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Serum Biomarker Panels for the Detection of Pancreatic Cancer

Randall E. Brand[1], **Brian M. Nolen**[2,3], **Herbert J. Zeh**[4], **Peter J. Allen**[5], **Mohamad A. Eloubeidi**[6], **Michael Goldberg**[7], **Eric Elton**[7], **Juan P. Arnoletti**[8], **John D. Christein**[9], **Selwyn M. Vickers**[10], **Christopher J. Langmead**[11], **Douglas P. Landsittel**[12,13], **David C. Whitcomb**[3,13,14], **William E. Grizzle**[15], and **Anna E. Lokshin**[2,13,16,17]

Randall E. Brand: reb53@pitt.edu; Brian M. Nolen: nolanb@upmc.edu; Herbert J. Zeh: zehxhx@upmc.edu; Peter J. Allen: allenp@mskcc.org; Mohamad A. Eloubeidi: Eloubeidi@uab.edu; Michael Goldberg: mgoldberg@northshore.org; Eric Elton: eelton@northshore.org; Juan P. Arnoletti: parnolet@uab.edu; John D. Christein: jdc16@uab.edu; Selwyn M. Vickers: Vickers@umn.edu; Christopher J. Langmead: cjl+@cs.cmu.edu; Douglas P. Landsittel: landsitteldp@upmc.edu; David C. Whitcomb: whitcomb@pitt.edu; William E. Grizzle: wgrizzle@uab.edu; Anna E. Lokshin: lokshina@upmc.edu

[1] Division of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh and University of Pittsburgh Medical Center, Hillman Cancer Center, Suite 2.32, 5117 Centre Ave, Pittsburgh, PA 15213

[2] University of Pittsburgh Cancer Institute, Hillman Cancer Center, 5117 Centre Avenue 1.18, Pittsburgh, PA, 15213

[3] Department of Human Genetics, Graduate School of Public Health, University of Pittsburgh, A300 Crabtree Hall, 130 Desoto Street, Pittsburgh, PA 15261

[4] Department of Surgery, Hillman Cancer Center, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213

[5] Department of Surgery, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065

[6] Department of Medicine, Gastroenterology/Hepatology, University of Alabama at Birmingham, 701 19th Street South, LHRB 408, Birmingham, AL 35294

[7] Department of Medicine, Section of Gastroenterology, NorthShore University HealthSystems, 2650 Ridge Ave, Evanston, IL 60021

[8] Department of Surgery, General Surgical Oncology Section, University of Alabama at Birmingham, 1922 7th Avenue South, KB 321, Birmingham, AL 35294

[9] Department of Surgery, General Surgical Gastrointestinal Section, University of Alabama at Birmingham, 1922 7th Avenue South, KB 417, Birmingham, AL 35294

[10] Department of Surgery, University of Minnesota, MMC195, 420 Delaware, Minneapolis, MN, 55455

[11] Department of Computer Science and Lane Center for Computational Biology, Carnegie Mellon University, 7401 Gates-Hillman Center, 5000 Forbes Avenue, Pittsburgh, PA 15213

[12] Center for Research on Health Care Data Center, Institute for Clinical Research Education, University of Pittsburgh, 200 Meyran Ave, Suite 300, Pittsburgh, PA 15213

[13] Department of Medicine, School of Medicine, University of Pittsburgh, 1218 Scaife Hall, 3550 Terrace Street, Pittsburgh, PA 15213

Request for reprints: Anna E. Lokshin, University of Pittsburgh Cancer Institute, Hillman Cancer Center, Suite 1.19d, 5117 Centre Avenue, Pittsburgh, PA 15213, Phone: 412-623-7706, Fax: 412-623-1415, lokshina@pitt.edu.

[14] Department of Cell Biology and Physiology, School of Medicine, University of Pittsburgh, S362 Biomedical Science Towers, 3500 Terrace Street, Pittsburgh, PA 15261

[15] Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama 35294

[16] Department of Pathology, School of Medicine, University of Pittsburgh, S-417 BST, 200 Lothrop Street, Pittsburgh, PA 15261

[17] Department of Ob/Gyn, School of Medicine, University of Pittsburgh, 300 Halket Street Pittsburgh, PA 15213

Abstract

Purpose—Serum biomarker-based screening for pancreatic cancer could greatly improve survival in appropriately targeted high-risk populations.

Experimental Design—Eighty-three circulating proteins were analyzed in sera of patients diagnosed with pancreatic ductal adenocarcinoma (PDAC) (n=333), benign pancreatic conditions (n=144), and healthy control individuals (n=227). Samples from each group were split randomly into training and blinded validation sets prior to analysis. A Metropolis algorithm with Monte Carlo simulation (MMC) was used to identify discriminatory biomarker panels in the training set. Identified panels were evaluated in the validation set and in patients diagnosed with colon (n=33), lung (n=62), and breast (n=108) cancers.

Results—Several robust profiles of protein alterations were present in sera of PDAC patients compared to the Healthy and Benign groups. In a training set (n=160 PDAC, 74 Benign, 107 Healthy), the panel of CA 19-9, ICAM-1, and OPG discriminated PDAC patients from Healthy controls with a sensitivity/specificity (SN/SP) of 88/90%, while the panel of CA 19-9, CEA, and TIMP-1 discriminated PDAC patients from Benign subjects with a SN/SP of 76/90%. In an independent validation set (n=173 PDAC, 70 Benign, 120 Healthy), the panel of CA 19-9, ICAM-1 and OPG demonstrated a SN/SP of 78/94 while the panel of CA19-9, CEA, and TIMP-1 demonstrated a SN/SP of 71/89%. The CA19-9, ICAM-1, OPG panel is selective for PDAC and does not recognize breast (SP=100%), lung (SP=97%), or colon (SP=97%) cancer.

Conclusions—The PDAC-specific biomarker panels identified in this investigation warrant additional clinical validation to determine their role in screening targeted high-risk populations.

