

Stroma composition and proliferative activity are related to therapy response in neoadjuvant treated pancreatic ductal adenocarcinoma

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Summary. Background. Tumor regression grading (TRG) based on histopathology is the main tool to assess therapy effects after neoadjuvant therapy (NAT) of pancreatic ductal adenocarcinoma (PDAC). However, reliable markers to distinguish therapy effects from pre-existing tumor features are lacking. The aim of this study was the characterization of PDAC after NAT, focusing on the stroma.

Material and Methods. Tissue samples from patients resected for PDAC after NAT (n=27) were analyzed. TRG was assessed using the Royal North Shore (RNS) system. Stromal composition was evaluated by Movat's stain. Immunohistochemistry (IH) for Ki-67 and five previously established stroma markers (alpha-Crystallin B, alpha-Smooth muscle actin (alpha-SMA), Neurotrophin-3 (NT-3), SPARC and Tenascin C) was also performed. Results were compared with therapy-naïve PDACs (n=10).

Results. Most cases showed a moderate response (RNS 2; 74%), while 15% displayed a poor response (RNS 3), and 11% a good response (RNS 1). No complete response was observed. Poor regression was associated with mucin-rich stroma, while good regression was associated with collagen-rich stroma. Cases with poorer therapy response had significantly higher proliferation. Higher peritumoral staining intensity for alpha-SMA and Tenascin C also showed a trend towards an association with poor regression.

Conclusions. Similar to the stroma in therapy-naïve

PDAC, the stroma of PDAC after NAT is heterogeneous. Distinguishing between desmoplastic stroma and therapy-induced fibrosis by single markers is not possible. Movat's pentachrome stain, IH for Ki-67, and to some extent for Tenascin C and alpha-SMA, can help detect poor histopathological response to NAT.

Key words: Movat's stain, Neoadjuvant therapy, Pancreatic cancer, Proliferation, Stroma, Tumor regression grading

Introduction

Neoadjuvant therapy (NAT) of pancreatic ductal adenocarcinoma (PDAC) is emerging, especially in borderline resectable or primarily unresectable PDAC (Dhir et al., 2017). In this context, tumor regression grading (TRG) based on histopathology is the main tool to evaluate therapy effects. The evaluation of therapy effects via TRG is essential for adequate patient stratification, and for the comparison of different NAT regimens.

To date, a multitude of different PDAC TRG

Abbreviations. μ m, Micrometer; AB, Alcian Blue; Alpha-SMA, Alpha-Smooth muscle actin; CAP, College of American Pathologists; DAB: 3'3'-Diaminobenzidine; FFPE, Formalin-fixed, paraffin-embedded; H&E, Hematoxylin & Eosin; ICCR, International Collaboration on Cancer Reporting; IH, Immunohistochemistry; min, Minute(s); mM, Millimolar; NAT, Neoadjuvant therapy; NT-3, Neurotrophin-3; PAS, Periodic acid-Schiff; PDAC, Pancreatic ductal adenocarcinoma; RNS, Royal North Shore; SPARC, Secreted protein acidic & rich in cysteine; TRG, Tumor regression grading; TUNEL, TdT-mediated dUTP-biotin nick end labeling

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systems exists. All are based on a semi-quantitative estimation of the amount of residual vital tumor cells/destroyed tumor cells and/or the extent of therapy-induced fibrosis (Ishikawa et al., 1989; Evans et al., 1992; White et al., 2005; Le Scodan et al., 2008; Chatterjee et al., 2012; Kakar et al., 2017). Some authors suggest the inclusion of additional features thought to represent regressive changes, such as necrosis, mucin pools, inflammation and cytopathic changes, e.g. cytoplasmic vacuolization (Kalimuthu et al., 2016).

Two main problems exist regarding TRG: first, many different TRG systems are currently in use, with varying criteria and varying arbitrarily assigned cut-offs. This results in limited data comparability. Second, all PDAC TRG systems are limited by the fact that they require a comparison with the pre-therapeutic tumor extent, which is almost impossible to estimate reliably, even with modern imaging modalities (Katz et al., 2012). Determination of the size of PDAC by imaging, and by pathological grossing, is hampered by ill-defined tumor borders, discontinuous growth, and extensive desmoplasia. In general, it is highly challenging to decide which tumor features are induced by NAT. This is not limited to fibrosis, but also includes other histopathological features mentioned above.

Therefore, the standardization of TRG for PDAC as well as finding additional, possibly more reliable markers to assess tumor regression in PDAC are urgent goals (Verbeke et al., 2018).

The aims of this study were a thorough characterization of PDAC after NAT with focus on the stroma, to define markers which might help to distinguish between desmoplastic stroma and therapy-induced fibrosis, and ultimately, to improve TRG in PDAC.

Materials and methods

Tissue samples

Tissue samples were collected from patients who underwent surgical resection after chemotherapy, alone or in combination with radiotherapy, at the Department of General Surgery of the Technical University in Munich, Germany, between September 2008 and January 2014 (n=27).

Resection specimens were processed according to a

standardized protocol: tumors/residual tumor beds were embedded either completely or sub-totally for TRG assessment (Esposito et al., 2008). Samples were fixed in 4% buffered formaldehyde and embedded in paraffin.

