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Combining conventional and stroma-derived tumour markers in pancreatic ductal adenocarcinoma

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Abstract.

BACKGROUND: A lack of disease-specific symptoms and good tumour markers makes early detection and diagnosis of pancreatic ductal adenocarcinoma (PDAC) challenging.

OBJECTIVE: To analyse the tissue expression and circulating levels of four stroma-derived substances (type IV collagen, endostatin/type XVIII collagen, osteopontin and tenascin C) and four conventional tumour markers (CA 19-9, TPS, CEA and Ca 125) in a PDAC cohort.

METHODS: Tissue expression of markers in normal pancreas and PDAC tissue was analysed with immunofluorescence. Plasma concentrations of markers were measured before and after surgery. Patients with non-malignant disorders served as controls.

RESULTS: The conventional and stromal substances were expressed in the cancer cell compartment and the stroma, respectively. Although most patients had increased levels of many markers before surgery, 2/12 (17%) of patients had normal levels of Ca 19-9 at this stage. High preoperative endostatin/type XVIII collagen, and postoperative type IV collagen was associated with short survival. Neither the pre- nor postoperative levels of TPS, Ca 125 or CA 19-9 were associated to survival.

CONCLUSIONS: PDAC is characterized by an abundant stroma. These initial observations indicate that the stroma can be a source of PDAC tumour markers that are found in different compartments of the cancer, thus reflecting different aspects of tumour biology.

Keywords: Pancreatic ductal adenocarcinoma (PDAC), tumour markers, stroma, type IV collagen, type XVIII collagen, endostatin, osteopontin, tenascin C, TPS, Ca 125, Ca 19-9, CEA

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a disease with poor prognosis and limited response to treatment [1]. The disease is the fourth leading cause of cancer related death, and the overall 5-year survival rate is merely 6%. Over the last decades the survival

rate has only improved marginally and radical surgical resection remains the sole curative treatment [2]. In addition, most patients have metastatic spread when presenting with symptoms and < 20% are eligible for surgery with curative intent [3]. There is an urgent need for novel diagnostic approaches in order to provide early diagnosis, correctly classify the disease as metastatic or localized, to detect early recurrences and monitor treatment effects.

The traditional view on tumours has been focused on malignantly transformed cancer cells. Consequently, evaluated tumour markers for PDAC have conventionally been cancer cell-derived. Cancer antigen 19-9 (Ca

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19-9), the only tumour marker currently used in clinical practice when managing pancreatic tumours is indeed expressed on cancer cell surfaces [4]. However, 5–10% of the population lack an enzyme enabling the expression of the Ca 19-9 antigen, and many benign and malignant diseases can cause elevated levels. As a marker, Ca 19-9 is useful in setting prognosis and when monitoring the effect of chemotherapy, but is of limited use in the diagnostic setting and is impotent as a screening marker in an asymptomatic population [4]. Tissue polypeptide specific antigen (TPS), a potential tumour marker that outperforms Ca 19-9 when differentiating between PDAC and chronic pancreatitis [5], is a soluble fragment of cytokeratin 18 (ck18), a cytoskeletal component in normal and cancer cells [6,7]. Carcinoembryonic antigen (CEA) used in colorectal cancer management and cancer antigen 125 (Ca 125) used in ovarian cancer are also cancer cell-derived, and have both been evaluated as markers in PDAC [8–15].

In recent years, the tumour stroma has been recognized as an important part of tumour progression and metastasis [16]. PDAC tumours are characterized by a prominent stroma outnumbering the malignant cells in the tumour mass, and stroma-derived proteins produced and secreted in tumour-stroma interactions promote tumour survival and migration [17–20]. Substances from different compartments of the tumour may reflect different aspects of the tumour biology, and stroma-derived substances can leak into the circulation and potentially serve as tumour markers [21]. In this study, we compare the tissue expression of four stroma-derived substances – the basement membrane (BM) derived fragments type IV and type XVIII collagen/endostatin and the matricellular proteins osteopontin (OPN) and tenascin C (TNC) with four conventional, cancer cell-derived markers – Ca 19-9, TPS, CEA and Ca 125 by immunofluorescence staining of PDAC and normal pancreatic tissue. In order to study whether stromal substances provide any advantages compared to conventional tumour markers, the circulating levels of the four stroma-derived substances and the four conventional markers were measured in the same patient cohort.

