Serum CA19-9 is significantly up-regulated up to 2 years prior to diagnosis with pancreatic cancer: implications for early disease detection

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TRANSLATIONAL RELEVANCE

Pancreatic ductal adenocarcinoma is a leading cause of cancer death and has the lowest survival rate for any solid cancer. Biomarkers for the early detection of pancreatic cancer are urgently needed to improve survival, although studies assessing biomarkers for early detection rarely use samples from patients with pre-clinical disease. For the first time, serum markers CA19-9, CA125, CEACAM1 and REG3A have been assessed in samples taken up to 6 years prior to clinical presentation of pancreatic cancer. We show that CA19-9 and CA125 are elevated many months prior to clinical presentation of pancreatic cancer and when used in combination, CA125 improved upon the performance of CA19-9 alone through the detection of CA19-9 negative cases. Moreover, both markers can be used as prognostic tools in pancreatic cancer. These markers have the potential, when combined, for screening high-risk groups, particularly if used longitudinally.

ABSTRACT

Purpose Biomarkers for the early detection of pancreatic cancer are urgently needed. The primary objective of this study was to evaluate whether increased levels of serum CA19-9, CA125, CEACAM1 and REG3A are present prior to clinical presentation of pancreatic cancer and to assess the performance of combined markers for early detection and prognosis.

Experimental Design This nested case control study within UKCTOCS included 118 single- and 143 serial-serum samples from 154 post-menopausal women who were subsequently diagnosed with pancreatic cancer and 304 matched non-cancer controls. Samples were split randomly into independent training and test sets. CA19-9, CA125, CEACAM1 and REG3A were measured using ELISA and/or CLIA. Performance of markers to detect cancers at different times prior to diagnosis and for prognosis was evaluated.

Results At 95% specificity, CA19-9 (>37 U/mL) had a sensitivity of 68% up to 1 year, and 53% up to 2 yrs before diagnosis. Combining CA19-9 and CA125 improved sensitivity as CA125 was elevated (>30 U/mL) in ~20% of CA19-9-negative cases. CEACAM1 and REG3A were late markers adding little in combined models. Average lead times of 20-23 months were estimated for test-positive cases. Pre-diagnostic levels of CA19-9 and CA125 were associated with poor overall survival (HR 2.69 and 3.15, respectively).

Conclusions CA19-9 and CA125 have encouraging sensitivity for detecting pre-clinical pancreatic cancer and both markers can be used as prognostic tools. This work challenges the prevailing view that CA19-9 is up-regulated late in the course of pancreatic cancer development.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a leading cause of cancer death and has the lowest survival rate for any solid cancer (~2%) (1, 2). Surgical resection is the only chance of cure, but due to advanced stage at presentation only 20% of patients have resectable tumours (3). Of these, only 15% will have early-stage cancers (4, 5). When resection is possible followed by adjuvant therapy, the 5-year survival is better at 20-30% (6). It is clear that early detection of smaller tumors is necessary to improve resectability rates and survival. Indeed it was shown that if tumor size at detection can be reduced from 3 to 2 cm, then there is an increase in resectability from 7% to 83% with increased median survival from 7.6 to 17.2 months (7).

The serum marker CA19-9 (8-10) is the only biomarker used routinely in the management of PDAC. It has a 79-81% sensitivity and 82-90% specificity for diagnosis (11), with false-positive results observed in benign pancreatico-biliary diseases such as pancreatitis, cholangitis and obstructive jaundice (12-14). Furthermore, CA19-9 is not expressed in 8-10% of the Caucasian population with the Lewis a-b- genotype, as the CA19-9 epitope is the sialylated Lewis A blood group antigen (14, 15). Despite this, CA19-9 has proved useful for disease management, where increased post-therapy levels indicate poor prognosis and poor therapy response (16, 17). Moreover, the levels of CA19-9 in the months and years prior to PDAC diagnosis have not previously been examined, leaving its capacity to contribute to early diagnosis untested. Other reported non-invasive diagnostic and/or prognostic markers of pancreatic cancer that have been tested alone or in combination, include CA125 (18-21), CEA (22), CEACAM1 (23), MUC1 (24), OPN/SPP1 (25), MIC1/GDF15 (26), REG3A/PAP1 (27, 28) and PKM2 (29). As yet, the clinical utility of these markers remains to be determined and most require further multi-centre validation.

A major shortcoming of cancer biomarker studies aiming to address early detection is a lack of appropriate samples. Biomarkers tested in samples taken from patients diagnosed with cancer and benign or healthy controls only address potential use for differential diagnosis. Samples collected prior to diagnosis are preferential, enabling early changes to be detected, with consistently rising levels in the lead up to diagnosis adding confidence to the discovery.

The serum samples used in this study come from a repository collected as part of the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS); a randomized controlled trial of ovarian cancer assessing impact of screening on mortality using transvaginal ultrasound and serum CA125 interpreted using the Risk of Ovarian Cancer Algorithm (ROCA). Preclinical samples from participating women who subsequently developed PDAC were available for evaluating CA19-9, CA125, CEACAM1 and REG3A for early diagnosis.

MATERIALS AND METHODS

Serum samples

This nested case control study was approved by the Joint UCL/UCLH Research Ethics Committee A (Ref. 05/Q0505/57). Written informed consent was obtained from donors and no data allowing identification of patients was provided. The study set comprised serum from women recruited to UKCTOCS between 2001 and 2005 and collected according to a SOP (30, 31). Trial participants at enrolment were post-menopausal women aged 50-74 who had no family history of ovarian cancer. All participants were 'flagged' with the national agencies for cancer registrations and deaths using their NHS number. Women subsequently diagnosed with pancreatic cancer (cases) were identified by cross-referencing with the Health and Social Care Information Centre cancer registry codes and death codes (ICD10 C25.0/1/2/3/9). Initially, 171 cases were identified (with 304 associated samples) that had not been registered as having any other cancer since randomization. Matched non-cancer controls (i.e. with no cancer registry code) from individual women (1 per case sample; n=304) were selected based on collection date and center to minimize variation due to handling. For the 171 cases, confirmation of diagnosis was sought from GPs and consultants through questionnaire and from the Hospitals Episode Statistics database. As a result, 17 cases were excluded that did not have a confirmed diagnosis of pancreatic ductal adenocarcinoma. The resulting study set was 261 serum samples from 154 women subsequently diagnosed with pancreatic ductal adenocarcinoma up to 6.5 years later and 304 matched control sera from 304 women. 119 cases provided single

5

samples and 35 provided 2 or more serial samples. Table 1 shows clinical, lifestyle and sample data for the study set. Samples were distributed randomly into discovery and validation sets and grouped by time to diagnosis as indicated in Table 2.

Serum measurements

All serum tests were executed and interpreted by trained and experienced staff. Samples were randomized for testing and blinded to the experimenters prior to interpretation. CA19-9 was measured in discovery set samples using the Mucin PC/CA19-9 ELISA kit (Alpha Diagnostic International) according to the manufacturer, using a 1:4 serum dilution. Values lower than the assay's limit of detection were given a 'low' value of 0.6 U/mL. Duplicate measurements gave an average CV of 6.9% (R^2=0.998). CA19-9 was measured in validation set samples using the Cobas CA19-9 CLIA with a CA19-9 Calibrator Set (Roche and Fujirebio Diagnostics), run on a Cobas E411 analyzer with PreciControl Tumour Marker to monitor assay imprecision. The average CV from 31 replicate measurements of serum standard run at the same time was 3.2%.

