Supplementary Online Content

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This supplementary material has been provided by the authors to give readers additional information about their work.

Supplemental Text

Patients

When we calculated the number of patients and controls to be included in this miRNA biomarker study, there were no studies regarding miRNA expression in whole blood in patients with PC. Power calculations could therefore not be performed. The "Discovery Study" included patients with PC from the Copenhagen University Hospitals (Center 1 (n = 122): July 2008 - June 2011; Center 6 (n = 21): January 2011 - December 2011 (only in patients operated on)) and 18 patients with chronic pancreatitis (July 2008 – June 2011). All six participating hospitals included patients in the "Training Study" (Center 1 (n = 36): July 2011 - June 2012; Center 2 (n=78): April 2009 - June 2012; Center 3 (n = 19): July 2009 - June 2012; Center 4 (n = 139): August 2010 - June 2012; Center 5 (n = 18): July 2010 - June 2012; and Center 6 (n = 16): January 2012 - June 2012). For the final "Validation Study," we used PC patients included in Centers 1-6 (1: n = 33; 2: n = 11; 3: n = 8; 4: n = 8; 5: n = 4; and 6: n = 22) from July 2012 - October 2012, and 7 patients with chronic pancreatitis included from June 2011 - October 2012.

We started the miRNA analysis in January 2012 when enough patients were included (143 patients with PC and 18 patients with chronic pancreatitis) according to the literature regarding miRNA studies in serum or plasma of patients with cancer. The number of healthy participants was approximately half of the number of patients with PC in the "Discovery Study" and similar to the number of patients with PC in the "Training Study" and "Validation Study".

Methods

Purification of miRNA in whole blood

Pretreatment whole-blood samples (2.5 ml) were collected in PAXgene Blood RNA tubes (Qiagen), which stabilize the RNA, and treated according to the manufacturer's instructions. Small RNAs were extracted from the PAXgene Blood RNA tubes in 22 fractions (1). The PAXgene Blood RNA

tubes were processed on the Biorobot MDx (Qiagen, Hilden, Germany) using a customized protocol that binds large RNAs and rescues the run-through from the RNA binding plate. The binding condition in the run-through was subsequently modified, enabling the miRNA to be purified on an RNeasy-96 plate. The concentration of the small RNA fractions was assessed by absorbance spectrometry on a DTX 880 (Beckman Coulter). The purification was performed at the biotech company AROS, Applied Biotechnology A/S, Denmark.

MiRNA expression analysis

All miRNA analyses were performed at the biotech company AROS, Applied Biotechnology A/S, Denmark.

"Discovery Study". The TaqMan® Human MicroRNA assay using A LDA cards (v2.0) and B LDA Cards (v3.0) (Part Number 4400238, Applied Biosystem) was used. This method used a set of 22 pre-configured micro fluidic cards that enable quantization of 754 preconfigured human miRNAs. Included on each card were 3 TaqMan® MicroRNA assay endogenous controls to aid in data normalization and 1 TaqMan® MicroRNA assay not related to human as a negative control. RNA was transcribed into cDNA in 2 multiplex reactions each containing 3 µl of the small RNA preparation and either Megaplex RT Primer A Pool or Pool B pool and using the TaqMan® MicroRNA Reverse Transcription Kit in a total volume of 14 µl. Prior to loading the arrays, a 12-cycle pre-amplification reaction was performed using 2.5 µl cDNA in a 25-µl reaction. Each of the arrays was loaded with 800 µl Universal PCR Master Mix assay containing 1/40 of the pre-amplification reaction and run on the 7900HT Fast Real-Time PCR System. The instructions provided by Applied Biosystems were carefully followed, including the use of pre-amplification (https://www.products.appliedbiosystems.com). Six samples could be analyzed in a day, and hence the duration of the experiment was 47 days. To reduce confounding and bias from technical variation, the samples were purified so that age, sex, and diagnosis were distributed in a balanced

way with respect to day of RNA purification and miRNA analysis and randomized within each day of RNA purification and miRNA analysis. We defined technical variation as differences in plates, purification, and analysis. An extra whole-blood sample, in 10 replicates, was included as internal control.

"Training Study": 38 miRNAs selected from the "Discovery Study" were analyzed using the FluidigmBioMark™ System. These miRNAs were analyzed in duplicate (n = 23) or triplicate (n = 15) at AROS. This system can perform multiple simultaneous real-time PCR experiments running gold-standard TaqMan® assays in nanoliter quantities. The instructions from Fluidigm were carefully followed (https://www.fluidigm.com). The sample RNA concentrations were normalized to 10 ng/µl on a Biomek pipetting station using method "Normalization from 96 to 96 v1.1." cDNA for each sample was prepared from 30 ng RNA in each of 2 20-µl reactions – 1 using Megaplex Primer Pool A and 1 using Megaplex Primer Pool B. A synthetic plant miRNA, ath-miR159a, was added as an internal control. The cDNA was put through a 14-cycle pre-amplification using a mixture of the specific primers/probe assays for 39 miRNAs species (38 selected miRNAs plus 1 internal control) to be profiled. The PCR analysis was performed on Fluidigm 96.96 dynamic arrays, with the assays applied in triplicate. The samples were balanced and randomized in a manner similar to that in the "Discovery Study," with the exception that the samples were balanced with respect to plates instead of analysis days.

"Validation Study": Thirteen miRNAs were selected based on the results of both the "Discovery Study" and "Training Study" including the miRNAs used in the Diagnostic indices. Each miRNA was replicated 8 times (except let-7g, 7 times), and analyzed using the FluidigmBioMark™ System. Please see text above regarding the details of this system.

Statistics

The statistical software R version 2.14.0 was used (2) (including the library stats version 2.14.0 for the logistic regression model and libraries ROCR version 1.0-4, pROC version 1.5 and "binom" for the analysis of the suggested indexes).

"Discovery Study". Data were checked for outliers by visual inspection, and samples with low RNA yield or absorbance were excluded. Three samples with very few detectable miRNAs and 1 sample with too low absorbance were excluded. The LDA card contains 1 sample per card, implying that sample and card are completely confounded, and thus technical variation is a problem that can be handled with normalization. To test the sensitivity of results due to plate variation and technical bias, analysis was done on raw values as well as normalized values.

Different normalization methods were applied to verify the sensitivity of results. Raw Ct values of each miRNA were pre-processed using either 1) rank normalization; 2) quantile normalization (3); 3) 120 most expressed microRNAs (4); 4) endogene normalization; or 5) kept as raw values. Rank normalization was done for each patient by ranking the Ct values for the miRNAs, such that the lowest Ct value was given rank 1 and so on. Normalization by endogene controls or by 120 most expressed miRNA was done by subtracting the mean value of the endogene controls (mean of RNU44 and RNU48) or the mean value of the 120 most expressed miRNAs for each patient from the Ct values. The described statistical analysis described in the following was applied separately for each normalization method. The association between miRNA expression and case-control status was bivariably analyzed by means of logistic regression with a logit link, and miRNAs were included additively as continuous linear variables (5-10).

The selection of miRNAs from the discovery data was based on bivariable models and did not include interaction terms or assessment of possible co-linearity. We tested whether the distribution of *P* values deviated significantly from a uniform distribution by applying a Kolmogorov-Smirnov test and thus tested whether any association between miRNA expression and incidence of cancer was present (11). Following this overall test, we included all miRNAs with a *P* value less than

0.001 for further analysis. Applying a significance level of 0.001 corresponds to an overall probability of making more than 1 type I error to be less than 0.05 (when testing 700 hypotheses each at 5% significance level). The choice of significance level of 0.001 for the individual test was thus corrected for multiple testing. We furthermore analyzed whether missing values were associated with outcome for each miRNA and tested these with a Kolmogorov-Smirnov test (P = 0.7040). Based on the bivariable analysis, all miRNAs that met the 0.001 significance level were included in a multivariable model, which was then reduced by means of backwards elimination and the Akaike's Information Criterion (AIC). In this analysis, only complete cases were included, i.e., samples in which all miRNAs included in the multivariable model were detectable. The final model was obtained by backwards elimination of the multivariate model using AIC (12). The estimated effects of the most significant miRNAs were presented as odds ratios (ORs) with 95% confidence intervals (CIs) corresponding to an interquartile range increase (to be able to compare different normalizations).

"Training Study". Data were checked for outliers, and samples with low RNA yield or absorbance were excluded from further analysis. miRNAs found significantly differentially expressed between PC and controls by more than 1 of the normalization methods in the "Discovery Study" were analyzed in triplicate. miRNAs that were found significantly differentially expressed by only 1 of the normalization methods were analyzed in duplicate. The mean Ct of the replicated measurement of each miRNA for each sample was included in the analysis. Association between miRNA expression and case-control status was estimated bivariably by means of logistic regression, with miRNA included as continuous variables, and the obtained effect estimates were compared with the ones found in the "Discovery Study". The estimated ORs per 1 unit increase in Ct of the 38 selected miRNAs were presented with 95% CI. Furthermore, we computed ORs adjusted for serum CA19-9 above/under 37 KU/l. In all analyses, missing values were excluded.

"Validation Study". The mean Ct of the replicated measurement of each miRNA for each sample was included in the analysis. Association between miRNA expression and case-control status was

estimated unadjusted as well as adjusted for serum CA19-9 by means of logistic regression as described under "Training study". The estimated effects of the 13 miRNAs were presented in terms of ORs with 95% CIs. In all analyses, missing values were excluded.

Diagnostic indices I and II. Based on the miRNAs found significant in the "Training Study" (all found significant in the "Discovery Study"), we suggested 2 diagnostic indices.

Index I: $Index_k = miRa_k + miRb_k - miRc_k - miRd_k$. Where $miRa_k$ and $miRb_k$ are the Ct values for 2 specific miRNAs, here denoted a and b, for the k^{th} sample that are significantly upregulated in cases. $miRc_k$ and $miRd_k$ are the Ct values for 2 specific miRNAs, denoted c and d, for the k^{th} sample that are significantly downregulated in cases. Thus, index I is a linear combination of 4 miRNAs. The index is designed to maximize the contrast between cases and controls, and simultaneously eliminate the influence from technical nuisance variation. The latter was obtained by having a balanced sum of signs with 2 plusses for miRNAs with OR > 1 and 2 minuses for miRNAs with OR < 1.

Index II. miRNAs that were significant in the "Training Study" ($P < 0.05$ cut-off) were included in a multivariable model, and a backwards elimination procedure was applied using Akaike's Information Criterion as optimality criterion to obtain a second index. Index II is the linear predictor from the logistic regression model, i.e., the log-odds of cancer. This data driven index II is given as:

$$Index_k = c + \sum_{i=1}^{p} \beta_i * miR_{i,k} = c + \beta_1 * miR_{1,k} + \beta_2 * miR_{2,k} + \cdots + \beta_{p-1} * miR_{p-1,k} + \beta_p * miR_{p,k}.$$

Where c is a constant and β_1-β_p are estimated coefficients from the multivariable logistic regression model. k identifies the k-th sample, and miR_1 to miR_p are p different miRNAs identified after applying the backwards elimination procedure. β_1-β_p can be interpreted as weights for the miRNAs in index II. This index is fitted from data but does not eliminate technical nuisance variation. Index II corresponds to the upper limit in terms of training and is thus potentially an over-fit. The index II can be seen as a benchmark index for the "Training Study", i.e., indicating the upper limit in terms of performance.

