Towards implementation of the tumour-stroma ratio in colorectal cancer

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Chapter 5

Fibroblast activation protein-expression in colorectal carcinomas and implications for clinical application

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Abstract

Background: Colorectal cancer is highly prevalent. The stromal tumour microenvironment significantly influences tumour behaviour, and cancer-associated fibroblasts (CAFs), as major component of tumour stroma, are increasingly studied. Specifically, CAF-marker fibroblast activation protein (FAP) is gaining interest as tracer for imaging using radiolabelled FAP-inhibitor (FAPI). We describe patterns of FAP-expression, and associations to intratumoural stroma amount, establishing a biological background and potential future reference to pathology assessment.

Materials and methods: Archival histological material from 125 stage-II/III CRC patients was collected. Haematoxylin-and-eosin staining was performed to determine the tumour-stroma ratio (TSR), indicating stromal percentages in primary tumours, lymph nodes (LNs) and biopsies. On immunohistochemistry stains, FAP-expression was semiquantitatively scored as little-no, heterogeneous, or moderate-high expression. Correlation of TSR and FAP-expression with Chi-square testing was assessed. Other patterns were also described, e.g. tumour epithelial FAP-expression.

Results: In total, 93 patients (40 stage-III colon [CC], 53 stage-II/III RC) were included. Correlation between 41 (44%) stroma-high CRC (18/40 CC, 45%; 23/53 RC, 43%) with high FAP-expression was not significant (P=0.428 CRC; P=0.470 CC; P=0.615 RC). The majority of CRC had any FAP-expression (78 CRC, 84%), mostly the invasive front, and in most associated LN metastases (87% CRC tumour-positive LN). However, FAP-expression was often heterogeneous, even staining healthy colon and lymphoid tissue.

Conclusions: CRCs and LN metastases generally express FAP, but levels vary significantly between and within tumours and have no direct correlation with TSR. Care has to be taken translating FAPI-PET/CT results with e.g. disease extent and activity, emphasizing the importance of multidisciplinary approach.

Introduction

Colorectal cancer (CRC) is a multifactorial and multifaceted disease [1]. Although the focus is increasing on prevention through life style improvements and early detection, such as nationwide screening programs, it still remains a highly prevalent disease, accounting for nearly 2 million new cases in 2022 worldwide [2-4]. Therapeutic guidelines are mainly based on clinicopathological variables, such as extent of disease as described by the tumour-node-metastasis (TNM) classification [5-9]. However, the last decades, a paradigm shift has occurred in oncology research. The traditional focus on tumour epithelial cells is increasingly redirected to the tumour microenvironment as a crucial influencer of tumour behaviour [10, 11]. Mainly composed of immune cells, including tumour-infiltrating T-cells and tumour-associated macrophages, vasculature and the angiogenesis process, the extracellular matrix including collagen, and activated quiescent fibroblasts, the tumour stroma is subject to extensive study [11-16]. Crosstalk between this dynamic entity and tumour epithelial cells disrupts the homeostasis and promotes tumorigenesis, in which the quiescent fibroblasts are activated. These cancer-associated fibroblasts (CAFs) have been identified as important contributors [11, 16-18].

CAFs comprise a significant proportion of cells within the tumour stroma and have a proliferative phenotype. Furthermore, they actively induce neoplastic cell growth and through e.g. epithelial-to-mesenchymal transition, recruit more CAFs [11, 16-18]. Studies moreover revealed the pivotal role of CAFs in the development and progression of CRC [19-21]. High levels of accumulated CAFs as well as a high amount of intratumoural stroma are known to predict the course of CRC [22-25]. Indeed, the tumour-stroma ratio (TSR), capturing this phenomenon, has iteratively proven to of predictive value to patient-related outcomes, with stroma-high tumours leading to worse survival [23, 26-28] and poor response to (neo)adjuvant therapy [29-31].

Hence, tumour stroma and especially CAFs constitute attractive targets for improved CRC diagnosis and prognosis. One approach currently being explored in nuclear medicine is the targeting of fibroblast activation protein (FAP), a universal CAF-marker, with the developed fibroblast activation protein-inhibitor (FAPI) [32, 33]. Coupled with a radioisotope label like [18F]Fluor or [68Ga]Gallium, FAPI is increasingly researched using PET/CT scanning to optimise tumour detection, tumour characterisation and even treatment strategies in CRC [34-36]. However, translational studies on the correlating histopathology level, determining FAP expression of CAFs in CRC, are scarce [37-40].

The aim of this study was therefore primarily to assess the levels and patterns of expression of FAP in CRC specimens, establishing a biological background for clinical implementation, which could potentially serve for future reference in pathology assessment. Secondly, we determine the association of the prognostic TSR parameter and the amount of FAP expression of CAFs in CRC. We hypothesized that FAP levels would correlate to TSR, as stroma-high tumours contain more CAFs, theoretically leading to a higher FAP expression. TSR combined with FAP expression could potentially then aid upfront selection of CRC patients for therapeutic strategies, e.g. identifying more aggressive and therapy-resistant stroma-high patients on imaging.

Materials and methods

Patients and material

For this retrospective study, we included 125 CRC patients operated between 2000-2016 at the Leiden University Medical Centre (LUMC): 50 stage III colon carcinoma (CC) and 75 stage II/III rectal carcinoma (RC). Paraffin blocks were collected of resection material from the most invasive part of the primary tumour (PT) of all CRC, of all resected lymph nodes (LNs) of stage III CRC, and of biopsy material from RC patients, as well as all associated clinicopathological data. Exclusion followed when blocks were missing, staining artefacts occurred, or if there was not enough tumour material present. All already available archival material and data were coded and handled according to the National Ethical Guidelines (“Code of proper secondary use of human tissue”). No informed consent was necessary under the legislation for this retrospective analysis. In short, a varied patient population was created to grant insight in potential FAP expression correlation to different histopathology patterns: Supplementary Figure 1 shows a flowchart of ultimately included CRC patients (N=93). Baseline characteristics, treatment types and patient-related outcomes of the cohorts are shown in Supplementary Table 1. General pathology characteristics of the cohorts are further summarized in Table 1.

Table 1. Overview of the general pathology variables of the CC and RC cohorts

Characteristic (unit)	**CC cohort (N=40)**	**RC cohort (N=53)**
Tumour diameter – millimetres		
Median maximum diameter (range)	50 (15-125)	30 (0-120)
Residual tumour*		
R0 resection	40 (100)	51 (96)
R1 resection	0 (0)	1 (2)
R2 resection	0 (0)	1 (2)
y(p)T-category**		
y(p)T-stage 0	0 (0)	3 (6)
y(p)T-stage I	0 (0)	2 (4)
y(p)T-stage II	2 (5)	19 (36)
y(p)T-stage III	32 (80)	27 (51)
y(p)T-stage IV	6 (15)	2 (4)
LN (median; range)		
LN examined	12 (4-50)	13 (3-27)
LN positive	2 (1-13)	0 (0-6)
LN – hurdle scoring		
N/A (no LN positive)	0 (0)	32 (61)
Positive LN	40 (100)	21 (40)
No hurdles, normal metastasis and scoring possible	15 (38)	12 (57)
Mucin abundantly present	7 (18)	2 (10)
Necrosis abundantly present	18 (45)	4 (19)
Fibrosis present	N/A	3 (14)
y(p)N-category**		
y(p)N-stage 0	0 (0)	30 (57)
y(p)N-stage I	27 (68)	20 (38)
y(p)N-stage II	13 (33)	3 (6)
(y)pTNM-stage**		
(y)pTNM-stage 0	0 (0)	3 (6)
(y)pTNM-stage I	0 (0)	16 (30)
(y)pTNM-stage II	0 (0)	11 (21)
(y)pTNM-stage III	40 (100)	23 (43)
Tumour morphology (PT or biopsy)		
Adenocarcinoma	31 (78)	51 (96)
Mucinous adenocarcinoma	9 (23)	2 (4)
Differentiation grade tumour		
Well to moderate	4 (10)	9 (17)
Moderate to poorly	22 (55)	31 (59)
Poor to undifferentiated	7 (18)	2 (4)
N/A (no tumour)	0 (0)	4 (8)
Not determined	7 (18)	7 (13)