Keywords

Pancreatic Cancer; PDAC; Early Detection; Diagnosis; Serum Biomarkers; CA 19-9; Multimarker Panel

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States. In 2010, an estimated 43,140 people will be diagnosed with pancreatic cancer with a staggering 36,800 perishing from the disease [1]. Although a variety of tumors can arise in the pancreas, the vast majority of pancreatic tumors, 85–90%, are represented by a specific histological subtype termed pancreatic ductal adenocarcinoma (PDAC) [2]. The poor prognosis from PDAC is largely due to our inability to detect the cancer at an early stage when the option of curative resection remains available. Factors contributing to this difficulty include the inaccessible location of the pancreas deep in the abdomen, late-presenting clinical manifestations (e.g., weight loss, epigastric pain, or obstructive jaundice), and the early development of metastasis. As a result, the majority of pancreatic cancer patients present with unresectable disease leading to a median survival of 6 months and an overall 5-year survival of <5%[3]. In contrast, those few who present with small, surgically-resectable

cancers have a realistic chance of cure and a 5-year survival rate of 20–30%[4]. Owing to the low prevalence of PDAC, it is currently neither advisable nor cost effective to screen the general population [5]. Efforts are focused on early screening of selected high-risk-cohorts (more than 10-fold increased risk), who account for approximately 10% of patients with PDAC. These mainly consist of patients with a genetic predisposition for developing PDAC including individuals with a family history in multiple family members, patients with hereditary pancreatitis, Peutz-Jeghers syndrome, hereditary breast-ovarian cancer syndrome, or familial atypical multiple mole melanoma [6]. It has been calculated that a screening test with a sensitivity (SN) and specificity (SP) above or near 90% would benefit high-risk groups [5].

There are no reliable screening tests, either molecular or imaging based, for detecting pancreatic cancer in asymptomatic persons and the deep anatomic location of the pancreas makes detection of small localized tumors unlikely during routine abdominal examination. Tumor resolution is a critical factor in the early detection of pancreatic cancer as tumors as small as 2cm in diameter are frequently associated with metastatic disease [7]. Commonly used imaging studies (e.g., abdominal CT or MRI) in the setting of a high clinical suspicion of having PDAC are inadequate for diagnosing pancreatic cancer at an early stage since they do not reliably detect pancreatic tumors <1–2 cm in size [8]. More accurate tests such as endoscopic retrograde cholangiopancreatography (ERCP) and endoscopic ultrasound (EUS) are inappropriate for screening asymptomatic patients due to their invasiveness, cost and attendant clinical risks [9]. The mucin-associated carbohydrate antigen CA 19-9 is a biomarker of PDAC with limited clinical utility in the screening setting. CA 19-9 has demonstrated modest effectiveness in the screening of symptomatic individuals on an outpatient basis with a median SN of 79% (range 70–90%) and median SP of 82% (range 68–91%), however it has been shown to be ineffective in the mass screening of asymptomatic subjects [10]. The principal limitations of CA 19-9 include its frequent elevation associated with non-malignant conditions such as pancreatitis and obstructive jaundice, and its inability to detect many early stage malignancies [11]. CA 19-9 is also unsuitable for use in the estimated 5 to 10% of patients who are carriers of the Lewis-negative genotype and develop tumors that do not express the antigen [12].

These limitations of CA 19-9 have led investigators to search for alternative biomarkers for use in screening for PDAC. Such alternative serum biomarkers include TPA/TPS, macrophage inhibitory cytokine-1 (MIC-1), IGFBP-1, haptoglobin, SAA, TIMP-1, osteopontin (OPN), HE4, NGAL and others, however none of these have been clinically proven to be superior to CA 19-9 [11, 13–19]. Several groups have also reported on the performance of combinations of these markers[19–21]. These multiplexed biomarker approaches have demonstrated improved SN and SP for the detection of pancreatic cancer. For example, in a recent study, a panel of seven proteins (ALCAM, ICAM-1, LCN2, TIMP-1, REG1A, REG3, and IGFBP-4) with or without the addition of CA 19–9, selected based on findings in a mouse model, was able to discriminate human pancreatic cancer cases from matched controls in a small group of pre-symptomatic and pre-diagnostic blood samples [19]. In efforts to diagnose pancreatic cancer from benign and healthy controls, TIMP-1 was evaluated along with its target MMP-9 [21], while serum levels of OPN were capable of discriminating resectable PDAC from controls with a SN/SP of 80/97 [20].

Biomarker profiles indicative of a specific cancer include not only those factors produced by the tumor itself but also represent the systemic response to the growing tumor including acute phase reactants, inflammatory cytokines, growth and angiogenic factors, etc. Additionally, it is likely that levels of proteins secreted or released by the tumor will correlate together and therefore mitigate the advantage of their use in combination. We hypothesize that combinations of biomarkers originating from multiple tumoral and

extratumoral sites could offer superior diagnostic ability. Proteins representing the systemic response to malignancy may also reflect advancements in tumor development since the tumor relies on these exogenous factors for growth and spreading. In this study we utilized an extensive array of bead-based assays for a broad range of circulating proteins to evaluate serological alterations present in a diverse group of patients diagnosed with PDAC, non-malignant pancreatic disease, and healthy controls. We identify a number of significant differences in biomarker profiles associated with each diagnosis. Our underlying objective was the identification of a panel of serum biomarkers capable of detecting PDAC with high SN and SP. Such a panel would offer a non-invasive means of screening in appropriately targeted high-risk populations.

Materials and Methods

Patient populations

The study population was comprised of 333 patients with histologically diagnosed pancreatic ductal adenocarcinoma (PDAC), 144 patients with benign pancreatic conditions including acute or chronic pancreatitis, benign pancreatic cysts, or other benign pancreatic neoplasms, 227 healthy controls without a history of pancreatic diseases, and patients diagnosed with colon (n=33), lung (n=62), and breast (n=108) cancer (Table 1). The diagnoses of the patients with benign pancreatic diseases were clinical and guided by standard radiological imaging tests and determined by an experienced pancreatic expert. None of the patients with benign pancreatic diseases or the healthy controls had a history of any malignancies. Samples were obtained prior to any treatments from multiple sources (University of Pittsburgh, Sloan-Kettering Cancer Center, NorthShore University HealthSystems (NUH) -formerly known as Evanston Northwestern Healthcare, University of Alabama Birmingham, Fox Chase Cancer Center, Gynecological Oncology Group, Duke University) and were annotated with information regarding age, diagnosis, disease stage, histology, and grade. Written informed consent was obtained from each subject and the local institutional review boards approved theprotocols for use of each sample collection. All collection sites utilized a standardized protocol for sample collection. Samples were stored at −70°C or colder and shipped on dry ice overnight to UPCI. No more than 2 freeze/thaws were allowed. The Healthy, Benign, and PDAC subjects were randomly assigned to either the training or validation sets (Table 1) and validation samples were blinded until completion of the multivariate analysis.