Using indices I and II the sensitivity, specificity, and area under the ROC curve (AUC) and corresponding 95% CIs were presented for the "Discovery Study", "Training Study", and "Validation Study". For the indices, we considered the performance by defining a cut-off corresponding to fixing the sensitivity to 0.85, i.e., choosing a value such that 85% of the cases were correctly classified to handle the difference in setup between the "Training Study" and the "Validation Study." Based on the cut-off, participants were classified as cases or controls, and subsequently, specificity, accuracy and AUC were computed.

For indices I and II, we also tested the performance by including serum CA19-9 in the indices. This was done by fitting a logistic regression model with the indices as offset (coefficient 1) and with log of serum CA19-9 as a continuous variable. Additionally, we tested serum CA19-9 alone in a similar way as done for indices I and II. Finally, we evaluated the indices in low-stage patients with PC and in patients with other periampullary cancers.

Results

Purification and microRNA assay quality

The mean yields of purified small RNAs were 4.40 μg, 2.99 μg, and 4.00 μg for patients with PC, healthy participants, and patients with chronic pancreatitis, respectively. The yield of small RNAs was significantly higher in patients with PC than in healthy participants ($P < 0.001$).

In the "Discovery Study" 1 sample had an absorbance (260/280) ratio less than 1.80 and was excluded from further analysis. Three outliers (1 according to many missing miRNAs and 2 according to a high mean Ct value, i.e., low miRNA expression) were excluded. In the "Training Study" let-7b and miR-374b* failed to show any expression in the PCR, leaving 36 of the selected miRNAs from "Discovery Study" for further analyses. eTable 1 shows the quality of the PCR assays for the "Training Study" and the "Validation Study," including numbers of missing miRNAs for cancers and coefficients of variance (CV) for the replicates of each miRNA.

Validation of diagnostic miRNAs described in the literature in whole blood, serum, and plasma

Our findings of significantly deregulated miRNAs in the "Discovery Study" were consistent through the "Training Study" and the "Validation Study". There was also good agreement with the only other study of whole blood miRNA expression profiles in patients with PC by Bauer et al., analyzed with another array, where let-7b, miR-9*(miR-9), -26b(miR-26b*)-34a,-122,-126*,-199b-5p,-223, -582-5p(-582-3p) -636, -769-5p(-769-3p) and -885-5p were also reported to be deregulated compared to healthy controls (13).

eTable 3 gives significantly differently expressed miRNAs between patients with PC and healthy participants (up-regulated or down-regulated) in the Discovery, Training, and Validation studies. The expression of miRNAs in whole blood, plasma, and serum reported in other studies is also shown in eTable 3.

Performance of serum CA 19-9 vs. bPANmiRC indices I and II in combination with CA 19-9

eTable 4 shows the results of a test for the performance in terms of AUC for serum CA 19-9 with and without indices I and II using the procedure suggested by Delong et al. (14). It should be noted that the CIs around the AUCs were found by means of the bootstrap method and are thus not necessarily symmetric. The combination of index I and serum CA 19-9 increased AUC significantly compared to CA 19-9 alone (patients with PC vs. healthy participants), whereas for index II the combination increased AUC but not significantly.

Other periampullary cancers

When index I was used to discriminate between the 33 other periampullary cancers (15 patients with ampullary adenocarcinomas, 6 patients with duodenal adenocarcinomas, and 12 patients with distal common bile duct cancer) and healthy participants in the "Validation Study", the AUC was 0.98 (95% CI, 0.94-1.00). Using the cut-off for index I, 33 cancers (100%) and 21 (48%) healthy

participants were correctly classified. When index II was used to discriminate between the 33 patients with other periampullary cancers (PAC) and healthy participants in the "Validation Study", the AUC was 0.93 (95% CI, 0.86-0.98). Thirty-one patients with cancer (94%) and 24 (55%) healthy participants were correctly classified. Serum CA 19-9 did not improve the indices for the other periampullary cancers. Box plots are shown in eFigure 7.

Robustness of effect estimates

Imputed values for missing Ct values were calculated with the use of standard procedures for imputation [15]. We applied 2 different imputation strategies. First, for each case and control with missing Ct values, we imputed the missing values with the corresponding 95% percentile for the same miRNA among cases and controls without missing values. This strategy makes the assumption that missing Ct values is a result of no or very low expression of that miRNA. Second, we repeated the procedure but imputed missing Ct values with randomly selected Ct values for that miRNA, assuming that missing Ct values were a result of a failed measurement process. *P* values of all miRNA on diagnosis were obtained for both imputation strategies by bootstrapping and compared to the *P* values obtained by the complete case analysis (eFigure 1). The results obtained by analyzing the data with imputed data did not change the findings, as the main part of the same miRNA turned out statistically significant in analysis with and without imputed values.

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eTable 1. Quality of Polymerase Chain Reaction Assays

	Training Study						Validation Study					
miRNA	No. of missing (HP)	No. of missing (PC)	Repli-cates	Mean Ct	Sds (σ)	Coeffi-cient of variance (CV) §	No. of missing (HP)	No. of missing (PC)	Repli-cates	Mean Ct	Sds (σ)	Coeffi-cient of variance (CV) §
Let-7b			2									
Let-7g	5	6	2	19.84	0.289	0.015	1	0	6	20.27	0.750	0.037
miR-9*	105	80	2	27.69	1.366	0.049						
miR-19b	0	0	2	9.60	0.079	0.008						
miR-23a	23	11	2	22.70	0.573	0.025						
miR-24-2*	163	116	2	28.27	1.731	0.061						
miR-26b	1	1	2	18.90	0.165	0.009	0	0	7	20.96	0.591	0.028
miR-30b	0	0	2	9.95	0.086	0.009	0	0	7	11.25	0.066	0.006
miR-31	2	3	3	22.88	0.576	0.025	0	1	7	22.31	0.656	0.029
miR-31*	101	127	3	27.12	1.086	0.040						
miR-34a	35	15	3	25.09	1.075	0.043	5	4	7	24.41	1.368	0.056
miR-93	1	0	3	15.40	0.073	0.005						
miR-122	93	37	3	26.98	1.370	0.051	27	28	7	24.33	1.044	0.043
miR-126*	4	1	3	22.16	0.377	0.017	10	1	7	23.05	0.844	0.037
miR-128	1	2	2	19.36	0.171	0.009						
miR-143	14	4	2	25.90	0.937	0.036			7			
miR-144*	13	19	2	18.62	0.245	0.013			7			
miR-145	1	2	3	18.25	0.161	0.009	0	0	7	16.57	0.136	0.008
miR-150	0	0	3	9.91	0.082	0.008	0	0	7	11.05	0.106	0.010
miR-186	2	1	2	19.16	0.120	0.006						
miR-199b-5p	96	61	3	26.81	1.231	0.046						
miR-223	0	3	2	5.93	0.054	0.009	0	0	7	6.66	0.073	0.011

	Training Study						Validation Study					
miR-223*	7	2	2	23.98	0.785	0.033						
miR-342-5p	67	72	2	24.61	0.848	0.034						
miR-345	1	0	2	18.08	0.134	0.007						
miR-362-3p	9	11	2	26.04	0.721	0.028						
miR-374b*			2									
miR-505	51	38	2	23.90	0.771	0.032	1	0		22.52	0.789	0.035
miR-508-3p	204	176	2	32.13								
miR-539	40	44	2	29.49	1.669	0.057						
miR-582-3p	145	83	3	32.07	1.266	0.039						
miR-625	3	6	2	27.05	0.929	0.034						
miR-628-3p	79	78	2	29.77	1.567	0.053						
miR-636	4	3	3	20.48	0.260	0.013	0	0		18.75	0.141	0.008
miR-769-5p	12	10	3	23.39	0.711	0.030						
miR-885-5p	13	8	3	23.20	0.617	0.027	0	1		22.64	0.748	0.033
miR-935	12	32	3	24.26	1.016	0.042						
miR-941	201	152	2	26.46	2.049	0.077						

§ CV= sds / mean Ct. Healthy participants (HP); Patients with pancreatic cancer (PC).

Two miRNAs, let-7b and miR-374b* failed to show any expression in the PCR.

eTable 2. Odds Ratios and 95% Confidence Intervals for MicroRNAs From the Discovery Study

miRNA	OR Raw Ct values	*P* value	OR Rank	*P* value	OR Quantile	*P* value	OR 120 most expressed	*P* value	OR Endogene controls	*P* value
miR-34a	0.16 (0.04-0.44)	0.001	0.27 (0.10-0.63)	0.004	0.39 (0.16-0.84)	0.023	0.16 (0.06-0.38)	<0.001		
miR-145	0.30 (0.11-0.73)	0.013	0.14 (0.04-0.40)	<0.001	0.70 (0.35-1.08)	0.231	0.15 (0.04-0.47)	0.002		
miR-769.5p	0.06 (0.01-0.22)	0.001	0.07 (0.02-0.20)	<0.001	0.28 (0.10-0.66)	0.006	0.12 (0.03-0.36)	<0.001		
miR-885.5p	0.20 (0.06-0.51)	0.002	0.22 (0.07-0.58)	0.004	0.22 (0.07-0.57)	0.004	0.25 (0.10-0.54)	0.002		
miR-935	8.72 (2.30-44.05)	0.004	13.84 (4.03-59.92)	<0.001	3.84 (1.54-11.72)	0.009			4.30 (1.88-11.15)	0.001
miR-31*	2.98 (1.07-9.74)	0.051			6.70 (2.56-21.50)	<0.001			2.64 (1.47-5.05)	0.002
miR-31			6.69 (2.12-25.21)	0.002			2.77 (0.99-8.40)	0.059	6.86 (2.42-21.53)	<0.001
miR-150	8.73 (2.71-39.46)	0.001	3.13 (1.41-7.78)	0.008			8.92 (2.21-44.00)	0.004		
miR-199b.5p			0.35 (0.12-0.78)	0.031			0.57 (0.20-1.14)	0.261	0.42 (0.20-0.87)	0.023
miR-93	17.60 (2.00-238.85)	0.017			9.36 (2.70-41.69)	0.001				
miR-122			0.43 (0.16-1.01)	0.067					0.25 (0.12-0.48)	0.001
miR-126*	0.11 (0.01-0.75)	0.032							0.13 (0.03-0.50)	0.005
miR-582.3p					0.57 (0.26-1.18)	0.133			0.52 (0.29-0.91)	0.025
miR-636					3.51 (1.39-9.96)	0.012			0.25 (0.08-0.70)	0.012
Let-7b	5.75 (1.53-27.85)	0.017								
Let-7g									12.96 (3.12-62.59)	<0.001
miR-9*	5.02 (1.42-21.64)	0.019								
miR-19b			2.02 (0.98-4.33)	0.061						
miR-23a					4.09 (1.46-13.23)	0.012				
miR-24.2*							1.98 (0.82-5.17)	0.145		

miRNA	**OR Raw Ct values**	***P* value**	**OR Rank**	***P* value**	**OR Quantile**	***P* value**	**OR 120 most expressed**	***P* value**	**OR Endogene controls**	***P* value**
miR-26b							0.36 (0.10-1.09)	0.082		
miR-30b					0.51 (0.25-0.89)	0.034				
miR-128							0.18 (0.05-0.56)	0.004		
miR-143			2.81 (1.03-8.33)	0.046						
miR-144*	8.61 (1.09-83.74)	0.049								
miR-186							0.26 (0.08-0.73)	0.018		
miR-223							0.21 (0.06-0.62)	0.007		
miR-223*									0.35 (0.09-1.24)	0.113
miR-342.5p					2.05 (0.93-4.65)	0.077				
miR-345							4.16 (1.44-13.51)	0.011		
miR-362.3p					2.12 (0.86-5.75)	0.116				
miR-374b*			2.82 (0.93-9.41)	0.075						
miR-505	0.39 (0.12-1.08)	0.081								
miR-508.3p			5.78 (2.00-19.71)	0.002						
miR-539									2.67 (1.28-6.00)	0.011
miR-625					0.40 (0.14-0.95)	0.049				
miR-628.3p									2.38 (0.89-6.89)	0.093
miR-941					0.46 (0.20-0.94)	0.048				