CA125 was measured using the Cobas CA125 II CLIA with a CA125 II Calibrator Set (Roche and Fujirebio Diagnostics) on a Cobas E411 analyzer with PreciControl Tumour Marker, as above. Assays were performed originally on 320 fresh study samples in UKCTOCS and on discovery set samples (after two freeze thaw cycles) where original values were missing. Assays were repeated on all validation set samples. For matched duplicate readings, average CV was 8.5 % (R^2=0.997). The average CV from 31 replicate measurements of serum standard was 4.1%.

For CEACAM1, a sandwich ELISA was established using the human CEACAM1/CD66a DuoSet kit (R&D Systems), as described in Supplementary Data. Replicate readings gave an average CV of 10.3%; (R^2=0.81). Serum REG3A/PAP was measured using the PANCREPAP ELISA kit (DynaBio) according to the manufacturer, using a 1:100 serum dilution. Replicate readings gave an average CV of 21.9 % (R^2=0.46).

6

Statistical Analysis

GraphPad Prism and MedCalc software were used for statistical analyses. For normally distributed data, the Student t test was used to assess significance of differences, otherwise the Mann-Whitney U-test was used. Fisher's exact test was used to assess significance of associations for non-continuous variables. Correlation analysis used Spearman's rank test. All P values <0.05 were considered significant. Receiver operating characteristic (ROC) curves were constructed for each marker and combinations to assess diagnostic accuracy. Kaplan-Meier analysis was used to examine biomarker levels in relation to survival using time from sample collection to death.

RESULTS

Study set characteristics

There was no significant difference in time to centrifugation between case control samples, whilst there was a difference in mean age (2.8 years; P<0.0001) and BMI (1 kg/m^2; P=0.041) between cases and controls (Table 1). Hormone replacement therapy (HRT) use at randomization was associated with lowered risk of pancreatic cancer (OR=0.49; 95%CI 0.27-0.88; P=0.022), whilst oral contraceptive pill use had no association. Smoking was not associated with pancreatic cancer, whilst alcohol consumption was negatively associated (OR=0.4; P=0.01). Notably, response rate for reporting smoking and alcohol consumption was poor, particularly from those volunteers who developed cancer. The death rate was 95.5% for cases with a median time from diagnosis to death of 4 months.

Serum CA19-9, CA125, CEACAM1 and REG3A in discovery set

CA19-9, CEACAM1 and REG3A were measured in all discovery samples using commercial ELISA kits, whilst CA125 was measured using a robust CLIA assay. There was a significant increase in level between all case and control samples for CA19-9 (P<0.0001) and CA125 (P=0.0004), but not for CEACAM1 or REG3A (Figure 1). Time to diagnosis plots showed

increasing CA19-9 and CA125 for cases in the lead up to diagnosis, whereas CEACAM1 and REG3A showed no such trend (Supplementary Data; Figure S1). For serial samples from the same women, CA19-9 and CA125 increased towards diagnosis in the majority of cases (Supplementary Data; Figure S2), whereas none showed increasing CEACAM1 or REG3A.

Markers were analyzed according to time to diagnosis (grouped as 0-0.5 yrs, 0.5-1 yrs, 1-2 yrs, 2-3, yrs, 3+ yrs, 0-4+ yrs (all samples), 0-1 yrs and 1-4 yrs). In cases where serum was obtained <12 months before diagnosis, median CA19-9 was 43.2 U/mL (IQR 5.7-136.2 U/mL) compared to 3.1 U/mL (IQR 0.6-6.9 U/mL) in controls ($P<0.0001$; AUC=0.82) (Figure 2). For CA125, median values were 24.1 U/mL (IQR 12.9-47.9) for cases and 12.8 U/mL (IQR 9.3-14.5) for controls ($P<0.0001$; AUC=0.78). CEACAM1 ($P=0.045$; AUC=0.71) and REG3A ($P=0.022$; AUC=0.73) were only significantly elevated in the 6 months before diagnosis. CA19-9 and CA125 were also raised in the 0.5-1 year pre-diagnosis group ($P=0.0016$; AUC=0.80 and $P=0.0167$; AUC=0.73, respectively), but not >1 year. There were no significant associations for any of the markers with respect to time to centrifugation, age, BMI, smoking, alcohol consumption, HRT or OCP use, except REG3A, which correlated with age ($\rho=0.31$; $P<0.001$) in the controls.

Sensitivities for detection of PDAC were calculated using selected cut-offs (Table 3). CA19-9 (>25 U/mL) was the best performing marker, discriminating cases from controls with SN/SP of 70.6%/95.0% and 64.7/95.5% in the 0-0.5 yrs and 0.5-1 yrs prior to diagnosis (Table 3). The SN/SP for detection of PDAC with CA125 (>20 U/mL) in these time periods were somewhat poorer at 70.6%/90.0% and 52.9%/86.4%, respectively. CEACAM1 and REG3A were poor at detecting cancer compared to CA19-9 or CA125; for the 0-0.5 yrs group, the SN/SP for CEACAM1 was 53.3/83.3% (>50 ng/mL), whilst for REG3A it was 29.4/90% (>13 ng/mL). Combining markers showed the model 'CA19-9>37 U/mL or CA125>30 U/mL' provided the highest sensitivities at >90% specificity; 57.1% for the 0-1 year group (PPV 90.9%; OR=26.67, 95%CI 5.6-128.2) and 44.2% for the 0-2 year time group (PPV 92.0%; OR=24.59, 95%CI 5.4-111.4), but were only marginally better (and not significantly so) than using CA19-9 alone (Table

3). Adding CEACAM1 and/or REG3A into models decreased specificity with little improvement in sensitivity (data not shown). Logistic regression showed the best combined model (CA19-9, CA125 and CEACAM1) had an AUC of 0.88 (SE=0.042; 95%CI 0.79-0.95) for the 0-1 year group, but was not significantly higher than using CA19-9 alone (AUC=0.82). Together, these data indicate that CA19-9, and possibly CA125, may be useful in predicting PDAC up to 24 months in advance of diagnosis.

Validation of CA19-9 and CA125 as early detection biomarkers

CA19-9 and CA125 were further assessed in a validation set comprising 168 samples from 101 cases. This was subsequently restricted to 138 samples by removing all but one serial samples (at random) from the same case/woman that fell within the same time group (Table 2). In this restricted set, CA19-9 was significantly higher in cases than controls for the 0-0.5, 1-2, 2-3 and 3+ year time groups, though failed significance for the 0.5-1 year group (Supplementary Data Figure S3). CA125 was significant for the 0-0.5 and 3+ year time groups. The simple cut-off model 'CA19-9>37 U/mL or CA125>30 U/mL' applied to this dataset gave 56.7% sensitivity and 90.6% specificity for the 0-1 year group (PPV=77.3%; OR=12.55, 95%CI 3.89-40.48), compared to 50% sensitivity and 94.3% specificity using CA19-9 alone (PPV=83.3%; OR=16.67, 95%CI 4.25-65.43) (Table 4). For the 0-2 year group, sensitivity was 40.6% and specificity 90.5% (PPV=74.3%; OR=6.54, 95%CI 2.80-15.28). Logistic regression combining CA19-9 and CA125 gave AUCs of 0.90 and 0.76 for the 0-0.5 and 0-1 year groups, respectively, but was not significantly higher than using CA19-9 alone. Thus, CA125 adds little discriminatory power in combined models. Despite this, 13 out of 53 positive cases in the validation set were detected solely using CA125.

