

## Research Article

# A study of tumor budding and the factors, affecting interpretability of peritumoral budding, based on endoscopic colorectal biopsies from the left and right sided colorectal carcinoma

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## Summary

In this paper we summarize the results of a retrospective investigation of tumor budding in endoscopic preoperative biopsies in patients with colorectal adenocarcinoma (CRC). The aim of this study was to assess the peritumoral budding evaluation interpretability, based on endoscopic colorectal biopsies in left and right sided colorectal carcinoma and some of the factors that influence it. A group of 100 patients, with preoperative endoscopic biopsies was selected and the tumor buds were counted on H&E stained slides, according to contemporary clinical recommendations. In the studied patient group, budding was identified in a total of 13 patients (13% of the studied cases). The primary localization of the tumor in the left or right colon was not associated with the reporting of budding in endoscopic biopsies of colorectal carcinoma. No budding was observed in highly differentiated tumors, and the presence of budding was reported less frequently in poorly differentiated tumors compared to moderately differentiated ones. Budding was accessible for evaluation in nearly 1/10 of the small biopsies from CRC. Artifacts from the sample management as well as factors related to the tumor characteristics predetermined the possibility for evaluation of tumor budding on small biopsies.

**Key words:** Colorectal adenocarcinoma, endoscopic biopsy, tumor budding



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## Introduction

In colorectal cancers (CRC), epithelial mesenchymal transition (EMT) is one of the mechanisms allowing tumor epithelial cells to undergo mesenchymal transformation and to acquire some mesenchymal characteristics. Due to EMT, tumor cells gain increased migration capacity, develop resistance to apoptosis and as a result their invasive potential increases (Zlobec and Lugli 2010; Lugli et al. 2017; Zlobec et al. 2020). Epithelial mesenchymal transition of cancer cells is one of the key factors for resistance to therapy and poor prognostic predictor in various cancer types, especially in CRC (Jäger et al. 2018; Maffei et al. 2019; Zlobec et al. 2020). This phenomenon is best presented at invasive

front of the tumor and is known as tumor budding (TB). The TB can be divided to peritumoral budding (PTB)- tumor buds at the invasive front of the tumor and intratumoral budding (ITB)- tumor buds within the tumor (Lugli et al. 2017; Haddad et al. 2021; Pour Farid et al. 2021).

The definition and consensus for implementation of tumor budding in colorectal carcinoma were accepted in 2016 in Bern, Switzerland and published in *Modern Pathology* journal in 2017 (Lugli et al. 2017).

Tumor buds are defined as a single cell or a cluster up to four cancer cells, located at the invasive front of the tumor, in addition tumor buds are graded in a three-tiered scale (Lugli et al. 2017):

- Bd1- low budding- 0–4 tumor buds;
- Bd2- intermediate budding- 5–9 tumor buds;
- Bd3- high budding- 10 or more tumor buds.

Some recent studies propose a new -“0- zero” tumor budding group but these propositions have not yet been accepted as a routine practice (Zlobec et al. 2021).

According to the consensus, the assessment of PTB is performed on H&E stained slides in a hotspot area with the highest bud numbers located at the invasive front of the tumor.

Tumor buds are counted at hotspots, using 20× objective within an area of 0.785 mm<sup>2</sup> (Lugli et al. 2017; Haddad et al. 2021).

Pursuant to the recommendations of ITBCC, the main goals of tumor budding assessment are:

- 1<sup>st</sup>- to predict the possibility of metastasis in local lymph nodes in pT1 CRC and to select patients for radical surgery after endoscopic polypectomy;
- 2<sup>nd</sup>- TB is an independent predictor of patients survival in Stage II CRC, allowing to make a decision for adjuvant therapy in high-risk patients, similar to lymphovascular, perineural and serosal invasion, positive margins, etc (Lugli et al. 2017; Zlobec et al. 2020; Haddad et al. 2021; NCCN 2023).

## **Aim**

To assess the tumor budding evaluation interpretability, based on endoscopic colorectal biopsies in left and right sided colorectal carcinoma and some of the factors that influence it.

## **Materials and methods**

### **Cohort selection**

We conducted a retrospective selection of 100 patients with CRC through random sampling, from the archive of the Pathology Department at “Georgi Stranski” University Hospital- Pleven.

The biopsy samples were obtained over two-year period, between 2020 and 2022.