The microRNAs from the **“**Discovery Study” were selected for further analysis after 5 different methods of normalization. Odds ratios (ORs) and 95% CIs (per interquartile range increase) for prediction of pancreatic cancer compared to healthy participants and patients with chronic pancreatitis. The following statistical analysis was applied separately for each normalization method. The association between miRNA expression and case-control status was bivariable analyzed by means of logistic regression. Based on the bivariable analysis, all miRNAs that met the 0.001 significance level were included in a multivariable model, which was then reduced by means of backwards elimination and the Akaike’s Information Criterion (AIC).

eTable 3. Differences in MicroRNAs in Patients With Pancreatic Cancer vs Healthy Participants in the 3 Cohorts and in Other Studies

hsa-miRNA	Discovery	Training **	Validation	Bauer et al. (19) Whole blood	Ali S et al. (22) Plasma	Liu R et al. (26) Serum
Let-7b	Down		Up	Down	Down	Up#
Let-7g	Down				(let-7 family Down)	Up#
miR-9*	Down			(miR-9 Up)		
miR-19b	Down					
miR-23a	Down				Down	
miR-24-2*	Down					
miR-26b	Up		Up	(miR-26b*Up)		
miR-30b	Up		Up #			
miR-31	Down	Down	Down#			
miR-31*	Down					
miR-34a	Up	Up	Up	Up		
miR-93	Down					
miR-122	Up	Up	Up#	Up		
miR-126*	Up		Up	Up		
miR-128	Up	Up				
miR-143	Down	Up				
miR-144*	Down					
miR-145	Up	Up	Up	(145* Down)		
miR-150	Down	Down	Down		(miR-150* Up)	Down
miR-186	Up					
miR-199b-5p	Up			Up		
miR-223	Up	Up	Up		Down	
miR-223*	Up	Up		Up		
miR-342-5p	Down					
miR-345	Down					
miR-362-3p	Down					
miR-374b*	Down			Up		
miR-505	Up	Up	Up			
miR-508-3p	Down			Up		

miR-539	Down			Up		
miR-582-3p	Up			(miR-582-5p Up)		
miR-625	Up	Up				
miR-628-3p	Down					
miR-636	Down*	Down	Down	Down		
miR-769-5p	Up	Up		(miR-769-3p Up)		
miR-885-5p	Up	Up	Up	Up		
miR-935	Down	Down				
miR-941	Up					

The MiRNAs significantly differently expressed in patients with PC and in healthy participants (up-regulated or down-regulated) in the Discovery, Training, and Validation Studies. Furthermore, the expression of these microRNAs in whole blood, plasma, and serum reported in other studies is shown. The reference numbers correspond to the articles listed in the reference list. Up-regulated: Up; Down-regulated: Down.

*Up-regulated with normalization to endogene controls, but down-regulated when the more robust quantile normalization was used in both the “Training Study” and “Validation Study”.

**Training Study: In this table, only significant miRNAs in unadjusted analysis are included.

Not significant.

eTable 4. Comparison of the Area Under the Curve for Serum Cancer Antigen (CA) 19-9 vs Indices I and II and Serum CA 19-9 vs Index I Plus CA 19-9 and Index II Plus CA 19-9*

Comparison	***P* value**	**Difference****
Index I vs. CA 19-9 (PC vs. HP)	0.044	-0.0773
Index I + CA 19-9 vs. CA.19.9 (PC vs. HP)	0.034	0.0313
Index II vs. CA19-9 (PC vs. HP)	0.022	-0.0932
Index II + CA 19-9 vs. CA 19-9 (PC vs. HP)	0.273	0.0215
Index I vs. CA 19-9 (PC vs. HP+PC)	0.094	-0.0661
Index I + CA 19-9 vs. CA19-9 (PC vs. HP+CP)	0.014	0.0344
Index II vs. CA 19-9 (PC vs. HP+CP)	0.047	-0.0832
Index II + CA19-9 vs. CA 19-9 (PC vs. HP+CP)	0.154	0.0292
Index I vs. CA19-9 (PC vs. CP)	0.965	0.0042
Index I + CA 19-9 vs. CA 19-9 (PC vs. CP)	0.068	0.0540
Index II vs. CA 19-9 (PC vs. CP)	0.861	-0.0208
Index II + CA 19-9 vs. CA 19-9 (PC vs. CP)	0.179	0.0772

*DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the area under 2 or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics.* 1988;44(3):837-845.

**Defined as first minus last, i.e., positive difference for "Index I + serum CA 19-9 vs. serum CA 19-9 (PC vs. HP)" means index I + serum CA 19-9 has the highest AUC.

PC = patients with pancreatic cancer; HP = healthy participants; CP = patients with chronic pancreatitis.

eTable 5. Performance of Indices I and II Alone, Serum Cancer Antigen (CA) 19-9 Alone, and Both Indices I and II in Combination With Serum CA 19-9

Study	Index	Sensitivity	Specificity	Accuracy	AUC	TP	TN	FP	FN
Stage I, PC vs. HP+CP	Index I	0.70 (0.35-0.93)	0.64 (0.59-0.69)	0.64 (0.59-0.69)	0.75 (0.63-0.87)	7	222	125	3
Stage II, PC vs. HP+CP	Index I	0.78 (0.66-0.88)	0.64 (0.59-0.69)	0.66 (0.61-0.71)	0.81 (0.72-0.88)	47	222	125	13
Stage I+II, PC vs. HP+CP	Index I	0.77 (0.66-0.86)	0.64 (0.59-0.69)	0.66 (0.61-0.71)	0.80 (0.72-0.87)	54	222	125	16
Stage I, PC vs. HP	Index I	0.70 (0.35-0.93)	0.66 (0.61-0.71)	0.66 (0.61-0.71)	0.76 (0.64-0.88)	7	213	109	3
Stage II, PC vs. HP	Index I	0.78 (0.66-0.88)	0.66 (0.61-0.71)	0.68 (0.63-0.73)	0.81 (0.73-0.89)	47	213	109	13
Stage I+II, PC vs. HP	Index I	0.77 (0.66-0.86)	0.66 (0.61-0.71)	0.68 (0.63-0.73)	0.80 (0.73-0.87)	54	213	109	16
Stage I, PC vs. CP	Index I	0.70 (0.35-0.94)	0.36 (0.18-0.57)	0.47 (0.29-0.63)	0.60 (0.38-0.80)	7	9	16	3
Stage II, PC vs. CP	Index I	0.78 (0.66-0.88)	0.36 (0.18-0.57)	0.66 (0.55-0.76)	0.76 (0.65-0.85)	47	9	16	13
Stage I+II, PC vs. CP	Index I	0.77 (0.66-0.86)	0.36 (0.18-0.57)	0.66 (0.56-0.76)	0.73 (0.63-0.83)	54	9	16	16
Stage I, PC vs. HP+CP	Index II	0.50 (0.19-0.81)	0.80 (0.76-0.84)	0.80 (0.75-0.84)	0.81 (0.71-0.90)	5	279	68	5
Stage II, PC vs. HP+CP	Index II	0.85 (0.73-0.93)	0.80 (0.76-0.84)	0.81 (0.77-0.85)	0.91 (0.87-0.95)	51	279	68	9
Stage I+II, PC vs. HP+CP	Index II	0.80 (0.69-0.89)	0.80 (0.76-0.84)	0.80 (0.76-0.84)	0.90 (0.85-0.94)	56	279	68	14
Stage I, PC vs. HP	Index II	0.50 (0.19-0.81)	0.82 (0.77-0.86)	0.81 (0.76-0.85)	0.84 (0.74-0.92)	5	264	58	5
Stage II, PC vs. HP	Index II	0.85 (0.73-0.93)	0.82 (0.77-0.86)	0.82 (0.78-0.86)	0.92 (0.88-0.96)	51	264	58	9
Stage I+II, PC vs. HP	Index II	0.80 (0.69-0.89)	0.82 (0.77-0.86)	0.82 (0.77-0.85)	0.91 (0.87-0.94)	56	264	58	14
Stage I, PC vs. CP	Index II	0.50 (0.19-0.81)	0.60 (0.39-0.79)	0.57 (0.39-0.74)	0.52 (0.26-0.71)	5	15	10	5
Stage II,	Index II	0.85	0.60	0.78	0.76	51	15	10	9

