Validation of Biomarkers That Complement CA19.9 in Detecting Early Pancreatic Cancer

Alison Chan¹,², Ioannis Prassas¹,², Apostolos Dimitromanolakis³, Randall E. Brand³, Stefano Serra¹,⁴, Eleftherios P. Diamandis¹,²,⁵, and Ivan M. Blasutig¹,⁵

Abstract

Purpose: Pancreatic ductal adenocarcinoma (PDAC) is a significant cause of cancer mortality. Carbohydrate antigen 19.9 (CA19.9), the only tumor marker available to detect and monitor PDAC, is not sufficiently sensitive and specific to consistently differentiate early cancer from benign disease. In this study, we aimed to validate recently discovered serum protein biomarkers for the early detection of PDAC and ultimately develop a biomarker panel that could discriminate PDAC from other benign disease better than the existing marker CA19.9.

Patients and Methods: We performed a retrospective blinded evaluation of 400 serum samples collected from individuals recruited on a consecutive basis. The sample population consisted of 250 individuals with PDAC at various stages, 130 individuals with benign conditions and 20 healthy individuals. The serum levels of each biomarker were determined by ELISAs or automated immunoassay.

Results: By randomly splitting matched samples into a training (n = 186) and validation (n = 214) set, we were able to develop and validate a biomarker panel consisting of CA19.9, CA125, and LAMC2 that significantly improved the performance of CA19.9 alone. Improved discrimination was observed in the validation set between all PDAC and benign conditions (AUCCA19.9+ = 0.80 vs. AUCCA19.9+CA125+LAMC2 = 0.87; P < 0.005) as well as between early-stage PDAC and benign conditions (AUCCA19.9+ = 0.69 vs. AUCCA19.9+CA125+LAMC2 = 0.76; P < 0.05) and between early-stage PDAC and chronic pancreatitis (CP; AUCCA19.9+ = 0.59 vs. AUCCA19.9+CA125+LAMC2 = 0.74; P < 0.05).

Conclusions: The data demonstrate that a serum protein biomarker panel consisting of CA125, CA19.9, and LAMC2 is able to significantly improve upon the performance of CA19.9 alone in detecting PDAC. Clin Cancer Res; 20(22); 5787–95. ©2014 AACR.

Introduction

Pancreatic cancer is the tenth most commonly diagnosed cancer in North America but it ranks fourth in cancer-related deaths (1, 2). In contrast to other major human malignancies (lung, breast, colon, and prostate) that have shown notable reductions in mortality rate over the past 30 years, pancreatic cancer has had minimal improvement in patients’ survival rate (1). The 5-year survival rate for pancreatic ductal adenocarcinoma (PDAC), the most common form of pancreatic cancer, improves from 2% to 23% if the disease is diagnosed at its localized stage compared with a distant metastatic stage (3). However, the late presentation of disease-specific symptoms often leads to missed or delayed diagnosis of PDAC, and at the time of diagnosis, approximately 80% of patients harbor aggressive and metastatic disease not suitable for surgical resection, the only potentially curative treatment available (4). These statistics emphasize the urgent clinical need to identify biomarkers that can detect PDAC early.

In terms of diagnosis, there are currently no sufficiently sensitive or specific screening tests for early detection of PDAC. Conventional imaging tools, including computerized tomography (CT) scanning, magnetic resonance imaging (MRI), endoscopic ultrasonography (EUS), and endoscopic retrograde cholangiopancreatography (ERCP), are not sensitive at detecting small premalignant lesions and are relatively costly, time-consuming, and invasive (5, 6). On the contrary, serum biomarkers are low cost, minimally invasive, and ideal for early diagnosis (7).

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

A. Chan and I. Prassas contributed equally to this article.

Corresponding Author: Ivan M. Blasutig, University Health Network, 200 Elizabeth Street, Room 3 EB 362, Toronto, ON, M5G 2C4, Canada. Phone: 416-340-4800, ext. 7199; Fax: 416-340-4215; E-mail:ivan.blasutig@uhn.ca
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standard serum biomarker carbohydrate antigen 19.9 (CA19.9) is used in the clinic only for disease monitoring and prognosis, has limited sensitivity in PDAC detection due to its absence in Lewis-negative individuals (5%–10% of Caucasian population), is minimally elevated in early pre-malignant disease, and is elevated in other benign conditions and multiple cancer types (2, 8, 9). Taken together, it is critical to discover novel biomarkers to complement CA19.9 to improve both its sensitivity and specificity.

