Diploma Thesis

IDENTIFICATION OF TUMOR ASSOCIATED PARAMETERS WHICH ARE RELATED TO LYMPH NODE SIZE IN COLORECTAL CANCER SPECIMENS

A histological, immunohistochemical and molecular analysis

submitted by
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and
OA. Dr. Karl Mrak, MSc

Graz, 26.4.2014

Affidavit

I hereby declare that the following thesis has been written only by myself and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

Graz, am 26.4.2014

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Glossary and Abbreviations

aHR	Adjusted Hazard Ratio
AJCC	American Joint Committee on Cancer
BAT-25	Microsatellite marker
BAT-26	Microsatellite marker
CI	Confidence interval
DAB	Diaminobenzidine
DNO	Deoxyribonucleic Acid
Fig.	Figure
G	Grading
H&E	Hematoxylin and eosin
KI	Konfidenzintervall
M	Distant metastases
MLH1	mutL homolog 1; mismatch repair protein
MMR	Mismatch repair protein
MONO-27	Microsatellite marker
MSH2	MutS homolog 2; mismatch repair protein
MSH6	MutS homolog 6; mismatch repair protein
MSI-H	Highly micro satellite instable
MSI-L	Low micro satellite instable
MSS	Micro satellite stable
N	Nodal
n	Amount
NCCN	National Comprehensive Cancer Network
NR-21	Microsatellite marker
NR-27	Microsatellite marker

p	Probability
PCR	Polymerase chain reaction
PMS2	Postmeiotic segregation increased 2 – mismatch repair protein
RA	Risikoadjustierung
ROC	Receiver-operator characteristic
T	Tumor
TIL	Tumor infiltrating lymphocytes
Tis	In situ tumor
TME	Total mesorectal excision
TNM	Tumor node metastasis
UICC	Union internationale contre le cancer; Union for International Cancer Control
WHO	World Health Organization

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Abstract

BACKGROUND: In patients with colorectal cancer, preoperative assessment of lymph node size cannot be regarded as reliable factor indicating presence or absence of metastatic cancer spread in regional lymph nodes. However, parameters determining lymph node size apart from metastatic infiltration yet are largely unknown.

METHODS: This study was carried out prospectively and cross-sectional. Investigation of the relationship between lymph node size and presence of metastatic cancer spread was conducted. In addition, a closer look was taken on the relationship between lymph node size and different primary tumor characteristics, such as depth of tumor penetration (T stage), tumor differentiation (tumor grade), tumor size, primary tumor location, intra- and peritumoral inflammation, lymphocytic anti-tumor reaction, tumor budding and tumor necrosis. Patients with neoadjuvant therapy as well as multiple, mono-segmental tumors had to be excluded.

RESULTS: The final study cohort included 148 patients with a mean age of 68 years (median 69, range 36-92). Dissection of the lymph nodes yielded 4167 nodes. Mean lymph node count was 28.2 (median 26, range 9-67). In 320 (8%) of all nodes, metastatic disease was detected and was related to lymph node size ($p<0.001$). Nodes measuring 1 or 2 mm caused upstaging within the N category in one third of the cases, but in none of these cases, such small nodes identified a patient as node positive. Lymph node enlargement was defined as at least 5 nodes ≥ 5 mm and showed an independent association with deep tumor penetration (adjusted hazard ratio [aHR] 3.38, 95% confidence interval [CI] 1.18-9.75; $p=0.024$), large tumor size (aHR 7.61, 95% CI 2.41-24.01; $p=0.001$), and right tumor location (aHR 5.92, 95% CI 2.35-14.71; $p<0.001$). However, high lymphocytic anti-tumor reaction just missed statistical significance (aHR 8.87, 95% CI 0.97-80.81; $p=0.053$).

CONCLUSION: Additional to metastatic infiltration primary tumor characteristics (i.e. tumor size, T stage and right tumor location) are significantly associated with the size of the regional lymph nodes, whereas high lymphocytic anti-tumor reaction just showed a trend for association.

Zusammenfassung

HINTERGRUND: Bei Patienten mit kolorektalem Karzinom kann die präoperative Beurteilung der Lymphknotengröße nicht als zuverlässiger Faktor für die Existenz von Tumorzellen in den regionalen Lymphknoten aufgefasst werden. Allerdings sind die Parameter welche außer der Tumorzellinfiltration die Lymphknotengröße beeinflussen bislang weitgehend unbekannt.

