Diploma Thesis

Tumour budding with and without admixed inflammation in colorectal cancer

A systematic clinicopathological analysis

submitted by

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Univ. Doz. Dr. Cord Langner

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Affidavit

I hereby confirm that the following diploma thesis has been written by myself without any support from third parties. For preparation no other sources than those indicated in the thesis have been used.

Please consider that this work has already been published in the journal "British Journal of Cancer":

Max N, Harbaum L, Pollheimer MJ, Lindtner RA, Kornprat P, Langner C. Tumour budding with and without admixed inflammation: two different sides of the same coin? Br J Cancer; 114: 368-71.
PMID: 26766735

Graz, 1st of December 2016 *Nicole Max eh.*

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Abbreviations

AJCC	American Joint Committee on Cancer
APC	Adenomatous polyposis coli
CI	Confidence Interval
CRC	Colorectal Cancer
EET	Epithelial Epithelial Transition
EMT	Epithelial Mesenchymal Transition
ESMO	European Society for Medical Oncology
HR	Hazard Ratio
H&E	Hematoxylin and Eosin
ITBCC	International Tumor Budding Consensus Conference
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
MHC	Major Histocompatibility Complex
MLH1	MutL Homolog 1
MMR	Mismatch Repair
MSH2	MutS Homolog 2
MSH6	MutS Homolog 6
MSI	Microsatellite Instability
MSI-H	High-level Microsatellite Instability
MUC1	Mucin-1
PMS1	PostMeiotic Segregation 1
PMS2	PostMeiotic Segregation 2
TNM	Tumour Node Metastasis
TRP53	Transformation-Related Protein 53
UICC	Union for International Cancer Control
WHO	World Health Organization

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Abstract

Background: The morphology at the invasive tumour margin is known to reflect the biology of disease in colorectal cancer, rendering important prognostic information. Tumour budding, defined as the presence of isolated single cells or small clusters of cells (composed of fewer than five cells), has been associated with adverse outcome in colorectal cancer. In contrast, peritumoral inflammation has been associated with favourable outcome. Of note, anti-tumour activity of inflammation may lead to the destruction of tumour glands and, ultimately, the presence of small clusters of cells scattered in the tumour stroma.

Methods: Colorectal cancers of 381 randomly selected patients were retrospectively evaluated for the extent of tumour budding and peritumoral inflammation. Both parameters were related to various clinicopathological features using univariate and multivariate analyses. Progression-free and cancer-specific survivals were determined using the Kaplan-Meier method.

Results: Tumour budding and overall inflammation in colorectal cancer was significantly associated with both progression-free and cancer-specific survival in our cohort. Cases with high grade budding and marked inflammation had a significant better outcome regarding progression-free (p<0.001) and cancer-specific survival (p<0.001) than high grade cases with only mild inflammation. Outcome in these cases was still worse compared to cases with low grade budding, in which the extent of peritumoral inflammation had no further prognostic effect.

Conclusions: Though tumours with marked inflammation at the invasive tumour margin may show destruction of cancer islands due to the anti-tumour effect of the inflammatory infiltrate, the presence of isolated tumour cells and small clusters of cells scattered in the stroma at the tumour margin does not *per se* imply favourable outcome in these cases. It is of note that tumours with high grade budding and marked inflammation at the invasion front still bear a significantly poorer outcome than tumours with low grade budding, in which the extent of peritumoral inflammation had no prognostic effect. Validation of our findings by other independent centres is desirable.

Zusammenfassung

Hintergrund: Über das kolorektale Karzinom ist bekannt, dass die Morphologie der Invasionsfront eines Tumors die Biologie der Krankheit wiederspiegelt und dadurch wichtige Informationen zur Prognose darstellt. Die Tumorzelldissoziation („Tumour budding“), definiert als der Übergang von glandulären Karzinomstrukturen in Einzelzellformationen oder Anhäufungen von bis zu vier Tumorzellen an der Invasionsfront, ist im kolorektalem Karzinom mit einem ungünstigerem PatientInnenüberleben assoziiert. Im Gegensatz dazu ist die tumorumgebende Entzündungsreaktion mit einem günstigeren PatientInnenüberleben assoziiert. Beachtenswert ist, dass die, gegen den Tumor gerichtete Entzündungsaktivität zu einer Zerstörung der Tumordrüsen und letztendlich zur Anwesenheit von einzelnen verstreuten Tumorzellen oder Zellformationen im Tumorstroma führen kann.

Methodik: Kolorektale Primärtumore von 381 zufällig ausgewählten PatientInnen wurden retrospektiv auf das Ausmaß der Tumorzelldissoziation und auf die tumorumgebende Entzündungsreaktion untersucht. Beide Parameter wurden, unter Verwendung von uni- und multivariaten Analysen, mit verschiedenen klinischen und pathologischen Tumorparametern in Zusammenhang gesetzt. Die prognostische Aussagekraft bezüglich progressionsfreien und krankheitsspezifischen Überleben wurde mit der Kaplan-Meier-Methode überprüft.

Ergebnisse: In unserem PatientInnenkollektiv wurde die Tumorzelldissoziation signifikant mit progressionsfreien und krankheitsspezifischen Überleben assoziiert. Fälle mit hochgradiger Tumorzelldissoziation und ausgeprägter Entzündungsreaktion hatten ein besseres Ergebnis in Bezug auf progressionsfreies (p<0.001) und krankheitsspezifisches (p<0.001) Überleben als Fälle mit hochgradiger Tumorzelldissoziation mit nur leichter Entzündungsreaktion. Das Überleben in diesen Fällen war jedoch trotzdem ungünstiger als in Fällen mit geringgradiger Tumorzelldissoziation, in denen das Ausmaß der tumorumgebenden Entzündungsreaktion keine weitere prognostische Aussagekraft hatte.

Schlussfolgerung: Auf Grund der Antitumorwirkung des entzündlichen Infiltrats können Tumore mit ausgeprägter Entzündungsreaktion an der Invasionsfront eine Zerstörung von Krebsinseln zeigen. In diesen Fällen macht die Anwesenheit von verstreuten Tumorzellen oder Zellformationen im Stroma kein besseres PatinetInnenüberleben *per se* aus. Tumore mit hochgradiger Tumorzelldissoziation und ausgeprägter Entzündungsreaktion an der Invasionsfront haben trotzdem ein signifikant schlechteres Überleben als Tumore mit geringgradiger Tumorzelldissoziation, in denen das Ausmaß der tumorumgebenden Entzündungsreaktion keine weitere prognostische Aussagekraft hat. Die Bestätigung unserer Ergebnisse durch andere unabhängige Zentren ist wünschenswert.

1. Introduction

In 2012, cancer was the main cause of death in both economically developed and developing countries accounting for about 14.1 million cancer cases and 8.2 million cancer related deaths (1). This progress will continue due to aging as well as cancer-associated lifestyle (i.e. smoking, physical inactivity and nutrition). Reproductive changes associated with urbanization and economic development reinforce this progress. Over the years, the burden of cancer has relocated to developing countries. (2) The most frequently diagnosed cancers are lung cancer in males and breast cancer in females. They are responsible for the majority of cancer related deaths for each sex worldwide, independent of the economic status (Figure 1A-B). (3-5)

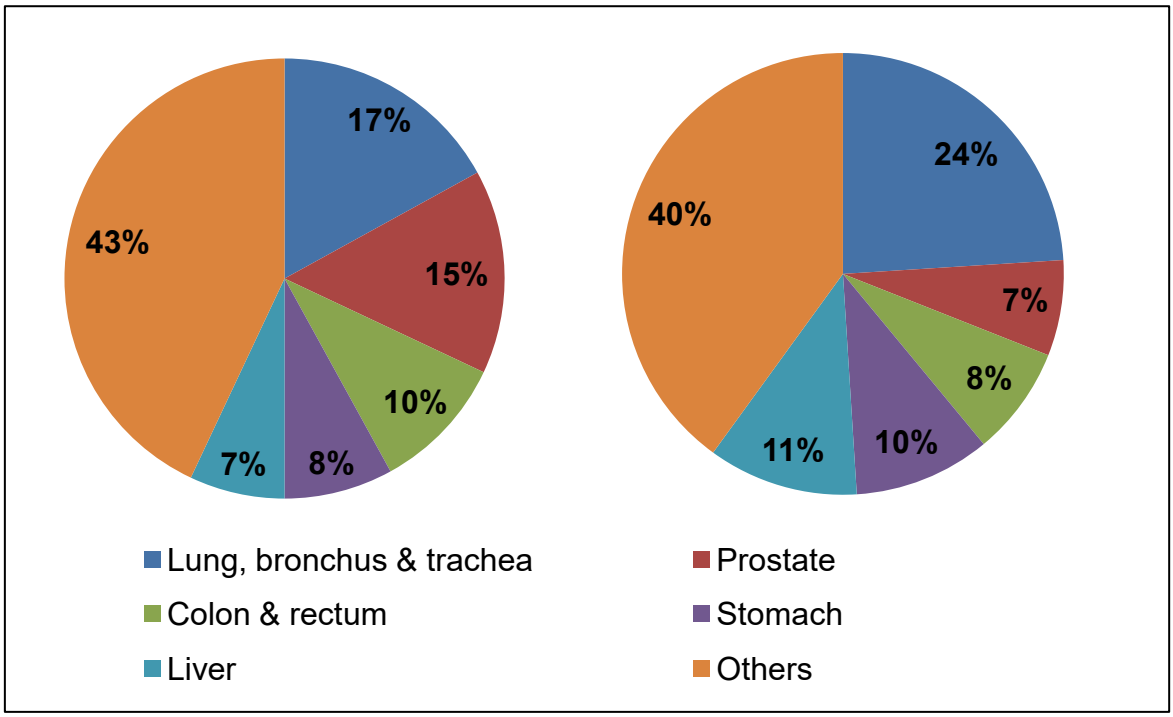


Figure 1A: Estimated new cases (left) and deaths (right) worldwide in men 2012; data from Lindsey et al. (1)

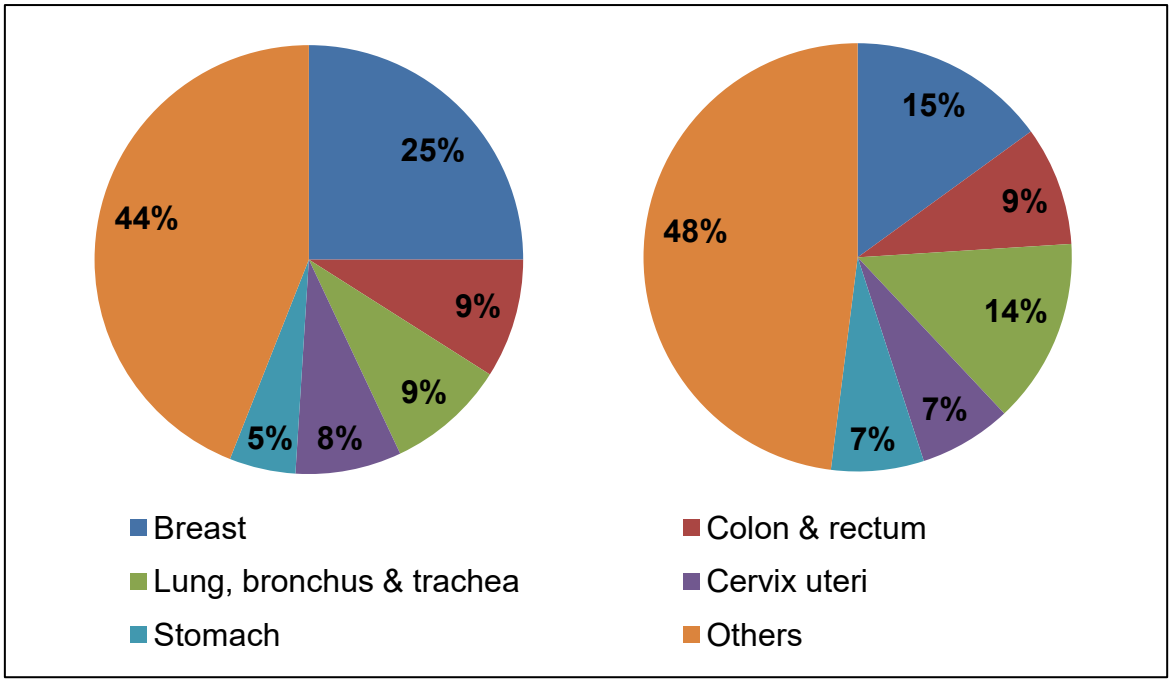


Figure 1B: Estimated new cases (left) and deaths (right) worldwide in women 2012; data from Lindsey et al. (1)

1.1 Epidemiology

Colorectal cancer (CRC) is the third most common cancer in males (after lung and prostate cancer) and second in females (after breast cancer). In 2012, 1.4 million new cases of CRC occurred and 693,900 patients died as result. (1) The incidence rates are higher in men than in women in the most parts of the world and strongly increase with age. In developed countries, the median age at diagnosis is about 70 years. in most cases the development of CRC is sporadic and arises slowly over several years through the adenoma–carcinoma sequence. (1, 2, 6) Countries with the highest incidence rates are Australia/New Zealand, Europe, and Northern America. Africa and South-Central Asia have the lowest rates. (1) Attributed to the so-called western lifestyle including unhealthy diet, obesity, and smoking a radip increase in previous low-risk countries has been observed. Trends in high-risk and high-incoming countries have varied in the last 20 years. (2) While incidence rates increased in Finland and Norway, stabilised in France and Australia, they declined in the United States in patients aged 50 years and older as a result of population-based screening programms and removal of precancerous lesions. (2) In contrast to the incidence rates, decreasing death rates in most countries of the world are noticed, due to reduced prevalence of risk factors, and/or improved treatments. (1)

In the European Union almost 92.900 cancer related deaths in men and 75.500 cancer related deaths in women have been estimated for 2014. (7) Investigating the major EU countries, cancer mortality persists heterogeneously, with a difference of over 70% between men in France and Poland. For women these development is more converging. Compared to 2009 data, age-standardised mortality rate, using the world standard population, of CRC are falling from 17.3/100 000 to 16.5/100 000 in men and from 10.2/100 000 to 9.5/100 000 in women. (7) This correspond to a decrease in death rates of 4% in men and 7% in women, respectivley. (7, 8) For 2015, it is predicted that CRC deaths represent 13% (172 600 projected deaths) of total cancer deaths in the european union. (8)

1.2 Anatomical Evaluation

The human gastrointestinal tract extends from the mouth to the anus and is divided into the upper and lower gastrointestinal tracts. The lower gastrointestinal tract includes the small and large intestine. The large intestine or colorectum extends from the Bauhin's valve to the anal canal and has the following divisions (9): Caecum, ascending colon, hepatic flexure, transverse colon, splenic flexure, descending colon, sigmoid colon, rectosigmoid junction, and rectum. The colon itself, without rectum and appendix vermiformis is split into four parts and termed as follows: the right or ascending colon, the middle or transverse colon, the left or descending colon, and the sigmoid colon. (9, 10) The caecum, ascending colon, hepatic flexure, and transverse colon is summarised as the proximal or right-sided colon, whereas the splenic flexure, descending colon, sigmoid colon and rectosigmoid junction is added to the distal or left-sided colon (Figure 2). (10)

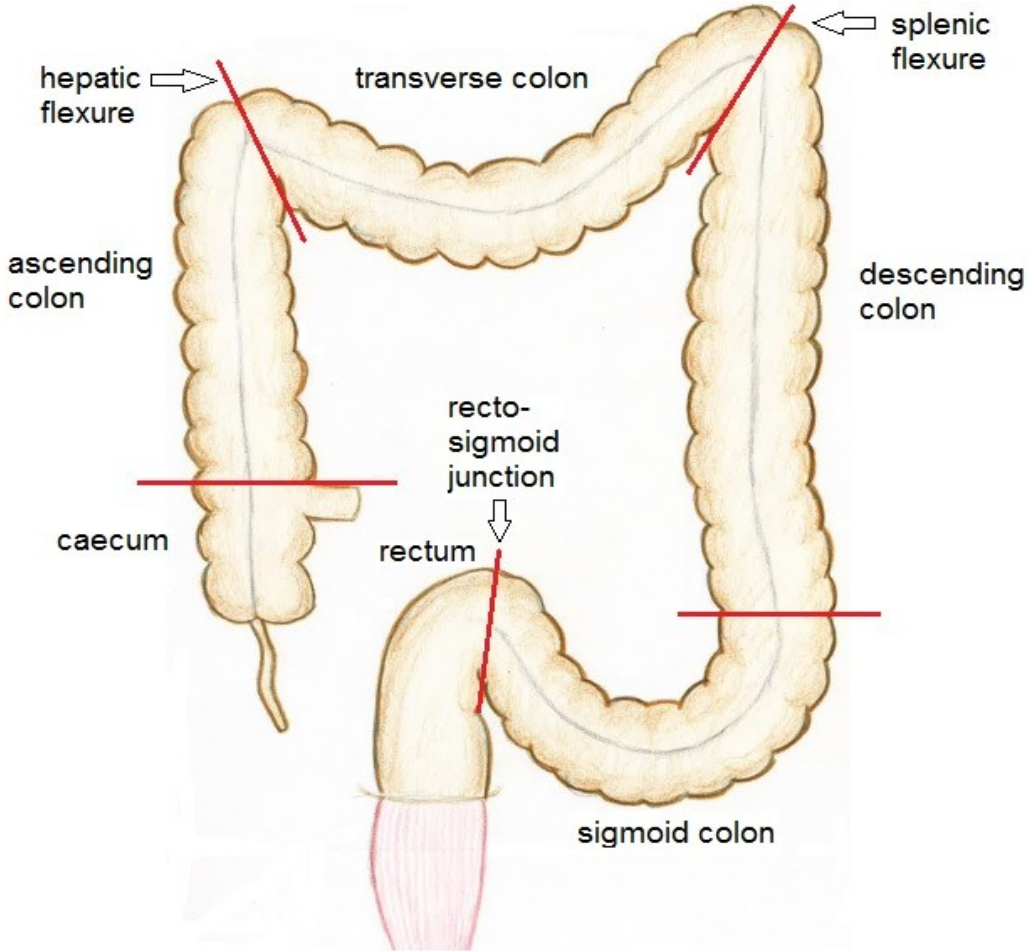


Figure 2: Overview of the large intestine with its four divisions and the rectum. Modified after Schünke et al. (10)

CRCs vary in matters to clinicopathological characteristics based on their location in the colon or rectum, suggesting definite carcinogenic processes and aetiologies. The most commonly diagnosed tumour location is the proximal colon with an average of men and women of 42%, followed by the rectum with 28% and the distal colon with 23%. (4) Meanwhile, this distribution varies by gender and age of diagnosis. Females have a higher percentage of proximal colon cancers, whereas males have a higher percentage to sicken with rectal cancer. (4) In relation to the age of diagnosis, a remarkable decrease in rectal cancers and increase in proximal cancers with advancing age is of note. This results in a younger median age at diagnosis for rectal cancer of 63 years in males and 65 years in females than that of colon cancer with 69 years in males and 73 years in females, respectively. Data are summarised in Table 1. (4)

Table 1: Distribution of CRC in the colon and rectum, United States, 2006-2010; data from Siegel et al. (4)

	All persons	**Male**	**Female**
Proximal colon	42%	38%	46%
Rectum	28%	31%	24%
Distal colon	23%	24%	22%
other	7%	7%	8%

Depending on localisation, the clinicopathological manifestation of CRC is various. Cancer of the distal colon and the rectum are associated with rectal bleeding and blood on the stool. Tumours of the proximal colon may typically cause a change in bowel habits, diarrhoea, constipation, or narrowing of the stool, lasting for more than a few days combined with losing weight. (11)

1.3 Pathology Report

1.3.1 Histology

The World Health Organisation (WHO) classification of carcinomas of the colon and rectum lists several distinct histological variants or subtypes with prognostic potential. Adenocarcinomas represent the greatest part, with approximately 90%, of CRCs. (11, 12) Adenocarcinomas considered malignant, if they have penetrated through the muscularis mucosae into submucosa and are termed ‘adenocarcinomas not other specified’.

