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Abbreviations:

AC	Ampullary carcinoma
AUC	Area under the ROC
BIOPAC	BIOmarkers in patients with pancreatic cancer
BMI	Body mass index
CI	Confidence interval
CP	Chronic pancreatitis
CRP	C-reactive protein
CV	Coefficient of variations
DBTC	Distal biliary tract cancer
ELISA	Enzyme-linked immunosorbent assay

FOLFIRINOX	5-FU, leucovorin, irinotecan, and oxaliplatin
ECM	Extracellular matrix
HA	Hyaluronic acid or hyaluronan
DA	Duodenal adenoma
HR	Hazard ratio
IFP	Interstitial fluid pressure
IL-6	Interleukin-6
IPMN	Intraductal papillary mucinous neoplasm
OS	Overall survival
PC	Pancreatic cancer
PS	Performance status
PRO-C3	Propeptide of type III collagen
ROC	Receiver operating characteristics curve
TME	Tumor microenvironment
YKL-40	Chitinase 3-like 1 protein (CHI3L1)

"Novelty and Impact" What's new? (max. 75 words)

Hyaluronan (HA) and collagen are highly expressed in pancreatic cancer (PC) stroma. HA and collagen accumulation increase tumor interstitial fluid pressure, compromising blood flow and drug penetration. High levels of serum HA and propeptide of type III collagen (PRO-C3) were associated with short overall survival. Serum HA and PRO-C3 were higher in PC patients than patients with benign conditions. The findings warrant further exploration to help clarify links between HA, collagen type III, and PC.

Abstract

Hyaluronan (HA) and collagen are highly expressed in pancreatic cancer (PC) stroma. HA and collagen accumulation increase tumor interstitial fluid pressure, compromising blood flow and drug penetration. The aim of this biomarker study was to determine the clinical utility of serum HA and the propeptide of type III collagen (PRO-C3) in patients with PC. A cohort from the Danish BIOPAC study (NCT03311776) including patients with histologically confirmed pancreatic ductal adenocarcinoma (n=809), ampullary carcinoma (n=44), distal biliary tract cancer (n=31), chronic pancreatitis (n=15), intraductal papillary mucinous neoplasm (n=41), duodenal adenoma (n=7), and no cancer (n=25). Healthy controls were available for serum HA (n=141) and PRO-C3 (n=8). The main outcome was overall survival (OS) of patients with PC in relation to pretreatment serum HA and PRO-C3 levels. Patients with PC had higher baseline serum HA and PRO-C3 than healthy subjects and patients with benign conditions. Pretreatment serum baseline HA and PRO-C3 in patients with PC were associated with poorer survival and PRO-C3 remained prognostic also after adjusting for age, performance status, stage, presence of liver and peritoneum metastasis, and CA19-9. Detection of HA and PRO-C3 may be useful in differentiating between malignant and benign pancreatic conditions. Serum HA and PRO-C3 were prognostic for OS in patients with PC.

Introduction

Pancreatic cancer (PC) is one of the most lethal of all major cancers[11, 36, 40]. Pancreatic ductal adenocarcinomas account for 90% of all PC tumors and are highly resistant to all standard treatments including chemotherapy, surgery, radiation, and targeted agents, partly due to the multifaceted and immunosuppressive tumor microenvironment (TME)[4, 6, 39, 49]. The prognosis has only improved slightly during the last decades, and the overall 5-year survival remains low at 8%[36]. PC is characterized by pronounced inflammation and disperse tumor cells in a dense desmoplastic stroma consisting of pancreatic stellate cells (PSCs), carcinoma-associated fibroblasts, extracellular matrix (ECM), endothelial cells, and immune cells. Interaction between stroma and inflammation is reported[3, 13, 33, 43, 46]. Stroma contributes to the hypoxic and avascular microenvironment in both primary tumors and metastases[10, 49]. However, stromal biology and its prognostic impact are still under discussion[44]. Multiple stroma-targeted therapies are under active investigation[21, 47].

Hyaluronic acid (HA), also called hyaluronan, and collagen are major components of the ECM of the tumor stroma in PC, and high levels of each are found in both primary tumors and metastatic lesions[17, 20, 49]. HA is a glycosaminoglycan that is synthesized at high levels by cancer cells and fibroblasts in the TME and promotes tumor growth, angiogenesis, inflammation, immunosuppression as well as preserves growth factors and cytokines[20, 23, 38, 42].

The collagen types I, III, and IV are concomitantly overexpressed together with HA in PC[49]. Collagen type IV is the major component of the basement membrane, a specialized ECM that underlies the epithelial and endothelial cell layer and plays a major role in tumor cell invasion and angiogenesis. Collagen type I and type III are the major components of the stromal ECM and the interstitial matrix. They are produced by PSCs within the desmoplastic stroma and promote PC cell adhesion, proliferation, migration, and chemo-resistance[2, 14, 24, 50, 54]. The ECM in the tumor stroma undergoes

excessive remodeling. The increased activity of PSC/fibroblasts leads to excessive formation and accumulation of interstitial collagens[27]. HA and collagen accumulation increase tumor solid stress and interstitial fluid pressure (IFP), compromising blood vessels, circulation, perfusion, and drug penetration[8, 25, 29, 37, 42]. The prognostic value of HA and collagen deposition in PC tissue has been described in a few studies[5, 7, 12, 35, 49]. HA was hypothesized to be a potential therapeutic target to decrease tumor IFP and enhance drug delivery and immune cells' access into the tumor[31, 34, 37]. Reprogramming of PSC and downregulation of collagen-related signals by the vitamin D receptor ligand, calcipotriol, have been shown to improve gemcitabine penetration into the tumor, resulting in prolonged survival in PC mouse models[35].