In the pursuit of deciphering PDAC biomarkers, we have combined the following approaches: (i) integrative proteomic analysis of cell line conditioned media, pancreatic ascites, and pancreatic juice (10, 11); (ii) comparative proteomic analysis of PDAC tissues with adjacent benign tissues (12); and (iii) bioinformatics analysis of publicly available gene and protein databases for identification of pancreatic-specific proteins (13). Our multiple approaches enabled us to identify numerous biomarker candidates including anterior gradient homolog 2 (AGR2), regenerin islet-derived 1B (REG1B), syncollin (SYCN), laminin-γ3 (LAMC2), and cancer antigen 125 (CA125), all of which were subsequently validated in more than 400 samples (11, 12, 14). Notably, CA125 was rediscovered as "CUZD1 protein"; ref. 15. In this study, we took our top five candidates: AGR2, REG1B, SYCN, LAMC2, and CA125, and used them to perform a large blinded validation study using 400 patient plasma samples to evaluate their performance, individually and combined, in detecting early-stage PDAC.

Materials and Methods

Study population

Patients and control subjects were recruited on a consecutive basis from participating investigators in two major hospitals of the University of Pittsburgh Medical Centre (UPMC; Pittsburgh, PA) system including the UPMC Presbyterian and UPMC Shadyside campus. Subjects with a histologically or CT scan confirmed diagnosis of PDAC or with an abnormal abdominal imaging study (CT, MRI, magnetic resonance cholangiopancreatography, and EUS) were eligible for the study. Control subjects with a clinical diagnosis of a pancreas, liver, or intestinal condition, or being evaluated for nonpancreatic malignancies, were included in the study. Subjects under the age of 18 years old and those without informed consent were excluded. Any patients with a prior history of any other malignancy except nonmelanoma skin cancers within 10 years of treatment were not included. Healthy controls were eligible volunteers without any pancreatic conditions or malignant diseases.

All samples used in this study were obtained within a 4-year period from April 2008 to June 2012. Blood was collected in acid citrate dextrose (ACD) anticoagulant vacuum tubes and plasma samples were processed within 24 hours of blood draw. Blood samples were centrifuged at room temperature for 10 minutes (at 1,000 × g) to pellet the cells. Immediately following centrifugation, the plasma samples were aliquoted into 1 mL cryotubes and stored at –80°C until analysis in October 2012.

A subset of patients was selected from the available subject pool based on desired characteristics (prospective specimen collection, retrospective blinded evaluation). A total of 400 blinded plasma samples were obtained and samples within each group were randomly split into a training set (n = 186) and an independent validation set (n = 214). Overall, the 400 samples comprised 20 healthy individuals, 130 benign condition patients, 51 stage IA and IB, 150 stage IIb, and 49 stage IV PDAC patients. Details about the patient population are shown in Table 1. All samples were collected before any treatment following informed consent with an Institutional Review Board–approved protocol.

Measurement of markers in blood samples

All samples (n = 400) were analyzed using ELISA assays on the same day for each candidate, according to the "Standards for the reporting of diagnostic accuracy studies (STARD) initiative" (Supplementary Table S1; ref. 16).

Using commercially available sandwich enzyme-linked immunosorbent assays (ELISA) for AGR2, REG1B, SYCN, and LAMC2, purchased from USCN Life Sciences, the levels of these proteins were measured in duplicates according to the manufacturer's protocols. CA19.9 levels were measured using the Abbott Architect CA19.9XR immunoassay. CA125 values were, first, obtained as "CUZD1 values" from a commercial CUZD1 ELISA kit obtained from USCN Life Sciences. When we discovered that this commercial kit was, in fact, measuring CA125, we remeasured all available samples (251 of 400) with the Abbott Architect CA125 immunoassay. As expected, a strong linear log_{CA125}/log_{CUZD1} correlation curve was observed (Supplementary Fig. S4). CA125 values for the remaining 149 samples (which were depleted) were determined by extrapolation.
Before all sample analyses, AGR2, REG1B, SYCN, and LAMC2 ELISAs were first tested to optimize the analytic performances, to select appropriate controls (low, medium, and high), and the sample dilution factor to be used for each of the ELISA kits. Controls were used to assess the interplate variability.