(continued) **Characteristic (unit)**	**CC cohort (N=40)**	**RC cohort (N=53)**
No risk factors present	3 (8)	15 (28)
Lymphatic invasion	1 (3)	1 (2)
Vascular invasion	3 (8)	2 (4)
Lymphangial invasion	2 (5)	1 (2)
Perineural invasion	1 (3)	5 (9)
Lymphatic and perineural invasion	0 (0)	1 (2)
All of the above	1 (3)	0 (0)
Not determined	29 (73)	29 (53)
Tumour response***		
N/A (no neoadjuvant therapy)	40 (100)	12 (23)
No or nearly no pathological response	N/A	28 (53)
Partial pathological response (pPR)	N/A	19 (18)
Complete pathological response (pCR)	N/A	3 (6)
Tumour regression grade (TRG)****		
N/A (no neoadjuvant therapy)	40 (100)	12 (23)
Absence of regressive changes (TRG5)	N/A	12 (23)
Residual tumour cells outgrowing fibrosis (TRG4)	N/A	16 (30)
More residual tumour cells but fibrosis predominates (TRG3)	N/A	5 (9)
Rare viable tumour cells (TRG2)	N/A	5 (9)
Complete regression, no viable tumour cells (TRG1)	N/A	3 (6)
Microsatellite status		
Not determined	31 (78)	36 (68)
Determined, and		
Microsatellite instable (MSI) and/or deficient mismatch repair (dMMR) enzymes	0 (0)	0 (0)
Microsatellite stable (MSS) and/or proficient mismatch repair (pMMR) enzymes	9 (22)	17 (32)
Mutational burden		
No mutational analysis performed	32 (80)	45 (85)
Performed, and		
No mutations found	4 (10)	2 (4)
KRAS mutation	4 (10)	5 (9)
BRAF mutation	0 (0)	0 (0)
APC mutation	0 (0)	1 (2)

All variables are given as absolute numbers with associated percentages or medians with ranges (minimum-maximum). Sum of percentages can be less or more than 100 due to rounding. CC, colon carcinoma; LN, lymph node; PT, primary tumour; RC, rectal carcinoma; TNM, tumour-node metastasis.

*Residual tumour according to Wittekind (2009). **Different versions of the TNM classification were used, here all variables are converted to the AJCC/UICC TNM version 5 (1997). ***Tumour response is roughly categorized in three; no/partial/complete response. ****Tumour regression grade as categorized by Mandard.

Staining protocols

Additional 5 µm thick paraffin sections were cut from all collected archival blocks, on which standard haematoxylin and eosin (H&E) staining and immunohistochemical staining (IHC) using a FAP antibody (rabbit monoclonal antibody IgG, ERP20021, ab207178, Abcam - dilution 1:200) was performed, according to Sandberg et al [20]. After deparaffinization and rehydration, antigen retrieval was performed using the PreTreatment Link Module (Dako), following mounting with antifade reagent, and lastly, counterstaining (DAPI, 2µg/ml Sigma-Aldrich).

Tumour stroma and pathology analysis

The H&E-stained slides were used for the TSR, scored by two trained independent researchers blinded for clinical data (MP and GWvP). Subsequently, categorisation into stroma-low (≤50% stroma) or stroma-high (>50% stroma) followed according to van Pelt et al.[41] (Supplementary Figure 2). Cohen's interobserver agreement kappa's were 0.87 for CC (almost perfect agreement; scored on PT), and 0.67 for RC (substantial agreement; scored on biopsy). As neoadjuvant treatment alters the tumour stromal compartment, e.g. creating fibrosis, the TSR in PT material in neoadjuvant treated RC is not representative [42]. However, as was proven in literature, the TSR of biopsy material is deemed representative for the TSR of the associated PTs [31, 43], hence, this was used to categorize RC. In CC, as these patients did not receive neoadjuvant treatment, the complete PT material was available, and no biopsy material was collected. Moreover, we had previously observed that the LN containing the highest stromal percentage was significantly associated to patient outcomes, thus this LN was deemed representative for all LNs, as well [30, 44].

FAP expression analysis

FAP expression was assessed by two observers, also blinded for clinical data (pathologist JHJMvK, researcher MP). Biopsies, PTs and associated LNs were all semi quantitatively scored on IHC slides, in stroma as well as tumour epithelium: (0) little to no, (1) heterogeneous, and (2) moderate to high expression. In LNs, distinction was made for assessments of the staining of metastases as well as healthy lymphoid tissue. For comparison and association to TSR for future implications, only moderate-high expression of FAP was deemed sufficient for potential outcomes.

Slide and statistical analysis

Slides were scanned with the Panoramic 250 scanner (3DHistech, Hungary) (tissue level pixel size ~0.33 µm/pixel; 20x magnification). Annotations and figures were made using the 3DHistech SlideViewer 2.7 software. Continuous variables were expressed in medians with interquartile ranges (IQR), and nominal and ordinal variables in number of frequencies with corresponding percentages. Statistical analysis was performed using Chi-square analyses with significance determined at two-tailed P-values <0.05, using IBM SPSS Statistics 29.0.

Results

TSR findings

TSR characteristics per cohort are summarized in Table 2. A majority of both CC (scored on PT; N=22, 55%) and RC (scored on biopsy; N=30, 57%) were stroma-low, similar to previous studies [23, 26-28]. The TSR of biopsies in all RC and their associated PT did not completely correlate (P=0.053; Supplementary Table 2), caused by a large insignificant effect on neoadjuvant treated RC PTs (P=0.785; Supplementary Table 2), illustrating that the TSR can not be scored in resected material due to extensive fibrosis induced by any neoadjuvant treatment [45]. The TSR of treatment-naïve RC biopsies did however significantly correlate to the TSR of the PT (P=0.007), which confirms the reliability and representativeness of biopsy-assessed TSR scores (Supplementary Table 2).

Table 2. Overview of the TSR and FAP variables of the CRC and separate CC and RC cohorts.

Characteristic (unit)	**CRC cohort (N=93)**	**CC cohort (N=40)**	**RC cohort (N=53)**
TSR – biopsy			
Stroma-low	30 (32)	N/A	30 (57)
Stroma-high	23 (25)	N/A	23 (43)
N/A (no biopsy)	40 (43)	40 (100)	N/A
TSR – PT*			
Stroma-low	38 (41)	22 (55)	16 (30)
Stroma-high	52 (56)	18 (45)	34 (64)
N/A (no tumour)	3 (3)	N/A	3 (6)
TSR – representative LN**			
Stroma-low	51 (55)	22 (55)	29 (55)
Stroma-high	20 (22)	18 (45)	2 (4)
N/A (no LN positive)	22 (24)	0 (0)	22 (42)
FAP overall expression – biopsy			
Little to no expression	6 (6)	N/A	6 (11)
Heterogeneous expression	26 (28)	N/A	26 (49)
Moderate to high expression	21 (23)	N/A	21 (40)
N/A (no biopsy)	40 (43)	40 (100)	N/A
FAP expression tumour epithelium – biopsy			
No, only tumour stroma	23 (25)	N/A	23 (43)
Heterogeneous expression	18 (19)	N/A	18 (34)
Yes, high expression in tumour epithelium	12 (13)	N/A	12 (23)
N/A (no biopsy)	40 (43)	40 (100)	N/A
FAP overall expression – PT			
Little to no expression	11 (12)	7 (18)	4 (8)
Any expression, of which	*82 (88)*	*33 (83)*	*49 (92)*
Heterogeneous expression	47 (61)	25 (63)	32 (60)
Moderate to high expression	25 (27)	8 (20)	17 (32)
FAP expression tumour epithelium – PT			
No, only tumour stroma	43 (46)	33 (83)	10 (19)
Yes, any FAP expression, of which	47 (51)	7 (18)	40 (75)
Heterogeneous expression	26 (28)	5 (13)	21 (40)
High expression in tumour epithelium	21 (23)	2 (5)	19 (36)
N/A (no tumour)	3 (3)	N/A	3 (6)