The prevailing WHO classification of tumours of the digestive system includes histological subtypes as follows: mucinous adenocarcinoma, signet-ring cell carcinoma, medullary carcinoma and micropapillary adenocarcinoma. (11)

Mucinous adenocarcinomas represent 4%-19% of CRC worldwide shown by population-based studies. (12-16) This term is used if more than 50% of the lesion is composed of pools of extracellular mucin containing malignant epithelium as acinar structures, layers of tumour cells, or isolated tumour cells including signet ring cells (Figure 3A). Carcinomas with mucinous areas of less than 50% are categorised as having a mucinous component. (11, 12, 17)

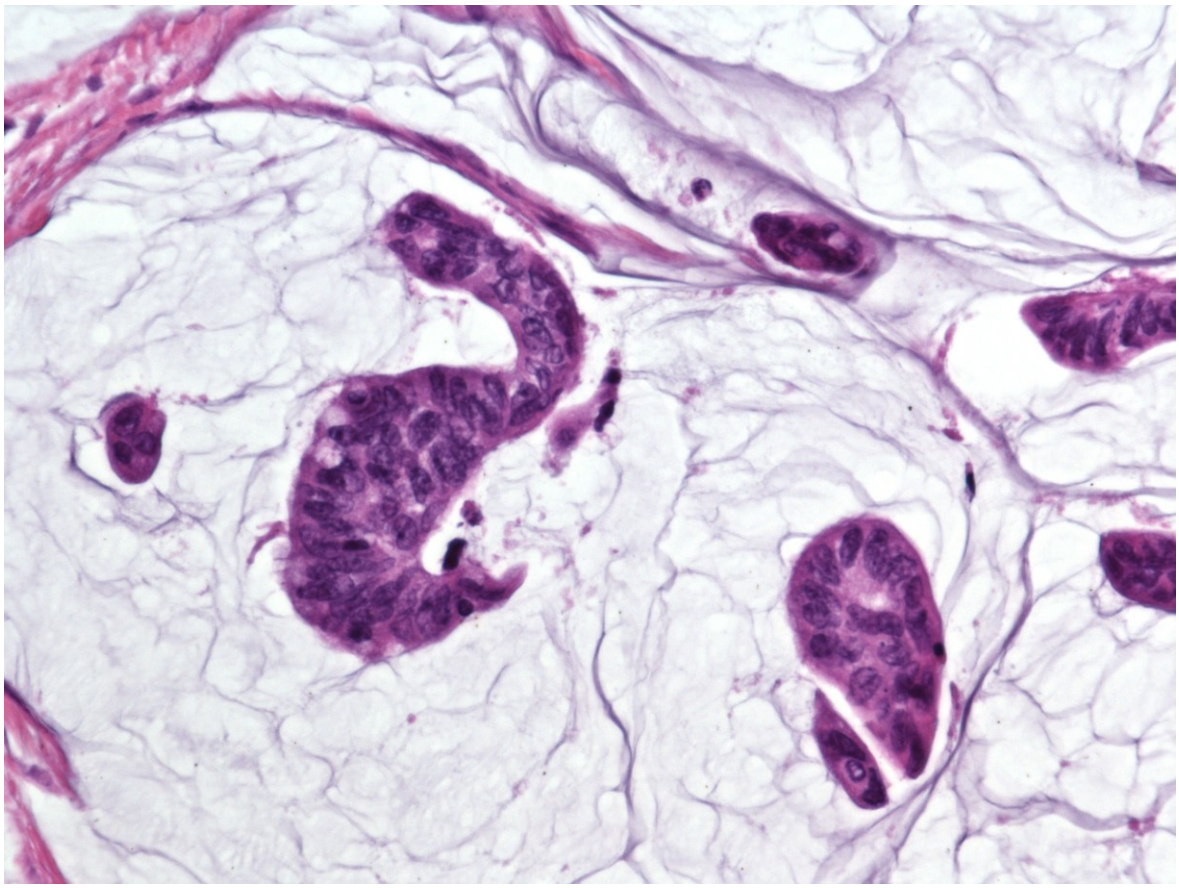


Figure 3A: Mucinous adenocarcinoma: pools of extracellular mucin represent more than 50% of the lesion (original x100)

Approximately 1% of CRC are signet-ring cell carcinomas. (18-20) This subtype is defined by the presence of more than 50% of tumour cells with prominent intracytoplasmic mucin, typically with displacement of the nucleus (Figure 3B). If less than 50% of the tumour cells are categorised as signet ring cells, the tumour is referred to as adenocarcinoma with a signet ring cell component. (11, 12, 17)

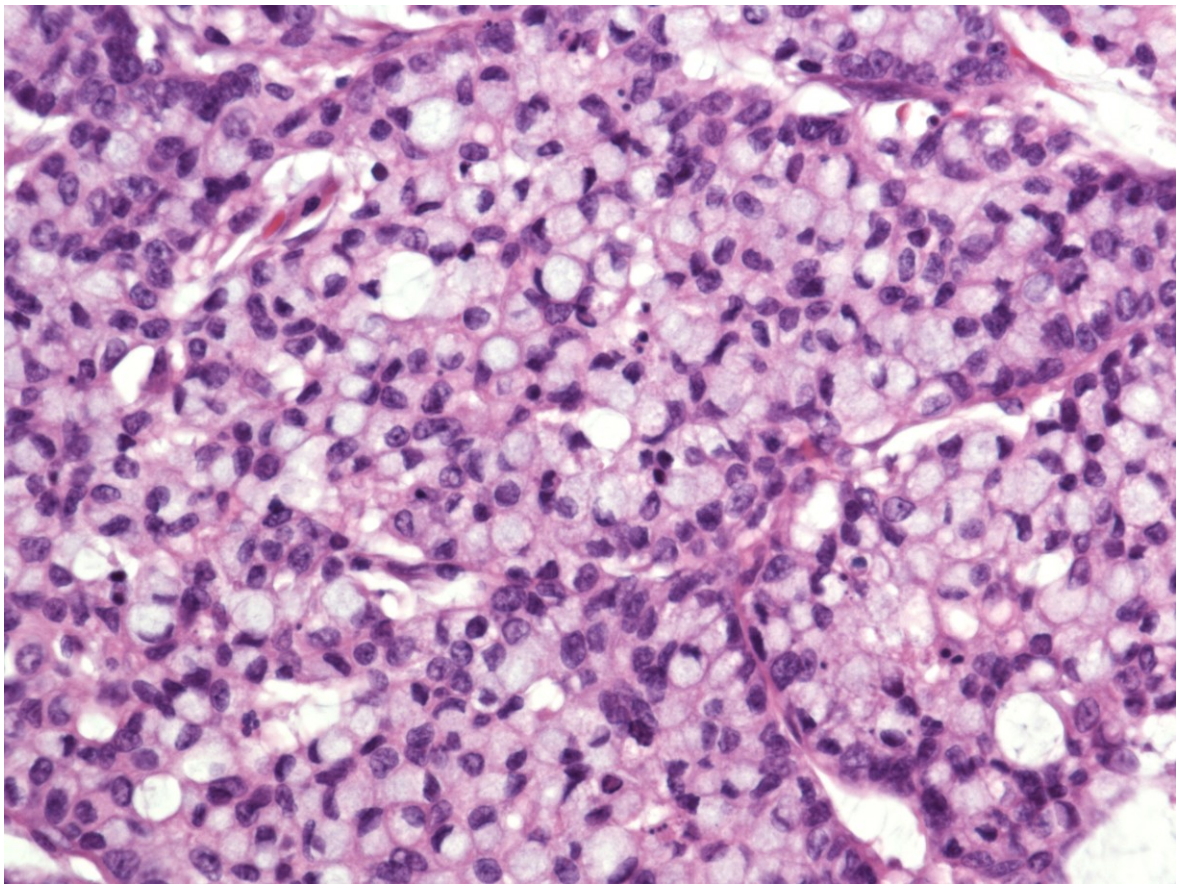


Figure 3B: Signet-ring cell carcinoma: prominent intracytoplasmic mucin is present in more than 50% of tumour cells (original x100)

The medullary carcinoma is a rare subtype. The tumour cells are usually disposed in syncytial sheets showing vesicular nuclei with prominent nucleoli, and abundant eosinophilic cytoplasm exhibiting prominent infiltration by intraepithelial lymphocytes (Figure 3C). (11, 12, 17)

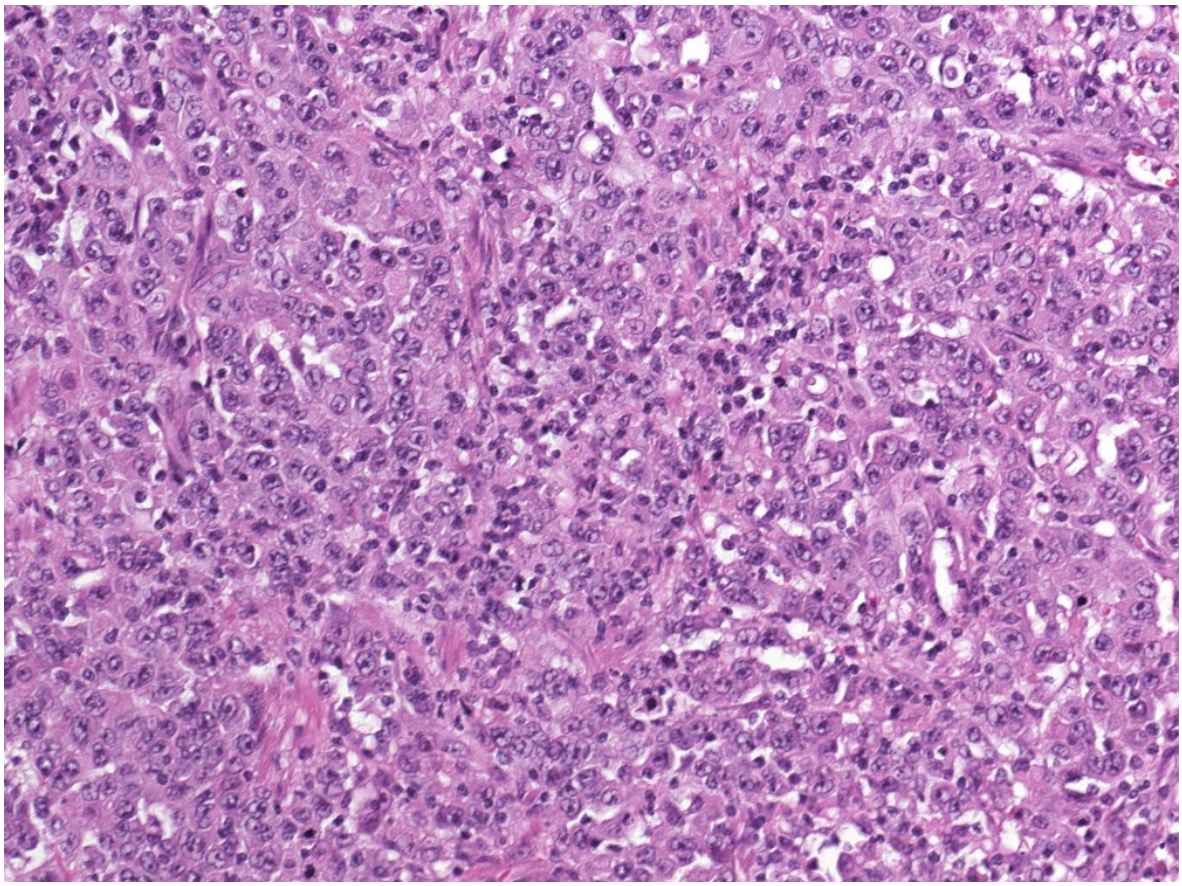


Figure 3C: Medullary carcinoma: the tumour is characterised by prominent intratumoral inflammation and sheets of malignant cells with vesicular nuclei (original x100)

The micropapillary adenocarcinoma is a rare tumour subtype that is defined by small angular and/or papillary tumour cell clusters within stromal spaces simulating vessels (Figure 3D). In conventional adenocarcinoma this pattern is mainly seen as a minor component. (12) Upon immunohistochemistry, micropapillary adenocarcinoma demonstrates usually an “inside-out” staining-pattern, i.e. reversed polarity for MUC1 and villin. (17, 21)

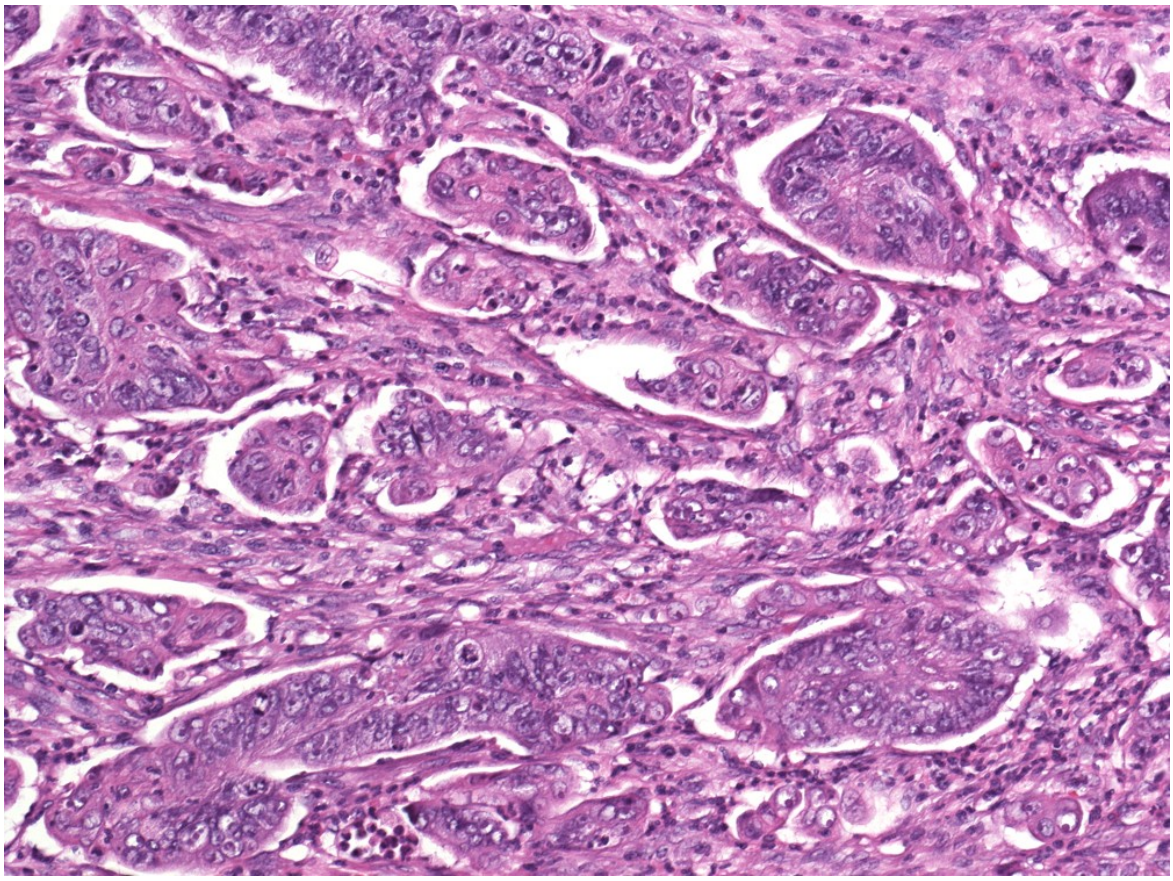


Figure 3D: Micropapillary adenocarcinoma: the tumour cells display small papillary and/or angular clusters, accompanied by stromal spaces mimicking vascular channels (original x100)

Further histological variants are the serrated adenocarcinoma and adenosquamos carcinoma. The serrated adenocarcinoma has architectural similarity to a sessile serrated adenoma/polyp, and characteristically shows glandular serration that may be accompanied by mucinous, cribriform, and trabecular areas. Adenosquamous carcinoma has features of both squamous cell carcinoma and adenocarcinoma, either as separated areas or admixed. (11, 12)

1.3.2 Tumour Staging

Tumour staging has the ambition to classify the extent of tumour spread in the patient's body. The tumour node metastasis (TNM) system was established and standardised by the American Joint Committee on Cancer (AJCC) / Union for International Cancer Control (UICC) to achieve a unique and globally recognised standard of tumour staging. (9) Thus, the TNM system procures standardised patient care worldwide and adds greater precision in the identification of prognostic subgroups. Cancer staging is performed by considering anatomic information of the primary tumour such as depth of invasion and/or size of the tumour (T), involvement of regional lymph nodes (N), and existent of distant metastases (M). In recent years selected nonanatomic prognostic factors were supplemented. (9)

In CRC, the T classification provides 5 categories (T0-T4) describing the depth of invasion. (9) In particular, T0 means that there is no evidence of a primary tumour in the colon or rectum, T1 describes the invasion of the submucosa by the tumour, T2 shows the invasion throughout the submucosa into the muscularis propria, T3 demonstrates the invasion beyond the muscularis propria into the subserosa or into non-peritonealised pericolic or perirectal tissues and T4 indicates the tumour penetration to the surface of the visceral peritoneum (T4a) and/or the direct invasion of other structures or organs (T4b) by the tumour. (9) The categories N0, N1 and N2 stratify the tumour according to the number of involved regional lymph nodes. Specifically, N0 describes the absence of lymph node metastasis, whereas N1a refers to 1 involved node, N1b to 2 or 3 involved nodes, N2a to 4 to 6 involved nodes, and N2b to seven or more involved nodes. The M category is subdivided into a category with distant metastasis limited to one organ (liver, lung, ovary, non-regional lymph nodes) and a second category refers to metastatic spread in more than one organ or the peritoneum. The combination of different T, N, and M values enable the stratification of patients into tumour stage I to IV, illustrated in Table 2. (9, 11, 22)

Table 2: Different values of T, N, and M and their corresponding prognostic group (I-IV) for carcinomas of the colon and rectum. Modified after AJCC/UICC staging system. (9)

Stage	T	N	M
0	Tis	N0	M0
I	T1	N0	M0
	T2	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1-T2	N1	M0
	T1	N2a	M0
IIIB	T3-T4a	N1	M0
	T2-T3	N2a	M0
	T1-T2	N2b	M0
IIIC	T4a	N2a	M0
	T3-T4a	N2b	M0
	T4b	N1-N2	M0
IVA	Any T	Any N	M1a
IVB	Any T	Any N	M1b

1.3.3 Prognostication

Clinicopathological staging according to the AJCC/UICC TNM system is considered as the most powerful prognostic parameter for patients with early and advanced stages of disease, whereas for intermediate stage it is less able to predict disease outcome. (9, 23-25) Especially, patients within the same stage of CRC may suffer from a substantially different clinical outcome. In particular, patients with AJCC/UICC stage II CRC may experience outcome inferior to AJCC/UICC stage III CRC. Due to a high risk of recurrence, adjuvant chemotherapy, which is primarily based on 5-fluorouracil, is recommended for all AJCC/UICC stage III patients, while patients with AJCC/UICC stage II CRC only receives adjuvant therapy, when they have a high risk of relapse. (2, 25) Because of this, it is of supreme importance to identify high-risk patients to optimise clinical decision making and treatment management. (26) It is of note, that tumour stage only indicates the level of tumour progression. Tumour stage, however, does not reflect the biological aggressiveness of the tumour. (27)

Stage independent prognostic markers may help to overcome this dilemma and may serve as valuable tools for outcome prediction. Therefore, additional prognostic markers are required. Helpful histopathological prognostic factors are readily assessable on hematoxylin and eosin (H&E) stained tumour slides and should easily integrate into pathologist's routine in clinical practice. (28)

Established histological parameters, which can be used for prognostication, include the histological type, lymph and blood-vessel invasion, perineural invasion, tumour necrosis, inflammatory response, and tumour budding. (6, 11, 12, 17) An overview of adverse and favourable prognostic markers is illustrated in Table 3.

