Towards implementation of the tumour-stroma ratio in colorectal cancer

Polack, M.

2

Chapter 2

Results from the UNITED study: a Multicentre Validation Study of the Tumour-Stroma Ratio in Colon Carcinoma

M. Polack, M.A. Smit, G.W. van Pelt, A.G.H. Roodvoets, E. Meershoek-Klein Kranenbarg, H. Putter, H. Gelderblom, A.S.L.P. Crobach, V. Terpstra, G. Petrushevska, G. Gašljević, S. Kjær-Frifeldt, E.M.V. de Cuba, N.W.J. Bulkmans, G.R. Vink, R. Al Dieri, R.A.E.M. Tollenaar, J.H.J.M. van Krieken, W.E. Mesker, and the UNITED collaboration

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Abstract

Background: The TNM Evaluation Committee (UICC) and College of American Pathologists (CAP) recommended to prospectively validate the cost-effective and robust tumour-stroma ratio (TSR) as independent prognostic parameter, as high intratumour stromal percentages have previously predicted poor patient-related outcomes.

Patients and methods: The UNITED study enrolled patients in 27 participating centers in 12 countries worldwide. The TSR, categorized as stroma-high (>50%) or stroma-low (≤50%), was scored through standardized microscopic assessment by certified pathologists, and effect on disease-free survival was evaluated with 3-year median follow-up. Secondary endpoints were benefit assessment of adjuvant chemotherapy and overall survival.

Results: A total of 1,537 patients were included, with 1,388 eligible stage II/III patients curatively operated between 2015-2021. Disease-free survival was significantly shorter in stroma-high (*N*=428) than in stroma-low patients (*N*=960) (3-year rates 70% vs. 83%; *P*<0.001). In multivariate analysis, TSR remained an independent prognosticator for disease-free survival (*P*<0.001, hazard ratio 1.49, 95% confidence interval 1.17–1.90). As secondary outcome, disease-free survival was also worse in stage II and III stroma-high patients despite adjuvant treatment (3-year rates stage II 73% vs. 92% and stage III 66% vs. 80%; *P*=0.008 and *P*=0.011, respectively). In stage II patients not receiving adjuvant chemotherapy (*N*=322), the TSR outperformed the ASCO-criteria in identifying patients at risk of events (event rate 21% vs. 9%), with a higher discriminatory 3-year disease-free survival rate (stroma-high 80% vs. ASCO high-risk 91%). A trend towards worse 5-year overall survival in stroma-high was noticeable (74% vs. 83% stroma-low; *P*=0.102).

Conclusion: The multicentre UNITED study unequivocally validates the TSR as independent prognosticator, confirming worse outcomes in stroma-high patients. The TSR improved current selection criteria for patients at risk of events, and stroma-high patients potentially experienced chemotherapy resistance. TSR implementation in international guidelines is highly recommended as aid in personalized treatment.

Trial Registration:

Dutch Trial Register NL7072; https://clinicaltrialregister.nl/en/trial/23560

International Registered Report Identifier (IRRID): DERR1-10.2196/13464

Introduction

Current treatment guidelines for colon cancer are traditionally based on extent of disease, expressed through the tumour-node-metastasis (TNM) classification, as well as risk assessments for patient outcome and expected benefits of adjuvant chemotherapy (ACT) [1-6]. However, the prognostic capacity of TNM staging remains suboptimal. Overtreatment, when patients do not or barely benefit from their ACT, as well as undertreatment, when patients actually could have benefited from additional treatment to prevent recurrences, therefore still occur at high rates [2, 3]. This supports the clinical need to improve individualized ACT indications upfront through additional prognostic biomarkers. Many pathological parameters have been discovered and implemented in guidelines, like tumour differentiation grade, tumour budding, or microsatellite instability (MSI) status [2, 3, 7, 8]. However, these mainly focus on the tumour epithelial compartment.

The past decades, the tumour stroma, a major component within the tumour microenvironment, emerged as an important influencer herein [9-14]. Abundance of intratumoural stroma has been demonstrated to lead to worse patient-related outcomes [15, 16]. The tumour-stroma ratio (TSR) is a histopathological parameter based on the amount of stroma expressed in percentages compared to the tumour epithelial component, and was initially developed in colon cancer, but has repeatedly been shown to be of prognostic value for almost all epithelial cancers. Patients with stroma-high tumours, i.e. >50% stroma, have a worse disease-free and overall survival (DFS and OS, respectively) than patients with stroma-low tumours, i.e. ≤50% stroma [17-21].

Implementation of the TSR in international guidelines and pathology diagnostics was advocated to the TNM Evaluation Committee, Union for International Cancer Control (UICC), and College of American Pathologists (CAP). Although these instances acknowledged the high potential of the TSR as prognostic parameter, validation was advised, including consensus on scoring of the TSR. Therefore, the present "Uniform Noting for International application of Tumour-stroma ratio as Easy Diagnostic tool" (UNITED) prospective multicentre study was initiated [22].

Herein, we hypothesized that patients with stroma-high tumours will have worse outcomes compared to patients with stroma-low tumours. Our primary endpoint was to determine the influence of the TSR on DFS, and secondary endpoints were influence of TSR on benefit of ACT, and on OS. The added value of the TSR in clinical treatment decision making could be, based on this prognostic information, to select patients with stage II stroma-high tumours for ACT, whereas the older patient with comorbidity

and a stage III stroma-low tumour could potentially be spared a burdensome and costly treatment. Validation of the TSR will result in unequivocally high-level evidence to accomplish implementation in international guidelines, aiding in shared-decision making through improved personalized treatment. Through this UNITED study, we aimed to validate the prognostic effect of the TSR in colon cancer patients.

Material and methods

Patients

The UNITED study was an investigator-driven, prospective, observational multicentre cohort study, enrolling patients in 27 centres from 12 countries. Approved and contracted centres could only start including after participating pathologists were certified through the official UNITED-study E-learning [23]. Coordination, including contract and database management, quality control and overall support, was done by the Clinical Research Center from the Leiden University Medical Centre (LUMC).

Patients ≥18 years of age with pathological stage II/III colon carcinoma and who had undergone a complete curative resection (R0) of their primary tumour were eligible. Patients were excluded in case of receiving neoadjuvant treatment, rectal cancer, multiple synchronous tumours, previous malignancies ≤10 years prior to the current cancer (except basal cell cancer or cervical cancer in situ) or any colon cancer in their medical history. Postoperative exclusion criteria were pathological stage I or IV and mortality within three months (Supplementary Table 1).

A sample size calculation was performed previously [15, 16]. For a 3-year median follow-up period, 114 and 97 recurrences were necessary for sufficient power for stage II and III colon cancer, respectively, requiring 722 and 450 evaluable patients in both groups. To obtain this minimum of 1,172 inclusions, approximately 1,500 patients (+25%) were to be registered in total, as inclusions in prospective cohort studies could ultimately be ineligible [22].

Materials and tumour-stroma analysis

Diagnostic haematoxylin-and-eosin (H&E)-stained slides of included patients, on which the T-stage of the tumour was already determined, were also used for scoring the TSR through conventional

microscopy. All participating pathologists were trained through the official UNITED study E-learning [23, 24]. This quality-controlled E-learning was supported by the European Society of Pathology (ESP) with official consensus on TSR scoring. High Cohen's interobserver agreement kappa's of >0.70 (at least substantial agreement) were previously observed, proving the reliability and efficiency in teaching the TSR scoring method, also long term [23]. The stromal percentage was scored on these H&E-stained slides according to the established method of van Pelt *et al.* [24], per 10 percent increments. Subsequently, categorization using the predefined cut-off value of 50% resulted in stroma-low (≤50%) and stroma-high (>50%) groups, similar to multiple previous studies [15, 20, 24] (Supplementary Figure S1).

Statistical analysis

DFS was defined as time between date of surgery and date of first event, i.e. recurrence (locoregional recurrence or distant metastasis) or death (by any cause). In the case of no event, DFS was calculated from date of surgery until censoring. Although accurate interpretation is only possible after five years, we also analyzed the preliminary effect of TSR on OS. OS was defined as time from date of surgery until date of death (by any cause) or until censoring. Censoring took place when patients were disease-free and/or alive at their last follow-up appointment.

Influence of TSR on benefit of ACT was assessed through comparison of TSR categories on DFS with treatment. As ACT is not routinely recommended in stage II colon cancer, the American Society of Clinical Oncology (ASCO)-criteria can be used to select those at high risk for events like recurrences, and thus could potentially benefit from this treatment: the stage II-HR. A pT4-tumour is deemed the most important ASCO risk criterium and commonly used in the Netherlands, but also sampling of <12 lymph nodes or emergency operation setting, presence of pathological risk factors like lymphovascular or perineural invasion (LVI and PnI, respectively), and poor tumour differentiation are risk factors [3]. First, for both pathological stages separately to minimize bias, influence of TSR will be determined in those receiving ACT to assess potential benefit. Subsequently, recurrence rates will be assessed for the TSR compared to ASCO criteria. To facilitate comparison and grouping of patients, all (sub)stages were recoded to the TNM-5 classification.

Statistical analysis was performed using the Chi-square test between ordinal or nominal variables. Through reversed Kaplan-Meier analysis, median follow-up time was calculated. Survival analyses were performed using Kaplan-Meier analysis with log-rank tests, and associated number needed to treat tables were added. Hazard ratio's (HR) with associated 95% confidence intervals (CI) were calculated

with the Cox proportional hazard model, using significant variables (P<0.05) from the univariate analysis for the multivariate analysis.

All continuous variables were expressed in medians with interquartile ranges (IQR), whereas nominal and ordinal variables were stated as number of frequencies and corresponding percentages. Two-tailed P-values <0.05 were considered statistically significant. Statistical analysis was performed in collaboration with the Department of Biomedical Data Sciences of the LUMC using IBM SPSS Statistics (version 29.0) and figures with GraphPad Prism (version 9.3.1).

Data storage

The Clinical Research Center coordinated data storage of the UNITED study, through the worldwide used and highly secured cloud-based platform Castor Electronic Data Capture (Castor EDC; Castor, Amsterdam, The Netherlands) [25]. Collection and supply of electronic Case Report Forms, central monitoring, quality control and generation of queries within the Castor database was performed by the Clinical Research Center, leading to high quality and reusable data. As per protocol, all data and documents are stored for a minimum of 15 years.