All available H&E slides were re-evaluated. One to three representative tumor samples were chosen for further analyses. In detail, three tumor samples were collected from 19 patients (tumor center, tumor periphery, intermediate area). In four cases, only two samples (central and peripheral in three, central and intermediate in one), and in further four, only one sample (central) were available. Pre-therapeutic biopsy samples were available from five patients.

Stromal composition of ten PDACs of patients who underwent up-front surgery was evaluated and used as control group.

Hematoxylin & Eosin (H&E) and Alcian Blue/Periodic acid-Schiff (AB/PAS) staining

Hematoxylin & Eosin (H&E) and Alcian Blue/Periodic acid-Schiff (AB/PAS) stains were prepared from 2-µm sections of formalin-fixed paraffin-embedded (FFPE) tissues. Histomorphology of the samples was thoroughly evaluated, with focus on the stromal composition.

Movat's pentachrome staining

Movat's pentachrome staining was performed using the MORPHISTO Movat's pentachrome Verhoeff staining kit according to the manufacturer's protocol. In Movat's stain, collagen fibers appear yellow, while mucin/ground substance components appear blue. Movat's stain of the stroma was analyzed using an automated image analysis software (Definiens Tissue Studio, Munich, Germany). Stromal areas that the software did not recognize as yellow or blue were not considered for statistical evaluations; these "mixed" areas represented only a small percentage of the tissue area in few cases.

Immunohistochemistry (NT-3, SPARC, Alpha-Crystallin B, Alpha-SMA, Tenascin C and Ki-67)

2-µm sections of FFPE tissues were subjected to immunohistochemistry (Table 1). NT-3 (Neurotrophin-

Table 1. Antibodies used for immunohistochemistry.

Antibody to	Antibody type	Dilution	Source
Alpha-Crystallin B	Mouse monoclonal	1:200	Enzo Life Sciences, Loerrach, GER
Alpha-Smooth muscle actin	Mouse monoclonal	1:200	Dako Deutschland, Hamburg, GER
Ki-67	Mouse monoclonal	1:50	Dako Denmark, Glostrup, DK
Neurotrophin-3	Rabbit polyclonal	1:100	Santa Cruz, Heidelberg, GER
SPARC	Mouse monoclonal	1:500	Invitrogen, Darmstadt, GER
Tenascin C	Mouse monoclonal	1:10	Leica Biosystems, Wetzlar, GER

GER: Germany, DK: Denmark.

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3), SPARC (Secreted protein acidic & rich in cysteine), alpha-Crystallin B and alpha-SMA (alpha-Smooth muscle actin) were previously established as markers for activated pancreatic stellate cells within the stromal reaction of PDAC (Haeberle et al., 2018).

Anti-NT-3, anti-SPARC and anti-alpha-Crystallin B stainings were performed using a semi-automated system (Dako, Hamburg, Germany). Epitope retrieval was achieved by pretreatment with 1:20 diluted Pronase E (Merck Millipore, Darmstadt, Germany) for 4 min at 37°C (anti-NT-3) or by boiling citrate buffer (20 mM citric acid, pH 6) for 20 min (anti-SPARC and anti-alpha-Crystallin B). Endogenous peroxidase and protein blocking were performed with 3% H₂O₂ diluted in methanol for 10 min and with 3% normal goat serum (Abcam, Cambridge, UK) for 30 min. After incubation with the respective primary antibody at the appropriate dilution for 60 min, slides were treated with biotinylated secondary antibody for 30 min and streptavidin horseradish peroxidase (KPL, Inc., Gaithersburg, MD, USA) for another 30 min (anti-SPARC) or with DakoEnVision detection system for 30 min (anti-NT-3 and anti-alpha-Crystallin B). DAB (Medac GmbH, Wedel, Germany) was used as a chromogen, counterstaining was performed with hemalaun (AppliChem GmbH, Darmstadt, Germany).

Anti-alpha-SMA, anti-Tenascin C and anti-Ki-67 staining were performed with the XT ultraView™ DAB v3 Detection Kit on the Ventana BenchMark XT automated IHC/ISH slide staining system (Ventana Medical Systems, Inc., Basel, Switzerland) according to the protocols established for routine diagnostics at the Institute of Pathology, Technical University Munich, Germany.

TUNEL apoptosis assay

TUNEL (TdT-mediated dUTP-biotin nick end labeling) apoptosis assay was performed using the Millipore detection kit according to manufacturer's protocol (ApopTag® Plus Peroxidase In Situ Apoptosis

Detection Kit S7101, Millipore S.A.S., Molsheim, France).

Evaluation of Movat's staining, Ki-67 immunohistochemistry, TUNEL apoptosis assay and stromal markers immunohistochemistry

Movat's staining was performed on all three tumor samples selected for the study (central, intermediate, peripheral), if available. From the percentages of yellow/blue-stained stromal area obtained by the automated image analysis software, a mean percentage value was calculated for each case.

Ki-67 immunohistochemistry and TUNEL apoptosis assay were performed on the central tumor samples of the resection specimens. To assess Ki-67 staining and TUNEL apoptosis assay, at least 1000 vital residual tumor cells were evaluated for positivity in resection specimens with substantial residual tumor. In resection specimens with little residual tumor and in biopsy samples, at least 500 vital residual tumor cells were evaluated for positivity.

For the evaluation of stromal immunohistochemical markers, two samples (central and peripheral) were stained per case, if available. Staining intensity was classified as weak (1+), moderate (2+) or strong (3+), and a mean value was calculated for each case.