Table 3: Prognostic factors not included in the TNM staging system of colorectal carcinomas. Data from Hamilton et al. (11)

Favourable host response	Adverse features of primary tumour	Adverse vessel invasion	Adverse surgical technique
➢ Intratumoral inflammation ➢ Peritumoral inflammation ➢ Desmoplasia ➢ Reactive lymph nodes	➢ Greater extent of circumferential involvement ➢ Bowel obstruction ➢ Bowel perforation ➢ Poor differentiation ➢ Infiltrative pattern of invasion/budding ➢ Selected molecular characteristics	➢ Muscular veins ➢ Lymphatic vessel ➢ Perineural spaces	➢ Short distance between margin and tumour ➢ Incomplete excision with residual tumour

As already mentioned above, histological subtypes, classified by the WHO not only have different microscopic appearance, but also a different prognostic value. Patients with mucinous adenocarcinoma showed worse outcome compared to patients with non-mucinous adenocarcinoma. Moreover, these patients were older, had a larger extent of tumour mass, higher T level, and increased occurrence of extrahepatic metastases. (29) Signet-ring cell differentiation is also an adverse prognostic factor, contributed to poor survival rates. Carcinomas with signet-ring cell differentiation are associated with higher tumour stage, higher rate of lymphatic invasion and poorer differentiation compared to conventional adenocarcinomas. (20, 30) Micropapillary adenocarcinomas sustain a high potential of malignancy. Higher rates of infiltrative growth pattern, deeper

infiltartion into the bowel wall, lymphovascular and perineural invasion, and a higher risk of lymph node, local and distant metastases compared to conventional adenocarcinomas was strongly related to micropapillary adenocarcinomas. (17) In contrast to the last three entities, the medullary carcinoma is a predictor for favourable outcome. Medullary carcinomas arose in older patients and usually manifested with AJCC/UICC stage II disease without lymph node involvement. (31, 32)

Lymph- and/or blood vessel invasion by tumour cells is an essential mechanism in the process of metastatic spread. Lymphatic invasion is defined as carcinoma being present in vessels with an unequivocal endothelial lining, lacking a thick muscular wall (Figure 4A). Blood vessel invasion is characterised histologically by isolated cancer cells being present in vessels with a thick muscular wall and red blood cells in the lumen (Figure 4B). (12, 17) Blood vessel invasion is independently related to adverse prognosis and hepatic metastasis, whereas the significance of lymphatic invasion is less known (33).

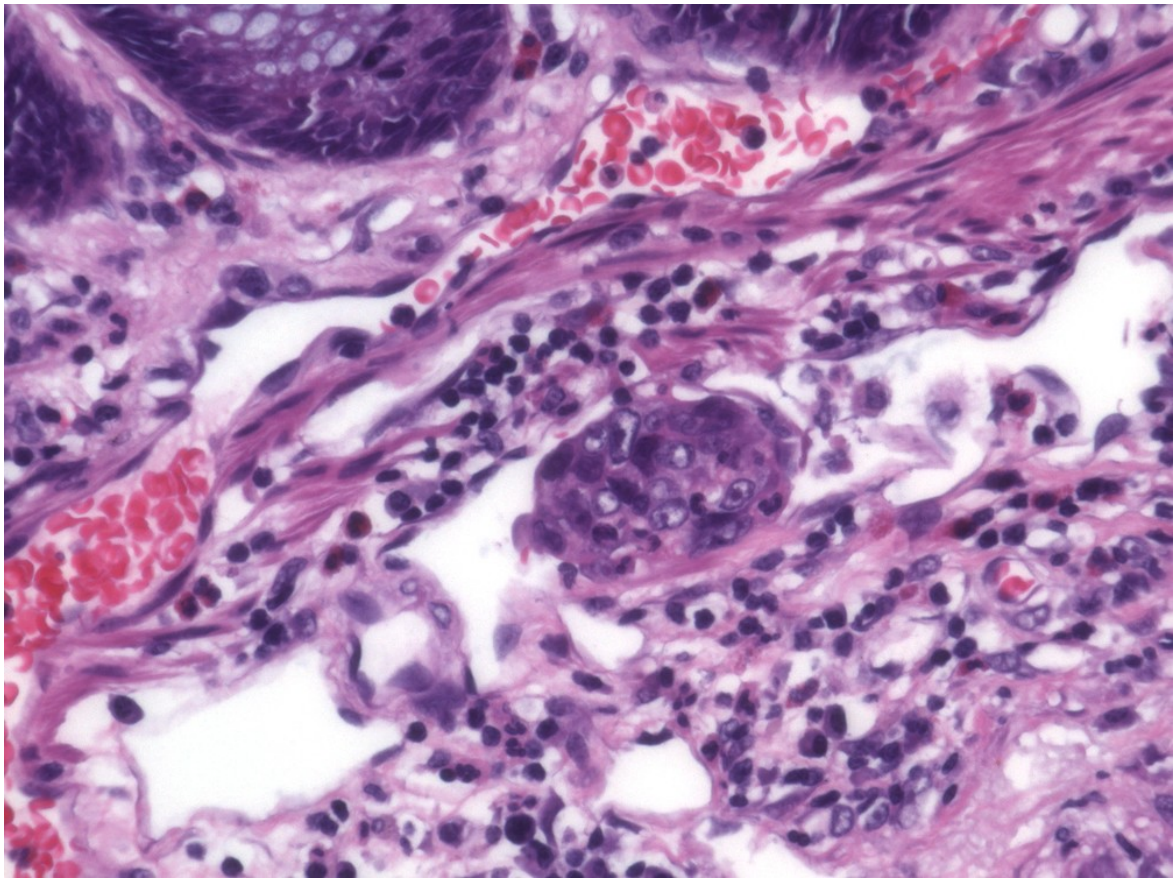


Figure 4A: Lymphatic invasion is defined as tumour cells presented in vessels with an unequivocal endothelial lining

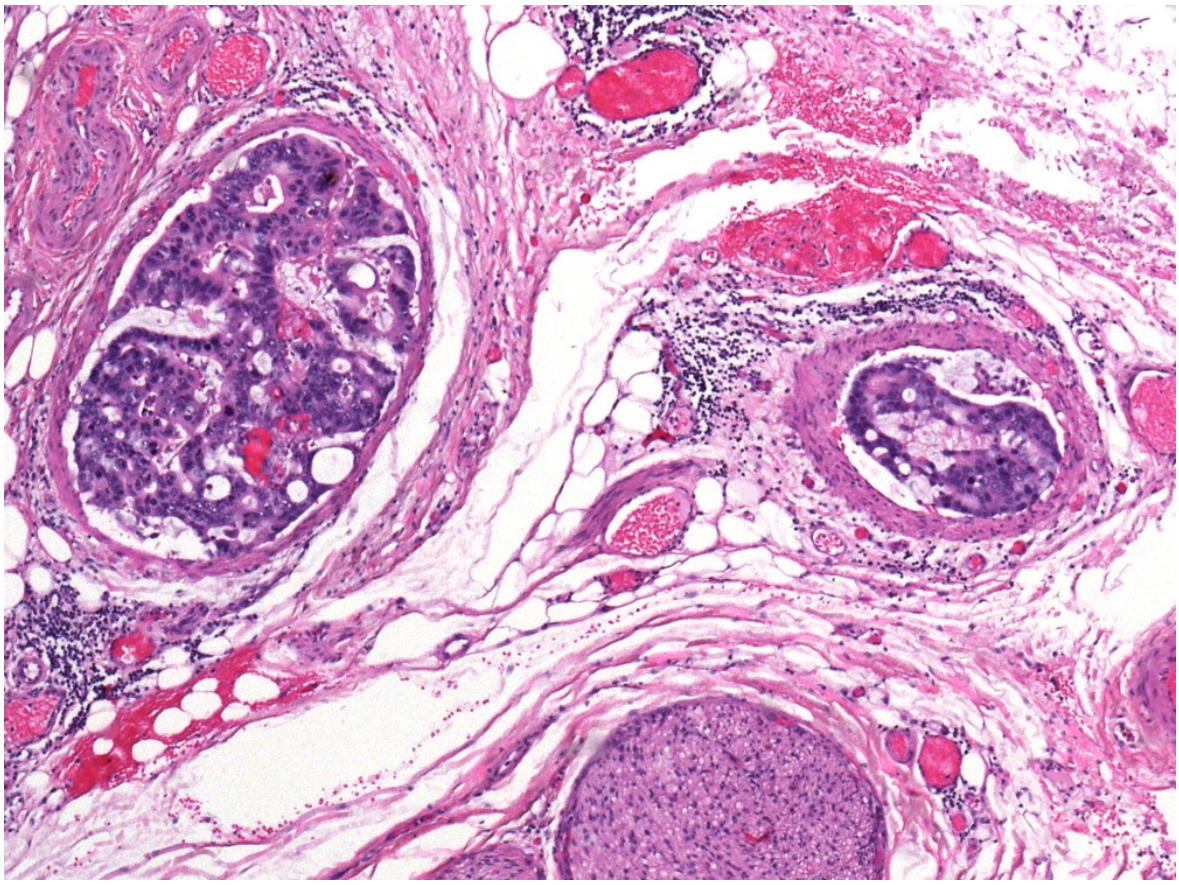


Figure 4B: Blood vessel invasion characterised by the presence of tumour cells in vessels with a thick muscular wall containing red blood cells

Perineural invasion is diagnosed by the neoplastic invasion of nervous structures or if at least one third of the nerve's circumference is surrounded by tumour cells (34). It could be a possible route of metastatic spread and is associated with poor prognosis. CRC with perineurial invasion is correlated to an aggressive tumour phenotype. Patients with perineural-invasion-negative CRCs had a fourfold-greater 5-year disease-free survival rate than patients with perineural-invasion-positive cancers. (12)

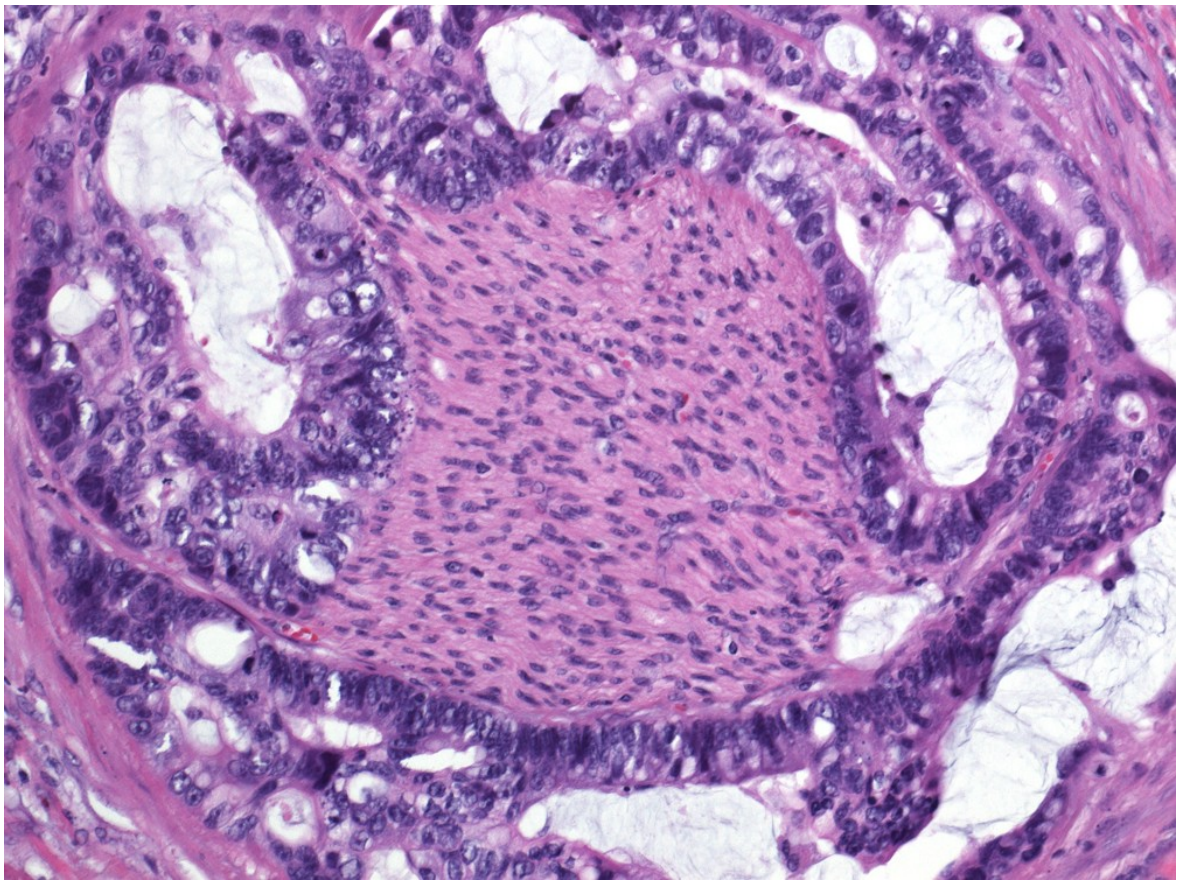


Figure 5: Perineural invasion is defined by neoplastic invasion of nerves and/or spread around the nerve with at least 33% of the circumference

It is believed that tumour necrosis is a consequence of chronic ischemic injury based on rapid tumour growth. Chronic ischemic injury has been related to levels of high cellular hypoxia due to increased metastatic potential, poor prognosis, and resistance to chemotherapy and radiotherapy. (35, 36) The extent of tumour necrosis is associated with large tumour size, AJCC/UICC stage, T and N classification, poor tumour differentiation, and angioinvasion. The presence of extensive tumour necrosis was identified as stage-independent prognostic marker of patients' outcome in multivariate analysis. (35, 36)

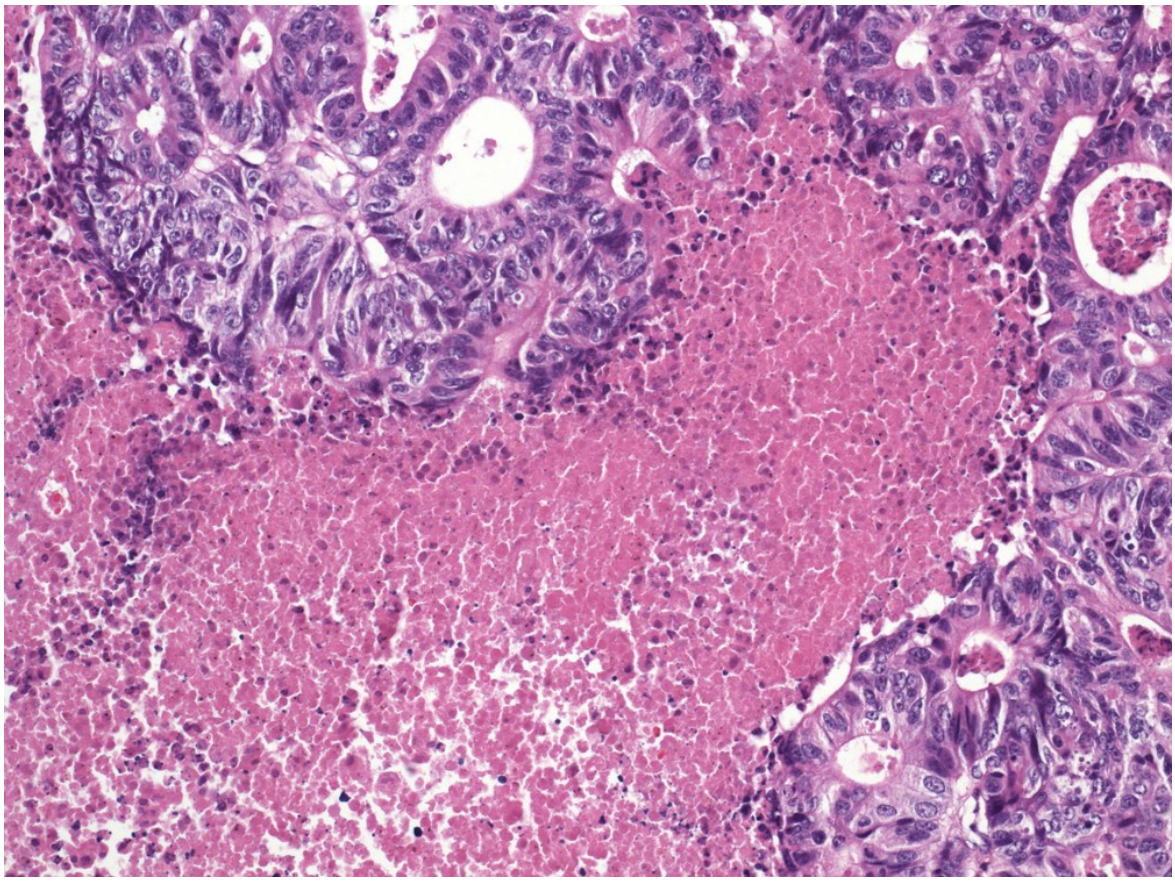


Figure 6: Tumour necrosis as consequence of chronic ischemic injury based on rapid tumour growth

1.3.4 Tumour Budding

In CRC, tumour budding is assumed to reflect cancer aggressiveness at the tumour margin and thus been proposed as predictive marker. (37) This phenomenon is defined as the presence of isolated single cells or small clusters of cells with maximum 4 cells scattered in the stroma of the tumour margin (Figure 7). (37) Tumour buds at the invasive front of the solid tumour tend to lose cohesion and remove as single cells, thereby promoting invasion and ultimately metastatic cancer spread. (38) The underlying theory is suggested to be the epithelial mesenchymal transition (EMT), which allows cancer cells to pass into cells with mesenchymal phenotype. These cells have the ability of migration, invasiveness, resistance to apoptosis and production of extracellular matrix molecules. The first step in the process of tumour budding appear to be the detachment from the solid

tumour by the loss of membranous E-cadherin expression. (12, 37, 39) Furthermore, tumour buds also express fibronectin within the cytoplasm underlining the shift to a mesenchymal phenotype and its interaction with the surrounding stroma. (38, 40)

Some studies identified tumour budding as a stage-independent marker for poor prognosis and high risk of recurrence. (37, 41-49) In particular, the extent of tumour budding in early lesions seems to have a strong predictor for metastatic lymph node spread. With this tool, it might be possible to select high-risk patients in AJCC/UICC stage II disease for adjuvant chemotherapy. (50)

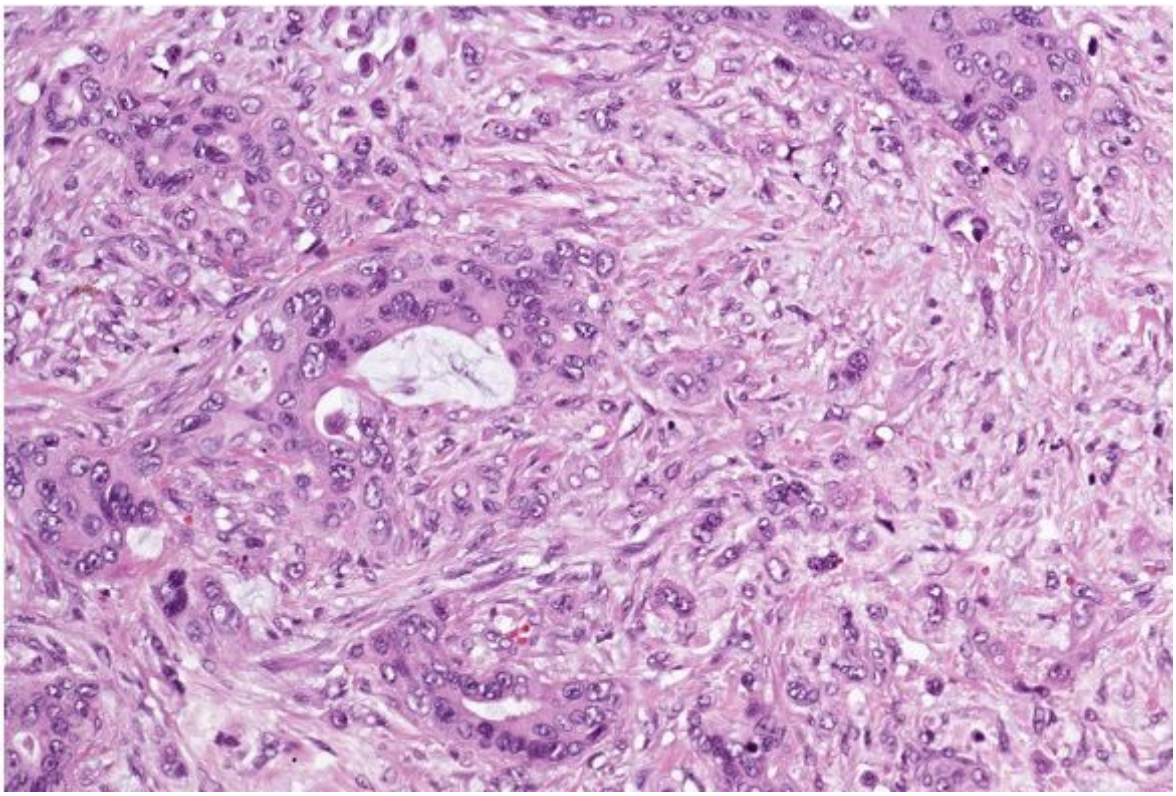


Figure 7: Tumour budding, defined as the presence of isolated single cells or small clusters of cells with maximum 4 cells on a haematoxylin and eosin stain slide (37)

