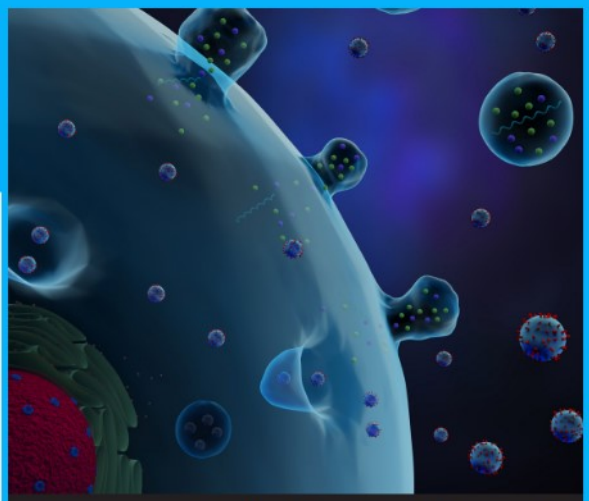
Exosome Production, Isolation and Applications: From Cancer Research to Theraputic Targets



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Relevance of shrinkage versus fragmented response patterns in rectal cancer

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Relevance of shrinkage versus fragmented response patterns in rectal cancer

Aims: Partial response to neoadjuvant chemoradiotherapy (CRT) presents with one of two main response patterns: shrinkage or fragmentation. This study investigated the relevance of these response patterns in rectal cancer, correlation with other response indicators, and outcome.

Methods and results: The study included a test (n = 197) and a validation cohort (n = 218) of post-CRT patients with rectal adenocarcinoma not otherwise specified and a partial response. Response patterns were scored by two independent observers using a previously developed three-step flowchart. Tumour regression grading (TRG) was established according to both the College of American Pathologists (CAP) and Dworak classifications. In both cohorts, the predominant response pattern was fragmentation (70% and 74%), and the scoring interobserver agreement was excellent (k = 0.85). Patients with a fragmented pattern presented with significantly higher pathological stage (ypTNM II-IV, 78% versus 35%; $P < 0.001$), less tumour regression with Dworak (P = 0.004), and CAP TRG (P = 0.005) compared to patients with a shrinkage pattern. As a predictor of prognosis, the shrinkage pattern outperformed the TRG classification and stratified patients better in overall (fragmented pattern, hazard ratio [HR] 2.04, 95% confidence interval [CI] 1.19–3.50, P = 0.008) and disease-free survival (DFS; fragmented pattern, HR 2.50, 95% CI 1.23–5.10, P = 0.011) in the combined cohorts. The multivariable regression analyses revealed pathological stage as the only independent predictor of DFS.

Conclusions: The heterogeneous nature of tumour response following CRT is reflected in fragmentation and shrinkage. In rectal cancer there is a predominance of the fragmented pattern, which is associated with advanced stage and less tumour regression. While not independently associated with survival, these reproducible patterns give insights into the biology of tumour response.

Keywords: neoadjuvant chemoradiation treatment, partial response, patterns of response, rectal cancer

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Introduction

Chemoradiotherapy (CRT) is one of the main neoadjuvant treatments in locally advanced rectal cancer management; it facilitates radical surgical resection due to tumour downstaging and decreases the risk of local recurrence.[1–3] To evaluate the effect of this therapy, resection specimens are assessed for response. Tumour regression grading (TRG) and tumour downstaging are well-known response indicators, that also have been suggested as surrogate outcomes of neoadjuvant therapies.[4–7]

After the introduction of the initial 5-tier tumour regression grading (TRG) classification by Mandard,[8] several modifications have been suggested consisting of 3-, 4-, or 5-tiers,[9–13] based on either tumour percentage or tumour–stroma ratio. In practice, considerable interobserver variation as well as the wide variation in classifications and definitions hamper a standardized approach. However, both pathologic complete response (pCR) and "no response" classifications are generally straightforward. The "partial response" group display profound heterogeneity, causing poor interobserver agreement.[14–16] Conflicting results about the clinical relevance of TRG have been reported.[6,14,17–22]

Previous small rectal cancer studies indicated "shrinkage" and "fragmentation" as two main response patterns after neoadjuvant therapy.[14,17,23,24] Shrinkage is defined as the downsizing of the residual tumour resulting in a luminal tumour bulk or defined discrete tumour mass. Fragmentation refers to the dissociation of the tumour mass forming different-sized groups spreading randomly into the tumour bed.[14] The presence of a fragmented pattern increases the possibility of tumour in the deeper layers of the bowel, resulting in an advanced ypT stage, more frequent lymph node metastasis, and poor outcome.[17,23,24] Although the clinical relevance of the pattern of response has been suggested, these terms are fairly new, ill-defined, and have not been used in daily practice yet.