Statistical analysis

Statistical analyses were performed using IBM SPSS statistic software, version 26 (SPSS, Inc., Chicago, IL, USA). Statistical significance was defined as $p < 0.05$.

Results

Clinico-pathological features

27 patients with PDAC were retrospectively selected for this study. All cases were considered unresectable

Table 2. Overview of NAT regimens used in this study.

NAT regimen*	Cases	RNS grades
Gemcitabine & oxaliplatin (GEMOX)	7/27 (25.9%)	RNS 2 (6/7, 86%) RNS 3 (1/7, 14%)
Folinic acid, fluorouracil, irinotecan & oxaliplatin (FOLFIRINOX)	5/27 (18.5%)	RNS 2 (5/5, 100%)
Gemcitabine & erlotinib / Gemcitabine, then erlotinib / Gemcitabine & erlotinib, then gemcitabine monotherapy	4/27 (14.8%)	RNS 1 (1/4, 25%) RNS 2 (2/4, 50%) RNS 3 (1/4, 25%)
Gemcitabine monotherapy	2/27 (7.4%)	RNS 2 (1/2, 50%) RNS 3 (1/2 (50%)
5-Fluorouracil (5-FU) & radiation	2/27 (7.4%)	RNS 2 (1/2, 50%) RNS 3 (1/2 (50%)
Cisplatin monotherapy	1/27 (3.7%)	RNS 2
Gemcitabine & radiation	1/27 (3.7%)	RNS 1
Cisplatin hyperthermia therapy & gemcitabine	1/27 (3.7%)	RNS 1
FOLFIRINOX & radiation, then 5-FU	1/27 (3.7%)	RNS 2
FOLFIRINOX, then gemcitabine, then FOLFIRINOX	1/27 (3.7%)	RNS 2
Cisplatin & gemcitabine, then gemcitabine & erlotinib, then 5-FU & radiation, then oxaliplatin & capecitabine	1/27 (3.7%)	RNS 2
Cisplatin, carboplatin & gemcitabine	1/27 (3.7%)	RNS 2

*Number of cycles varied for chemotherapy; radiation doses varied for radiotherapy. NAT: Neoadjuvant therapy. RNS: Royal North Shore tumor regression grading system.

and therefore treated with individual neoadjuvant protocols for an average period of 12 months (range: 2–22 months) (Table 2). Clinico-pathological features of all patients are reported in Table 3.

Histomorphological features

None of the 27 cases showed complete regression. The percentage of residual viable tumor ranged from 5–100%. Four cases (15%) showed no regression. In eight cases (30%), regression was heterogeneously distributed, with areas of distinct regression and areas of abundant vital tumor residuals.

Nearly all tumors (26 cases, 96%) had a prominent stromal reaction/fibrosis. Further morphological features included acellular mucin pools, cytoplasmic vacuoles, necrosis, and inflammation (Fig. 1). Mucin pools were detected in eleven cases (40.7%) in at least one representative sample, accounting for up to 50% of the tumor mass. Cytoplasmic vacuolization was found in 25 cases (92.6%). Necrosis was detected in 17 cases (70%), accounting for up to 30% of the entire tumor. Relevant inflammation was seen in five cases (19%).

Tumor regression grading

Tumor regression grading was done for each case according to the three-tiered Royal North Shore (RNS) TRG system (Table 4) (Chou et al., 2021), taking into consideration all individual slides per case.

RNS grades were distributed as follows: most cases were RNS 2 (11–75% residual vital tumor cells) (20/27, 74%), while RNS 3 (>75% residual vital tumor cells) was the second most common grade (4/27, 15%), followed by RNS 1 ($\leq 10\%$ residual vital tumor cells) (3/27, 11%) (Table 4). No cases of complete response were observed.

Movat's staining

For the evaluation of Movat's pentachrome stain, three different staining patterns were observed.

Pattern 1 was characterized by a homogeneous blue staining of the stroma, indicating mucin-rich/collagen-poor extracellular matrix (loose/immature/active stroma). Pattern 2 displayed a homogeneous yellow staining, indicating collagen-rich stroma