(continued) **Characteristic (unit)**	**CRC cohort (N=93)**	**CC cohort (N=40)**	**RC cohort (N=53)**
N/A (no LN positive)	32 (34)	0 (0)	32 (60)
Positive LN, of which	61 (66)	40 (100)	21 (40)
Little to no expression	15 (25)	14 (35)	1 (2)
Heterogeneous expression	21 (32)	17 (43)	4 (8)
Moderate to high expression	25 (41)	9 (23)	16 (30)
FAP expression tumour epithelium – representative LN**			
N/A (no LN positive)	32 (34)	0 (0)	32 (60)
Expression, of which	61 (66)	40 (100)	21 (40)
No, only expression in tumour stroma	35 (57)	32 (80)	3 (14)
Heterogeneous expression	15 (25)	8 (20)	7 (33)
Yes, moderate to high expression in tumour epithelium	11 (18)	0 (0)	11 (52)
FAP expression – general			
Equal distribution of FAP expression	15 (16)	7 (18)	8 (15)
High expression in remodelling areas	78 (84)	33 (83)	45 (85)
FAP discrepancy in expression – biopsy and PT			
No discrepancy, similar expression	25 (27)	N/A	25 (47)
Slight discrepancy, biopsy higher	14 (15)	N/A	14 (26)
Slight discrepancy, PT higher	12 (13)	N/A	12 (23)
Major discrepancy	2 (2)	N/A	2 (4)
N/A (no biopsy)	40 (43)	40 (100)	N/A
FAP discrepancy in expression – PT and representative LN**			
No discrepancy, similar expression	27 (29)	16 (40)	11 (21)
Slight discrepancy, PT higher	9 (10)	9 (23)	0 (0)
Slight discrepancy, LN higher	19 (20)	11 (28)	8 (15)
Major discrepancy	6 (6)	4 (10)	2 (4)
N/A (no LN positive)	32 (34)	0 (0)	32 (60)

All variables are given as absolute numbers with associated percentages or medians with IQR. Sum of percentages can be less or more than 100 due to rounding. CC, colon carcinoma; FAP, fibroblast activation protein; IQR, interquartile range; LN, lymph node; N/A, not applicable; PT, primary tumour; RC, rectal carcinoma; TSR, tumour-stroma ratio.

*All primary tumours are shown here, including those with neoadjuvant treatment.

**Representable lymph node is the lymph node with most tumour stroma.

In total, we analysed 288 CRC LNs, of which 125 (43%) were positive for metastasis, accounting for 61 patients (66%; 40 CC and 21 RC patients). The TSR of LNs with metastases was not associated with the TSR score in the PT (P=0.529 in CRC; Table 3), emphasising their heterogeneity. A trend was seen in stroma-high CC patients that their LNs were more often difficult to score due to e.g. necrosis (P=0.071), whereas in RC merely 2 patients had stroma-high tumour-positive LNs, both potentially skewing results (Supplementary Table 3).

FAP expression

General FAP characteristics are given in Table 1. During microscopic analysis of FAP expression, various patterns were noted and scored accordingly, illustrated by Figure 1. FAP was expressed in the majority of CRC patients (78 in CRC, 33 in CC, 83%; and 47 in RC, 89%) (Figure 1A-B), as well as in associated LN metastases (87% of all CRC tumour-positive LN) (Figure 1C-D). However, levels of FAP expression varied and could also be heterogeneous throughout the tumour (61% in CRC PT) (Figure 1E-F). In some cases, also some FAP expression was seen in tumour epithelium of PT as well (51% in CRC, mostly in RC after neoadjuvant treatment [75%]) (Figure 1G-H). FAP was expressed mostly in areas of active remodelling, i.e. the invasive front (78 in CRC; 84%). Overall, there was often a discrepancy in intensity of FAP expressed by biopsy and PT, as well as between PT and LN (Table 1).

Correlations FAP expression and TSR and other findings

In ascertaining the correlation between TSR and FAP expression in CRC, although stroma-high CRC more often expressed higher levels of FAP, this did not reach significance (P=0.428 in CRC; P=0.470 in CC and P=0.615 in RC, respectively) in their counterpart nor in their LNs (Table 3). Despite the fact that the majority of PTs and LN metastases express FAP, still 22% of all analysed CRC tumour-positive LN did not stain for FAP (Supplementary Table 3). Moreover, in 22% of tumour-negative CRC LNs, FAP conversely did get expressed, e.g. in overactive germinal centres (Figure 2A-B). A mean percentage of 69% of all analysed CRC LNs per patient was found that was tumour-positive and had corresponding FAP-positivity, whereas 31% of tumour-positive LNs did not express FAP, and 14% of tumour-negative LNs were actually FAP-positive within the patient (Supplementary Table 3).

Table 3. Comparison of stroma-low and stroma-high tumours within the CC and RC cohorts

Characteristic (unit)	**CRC cohort (N=93)**			**CC cohort (N=40)**			**RC cohort (N=53)**		
	Stroma-low (N=52)	Stroma-high (N=41)	P-value	Stroma-low (PT; N=22)	Stroma-high (PT; N=18)	P-value	Stroma-low (biopsy; N=30)	Stroma-high (biopsy; N=23)	P-value
Clinical TNM-stage*			0.705#			0.720#			0.332#
cTNM-stage I	1 (2)	0 (0)		0 (0)	0 (0)		1 (3)	0 (0)	
cTNM-stage II	17 (33)	12 (29)		6 (27)	7 (39)		11 (37)	5 (22)	
cTNM-stage II/III**	12 (23)	8 (20)		11 (50)	8 (44)		1 (3)	0 (0)	
cTNM-stage III	22 (42)	21 (51)		5 (23)	3 (17)		17 (57)	18 (78)	
Treatment type, grouped			0.141#			0.676#			**0.022#**
Neoadjuvant therapy and surgery	20 (39)	21 (51)		0 (0)	0 (0)		20 (67)	21 (91)	
Surgery and adjuvant therapy	12 (23)	12 (29)		12 (55)	11 (61)		0 (0)	1 (4)	
Surgery alone	20 (39)	8 (20)		10 (45)	7 (39)		10 (33)	1 (4)	
Pathology risk factors***									
Not determined	31 (60)	26 (63)		18 (82)	11 (61)		13 (43)	15 (65)	
Determined, and	21 (40)	15 (37)	0.735#	4 (18)	7 (39)	0.125#	17 (57)	8 (35)	0.861#
No pathology risk factors present	10 (48)	8 (53)		0 (0)	3 (43)		10 (59)	5 (63)	
Yes, 1 or more risk factors present	11 (52)	7 (47)		4 (100)	4 (57)		7 (41)	3 (38)	
TSR									
TSR in PT			N/A			N/A			0.053#
Stroma-low	N/A	N/A		N/A	N/A		13 (43)	3 (13)	
Stroma-high	N/A	N/A		N/A	N/A		16 (53)	18 (78)	
N/A (no tumour)	N/A	N/A		N/A	N/A		1 (3)	2 (9)	

