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Improving diagnostic, prognostic and predictive biomarkers in colorectal cancer: the role of proteomics and stromatogenesis

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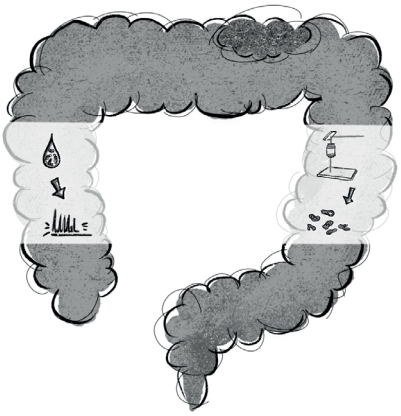

The proportion of tumor-stroma as a strong prognosticator for stage II and III colon cancer patients; validation in the VICTOR trial.

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SUMMARY

Background: The intra-tumor stroma percentage in colon cancer (CC) patients has previously been reported by our group as a strong independent prognostic parameter. Patients with a high stroma percentage within the primary tumor have a poor prognosis.

Patients and Methods: Tissue samples from the most invasive part of the primary tumor of 710 patients (52% Stage II, 48% Stage III) participating in the VICTOR trial were analyzed for their tumor-stroma percentage. Stroma-high (>50%) and stroma-low (≤50%) groups were evaluated with respect to survival times.

Results: Overall and disease free survival times (OS and DFS) were significantly lower in the stroma-high group (OS $p<0.0001$, Hazard ratio (HR)=1.96; DFS $p<0.0001$, HR=2.15). The five year OS was 69.0% versus 83.4% and DFS 58.6% versus 77.3% for stroma-high versus stroma-low patients.

Conclusion: This study confirms the intra-tumor stroma ratio as a prognostic factor. This parameter could be a valuable and low cost addition to the TNM-status and next to current high-risk parameters such as Microsatellite instability (MSI) status used in routine pathology reporting. When adding the stroma-parameter to the ASCO criteria the rate of "undertreated" patients dropped from 5.9% to 4.3%, the "overtreated" increased with 6.8% but the correctly classified increased with an additional 14%.

BACKGROUND

Traditional pathological staging systems are still the most important tool for therapeutic decision making in colorectal cancer. However, pathological variables are only moderate indicators of outcome and therapy response. Twenty-five percent of stage II colorectal cancer patients (CRC) have recurrence of disease within 5 years. Current research focuses on the identification of this high risk group within the stage II CRC patients who would benefit from additional therapy. The Quasar collaborative group et al (1) reported a small benefit (3.6%) for chemotherapy (CT) treatment (fluorouracil and folinic acid) compared to observation within stage II CRC patients (1,2). This percentage is below the accepted level of 5% and therefore CT for the entire stage II groups is not advised.

Additional parameters of CRC, e.g. microsatelite-instability (MSI-high), have become of greater importance. MSI-high patients have been reported in several studies to have better prognosis compared to MSI-low.

Former studies have shown that a high intra-tumor stroma percentage predicts for CC patients with worse prognosis (3-5) and we postulated those patients would benefit from additional therapy. The intra-tumor stromal parameter has also been evaluated for esophageal and breast cancer and found to be an independent prognostic factor (6,7). For breast cancer the intra-tumor stromal percentage showed to be of additional predictive value for systemic therapy. The importance of intra-tumor stromal percentage and its use in therapy selection should be further examined.

Despite the frequency of colon cancer, the cellular and molecular characteristics of the target cells for oncological transformation and tumor-initiation at the primary site and distant metastasis is largely unknown. It is becoming increasingly clear that metastases develop when distant organs are seeded with this subpopulation of cancer cells with a stem/progenitor phenotype that arise from the primary tumor. The stroma is not an innocent bystander, but actively involved in formation and progression of malignant tumors. We hypothesize that disruption of these tumor-stroma interactions will inhibit or help to eliminate tumor progression and metastasis.

The current study presents a validation of our previous findings in colon cancer patients in a large independent series, the VICTOR trial (8,9). This trial was initially designed to monitor recurrence prevention by VIOXX in stage II-III CRC patients after potentially curative therapy.