2. Materials and methods

2.1. Patients and samples

The study cohort is based on patients with suspected pancreatic cancer that were admitted to the Department

of Surgery at Umeå University Hospital for further diagnostic evaluation with computed tomography scan and/or magnetic resonance imaging and laparoscopy during the years 2000–2009 and selected based on the availability of samples. Patients considered operable (with no signs of metastases) underwent pancreaticoduodenectomy (Whipple's procedure) with curative intent. Patients with histologically verified PDAC were included retrospectively in this study ($n = 19$). Plasma was collected both pre- and postoperatively from most of these patients ($n = 8$), but for some of these patients only preoperative ($n = 4$) or postoperative samples were available ($n = 7$). Thus, paired pre- and postoperative samples were available from eight individuals ($n = 8$). All plasma samples were stored at -80°C until analysis. Preoperative levels of serum creatinine, bilirubin, transaminases and C-reactive peptide (CRP) as well as patient survival data was obtained by reviewing the clinical records. At the time of analysis all the patients included in the study had died. Control samples were collected from patients admitted to the department for non-malignant diseases ($n = 8$). Patient characteristics are summarized in Table 1. The Research Ethics Review Board (EPN) of Northern Sweden approved this study.

Tissue samples from PDAC ($n = 8$) and normal pancreas ($n = 4$) were snap frozen in liquid nitrogen after collection, and kept at -80°C until analysis. The tumour differentiation grade was extracted from the final pathology report, and the cohort consisted of two cases with well differentiated (G1), four moderately differentiated (G2) and two poorly differentiated (G3) pancreatic adenocarcinomas. 5 μm tissue sections were cut using a cryostat microtome and stained by haematoxylin and eosin (H&E). Tissue samples were evaluated by a clinical pathologist (W.W.) prior to further staining in order to verify the accuracy of the cancer diagnosis and histological grade.

2.2. Immunofluorescence and microscopy

PDAC tissue and normal pancreatic tissue were examined to determine the expression pattern of the tumour markers. Slides were fixated in -20°C acetone for 10 minutes, and dried in room temperature (RT) for 1 minute. Slides were washed with phosphate buffered saline (PBS), and blocked with 3% bovine serum albumin (BSA) in PBS for 1 hour at RT, followed by incubation with primary antibodies (supplemental Table 1) for 1 h at RT or at 4°C overnight. After PBS washing the secondary antibodies

Table 1
Patient characteristics

	Controls	Patients
Number	8	19
Sex (females/males)	5/3	12/7
p-value versus control	–	$p = 1.0^a$
Average age (years)	51	67
p-value versus control	–	0.012 ^b
Primary diagnose (%)	Gallstone disease ($n = 6$) Diverticular disease ($n = 2$)	Pancreas adenocarcinoma ($n = 19$)
Differentiation grade (%)	–	Poorly differentiated ($n = 2$) Moderately differentiated ($n = 15$) Well differentiated ($n = 2$)

^aFischer's exact test, ^bMann-Whitney test.

were added, and incubated in the dark for 1 hour in RT. The slides were washed, and cover slides were mounted with VECTASHIELD® Hard Set™ Mounting Medium with DAPI (Vectashield, Vector Laboratories Inc.). When double staining with the Rabbit-anti-OPN antibody the tissue was instead fixated in 4% paraformaldehyde for 10 minutes at RT, and 10% BSA in PBS was used for blocking and antibody dilutions. Cytokeratin 18 (ck18) is expressed in both normal and malignant epithelial cells, and thus ck18 was chosen as a marker for the epithelial and cancer cells in all double stainings.

The expression was analysed in cancer cells and adjacent tumour stroma in PDAC tissue, and compared to ductal and acinar cells and adjacent stroma in normal pancreas. Both the basement membrane staining and staining of adjacent stroma were included in the stromal compartment. Staining intensity was scored as 0 = negative, 1 = weak, 2 = strong. The distribution of the staining was categorized in three intervals; > 66%, 33–66% and > 33% of the total area in the section.

