7

Biomarkers for the Early Detection of Pancreatic Ductal Adenocarcinoma

Carl A.K. Borrebaeck • Linda D. Mellby • Thomas C. King

Department of Immunotechnology, Lund University, Lund, Sweden; Immunovia AB, Lund, Sweden; Immunovia, Inc., Marlborough, Massachusetts, USA.

Author for Correspondence: Thomas King, Immunovia, Inc., Marlborough, Massachusetts, USA. Email: Thomas.king@immunovia.com

Cite this chapter as: Borrebaeck CAK, Mellby LD, King TC. Biomarkers for the Early Detection of Pancreatic Ductal Adenocarcinoma. In: Morgado-Diaz JA, editor. *Gastrointestinal Cancers*. Brisbane (AU): Exon Publications. Online first 27 Jun 2022.

Doi: https://doi.org/10.36255/exon-publications-gastrointestinal-cancers-biomarkers-pancreatic-cancer

Abstract: Pancreatic ductal adenocarcinoma has one of the worst survival rates among adult cancers, with only 11% in the United States surviving five years after diagnosis. The majority of patients are diagnosed with late-stage disease, since early-stage pancreatic ductal adenocarcinoma is typically either asymptomatic or presents with non-specific symptoms. Pancreatic ductal adenocarcinoma thus remains a highly fatal disease. Today, surgical resection (removal of the pancreas) is the only potentially curative modality of treatment available. Detecting pancreatic cancer lesions early enough to perform surgery is, however, beset with difficulties. Nevertheless, the timeline of progression from low-grade precursor lesions to invasive cancer does offer a window of opportunity to detect the disease earlier than is currently possible. By providing physicians with actionable information early enough for the cancer to be removed surgically, the overall 5-year pancreatic ductal adenocarcinoma survival rate could increase from 11% to over 50%. In this chapter, we describe the development and clinical implementation of a proteomic, multi-biomarker blood test for the early detection of pancreatic ductal adenocarcinoma.

In: Morgado-Diaz JA (Editor). *Gastrointestinal Cancers*. Cellular and Molecular Oncobiology Program, Cellular Dynamic and Structure Group, National Cancer Institute-INCA, Rio de Janeiro, Brazil. ISBN: 978-0-6453320-6-3. Doi: https://doi.org/10.36255/exon-publications-gastrointestinal-cancers

Copyright: The Authors.

License: This open access article is licenced under Creative Commons Attribution-NonCommercial 4.0 International (CC BY-NC 4.0) https://creativecommons.org/licenses/by-nc/4.0/

Keywords: biomarkers for pancreatic adenocarcinoma; blood test for pancreatic cancer; CA19-9 in pancreatic cancer; early detection of pancreatic cancer; pancreatic ductal adenocarcinoma

INTRODUCTION

With an increasing incidence and a 5-year survival rate in the United States of just 11%, pancreatic ductal adenocarcinoma is one of the deadliest cancers (1, 2). Projections estimate that pancreatic cancer will surpass colon cancer by 2030 as the second leading cause of cancer death in the United States (3). As with any cancer, improving survival relies on detecting it at a potentially curable stage, which is particularly challenging with pancreatic ductal adenocarcinoma, given that early-stage cancers are typically asymptomatic or present with only non-specific symptoms (4). As a result, most patients are diagnosed with advanced non-resectable disease, and only 20% of sporadic pancreatic cancers are diagnosed during a potentially resectable stage (1, 2). A recent study of high-risk, asymptomatic individuals with germline cyclin dependent kinase inhibitor 2A (CDKN2A) mutations undergoing pancreatic carcinoma surveillance reported that 75% of tumors detected were resectable, resulting in a 5-year survival rate of 24%, a substantial increase over the typical 5-year survival rate (5). These results suggest that survival in pancreatic ductal adenocarcinoma patients can be significantly increased with earlier diagnosis (6–9), and that active surveillance of high-risk individuals can significantly improve their survival (6, 10, 11). Unfortunately, only a minority of individuals who qualify for high-risk surveillance (21% in a recent study) are enrolled in such programs (10).

Diagnosing pancreatic ductal adenocarcinoma, particularly in early stages, remains challenging and there is no gold standard. Reliance rests on imaging modalities such as magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS), but neither is very sensitive or specific. Small lesions require precise targeting for successful fine needle aspiration or needle biopsy, and this may not be possible if lesions are hard to visualize by imaging (9). CA19-9, a blood biomarker used to monitor pancreatic ductal adenocarcinoma disease progression, has limited specificity in diagnosing or detecting pancreatic ductal adenocarcinoma, since other cancers and conditions (such as cirrhosis) can cause elevated CA19-9 levels (12, 13). Additionally, individuals who are genotypically Lewis antigen null (i.e., le/le with inactivating mutations in both copies of the FUT3 gene) (12–16) have low or no expression of CA19-9. Different ethnic groups have varying frequency of the Lewis null phenotype, ranging from 6% to more than 20% (17), further reducing the ability to rely on CA19-9 as an accurate biomarker for pancreatic ductal adenocarcinoma detection. Although CA19-9 is not currently recommended for surveillance (12, 13, 18), it has been recently suggested to have value as "an anchor marker" for detection of pancreatic cancer (16, 19).

Identifying minimally invasive, reliable, and effective methods for detecting pancreatic ductal adenocarcinoma at an early stage is an important unmet clinical need. In this chapter, the development and validation of the IMMray™ PanCan-d assay, a multi-biomarker signature for pancreatic ductal adenocarcinoma, is described. Intended for individuals at high risk for developing pancreatic ductal

adenocarcinoma, IMMray™ PanCan-d encompasses both immunoregulatory and cancer-associated biomarkers (20–31).

