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Spatial and phenotypic characterization of pancreatic cancer-associated fibroblasts after neoadjuvant treatment

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Short running title: CAF heterogeneity in pancreatic cancer
Summary

Pancreatic ductal adenocarcinoma (PC) is characterized by a highly fibrotic desmoplastic stroma. Subtypes of cancer-associated fibroblasts (CAFs) have been identified in chemotherapy-naïve PC (CTN-PC), but their precise functions are still unclear. Our knowledge regarding the properties of CAFs in the regressive stroma after neoadjuvant treatment (NAT) of PC (NAT-PC) is particularly limited. We aimed to examine the marker phenotypic properties of CAFs in the regressive stroma of PC.

Surgical specimens from patients with CTN-PC (n=10) and NAT-PC (n=10) were included. Juxtatumoural, peripheral, lobular, septal, peripancreatic, and regressive stromal compartments were manually outlined using digital imaging analysis (DIA) for area quantification. The compartment-specific expression of CD271, cytoglobin, DOG-1, miR-21, osteonectin, PDGF-Rβ, and tenascin C was evaluated by immunohistochemistry or in situ hybridization, using manual scoring and automated DIA.

The area fraction of the regressive stroma was significantly higher in NAT-PC than in CTN-PC (P = 0.0002). CD271 (P < 0.01), cytoglobin (P < 0.05), DOG1 (P < 0.05), miR-21 (P < 0.05), and tenasin C (P < 0.05) exhibited significant differences in their expression profiles between the juxtatumoural compared to the peripheral and regressive stroma. PDGF-Rβ expression was significantly higher in juxtatumoural than in peripheral CAFs (P < 0.05).

Our data provide further support of the concept of stromal heterogeneity and phenotypic different CAF subtypes in PC. CAFs in the regressive stroma of NAT-PC show a marker phenotype similar to some (namely, peripheral) and different from other (namely, juxtatumoural) previously defined CAF subtypes. It may be hypothesized that phenotypic CAF subtypes, at least in part, also may share functional properties. Studies examining the precise functional characteristics of CAF subtypes in PC are needed.

Key words: Pancreatic cancer, neoadjuvant treatment, FOLFIRINOX, tumour stroma, cancer-associated fibroblasts
Introduction

Pancreatic ductal adenocarcinoma (PC) has a dismal prognosis, with data from Danish patients indicating an overall 5-year survival rate of 5% and a median survival time of only 5.9 months (Database, 2017). PC is characterized by a prominent desmoplastic stroma produced by cancer-associated fibroblasts (CAFs) (Yen et al., 2002; Erkan et al., 2008; Farrow et al., 2009; Nielsen et al., 2016). Fibrosis is a hallmark of PC visible by microscopy, and the fibrotic stroma can occupy up to 80% of the total tumour volume (Kadaba et al., 2013). However, the exact role of the desmoplastic stroma and CAFs in PC progression has not been fully elucidated (Gore and Korc, 2014).

The most important precursor cells for CAFs in PC are quiescent pancreatic stellate cells (qPSCs) (Apte et al., 1998; Bachem et al., 1998; Erkan et al., 2012). It has previously been demonstrated that qPSCs express cytoglobin and that the expression of this marker is sustained upon activation in CAFs (Nielsen et al., 2017; 2018). Previous studies indicate that spatially and phenotypically distinct subtypes of CAFs exist in PC (Iacobuzio-Donahue et al., 2002; Sugimoto et al., 2006; Öhlund et al., 2017; Haeberle et al., 2018; Nielsen et al., 2018). One subtype of CAFs, namely, juxtatumoral/perilesional CAFs, are located in close vicinity to the PC cells, while another, the peripheral CAFs, are found at a greater distance from them (Iacobuzio-Donahue et al., 2002; Öhlund et al., 2017; Haeberle et al.; 2018, Nielsen et al., 2018). It was reported that the marker phenotype of juxtatumoural CAFs is characterized by high expression of CD10, cytoglobin, discovered on gastrointestinal stromal tumours 1 (DOG1), MMP11, Apolipoprotein C-1, Apolipoprotein D, and miR-21 and by low expression of CD271 (Iacobuzio-Donahue et al., 2002; Nielsen et al., 2018). Furthermore, peripheral CAFs display the opposite antigen expression, with high expression of CD271 and limited expression of CD10, cytoglobin, DOG1, and miR-21 (Nielsen et al., 2018). Tenascin C is predominantly expressed in the juxtatumoural extracellular matrix compared to the peripheral ECM in PC (Nielsen et al., 2018). Several prognostic studies show that juxtatumoural CAF markers, such as CD10 and miR-21, hold negative prognostic value in PC, while high stromal expression of CD271 is associated with a better prognosis (Dang et al., 2006; Ikenaga et al., 2010; Fujiwara et al., 2012; Kadera et al., 2013). Overall, the functional role of CAF subtypes in PC has not been elucidated. However, it is tempting to speculate whether these data may indicate that (some of the) juxtatumoural CAFs and the juxtatumoural stroma may play a more growth-promoting role in PC progression than (some of the) peripheral CAFs and the peripheral stroma (Iacobuzio-Donahue et al., 2002; Nielsen et al., 2018).
It is generally accepted that “regressive fibrosis” is a hallmark of the histological response to neoadjuvant treatment (NAT) with FOLFIRINOX in PC (Chatterjee et al., 2013; Verbeke et al., 2015; Pai and Pai, 2018). However, the marker phenotypic properties of fibroblasts in regressive fibrosis (regressive CAFs) are currently unknown. Several case studies have described histological responses, such as clusters of multinucleated foreign body-type giant cells, cholesterol clefts, fibrosis and repair reactions, to FOLFIRINOX treatment in PC, but the antigen expression of fibroblasts located in this regressive type of stroma has, to the best of our knowledge, not been elucidated (Jones et al., 2013; Gostimir et al., 2016; Haeberle et al., 2017). Haeberle et al. tested whether biomarkers are useful for the differentiation of tumour regression from tumour desmoplasia, but concluded that this distinction remains challenging when based on single markers (Haeberle et al., 2017).

In this study, we aimed to examine the marker phenotype of CAFs in the regressive stroma of NAT-PC, with special emphasis on similarities or dissimilarities with juxtatumoural and peripheral CAFs. We defined six stromal compartments in resection specimens with PC: the juxtatumoural, peripheral, regressive, lobular, septal, and peripancreatic stroma. The compartment-specific expression of CD271, cytoglobin, DOG1, miR-21, osteonectin (SPARC), platelet-derived growth factor receptor β (PDGF-Rβ), and tenascin C was evaluated semiquantitatively and using automated digital image analysis (DIA) in PC tissues from patients who had received NAT (NAT-PC) and from patients who were chemotherapy-naïve (CTN-PC) using immunohistochemistry (IHC) and in situ hybridization (ISH).
Materials and methods

Tissue specimens

Ten CTN-PC patients and 10 NAT-PC patients treated with FOLFIRINOX (Folinic acid, Fluorouracil, Irinotecan, and Oxaliplatin) prior to surgery for the purpose of downstaging were included in this study (Table 1). From each surgical specimen, one formalin-fixed paraffin-embedded (FFPE) tissue block was included.