1.3.5 Peritumoral Inflammation

The impact of tumour-related inflammatory cell reaction has been proposed as a beneficial prognostic predictor in CRC. It is supposed that multiple different cell types of the adaptive and innate immune system within the tumour and/or at the invasive margin interact with cancer cells and promote cancer-associated apoptosis. This local inflammatory cell reaction represents the immune response of the host against the malignant tumour. (23, 51, 52) It is of evidence, that an increasing number or density of peri- and/or intratumoral immune cells is combined with an improved clinical outcome in CRC independent of AJCC/UICC stage. In contrast to the histological prognostic predictors mentioned above, which all reflect tumour aggressiveness, the inflammatory cells act as “defenders”, indicating favourable outcome. (51)

Since 1987, the Jass and Morson classification has been using the predictive value of the inflammatory cell. (53) Today, the intensity of inflammation at the invasive margin is widely scored by Klintrup’s criteria using a four-degree scale (Figure 8A).(51) Klintrup et al. (51) revealed that high-grade inflammation at the invasive margin and central region in lymph node-negative CRC is accompanied with favourable 5-year-survival compared to low-grade inflammation, confirmed by several studies. (54, 55) Another investigation with respect to tumour-infiltrating lymphocytes, lymphocytic infiltration of the intra- and peritumoral stroma, and Crohn’s-like lymphoid reaction (anti-tumour immune response) also revealed beneficial effects on prognosis (Figure 8B). (56) The anti-tumour immune response is frequently addicted to high-level microsatellite instability (MSI-H), which might be an explanation for the immune response by producing immunogenic truncated peptides. (56)

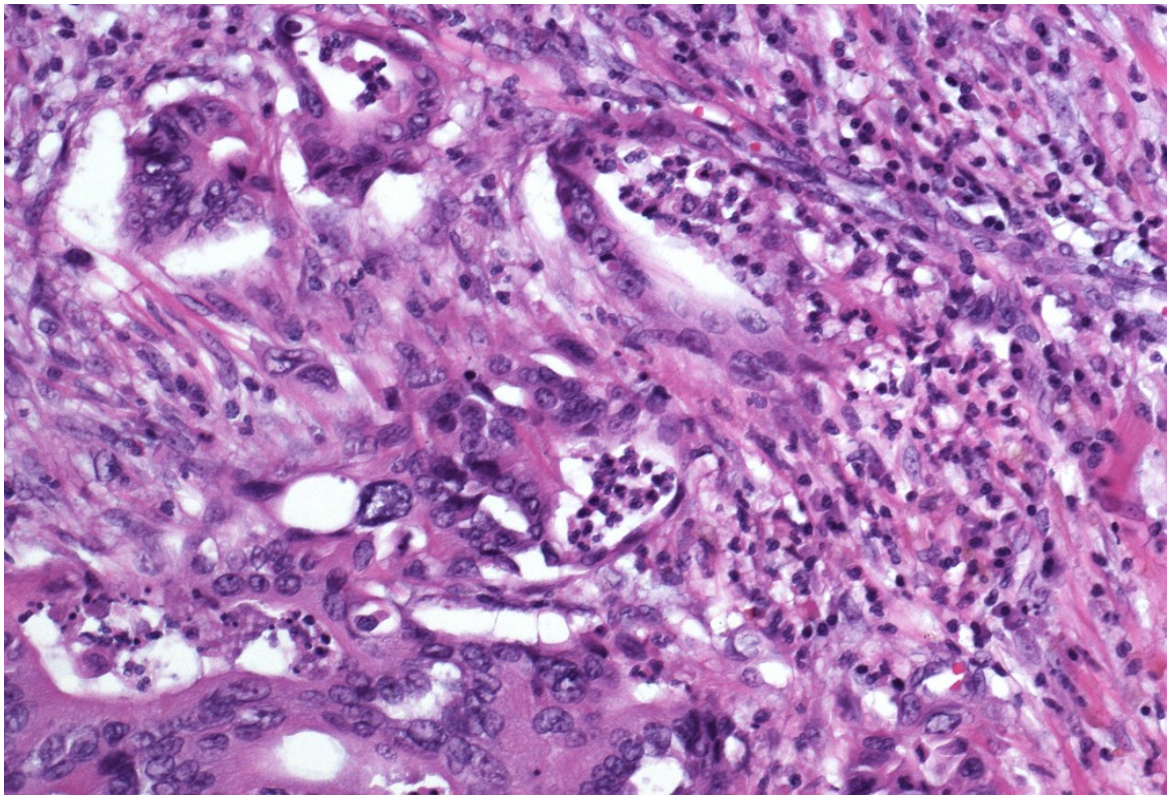


Figure 8A: Marked peritumoral inflammation at the invasive front with admixed Inflammation

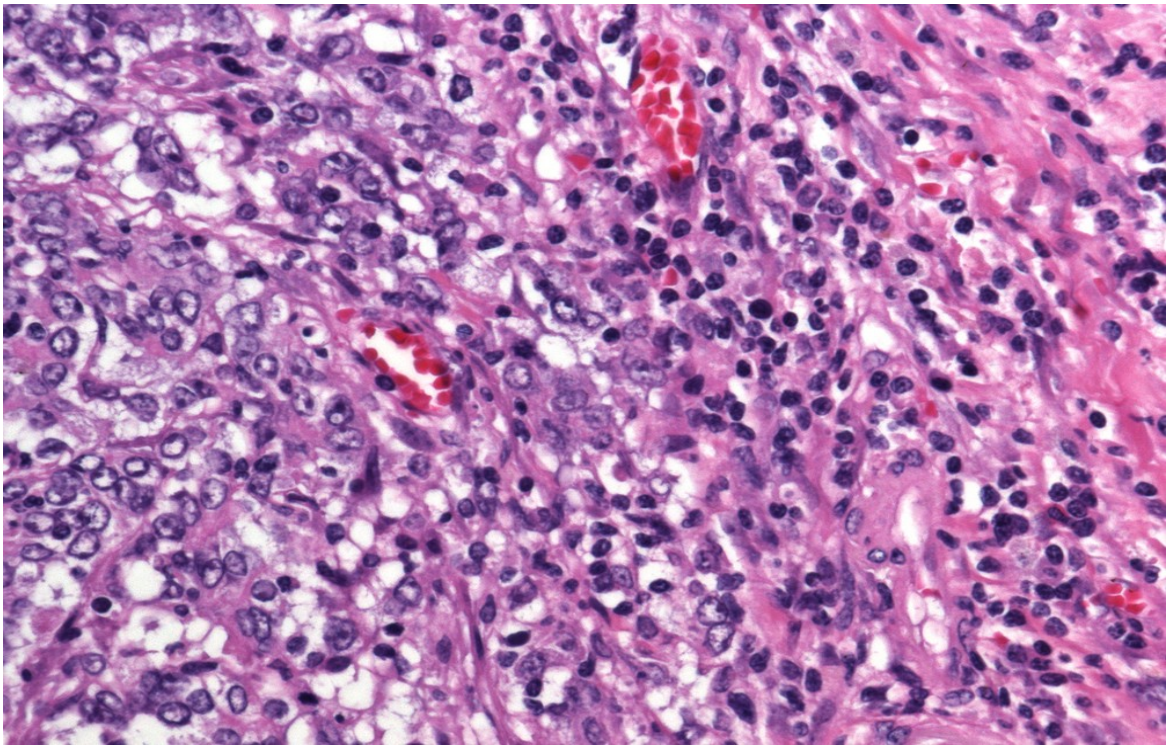


Figure 8B: Dense peritumoral lymphocytic infiltration designated as anti-tumour immune response

1.4 Aims

The aim of the current study was to assess the relationship between tumour budding and the overall inflammatory reaction in a large cohort of CRC patients. In particular, we were interested in the prognostic values of high-grade and low-grade budding in combination with high-grade and low-grade inflammation. We were keen on answering the question whether patients with both high-grade budding and high-grade inflammation have a prognosis comparable to patients with low-grade budding.

In addition, we investigated the potential prognostic values of tumour budding and overall inflammatory reaction separately. We compared the prognostic efficiency of both parameters with that of other well-established prognostic markers, like lymphatic and venous invasion, T and N classification, tumour grade, and tumour size. Both, univariate and multivariate analyses were implemented.

2. Patients and Methods

2.1 Case selection and follow-up assessment

In the time period from 1st of January, 1984 to 31st of December, 2005, all in all 7909 CRCs from 7564 individuals (4095 men, 3469 women; ratio 1.2:1) were identified in the local database of CRC of the Institute of Pathology (Medical University of Graz, Austria). Out of these, 400 (5%) patients were randomly selected from January 1992 to December 2000 with the aim to obtain at least 5 years' follow-up as well as identical adjuvant treatment modalities. (15, 23, 37, 41)

AJCC/UICC stage guided the decision for treatment for our patients: stage III patients were treated with 5-fluorouracil/folinic acid based chemotherapy according to the Mayo Clinic regime, whereas patients with stage I and II disease did not receive adjuvant treatment. (15, 23, 37, 41, 57)

Nineteen patients were excluded:

(i) those who were treated wtih endoscopic polypectomy for low-risk T1 cancer due to lacking data regarding lymph node status (n = 2);

(ii) patients who received neoadjuvant chemotherapy due to presumptive treatment-related changes in T classification (n = 8);

(iii) patients with synchronous or metachronous secondary CRC due to disease progression cannot be refered dependably to one of the tumours (n = 5); and

(iv) patients with competitive invasive carcinomas originating from other sites if metastatic deposits were not assessed by histology (n = 4). (15, 23, 35, 41)

The study cohort is illustrated in Figure 9.

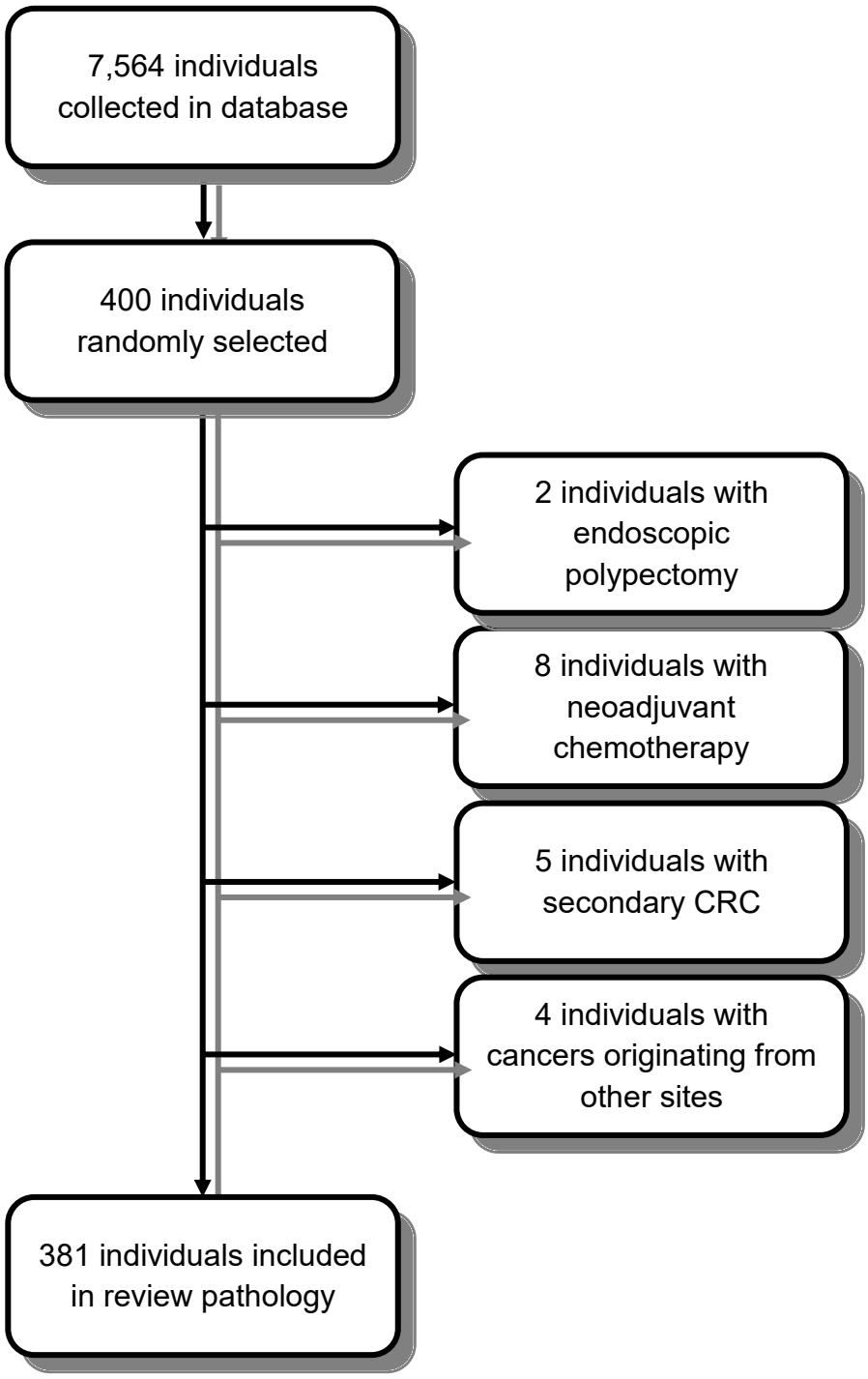


Figure 9: Study cohort of 381 colorectal cancer patients suitable for further investigation concerning tumour budding and peritumoral inflammation

In all, 381 specimens out of 400 (95%) were included for review pathology. Among these, 215 (56%) were males and 166 (44%) were females (ratio 1:1.5) with mean and median ages of 68.5 and 70.1 years. The youngest patient was 27.6 years old and the oldest one 93.1 years. (41) The age and gender distribution is summarised in Figure 10.

CRCs were located in the proximal/right-sided colon in 107 (28%) (caecum to transverse colon), in the distal/left-sided colon in 110 (29%) (splenic flexure to rectosigmoid junction) and in the rectum in 164 (43%) cases, respectively (Figure 11).

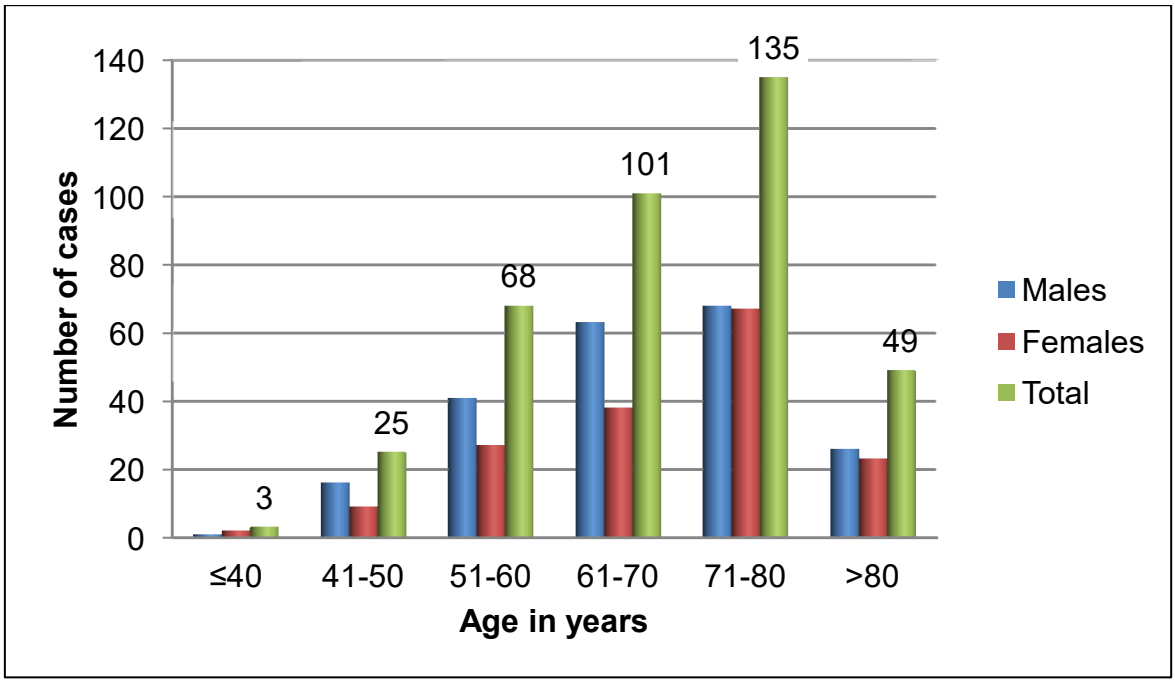


Figure 10: Age and gender distribution in our cohort of 381 patients with CRC.

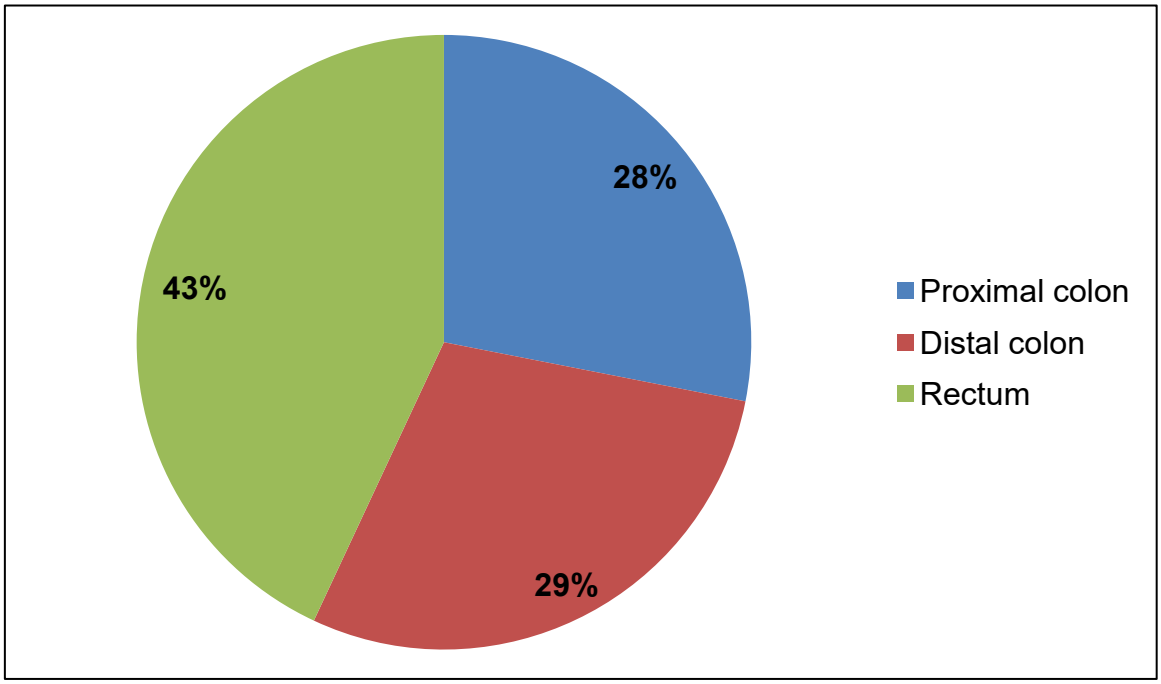


Figure 11: Tumour location of primary colorectal cancer in our cohort of 381 patients.