METHODEN: Es wurde untersucht, ob ein Zusammenhang zwischen der Lymphknotengröße und der Tumorzellinfiltration in den Lymphknoten vorliegt. Weiters wurde der Zusammenhang zwischen der Lymphknotengröße und verschiedenen tumorspezifischen Parametern untersucht, im Speziellen: Tiefe der Tumorinfiltration (T Stadium), Tumorzelldifferenzierung (Grading), Tumorgröße, Tumorlokalisation, intra- und peritumorale Entzündungsreaktion, lymphozytäre anti-Tumorreaktion, Tumorbudding und Tumornekrose. Patienten, die eine neoadjuvante Chemotherapie erhalten oder an multiplen, im gleichen Segment auftretenden Tumoren gelitten haben, wurden aus der Studie ausgeschlossen.

ERGEBNISSE: Die Studienkohorte umfasste 148 Patienten, mit einem mittleren Alter von 68 Jahren (Median 69, Spanne 36-92). Insgesamt wurde 4167 Lymphknoten geborgen, deren mittlere Zahl 28,2 pro Resektat (Median 26, Spanne 9-67) betrug. In 320 (8%) aller Knoten konnten Metastasen gefunden werden. Ihre Existenz hatte einen signifikanten Einfluss auf die Lymphknotengröße (p=0,001). Eine Erhöhung der N Kategorie durch Knoten von 1 oder 2 mm wurde in einem Drittel der Fälle beobachtet. Allerdings wurde in keinem Fall ein Patient durch so kleine Knoten alleine als N positiv kategorisiert. Eine Lymphknotenvergrößerung wurde definiert als mindestens 5 Knoten ≥ 5 mm. Sie ist unabhängig assoziiert mit der T Kategorie (Risikoadjustierung [RA] 3.38, 95% Konfidenzintervall [KI] 1.18-9.75; p=0.024), Tumorgröße (RA 7.61, 95% KI 2.41-24.01; p=0.001) und rechtsseitige Tumorlokalisation (RA 5.92, 95% KI 2.35-14.71; p<0.001). Allerdings war die lymphozytäre anti Tumorreaktion nicht statistisch signifikant (RA 8.87, 95% KI 0.97-80.81; p=0.053).

SCHLUSSFOLGERUNGEN: Zusätzlich zu Lymphknotenmetastasen sind tumorspezifischen Parameter (Tumorgröße, T Stadium, rechtsseitige Tumorlokalisation) signifikant assoziiert mit der Lymphknotengröße, allerdings zeigt die lymphozytäre anti- Tumorreaktion nur eine trendmäßige Assoziation.

1 Introduction

1.1 General facts about colorectal carcinomas

Colorectal cancer is one of the most common cancers worldwide. The American Cancer Society predicates colorectal cancer on third place of the estimated incidence of all cancer types. Only prostate cancer in men and breast cancer in women as well as lung cancer in both sexes will occur more often. In the United States, approximately 96,830 new cases of colon cancer and 40,000 new cases of rectal cancer have been estimated for 2014. For the same period, 50,280 deaths from these cancer types have been calculated. Overall colorectal cancer accounts for 8.6% of all estimated cancer deaths. [1]

1.1.1 Etiology and risk factors

Occurrence as well as prevention of many illnesses can be performed by diet and lifestyle. [2] Populations with a Western type of diet tend to show a high incidence of colorectal tumors. This diet includes highly caloric food, frequent intake of animal fat, and a sedentary lifestyle. Further potential risk factors are frequent like consumption of meat, smoking and frequent intake of alcohol, whereas vegetable consumption, estrogen replacement therapy, prolonged use of non-steroidal anti-inflammatory drugs, and physical activity are known to be protective against colorectal carcinomas. [3]

Chronic inflammatory bowel disease seems to be an important factor in the development of colorectal cancer. Increase in the risk to harbor colorectal carcinoma is seen after 8 to 10 years of chronic inflammation. An even higher risk of developing colorectal cancer can be seen in patients with early-onset of inflammation or widespread manifestation of inflammation due to affection of the whole large intestine (pancolitis). [3]

Ulcerative colitis is one of those mentioned chronic inflammatory bowel diseases. Its etiology is still unknown; further, it affects both, children and adults. The peak incidence is known to lie in the early third decade. Its major

risk factors are duration and extent of the disease. Thus, it is regarded as premalignant disorder. There is a strong association between involvement of more than one-half of the colon and risk of carcinoma development. In this case, the risk to bear neoplasia is approximately 15%. However, left sided disease seems to cause malignancy in 5% of all cases. Notably, there is no association between ulcerative proctitis and increasing carcinoma risk. [3]

Crohn's disease provides similar data. This illness develops carcinomas in both, the small and the large intestine. Colorectal carcinoma risk is known to be threefold above normal. Risk factors for malignancy are similar to ulcerative colitis: early onset and long duration of the disease. [3]