The database of the Department of Pathology, Odense University Hospital, was searched for pancreatic surgical specimens that were obtained beginning on January 1, 2015, and thereafter. All included cases were re-examined by a pancreatic pathologist, and only pancreatic ductal adenocarcinomas (PDACs) were included in the study. Tumours originating from an intraductal papillary mucinous neoplasm (IPMN); originating from the ampulla of Vater, distal common bile duct or duodenum; and secondarily involving the pancreas were excluded. In this report, we use the term PC to indicate PDAC throughout. The FFPE tissue block that was most representative for each case was selected. Regarding the CTN-PC specimens, special emphasis was on the histologic grade (G2) and growth pattern. Regarding the NAT-PC specimens, special emphasis was on the presence of regressive features, such as regressive fibrosis, hyalinosis, acellular mucin lakes, and accumulation of macrophages.

This study was approved by the Ethics Committee of the Region of Southern Denmark (project-ID: S-20140168) and the Danish Data Protection Agency (project-ID: 15/33101). We ensured that patients had not advocated against the use of their tissue in the Danish registry for the use of tissue in research (‘Vævsanvendelsesregisteret’).

Immunohistochemistry (IHC)

Four-micron-thick sections were cut on a microtome and mounted on FLEX IHC slides (Dako, Glostrup, Denmark). The IHC staining procedures for some antigens were automated, and some antigens were stained manually. Heat-induced epitope retrieval (HIER) and non-HIER protocols were tested for antigen retrieval to obtain the highest signal-to-noise ratio. Details regarding antibody specifications, dilutions, incubation times, and epitope retrieval procedures for each antigen are presented in Table 2.
Manual IHC staining procedures were used for cytoglobin and PDGF-Rβ. Tissue sections were dewaxed with xylene and rehydrated with an ethanol gradient in water. Incubation in 1.5% H2O2 (Merck, Darmstadt, Germany) was performed to block endogenous peroxidase activity. Tissue sections were placed in HIER buffer and exposed to three successive steps using a microwave oven (NN-SD450W, Panasonic, Osaka, Japan): (1) 9 min at 900 W, (2) 15 min at 440 W, and (3) 15 min at room temperature (RT). Nonspecific binding was blocked by incubation for 30 min in 2% BSA. The sections were incubated with primary antibody diluted in antibody diluent S2022 (Dako, Glostrup, Denmark) for 60 min at RT or overnight at 4°C. Unbound primary antibodies were washed away, and the EnVision+ peroxidase/DAB detection system (Dako, Glostrup, Denmark) was used for detection of antigen-bound antibodies. Nuclear counterstaining was performed with Mayer’s haematoxylin (Fagron Nordic, Copenhagen, Denmark). Slides were washed, dried and mounted with coverslips using Pertex (Histolab, Gothenburg, Sweden).

Automated protocols were used for α-smooth muscle actin (α-SMA), caldesmon, CD163, CD271, DOG1, ETS-related gene (ERG), maspin, osteonectin (SPARC), and tenasin C. The staining process, including deparaffinization, epitope retrieval and blocking of endogenous peroxidase activity, were performed using either a BenchMark Ultra Immunostainer (Ventana Medical Systems, Tucson, AZ) with the OptiView Detection Kit (Ventana Medical Systems, Tucson, AZ) or a Dako Omnis instrument (Agilent, Santa Clara, US) with the Dako EnVision FLEX visualization system (Agilent, Santa Clara, US).

**In situ hybridization**

The probe sequences for miR-21, miR-126 (positive control, endothelial marker), and scramble (negative control) are listed in Table 3. The listed probes were all double-labelled with digoxigenin (DIG), and ISH was generally performed on 5-µm-thick paraffin sections as described elsewhere (Nielsen et al., 2011; Nielsen et al., 2018). The ISH analyses were performed in collaboration with Boye Schnack Nielsen, Bioneer A/S, Hørsholm, Denmark. Briefly, DIG-labelled locked nucleic acid (LNA) probes were detected with alkaline phosphatase-conjugated anti-DIG antibodies followed by incubation with 4-nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as the substrate. All slides were counterstained with nuclear fast red, dehydrated and mounted using Entellan mounting medium (Fisher Scientific). miR-126 was positively expressed in endothelial
cells in all examined tissue specimens, which indicate that the included tissue specimens were suitable for ISH analyses.

Sirius Red

The procedures for the sectioning, mounting and deparaffinization steps were similar to those performed for the IHC analyses. Deparaffinized sections were first stained in Weigert’s iron haematoxylin (15 min, Fagron Nordic). Following a washing step, the sections were stained with 0.1% Sirius Red (Ampliqon, Odense, Denmark) in picric acid (15 min, VWR, Søborg, Denmark). The slides were dehydrated and mounted with coverslips using Pertex.

Definition of stromal compartments in pancreatic resection specimens with PC and NAT-PC

We used a slightly modified and extended definition of the stromal compartments in pancreatic resection specimens with PC that we used in a previous study (Nielsen et al., 2018). This definition comprises six compartments, and takes also compartments that are characteristic of PC after NAT into account: The juxtatumoural, peripheral, lobular, septal, peripancreatic, and regressive stroma (Fig. 1) (Nielsen et al., 2018). It has to be emphasized that the juxtatumoural and peripheral compartments were considered an essential part of the malignant tumour, while the lobular, septal, and peripancreatic compartments were considered part of an inflammatory process in the vicinity of the tumour (“peritumoural pancreatitis”).

The 1) juxtatumoural stroma was defined as the stroma located between 0 and 50 µm from the cancer cells (Fig. 1). The 2) peripheral stroma fulfilled the following criteria: It was 50-500 µm away from cancer cells, it was not inside lobuli, it was not inside septa, and it was not related to peripancreatic (fat tissue necrosis-associated) stroma. Of note, when cancer cells were present within the lobular, septal or peripancreatic compartments, the juxtatumoural and peripheral stroma were defined as just described also at these locations. The stroma in the peritumoural pancreatic tissue was divided into lobular and septal stroma: The 3) lobular stroma was defined as the stroma located in intralobular areas, no further than 200 µm away from acinar cells, intralobular ducts and/or islets. The 4) septal stroma was defined as the stroma located in perilobular (septal) areas surrounding the pancreatic lobuli (Fig. 1). The width of the septa was defined as half the maximum
diameter of the accompanying lobule. The 5) peripancreatic stroma was defined as the border between the pancreatic parenchyma and peripancreatic fat and contained fat cells, blood vessels, nerves, inflammatory cells, fibroblasts and ECM. Importantly, the peripancreatic stroma had to be devoid of acinar cells, islets of Langerhans, and ducts – if these structures were present, the stroma was considered to be lobular stroma. The 6) regressive stroma was defined as the stroma > 500 \( \mu \text{m} \) away from cancer cells and had to be devoid of acinar cells, intralobular ducts, islets, and fat cells – if these structures were present, the stroma was considered to be lobular stroma. When cancer cells were present within the lobular, septal, peripancreatic or regressive stroma, the juxtatumoural and peripheral compartments were defined as described above also at these sites.