Follow-up included laboratory testing (blood count, liver enzymes and the tumour markers carcinoembryonic antigen and carbohydrate antigen 19-9) at 3-month intervals for the first 3 years and 6-months intervals thereafter. Chest X-ray and abdominal ultrasound were performed at 6-month intervals for the first 3 years and 12-months intervals thereafter. Patients with rectal cancer underwent pelvic computerised tomography every 12 months. (15, 23, 35, 41, 58)

Progressive disease was defined as either local recurrence, that is any detectable local disease, occurring either alone or in conjunction with generalised recurrence or systemic recurrence, that is any detectable disease except local recurrence. (15, 23, 35, 41)

Institutional review board approval was received from the Ethic's Committee of the Medical University of Graz, Austria.

2.2 Histopathological Evaluation

All available histological slides were independently re-evaluated by two pathologists with special interest in gastrointestinal pathology (Dr. Marion J. Pollheimer and Dr. Cord Langner) who were blinded to clinical data, in particular follow-up information. Discrepancies were discussed by concurrent re-examination of the histopathological slides using a double-headed microscope. (15, 23, 35, 41) According to the situation in daily routine, no immunohistochemistry was used to evaluate the extent of tumour budding and peritumoral inflammation in this study.

T and N classification were assessed according to the AJCC/UICC 2009 edition of the TNM classification. (22) Histological tumour type and grade were analysed by the WHO guidelines of 2010. (11)

The extent of tumour budding was evaluated on H&E stained slides as described previously by Ueno et al. (49): In a field in which budding intensity was maximal, the number of isolated single cells or small clusters of maximum 4 cells scattered in the stroma at the invasive tumour margin was counted using a x20 objective lens in a field measuring 0.95 mm^2 (Olympus BX45, Tokyo, Japan). (15, 23, 41) The intensity of budding was listed as follows: score 1 (<5 budding foci), score 2 (5-9 budding foci), score 3 (10-19 budding foci), and score 4 (≥20 budding foci). For statistical comparison, tumours were then divided into two groups based upon the extend of tumour budding: counts of 0–9 budding foci were termed low-grade (score 1 and 2) while counts of 10 or more budding foci were termed high-grade budding (score 3 and 4). (41, 49)

The overall inflammatory reaction and the number of lymphoid cells, neutrophilic and eosinophilic granulocytes as well as macrophages were analysed using the four-degree scale introduced by Klintrup et al. (51): a score of 0 indicated ‘no increase of inflammatory cells’. Score 1 denoted ‘mild and patchy increase of inflammatory cells at the invasive margin, but no destruction of invading cancer cell islets by the inflammatory cells’. A score of 2 was given when inflammatory cells formed a ‘band-like infiltrate at the invasive margin with some destruction of cancer cell islets by inflammatory cells’. A score of 3 indicated a ‘very prominent inflammatory reaction, forming a cup-like zone at the invasive margin, and destruction of cancer cell islets was frequent and invariably present’. (51)

2.3 Statistical analysis

Associations between tumour budding and other tumour parameters, such as, tumour grade, T and N classification, inflammation, tumour location and size were analysed using χ^2-test. Progression-free survival was defined as the period (measured in months) between the date of surgical resection of the primary carcinoma to the date of diagnosis of disease recurrence, considering both local and distant recurrences. Cancer-free survival was defined as the period from the date of surgical resection to tumour-related death. Cause of death was determined by treating physicians and/or by chart review and was corroborated by death certificates if available. Progression-free and cancer-specific survival was determined using the Kaplan–Meier method and compared by the log-rank test. For multivariable testing, Cox's proportional hazards regression models were performed. (15, 23, 35, 37, 41)

All reported tests were two-sided and *P*-values with significance at $P<0.05$. All statistical evaluations were performed by Dr. Lars Harbaum, Department of Oncology, Hematology, Bone Marrow Transplantation with Section Pneumology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; using SPSS statistics version 20 (IBM, Armonk, New York, United States).

3. Results

3.1 Histopathology

Basic tumour characteristics including T and N classification, AJCC/UICC stage and tumour grade are summarised in Table 4. Specifically, 28 (7%) patients manifested a pT1, 70 (18%) a pT2, 218 (57%) a pT3, 15 (4%) a pT4a and 50 (13%) a pT4b CRC. Lymph node involvement was observed in 168 (44%) patients. In 23 (6%) cases cancer spread to distant organs (M1) was found. AJCC/UICC stage was I in 81 (21%), II in 125 (33%), III in 151 (40%) and IV in 23 (6%) patients, respectively. 121 (32%) tumours displayed high (G1), 138 (36%) moderate (G2) and 122 (32%) low (G3) differentiation, respectively.

Table 4: Tumour distribution according to T/N/M classification, AJCC/UICC stage and tumour grade

T	n (%)	N	n (%)	M	n (%)	Stage	n (%)	G	n (%)
T1	28 (7%)	N0	213 (56%)	M0	358 (94%)	I	82 (22%)	G1	121 (32%)
T2	70 (19%)	N1a	43 (11%)	M1	23 (6%)	II	125 (33%)	G2	138 (36%)
T3	218 (57%)	N1b	40 (11%)			III	151 (40%)	G3	122 (32%)
T4a	15 (4%)	N2a	39 (10%)			IV	23 (6%)		
T4b	50 (13%)	N2b	46 (12%)						

The distribution of histological subtypes was the following: 316 (83%) tumours presented as adenocarcinomas (not otherwise specified), 45 (12%) as mucinous adenocarcinomas, 13 (3%) as undifferentiated carcinomas, 3 (1%) as signet-ring cell carcinomas, 2 (0.5%) as medullary carcinomas, 1 (0.3%) as adenosquamous carcinoma and 1 (0.3%) as amphicrine carcinoma. These data are illustrated in Figure 12.

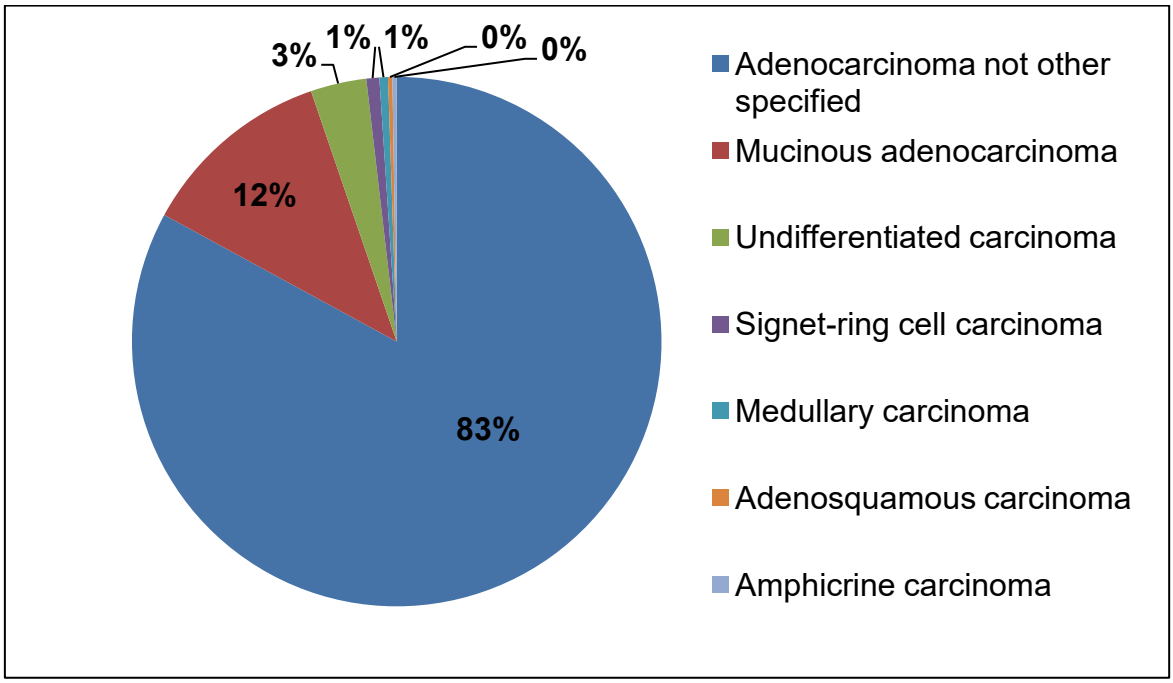


Figure 12: Distribution of histological subtypes in the cohort of 381 primary colorectal cancers.

3.2 Tumour characteristics

Overall, 221 (58%) tumours showed low-grade budding and 160 (42%) high-grade budding, respectively. In detail, 125 (33%) tumours showed less than five budding foci (score 1), 96 (25%) had five to nine budding foci (score 2), 98 (26%) tumours presented with ten to 19 budding foci (score 3) and 62 (16%) showed 20 or more budding foci (score 4) (Figure 13A).

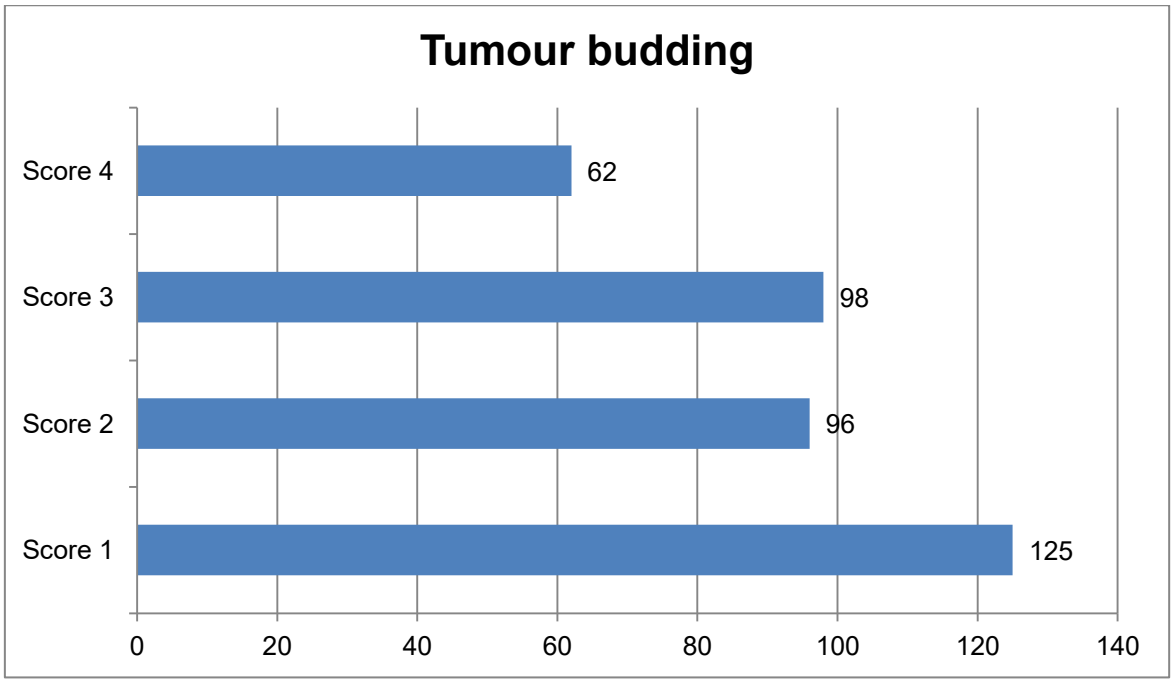


Figure 13A: The extent of tumour budding in the cohort of 381 primary colorectal cancers.

167 (44%) tumours showed no increase or only a mild and patchy increase of inflammatory cells at the invasive tumour margin, but no destruction of invading cancer cell islets (scores 0 and 1), whereas in 214 (56%) tumours presented with a band-like infiltrate of inflammatory cells with at least some destruction of cancer cell islets (scores 2 and 3) (Figure 13B).

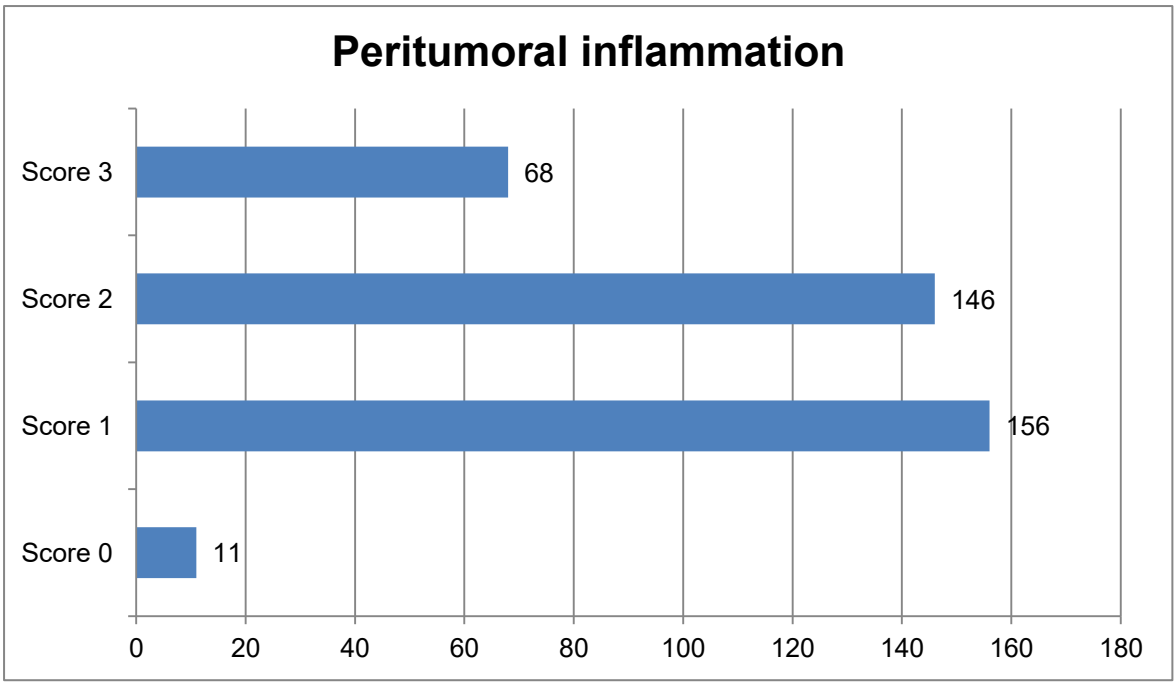


Figure 13B: The extent of peritumoral inflammation in the cohort of 381 primary colorectal cancers.

When the analysis of inflammation at the invasive front was restricted to tumours with high-grade budding, 82 (51%) cases showed mild inflammation (scores 0 and 1) and 78 (49%) marked inflammation (scores 2 and 3).

Figure 14 illustrates the different histopathological appearance of high-grade tumour budding with (A and B) and without (C and D) peritumoral inflammation. This phenomenon is shown in serial sections stained with H&E (A, C) and anti-cytokeratin immunohistochemistry (B, D).

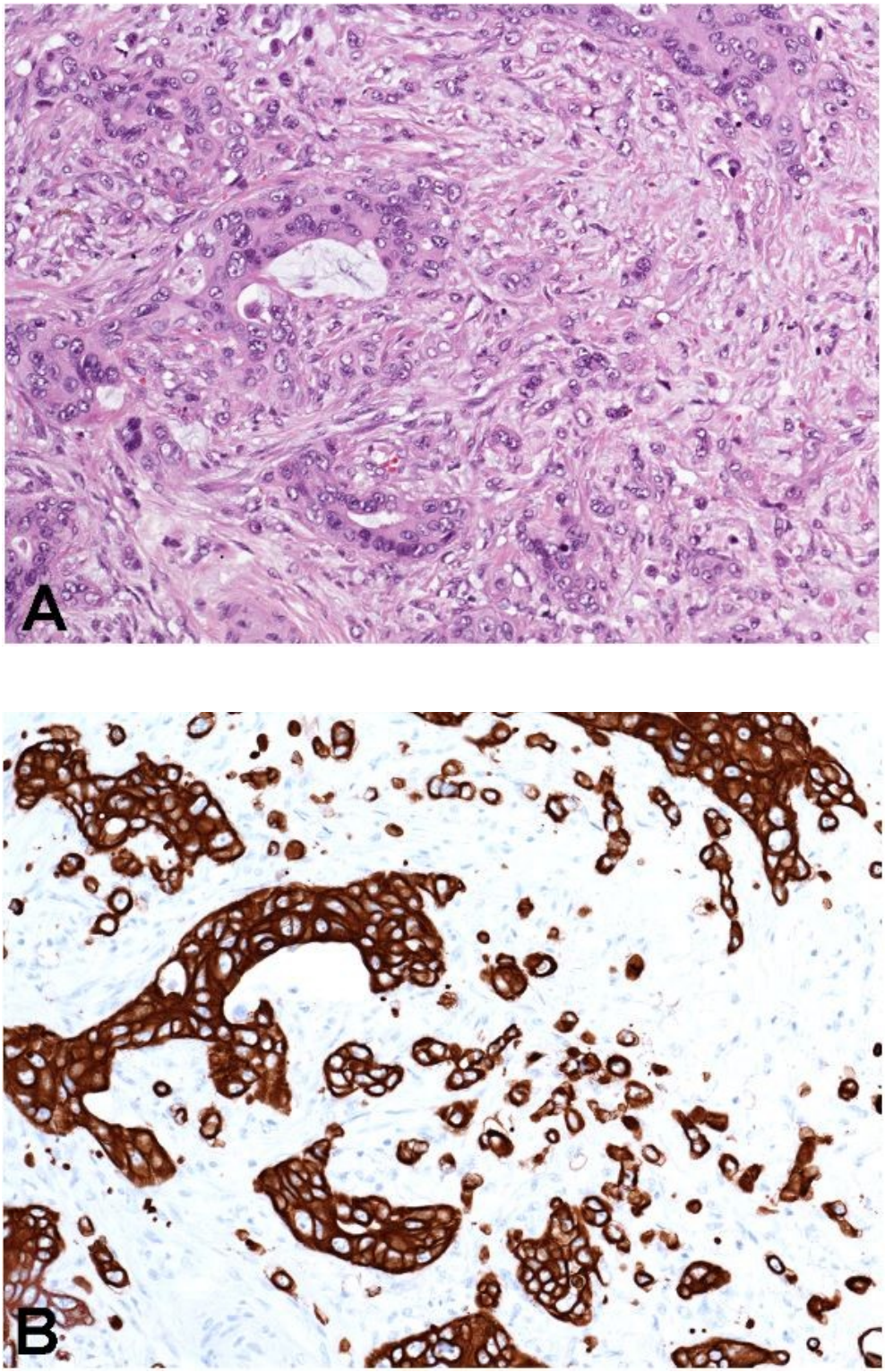


Figure 14 A-B: Tumour budding, defined as the presence of isolated single cells or small clusters of cells (composed of fewer than five cells), without peritumoral inflammation is shown in serial sections stained with haematoxylin and eosin (A; original x100) and anti-cytokeratin immunohistochemistry (B; original x100).