(continued)									
TSR in representative LN****									
N/A (missing, no LN positive)	18 (35)	14 (34)		0 (0)	0 (0)		18 (60)	14 (61)	
Positive LN, of which	34 (65)	27 (66)	0.529#	22 (100)	18 (100)	0.565#	12 (40)	9 (39)	0.830#
Stroma-low	24 (71)	17 (63)		13 (59)	9 (50)		11 (92)	8 (89)	
Stroma-high	10 (29)	10 (37)		9 (41)	9 (50)		1 (8)	1 (11)	
FAP expression – with counterpart			0.428#			0.470#			0.615#
Little expression (no - heterogeneous expression)	37 (71)	26 (63)		18 (82)	13 (72)		19 (63)	13 (57)	
Moderate to high expression	15 (29)	15 (37)		4 (18)	5 (28)		11 (37)	10 (44)	
FAP in representative LN****									
N/A (missing, no LN positive)	18 (35)	14 (34)		0 (0)	0 (0)		18 (60)	14 (61)	
Positive LN, of which	34 (65)	27 (66)	0.191#	22 (100)	18 (100)	0.714#	12 (40)	9 (39)	0.055#
Little expression (no - heterogeneous expression)	17 (50)	18 (67)		16 (73)	14 (78)		1 (8)	4 (44)	
Moderate to high expression	17 (50)	9 (33)		6 (27)	4 (22)		11 (92)	5 (56)	
Any FAP expression in tumour epithelium – with counterpart			0.576#			0.477#			0.259#
No, only in tumour stroma	30 (58)	26 (63)		19 (86)	14 (78)		11 (37)	12 (52)	
Yes, also some in tumour epithelium	22 (42)	15 (37)		3 (14)	4 (22)		19 (63)	11 (48)	
FAP expression in remodelling areas			0.431#			0.477#			0.683#
Equal distribution of FAP expression	7 (14)	8 (20)		3 (14)	4 (22)		4 (13)	4 (17)	
High expression in remodelling areas	45 (87)	33 (81)		19 (86)	14 (78)		26 (87)	19 (83)	
FAP discrepancy – biopsy-PT			N/A			N/A			0.657#
No discrepancy, similar expression	N/A	N/A		N/A	N/A		15 (52)	10 (46)	
Yes, any discrepancy	N/A	N/A		N/A	N/A		14 (48)	12 (55)	

(continued) FAP discrepancy – PT-representative LN****									
N/A (missing, no LN positive)	18 (35)	14 (34)		0 (0)	0 (0)		18 (60)	14 (61)	
Positive LN, of which	34 (65)	27 (66)	0.586#	22 (100)	18 (100)	0.604#	12 (40)	9 (39)	0.801#
No discrepancy, similar expression	14 (41)	13 (48)		8 (36)	8 (44)		6 (50)	5 (56)	
Yes, any discrepancy	20 (59)	14 (52)		14 (64)	10 (56)		6 (50)	4 (44)	
Tumour response*****			N/A			N/A			0.864#
No or nearly no pathological response	N/A	N/A		N/A	N/A		9 (45)	10 (46)	
Partial pathological response (pPR)	N/A	N/A		N/A	N/A		10 (50)	10 (46)	
Complete pathological response (pCR)	N/A	N/A		N/A	N/A		1 (5)	2 (9)	
Pathological TNM-stage*			0.828#			N/A			0.829#
(y)pTNM-stage 0	1 (2)	2 (5)		0 (0)	0 (0)		1 (3)	2 (9)	
(y)pTNM-stage I	9 (17)	7 (17)		0 (0)	0 (0)		9 (30)	7 (30)	
(y)pTNM-stage II	7 (14)	4 (10)		0 (0)	0 (0)		7 (23)	4 (17)	
(y)pTNM-stage III	35 (67)	28 (68)		22 (100)	18 (100)		13 (43)	10 (44)	

All variables are given as absolute numbers with associated percentages. Sum of percentages can be less or more than 100 due to rounding. CC, colon carcinoma; FAP, fibroblast activation protein; LN, lymph node; N/A, not applicable; PT, primary tumour; RC, rectal carcinoma; TNM, tumour-node-metastasis; TSR, tumour-stroma ratio.

*Different versions of the TNM classification were used, here all variables are converted to the AJCC/UICC TNM version 5 (1997).

**Stage II/III is given in case no imaging is performed and locoregional extent of disease is uncertain.

*** Pathology risk factors include lymphatic invasion, venous invasion, perineural invasion.

****Representable lymph node is the lymph node with most tumour stroma.

*****Tumour response is roughly categorized in three; no/partial/complete response.

Calculated with the Chi-square test.

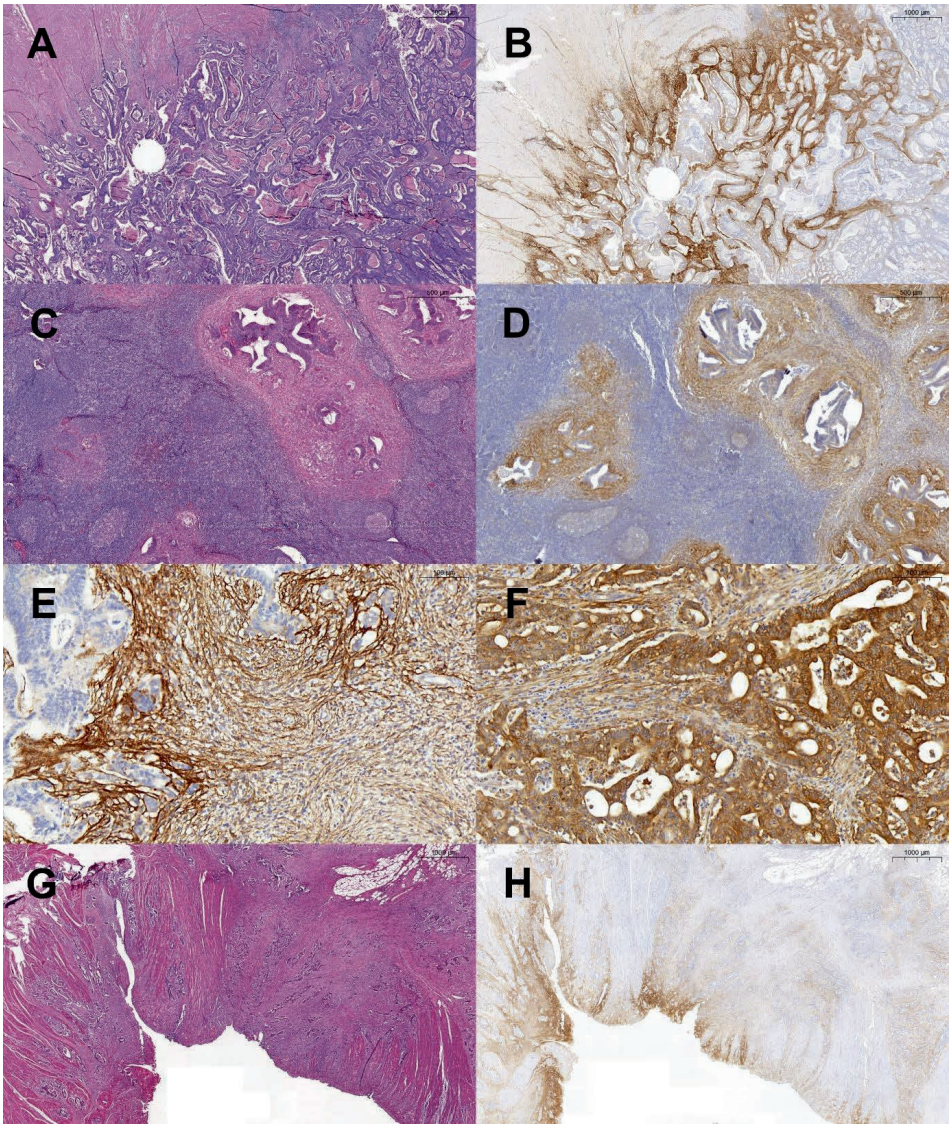

Figure 1. (A-B) Haematoxylin-and-eosin (H&E)-stained slide and immunohistochemistry-stained fibroblast activation protein (FAP) slide examples of a primary tumour and expression towards the invasive front. Punch hole for localisation (2.0x magnification). (C-D) H&E and FAP examples of lymph node metastases (5.0x magnification). (E-F) FAP expression in stroma with stained cancer-associated fibroblasts, compared to strong staining of FAP in tumour epithelial cells more than in stroma (20.0x magnification). (G-H) H&E and FAP examples of a primary tumour with heterogeneous expression (2.0x magnification).