Samples were diluted in assay buffer diluent as follows: 1:10 dilution for AGR2, 1:10,000 dilution for REG1B, 1:20 dilution for SYCN, 1:5 dilution for CUZD1, and 1:100 dilution for LAMC2. One hundred microliters of diluted sample was incubated in precoated ELISA 96-well plates along with standards for 2 hours in 37°C. After washing the wells, 100 μL of biotin-labeled polyclonal secondary antibody (detection reagent A) was added and incubated for another hour at 37°C. After washing, 100 μL of avidin-conjugated horseradish peroxidase (detection reagent B) was added and incubated for 30 minutes at 37°C. After a final washing step, 90 μL of tetramethylbenzidine (TMB) substrate was added to each well and incubated for approximately 10 to 15 minutes in the dark at 37°C until the second lowest standard could be distinguished from the blank by a change of color. Fifty microliters of stopping solution (sulfuric acid solution) was then added and the absorbance was measured using the PerkinElmer Envision 2103 Multilabel Reader at 450 nm wavelength, standardized with a background absorbance at 540 nm.

Interplate assay imprecision was assessed across the 12 plates used for each marker using three controls (low, medium, and high). The coefficient of variation (CV) was calculated for each marker. Overall, LAMC2, AGR2, and SYCN assays demonstrated acceptable reproducibility across 12 plates, with <20% CVs for all controls. REG1B assays were relatively poor, showing medium and high control CVs of 36% and 58%, respectively. As an additional quality control step, all samples were analyzed in duplicate to assess the intraplate variations. The mean and median CVs among duplicate samples ranged from 5% to 12% for all markers, which is indicative of good intraplate performance of the assays (Supplementary Table S2).

### Statistical analysis

Comparisons of levels of markers between groups were performed using the Mann–Whitney–Wilcoxon test. Mean level comparisons were performed using a t test and/or an ANOVA test.

The discriminatory ability of the biomarkers was assessed by building receiver operating characteristic curves (ROC) for individual markers and combined predictors. The diagnostic value of the markers was evaluated on the basis of area under the curve (AUC) and the evaluation of sensitivity and specificity at an optimal cutoff obtained by minimizing the total prediction error, by the following formula:

\[
\sqrt{\frac{1 - \text{sensitivity}}{\text{specification}}}^2 + (1 - \text{specificity})^2
\]

Confidence intervals (95% CI) for AUCs and P value for comparison between related ROC curves were performed using the method described by DeLong and colleagues (17).

### Logistic regression model building

Multiparametric models explored included logistic regression models using log2-transformed markers (see Supplementary Table S4 for model fit diagnostics). Logistic regression models with interaction terms and more advanced nonlinear classifiers such as Random Forests and Support Vector Machines (data not shown). Despite its

| Table 1. Sample characteristics and numbers in training and validation sets |
|---------------------------------|--------|--------|--------|
| Sample characteristics         | Training | Validation | Total |
| Healthy control                | 10      | 10      | 20     |
| Acute pancreatitis             | 6       | 23      | 29     |
| CP                             | 25      | 25      | 50     |
| CBDstones                      | 19      | 0       | 19     |
| Other benign conditions        | 15      | 17      | 32     |
| PDAC, stage IA                 | 4       | 5       | 9      |
| PDAC, stage IB                 | 3       | 5       | 8      |
| PDAC, stage II A               | 17      | 17      | 34     |
| PDAC, stage II B               | 62      | 88      | 150    |
| PDAC, stage IV                 | 25      | 24      | 49     |
| Total                          | 186     | 214     | 400    |
| Number of females/males        | 84/101  | 110/104 | 194/205|
| Median (mean) age              | 66.0 (63.0) | 64.0 (63.1) | 65.0 (63.1) |
| Smoking history<sup>b</sup>    | 35C/62P/88NE (1 unknown) | 43C/70P/74NE (2 unknown) | 78C/132P/162NE |
| Diabetic history<sup>c</sup>   | 53Y/131N (2 unknown) | 25Y/189N | 78Y/320N |

<sup>a</sup>CBD, common bile duct.
<sup>b</sup>C, current; P, past; NE, never.
<sup>c</sup>Y, yes; N, no.
Figure 1. Scatter plots of CA19.9, CA125, and LAMC2 in the training and validation cohorts. CA19.9 (A and B), CA125 (C and D), and LAMC2 (E and F) for training and validation cohorts, respectively. Black horizontal lines are medians. The clinical groups are shown on the x-axis and further described in the text.
simplicity, the logistic regression model demonstrated the best performance and was chosen as our main model for this article. Summary of model fitting diagnostics and parameters for our chosen models are shown in Supplementary Tables S4 and S5. FDR-adjusted \( P \)-values for model comparisons in the training set are shown in Supplementary Table S6. The reduced coefficient models evaluated for diagnostic performance are: (i) \( \text{CA19.9} + 1.11 \cdot \text{CA125} \), (ii) \( \text{CA19.9} + 0.202 \cdot \text{LAMC2} \), and (iii) \( \text{CA19.9} + 1.13 \cdot \text{CA125} + 0.143 \cdot \text{LAMC2} \).