In the present study we investigate response patterns in rectal cancer in four large international multicentre cohorts, focusing on clinical relevance of response patterns compared with well-known response indicators.

Materials and methods

PATIENTS

We included patients with clinical stage I–III rectal cancer receiving long-term CRT and diagnosed with adenocarcinoma not otherwise specified and a partial response to treatment. Patients with multiple malignancies, tumours other than adenocarcinoma, and specific subtypes (mucinous adenocarcinoma, signet ring cell carcinoma, medullary carcinoma) were excluded. Clinical and follow-up data including demographic features, cTNM staging, locoregional or distant disease recurrence dates, and vital status were retrieved. Follow-up was performed according to national guidelines and trial protocols. Relevant pathologic data including pathological stage were obtained from the institutional pathology databases. The ethical standards of the research Ethics Committee and the Helsinki Declaration of 1964 and later versions were thoroughly met.

The test cohort was retrieved from the local pathology databases of two centres, namely, the Radboud University Medical Centre, the Netherlands, and St. Vincent's University Hospital, Ireland, and patients underwent surgery in the period 2003–2020.

A combined external cohort from the standard arm of the prospective RAPIDO trial (NCT01558921)[25] and the Erasmus University Medical Centre, the Netherlands, was used as a validation cohort. All of the patients selected were operated on between 2006 and 2022.

ASSESSMENT OF TUMOUR RESPONSE

After histological review, all tumour representative haematoxylin-eosin (H&E) slides per case were selected and digitized to assess response. Tumour response was assessed in two ways. First, tumour regression was graded by a pathologist (S.K.O.) in the primary tumour slides according to 5-tiered Dworak and 4-tiered Modified Ryan Scheme, as suggested in the AJCC/College of American Pathologists (CAP).[9] Dense tumoral mass without regressive changes, that is, fibrosis, acellular mucin, was classified as no response. Second, patterns of response were evaluated. Two main response patterns, i.e. tumour shrinkage and fragmentation, were assessed using a previously developed flowchart by two independent observers (S.K.O. and C.G.M.; Figure 1).[26] Difficult cases were discussed among the same researchers to reach consensus and, in case of discordance, consultation with an expert gastrointestinal pathologist (I.D.N.) was carried out. After scoring all the tumour slides per case, an overall response pattern was attributed to each rectal cancer.

Fragmentation was identified as dissociated tumour groups without a discrete tumour mass or tumours containing fragments at least 3 mm away from the bulk (Figure 2C–F). The remnants in the fragmented pattern were heterogeneous in size. While some cases