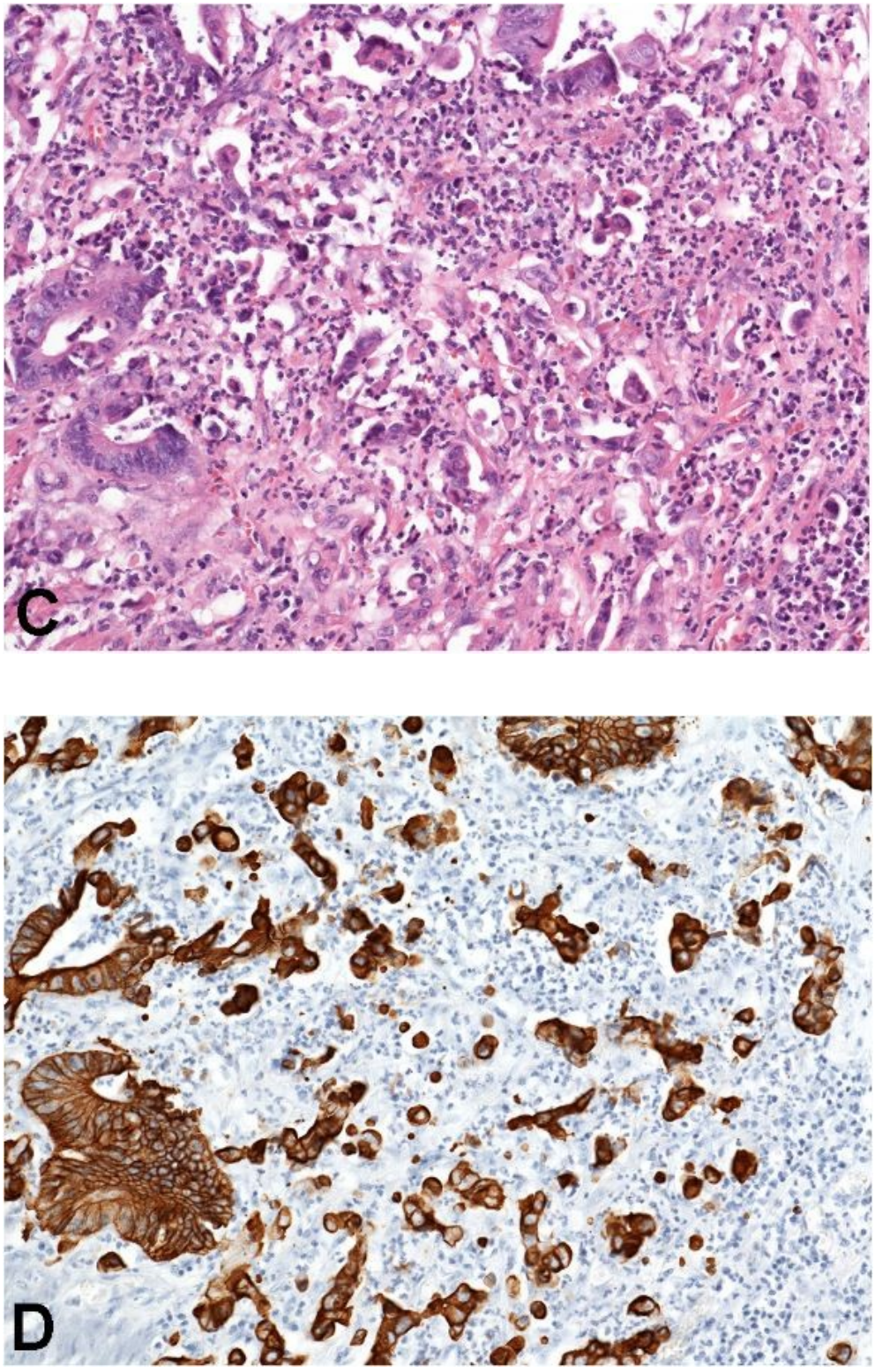


Figure 14 C-D: Marked peritumoral inflammation, leading to the presence of isolated cancer cells due to destruction of invading cancer cell islets, is shown in serial sections stained with haematoxylin and eosin (C; original x100) and anti-cytokeratin immunohistochemistry (D; original x100).

In the next step, the extent of tumour budding was associated with other tumour parameters. The following results were obtained: High-grade budding was significantly associated with high T classification ($p<0.001$), high N classification ($p<0.001$) and poor tumour differentiation ($p<0.001$). Interestingly, budding was inversely associated with peritumoral inflammation ($p=0.016$). Data are summarised in Table 5.

Likewise, the extent of peritumoral inflammation was analysed with respect to possible associations with other tumour parameters. Hereby, significant associations were detected for T classification ($p<0.001$), tumour grade ($p<0.001$), and for the extent of tumour budding ($p<0.001$). It is of note that 132 of 213 (62%) of node-negative cancers showed high-grade budding compared to 82 of 168 (49%) of node-positive cancers ($p=0.013$). Results are summarised in Table 6.

Table 5: Association of tumour budding with other pathological parameters (tumour size was known for 360 patients)

		N	Low-grade budding (n=221)	High-grade budding (n=160)	*P*-value
T classification	T1	28	27 (96.4%)	1 (3.6%)	<0.001
	T2	70	55 (78.6%)	15 (21.4%)	
	T3	218	119 (54.6%)	99 (45.4%)	
	T4a	15	7 (46.7%)	8 (53.3%)	
	T4b	50	13 (26%)	37 (74%)	
N classification	N0	213	164 (77%)	49 (23%)	<0.001
	N1a	43	27 (62.8%)	16 (37.2%)	
	N1b	40	12 (30%)	28 (70%)	
	N2a	39	8 (20.5%)	31 (79.5%)	
	N2b	46	10 (21.7%)	36 (78.3%)	
Grade	G1	121	103 (85.1%)	18 (14.9%)	<0.001
	G2	138	67 (48.6%)	71 (51.4%)	
	G3	122	51 (41.8%)	71 (58.2%)	
Inflammation	Score 0	11	5 (45.5%)	6 (54.5%)	0.016
	Score 1	156	80 (51.3%)	76 (48.7%)	
	Score 2	146	86 (58.9%)	60 (41.1%)	
	Score 3	68	50 (73.5%)	18 (26.5%)	
Tumour size	≤4.5 cm	202	123 (60.9%)	79 (39.1%)	0.33
	>4.5 cm	158	88 (55.7%)	70 (44.3%)	
Tumour location	Right	107	60 (55.7%)	47 (44.3%)	0.75
	Left	110	67 (60.9%)	43 (39.1%)	
	Rectum	164	94 (57.3%)	70 (42.7%)	

Table 6: Association of peritumoral inflammation with other pathological parameters (tumour size was known for 360 patients)

		N	Low-grade inflammation (n=167)	High-grade inflammation (n=214)	*P*-Value
T classification	T1	28	11 (39%)	17 (61%)	<0.001
	T2	70	16 (23%)	54 (77%)	
	T3	218	99 (45%)	119 (55%)	
	T4a	15	14 (93%)	1 (7%)	
	T4b	50	27 (54%)	23 (46%)	
N classification	N0	213	81 (38%)	132 (62%)	0.073
	N1a	43	20 (46%)	23 (54%)	
	N1b	40	18 (45%)	22 (55%)	
	N2a	39	22 (56%)	17 (44%)	
	N2b	46	26 (57%)	20 (43%)	
Grade	G1	121	40 (33%)	81 (67%)	<0.001
	G2	138	55 (40%)	83 (60%)	
	G3	122	72 (59%)	50 (41%)	
Tumour budding	Score 1	125	53 (42%)	72 (58%)	<0.001
	Score 2	96	32 (33%)	64 (67%)	
	Score 3	98	38 (39%)	60 (61%)	
	Score 4	62	44 (71%)	18 (29%)	
Tumour size	≤4.5 cm	202	82 (41%)	120 (59%)	0.56
	>4.5 cm	158	69 (44%)	89 (56%)	
Tumour location	Right	107	51 (48%)	56 (52%)	0.35
	Left	110	51 (46%)	59 (54%)	
	Rectum	164	65 (40%)	99 (60%)	

3.3 Survival Analysis

Data with respect to patients' progression-free and cancer-specific survival were available for 350 of 381 (92%) patients. Median follow-up was 45 months with range from 1 to 182 months. After a median of 15 months (range 0-88), 141 (37%) patients developed progressive disease. Of these, 118 (84%) patients died from cancer, 11 (8%) patients were alive with metastatic disease at the time of the investigation. Another 7 (5%) patients had no evidence of additional tumour progression after metastasectomy (liver, lungs). Five (3%) patients had died within 30 days after surgery. Follow-up data are illustrated in Figure 15.

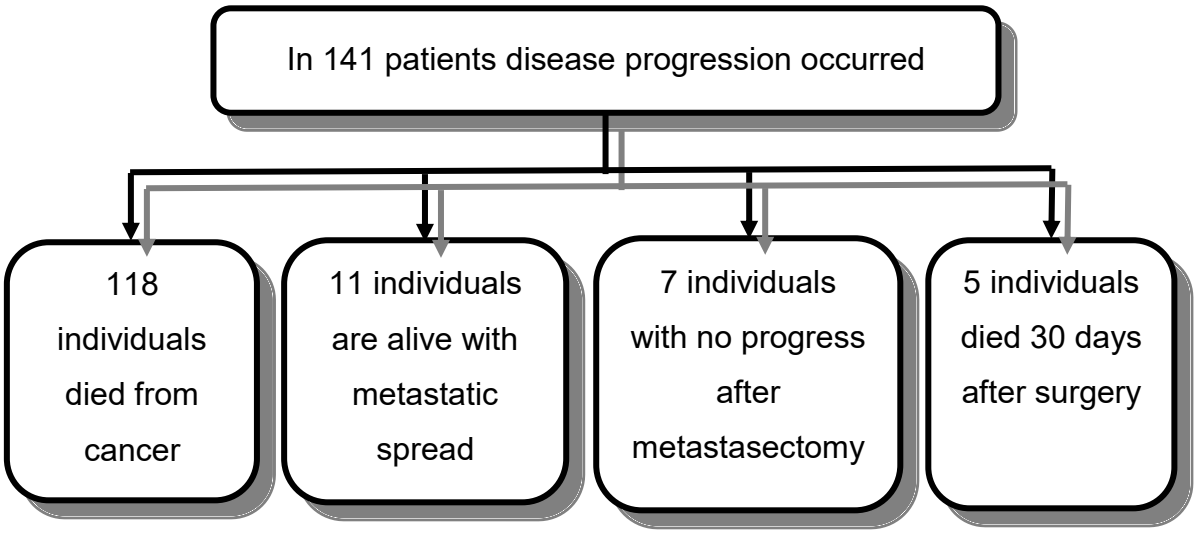


Figure 15: Disease progression occurred in 141 (37%) out of 350 patients of the study cohort.

3.3.1 Tumour budding

High-grade tumour budding was significantly associated with poor progression-free (p<0.001) and cancer-specific (p<0.001) survival in comparison to low-grade budding in our cohort (Figure 16).

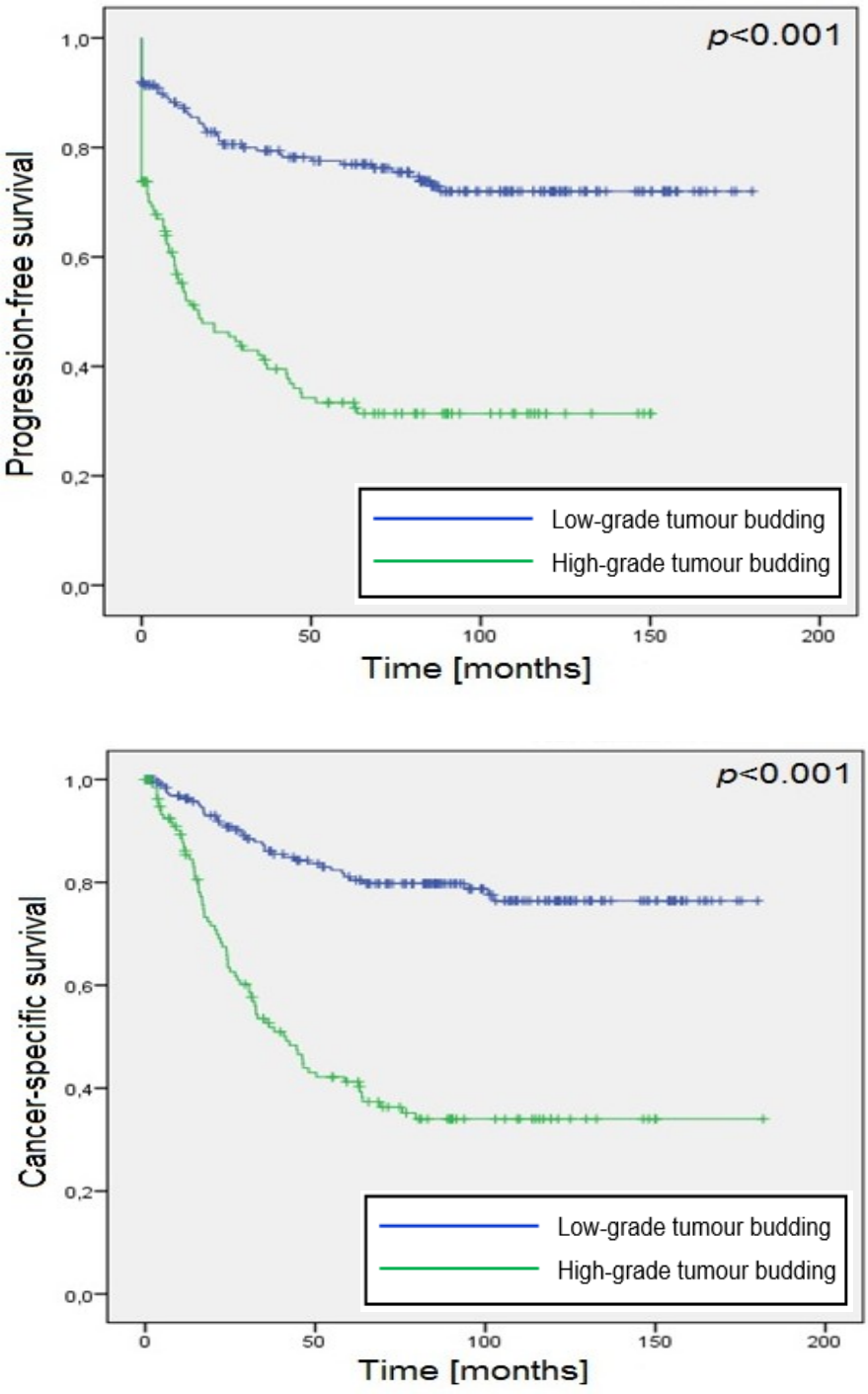


Figure 16: Survival analysis of tumour budding using the Kaplan-Meier method in the cohort of 381 primary colorectal cancers.

In the next step, we performed multivariate testing, which proved tumour budding to be an independent predictor of both progression-free (p=0.014) and cancer-specific (p=0.003) survival. Data are summarised in Table 7.

Table 7: Cox's proportional hazards regression models comparing the prognostic significance of age, gender, T and N classification, tumour grade, tumour budding, lymphatic and venous invasion.

		HR	95% CI	*P*-value
Progression-free survival	Age	1.05	0.75 – 1.47	0.780
	Gender	1.26	0.89 – 1.79	0.190
	T classification	2.03	1.11 – 3.71	0.021
	N classification	2.94	1.90 – 4.56	0.001
	Tumour grade	1.06	0.74 – 1.53	0.741
	Budding (high-grade versus low-grade)	1.67	1.11 – 2.51	0.014
	Lymphatic invasion	1.30	0.89 – 1.89	0.177
	Venous invasion	1.66	1.12 – 2.46	0.011
Cancer-specific survival	Age	1.48	1.02 – 2.13	0.040
	Gender	1.34	0.91 – 1.98	0.138
	T classification	2.12	1.08 – 4.17	0.029
	N classification	2.79	1.74 – 4.49	0.001
	Tumour grade	1.64	1.13 – 2.40	0.010
	Budding (high-grade versus low-grade)	1.93	1.25 – 2.97	0.003
	Lymphatic invasion	1.23	0.82 – 1.84	0.314
	Venous invasion	2.04	1.35 – 3.07	0.001

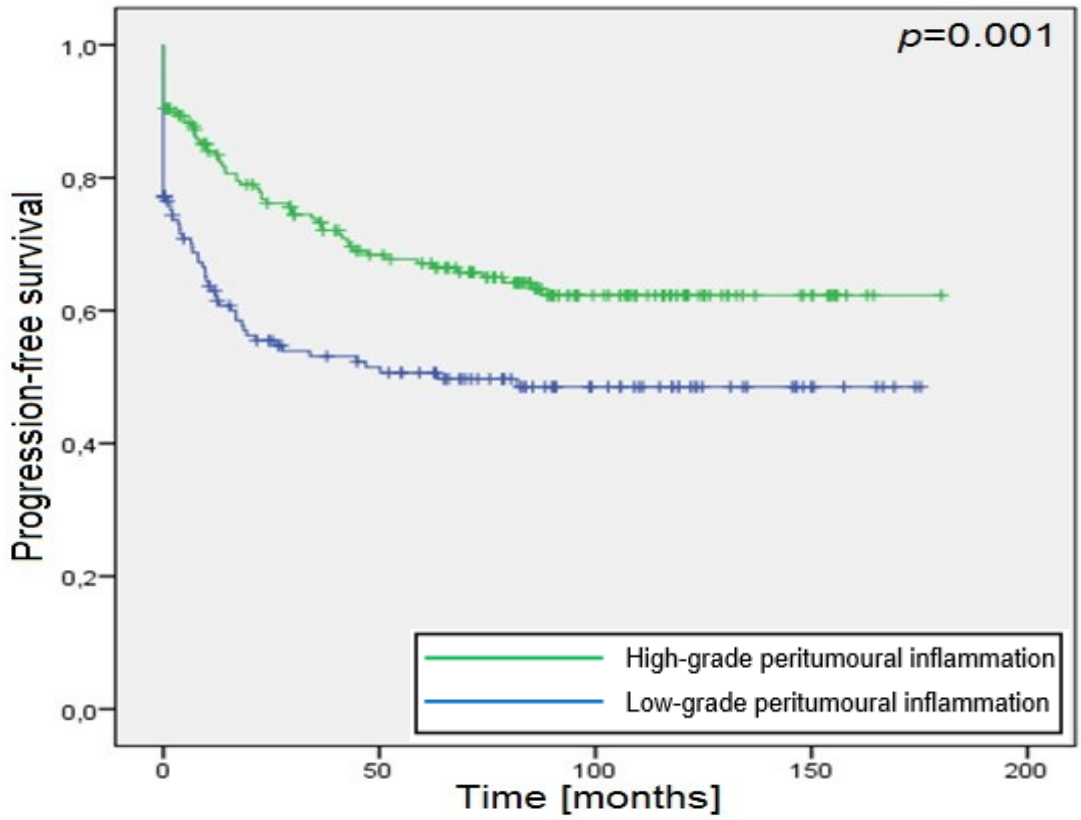
When peritumoral inflammation was included in multivariate testing, tumour budding still proved to be statistically significant, whereas for inflammation no independent impact on patients' outcome was noted (Table 8).

Table 8: Cox's proportional hazards regression models comparing different prognostic markers including tumour budding and inflammation.

		HR	95% CI	*P*-value
Progression-free survival	Age	1.03	0.74 – 1.49	0.851
	Gender	1.26	0.89 – 1.79	0.197
	T classification	1.92	1.05 – 3.52	0.035
	N classification	2.95	1.91 – 4.57	0.001
	Tumour grade	1.06	0.74 – 1.52	0.752
	Budding (high-grade versus low-grade)	1.67	1.11 – 2.51	0.013
	Inflammation (high-grade versus low-grade)	0.75	0.53 – 1.06	0.103
	Lymphatic invasion	1.30	0.89 – 1.90	0.177
	Venous invasion	1.60	1.08 – 2.37	0.020
Cancer-specific survival	Age	1.45	1.00 – 2.10	0.047
	Gender	1.34	0.91 – 198	0.137
	T classification	1.93	0.98 – 3.83	0.059
	N classification	2.90	1.86 – 4.65	0.001
	Tumour grade	1.69	1.16 – 2.46	0.007
	Budding (high-grade versus low-grade)	1.93	1.25 – 2.97	0.003
	Inflammation (high-grade versus low-grade)	0.70	0.48 – 1.01	0.058
	Lymphatic invasion	1.20	0.80 – 1.80	0.370
	Venous invasion	1.93	1.27 – 2.92	0.002

3.3.2 Overall peritumoral inflammation

Overall peritumoral inflammation was significantly associated with better progression-free (p<0.001) and cancer-specific (p<0.001) survival in our cohort (Figure 17).