Extern impact factors are also known to increase the risk of developing a colorectal carcinoma. To mention one, therapeutic irradiation of the pelvis is a well known, yet less common etiological parameter. [3]

1.1.2 Signs and symptoms

One common sign of colorectal carcinomas is haematochezia that is caused by bleeding from the tumor. Due to the loss of blood, patients often show anemia. In addition, patients often experience changes in their bowel habits. Feces in the right part of the large intestine are usually of fluid consistence. Thus, they can easily pass exophytic growing tumors. Feces of the left side of the large intestine tend to be more solid. Thereby, passing exophytic growing tumors is less possible due to the resistance caused by the tumor. Therefore, those patients often experience constipation and, sometimes, abdominal distension. Tumors of the rectosigmoid are known to cause tenesmuses. [4]

As general symptoms, colorectal carcinomas often produce fever, weight-loss, malaise, and abdominal pain. Very advanced tumors also tend to cause complications like perforation or obstruction, but unfortunately, some patients do not suffer from any of these symptoms. Thus, their colorectal tumor is first identified by screening or surveillance. [4]

1.1.3 Selected cancer types

1.1.3.a Adenocarcinoma - ICD-O Code: 8140/3

Most of these carcinoma types are gland-forming. Size and configuration of the glandular structures varies within each tumor and each patient. Epithelial cells of well and moderately differentiated tumors usually are large and tall. On the luminal side, the structures often contain cellular debris. [5]

1.1.3.b Mucinous adenocarcinoma - ICD-O Code: 8480/3

This subtype of tumor is defined if > 50% of the lesion is composed of mucin that pools in the extracellular space. Within this mucin, malignant epithelium can be found as acinar structure, strips of tumor cells or single tumor cells. These carcinomas are known to show high frequency microsatellite instability. [5]

1.1.3.c Signet-ring cell carcinoma - ICD-O Code: 8490/3

This subtype of tumor is defined if > 50% of the lesion is composed of tumor cells with prominent intracytoplasmic mucin. Typically, signet-ring cells have one large mucin vacuole. Usually, this vacuole fills the cytoplasm and displaces the nucleus. These cells can occur as diffusely infiltrating the tumor that produces minimal extracellular mucin. Sometimes, those cells also appear in mucin pools of mucinous adenocarcinomas. There is a coincidence between this tumor type and microsatellite instability. [5]

1.1.3.d Micropapillary carcinoma - ICD-O Code: 8265/3

This tumor type has the status of being a very aggressive variant of the adenocarcinomas of the colon and rectum. Small papillary cell clusters are the main characteristic of this cancer type. Those cell clusters are surrounded by lacunar spaces. According to recent findings, usually less than 30% of an entire lesion contains this tumor subtype. The sigmoid colon and the rectum are the preferred location of this tumor type. Aggression of this carcinoma is based on the higher frequency of lymphovascular invasion, lymph node metastasis and results in more aggressive biological and clinical behavior. [6]

1.2 Therapy strategies

1.2.1 Treatment choices

Clinical decision-making in patients with colorectal cancer is primarily based on tumor stage as main predictor for patients' outcome. Tumor stage itself is reflected by the AJCC (American Joint Committee on Cancer) / UICC (Union for International Cancer Control) tumor node metastasis (TNM) system. [7,8] This system describes the anatomical extent of illness. T stands for the extend of the primary tumor, N stands for presence or absence as well as extend of regional lymph node metastasis, and M stands for presence or absence of distant metastasis. By adding numbers to these components, the extent of disease is described. [9]

Combining the T, N and M classification, the AJCC/UICC provides a staging system that refers to the patient's treatment and prognosis [10]. The higher the number, the more advanced the tumor stage is. In conclusion, high numbers indicate a worse outcome than low numbers. [9,11]

The NCCN (National Comprehensive Cancer Network) guidelines suggest primary surgical treatment for patients who suffer from Tis (in situ tumor) and T1-2, nodal negative, non-metastatic spread colon cancer. At this stage, there is no indication for adjuvant treatment, which is primarily based on 5-flourouracil. Adjuvant chemotherapy combined with surgical treatment is suggested for any patients suffering T3-4, nodal negative, non-metastatic spread cancer. [12]

In case, regional lymph node metastases are present in the resection specimen, adjuvant treatment is indicated in any stage (Tis, T1-4) of illness because adjuvant chemotherapy has decreased tumor recurrence in patients with AJCC/UICC stage III colon cancer. [12,13]

