PAM4 Immunoassay Alone and in Combination with CA19-9 for the Detection of Pancreatic Adenocarcinoma

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Abstract

Background—Monoclonal antibody PAM4 has high specificity for pancreatic ductal adenocarcinoma (PDAC), as well as its precursor lesions, but is not reactive with normal and benign pancreatic tissues. Our purpose was to evaluate a PAM4-based serum-immunoassay alone, and in combination with the CA19-9 assay, for the detection of PDAC with particular attention to early-stage disease.

Methods—Sera from patients with confirmed PDAC (N=298), other cancers (N=99), benign disease of the pancreas (N=120), and healthy adults (N=79) were evaluated by specific enzyme-immunoassay for concentration of PAM4 and CA19-9 antigen levels by blinded analyses. All tests for statistical significance were two-sided.

Results—Overall sensitivity for PAM4 detection of PDAC was 76%, with 64% of stage-1 patients also identified. The detection rate was considerably higher (85%) for advanced disease. The assay showed high specificity compared to benign pancreatic disease (85%), with a positive likelihood ratio (+LR) of 4.93. CA19-9 provided an overall sensitivity of 77%, and was positive in 58% of patients with stage-1 disease; however, specificity was significantly lower for CA19-9 (68%) with a +LR=2.85 (P=0.026 compared to PAM4). Importantly, a combined PAM4/CA19-9 assay demonstrated an improved sensitivity (84%) for overall detection of PDAC without significant loss of specificity (82%), as compared to either arm alone.

Conclusions—The PAM4-immunoassay identified approximately two-thirds of stage-1 PDAC patients with high discriminatory power with respect to benign, non-neoplastic pancreatic disease. These results provide a rationale for testing patient groups considered at high-risk for PDAC with a combined PAM4/CA19-9 biomarker assay for detection of early-stage PDAC.

Keywords

pancreatic cancer; pancreatitis; diagnosis; early detection; PAM4; CA19-9
Although imaging methods play an important role in the detection of pancreatic cancer,\textsuperscript{1–4} especially in selecting a course of therapy, there are limitations with respect to detection of small lesions,\textsuperscript{1} as well as for discriminating pancreatic cancer from pancreatitis and other benign lesions.\textsuperscript{2,5,6} Thus, at present only 7\% of all pancreatic cancers are detected early.\textsuperscript{7} With no effective treatment for advanced pancreatic cancer, the prognosis for most patients is dismal.

Biomarkers potentially can provide a more cost-effective means for early detection and diagnosis, and a great deal of effort has been devoted to biomarker research.\textsuperscript{8–16} Such biomarkers also are in high demand for the detection and diagnosis of pancreatic cancer, especially early-stage disease, but as yet no single assay has achieved this role.

We have been engaged in the preclinical and clinical evaluation of monoclonal antibodies (mAbs) for the detection, diagnosis, imaging, and treatment of cancer, and have focused on the assessment of the PAM4 antibody, which binds to a large-size mucin\textsuperscript{17} for the detection and diagnosis of pancreatic ductal adenocarcinoma (PDAC),\textsuperscript{18–21} for imaging,\textsuperscript{22,23} and for therapy as the clinical reagent $^{90}$Y-clivatuzumab tetraxetan.\textsuperscript{24,25} Our data at the tissue level provide strong evidence that reactivity of the PAM4 antibody is highly restricted to PDAC, and that the specific PAM4-epitope is expressed (or becomes accessible) at the earliest stages of PDAC development; that is, the precursor lesions pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasia (IPMN), and mucinous cystic neoplasm (MCN).\textsuperscript{17,18} The PAM4-antigen is absent from normal pancreatic tissues and non-neoplastic disease of the pancreas. The antigen was not detected within ducts, acinar cells, nor isolated acinar-ductal metaplasia (ADM) within surgical specimens obtained from over 50 patients with chronic pancreatitis (CP).\textsuperscript{20–22} However, the PAM4-antigen was expressed by invasive PDAC identified in two cases, and in PanIN precursor lesions found in several of the specimens.