All patients provided signed informed consent at the respective hospital department.

The Ethics Committee of Medical University – Pleven approved the current study before its commencement.

The selection was made according to our predefined inclusion and exclusion criteria shown in Table 1.

All patient data were anonymized and used without any patient identification.

The collected information, including patient gender, age, tumor localization, histological type and grade, tumor budding, reasons for non-reporting and all additional data, was summarized and recorded in a protocol form.

**Table 1.** Criteria for patient selection.

Inclusion criteria:	Exclusion criteria:
- patients with primary CRC	- metastases to the colon and rectum from other primary tumors (based on clinical data)
- preoperative endoscopic biopsy	- preoperative neoadjuvant therapy
	- other types of primary colorectal tumors, different from CRC
	- recurrent CRC
	- non-endoscopically obtained biopsy sample
	- biopsy samples at risk of depletion

## Methods

During our study, we strictly adhered to the protocol for tumor budding assessment, published by ITBCC (Lugli et al. 2017; Haddad et al. 2021).

1. In our study, we were focused on peritumoral budding only.
2. Biopsy samples were fixed in 10% buffered formalin, embedded in paraffin blocks, cut at 4  $\mu\text{m}$  and stained with H&E.
3. Tumor buds were evaluated on H&E stained slides according to the following algorithm:
  - scanning 10 fields at 10 $\times$  objective to identify the invasive front of a tumor and the TB hotspot area;
  - counting PTB in a hotspot area at 20 $\times$  objective;
  - dividing the result by the normalization factor to receive TB count at 0.785 mm<sup>2</sup>, in our work the normalization factor was 0.810;
  - tumor budding grading:
    - Bd1- low budding- 0–4 tumor buds;
    - Bd2- intermediate budding- 5–9 tumor buds;
    - Bd3- high budding- 10 or more tumor buds;
4. Evaluation and classification of factors preventing the determination of tumor budding.

## Immunohistochemical examination

Immunohistochemical staining was performed on selected tumor slides exclusively to compare and validate the tumor budding (TB) results obtained from the H&E slides.

We did not employ immunohistochemically stained slides for TB quantification.

A Dako Agilent Autostainer Link 48 slide stainer was used in accordance with the manufacturer's protocol.

The used primary antibodies were: Cytokeratin, Clone AE1/AE3, FLEX RTU. Monoclonal Mouse Anti-Human, ready-to-use antibody (Dako, Agilent Technologies, Inc.).

### Statistical analysis

The tumor budding results, along with clinicopathological data, were summarized, recorded in protocol forms, and analyzed using IBM SPSS Statistics.

The Chi-Square test was used to compare categorical data.

Values of  $p < 0.05$  were considered statistically significant.

Parametric data were assessed using the One-way ANOVA test, and for data that did not follow a normal distribution, the Kruskal- Wallis test was employed.

Values of  $p < 0.05$  were considered statistically significant.

## Results

### Population data

A total number of 100 patients with endoscopic biopsies from colorectal carcinoma were investigated with a median age of 71 years (range: 35 to 87 years).

Men - 63 with a median age of 70 years (range: 48 to 87 years) and women - 37 with a median age of 72 years (range: 35 to 87 years).

There is no significant difference in age between the two gender groups (Kruskal-Wallis test:  $K-W = 1.2500$ ;  $p = 0.2635$ ), (Table 2).

**Table 2.** Distribution of patients by gender and age.

Gender	Number	Median age	Range years
Male	63	70.27	48 to 87
Female	37	71.86	35 to 87
Total	100	70.86	35 to 87

Patients with carcinoma of the left colon were 24, with a median age of 74 years (range: 62 to 87 years), and patients with carcinoma of the right colon were 76, with a median age of 70 years (range: 35 to 87 years), where the difference in age is not statistically significant (Kruskal-Wallis test:  $K-W = 3.2911$ ;  $p = 0.0696$ ), (Table 3).