The therapy of patients suffering from rectal cancer is more difficult due to higher complexity. Analogue to colon cancer clinical stage of the disease is the main criterion of choosing primary treatment. However, considerations must not only be given to surgical intent (e.g., curative or palliative) but also

to functional results. These include restoring or maintaining normal bowel function as well as anal continence and keeping genitourinary functions. Altogether, these coefficient parameters lead to optimized therapy and therefore recommendation of preoperative chemoradiation. [14]

1.2.2 Radiological assessment strategies and troubles

Preoperative gathering of information about T status and N status of patients is based on common imaging modalities such as endoluminal ultrasound and magnetic resonance imaging. Occurrence of distant metastases (M status) is confirmed by computed tomography scans. [14]

Depth of tumor penetration is assessed by preoperative endoluminal ultrasound and magnetic resonance imaging. Both imaging modalities are known therefore to provide very accurate results. [14]

In comparison, preoperative assessment of nodal status is much more challenging. In order to detect metastatic nodal spread radiologic images are checked for lymph nodes ≥ 10 mm. Lymph nodes of this size are considered as highly suspicious. [14] Potential understaging or overstaging in patients has a substantial consequence on the clinical proceed. In fact Bipat et al. [15] reported that evaluation of lymph node metastases by endoluminal ultrasound, computed tomography, and magnetic resonance imaging is inaccurate. Sensitivities and specificities of those imaging modalities range from 55% to 67% and 74% to 78%, respectively. Furthermore, neither iliac nodes nor mesenteric and retroperitoneal nodes can be evaluated by endoluminal ultrasound, thus, N status cannot riskless be determined with these methods. [14,15] Moreover, none of these mentioned imaging modalities is significantly superior to another method. In conclusion, these modalities do not provide an accurate determination of tumor N status. [16]

Sauer et al. [17] published the German rectal cancer study in 2004. They demonstrated that 18% of patients who underwent endoluminal ultrasound for considering preoperative chemoradiation might be overstaged. Guillem et al. [18] performed a recent retrospective multicenter study. They

collected 188 patients who were clinically staged T3 N0 by either endoluminal ultrasound or magnetic resonance imaging and subsequently received preoperative chemoradiation. Unfortunately, 22% of those patients showed positive lymph nodes in the resection specimen. That indicates a respectable amount of patients possibly being understaged.

1.3 Lymph node size and its influencing parameters

Summing up these data lymph node size does not seem to be a reliable marker for the diagnosis of lymph node metastases, although it is known that the mean diameter of positive nodes is larger than that of negative nodes. [19] Notably, however, metastatic spread is detected in only 25% to 40% of lymph nodes larger than 10mm. [19,20] According to these data most of the enlarged lymph nodes are free of cancer.

Up to now parameters effecting lymph node size in absence of metastatic infiltration are yet not determined. So far, there is only one study available, which tried to identify possible influencing factors. According to them histological antitumor response is suspected to play a central role in lymph node enlargement. [21] But this study is hampered due to low lymph node yield and retrospective nature.

Interestingly, Gafá et al. [22] published in their study, that histological antitumor response, especially Crohn's-like lesions, occurred much more often in highly micro satellite instable (MSI-H) tumors than in low micro satellite instable (MSI-L) tumors and micro satellite stable (MSS) tumors. In addition, MSI-H tumors are known more likely to be poorly differentiated [23]. Thus, attention was paid to possible influence of micro satellite status on lymph node size.

1.4 Study Aim

With radiological imaging modalities being not reliable enough to detect (positive) lymph nodes in patients with colorectal cancer, and lymph node size itself as possibly multiply influenced parameter, a strong need for determining relation between lymph node size, metastatic cancer spread and other tumor associated factors are given. Up to now, still too less information about these factors was collected. In addition, existing data need to be verified and specified. The aim of this study was to indentify parameters that apart from metastatic cancer spread might be responsible for lymph node enlargement.

2 Materials and Methods

This prospective cross-sectional study was conducted to investigate the relation between lymph node size and involvement by metastatic cancer tissue. Therefore in consecutively recruited patients a large number of both macroscopically and histological tumor features were assessed. In particular it was focused on tumor size, tumor grade, location of the primary tumor as well as intratumoral necrosis, peritumoral inflammation, and tumor budding. Those findings were related to the size of the resected lymph nodes.