Sources of bead-based immunoassays

The xMAP™ bead-based technology (Luminex Corp., Austin, TX) permits multiplexed analysis of several analytes in one sample. Eighty-three bead-based xMAP™ immunoassays for a diverse set of serum biomarkers were utilized in this study (Table 2). The immunoassays were either obtained from commercial suppliers or developed by the UPCI Luminex Core Facility as described previously [22]. Overall, 14 different multiplexed panels were used. For the UPCI core-developed assays, the intra-assay variability of each assay was 3.5–5% and inter-assay variability was 11–15%. Additional quality control data for each core-developed assay, including correlation with commercial ELISA can be found on the UPCI Luminex Core Facility website. All assays were performed at UPCI.

Multiplex biomarker analysis

The bead-based multiplex serum assays were performed in 96-well microplate format. All purchased assays were performed according to appropriate manufacturer's protocols. In-house assays, sample analysis, and curve-fitting were performed as previously described [22]. All biomarker data was normalized prior to statistical analysis according to a scaling procedure developed by our group to specifically account for variation acquired throughout

repeated experiments. A complete description of this procedure is provided in the Supplemental Material.

Statistical analysis of data

Descriptive statistics for serum concentrations of each of the tested biomarkers were calculated for each subject group using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). A 1-way Analysis of Variance (ANOVA) with Tukey's multiple comparison test was used to determine significance of any observed differences in serum biomarker concentrations between the groups. The minimum level of significance was taken as $p<0.05$. The False Discovery Rate (FDR) was controlled at 5% according to the method described by Benjamini and Hochberg[23]. Briefly, the individual p-values for each biomarker comparison were ranked from most to least significant. The ranked, unadjusted p-values were then compared to the statistic i*q/m, where i is the p-value rank, q is the FDR (0.05), and m is the total number of biomarker comparisons tested.

Multivariate analysis

All development of statistical models for distinguishing cases from controls was restricted to the training set until one panel and one model of combining the candidate biomarkers in the panel were selected. A Metropolis algorithm with Monte Carlo simulation (MMC) was utilized for analysis of the data as previously described [22]. Using this algorithm, all possible panels consisting of 2, 3 and 4 biomarkers were evaluated for SN at 90% SP in the training set. For each panel size, the 500 panels with the best SN at 95% SP on the full data set we re-estimated the SN with cross-validation. For cross-validation, 20% of subjects were randomly excluded from the data set and the rest used as at training set to build the optimal Scoring Function (SF). The resultant model was applied to the excluded subjects, and this process was repeated 400 times which was sufficient to obtain a smooth averaged ROC curve. For each comparison (PDAC vs. Healthy, PDAC vs. Benign), a single multimarker panel demonstrating the best SN at 90% SP as determined from the ROC curves was validated in an independent, blinded validation set. Following the classification of each sample in the validation set, the samples were unblinded and the diagnosis assigned by the MMC algorithm was compared to the actual diagnosis for calculation of SN/SP. The top performing multimarker panels for the discrimination of PDAC vs. Healthy were further evaluated for cancer selectivity in patients diagnosed with colon, lung, and breast cancer.

Results

Univariate analysis of biomarker levels in patients diagnosed with PDAC, benign pancreatic disease, and healthy controls

Serum levels of each biomarker were compared between the PDAC, Benign, and Healthy subject groups and the presented data reflect the inclusion of all subjects, after unblinding, in order to increase the statistical power of comparisons. Of the 83 biomarkers evaluated, 42 were found to differ significantly between the PDAC patients and the Healthy and Benign groups (Table 3). Of these 42 biomarkers, 33 were found to be increased in the PDAC group in comparison to the Healthy group, while 9 were found to be decreased. In the comparison between the Benign and PDAC patient groups, 15 biomarkers were observed at higher concentrations in the PDAC group while 5 biomarkers were found in lower levels. With the exception of CEA and TIMP-3 all observed trends in biomarker levels were consistent between the two comparisons.

We noted a discrepancy in the age distribution between the Healthy, Benign, and PDAC subjects with the Healthy and Benign groups tending to be younger (Table 1). To further assess this discrepancy we conducted an analysis of age-related biomarker levels within the

Healthy and Benign subjects. None of the biomarkers were differed significantly between the age-defined subgroups (cutoff of 55 years) of Benign subjects. Significant differences were identified between subgroups of healthy controls defined as ≥55 years of age and <55 years of age for endostatin, LH, MIF, and TNF-RI (Supplemental Table S1, Figure S1).

Evaluation of Source Bias

To analyze bias associated with variations in sample collection procedures at different centers, circulating levels of the 12 most informative biomarkers: CA 19-9, OPG, OPN, ICAM-1, TIMP-1-4, SAA, ApoA1, TIMP-2, and CRP were analyzed in serum samples obtained from healthy individuals by 1-Way ANOVA with Tukey's Multiple Comparison Test (Supplementary Figure S2). Only CRP was significant in this analysis, however the magnitudes of the biases (6–7%) were small compared to the differences between cancers and control subjects (86%). All observed trends in biomarker differences observed in the univariate analysis of PDAC vs. Healthy were consistently present throughout the sampling sites. We concluded from this analysis that source bias was a minimal factor in our analysis.