Promising results of targeting of HA in combination with chemotherapy were reported in a randomized phase II study, which showed that patients with high HA levels in the tumor microenvironment had a largest improvement in progression free survival after addition of pegvorhyaluronidase alfa (PEGPH20) to a standard combination of nab-paclitaxel/gemcitabine[15]. Preliminary data based on retrospective analysis of this trial suggested that type III collagen turnover measured in serum was able to identify patients that would benefit from the addition of PEGPH20[48]. A phase III study targeting HA is ongoing (NCT02715804). Although HA and collagen expression in the TME are considered prognostic biomarkers, there are no/few studies examining serum concentrations of HA and type III collagen in patients with PC. Previous studies conducted in other malignancies have shown a negative correlation between serum HA and outcome[1, 19, 28, 45, 52, 53] and serum collagen fragments and outcome[7, 18, 22, 51]. The aim of this biomarker study was to investigate the clinical utility of serum HA and propeptide of type III collagen (PRO-C3) in patients with PC.

Materials and Methods

Patients

We analyzed pretreatment serum samples from a cohort of patients included in the Danish BIOPAC study "Biomarkers in Patients with Pancreatic Cancer" (NCT03311776) from six hospitals in Denmark admitted from December 2008 until September 2017. A total of 972 participants, including consecutive BIOPAC patients with histologically confirmed PC, ampullary carcinoma (AC), distal biliary tract cancer (DBTC), chronic pancreatitis (CP), intraductal papillary mucinous neoplasm (IPMN), patients subjected to surgery in whom PC was suspected but not histologically confirmed (no pathologic finding), and patients with duodenal adenomas (DA), were studied.

Serum samples and clinical data from patients were collected prospectively. Retrospective HA and PRO-C3 analyses and the clinical data review of electronic medical records were performed separately (or independent of each other). Cancer patients were treated with various types of chemotherapy according to national guidelines www.gicancer.dk.

Biological samples

Blood samples were obtained at time of diagnosis and/or before operation if available. Sequential samples collected during and after treatment (before 2nd cycle and at the time of first CT scan) were available for the group of patients receiving palliative chemotherapy. Samples were processed according to the nationally approved standard operation procedures for blood (www.herlevhospital.dk/biopac.dk).

Serum HA analysis

Pre-treatment and longitudinal serum concentrations of HA were determined by a human HA high-sensitive solid-phase immunoassay (Quantikine® Immunoassay, R&D Systems, Abingdon, OX, UK) in accordance with the manufacturer's instructions. The lower level of detection (LLOD) for HA was 0.068 ng/mL. The inter-assay coefficient of variation (CV) was ≤7.2% and the intra-assay CV was ≤4.9%. The molecular weight range of HA detected by the Quanitkine kit was 0.2 – 40.0 ng/mL. The median serum HA in 141 healthy blood donors was 41 ng/ml.

Serum PRO-C3 analysis

Serum PRO-C3 was determined by enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody to detect the N-protease mediated cleavage of the N-terminal propeptide of type III collagen (Nordic Bioscience, PRO-C3 assay)[26]. The LLOD was 4.0 ng/mL. The inter- and intra-CVs were 11.0% and 4.1%. The molecular weight range of PRO-C3 detected by the Nordic Bioscience ELISA kit was 1.1 – 8.5 ng/mL. The median serum PRO-C3 level in eight healthy subjects was 7 ng/mL.

ETHICAL ISSUES

The BIOPAC study was approved by the Regional Ethics Committee (VEK ref. KA-20060113) and the Danish Data Protection Agency (j.nr. 2006-41-6848). Informed consent for research use was obtained from all patients at the enrolling hospitals before prospective serum banking. Serum HA and PRO-C3 assessments were not predefined in the protocol but approval was obtained before analysis. The responsible investigators ensured that the clinical studies were conducted in agreement with the Declaration of Helsinki, version 8.

Statistical analysis

Results are reported in accordance with the REMARK (reporting recommendations for tumor marker prognostic study) guidelines[32]. The objective was to examine the clinical value of serum HA and PRO-C3 and the association between baseline and longitudinal serum HA levels and overall survival (OS) in the PC patients. Patients were followed from the date of inclusion in the BIOPAC study to the end of follow-up (October 24, 2018) or until death from any cause, whichever came first.

Descriptive statistics were performed to describe patients' demographics and clinical baseline characteristics. Receiver operating characteristic (ROC) analysis was conducted to assess the diagnostic values of serum HA. Changes of serum HA and PRO-C3 over time during and after systemic treatment and their prognostic value were investigated. Hazard ratios (HRs) adjusted for baseline characteristics (age, gender, performance status (PS), stage, history of diabetes, smoking and alcohol abuse, serum CA19-9, as well as chemotherapy regimen (gemcitabine, mFOLFIRINOX, gemcitabine with nab-paclitaxel, or other), and body mass index (BMI) were estimated with Cox proportional-hazard regression. Kaplan–Meier curves were used to illustrate the survival curves in patients with PC in relation to measured values of serum HA and PRO-C3 levels, comparing patients with the 25% highest values with those with the 75% lowest for each biomarker separately. The association between changes in serum HA and PRO-C3 during palliative chemotherapy and OS was analyzed in patients in whom sequential (baseline, before 2nd cycle, and at the time of first CT scan) data were available. Differences in serum concentration over time from baseline were measured; negative values correspond to a decrease in serum HA and PRO-C3. Patients were grouped according to change from baseline. Statistical analyses were conducted using the latest version of R (currently 3.2.4).