**Table 3.** Distribution of CRC by localization, number of patients and age.

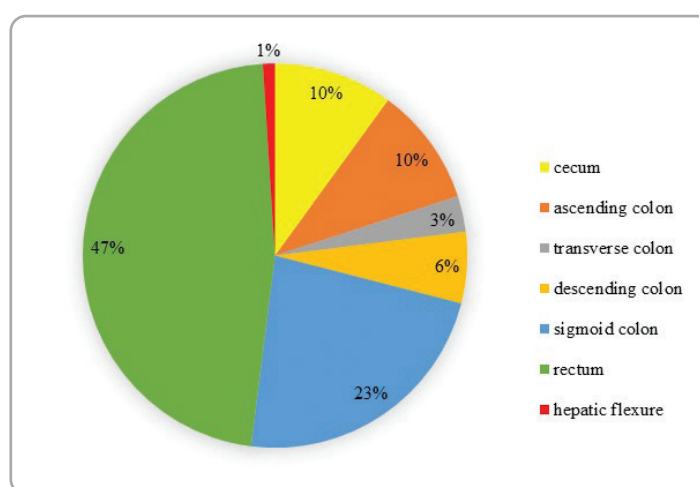
Localisation	Patients Number	Median age	Range years
Left colon	24	74.17	62 to 87
Right colon	76	69.82	35 to 87

Distribution of CRC according to the primary tumor localization and tumor histology (Fig. 1). Most often, the pathology occurred in the rectum ( $n = 47$ ), followed by the sigmoid colon ( $n = 23$ ), cecum ( $n = 10$ ) and ascending colon ( $n = 10$ ).

Distribution of CRC according to the primary tumor histology is given in Table 4.

**Table 4.** Distribution of CRC according to the primary tumor localization and tumor histology.

Localization	Tumor histology						Total
	AdenoCa NOS	AdenoCa with extracellular mucin production	AdenoCa with intra- and extracellular mucin production	AdenoCa with poorly cohesive component	Medullary adenocarcinoma	Poorly cohesive adenoCa	
Cecum	8	0	1	0	1	0	10
Ascending	9	0	0	0	0	1	10
Hepatic flexure	1	0	0	0	0	0	1
Transverse	2	1	0	0	0	0	3
Descending	5	0	0	1	0	0	6
Sigmoid	22	0	1	0	0	0	23
Rectum	45	1	1	0	0	0	47
Total	92	2	3	1	1	1	100

**Figure 1.** Distribution of CRC according to the primary tumor localization.

### Peritumoral budding assessment

In the studied patient group, budding was identified in a total of 13 patients (13% of the studied cases).

Among these cases, 10 (10%) exhibited optimal biopsy quality and tumor morphology, facilitating the detection of peritumoral budding. Within this subgroup, three cases (3%) were categorized as showing low budding, five cases (5%) as moderate budding, and two cases (2%) as high budding.

Eight biopsies contained poorly differentiated tumors, and among them, only one case (1%) demonstrated moderate budding.

In a group with significant fragmentation of the biopsy samples, a total of 44 cases (44%), budding was identified in only one case (1%). In this particular case, a high tumor budding was observed (>10 individual cells and cell clusters).

In twelve cases, the material was insufficient, preventing the assessment of TB.

Furthermore, various factors affected the assessment of tumor budding: necrosis was observed in 10 cases (10%), one case (1%) represented a poorly cohesive tumor with High PTB, one case (1%) had significant inflammation, an invasive front was absent in six cases (6%), and the presence of well-differentiated tumors was noted in eight cases (8%). All of these factors prevented an objective evaluation of tumor budding.

The possibilities for detecting budding in the studied endoscopic biopsies and the artifacts complicating PTB assessment were presented in Table 5.

**Table 5.** Artifacts complicating assessment: types and effect on reproducibility.

	Budding not / cannot be reported	Reported tumor budding
Endoscopic biopsies suitable for assessment (optimal)	0 (0%)	10 (10%)
Endoscopic biopsies with artifacts complicating assessment *	33 (33%)	1 (1%)
Assessment challenges related to tumor characteristics **	54 (54%)	2 (2%)

Legend:

\* Fragmented and/or scant material;

\*\* Tumors with extensive necrosis, poorly differentiated tumors, poorly cohesive tumors, tumors with marked inflammation, tumors lacking an invasive front, highly differentiated tumors.

Factors influencing peritumoral tumor budding assessment in endoscopic colorectal biopsies are shown in Table 6.