Our intention was also to build algorithms based on available serial/longitudinal data. ROCA used in UKCTOCS (32, 33) could not be developed for CA19-9 due to lack of serial control samples. A Parametric Empirical Bayes algorithm (34) applied to the combined dataset gave sensitivities of 19% and 17% (at 95% specificity) for CA19-9 and CA125, respectively, and was

thus poorer than the threshold models. This again is likely due to an insufficient longitudinal data for accurate model building.

Lead time estimation

In two cases with sufficient longitudinal samples, increasing CA19-9 was apparent as early as 3 years prior to diagnosis of PDAC (Supplementary Data; Figure S2; Cases 5 and 6). However, most marker 'change-points' occurred within 12 months of diagnosis. CA125 tended to rise later than CA19-9, but was diagnostic alone in ~20% of cases where CA19-9 was not elevated (Figure S2; Case 40). Taking the earliest point of detection for cases with longitudinal samples, the model 'CA19-9>30 or CA125>25' gave an average lead time of 22.9 months (median 18.5 months; IQR 8.0-32.8 months). Whilst estimated lead times were slightly higher for combined models *versus* CA19-9 alone, differences were not significant.

Prognosis

The four markers were investigated as prognostic factors using time from sample collection to death in cases where pancreatic cancer was cited as the primary or contributory cause of death. Firstly, poor and good prognosis case samples were respectively defined as falling below and above the median time from sample collection to death (30.5 months). Both CA19-9 and CA125 were significantly elevated in the poor prognosis group, whilst CEACAM1 and REG3A were not (data not shown). Kaplan-Meier analysis confirmed a significant difference in survival curves for CA19-9 (cut-off 40 U/mL; Log-rank test P<0.0001; HR=2.69, 95%CI 1.84-3.91) and CA125 (cut-off 25 U/mL; Log-rank test P<0.0001; HR= 3.15, 95%CI 2.11-4.69), confirming them as prognostic markers (Figure 3). Median survival times from collection were 14.5 *versus* 36.0 months for CA19-9 and 14.0 *versus* 35.0 months for CA125.

DISCUSSION

To our knowledge this is the first study to show serum CA19-9, CA125, CEACAM1 and REG3A are significantly elevated prior to PDAC diagnosis. To our surprise, in 16% of cases, CA19-9

10

was elevated (>37 U/mL) 2-3 years before diagnosis, with sensitivity increasing towards diagnosis. The PPV of CA19-9 was 90% up to 1 year before diagnosis and >80% up to 2 years, depending on the cut-off level used. CA125 also increased in pre-clinical disease and although combined models gave only modest increases in performance *versus* CA19-9 alone, CA125 detected 13% of CA19-9-negative cases; some of which are likely to be Lewis antigen negative. Notably, combined models gave average lead times up to 23 months. Together, our data support the notion that a panel of markers including CA19-9 may be beneficial for earlier detection of pancreatic cancer with potential use in screening.

Three studies have explored the utility of serum CA19-9 levels as a screening tool for pancreatic cancer (35-37). Kim *et al* assessed CA19-9 in 70,940 asymptomatic individuals identifying 4 cancers among 1,063 individuals with elevated CA19-9 (>37 U/mL; PPV= 0.9%; sensitivity 100%; specificity 98.5%). Notably, a higher proportion of women (2.5%) compared to men (0.5%) had elevated CA19-9. This did not increase with age in women, as was observed here. Given the low predictive value of CA19-9 and low prevalence of pancreatic cancer in the general population, it was concluded from these studies that CA19-9 testing alone has no utility as a screening tool. However, here CA19-9 elevations were noted up to 36 months prior to diagnosis, indicating its potential as a first line test for early detection that may increase the number of patients with resectable disease. These results need to be independently validated. Annual CA19-9 blood testing may also benefit high-risk populations such as those from kindred with familial pancreatic cancer. Guidelines for the surveillance of these family members are not established, although there are recent studies assessing outcomes of screening these high-risk populations (38, 39).

Serum CA125 has been evaluated as a marker for detecting malignant *versus* benign pancreatic tumours with a reported sensitivity of 60.8% and specificity of 83.3% (21). Combining CA19-9 and CA125 gave values of 87.8% and 77.8% respectively, with the authors concluding that test results should be interpreted in reference to imaging. A similar study reported a sensitivity of 56.9% and specificity of 77.6%, with CA125 providing a limited contribution in

11

jaundiced patients (40). Herein, in the pre-diagnosis setting, we show that CA125 performed with higher specificity, providing additional sensitivity in combination with CA19-9.

CEACAM1 is expressed in pancreatic carcinoma *in situ* lesions, and thus has early stage marker potential (23). It was further shown in this previous study that serum CEACAM1 had superior accuracy to CA19-9 in clinical samples from PDAC patients. Whilst the function of CEACAM1 remains unclear, several studies have identified its aberrant expression in a variety of cancers often with conflicting reports (41-43). Herein, CEACAM1 appears to be up-regulated in pancreatic adenocarcinoma, although is elevated in serum closer to diagnosis as compared to CA19-9. CEACAM1 did not significantly improve classification in combined models and it was not a prognostic factor. We conclude that serum CEACAM1 is not an early marker of pancreatic cancer.

REG3A expression has been associated with pancreatic inflammation (44) and cancer (27, 28, 45). In the latter study, REG3A had 90% sensitivity and 82.8% specificity for discriminating PDAC cases from healthy controls and was not correlated with CA19-9 level or associated with concomitant pancreatitis or jaundice. In the present study, REG3A was discriminatory within 6 months of diagnosis, but was rarely elevated independently of CA19-9. In summary, REG3A is a poor early marker, adding little in combined models.

Our data also confirm CA19-9 as a prognostic marker since we show that pre-diagnosis cases with levels <40 U/mL had a prolonged median survival (from 14.5 to 36 months using time from blood draw), and is in agreement with published data (reviewed in (11)). CA125 was similar in predicting overall survival, and thus also appears to be a prognostic factor in pancreatic cancer. This is supported by the notion that CA125 plays a direct role in the progression and dissemination of pancreatic tumour cells (46).

Our study has several limitations. Firstly, only post-menopausal women were studied, and hence may not reflect the utility of pancreatic tumor markers in the general population. Secondly, there was insufficient information on grading, staging, tumor size and treatment to be able to examine correlations with the markers. Indeed, it is likely that many of the test-positive cases were at an advanced stage of disease when tested, in agreement with observed

12

correlations between serum CA19-9 levels and pancreatic tumor burden (47); although this may not always hold true (48). Thirdly, the number of cases (and controls) with serial samples was insufficient to accurately build and assess longitudinal algorithms. Fourthly, there was a lack of data on benign morbidities in both the case and control groups for which CA19-9 may be elevated. Despite these limitations, the study is unique in that pre-clinical samples were investigated, allowing an objective assessment of how serological markers change during disease progression. Indeed, our findings suggest that raised CA19-9 can be detected early in the course of PDAC development when tumor size is likely to be smaller and when survival outcomes are improved (7). Importantly, although the majority of test positive cases in our study had elevated CA19-9 (and/or CA125) within 1 year of diagnosis, likely indicating advanced disease, these patients still went undiagnosed. This alludes to the non-specific nature of the symptoms of pancreatic cancer, but also raises the possibility of an earlier diagnosis that may improve outcome. Whilst one recent study has assessed pre-diagnostic PDAC samples (from the prospective EPIC cohort), reporting that autoantibodies against ezrin appear early in PDAC development, CA19-9 levels were unfortunately not reported in the 16 cases examined (49).