Data availability

The data that support the findings of this study are available upon reasonable request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Results

Characteristics of study population

The study included 972 participants with the following diagnoses: histologically confirmed PC (n=809), AC (n=44), DBTC (n=31), CP (n=15), IPMN (n=41), no pathologic finding (n=25), DA (n=7 including six patients with high-grade dysplasia and one patient with ungraded dysplasia). Clinicopathological data of PC patients are shown in Table 1. The median age was 67 years (range 37–89 years), and most of the PC patients had PS 0 or 1. The majority of PC patients had advanced stage III or IV disease, had normal weight, and were registered as present or earlier smokers. Fifty-one patients across all stages were never treated with chemotherapy. Of 598 patients who received palliative chemotherapy, 404 (67.6%) were treated with gemcitabine, 120 (20.1%) with FOLFIRINOX (5-FU, leucovorin, irinotecan, and oxaliplatin), 55 (9.2%) with combination of gemcitabine and nab-paclitaxel, and 19 (3.2%) with other combinations.

Pretreatment serum HA and PRO-C3 and clinical characteristics and overall survival

First, we compared serum HA levels between the study groups (Table 2). The median pretreatment HA level was 84 ng/mL (IQR: 48-131). Pretreatment serum HA in patients with cancer including PC, AC, DBTC was increased compared to patients with benign conditions or healthy controls. The median serum HA level in PC patients was about two-fold higher than in healthy controls ($p<0.001$)

and was increased compared to patients with benign conditions including CP, IPMN, DA and those subjects without pathological findings ($p<0.001$). No statistically significant difference in HA was observed comparing PC with other malignant diseases. We further stratified the PC patients according to stage, PS, and presence of liver metastases. Higher serum HA levels were associated with metastatic disease, presence of liver metastases, and worse PS (Table 2). Thirty-four percent of patients with liver metastases had the highest quartile of serum HA compared to 19% patients without liver metastases ($p<0.001$). Correlation analyses displayed a pairwise positive relation between baseline serum HA and PRO-C3, CA19-9 and inflammation markers in patients with PC (Table S1). ROC analysis showed that serum HA level had a better potential to discriminate PC patients from healthy controls (AUC=0.75, 95% CI 0.72–0.79, Figure 1A) than from patients with benign conditions (AUC=0.63, 95% CI 0.57–0.69, Figure 1B).

We subsequently investigated the clinical value of serum PRO-C3 in the same cohort. The median pretreatment PRO-C3 level was 18 ng/mL (IQR: 11-33). Serum PRO-C3 was higher in patients with malignant diseases than in those with benign conditions (Table 2). PC patients had higher median serum PRO-C3 levels compared to patients with benign conditions ($p<0.001$). PRO-C3 was positively correlated with higher stage and presence of liver metastases ($p<0.001$). Double as many patients with liver metastases had serum PRO-C3 in the highest quartile compared to patients without liver metastases (37% versus 18%, $p<0.001$). PS≥2 was associated with higher serum PRO-C3 values. Detection of PRO-C3 performed slightly better as a diagnostic marker than serum HA. ROC analyses comparing PC patients with healthy controls and patients with benign conditions yielded an AUC=0.87, 95% CI 0.71–1.00 (Figure 1C) and AUC=0.71, 95% CI 0.65–0.76 (Figure 1D).

Overall survival according to levels of HA and PRO-3C is shown in Figure 2. Univariate analysis showed that high baseline serum HA (1.18, 95% CI: 1.13–1.25) and PRO-C3 (1.28, 95%: CI 1.11–

1.49) were associated with worse prognosis. After allocating patients into stages, a significance association was still seen for patients with stage IV (Figure 2). High baseline HA and PRO-C3 levels according to types of treatments in 579 patients undergoing palliative chemotherapy were associated with short survival (Figures S1). After adjusting factors associated with OS, the HRs for serum HA and PRO-C3 were 1.04 (95% CI: 0.97–1.13) and 1.42 (95% CI: 1.14–1.76), respectively (Table 3). Further adjustment for inflammatory markers (CRP, IL-6, and YKL-40) changed the HRs to 1.02 (95% CI: 0.93–1.11) and 1.35 (95% CI: 1.05–1.73) for serum HA and PRO-C3, respectively (Table S2 and Supplementary Materials & Methods). Patients with combined 25% highest levels of both serum HA and PRO-C3 had the poorest survival (Figure S2).

Prognostic value of changes in serum HA and PRO-C3 over time

Serum HA levels decreased after the first treatment cycle, with a median difference of −11.50 [IQR, −41.98, 12.23] ng/mL, and at the time of first CT evaluation 2 or 3 months after chemotherapy start of −34.10 [IQR, −72.95, −5.25] ng/mL compared to the HA level at baseline. In contrast, no decrease was observed for longitudinal serum PRO-C3 concentrations: −0.90 [IQR, −3.80, 3.05] and −1.90 [IQR, −9.30, 4.30] after the first treatment cycle and at the time of first CT evaluation, respectively. Changes in HA and PRO-3C were not associated with overall survival (Figure 3).

Discussion

PC remains one of the most aggressive tumors and is known to be highly enriched with ECM in the desmoplastic stroma, and HA and collagen are essential components of the ECM. Biomarker availability, reliability, and function of the serum levels of HA and N-terminal propeptide of type III collagen (PRO-C3) in patients with PC have not been extensively investigated so far. In this study, we demonstrated that higher values of serum PRO-C3 and HA at the time of diagnosis are associated with an increased mortality. However, serum HA was not associated with OS after adjusting for potential confounding factors. This suggests that serum PRO-C3 may serve as prognostic marker in patients with PC. In support for this hypothesis, collagen turnover rate needs to be maintained in normal tissue homeostasis, and changes in the deposition of collagens result in the tissue fibrosis that drives tumorigenesis[41]. Hence type III collagen synthesis may be a major component of the dense stroma (desmoplastic reaction) in the tumor and linked to tumor growth, metastasis, and drug resistance.