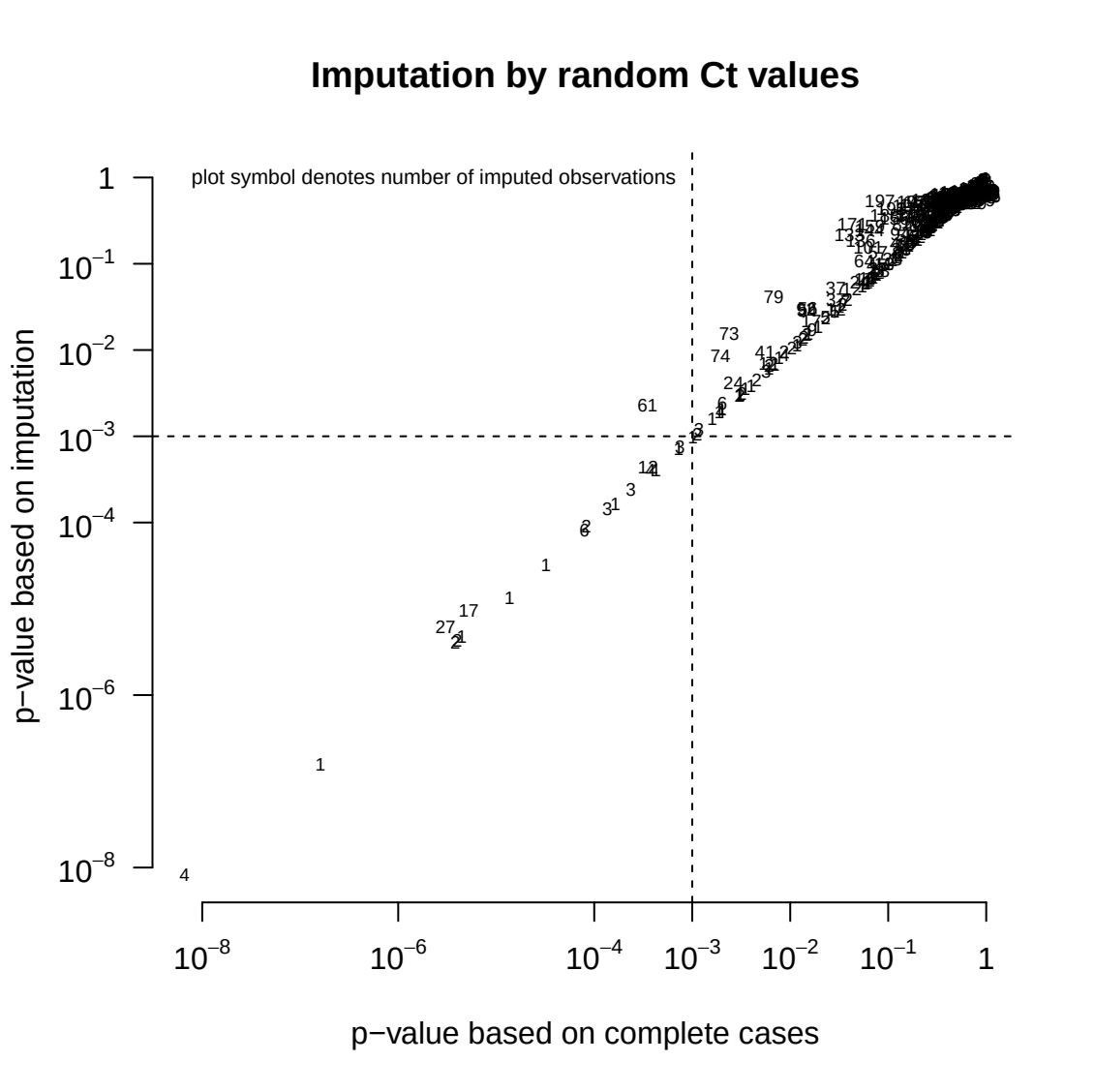
Study	Index	Sensitivity	Specificity	Accuracy	AUC	TP	TN	FP	FN
PC vs. CP		(0.73-0.93)	(0.39-0.79)	(0.67-0.86)	(0.65-0.86)				
Stage I+II, PC vs. CP	Index II	0.80 (0.69-0.89)	0.60 (0.39-0.79)	0.75 (0.65-0.83)	0.73 (0.62-0.83)	56	15	10	14
Stage I, PC vs. HP+CP	CA19-9	0.30 (0.07-0.65)	0.95 (0.93-0.97)	0.94 (0.90-0.96)	0.45 (0.31-0.76)	3	331	16	7
Stage II, PC vs. HP+CP	CA19-9	0.82 (0.67-0.90)	0.95 (0.93-0.97)	0.93 (0.90-0.96)	0.86 (0.79-0.93)	49	331	16	11
Stage I+II, PC vs. HP+CP	CA19-9	0.74 (0.62-0.84)	0.95 (0.93-0.97)	0.92 (0.89-0.94)	0.80 (0.72-0.88)	52	331	16	18
Stage I, PC vs. HP	CA19-9	0.30 (0.07-0.65)	0.99 (0.97-1.00)	0.97 (0.94-0.98)	0.46 (0.31-0.76)	3	318	4	7
Stage II, PC vs. HP	CA19-9	0.82 (0.70-0.90)	0.99 (0.97-1.00)	0.97 (0.94-0.98)	0.87 (0.80-0.94)	49	318	4	11
Stage I+II, PC vs. HP	CA19-9	0.74 (0.62-0.84)	0.99 (0.97-1.00)	0.94 (0.92-0.96)	0.81 (0.73-0.88)	52	318	4	18
Stage I, PC vs. CP	CA19-9	0.30 (0.07-0.65)	0.52 (0.31-0.72)	0.46 (0.29-0.63)	0.35 (0.45-0.81)	3	13	12	7
Stage II, PC vs. CP	CA19-9	0.82 (0.70-0.90)	0.52 (0.31-0.72)	0.73 (0.62-0.82)	0.77 (0.65-0.87)	49	13	12	11
Stage I+II, PC vs. CP	CA19-9	0.74 (0.62-0.84)	0.52 (0.31-0.72)	0.68 (0.58.0.78)	0.71 (0.59-0.81)	52	13	12	18
Stage I, PC vs. HP+CP	Index I + CA 19-9	0.30 (0.07-0.65)	0.93 (0.90-0.96)	0.91 (0.88-0.94)	0.51 (0.28-0.68)	3	323	24	7
Stage II, PC vs. HP+CP	Index I + CA 19-9	0.82 (0.70-0.90)	0.93 (0.90-0.96)	0.91 (0.88-0.94)	0.88 (0.81-0.94)	49	323	24	11
Stage I+II, PC vs. HP+CP	Index I + CA 19-9	0.74 (0.62-0.84)	0.93 (0.90-0.96)	0.90 (0.87-0.93)	0.83 (0.75-0.90)	52	323	24	18
Stage I, PC vs. HP	Index I + CA 19-9	0.30 (0.07-0.65)	0.96 (0.93-0.98)	0.94 (0.91-0.96)	0.52 (0.27-0.67)	3	309	13	7
Stage II, PC vs. HP	Index I + CA 19-9	0.82 (0.70-0.90)	0.96 (0.93-0.98)	0.94 (0.91-0.96)	0.89 (0.82-0.95)	49	309	13	11
Stage I+II, PC vs. HP	Index I + CA 19-9	0.74 (0.62-0.84)	0.96 (0.93-0.98)	0.92 (0.89-0.95)	0.83 (0.76-0.90)	52	309	13	18
Stage I, PC vs. CP	Index I + CA 19-9	0.30 (0.07-0.65)	0.56 (0.35-0.76)	0.49 (0.31-0.66)	0.34 (0.43-0.86)	3	14	11	7

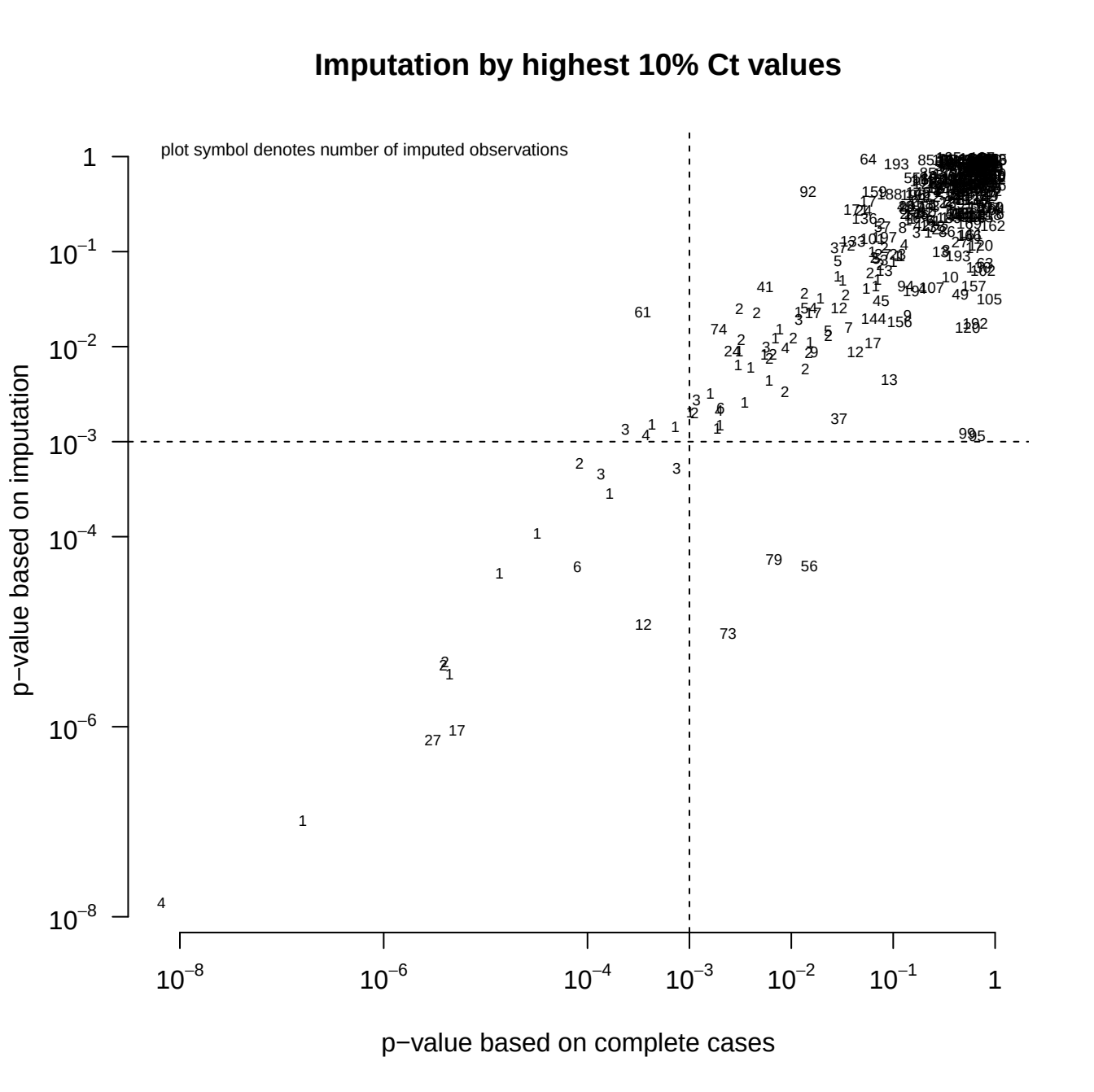
Study	Index	Sensitivity	Specificity	Accuracy	AUC	TP	TN	FP	FN
Stage II, PC vs. CP	Index I + CA 19-9	0.82 (0.70-0.90)	0.56 (0.35-0.76)	0.74 (0.63-0.83)	0.80 (0.69-0.88)	49	14	11	11
Stage I+II, PC vs. CP	Index I + CA 19-9	0.74 (0.64-0.84)	0.56 (0.35-0.76)	0.69 (0.59-0.79)	0.73 (0.62-0.82)	52	14	11	18
Stage I, PC vs. HP+CP	Index II + CA 19-9	0.30 (0.07-0.65)	0.96 (0.94-0.98)	0.94 (0.91-0.96)	0.74 (0.57-0.89)	3	333	14	7
Stage II, PC vs. HP+CP	Index II + CA 19-9	0.80 (0.68-0.89)	0.96 (0.94-0.98)	0.94 (0.91-0.96)	0.93 (0.88-0.97)	48	333	14	12
Stage I+II, PC vs. HP+CP	Index II + CA 19-9	0.73 (0.61-0.83)	0.96 (0.94-0.98)	0.92 (0.89-0.94)	0.90 (0.85-0.95)	51	333	14	19
Stage I, PC vs. HP	Index II + CA 19-9	0.30 (0.07-0.65)	0.97 (0.94-0.99)	0.95 (0.92-0.97)	0.76 (0.59-0.90)	3	312	10	7
Stage II, PC vs. HP	Index II + CA 19-9	0.80 (0.86-0.89)	0.97 (0.94-0.99)	0.94 (0.91-0.96)	0.93 (0.87-0.97)	48	312	10	12
Stage I+II, PC vs. HP	Index II + CA 19-9	0.73 (0.61-0.83)	0.97 (0.94-0.99)	0.93 (0.90-0.95)	0.91 (0.86-0.95)	51	312	10	19
Stage I, PC vs. CP	Index II + CA 19-9	0.30 (0.07-0.65)	0.84 (0.64-0.95)	0.69 (0.51-0.83)	0.49 (0.27-0.76)	3	21	4	7
Stage II, PC vs. CP	Index II + CA 19-9	0.80 (0.68-0.89)	0.84 (0.64-0.95)	0.81 (0.71-0.89)	0.85 (0.77-0.92)	48	21	4	12
Stage I+II, PC vs. CP	Index II + CA 19-9	0.73 (0.61-0.83)	0.84 (0.64-0.95)	0.76 (0.66-0.84)	0.80 (0.71-0.88)	51	21	4	19