METHODS

Patients

Tissue samples were collected within the study population of the VICTOR trial (8,9). Patients entering the VICTOR trial had undergone complete potential curative treatment including surgery alone or surgery plus radiation and/or chemotherapy within 12 weeks before entering the study. Inclusion criteria were: histologically proven Dukes B (Stage II; T3 or T4, N0, M0) or Dukes C (Stage III: any T, N1 or N2, M0) without gross or microscopically evidence of residual disease. Patients were randomized in a double blind design to receive rofecoxib or placebo for 2 or 5 years. They were recruited in 151 hospitals in the United Kingdom. For detailed trial design see Pendlebury et al. (9).

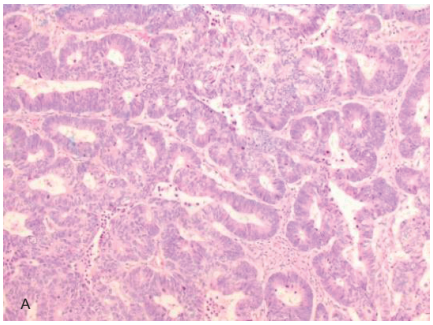
Initially the study was to have been completed in 2012 and aimed to recruit 7000 patients. Unfortunately the trial was closed to recruitment on 30 September 2004. Due to cardiovascular adverse effects of rofecoxib reported in the APPROVe trial (10-12) all patients were taken off the study drug. All randomized patients continued to be followed-up conform protocol. Kerr et al. describe no significant difference in mortality between patients with and without cardiovascular events within the VICTOR trial (13). Thus it may be expected that this does not influence OS in our analysis.

Histopathological scoring

Tissue samples consisting of 5µm Haematoxylin and Eosin (H&E) stained sections from the most invasive part of the primary tumor were used for analysis using conventional microscopy. The invasive front was chosen from the tissue block the pathologist selects as most invasive part and uses to determine the T-status. The most invasive tumor area on each slide was selected using a 2.5x or 5x objective. A part of the sample was selected where both tumor and stromal tissue were available using a 10x objective. Tumor cells must be present at all borders of the image field (north-east-south-west) (Figure 1). When mucinous tissue was present within a field that matched our scoring criteria, the mucinous tissue was visually excluded for the scoring. Two investigators (WM, GvP) estimated the stromal percentage in a blinded manner. In case of an inconclusive score a third observer was decisive (VS). Scoring percentages were given per tenfold (10, 20, 30% etc.) per image-field. For statistical analysis stromal ratio groups were divided into 'stroma-high (>50%)' and 'stroma-low (≤50%)' as determined a priori to have maximum discriminative power (4).

MSI status

For additional analyses MSI status was determined using initially 3 Bethesda microsatellites (Bat25, Bat26 and D2S123). Tumours with two unstable markers were classified



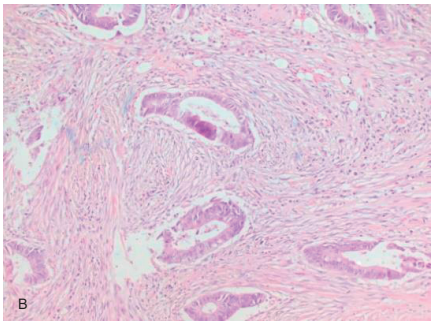


Figure 1. Haematoxylin and Eosin (H&E) stained 5 µm paraffin sections examined of the most invasive part of primary colon tumors. a) Stroma Low (20%) / b) Stroma High (80%)

as MSI and tumours without any unstable marker as MSS. Tumours with one single unstable marker were further analysed with the Bethesda marker D5S346 and the mononucleotide Bat40, which has been proven to be very useful for MSI identification (14). These tumours were classified as MSI if one of these two markers also displayed instability, otherwise they were classified as MSS.

Statistical analysis

Statistical analysis was performed using SPSS software version 17.0. Overall-Survival (OS) was defined as the time period between the randomization date and the date of death from any cause or the date of the last follow-up. Disease free survival (DFS) was defined as the time between the randomization date and the date of death or the date of first loco-regional or distant recurrence. If no recurrence occurred DFS was calculated as the time period until the date of last follow-up (15). Unfortunately no data were available on new primary tumors. Analysis of the survival curves was performed using Kaplan-Meier Survival Analysis and differences in survival distributions were tested using Log Rank Statistics. The Cox proportional hazard model was used to determine the Hazard Ratio (HR) of explanatory variables for OS and DFS. MSI statistical analysis was performed using STATA 11.2.