2.1 Ethics board approval

This survey was carried out according to the Declaration of Helsinki and obtained approval by the Institutional Review Board of the Medical University of Graz, Austria. Data will be presented following the STROBE Statement aimed at strengthening the reporting of observational studies. [24]

2.2 Collection of the study subjects

Between January 2011 and December 2013, 175 patients underwent curative resection for colorectal cancer at the Department of Surgery, Krankenhaus der Barmherzigen Brüder, St. Veit an der Glan, Austria and were prospectively included in this investigation. Respecting the standards in surgical oncology different techniques were performed, depending on the location of the primary tumor. Most of the patients were operated on by two surgeons only and therefore procedure-related factors showing an impact on the extent of lymph node dissection were controlled. The remaining patients were operated on under supervision by other surgeons applying the same surgical technique under supervision of these two senior-surgeons Prim. Univ.-Prof. Dr. Jörg Tschmelitsch FACS and OA Dr. Karl Mrak, MSc.

2.2.1 Overview of anatomical features

For understanding the different surgical procedures and macroscopic examinations, a short overview of anatomy of the large intestine is essential. Thus, it is focused on the vascular supply as well as the lymphatic drainage system.

2.2.1.a Arterial and venous System

The main blood supply is delivered through the superior and inferior mesenteric artery ***(Fig. 1)***. The branches of the superior mesentery artery supply the cecum (ileocaecal artery), the appendix (appendicular artery), the ascending colon (left colon artery) and approximately the right two-thirds of the transverse colon (middle colon artery) with blood. The branches of the inferior mesentery artery supply approximately the left third of the transverse colon (left colon artery), the descending colon (left colon artery), the sigmoid (sigmoid arteries), and the first third of the rectum (superior rectal arteries) with blood. Between theses arteries, many unnamed branches ramify building a dense anastomosis system. The branches of the internal iliac artery (middle rectal arteries) and of the internal pudendal artery (inferior rectal arteries) supply the remaining parts of the rectum and the upper anal canal with blood. [25,26]

In brief, the venous drainage is mostly performed by the superior and inferior mesenteric veins, flowing to the portal vein. However, a small part of the rectum drains through the middle rectal veins into the internal iliac vein, and the inferior rectal veins drain through the pudendal vein. [25,27]

2.2.1.b Lymphatic drainage System

Lymph nodes are tiny nodes that are intercalated in the lymphatic drainage system. They are surrounded by a taut capsule made of collagenous connective tissue, which guarantees fixation of the nodes with the surrounding tissues, vessels and/ or organs. Their main function is stimulation of several immune competent cells with different foreign antigens. Further, they act as filter for different foreign particles and noxious substances like bacteria or metastatic tumor spread. [28]

The lymphatic drainage mirrors the arterial circulation ***(Fig 1)***, in contrast to many other organs, where the lymphatic vessels are following the venous system. [25] Thus, the lymphatic vessels of the cecum drain into the ileocolic lymph nodes, those of the ascending colon drain into the left colic lymph nodes, and those of the transverse colon drain into the middle colic lymph nodes. These lymph nodes drain into the superior mesentery nodes. The lymphatic vessels of the descending colon drain into the right colic lymph nodes, those of the sigmoid drain into the sigmoid lymph nodes, and those of the upper part of the rectum drain into the superior rectal nodes. All these nodes drain into the inferior mesenteric nodes. The remaining parts of the rectum and the anal verge drain into the internal iliac nodes and the superficial inguinal nodes, respectively. [25,29]

Furthermore, the lymph nodes can be classified into four groups, which are essential for understanding the drainage pattern of the large intestine: epicolic lymph nodes, paracolic lymph nodes, intermediate lymph nodes and preterminal lymph nodes. The epicolic lymph nodes are small nodes localized on the serosal surface of the colon. The paracolic nodes are lying along all segments of the large intestine. The intermediate lymph nodes lie along the large supplying colic arteries and their origins. These nodes include the ileocolic, left colic, middle colic, right colic, sigmoid lymph nodes and superior rectal nodes. Finally, the preterminal lymph nodes are located at the main trunks of the superior and inferior mesenteric artery. [25,29]

The number of nodes in each group decreases the nearer the lymph node group is located to the aorta. Notably, up to now no study has been performed that attempted defining the average lymph node number of the large intestine [25], which causes troubles defining the number of lymph nodes that must at minimum be achieved to stage colorectal cancer specimen. [30] Yet, a number of 12 is sufficient to guarantee a proper staging. [31] Situation is better in the rectum. There, Miscusi et al. [32] published that approximately 34 nodes can normally be found within the mesorectum, though they had a small study population. [25]

The drainage pattern of the lymphatic fluid starts at the multiple epicolic nodes, progresses from the paracolic nodes to the intermediate lymph nodes and finally to the few preterminal lymph nodes, from where the lymphoid fluid progresses to para-aortic nodes and onwards. [25]

Between those nodal groups, the lymphatic vessels often build multiple anastomoses, which differ from person to person. Thus, there is no possibility to define the concrete way of the lymphoid fluid drained from the first (epicolic) to the last, (preterminal) passed by lymph node. [33] This circumstance is the main reason why it is difficult to determine sentinel lymph nodes for biopsies in order to scan for possible lymph node metastases. [25] Also it indicates an expanded surgical specimen to achieve removal of the highest possible draining lymph nodes of the tumor located colon area to avoid missing a possible infiltrated node [25]. Furthermore this indicates a dedicate search for lymph nodes in the resection specimen to recover all possible lymph nodes in order to achieve at least 12 lymph nodes, as recommended. [8,31].