Performance of indices I and II alone, serum CA 19-9 alone and both indices I and II in combination with serum CA 19-9 in the differential diagnosis of patients with pancreatic cancer stages I-II (PC) from healthy participants (HP) and patients with chronic pancreatitis (CP). 95% confidence intervals are given in parentheses. TP, true positive; TN, true negative; FP, false positive; and FN, false negative.

eFigures

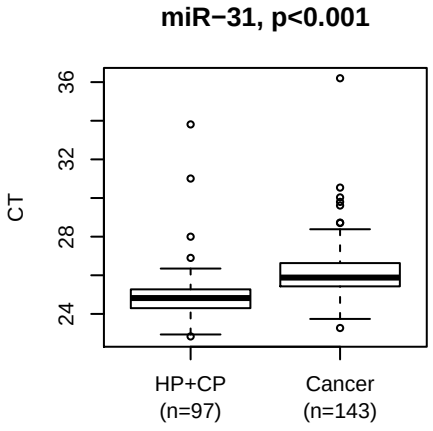
eFigure 1. Cycle Threshold Values of Specific MiRNA Imputed by Random Sampling

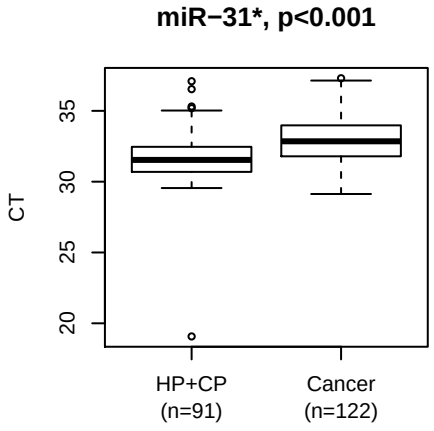

Imputation by random Ct values
plot symbol denotes number of imputed observations
p−value based on imputation
p−value based on complete cases
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-6}
10^{-8}

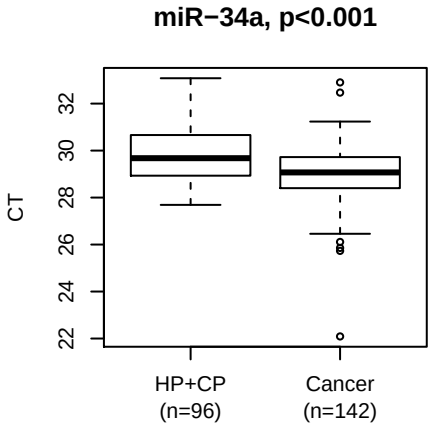

Imputation by highest 10% Ct values
plot symbol denotes number of imputed observations
p−value based on imputation
p−value based on complete cases
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-6}
10^{-8}

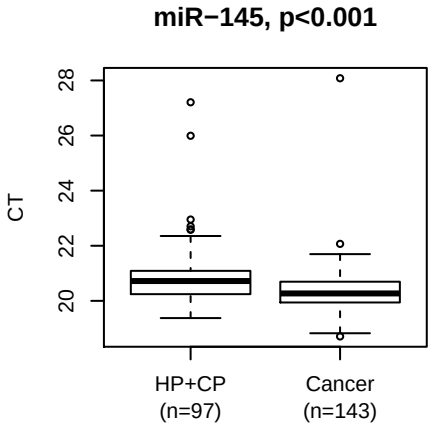
In eFigure 1a missing Ct values of a specific miRNA are imputed by random sampling of Ct values of that miRNA. In eFigure 1b missing Ct values of a specific miRNA are imputed by sampling among the 10 percent highest non-missing Ct values of that miRNA. Dotted lines indicate significance level of 0.001 in the analysis.

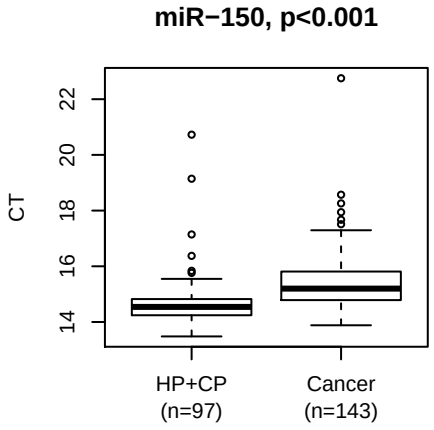
eFigure 2. Box Plots of Relative Expression of Several MiRNAs

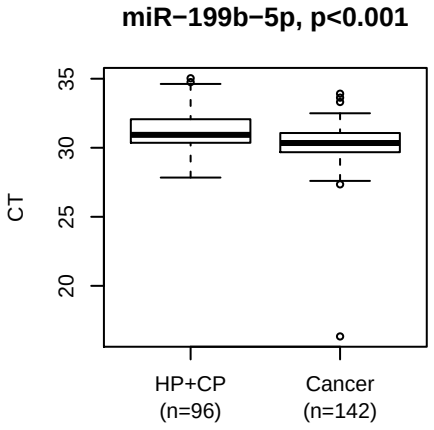

miR−31, p<0.001
CT
HP+CP
(n=97)
Cancer
(n=143)

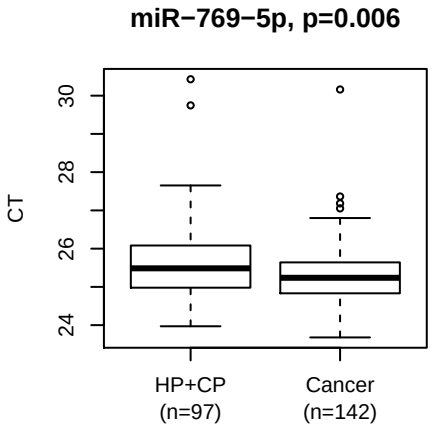

miR−31*, p<0.001
CT
HP+CP
(n=91)
Cancer
(n=122)

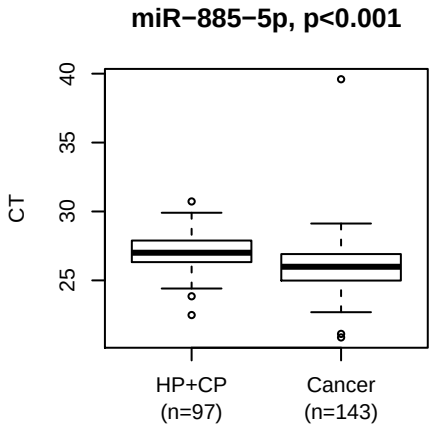

miR−34a, p<0.001
CT
HP+CP
(n=96)
Cancer
(n=142)

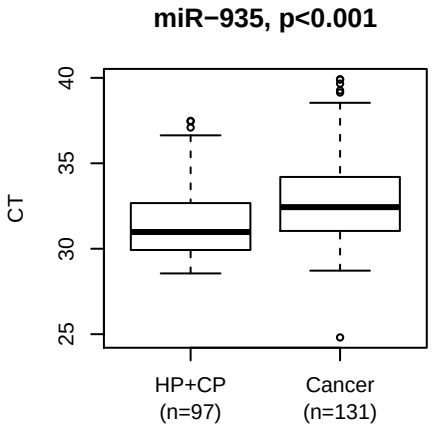

miR−145, p<0.001
CT
HP+CP
(n=97)
Cancer
(n=143)


miR−150, p<0.001
CT
HP+CP
(n=97)
Cancer
(n=143)


miR−199b−5p, p<0.001
CT
HP+CP
(n=96)
Cancer
(n=142)