We reported recently that a serum-based enzyme immunoassay (EIA) was able to correctly identify 82\% of patients with known PDAC and, importantly, that this assay had promising sensitivity for detection of early-stage disease (62\% of stage-1 and 86\% of stage-2 patients).\textsuperscript{20} In the present study, we confirm and extend these findings in a much larger patient population that includes both malignant and benign diseases of the pancreas and surrounding tissues. Importantly, we also compare and combine the PAM4 assay with CA19-9 assay results, the latter considered the standard for use in the management of pancreatic cancer, and demonstrate that the unique combination of these two biomarkers provides significantly greater sensitivity for detection of PDAC with high diagnostic value.

**MATERIALS AND METHODS**

**Human specimens**

Sera from a total of 517 patients with histologically- or cytologically-confirmed diagnoses (assessed at each of the individual medical centers involved) of the pancreaticobiliary and peripancreatic tissues were received from the Universitätsmedizinen Göttingen (UMG, Göttingen, Germany), Johns Hopkins Medical Institutions (JHMI, Baltimore, MD), Immunomedics, Inc. (IMMU, Morris Plains, NJ), and the Cooperative Human Tissue Network (CHTN, Philadelphia, PA). All sera from UMG, JHMI, and CHTN were obtained from patients who had undergone surgical resection. In total, patients with early stage-1 and -2 disease comprised 40\% of the PDAC population within this study. In addition, 32 specimens from a control group of patients diagnosed with colon cancer were purchased from ZeptoMetrix Corp. (Buffalo, NY), and 79 specimens from healthy adults were purchased from ZeptoMetrix (Buffalo, NY; N=29) and SeraCare Life Sciences (Milford, MA; N = 50). Separate institutional review boards approved this study, and the New Cancer. Author manuscript; available in PMC 2014 February 01.
England Institutional Review Board approved the protocol of the Center for Molecular Medicine and Immunology. All specimens were deidentified and the immunoassays were performed in a blinded fashion.

**Enzyme immunoassays (EIA)**

The PAM4-immunoassay was conducted by the procedure described in a prior report. The anonymous serum specimens (300 μL) were first extracted with an equal volume of n-butanol, followed by mixture with an equal volume of chloroform to invert the organic and aqueous layers. The aqueous layer was then evaluated in the PAM4-assay. Nonlinear regression was performed with Prism 4.0 software (GraphPad, La Jolla, CA) to interpolate unknowns.

CA19-9 immunoassays were performed with a commercial kit (BioCheck, Inc., Foster City, CA) according to the manufacturer’s procedure. A value greater than 37 units/mL was considered a positive test. For combination of the PAM4 and CA19-9 immunoassay results, we employed the algorithm: Result = [PAM4]+0.01*[CA19-9]; a value >2.4 units/mL was considered a positive interpretation. This algorithm was chosen by review of ROC curves for maximum area under the curve.

**Statistical analyses**

ROC curves were evaluated by use of Med-Calc, version 7.5. Regression analyses, non-parametric log-rank comparisons, Student’s t-test, and ANOVA for comparison of test groups were evaluated by use of Prism software, version 4.0 (GraphPad, La Jolla, CA). All tests for statistical significance were two-sided.

**RESULTS**

**Performance characteristics of the PAM4-based EIA**

To evaluate immunoassay performance, normal human serum (pool of 5 individuals) was spiked with PAM4-antigen at nominal concentrations surrounding the critical 2.4 units/mL cutoff point (8.26, 3.30, 1.32, and 0 units/mL) and then assayed 14 times over a 2-month period. Linear regression of nominal vs. measured gave a trendline with $r^2=0.997$, indicating a consistent linear relationship over the range of concentrations surrounding the cutoff value (Figure 1). Values for the CV were 11.30% and 12.76% for the 8.26 and 3.30 units/mL spiked sera above the cutoff value, and 20.12% for the 1.32 units/mL below the cutoff, well within the guidelines for reproducibility of 15% above the cutoff and 20% at the cutoff value. A cutoff value of 2.4 units/mL, determined by receiver operating characteristic curve analysis in a prior learning set, was used as an indicator of positive response.