**Blinded pathological evaluation of pancreatic resection specimens with NAT-PC and CTN-PC**

A board-certified pathologist (MW) examined the H&E-stained slides of the ten NAT-PC and ten CTN-PC specimens without any prior information on the treatment history of the patients. The histological response was evaluated using a slightly modified CAP (College of American Pathologists) tumour regression grading (TRG) system (Washington et al., 2013). We used a system allowing for a more detailed evaluation of histological regression, consisting of 5 different grades of regression (0 to 4), while the original CAP TRG system was based on 4 different grades (0 to 3). TRG score 0 indicated a complete response, with no residual cancer (only regressive features); TRG score 1 indicated a near-complete response, with minimal residual cancer cells; TRG score 2 indicated a major response, with more regressive features than cancer cells; TRG score 3 indicated a minimal response, with more cancer cells than regressive fibrosis; and TRG score 4 indicated no response, characterized by a lack of any regressive features. Notably, regressive features, such as regressive fibrosis, hyalinosis, acellular mucin lakes, and the accumulation of macrophages could sometimes be observed focally in CTN-PC, possibly due to an inflammatory or immunological host-versus-tumour response.

**Quantification of stromal compartments**

Area quantification of the stromal compartments was performed with the VIS Image Analysis Software, version 2018.4 (Visiopharm, Hørsholm, Denmark). Digitalized (40x objective) H&E-
stained slides of the ten NAT-PC specimens and ten CTN-PC specimens were imported to the program for subsequent analysis. According to the definitions above, the six compartments were manually outlined and defined as regions of interest (ROIs) (Fig. 1). In addition, areas with tumour cells, ductal structural collagen, periductal inflammation, and dysplastic cells/PanINs were outlined and defined as individual ROIs (Fig. 2A-B). The area of each ROI was determined by the VIS Image Analysis Software. All remaining structures (excluded regions), including fat, blood vessels, nerves, duodenum, lymph nodes, and the common bile duct, were combined into and outlined as one region but were not included in the total ROI area. The regressive stroma score (RS) was calculated by dividing the area of regressive stroma by the sum of the area of the juxtatumoural, peripheral and regressive stroma, multiplied by 100.

Evaluation of interobserver variability for identification and quantification of stromal compartments was performed as follows: Random sampling (meander-based sampling) of three digital images (5x objective) per H&E-stained slide was performed for all 20 tissue specimens using the VIS Image Analysis Software. Two independent observers (MFBN and MDS) manually outlined ROIs on the sampled images according to the abovementioned definitions. Areas were determined using the VIS Image Analysis Software for subsequent RS calculations.

**Semiquantitative evaluation of IHC and ISH markers**

Stained slides were scanned using a 40x objective on a NanoZoomer 2.0HT whole-slide scanner (Hamamatsu Photonics, Hamamatsu, Japan). All quantitative evaluation was performed on digitalized slides using NanoZoomer Digital Pathology (NDP).view2 software (Hamamatsu Photonics).

IHC and ISH stains were assessed using a semiquantitative scoring system, as described previously (Nielsen et al., 2018). Briefly, the expression of each individual marker was assessed in myofibroblasts (MFBs) and ECM in the juxtatumoural, peripheral, lobular, septal, peripancreatic, and regressive stroma using a labelling score (LS) from 0 to 4. In this LS scale, 0 indicated no expression, 1 indicated barely detectable expression, 2 indicated weak expression, 3 indicated moderate expression, and 4 indicated strong expression. Each score was based on the intensity as well as the distribution of the respective marker. α-SMA was used as a reference marker for MFBs, and Sirius Red was used as a reference stain for ECM.
**Digital image analysis based quantitation of IHC and ISH markers**

For validation of the semiquantitative evaluation of IHC and ISH markers, automated DIA and quantitation were performed using VIS Image Analysis Software, version 2018.4. Briefly, ROIs containing juxtatumoural, peripheral, and regressive stroma were manually outlined, according to the definitions described above, in 5 NAT-PC and 5 CTN-PC tissue specimens followed by random sampling (meander-based sampling) of 10 digital images (20x objective) per marker per compartment in each specimen. CD271, cytoglobin, DOG1, miR-21, tenascin C, PDGF-Rβ, and osteonectin expression then were quantified by developing pixel-based algorithms to detect immunopositive staining signals. The method used for each algorithm was threshold-based classification using a hematoxylin/DAB and DAB (HDAB-DAB) feature or a Contrast Red-Blue feature from the RGB colour band. For each marker, the mean intensity and area fraction (defined as the immunopositive area as a fraction of the total area of specific ROI) was quantified. The labelling score for each marker was defined as the product of the area fraction and mean intensity.

**Statistical analysis**

Pie charts illustrating the area fraction of each of the different compartments of the total ROI were constructed in Microsoft Excel 2010 (Microsoft, Redmond, US). Statistical analysis of the RSs was performed in GraphPad Prism, ver. 5.01 (GraphPad Software, La Jolla, CA, USA). Scatter plots were constructed in GraphPad Prism, ver. 5.01, illustrating the mean RS with the standard error of the mean (SEM). The Mann-Whitney test was used to compare the RSs of CTN-PC with NAT-PC. To correlate the RS with the pathological TRG evaluation, a linear regression analysis was performed in GraphPad Prism, ver. 5.01. Pearson’s correlation coefficient (r) and the R squared value (R²) were calculated to determine how well the data fit the regression line.

The mean labelling score (MLS) for CD271, cytoglobin, DOG1, miR-21, osteonectin, PDGF-Rβ, or tenascin C was calculated from the average LS. Scatter plots were constructed in GraphPad Prism, ver. 5.01, illustrating the MLS with the SEM. The Mann-Whitney test was used to compare the compartmentalized expression of the markers in CTN-PC versus NAT-PC. Ordinal data were
compared using the nonparametric Kruskal-Wallis test followed by Dunn’s multiple comparisons test for comparisons of marker expression in the juxtatumoural, peripheral, and regressive stroma.

Evaluation of interrater agreement for quantification of stromal compartments was performed by calculating Fleiss' kappa in GraphPad Prism, quickcalcs online tool (GraphPad Software, San Diego, CA, USA). RSs were divided in 10 different categories representing 10-point intervals (RS 0-10, 10-20, etc.) and Fleiss' kappa was calculated by comparing the data from the two independent observers. The level of statistical significance was set at P < 0.05. In the graphs, * P < 0.05, ** P < 0.01, and *** P < 0.001.
Results

Quantification of the different stromal compartments in NAT-PC and CTN-PC

Quantitative data of the stromal compartments are presented in Fig. 2A-C. When the compartments of the CTN-PC specimens (Fig. 2A) were compared with those of the NAT-PC specimens (Fig. 2B), the area fraction of the regressive stroma was found to be higher in NAT-PC than in CTN-PC (32.3% vs 7.8%, P < 0.0001) (Fig. 2C). Conversely, the area fractions of tumour cells (5.6% vs 24.9%, P = 0.002), juxtatumoural stroma (3.9% vs 13.9%, P = 0.003), and peripheral stroma (11.3% vs 27.5%, P = 0.03) were smaller in the NAT-PC specimens than in the CTN-PC specimens, respectively (Fig. 2C).