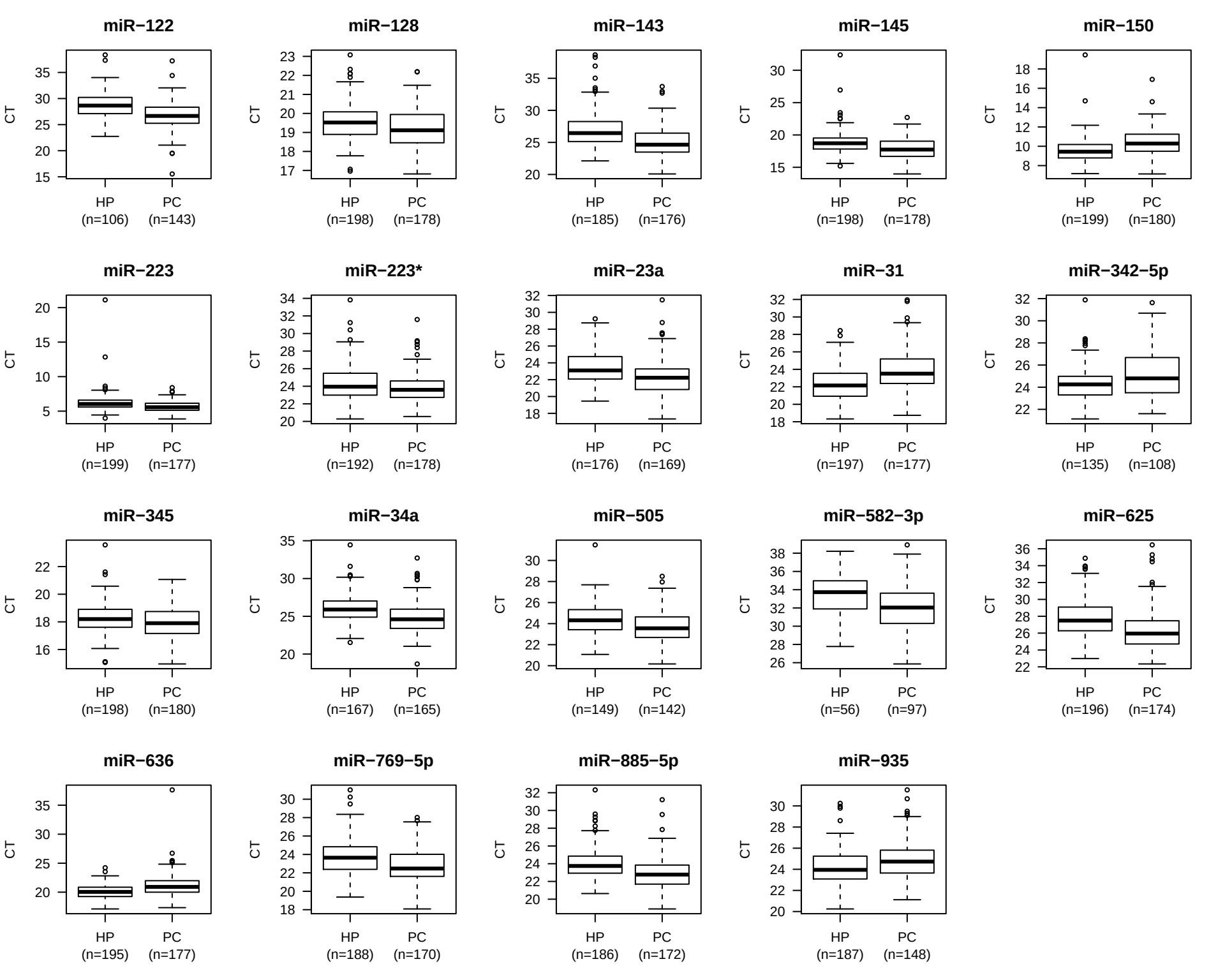

miR−769−5p, p=0.006
CT
HP+CP
(n=97)
Cancer
(n=142)


miR−885−5p, p<0.001
CT
HP+CP
(n=97)
Cancer
(n=143)


miR−935, p<0.001
CT
HP+CP
(n=97)
Cancer
(n=131)

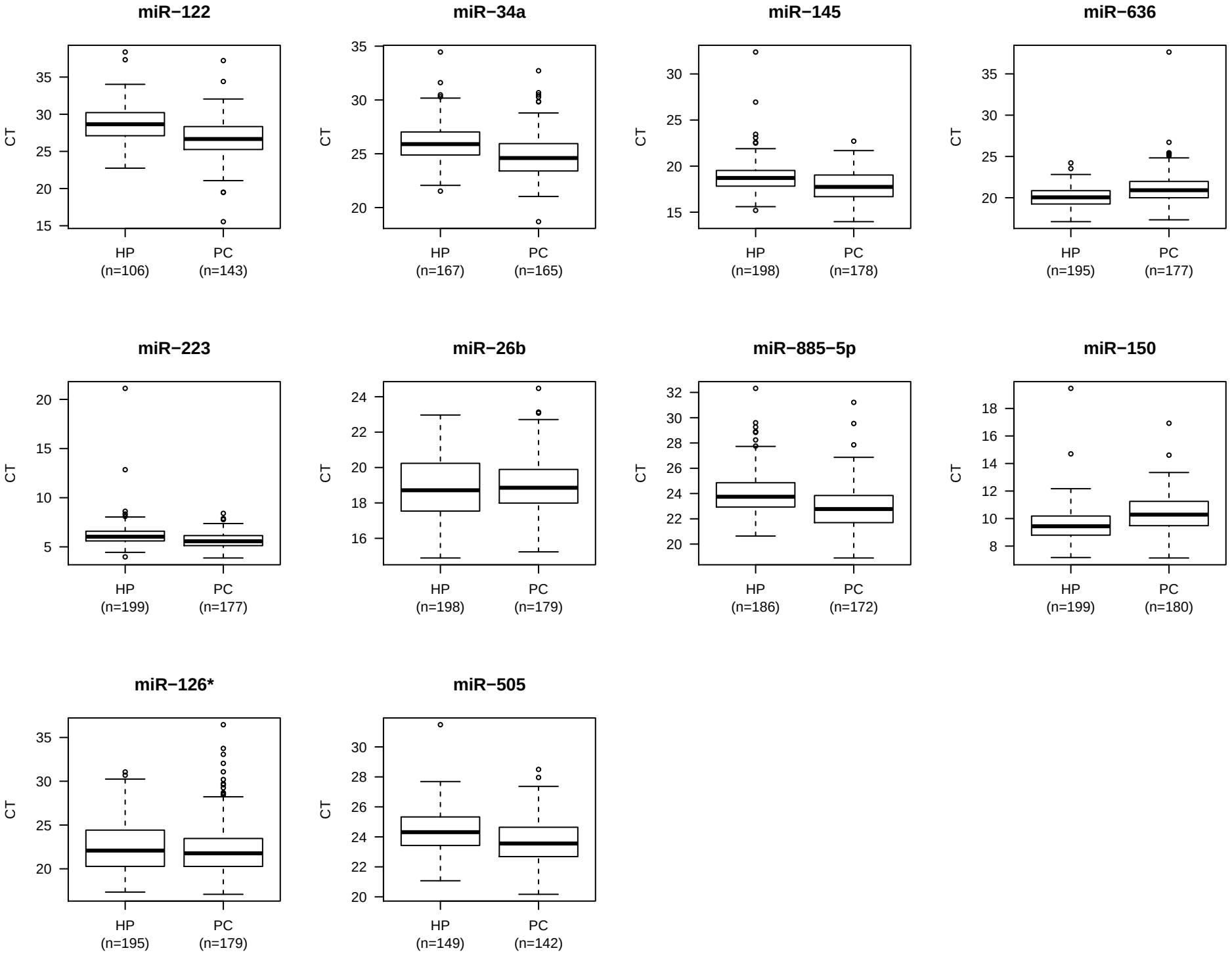
Box-plot of relative expression of miR-31, miR-31*, miR-34a, miR-145, miR-150, miR-199b.5p, miR-769.5p, miR-885.5p, miR-935 in whole blood from patients with PC and controls (patients with chronic pancreatitis (CP) and healthy participants combined (HP)) in the “Discovery Study” population. Ct values were used without normalization.The median score is the line in the middle of the box and the 25^{th} and 75^{th} percentile are the lower and upper part of the box. The whiskers extend to the most extreme point no longer than 1.5 times the interquartile range away from the box. Outliers are given as dots.

eFigure 3. Box Plots of 19 MiRNAs Significantly Differently Expressed in Whole Blood in Patients With Pancreatic Cancer vs Healthy Participants


miR−122
CT
HP (n=106)
PC (n=143)
miR−128
HP (n=198)
PC (n=178)
miR−143
HP (n=185)
PC (n=176)
miR−145
HP (n=198)
PC (n=178)
miR−150
HP (n=199)
PC (n=180)
miR−223
HP (n=199)
PC (n=177)
miR−223*
HP (n=192)
PC (n=178)
miR−23a
HP (n=176)
PC (n=169)
miR−31
HP (n=197)
PC (n=177)
miR−342−5p
HP (n=135)
PC (n=108)
miR−345
HP (n=198)
PC (n=180)
miR−34a
HP (n=167)
PC (n=165)
miR−505
HP (n=149)
PC (n=142)
miR−582−3p
HP (n=56)
PC (n=97)
miR−625
HP (n=196)
PC (n=174)
miR−636
HP (n=195)
PC (n=177)
miR−769−5p
HP (n=188)
PC (n=170)
miR−885−5p
HP (n=186)
PC (n=172)
miR−935
HP (n=187)
PC (n=148)

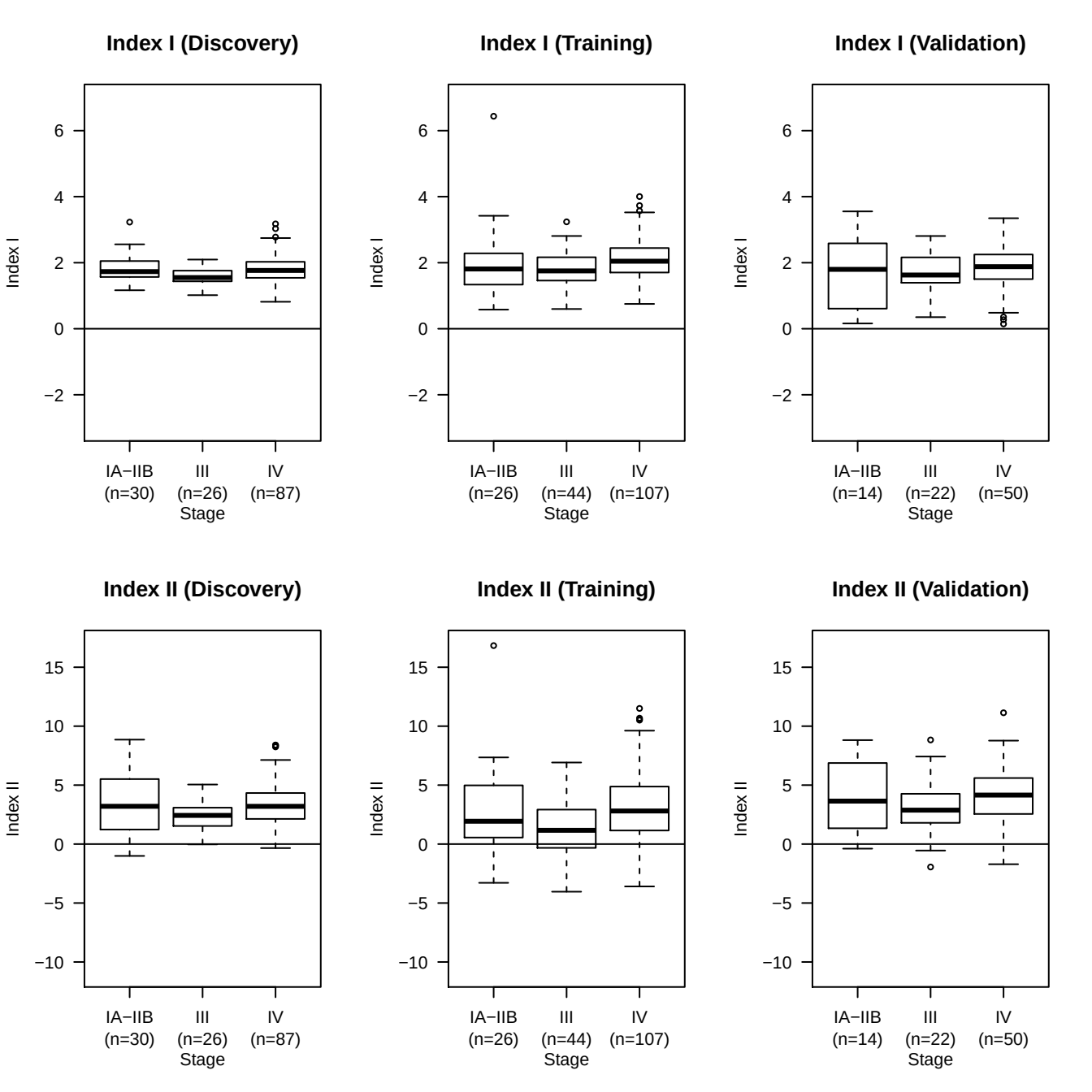
Box-plot of the 19 miRNAs significantly differently expressed in whole blood between pancreatic cancer patients (PC) and healthy participants (HP) in the “Training Study” population. The median score is the line in the middle of the box and the 25^{th} and 75^{th} percentile are the lower and upper part of the box. The whiskers extend to the most extreme point no longer than 1.5 times the interquartile range away from the box. Outliers are given as dots.