Multivariate Analysis of Biomarker Levels

The PDAC, Benign, and Healthy subject groups were each split into two sets termed training and validation on a random basis (Table 1). All development of multimarker panels using the MMC algorithm was restricted to the training sets. Our analysis identified the highest performing 2, 3, and 4-biomarker panels trained on either the PDAC (cases) vs. Healthy (controls) groups or the PDAC (cases) vs. Benign (controls) groups. The 21 best 3-biomarker panels for each comparison are shown in Table 4 along with the performance of CA19-9 alone. Each of these panels outperformed all possible 2-biomarker combinations while the addition of a fourth biomarker did not improve performance (Supplementary Table S2). Seven of the panels were identified in both comparisons and these are designated by italics in Table 4. One optimal panel was chosen for each comparison which demonstrated the highest SN at 90% SP. The performance of each of these panels in the training set was compared to CA 19-9 alone by ROC analysis (Figure 1A, B). In the comparison of PDAC vs. Healthy, the selected combination of CA19-9, ICAM-1 and OPG demonstrated improved performance over CA 19-9 alone in terms of AUC (0.93 vs. 0.83) and SN (87.5% vs. 57.2%) at 90% SP. In the comparison of PDAC vs. Benign, the selected combination of CA 19-9, CEA and TIMP-1 demonstrated an improvement over CA 19-9 alone in terms of AUC (0.86 vs. 0.82) and SN (75.8% vs. 56.4%) at 90% SP.

Next, the two optimal panels were validated in an independent blinded validation set (Table 1) containing Healthy, Cancer, and Benign samples (Figure 1C, D). Each validation sample was diagnosed as cancer or control (non-cancer) using the scoring function (SF) assigned to each biomarker panel following the MMC training analysis, and each diagnosis was compared with the clinical diagnosis after unblinding. The diagnostic performance of each panel in the validation set was compared to CA 19-9 alone by ROC analysis (Figure 1C, D). In the independent validation set, the combination of CA19-9, ICAM-1 and OPG offered SN=78% at 94.1% SP (AUC=0.91) for the discrimination of PDAC vs. Healthy compared to SN=51.4% at 90% SP (AUC=0.82) for CA 19-9 alone. In the comparison of PDAC vs. Benign, the combination of CA 19-9, CEA and TIMP-1 demonstrated an improvement over CA 19-9 alone in terms of AUC (0.83 vs. 0.78) and SN/SP (71.2%/88.6% vs. 52.1%/90.2%).

Cancer Selectivity of Multimarker Panels for Pancreatic Cancer

We utilized the MMC algorithm to evaluate the cancer specific selectivity of the biomarker panels identified in the multivariate analysis of PDAC vs. Healthy (Table 4). Each panel was applied to several groups of sera obtained from patients diagnosed with colon (n=33), lung (n=62), and breast (n=108) cancer and the percentage of each group diagnosed as non-

PDAC was determined using the MMC algorithm (Table 5). In this analysis, the validated panel of CA 19-9, ICAM-1, and OPG classified 97% of colon cancer sera, 97% of lung cancer sera, and 100% of breast cancer sera as non-PDAC. Several additional panels performed equally well in this analysis.

Discussion

In the present study, we identify several 3-biomarker panels offering high SN/SP and significant improvement over CA 19-9 alone for the discrimination of PDAC from healthy controls and benign subjects. To the best of our knowledge, these results represent the most advanced validated biomarker discovery effort aimed at the development of improved screening methodologies for the detection of pancreatic cancer. Since the incidence of pancreatic cancer in the general population (~1% lifetime risk) is too low to warrant screening, strategies are being investigated to define patient cohorts in which the positive predictive value for early-stage pancreatic cancers and advanced precursor lesions is high enough to justify more costly and invasive testing [24]. Therefore, our study intended to develop a panel of biomarkers that can be used to screen populations at an increased risk for the development of pancreatic cancer.

At present, high risk populations are not readily available for the purpose of pancreatic cancer screening. Pancreatic cancer-prone families are the most widely accepted population of this type to date, but only account for 5 to 10% of all pancreatic adenocarcinomas. Furthermore, the genetic factors underlying this predisposition are uncharacterized in the majority of these families. As it would take many years to follow a cohort of these patients to determine which unaffected individuals in these familial pancreatic cancer kindreds would develop PDAC, it is not practical to use this population for biomarker discovery. However, many centers are actively investigating risk stratification models that will take into account genetic factors along with epidemiological information, such as smoking and alcohol use, or clinical history including diabetic status or history of gastric ulcer. This should provide appropriate populations with a high enough incidence of pancreatic adenocarcinoma to warrant surveillance.

The multimarker panels identified in our analysis were highly discriminatory for PDAC vs. Healthy subjects demonstrating sensitivities ranging from 77–88% with 90% SP and a lower discriminatory power of 70–76% with 90% SP for PDAC vs. Benign. The optimal panel identified in the comparison of PDAC vs. Healthy comprised of CA 19-9, ICAM-1 and OPG demonstrated significant improvement over CA 19-9 by ROC analysis, whereas the best panel for discrimination of Benign from PDAC did not offer a substantial improvement in terms of SN over CA 19-9 at a level of SP above 80% (Figure 1). The AUC values we observed for CA 19-9 were within the reported range (0.72–0.84) of several recent studies[25–26]. The panel of CA 19-9, ICAM-1 and OPG also demonstrated a high level of cancer selectivity when applied to colon, lung, and breast cancers. The relative performance of these biomarker panels coupled with the small number of analytes required to achieve that performance make them attractive candidates in the development of early detection strategies.

In the course of our evaluation, we have identified number of proteins that differ significantly in the sera of patients diagnosed with PDAC and healthy controls, and many of these were also significant in comparison to the Benign group with considerable reproducibility in biomarker trends across the multiple sampling sites. To the best of our knowledge, 19 of the observed associations between serum biomarker levels and PDAC have not been described previously: GH, PRL, PTH, sFas, sFasL, MMP-2, TIMP 2-4, MPO, EGFR, ApoAI-II, ApoCIII, OC, GLP-1, HE4, and TGII. Although recent reports have

described significant associations between serum levels of OPG [27] and pancreatic cancer, to the best of our knowledge, our report is the first to characterize its diagnostic capacity. The significant biomarkers include representatives from a diverse set of biological families, particularly proteins with functions in such critical aspects of tumor development as growth, angiogenesis, metastasis, inflammation, etc., and encompass an array of factors likely to originate from the developing tumor, the tumor microenvironment and components of the systemic host response to the malignancy.

