



LETTER

Blood-based prediction and early detection of pancreatic cancer recurrence after tumor resection

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The 5-year survival rate of patients with pancreatic ductal adenocarcinoma (PDAC) is < 8%. The best therapeutic option for PDAC is tumor resection. However, most patients experience recurrence within 2 years after surgery¹. Postoperative monitoring includes CT imaging and determining CA19-9 levels² but the diagnosis is frequently late with dismal consequences for the patient³. In this study variations of proteins and microRNAs in peripheral blood were analyzed for the definition of classifiers that facilitate the early and accurate diagnosis of recurrences and prediction of how soon the recurrence will occur. Many blood-based markers for diagnosing PDAC have been reported⁴ but little is known about detecting recurrences⁵.

A total of 149 serum samples from patients who had undergone PDAC resection were studied. An immunoassay with 2953 antibodies was used to measure protein levels. The abundances of micro(mi)RNA were detected by next-generation sequencing. Support vector machine (SVM) classifiers were trained on the data. SVM classifier performance was validated on a separate set of 60 prospectively collected sera. In addition, there was technical validation by ELISA or qPCR. The results of the study are shown below. Flow charts, methods, actual data, and additional information are provided in the Supplementary Material.

Protein analysis

The design of the overall protein analysis is shown in **Figure S1**. Details on the data analysis are provided in the Methods section of the Supplementary Material. A total of 957 serum proteins exhibited significant abundance differences between patients with and without a recurrence based on the immunoassay analysis. The results were compared to variations observed in the secretomes of six PDAC cell lines and non-cancerous HPDE cells to focus on variations directly associated with the tumor. The cancer cell lines collectively represent diverse clinical backgrounds (primary vs. metastatic tumors), different differentiation states (well-, moderately, and poorly differentiated carcinomas), and diverse genetic profiles (*KRAS*, *BRAF*, and *TP53* mutations) that commonly occur in PDAC. A panel of six cell lines was selected over patient-derived organoids or primary cell lines because the cell lines capture major axes of PDAC heterogeneity, while providing a clean, stromal- and Matrigel-free secretome essential for unbiased biomarker discovery. All cells were grown individually or co-cultured with pancreatic stellate cells or peripheral blood mononuclear cells (PBMCs). This analysis yielded 617 proteins with significant differences between PDAC and HPDE cell secretomes. Some proteins are linked to immune cell trafficking and inflammatory responses, suggesting that biomarker candidates may not only originate from the tumor cells but might also be released by other cell types in the co-cultures.

Fifty-one proteins were differentially abundant in the serum and secretome with only concurrent changes considered. Receiver operating characteristic curve analysis was performed and the resulting area under the curve (AUC) value was designated as a measure of performance. No single protein produced an AUC > 90%. Furthermore, enormous variation in the performance of many proteins became apparent upon validation (**Figure S2**). Therefore, least absolute shrinkage and

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Received September 25, 2025; accepted January 6, 2026;

published online February 16, 2026.

Available at www.cancerbiomed.org

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selection operator (LASSO) regression and recursive feature elimination (RFE) with 5-fold cross-validation were applied, which are robust procedures for marker selection and deleting unnecessary covariates⁶. The resulting protein panel was used to train an SVM classifier, which consisted of 8 proteins and distinguished patients with or without recurrence with an AUC value of 76% upon validation (**Table S1**).

The same analysis process was performed with the 957 serum proteins. The rationale was that the entire body reacts to the re-emergence of a tumor so that protein changes released by cells and tissues other than the actual tumor could be indicative of disease⁷. Again, no individual molecule exhibited robust accuracy (**Figure S2**). The biomarker selection process resulted in an SVM classifier of eight proteins. Remarkably, only the protein GCA was part of this classifier and the classifier derived from the tumor-associated proteins. On validation the classifier had an AUC of only 68% (**Table S1**).

With both approaches yielding classifiers of limited performance, we hypothesized that a combination may improve accuracy. Therefore, another round of RFE and SVM training was performed. The resulting classifier, consisting of 10 proteins, yielded a validated AUC of 85% (**Table S1**). This improvement was due to a much-increased sensitivity. Interestingly, GCA was not included despite being part of both initial classifiers, indicating that a combination of complementary rather than supplementary factors is critical for improving the quality of diagnostics.

miRNA classifier

The miRNA content of the same samples analyzed for protein variations were studied by small RNA sequencing to define a miRNA signature capable of detecting tumor recurrence (**Figure S3**). The details on data analysis are provided in the Methods section of the Supplementary Material. One hundred miRNAs exhibited significant differences upon logistic regression analysis. Individual miRNAs were insufficient for reproducible diagnosis, as with proteins (**Figure S4**). A classifier of 7 miRNAs was constructed using LASSO and RFE that yielded an AUC of 90% upon validation (**Table S1**).