Our data also suggest that that HRT use at randomization was associated with lowered risk of pancreatic cancer. The apparent protective effect of HRT use is at odds with two large prospective cohort studies showing no association (50, 51). The reason for this discrepancy is unclear, but may be a chance association given the much lower number of controls used in the present study.

In conclusion, CA19-9 may have clinical utility in screening for pancreatic cancer as a first line test, particularly if used longitudinally in higher risk or symptomatic populations, whilst CA125 measurements may improve its performance and increase its prognostic value. It is unclear whether the use of other screening investigations (EUS, CT scan) would have allowed earlier diagnosis (i.e. at a still resectable state) when applied at the time of a CA19-9 or CA125 rise and this would need to be trialed in future studies.

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13

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Table 1 Clinical, lifestyle and sample characteristics data for whole study set. [a]*P* values determined using Mann Whitney or Fisher's Exact test. BMI = body mass index at recruitment; HRT = hormone replacement therapy; OCP = oral contraceptive pill; na = not applicable.

Variable	Cases	Controls	[P] *value*
No. individuals	154	304	
No. samples	261	304	
Tumor site			
tail	8	na	
body	10	na	
head	65	na	
unspecified	71	na	
Mean time to spin (hrs) (range)	21.8 (0.5-47)	22.0 (6.9-47)	0.62
Mean age at sample draw (yrs) (range)	65.3 (51.2-74.9)	62.5 (50.4-77.5)	<0.0001
Mean BMI (kg/m2) (range)	27.6 (17.8-43.7)	26.6 (17.9-44.4)	0.041
Mean time from sample collection to diagnosis (months) (range)	25.5 (0-79)	na	
HRT use (at randomisation)			
yes	16	58	0.022
no	138	246	(OR 2.03)
OCP use (ever)			
yes	79	163	0.69
no	75	141	
Smoker			
yes	15	75	1.00
no	28	136	
no response	111	93	
Alcohol			
yes	25	182	0.01
no	20	58	(OR 2.5)
no response	109	64	
Deaths as of 03/2013 (%)	95.45%	1.32%	
Median time from diagnosis to death (months) (range)	4.04 (1-45)	na	

Table 2 Sample sets and case controls used in study. [a] In the 'restricted' validation set, serial samples from the same woman falling in the same time group were removed at random to leave a single representative sample. There were no such samples in the discovery set.

Time group (yrs)	Total case samples	Total control samples	Discovery case samples	Discovery control samples	Validation case samples (all)	Validation case samples (limited)	Validation control samples
0-0.5	43	47	17	20	26	12	27
0.5-1	42	48	18	22	24	18	26
1-2	57	64	17	22	40	34	42
2-3	46	54	13	17	33	32	37
3-4	30	41	12	18	18	18	23
4+	43	50	16	21	27	24	29
0-1	85	95	35	42	50	30	53
0-2	142	159	52	64	90	64	95
0-3	188	213	65	81	123	96	132
0-4+	261	304	93	120	168	138	184
1-3	103	118	30	39	73	66	79
3+	73	91	28	39	45	42	52
Total samples	**261**	**304**	**93**	**120**	**168**	**138**	**184**
Total cases	**154**		**53**		**101**	**101**	

Table 3 Performance of cut-off models for discovery set. Darker shading denotes higher values. See Supplementary Data; Table S1 for numbers of test positive cases and controls for using the CA19-9 >37 U/mL and CA125 >30 U/mL models.

Time group	Mean time to Dx (months)	Parameter	CA19-9 >25	CA125 >20	CEACAM1 >50	REG3A >13	CA125 or CA19-9	CA19-9 >30	CA125 >25	CA125 or CA19-9	CA19-9 >37	CA125 >30	CA125 or CA19-9	CA19-9 >40	CA125 >25	CA125 or CA19-9
0-0.5 yrs	2.94	Sensitivity	70.6	70.6	53.3	29.4	88.2	58.8	52.9	64.7	52.9	47.1	58.8	52.9	52.9	58.8
		Specificity	95.0	90.0	83.3	90.0	85.0	95.0	100.0	95.0	100.0	100.0	100.0	100.0	100.0	100.0
0.5-1 yrs	9.11	Sensitivity	64.7	52.9	23.5	37.5	77.8	64.7	35.3	66.7	58.8	17.6	55.6	47.1	35.3	50.0
		Specificity	95.5	86.4	81.0	90.9	81.8	95.5	90.9	86.4	95.5	95.5	90.9	95.5	90.9	86.4
1-2 yrs	18.24	Sensitivity	23.5	17.6	46.7	18.8	29.4	23.5	11.8	23.5	17.6	5.9	17.6	17.6	11.8	17.6
		Specificity	100.0	86.4	55.0	90.5	86.4	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
2-3 yrs	28.76	Sensitivity	8.3	7.7	33.3	7.7	15.4	8.3	0.0	7.7	8.3	0.0	7.7	8.3	0.0	7.7
		Specificity	88.2	94.1	68.8	94.1	82.4	88.2	100.0	88.2	94.1	100.0	94.1	94.1	100.0	94.1
3+ yrs	52.54	Sensitivity	10.7	8.0	28.6	21.4	14.3	3.6	8.0	10.7	3.6	4.0	7.1	3.6	8.0	10.7
		Specificity	89.7	87.2	55.6	81.6	79.5	89.7	89.7	82.1	94.9	94.9	92.3	94.9	89.7	87.2
0-4+ (all)	25.44	Sensitivity	34.1	30.3	35.6	23.3	43.0	29.7	21.3	33.3	26.4	14.6	28.0	24.2	21.3	28.0
		Specificity	93.3	88.3	66.7	88.1	82.5	93.3	95.0	89.2	96.7	97.5	95.0	96.7	95.0	92.5
0-1 yrs	6.11	Sensitivity	67.6	61.8	37.5	33.3	82.9	61.8	44.1	65.7	55.9	32.4	57.1	50.0	44.1	54.3
		Specificity	95.2	88.1	82.1	90.5	83.3	95.2	95.2	90.5	97.6	97.6	95.2	97.6	95.2	92.9
0-2 yrs	12.18	Sensitivity	52.9	47.1	40.4	28.6	65.4	49.0	33.3	51.9	43.1	23.5	44.2	39.2	33.3	42.3
		Specificity	96.9	87.5	72.9	90.5	84.4	96.9	96.9	93.8	98.4	98.4	96.9	98.4	96.9	95.3
1-4+ yrs	37.10	Sensitivity	14.0	10.9	34.5	17.5	19.0	10.5	7.3	13.8	8.8	3.6	10.3	8.8	7.3	12.1
		Specificity	92.3	88.5	58.3	86.8	82.1	92.3	94.9	88.5	96.2	97.4	94.9	96.2	94.9	92.3

Table 4 Performance of cut-off models for restricted validation set. Darker shading denotes higher values. See Supplementary Data; Table S1 for numbers of test positive cases and controls for using the CA19-9 >37 U/mL and CA125 >30 U/mL models.