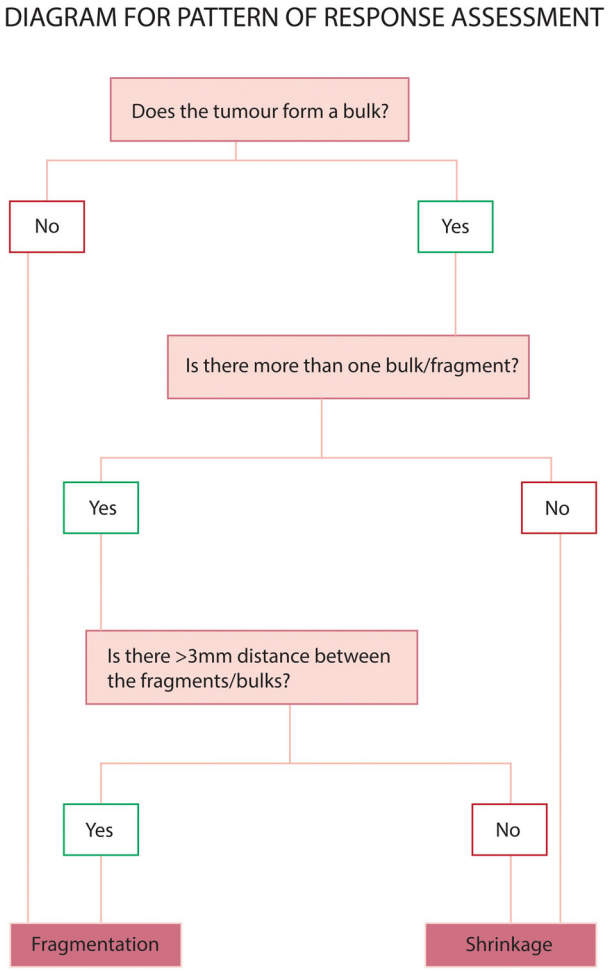

Figure 1. Flow chart.[26]

purely consisted of clusters of more than 10 cells (clustered fragmentation), others were composed of fewer than 10 cell groups (scattered fragmentation), or a mixture of different-sized tumour groups (mixed fragmentation).

Shrinkage was characterized by bulk-forming tumour residue without fragments or with fragments within 3 mm of the tumour bulk (Figure 2A, B). The main tumour bulk may reside in the luminal (luminal shrinkage) or deeper layers (irregular shrinkage).

The distribution of residual tumour cells among the layers of the bowel was also assessed. Tumour downstaging was defined as a decrease between the preoperative clinical and postoperative pathological stage.

STATISTICAL ANALYSIS

The two main response patterns were compared according to their baseline characteristics. Pearson's χ^2 and analysis of variance (ANOVA) tests were used for qualitative and quantitative comparisons, respectively. The correlation between patterns of response and ypTNM was tested with Spearman's ρ rank correlation test. Cohen's kappa coefficient (κ) was used to measure the interobserver agreement rate for the classification of patterns of response. A κ score of 0.60–0.79 indicated moderate agreement and a κ score above 0.80 was considered strong interobserver agreement.

The interval between surgical resection and death or last follow-up time was defined as overall survival (OS) time. Disease-free survival (DFS) time was defined as the time from surgical resection to disease recurrence and/or death or the last follow-up time. Kaplan–Meier survival curves and log-rank tests were used to visualize OS and DFS. Univariable and multivariable Cox regression analyses were performed to demonstrate survival-associated clinicopathological variables in the test cohort and validation cohort separately. Multivariable analysis was repeated after the combination of two cohorts. $P < 0.05$ was considered statistically significant. Hazard ratios (HRs) and risk ratios (RRs) are presented with a 95% confidence interval (CI).

For all analyses, RStudio (2020) was used. Results were confirmed by a second researcher using SPSS Statistics v25.0 (IBM, Armonk, NY, USA).

Results

TEST COHORT

From the original 283 patients, we excluded patients with special type adenocarcinomas ($n = 36$), pCR ($n = 27$), and no response to CRT ($n = 23$), resulting in the inclusion of 197 patients with partial response.

PATTERNS OF TUMOUR RESPONSE

Fragmentation (70%) was the predominant main response pattern observed in the test cohort. An excellent interobserver agreement was reached ($\kappa = 0.85$) by use of the flowchart in the response pattern evaluation. There was full agreement on tumour response patterns in 183 out of 197 cases (93%). More than half of the shrinkage pattern cases presented with ypTNM I. Advanced stages showed more frequently a fragmented pattern ($P < 0.001$, Table 1). Indeed, there was a significant positive correlation between ypTNM and response patterns (Spearman's rank correlation coefficient,