Statistical analysis in the training set was performed while being blinded to clinical annotations of the validation set. Multiparametric prediction models were built based on the comparison of the benign vs. all PDAC groups in the training set, with \( P \) values adjusted for FDR by the Benjamini–Hochberg procedure (Supplementary Table S6). Once the optimal models were identified, clinical information for the validation samples were unblinded and the model predictions were evaluated. The primary measure for the three models was the comparison of the benign vs. all PDAC groups. Hypothesis testing was two-tailed, and \( P \) values of less than 0.05 were considered significant. Statistical analysis was performed in the R environment (version 2.15.2) available from http://www.R-project.org. ROC curve analysis and comparisons between ROC curves was performed using the pROC package (18).

**Association of markers with age and gender**

Pearson correlation was used to evaluate the correlation of markers with age, separately in the healthy and benign groups (Supplementary Table S7). Gender association was evaluated on the basis of a \( t \) test of marker values between males and females (Supplementary Table S7).

**Results**

**Performances of markers in the training and validation sets**

As individual markers, the performances of the five candidates were compared with CA19.9 in discriminating benign conditions versus PDAC and healthy controls versus PDAC in both training and validation cohorts (Figs. 1 and 2; Supplementary Table S3; Supplementary Figs. S1–S3). As single markers, CA125 and LAMC2 were the most promising of the five candidates. Their concentrations were significantly increased in PDAC cases compared with benign controls in both training and validation cohorts (\( P < 0.0001; \) Fig. 1 and Supplementary Table S3). The remaining three proteins, AGR2, REG1B, and SYCN, demonstrated poor discriminatory performances, both individually and as part of a marker panel, and were left out of subsequent analyses (Fig. 2; Supplementary Table S3; Supplementary Figs. S1 and S2). As shown in Table 2, the AUCs for CA19.9 and CA125 in discriminating all benign from all PDAC samples were comparable in the training (AUC_{CA19.9} = 0.85; AUC_{CA125} = 0.77) and validation sets (AUC_{CA19.9} = 0.80; AUC_{CA125} = 0.78). LAMC2 also showed comparable performance in the training set (AUC_{LAMC2} = 0.81); however, it demonstrated poorer performance in the validation set (AUC_{LAMC2} = 0.69). Similarly, in discriminating

![Figure 2](image_url)
benign from early-stage PDAC, the performance of the three markers was comparable in both the training (AUCA19.9 = 0.82; AUCA125 = 0.78; AUCLAMC2 = 0.73) and validation sets (AUCA19.9 = 0.69; AUCA125 = 0.72; AUCLAMC2 = 0.68). Finally, in discriminating patients with CP from patients with early-stage PDAC, both markers had similar performance to CA19.9 in the training (AUCA19.9 = 0.76; AUCA125 = 0.79; AUCLAMC2 = 0.64) and validation sets (AUCA19.9 = 0.59; AUCA125 = 0.75; AUCLAMC2 = 0.69).

Optimal cutoffs for each marker were obtained by minimizing the total prediction error as described in Materials and Methods. On the basis of the ROC analysis in the training set comparing all PDAC (n = 111) versus all benign conditions (n = 65), the optimum diagnostic cutoff for CA19.9 was 20.3 U/mL (sensitivity, 77.5%; specificity, 83.1%; Table 2). The optimum cutoff for CA125 was 17.9 U/mL (sensitivity, 70.3%; specificity, 75.4%) and for LAMC2 was 123.2 ng/mL (sensitivity, 70.3%; specificity, 87.7%).