2.3. Analysis of circulating tumour markers

Circulating levels of tumour markers were measured in plasma before and after surgery in patients and controls by using commercially available ELISA and Luminex based assays. Type IV collagen was measured by using Serum Collagen IV EIA (Argutus Medical, Dublin, Ireland), TPS with TPS™ ELISA (IDL Biotech AB, Bromma, Sweden), endostatin/type XVIII collagen with Human Endostatin Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA), OPN with Human Osteopontin Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA) and TNC with Tenascin-C Large (FN III-B) ELISA Kit (IBL, Gunma, Japan). AFP, CA 125, CA 15-3, CA 19-9, CEA and prolactin were measured with

WideScreen™ Human Cancer Panel 1 (Merck KGaA, Darmstadt, Germany). All samples were run in duplicates and according to the manufacturers' instructions. For all assays CV% < 15 was considered acceptable. If a duplicate showed CV% > 15 the sample was measured again.

2.4. Statistics

Non-parametric Mann-Whitney test tests were used when comparing means. P-values ≤ 0.05 were considered significant. Data is presented as median and range (within square brackets). Survival analysis (COX-regression) and correlation analysis (Spearman's rank correlation test) between the different tumour markers were performed in IBM PASW Statistics 20 (Chicago, IL, USA). In the COX-model all variables (tumour markers) were transformed by the division of 10, so that 10 units in the original scales corresponded to 1 unit in the scales used in the analysis. The hazard is presented with 95% confidence interval within square brackets.

3. Results

3.1. Stromal and conventional markers are expressed in different compartments of the tumour

Tissue expression of the studied markers was analysed in PDAC ($n = 8$) and normal pancreas ($n = 4$) by immunofluorescence on frozen sections. Cytokeratin 18 (ck18) was expressed strongly by acinar and ductal cells in all normal pancreas samples (4/4), and by cancer cells in all PDAC samples (8/8). Ck18 was chosen as marker for compartments of epithelial origin, and all other antibodies were subsequently double stained with a ck18 antibody (Fig. 1).