RESULTS

Patients

In the VICTOR trial a total of 2434 patients were recruited between 2002 and 2004. A total of 959 histological samples were obtained from the participating clinics. Some of the samples were of poor histological quality and therefore excluded (N=20). After scoring all samples for the stromal parameter, additional patient information was collected. Due to the fact that most rectal cancer patients receive radiotherapy (RT) and

the known effect of RT on stromal formation in tissue we excluded all rectal cancer patients (N= 229). The stromal study cohort thus comprised of 710 patients.

Study population

Since only a part of the total study population was included for stromal analysis we compared our study population with the total VICTOR population. Between both groups no statistically significant differences were seen in gender, age, stage distribution, tumor localization, chemotherapeutic treatment or study-treatment arm (Rofecoxib/Placebo) (Table 1). Only a small difference in length of follow-up (FUP) was seen; total population mean FUP 52.1 (0-84.2) months compared to 55.4 (0-84.9) within the stromal study group ($p<0.0001$). Additionally no differences in number of deaths or recurrences were seen.

As can be found in Table 1 the stromal study consists of 438 men and 272 women, with a mean age of 65 years (range 25-86 years). Since patients had to first complete primary curative treatment, 61.0% (433) of them received adjuvant chemotherapy (CT) before randomization. After randomization 354 patients received rofecoxib and 356 were in the placebo treatment group. A total of 368 patients were stage II and 342 stage III (Supplementary Table S1).

Table 1. Comparison patient characteristics study and total population.

	Total study population, *N* = 1063	Stromal study population, *N* = 710
Males	646 (60.8%)	438 (61.7%)
Females	417 (39.2%)	272 (38.3%)
Stage II	526 (49.5%)	368 (51.8%)
Stage III	537 (50.5%)	342 (48.2%)
Colon	955 (89.8%)	637 (89.7%)
Rectosigmoid	108 (10.2%)	73 (10.3%)
Chemotherapy	687 (64.6%)	433 (61.0%)
No chemotherapy	376 (35.4%)	277 (39.0%)
Rofecoxib	534 (50.2%)	354 (49.9%)
Placebo	529 (49.8%)	356 (50.1%)
Age	64.1 (29–89)	64.8 (25–86)

Scoring stroma percentage

In 676 (95.2%) cases observers agreed on classification. Only in 34 (4.8%) cases there was no agreement between the observers; in those cases a third observer was decisive. Cohen's kappa coefficient revealed an almost perfect agreement in classification (Kappa = 0.89) (Figure 1).

Survival analysis

Out of 710 analyzed samples 207 (29.2%) were scored as stroma-high and 503 (70.8%) as stroma-low. In the stroma-high population the five year survival rate for OS was 69.0% versus 83.4% within the stroma-low population. For the DFS the five year survival rates for stroma-high and stroma-low were 58.6% versus 77.3% respectively. OS and DFS within the stroma-high group were as expected significantly lower than in the stroma-low group (OS p<0.0001, HR=1.96 (95%CI:1.41 to 2.74); DFS p<0.0001, HR=2.15 (95% CI:1.61 to 2.86)) (Figure 2). In uni,- and multivariate analysis, after adjusting for age, sex, stage, chemotherapy, tumor site, stroma percentage, vioxx treatment and MSI status, the tumor-stroma ratio was an independent prognostic factor for both OS (p = 0.002, HR 1.7 (95%CI: 1.2 to 2.4)) and DFS (p<0.001, HR 1.9 (95%CI: 1.4 to 2.6)) (Table 2). Because left and right sited tumors are known to have a different prognosis, a uni,- and multivariate analysis is repeated with this subdivision (Table 3). Descending colon and sigmoid were considered left sited and caecum, ascending colon, hepatic flexure, transverse colon and splenic flexure as right sited tumors. Unfortunately additional pathological information for 72 patients is lacking (in these cases site is classified as colon without further specifications). For this reason the analysis is performed for both total population and this subset of patients with more specific tumor-site status.

