer cell lines. The apolipoproteins appear to have important roles in the biology of pancreatic cancer. ApoA-II is also depressed in pancreatic cancer (Xue *et al*, 2010). Although ApoC-II has a different function from ApoA-II, their involvement is consistent with pancreatic cancer's reliance on lipid metabolism. Further, RELN (Reelin) pathway signalling via the very low-density lipoprotein (VLDL) receptor, to which ApoC-II is known to bind, influences cell motility in pancreatic cancer (Sato *et al*, 2006). We speculate that ApoC-II levels may be involved with tumour progression.

In conclusion, this study shows that serum levels of ApoC-II, and CA 19-9 along with major vessel invasion, which can be determined preoperatively with a CT scan, allow for improved prediction of prognosis of a pancreatic resection. These prognostic indicators selected a small group of short-term survivors, who might have been better served by a palliative procedure, while the remaining patients benefitted from the surgery. Furthermore, these measures provide information about factors leading to a poor prognosis by highlighting the stimulation of cancer tissue growth by apolipoproteins. These factors are so strong that even after the inclusion of the influence of standard histopathological measures and CA 19-9, the blood level of ApoC-II remains a strong independent predictor of survival.

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