SUPPLEMENTARY MATERIAL

Supplementary Methods

Sample collection and storage

Serum samples from 11 hospital biobanks were collected according to standard serum collection protocols with minor variations as stated in the following. Blood was collected into serum-separating tubes and centrifuged at 2500−3500 rpm for 10 to 20 minutes at 4°C. The serums were moved into cryovials and stored at −80°C deep freezer or liquid nitrogen vapor tank at the individual biobanks. Serum samples were transported to the measurement laboratory in boxes filled with dry ice. Freeze-thaw cycles were minimized before asprosin measurement for sample stability.

Cell lines

Five human pancreatic cancer cell lines (AsPC-1, BxPC-3, HPAC, MIA PaCa-2, and PANC-1) and a normal pancreas cell line (HPNE) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in DMEM or RPMI supplemented with 10% FBS and 1% penicillin-streptomycin (all from Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a 5% CO_2 humidified incubator.

Measurement of asprosin and CA19-9

Asprosin from media (from 5×10^4 cells at 48 h incubation) or serum was measured using an ELISA kit (MBS2707373, MyBioSource, San Diego, CA, USA) with a modified protocol. Fifty microliters of sample or the standard protein was added to a 96-well plate pre-coated with the manufacturer's anti-asprosin antibody and incubated at 37°C for 2 h. The solution was aspirated, and 100 μL of detection reagent A was added, after which the plate was incubated at 37°C for 1 h. Upon removing the solution, the plate was washed with the washing buffer three times and incubated with 100 μL of detection reagent B at 37°C for 1 h. The solution was removed, and the plate was washed five times with the washing buffer. The TMB solution (90 μL) was added, and the plate was incubated

for 10–30 minutes in the dark. The stop solution (50 μL) was added, and the asprosin concentrations were assessed with the absorbance at 450 nm.

CA19-9 concentrations from human serum were measured using an ELISA kit (MBS494411, MyBioSource). Twenty-five microliters of serum or the standard protein was added to a 96-well plate coated with streptavidin, reacted with 100 μL of anti-CA-19-9-biotin reagent, mixed thoroughly, and incubated for 1 h 37℃. The solution was aspirated, and the plate was washed three times with 350 μL of the washing buffer. One hundred microliters of anti-CA19-9-HRP enzyme conjugate was added, and the plate was incubated at 37℃ for 1 h, followed by three times of wash. Then, the TMB solution (100 μL) was added, and the plate was incubated for 10–30 minutes. The stop solution (50 μL) was added, and the CA19-9 concentrations were assessed with the absorbance measurement at 450 nm.

Orthotopic xenograft

Male BALB/c nude mice (5-week-old) were obtained from the Orient Bio Laboratory Animal Research Center Co., Ltd (Seoul, Korea). All mice were bred in laminar-flow cabinets under specific pathogen-free conditions. To establish MIA PaCa-2 tumor orthotopic mouse models, the pancreatic tail was exposed, and then 5×10^6 cells/100 μL were orthotopically implanted in the pancreas tail using a 31G syringe. To prevent any leakage of tumor cells, a cotton swab was held over the injection site for 1 minute. Following the procedure, the mice were closely monitored to ensure the procedure was well tolerated. The mice were sacrificed one month later.

Immunochemistry

For cell lines, 5×10^4 cells were seeded and cultured for 48 h. Dishes were fixed with ethanol and washed gently with PBS, and the cells were treated with 0.2% (v/v) Triton X-100 at room temperature. After overnight incubation at 4℃ with primary anti-asprosin antibody (MBS7607159, MyBioSource; 1:30 diluted with 3% BSA), cells were washed with PBS and incubated for 1 h at room temperature with secondary biotinylated antibody (1:200, BA-1000, Vector Laboratories, Inc., Burlingame, CA, USA.) Cells were washed with PBS three times and conjugated with avidin-biotin

complex (ABC) solution (PK-4000, Vector Laboratories, Inc.) at room temperature for 1 h, followed by development with 3,3′-diaminobenzidine (DAB) solution (Invitrogen 750118, Thermo Fisher Scientific). Staining of asprosin in cells were observed with an optical microscope.

Human tissue staining

A human tissue microarray with normal (n = 20, 10 cases) and pancreatic adenocarcinoma (n = 74, 37 cases) tissues (PA1001c, US Biomax, Inc., Derwood, MD, USA) was used to examine the asprosin expression and its relationship with PC stages. The tumor stage information was provided by the microarray manufacturer. Asprosin staining was scored by four levels, as reported previously (1) by an experienced personnel; 0 for no, 1 for weak, 2 for moderate, and 3 for strong staining.