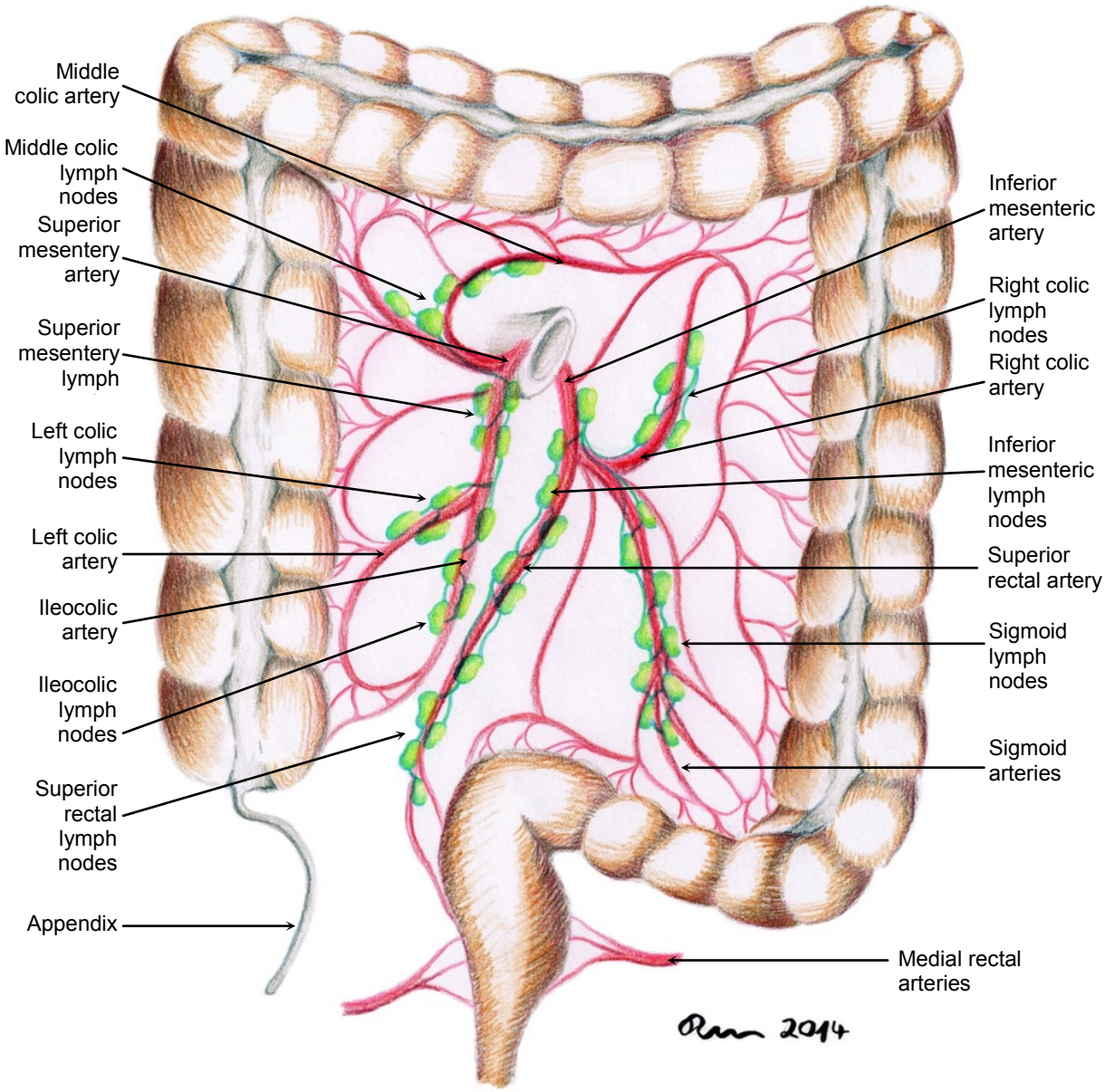

Figure 1 Schematic illustration[1] of the large intestine

Ventral view of the large intestine, the ascending colon, the transverse colon, the descending colon, the sigmoid colon and the rectum are illustrated. Further, the appendix can be seen.