**PAM4-antigen levels in the circulation**

Overall sensitivity for detection of PDAC (N=298, with early stages 1 and 2 comprising 40% of cases) was 76%, with a median value of 10.40 units/mL at a specificity of 96% with respect to healthy adults (Table 1). The detection rate for patients with stage-1 disease was 64% (N=28) and, as expected, was considerably higher for advanced disease (85%). For the most part, sera from patients with pancreatic cancers arising from other tissues of origin (squamous, GIST, clear-cell, etc.) did not have detectable levels of the PAM4-antigen. Further, several non-pancreatic cancers with metastases to the pancreas (N=11) were amongst the group of sera evaluated, but only 2, an ovarian and a breast carcinoma, had elevated levels of PAM4-antigen. PAM4-negative metastatic disease to the pancreas included cervical, prostate, and renal cancers, along with melanoma and lymphoma.
Approximately half of the patients with ampullary (48%) and extrahepatic biliary (50%) adenocarcinomas had positive levels of circulating antigen by this assay. Nine of the 13 (69%) PAM4-positive biliary adenocarcinomas were located within the distal bile duct and 4 at the junction of the left and right hepatic ducts (Klatskin tumor). Although small patient numbers were evaluated, sera from patients with duodenal adenocarcinomas were mostly positive for PAM4-antigen, while sera from patients with intrahepatic cholangiocarcinomas were mostly negative. Patients with colon carcinoma showed little reactivity in this EIA, with only 5 of 32 (16%) considered positive. PAM4-antigen levels were significantly higher in PDAC than all other patient groups; \( P<0.0001 \) for all groups, except extrahepatic biliary adenocarcinoma (\( P=0.0257 \)).

Of 120 patients diagnosed with benign, non-neoplastic conditions of the pancreas, only 24 (19%) were positive for the PAM4-antigen, and in particular, 18 of 80 (23%) patients with CP were positive. As shown in Figure 2, ROC curve analyses demonstrated a statistically significant difference between the PDAC and CP groups (\( P<0.0001 \)), with an area under the curve of 0.84±0.02 (95% CI: 0.79–0.89). A similar finding was demonstrated for the comparison of PDAC with all benign specimens taken together (0.85±0.02; 95% CI: 0.82–0.89). The positive and negative likelihood ratios for differentiating PDAC from benign conditions of the pancreas were 4.00 and 0.30, respectively, which were significant (\( P<0.0001 \)).

Circulating levels of CA19-9 are known to be influenced by serum bilirubin.\(^{26,27}\) Thus, we sought to determine if a relationship existed between PAM4-antigen elevation and bilirubin levels in patients with PDAC. There was no such relationship, with PAM4-antigen levels being elevated independent of bilirubin concentration (\( N=44 \) cases from a single institution, \( r^2=0.0024, P=0.789 \)).

Comparison of PAM4 and CA19-9 immunoassays

Of the 628 total serum samples, sufficient volume was available to evaluate both PAM4 and CA19-9 antigens in 474 specimens. Overall sensitivity for detection of PDAC was similar for the two assays, 74% and 77% for PAM4 and CA19-9, respectively (Table 2). Sensitivity for detection of stage-1 disease, although somewhat higher for the PAM4-antigen, was also similar, 65% and 58% for PAM4 and CA19-9, respectively (\( P=0.578 \)). However, specificity was significantly greater for PAM4-antigen, particularly with respect to CP, 86% compared to 68% for the PAM4 and CA19-9 assays, respectively (\( P=0.014 \)). Also, as expected, CA19-9 results showed considerably higher detection rates for non-PDAC neoplasia, including patients with non-pancreatic cancers that metastasized to the pancreas. Thus, the positive likelihood ratio (+LR) was significantly higher for the PAM4 assay (+LR=5.29) than the CA19-9 assay (+LR=2.41).

Concentrations of circulating PAM4 and CA19-9 antigens in patients with PDAC were independent of each other (\( r^2=0.003, P=0.410 \)); the positive and negative interpretations were concordant in only 68% of the cases. This led us to investigate the potential use of a combined biomarker analysis that ultimately provided an improved overall PDAC detection rate (84%) than either arm alone. Importantly, this increased sensitivity did not have a commensurate significant decrease in specificity. ROC curves and statistical comparisons are presented in Figure 3 and Table 3.