Immunostaining was performed on 8-μm thick sections after deparaffinization. Microwave antigen retrieval was performed in citrate buffer (pH 6.0) for 10 min before peroxidase quenching with 3% H_2O_2 in PBS for 10 min. Then, the sections were washed in water and pre-blocked with normal goat or rabbit serum for 10 min. For primary antibody reaction, slides were incubated for 1 h at room temperature in a 1:30 dilution of anti-asprosin antibody (MBS7607159, MyBioSource). The sections were then incubated with biotinylated secondary antibodies (1:60, BA-1000, Vector Laboratories, Inc.,) for 1 h. After washing with PBS, the ABC solution was applied. Finally, the sections were developed with DAB solution for 10 min and then counterstained with hematoxylin.

Reference

1. Taylor NJ, Nikolaishvili-Feinberg N, Midkiff BR, Conway K, Millikan RC, Geradts J. Rational Manual and Automated Scoring Thresholds for the Immunohistochemical Detection of TP53 Missense Mutations in Human Breast Carcinomas. *Appl Immunohistochem Mol Morphol* 2016;24:398–404.

Supplementary Table 1. Sample information of tumor tissues and matched normal tissues used in the bioinformatics screening.[a]

Tumor			Matched Normal				
Sample source from TCGA (project name)	Full name of TCGA project	No. of samples	Sample source from TCGA (project name)	No. of samples	Sample source from GTEx	No. of samples	Total No. of samples
ACC	Adrenocortical carcinoma	77	–	0	Adrenal Gland	125	125
BLCA	Bladder Urothelial Carcinoma	413	BLCA	19	Bladder	9	28
BRCA	Breast invasive carcinoma	1139	BRCA	114	Breast – Mammary Tissue	178	292
CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma	305	CESC	3	Cervix – Ectocervix Cervix – Endocervix	10	13
CHOL	Cholangiocarcinoma	36	CHOL	9	–	0	9
COAD	Colon adenocarcinoma	334	COAD	41	Colon – Sigmoid Colon – Transverse	303	344
DLBC	Lymphoid Neoplasm Diffuse Large B-cell Lymphoma	47	–	0	Whole Blood	337	337
ESCA	Esophageal carcinoma	182	ESCA	13	Esophagus – Gastroesophageal Junction Esophagus – Mucosa Esophagus – Muscularis	650	663
GBM	Glioblastoma multiforme	168	GBM	5	Brain – Cortex Brain – Frontal Cortex (BA9)	205	210
HNSC	Head and Neck squamous cell carcinoma	520	HNSC	44	–	0	44
KICH	Kidney Chromophobe	66	KICH + KIRC + KIRP	129	Kidney – Cortex	27	156
KIRC	Kidney renal clear cell carcinoma	543	KICH + KIRC + KIRP	129	Kidney – Cortex	27	156
KIRP	Kidney renal papillary cell carcinoma	289	KICH + KIRC + KIRP	129	Kidney – Cortex	27	156
LAML	Acute Myeloid Leukemia	173	–	0	Whole Blood	337	337
LGG	Brain Lower Grade Glioma	527	–	0	Brain – Cortex	205	205