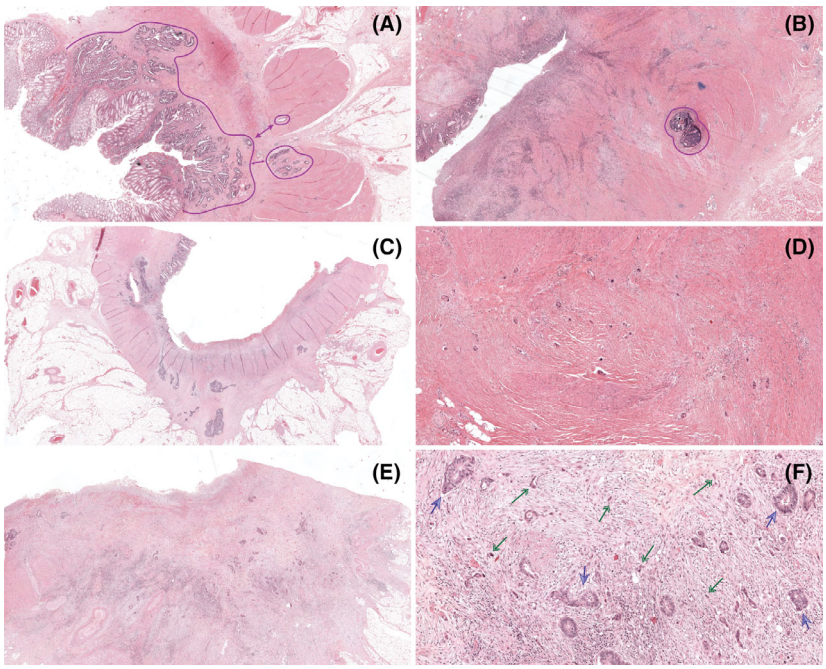

Figure 2. **A**: Luminal shrinkage, **B**: Irregular shrinkage, **C**: fragmented clustered, **D**: fragmented scattered, **E,F**: Fragmented mixed (thin arrows indicate scattered, thick arrows indicate clustered component).

rho = 0.36, $P < 0.001$). Residual tumour in deeper layers (muscularis propria: fragmented 96% versus shrinkage 49%, $P < 0.001$ and subserosa: fragmented 71% versus shrinkage 18%, $P < 0.001$) was more common in the fragmented pattern. Patients with a fragmented pattern had less tumour regression in both Dworak ($P = 0.004$), and CAP TRG classifications ($P = 0.005$). Clinical stage ($P = 0.85$) and clinical T stage ($P = 0.13$) were similar among the two main response patterns (Table 1).

PROGNOSIS

The median follow-up time was 56 months (interquartile range [IQR] 36–104) in the test cohort. Patients with a shrinkage pattern had a trend of better OS, although the difference was not significant (5-year; 85% versus 70%, $P = 0.094$). The risk of disease recurrence (RR: 1.14, 95% CI: 0.86–1.51, $P = 0.40$) was similar among the two main response patterns.

In the univariable survival analysis, advanced pathological stage (HR 1.9, 95% CI 1.0–3.6, $P = 0.05$) was an indicator of poor OS and DFS. Absence of downstaging and poor tumour regression according to Dworak and CAP TRGs were also indicators of poor OS (Figure 3). In multivariable regression analyses, none of the aforementioned variables proved to be an independent prognostic factor of survival.

VALIDATION COHORT

From the original 293 patients in the validation cohort, we excluded 75 patients (13 pCR, 19 no response, and 43 mucinous adenocarcinomas), resulting in 218 patients. Compared to the test cohort, the validation cohort presented less advanced tumours (ypTNM III 32% validation cohort versus 35% test cohort, $P = 0.006$) and a higher prevalence of cases with high tumour regression according to the Dworak classification (TRG 2–3, 86% versus 76%, $P = 0.03$; Table 1). The fragmented pattern remained the predominant pattern and the proportions of shrinkage (26%) and fragmented pattern (74%) were similar to the test cohort ($P = 0.68$). When scoring patterns of response, the interobserver agreement was again excellent ($\kappa = 0.83$).

The validation cohort's median follow-up time was 65 months (IQR 50–85). Patients with a shrinkage pattern had a better OS than those with a fragmented pattern (5-year; 88% versus 72%, $P = 0.045$). Furthermore, patients with a shrinkage pattern had a lower risk of recurrence (RR: 2.37, 95% CI: 1.06–5.28, $P = 0.02$) and a better DFS compared to patients with a fragmented pattern of response (5-year; 80% versus 62%, $P = 0.01$).