DISCUSSION

There are several important findings from this study. The primary conclusion is that the PAM4-immunoassay is able to identify approximately two-thirds of stage-1 PDAC patients, and does so with high discriminatory power with respect to benign pancreatic disease. To
the best of our knowledge, there are only a few reports that describe the use of a noninvasive biomarker assay to detect stage-1 disease, and the majority of these discuss the performance characteristics of CA19-9. The sensitivity reported for CA19-9 in stage-1 PDAC ranges from 40%–64%, with our results showing a detection rate of 58%. However, the specificity reported for CA19-9 in the literature is considerably lower than reported for the PAM4-antigen, as is also true for the paired study described herein, particularly with respect to discrimination of PDAC and CP. Positive likelihood ratios were significantly higher for the PAM4-immunoassay.

The data suggest that the PAM4-antigen is not expressed by pancreatic tumors originating from non-epithelial tissues. However, PAM4-antigen is expressed and released by biliary and periampullary adenocarcinomas. Detection of these latter cancers, although rare (approximately 3500 new cases/year altogether in the U.S.), is likely to prove of clinical value, with follow-up imaging studies providing confirmation of tumor mass and location. That these latter cancers express the PAM4-antigen and are detectable by the PAM4-immunoassay was not unexpected, considering that these tissues are derived from closely-related structures in early embryonic development. Indeed, many of the reported biomarkers for PDAC are reactive with these tumors as well. The limited expression of PAM4 in the control colon cancer group confirms our prior serum assay and immunohistochemical studies indicating that PAM4 has limited elevation with other gastrointestinal and non-gastrointestinal cancers.

Also of importance is the ability of the PAM4-immunoassay to discriminate PDAC (and adjacent carcinomas) from benign, non-malignant disease of the pancreas, and in particular, the discrimination of PDAC and CP. In a prior study, we reported a discordance between the PAM4-antigen levels in the serum of patients with CP (N=29 with 38% FP) and immunohistochemical data on a separate group of patients with CP, where only 2 of 19 specimens had evidence of weak, focal expression of PAM4-antigen localized to ADM. In a followup immunohistochemical study of an additional 32 surgically resected specimens from CP patients, PAM4 labeling was observed in 6 of 32 (19%); however, PAM4 reactivity was restricted to the PanIN precursor lesions associated with CP, and was not observed in non-neoplastic inflamed pancreatic tissue. Taken together, the data from immunohistochemical labeling of over 50 specimens of chronic pancreatitis demonstrate that mAb-PAM4 does not react with the inflamed, non-neoplastic tissues. Thus, the question of the biological and clinical significance of a positive serum PAM4-antigen level in this patient group is of great importance. Are these assay results false positives or do they provide evidence of incipient PDAC (and/or precursor lesions)? Unfortunately, clinical followup for this patient group was unavailable. However, at the very least, the data suggest the need to undertake a prospective investigation to identify PAM4-positive CP patients, and/or others considered to be at high-risk for PDAC, for whom followup surveillance could be performed for potential discovery of early PDAC.

Based on the limitations of the CA19-9 assay (the CA19-9 assay cannot be performed in Lewis antigen-negative patients and is altered by bilirubin levels), we determined that a combined PAM4 and CA19-9 biomarker assay would provide a superior detection and diagnostic tool than either assay alone. Overall sensitivity was improved without loss of specificity. Thus, a positive result provides a rationale for proceeding to diagnostic imaging for confirmation of disease. Further, this combined biomarker assay could play a role in monitoring of treatment and perhaps prove useful for earlier detection of recurrent disease.