Our findings are in line with reports on the facilitating role of HA and PRO-C3 in the malignant behavior of tumor cells and the IFP increase within a tumor, limiting perfusion and compromising drug delivery[8, 25, 29, 37, 42]. Analyses of HA and collagen levels in tumors from patients with PC revealed that the survival was worse among patients with high HA and collagen accumulation compared to subjects with low HA and collagen expression, suggesting that HA and collagen levels in tumors of patients with PC may be predictive of survival[5, 49]. In contrast, prognostic significant relevance was not observed for HA accumulation, found in the majority of the PDAC-resected tumors[12]. Surgical examination of tissues from 114 PC patients identified a particular rearrangement of collagen fibers surrounding the tumor as a "biomarker" of early death[7]. By staining 53 patients' primaries and 57 patients' metastases, Whatcott et al. found highly fibrotic stroma with high levels of HA and collagen type I in both primary tumors and metastases[49]. PDAC patients with a high

expression of HA were more susceptible to therapy targeting HA[15]. Addition of HA blockade to FOLFIRINOX did not shown any benefit in patients unselected for tumor HA status[30]. Interestingly, in our study the more pronounced association with survival of baseline HA according to types of treatments in patients undergoing palliative chemotherapy was observed in participants treated with gemcitabine and nab-paclitaxel combination (Figures S1). However, due to the small number of patients undergoing combination palliative chemotherapy the survival data should be viewed with caution. A phase III trial investigating the efficacy of HA blockade in combination with gemcitabine and nab-paclitaxel in PDAC is pending (https://clinicaltrials.gov/ct2/show/NCT02715804). Collagen type III in PC was found to be linked to fibrosis within ECM and associated with poor prognosis and tumorigenesis[16, 26]. Targeting of collagen compartments in combination with various drugs is under extensive investigation.

We demonstrated that serum HA was increased in PC patients from the BIOPAC cohort compared with patients with benign conditions and healthy subjects. Likewise, a significantly higher concentration of serum PRO-C3 was observed in PC patients compared to patient with non-malignant conditions. Specific protease-generated fragments of collagen were significantly elevated in serum from patients with PC compared to controls, and a combination of serum biomarkers reflecting matrix metalloproteinase-mediated collagen turnover resulted in complete separation between healthy controls and patients with PC[50].

We also demonstrated that high serum HA and PRO-C3 levels at diagnosis were not restricted to patients with metastatic PC but were present in all PC patients regardless of stage. However, our study suggests an association between higher concentrations of serum HA and PRO-C3 levels and advanced stage of disease, many metastatic sites, as well as presence of liver metastases. This is in agreement with the suggestion that the increase in matrix components is due to the ECM degradation that occurs

during metastasizing[9]. Furthermore, our findings were compared to patients with other upper gastrointestinal cancer diagnoses, including AC and DBTC. No significant difference was observed for AC and DBTC compared to patients with PC. Finally, serum HA and PRO-C3 also correlated with serum CRP, IL-6, and YKL-40, suggesting mutual interplay between inflammation and stroma. While treatment of patients with PC is based on a balance between tolerability and benefit, the optimization of non-invasive cost-effective monitoring would be of useful clinical value. Prognosis of PC patients often displays a dynamic pattern during systemic treatment. We observed a reduction in serum HA but not PRO-C3 levels in longitudinal samples; however, neither a decrease in HA nor in PRO-C3 was associated with survival. Pretreatment serum HA and PRO-C3 in patients undergoing palliative chemotherapy are more robustly associated with survival than serum HA and PRO-C3 after first cycle chemotherapy and at the time of the first evaluation CT scanning. This suggests that pretreatment serum HA and PRO-C3 would be more useful in identifying patients at risk of poor survival due to primary aggressive biology. However, due to the small number of patients with available longitudinal serum samples, natural selection, and the limited number of patients who received gemcitabine with nab-paclitaxel or FOLFIRINOX, the survival data according to serum HA and PRO-C3 changes should be interpreted with caution. To our knowledge, this is the first large-scale study to describe serum HA and PRO-C3 in samples derived from population-based consecutive patients with PC that can be applied to future research addressing stroma and ECM.

Several limitations must be noted. First, causal relationships cannot be determined in an observational study, and residual confounding is always a concern. However, prospective well-characterized data sets with clinico-pathological characteristics and treatment data on consecutive patients across the different centers including patients with PC in the BIOPAC study in a well-defined period of time reduce residual confounding. Second, staging is based on established routine practice, and it is well

known that clinical staging systems are not as clear-cut. However, we demonstrated consistent associations between serum HA and PRO-C3 levels with survival across all stages. Third, selection bias may affect the generalizability of the results since only around 20% of all registered Danish patients with PC are included in the BIOPAC study. Thus, many patients are not referred to systemic therapy due to rapid deterioration and die within a short time after diagnosis. Enrolled patients were more likely to have better PS, and more likely to receive chemotherapy. Thus, it is possible that the relationship between serum HA and PRO-C3 and survival in this subset is not generalizable to all patients with PC. Finally, whether serum HA and PRO-C3 values are correlated with HA and PRO-C3 expression in tumors has not yet been determined.

In conclusion, we have shown that high serum HA and PRO-C3 levels are associated with increased mortality in patients with PC. Moreover, compared to healthy subjects and a group of patients with benign conditions, high values of these parameters are measured in patients with malignant lesions, including PC, AC, and DBTC. Serum HA and PRO-C3 were associated with PC stage. Our findings suggest that serum HA and PRO-C3 levels may have a clinical value and motivate further research on serum HA and PRO-C3 as novel, minimally invasive and cost-effective prognostic and diagnostic markers of PC. Our study also supports evidence that encourages ongoing investigation of stroma targeting strategies.