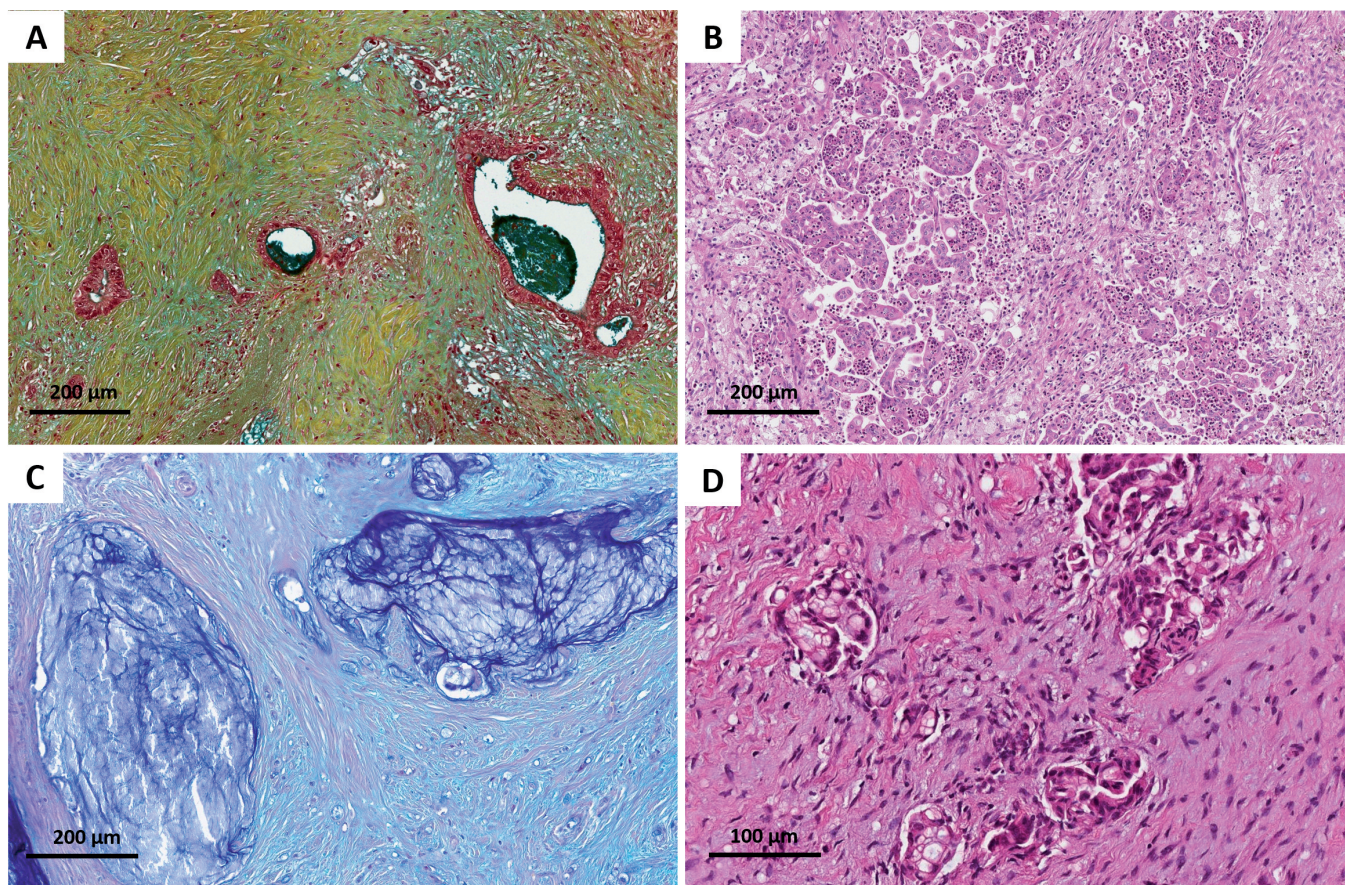


Fig. 1. Histomorphologic changes observed in the presented cohort of PDAC after NAT. **A.** Stromal fibrosis rich in collagen, indicated by yellow-stained fibers, especially in the periphery of residual vital tumor cells (Movat's pentachrome stain). **B.** Necrosis and inflammatory infiltration (H&E). **C.** Large mucin pools (AB/PAS). **D.** Cytoplasmic vacuolization of tumor cells (H&E).

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(dense/mature/inactive stroma). Pattern 3 consisted of a combination of pattern 1 and 2, with blue-stained stroma around tumor residuals and yellow-stained stroma in the periphery of the tumor.

In 26 cases, the staining pattern could be assigned by light microscopy: 14 cases (54%) showed pattern 3, eight cases (31%) showed pattern 1, and four cases (15%) showed pattern 2.

Percentages of yellow-stained stroma and blue-

stained stroma were determined by software supported evaluation. In cases of poor regression (RNS 3), the median percentage of blue-stained stroma (mucin-rich/collagen-poor) was 56%, compared to a median percentage of blue-stained stroma of 47% in cases of moderate regression (RNS 2), and of 21% in cases of good regression (RNS 1). The median percentage of yellow-stained stroma (collagen-rich/mucin-poor) was 33% in cases of poor regression (RNS), while it was 22% in cases of moderate regression (RNS 2), and 44% in cases of good regression (RNS 1). This indicates a trend towards an association between ground-substance-rich stroma and poor regression and collagen-rich stroma and good regression (Fig. 2, Table 5).

Table 3. Clinico-pathological data of 27 pre-treated patients with PDAC.

	Characteristic	n=27	%
Sex	Male	16	59.3
	Female	11	40.7
Average age (years)	Males	62.4	
	Females	62.8	
Tumor localisation in the pancreas	Head	19	70.4
	Head and Body	3	11.1
	Body and Tail	1	3.7
	Tail	4	14.8
ypT category*	ypT1c/ypT2	17	63.0
	ypT3/ypT4	10	37.0
ypN category**	ypN0	14	51.9
	ypN1/ypN2	13	48.1
ypM category	ypM1	5	18.5
	Hepatic	2	40
	Peritoneal	3	60
	ypM0	22	81.5
R status	R0	7	25.9
	R1	16	59.3
	Rx	4	14.8

*Average maximum diameter of tumor: 34 mm (range: 15-80 mm).

** Average number of lymph nodes evaluated: 37 (range: 6-43). ypT, ypN and ypM category according to 8th edition of the AJCC tumor staging system.