Due to the asymptomatic nature of PDAC, and its relatively low incidence, both medical and economic concerns have argued against screening of the general population at large. However, with recent studies providing the ability to identify specific patient groups at high
risk for PDAC, a rationale exists for longitudinal surveillance as a means to improve early detection specifically in these risk-settings. Individuals with a family history of PDAC,\(^2,36,37\) those patients with long-standing CP,\(^38,39\) patients with new-onset diabetes who meet certain other conditions,\(^40,41\) or those with FAMMM syndrome,\(^42\) etc., could be evaluated on a long-term basis for the detection of early malignant changes by use of the combined PAM4/CA19-9 assay procedure. Imaging has played an important role in these surveillance programs, the most significant being endoscopic ultrasound (EUS) scanning of the pancreas. EUS offers the additional ability to obtain fine-needle aspirates and/or fluids (pancreatic juice and cyst fluids) that can be examined for specific morphologic and biomarker changes representative of malignancy. Several reports have identified increased levels of CEACAMs and CA19-9 antigen,\(^43–45\) or have observed mutations in KRAS and other genes potentially indicative of cancer from these endoscopically-retrieved materials.\(^15,46,47\) Likewise, it is of interest to evaluate these fluids for the PAM4-antigen. However, it is obvious that use of a serum-based biomarker to detect early-stage PDAC would provide a clinically more valuable and cost-effective tool for monitoring patients at high-risk for this disease.

In summary, a blinded analysis of over 600 patient specimens demonstrated that the PAM4-assay could detect two thirds of patients with early PDAC, and did so with high specificity by discriminating PDAC from benign diseases of the pancreas. Further, inclusion of a second biomarker, CA19-9, significantly enhanced overall, positive identification of PDAC patients without loss of specificity. Although we have examined the CA19-9 assay in combination with the PAM4-assay, we appreciate that other biomarkers may prove of equal or greater value in combination with PAM4. Thus, our results provide a basis for future studies of biomarker combinations with PAM4 for surveillance of patients at high risk for PDAC, and as a potential means for monitoring patients with more advanced disease. Finally, the ability of the PAM4-immunoassay to identify a significant number of patients with biliary and periampullary adenocarcinomas, although relatively rare, may provide an additional means for improving the management of patients with these cancers.

Acknowledgments

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References


Figure 1.
Accuracy for quantitation of PAM4-antigen spiked into normal human serum. A consistent linear relationship was observed for nominal versus measured at antigen concentrations at or near the cutoff value of 2.4 units/mL.
Figure 2.
ROC curves for the performance of the PAM4-based immunoassay; pancreatic ductal adenocarcinoma vs chronic pancreatitis on the left, and pancreatic ductal adenocarcinoma vs all patients with diagnoses of benign pancreatic disease (including chronic pancreatitis) on the right. Values for the area under the curves (AUC) and 95% confidence limits are provided.
Figure 3.
ROC curves for the performance of the PAM4-immunoassay (—— bold black), CA19-9-immunoassay (– – – dashed black), and the combined-biomarker assay (………… dotted gray). On the left are the ROC curves for discrimination of pancreatic ductal adenocarcinoma vs chronic pancreatitis, and on the right, ROC curves for discrimination of pancreatic ductal adenocarcinoma vs all patients with diagnoses of benign pancreatic disease (including chronic pancreatitis). Descriptive statistical values are provided in Table 3. For each of the comparisons evaluated, the combined-biomarker assay had significantly greater sensitivity for detection of pancreatic cancer when compared to either the PAM4 or CA19-9 immunoassays alone ($P<0.0257$ or better).
Table 1