Declarations

Consent for publication

Not applicable.

Competing interests

NWI, MK are employees at Nordic Bioscience involved in biomarker discovery and development. MK holds stocks of Nordic Bioscience. All the other authors do not have any conflict to declare.

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Authors' contributions

All authors contributed to inclusion of patients in the BIOPAC study. AZJ, IMC, and JSJ entered the clinical data in the database. IMC drafted and wrote the manuscript and revised the manuscript after feedback from all authors. All authors contributed to review of the present manuscript and approved the final version of the manuscript.

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Table 1. **Clinical characteristics of 809 patients with pancreatic cancer.**

Characteristic	Number (%)
Age, median (range) years	67 (37-89)
>75 years	135 (16.7)
Sex	
Male	440 (54.4)
Female	369 (45.6)
ECOG PS	
0	283 (35.0)
1	346 (42.8)
2+	103 (12.7)
unknown	77 (9.5)
Stage	
I+II	197 (24.4)
III+IV	595 (73.5)

unknown	17 (2.1)
Diabetes	
yes	210 (26.0)
no	599 (74.0)
Smoking	
yes	483 (59.7)
no	251 (31.0)
unknown	74 (9.1)
Alcohol	
yes	179 (22.1)
no	554 (68.5)
unknown	76 (9.4)
BMI	
underweight	63 (7.8)
normal weight	434 (53.6)
overweight	252 (31.1)
unknown	60 (7.4)
Metastatic sites	
Liver	337 (41.7)
Peritoneum	83 (10.3)
Chemotherapy type	
Never received chemotherapy	51 (6.3)
Adjuvant gemcitabine	160 (19.8)
1st line palliative	
Gemcitabine	404 (49.9)
FOLFIRINOX	120 (14.8)
Gemcitabine and nab-paclitaxel	55 (6.8)
Other	19 (2.3)
CA19-9 median [IQR] U/ml	519 [76, 3230]

Abbreviations: BMI, body mass index; ECOG PS, Eastern Cooperative Oncology Group performance status; IQR, interquartile range.

Table 2. Baseline serum levels of HA and PRO-C3 in patients with PC stratified by stage, presence of liver metastases, and ECOG PS compared to patients with other upper gastrointestinal cancer, benign diseases, and healthy controls.

Characteristic	**HA median [IQR] (ng/mL)**	**PRO-C3 median [IQR] (ng/mL)**

Pancreatic cancer (n=809)	84 [48, 131][a,b]	18 [11, 33][a,b]
Stage		
I+II	75 [46, 126]	15 [10, 29]
III	69 [39, 101]	14 [10, 22]
IV	94 [51, 140][c]	20 [13, 41][c]
Liver metastases		
No	74 [43, 114]	15 [10, 25]
Yes	100 [57, 148][d]	22 [14, 49][d]
ECOG PS		
0	76 [44, 121]	17 [11, 35]
1	87 [48, 132]	17 [11, 31]
2+	98 [57, 140][e]	19 [15, 36][f]
Ampullary carcinoma (n=44)	102 [70, 154]	17 [12, 38]
Distal biliary tract cancer (n=31)	95 [66, 147]	30 [18, 52]
Chronic pancreatitis (n=15)	72 [36, 105]	11 [10, 17]
Intraductal papillary mucinous neoplasm (n=41)	66 [32, 101]	11 [8, 15]
Duodenal adenoma (n=7)	47 [32, 53]	14 [11, 17]
No pathologic finding (N=25)	53 [37, 100]	11 [9, 14]
Healthy controls (n=141 and n=7)	41 [27, 60]	7 [5, 9]

[a]P<0.001 compared with healthy controls.
[b]P<0.001 compared with benign conditions.
[c]P<0.001 across different stages.
[d]P<0.001 compared with no liver metastases.
[e]P=0.02 across different PS.
[f]P<0.05 across different PS.
Abbreviations: HA, hyaluronan; IQR, interquartile range; PRO-C3, propeptide of type III collagen; ECOG PS, Eastern Cooperative Oncology Group performance status.

Table 3. Univariate and multivariate Cox analyses for overall survival according to clinical characteristics and pretreatment serum HA and PRO-C3 (HRs are reported per 100 unit increases). The significant terms are included in the multivariate analyses.

	Univariate analysis		**Multivariate analysis**	
Variable	**HR (95% CI)**	**P-value**	**HR (95% CI)**	**P-value**
Age ≥ 75 years (vs. <75 years)	1.42 (1.18-1.72)	<0.01	1.33 (1.06-1.66)	<0.01
Sex (female vs. male)	1.01 (0.88-1.17)	0.88	-	-
PS 1 (vs. PS 0)	1.60 (1.35-1.89)	<0.01	1.34 (1.12-1.61)	<0.01
PS 2 (vs. PS 0)	2.71 (2.15-3.43)	<0.01	2.58 (1.99-3.35)	<0.01
BMI (underweight vs. normal weight)	1.27 (0.97-1.66)	0.09	-	-
BMI (overweight vs. normal weight)	1.02 (0.87-1.20)	0.82	-	-
Diabetes (yes vs. no diabetes)	1.14 (0.97-1.34)	0.11	-	-
Smoking ((ever vs. never)	1.08 (0.92-1.26)	0.37	-	-