Time group	Ave time to Dx (months)	Parameter	CA19-9 >25	CA125 >20	CA19-9 or CA125	CA19-9 >30	CA125 >25	CA19-9 or CA125	CA19-9 >37	CA125 >30	CA19-9 or CA125	CA19-9 >40	CA125 >25	CA19-9 or CA125
0-0.5 yr	3.42	Sensitivity	75.0	75.0	83.3	75.0	58.3	83.3	75.0	41.7	83.3	75.0	58.3	83.3
		Specificity	77.8	77.8	63.0	85.2	92.6	85.2	92.6	96.3	88.9	100.0	92.6	92.6
0.5-1 yr	9.27	Sensitivity	24.0	24.0	22.0	43.8	13.3	50.0	37.5	12.5	43.8	31.3	13.3	37.5
		Specificity	50.0	20.0	62.5	96.2	96.2	92.3	96.2	96.2	92.3	96.2	96.2	92.3
1-2 yr	18.68	Sensitivity	32.4	20.6	41.2	32.4	11.8	35.3	23.5	8.8	26.5	20.6	11.8	23.5
		Specificity	85.7	78.6	69.0	92.9	97.6	90.5	92.9	97.6	90.5	95.2	97.6	92.9
2-3 yr	29.24	Sensitivity	34.4	25.0	50.0	28.1	18.8	43.8	18.8	15.6	31.3	18.8	18.8	34.4
		Specificity	94.4	80.6	77.8	94.4	97.2	91.7	97.2	97.2	94.4	97.2	97.2	94.4
3+ yr	50.07	Sensitivity	23.8	35.7	45.2	14.3	14.3	26.2	9.5	7.1	14.3	9.5	14.3	21.4
		Specificity	94.2	78.8	75.0	98.1	88.5	86.5	98.1	92.3	90.4	98.1	88.5	86.5
0-4+ (all)	25.13	Sensitivity	35.5	30.4	50.0	30.4	18.1	39.9	23.9	13.0	30.4	22.5	18.1	31.9
		Specificity	89.6	80.9	73.8	94.0	94.0	89.1	95.6	95.6	91.3	97.3	94.0	91.3
0-1 yr	6.93	Sensitivity	56.7	40.0	66.7	53.3	30.0	60.0	50.0	23.3	56.7	46.7	30.0	53.3
		Specificity	84.9	84.9	73.6	90.6	94.3	88.7	94.3	96.2	90.6	98.1	94.3	92.5
0-2 yr	13.17	Sensitivity	43.8	29.7	53.1	42.2	20.3	46.9	35.9	15.6	40.6	32.8	20.3	37.5
		Specificity	85.3	82.1	71.6	91.6	95.8	89.5	93.7	96.8	90.5	96.8	95.8	92.6
1-4+ yr	34.02	Sensitivity	29.6	27.8	45.4	24.1	14.8	34.3	16.7	10.2	23.1	15.7	14.8	25.9
		Specificity	91.5	79.2	73.8	95.4	93.8	89.2	96.2	95.4	91.5	96.9	93.8	90.8

FIGURE LEGENDS

Figure 1 Box and whisker plots showing serum levels of CA19-9, CA125, CEACAM1 and REG3A for all case and control discovery set samples. Whisker limits represent the 5th and 95th percentiles, the box limits represent interquartile range, the horizontal line the median and the cross the mean. Case and control groups were compared using the Mann-Whitney test; P values are shown above the plots.

Figure 2 Box and whisker plots showing serum levels of CA19-9, CA125, CEACAM1 and REG3A for case control discovery samples grouped into different time to diagnosis groups. Whisker limits represent the 5th and 95th percentiles, the box limits represent interquartile range, the horizontal line the median and the cross the mean. Case and control groups were compared using the Mann-Whitney test; P values are shown above the plots.