Table 1. Clinicopathologic characteristics of the patients

	Test cohort (n = 197)				Validation cohort (n = 218)				P (T versus V)
	Total	Shrinkage, n = 55 30%	Fragmented, n = 142 70%	P	Total	Shrinkage, n = 56 26%	Fragmented, n = 162 74%	P	0.68
Age, median (min-max)		61 (42–80)	62 (28–81)	0.61		60 (18–83)	63 (32–86)	0.13	0.66
Gender (%)									
Male	58	31	69	0.69	64	27	73	0.73	0.39
Female	42	26	74		36	30	70		
Medical centre (%)									
Centre 1	57	29	71	0.94	26	37	63	**0.04**	
Centre 2	43	27	73		74	22	78		
Clinical stage (%)									
I	5	5	5	0.85	1	2	—	0.27	**0.05**
II	18	14	20		17	16	17		
III	77	81	75		82	82	83		
Pathological stage (%)									
I	34	65	22	**<0.001**	23	55	10	**<0.001**	**0.006**
II	31	15	37		45	20	55		
III	33	20	38		32	25	35		
IV	2	—	3		—	—	—		
Recurrence (%)									
Yes	19	13	21	0.60	23	12	28	**0.03**	0.50
No	81	87	79		77	88	72		
Downstaging (%)									
Yes	67	81	62	0.19	61	71	58	0.15	0.46
No	33	19	38		39	29	42		
Dworak TRG (%)									
TRG1	24	14	27	**0.004**	14	—	19	**<0.001**	**0.03**
TRG2	64	62	65		74	64	77		
TRG3	12	24	8		12	36	4		
CAP TRG (%)									
Score 1	13	25	8	**0.005**	12	36	4	**<0.001**	0.68
Score 2	71	64	74		75	64	78		
Score 3	16	11	18		13	—	18		

The comparisons that were statistically significant are highlighted with bold.