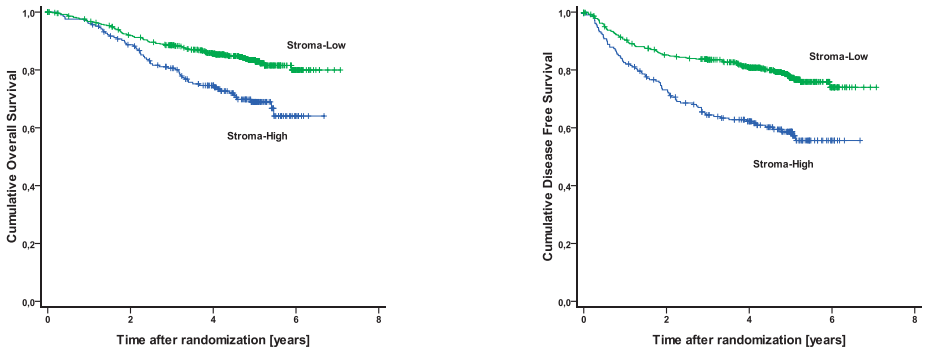


Figure 2. Kaplan-Meier survival curves of overall survival and disease free survival of stroma-high versus stroma-low in the total patient population (stage II and III) N=710 (OS p<0.0001, HR=1.96 (95%CI:1.41 to 2.74); DFS p<0.0001, HR=2.15 (95%CI:1.61 to 2.86)).

To account for systemic therapy effects the tumor stroma ratio was analyzed in a subgroup of patients treated with and without chemotherapy. The traditional pathological staging system (15) was used in combination with the ASCO criteria (16) to categorize patients as high risk or low risk within the stage II and III group. Patients with high risk are considered for adjuvant chemotherapy. In our study group 433 patients

received CT. Although this decision was made before randomization we assessed the stroma value of the high and low risk patients within our analysis. From all patients receiving CT, OS and DFS between stroma-high and stroma-low differed significantly

Table 2. Univariate & Multivariate analysis including age, sex, stage, chemotherapy, tumor site, stroma percentage, vioxx treatment and MSI status OS and DFS of total study population N=710.

	Univariate analysis						Multivariate analysis					
	Overall survival			Disease-free survival			Overall survival			Disease-free survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age												
<75 years	1		**0.005**	1		0.08	1		**0.001**			
≥75 years	1.71	1.18–2.49		1.35	0.96–1.90		1.88	1.28–2.77				
Sex												
Male	1		0.20	1		0.09						
Female	0.80	0.56–1.13		0.77	0.56–1.04							
Stage												
II	1		**<0.001**	1		**<0.001**	1		**<0.001**	1		**<0.001**
III	2.71	1.89–3.88		2.36	1.75–3.19		2.77	1.67–4.59		2.41	1.58–3.69	
Chemotherapy												
No chemotherapy	1		**0.001**	1		**<0.001**	1		0.75	1		0.45
Chemotherapy	1.88	1.30–2.73		1.78	1.30–2.45		0.92	0.53–1.57		0.84	0.54–1.32	
Site												
Colon	1		0.30	1		0.24						
Rectosigmoid	1.30	0.79–2.13		1.29	0.84–1.98							
Stroma												
Stroma-low	1		**<0.001**	1		**<0.001**	1		**0.002**	1		**<0.001**
Stroma-high	1.96	1.40–2.74		2.15	1.60–2.90		1.71	1.22–2.41		1.95	1.45–2.61	
VIOXX												
No VIOXX	1		0.58	1		0.50						
Vioxx	1.10	0.79–1.53		0.87	0.65–1.15							
MSI status												
MSS	1		0.66	1		0.09						
MSI	0.89	0.55–1.45		0.67	0.42–1.06							

Table 3. Univariate & Multivariate analysis including age, sex, stage, chemotherapy, tumor site, stroma percentage, vioxx treatment and MSI status OS and DFS of subpopulation with additional pathological information of tumor site N=638.