Figure 3 Survival curves for CA19-9 and CA125 using time from sample collection to death due to pancreatic cancer.
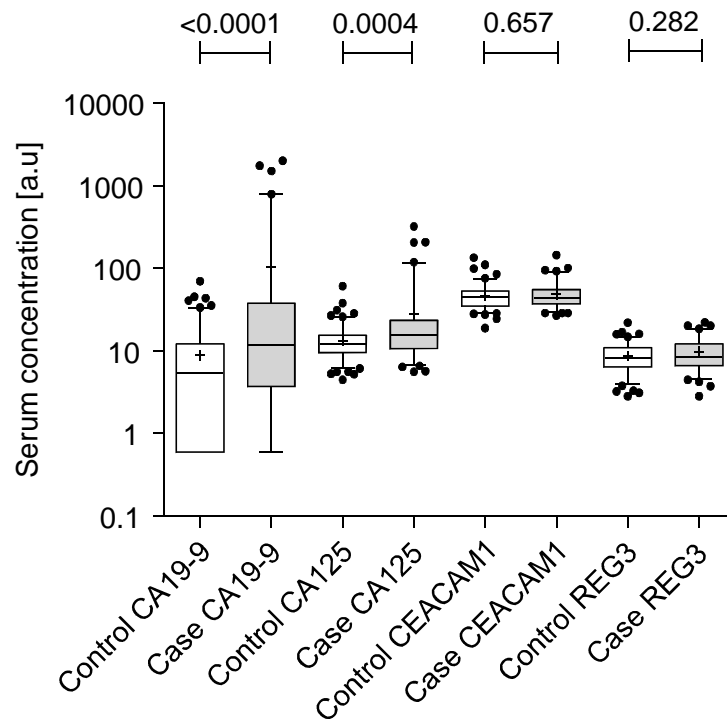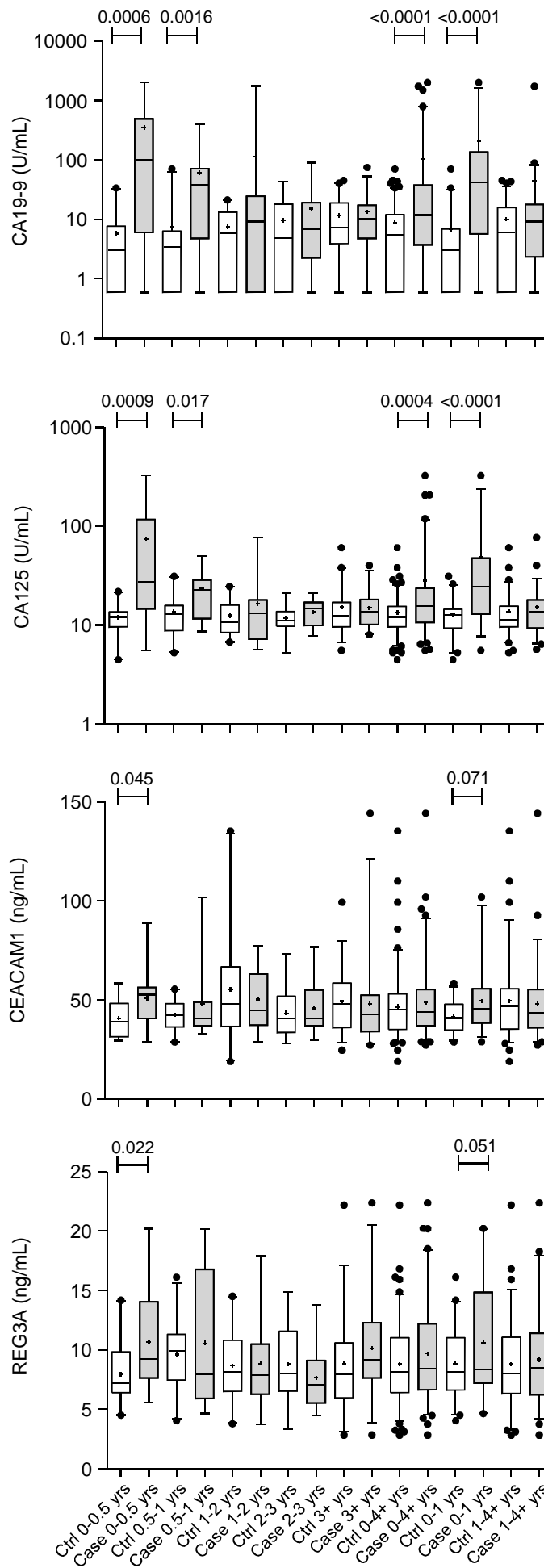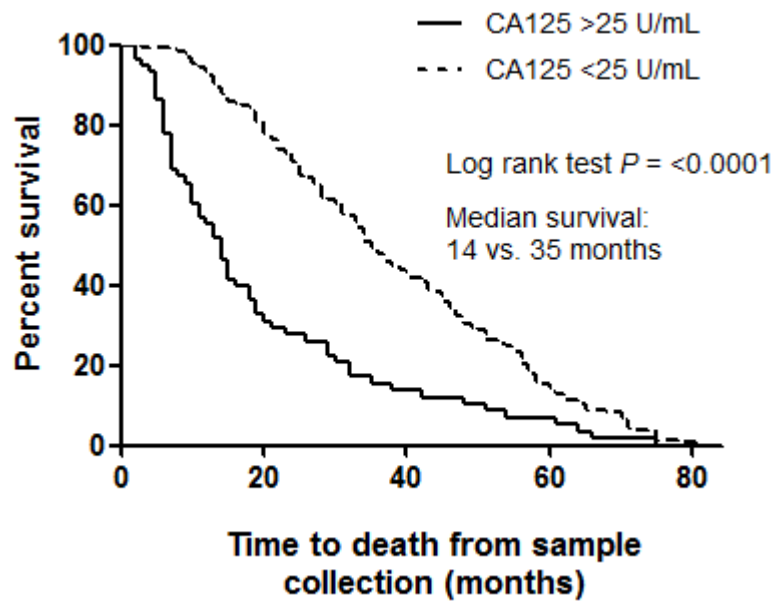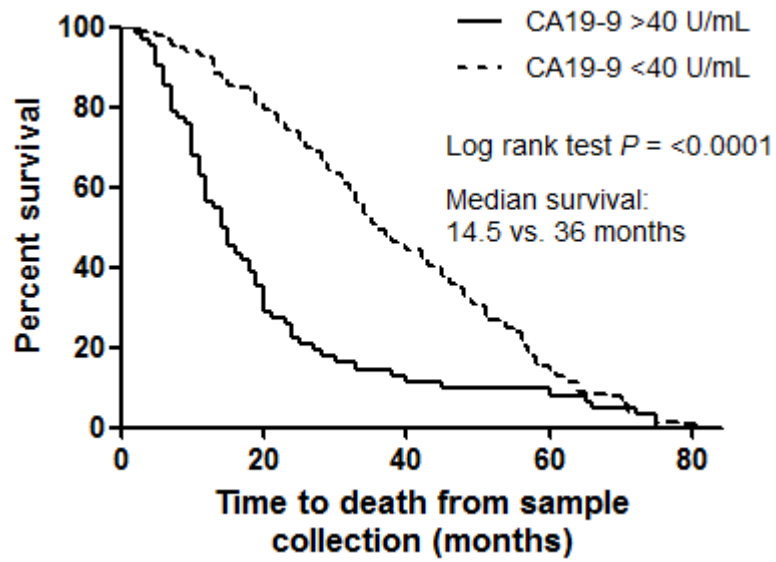
Figure 1

Figure 2

Figure 3

CEACAM1 ELISA

For serum CEACAM1 measurements, a sandwich ELISA was established using the human CEACAM1/CD66a DuoSet kit (R&D Systems) as follows. Mouse anti-human CEACAM1 monoclonal antibody was coated onto ELISA plates at 4 μg/mL overnight at room temperature (RT). Plates were washed 3 times in PBS containing 0.05% Tween-20 and then wells were blocked with 1% BSA in dilution buffer (PBS) for 1 hr at RT. After washing three times, 100 mL of diluted sample (1:50) was added as duplicates with a standard dilution series (recombinant human CEACAM1; 23.4-6,000 pg/mL) and blanks. After incubation for 2 hrs at RT, plates were washed 3 times and 100 μL of biotinylated goat anti-human CEACAM1 detection antibody (100 ng/mL) was added and incubated for a further 2 hrs at RT. After 3 washes, streptavidin-HRP was added and plates incubated for 20 min at RT. After a further 3 washes, 100 μL of a 1:1 mixture of H_2O_2 and tetramethylbenzidine was added and incubated for 20 min at RT in the dark. Finally, 50 μL of stop solution (2N H_2SO_4) was added and the plates read in a blanked microtitre plate reader at 450 nm.