Ethical considerations

The UNITED study protocol was approved by the Medical Research Ethics Committee (MREC) of LUMC. All participating centres had their local MREC approve the protocol before inclusion could commence. In the Netherlands, centres were contracted through the Prospective Dutch ColoRectal Cancer cohort (PLCRC) [26, 27]. Patients from this prospective registration study, fulfilling the UNITED study eligibility criteria and treated in one of the participating centres from 2015 onward, were included. The workflow for retrieval of data through PLCRC is illustrated in Supplementary Figure S2. PLCRC explicitly included the possibility of a cohort-within-a-cohort format in their study design; patients signed broad official informed consent forms for the use of their histopathological data by other studies [27]. The UNITED study was conducted according to the Declaration of Helsinki (2013).

Results

Between January 8th, 2019 and September 9th, 2022, a total of 1,537 patients were registered. An overview of inclusion numbers per participating centre is provided in Supplementary Table 2. Baseline characteristics of all study patients are added in Supplementary Table 3. Due to the in part prospective nature of the study, 31 patients (2%) were ineligible at registration, e.g. based on medical history with another malignancy ≤10 years of their current colon cancer (N=26). Exclusion followed subsequently in 83 (5%) patients, due to presence of multiple tumours (N=21) or other pathology exclusions like pathological stage 0 – I (N=42) or IV colon cancer (N=9). Lastly, 35 (2%) were excluded during follow-up, mostly caused by postoperative mortality within 3 months (N=26).

In total, 1,388 stage II/III colon cancer patients were included in the final analysis (Figure 1). Of these, 770 patients (55%) were of male sex, and 453 patients were aged ≥75 years (33%). In 1,210 (87%) cases, a preoperative endoscopic biopsy was taken, highly indicative of an absence of emergency setting and indicating an elective operation. A total of 723 patients (52%) had stage II colon cancer. The tumour was categorized as stroma-high, i.e. >50% stroma, in 428 patients (31%), conform previous literature. Patient characteristics of the eligible cohort are summarized in Table 1.

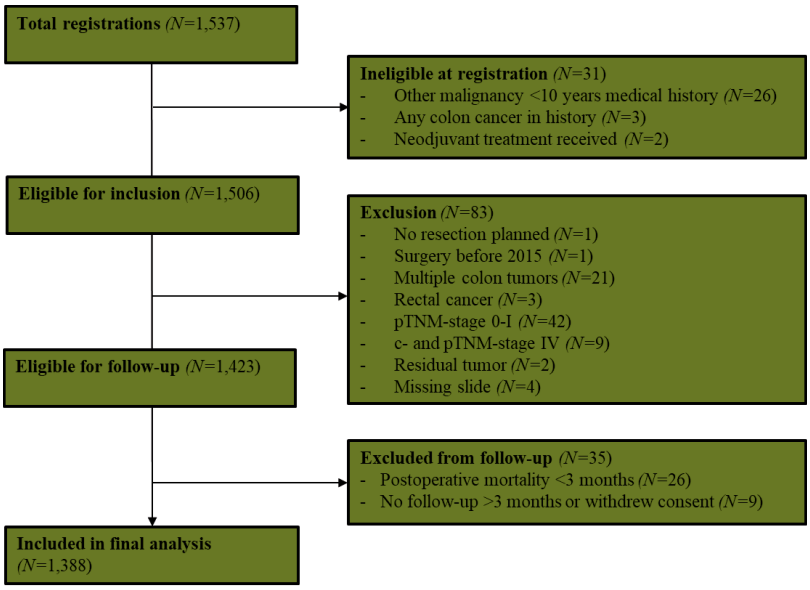


Figure 1. CONSORT diagram of the UNITED cohort. CONSORT, Consolidated Standards of Reporting Trials; UNITED, Uniform Noting for International application of Tumour-stroma ratio as Easy Diagnostic tool.

Table 1. Baseline characteristics of the eligible patients in the UNITED cohort.

Baseline characteristics	**UNITED cohort (N=1,388)**
Sex	
Female	618 (45)
Male	770 (55)
Age at surgery	
Median age (years)	69 (61 – 77)
≥75 years of age	453 (33)
Biopsy taken	
Yes, preoperative endoscopy	1,210 (87)
Yes, other method*	13 (1)
No**	165 (12)
Surgery	
Surgery year	2019 (2018 – 2020)
Pathological stage	
Stage II	723 (52)
Stage III	665 (48)
Lymph nodes	
Examined *(in total group)*	20 (15 – 28)
Positive *(in pTNM-stage III)***	2 (1 – 4)
Tumour-stroma ratio	
Stroma-low (≤50%)	960 (69)
Stroma-high (>50%)	428 (31)

All variables are given as absolute numbers with associated percentages or medians with interquartile ranges. Sum of percentages can be less or more than 100 due to rounding. TNM, tumour-node-metastasis stage.

*Other methods for biopsy are e.g. during surgery.

**Reasons why biopsy was not taken, is e.g. in emergency setting (obstructive ileus).

***Using the Union for International Cancer Control (UICC) TNM version 8, a tumour deposit (leading to stage N1c) will also lead to a pTNM-stage III, also when there are no positive lymph nodes.

Demographics, surgery type, tumour morphology and differentiation grade were equally distributed amongst stroma-low (N=960) and stroma-high (N=428) groups. Stroma-low tumours were more often right-sided (P=0.049) and had more often <12 lymph nodes sampled (P<0.001), however, in MSI or mismatch repair (MMR) analysis (MSI/MMR), stroma-low tumours were also more prone to MSI or MMR deficiency (MSI/dMMR; P=0.012). Stroma-high tumours ultimately had more risk factors, as these were more often stage III (P<0.001), pT4-stage (P<0.001), higher pN-stage (P<0.001), and more

often pathology risk factors like extramural venous invasion (EMVI) were present (P<0.001), illustrating their aggressiveness (Table 2). In merely a small subset (N=153, 11%), mutational status like KRAS or BRAF was determined, which was not significantly associated to TSR (P=0.150; Supplementary Table 4).

Table 2. Analysis of the variables of surgery, pathology and adjuvant chemotherapy in the eligible UNITED cohort, stroma-low compared to the stroma-high patients.

Variables (unit)	**Stroma-low (N=960)**	**Stroma-high (N=428)**	**P-value**
Sex			0.385#
Female	420 (44)	198 (46)	
Male	540 (56)	230 (54)	
Age at surgery - older category			0.185#
<75 years of age	636 (66)	299 (70)	
≥75 years of age	324 (34)	129 (30)	
Biopsy taken			0.530#
Yes	843 (88)	380 (89)	
No*	117 (12)	47 (11)	
Surgery type			0.309ǂ
Hemicolectomy right	435 (45)	176 (41)	
Hemicolectomy left	121 (13)	59 (14)	
Sigmoidectomy	277 (29)	136 (32)	
Other**	127 (13)	57 (13)	
Tumour-sidedness***			**0.049#**
Right-sided tumour	473 (49)	186 (44)	
Left-sided tumour	487 (51)	241 (56)	
Missing	0 (0)	1 (0)	
Lymph nodes			**0.004#**
Lymph nodes <12 examined	117 (12)	30 (7)	
Lymph nodes ≥12 examined	843 (88)	398 (93)	
Pathological tumour (pT)-stage****			**<0.001#**
pT1-3	810 (84)	305 (71)	
pT1	*10 (1)*	*1 (0)*	
pT2	*61 (6)*	*6 (1)*	
pT3	*739 (77)*	*298 (70)*	
pT4	150 (16)	123 (29)	
Pathological nodal (pN)-stage****			**<0.001#**
pN0	541 (56)	182 (43)	
pN1	292 (30)	152 (36)	
pN2	127 (13)	94 (22)	

(continued) **Variables (unit)**	**Stroma-low (N=960)**	**Stroma-high (N=428)**	**P-value**
Tumour morphology			
Adenocarcinoma	855 (89)	390 (91)	
Mucinous carcinoma	90 (9)	33 (8)	
Other, including signet cell carcinoma	15 (2)	5 (1)	
Differentiation grade*****			0.062#
Well-Moderate	809 (84)	378 (88)	
Poor-Undifferentiated	125 (13)	41 (10)	
Grade cannot be assessed	26 (3)	9 (2)	
Pathology risk factors******			**<0.001#**
No pathology risk factors present	566 (59)	197 (46)	
Presence of ≥1 pathology risk factor	394 (41)	231 (54)	
Extramural venous invasion (EMVI)			
Not reported	162 (17)	55 (13)	
Reported, of which	798 (83)	373 (87)	**<0.001#**
Yes (EMVI+)	86 (9)	98 (23)	
No (EMVI-)	712 (74)	275 (64)	
Venous invasion			
Not reported	99 (10)	56 (13)	
Reported, of which	861 (90)	372 (87)	**<0.001#**
Yes (V1)	100 (12)	69 (19)	
No (V0)	761 (88)	303 (81)	
Lymphatic invasion			
Not reported	28 (3)	20 (5)	
Reported, of which	932 (97)	408 (95)	0.193#
Yes (L1)	304 (33)	148 (36)	
No (L0)	628 (67)	260 (64)	
Perineural invasion			
Not reported	423 (44)	186 (44)	
Reported, of which	537 (56)	242 (57)	**<0.001#**
Yes (Pn1)	68 (13)	58 (24)	
No (Pn0)	469 (87)	184 (43)	
Microsatellite instability/Mismatch repair (MMR) status			
Not determined	439 (46)	213 (50)	
Determined, of which	521 (54)	215 (50)	**0.012#**
Microsatellite stable (MSS)/MMR proficient (pMMR)	403 (77)	184 (86)	
Microsatellite instable (MSI)/MMR deficient (dMMR)	118 (23)	31 (14)	
Pathological TNM-stage			**<0.001#**
Stage II	541 (56)	182 (43)	
Stage III	419 (44)	246 (57)	

(continued) **Variables (unit)**	**Stroma-low (N=960)**	**Stroma-high (N=428)**	**P-value**
Adjuvant chemotherapy - received			**<0.001#**
No	539 (56)	174 (41)	
Yes	421 (44)	254 (59)	
Adjuvant chemotherapy – per pathological TNM-stage			**<0.001#**
Stage II + No adjuvant therapy	434 (45)	125 (29)	
Stage II + Adjuvant therapy	107 (11)	57 (13)	
Stage III + Adjuvant therapy	314 (33)	197 (46)	
Stage III + No adjuvant therapy	105 (11)	49 (11)	

All variables are given as absolute numbers with associated percentages or medians with interquartile ranges. Sum of percentages can be less or more than 100 due to rounding. N/A, not applicable; TNM, tumour-node-metastasis stage.