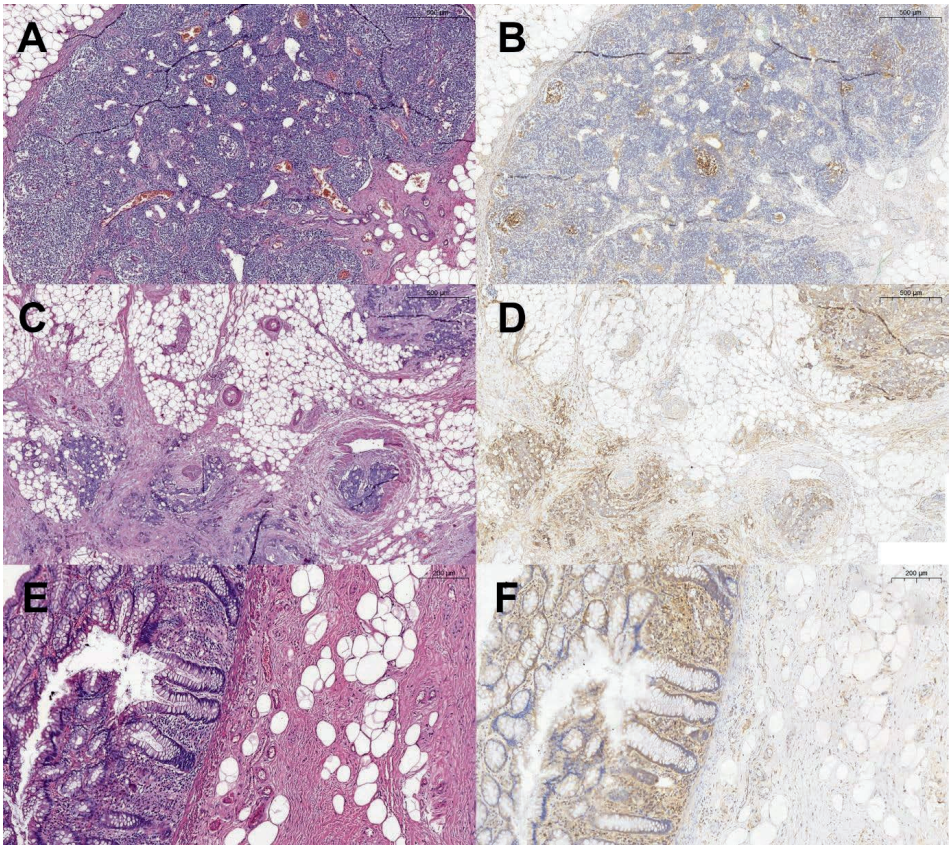

Figure 2. (A-B) H&E and FAP of a tumour-negative lymph node metastasis but positive FAP expression (10.0x magnification). (C-D) H&E and FAP examples of a tumour deposit and extramural venous invasion, strongly staining for FAP, illustrating aggressiveness (5.0x magnification). (E-F) H&E orientation for FAP expression in post-radiation mucosa in a rectal cancer patient with a pathological complete response (10.0x magnification). FAP, fibroblast activation protein; H&E, haematoxylin and eosin staining.

Interestingly, the sporadically present tumour deposits often strongly expressed FAP, potentially illustrating tumour aggressiveness (Figure 2C-D). In this study, no correlation could be found between TSR scores of biopsies and predicted tumour regression grade or response of pathological TNM stage (ypTNM) in RC, although a large bias exists in treatment type (P=0.022) and a trend towards higher a priori stages in stroma-high RC (P=0.332) (Table 2). We also observed, of important note, in mucosa, for instance post-radiation, expression of FAP as well, i.e. in one RC patient with a pathological complete response (Figure 2E-F).

Discussion

In this study, we hypothesized that stroma-high CRC, composed of more CAFs, would have higher FAP-marker expression. Theoretically, this could aid upfront selection of patients for treatment strategies, where for instance on a future FAPI PET/CT scan, more tracer uptake would indicate more aggressive and therapy-resistant stroma-high tumours. However, no direct correlation between the well-established TSR parameter and FAP expression was observed. Moreover, although FAP was expressed in the majority of CRC patient material, this was not ubiquitous and more importantly, also non-tumourous tissue stained positive for FAP. In conclusion, our results elucidate a biological background in contrast to current literature, necessitating care on the direct translation of FAPI as radiopharmaceutical for CRC detection and staging, which is increasingly researched, emphasizing the importance of a multidisciplinary approach.

FAP is a proteolytic enzyme on the cell surface of CAFs, promoting tumour invasion through processes like active extracellular matrix degradation and tissue remodelling [46]. Most tumour types contain CAFs expressing FAP, including gastrointestinal cancers as also described by Mona et al [37]. Our results confirm that the majority of CRC indeed expressed FAP, however, that this was more intense towards the invasive front. This most likely explains the discrepancy with TSR scores, which are generally acquired from the tumour centre [20, 41, 47]. Interestingly, we observed strong staining for FAP in the sporadically present tumour deposits and extramural venous invasion. This is corroborated with other literature, stating that higher FAP expression or FAP expression in tumour epithelium was associated with more invasive tumours [46, 48], emphasizing the correlation of FAP expression and tumour aggressiveness.

Traditionally, after an initial diagnosis with an endoscopic biopsy, determining extent of disease as major characteristic of tumour behaviour, is important for diagnosis and prognosis prediction, also in CRC [6, 7]. This assessment of PT, LNs and potential distant metastases, using the TNM stage classification, is concurrently performed through imaging [5]. However, especially accurate nodal staging remains challenging despite current high-standard imaging modalities and techniques [49-51]. Since FAPI PET/CT scanning could improve staging as described in literature [52, 53], and as we had previously seen that most LN metastases, independent from size, contain tumour stroma [54], we aimed to determine in this present study whether FAP, as a proven derivative of FAPI PET/CT imaging, might indeed theoretically, biologically, improve diagnostic accuracy [37].

However, our results illustrate that FAP expression was prone to variation. Not all CRC expressed FAP, and moreover, non-cancerous tissue could also exhibit FAP expression. Although this could in part be due to general intratumoural heterogeneity [55], the clinical consequences are far-reaching. In a few cases, when directly translating FAP expression to future FAPI tracer uptake in LNs for instance, this could have potentially lead to understaging in a patient. As an observed FAP-negative but tumour-positive LN could in turn lead to a clinical stage II but pathological stage III tumour, this could have necessitated other (neo)treatment strategies [6, 7]. Examples from literature also mention specifically FAP staining in post-radiation mucosa, dysplasia or fibrosis [39, 56], which our results corroborated, e.g. FAP expression in some patients with pathological complete response. Consequently, RC watch-and-wait strategies [57] could be rendered insufficient with mere FAPI PET/CT in follow-up, as these patients could be subjected to surgeries while potentially have reached a complete regression of their tumour. Especially with the rise of newer neoadjuvant chemoradiotherapy combination regimens, like the total neoadjuvant treatment strategy or RAPIDO trail [58], this could influence diagnostic results. Also, non-oncologic diseases leading to fibrosis, scarring or inflammation like Crohn's disease have been found to influence FAPI-uptake [59, 60], rendering assessment in patients with chronic illnesses inaccurate and requiring further research [61].