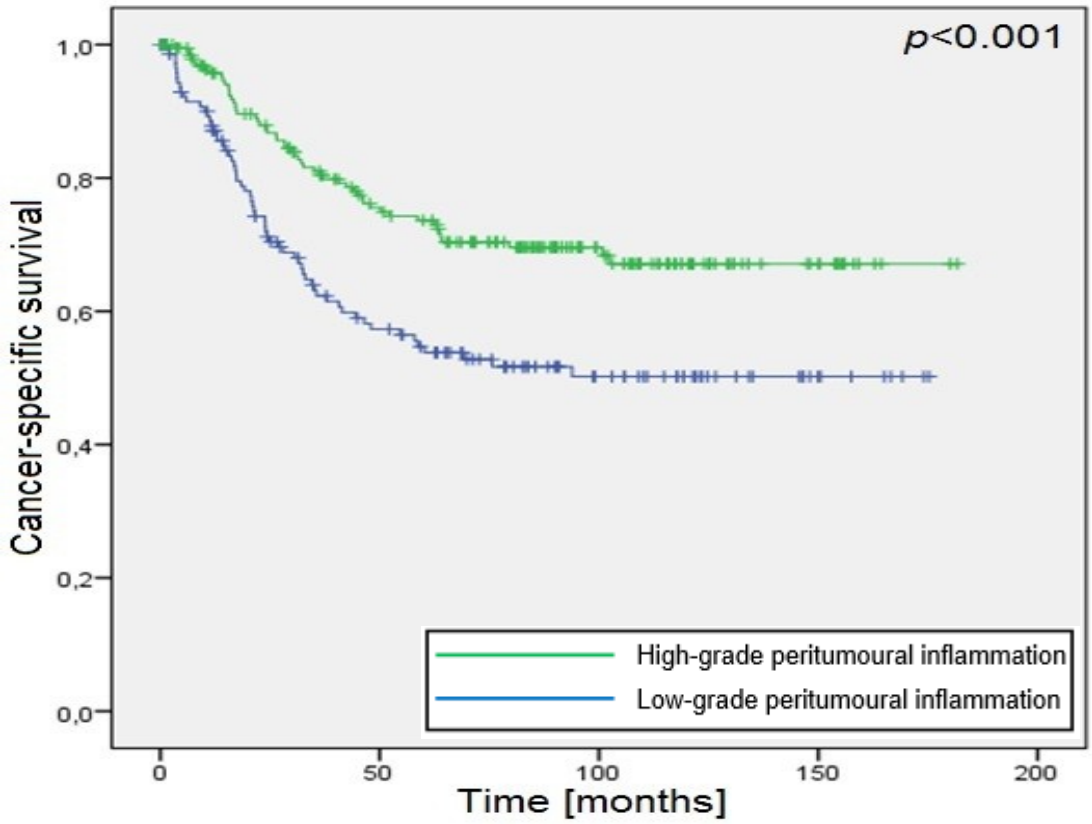


Figure 17: Survival analysis of peritumoral inflammation using Kaplan-Meier method in the cohort of 381 primary colorectal cancers.

3.3.3 Tumour budding and overall peritumoral inflammation

Combined analysis of tumour budding and overall peritumoral inflammation led to the following results: Patients with high-grade budding and marked inflammation had a better outcome, with respect to both progression-free (p<0.001) and cancer-specific survival (p<0.001), compared to patients with high-grade budding and only mild inflammation. Outcome in these cases, however, was still worse compared to patients with low-grade budding, in which the extent of peritumoral inflammation had no further prognostic effect. Data are illustrated in Figure 18.

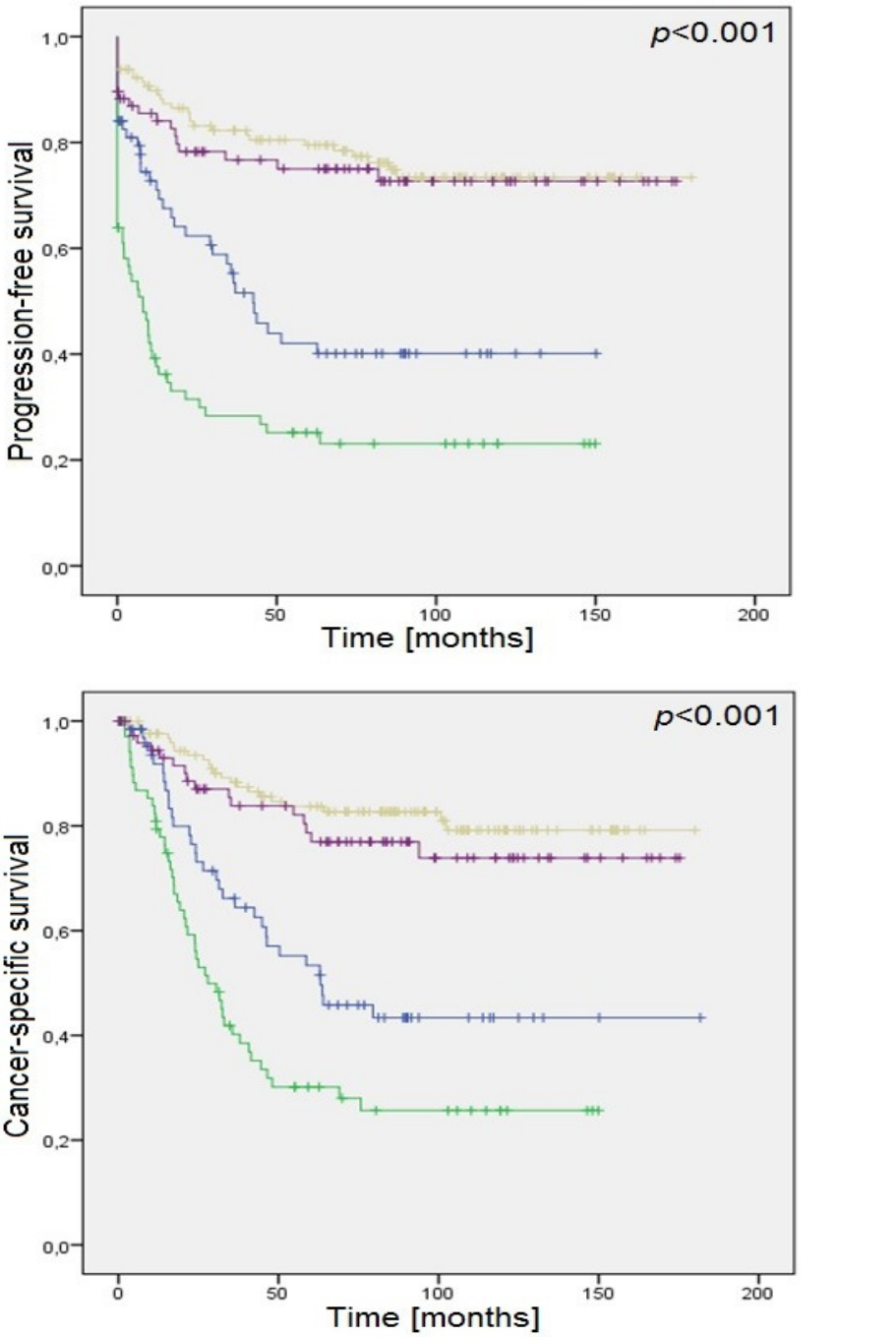


Figure 18: Survival analysis of both tumour budding and overall peritumoral inflammation using Kaplan-Meier method in the cohort of 381 primary colorectal cancers.

These results were confirmed in multivariate analysis, which proved high-grade inflammation to be an independent predictor of favourable progression-free (p=0.021) and cancer-specific (p=0.024) survival. Data are summarised in Table 9.

Table 9: Cox's proportional hazards regression models comparing the prognostic significance of age, gender, T and N classification, tumour grade, inflammation, lymphatic and venous invasion; analysis restricted to patients with tumours with high-grade budding (n=160).

		HR	95% CI	*P*-value
Progression-free survival	Age	0.98	0.64 – 1.50	0.931
	Gender	1.30	0.83 – 2.04	0.249
	T classification	1.16	0.49 – 2.78	0.732
	N classification	2.09	1.20 – 3.63	0.009
	Tumour grade	1.16	0.74 – 1.80	0.515
	Inflammation (high-grade versus low-grade)	0.59	0.38 – 0.92	0.021
	Lymphatic invasion	1.28	0.81 – 2.02	0.291
	Venous invasion	1.59	1.00 – 2.53	0.051
Cancer-specific survival	Age	1.47	0.93 – 2.34	0.103
	Gender	1.44	0.88 – 2.36	0.150
	T classification	1.46	0.56 – 3.77	0.439
	N classification	2.13	1.19 – 3.81	0.011
	Tumour grade	2.06	1.30 – 3.26	0.002
	Inflammation (high-grade versus low-grade)	0.58	0.36 – 0.93	0.024
	Lymphatic invasion	1.23	0.75 – 2.00	0.410
	Venous invasion	2.07	1.25 – 3.44	0.005

In tumours with low-grade budding no independent impact of inflammation on progression-free (p=0.785) or cancer-specific (p=0.865) survival was detected. Data are summarised in Table 10.

Table 10: Cox's proportional hazards regression models comparing the prognostic significance of age, gender, T and N classification, tumour grade, inflammation, lymphatic and venous invasion; analysis restricted to patients with tumours with low-grade budding (n=221).

		HR	95% CI	*P*-value
Progression-free survival	Age	1.01	0.56 – 1.82	0.985
	Gender	1.18	0.67 – 2.09	0.569
	T classification	2.52	1.08 – 5.87	0.032
	N classification	4.33	2.29 – 8.18	0.001
	Tumour grade	0.84	0.42 – 1.67	0.617
	Inflammation (high-grade versus low-grade)	1.09	0.60 – 1.96	0.785
	Lymphatic invasion	1.21	0.59 – 2.49	0.598
	Venous invasion	2.04	0.89 – 4.68	0.094
Cancer-specific survival	Age	1.34	0.69 – 2.60	0.390
	Gender	1.09	0.56 – 2.10	0.809
	T classification	2.68	0.99 – 7.22	0.052
	N classification	4.22	2.04 – 8.73	0.001
	Tumour grade	0.93	0.43 – 2.01	0.855
	Inflammation (high-grade versus low-grade)	0.94	0.49 – 1.83	0.865
	Lymphatic invasion	1.26	0.55 – 2.87	0.580
	Venous invasion	1.90	0.76 – 4.75	0.170

4. Discussion

In future years, cancer cases are predicted to occur more frequently and on a global scale, contributing to the growing health problem that we are currently witnessing. In the United States, cancer is expected to be the leading cause of death and CRC is set to be the second major cause of cancer related deaths. (59) Consequently, new achievements in cancer research are fundamental to improve personalised patient management.

Therefore, knowledge of development and behaviour of CRC is a basic requirement for the improvement of patients' treatment and outcome. In more than 70% of cases CRC develops through premalignant precursor lesions called adenomas. Adenomas are defined by the presence of glandular structures with intraepithelial neoplasia showing different degrees of severity. The risk of cancer development within a given adenoma depends on its histopathological appearance (tubular vs. tubulovillous vs. villous), degree of intraepithelial neoplasia, growth habit (sessile vs. pedunculated) and size. Villous adenomas carry the highest risk of progressing into CRC, followed by tubulovillous and tubular adenomas. (11)

In contrast to other cancer entities, CRC has a well-known pathogenesis. As mentioned above, the multistep process of cancer formation, developing over years, is identified as the adenoma-carcinoma sequence. (60, 61) The adenoma-carcinoma sequence, firstly described by Fearon and Vogelstein (62), illustrates the transition of a benign adenoma into a malignant carcinoma through the successive accumulation of genetic alterations, that is, somatic mutations. As first step, APC-gene mutations appear, followed by activating mutations of the KRAS oncogene and inactivating mutations of the tumour suppressor gene TPR53. The multistep process of carcinogenesis with its genetic alterations is shown in Figure 19. (63)

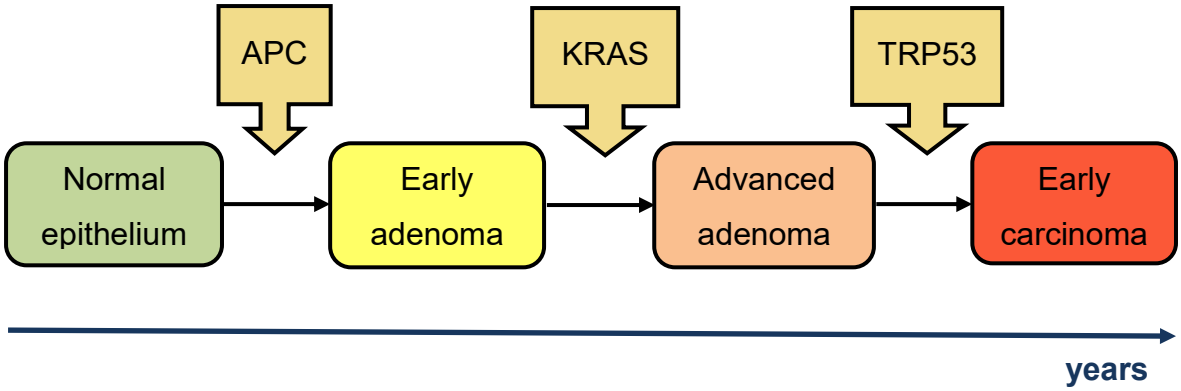


Figure 19: Adenoma-carcinoma sequence. Modified after Fearon and Vogelstein. (62)

Due to the prolonged time interval between intraepithelial neoplasia and invasive carcinoma, early detection of precursor lesions is possible and makes local resection of preinvasive lesions achievable. For this reason, colonoscopy and faecal occult blood tests are used in many countries as early-detection tools for CRC in population-based screening programs, allowing for early diagnosis, thereby decreasing disease-related morbidity and mortality. (2, 64)

It is of notable interest that not every CRC develops and proceeds in the same manner. 80% of CRCs develop sporadically, in contrast to 20% that develop with familial clustering. For a total of 5% of all CRCs distinct germline mutations have been identified that prompt tumour development. (65) Examples include Lynch Syndrome (hereditary non-polyposis colorectal cancer syndrome), familial adenomatous polyposis (FAP), Li-Fraumeni-Syndrome, Peutz-Jeghers-Syndrome and Cronkhite-Canada-Syndrome. (2, 65)

Approximately 20% of sporadic CRC do not originate from classical adenomas as illustrated above. These cancers arise from serrated adenomas, most commonly in the proximal colon. Tumorigenesis occurs through a fundamentally different molecular pathway, known as the “serrated route to cancer”. Upon endoscopy, serrated adenomas are flat and show ill-defined borders. In addition, they are often hidden underneath a mucus cap. Therefore, endoscopic diagnosis may be challenging. (63)

Cancer management nowadays includes the discussion of patients in a multidisciplinary team, which generally includes colorectal surgeons, oncologists,

gastroenterologists, radiologists and radiotherapists as well as pathologists. The discussion must consider the patient's individual risk profile. (2, 25)

This profile is mainly based upon AJCC/UICC tumour stage, which represents the most important predictor of outcome nowadays. Patients with localised, regional and distant disease have five-year relative survival rates of 90.3%, 70.4%, and 12.5% and an overall five-year survival rate of 66% for both sexes. (4, 59) According to the consensus guidelines of the European Society for Medical Oncology (ESMO) (25), AJCC/UICC stage II disease, defined by the presence of transmural tumour penetration (T3, T4a and T4b) with the absence of lymph node or distant metastasis (N0 and M0), is treated with tumour resection alone. Adjuvant therapy is not routinely recommended, except for patients with additional risk factors, such as serosal penetration or pT4a disease. (9, 25) Patients with AJCC/UICC stage III disease (T1-T4b, N1-N2b and M0) usually receive adjuvant treatment; but only 15 to 50% show disease progression within five years. (2) The treatment algorithm of the ESMO consensus guidelines for localised and regional colon cancer is illustrated in Figure 20.

AJCC/UICC tumour stage is the strongest determinant of long-term outcome in CRC, and outcome prediction is particularly valid for early (stage I) and late (stage IV) disease. However, patients with intermediate levels of disease, that is, stages II and III, may experience entirely different outcomes, illustrating that the TNM classification is unsatisfactory for prognostication in this subset. (41, 66, 67)

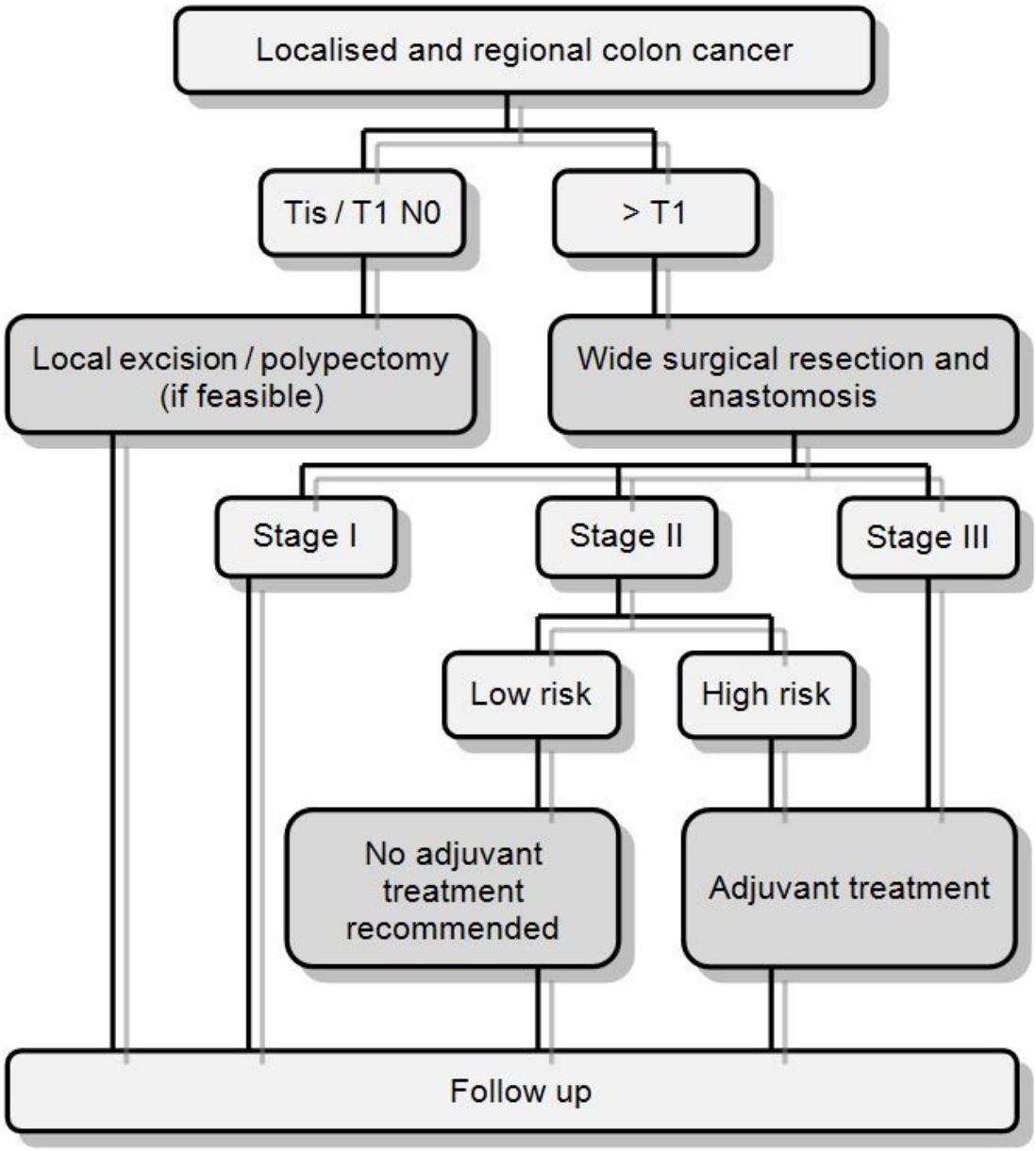


Figure 20: Treatment algorithm for localised and regional colon cancer. Modified after Schmoll et al. (25)

The study of Moertel et al. (57) shows that 45% of patients with AJCC/UICC stage III disease treated only with tumour resection had no disease progression within 5 years compared to 65% of patients treated with both surgery and adjuvant therapy using 5-fluorouracil and levamisole. This implies that, 45% stage III CRC patients did not require adjuvant treatment and that 35% will die even with systemic treatment. (57) These data highlight that a significant number of patients receive aggressive treatment, which implies a high risk of complications and severe side effects, even though they do not benefit. It must be taken into consideration that the global population is ageing and naturally suffers from concomitant diseases. Moreover, adjuvant treatment is expensive, in particular, when it is not indicated, due to limited financial resources available for public health care systems.