	Univariate analysis						Multivariate analysis					
	Overall survival			Disease-free survival			Overall survival			Disease-free survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age												
<75 years	1		**0.003**	1		**0.050**	1		**0.001**	1		0.014
≥75 years	1.79	1.22–2.62		1.41	1.00–1.99		1.98	1.34–2.93		1.56	1.10–2.23	
Sex												
Male	1		0.36	1		0.25						
Female	0.84	0.59–1.21		0.83	0.61–1.14							
Stage												
II	1		**<0.001**	1		**<0.001**	1		**<0.001**	1		**<0.001**
III	2.69	1.86–3.90		2.38	1.75–3.25		3.07	1.83–5.15		2.40	1.55–3.68	
Chemotherapy												
No chemotherapy	1		**0.002**	1		**0.001**	1		0.33	1		0.74
Chemotherapy	1.83	1.25–2.67		1.75	1.26–2.42		0.92	0.54–1.60		0.92	0.58–1.47	
Site												
Left	1		***0.051***	1		0.24	1		**0.004**			
Right	1.41	1.00–1.99		1.20	0.89–1.61		1.66	1.17–2.36				
Stroma												
Stroma-low	1		**<0.001**	1		**<0.001**	1		**0.008**	1		**<0.001**
Stroma-high	1.86	1.31–2.63		2.09	1.55–2.82		1.66	1.17–2.29		1.88	1.38–2.54	
VIOXX												
No VIOXX	1		0.59	1		0.34						
VIOXX	1.10	0.78–1.55		0.87	0.64–1.16							
MSI status												
MSS	1		0.66	1		0.09						
MSI	0.89	0.55–1.45		0.67	0.42–1.06							

(OS p=0.002, HR=1.85 (95%CI: 1.25 to 2.72); DFS p<0.0001, HR=2.03 (95%CI: 1.45 to 2.86)) with 5 year survival rates of stroma-high: OS 65.5%, DFS 54.5% compared to stroma-low: OS 80.8%, DFS 74.2%. Within the 'low-risk' group of patients not receiving CT, there was no significant difference of OS and DFS comparing stroma values (OS p=0.210, HR=1.58 (95%CI:0.77 to 3.26); DFS p=0.048, HR=1.81 (95%CI:0.99 to 3.28)) (Supplementary Figure S1 and S2).

From 368 stage II CC patients analyzed, 83 were scored as stroma-high and 285 as stroma-low. The differences for OS and DFS between stroma-high and stroma-low were OS p=0.034, HR=1.95 (95%CI:1.04 to 3.65); DFS p=0.0005, HR=2.04 (95%CI:1.23 to 3.40). Five year survival rates for overall and disease free survival time respectively were 79.8% versus 89.1% and 71.1% versus 83.3% for stroma-high versus stroma-low (Supplementary Figure S3).

The stage III CC group consisted of 342 patients of which 124 were scored stroma-high and 218 as stroma-low. There were significant differences in survival time for this group of patients when comparing stroma-high and stroma-low (OS p=0.019, HR=1.61 (95%CI: 1.07 to 2.39); DFS p<0.0001, HR=1.86 (95%CI: 1.30 to 2.64)). Five year overall and disease free survival rates for the stroma-high group versus the stroma-low group were 61.7% versus 76.1% and 50.2% versus 69.4% respectively (Supplementary Figure S4).

Relation between MSI status and intra-tumour stroma proportion

To evaluate whether there could be a relation between MSI status and the stroma percentage additional analyses were performed. Within our study population (N=710) MSI data of 662 patients were available. Within this group 558 patients were classified as MSS and 104 as MSI. Within the MSS group 178 (31.9%) are stroma-high and 389 (69.7%) stroma low. The MSI group consists of 20 (19.2%) stroma-high and 84 (80.7%) stroma-low. Stroma and MSI were found to be associated; Chi-square p=0.010.

Correlation of T stage and N stage to the intra-tumour stroma proportion

The relation between TNM stage and intra-tumour stroma patients is evaluated. TNM data of 661 patients were available. Because all patients included in this study are stage II or stage III patients, only T and N stage were considered. Therefore the stroma percentages within the T stage and N stage groups were compared with a chi-squared test. Both the T and the N status were significantly related to the stroma percentage (T-status p <0.0001 and N-status p=0.005). All T1 (n=4) patients were stroma-low. 96.2% of the T2 (n=26) patients were stroma-low. In the T3 group (n=460) this per-

centage decreased to 74.8% and in the T4 group (n=171) it was only 55%. For the N status the stroma low percentage in the N0 group (n=348) was 76.1%, in the N1 group (n=210) 64.3% and in the N2 group (n=67) 65% (Figure 3).