Importantly, while FAP is widely researched and deemed the universal CAF-marker, there are however, many CAF subtypes. Derived from a variety of origins through direct recruitment or an epithelial-to-mesenchymal transition, a concrete molecular consensus is thus lacking for this heterogeneous group and merely distinguishable from tumour epithelial cells by using panels with different markers [20, 62]. CAFs are generally associated with tumour invasion and other hallmarks of tumour aggressiveness, such as treatment resistance, angiogenesis and immunosuppression, e.g. by producing growth factors and inflammatory cytokines (inflammatory CAFs; iCAFs). Contrarily, some exhibit antitumoural properties, for instance in antigen-presenting (apCAF) subtypes, or even dual functions, like alpha-

smooth muscle actin-expressing CAFs, identifying a more myofibroblast-like (myCAF) cell [63, 64]. Solely focussing on FAP could thus possibly lead to underrepresentation of fibroblast presence or even misinterpretation of tumour behaviour. Furthermore, CAF subtypes in metastases have been shown to vary from those remaining in the PT, more actively inducing cell invasion and are of deleterious influence on patient survival [65, 66]. Crucial research is therefore currently being initiated to unravel this complex entity [67].

This study has some limitations. We performed a single-centre study with a relatively small patient cohort, also hindering patient-related outcome analyses, with inclusions up until 2016. Novel treatment strategies are hence not taken into account, and moreover, data on currently per protocol analysed microsatellite or mutational status [6, 7], was scarce or not available. Additionally, a limitation also pertained to our semi quantitative scoring methodology. Although these semi quantitative measures are often used [68], e.g. in Mona et al. [29], visual assessment could potentially lead to suboptimal scores [69]. Standardisation or automatic analysis using artificial intelligence could overcome this hurdle in the future and support the increasing workload for pathologists, as is already being endeavoured with the TSR [70]. Lastly, despite the possibility of some variations of the chemical compounds within the FAPI-tracer, which could in theory lead to a minor discrepancy with standard IHC staining with the FAP antibody [34], the biodistribution did previously correlate, granting a representative insight in CRC [37, 38]. Results of future studies on the correlation between FAPI uptake on imaging and pathology assessment are awaited, like the upcoming clinical trials FAPI-CRC1 (NCT05209750) and FoCus (NCT06191120).

However, this study has strengths as well, including the in-depth analysis of a wide variety of CRC patients, hence establishing a literal biological framework for current trends in research. Although our study did not find a direct correlation between biopsy-scored TSR and the response on treatment and/or tumour regression grade in RC, most likely due to bias, we aim to analyse this specifically in a larger future study. Moreover, as our research group had previously found that the TSR in LN metastases formed an additional prognostic parameter, we assessed whether FAP expression in LNs could be predicted by the PT [44]. The TSR scores, however, like FAP expression, show high heterogeneity between the PT and LNs, highlighting once again the importance of analysing all LNs in pathology, as stroma-high LN metastases indicate more aggressive tumours and lead to worse patient-related outcomes [30, 44].

In conclusion, CRC and associated positive LNs can express FAP, though this is subject to variation, necessitating care in translating future intensity of FAPI-PET/CT directly into clinical results, e.g. tumour characterisation. No clear agreement between TSR and FAP expression exists, hindering prediction of a TSR score in patients from mere imaging of uptake through FAPI PET/CT scanning and underlining the importance of collaboration for pathology assessment. In light of the increasing interest and research the past years, these findings need to be considered when FAPI as radiopharmaceutical in PET/CT scanning is implemented to improve CRC staging, emphasizing the need for a multidisciplinary approach.

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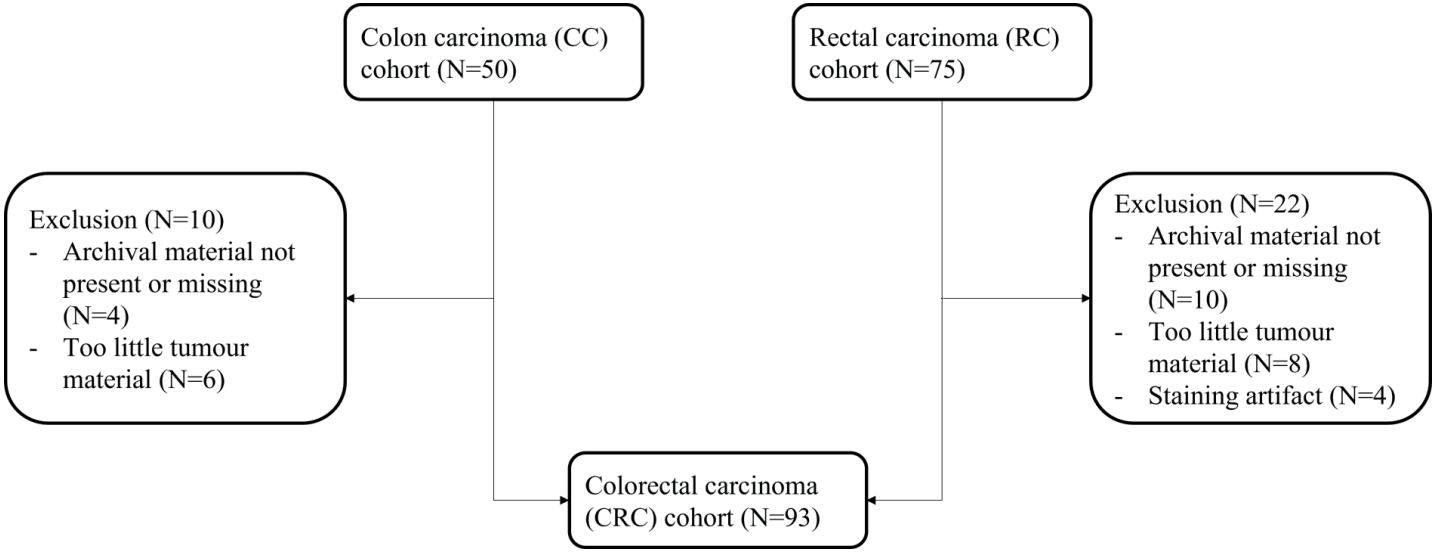
Supplementary Material



Supplementary Figure 1. Flowchart of ultimately included patients in separate and combined cohorts.

Supplementary Table 1. Baseline characteristics of the complete CRC cohort, and the CC and RC cohorts shown separately

Characteristic (unit)	Total CRC cohort (N=93)	CC cohort (N=40)	RC cohort (N=53)
Sex			
Male	57 (61)	22 (55)	35 (66)
Female	36 (39)	18 (45)	18 (34)
Age at diagnosis – years			
Median age (range)	67 (39-95)	70 (39-91)	67 (45-95)
Age ≥75 years old	26 (28)	13 (33)	13 (25)
Diagnosis year			
Median (range)	2008 (2000-2016)	2005 (2000-2011)	2013 (2005-2016)
Median period until surgery (weeks)	6 (0-29)	2 (0-11)	7 (0-29)
Clinical TNM-stage*			
cTNM-stage I	1 (1)	0 (0)	1 (2)
cTNM-stage II	29 (31)	13 (33)	16 (30)
cTNM-stage II/III**	20 (22)	19 (48)	1 (2)
cTNM-stage III	43 (46)	8 (20)	35 (66)
cTNM-stage IV	0 (0)	0 (0)	0 (0)
Treatment			
Neoadjuvant therapy and surgery	41 (44)	0 (0)	41 (77)
Surgery alone	28 (30)	17 (43)	11 (21)
Neoadjuvant, surgery and adjuvant therapy	0 (0)	0 (0)	0 (0)
Surgery and adjuvant therapy	24 (26)	23 (57)	1 (2)
Operation setting			
Elective	81 (87)	28 (70)	53 (0)
Emergency***	12 (13)	12 (30)	0 (0)
Location tumour****			
Right-sided colon	24 (26)	24 (60)	N/A
Left-sided colon	15 (16)	15 (38)	N/A
Rectosigmoid	2 (2)	1 (2)	1 (2)
High rectum (>10cm anal verge)	15 (16)	N/A	15 (28)
Mid rectum (5-10cm anal verge)	16 (17)	N/A	16 (30)
Low rectum (<5cm anal verge)	21 (23)	N/A	21 (40)
Operation type			
Subtotal colectomy	3 (3)	3 (8)	0 (0)
Hemicolectomy right (also extended)	21 (21)	21 (53)	0 (0)
Transversectomy	2 (2)	2 (5)	0 (0)
Hemicolectomy left	5 (5)	5 (13)	0 (0)
Sigmoidectomy, Hartmann	9 (10)	8 (20)	1 (2)
Low anterior resection	33 (36)	1 (3)	32 (60)
Abdominal perineal resection	20 (22)	0 (0)	20 (38)
Disease-free survival – years			
Median survival (range)	5.1 (0.1-21.5)	4.6 (0.2-21.5)	5.3 (0.1-17.8)