For this reason, identification of additional prognostic markers is urgently needed, in order to indicate disease progression or favourable outcome. Ideal outcome predictors should be easily assessable in daily routine, e.g. on H&E stained slides. The information rendered from these additional prognostic parameters might facilitate the identification of patients who would benefit from tumour resection alone or, on the contrary, who would require adjuvant treatment.

The tumour-host interaction at the invasive tumour margin has achieved major attention in recent years and both tumour growth characteristics and features of the tumour stroma, including the so-called tumour microenvironment may be evaluated. Tumour characteristics define tumour aggressiveness, both tumour border configuration as well as the extent of tumour budding deserve special attention. In contrast, stroma-related features mainly include peritumoral inflammation, which generally shows a beneficial effect (tumour defence or anti-tumour host response). (41) In the following, tumour growth characteristics will be discussed followed by anti-tumour host response.

Jass et al. (68) introduced a classification system that defines the configuration of the leading edge of invasion as follows: The loss of strictly delimited tumour-host interface with a diffuse invading tumour manner is defined as “infiltrating” and has been linked to poor prognosis. As opposed to this, a round circumscribed configuration of the infiltrative margin with a clear boundary between tumour and host tissue is termed “expanding”. Tumours with an “expanding” invasive border are frequently associated with low risk of progression.

In contrast, CRCs with high aggressiveness often display an infiltrative growth pattern with dissection of tumour tissue through the anatomic structures of the bowel wall with slight or absent desmoplastic stromal response. (69, 70) Methodically, the tumour border configuration should be diagnosed at low magnification and has to be differentiated from other diagnostic features that are assessed on high magnification, such as vascular invasion. (69)

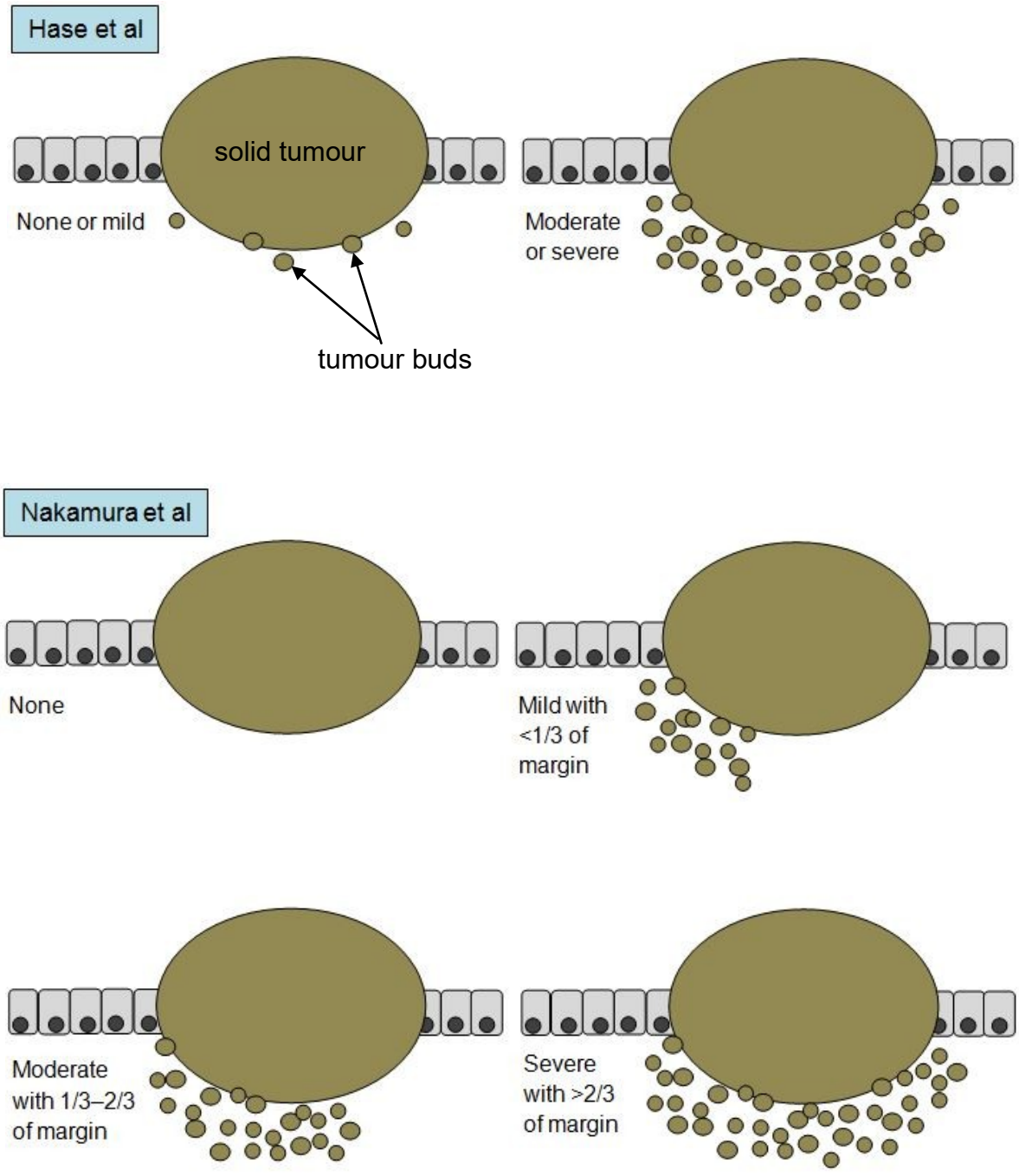
Tumour budding, which likewise has to be assessed under high magnification, represents another prognostic marker, which was introduced to assess growth characteristics at the leading edge of tumour invasion. It should be noted that budding is frequently associated with an infiltrative growth pattern, according to the Jass classification mentioned above, but it should be handled as individual prognostic tool. (71)

Ueno et al. (49) defined budding as the presence of single tumour cells or small clusters composed of fewer than five cells. The presence of budding cancer cells indicates an aggressive phenotype with the potential of dissemination. It has also been repeatedly related to adverse long-term outcome in patients with CRC. (37, 41-49) On the molecular level, budding is believed to reflect EMT, which represents a crucial step for cancer progression and spreading. (38) Ki67, caspase-3 and M30 are usually absent in tumour buds, indicating decreased proliferation and apoptosis. (72) Budding cancer cells usually gain a mesenchymal phenotype, indicated by their frequent positivity for vimentin. Epithelial markers are de-regulated: While adhesion molecules, such as E-cadherin, are usually downregulated, other epithelial markers, such as keratin 7, may be upregulated. This process has been referred to as “epithelial-epithelial transition” (EET). (73)

It is important to note that the criteria for the assessment of tumour budding vary from study to study. The obvious lack of consensus, that is, a standardised scoring system, so far has prevented the implementation of the marker into practice guidelines. (74) Figure 21 illustrated the most commonly applied histological criteria for the assessment of budding, following the publications by Hase et al., (75) Nakamura et al. (76) and Ueno et al. (49). (38, 44, 77)

Interobserver variation is believed to be another important limitation. The study by Puppa et al. (78) demonstrated an overall fair level of diagnostic agreement, which was significantly higher in early cancers and also among expert gastrointestinal pathologists. Cytokeratin immunostaining facilitated the detection

of budding cancer cells, but did not result in improved interobserver agreement. (78) Despite these shortcomings, many studies confirmed tumour budding as a marker of cancer progression, independent of the used scoring system. (38, 41-44, 46-49, 75-77)



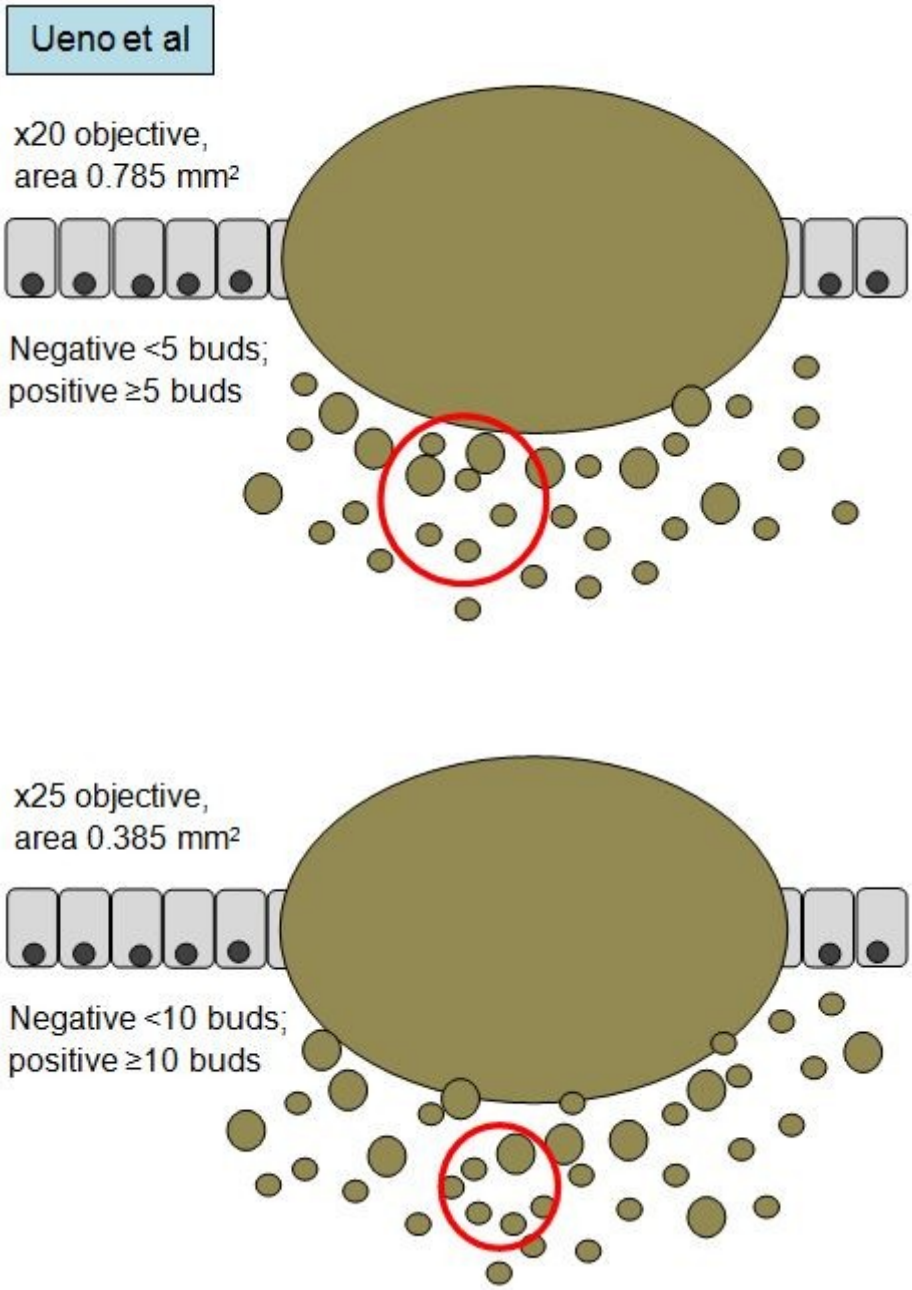


Figure 21: Prognostic scoring system of tumour budding by Hase et al., (75) Nakamura et al. (76) and Ueno et al. (49) Modified after Lugli et al. (38)

Overall, the interaction between cancer and its surrounding cells bears new possibilities to stage CRC. Thus, active immunosurveillance in the tumour microenvironment has been related to low levels of tumour budding and is associated with improved outcome. (37, 45, 79) According to Koelzer et al. (79), tumour buds may evade immune recognition through down-regulation of membranous major histocompatibility complex (MHC) class I. Specifically, tumour buds are able to escape $CD8^+$ T-cell driven antitumoral host response. It is of note that MHC class I positivity influence patients' outcome only in the absence of tumour budding. This supports the notion that mostly MHC class I negative tumour buds determine the biological behaviour of CRC. (37, 79)

As indicated above, the anti-tumour activity of the overall inflammatory cell reaction, driven mainly by neutrophils and macrophages, may lead to unspecific destruction of cancer cell islets, thereby generating isolated single cells or small cell clusters. (37) Pathogenetically, this phenomenon needs to be differentiated from the specific anti-tumour response, which is characterised by tumour-infiltrating T lymphocytes. These T-cells specifically target (pre-existing) tumour buds, described by Lugli et al. (45) as 'nipping in the bud'.

On the histological level, peritumoral inflammatory cells, including histiocytes, can be difficult to differentiate from tumour buds, and may sometimes obscure underlying budding. Immunohistochemistry for anticytokeratin may help to highlight tumour buds in this setting, as discussed in (80), and may also improve interobserver agreement (81). Nevertheless, the presence of cytokeratin-positive membrane-fragments and microvesicles mimicking tumour buds has been identified as a potential diagnostic pitfall that may result in over-counting. (81) The use of immunohistochemistry remains a critical task that needs further elaboration and was therefore not considered in our study, which is in keeping with standard practice. (41)

Our data clearly support the importance of tumour budding as a prognostic tool: Patients with tumours with low-grade budding showed better prognosis, regarding both progression-free and cancer-specific survival, compared to patients with tumours with high-grade budding. In multivariate analyses, tumour budding turned out to be a predictor of progression-free and cancer-specific survival, independent of lymphatic and venous invasion, T and N classification, and tumour grade. When peritumoral inflammation was included in analysis, tumour budding

kept statistical significance, whereas for inflammation no independent impact on patient outcome was identified.

In order to standardise tumour budding, but also to stress its eminent importance as a prognostic tool in the diagnostic histopathology of solid malignancies an International Tumour Budding Consensus Conference (ITBCC) was initiated and held its first meeting in April 2016. Experts from eleven countries all over the world discussed how to standardise budding assessment and implement the method into standard pathology reporting. (82) The results of this meeting have so far only been reported as meeting abstract (83): Budding has been defined as an isolated cancer cell or a cell cluster of maximum 4 cells. It is recommended to assess tumour budding on H&E stained slides using the hot spot method in a high power field of 0.785mm^2 at the invasive tumour edge. To facilitate risk stratification a trinomial system has been introduced along with the tumour budding count. (83)

Tumour-related inflammation plays an important role in tumour progression and has been described as the 'seventh hallmark of cancer'. (84) In CRC, marked peritumoral overall inflammation as well as T-cell mediated anti-tumour immune response have been identified as parameters of favourable outcome. (51, 55) Studies by Di Caro et al. (85) and Roxburgh et al. (52) demonstrates the correlation of prominent intra- and peritumoral inflammatory cell infiltrate with improved prognosis. Galon et al. (86) demonstrated that CRCs with high numbers of intratumoral CD3$^+$ T-cells and CD8$^+$/CD45RO$^+$ memory T-cells share improved outcome compared to tumours lacking these features. Notably, the prognostic effect was independent of AJCC/UICC tumour stage. Thus, immune activation seems to reduce disease progression and therefore offers prognostic information to be used in clinical decision making. (86)

For the improved description of the prognostic value of the lymphocytic infiltrate, especially the role of T-cells in CRC, a host-dependent immunoscore has recently been developed by international endeavour. (23, 87) The score is based on the assessment of two T-cell subpopulations: CD3$^+$ and CD8$^+$. (23, 87) The immunoscore describes the extent of CD3$^+$ and CD8$^+$ lymphocytic infiltration at the invasive margin and in the centre of resected tumours, visualised by immunohistochemistry. Scoring ranges between 0, when low densities of both cell types are found in both regions and a score of 4, when high densities of both cell

types are found. A study of Pagès et al. (88) has shown an association of the immunoscore with disease progression independent of AJCC/UICC tumour stage. An international working group consisting of 23 centres in 17 countries, including the Medical University of Graz, Austria, is currently working on the immunoscore to promote its implementation in routine cancer assessment. (87)

In contrast to the immunoscore, Klintrup et al. (51) investigated the prognostic value of an overall inflammatory cell reaction in CRC, involving lymphoid cells, neutrophilic and eosinophilic granulocytes as well as macrophages. They assessed the overall inflammation at the invasive margin and in central areas of the tumour. (51) The inflammatory cell reaction is assessed on H&E stained slides and represents an easy-to-use and reproducible grading system that can be applied easily in pathologists' daily routine work. The study by Klintrup et al. (51) included a large cohort of cases (n = 374). High-grade overall inflammatory cell reaction proved to be an independent predictor of favourable outcome. (51) Notably, high-grade inflammation, especially at the invasive margin, proved to be superior to AJCC/UICC tumour stage when patients with distant metastatic spread (AJCC/UICC stage IV) were excluded. (51) According to Klintrup et al. (51), assessing the overall inflammatory reaction is superior to the assessment of single inflammatory cell types with respect to prediction of progression-free and cancer-specific survival. (51)

Harbaum et al. (23) investigated the prognostic significance of peri- and intratumoral eosinophil count and showed that the eosinophil count goes hand in hand with the intensity of the overall inflammatory cell reaction. In that study, only peritumoral eosinophils were associated with improved progression-free and cancer-specific survival independent of the overall inflammatory cell reaction. (23)

In univariate analyses our data confirm the prognostic value of overall peritumoral inflammation. Patients with high-grade inflammation showed favourable outcome compared to patients with low-grade inflammation, considering both progression-free and cancer-specific survival.

It is generally believed, that in CRC disease progression or, on contrary, disease regression depends on the interplay of tumour-promoting and anti-tumour host factors, preferably at the leading edge of invasion. As illustrated in detail above, a growing body of evidence suggests that tumour budding and an infiltrating growth pattern favour disease progression, whereas the hosts' immune

response or the unspecific overall inflammatory cell reaction protect the host from invading cancer cells, thereby preventing local or distant metastatic spread.

This study is the first to investigate the relationship between both tumour budding and the overall inflammatory response. The two phenomena can exist in parallel, but run in opposite directions with respect to the impact on patients' outcome. Our data clearly show that patients with high-grade budding (anti-tumour factor) and marked inflammation (pro-tumour factor) have a significantly better outcome compared to patients with high-grade budding and only mild inflammation, which is attributable to inflammation-related destruction of cancer cell islets at the invasive tumour margin. Outcome of cases with high-grade budding and marked inflammation, however, was still significantly worse compared to patients with low-grade budding. These data, which were evident in univariate and multivariate analyses, clearly confirm the value of tumour budding as a prognostic tool, independent of the inflammatory response. (37)

It must be noted that this study carries limitations, foremost due to the retrospective character of our analysis. Tumour budding was assessed only on H&E stained slides, and anticytokeratin immunohistochemistry was not applied in this study. Another limitation of our study is the fact that patients received tumour resection by various surgeons from both academic and community settings.

Strengths of this study include review pathology, systematically performed by two experts in gastrointestinal pathology. In addition, the investigated cohort is large, with a comparably long follow-up time. It represents a random sample of more than 7,500 CRC cases diagnosed at the Institute of Pathology, Medical University of Graz, Austria.

In conclusion, tumour budding and the overall inflammatory cell reaction are powerful predictors of outcome in patients with CRC. The inflammation-dependent destruction of invading cancer islets has the ability to stratify cases with high-grade budding into two distinct prognostic groups. When compared to tumour budding however it plays a minor role in prognostication, having low significance in patients with low-grade budding tumours.

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