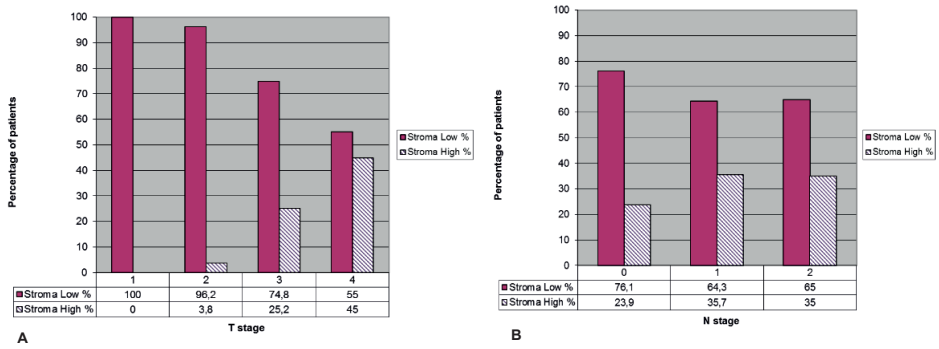


Figure 3. Correlation of T stage and N stage to the intra-tumour stroma proportion.

Comparing the intra-tumor stroma ratio with the ASCO high risk criteria

To identify high risk stage II CC patients that might benefit from adjuvant CT, ASCO proposed several high risk criteria for clinical implementation. These criteria include T4 tumor stage, a lymph node yield less than 10 nodes in the resection specimen, poor tumor differentiation, vascular invasion or perforation of the bowel wall at presentation. We compared the efficiency of these ASCO criteria in the identification of high risk patients to our stroma parameter. For this we used a subset of our study population consisting of 256 Stage II CC patients that did not receive any adjuvant therapy. Based on the ASCO criteria 119 patients were classified as high risk. With the addition of the stroma parameter to the ASCO criteria 140 patients were classified as high risk. The addition of the stroma parameter improved the false negative rate of ASCO criteria and correctly identified 14% (N=4) more patients (i.e. of patients that were not classified as high risk by the ASCO criteria but indeed developed a distant metastasis or died due to CC in the follow up period). As a conclusion the rate of "undertreated" patients based on the ASCO criteria dropped from 5.9% to 4.3% and the correctly classified increased with an additional 14% when using the ASCO-stroma parameter combination.

DISCUSSION

Our study confirms previous findings that the intra-tumor stroma percentage is an independent factor for prognosis of CC patients. Patients with a high intra-tumor stroma percentage have a significantly worse prognosis than those with a low stroma percentage, with a consistent hazard ratio of about two. In multivariate analysis, even after correction for TNM stage, the tumor-stroma ratio remained an independent prognostic factor for both OS ($p = 0.002$, HR 1.7 (95%CI: 1.2 to 2.4)) and DFS ($p<0.001$, HR 1.9 (95%CI: 1.4 to 2.6)).

To our knowledge we are the first group to describe the intra-tumor stroma ratio as a independent prognostic parameter (3,4). This method was applied for automation by West et al. (5) and they validated our findings with similar results: (HR)2.087, 95%CI: 1.08 to 4.00, P=0.024 using a cut-off value of 47%.

Our study suggests that an increased amount of stromal involvement, even if it is detected in only a small part of the total tumor mass, can be linked to an unfavorable prognosis, independent of other prognostic parameters. Possibly, this particular part of the tumor has obtained the capability to orchestrate its direct environment to facilitate its invasive and metastatic behavior.

Currently, next to traditional histopathological staging, MSI status is advised as an indicator for therapy choice and possible predictor for prognosis (17-21). In this study MSI status showed no significant differences in OS and DFS. Stroma and MSI were found to be associated; Chi-square p=0.010. As expected in relation to survival, within the MSI group the number of stroma-low patients was higher (80.7% vs. 19.2%). The same was seen in the MSS group, this group consisted of a higher number of stroma-high patients (69.7% vs. 31.9%).