*Reasons why biopsy was not taken, is e.g. in emergency setting (obstructive ileus).

**Other operation types include a (sub)total colectomy, high anterior resection, or transversectomy.

***A right-sided tumour is defined as a colon carcinoma in the coecum, colon ascendens, flexura hepatica or colon transversum.

****Different versions of the Union for International Cancer Control (UICC) TNM classification were used, here all variables are converted to the UICC TNM version 5 (1997).

*****Differentiation grade is variously registered as separate or combined subgroups, this is than categorized in combined grades, i.e. well – moderate or poor – undifferentiated.

******Pathology risk factors are stated below, presence of a risk factor is defined as at least one of registered risk factors.

Absence is the absence of registered risk factors, as not all risk factors are registered.

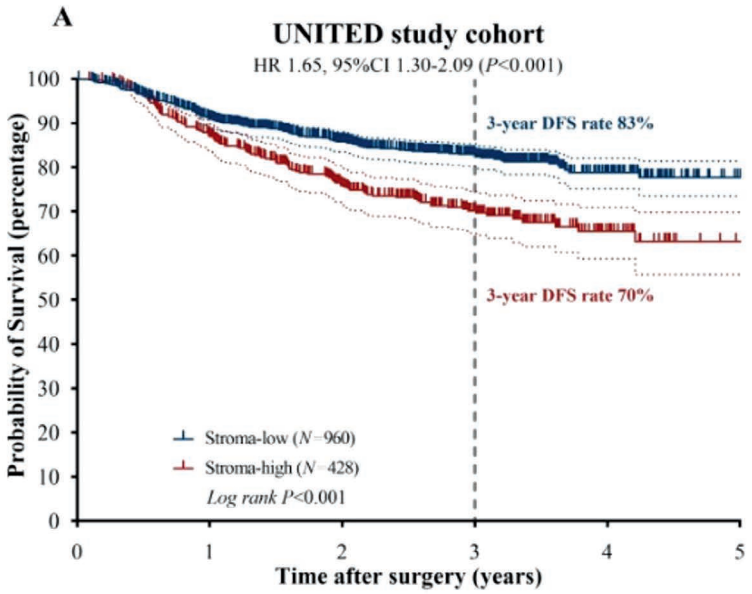
*******Multiple mutations can occur simultaneously, hence the number of added percentages can be higher than 100.

Calculated with the Chi-square test.

ǂ Calculated using the Chi-square test for the three most common and here presented operation types:

hemicolectomy right, hemicolectomy left and sigmoidectomy.

Median follow-up time was 36.2 months (95%CI 35.9 – 36.5) at time of database lock (January 31st, 2023), and comparable between both groups (P=0.469) (Supplementary Figure S3). Generally, follow-up was according to daily clinical practice, differing per country and centre, but approximately at 6, 12 and 24 – 36 months postoperatively. A total of 286 events occurred, of which 123 in the stroma-high group (29% of stroma-high patients; P<0.001). Mostly, this concerned distant metastases (92 stroma-high patients, 75% of events) (Supplementary Table 4). Hence, a statistically significantly worse DFS was observed in stroma-high patients with 3-year DFS rates of 70%, compared to 83% in stroma-low patients (HR 1.78, 95%CI 1.41 – 2.26; P<0.001; Figure 2).



Number at risk* (number censored)						
Stroma-low	960 (15)	860 (163)	654 (204)	431 (310)	109 (77)	31 (30)
Stroma-high	427 (8)	364 (65)	257 (89)	151 (103)	43 (29)	13 (13)

Figure 2. Disease-free survival effect of TSR in the UNITED cohort and subgroup analyses. A) Kaplan-Meier analysis and log rank test showing worse 3-year disease-free survival rates for stroma-high patients in the whole UNITED cohort (70 vs. 83%, respectively; P<0.001)

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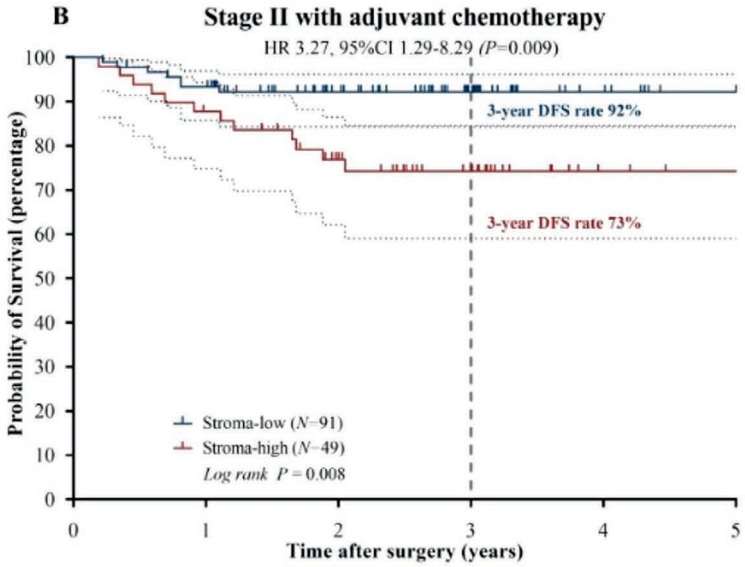
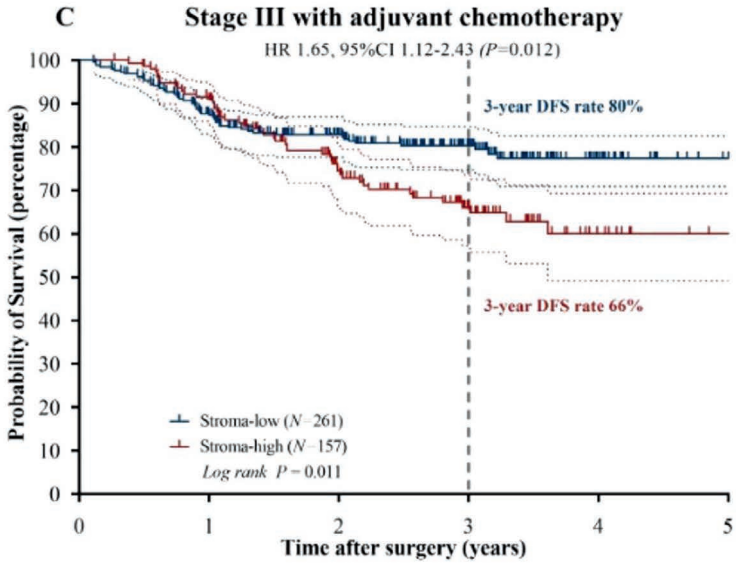
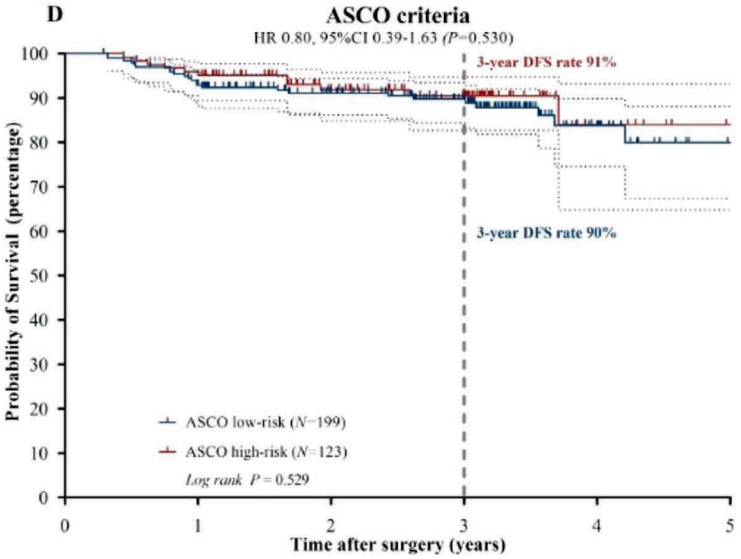


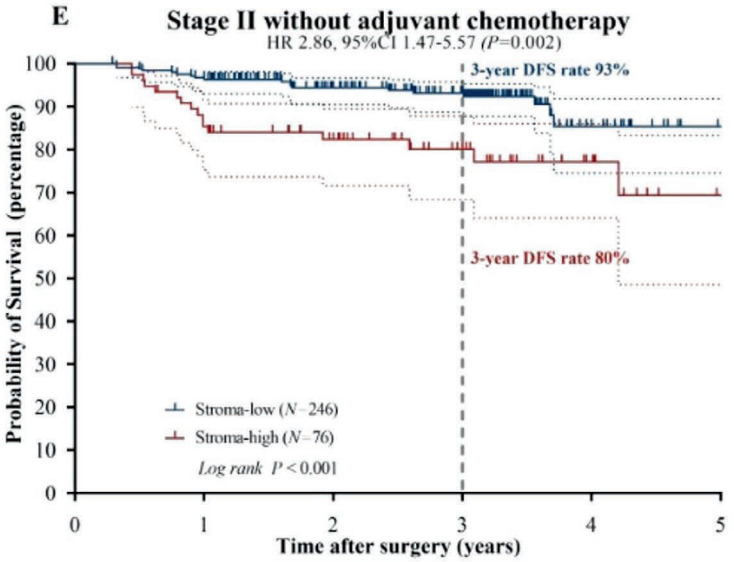
Number at risk (number censored)						
Stroma-low	91 (1)	84 (21)	62 (25)	37 (25)	12 (7)	5 (5)
Stroma-high	49 (1)	42 (7)	30 (11)	18 (15)	3 (2)	1 (1)



Number at risk (number censored)						
Stroma-low	261 (4)	225 (39)	174 (64)	105 (72)	30 (23)	7 (7)
Stroma-high	157 (5)	138 (24)	91 (29)	53 (36)	14 (9)	5 (4)



Number at risk (number censored)						
ASCO low-risk	199 (4)	181 (32)	146 (40)	104 (70)	30 (19)	10 (10)
ASCO high-risk	123 (0)	117 (34)	80 (25)	54 (43)	10 (6)	4 (4)



Number at risk (number censored)						
Stroma-low	246 (3)	234 (52)	178 (47)	129 (98)	27 (17)	10 (10)
Stroma-high	76 (1)	64 (14)	48 (18)	29 (15)	13 (8)	4 (4)