The RSs for the individual PC specimens were calculated from the juxtatumoural, peripheral and regressive areas (Table 4). The mean RSs of the NAT-PC and CTN-PC specimens were 70.3 (range 30.9 - 100) and 12.9 (range 1.3 - 30.3), respectively. Interobserver variability analyses of identification and quantification of stromal compartments demonstrated a very good strength of agreement between two independent observers (κ = 0.85, Table 5). The comparison of the RS of CTN-PC with that of NAT-PC demonstrated that the RS was significantly higher in NAT-PC than in CTN-PC (P = 0.0002) (Fig. 2D). The RS was validated by comparing it to a score determined with a conventional histopathological TRG scoring system (modified version of the CAP TRG system) (Washington et al., 2013). The TRG scores for each individual specimen are listed in Table 4. Correlation analyses demonstrated that the RS correlated highly significantly with the TRG scores (r = -0.93, R² = 0.86, p < 0.0001) (Fig. 3).

Evaluation of the marker phenotype of the different stromal compartments in CTN-PC and NAT-PC

IHC expression of CD271, cytoglobin, DOG1, osteonectin, PDGF-Rβ, and tenascin C and ISH of miR-21 were semiquantitatively evaluated using a four-tiered scoring system to characterize these markers in the juxtatumoural, peripheral, regressive, lobular, septal, and peripancreatic stroma. The detailed results for each specific compartment are summarized in Table 6.
When evaluating the expression of each of the seven markers mentioned above, no difference was found when the stromal compartments were individually compared between NAT-PC and CTN-PC (Figure 4). This suggests that NAT does not affect the biomarker profile in the individual compartments. However, as shown in Fig. 2, the amount of regressive stroma was much larger in NAT-PC than in CTN-PC. Thus, for subsequent analyses, we combined the compartmentalized expression data from the NAT-PC and CTN-PC specimens. In general, semiquantitative evaluation and automated DIA-based quantitation of IHC and ISH markers displayed the same compartmentalized expression profiles of the examined markers (Fig. 5). CD271 expression was significantly higher in peripheral than in juxtatumoural CAFs (P < 0.01) (Fig. 5A1-5A4). The expression of CD271 in regressive CAFs was moderate/strong (Fig. 5A2). There was no significant difference in the expression of CD271 in regressive and peripheral CAFs, but CD271 expression was significantly higher in regressive CAFs than in juxtatumoural CAFs (P < 0.001) (Fig. 5A3-5A4).

In contrast to CD271, the cytoglobin (P < 0.05) (Fig. 5B1-5B4), DOG1 (P < 0.05) (Fig. 5C1-5C4), and miR-21 (P < 0.05) (Fig. 5D1-5D4) expression levels were all significantly higher in juxtatumoural than in peripheral and regressive CAFs. There were no significant differences in the expression levels of these markers in peripheral compared to those in regressive CAFs. Tenascin C was strongly expressed in juxtatumoural ECM, and its expression was significantly higher in juxtatumoural than in peripheral and regressive ECM (P < 0.05) (Fig. 5E1-5E4). The expression of tenasin C in peripheral ECM did not differ significantly from that in regressive ECM.

PDGF-Rβ expression was most prominent in the juxtatumoural stroma but was also moderate/strong in both the peripheral and regressive stroma (Fig. 5F1-5F4). When the expression of PDGF-Rβ was compared between the compartments, PDGF-Rβ was found to be significantly higher in juxtatumoural than in peripheral CAFs (P < 0.01), whereas no significant differences were observed between regressive CAFs and the other two CAF subtypes based on the semiquantitative evaluation (Fig. 5F3), while PDGF-Rβ expression was significantly higher in juxtatumoural than in regressive CAFs based on DIA (P < 0.001) (Fig. 5F4). Osteonectin displayed moderate expression in all three compartments, and there were no significant differences in osteonectin expression among juxtatumoural, peripheral, and regressive CAFs (Fig. 5G1-5G4).

A summary of the compartmentalized expression profile of the seven markers is listed in Table 7. Among the tumoural compartments, the antigen expression of CD271, cytoglobin, miR-21, PDGF-
Rβ, and tenasin C in the regressive stroma was identical to the peripheral and different from the juxtatumoural stroma. Furthermore, while the juxtatumoural stroma was characterized by moderate DOG1 expression, this marker was only barely perceptible in the peripheral and absent in the regressive stroma. Regarding the peritumoural compartments (lobular, septal and peripancreatic stroma), the antigen expression profiles were generally very similar (Fig. 4 and Table 7). The only notable exception was cytoglobin, a marker that was expressed strongly in the lobular stroma, similar to the juxtatumoural stroma but different from the remaining compartments, where only weak cytoglobin expression was found. In summary, these data indicate that the antigen expression of regressive CAFs and regressive ECM are similar to the antigen expression of peripheral CAFs and peripheral ECM (CD271<sup>high</sup>, cytoglobin<sup>low</sup>, DOG1<sup>low</sup>, miR-21<sup>low</sup>, PDGF-Rβ<sup>low</sup>, and tenasin C<sup>low</sup>). Furthermore, the marker profiles of regressive CAFs and regressive ECM differ from the marker profiles of juxtatumoural CAFs and juxtatumoural ECM (CD271<sup>low</sup>, cytoglobin<sup>high</sup>, DOG1<sup>high</sup>, miR-21<sup>high</sup>, PDGF-Rβ<sup>high</sup>, and tenasin C<sup>high</sup>). Juxtatumoural CAFs and juxtatumoural ECM, on the other hand, are distinguished from all other compartments by the following antigen expression profile: CD271<sup>low</sup>, DOG1<sup>high</sup>, miR-21<sup>high</sup>, PDGF-Rβ<sup>high</sup>, and tenasin C<sup>high</sup>.  


**Discussion**

In this study, we characterized the marker phenotypic properties of the regressive stroma in NAT-PC and compared it with the juxtatumoural and peripheral stroma that is found in CTN-PC. We found that the amount of regressive stroma was much higher in NAT-PC compared to CTN-PC. This was found semiquantitatively by manual scoring and also when using digital imaging analysis (DIA), supporting the utility of our method to identify the regressive stroma in PC. Next, we examined the marker phenotype of CAFs in the regressive stroma, with special emphasis on similarities or dissimilarities with juxtatumoural and peripheral CAFs. We found that the antigen expression characteristics of regressive CAFs were similar to those of peripheral CAFs, which are located at a greater distance from the cancer cells, and different from those of juxtatumoural CAFs, which are located in close vicinity to the cancer cells. Moreover, the present study confirms that different subtypes of CAFs with different antigen expression exist in PC. Moreover, our data provide indirect support of the hypothesis that phenotypically different subtypes of CAFs, at least in part, also may hold different functional properties (Iacobuzio-Donahue et al., 2002; Öhlund et al., 2017; Nielsen et al., 2018).