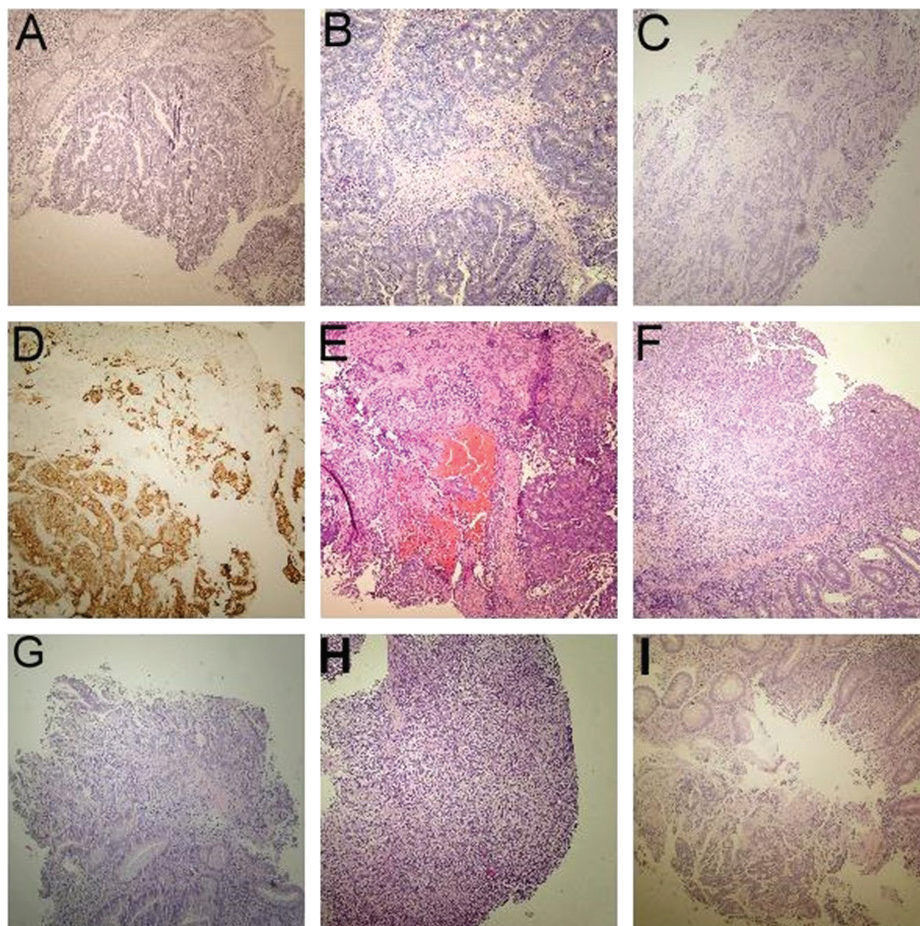
**Table 6.** Factors influencing tumor budding assessment in endoscopic colorectal biopsies.

Factors influencing PTB assessment	Assessed peritumoral budding				Total
	0	1	2	3	
Optimal sample	0	3	5	2	10
Lacking invasive front	6	0	0	0	6
Well-differentiated	8	0	0	0	8
Marked inflammation	1	0	0	0	1
Necrosis	10	0	0	0	10
Poorly differentiated	7	0	1	0	8
Insufficient	12	0	0	0	12
Poorly cohesive	0	0	0	1	1
Fragmented	43	0	0	1	44
Total	87	3	6	4	100
Optimal sample	0	3	5	2	10

Factors affected the assessment of peritumoral tumor budding (PTB) were illustrated in the Fig. 2. For comparison purposes only, certain slides were stained using the CK AE1/AE3 antibody.

The presented data suggests that in over half of the examined cases, tumor budding cannot be evaluated due to the characteristics of the tumor component. Furthermore, in one-third of cases, artificial alterations in the biopsy material complicate the interpretation and reporting of tumor budding. Only 1 in 10 biopsies were found optimal for reliable assessment and interpretation of tumor budding in endoscopic biopsies from colorectal carcinoma, making it challenging to establish this method as a routine standard.

In the context of tumor differentiation, it was observed that budding most frequently was reported in moderately differentiated tumors. No budding was observed in well-differentiated tumors, and the presence of budding is reported less frequently in poorly differentiated tumors compared to moderately differentiated ones. However, these differences do not exhibit statistically significant dependence (Table 7).