PANCREATIC DUCTAL ADENOCARCINOMA PRECURSOR LESIONS (EARLY PANCREATIC NEOPLASIA)

Three types of precursor lesions are thought to give rise to pancreatic ductal adenocarcinoma, with varying rates and probabilities of progression. Two of these lesions are associated with cyst formation in the pancreas: intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs). The third, and perhaps the most common type of precursor lesions, are pancreatic intraepithelial neoplasia (PanINs): flat epithelial dysplasia of pancreatic ducts similar to dysplasia in other sites such as the esophagus or uterine cervix. Higher grades of dysplasia are associated with a greater risk of pancreatic ductal adenocarcinoma. PanINs are usually not detectable by diagnostic imaging and may be multifocal. Effective evaluation of all these precursor lesions requires a multidisciplinary approach (imaging and cytological sampling) that could be greatly improved by the availability of sensitive and specific serum biomarkers. As the NCCN guidelines state: “Decisions about diagnostic management and resectability should involve multidisciplinary consultation at a high-volume center with use of appropriate imaging studies” (32).

Identifying individuals at high risk for pancreatic ductal adenocarcinoma

The known non-genetic risk factors for pancreatic ductal adenocarcinoma are listed in the top panel of Table 1 (4, 13). Although in combination these risk factors may produce a substantial increase in pancreatic ductal adenocarcinoma risk, individually they are modest in magnitude and are not sufficient to warrant active surveillance. Genetic risk factors for pancreatic ductal adenocarcinoma (4, 33) are listed in the bottom panel of Table 1 and many of these are now considered sufficient to offer active surveillance. An individual with multiple close relatives diagnosed with pancreatic ductal adenocarcinoma (familial pancreatic ductal adenocarcinoma) has a 10-fold increased lifetime risk of developing pancreatic ductal adenocarcinoma compared with the general population.

While heritable single gene mutations are responsible for some familial cases, in many cases the genetic causes are unknown. Individuals with hereditary pancreatitis as well as individuals with a history of acute and/or chronic pancreatitis are also at markedly elevated lifetime risk of pancreatic ductal adenocarcinoma. Developing type 2 diabetes after 50 years of age is also associated with an 8-fold risk for pancreatic ductal adenocarcinoma in the first 3 years after diagnosis. Individuals with pancreatic abnormalities detected by diagnostic imaging are also at increased risk. Like many other cancers, an individual’s risk for developing pancreatic ductal adenocarcinoma increases with age, with the majority of cases occurring after age 60 (9). Many of these groups at higher risk may benefit from annual surveillance using a sensitive and specific test that can detect early pancreatic neoplasia.

TABLE 1 Identified Risk Factors Associated with Developing Pancreatic Ductal Adenocarcinoma

Non-Genetic Risk Factors (4, 13)	Relative Risk
Current cigarette use	1.7–2.2
Current pipe or cigar use	1.5
> 3 alcoholic drinks per day	1.2–1.4
Chronic pancreatitis	13.3
Body Mass Index > 40 kg/m^2, male	1.5
Body Mass Index > 40 kg/m^2, female	2.8
Diabetes mellitus, type 1	2
Diabetes mellitus, type 2	1.8
Cholecystectomy	1.2
Gastrectomy	1.5
Helicobacter pylori infection	1.4
Genetic Risk Factors (4, 33)	**Relative Risk**
Hereditary breast and ovarian cancer syndrome: BRCA1, BRCA2, PALB2	2–3.5
Lynch syndrome (hereditary non-polyposis colorectal cancer): MLH1, MSH2, MSH6, PMS2, EPCAM	8.6
Familial adenomatous polyposis: APC	4.5–6
Peutz-Jeghers syndrome: STK11/LKB1	132
Familial atypical multiple mole melanoma (FAMMM): P16INK4A/CDKN2A	47
Hereditary pancreatitis: PRSS1, SPINK1	69
Ataxia-telangiectasia: ATM	Increased
Familial pancreatic cancer (2 or more first-degree relatives with pancreatic cancer): gene(s) unknown	9–32
First-degree relative of person with sporadic pancreatic cancer: gene(s) unknown	2–4

Shortfalls of current pancreatic ductal adenocarcinoma diagnostic tools

Although diagnostic imaging is useful in assessing pancreatic abnormalities, it often cannot differentiate between benign conditions (e.g., chronic pancreatitis) and pancreatic neoplasia with certainty (34). In addition, diagnostic imaging fails to detect many (perhaps most) early pancreatic ductal adenocarcinomas when they are of a stage amenable to surgical cure (9, 34). The serum tumor marker CA19-9 (reference range < 37 U/ml) has been useful for monitoring patients with advanced pancreatic ductal adenocarcinoma, but its clinical utility in early detection has not been demonstrated. Additionally, it's been found to be non-specific as it can also be elevated in unrelated conditions. Unfortunately, no other single serum tumor biomarker has shown performance even as good as CA19-9. A different approach to biomarker development has been undertaken to create a more

sensitive and specific assay for pancreatic ductal adenocarcinoma by combining the results of multiplex immunoassays for specific serum proteins.

BIOMARKER ASSAY DEVELOPMENT FOR THE EARLY DETECTION OF PANCREATIC DUCTAL ADENOCARCINOMA: PROTEIN MICROARRAY TECHNOLOGY

Human blood serum contains a very large amount of potentially useful diagnostic information. Affinity proteomics has now been developed as an accurate approach that can generate actionable information that can result in more precise and evidence-based options to manage cancer (29). To achieve this, there is clearly a need to move from a single biomarker to multiplex biomarkers, a so-called signature, that can provide significantly increased diagnostic accuracy. Protein biomarker discovery has been driven more by technology rather than focusing on specific clinical needs. Proteomic technology platforms have developed rapidly with substantially increased resolution in terms of depth of proteome coverage and speed. Multiplexed enzyme-linked immunosorbent assays (ELISA) have also demonstrated clinical applicability and have paved the way for next-generation multiparametric diagnostics, in particular high-density antibody microarrays (35). Such protein or antibody microarrays can theoretically display almost unlimited resolution of the most complex proteomes. However, in contrast to the more mature transcriptional profiling technologies, the proteome coverage of antibody microarrays is limited by the number of available well-characterized antibodies. Improved accuracy has been achieved using antibody microarrays, reverse phase protein arrays and bead-based arrays, demonstrating the feasibility of multiparametric proteomic analysis. Although novel technologies open new avenues for clinical proteomics by introducing substantially improved proteome coverage, the quality of available samples can also be problematic in terms of their analytical quality.