Time group	Parameter	Discovery set			Validation set		
		CA19-9 >37	CA125 >30	CA125 or CA19-9	CA19-9 >37	CA125 >30	CA125 or CA19-9
0-0.5 yr	Case positive	9	8	10	9	5	10
	Case negative	8	9	7	3	7	2
	Control positive	0	0	0	2	1	3
	Control negative	20	20	20	25	26	24
	Sensitivity	52.94	47.06	58.82	75.00	41.67	83.33
	Specificity	100.00	100.00	100.00	92.59	96.30	88.89
	No. cases	17	17	17	12	12	12
	No. controls	20	20	20	27	27	27
0.5-1 yr	Case positive	10	3	10	6	2	7
	Case negative	7	14	8	10	14	9
	Control positive	1	1	2	1	1	2
	Control negative	21	21	20	25	25	24
	Sensitivity	58.82	17.65	55.56	37.50	12.50	43.75
	Specificity	95.45	95.45	90.91	96.15	96.15	92.31
	No. cases	17	17	18	16	16	16
	No. controls	22	22	22	26	26	26
1-2 yr	Case positive	3	1	3	8	3	9
	Case negative	14	16	14	26	31	25
	Control positive	0	0	0	3	1	4
	Control negative	22	22	22	39	41	38
	Sensitivity	17.65	5.88	17.65	23.53	8.82	26.47
	Specificity	100.00	100.00	100.00	92.86	97.62	90.48
	No. cases	17	17	17	34	34	34
	No. controls	22	22	22	42	42	42
2-3 yr	Case positive	1	0	1	6	5	10
	Case negative	11	13	12	26	27	22
	Control positive	1	0	1	1	1	2
	Control negative	16	17	16	35	35	34
	Sensitivity	8.33	0.00	7.69	18.75	15.63	31.25
	Specificity	94.12	100.00	94.12	97.22	97.22	94.44
	No. cases	12	13	13	32	32	32
	No. controls	17	17	17	36	36	36
3+ yr	Case positive	1	1	2	4	3	6
	Case negative	27	24	26	38	39	36
	Control positive	2	2	3	1	4	5
	Control negative	37	37	36	51	48	47
	Sensitivity	3.57	4.00	7.14	9.52	7.14	14.29
	Specificity	94.87	94.87	92.31	98.08	92.31	90.38
	No. cases	28	25	28	42	42	42
	No. controls	39	39	39	52	52	52
0-4+ yr	Case positive	24	13	26	33	18	42
	Case negative	67	76	67	105	120	96
	Control positive	4	3	6	8	8	16
	Control negative	116	117	114	175	175	167
	Sensitivity	26.37	14.61	27.96	23.91	13.04	30.43
	Specificity	96.67	97.50	95.00	95.63	95.63	91.26
	No. cases	91	89	93	138	138	138
	No. controls	120	120	120	183	183	183
0-1 yr	Case positive	19	11	20	15	7	17
	Case negative	15	23	15	15	23	13
	Control positive	1	1	2	3	2	5
	Control negative	41	41	40	50	51	48
	Sensitivity	55.88	32.35	57.14	50.00	23.33	56.67
	Specificity	97.62	97.62	95.24	94.34	96.23	90.57
	No. cases	34	34	35	30	30	30
	No. controls	42	42	42	53	53	53
0-2 yr	Case positive	22	12	23	23	10	26
	Case negative	29	39	29	41	54	38
	Control positive	1	1	2	6	3	9
	Control negative	63	63	62	89	92	86
	Sensitivity	43.14	23.53	44.23	35.94	15.63	40.63
	Specificity	98.44	98.44	96.88	93.68	96.84	90.53
	No. cases	51	51	52	64	64	64
	No. controls	64	64	64	95	95	95
1-4+ yr	Case positive	5	2	6	18	11	25
	Case negative	52	53	52	90	97	83
	Control positive	3	2	4	5	6	11
	Control negative	75	76	74	125	124	119
	Sensitivity	8.77	3.64	10.34	16.67	10.19	23.15
	Specificity	96.15	97.44	94.87	96.15	95.38	91.54
	No. cases	57	55	58	108	108	108
	No. controls	78	78	78	130	130	130