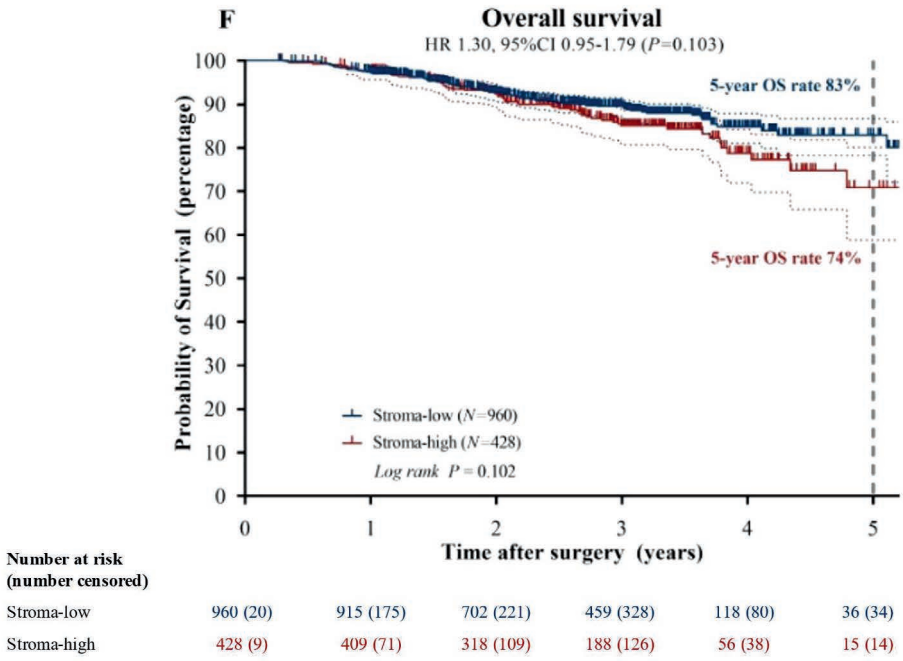


Figure 2. (*continued*) B) Kaplan-Meier analysis with log rank test in stage II patients receiving adjuvant chemotherapy, illustrating the worse 3-year survival rates for stroma-high patients despite treatment, indicating potential resistance to adjuvant chemotherapy (stroma-high 73% vs. stroma-low 92%; *P*=0.008). C) Kaplan-Meier analysis with log rank test in stage III patients receiving adjuvant chemotherapy, again illustrating the worse 3-year survival rates for stroma-high patients despite treatment (stroma-high 66% vs. stroma-low 80%; *P*=0.011); D) The ASCO criteria (high-risk vs. low-risk) not distinguishing any disease-free survival difference (high-risk 91% vs. low-risk 90%; *P*=0.529). E) Kaplan-Meier analysis with log rank test in stage II patients not receiving adjuvant chemotherapy, showing significant worse 3-year survival rates in stroma-high patients compared to stroma-low patients (stroma-high 80% vs. stroma-low 93%; *P*<0.001). F) Kaplan-Meier analysis with log rank test, showing overall worse survival in the stroma-high groups despite the short median follow-up of 3 years instead of 5 years, with the curves already diverging at 3 years. The 5-year overall survival rates are 74% vs. 83%, respectively (*P*=0.102). *For Disease-free survival, number of patients starting can be lower due to missing data. ASCO, American Society for Clinical Oncology; TNM, Tumour-node-metastasis stage; TSR, Tumour-stroma ratio; UNITED, Uniform Noting for International application of the Tumour-stroma ratio as Easy Diagnostic tool

Table 3. Univariate and multivariate analysis of on disease-free survival in UNITED cohort with Cox regression analysis

Variable (unit)	Univariate analysis			Multivariate analysis		
	Disease-free survival			Disease-free survival		
	Hazard ratio	95% Confidence interval	P-value	Hazard ratio	95% Confidence interval	P-value
Sex						
Female	1	..	0.753	..	..	..
Male	1.04	0.82 – 1.31	..	..	..	..
Age at surgery – older category						
<75 years of age	1	..	**0.036**	1	..	**<0.001**
≥75 years of age	1.29	1.02 – 1.63	..	1.54	1.21 – 1.96	..
Biopsy taken						
Yes	1	..	**<0.001**	1	..	**<0.001**
No*	2.60	1.96 – 3.46	..	2.33	1.72 – 3.16	..
Tumour-sidedness**						
Left-sided tumour	1	..	0.404	..	..	..
Right-sided tumour	0.91	0.72 – 1.14	..	..	..	..
Lymph nodes						
Lymph nodes ≥12 examined	1	..	**0.009**	1	..	0.067
Lymph nodes <12 examined	1.54	1.11 – 2.14	..	1.38	0.98 – 1.95	..
pT-stage***						
pT1-3	1	..	**<0.001**	1	..	**<0.001**
pT4	2.25	1.76 – 2.89	..	1.59	1.22 – 2.07	..
pN-stage***						
pN0	1	..	**<0.001**	1	..	**<0.001**
pN1	1.88	1.43 – 2.48	..	1.71	1.28 – 2.30	..
pN2	3.33	2.48 – 4.47	..	2.66	1.91 – 3.70	..

(continued)						
Tumour morphology						
Adenocarcinoma	1	..	0.514	..	..	..
Other, including mucinous and signet cell carcinoma	1.13	0.79 – 1.62	..	..	..	..
Differentiation grade						
Well-moderate (high grade)	1	..	0.191	..	..	..
Poor-undifferentiated (low grade)	1.25	0.90 – 1.75	..	..	..	..
Pathology risk factors						
No pathology risk factors present****	1	..	**<0.001**	1	..	**0.018**
Presence of ≥1 pathology risk factor	2.17	1.71 – 2.76	..	1.38	1.06 – 1.81	..
Adjuvant chemotherapy – received						
No	1	..	0.267	..	..	..
Yes	1.14	0.90 – 1.44	..	..	..	..
Tumour-stroma ratio						
Stroma-low	1	..	**<0.001**	1	..	**0.001**
Stroma-high	1.78	1.41 – 2.26	..	1.49	1.17 – 1.90	..
Microsatellite stability******						
Microsatellite instable (MSI)	1	..	0.070	..	..	..
Microsatellite stable (MSS)	1.55	0.97 – 2.48	..	..	..	..

All variables are given as absolute numbers with associated percentages or medians with interquartile ranges.

*Reasons why biopsy was not taken, is e.g. in emergency setting (obstructive ileus).

**A right-sided tumour is defined as a colon carcinoma in the coecum, colon ascendens, flexura hepatica or colon transversum.

***Different versions of the Union for International Cancer Control (UICC) classification were used. Here, all variables are converted to the UICC version 5 (1997).

****Pathology risk factors are stated below, presence of a risk factor is defined as at least one of registered risk factors. Absence is the absence of registered risk factors, as not all risk factors are registered.

******Dependent covariate, excluded in analysis.

In multivariate analysis, after correcting for significant univariate variables, DFS remained worse for stroma-high compared to stroma-low patients, confirming the independent prognostic effect of the TSR on DFS (HR 1.49, 95%CI 1.17 – 1.90; P<0.001) (Table 3). Forest plots for these univariate and multivariate analyses are provided in Supplementary Figures S4 and S5. Effect of TSR on DFS per stage is illustrated in Supplementary Figure S6. In stage II, the worse DFS for stroma-high patients remained significant with 3-year DFS rates of 77% vs. 91% in stroma-low (P<0.001), but for stage III this was not the case (3-year DFS rates 65% vs. 72%, respectively; P=0.055). However, a significant bias occurred in stage III patients due to the difference in median age in stage III for patients who received ACT (65 years, IQR 57 – 72 years) and those who did not (79 years, IQR 71 – 84 years; Student's Independent T-test P<0.001). As this led to skewed results, stratification for age was deemed necessary here. After stratifying for age (<75 years), stroma-high stage III colon cancer led to significantly worse 3-year DFS rates as well (64% vs. 78%; P=0.008). The predictive potential of the TSR on benefit of ACT was investigated as secondary outcome in these groups.

A total of 676 patients (49%) of the UNITED study started with ACT, mostly intravenous oxaliplatin combined with oral capecitabine (CAPOX/XELOX; N=394, 58%). A detailed overview of treatment regimens is given in Supplementary Table 5. Although treatment guidelines can differ between countries, centres, and can be dependent on the decision of the physician and/or patient, we also looked within stages at those receiving ACT or not, to ascertain the benefit of patients who received additional treatment on DFS in reducing risk of recurrences.

Specifically, we analysed potential added benefit from ACT in stage II and III patients and influence of the TSR herein on DFS, after correction for age (<75 years). Within the stage II patients who did receive ACT (N=140), mostly stage II-HR, a significantly worse DFS was seen (3-year DFS rates stage II with ACT 73% stroma-high vs. 92% stroma-low; P=0.008). In the stage III group receiving standard-of-care ACT (N=418), 3-year DFS rates were significantly worse for stroma-high patients than their stroma-low counterparts despite their ACT, too (66% vs. 80%; P=0.011). (Figure 2B–C). This illustrates that stroma-low patients could benefit from ACT, but that stroma-high patients exhibit a lack of benefit or even potential resistance to ACT. Supplementary Figures S7A–B show per TSR category the different groups, with indeed worse DFS rates in all stroma-high groups not significantly increasing despite ACT (P=0.080) compared to stroma-low patients (P<0.001).

To assess which parameter could potentially have identified more patients at risk for an event, in-depth analysis on the subgroup of stage II patients <75 years not receiving ACT (N=322) was performed,

comparing the TSR to the ASCO criteria. According to the ASCO-criteria, in these stage II patients, 123 (38%) patients fulfilled one or more high-risk criteria. In this ASCO high-risk group, a 9% (N=11) event rate was observed, illustrating the percentage of undertreated patients. In the ASCO low-risk group however, in 24 cases (12%) an event occurred. The ASCO-criteria did thus not correctly identify patients at risk for events nor show differences in DFS rates (P=0.383 in Supplementary Table 6; 3-year DFS rates 90% low-risk vs. 91% high-risk, P=0.529 in Figure 2D, respectively). For TSR analysis, in this group of stage II patients not receiving ACT (N=322, 246 stroma-low and 76 stroma-high), a total of 35 events (11% event rate) occurred. Of these events, 16 occurred in the stroma-high (21% event rate in stroma-high group) and 19 in the stroma-low group (8% event rate in stroma-low group; P=0.001). DFS rates plotted in this subgroup show similar differences (80% vs. 93%; P<0.001) (Figure 2E). Compared to the ASCO criteria, the TSR thus identified an additional 12% patients at risk for events (21% vs. 9%) with a 91% 3-year DFS rate for ASCO high-risk patients in comparison to the 80% in stroma-high patients.