Sample acquisition procedures must be strictly standardized to achieve accurate and reproducible proteomic data. However, in most retrospective studies, standard operating procedures for sample collection did not exist or were highly variable. The introduction of such pre-analytical variables can not only affect sample integrity but also introduce bias, due to differences in the acquisition of different sample cohorts. Furthermore, comprehensive information about patient demographics, such as gender, age, tumor stage and treatment schemes, as well as lifestyle factors, such as smoking and alcohol habits, is important to design the proper case-control studies. Each sample subgroup should be clearly defined, and enough samples must be collected from each cohort, since differences in treatment modalities can have a major impact on the results of a proteomic analysis. Therefore, sample quality, as well as their clinical documentation is essential for high quality clinical proteomics (36), which has become more evident in comparison to the more robust genomic approaches.

During biomarker analysis, the main challenge is to define biomarker combinations that deliver optimal clinical accuracy. This cannot be based simply on the p values for each biomarker, since this approach discards information about synergistic contributions among the biomarkers that could improve classification accuracy.

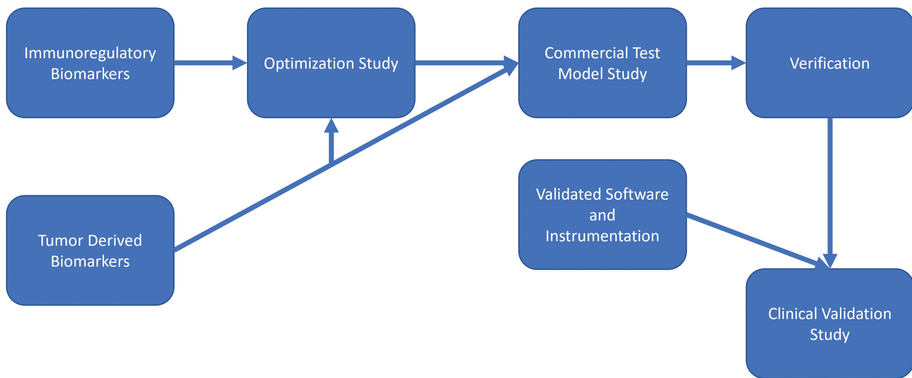

Figure 1. Biomarker development pathway. Development pathway for a biomarker signature for the early detection of pancreatic ductal adenocarcinoma.

A combination of 'orthogonal biomarkers' that do not depend on each other is optimal. In this case, the information contributed by each biomarker provides independent information about the disease process. To achieve this, an ordered approach is needed to select the biomarkers with the largest impact on accuracy, and to eliminate biomarkers with the lowest impact on the accuracy. This can be achieved by combining the leave-one-out cross-validation procedure with a backward elimination algorithm or using feature selection based on binary classification algorithms, such as Random Forest. For example, eliminating biomarkers one by one and identifying those that contributed the least to a correct sample classification produces a ranking of the biomarkers. This enables the selection of a biomarker signature displaying optimal accuracy for each application.

Advanced cancer diagnostics based entirely on proteomics has only recently delivered biomarker signatures with the required clinical accuracy (20). Both technological difficulties in decoding complex proteomes, as well as lack of rigorous validation, have been barriers to realize this potential. Recently, antibody microarrays have reached the point at which clinically relevant information related to risk classification and/or prognosis can be routinely generated (35). Here we describe the iterative approach that was used to develop and validate a multiplex assay for the early detection of pancreatic ductal adenocarcinoma using serum samples, as outlined in Figure 1.

IMMUNOREGULATORY PROTEINS AS BIOMARKERS FOR PANCREATIC DUCTAL ADENOCARCINOMA

Blood samples from patients diagnosed with a lesion in the pancreas were collected and processed before resection or start of chemotherapy. Pancreatic cancer staging was performed according to the American Joint Committee on Cancer guidelines (37). Blood samples from controls were collected, using the same standard operating procedure. 5 µL of the serum samples was used for the analysis, with a recombinant antibody microarray composed of 349 human recombinant single-chain variable

fragments (scFvs) that were directed against 156 antigens. The concept was to utilize the body's response to disease rather than the tumor secretome. Consequently, the selected biomarkers were involved mainly in immunoregulation (30).

In order to develop a final biomarker signature, data were divided into a training set including two thirds of the samples (~1,000 samples) and a test set including one third of the samples (~340 samples). The same ratio of case versus control samples was preserved within the randomly generated data sets. Four unique test and training sets were generated using this approach. A backward elimination algorithm was applied to each training set in R (open-source statistical software from The R Foundation), excluding one antibody at a time. This approach allows an unbiased selection of biomarkers contributing orthogonal information, compared with other biomarkers.

The generated biomarker signature was used to build a model by support vector machine in R, utilizing only data from the training set. The model was tested on the corresponding test set (to avoid overfitting) and its performance was measured using area under the curve (AUC) values from receiver operating characteristic (ROC) curves. Analyte detection was based on the recombinant antibody microarray platform as described above. Because the focus was to interrogate the body's response to pancreatic ductal adenocarcinoma, the selected antibodies targeted predominantly immunoregulatory proteins. The obtained AUC value for differentiating stage I and II pancreatic ductal adenocarcinoma versus normal controls was 0.96. The corresponding value for normal controls versus stage III and IV pancreatic ductal adenocarcinoma was 0.99.

Although some of the biomarkers included in the final signature had been previously reported as potential pancreatic ductal adenocarcinoma biomarkers, no obvious mechanistic model explains the composition or possible interaction of these biomarkers with pancreatic ductal adenocarcinoma development. Perhaps gradual changes in the tumor microenvironment can be reflected in the biomarker content in blood. The result of this study was identification and validation of a biomarker signature, based on two large, case-control studies of patients with pancreatic ductal adenocarcinoma, which was able to detect stage I and II cancer samples with high accuracy.