Schematically the main arterial supply (colored in red) and intermediate lymph node subgroups with their vessels (colored in green) are illustrated. For clarification, lymphoid vessels are thickened. Further, differences in lymph node size as well as in their actual count were not considered. [34] The venous system is not shown in this figure.

[1] Illustration was drawn by Cand.med. Ortrun Rössler, author of this thesis.

2.2.2 Overview of the surgical techniques

Surgical treatment of colorectal cancer intends to remove the primary tumor as well as potential metastatic infiltrated colonic or rectal attachments like vessels (arteries and accompanying veins) as well as lymph nodes. Depending on the location of the primary tumor, different surgical techniques were performed respecting oncological standards [35]:

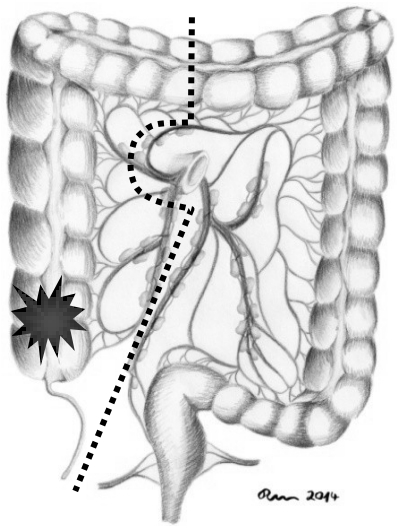

Figure 2 Resection line adjusted for tumors of the cecum and ascending colon

In case of tumor location at the appendix, cecum or ascending colon patients underwent right hemicolectomy as standard procedure. ***(Fig. 2)*** The proximal resection line was located about 20 to 30 cm proximal of the ileocaecal valve; the distal resection line was located in the middle of the transverse colon. Those parts of the large intestine were removed together with the ileocaecal artery, the right colic artery and the right pedicle of the middle colic artery. Further, the corresponding lymph nodes were removed en-bloc. After resection an end-to-side ileotransversostomy in a two-layer hand-sewn technique was performed. In selected cases of patients with tumors located at the cecum or near the ileocaecal valve, an ileocaecal-resection with consecutive ileoascendostomy was chosen, depending on age and comorbidities of the patients. [35]

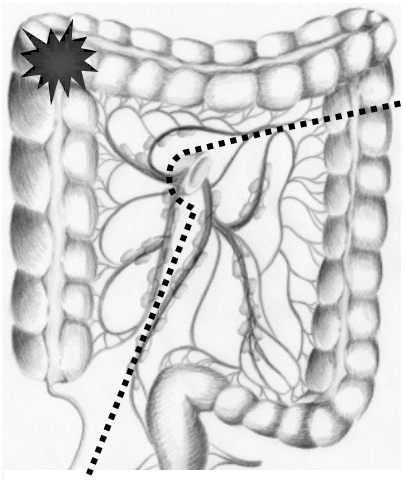

Figure 3 Resection line adjusted for tumors of the right colonic flexure

Tumors located at the right flexure or right transverse colon were resected by extended right hemicolectomy ***(Fig 3)*** including ligation of the ileocolic, right colic and middle colic artery including lymph-node-resection en-bloc. Also in this technique an end-to-side hand-sewn ileocolic anastomosis was performed. [35]

Figure 2 shows the resection line adjusted for a right hemicolectomy, Figure 3 the resection line adjusted for a patient undergoing extended right hemicolectomy. Figure 4 shows a transverse colon resection specimen.