Ki-67 and TUNEL apoptosis assay

Proliferation index and presence of apoptosis of the residual tumor cells were evaluated in the central representative sample of 25 cases by Ki-67 immunostaining and TUNEL assay, respectively. The Ki-67 index values ranged from 1.4% to 48% (mean: 23.3%). Cases with higher response to therapy in the central sample (arbitrarily defined as residual tumor cells $\leq 20\%$) had a median Ki-67 value of 9% compared to cases with poorer therapy response (residual tumor cells

Table 4. Tumor regression grading (TRG) according to Royal North Shore (RNS).

Grade of regression	Description	Cases in this study
Grade 1	$\leq 10\%$ residual vital tumor cells	3/27 (11%)
Grade 2	11-75% residual vital tumor cells	20/27 (74%)
Grade 3	$>75\%$ residual vital tumor cells	4/27 (15%)

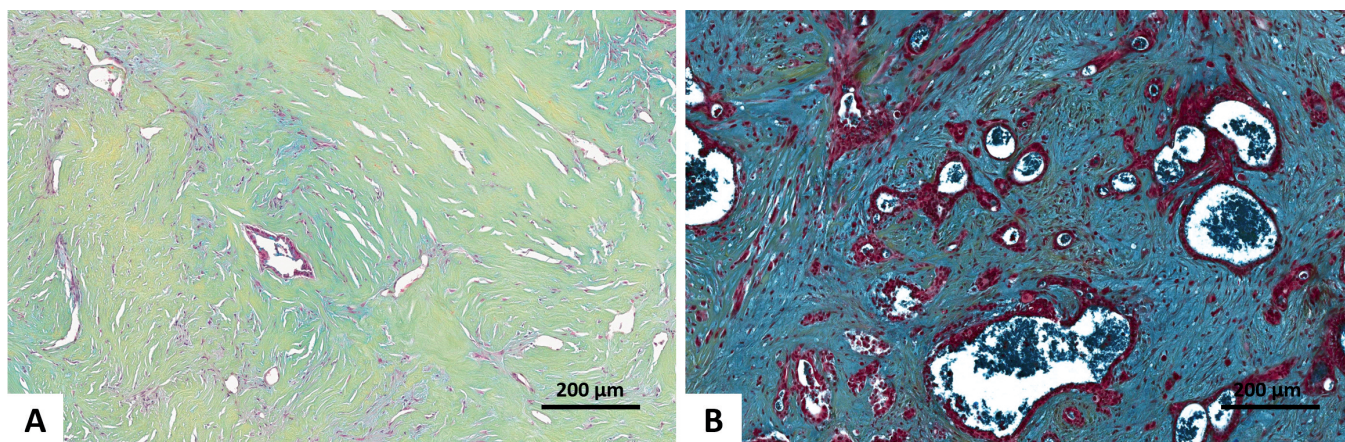


Fig. 2. Stromal composition of PDAC after NAT highlighted by Movat's pentachrome stain. **A.** Mature collagen-rich paucicellular fibrosis stained yellow by Movat's stain is found in areas/cases of good regression (Movat's stain). **B.** Immature collagen-lacking cell-rich fibrosis stained blue by Movat's stain is found in areas/cases of poor regression, while mature collagen-rich fibrosis is rarer in these cases (Movat's stain).

>20% in the central sample), which had a median Ki-67 value of 30% respectively. This difference was statistically significant (Mann-Whitney U, 2-sided, $p=0.006$) (Table 5).

TUNEL assay was homogeneous, with only minimal

rates of apoptosis observed in all cases, ranging from 0.1% to 1.4%. In three cases out of 25 (12%), no apoptotic tumor cells were identified. Due to the very low apoptosis rates encountered, no further statistical evaluations were performed.