Only a few studies have examined the marker phenotypic properties of the regressive stroma in PC. One study described the intratumour stromal expression of tenascin C in resected PC specimens after receiving chemoradiation therapy (CRT), using either a gemcitabine-based or a S-1 (tegafur, gimeracil and oteracil) and gemcitabine-based protocol (Hayasaki et al., 2018). After dividing the patients in high and low responder groups according to the Evans grading system (Evans et al., 1992), the intratumour stromal expression of tenascin C was significantly higher in low responders than in high responders (Hayasaki et al., 2018). This finding is in line with our results, as the phenotypic signature of regressive stroma was characterized by low expression of tenascin C compared to that of the juxtatumoural stroma. It may be speculated that markers such as CD271, cytoglobin, DOG1, and miR-21 could also aid in the evaluation of response to NAT along with tenascin C. A published abstract reported the expression of tenascin C in PC resection specimens after preoperative chemotherapy or CRT, along with other IHC markers, such as pan-cytokeratin, α-crystallin B, α-SMA, neurotrophin-3, Ki-67, and osteonectin (Haeberle et al., 2017). The authors concluded that differentiating between the desmoplastic stroma and the regressive stroma based on the examined list of markers is very challenging. However, it seems that the juxtatumoural and peripheral tumour stroma were not examined separately in this study. Another study focused on the CAF density in the regressive stroma (Miyashita et al., 2018). NAT with gemcitabine and nab-
paclitaxel resulted in a reduction of the total number of CAFs. Together with our own results, these
data highlight that NAT may affect not only the phenotypic composition of CAFs but also the
overall number of CAFs in the PC stroma.

Stromal compartmentalization is not a novel concept in PC. A study from 2002 used ISH and found
that three genes (MMP11, Apolipoprotein C-1 and Apolipoprotein D) were expressed in the
juxtatumoural stroma only (Iacobuzio-Donahue et al., 2002). In a later study in mice, the markers α-
SMA, neuron-glial antigen 2 (NG2), PDGF-Rβ, and S100A4 indicated a mixed population of CAFs
in the tumour microenvironment (Sugimoto et al., 2006). More recent studies also support the
concept of stromal compartmentalization in PC, demonstrating prominent expression of fibroblast
activation protein (FAP), α-SMA, NT-3, osteonectin, and tenasin C in the perilesional (=
juxtatumoural) compartment (Öhlund et al., 2017; Haeberle et al., 2018). Moreover, CD10,
cytoglobin, DOG1, nestin, miR-21, and tenasin C could be used to distinguish the marker
phenotype of the juxtatumoural from that of the peripheral stroma (Nielsen et al., 2018). The
signature of the lobular and septal fibroblasts (FBs) was less pronounced, but lobular FBs were
characterized by a high expression of cytoglobin and CD271, and the septal FBs by a high
expression of CD271 (Nielsen et al., 2018).

Stromal heterogeneity may in part explain the previously published, and partially contradictory,
reports on the role of the tumour stroma in PC progression. While high numbers of α-SMA-positive
CAFs were reported to hold negative prognostic value in human PC and while CAFs were found to
constrain the effect of CRT while supporting tumour progression, depletion of the whole population
of CAFs was associated with tumour progression and reduced survival of mice (Erkan et al., 2008;
Hwang et al., 2008; Vonlaufen, Phillips, et al., 2008; Mantoni et al., 2011; Rhim et al., 2014;
Özdemir et al., 2014). It has been suggested that (some of the) juxtatumoural CAFs, located in close
contact with PC cells, may play a supportive role in PC progression, in contrast to (some of the)
CAFs located at a greater distance from cancer cells (peripheral CAFs) (Iacobuzio-Donahue et al.,
2002; Nielsen et al., 2018). In the present study, the marker phenotypic signature of regressive
CAFs in neoadjuvantly treated PC was similar to that of peripheral CAFs, supporting the hypothesis
that these cells also, at least in part, may share functional properties. It is tempting to speculate that
juxtatumoural CAFs are also destroyed in tumour areas where NAT effectively eliminates PC cells
(= in the regressive stroma). An obvious alternative hypothesis could be that NAT changes the
functional (and marker phenotypic) properties of juxtatumoural CAFs to a peripheral CAF-
regressive CAF-like type of cell. Taken together, it is tempting to speculate that our findings may support the proposed close association between juxtatumoural CAFs and PC cells, as indicated by several studies (Bachem et al., 2005; Hwang et al., 2008; Vonlaufen, Joshi, et al., 2008).

We defined six stromal compartments in resection specimens with PC. One can argue that such an approach is too simplistic when evaluating complex biological conditions. Intra- and inter-tumour heterogeneity further add to the complexity of the PC architecture (Verbeke, 2016). Additionally, one can argue that the appointed cut-off values between compartments (e.g., 500 µm away from cancer cells as the cut-off between the peripheral and regressive stroma) are debatable. However, this strategy provides a framework for separately evaluating stromal compartments, and similar definitions of the stromal compartments in PC have been used in previous studies (Ene-Obong et al., 2013; Nielsen et al., 2018). We and others have previously utilized a cut-off of 100 µm to distinguish the juxtatumoural from the peripheral stroma (Ene-Obong et al., 2013; Nielsen et al., 2018). However, based on the results from our previous study, we concluded that the cut-off between the juxtatumoural and peripheral stroma should rather be 50 µm, and therefore this cut-off was used in the present study (Nielsen et al., 2018).

It can be challenging for pathologists to objectively evaluate histological tumour regression as a response to NAT in PC due to the high amounts of desmoplastic stroma characterizing these neoplasms (Verbeke et al., 2015). Hence, it was crucial for this study to develop a method that allowed us to identify the regressive stroma in NAT-PC specimens with certainty. We therefore used two different methods for definition and quantification of the regressive stroma. The NAT-PC specimens contained significantly higher amounts of regressive stroma than the CTN-PC specimens, and there was a highly significant correlation between the two different methods in evaluating the amount of regressive stroma. The methods used were 1) the manual TRG score based on a slightly modified CAP system and 2) manual outlining of the regressive stroma together with five other stromal compartments using DIA (Washington et al., 2013). The utility of this latter technique was strengthened by interobserver variability analyses that demonstrated a very good correlation between two independent observers. These findings supported our approach using stromal compartmentalization for the evaluation of the marker phenotypic properties of regressive stroma. We performed both a semiquantitative evaluation and an automated DIA-based quantitation of IHC and ISH markers that displayed the same compartmentalized expression profiles. Of note, we also identified small quantities of regressive stroma in CTN-PC specimens. This may be due to a
host immunological response against tumour cells (Chin et al., 2018). An alternative explanation could be that the cut-off that we used for the differentiation of peripheral stroma from regressive stroma may have been too conservative. Future studies with a higher number of patients are needed to evaluate our method, using varying cut-offs. Such studies should also determine whether this method holds prognostic value in PC after NAT.

In conclusion, our data provide further support of the concept of stromal heterogeneity and the existence of different CAF subtypes in PC. Our findings indicate that the antigen expression of CAFs in the regressive stroma in NAT-PC is similar to that of peripheral CAFs but different from that of juxtatumoural CAFs, supporting the hypothesis that these cells, at least in part, also may share functional properties. It is possible that additional (functional) CAF subtypes may be identified in the future, for example when single-cell transcriptomics is used. Studies evaluating the precise functional properties of CAF subtypes in PC are needed.