ADDITION OF A TUMOR-DERIVED BIOMARKER (CA19-9) TO THE ASSAY (OPTIMIZATION STUDY)

The Optimization Study aimed to evaluate how a biomarker signature could separate patients with pancreatic ductal adenocarcinoma (stages I–IV) from individuals with various symptomatic conditions not caused by pancreatic ductal adenocarcinoma. These controls were selected to mirror the relevant clinical settings (benign biliary obstructions, cirrhosis, etc.) encountered by healthcare professionals. In addition, it evaluated the utility of adding the established pancreatic ductal adenocarcinoma biomarker CA19-9 to a biomarker signature. The IMMray™ platform is designed to detect protein biomarkers so that CA19-9, which is an oligosaccharide, was measured separately using an electrochemiluminescence immunoassay (Roche Cobas®). In total, 923 serum samples were analyzed with the IMMray™ discovery set up and a CA19-9 ELISA. Patient samples from

136 pancreatic ductal adenocarcinoma (stage I–IV), 570 symptomatic individuals and 217 healthy controls were tested in a randomized manner. All pancreatic ductal adenocarcinomas were histologically confirmed. Based on one year follow-up data, none of the symptomatic controls (back pain, unexplained weight loss, etc.) developed pancreatic cancer. To minimize confounding and pre-analytical variables, all patient samples were collected and processed using the same standard operating procedures, stored at −80°C and tested within a year of collection. Data analysis for each group was performed using the support vector machine algorithm. Data was divided into a training and test set, and test performance given as ROC AUC values were then evaluated for the test set.

The biomarker signature together with CA19-9 had the capacity to differentiate pancreatic ductal adenocarcinoma (stages I–IV) from symptomatic individuals without cancer, including individuals with type 2 diabetes. Importantly, early-stage pancreatic ductal adenocarcinoma (stages I & II) was discriminated from controls with an unprecedented accuracy of 0.984. These findings needed to be further validated but have significant clinical implications for individuals in primary and secondary care settings with non-specific but concerning symptoms where pancreatic ductal adenocarcinoma may be suspected. This study paved the way for the IMMray™ PanCan-d Commercial Test Model Study, in which the final biomarker signature was selected, and the commercial test model built.

COMMERCIAL TEST MODEL STUDY (CTMS)

The IMMray™ PanCan-d Commercial Test Model Study (CTMS) aimed to select and lock the IMMray™ PanCan-d biomarker signature and evaluate its performance in differentiating pancreatic ductal adenocarcinoma (stages I–IV) vs. controls that simulate clinical test situations. Serum samples obtained from patients in Europe and the United States with non-specific but concerning symptoms, including diabetics, as well as samples from healthy individuals were analyzed. In total, 1113 patient serum samples were analyzed with a focused IMMray set up and CA19-9 assay. Patient samples from 315 pancreatic ductal adenocarcinoma (stage I–IV), 488 symptomatic individuals who did not have pancreatic cancer (including 79 with diabetes and 56 with chronic pancreatitis) and 310 healthy controls were tested. All these samples were freshly collected through eight reference sites in USA and Europe. Data analysis was performed using Immunovia's software algorithms and the data were divided into training and test sets. The test performance was evaluated using ROC AUC curves. In this CTMS study, we showed for the first time that the IMMray™ PanCan-d 9-plex signature, including CA19-9, had the capacity to differentiate between pancreatic ductal adenocarcinoma stage I & II and all controls, including diabetes, symptomatic, healthy individuals, with a ROC AUC of 0.950. We also locked and tested the model algorithms as part of this study, which were subsequently incorporated in the final IMMray™ PanCan-d test process.

CLINICAL VALIDATION OF IMMRAY™ PANCAN-D

IMMray™ PanCan-d is a multiplex micro-immunoassay that combines measurements of 9 serum biomarkers including CA19-9 using a mathematical algorithm (31).

After its development, this signature was created and locked during the Commercial Test Model Study, as described above. The algorithm can be expressed as a linear equation which includes the levels of 9 serum biomarkers (log2 transformed fluorescence intensity) multiplied by positive or negative real number coefficients:

$$A1^*(\log 2 \text{ intensity } 1) + A2^*(\log 2 \text{ intensity } 2) + \ldots + A9^*(\log 2 \text{ intensity } 9) + C = \text{Decision Value}$$

A1–A9 are real number coefficients determined from the support vector machine and *C* is the *Y* intercept for this linear equation. The IMMray PanCan-d single chain variable fragment (scFv) antibodies included in the IMMray™ PanCan-d microarray are listed in Figure 2.

The clinical validation study analyzed samples collected from multiple sites across Europe and the United States, including 57 early-stage (stage I and II) pancreatic ductal adenocarcinoma patients, 110 stage III and IV pancreatic ductal adenocarcinoma patients, 203 individuals at high risk for developing familial/hereditary pancreatic ductal adenocarcinoma enrolled in a surveillance program, and 216 healthy controls. All serum samples were collected in red top tubes and allowed to clot for 30–60 min before centrifugation for 10 minutes at 3,000 xg. Serum was then aliquoted and immediately frozen at –80°C. Samples were transported on dry ice and then thawed for analysis. All samples were analyzed within 2 years of their collection, and all were stored at –80°C until thawed for analysis. Pancreatic ductal adenocarcinoma staging was performed according to the American Joint Committee on Cancer Guidelines (37). Blood samples from patients with confirmed pancreatic ductal adenocarcinoma were collected and processed before treatment. Samples were blinded to laboratory staff and randomized using an Excel template designed to avoid an imbalance of any cohort in any assay batch (maximum batch size = 62 samples).

Sample cohort characteristics

Pancreatic ductal adenocarcinoma patients had a median age of 70 years, which is 11 years older than the high-risk population. Both cohorts were older than the healthy cohort with a median age of 49. Women were more frequent in the high-risk cohort, while the pancreatic ductal adenocarcinoma cohort had more men than women, as expected. 28% of the high-risk cohort had prior cancers and they were either cured or were in remission at the time that they were inducted into the study. This large number of prior neoplasms is not unexpected as many individuals in this cohort had documented germline mutations predisposing to pancreatic carcinoma as well as other tumor types. Together, the 203 high-risk subjects were receiving 619 prescription medications, some of which were adjuvant therapy for

Tumor-Associated	Hormone Transport	Bone Metabolism	Protease Inhibitor	Coagulation	Complement
• MUC16 (CA125)	• IGFBP3	• OPG	• Cystatin C	• GSN	• C5 • CFB • C4

Figure 2. Antibodies included on microarray. Identities of IMMray™ PanCan-d single chain variable fragment antibodies included on the microarray.

prior cancers (e.g., aromatase inhibitors). All individuals in the high-risk cohort underwent active imaging surveillance. 25% of this cohort had presumptive IPMNs and 27% had other imaging abnormalities in their pancreas. Detected IPMNs ranged from 1 to 10 in number (median 2) and from 0.2 to 2.2 cm in size (median 0.6). None had main duct IPMNs and none had worrisome features.