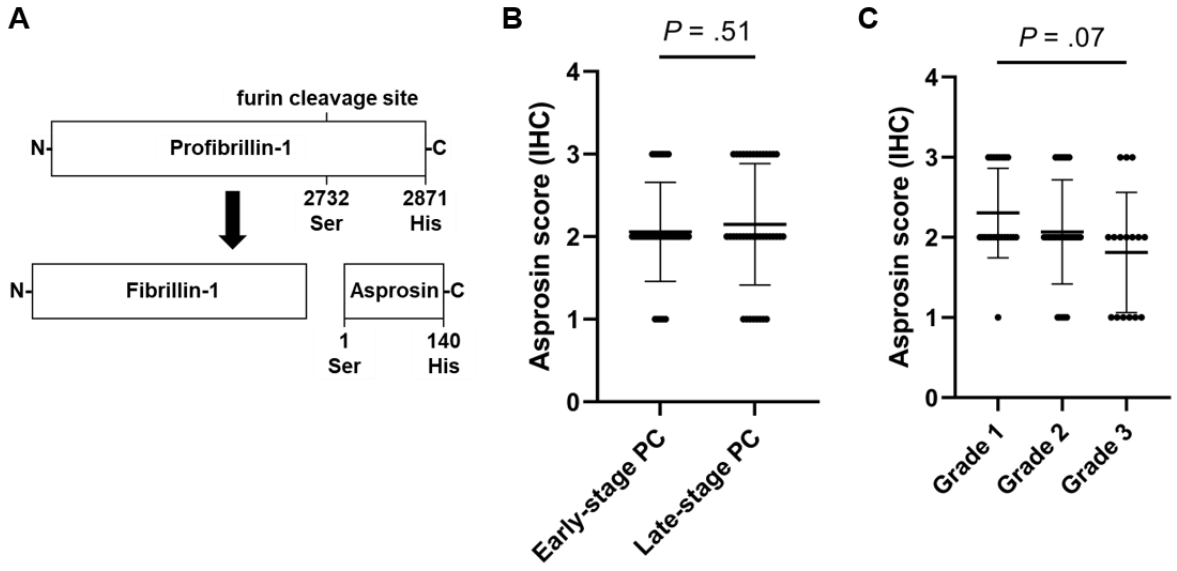
					Brain – Frontal Cortex (BA9)		
LIHC	Liver hepatocellular carcinoma	372	LIHC	50	Liver	110	160
LUAD	Lung adenocarcinoma	539	LUAD + LUSC	109	Lung	287	396
LUSC	Lung squamous cell carcinoma	501	LUAD + LUSC	109	Lung	287	396
OV	Ovarian serous cystadenocarcinoma	428	–	0	Ovary	88	88
PAAD	Pancreatic adenocarcinoma	179	PAAD	4	Pancreas	165	169
PCPG	Pheochromocytoma and Paraganglioma	182	PCPG	3	–	0	3
PRAD	Prostate adenocarcinoma	503	PRAD	51	Prostate	100	152
READ	Rectum adenocarcinoma	93	READ	10	–	0	10
SARC	Sarcoma	262	SARC	2	–	0	2
SKCM	Skin Cutaneous Melanoma	469	SKCM	1	Skin – Not Sun Exposed (Suprapubic)	556	557
STAD	Stomach adenocarcinoma	414	STAD	36	Skin – Sun Exposed (Lower leg) Stomach	173	209
TGCT	Testicular Germ Cell Tumors	154	–	0	Testis	165	165
THCA	Thyroid carcinoma	512	THCA	59	Thyroid	278	337
THYM	Thymoma	119	THYM	2	Whole Blood	337	339
UCEC	Uterine Corpus Endometrioid Carcinoma	190	UCEC	23	Uterus	78	101
UCS	Uterine Carcinosarcoma	57	UCEC	23	Uterus	78	101

[a] TCGA = The Cancer Genome Atlas; GTEx = The Genotype-Tissue Expression; ACC = Adrenocortical carcinoma; BLCA = Bladder Urothelial Carcinoma; BRCA = Breast invasive carcinoma; CESC = Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL = Cholangiocarcinoma; COAD = Colon adenocarcinoma; DLBC = Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; ESCA = Esophageal carcinoma; GBM = Glioblastoma multiforme; HNSC = Head and Neck squamous cell carcinoma; KICH = Kidney Chromophobe; KIRC = Kidney renal clear cell carcinoma; KIRP = Kidney renal papillary cell carcinoma; LAML = Acute Myeloid Leukemia; LGG = Brain Lower Grade Glioma; LIHC = Liver hepatocellular carcinoma; LUAD = Lung adenocarcinoma; LUSC = Lung squamous cell carcinoma; OV = Ovarian serous cystadenocarcinoma; PAAD = Pancreatic adenocarcinoma; PCPG = Pheochromocytoma and Paraganglioma; PRAD = Prostate adenocarcinoma; READ = Rectum adenocarcinoma; SARC = Sarcoma; SKCM = Skin Cutaneous Melanoma; STAD = Stomach adenocarcinoma; TGCT = Testicular Germ Cell Tumors; THCA = Thyroid carcinoma; THYM = Thymoma; UCEC = Uterine Corpus Endometrioid Carcinoma; UCS = Uterine Carcinosarcoma; BA9 = Brodmann area 9.

Supplementary Table 2. Diagnostic performance of asprosin in the validation set of normal versus pancreatic cancer (PC) groups.