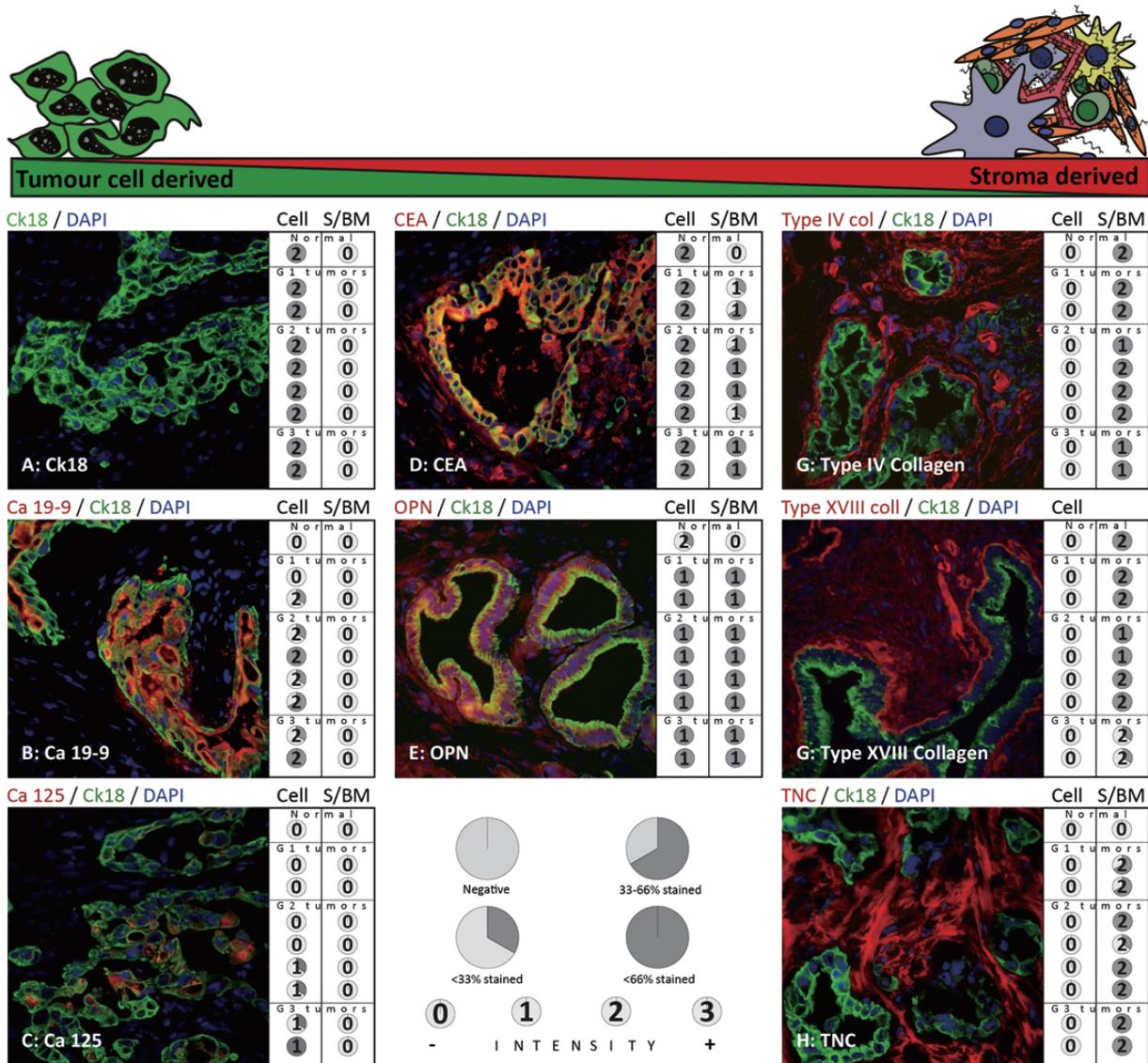


Fig. 1. Immunofluorescence staining of PDAC samples showing the distribution in cellular compartments (C) or in the tumour stroma/basement membrane like structures (S/BM). The staining scores for the 8 included tumour samples are presented and grouped in different tumour grades (G1-3). The score for the normal samples is the mean of the four analysed samples. Stainings A-C are solely expressed in tumour cell compartments, F-H show an exclusive stromal staining. D and E show expression in both compartments. Staining positivity is presented as 0 = no staining, 1 = weak positive staining, 2 = strong positive staining. The staining distribution is presented with pie charts.

In the normal pancreas, both type IV collagen and endostatin/type XVIII collagen stained strongly and continuously in the basement membrane (BM) around ductal and acinar cells. OPN exclusively stained central areas of acini. The majority of the normal pancreas samples were devoid of CEA expression, with only one sample showing a weak apical expression of CEA in a pancreatic duct. TNC, Ca 19-9 and Ca 125 did not stain in any of the normal pancreas samples.

In the PDAC samples (Fig. 1), the type IV collagen

staining presented with a generally strong staining in BM-like structures (when preserved), and a widely distributed staining in the stroma surrounding the cancer cells (5/8). In the two G3 tumours and in one G2 tumour BM-like structures were resolved, and the type IV collagen staining was confined to a widely distributed but weak stromal staining (Fig. 1). All PDAC samples also stained positive for endostatin/type XVIII collagen, in either the BM-like structures, the vascular BM or in the surrounding stroma. In all but one

G1 and one G2 tumour, there was a strong staining in the BM-like structures that was virtually absent in the two G3 tumours. A weaker expression in the surrounding stroma was observed for endostatin/type XVIII collagen in most samples, with a tendency towards loss of stromal expression with loss of differentiation. All PDAC samples stained strongly for TNC, 6 out of 8 stained the cancer cell-surrounding stroma, and 2 out of 8 stained only the BM-like structures around the cancer cells. OPN presented with a weak intracellular staining in all cancer cells of the examined samples. The stroma stained positive for OPN in all samples, however in 6 out of 8 samples this staining was weaker in intensity than the cancer cell staining. No intracellular staining in cancer cells was observed for type IV collagen, endostatin/type XVIII collagen or TNC.

Ca 19-9 strongly stained the cancer cells in 7 out of 8 samples, however in most samples not all cancer cells were stained. CEA presented an intracellular staining seen in most cancer cells of all samples, but some staining was also observed diffusely in the stroma surrounding the neoplastic glands. Ca125 stained weakly and focally in two G2 and in the G3 tumour samples, but was negative in the G1 samples and in two G2 samples.