The healthy cohort was recruited from multiple sites in Europe and North America. This group was more ethnically diverse than the other cohorts and had no history of cancer. The familial/genetic high-risk cohort was collected from 3 US sites (University of Pittsburgh Medical Center, Massachusetts General Hospital, and University of Pennsylvania) participating in the PanFAM prospective clinical trial (ClinicalTrials.gov Identifier: NCT03693378) and was made up of individuals with a strong family history of pancreatic cancer and/or individuals with known genetic mutations predisposing to pancreatic ductal adenocarcinoma who meet current criteria for active surveillance listed in Table 2. None of the individuals tested were known to have developed pancreatic ductal adenocarcinoma at the time of sample collection.

IMMray™ PanCan-d results (frozen signature and predefined classification cutoffs)

A histogram showing the distribution of decision values for the three sample cohorts is shown in Figure 3A. The decision values for the healthy and high-risk cohorts are clustered and are generally similar to one another (although they were

TABLE 2 Inclusion Criteria for the High-Risk Cohort Study

	Age
Two or more relatives with pancreatic adenocarcinomas (PDAC) on the same side of the family, where two PDAC-affected individuals are first degree related (FDR) + at least one PDAC-affected individual is an FDR of the Participant	≥50 years old OR 10 years before onset in family
Two affected FDRs with PDAC	≥50 years old OR 10 years before onset in family
Any of *BRCA1, BRCA2, PALB2, ATM* mutations confirmed pathogenic or likely pathogenic + one FDR or secondary degree related (SDR) with PDAC	≥50 years old OR 10 years before onset of an FDR and SDR
Familial atypical multiple mole-melanoma (FAMMM) with confirmed pathogenic or likely pathogenic mutation variants in: *p16, CDKN2A*	≥50 years old
Known mutation carrier for *STK11* (Peutz Jeghers Syndrome)	≥35 years old
Lynch syndrome (HNPCC) with confirmed pathogenic or likely pathogenic variants in: *MLH1, MSH2, MSH6, PMS2*, or *EPCAM* + one FDR or *SDR* with PDAC	≥50 years old OR 10 years before onset of an FDR or SDR
Hereditary pancreatitis with confirmed *PRSS1* pathogenic or likely pathogenic history of pancreatitis	≥40 years old

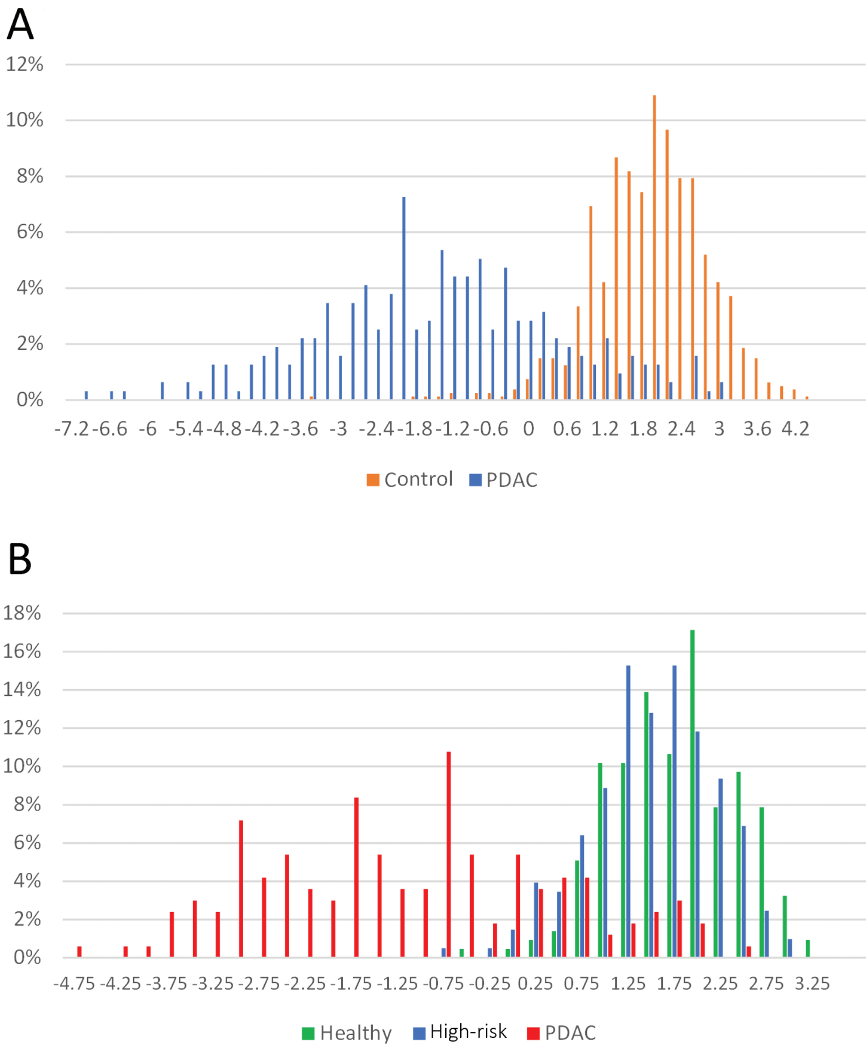

Figure 3. Decision values. Histograms showing the distribution of observed Decision Values for different subject cohorts from the Clinical Test Model Building Study (Figure 3A) and the Clinical Validation Study (Figure 3B).

statistically different by t test, $p < 0.001$). The mean decision values for the high-risk and healthy cohorts are 1.40 and 1.65, respectively. The corresponding standard deviations were 0.67 and 0.68. Both cohorts differed substantially from the pancreatic ductal adenocarcinoma cohort that showed a much greater variation in decision values (–4.75 to 2.5) and had a strong negative bias (mean value = –1.26 with a standard deviation of 1.58).