In the current study, PDAC was associated with circulating alterations of a number of known mediators of inflammatory processes and acute phase reactants. The relationship between tumorigenesis and inflammation has become a central theme in anticancer research and is the focus of increasing interest within the setting of pancreatic cancer. Malignant transformation can be closely associated with chronic infection and inflammation, while elevated levels of pro-inflammatory proteins could play a role as tumor promoters [28]. It has also been clear for some time now that a number of pro-inflammatory gene products including acute-phase reactants can also be produced by components or tumor microenvironment and tumor cells themselves to further mediate tumor growth [29]. For example, elevated circulating levels of SAA could reflect not only hepatic synthesis as part of the acute-phase response, but also increased release of these proteins by cancer cells, and a possible role for SAA in tumor growth, metastasis, and neovascularization has been investigated [30–31]. Therefore, these pro-inflammatory proteins could potentially be utilized as cancer biomarkers. In fact, it has been demonstrated that serum SAA levels are elevated in a broad spectrum of neoplastic diseases [5, 32] suggesting that it could act at least as a non-specific tumor marker. The combination of CRP, another acute-phase reactant, and sIFNα/βR was demonstrated to diagnose gastrointestinal and hepatobiliary-pancreatic cancer with a SN/SP of 94.6/88 [33]. The production of pro-inflammatory cytokines by tumor and stromal cells, regulated by NF-kB, has been observed to mediate tumorigenic effects in the pancreas as part of a characteristic desmosplastic reaction [34]. We observed altered levels of the NF-kB responsive cytokines TNFα and IL-8 which have been specifically shown to inhibit apoptosis and increase invasiveness of pancreatic cancer cells, respectively [35–36]. Also notably altered was the ROS-mediating enzyme, MPO. MPO has demonstrated involvement in the generation of DNA strand breaks, sister chromatid exchanges, mutations, and the formation of DNA adducts [37], and is associated with smoking, a known risk factor of pancreatic cancer [38].

Our findings also support several hypotheses regarding the role of specialized pathways related to obesity, bone homeostasis, and tissue remodeling, in pancreatic cancer. These findings include alterations in number of apolipoproteins and adipokines, modulators of lipid and insulin metabolism. ApoA1 may represent an emerging biomarker of malignancy as circulating levels of ApoA1 have recently been linked to several cancer types including pancreatic [39]. The adipokines, Adiponectin and Leptin are currently under intense scrutiny stemming from links to obesity, inflammation and malignancy [40]. We observed altered serum levels of OPN, a biomarker previously associated with pancreatic cancer [11], and several other bone related factors, OC and OPG. OPG is a secreted member of the tumor necrosis factor receptor superfamily, and altered serum levels of OPG have been associated with several malignancies including colorectal cancer, pancreatic cancer, liver metastases, multiple myeloma, Hodgkin's disease, and non-Hodgkin's lymphoma [27]. We report dysregulation of several metastasis-related proteins in PDAC. The MMPs and their natural inhibitors, TIMPs, have well-described tumorigenic roles and our observations regarding MMP-2 and TIMPs 1-4 in pancreatic cancer patients are consistent with previous findings [41]. The cellular adhesion mediator ICAM-1 has been previously linked to the development of PDAC [42] and was utilized as a part of multimarker panel for the classification of PDAC from healthy controls [19]. The expression of ICAM-1 is associated with PDAC cell

sensitivity to T-cell-based immunotherapy *in vitro* [43], and prognostic significance of preoperative sICAM-1 levels in several cancers has been demonstrated [44–46]. Correlation of TGII with metastatic properties of several cancers has been suggested [47–48], and its role as a tumor marker in the development and progression of pancreatic cancer has been well documented [49–50]. We also observed the dysregulation of a number of growth factors, receptors and mediators of angiogenesis including EGFR, ErbB2, Angiostatin, Endostatin, Thrombospondin, and IGFBP-1.

In summary, an extensive analysis of circulating biomarkers in patients diagnosed with PDAC and benign pancreatic conditions resulted in a robust profile of alterations in biomarkers in PDAC patients, offering improved insights into the network of factors involved in the process of pancreatic tumorigenesis. A number of the alterations we identify are novel with regard to associations with PDAC as is the expanded use of several previously characterized serum biomarkers for diagnostic purposes. Although the ideal biomarker test would recognize premalignant conditions, to the best of our knowledge, such requisite retrospective samples are presently not available for biomarker discovery. Therefore, the current study was guided by the assumption that a subset of those biomarkers demonstrating differential expression in developed cancer would also be altered in premalignant conditions. Thus, our analysis of biomarker alterations present in serum at the time of diagnosis should pave the way for the subsequent identification of biomarkers of preneoplastic disease. Additionally, biomarker panels discriminating PDAC from benign disease may offer a high level of clinical utility in combination with conventional imaging modalities to provide a diagnostic role as opposed to the aforementioned screening application. This study presents proof-of-principle of the utility of a multiplexed evaluation of circulating biomarkers for the identification and validation of multimarker panels with high classification power for PDAC. Further studies employing this type of approach may result in the identification of more robust panels for both screening and diagnosis of PDAC.

These findings represent an evaluation of biomarkers potentially related to pancreatic cancer and do not promote the use of any individual biomarker considered herein for diagnostic purposes. Moreover, while the nature of our investigation does not permit the identification of specific mechanistic links between any particular biomarker and the development of pancreatic cancer, our results do provide a sound basis for subsequent targeted analyses of pancreatic cancer biomarkers.

Statement of Translational Relevance

Specific challenges associated with pancreatic cancer including ubiquitous symptomatic presentation, deep anatomical location, and aggressive etiology, have greatly hindered efforts to combat the disease. Although the low incidence of the disease in the general population renders population-based screening impractical, screening could greatly improve survival in appropriately targeted high-risk populations. The current absence of reliable biomarker testing for pancreatic cancer mandates the development of novel strategies for identifying and characterizing additional biomarkers. The evaluation of serum biomarker levels in patients diagnosed with pancreatic cancer and a spectrum of benign pancreatic conditions presented here provides compelling evidence for the emerging role of blood-based screening in the clinical management of this disease. These findings not only include the identification of multimarker panels capable of discriminating pancreatic cancer from benign conditions and healthy controls with high sensitivity and specificity, but also offer improved insight into the complex network of factors involved in pancreatic tumorigenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of Abbreviations