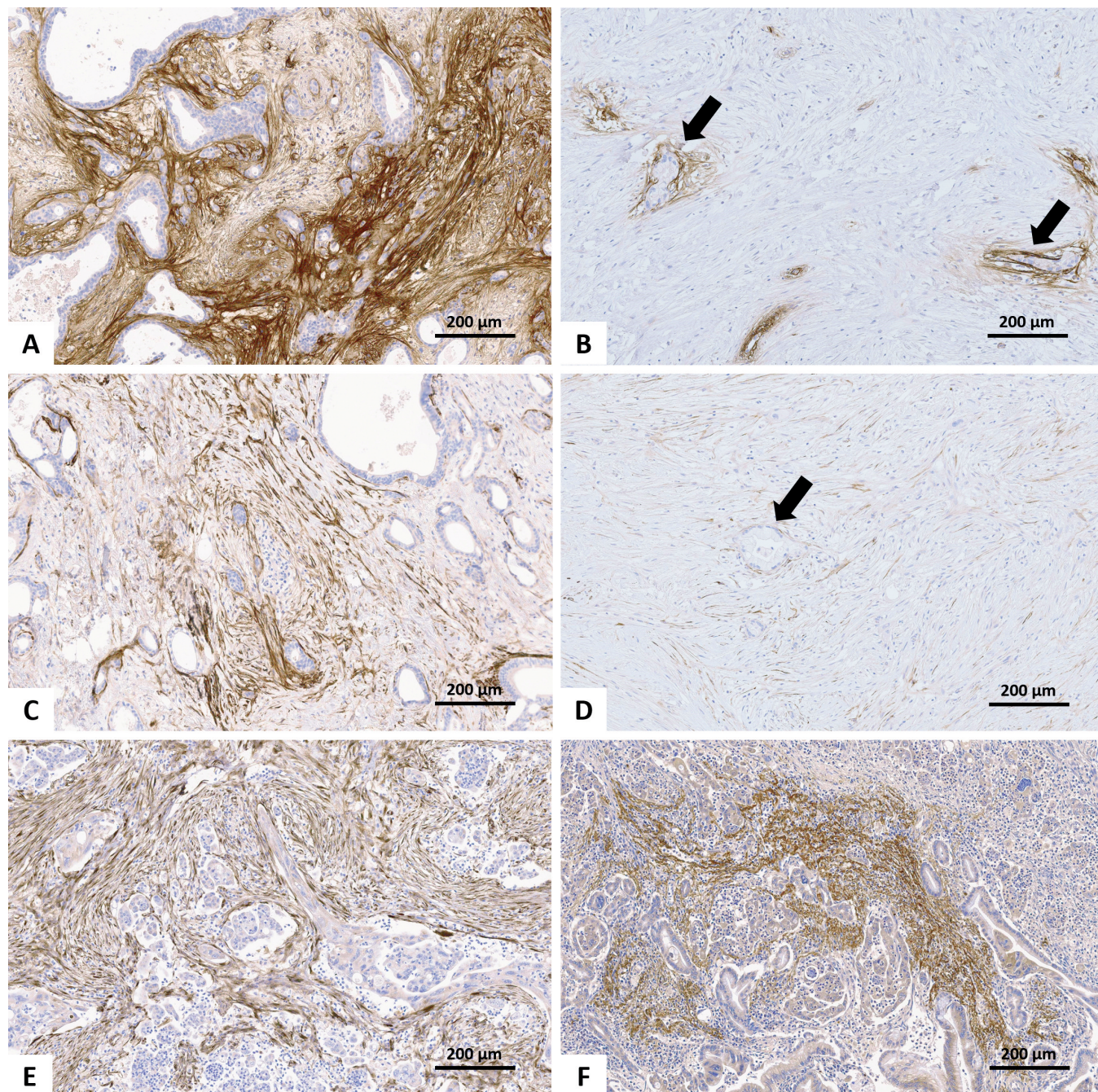


Fig. 3. Immunohistochemical analysis of PDAC after NAT. **A.** Area with no regression showing staining for Tenascin C in the peritumoral stroma. **B.** Area with marked regression displaying weaker peritumoral (arrows) Tenascin C staining. **C.** Area with no regression exhibiting strong peritumoral staining for alpha-SMA. **D.** Area with high regression showing peritumoral (arrow) alpha-SMA staining with weaker staining intensity. Peritumoral stromal staining was also found for SPARC (**E**) and NT-3 (**F**), although no marked differences in cases/areas of different regression grades could be observed.

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Alpha-SMA, Tenascin C, SPARC, NT-3 and alpha-Crystallin B immunohistochemistry

Alpha-SMA, Tenascin C, SPARC and NT-3

immunohistochemistry resulted in a staining of the peritumoral stroma in all 27 cases, with varying percentages of the stained stromal area and varying staining intensities (Fig. 3). This is in accordance with

Table 5. Overview of histochemical and immunohistochemical findings.

Neoadjuvant cases (n=27)				
Parameter	RNS 1 n=3 (11%)	RNS 2 n=20 (74%)	RNS 3 n=4 (15%)	Significance level
Collagen-poor stroma in Movat's stain (median [range])	21% [18-51%]	47% [1-73%]	56% [45-64%]	p=0.353 (Kruskal-Wallis, 2-sided)
Collagen-rich stroma in Movat's stain (median [range])	44% [8-64%]	22% [1-79%]	33% [3-65%]	p=0.683 (Kruskal-Wallis, 2-sided)
	22% [12-26%]	27% [1-48%]	18% [2-43%]	p=0.768 (Kruskal-Wallis, 2-sided)
Proliferation index/Ki-67 (median [range])	Case with ≤ 20% vital residuals in central sample: median Ki-67: 9% Cases with >20% vital residuals in central sample: median Ki-67: 30%			p=0.006 (Mann-Whitney U, 2-sided)
Tenascin C staining (mean)	2.3 (SD=0.29)	2.5 (SD=0.44)	2.9 (SD=0.25)	p=0.171 (one-way ANOVA)
Alpha-SMA staining (mean)	1.7 (SD=0.29)	2.2 (SD=0.52)	2.6 (SD=0.48)	p=0.06 (one-way ANOVA)
Therapy-naïve cases (n=10)				
Collagen-poor stroma (Movat's stain) (median [range])	52% [37-82%]			
Collagen-rich stroma (Movat's stain) (median [range])	6% [3-21%]			
Tenascin C staining (mean)	2.8 (SD=0.67)			
Alpha-SMA staining (mean)	2.0 (SD=0.42)			

RNS: Royal North Shore tumor regression grading system, SD: Standard deviation.