Excluding borderline results, these decision values correspond to a test sensitivity of 85% for early stage (stages I & II) pancreatic ductal adenocarcinoma and 87% for all-stage pancreatic ductal adenocarcinoma with a specificity of 98%

compared with the high-risk cohort and 99% compared with the healthy cohort. Using its clinical reference range cutoff, CA19-9 alone demonstrated 75.8% sensitivity and 97.6% specificity in these cohorts. 10% of samples were classified as borderline, and there was a higher percentage of borderline results among the pancreatic ductal adenocarcinoma cohort than in the control cohorts. Evaluation of the test classifications in the context of gender or smoking status did not demonstrate statistical significance by Chi Square ($p = 0.48$ and $p = 0.61$, respectively). The median age for individuals with negative and borderline classifications in the high-risk cohort were 59 and 60. The median age of individuals with negative, borderline, and positive classifications in the pancreatic ductal adenocarcinoma cohort were 68, 71, and 71 respectively. The distribution of decision values in this study is very similar to that obtained in the CTMS study in which the algorithm was developed and locked in 2019 (see Figure 3B).

CA19-9 and IMMray™ PanCan-d results

Prior publications have shown that individuals with very low baseline CA19-9 values are frequently deficient in Fucosyltransferase 3 (the enzyme FUT3) which normally adds the terminal sugar to form CA19-9. Based on these published findings and the fact that CA19-9 is a component of decision values for IMMray™ PanCan-d, we evaluated IMMray™ PanCan-d performance in the subsets of each study cohort that expressed CA19-9 levels greater than 2.5 U/ml. Excluding samples with CA19-9 values equal to or less than 2.5 U/ml excluded 55 samples from the analysis but improved assay sensitivity from 85% to 89% for early-stage pancreatic ductal adenocarcinoma and from 87% to 92% for all-stage pancreatic ductal adenocarcinoma (Figure 4).

Recent work has revived interest in CA19-9 as an important pancreatic ductal adenocarcinoma biomarker. CA19-9 has been largely relegated to a limited role in measuring tumor progression and/or response to therapy. One recent article brings forward the concept of CA19-9 as an "anchor" biomarker that can be combined with other biomarkers to achieve superior diagnostic performance (16). An important limitation of CA19-9 as a pancreatic ductal adenocarcinoma biomarker results from genetic variation in one of the enzymes that is required for its synthesis. FUT3 (also called the Lewis Antigen) catalyzes the addition of terminal sugar to DuPan2 to form CA19-9. The prevalence of FUT3 deficiency (both alleles nonfunctional) has been reported to vary in different ethnic groups and these findings were supported by this study. The following rates of CA19-9 values below 2.5 U/ml in the subjects from different ethnic backgrounds were observed: 8% of US Caucasians, 24% of US Hispanics, and 26% of African Americans were presumptively deficient. These observed frequencies are similar to those reported for FUT3 negative individuals in the US Caucasian and African American populations (17). Since the 8 biomarkers measured on the IMMray platform contribute significantly to discrimination between samples from individuals with pancreatic ductal adenocarcinoma and controls, we re-evaluated the decision values for samples with CA19-9 values less than 2.5 U/ml by removing the CA19-9 contribution from them and obtained a ROC AUC of 0.87.

The improvement in IMMray™ PanCan-d test sensitivity to 92% by excluding samples with very low CA19-9 values is clinically important. This exclusion also avoids the likelihood of under-diagnosing pancreatic ductal adenocarcinoma in

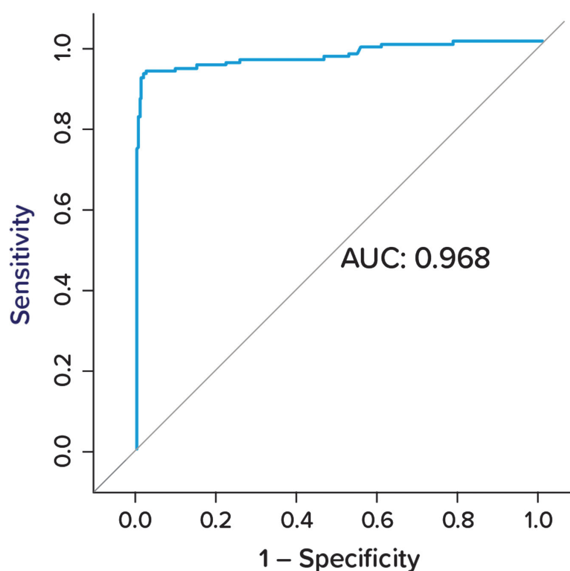

Figure 4. Test performance. Receiver operating characteristic (ROC) curves and area under the curve (AUC) for IMMray™ PanCan-d test performance in pancreatic ductal adenocarcinoma (PDAC) vs all controls, excluding samples with CA19-9 values of 2.5 U/ml or less.

ethnic groups with a higher prevalence of FUT3 (Lewis null) genotypes (e.g., Hispanics and African Americans in this study). The discrimination of the signature without CA19-9 in these samples is encouraging and provides a starting point for developing a companion assay to better address this population.

PAN-CANCER BIOMARKER ASSAYS

Although pan-cancer biomarker assays are a subject in and of themselves and a detailed discussion is beyond the scope of this chapter, a few comments are needed here since some of these tests can potentially detect pancreatic ductal adenocarcinoma as well as a variety of other tumors. Several pan-cancer assays are currently under development and one (Galleri™ from GRAIL, Inc.) is available clinically. Most of these assays, including Galleri™, depend heavily or exclusively on the detection of novel DNA methylation patterns from cell-free DNA. Since these tests depend predominantly on the release of DNA from tumor cells, their ability to detect small cancers is limited. The one pan-cancer commercial assay currently available, Galleri™, has reported a sensitivity for stage I and II pancreatic ductal adenocarcinoma of 61% compared with the IMMray PanCan-d sensitivity of 89%, most probably reflecting on the value of including nontumor cell derived biomarkers in the latter assay.