Diagnostic performance by combining miRNA and protein data

The 17 biomarkers of the protein and miRNA classifiers were combined and RFE was used to find the best combination because multi-parametric diagnosis with different biomarker types might be superior in both accuracy and robustness⁸, supplementing and complementing each other. Remarkably, a classifier made of only 5 molecules yielded an AUC of 89% upon validation. The overall highest AUC with 93% was achieved with all 7 miRNAs of the miRNA classifier and 3 proteins (**Figure 1A and Table S1**). Adjuvant chemotherapy had

been prescribed to 87% of the patients, from whom samples were collected. The relative abundance of markers between the samples from the patients who did and did not receive chemotherapy was compared to test whether adjuvant chemotherapy was a confounding factor; no significant difference was detected. In addition, the classifier performance was not affected by chemotherapy (**Figure S5**).

The diagnostic performance of CA19-9 variations with the same sera was studied for benchmarking. This resulted in AUC values of 76% or 77% in the discovery or validation sera, respectively (**Figure 1A**). The following clinicopathologic predictors of recurrence were also considered: age; tumor location; tumor stage; TNM status; and margin status. A SVM model built on this basis produced a validated AUC of 71%.

Early detection of recurrence

Detecting tumor recurrence earlier than currently possible could strongly affect outcome. Systemic treatment could be started earlier and a recurrent tumor might be removed by another surgical intervention, resulting in a substantially better prognosis⁹. Samples from 45 patients, who had blood collected several times during clinical follow-up evaluations, were tested. The blood-based protein/miRNA classifier detected recurrence earlier than current diagnostics in all cases (**Figure 1B**). The median gain was approximately 5 months. While promising, this data represents only an initial performance indicator because the sample number was limited and the time gained differed substantially between patients, making a thorough statistical analysis unfeasible.

Prediction of time-to-tumor recurrence

In addition to diagnostics a classifier that could predict the time-to-recurrence was defined. To this end, the advantage of consecutively collected samples was leveraged. The date of clinically confirmed recurrence was defined as the endpoint and the time from that endpoint backward was calculated. Pairwise comparisons of samples collected at the time of diagnosing recurrence versus samples collected earlier were performed. The same analysis approach used for diagnostics was followed. Three to five molecules were required for classifiers with AUC values of 93%–100% in validation (**Figure 1C and Table S2**).

Confirmation of biomarker performance

Commercial ELISA kits were used to confirm results obtained with the microarray-based immunoassay to test the robustness of results. Proteins, the serum levels of which vary, as well as molecules for which no significant change was recorded, were studied. More than 50 patient samples were analyzed with ELISA tests. High concordance with