3.2. Multiple circulating markers are elevated in PDAC patients

Circulating levels of the tumour markers were measured in controls and in patients before and after surgery. Compared to the controls, the levels of all the stromal proteins were elevated in PDAC patients before surgery (Fig. 2a and Table 2). For the conventional tumour markers, significantly elevated levels of TPS, Ca 125 and Ca 19-9 were found in PDAC patients before surgery (Fig. 2b and Table 2). After surgery the levels were normalised only for endostatin/type XVIII collagen, although the levels of TPS also decreased after surgery. For type IV collagen, TNC, OPN, Ca 125, and Ca 19-9 the levels remained elevated after surgery when compared to controls. As shown in Fig. 2b, when compared to the controls no significant changes in CEA levels were found neither pre- nor postoperatively. As expected, no significant changes for the tumour markers Ca 15-3, AFP and prolactin could be observed in PDAC patients pre- nor postoperatively (data not shown).

The range of marker levels appeared narrower for the stroma derived tumour markers, when compared to TPS, Ca 125 and Ca 19-9, for which the values spanned from normal to extremely high, thus causing the large

ranges and the extreme outlier values (marked with asterisk in the boxplots (Fig. 2b). The same was not observed for type IV collagen, endostatin/type XVIII collagen, TNC or OPN (Fig. 2a).

3.3. The combination of multiple circulating markers in the preoperative setting

To investigate the potential value of combining markers to differentiate disease preoperatively, we investigated whether the preoperative levels of the markers were elevated compared to healthy controls. Since there is no verified clinical cut off for the stromal markers (type IV collagen, endostatin/type XVIII collagen, TNC and OPN), as there is for the conventional markers (TPS, Ca 125, CEA and Ca 19-9), we used the 95th percentile of the healthy controls as the cut off for all markers (Table 3).

Only one patient presented with elevated levels of all eight markers analysed. The majority of the patients presented with elevated levels in four or more markers. One patient presented with elevation in only one marker (OPN), this was also the only patient with a G1 tumour, and the patient with the longest survival (28 months, data not shown). The patients presenting with normal Ca 19-9 levels, the only clinically used marker currently, had increased levels of at least one stromal marker.

3.4. Correlation between circulating tumour markers

To evaluate if any associations were present among the studied tumour markers, a correlation analysis was done between the preoperative values of TPS, type IV collagen, endostatin/type XVIII collagen, TPS, OPN, Ca 125 and Ca 19-9. A significant correlation between Ca 125 and Ca 19-9 was found ($r = 0.606, p = 0.037$), which indicates that high levels of Ca 125 are reflected in high Ca 19-9 levels. TNC was correlated with Ca 125 ($r = 0.588, p = 0.044$), and CEA with OPN ($r = 0.600, p = 0.039$). No other correlations could be detected (data not shown), which indicate that TPS, type IV collagen and endostatin/type XVIII collagen are not connected with the other markers tested. All markers were also tested for correlations against serum creatinine, bilirubin, transaminases, and C-reactive peptide (CRP) levels. High levels of TPS were associated to high levels of transaminases, which is well in line with previous reports [22]. No other correlations could be observed for the conventional or the stromal derived tumour markers.