(continued) **Characteristic (unit)**	**Total CRC cohort (N=93)**	**CC cohort (N=40)**	**RC cohort (N=53)**
No event occurred	43 (46)	14 (35)	29 (55)
Events, of which type	50 (54)	26 (65)	24 (45)
Locoregional recurrence	3 (6)	1 (4)	2 (8)
Distant metastasis	23 (46)	12 (46)	11 (46)
Death	24 (48)	13 (50)	11 (46)
Overall survival – years			
Median survival (range)	7.4 (0.4-23.1)	7.3 (0.5-23.1)	7.4 (0.4-18.0)
Overall survival status			
Alive	46 (50)	15 (38)	31 (59)
Dead, due to the cause of	47 (51)	25 (63)	22 (42)
Current colorectal carcinoma	22 (47)	11 (46)	11 (48)
Other, e.g. comorbidity	18 (38)	9 (38)	9 (39)
Unknown	7 (15)	4 (17)	3 (13)

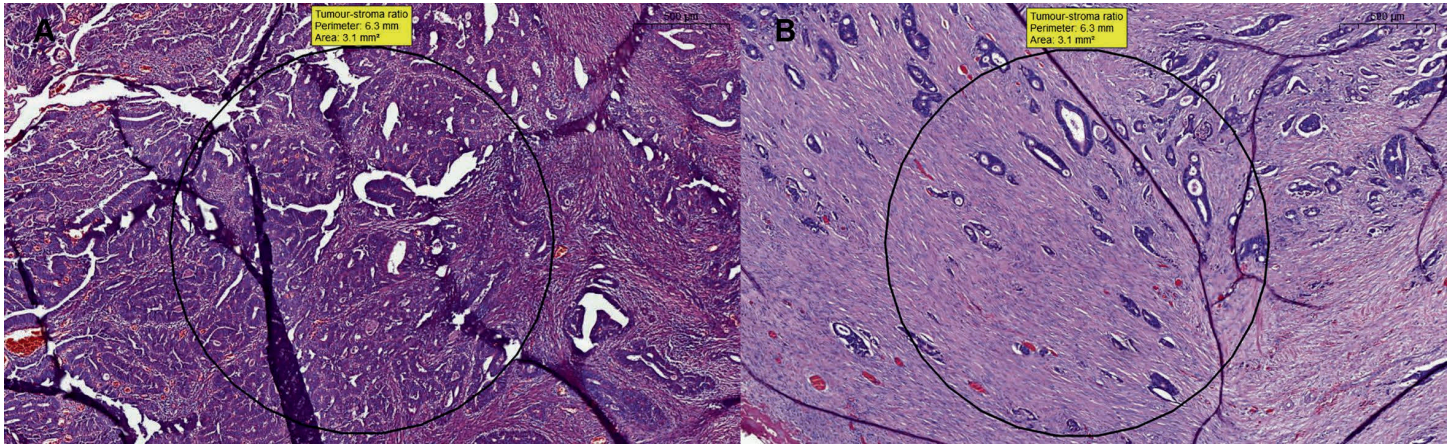
All variables are given as absolute numbers with associated percentages or medians with ranges (minimum-maximum). Sum of percentages can be less or more than 100 due to rounding. CC, colon carcinoma; N/A, not applicable; RC, rectal carcinoma; TNM, tumour-node metastasis.

*Different versions of the TNM classification were used, here all variables are converted to the AJCC/UICC TNM version 5 (1997).

**Stage II/III is given in case no imaging is performed and locoregional extent of disease is uncertain.

***Emergency setting includes the presenting of patients with obstructive ileus and e.g. during operation a tumour is found.

****Location is determined through imaging primarily. A right-sided tumour is defined as a colon carcinoma in the coecum, colon ascendens, flexura hepatica or colon transversum until the fluxura lienalis.



Supplementary Figure 2. Zoomed in examples of the tumour-stroma ratio annotations on haematoxylin and eosin-stained slides in colorectal cancer patients. (A) Stroma-low (≤50%); (B) Stroma-high (>50%). General overview 5.0x magnification, annotation 10.0x magnification.

Supplementary Table 2. Comparison of biopsy-scored stroma-low and stroma-high tumours within the RC cohort

Characteristic (unit)	RC cohort (N=53)		
	Stroma-low (biopsy; N=30)	**Stroma-high (biopsy; N=23)**	**P-value**
FAP expression – biopsy			
No expression	4 (13)	2 (9)	0.814#
Any expression (heterogeneous - high expression)			
Heterogeneous expression	15 (50)	11 (48)	
Little expression (no - heterogeneous expression)	19 (63)	13 (57)	0.615#
Moderate to high expression	11 (37)	10 (44)	
Treatment type – all treatments			
TSR – PT			0.053#
Stroma-low	13 (43)	3 (13)	
Stroma-high	16 (53)	18 (78)	
N/A (no tumour)	1 (3)	2 (9)	
FAP expression – PT			0.712#
Little expression (no - heterogeneous expression)	21 (70)	15 (65)	
Moderate to high expression	9 (30)	8 (35)	
Representative LN*			
N/A (missing, no LN positive)	18 (60)	14 (61)	
Positive LN, of which	12 (40)	9 (39)	
TSR			0.830#
Stroma-low	11 (92)	8 (89)	
Stroma-high	1 (8)	1 (11)	
FAP expression – LN*			0.055#
Little expression (no - heterogeneous expression)	1 (8)	4 (44)	
Moderate to high expression	11 (92)	5 (56)	
Treatment type – no neoadjuvant treatment			
No neoadjuvant treatment received	10 (33)	2 (9)	
TSR – PT**			**0.007#**
Stroma-low	9 (90)	0 (0)	
Stroma-high	1 (10)	2 (100)	
FAP expression – PT			0.584#
Little expression (no - heterogeneous expression)	7 (70)	1 (50)	
Moderate to high expression	3 (30)	1 (50)	
Representative LN*			
N/A (missing, no LN positive)	9 (90)	1 (50)	
Positive LN, of which	1 (10)	1 (50)	
TSR			N/A
Stroma-low	0 (0)	0 (0)	
Stroma-high	1 (100)	1 (100)	

(continued)			N/A
FAP expression – LN*			
Little expression (no - heterogeneous expression)	1 (100)	1 (100)	
Moderate to high expression	0 (0)	0 (0)	
Treatment type – neoadjuvant treatment* **			
Neoadjuvant treatment received	20 (67)	21 (91)	
TSR – PT			0.785#
Stroma-low	4 (20)	3 (14)	
Stroma-high	15 (75)	16 (76)	
N/A (no tumour)	1 (5)	2 (10)	
FAP expression – PT			0.819#
Little expression (no - heterogeneous expression)	14 (70)	14 (67)	
Moderate to high expression	6 (30)	7 (33)	
Representative LN*			
N/A (missing, no positive LN)	9 (45)	13 (62)	
LN positive, of which	11 (55)	8 (38)	
TSR			N/A
Stroma-low	11 (100)	8 (100)	
Stroma-high	0 (0)	0 (0)	
FAP expression – representative LN*			0.027#
Little expression (no - heterogeneous expression)	0 (0)	3 (38)	
Moderate to high expression	11 (100)	5 (63)	

All variables are given as absolute numbers with associated percentages. Sum of percentages can be less or more than 100 due to rounding. FAP, fibroblast activation protein; LN, lymph node; N/A, not applicable; PT, primary tumour; RC, rectal carcinoma; TSR, tumour-stroma ratio.