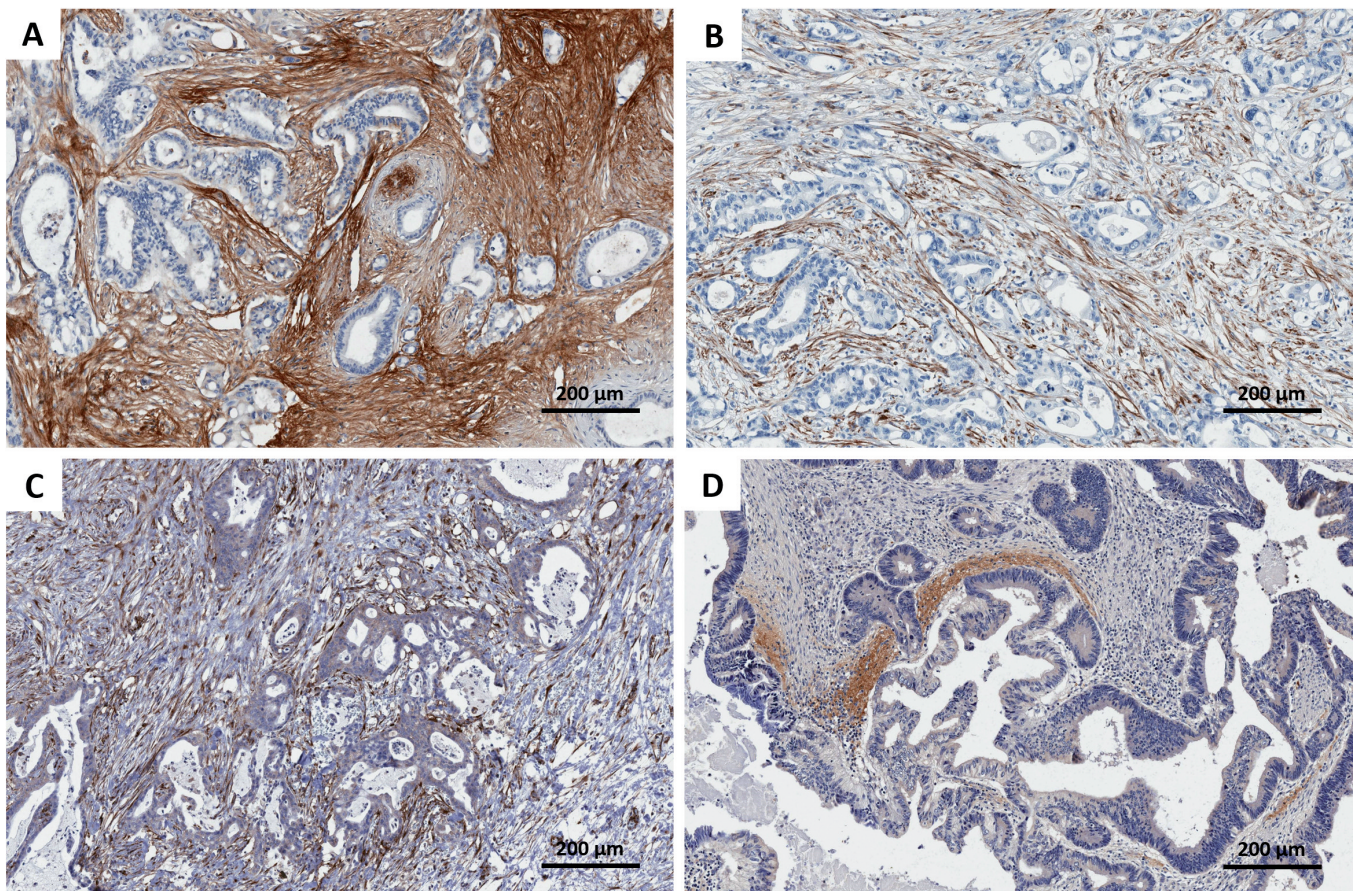


Fig. 4. Immunohistochemistry of therapy-naïve PDAC. Staining of the peritumoral stroma for Tenascin C (A), alpha-SMA (B), SPARC (C) and NT-3 (D).

data previously published regarding therapy-naïve PDAC (Haeberle et al., 2018). Higher staining intensity for alpha-SMA and Tenascin C each showed a trend towards an association with poor regression (Fig. 3A-D, Table 5).

For SPARC and NT-3, a staining around the tumor glands was observed, however, no association between the staining and the grade of tumor regression was seen (Fig. 3E,F).

Alpha-Crystallin B showed no significant peritumoral staining.

Pre-/post-therapy comparison

A pre-therapeutic biopsy was available in 5/27 cases (18.5%). Analyses were limited by this small number of available pre-therapeutic biopsies and by the scarce biopsy material in these cases. Stainings performed in biopsy samples included H&E, Movat's pentachrome, Ki-67, Tenascin C, and SPARC. Biopsy samples showed a dominance of blue-stained immature stroma in Movat's stain, as well as larger areas of Tenascin-C-stained stroma when compared to post-therapeutic material. Regarding Ki-67 and SPARC staining, no differences were observed.

Comparison with a cohort of therapy-naïve PDAC

H&E, Movat's pentachrome staining, and immunohistochemistry for alpha-SMA, Tenascin C, SPARC, NT-3 and alpha-Crystallin were also performed on a small cohort (n=10) of therapy-naïve PDAC (Table 5). Upon H&E staining of one representative tumor block, all cases showed prominent desmoplastic stroma. Interestingly, cytoplasmic vacuolization was also found in all cases, although only focally (3-5% of total tumor mass). Necroses were found in 90% of cases, accounting for about 5-10% of the total tumor mass. Mucin pools were not found. Movat's stain software-supported analysis showed a predominance of blue-stained immature stroma (median: 52% of stromal area) compared to yellow-stained mature stroma (median: 6% of stromal area), indicating a similarity of stroma composition between therapy-naïve cases and cases with poor regression. For alpha-SMA, Tenascin C, SPARC and NT-3, a peritumoral staining was observed in all cases (Fig. 4). Alpha-Crystallin B showed no significant peritumoral staining.