As expected, CA19.9 displayed strong discriminatory performance in both the training and validation cohorts (Fig. 2; Table 2; Supplementary Fig. S2). However, if used at its clinically used cutoff (>37 U/mL), a total of 22 out of 130 patients (~17%) with benign disease would be falsely positive for CA19.9 (>37 U/mL), and 75 out 250 (30%) patients with PDAC would be missed (false negatives) by this marker. To compare the performances of CA19.9 (as a single marker) with the three-marker panel, multivariate models for various combinations of the three proteins (CA19.9, CA125, and LAMC2) were constructed on the basis of the comparison of all patients with PDAC versus benign controls in the training set and applied to the blinded validation set. Our proposed panel significantly improved the performance of CA19.9 in the primary measure (benign vs. all PDAC; Table 2 and Fig. 3) as well as the secondary measures (Table 2 and Supplementary Fig. S3). The power of distinguishing benign conditions from all PDAC cases increased from AUCA19.9 = 0.85 to AUCA19.9+CA125+LAMC2 = 0.93 in the training cohort and from AUCA19.9 = 0.80 to AUCA19.9+CA125+LAMC2 = 0.87 in the validation cohort (P < 0.005). Significant improvements were also shown in the validation cohort in discriminating all benign patients from those with early-stage PDAC (AUCA19.9 = 0.69 vs. AUCA19.9+CA125+LAMC2 = 0.76; P < 0.05) and in discriminating CP cases from patients with early-stage PDAC (AUCA19.9 = 0.59 vs. AUCA19.9+CA125+LAMC2 = 0.74, P < 0.05). In the last subgroup (CP vs. early-stage PDAC), the addition of CA125

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<th>Table 2. Performances of CA19.9, CA125, LAMC2, two- and three-marker models for diagnosis of PDAC</th>
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<td>Benign vs. all PDAC</td>
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<td>Benign vs. early-stage PDAC (stage IA, IB, and IIA)</td>
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<td>CP vs. early-stage PDAC (stage IA, IB, and IIA)</td>
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<td>Training</td>
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<td>AUC (95% CI)</td>
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NOTE: When used as single markers, the specificity/sensitivity for each protein was estimated on the basis of the following cutoffs: CutoffCA19.9 = 20.3 U/mL, CutoffCA125 = 17.9 U/mL, and CutoffLAMC2 = 123.2 ng/mL.

aP < 0.05 in comparison with CA19.9.
bP < 0.005 in comparison with CA19.9.
alone seems to account for most of the improvement displayed by the panel as the addition of LAMC2 did not add significant diagnostic information (Table 2).

To further investigate the complementarity of CA125 and LAMC2 with CA19.9, we assessed the performance of these two markers (individually or combined) in all patients with PDAC that were missed by CA19.9 based on the clinically used threshold of 37 U/mL. As shown in Table 3, CA125 and LAMC2 retained their ability in discriminating benign from patients with PDAC in this subpopulation of patients with PDAC lacking elevated CA19.9 in both the training (AUCCA19.9 = 0.59; AUCCA125+LAMC2 = 0.81; P < 0.0001) and validation cohorts (AUCCA19.9 = 0.54; AUCCA125+LAMC2 = 0.76; P < 0.0001). Discriminatory ability was also noticed between patients with CP and early-stage PDAC in both the training (AUCCA19.9 = 0.53; AUCCA125+LAMC2 = 0.84; P < 0.0001) and validation cohorts (AUCCA19.9 = 0.52; AUCCA125+LAMC2 = 0.73; P = 0.01).

Discussion

CA19.9 remains the only clinically used marker for management of PDAC (FDA-approved as a disease monitoring marker). In terms of disease detection, CA19.9 is neither

| Table 3. Performances of CA125, LAMC2 in diagnosis of CA19.9-negative PDAC patients |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                 | Training set    | Validation set  |                  |                  |                  |
|                                 | AUC (95% CI)    | P               | AUC (95% CI)    | P               |
| Benign vs. all PDAC             |                 |                 |                  |                  |                  |
| CA19.9                         | 0.59 (0.46–0.72)| 0.2             | 0.54 (0.41–0.66)| 0.5             |
| CA125                          | 0.73 (0.62–0.84)| 0.0003          | 0.78 (0.67–0.88)| <0.0001         |
| LAMC2                          | 0.76 (0.65–0.86)| <0.0001         | 0.64 (0.53–0.76)| 0.02            |
| CA125 + LAMC2                  | 0.81 (0.71–0.90)| <0.0001         | 0.76 (0.66–0.86)| <0.0001         |
| CP vs. early-stage PDAC        |                 |                 |                  |                  |                  |
| CA19.9                         | 0.53 (0.37–0.69)| 0.7             | 0.52 (0.35–0.70)| 0.8             |
| CA125                          | 0.72 (0.58–0.86)| 0.007           | 0.77 (0.64–0.90)| 0.002           |
| LAMC2                          | 0.79 (0.67–0.91)| 0.0002          | 0.62 (0.44–0.79)| 0.2             |
| CA125 + LAMC2                  | 0.84 (0.73–0.95)| <0.0001         | 0.73 (0.57–0.89)| 0.01            |