*Representable lymph node is the lymph node with most tumour stroma.

**The TSR scored in biopsies is representable for the primary tumour TSR, as seen in literature and here proven in not neoadjuvantly treated tumours.

***Neoadjuvant treatment includes either short course radiotherapy (SCRT, 5x5 Gray) (N=30) or chemoradiation (CRT, 25x2/28x1.2 Gray + capecitabine monotherapy) (N=11).

#Calculated with the Chi-square test.

Supplementary Table 3. Comparison of the tumours with stroma-low and stroma-high lymph node metastases within the CRC and separate CC and RC cohorts.

Characteristic (unit)	CRC cohort (N=61)				CC cohort (N=40)				RC LN cohort (N=21)			
	All stage III patients (N=61)	Stroma-low LN patients* (LN, N=41)	Stroma-high LN patients* (N=20)	P-value	All stage III patients (N=40)	Stroma-low LN patients* (N=22)	Stroma-high LN patients* (N=18)	P-value	All stage III patients (N=21)	Stroma-low LN patients* (N=19)	Stroma-high LN patients* (N=2)	P-value
All LN analysed												
Total LN and tumour deposits scored, of which	288 (100)	169 (100)	119 (100)		189 (66)	76 (40)	113 (60)		99 (34)	93 (94)	6 (6)	
Mean total examined LN (SD)	*5 (4)*	*4 (3)*	*6 (5)*	*0.151$*	*5 (4)*	*4 (3)*	*6 (5)*	*0.049$*	*2 (3)*	*5 (4)*	*3 (0)*	*0.048$*
Total LN with metastasis (tumour-positive)	*125 (43)*	*78 (46)*	*47 (39)*		*77 (41)*	*34 (45)*	*44 (39)*		*47 (47)*	*44 (47)*	*3 (50)*	
Mean LN tumour-positive (SD)	*2 (2)*	*2 (1)*	*2 (2)*	*0.397$*	*2 (2)*	*2 (1)*	*2 (2)*	*0.122$*	*2 (2)*	*2 (2)*	*2 (1)*	*0.304$*
Total LN without metastasis (tumour-negative)	*163 (57)*	*91 (54)*	*72 (61)*		*112 (59)*	*42 (55)*	*69 (61)*		*52 (53)*	*49 (53)*	*3 (50)*	
Mean LN tumour-negative (SD)	*3 (3)*	*2 (3)*	*4 (3)*	*0.109$*	*3 (3)*	*2 (3)*	*4 (3)*	*0.051$*	*3 (3)*	*3 (3)*	*2 (1)*	*0.243$*
FAP expression in LN												
All LNs analysed, of which	288 (100)	169 (100)	119 (100)		189 (100)	76 (100)	113 (100)		99 (100)	93 (100)	6 (100)	
Total LN with any FAP expression (FAP-positive)	112 (39)	75 (46)	37 (31)		53 (28)	19 (25)	34 (30)		59 (60)	56 (60)	3 (50)	
Mean all LNs FAP-positive (SD)	2 (2)	2 (2)	2 (2)	0.968$	1 (2)	1 (1)	2 (2)	0.050$	3 (2)	3 (2)	2 (1)	0.114$
LN tumour-positive and FAP-positive	87 (78)	56 (75)	31 (84)		45 (85)	17 (89)	28 (82)		42 (71)	39 (70)	3 (50)	
Mean LN tumour-positive and FAP-positive (SD)	1 (1)	1 (1)	2 (2)	0.649$	1 (1)	1 (1)	2 (2)	0.074$	2 (1)	2 (2)	2 (1)	0.453$
LN tumour-negative, but FAP-positive (e.g. FAP expression in healthy lymphoid tissue)	25 (22)	19 (25)	6 (16)		8 (15)	2 (11)	6 (18)		17 (29)	17 (30)	0 (0)	
Mean LN tumour-negative, but FAP-positive (SD)	0 (1)	0 (1)	0 (1)	0.442$	0 (0)	0 (0)	0 (0)	0.127$	1 (1)	1 (2)	0 (0)	0.015$

(continued)												
Total LN without FAP expression (FAP-negative)	176 (61)	94 (56)	82 (69)		136 (72)	57 (75)	99 (88)		40 (40)	37 (40)	3 (50)	
Mean all LNs FAP-negative (SD)	3 (3)	2 (2)	4 (4)	0.059$	3 (3)	3 (3)	4 (4)	0.099$	2 (2)	2 (2)	2 (1)	0.571$
LN tumour-negative and FAP-negative	138 (78)	72 (77)	66 (80)		103 (76)	40 (70)	63 (64)		35 (88)	32 (86)	3 (50)	
Mean LN tumour-negative and FAP-negative (SD)	2 (3)	2 (2)	3 (3)	0.054$	3 (3)	2 (2)	4 (3)	0.070$	2 (2)	2 (2)	2 (1)	0.811$
LN tumour-positive, but FAP-negative	38 (22)	22 (23)	16 (20)		33 (24)	17 (30)	16 (16)		5 (13)	5 (14)	0 (0)	
Mean LN tumour-positive, but FAP-negative (SD)	1 (1)	1 (1)	1 (1)	0.369$	1 (1)	1 (1)	1 (1)	0.740$	0 (1)	0 (1)	0 (0)	0.056$
Intraindividual FAP expression												
Tumour-positive LN analysed, patients**	61 (100)	41 (67)	20 (33)	0.799$	40 (66)	22 (55)	18 (45)	0.433$	21 (34)	19 (90)	2 (10)	0.113$
Mean percentage LNs tumour-positive and FAP-positive (SD)	69 (42)	70 (43)	67 (42)		57 (46)	52 (48)	63 (43)		91 (23)	91 (24)	100 (0)	
Mean percentage LNs tumour-positive and FAP-negative (SD)	31 (42)	30 (43)	33 (42)		43 (46)	48 (48)	37 (43)		8 (23)	9 (24)	0 (0)	
Tumour-negative LN analysed, patients**	43 (72)	26 (60)	17 (40)	0.099$	27 (63)	12 (44)	15 (66)	0.413$	16 (37)	14 (87)	2 (13)	0.013$
Mean percentage LNs tumour-negative and FAP-positive (SD)	14 (29)	19 (35)	7 (13)		6 (12)	4 (10)	7 (14)		29 (41)	33 (42)	0 (0)	
Mean percentage LNs tumour-negative and FAP-negative (SD)	87 (28)	81 (35	93 (13)		94 (12)	96 (10)	93 (14)		71 (41)	67 (42)	100 (0)	

All variables are given as absolute numbers with associated percentages or means with SD. Sum of percentages can be less or more than 100 due to rounding. CC, colon carcinoma; FAP, fibroblast activation protein; LN, lymph node; N/A, not applicable; PT, primary tumour; RC, rectal carcinoma; SD, standard deviation; TSR, tumour-stroma ratio.

*Representable lymph node is the lymph node with most tumour stroma.

**Not all patients had negative lymph nodes which were examinable or available.

#Calculated with the Chi-square test.

$Calculated with Independent Samples T-test, equal variances not assumed.