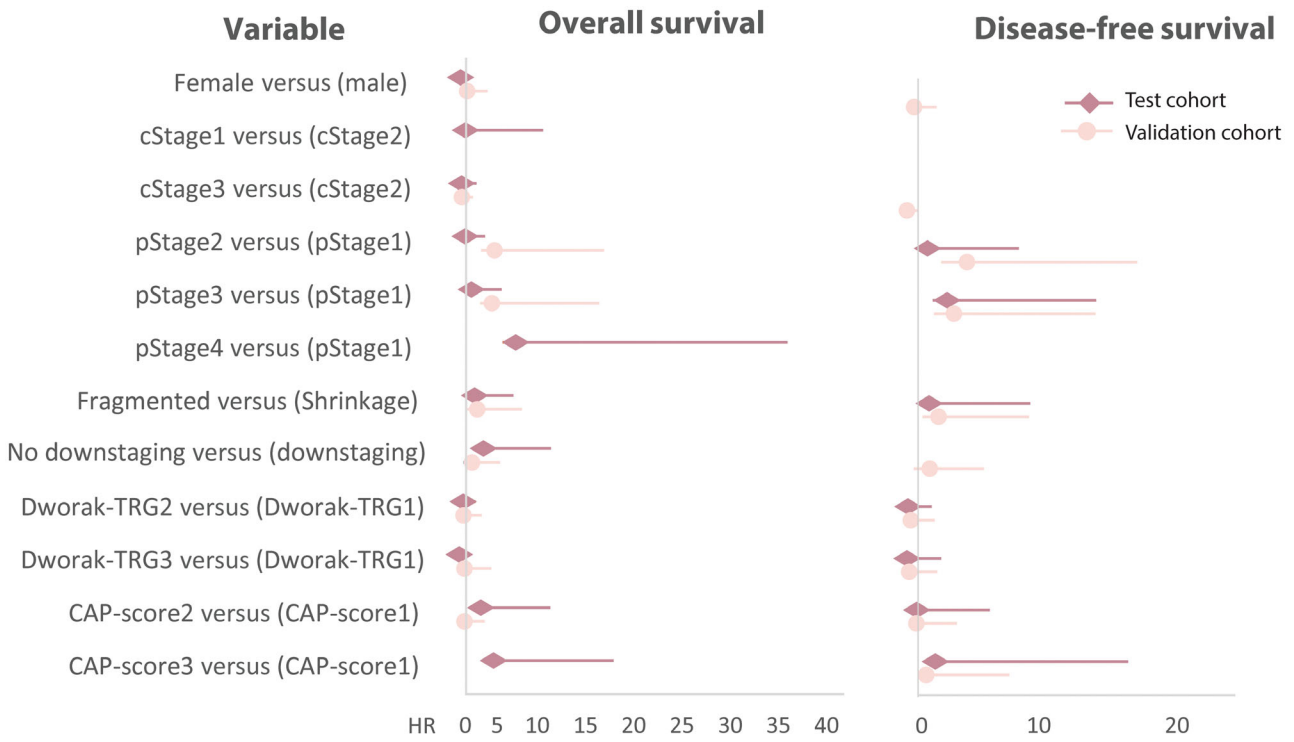

Figure 3. Univariate regression analyses of test and validation cohorts. The reference variable is indicated between brackets (e.g. female versus [male]).

Univariable Cox regression analysis revealed worse OS and DFS rates in patients with a fragmented pattern (HR 2.15, 95% CI 0.99–4.65, $P = 0.05$ and HR 2.75, 95% CI 1.16–6.53, $P = 0.02$, respectively) and advanced ypTNM. Absence of downstaging was also an indicator of poor DFS (Figure 3). Only the ypTNM was an independent prognostic factor of OS (HR 3.14, 95% CI 0.95–10.33, $P = 0.05$) and DFS (HR 3.69, 95% CI 1.31–10.35, $P = 0.01$) in the multivariable regression analyses.

UNIVARIABLE AND MULTIVARIABLE ANALYSES IN THE COMBINED COHORTS

In order to increase the statistical power for the multivariable analysis, both cohorts were pooled together. Patients with a shrinkage pattern had a better OS (5-year; 87% versus 73%, $P = 0.008$; Figure 4) and DFS (5-year; 80% versus 63%, $P = 0.004$; Figure 4B). Neither Dworak nor CAP TRG classifications significantly predicted OS or DFS.

Univariable regression analysis confirmed the poor prognostic effect of the fragmented pattern in OS (HR 2.04, 95% CI 1.19–3.50, $P = 0.008$) and DFS (HR 2.50, 95% CI 1.23–5.10, $P = 0.011$). Advanced ypTNM and the absence of downstaging were other prognostic indicators associated with worse OS and DFS. Multivariable regression analysis revealed ypTNM as the only significant independent prognostic factor of OS and DFS (Tables 2 and 3).

Discussion

Our study confirms that, following CRT, two major response patterns are observed in rectal cancer. The most frequent pattern is fragmentation. However, shrinkage is associated with more regression, and a better outcome. The strong association with ypTNM limits its impact in the multivariable analysis.

This study is in line with earlier observations in oesophageal cancers, suggesting that there are universal patterns of response in gastrointestinal tract tumours.[26] In rectal cancer, we observe a similar, although less pronounced, prognostic impact. A reason behind this might be the relatively favourable life expectancy of rectal cancer compared to oesophageal cancer,[29,30] even in tumours with a fragmented pattern.

Both the poor interobserver agreement in TRG and its discrepancies with ypTNM[4,27,28] hamper clinical