Although the UNITED study was powered specifically for DFS with a median 3-year follow-up period, as secondary endpoint the preliminary effect of the TSR on 5-year OS was estimated. A total of 163 deaths were recorded, of which 61 in the stroma-high group (14% of stroma-high patients; P<0.001). In the plotted Kaplan-Meier analysis and log rank analysis, effect on OS was not statistically significant, despite the relatively short median follow-up (HR 1.30, 95%CI 0.95 – 1.79; P=0.103). At 5 years, OS rates of 83% vs. 74%, respectively, were observed (P=0.102; Figure 2F).

Discussion

The UNITED study was initiated to prospectively validate the TSR as prognostic parameter in colon cancer patients. This study not only confirms that patients with stroma-high tumours indeed have significantly worse DFS, but also proves that this effect is independent from other prognostic high risk parameters, such as sampling of <12 lymph nodes and pathological T-stage or N-stage. Moreover, the TSR also outperformed the current ASCO criteria in identification of stage II colon cancer patients at risk for events. We had hypothesized that fit, stage II stroma-high patients could benefit from additional ACT and frail stage III stroma-low patients with better outcomes could perhaps be spared this treatment. However, our secondary findings contrarily indicate that all stroma-low patients benefit from ACT, whereas stroma-high patients do not and thus could actually be considered to not be selected for ACT. This study illustrates the aggressive behaviour of stroma-high tumours and the potential resistance to

(neo)adjuvant treatment of tumour stroma, which was also noticed in other studies by our research group [17, 28-30].

Despite the relatively short follow-up period of three instead of five years, a trend towards worse OS is already seen for stroma-high colon cancer. The curves diverge after three years, probably since most events, i.e. recurrences, occur within the first three years after primary diagnosis [2]. Additionally, due to the increase in sequential treatment options that may have extended survival in patients with recurrences or metastases, events mainly lead to an effect on DFS but not immediately on OS [2, 3, 31]. Therefore, we aim to collect longer follow-up data of UNITED study patients in the future, to adequately evaluate the effect of the TSR on OS after five to ten years.

In 2007, our research group was the first to describe the phenomenon of a high intratumoural stroma percentage and the associated worse patient-related outcomes in colon cancer [15]. Since then, much research has been performed regarding the role of tumour stroma, aiming to elucidate the biological mechanism. The intricate and dynamic tumour-stroma crosstalk has been observed to include the CAFs as important players, potentially also enabling the seed-and-soil principle of Paget and causing stromal metastases in lymph nodes [14, 32]. The TSR can be scored on these metastases as well, and patients with stroma-high primary tumours and stroma-high lymph node metastases have been observed to have the worst survival [33, 34]. Even small lymph nodes ≤5 millimetres in diameter, during routine radiologic imaging not suspected of malignancy, can contain metastases. Scoring the TSR in lymph nodes in the future, as well as more research improving positive lymph node detection, is pertinent for an even more tailored treatment [32].

Many biomarkers have emerged the past decades, as researchers are aiming to better predict tumour behaviour and patient outcomes. One such emerging biomarker is liquid biopsy, measuring circulating tumour DNA strands (ctDNA) in blood as a marker for minimal residual disease [35, 36]. Even more of interest, is the study on tumour stromal liquid biopsy panels, capturing the tumour microenvironment [37, 38]. However, not only do these increasing number of biomarkers add to existing high work load and are time consuming, often, more patient material or resources are necessary. Moreover, some biomarkers have variance in analyses, like the consensus molecular subtypes (CMS). CMS type 4, the mesenchymal type, mostly covers stroma-high tumours, but low reproducibility prohibited accurate analysis [39, 40]. Also tumour budding, now implemented in guidelines as an additional prognosticator, is known to have a less optimal interobserver agreement [41]. Although the TSR strictly does not capture the qualitative histopathological heterogeneity of the complete tumour-stroma entity, the UNITED

study shows that the quantitively determined TSR still is an independent prognostic parameter, robust and simple, determined by pathologists during routine diagnostic microscopy assessment under two minutes without extra resources or costs, and therefore cost-effective [15, 17, 20, 24]. Potentially additional analyses, e.g. organization or maturity determination, can be done and would be of interest to perform on UNITED study material for further characterization of the tumour stroma [10, 42].

Also, the tumour immune microenvironment has been proven to affect tumour behaviour, as for instance seen in the Immunoscore [43]. High influx of tumour-infiltrating immune cells (TIICs) is often indeed correlated with decreased rates of recurrence. Hence, increasing amounts of research demonstrate the high potential of certain immunotherapeutic regimens in cancer. Although there are various potential analyses to ascertain the tumour immune microenvironment, often also requiring additional resources and patient material like with the Immunoscore method, future analysis of TIICs in all UNITED study patients would be of interest [43]. Previous research namely shows that the amount of influx of TIICs combined with TSR forms a potentially even more accurate prognosticator, as stroma-high/immune-low tumours are associated with the worst patient-related outcomes [44].

There are some limitations to the present study. MSI/MMR status, has only been obligatory in current treatment guidelines, and thus was not determined in the included older first half of patients [2]. Additionally, our analyses on ACT, as the UNITED study was not powered nor set up for this specifically, may be biased despite stratification for risk factors like age, e.g. due to the powerful a priori prognostic effect. Although the association seen in our analyses is already significant, we are currently initiating a well-balanced, matched cohort specifically powered for this endpoint to confirm the apparent resistance to ACT in stroma-high patients. Also, median three years of follow-up is relatively short, but fulfils the standard to adequately ascertain DFS, as previously taken into account in our power calculation, and can even be interpreted as a valid surrogate for OS [22, 45].

Strengths of this study include foremost the prospective nature, to minimize bias and adequately evaluate the causal effect of the TSR on survival. After the initial proposal of implementation of the TSR in TNM-based guidelines to the UICC and CAP, various supportive collaborations have been established for this study. Participating pathologists were trained with the reliable and quality-controlled UNITED study E-learning [22, 23]. Support grew, ultimately leading to the completion of this study. Not only has the TSR thus been validated, but the UNITED study also led to the foundation of an international system for pathologists to potentially implement the TSR in daily routine diagnostics. Moreover, international guidelines regarding ACT can be re-evaluated. As stroma-high patients have a

worse DFS and exhibit resistance to ACT, they could be discussed in multidisciplinary settings and potentially be spared this treatment.

As part of this study, centres were requested to send scans of the scored H&E-stained slides. This collection will be used for future development of artificial intelligence algorithms for TSR automatization. Not only can the interobserver agreement be increased even more, also analysis of difficult cases with for instance high amounts of mucin or necrosis can be facilitated, and automatization supports the increasing interest in digital pathology as well, including possibilities for even further research. Deep learning models especially can discern even more and potentially novel features in the tumour stroma, e.g. stromal organization like previously analysed by our research group, and correlations to patient-related outcomes can be assessed [42, 46].

Importantly, future studies should focus on tumour stroma-targeted therapeutic regimens or strategies, as this study shows a lack of benefit of stroma-high colon cancer to ACT, revealing a clear clinical need for new treatment options for these patients. Similar to ACT, tumours with high amounts of tumour stroma also have been observed to respond less to immunotherapeutic strategies [47]. Although confirming this potential resistance to adjuvant treatment in a powered series is necessary, fundamental research and pharmacological phase I studies should be initiated to uncover specific targets.

Conclusions

In conclusion, the UNITED study hereby unequivocally confirms the independent prognostic effect of the TSR on DFS in colon cancer patients as per request of the UICC and CAP. As stroma-high patients have worse DFS and appear to benefit less from ACT than stroma-low patients, the TSR can herein aid in clinical shared decision-making and personalized treatment. Therefore, implementation of the TSR in standard of care pathology diagnostics and reporting in addition to currently used elements as the TNM classification and ultimately in international guidelines is highly recommended.