ACTH	adrenocorticotropic hormone
AFP	alpha-fetoprotein
CRP	C-reactive protein
FSH	follicle stimulating hormone
GH	growth hormone
GIP	gastric inhibitory polypeptide
GLP-1	glucagon-like peptide
GM-CSF	granulocyte macrophage colony stimulating factor
ICAM-1	inter-cellular adhesion molecule 1
IGFBP-1	insulin-like growth factor binding protein 1
LH	luteinizing hormone
MIF	macrophage inhibitory factor
MMC	Metropolic algorithm with Monte Carlo simulation
MMP	matrix metalloproteinase
MPO	myeloperoxidase
OC	osteocalcin
OPG	osteoprotegerin
OPN	osteopontin
PDAC	pancreatic ductal adenocarcinoma
PP	pancreatic polypeptide
PRL	Prolactin
PTH	parathyroid hormone
PYY	peptide YY
ROC AUC	receiver operator characteristic area under curve
SAA	serum amyloid A
SAP	serum amyloid P
SN	sensitivity
SP	specificity
TGII	tissue transglutaminase (transglutaminase II)

TIMP	tissue inhibitor of metalloproteinases
tPAI-1	tissue plasminogen activator inhibitor 1
TSH	thyroid stimulating hormone
VCAM-1	vascular cell adhesion molecule 1

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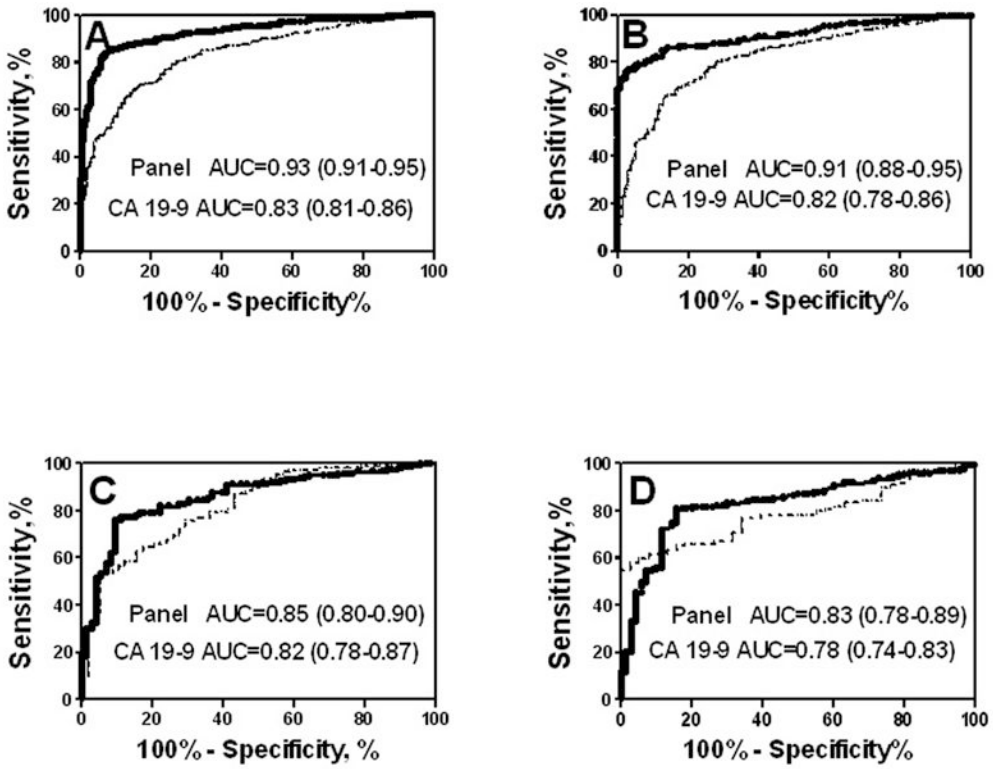

Figure 1. Receiver operator characteristic curves (ROC) for diagnosis of PDAC vs. Healthy controls and Benign cases

A,B, The diagnostic performance of the CA 19-9, ICAM-1, OPG combination (solid line) and CA 19-9 alone (dotted line) for the discrimination of PDAC vs. Healthy in the training set **(A)** and in the independent validation set **(B). C,D,** The diagnostic performance of the CA 19-9, CEA, TIMP-1 combination (solid line) and CA 19-9 alone (dotted line) for the discrimination of PDAC vs. Benign disease in the training set **(A)** and in an independent validation set **(B).** Areas under curve (AUC) with 95% CI are presented.

Table 1

Clinical Characteristics of Study Population

Training Set										
Healthy			**Benign**				**PDAC**			
Source	**Age Range (Median)**	**Gender**	**Source**	**Age Range (Median)**	**Gender**	**Dx**	**Source**	**Age Range (Median)**	**Gender**	**Stage**
UPCI n = 45	24–69 (53)	M = 36 F = 9	UAB n = 57	32–83 (55)	M = 23 F = 34	C = 31 P = 17 O = 9	NUH n = 57	49–91 (73)	M = 29 F = 28	I = 2 II = 18 III = 1 IV = 34 U = 2
FCCC n = 35	39–87 (66)	M = 1 F = 34	NUH n = 17	23–83 (66)	M = 6 F = 11	C = 1 P = 8 O = 8	UAB n = 49	42–89 (68)	M = 20 F = 29	I = 1 II = 3 III = 2 IV = 3 U = 40
NUH n = 19	33–77 (55)	M = 6 F = 13					SKCC n = 36	45–83 (64)	M = 17 F = 19	I = 0 II = 12 III = 5 IV = 6 U = 13
GOG n = 8	18–61 (44)	M = 0 F = 8					UPCI n = 18	49–87 (70)	M = 9 F = 9	I = 1 II = 0 III = 8 IV = 1 U = 8
Total Healthy n = 107	18–87 (58)	M = 43 F = 64	Total Benign n = 74	23–83 (56)	M = 29 F = 45	C = 32 P = 25 O = 17	Total PDAC n = 160	42–91 (69)	M = 75 F = 85	I = 4 II = 33 III = 16 IV = 44 U = 63
Validation Set										
Healthy			**Benign**				**PDAC**			
Source	Age Range (Median)	Gender	Source	Age Range (Median)	Gender	Dx	Source	Age Range (Median)	Gender	Stage
UPCI n = 46	20–76 (51)	M = 34 F = 12	UAB n = 54	43–85 (57)	M = 35 F = 19	C = 16 P = 19 O = 19	UAB n = 55	44–91 (67)	M = 36 F = 29	I = 0 II = 6 III = 4 IV = 6 U = 39