P values are calculated by the Wilcoxon test in the comparison between benign and cancer groups.
very sensitive (it is elevated mainly in late-cancer stages and up to 10% of the population genetica
ly negative) nor specific (elevated in nonpancreatic cancers and several benign conditions). Therefore, the identification of serum
markers that could aid in the detection of early-stage PDAC
remains a clear unmet need. Our group has used various
technologies to discover novel PDAC biomarkers and identi-
fied five proteins (AGR2, REG1B, SYCN, LAMC2, and
CA125) that carry significant diagnostic information for
the detection of PDAC (10–15). This current study is an
extensive blinded validation of these five markers, in addi-
tion to CA19.9, in a single set of patient samples with a focus
on their complementarity in the early detection of PDAC.
Our retrospective analysis revealed that CA125 and LAMC2
display strong diagnostic performances as individual serum
PDAC markers, but more importantly, our multiparametric
models demonstrated significant complementarity of these
two markers with CA19.9, especially in the detection of
early-stage PDAC (up to stage IIB) from benign conditions
(e.g., CP).

LAMC2 belongs to the laminin family of extracellular
matrix glycoproteins, which are major constituents of base-
ment membranes and have been implicated in many
tumor-related processes including cell adhesion, migration,
differentiation, and metastasis. At the gene level, LAMC2
expression has been inversely related to overall patient
survival (19). Moreover, LAMC2 overexpression has been
proposed as a poor prognostic indicator in patients with
late-stage PDAC (20). According to the Human Protein
Atlas (http://www.proteinatlas.org/), LAMC2 demonstrates
a very strong positivity in PDAC tissue sections. Further-
time, tissue expression databases, such as BioGPS
(http://biogps.org/#goto=welcome) and Tiger Expression Data-
base (http://bioinfo.wilmer.jhu.edu/tiger/), demonstrate
that pancreas is among the main LAMC2-expressing tissues.

CA125 is a high-molecular weight protein that in humans
is encoded by the MUC16 gene. It belongs to the mucin
superfamily, many members of which have been tested as
candidate markers for a plethora of cancer types. CA125 is
primarily known as a useful marker for the clinical man-
agement of ovarian cancer; however, accumulating evi-
dence reveals an increased expression of this antigen in the
serum of patients with PDAC (for example, see ref. 21).

Recent PDAC-related research suggests that it takes up to
da decade before the initial tumor acquires metastatic ability,
offering a long window of opportunity for early detection of
pancreatic cancer (22, 23). Considering the possibility that
no single marker possesses sufficient sensitivity and speci-
ficity for early diagnosis of PDAC, research interest has been
shifted into the development of biomarker panels
(7, 24, 25). In this study, we identify and validate a bio-
marker panel consisting of CA19.9, CA125, and LAMC2
that is better at detecting patients with PDAC than CA19.9
alone, most notably at early disease stages.

The journey for a biomarker from bench to clinic is long
and arduous and there remains many obstacles to overcome
(26, 27). Independent validation studies, using samples
collected and analyzed at multiple centers, will be necessary
before this panel can be brought into clinical use. Such
studies, as well as investigation of whether these two mar-
kers have the ability to complement CA19.9 in prognosis or
therapeutic PDAC monitoring are the main focus of our
ongoing research.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

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and do not necessarily represent the official views of National Cancer
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Authors’ Contributions

Conception and design: A. Chan, I. Prassas, R. Brand, I.M. Blasutig
Development of methodology: I. Prassas
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Chan, R. Brand
Analysis and interpretation of data (e.g., statistical analysis, biosta-
tistics, computational analysis): A. Chan, A. Dimitrakopoulou-Sktokou, L. Serra
Writing, review, and/or revision of the manuscript: A. Chan, I. Prassas, R. Brand, E.P. Diamandis, I.M. Blasutig
Administrative, technical, or material support (i.e., reporting or organi-
zation of data, constructing databases): I. Prassas, L. Serra
Study supervision: I. Prassas, E.P. Diamandis, I.M. Blasutig

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Validation of Biomarkers That Complement CA19.9 in Detecting Early Pancreatic Cancer

Alison Chan, Ioannis Prassas, Apostolos Dimitromanolakis, et al.


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