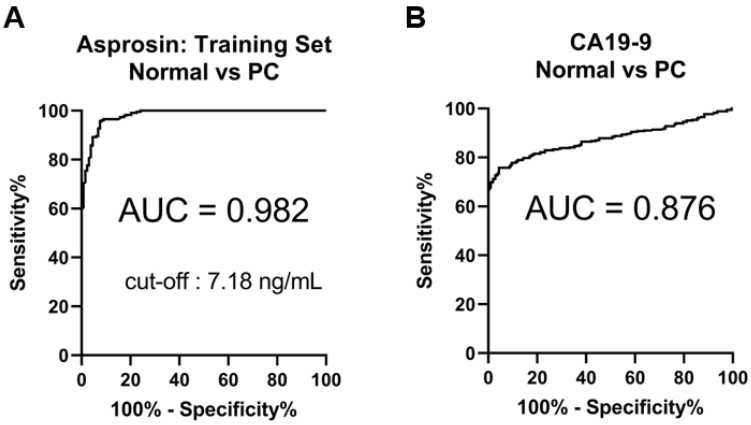
Type	PC	Normal	Predictive Value[a]
Positive	110	5	PPV = 0.957 (110/115) NPV = 0.924 (61/66) Sensitivity = 0.957 (110/115) Specificity = 0.924 (61/66)
Negative	5	61	

[a] PPV: Positive Predictive Value, NPV: Negative Predictive Value



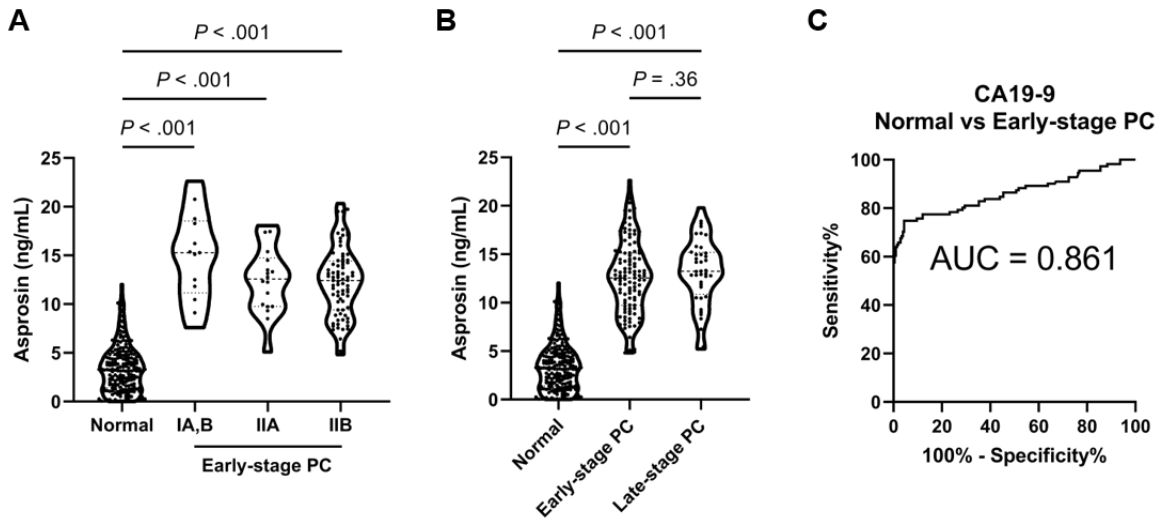
Supplementary Figure 1. Asprosin formation and its levels in different stages and grades of PC in human patients.

A, Schematic diagram of asprosin formation from profibrillin-1, the gene product of FBN1 is shown and the diagrams are not drawn to scale. **B**, Immunohistochemistry scores of asprosin in early- (n = 34, 17 cases) and late-stage PC tissues (n = 40, 20 cases) from human patients are graphed; two-sided Mann-Whitney test was used. **C,** Immunohistochemistry scores of asprosin in PC tissues with grade 1 (n = 23, 12 cases), grade 2 (n = 29, 16 cases) and grade 3 (n = 16, 9 cases) from human patients are graphed; ordinary one-way ANOVA was used. Data are presented as the mean ± SD. PC = pancreatic cancer; ANOVA = analysis of variance; SD = standard deviation.