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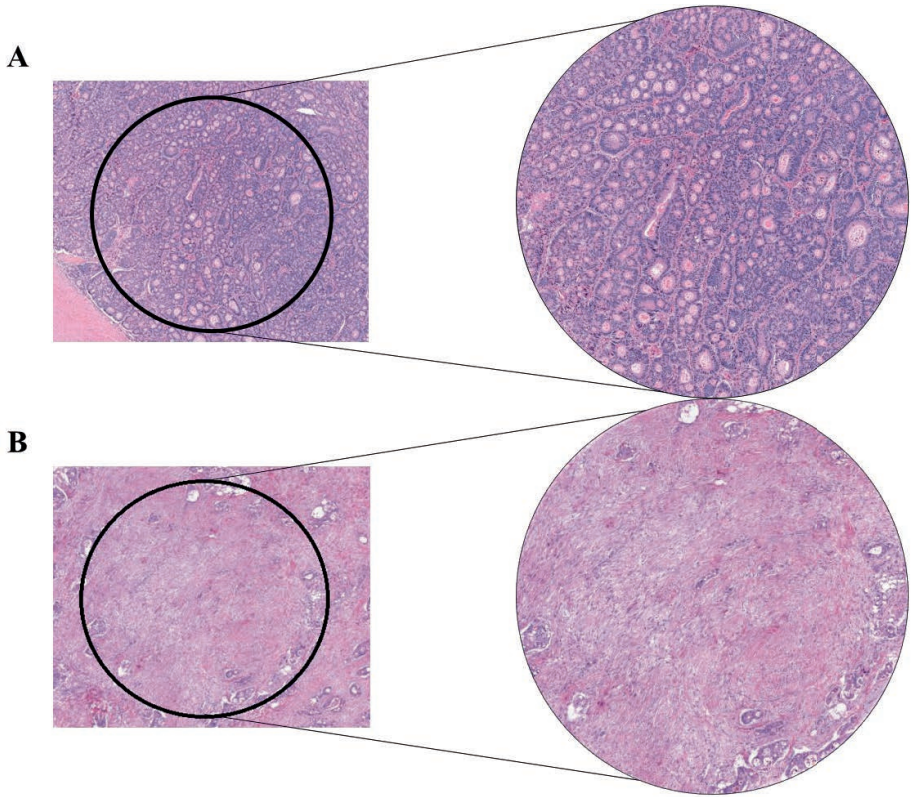
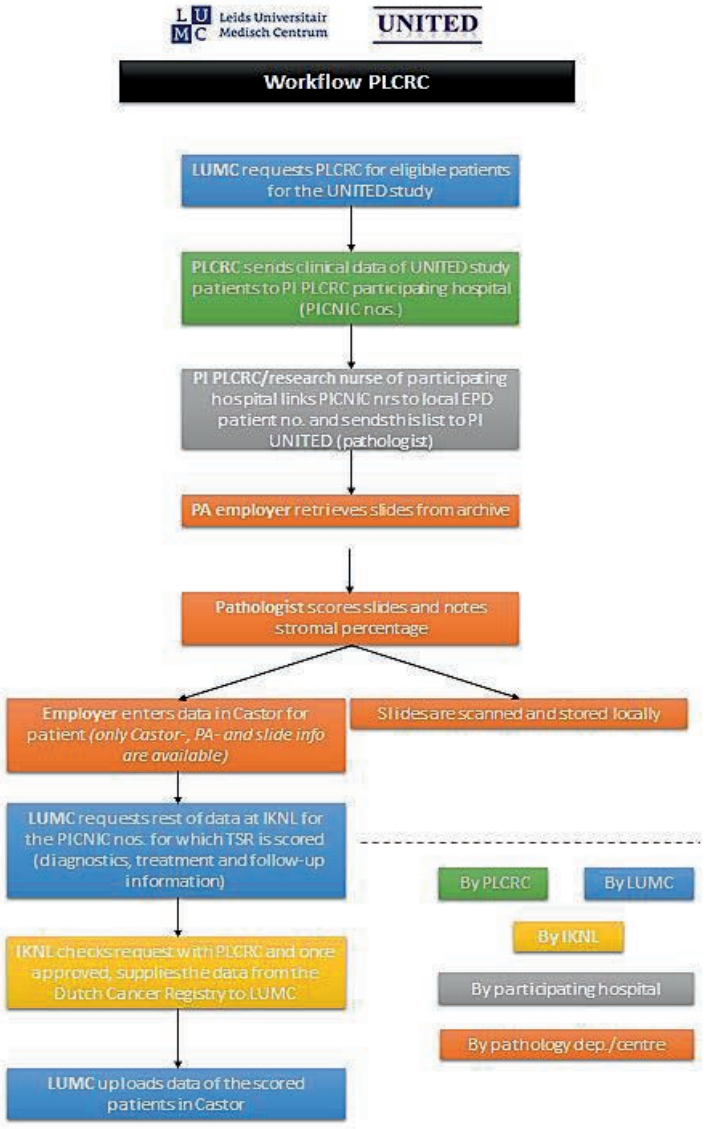
Supplementary Material

Supplementary Table 1. Final inclusion and exclusion criteria of the UNITED study

UNITED study	Criteria
Inclusion criteria *(eligible for inclusion and follow-up)*	• Operation date after 2015 • Histologically proven colon cancer • Pathological stage II (T3-4, N0, M0) or III (every T, N1-2, M0) • Tumour-stroma ratio score • Age ≥ 18 years and signed informed consent
Exclusion criteria *(ineligible)*	• Neoadjuvant therapy • Other malignancy 10 year prior to current colon cancer (except basal cell carcinoma or cervix carcinoma in situ) or in the complete medical history a colon carcinoma • Rectal cancer • Multiple synchronous malignant colon cancer • No complete curative resection (R1 of R2 resection) • Postoperative mortality within 3 months of operation



Supplementary Figure S1. Zoomed in examples of the scoring of the tumour-stroma ratio on haematoxylin and eosin-stained slides. (A) Stroma-low (≤50%) colon cancer; (B) Stroma-high (>50%) colon cancer. 10x magnification



Supplementary Figure S2. Workflow patient inclusion through PLCRC collaboration.

PA, Pathology; PI, Principle investigator; PLCRC, Prospective Dutch ColoRectal Cancer cohort; LUMC, Leiden University Medical Centre; IKNL, Institute for Cancer in the Netherlands.

Supplementary Table 2. Participating centres and collaborative investigators with associated inclusion rates

No. inclusions	Location (country)	Institute	Investigator (department)
230	Skopje (Macedonia)	Medical Faculty of Ss. Cyril and Methodius University	Gordana Petrushevska# (pathology) Magdalena Bogdanovska (pathology) Panche Zdravkoski (pathology) Svetozar Antovic (surgery) Darko Dzambaz (surgery) Panche Karagjozov (surgery)
133	Rotterdam region (Netherlands)	PATHAN Laboratories$ *(Franciscus Gasthuis & Vlietland; Admiraal De Ruyter Ziekenhuis; IJsselland Ziekenhuis)*	Erienne M.V. de Cuba#* (pathology) Frédérique Beverdam# (surgery; Franciscus Gasthuis & Vlietland) Jan Jansen# (surgery; Admiraal de Ruyter Ziekenhuis) Maarten Vermaas# (surgery; IJsselland Ziekenhuis)
117	Ljubljana (Slovenia)	Onkološki Inštitut	Gorana Gašljević# (pathology)
114	Vejle (Denmark)	Vejle Sygehus – Sygehus Lillebælt	Sanne Kjær-Frifeldt# (pathology) Jan Lindebjerg (pathology)
111	Venlo (Netherlands)	VieCuri Medisch Centrum	Maud Strous# (pathology) Jeroen F. Vogelaar (surgery)
101	Hoofddorp and Haarlem (Netherlands)	Spaarne Gasthuis$	Nicole W.J. Bulkmans#* (pathology)
83	Enschede region (Netherlands)	LabPON$ *(Medisch Spectrum Twente; Ziekenhuisgroep Twente; ZorgSaam Terneuzen)*	Joop van Baarlen# (pathology; retired)

			Leonie Mekenkamp (medical oncology; Medisch Spectrum Twente) Ronald Hoekstra (medical oncology; Ziekenhuisgroep Twente) Mark Sie (medical oncology; ZorgSaam Terneuzen)
80	Barcelona (Spain)	Hospital Clinic	Miriam Cuatrecasas# (pathology) Sara Simonetti (pathology) María Teresa Rodrigo (pathology) Iván Archilla Sanz (pathology) Jose Guerrero Pineda (pathology)
75	Deventer (Netherlands)	Deventer Ziekenhuis$	Natalja E. Leeuwis-Fedorovich# (pathology) Koen A. Talsma (surgery)
70	João Pessoa (Brazil)	Napoleão Laureano Hospital	Ricella M. Souza da Silva# (pathology)
57	Utrecht (Netherlands)	Universitair Medisch Centrum Utrecht$	Miangela M. Lacle# (pathology) Miriam Koopman (medical oncology)
55	Delft (Netherlands)	Reinier de Graaf Gasthuis$	Jan Willem T. Dekker# (surgery) Arjan van Tilburg (pathology)
53	Barcelona (Spain)	Vall d'Hebron Institute of Oncology	Paolo Nuciforo# (pathology) Xenia Villalobos Alberú (pathology) Stefania Landolfi (pathology) Adriana Zucchiatti (pathology)
42	Alkmaar (Netherlands)	Symbiant Laboratories$ *(Noordwest Ziekenhuisgroep Alkmaar)*	Emma Witteveen# (pathology) Arad Bordbar (pathology) Mathijs P. Hendriks (medical oncology)

41	Amersfoort (Netherlands)	Meander Medisch Centrum$	René Arensman# (pathology)
38	Hardwick (United Kingdom)	University Hospital of North Tees	Shonali Natu# (pathology)
34	Glasgow regio (United Kingdom)	NHS Greater Glasgow and Clyde	Noori Maka# (pathology)
28	Leiden (Netherlands)	Leids Universitair Medisch Centrum	Wilma E. Mesker# (surgery) Rob A.E.M. Tollenaar (surgery) Meaghan Polack (surgery) Marloes A. Smit (surgery) Gabi W. van Pelt (surgery) Hein Putter (biomedical data sciences) Elma Meershoek-Kleinenbarg (clinical research center, surgery) Annet G.H. Roodvoets (clinical research center, surgery) Augustinus S.L.P. Crobach (pathology) Hans Gelderblom (medical oncology)
28	Lisbon (Portugal)	Hospital CUF Tejo	Mário Fontes e Sousa# (medical oncology) Paula Borralho Nunes (pathology) João Cruz (pathology) Ana Raimundo (medical oncology) Nelson Silva (surgery)
24	Almada (Portugal)	Hospital Garcia de Orta	Maria J. Brito# (pathology)
19	The Hague (Netherlands)	Haaglanden Medisch Centrum	Valeska Terpstra# (pathology)
4	Kiev (Ukraine)	Bogomolets - Kyiv Oncology Center	L.M. Zakhartseva (pathology)

N/A	Brussels (Belgium)	European Society for Pathology	Raed Al Dieri (pathology) Jean-François Fléjou (pathology) Roger Feakins (pathology) Els Dequeker (pathology)
N/A	Utrecht (Netherlands)	Netherlands Comprehensive Cancer Organisation (IKNL)$	Geraldine R. Vink (research and development)
N/A	Nijmegen (Netherlands)	Radboud University Medical Center	J. Han J.M. van Krieken (pathology)

N/A, Not applicable.

Local principal investigator.

$ Part of the PLCRC collaboration.

* Currently employed elsewhere.