CONCLUSION

Antibody microarray technology displays great promise for protein expression profiling of complex proteomes. In some cases, the addition of biomarkers to an anchor biomarker, such as CA19-9, can improve the accuracy of that biomarker sufficiently to substantially alter its clinical utility. Microarray technology has now evolved from proof-of-concept designs to established high-performing technology platforms capable of evaluating non-fractionated complex proteomes from human samples. A variety of platforms, displaying a wide range of performances, based on monoclonal, polyclonal, and recombinant antibodies, are available from both academic laboratories and commercial vendors. To date, the technology has been used to detect disease-associated (biomarker) signatures for bladder cancer, colorectal cancer, gastric adenocarcinoma, lung cancer, breast cancer, pancreatic adenocarcinoma, prostate cancer. We feel that the most important clinical uses of these antibody microarrays will be in disease diagnosis, prognosis, and therapy selection for complex polygenic diseases. Efforts have now been launched to extend the technology beyond the current state-of-the-art, to be able to perform true global proteome analysis, setting a new standard for disease proteomics. This chapter has attempted to describe the clinical landscape for developing a multiplex proteomic test for early cancer detection and provide a paradigm for the iterative selection of an optimal biomarker signature that may include not only tumor cell products but also components of the tumor microenvironment and the host immune and inflammatory response to a tumor. In this context, we feel that IMMray™ PanCan-d can now have a significant clinical impact on individuals at risk for pancreatic ductal adenocarcinoma and perhaps ultimately in managing pancreatic ductal adenocarcinoma patients' care through optimal selection of therapeutic modalities and detection of tumor recurrence. The WHO has proposed that millions of patients with cancer could be saved from premature death if diagnosed and treated earlier. To achieve this, more advanced diagnostic approaches must be developed for multiple tumor types and applied to detect lethal cancers, such as pancreatic cancer, earlier in their clinical course.

Acknowledgment: Research conducted for the development of the IMMray™ PanCan-d test was funded by Immunovia. Carrie Mansfield provided expert editorial assistance.

Conflicts of Interest: Linda Mellby and Thomas King are employees of Immunovia. Carl Borrebaeck is Chairman of the Board of Directors of Immunovia.

Copyright and Permission Statement: The authors confirm that the materials included in this chapter do not violate copyright laws. Where relevant, appropriate permissions have been obtained from the original copyright holder(s), and all original sources have been appropriately acknowledged or referenced.

REFERENCES

1. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer Statistics 2022. CA Cancer J Clin. 2022;72:7–33. https://doi.org/10.3322/caac.21708

2. American Cancer Society. Cancer Facts & Figures 2022. Atlanta: American Cancer Society; 2022.
3. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: The unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res.74(11):2913–2921. https://doi.org/10.1158/0008-5472.CAN-14-0155
4. Becker AE, Hernandez YG, Frucht H, Lucas AL. Pancreatic ductal adenocarcinoma: Risk factors, screening, and early detection. World J Gastroenterol. 2014;20(32):11182–11198. https://doi.org/10.3748/wjg.v20.i32.11182
5. Vasen H, Ibrahim I, Ponce CG, Slater EP, Matthäi E, Carrato A, et al. Benefit of surveillance for pancreatic cancer in high-risk individuals: Outcome of long-term prospective follow-up studies from three European expert centers. J Clin Oncol. 2016;34(17):2010–2019. https://doi.org/10.1200/JCO.2015.64.0730
6. Canto MI, Almario JA, Schulick RD, Yeo CJ, Klein A, et al. Risk of neoplastic progression in individuals at high risk for pancreatic cancer undergoing long-term surveillance. Gastroenterol. 2018;155(3):740–751. https://doi.org/10.1053/j.gastro.2018.05.035
7. Sohn TA, Yeo CJ, Cameron JL, Koniaris L, Kaushal S, Abrams RA, et al. Resected adenocarcinoma of the pancreas-616 patients: Results, outcomes, and prognostic indicators. J Gastrointest Surg. 2000;4(6):567–579. https://doi.org/10.1016/S1091-255X(00)80105-5
8. Hanada K, Okazaki A, Hirano N, Izumi Y, Minami T, Ikemoto J, et al. Effective screening for early diagnosis of pancreatic cancer. Best Pract Res Clin Gastroenterol. 2015;29(6):929–939. https://doi.org/10.1016/j.bpg.2015.09.017
9. Chari ST, Kelly K, Hollingsworth MA, Thayer SP, Ahlquist DA, Andersen DK, et al. Early detection of sporadic pancreatic cancer: Summative review. Pancreas. 2015;44(5):693–712. https://doi.org/10.1097/MPA.0000000000000368
10. Everett JN, Burgos G, Chun J, Baptiste A, Khanna LG, Oberstein PE, et al. Cancer surveillance awareness and practice among families at increased risk for pancreatic adenocarcinoma. Cancer. 2021;127:2271–2278. https://doi.org/10.1002/cncr.33500
11. Yachida S, Jones S, Bozic I, Antal T, Leary R, Fu B, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature. 2010;467(7319):1114–1117. https://doi.org/10.1038/nature09515
12. Stoffel EM, McKemin SE, Brand RE, Canto M, Goggins M, Moravek C, et al. Evaluating susceptibility to pancreatic cancer: ASCO provisional clinical opinion. J Clin Oncol. 2018;37:153–164. https://doi.org/10.1200/JCO.18.01489
13. Ducreux M, Cuhna AS, Caramella C, Hollebecque A, Burtin P, Goéré D, et al. Cancer of the pancreas: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2015;26(Supplement 5):v56–v68. https://doi.org/10.1093/annonc/mdv295
14. Kawai S, Suzuki K, Nishio K, Ishida Y, Okada R, Goto Y, et al. Smoking and serum CA19-9 levels according to Lewis and secretor genotypes. Int J Cancer. 2008;123:2880–2884. https://doi.org/10.1002/ijc.23907
15. Narimatsu H, Iwasaki H, Nakayama F, Ikehara Y, Kudo T, Nishihara S, et al. Lewis and secretor gene dosages affect CA19-9 and DU-PAN-2 serum levels in normal individuals and colorectal cancer patients. Cancer Res. 1998;58(3):512–518.
16. Fahrmann JF, Schmidt CM, Mao X, Irajizad E, Loftus M, Zhang J, et al. Lead-time trajectory of CA19-9 as an anchor marker for pancreatic cancer early detection. Gastroenterol. 2021;160(4):1373–1383. https://doi.org/10.1053/j.gastro.2020.11.052
17. Roback JD, Combs MR, Grossman BJ, Hillyer CD (eds). Technical Manual: 16th Edition, Bethesda, MD: AABB; 2008.
18. Galli C, Basso D, Plebani M. CA 19-9: handle with care. Clin Chem Lab Med. 2013;51(7):1369–1383. https://doi.org/10.1515/cclm-2012-0744
19. Modi S, Kir D, Saluja AK. Old dog, new tricks: Use of CA 19-9 for early diagnosis of pancreatic cancer. Gastroenterol. 2021;160(4):1019–1021. https://doi.org/10.1053/j.gastro.2021.01.001
20. Borrebaeck CAK. Precision diagnostics: Moving towards protein biomarker signatures of clinical utility in cancer. Nat Rev Cancer. 2017;17(3):199–204. https://doi.org/10.1038/nrc.2016.153
21. Hanash SM, Pitteri SJ, Faca VM. Mining the plasma proteome for cancer biomarkers. Nature. 2008;452(7187):571–579. https://doi.org/10.1038/nature06916