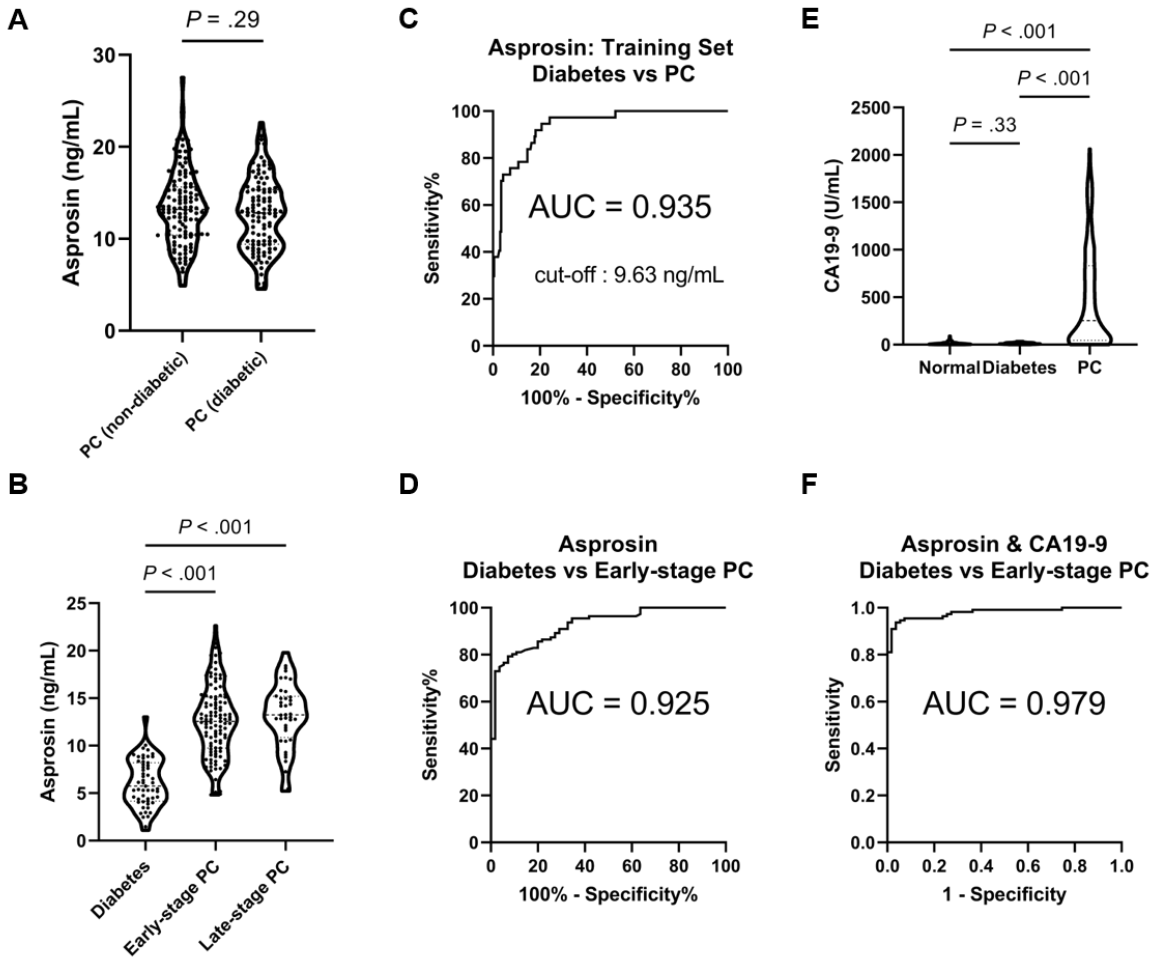
Supplementary Figure 2. ROC curves for asprosin and CA19-9 in the normal and PC groups.

A, ROC curve for serum asprosin was obtained for the training set (normal, n = 133; PC, n = 232). The cut-off value was obtained with the shortest Euclidean distance method. **B**, ROC curve for serum CA19-9 was obtained for the entire cohort (normal, n = 207; PC, n = 347). PC = pancreatic cancer; ROC = receiver operating characteristic; AUC = area under the curve.



Supplementary Figure 3. Asprosin levels in PC at different stages and ROC curve for CA19-9 in the normal and early-stage PC groups.