Training Set

Healthy			Benign				PDAC			
Source	**Age Range (Median)**	**Gender**	**Source**	**Age Range (Median)**	**Gender**	**Dx**	**Source**	**Age Range (Median)**	**Gender**	**Stage**
FCCC n = 37	37–84 (65)	M = 6 F = 31	NUH n = 16	35–86 (75)	M = 7 F = 9	C = 2 P = 8 O = 6	SKCC n = 52	45–83 (65)	M = 22 F = 30	I = 0 II = 16 III = 10 IV = 2 U = 24
NUH n = 27	34–87 (54)	M = 5 F = 22					NUH n = 46	42–92 (74)	M = 22 F = 24	I = 3 II = 14 III = 0 IV = 29 U = 0
GOG n = 10	39–56 (51)	M = 0 F = 10					UPCI n = 20	29–82 (64)	M = 8 F = 12	I = 1 II = 1 III = 6 IV = 4 U = 8
Total Healthy n = 120	20–87 (54)	M = 45 F = 75	Total Benign n = 70	35–86 (66)	M = 42 F = 28	C = 18 P = 27 O = 25	Total PDAC n = 173	29–92 (68)	M = 88 F = 95	I = 4 II = 37 III = 20 IV = 41 U = 71

Non-PDAC Cancer Set

Colon			Lung				Breast			
Source	Age Range (Median)	Gender	Source	Age Range (Median)	Gender	Dx	Source	Age Range (Median)	Gender	Dx
UPCI n = 33	47–89 (64.5)	M = 18 F = 15	UPCI n = 62	46–99 (68)	M = 30 F = 32	Ad = 33 Sq = 29	Duke n = 108	52–82 (63)	F = 108	IDC = 108

Sources: UPCI – University of Pittsburgh Cancer Institute; FCCC – Fox Chase Cancer Center; NUH – NorthShore University HealthSystems; GOG – Gynecological Oncology Group; UAB – University of Alabama-Birmingham; SKCC – Sloan-Kettering Cancer Center; Duke – Duke University; **Benign Dx:** C – Benign Cyst; P – Pancreatitis;O – Other benign neoplasm; **PDAC Dx:** U – stage unknown; Ad – adenocarcinoma; Sq – squamous cell carcinoma; IDC – invasive ductal carcinoma

Table 2

Complete List of Multiplexed Biomarker Assays

Biological Groups	Markers
Tumor Markers	Alpha-fetoprotein (AFP), CA 19-9, CA 125, CA 15-3, CA72-4, CEA
Hormones	adrenocorticotropic hormone (ACTH), follicle stimulating hormone (FSH), Growth hormone (GH), Insulin, luteinizing hormone (LH), Prolactin (PRL), parathyroid hormone (PTH), thyroid stimulating hormone (TSH), Pancreatic Polypeptide (PP), Peptide YY (PYY) (total)
Apoptotic Factors	Cytokeratin 19 (Cyfra 21-1), DR5, sFas, sFasL
Cell Adhesion	ICAM-1, VCAM-1
Proteases/Inhibitors	Kallikrein 10, MMP-2, 3, 9, TIMP-1-4
Cytokines/Chemokines/Receptors	CD40L (TRAP), Eotaxin-1, Fractalkine, IFNγ, IL-10, IL-12p70, IL-13, IL- 1b, IL-1Rα, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-6R, IL-7, IL-8, IP-10, Macrophage inhibitory factor (MIF), MIP-1b, TGFα, TNFα, TNF-RI, TNF- RII
Growth/Angiogenesis Factors/Receptors	Angiostatin, EGFR, Endostatin, ErbB2, GM-CSF, IGFBP-1, Thrombospondin
Adipokines	Adiponectin, Leptin
Apolipoproteins	ApoAI, ApoAII, ApoB, ApoCII, ApoCIII, ApoE
Other	CRP, Ghrelin (active), Gastric inhibitory polypeptide (GIP) (total), Glucagon like peptide 1(GLP-1) (active), HE4, Mesothelin, Osteocalcin (OC), osteoprotegerin (OPG), Osteopontin (OPN), Serum amyloid A (SAA), Serum amyloid P (SAP), Transglutaminase II (TG II), total Plasminogen activator inhibitor 1 (tPAI 1), myeloperoxidase (MPO)

Table 3

Circulating Levels of Significant Biomarkers in Patients Diagnosed with Pancreatic Cancer, Benign Pancreatic Disease, and Healthy Control Individuals

Class	Biomarker	Healthy	Benign	PDAC[1]		
		Mean	Mean	Mean	vs. Healthy	vs. Benign
		pg/ml	pg/ml	pg/ml	p-value[2] (Trend)	
Tumor Markers	**CA 19-9**	5.424	2.608	56.04	< 0.001 (I)	< 0.001 (I)
	CA-125	27.38	26.94	1073	< 0.001 (I)	ns
	CEA	33271	621444	474737	< 0.01 (I)	< 0.05 (D)
Hormones	**GH**	1932	1167	2398	< 0.001 (I)	< 0.001 (I)
	Prolactin[4]	618.1	2366	1410	< 0.01 (I)	ns
	PTH	27.22	43.48	38.6	< 0.001 (I)	ns
Apoptosis Markers	**Cytokeratin 19**	20.16	122.8	7579	< 0.01 (I)	ns
	sFas	8877	9273	13621	< 0.001 (I)	< 0.05 (I)
	sFasL	200.2	190.1	162	< 0.001 (D)	ns
Adhesion Mediators	**MMP-2**[4]	37770	38260	44830	< 0.05 (I)	ns
	ICAM-1[3]	177.8	535.1	1011	< 0.001 (I)	< 0.05 (I)
	TIMP-1[3]	116.2	184	331.5	< 0.001 (I)	< 0.001 (I)
	TIMP-2[3]	9277	14680	12400	< 0.01 (I)	ns
	TIMP-3	707186	3756000	823150	< 0.01 (I)	< 0.01 (D)
	TIMP-4	77019	87711	106717	< 0.001 (I)	< 0.001 (I)
Cytokines/Chemokines/Receptors	**IL-2R**	1124	1938	3214	< 0.001 (I)	ns
	IL-8	10.52	16.73	37.68	< 0.001 (I)	ns
	IP-10	189.5	187.6	230.3	< 0.001 (I)	< 0.05 (I)
	MPO[3]	289	481.7	501.7	< 0.001 (I)	ns
	TNF-a	6.135	7.603	7.141	< 0.05 (I)	ns
	TNF-RI	6093	8102	10604	< 0.001 (I)	< 0.05 (I)
	TNF-RII	4255	5309	9016	< 0.001 (I)	ns
Growth/Angiogenesis Factors	**Angiostatin**[3]	6604000	6005000	6037000	< 0.01 (D)	ns