Acknowledgements
We are thankful to senior histotechnologist and project coordinator Ole Nielsen and to histotechnologists Lisbet Mortensen, Lone Christiansen, and Christian Enggaard, Department of Pathology, Odense University Hospital, for their assistance with the IHC staining. We thank Claus Fristrup, Department of Surgery, Odense University Hospital, for his advice regarding the statistical analyses.
References


Table 1: Patient and tumour characteristics in 10 neoadjuvantly treated pancreatic cancer patients (NAT-PC) and 10 chemotherapy-naïve pancreatic cancer patients (CTN-PC).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CTN-PC</th>
<th>NAT-PC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td>n</td>
<td>10</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>Mean, years (range)</td>
<td>69.9 (52.8-79.8)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td>Female/male</td>
<td>6/4</td>
</tr>
<tr>
<td><strong>Neoadjuvant treatment with FOLFIRINOX</strong></td>
<td>N</td>
<td>0</td>
</tr>
<tr>
<td><strong>Time from completion of neoadjuvant treatment until tissue retrieval</strong></td>
<td>Mean, days (range)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Tumour differentiation grade</strong></td>
<td>G1/G2/G3</td>
<td>2/7/1</td>
</tr>
<tr>
<td><strong>pT stage</strong></td>
<td>T1/T2/T3/T4</td>
<td>1/0/9/0</td>
</tr>
<tr>
<td><strong>pN stage</strong></td>
<td>N0/N1</td>
<td>0/10</td>
</tr>
<tr>
<td><strong>Surgical procedure</strong></td>
<td>Whipple resection</td>
<td>n</td>
</tr>
<tr>
<td><strong>Survival data</strong></td>
<td>Patients alive at time of analysis</td>
<td>n</td>
</tr>
<tr>
<td><strong>Observational time for patients alive</strong></td>
<td>Mean, months (range)</td>
<td>34.9 (16.2 – 55.5)</td>
</tr>
<tr>
<td><strong>Median survival</strong></td>
<td>Median, months (SE)</td>
<td>32.2 (6.4)</td>
</tr>
</tbody>
</table>

#According to the UICC pathological Tumour-Node-Metastasis (pTNM) classification version 7 (Sobin et al., 2009).

Table 2: List of antibodies, retrieval methods, incubation times, dilutions and staining platforms used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species and clonality</th>
<th>Company</th>
<th>Clone/Product ID</th>
<th>Epitope retrieval</th>
<th>Incubation</th>
<th>Dilution</th>
<th>Platform</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-SMA</td>
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<td>Nordic BioSite</td>
<td>BS66</td>
<td>HIER: CC1_32_100</td>
<td>16min / 36°C</td>
<td>1:1000</td>
<td>BenchMark</td>
<td>OptiView</td>
</tr>
<tr>
<td>Caldesmon</td>
<td>Mouse, mAb</td>
<td>Dako</td>
<td>M3557</td>
<td>Non-HIER: Protease 3/4 + HIER: CC1_32_100</td>
<td>16min / 36°C</td>
<td>1:100</td>
<td>BenchMark</td>
<td>OptiView</td>
</tr>
<tr>
<td>CD163</td>
<td>Mouse, mAb</td>
<td>Ventana Medical Systems</td>
<td>MRQ-26</td>
<td>HIER: CC1_32_100</td>
<td>32min / 36°C</td>
<td>RTU</td>
<td>BenchMark</td>
<td>OptiView</td>
</tr>
<tr>
<td>CD271</td>
<td>Mouse, mAb</td>
<td>Cell Marque</td>
<td>MRQ-21</td>
<td>HIER: TRS_30_97</td>
<td>20min / 32°C</td>
<td>1:2000</td>
<td>Omnis</td>
<td>EnVision</td>
</tr>
<tr>
<td>Cytoglobin</td>
<td>Rabbit, pAb</td>
<td>Sigma Aldrich</td>
<td>HPA017757</td>
<td>HIER: EDTA / MWO 15min</td>
<td>O/N / 4°C</td>
<td>1:100</td>
<td>Manuel</td>
<td>EnVision</td>
</tr>
<tr>
<td>DOG1</td>
<td>Rabbit, mAb</td>
<td>Ventana Medical Systems</td>
<td>SP31</td>
<td>HIER: CC1_32_95 + Non-HIER: Protease (3/4)</td>
<td>8min/. 36°C</td>
<td>RTU</td>
<td>BenchMark</td>
<td>OptiView</td>
</tr>
<tr>
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<td>Ventana Medical Systems</td>
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<td>16min / 36°C</td>
<td>RTU</td>
<td>BenchMark</td>
<td>OptiView</td>
</tr>
<tr>
<td>Maspin</td>
<td>Mouse mAb</td>
<td>Pharmingen</td>
<td>G167-70</td>
<td>HIER: CC1_32_100</td>
<td>32min / 36°C</td>
<td>1:100</td>
<td>BenchMark</td>
<td>OptiView</td>
</tr>
<tr>
<td>Osteonectin</td>
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<td>NovoCastra</td>
<td>15G12</td>
<td>HIER: CC1_48_100</td>
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<td>1:200</td>
<td>BenchMark</td>
<td>OptiView</td>
</tr>
<tr>
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<td>Cell Signaling</td>
<td>28E1</td>
<td>HIER: EDTA / MWO 15min</td>
<td>60min / RT</td>
<td>1:100</td>
<td>Manuel</td>
<td>EnVision</td>
</tr>
<tr>
<td>Tenascin C</td>
<td>Mouse, mAb</td>
<td>NovoCastra</td>
<td>49</td>
<td>HIER: CC1_32_95 + Non-HIER: Protease (3/4)</td>
<td>32min / 36°C</td>
<td>1:100</td>
<td>BenchMark</td>
<td>OptiView</td>
</tr>
</tbody>
</table>

Table 3: List of LNA probes and experimental conditions used for *in situ* hybridization analyses

<table>
<thead>
<tr>
<th>Target RNA</th>
<th>nt</th>
<th>Probe sequence</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
<th>T&lt;sub&gt;hyb&lt;/sub&gt; (°C)</th>
<th>Probe concentration (nM)</th>
</tr>
</thead>
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<tr>
<td>miR-21-5p</td>
<td>22</td>
<td>TCAACATCAGTCTGATAAGCTA</td>
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<td>miR-126-3p</td>
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<td>CATTATTACTCACGGTACGA</td>
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<td>10</td>
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<td>Scramble</td>
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<td>TGTAACACGTCTATACGCCCA</td>
<td>87</td>
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<td>10</td>
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Table 4: Areal quantification and histological tumour regression grading (TRG) in neoadjuvantly treated pancreatic cancer (NAT-PC) and chemotherapy-naïve pancreatic cancer (CTN-PC). The stromal compartments were manually outlined on digitalized slides using Visiopharm software for area quantification. The regressive stroma score (RS) was calculated by dividing the area of regressive stroma by the sum of the area of the juxtatumoural, peripheral and regressive stroma multiplied by 100. TRG scores were assessed by a trained pancreatic pathologist using a modification of the CAP TRG system.