PAM4-antigen levels in patient sera.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>N</th>
<th>Median (units/mL)</th>
<th>Number of Positive Cases</th>
<th>Percentage of Positive Cases</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic Adenocarcinoma</td>
<td>298</td>
<td>10.40</td>
<td>225</td>
<td>76</td>
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<tr>
<td>Pancreatic Neuroendocrine</td>
<td>20</td>
<td>0.08</td>
<td>2</td>
<td>10</td>
<td>0.0001</td>
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<tr>
<td>Pancreatic - Other Morphology</td>
<td>7</td>
<td>0.51</td>
<td>1</td>
<td>14</td>
<td>0.0001</td>
</tr>
<tr>
<td>Non-PC, Mets to the Pancreas</td>
<td>11</td>
<td>0.00</td>
<td>2</td>
<td>18</td>
<td>0.0001</td>
</tr>
<tr>
<td>Ampullary Adenocarcinoma</td>
<td>21</td>
<td>1.52</td>
<td>10</td>
<td>48</td>
<td>0.0001</td>
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<tr>
<td>Biliary Adenocarcinoma</td>
<td>26</td>
<td>4.41</td>
<td>13</td>
<td>50</td>
<td>0.0257</td>
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<td>Cholangiocarcinoma</td>
<td>7</td>
<td>1.07</td>
<td>2</td>
<td>29</td>
<td>0.0001</td>
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<td>Duodenal Adenocarcinoma</td>
<td>7</td>
<td>2.80</td>
<td>4</td>
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<td>All Biliary and Periampullary</td>
<td>61</td>
<td>1.78</td>
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<td>Colon Carcinoma</td>
<td>32</td>
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<td>5</td>
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<td>Chronic Pancreatitis</td>
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<td>0.41</td>
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<td>23</td>
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<td>Benign Cystadenoma</td>
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<td>0.18</td>
<td>1</td>
<td>7</td>
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</tr>
<tr>
<td>Benign - Other</td>
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<td>0.20</td>
<td>5</td>
<td>20</td>
<td>0.0001</td>
</tr>
<tr>
<td>All Benign Disease</td>
<td>120</td>
<td>0.26</td>
<td>24</td>
<td>20</td>
<td>0.0001</td>
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<tr>
<td>Healthy Volunteers</td>
<td>79</td>
<td>0.27</td>
<td>3</td>
<td>4</td>
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</table>

*p values are in comparison to pancreatic adenocarcinoma; Mann-Whitney nonparametric test.
Table 2

Comparison of PAM4 and CA19-9 antigen levels in patient sera.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>PAM4</th>
<th>CA19-9</th>
<th>Combined</th>
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<tr>
<td></td>
<td>N</td>
<td>Number of Positive Cases</td>
<td>Percentage of Positive Cases</td>
</tr>
<tr>
<td>Pancreatic Adenocarcinoma</td>
<td>234</td>
<td>173</td>
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<td>Pancreatic Neuroendocrine</td>
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<td>Pancreatic - Other Morphology</td>
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<td>1</td>
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<td>Non-PC, Mets to the Pancreas</td>
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<tr>
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<td>13</td>
<td>57</td>
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<td>1</td>
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<tr>
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<td>Chronic Pancreatitis</td>
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<td>All Benign Disease</td>
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<td>18</td>
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<tr>
<td>Healthy Volunteer</td>
<td>50</td>
<td>3</td>
<td>6</td>
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Table 3

Diagnostic performance for PAM4 and CA19-9 biomarkers (ROC curve analyses).

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>AUC</th>
<th>P a</th>
<th>+LR</th>
<th>−LR</th>
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<td><strong>PDAC vs. CP</strong></td>
<td></td>
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<tr>
<td>PAM4</td>
<td>74</td>
<td>86</td>
<td>0.87 + 0.02</td>
<td>0.0001</td>
<td>5.29</td>
<td>0.3</td>
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<tr>
<td>CA19-9</td>
<td>77</td>
<td>68</td>
<td>0.84 + 0.03</td>
<td>0.0073</td>
<td>2.41</td>
<td>0.34</td>
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<tr>
<td>Combined</td>
<td>84</td>
<td>82</td>
<td>0.91 +0.02</td>
<td></td>
<td>4.67</td>
<td>0.2</td>
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<td><strong>PDAC vs. Benign</strong></td>
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<tr>
<td>PAM4</td>
<td>74</td>
<td>85</td>
<td>0.87 + 0.02</td>
<td>0.0001</td>
<td>4.93</td>
<td>0.31</td>
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<td>CA19-9</td>
<td>77</td>
<td>73</td>
<td>0.85 + 0.02</td>
<td>0.0257</td>
<td>2.85</td>
<td>0.32</td>
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<td>Combined</td>
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<td>83</td>
<td>0.91 +0.02</td>
<td></td>
<td>4.94</td>
<td>0.19</td>
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</table>

aAll P values are for comparison to PAM4 immunoassay. P values for CA19-9 versus Combined were identical to P values for PAM4 versus Combined.