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Class	Biomarker	Healthy	Benign	PDAC[1]		
		Mean	Mean	Mean	vs. Healthy	vs. Benign
		pg/ml	pg/ml	pg/ml	p-value[2] (Trend)	
	EGFR	103980	100387	89214	< 0.001 (D)	ns
	Endostatin	146542	169267	195388	< 0.001 (I)	< 0.05 (I)
	ErbB2	11018	12061	16458	< 0.001 (I)	ns
	IGFBP-1[4]	16040	39060	50730	< 0.001 (I)	ns
	Thrombospondin[4]	16050	16730	14070	< 0.01 (D)	ns
Adipokines	**Adiponectin**	78930	72840	100400	< 0.01 (D)	ns
	Leptin	5466	3803	3099	< 0.05 (D)	ns
Apolipoproteins	**ApoAI**	226872	211750	103597	< 0.001 (D)	< 0.001 (D)
	ApoAII	3226	2596	2100	< 0.001 (D)	< 0.01 (D)
	ApoCIII	231.3	223.3	195.6	< 0.001 (D)	ns
	ApoE	63	66.48	95.88	< 0.001 (I)	< 0.05 (I)
Bone Regulation	**OC**	5622	7366	4869	< 0.001 (D)	< 0.001 (D)
	OPG	441.7	719.5	824.1	< 0.001 (I)	< 0.01 (I)
	OPN	1483	6520	16151	< 0.001 (I)	< 0.001 (I)
Other	**CRP**[3]	16450	67870	120500	< 0.001 (I)	< 0.001 (I)
	GLP-1 (active)	18.59	17.91	24.07	< 0.05 (I)	ns
	HE4	36257	20677	51142	< 0.001 (I)	< 0.05 (I)
	SAA[3]	100.2	1012	2769	< 0.001 (I)	< 0.001 (I)
	Tg II	24674	48184	62525	< 0.05 (I)	ns

[1] Pancreatic ductal adenocarcinoma;

[2] significance determined by 1-way ANOVA with Tukey's multiple comparison test;

[3] ug/ml;

[4] ng/ml; ns-not significant; Trend: (I) – increased in PDAC, (D) – decreased in PDAC

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Table 4

Top Performing Biomarker Panels Identified by MMC Algorithm Applied to the Training Set

PDAC vs. Healthy				PDAC vs. Benign			
			%SN at 90% SP				%SN at 90% SP
CA 19-9			57.2	CA 19-9			56.4
CA 19-9	**ICAM-1**	**OPG**	88.1	**CA 19-9**	**CEA**	**TIMP-1**	75.8
CA 19-9	TIMP-1	OPG	87.5	CA 19-9	TIMP-3	TIMP-4	75.6
CA 19-9	OPN	TIMP-1	86.9	CA 19-9	TIMP-4	CRP	74.4
CA 19-9	*ICAM-1*	*TIMP-1*	86.3	CA 19-9	SAA	ApoAI	73.1
CA 19-9	*CA-125*	*TIMP-1*	85	CA 19-9	SAA	CRP	72.5
CA 19-9	ICAM-1	OPN	85	CA 19-9	Tg II	TIMP-4	72.5
CA 19-9	*SAA*	*TIMP-1*	84.4	CA 19-9	ApoAI	TIMP-3	71.9
CA 19-9	*CEA*	*TIMP-1*	84.4	CA 19-9	TIMP-3	CRP	71.9
CA 19-9	TIMP-1	CRP	83.8	CA 19-9	SAA	CA-125	71.3
CA 19-9	TIMP-1	TIMP-2	83.8	CA 19-9	CEA	TIMP-4	71.3
CA 19-9	Tg II	TIMP-1	83.8	*CA 19-9*	*ICAM-1*	*SAA*	71.3
CA 19-9	*OPG*	*CRP*	83.1	*CA 19-9*	*TIMP-1*	*TIMP-4*	71.3
CA 19-9	*TIMP-1*	*TIMP-4*	82.5	*CA 19-9*	*CA-125*	*TIMP-1*	70.6
CA 19-9	ApoAI	TIMP-1	82.5	CA 19-9	CEA	SAA	70.6
CA 19-9	OPN	CRP	82.5	*CA 19-9*	*SAA*	*TIMP-1*	70.6
CA 19-9	TIMP-1	TIMP-3	81.9	*CA 19-9*	*OPG*	*CRP*	70.6
CA 19-9	*ICAM-1*	*SAA*	81.3	CA 19-9	SAA	TIMP-4	70.6
CA 19-9	ICAM-1	Apo AI	80.6	*CA 19-9*	*ICAM-1*	*TIMP-1*	70
CA 19-9	ICAM-1	CRP	80	CA 19-9	TIMP-2	TIMP-4	70
CA 19-9	ICAM-1	Tg II	78.8	CA 19-9	SAA	TIMP-3	70
CA 19-9	ICAM-1	TIMP-4	77.5	CA 19-9	ApoAI	TIMP-4	69.4

Panels listed in *italics* identified in both comparisons, panels listed in **bold** selected for independent validation

Table 5

Cancer Selectivity of Multimarker Panels for PDAC (%)

Panel			Colon N=33	Lung N=62	Breast N=108
CA 19-9	ICAM-1	OPG	97.0	97.0	100
CA 19-9	TIMP-1	OPG	97.0	97.0	100
CA 19-9	OPN	TIMP-1	97.0	97.0	100
CA 19-9	ICAM-1	TIMP-1	97.0	97.0	100

Values List are % classified as Non-PDAC