**Figure 2.** Factors affected the assessment of peritumoral tumor budding (PTB) **A** well differentiated CRC located at the periphery of the biopsy sample, with lacking of the tumor invasive front **B** well differentiated CRC without PTB **C, D** (CK AE1/AE3) – CRC with marked inflammation and necrosis **E** biopsy sample with marked necrosis **F** poorly differentiated CRC with marked inflammation at the invasive front **G** insufficient and peripherally located CRC at the biopsy **H** poorly cohesive CRC **I** fragmented CRC at the biopsy sample. The slides A, B, C, E, F, G, H, I, were H&E stained. Slide D were stained using CK AE1/AE3 antibody. All slides were photographed at original magnification at 20×.

**Table 7.** Dependence between CRC differentiation degree, peritumoral budding grade and PTB assessment in endoscopically obtained CRC biopsies.

Grade	Budding not / cannot be reported	Reported tumor budding
G1	10 (10%)	0 (0%)
G2	64 (64%)	9 (9%)
G3	13 (13%)	4 (4%)

In relation to the histological variant of the tumor, the analysis indicated that budding was most frequently reported in adenocarcinomas NOS- 10 of 92 cases. Furthermore, budding was detected in one of three cases of adenocarcinoma with intra- and extracellular mucus production, as well as in the only cases of adenocarcinomas with a poorly cohesive component and poorly cohesive adenocarcinoma. However, budding was not reported in other variants, such as medullary adenocarcinoma and adenocarcinoma with extracellular mucus production (Table 8).

**Table 8.** Evaluation of peritumoral budding (PTB) in various histological types of colorectal carcinoma (CRC).

Tumor histology	Assessed peritumoral budding				Total
	0	1	2	3	
Adenocarcinoma NOS	82	3	5	2	92
Adenocarcinoma with extracellular mucin production	2	0	0	0	2
Adenocarcinoma with intra-and extracellular mucin production	2	0	1	0	3
Adenocarcinoma with poorly cohesive component	0	0	0	1	1
Medullary adenocarcinoma	1	0	0	0	1
Poorly cohesive adenocarcinoma	0	0	0	1	1
Total	87	3	6	4	100

As expected, gender was not associated with the reporting of tumor budding in endoscopic biopsies of colorectal carcinomas (Table 9).

**Table 9.** Gender distribution and presence of tumor budding in endoscopic biopsies from colorectal carcinoma cases.

Gender	Budding not / cannot be reported	Reported tumor budding
Male	54 (54%)	9 (9%)
Female	33 (33%)	4(4%)

The primary localization of the tumor in the left or right colon was not associated with the reporting of budding in endoscopic biopsies of colorectal carcinoma ( $\chi^2 = 0.00$ , Df = 1, p = 1.0000), (Table 10).

**Table 10.** Distribution of colorectal carcinomas in the left and right colon and presence of tumor budding in endoscopic biopsies.

	Budding not / cannot be reported	Reported tumor budding
Left colon	20 (20%)	4(3%)
Right colon	67 (67%)	9(9%)

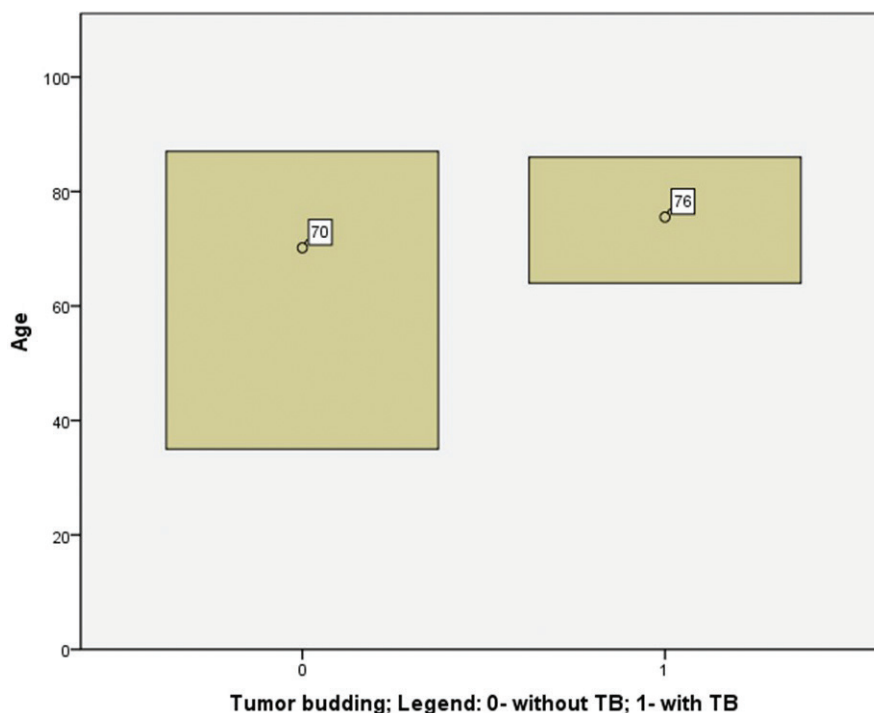
A significant difference was observed in the age of patients in whom budding was reported compared to those in whom it was not reported. Among the patients in whom no budding was reported (n = 87), the median age was 70 years (ranging from 35 to 87 years), which was significantly lower than the median age of 76 years (ranging from 64 to 86 years) observed in the thirteen patients with reported tumor budding (K-W = 4.2668, p = 0.0389) (Fig. 3).