eFigure 4. Box Plots of 10 MiRNAs Included in Indices I and II


miR−122
CT
HP
(n=106)
PC
(n=143)
miR−34a
HP
(n=167)
PC
(n=165)
miR−145
HP
(n=198)
PC
(n=178)
miR−636
HP
(n=195)
PC
(n=177)
miR−223
HP
(n=199)
PC
(n=177)
miR−26b
HP
(n=198)
PC
(n=179)
miR−885−5p
HP
(n=186)
PC
(n=172)
miR−150
HP
(n=199)
PC
(n=180)
miR−126*
HP
(n=195)
PC
(n=179)
miR−505
HP
(n=149)
PC
(n=142)

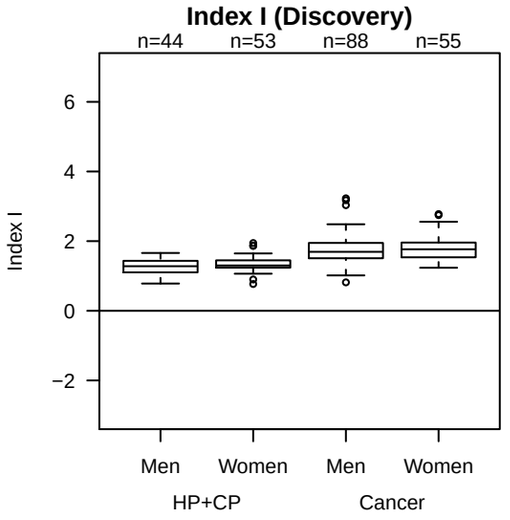
Box-plots of the 10 miRNAs included in Index I and II (miR-26b, miR-34a, miR-122, miR-126*, miR-145, miR-150, miR-223, miR-505, miR-636, and miR-885.5p) in patients with pancreatic cancer (PC) vs. healthy participants (HP) included in the "Training Study". The median score is the line in the middle of the box and the 25^{th} and 75^{th} percentile are the lower and upper part of the box. The whiskers extend to the most extreme point no longer than 1.5 times the interquartile range away from the box. Outliers are given as dots.

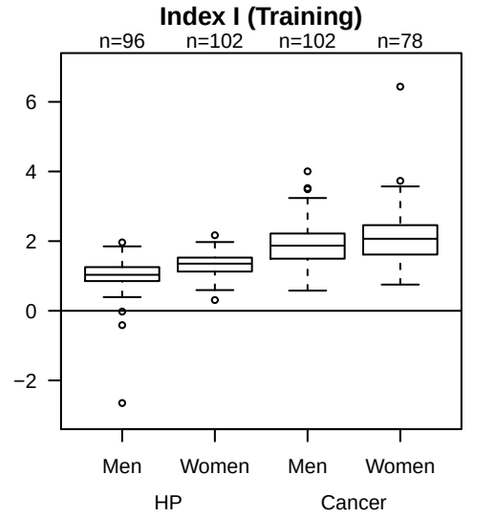
eFigure 5. Indices I and II by Tumor Stage in the 3 Cohorts

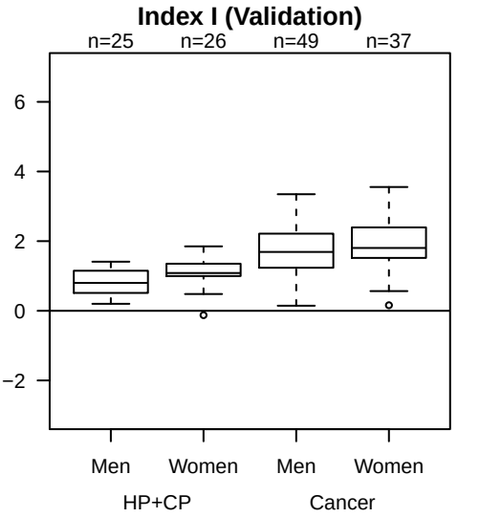

Index I (Discovery)
Index I
6
4
2
0
−2
IA−IIB
(n=30)
III
(n=26)
IV
(n=87)
Stage
Index I (Training)
Index I
6
4
2
0
−2
IA−IIB
(n=26)
III
(n=44)
IV
(n=107)
Stage
Index I (Validation)
Index I
6
4
2
0
−2
IA−IIB
(n=14)
III
(n=22)
IV
(n=50)
Stage
Index II (Discovery)
Index II
15
10
5
0
−5
−10
IA−IIB
(n=30)
III
(n=26)
IV
(n=87)
Stage
Index II (Training)
Index II
15
10
5
0
−5
−10
IA−IIB
(n=26)
III
(n=44)
IV
(n=107)
Stage
Index II (Validation)
Index II
15
10
5
0
−5
−10
IA−IIB
(n=14)
III
(n=22)
IV
(n=50)
Stage

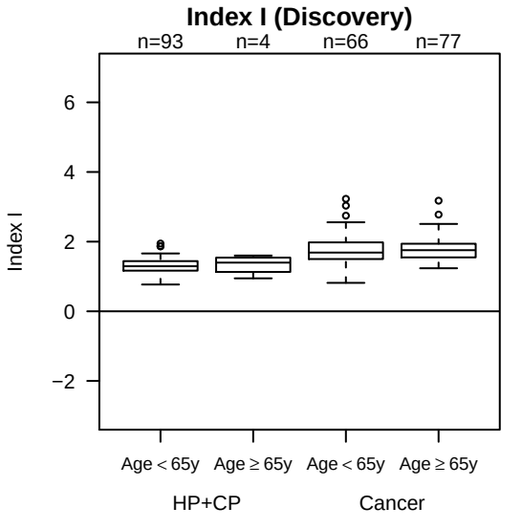
Index I and II by tumor stage (stage IA-IIB, III and IV) “Discovery Study”, “Training Study” and “Validation Study”. The median score is the line in the middle of the box and the 25^{th} and 75^{th} percentile are the lower and upper part of the box. The whiskers extend to the most extreme point no longer than 1.5 times the interquartile range away from the box. Outliers are given as dots.

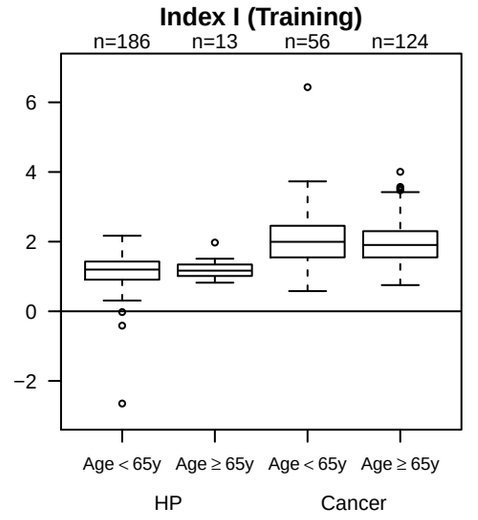
eFigure 6. Indices I and II by Sex and Age in the 3 Cohorts

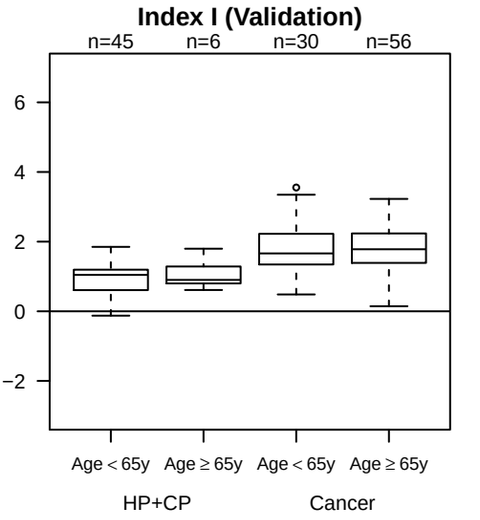

Index I (Discovery)
n=44 n=53 n=88 n=55
Index I
Men Women Men Women
HP+CP Cancer

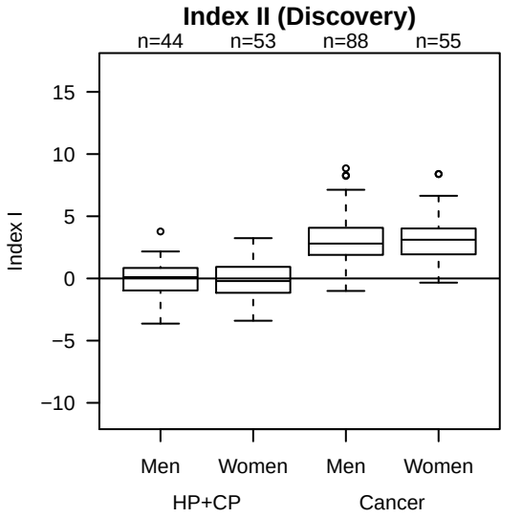

Index I (Training)
n=96 n=102 n=102 n=78
Men Women Men Women
HP Cancer

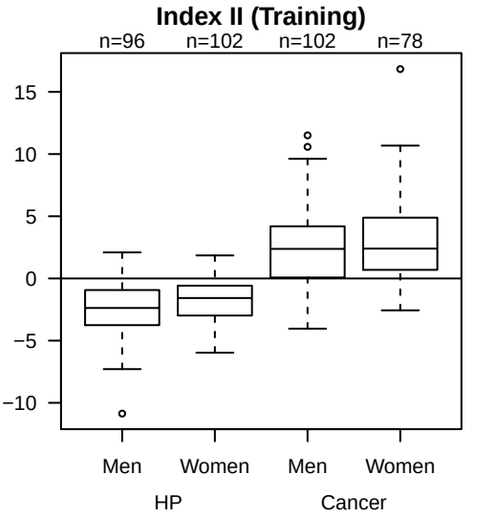

Index I (Validation)
n=25 n=26 n=49 n=37
Men Women Men Women
HP+CP Cancer

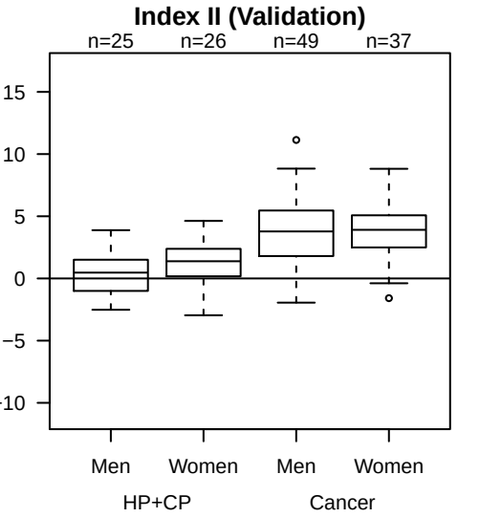

Index I (Discovery)
n=93 n=4 n=66 n=77
Index I
Age < 65y Age ≥ 65y Age < 65y Age ≥ 65y
HP+CP Cancer