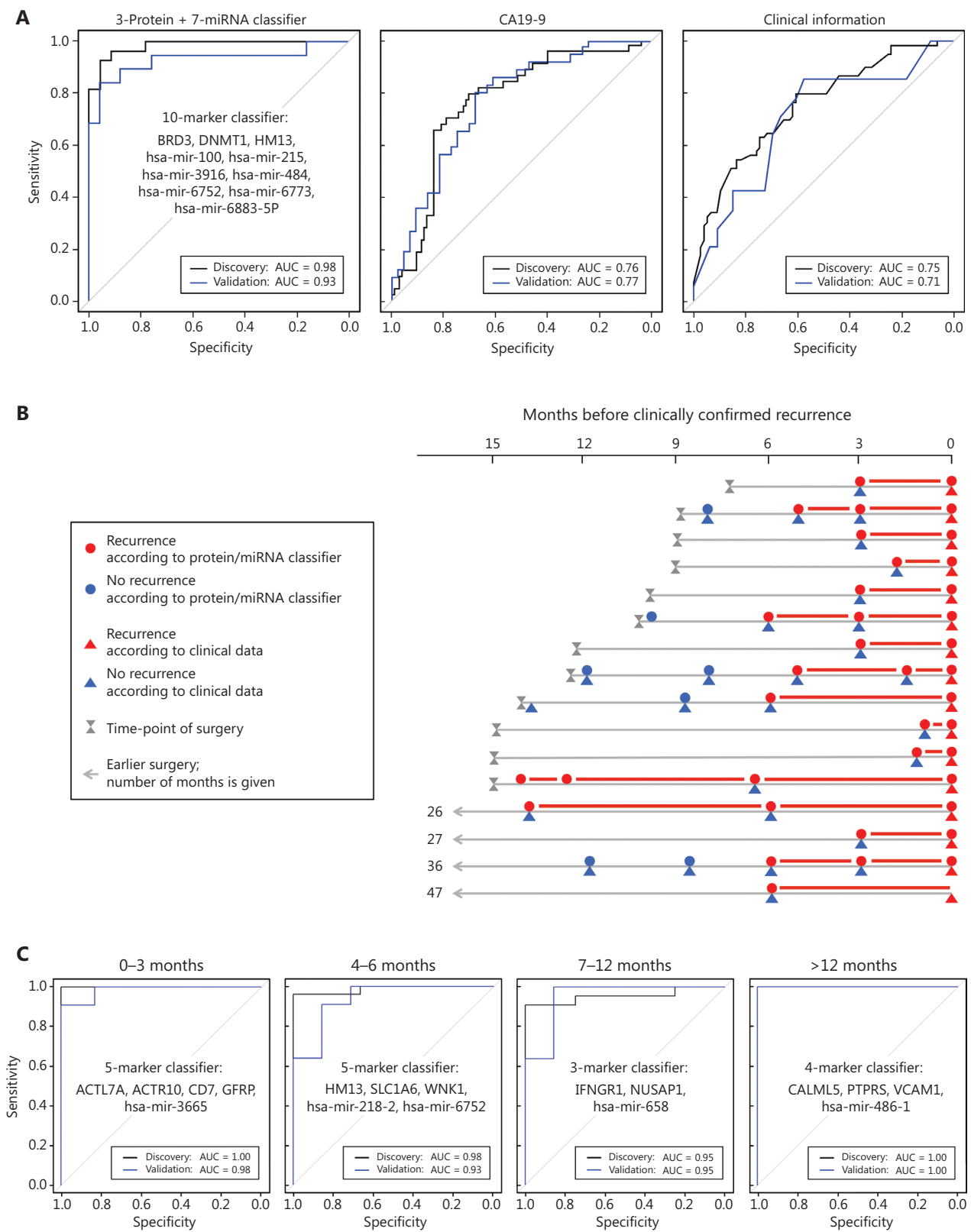


Figure 1 SVM classifiers for diagnosing and predicting pancreatic cancer recurrence. Results in (A) and (C) are presented as receiver operating characteristic curves and the corresponding AUC values are given as determined with the discovery and validation samples, respectively. (A) The overall best diagnostic result was obtained by a classifier that combined three proteins and seven miRNAs. For benchmarking,

diagnosis was performed on the same samples by means of CA19-9 measurements and clinicopathologic predictors. (B) Blood samples were drawn from individual patients after tumor resection for early detection of tumor recurrence. In parallel, standard diagnostics was applied. The time span is shown in red between classifier-based diagnosis and clinically confirmed recurrence for each patient. (C) PDAC recurrence prediction was as follows: pairwise comparisons were performed between the protein and miRNA content of serum samples collected at the time of recurrence versus samples, which had previously been collected at 1–3 months, 4–6 months, 7–12 months, or >12 months for the identification of predicting classifiers.

the antibody microarray data was demonstrated in all cases (Figure S6). The miRNA results were confirmed using qPCR instead of small RNA sequencing; a commercial system was utilized. There was high concordance, as with the proteins (Figure S6).

Conclusions

Protein and miRNA liquid-biopsy classifiers were defined and validated that permit detection of PDAC recurrence with an AUC of 93%, substantially outperforming current processes. While this accuracy would be insufficient for screening individuals with no risk or no strong suspicion of disease, it could make a significant difference for resection patients, since basically all will experience recurrence. Also, diagnosis might be substantially earlier than currently possible. In addition, abundance variations allow predicting the period until recurrence is likely to occur. In combination, this could have substantial consequences on patient management and prognosis.

Grant support

F.N.A. was supported by a long-term fellowship of the German Academic Exchange Service (DAAD). C.Z. is gratefully acknowledging a scholarship of the China Scholarship Council. The work was in part funded by BMBF grant 01KT1506 Bio-PaC (Grant No. EU TRANSCAN-2) to N.A.G. and T.H.

Conflict of interest statement

No potential conflicts of interest are disclosed.

Author contributions

Conceived and designed the analysis: Fawaz N. Al-Shaheri, Natalia A. Giese, Jörg D. Hoheisel, Ulrike Heger, Thilo Hackert. Collected the data: Fawaz N. Al-Shaheri, Teresa Colbatzky, Lucas Sperling, Miriam Schenk, Christin Tjaden, Ulrike Heger. Contributed data or analysis tools: Mohamed S. S. Alhamdani, Andrea S. Bauer, Henning Boekhoff, Liang Xu, Christin Tjaden, Thilo Hackert, Markus W. Büchler. Performed the analysis: Chaoyang Zhang, Fawaz N. Al-Shaheri, Teresa Colbatzky, Andrea S. Bauer, Natalia A. Giese, Jörg D. Hoheisel, Ulrike Heger. Wrote the paper: Fawaz N. Al-Shaheri, Jörg D. Hoheisel.

Data availability statement

The data generated in this study are available in the Supplementary Material of this article and from the authors upon request.

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Cite this article as: Al-Shaheri FN, Colbatzky T, Bauer AS, Zhang C, Alhamdani MSS, Boekhoff H, et al. Blood-based prediction and early detection of pancreatic cancer recurrence after tumor resection. *Cancer Biol Med*. 2026; x: xx-xx. doi: 10.20892/j.issn.2095-3941.2025.0495