Discussion

To date, most TRG for PDAC fail to deliver reproducible results and do not yield a high prognostic value, as inter-observer variability for many common TRG systems is high (Kalimuthu et al., 2016). Proposed regression features are unreliable, as they may indeed be induced by NAT, but can also be seen in therapy-naïve PDAC (Hartman and Krasinskas, 2012). This was confirmed in this study, except for mucin pools, which

were not observed in therapy-naïve cases in this study (but are known to exist in therapy-naïve PDAC).

A distinct feature of both therapy-naïve and post-therapeutic PDAC is the stromal reaction/therapy-induced fibrosis. However, markers to distinguish pre-existing stroma from therapy-induced fibrosis are lacking.

In this study, the stroma of PDAC after NAT displayed similar heterogeneity as the stroma of therapy-naïve PDAC, which has been previously characterized by our group (Haeberle et al., 2018). As in therapy-naïve PDAC, this was true not only between different cases, but also within single cases. We did not find markers to clearly distinguish between the pre-existing stromal reaction and therapy-induced fibrosis. However, immature collagen-lacking stroma detected via Movat's stain and strong peritumoral staining for Tenascin C and alpha-SMA were associated with the presence of residual vital cancer/poor regression in this study, indicating the possible utility of Movat's staining to assess regression in neoadjuvant treated PDAC.

The fact that the desmoplastic stroma of PDAC is rich in cells and ground substance components, e.g. hyaluronic acid, which may be linked to increased aggressive behavior, has already been described (Theocharis et al., 2000; Toole and Slomiany, 2008; Haeberle et al., 2018). It therefore makes sense that this type of stroma was also found in the stromal areas surrounding residual viable tumor glands after NAT, while areas/cases with better regression displayed a collagen-rich inactive stroma. The lack of a significant correlation between this finding and TRG can possibly be attributed to the low case number, requiring confirmation in larger studies.

Immunohistochemical findings also require confirmation in larger studies. Association of poor regression with the presence/persistence of higher-intensity alpha-SMA and Tenascin C staining may reflect persisting cross talk between cancer cells and activated stromal myofibroblasts, as both proteins are secreted by these cells and play a role in pancreatic carcinogenesis (Esposito et al., 2006; Erkan et al., 2012).

Focusing on the characterization of residual tumor (and its associated stroma) seems useful in PDAC, since most patients do not show abundant regression. As residual cancer is what influences possible local recurrence or metastasis, its biological behavior is of great interest. In patients with neoadjuvant treated breast cancer, proliferation has been shown to be an independent prognostic factor (Jones et al., 2009; Tanei et al., 2011; von Minckwitz et al., 2013; Enomoto et al., 2016). In the present study, a significant correlation between tumor regression in the tumor center and proliferative activity in this area could be confirmed for PDAC, suggesting the usefulness of Ki-67 staining to identify unfavorable biological behavior of PDAC after NAT. Other factors to be considered in the future include, for example, the molecular profile or the immune microenvironment of residual cancer (Verbeke

et al., 2018).

While a survival analysis was not useful in the present study due to the small case number, larger studies focusing on clinico-pathological features of PDAC patients after NAT were unable to show correlations between most TRG systems and survival, with the exception of the three-tiered MD Anderson TRG system (Chatterjee et al., 2012). The MD Anderson TRG system also possesses higher inter-observer concordance compared to other established TRG systems for PDAC (Kalimuthu et al., 2016; Matsuda et al., 2020; Chou et al., 2021). However, the prognostic significance and inter-observer concordance of the MD Anderson TRG was achieved at the expense of its preciseness: setting the cut-off between marked regression and poor regression at 5% means pooling most patients into one large group. The advantage of a three- or four-tiered TRG system with a more “moderate” cut-off, such as the RNS or CAP system, which has also been recommended by the International Collaboration on Cancer Reporting (ICCR) (Verbeke et al., 2020) and by a large panel of experts in the Amsterdam International Consensus Meeting (Janssen et al., 2021), is a more even contribution of cases into the different categories. Avoiding terms like “rare small groups of cancer cells” and using clear numerical cut-off values, even when those are only based on empirical considerations, can also help reduce inter-observer variability, as has been shown in other GI cancers (Mirza et al., 2012).

The main limitations of this study include its retrospective character, the small case number and the cases’ heterogeneity, e.g. concerning the type and length of neoadjuvant treatment (Table 2). While a clear distinction between therapy-naïve and neoadjuvant treated stroma of PDAC could not be proven, trends observed in this study point to differences in the stromal composition. Moreover, other significant differences may exist and could be unraveled in further studies using larger, more homogeneous cohorts.

In the future, larger, possibly international studies focusing on residual cancer and its biological behavior, based on homogenous series and using standardized reproducible TRG systems are needed to verify the utility of Movat’s and Ki-67 staining to assess therapy response and predict the biological behavior of neoadjuvant treated PDAC.

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