Our high intra-observer agreement with a kappa value of 0.89 in this study and scoring in previous studies indicates that the intra-tumor stroma proportion is a highly reproducible measurement. The previously published stromal study in a CC patient group showed kappa values between three different observers varying between 0.60 and 0.70 (concordance 93%) (3,4). For esophageal cancer and breast cancer Cohen's kappa coefficient for two observers was respectively 0.86 and 0.85 (6,7).

The relation between TNM stage and intra-tumour stroma patients is evaluated. It shows that with the increase of T and N stage the number of stroma-high patients grows. This is as expected.

ASCO proposed guidelines to identify high risk stage II CC patients that might benefit from adjuvant CT (16). Our study showed that with adding the stroma-parameter to the ASCO criteria the rate of "undertreated" patients dropped from 5.9% to 4.3% and the correctly classified increased with an additional 14% when using the ASCO-stroma parameter combination. This comparison is a good parameter to measure how addition of the stroma parameter can improve current high risk stratification methods. However to compare the efficiency of adding the stroma parameter to the ASCO criteria should ideal be tested in a prospective study instead of a subset of untreated stage II CC patients like in this case.

A secondary aim of stromal analysis within the VICTOR trial was to investigate association with therapy response. Therefore the different treatment arms were compared for OS and DFS. There is a statistical drawback with this analysis. Within the study population 'high-risk' patients were selectively treated following current treatment protocols with CT before randomization and 'low risk' patients did not receive CT.

Within the low-risk treated patients the stroma-parameter showed no difference. Although we have found in former studies that a small number of patients with low-risk have a stroma-high tumor, probably in this study the number is too low to reach statistical significance.

In conclusion, we found the stroma parameter to be a simple and reproducible prognostic parameter which may indicate important differences in biology. It is remarkable that a simple cell based parameter using conventional microscopy can possess such a high predictive power without any additional costs. This parameter does not seem to be limited to CC but is also relevant as new prognostic factor for esophageal and breast cancer.

In this manuscript we validated the stroma parameter to select patients at risk for death or recurrence of disease for additional therapy. This parameter is to be expected to be used in clinical practice for better risk-classification and should therefore be considered for implementation in standard pathology reports together with the MSI status in addition to the current TNM classification.

SUPPLEMENTARY TABLE & FIGURES

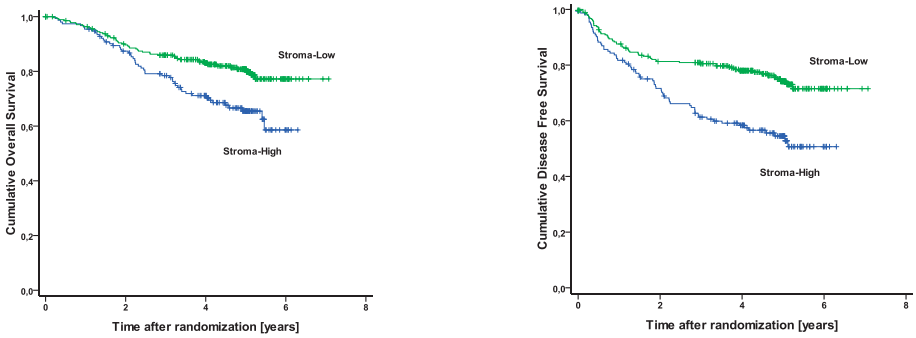


Figure S1. Kaplan-Meier survival curves of overall survival and disease free survival of stroma-high versus stroma-low in all patients receiving CT N=433 (OS p=0.002, HR=1.85 (95%CI: 1.25 to 2.72); DFS p<0.0001, HR=2.03 (95%CI: 1.45 to 2.86)).