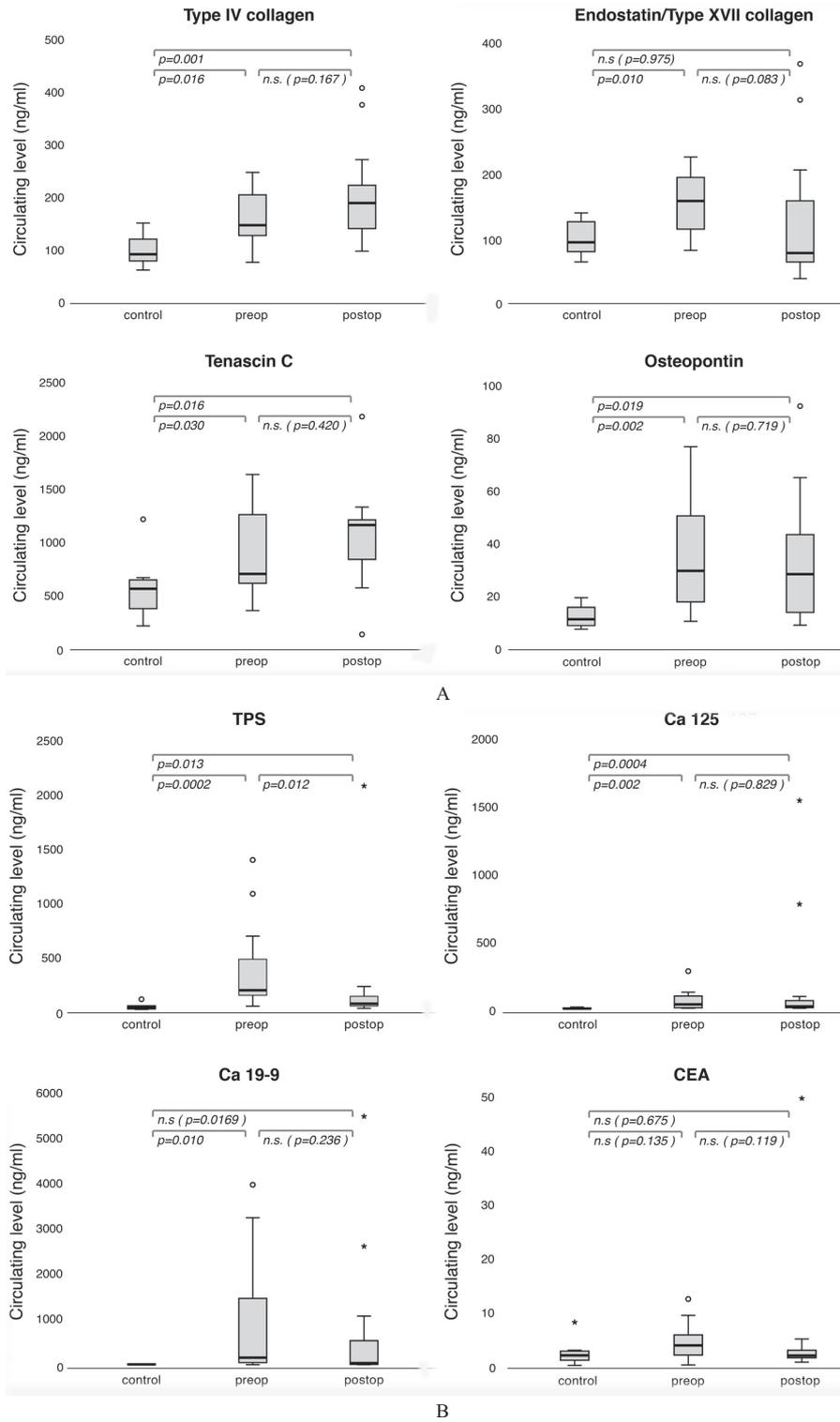


Fig. 2. Levels of stroma derived (A), and conventional (B) tumour markers. Boxplot of circulating levels in controls and patients before (preop) and after (postop) surgery. P-values are presented in the figure. n.s. = no significant difference. (*) indicates extreme outlier and (°) mild outlier.

Table 2
Pre- and postoperative circulating levels in PDAC patients compared to controls

Marker		Levels		
		Preop (n = 12)	Postop (n = 15)	Con (n = 8)
Type IV Collagen	ng/mL	149 [78.4–249]	191 [99.5–410]	93.7 [63.6–153]
Endostatin	ng/mL	161 [86.2–228]	82.1 [43.2–370]	98.4 [68.5–143]
Tenascin C	ng/mL	711 [367–1645]	1170 [143.4–2187.8]	572 [223–1224]
Osteopontin	ng/mL	30.0 [10.8–77.2]	28.7 [9.3–92.6]	11.6 [7.80–19.8]
TPS	U/L	207 [59.8–1409]	83.4 [39.9–2092]	47.6 [28.6–125]
Ca 125	U/mL	36.3 [7.4–281]	20.9 [6.9–1540]	4.9 [3.1–16.0]
Ca 19-9	U/mL	157 [3.5–3985]	38.4 [0.75–5499]	9.6 [4.7–20.1]
CEA	ñjg/L	4.1 [0.5–12.7]	2.2 [1.0–49.9]	2.2 [0.4–8.4]

Abbreviations: preop = preoperative, postop = postoperative, con = control.