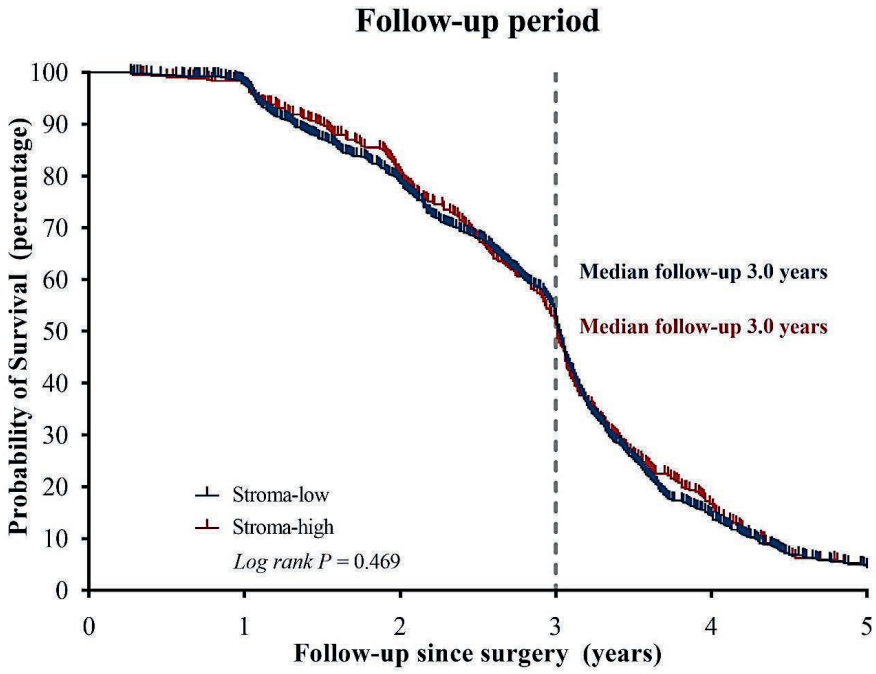
Supplementary Table 3. Baseline characteristics of the total UNITED cohort

Baseline characteristics	**Total UNITED cohort (N=1,537)**
Sex	
Female	686 (45)
Male	851 (55)
Age at surgery – years	
Median age	70 (61 – 77)
≥75 years of age	509 (33)
Ineligible at registration	31 (2)
Biopsy taken	
Yes	1,353 (88)
No*	179 (12)
Rectal cancer	3 (0)
Surgery	
Surgery year	2019 (2018 – 2020)
Surgery before 2015	1 (0)
No surgery	1 (0)
Pathological TNM-stage	
Stage 0 - I	42 (3)
Stage II	756 (49)
Stage III	694 (45)
Stage IV	9 (1)
Multiple colon tumours	21 (1)
Lymph nodes - number	
Examined *(in total group)*	20 (14 – 28)
Positive *(in pTNM-stage III)***	2 (0 – 33)
Tumour-stroma ratio	
Stroma-low (≤50%)	969 (63)
Stroma-high (>50%)	433 (28)
Residual tumour	2 (0)
Missing slide	4 (0)
Follow-up	
Postoperative mortality <3 months	26 (2)
Not started follow-up	5 (0)
Withdrew consent	4 (0)
Included in final analysis	1,388 (90)

All variables are given as absolute numbers with associated percentages or medians with interquartile ranges. Sum of percentages can be less or more than 100 due to rounding. TNM, tumour-node-metastasis stage.

* Reasons why biopsy was not taken, is almost always in emergency setting (obstructive ileus).

**Although there are no positive lymph nodes, using the UICC version 8, a tumour deposit (leading to stage N1c) will also lead to a pathological TNM-stage III.



Supplementary Figure S3. Median follow-up period.

Follow-up times calculated with reverse Kaplan-Meier analysis and log rank test, showing similar follow-up curves for the stroma-high group (median follow-up 3.0 years, 95% confidence interval 2.9 – 3.1) and stroma-low (median follow-up 3.0 years, 95% confidence interval 3.0 – 3.1) (P=0.469).

Supplementary Table 4. General overview of patient outcomes

Patient outcome	**Stroma-low (N=960)**	**Stroma-high (N=428)**	**P-value**
Follow-up time – years			0.469$
Median follow-up time	3.0 (3.0 – 3.1)	3.0 (2.9 – 3.1)	
Mutational status determined			
Not determined	864 (90)	371 (87)	
Determined, of which	96 (10)	57 (13)	0.068#
No mutations	38 (40)	16 (28)	
Mutations present, of which*	58 (60)	41 (72)	0.150#
KRAS	21 (36)	21 (51)	
BRAF	31 (53)	16 (39)	
NRAS	1 (2)	2 (5)	
PIK3A	4 (7)	2 (5)	
TP53	6 (10)	8 (20)	
Other	8 (14)	7 (17)	
Disease-free survival – years			
Disease-free survival time	5.2 (5.0 – 5.3)	4.8 (4.4 – 5.1)	**<0.001ǂ**
Stage and treatment group			**<0.001#**
Stage II - No adjuvant therapy	434 (45)	125 (29)	
Number of events	47 (11)	27 (22)	
Stage II + Adjuvant therapy	107 (11)	57 (13)	
Number of events	9 (8)	14 (25)	
Stage III + Adjuvant therapy	314 (33)	197 (46)	
Number of events	63 (21)	60 (31)	
Stage III - No adjuvant therapy	105 (11)	49 (11)	
Number of events	44 (42)	22 (45)	
Disease-free status			
No event	797 (83)	305 (71)	**<0.001#**
Event	163 (17)	123 (29)	
Type of event			**<0.001#**
No event	797 (83)	305 (71)	
Death by any cause	39 (4)	15 (4)	
Distant metastasis	105 (11)	92 (22)	
Locoregional recurrence	10 (1)	7 (2)	
Simultaneous distant metastasis and locoregional recurrence	9 (1)	9 (2)	

(continued) **Patient outcome**	**Stroma-low (N=960)**	**Stroma-high (N=428)**	**P-value**
Location of distant metastasis			**0.007#**
Liver	37 (35)	26 (29)	
Lung	22 (21)	6 (7)	
Liver and lung	14 (13)	6 (7)	
Bone	2 (2)	1 (1)	
Brain	1 (1)	3 (3)	
Peritoneal metastases	10 (10)	12 (13)	
Abdominal lymph nodes	2 (2)	2 (2)	
Two or more locations	16 (15)	29 (32)	
Other	1 (1)	5 (6)	
Overall survival – years			
Overall survival time	5.5 (5.3 – 5.7)	5.6 (5.3 – 5.9)	0.102ǂ
Overall survival status			0.053#
Alive	858 (89)	367 (86)	
Died	102 (11)	61 (14)	
Cause of death			**0.020#**
(Metastases of) current colon cancer	58 (57)	48 (79)	
Second primary malignancy	5 (5)	4 (7)	
Other, including pre-existing comorbidity	27 (26)	6 (10)	
Missing	12 (12)	3 (5)	

All variables are given as absolute numbers with associated percentages or medians with interquartile ranges. N/A, not applicable.

*Multiple mutations can occur simultaneously, hence the number of added percentages can be higher than 100 and no analysis is performed.

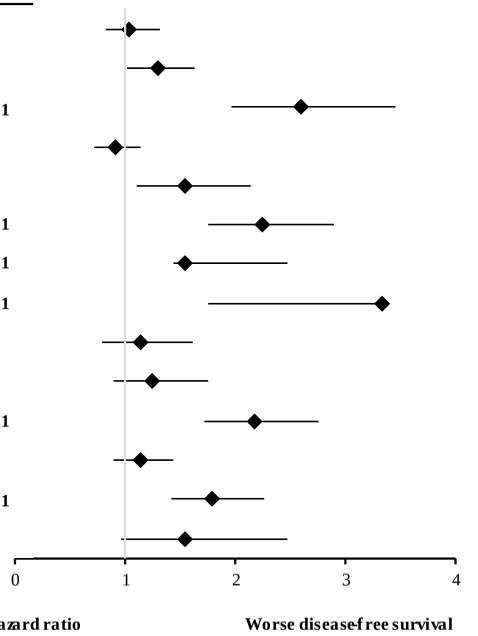
Calculated with the Chi-square test.

$ Calculated with a reverse Kaplan-Meier analysis and log rank test.

ǂ Calculated with Kaplan-Meier analysis and log rank test.

Supplementary Figure S4. Forest plot of the effect of risk factors on disease-free survival (univariate Cox regression analysis).

Risk factor	Events/patients	Hazard ratio	95% Confidence interval (lower – upper limit)	*P*-value
Male sex	770 (55%)	1.04	0.82 – 1.31	0.753
Age ≥75 years	453 (33%)	1.29	1.02 – 1.63	**0.008**
No biopsy taken	165 (12%)	2.60	1.96 – 3.46	**<0.001**
Right-sided tumor	659 (47%)	0.91	0.72 – 1.14	0.404
Lymph nodes <12 examined	147 (11%)	1.54	1.11 – 2.14	**0.009**
pT4-category	273 (20%)	2.25	1.76 – 2.89	**<0.001**
pN1-category	444 (32%)	1.54	1.43 – 2.48	**<0.001**
pN2-category	221 (16%)	3.33	1.76 – 2.89	**<0.001**
Other tumor morphology*	143 (10%)	1.13	0.79 – 1.62	0.514
Poor-undifferentiated tumor grading	166 (12%)	1.25	0.90 – 1.75	0.191
Presence of ≥1 pathology risk factor**	625 (45%)	2.17	1.71 – 2.76	**<0.001**
Adjuvant chemotherapy received	675 (49%)	1.14	0.90 – 1.44	0.267
Stroma-high tumor	428 (31%)	1.78	1.41 – 2.26	**<0.001**
Microsatellite stable (MSS) tumor	587 (42%)	1.55	0.97 – 2.48	0.070



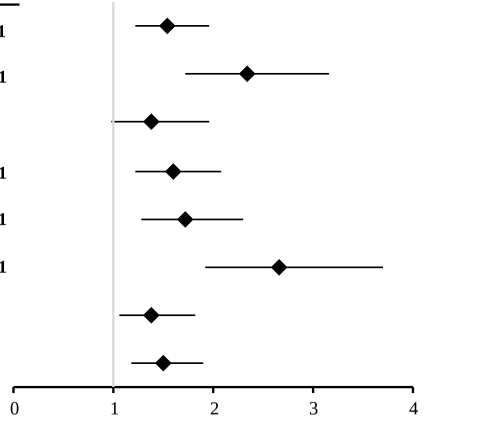
***** Other tumour morphology include signet cell carcinoma or medullary carcinoma.

** Pathology risk factors are stated below, presence of a risk factor is defined as at least one of registered risk factors.

Absence is the absence of registered risk factors, as not all risk factors are registered. Risk factors include extramural vascular invasion (EMVI), perineural invasion (PnI), etc.