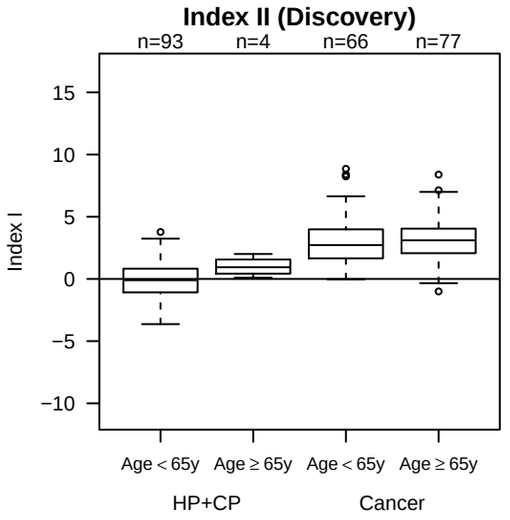

Index I (Training)
n=186 n=13 n=56 n=124
Age < 65y Age ≥ 65y Age < 65y Age ≥ 65y
HP Cancer

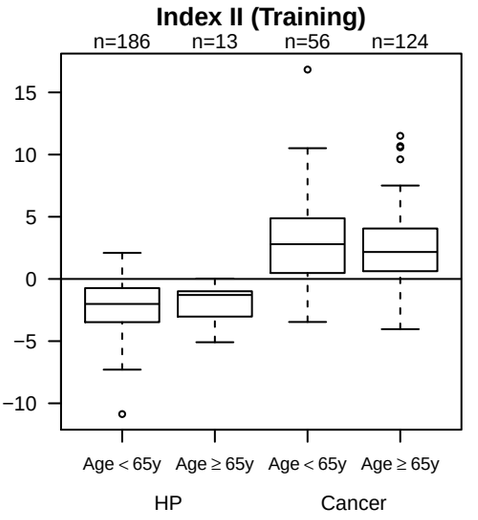

Index I (Validation)
n=45 n=6 n=30 n=56
Age < 65y Age ≥ 65y Age < 65y Age ≥ 65y
HP+CP Cancer

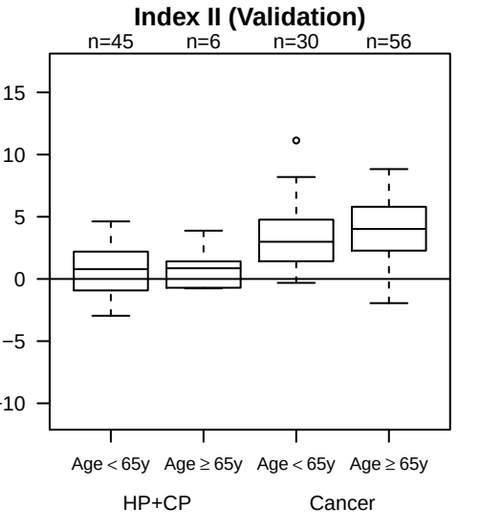

Index II (Discovery)
n=44 n=53 n=88 n=55
Index I
Men Women Men Women
HP+CP Cancer


Index II (Training)
n=96 n=102 n=102 n=78
Men Women Men Women
HP Cancer


Index II (Validation)
n=25 n=26 n=49 n=37
Men Women Men Women
HP+CP Cancer

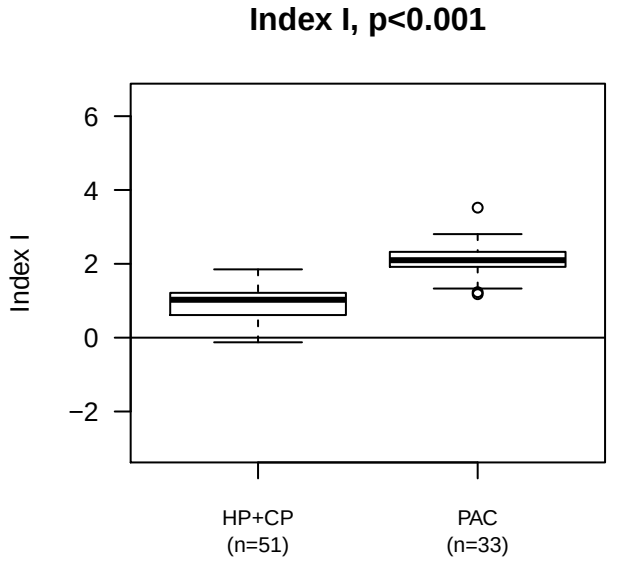

Index II (Discovery)
n=93 n=4 n=66 n=77
Index I
Age < 65y Age ≥ 65y Age < 65y Age ≥ 65y
HP+CP Cancer

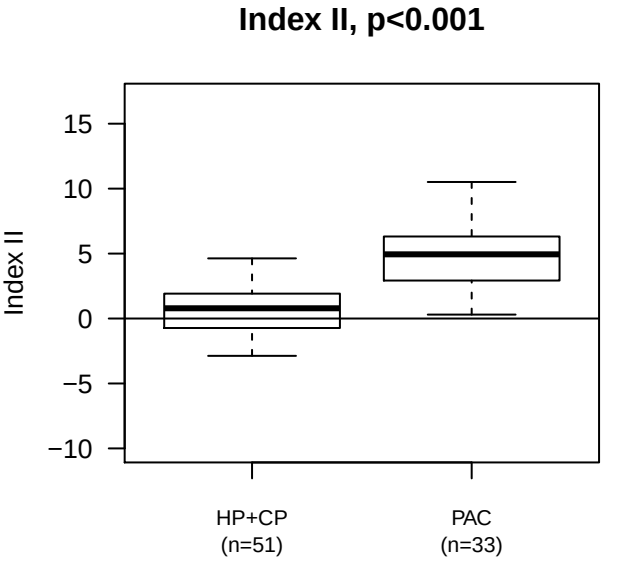

Index II (Training)
n=186 n=13 n=56 n=124
Age < 65y Age ≥ 65y Age < 65y Age ≥ 65y
HP Cancer

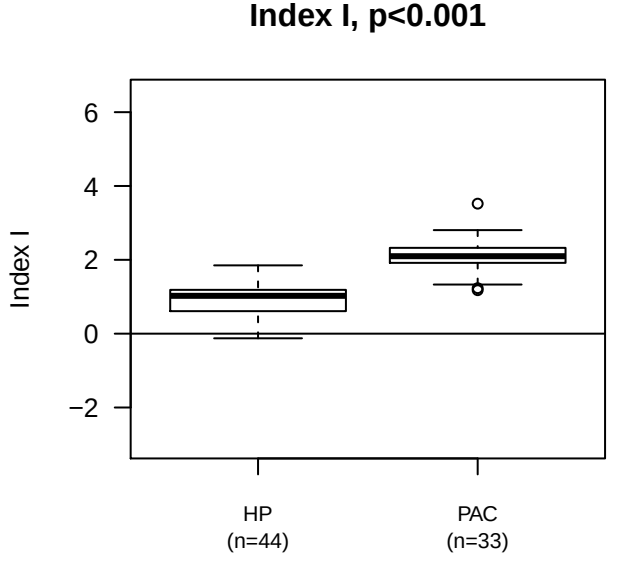

Index II (Validation)
n=45 n=6 n=30 n=56
Age < 65y Age ≥ 65y Age < 65y Age ≥ 65y
HP+CP Cancer

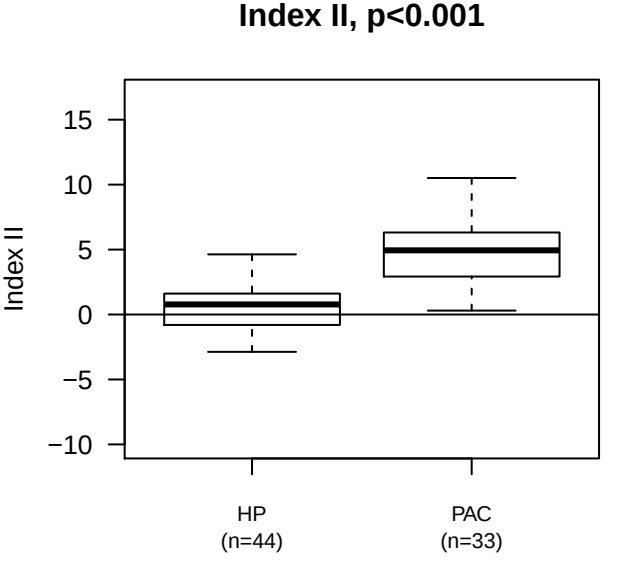
Index I and II by sex and age (< 65 years and ≥ 65 years) in the “Discovery Study”, “Training Study” and “Validation Study” for pancreatic cancer, healthy participants (HP) and chronic pancreatitis (CP). The median score is the line in the middle of the box and the 25^{th} and 75^{th} percentile are the lower and upper part of the box. The whiskers extend to the most extreme point no longer than 1.5 times the interquartile range away from the box. Outliers are given as dots.

eFigure 7. Box Plots of Indices I and II Using the Other Periampullary Cancer Sample


Index I, p<0.001
Index I
6
4
2
0
−2
HP+CP
(n=51)
PAC
(n=33)


Index II, p<0.001
Index II
15
10
5
0
−5
−10
HP+CP
(n=51)
PAC
(n=33)


Index I, p<0.001
Index I
6
4
2
0
−2
HP
(n=44)
PAC
(n=33)


Index II, p<0.001
Index II
15
10
5
0
−5
−10
HP
(n=44)
PAC
(n=33)

Box-plot of Index I and II using the other periampullary cancer (PAC) sample, healthy participants (HP) and chronic pancreatitis (CP) from the “Validation Study”. The median score is the line in the middle of the box and the 25^{th} and 75^{th} percentile are the lower and upper part of the box. The whiskers extend to the most extreme point no longer than 1.5 times the interquartile range away from the box. Outliers are given as dots.