Table 3
Circulating preoperative levels compared to the 95th percentile of the controls

Patient #	Stroma-derived tumor markers				Conventional tumor markers				# of markers > 95th perc
	Type IV Coll	Endostatin	TNC	OPN	TPS	Ca 125	Ca 19-9	CEA	
1	↑	↑	↑	↑	↑	↑	↑	↑	
2	–	–	–	–	–	↑	↑	–	2
3	–	–	–	↑	–	–	–	–	1
4	↑	–	–	↑	↑	–	↑	–	4
5	↑	↑	↑	↑	↑	↑	↑	–	7
6	–	↑	↑	↑	↑	↑	↑	–	6
7	↑	↑	–	↑	↑	↑	↑	–	6
8	↑	↑	–	↑	↑	–	↑	–	5
9	–	↑	–	–	↑	–	↑	–	3
10	–	–	↑	↑	↑	↑	–	–	4
11	↑	–	–	↑	↑	–	↑	↑	5
12	↑	↑	–	–	↑	↑	↑	–	5
95th percentile of the controls	142.7	140	1032.3	19	103.7	14.6	19.7	6.6	

↑Circulating levels higher than the 95th percentile of the controls; –Circulating levels equal as, or lower than the 95th percentile of the controls.

3.5. Association to survival

Whether the analysed tumour markers provide any prognostic information concerning overall survival was tested with COX-regression both in the pre- and postoperative setting for each marker. As previously shown [28], high postoperative levels of type IV collagen were associated to shorter survival. In this study, an increase of 10 ng/ml of the marker in the postoperative situation increases the hazard by 1.093 [95% CI 1.015–1.177] for type IV collagen, by 1.073 [95% CI 1.001–1.150] for endostatin/type XVIII collagen, and by 1.502 [95% CI 1.113–2.027] for OPN. These associations to survival were still significant after adjustment for age and sex. Furthermore, with COX-regression, high preoperative levels of endostatin/type XVIII collagen and Ca 125 indicated shorter survival with a hazard of 1.227 [1.020–1.476] for endostatin/type XVIII collagen and a hazard of 1,141 [95% CI 1.021–1.274] for Ca 125. These associations remained significant when adjusted for age and sex. No associations to survival were found for TPS, Ca 19-

9, TNC and CEA. Moreover, in the COX-analysis, the differentiation grade showed an association to survival (hazard of 0.32 [95% CI 0.109–0.959]), but age and sex did not.

4. Discussion

Despite several potential PDAC tumour markers have been presented in the literature, including combinations of novel markers with Ca 19-9, no single marker or combination has reached everyday clinical practice beyond Ca 19-9 [23,24]. Promising tumour markers derived from the PDAC tumour stroma, including matricellular proteins such as OPN, have previously been reported [24–29]. We have previously reported that collagen fragments derived from the basement membrane (BM) are elevated in PDAC patients at the time of diagnosis [28,29], and that type IV collagen has a role in PDAC cell survival and migration [20]. The aim of this study was to compare BM derived fragments (type IV collagen and endostatin/type XVIII

collagen) and matricellular proteins (OPN and TNC) from the tumour stroma with conventional, cancer-cell derived tumour markers in the same PDAC patient cohort. As has been shown earlier, the levels of type IV collagen, endostatin/type XVIII collagen, OPN and TNC are elevated in the circulation at the time of diagnosis. The same can be found for TPS and Ca 19-9, the currently most commonly used and evaluated tumour markers in PDAC patients. Interestingly, the range of the levels for the conventional markers was clearly broader when compared to the range of type IV collagen and endostatin/type XVIII collagen levels. Less extreme outlier values were seen for the stromal markers, when compared to the conventional markers. Furthermore, in our patient cohort, in the post-operative setting, only stromal markers were associated with overall survival, with high levels of type IV collagen, endostatin/type XVIII collagen and OPN indicating poor prognosis. In the same patients, no such associations could be observed for TPS, Ca 19-9 or Ca 125, and this is well in line with the findings by Sandblom et al. [30]. However, in other studies with much larger patient materials, high levels of Ca 19-9 preoperatively [31,32] and postoperatively [33] have been shown to be associated with poor prognosis. These associations could not be reproduced in our patient material, which most likely is due to the small sample size in this study. Nevertheless, and despite the relatively low number of patients included in this study, the associations to survival for the stroma-derived tumour markers were significant. This is of particular interest and indicates a strong association for these substances to survival. The high preoperative levels of endostatin/type XVIII collagen likely reflects the degradation and remodelling of the stroma during tumour growth, whereas the remaining high postoperative type IV collagen, TNC and OPN levels could reflect the continuing production of these proteins by cells in tumour metastases.