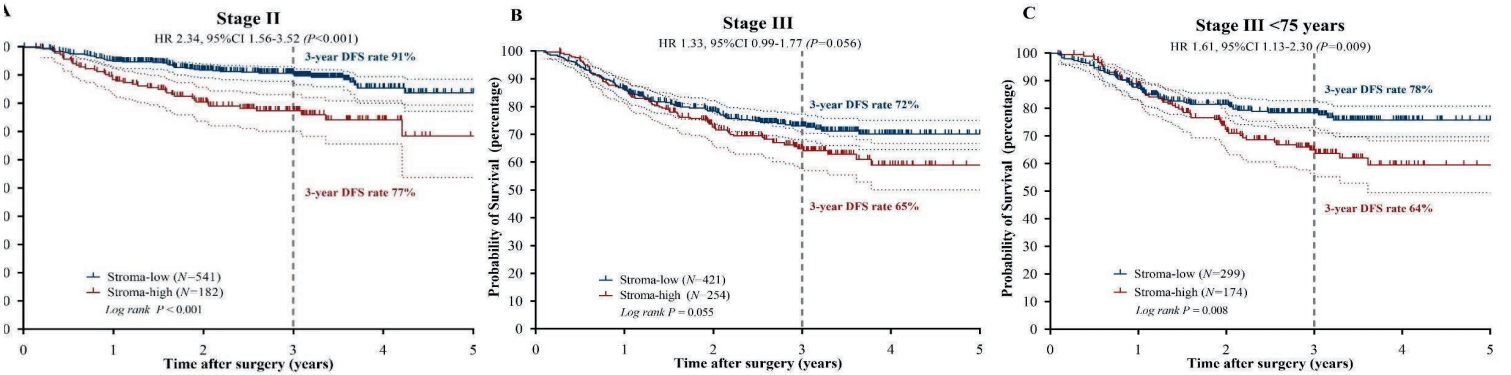
Supplementary Figure S5. Forest plot of the effect of risk factors on disease-free survival using significant variables from the univariate analysis (multivariate Cox regression analysis).

Risk factor	Events/patients	Hazard ratio	95% Confidence interval (lower – upper limit)	*P*-value
Age ≥75 years	453 (33%)	1.54	1.21 – 1.96	**<0.001**
No biopsy taken	165 (12%)	2.33	1.72 – 3.16	**<0.001**
Lymph nodes <12 examined	147 (11%)	1.38	0.98 – 1.95	0.067
pT4-category	273 (20%)	1.59	1.22 – 2.07	**<0.001**
pN1-category	444 (32%)	1.71	1.28 – 2.30	**<0.001**
pN2-category	221 (16%)	2.66	1.91 – 3.70	**<0.001**
Presence of ≥1 pathology risk factor*	625 (45%)	1.38	1.06 – 1.81	**0.018**
Stroma-high tumor	428 (31%)	1.49	1.17 – 1.90	**0.001**



* Pathology risk factors are stated below, presence of a risk factor is defined as at least one of registered risk factors.

Absence is the absence of registered risk factors, as not all risk factors are registered. Risk factors include extramural vascular invasion (EMVI), perineural invasion (PnI), etc.



Supplementary Figure S6. TSR on Disease-free survival per TNM-stage.

Kaplan-Meier curve and log rank analysis of TSR category and plotted 95% confidence intervals. A) Stage II with 3-year survival rates of 77% vs. 91%, respectively (P<0.001); B) Stage III, with 3-year survival rates of 65% vs. 72%, respectively (P=0.055). TNM-stage III is only nearly significant due to bias through high number of patients with comorbidities or high age and not treated with standard adjuvant chemotherapy. C) After stratifying for age <75 years, stage III stroma-high colon cancer also leads to significantly worse disease-free survival, with 3-year rates of 64% vs. 78% (P=0.008).

TNM, tumour-node-metastasis stage; TSR, tumour-stroma ratio.

Supplementary Table 5. Detailed overview of the adjuvant chemotherapy regimens and durations

Variables	Total eligible (N=1,388)	Stroma-low (N=960)	Stroma-high (N=428)	P-value
Adjuvant chemotherapy – not started				
Total not started adjuvant treatment	713 (51)	539 (56)	174 (41)	**<0.001#**
Stage II	559 (78)	434 (80)	125 (72)	**<0.001#**
Stage III	154 (22)	105 (20)	49 (28)	**<0.001#**
Reasons not started adjuvant treatment*				N/A
Not indicated	509 (71)	401 (76)	108 (62)	
Comorbidity, age	100 (14)	70 (14)	30 (17)	
Other, including patients wish	88 (16)	56 (10)	32 (18)	
Missing	20 (3)	15 (3)	5 (3)	
Adjuvant chemotherapy – started				
Total started treatment	675 (49)	421 (44)	254 (59)	**<0.001#**
Stage II	164 (24)	107 (26)	57 (22)	**<0.001#**
Stage III	511 (76)	314 (74)	197 (78)	**<0.001#**
Regimen started adjuvant chemotherapy**				N/A
CAPOX/XELOX***	394 (58)	238 (57)	156 (61)	
Capecitabine monotherapy	173 (26)	112 (27)	61 (24)	
FOLFOX	33 (5)	26 (6)	7 (3)	
Other, including 5FU monotherapy	32 (5)	18 (4)	14 (6)	
Missing	43 (6)	27 (6)	16 (6)	
Duration adjuvant chemotherapy – months				0.118$
Median duration	3 (2 - 5)	3 (2 - 5)	2 (2 - 5)	
Missing	52 (8)	32 (8)	20 (8)	

All variables are given as absolute numbers with associated percentages or medians with interquartile ranges. 5FU, 5-fluorouracil intravenous chemotherapy; CAPOX/XELOX, oral capecitabine with intravenous oxaliplatin chemotherapy; FOLFOX, intravenous 5-fluorouracil and oxaliplatin; TNM, tumour-node-metastasis stage.

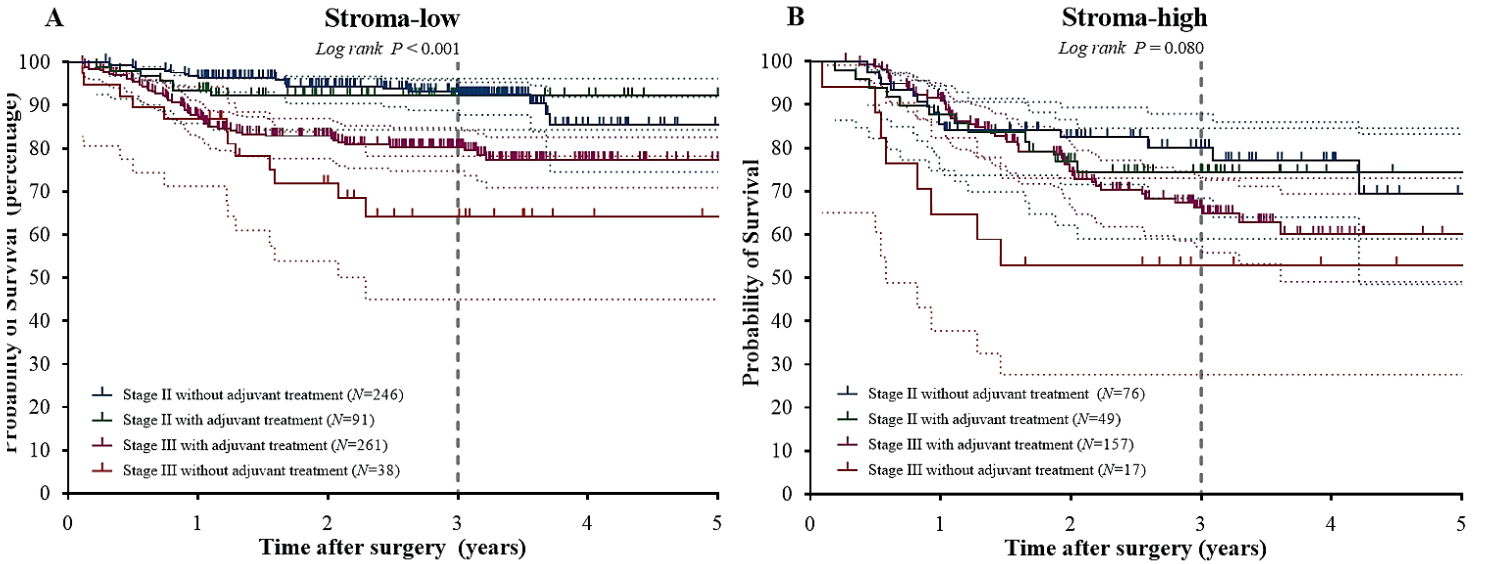
*Multiple reasons can occur, hence the number of added percentages can be higher than 100. The first given reason is shown here.

**Switch in regimens can occur, hence the number of added percentages can be higher than 100. The initially started regimen is mostly shown here.

***CAPOX/XELOX regimens here are both the 3 and 6 cycles.

Calculated with the Chi-square test.

$ Calculated with an Independent Student's T-test.



Supplementary Figure S7. Disease-free survival per TSR category and benefit of adjuvant treatment per TNM-stage.

Kaplan-Meier curve and log rank analysis of TSR category and plotted 95% confidence intervals. A) Stroma-low patient groups showing an significant influence of adjuvant treatment, with 3-year survival rates of 93% vs. 92% vs. 80% vs. 64%, respectively (P<0.001); B) Stroma-high patient groups showing worse outcomes than stroma-low groups in A but also within groups no significant difference despite adjuvant treatment, with 3-year survival rates of 80% vs. 73% vs. 66% vs. 52% (P=0.080). In TNM-stage III patients not receiving adjuvant treatment, bias occurs e.g. due to small numbers.

TNM, tumour-node-metastasis stage; TSR, tumour-stroma ratio.

Supplementary Table 6. Overview of ASCO criteria and TSR categories

Variables	**Total no. (%)**	**No. events (%)**	**P-value**
Stage II - No adjuvant chemotherapy			**<0.001#**
Patients <75 years of age*	322 (100)	35 (11)	
Tumour-stroma ratio			**0.001#**
Stroma-low	246 (76)	19 (8)	
Stroma-high	76 (24)	16 (21)	
ASCO-criteria**			0.383#
ASCO low-risk	199 (62)	24 (12)	
ASCO high-risk	123 (38)	11 (9)	

All variables are given as absolute numbers with associated percentages or medians with interquartile ranges. ASCO, American Society for Clinical Oncology; TSR, tumour-stroma ratio.

*Compared to the category as defined in Supplementary Table 4.

**ASCO-criteria include a pT4 tumour, sampling of <12 lymph nodes or emergency setting of surgery, presence of pathological risk factors like lymphovascular or perineural invasion, and poor tumour differentiation as risk factors. If one is present, categorization as ASCO high-risk followed.

Calculated with the Chi-square test.