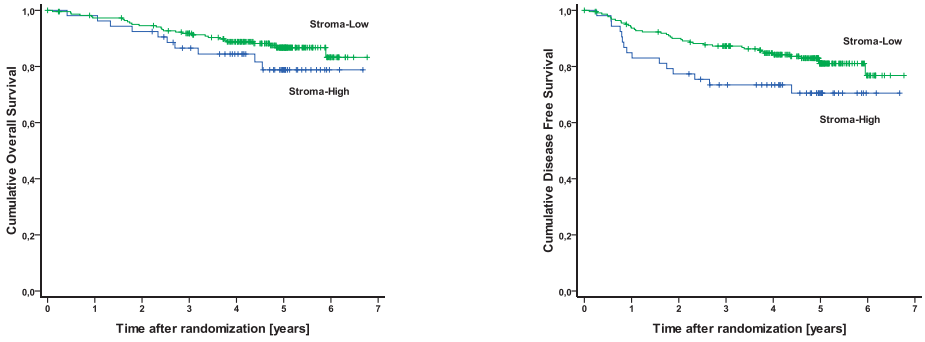


Figure S2. Kaplan-Meier survival curves of overall survival and disease free survival of stroma-high versus stroma-low in all patients not receiving CT N=277 (OS p=0.210, HR=1.58 (95%CI:0.77 to 3.26); DFS p=0.048, HR=1.81 (95%CI:0.99 to 3.28)).

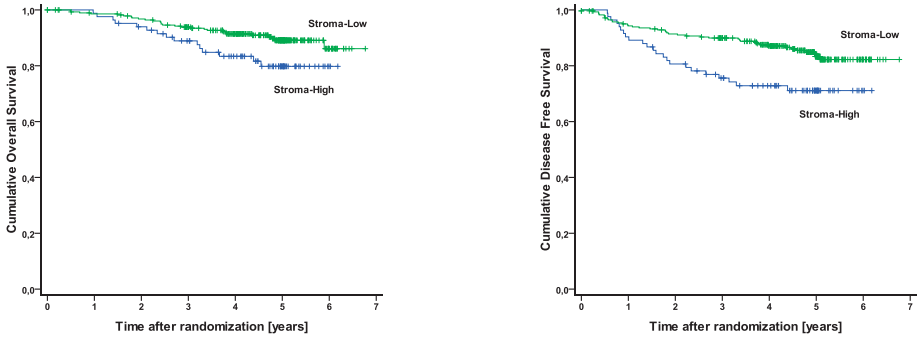


Figure S3. Kaplan-Meier survival curves of overall survival and disease free survival of stroma-high versus stroma-low in stage II CC patients N=368 (OS p=0.034, HR=1.95 (95%CI:1.04 to 3.65); DFS p=0.0005, HR=2.04 (95%CI:1.23 to 3.40)).

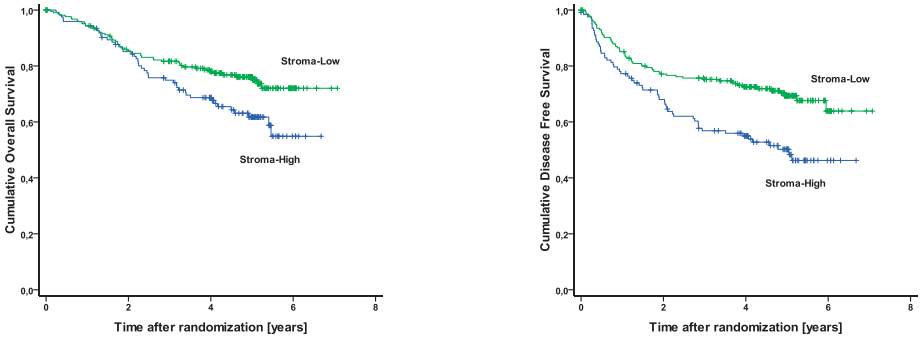


Figure S4. Kaplan-Meier survival curves of overall survival and disease free survival of stroma-high versus stroma-low in stage III CC patients N=342 (OS p=0.019, HR=1.61 (95%CI: 1.07 to 2.39); DFS p<0.0001, HR=1.86 (95%CI: 1.30 to 2.64)).

Table S1. Patient characteristics stroma-high versus stroma-low group. Chi-squared p<0.0001.

	Stroma-high	Stroma-low	Total
Stage II	**83 (22.6%)**	**285 (77.4%)**	**368**
Stage III	**124 (36.3%)**	**218 (63.7%)**	**342**
Total	**207 (28.2%)**	**503 (70.8%)**	**710**

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