We have verified in tissue samples that the substances included in this study are expressed in different compartments of the tumour. The expression patterns of Ca 19-9, CEA, Ck18 and Ca 125 was in line with previous observations, including the weak stromal CEA staining, and the tendency of increasing Ca 125 expression with loss of differentiation [6,34–36]. The absence of Ca 19-9 staining in two tumours probably stem from the fact that 5–10% of the population does not express the antigen. Type IV collagen, type XVIII collagen/endostatin and TNC stained the tumour stroma exclusively, with a tendency of loss of the BM-

associated collagens with loss of differentiation. OPN stained both in the tumour stroma and in the cancer cells, this is in line with previous studies [26,37]. It is our hypothesis that combinations of tumour markers that reflect different aspects and pathophysiological processes of the tumour biology will increase the diagnostic and predictive strength. All ductal adenocarcinomas share some features, but malignancies are nevertheless clonal diseases. It is thus unlikely that a single tumour marker, which is expressed unconditionally in all pancreatic cancers, can be found. It is of importance to identify pairs or groups of tumour markers suitable for being combined, and also to identify markers not suitable for combination. In this study we show a correlation between the tumour markers Ca 125 and Ca 19-9, indicating that these two markers might reflect the same biological processes and therefore not suitable for combination, as the combination most likely will not yield more information than one marker alone. This has been implied earlier, when specificity and sensitivity was evaluated for the combination of Ca 125 and Ca 19-9 [13]. Instead, the combination of a stroma-derived marker (such as type IV collagen, endostatin/type XVIII collagen, TNC or OPN) and a cancer cell specific marker (such as TPS or Ca 19-9) will most likely give more diagnostic and prognostic accuracy. Interestingly, the patients with normal Ca 19-9 levels in this study had increased levels of stromal markers.

The idea of increased diagnostic power by combining several markers is underlined by the fact that all patients had increases levels of at least one marker, and that 9 out of 12 patients (75%) had increased levels of at least 4 out of 8 markers. As no reference values are available for the stromal markers in PDAC, we used as the cut-off the 95th percentile of the controls, which could provide a too low or even too high reference value compared to the actual normal distribution in a healthy population. Therefore, an algorithm that combines levels of tumour markers in the preoperative setting for PDAC patients needs to be tested and validated in a larger population to evaluate the full clinical potential of the combined markers.

In this study we have showed that substances derived from the tumour stroma are elevated in the circulation in PDAC patients. Furthermore, included stroma-derived markers have potential as prognostic markers. However, elevations of the stromal markers are not specific for PDAC or even specific for malignancies as such. There are other conditions, such as liver cirrhosis and inflammation that will affect the levels of cir-

culating fragments from the stromal compartment. On the other hand, the same is true for the conventional cancer cell specific markers, as levels of these are increased by many non-malignant conditions. However, all tumours affect the surrounding stroma during tumour progression. This is illustrated by the results presented in this study, where the levels of the stroma-derived tumour markers in the PDAC patients showed less variation when compared to conventionally used markers. Therefore, measuring both tumour stroma derived and cancer cell specific proteins might improve sensitivity and specificity. The tumour stroma is an interesting future source of novel tumour markers, on their own, or in combination with conventional tumour markers.

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Conflict of interest

No conflicts declared.

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Supplemental material

Supplemental Table 1
Primary antibodies and dilutions

Antibody	Dilution	Manufacturer	Positive Control
Mouse-anti-Ck18	1:100	DakoCytomation, Glostrup, Denmark	Normal Pancreas
Rabbit-anti-Ck18 (ab52948)	1:50	Abcam, Cambridge, UK	Normal Pancreas
Goat-anti-Endostatin (AF1098)	1:50	R&D Systems, Abingdon, UK	Normal Pancreas
Goat-anti-Type IV Collagen (AB769)	1:400	Millipore, Temecula, USA	Normal Pancreas
Rabbit-anti-CEA (ab15987)	1:400	Abcam, Cambridge, UK	Liver
Rabbit-anti-OPN (ab8448)	1:50	Abcam, Cambridge, UK	Colon
Mouse-anti-Ca125 (ab693)	1:50	Abcam, Cambridge, UK	Serous Ovarial Carcinoma
Mouse-anti-Ca 19-9 (ABIN120086)	1:40	Antibodies-online GmbH, Atlanta, USA	Colon Adenocarcinoma
Mouse-anti-TNC (NCL-TENAS-C)	1:50	Novocastra, Leica Biosystems, Newcastle, UK	Adenoid