<table>
<thead>
<tr>
<th>Nb</th>
<th>Tumour cells Area (mm²)</th>
<th>Juxtatumoural stroma Area (mm²)</th>
<th>Peripheral stroma Area (mm²)</th>
<th>Lobular stroma Area (mm²)</th>
<th>Septal stroma Area (mm²)</th>
<th>Peripancreatic stroma Area (mm²)</th>
<th>Periductal connective tissue Area (mm²)</th>
<th>Periductal inflammation Area (mm²)</th>
<th>Dysplastic cells/ PanINs Area (mm²)</th>
<th>Regressive stroma Area (mm²)</th>
<th>Excluded region Area (mm²)</th>
<th>Total region of interest Area (mm²)</th>
<th>Regressive score (0-100)</th>
<th>TRG score (0-4)</th>
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<tr>
<td>1</td>
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<td>0.00</td>
<td>1.74</td>
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<td>45.52</td>
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<td>0.93</td>
<td>0.00</td>
<td>106.71</td>
<td>172.64</td>
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<td>100</td>
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<tr>
<td>2</td>
<td>0.00</td>
<td>0.00</td>
<td>0.82</td>
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<td>40.23</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>42.61</td>
<td>133.40</td>
<td>85.0</td>
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<tr>
<td>3</td>
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<td>0.26</td>
<td>0.48</td>
<td>29.63</td>
<td>13.73</td>
<td>22.91</td>
<td>1.34</td>
<td>0.11</td>
<td>0.43</td>
<td>31.26</td>
<td>235.71</td>
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<tr>
<td>4</td>
<td>17.92</td>
<td>9.71</td>
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<td>9.05</td>
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<td>23.47</td>
<td>63.83</td>
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<tr>
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<td>6.88</td>
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<td>2.68</td>
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<td>0.83</td>
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<td>1.96</td>
<td>18.56</td>
<td>90.5</td>
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</table>

Table 5: Evaluation of interobserver variability for digital imaging analysis (DIA)-based quantification of stromal compartments. The stromal compartments were manually outlined on digitalized slides using Visiopharm software for area quantification. Two independent observers re-evaluated all 20 included PC tissue specimens. Area quantification of stromal compartments was performed for subsequent calculation of regressive scores (RS). Interobserver agreement was evaluated by calculating Fleiss' kappa.

<table>
<thead>
<tr>
<th>Observer 1</th>
<th>RS 0-10</th>
<th>10-20</th>
<th>20-30</th>
<th>30-40</th>
<th>40-50</th>
<th>50-60</th>
<th>60-70</th>
<th>70-80</th>
<th>80-90</th>
<th>90-100</th>
<th>Total</th>
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<tbody>
<tr>
<td>0-10</td>
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<td>0</td>
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</tr>
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<td>0</td>
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<td>4</td>
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</tbody>
</table>

Weighted $\kappa$: 0.850

Strength of agreement: Very good
Table 6: Semiquantitative evaluation of the expression of seven markers in cancer-associated fibroblasts (CAFs) and extracellular matrix (ECM) in the juxtatumoural, peripheral, regressive stroma, lobular, septal, and peripancreatic stroma of neoadjuvantly treated pancreatic cancer (NAT-PC) and chemotherapy-naïve pancreatic cancer (CTN-PC).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Juxtatumoural stroma MLS (SEM)</th>
<th>Peripheral stroma MLS (SEM)</th>
<th>Regressive stroma MLS (SEM)</th>
<th>Lobular stroma MLS (SEM)</th>
<th>Septal stroma MLS (SEM)</th>
<th>Peripancreatic stroma MLS (SEM)</th>
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</thead>
<tbody>
<tr>
<td>CD271</td>
<td>0.88 (0.23)</td>
<td>2.25 (0.37)</td>
<td>2.89 (0.20)</td>
<td>3.30 (0.15)</td>
<td>3.20 (0.20)</td>
<td>2.90 (0.18)</td>
</tr>
<tr>
<td>Cytoglobin</td>
<td>3.50 (0.27)</td>
<td>2.25 (0.31)</td>
<td>2.20 (0.29)</td>
<td>3.40 (0.16)</td>
<td>2.10 (0.23)</td>
<td>2.40 (0.31)</td>
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<tr>
<td>DOG1</td>
<td>2.38 (0.26)</td>
<td>0.25 (0.16)</td>
<td>0.20 (0.13)</td>
<td>0.50 (0.22)</td>
<td>0.40 (0.22)</td>
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<td>miR-21</td>
<td>1.86 (0.51)</td>
<td>0.71 (0.29)</td>
<td>0.60 (0.27)</td>
<td>0.1 (0.1)</td>
<td>0.40 (0.22)</td>
<td>0.60 (0.27)</td>
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<td>Osteonectin</td>
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<td>2.75 (0.41)</td>
<td>2.70 (0.34)</td>
<td>1.60 (0.27)</td>
<td>1.80 (0.31)</td>
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<tr>
<td>PDGF-Rβ</td>
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<td>2.50 (0.27)</td>
<td>2.70 (0.26)</td>
<td>2.90 (0.23)</td>
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<tr>
<td>Tenascin C</td>
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<td>1.63 (0.38)</td>
<td>1.30 (0.37)</td>
<td>1.40 (0.31)</td>
<td>1.0 (0.15)</td>
<td>0.90 (0.31)</td>
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<th>Marker</th>
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<th>Peripheral stroma MLS (SEM)</th>
<th>Regressive stroma MLS (SEM)</th>
<th>Lobular stroma MLS (SEM)</th>
<th>Septal stroma MLS (SEM)</th>
<th>Peripancreatic stroma MLS (SEM)</th>
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<td>Osteonectin</td>
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<td>PDGF-Rβ</td>
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<tr>
<td>Tenascin C</td>
<td>3.70 (0.21)</td>
<td>2.10 (0.28)</td>
<td>1.50 (0.40)</td>
<td>1.50 (0.37)</td>
<td>1.50 (0.31)</td>
<td>1.13 (0.35)</td>
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CD: cluster of differentiation, DOG1: discovered on gastrointestinal stromal tumours 1, MLS: mean labelling score, PDGF-Rβ: platelet-derived growth factor receptor β, SEM: standard error of the mean.
Table 7 – Simplified scheme of the expression profiles of CD271, cytoglobin, DOG1, miR-21, osteonectin, PDGF-Rβ, and tenascin C in different stromal compartments of neoadjuvantly treated and chemotherapy-naïve pancreatic cancer (PC). The intratumoural compartments (juxtatumoural, peripheral and regressive stroma) represent the tumour per se, while the peritumoural compartments (lobular, septal, peripancreatic stroma) represent compartments that surround the tumour.

<table>
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<tr>
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<td>CD271</td>
<td>Cytoglobin</td>
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<td><strong>Intratumoural compartments</strong></td>
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<tr>
<td>Juxtatumoural stroma</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Peripheral stroma</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Regressive stroma</td>
<td>+++</td>
<td>++</td>
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<td><strong>Peritumoural compartments</strong></td>
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<tr>
<td>Lobular stroma</td>
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<tr>
<td>Septal stroma</td>
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<tr>
<td>Peripancreatic stroma</td>
<td>++++</td>
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</table>

**Symbol key:** (-) Not expressed in the cell type; (+) barely detectable expression; (++) weak expression; (+++) moderate expression; (++++) strong expression.

DOG1: discovered on gastrointestinal stromal tumours 1, ECM: extracellular matrix, PDGF-Rβ: platelet-derived growth factor receptor β.
Figure 1 - Simplified, schematic depiction of six stromal compartments in surgical specimens with PC after neoadjuvant treatment (NAT-PC).