## Discussion

The concept proposing a potential correlation between patients' survival, tumor cells growth activity, the presence of clusters of poorly differentiated cells within the tumor, and interactions between tumor stromal cells, the host immune system, and cancer cells was initially introduced by Broders in 1920 (Broders 1920).

In 1949, Imai mentioned for the first time the term "tumor sprouting" in the context of stomach cancers (Imai 1949).





**Figure 3.** Age differences between patients with or without reported TB.

Jass et al., in 1987, introduced new prognostic criteria for rectal cancers and proposed a scoring system for several histologic tumor variables. One of these variables was the “infiltrating” pattern of the tumor margins growth (Jass et al. 1987).

The term “budding” was first used by Morodomi et al. in 1989. They examined the relationship between tumor budding, lymphatic and venous invasion, and lymph node metastasis in preoperative biopsies from patients with rectal adenocarcinomas. Additionally, they proposed a quantification scoring system for the tumor buds (Morodomi et al. 1989).

Tumor budding has been identified as a prognostic factor not only in colorectal adenocarcinomas but also in wide range of other cancer types, including esophageal (Almangush et al. 2015; Davison et al. 2016; Thies et al. 2016) and gastric carcinomas (Gulluoglu et al. 2015; Guo et al. 2019; Zlobec et al. 2020; Gorcheva 2022), cholangiocarcinoma (Tanaka et al. 2019), oral (Ho et al. 2019) and lung cancers (Kadota et al. 2015; Nepl et al. 2020), urinary bladder (Fukumoto et al. 2016; Eckstein et al. 2021; Raventós Busquets et al. 2021; Seker et al. 2021), uterine cervix (Jesinghaus et al. 2018), and breast carcinomas as well (Li et al. 2017).

The understanding of tumor budding in colorectal cancer (CRC) underwent significant development with the Consensus Conference held in Bern, Switzerland in 2016. This Consensus was later published in the *Modern Pathology* journal in 2017 and played a pivotal role in refining the definition, assessment methods, and clinical significance of tumor budding in CRC (Lugli et al. 2017).

The Delphi consensus study conducted between 2019 and 2021 involving 14 pathologists with expertise in colorectal carcinoma represents a significant advancement in the understanding of tumor budding (TB). They not only reaffirmed the conclusions of the International tumor budding consensus con-

ference (ITBCC) but also introduced new important topics that require further investigation in TB assessment. Some of these topics include:

1. TB and post-neoadjuvant therapy;
2. TB and IHC Assessment Method;
3. High TB and prediction of response to adjuvant therapy in Stage II CRC;
4. Relations between TB and PDC (Poorly Differentiated Clusters).

In addition to these new topics, the consensus study also highlighted the need for education in TB assessment, addressed the difficulties and causes of TB misinterpretation, and explored the potential for automating the TB assessment process. These findings not only contribute to a deeper understanding of tumor budding's clinical implications but also pave the way for more accurate and effective diagnostic and treatment strategies in colorectal cancer patients (Haddad et al. 2021).