Alcohol abuse (ever vs. never)	1.02 (0.86-1.21)	0.84	-	-
Stages 3 and 4 (vs. stage 1 and 2)	3.67 (3.03-4.45)	<0.01	3.71 (2.27-6.09)	<0.01
Liver metastasis (yes vs. no met)	2.42 (2.08-2.81)	<0.01	1.69 (1.40-2.04)	<0.01
Peritoneum metastasis (yes vs. no met)	1.58 (1.25-1.99)	<0.01	1.28 (0.99-1.66)	0.06
Never received chemotherapy (vs. Res)	3.39 (2.41-4.78)	<0.01	4.21 (2.09-8.50)	<0.01
Gemcitabine (P) (vs. Res)	4.24 (3.43-5.26)	<0.01	1.05 (0.64-1.72)	0.85
FOLFIRINOX (P) (vs. Res)	2.48 (1.91-3.22)	<0.01	0.64 (0.38-1.09)	0.10
Gemcitabine+nab-paclitaxel (P) (vs. Res)	2.86 (2.02-4.04)	<0.01	0.63 (0.35-1.13)	0.12
Other combinations (P) (vs. Res)	3.31 (2.03-5.41)	<0.01	0.94 (0.49-1.82)	0.86
CA19-9 >median	1.93 (1.65-2.26)	<0.01	1.39 (1.17-1.65)	<0.01
HA (per 100 ng/mL)	1.18 (1.13-1.25)	<0.01	1.04 (0.97-1.13)	0.27
PRO-C3 (per 100 ng/mL)	1.28 (1.11-1.49)	<0.01	1.42 (1.14-1.76)	<0.01

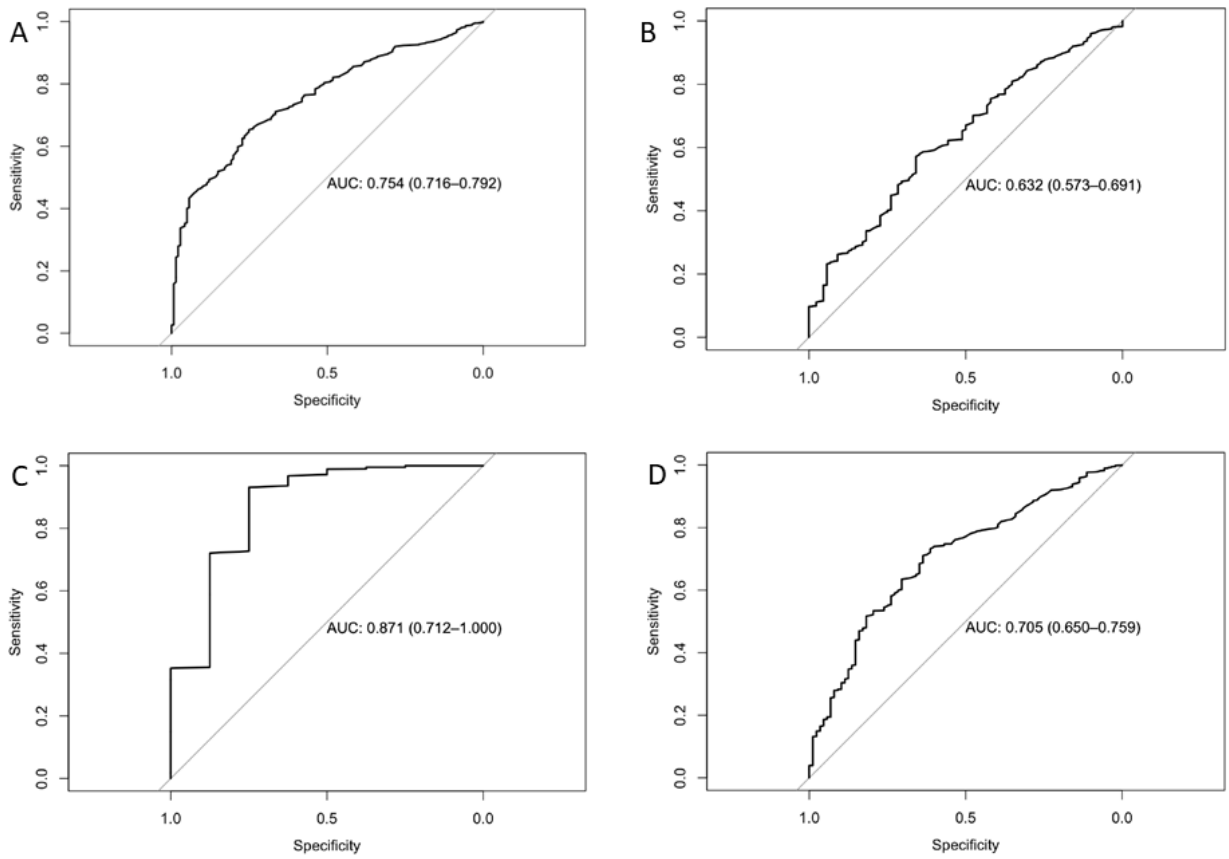
Abbreviations: BMI, body mass index; PS, performance status; HA 100, additional risk of death for every 100-unit increase in hyaluronan; HR, hazard ratio; CI, confidence interval; met, metastasis; P, palliative; PRO-C3 100, additional risk of death for every 100-unit increase in pro-peptide of type III collagen; Res, resection.