Table S1 Numbers of test positive cases and controls using CA19-9 37 U/mL and CA125 30 U/mL cut-offs
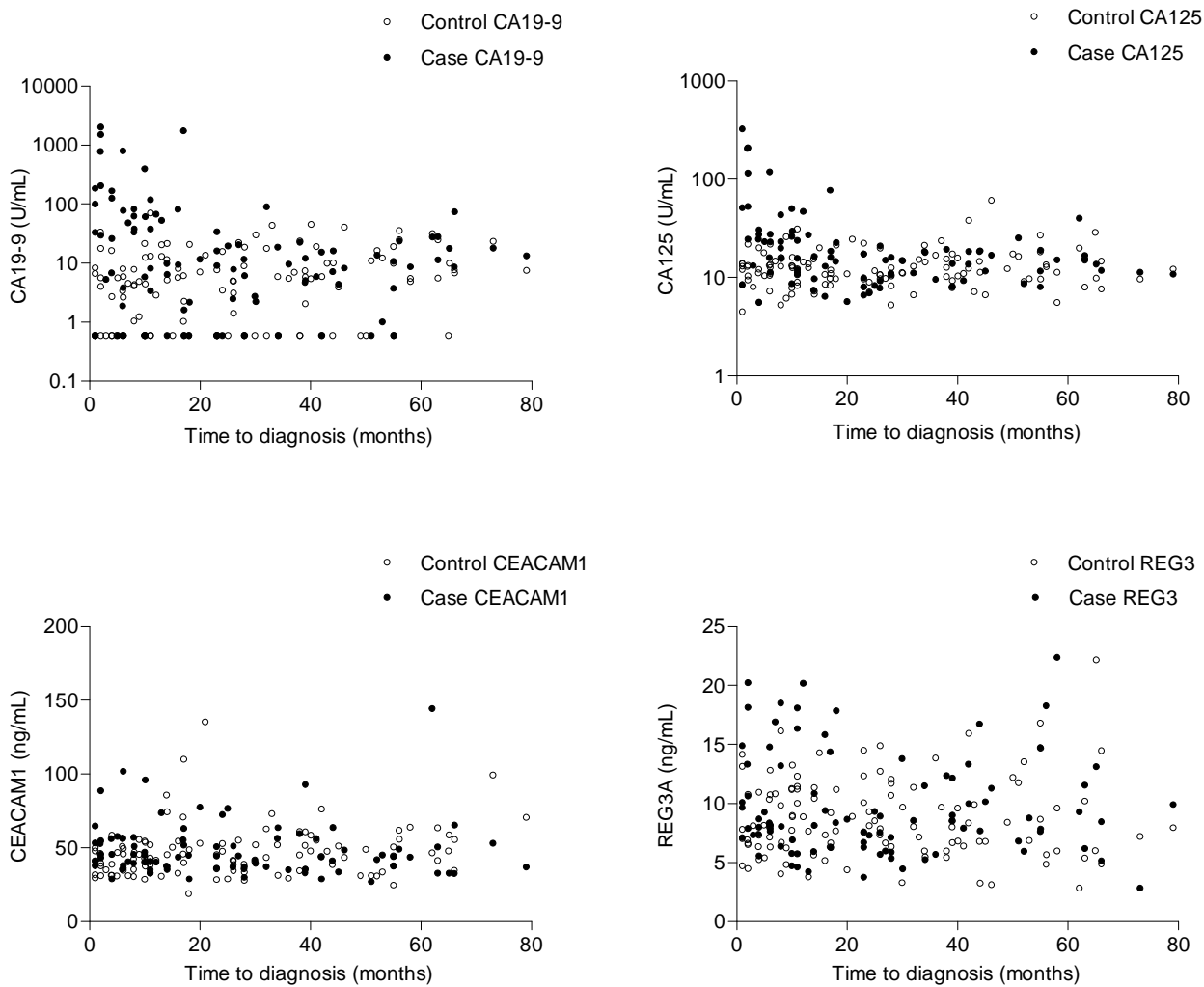
Figure S1 Scatter plots showing distribution of CA19-9, CA125, CEACAM1 and REG3A levels against time to diagnosis for discovery set. Zero represents the point of clinical diagnosis.
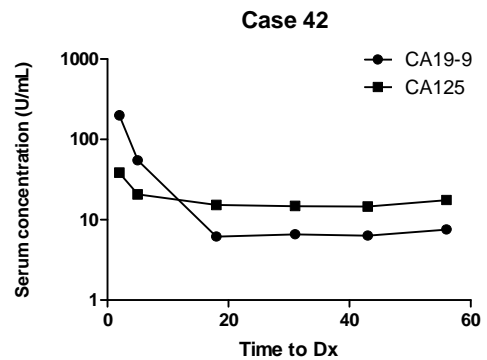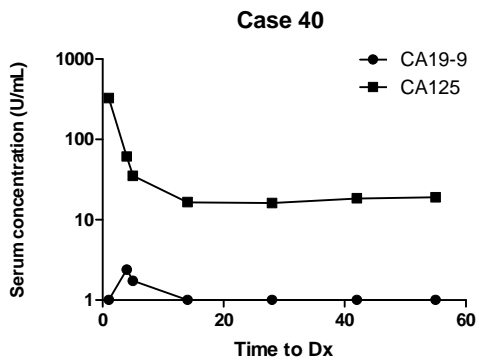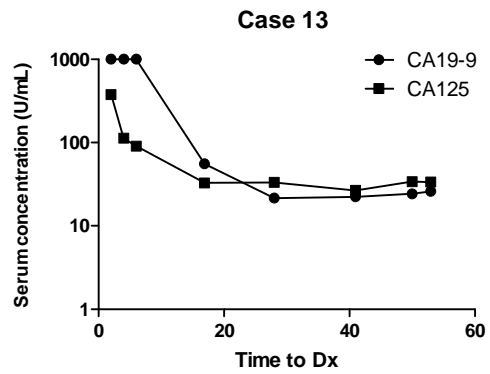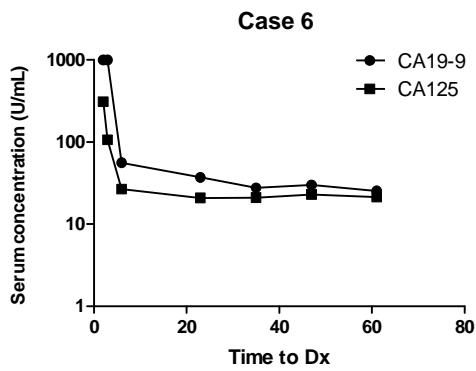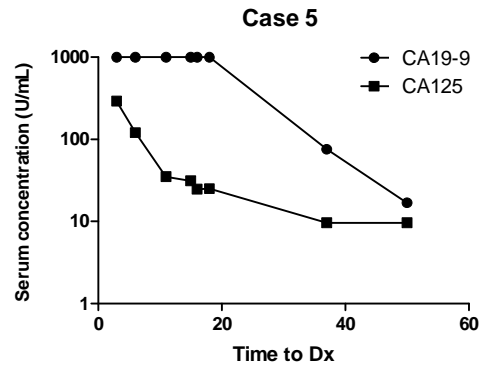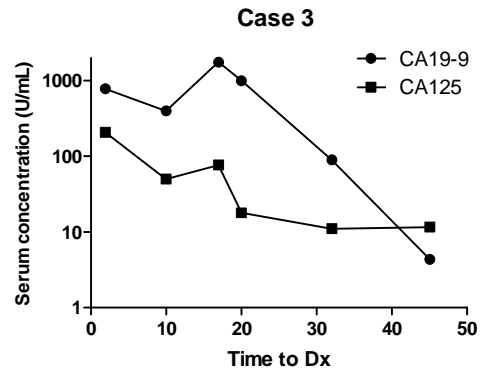
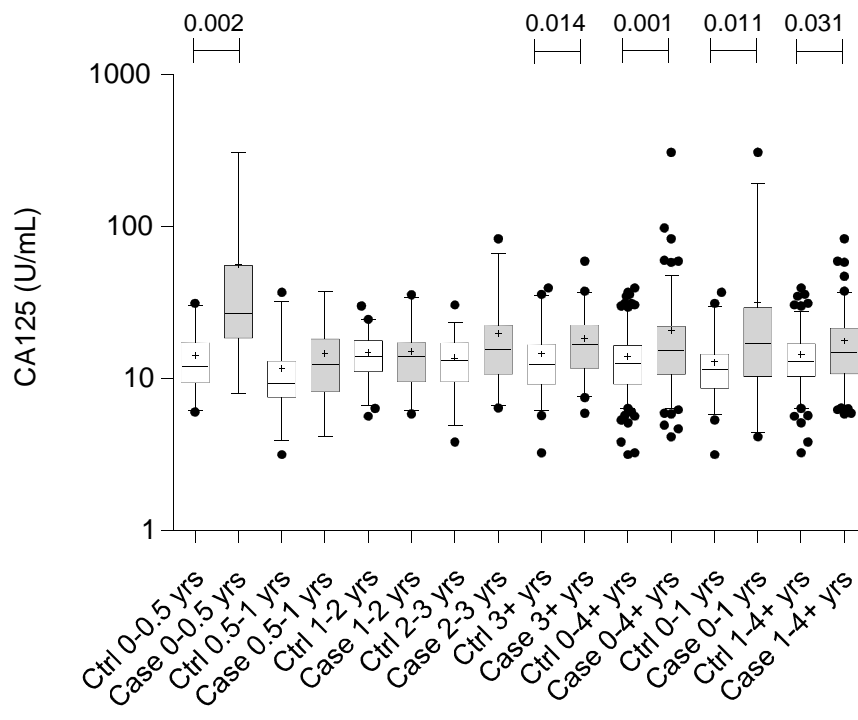**Figure S2** Examples of CA19-9 and CA125 levels in individual cases with serial/longitudinal samples.
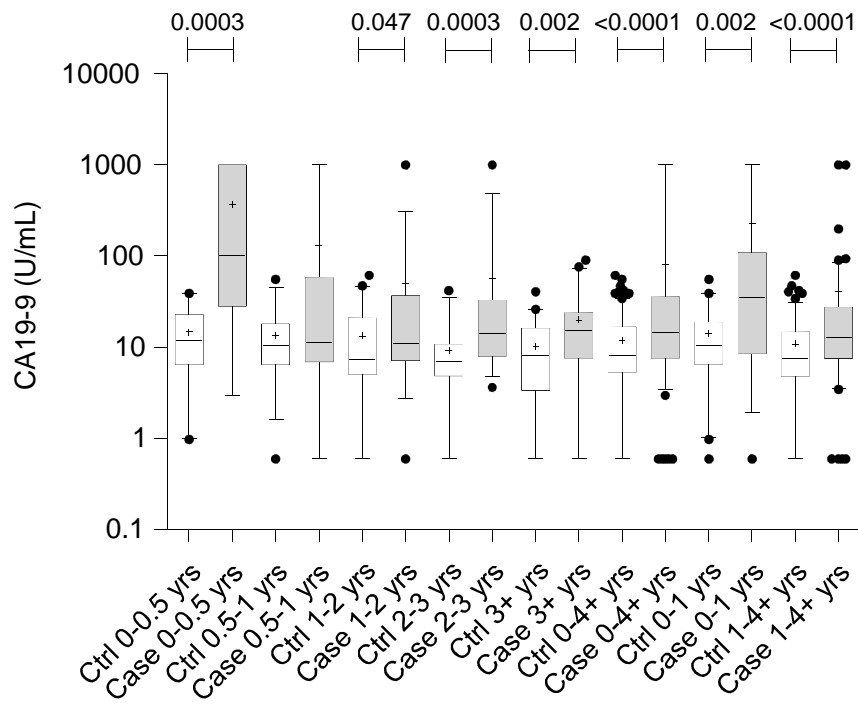
Figure S3 Box and whisker plots showing serum levels of CA19-9 and CA125 for case control validation samples grouped into different time to diagnosis groups. Whisker limits represent the 5th and 95th percentiles, the box limits represent interquartile range, the horizontal line the median and the cross the mean. Case and control groups were compared using the Mann-Whitney test; significant *P* values (<0.05) are shown above the plots.