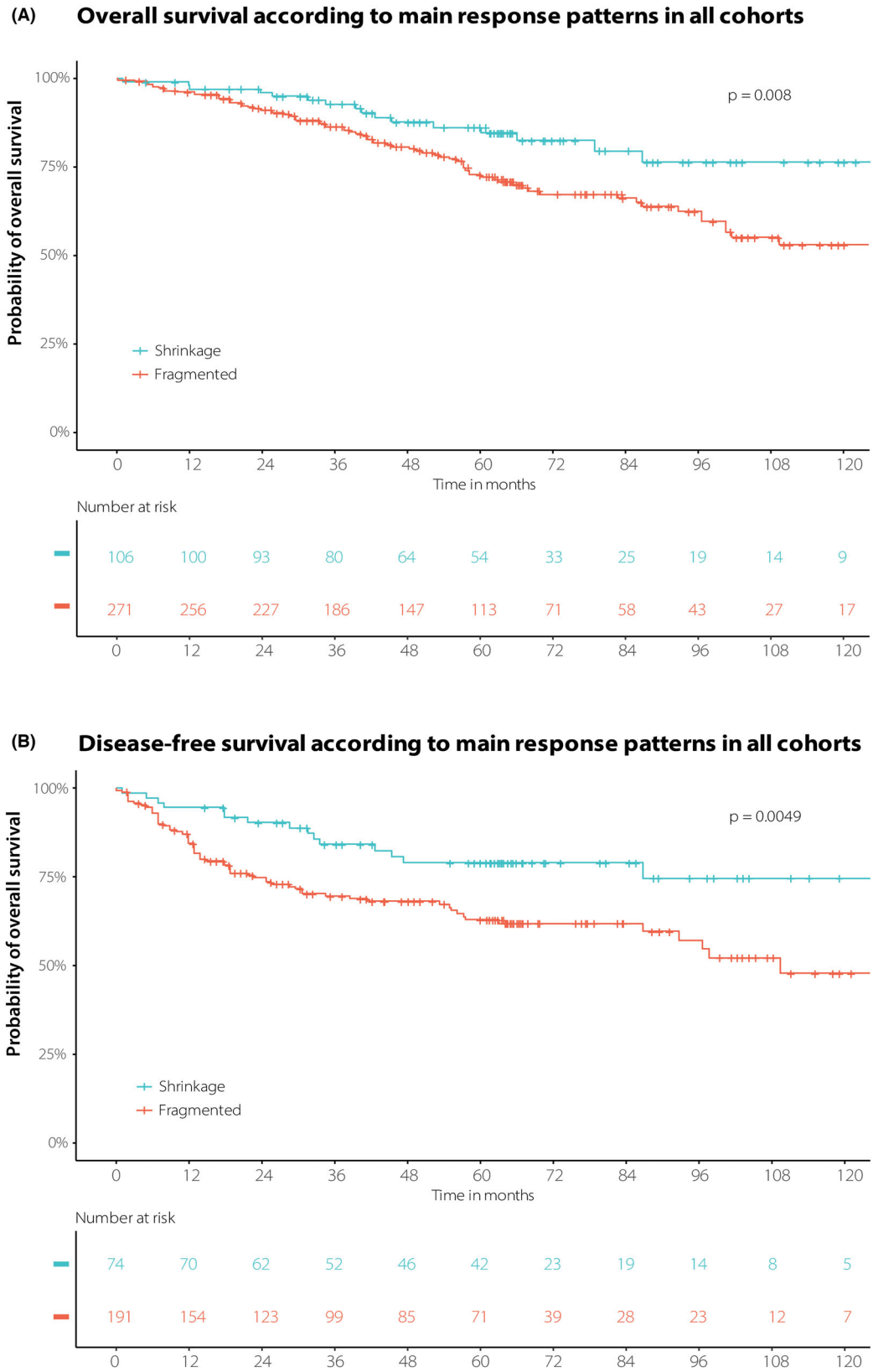

Figure 4. **A,B:** Overall survival and disease-free survival curves according to two main response patterns in combined cohorts.

Table 2. Univariate and multivariate analysis to identify risk factors of overall survival in combined cohorts

		Univariate analysis		Multivariate analysis	
Covariate	*n*	HR	95% CI	HR	95% CI
Gender					
Male	180	1.00			
Female	112	0.74	0.46–1.19		
Cohort					
Test	197	1.00			
Validation	180	0.90	0.60–1.37		
Clinical stage					
I	5	1.00			
II	45	1.79	0.23–13.55		
III	212	1.01	0.13–7.30		
Pathological stage					
I	107	1.00		1.00	
II	140	**1.79**	**0.99–3.24**	1.90	0.83–4.35
III	121	**2.11**	**1.17–3.82**	1.73	0.68–4.40
IV	4	**7.73**	**1.75–34.08**	—	—
Response pattern					
Shrinkage	106	1.00		1.00	
Fragmentation	271	**2.04**	**1.19–3.50**	1.82	0.87–3.79
Downstaging					
Yes	165	1.00		0.63	0.33–1.19
No	96	**1.85**	**1.12–3.05**	1.00	
Dworak TRG					
TRG1	68	1.00			
TRG2	262	0.67	0.40–1.11		
TRG3	47	0.48	0.22–1.04		
CAP TRG					
Score 1	49	1.00		1.00	
Score 2	278	1.48	0.76–2.88	0.85	0.36–2.02
Score 3	50	**2.45**	**1.10–5.48**	1.01	0.34–2.98

The comparisons that were statistically significant are highlighted with bold. HR, hazard ratio; CI, confidence interval.