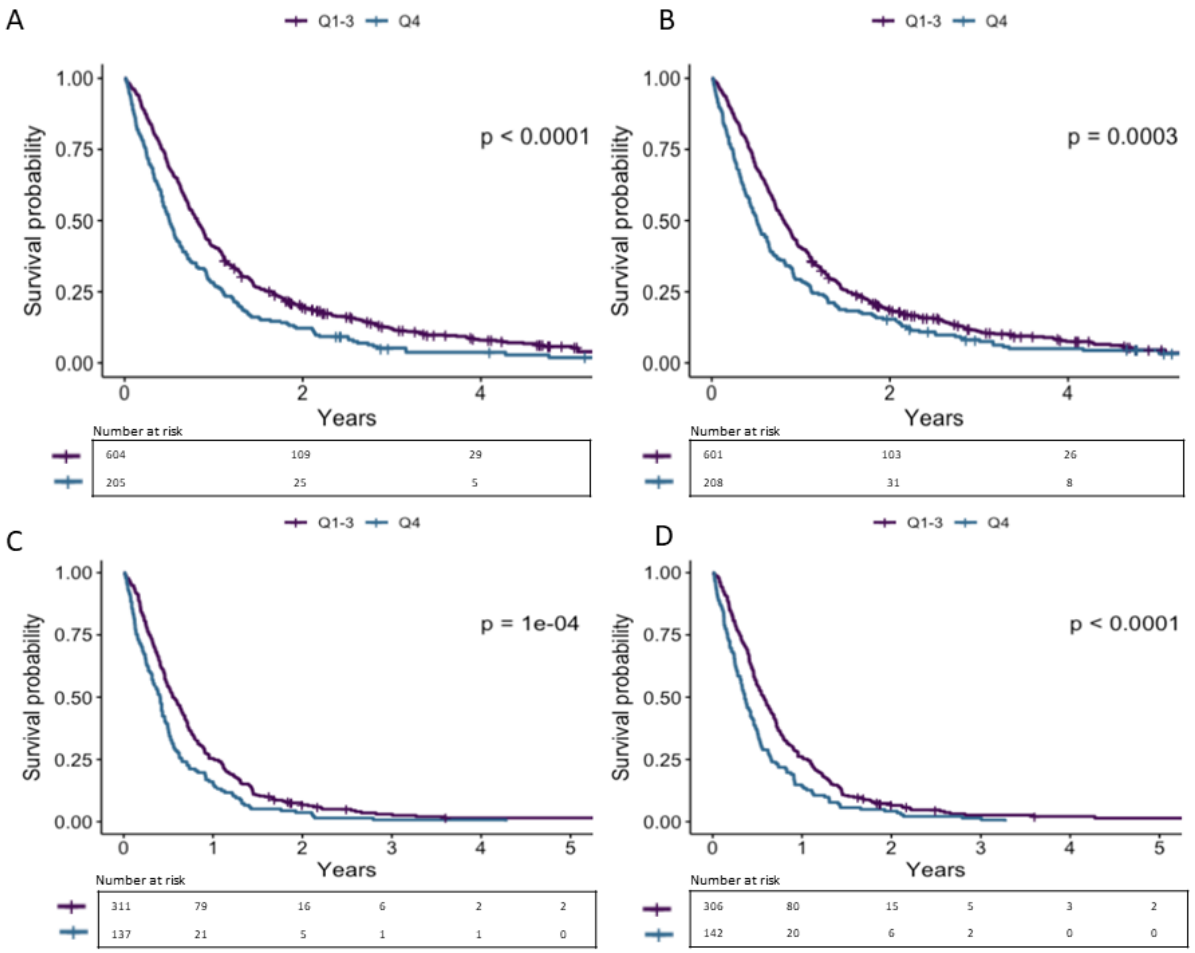
Figure Legends

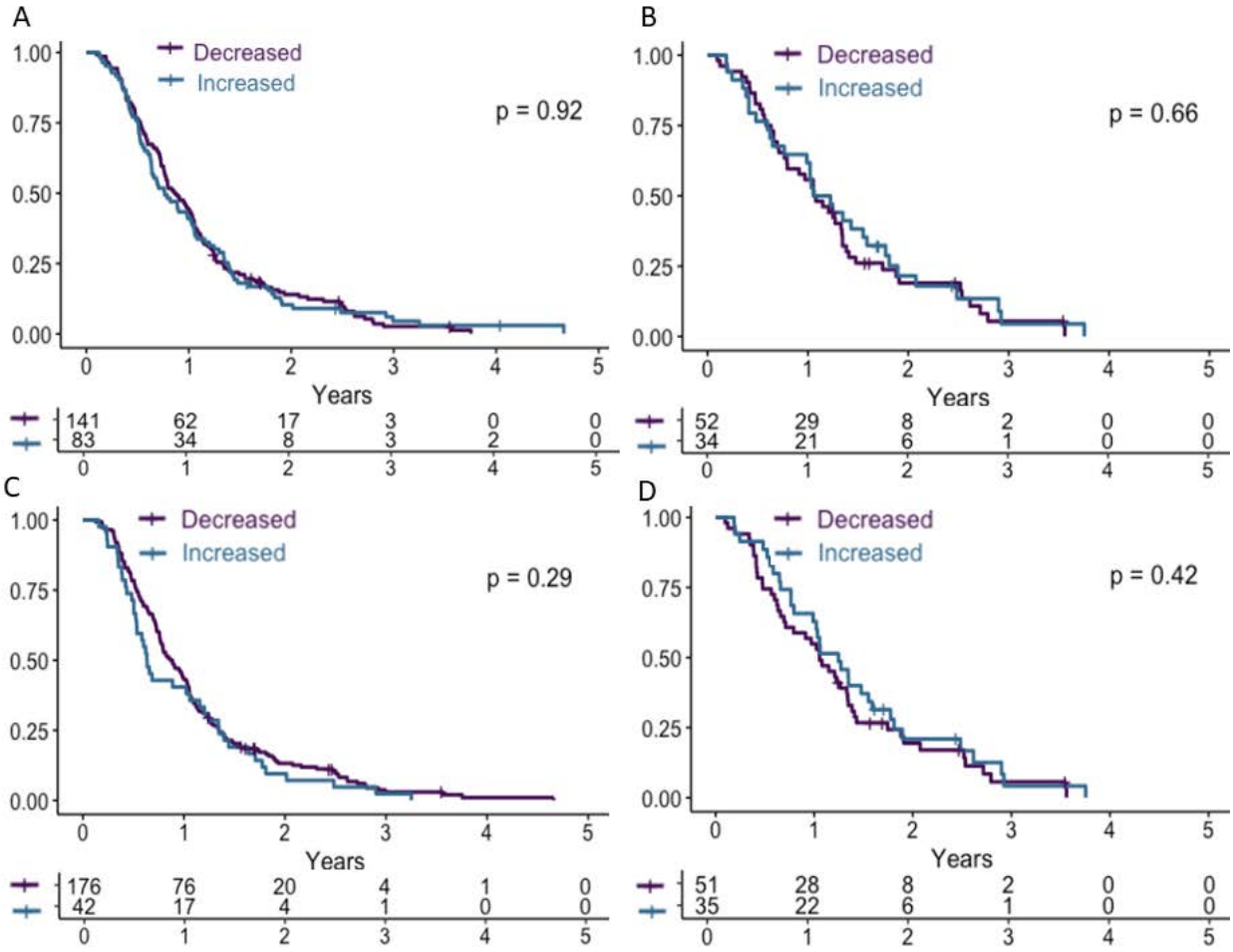
Figure 1. Receiver operating characteristic (ROC) curve of baseline serum HA (A, B) and PRO-C3 (C, D) detection in discriminating PC patients from healthy controls and from patients with benign conditions. Abbreviations: AUC, area under the ROC; CI, confidence interval; HA, hyaluronan; PRO-C3, propeptide of type III collagen.