The challenges associated with assessing tumor budding (TB) in preoperative biopsies have been thoroughly explored by various authors (Lugli et al. 2017; Almangush et al. 2019; Haddad et al. 2021). These challenges include factors such as extensive tumor necrosis and inflammation, tumor gland fragmentation, the absence of an invasive tumor front, well-differentiated tumors, and undifferentiated tumors. In our study, we also aimed to identify some of these factors, and the most significant ones were as follows: fragmentation of the biopsy material: 43%, insufficient biopsy sample: 12%, tumor necrosis: 10%, well-differentiated tumors: 8%, poorly differentiated tumors: 7%, lack of an invasive tumor front: 6%, marked inflammation: 1%. The challenges, encountered in the present study were in accordance with these, described in literature both as entities and to some extent as incidence.

Understanding and addressing these factors are crucial for accurate and reliable assessment of TB in preoperative biopsies. These challenges highlight the complexity of evaluating TB and emphasize the need for standardized protocols and guidelines to ensure consistent and meaningful results.

In 2021, Zlobec et al. introduced a novel scoring system for tumor budding (TB) which included a new category called "zero" budding. Their study revealed that over 10% of colorectal carcinomas exhibited this new "0" budding score.

In our study we observed the same phenomenon but we put all cases with "zero" budding to BD1 category, according to the recent rules of ITBCC.

However, it's important to note that this new grading system has not yet been widely accepted or adopted within the medical community. The proposed system suggests a more comprehensive approach to TB assessment, possibly encompassing a broader range of budding patterns including those with no budding activity. As with any new approach, further research, validation, and consensus among experts are necessary before such modifications become widely integrated into clinical practice (Zlobec et al. 2021).

Besides peritumoral budding, many studies have concentrated on investigating two other morphological characteristics of tumors and their predictive significance in patients with colorectal carcinoma. These characteristics encompass intratumoral budding and poorly differentiated tumor clusters. Intratumoral budding (ITB) shares similar characteristics with peritumoral budding (PTB), but it is observed within the central region of the tumor. In this context,

the endoscopic biopsies are limited in size and taken from rather superficial periphery of the tumor and occasionally pericentral (deeper) parts. This will formally classify the evaluated budding in another category or make it a mixture of intra- and peritumoral. There have been extensive investigations aimed at understanding the clinical implications and prognostic value of ITB and PTB. On the other hand, poorly differentiated tumor clusters (PDC) are defined as clusters containing five or more tumor cells that lack the glandular formation and they also can be observed at the invasive front of the tumor. Both intratumoral budding and poorly differentiated clusters exhibit similar biological features as peritumoral budding. ITB and PDC can also play a crucial role in determining prognosis and guiding treatment strategies for patients and need further investigation (Zlobec and Lugli 2010; Giger et al. 2012; Semba 2012; Lugli et al. 2017; Fujiyoshi et al. 2020; Smit et al. 2021; Jurescu et al. 2022; Sarkar et al. 2022).

In our study, we did not find any significant correlations between tumor budding (TB) and patients' sex or the primary tumor localization. This suggests that these factors may not have a substantial influence on the occurrence or extent of TB in the cases studied. It's important to consider that the absence of such associations could be due to various factors, including the sample size, small number of patients, and tumor characteristics within the specific cohort studied.

Interestingly, we observed a difference in age between patients with reported tumor budding (TB) and those in whom TB could not be assessed. However, due to the small size of our patient cohort, we refrain from making speculative interpretations about this phenomenon. Further studies with larger cohorts may be needed to better understand any potential age-related differences in tumor budding assessment.

## Conclusions

Peritumoral budding is recognized as an independent prognostic factor in patients with colorectal cancer (CRC) for several key aspects:

- Predicting lymph node metastasis in pT1 CRC.
- Predicting patient survival in Stage II CRC.
- Determining the necessity of neoadjuvant therapy in Stage II CRC.

These significant associations have led to the incorporation of tumor budding (TB) as an important histological feature in the evaluation of CRC (Nagtegaal et al. 2019; CAP 2023; NCCN 2023).

According to our experience, evaluation of budding on preoperative materials is achievable in less than a half of the cases, and even fewer were the samples optimal for evaluation. A question rises if the budding found is peri- or intra-tumoral and thus its clinical meaning is remaining subject of discussions.

Given these findings, it might be necessary to adapt the scoring of budding on small biopsy samples by introducing an interpretability category. This recognition acknowledges that not all cases with low budding scores on small biopsies are necessarily representative of the true tumor biology. Furthermore, there may be a need to establish criteria for categorizing peri- or intra-tumoral budding in specimens obtained from small biopsies. This approach could enhance the accuracy and reliability of budding assessment in such cases.

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