Table 3. Univariate and multivariate analysis to identify risk factors of disease-free survival in combined cohorts

		Univariate analysis		Multivariate analysis	
Covariate	*n*	HR	95% CI	HR	95% CI
Gender					
Male	115	1.00			
Female	65	0.97	0.57–1.64		
Cohort					
Test	85	1.00			
Validation	180	1.31	0.79–2.15		
Clinical stage					
I	1	—			
II	30	1			
III	149	0.46	0.26–0.80		
Pathological stage					
I	72	1.00		1.00	
II	108	**3.67**	**1.79–7.53**	**3.69**	**1.31–10.35**
III	83	**3.42**	**1.63–7.18**	2.09	0.64–6.85
Response pattern					
Shrinkage	74	1.00		1.00	
Fragmentation	191	**2.50**	**1.23–5.10**	1.36	0.66–2.79
Downstaging					
Yes	110	1.00		0.58	0.29–1.15
No	69	**2.12**	**1.16–3.90**	1.00	
Dworak TRG					
TRG1	36	1.00			
TRG2	195	0.68	0.34–1.36		
TRG3	34	0.59	0.22–1.55		
CAP TRG					
Score 1	34	1.00			
Score 2	198	1.19	0.51–2.52		
Score 3	33	1.92	0.73–5.05		

The comparisons that were statistically significant are highlighted with bold. HR, hazard ratio; CI, confidence interval.

decision-making. By application of response patterns, we can link response to the stage and the presence of residual tumour cells in deeper layers of the bowel wall. In this study, as well as in oesophageal carcinoma,[26] we have shown that implementing our definition and the three-step flowchart classification provided a more reliable and reproducible classification of response patterns, thus solving a practical issue with response classification. Moreover, its clinical relevance is consistent with the previous literature.[17,23,24,26]

Understanding response to treatment is important on several levels, directly, to determine further treatment strategies but also, indirectly, to understand underlying biological processes. Both pathological and clinical/radiological examinations generally lead to a straightforward diagnosis in case of pCR and no response. However, the evaluation of partial response remains problematic due to the variation and irreproducibility of the methodologies used.[8–13,21,24,25] On the other hand, partial responders are heterogeneous in terms of outcome. Therefore, there is an urgent need to define solid strategies to improve decision-making and differentiate the poor prognostic subgroups in partial response diagnosis. From that point of view, tumour response patterns may be a strong candidate to investigate tumour behaviour in partial responders.

To the best of our knowledge, there is no explanation yet as to why some tumours shrink while others break into fragments. Indeed, Graham Martinez *et al*. observed a difference in stromal immune cell populations between shrinkage and fragmented patterns in rectal cancer patients.[31] Yet, more research is needed to provide valuable information about tumour biology behind these patterns. With the increasing trend for organ preservation in both oesophageal and rectal cancer, there is an urgent need for understanding tumour response, and reliable prediction of downstaging and residual tumour. It might be interesting to compare presurgical radiologic findings of two main response patterns and even to make an effort to establish a radiological classification based on these patterns, to guide the extent of surgical treatment. Since patients with a fragmented pattern might not be cured by local excision, it is essential to identify those patients early and treat them accordingly. This should be the next step in predictive research.

Our study had a retrospective nature that may cause bias in case selection. However, including international cohorts and validating our findings with an external cohort strengthen our results.

In conclusion, we confirmed the reproducibility and replicability of the classification for patterns of response in rectal cancer. This classification can be easily implemented in clinical practice in addition to routinely used regression schemes. Patients with a shrinkage pattern presented with a favourable overall and DFS in rectal adenocarcinoma cohorts. These patterns provide insight in the heterogeneity of tumour response and form the basis for future novel treatment strategies.

Author contributions

S.K.O. and C.G.M. performed the research. S.K.O., C.G.M., R.S.P., and I.D.N. designed the research study. S.K.O., C.G.M., K.S., S.A., M.D., and D.M. collected the data. S.K.O. and C.G.M. analysed the data. S.K.O. wrote the article. All authors provided critical feedback on the article.

Acknowledgements

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Conflict of interest

All authors declare that they have no conflicts of interest.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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