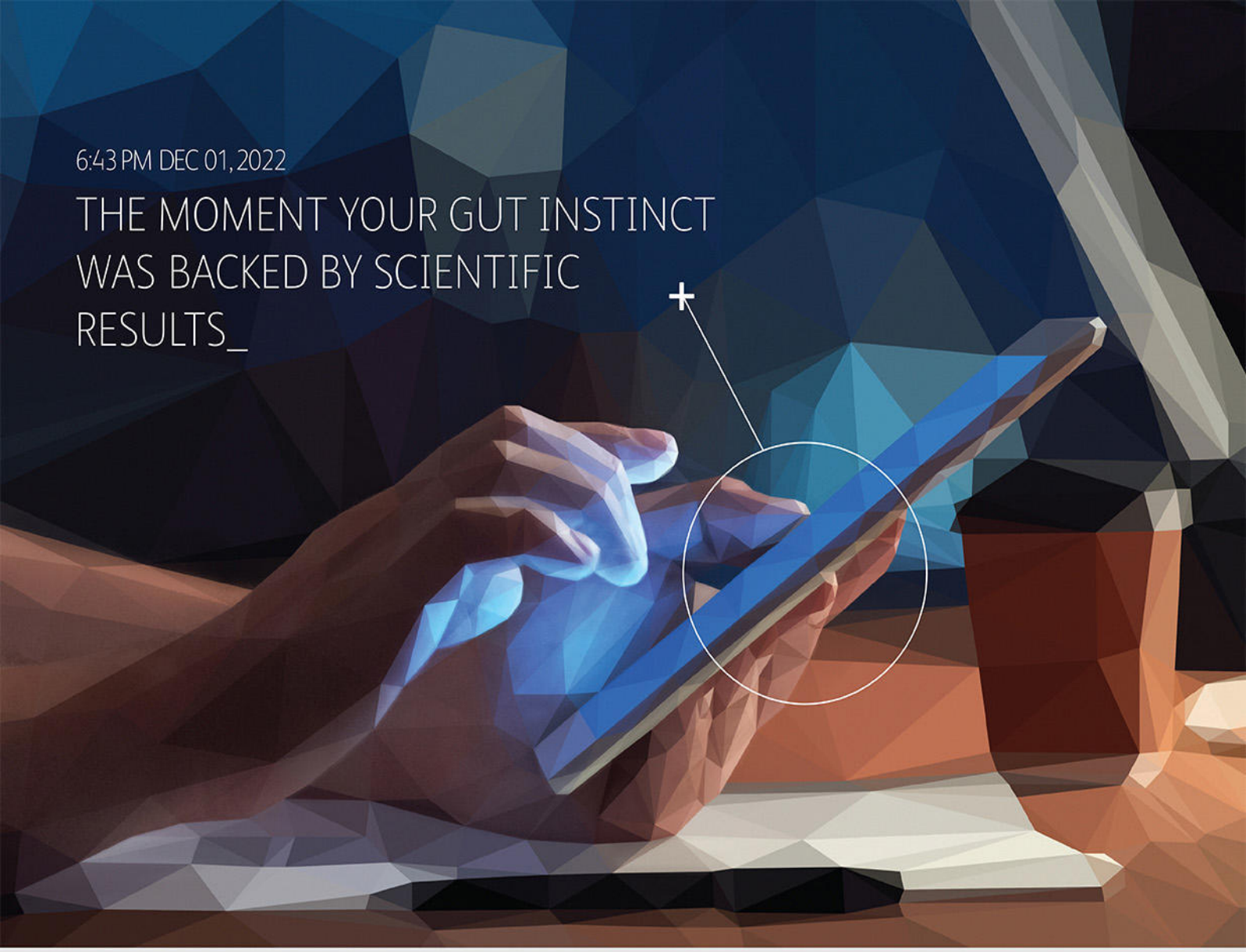
Figure 2. Kaplan–Meier survival curves showing the association between pre-treatment serum HA (2A) and PRO-C3 (2B) in patients with PC across all stages and for stage 4 (Figures 2C and 2D, HA and PRO-C3, respectively). Cut-off is the 75 percentile.

Figure 3. Kaplan–Meier survival curves in patients with PC receiving palliative chemotherapy, grouped according to decreased versus increased serum levels of HA and PRO-C3 after the first treatment cycle (Figures 3A and 3B) and at the time of first CT evaluation (Figures 3C and 3D).









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