Different stromal compartments in pancreatic resection specimens with NAT-PC, using a modification of a previous definition, also taking into account the compartment consisting of regressive stroma (Nielsen et al., 2018). The intratumoural compartments (juxtatumoural, peripheral and regressive stroma) were considered to represent the tumour per se, while the peritumoural compartments (lobular, septal, peripancreatic stroma) were considered compartments that surrounded the tumour. This figure is modified from Nielsen MFB, Mortensen MB, Detlefsen S (2018). Typing of pancreatic cancer-associated fibroblasts identifies different subpopulations. World Journal of Gastroenterology; 24 (41):4663-4678. With permission from Baishideng Publishing Group.
Figure 2 – The regressive stroma scores in NAT-PC and CTN-PC specimens.

Digitalized H&E sections of (A) CTN-PC (scale bar = 6.5 mm) and (B) NAT-PC specimens (scale bar = 8.5 mm) were imported into the Visiopharm software (upper part). Regions of the six stromal compartments (juxtatumoural, peripheral, regressive, lobular, septal, and peripancreatic stroma), tumour cells, periductal connective tissue, periductal inflammation, and dysplastic cells/PanINs were manually outlined and defined as regions of interest (ROIs) (lower part). Fat tissue, blood vessels, nerves, duodenum, lymph nodes and the common bile duct were combined into one region that was defined as an exclusion region, i.e., that was not included in the total ROI. (C) Pie charts illustrating the area fractions of the different compartments of the total ROI in ten NAT-PC and ten CTN-PC specimens. (D) The mean regressive stroma scores (SEM) were 70.3 (9.5) in the 10 NAT-PC specimens and 12.9 (3.2) in the 10 CTN-PC specimens. The regressive stroma scores were significantly higher in the NAT-PC group than in the CTN-PC group. *** P < 0.001. CTN-PC: chemotherapy-naïve pancreatic cancer, NAT-PC: neoadjuvantly treated pancreatic cancer, PanIN: Pancreatic intraepithelial neoplasia, ROI: region of interest.
Figure 3 – Correlation between the regressive stroma score and TRG score

Linear regression and correlation analyses demonstrated a significant correlation ($r = -0.93, R^2 = 0.86, p < 0.0001$) between two different methods to evaluate the amount of regressive stroma in pancreatic cancer (PC) specimens. The histological tumour regression grading (TRG) score is shown on the y-axis, and the regressive stroma score is on the x-axis.
Figure 4 – Comparison of the compartmentalized marker profile of neoadjuvantly treated pancreatic cancer (NAT-PC) compared to that of chemotherapy-naïve pancreatic cancer (CTN-PC).

The expression levels of (A) CD271, (B) cytoglobin (C) DOG1, (D) miR-21, (E) osteonectin, (F) PDGF-Rβ, and (G) tenascin C were semiquantitatively evaluated separately in NAT-PC and CTN-PC specimens in the juxtatumoural, peripheral, regressive, lobular, septal, and peripancreatic cancer-associated fibroblasts (CAFs), fibroblasts (FBs) and extracellular matrix (ECM). Statistical analyses demonstrated that there were no statistically significant differences in the expression of any marker when the expression of each was examined in each specific compartment in NAT-PC versus CTN-PC. The graphs illustrate mean labelling scores (MLS) with standard error of the mean (SEM). CTN-PC: chemotherapy-naïve pancreatic cancer, DOG1: discovered on gastrointestinal stromal tumours 1, NAT-PC: neoadjuvantly treated pancreatic cancer, PDGF-Rβ: platelet-derived growth factor receptor β.
Figure 5 – Immunohistochemical (IHC) expression of CD271 (A1-A4), cytoglobin (B1-B4), DOG1 (C1-C4), miR-21 (D1-D4), tenascin C (E1-E4), PDGF-Rβ (F1-F4), and osteonectin (G1-G4) in cancer-associated fibroblasts (CAFs) and extracellular matrix (ECM) in the juxtatumoural stroma, peripheral stroma, and regressive stroma in pancreatic cancer.

(\textbf{A1-A4}) CD271 was weakly expressed in juxtatumoural CAFs (mean labelling score (semiquantitative evaluation) (MLS (SE)) = 0.9, MLS (digital image analysis (MLS (DIA)) = 1.8) but highly expressed in peripheral CAFs (MLS (SE) = 2.3, MLS (DIA) = 39.0) and regressive CAFs (MLS (SE) = 2.7, MLS (DIA) = 38.4). (\textbf{B1-B4}) Cytoglobin was highly expressed in juxtatumoural CAFs (MLS (SE) = 3.5, MLS (DIA) = 51.6) with lower expression levels in peripheral CAFs (MLS (SE) = 2.3, MLS (DIA) = 7.0) and regressive CAFs (MLS (SE) = 2.3, MLS (DIA) = 5.6). (\textbf{C1-C4}) DOG1 expression was high in juxtatumoural CAFs (MLS (SE) = 2.4, MLS (DIA) = 12.3), but almost absent in peripheral CAFs (MLS (SE) = 0.5, MLS (DIA) = 0.1) and regressive CAFs (MLS (SE) = 0.2, MLS (DIA) = 0.03). (\textbf{D1-D4}) Moderate miR-21 expression was observed in juxtatumoural CAFs (MLS (SE) = 1.9, MLS (DIA) = 4.1), and miR-21 expression was mostly absent in peripheral CAFs (MLS (SE) = 0.6, MLS (DIA) = 0.2) and regressive CAFs (MLS (SE) = 0.5, MLS (DIA) = 0.1). (\textbf{E1-E4}) Tenascin C was strongly expressed in the juxtatumoural ECM (MLS (SE) = 3.6, MLS (DIA) = 96.4), weakly expressed in the peripheral ECM (MLS (SE) = 1.9, MLS (DIA) = 4.4) and almost undetectable in the regressive ECM (MLS (SE) = 1.4, MLS (DIA) = 1.0). (\textbf{F1-F4}) PDGF-Rβ expression was strong in juxtatumoural CAFs (MLS (SE) = 3.3, MLS (DIA) = 17.6) and moderate in peripheral CAFs (MLS (SE) = 2.6, MLS (DIA) = 6.6) and regressive CAFs (MLS (SE) = 2.8, MLS (DIA) = 6.8). (\textbf{G1-G4}) Osteonectin was moderately expressed in all three CAF subtypes: juxtatumoural CAFs (MLS (SE) = 2.9, MLS (DIA) = 23.7), peripheral CAFs (MLS (SE) = 2.6, MLS (DIA) = 16.3), and regressive CAFs (MLS (SE) = 2.7, MLS (DIA) = 13.0). Black scale bar = 100 \mu m and red scale bar = 50 \mu m. * indicates P < 0.05, ** indicates P < 0.01, and *** indicates P < 0.001.
HISTOLOGY AND HISTOPATHOLOGY

Fibroblast
ECM
Cancer cells
Acini
Fat cell

Lobular stroma
Septal stroma
Juxtatumoural stroma
Peripancreatic stroma
Perivascular stroma
Regressive stroma

500μm
50μm
The correlation between TRG score and Regressive stroma score is shown in the following graph. The correlation coefficient (r) is -0.93, and the coefficient of determination (R²) is 0.86. The p-value is less than 0.001, indicating a statistically significant relationship. The data points are split into two groups: CTN-PC (red squares) and NAT-PC (blue circles).