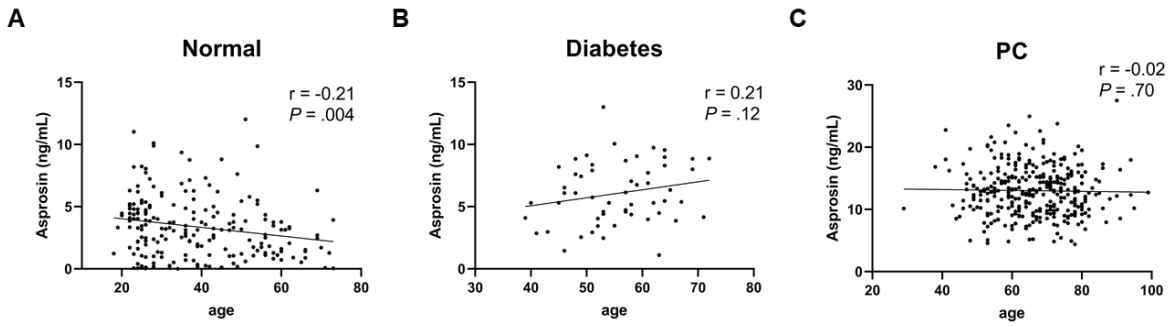
A, Violin plot was obtained for serum asprosin levels in normal and early-stage PC patients (normal, n = 199; PC stage IA or IB n = 13; PC stage IIA, n = 18; PC stage IIB, n = 80); two-sided Welch's t-test was used. **B**, Violin plot was obtained for serum asprosin levels in normal, early- and late-stage PC patients (normal, n = 199; early-stage PC, n = 111; late-stage PC, n = 40); two-sided Welch's t-test was used. **C**, ROC curve for serum CA19-9 was obtained for normal and ealy-stage PC patients (normal, n = 207; PC, n = 111). Violin plots are presented with the median and quartiles. PC = pancreatic cancer; ROC = receiver operating characteristic; AUC = area under the curve.



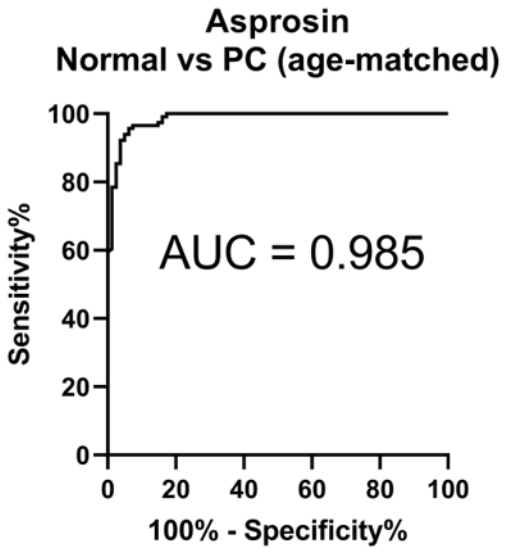
Supplementary Figure 4. Asprosin levels in diabetes and distinction between diabetes and entire or early-stage PC with asprosin or asprosin-CA19-9 combination.

A, Violin plot was obtained for serum asprosin levels in PC patients; according to diabetic status, patients with hemoglobin A1c ≥ 6.5% were classified as diabetic (PC (non-diabetic), n = 121; PC (diabetic), n = 110); two-sided Welch's t-test was used. **B,** Violin plot was obtained for serum asprosin levels in diabetes, early- and late-stage PC patients (diabetes, n = 55; early-stage PC, n = 111; late-stage PC, n = 40); two-sided Welch's t-test was used. C**,** ROC curve for serum asprosin was obtained for the training set (diabetes, n = 37; PC, n = 232). The cut-off value was obtained with the shortest Euclidean distance method. **D**, ROC curve for serum asprosin was obtained for diabetes versus early-stage PC patients (diabetes, n = 55; early-stage PC, n = 111). **E**, Violin plot was obtained for serum CA19-9 levels in normal, diabetes and PC patients. (normal, n = 207; diabetes, n = 55; PC, n = 347); two-sided Welch's t-test was used. **F,** ROC curve for the combination of serum asprosin and CA19-9 was obtained for

diabetes versus early-stage PC comparison. Logistic regression was used for asprosin and CA19-9 as continuous variables. The resulting probability variable was used to construct the ROC curve. Violin plots are presented with the median and quartiles. PC = pancreatic cancer; ROC = receiver operating characteristic; AUC = area under the curve.



Supplementary Figure 5. Correlation analysis of age and asprosin in normal, DM and PC groups. Distributions of asprosin and age in **A,** normal (n = 199) **B,** diabetes (n = 55), and **C,** PC (n = 347) groups are presented as scatter plots. Pearson correlation coefficient and two-sided *P* values from correlation analysis are presented. PC = pancreatic cancer.



Supplementary Figure 6. ROC curve for asprosin in the age-matched subset of normal and PC groups. ROC analysis was performed for the age-matched subset (normal: n = 81 with median age 53; PC: n = 116 with median age 55). Age was not statistically significantly different in each subset (*P* = .40, two-sided Welch's t-test was used). ROC = receiver operating characteristic; PC = pancreatic cancer; AUC = area under the curve.