22. Radon TP, Massat NJ, Jones R, Alrawashdeh W, Dumartin L, Ennis D, et al. Identification of a three-biomarker panel in urine for early detection of pancreatic adenocarcinoma. Clin Cancer Res. 2015;21(15):3512–3521. https://doi.org/10.1158/1078-0432.CCR-14-2467
23. Shaw VE, Lane B, Jenkinson C, Cox T, Greenhalf W, Halloran CM, et al. Serum cytokine biomarker panels for discriminating pancreatic cancer from benign pancreatic disease. Mol Cancer. 2014;13:114. https://doi.org/10.1186/1476-4598-13-114
24. Mayers JR, Wu C, Clish CB, Kraft P, Torrence ME, Fiske BP, et al. Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development. Nat Med. 2014;20(10):1193–1198. https://doi.org/10.1038/nm.3686
25. Jenkinson C, Elliott VL, Evans A, Oldfield L, Jenkins RE, O'Brien DP, et al. Decreased serum thrombospondin-1 levels in pancreatic cancer patients up to 24 months prior to clinical diagnosis: Association with diabetes mellitus. Clin Cancer Res. 2016;22(7):1734–1743. https://doi.org/10.1158/1078-0432.CCR-15-0879
26. Brand RE, Nolen BM, Zeh HJ, Allen PJ, Eloubeidi, Goldberg M, et al. Serum biomarker panels for the detection of pancreatic cancer. Clin Cancer Res. 2011;17(4):805–816. https://doi.org/10.1158/1078-0432.CCR-10-0248
27. Kim J, Barnlet WR, Oberg AL, Chaffee KG, Donahue G, Cao X, et al. Detection of early pancreatic ductal adenocarcinoma with thrombospondin-2 and CA19-9 blood markers. Sci Transl Med. 2017;9(398):eaah5583. https://doi.org/10.1126/scitranslmed.aah5583
28. Gerdtsson AS, Malats N, Säll A, Real FX, Porta M, Skoog P, et al. A multicenter trial defining a serum protein signature associated with pancreatic ductal adenocarcinoma. Int J Proteomics. 2015;2015:587250. https://doi.org/10.1155/2015/587250
29. Wingren C, Sandström A, Segersvärd R, Carlsson A, Andersson R, Löhr M, et al. Identification of serum biomarker signatures associated with pancreatic cancer. Cancer Res. 2012;72(10):2481–2490. https://doi.org/10.1158/0008-5472.CAN-11-2883
30. Mellby LD, Nyberg AP, Johansen JS, Wingren C, Nordestgaard BG, Bojesen SE, et al. Serum biomarker signature-based liquid biopsy for diagnosis of early-stage pancreatic cancer. J Clin Oncol. 2018;36(28):2887–2894. https://doi.org/10.1200/JCO.2017.77.6658
31. Brand RE, Persson J, Svein OB, Chung DC, Katona BW, Carrato A, et al. Detection of early-stage pancreatic ductal adenocarcinoma from blood samples: Results of a multiplex biomarker signature validation study. Clin Transl Gastroenterol. 2022;13(3):e00468. https://doi.org/10.14309/ctg.0000000000000468
32. Herman J, Ko AH, Komanduri S, Koong A, LoConte N, Lowy AM, et al. NCCN Clinical Practice Guidelines in Oncology: Pancreatic Adenocarcinoma, Version 2.2017. J Natl Compr Canc Netw. 2017 Aug;15(8):1028–1061. https://doi.org/10.6004/jnccn.2017.0131
33. Klein AP, Brune KA, Petersen GM, Goggins M, Tersmette AC, Offerhaus GJA, et al. Prospective risk of pancreatic cancer in familial pancreatic cancer kindreds. Cancer Res. 2004;64:2634–2638. https://doi.org/10.1158/0008-5472.CAN-03-3823
34. Costache MI, Costache CA, Dumitrescu CI, Tica AA, Popescu M, Baluta EA, et al. Which is the best imaging method in pancreatic ductal adenocarcinoma diagnosis and staging-CT, MRI or EUS? Curr Health Sci J. 2017;43(2):132–136.
35. Borrebaeck CAK, Wingren C. Developing and applying recombinant antibody microarrays for high-throughput disease proteomics. Eur Pharma Rev. 2010;6:45–49.
36. Wingren and Borrebaeck, Antibody-Based Microarrays. In Microchip Methods in Diagnostics, Ursula Bilitewski (ed.), Vol. 509, New York: Humana Press, 2008. https://doi.org/10.1007/978-1-59745-372-1_5
37. Amin MB, Edge SB, Greene FL, Byrd DR, et al, editors. AJCC Cancer Staging Manual. 8th ed, Springer International Publishing: American Joint Committee on Cancer, 2017.