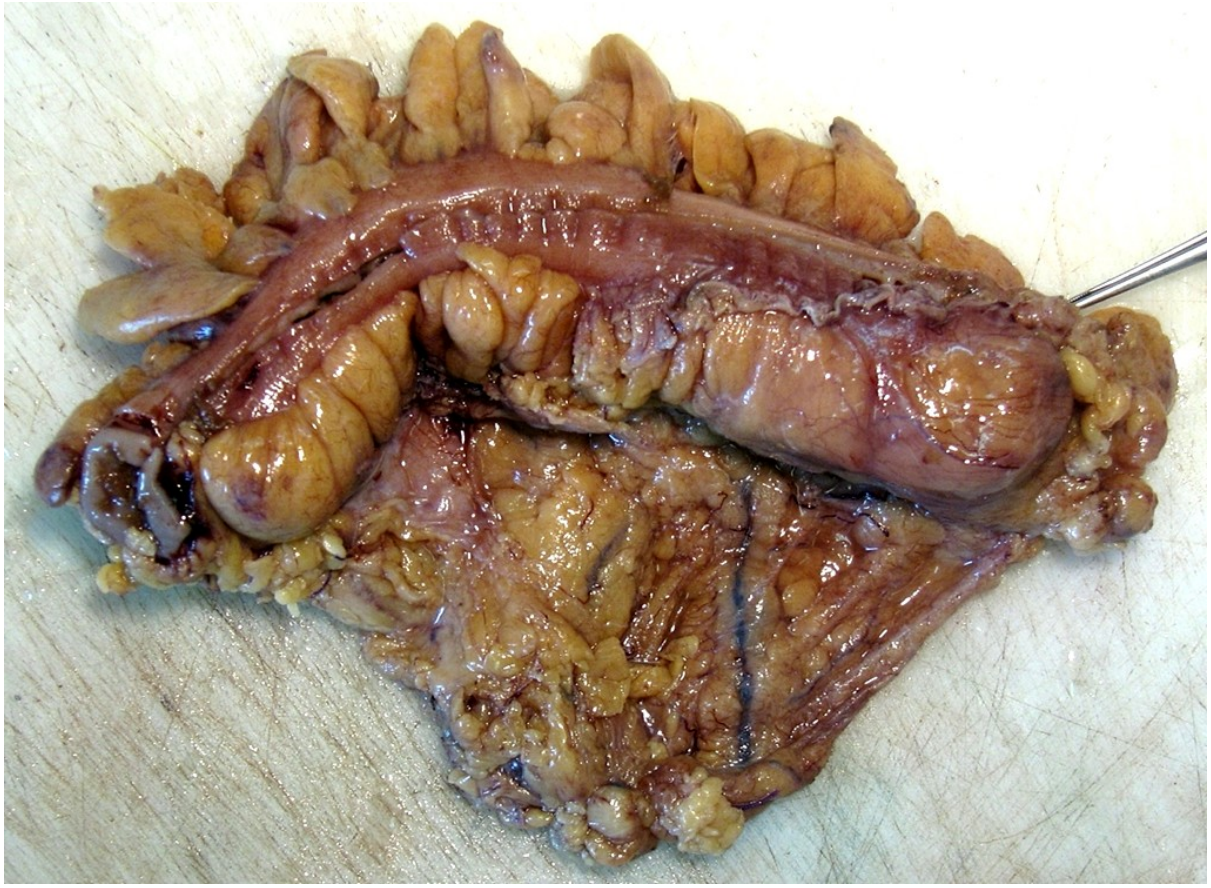

Figure 4 Resection specimen of a transverse colectomy.

Resection specimen is opened along the antimesenteric border. The transverse colon and the adjusted fatty tissue can be seen.

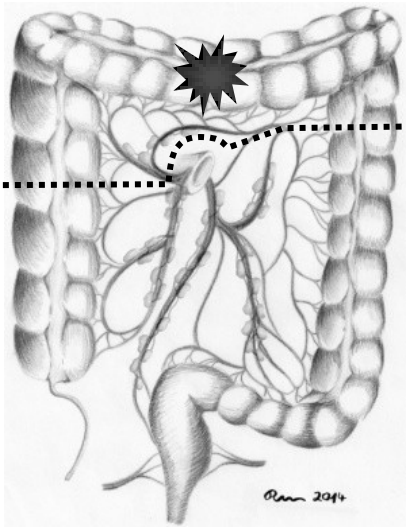

Figure 5 Resection line adjusted for tumors of the transverse colon

Resection of the transverse colon was conducted in presence of centric cancer species located at the transverse colon. ***(Fig 5)*** The resection lines were determined proximal of the right colonic flexure and distal of the left colonic flexure. The middle colic artery and the middle colic lymph nodes were removed en-bloc. [35]

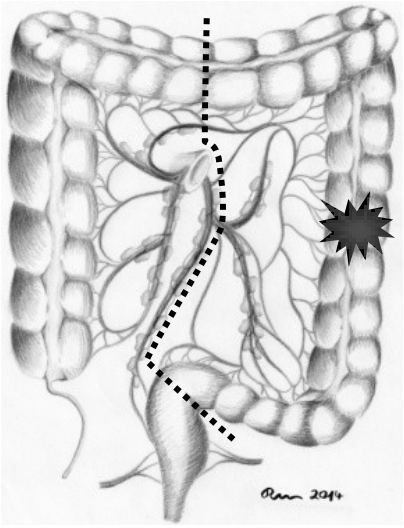

Figure 6 Resection line adjusted for tumors of the descending colon

Left hemicolectomy was performed in presence of cancer tissue located at the descending colon or sigmoid colon ***(Fig. 6)***. Therefore dissection of the inferior mesenteric artery and en-bloc lymph-node resection was performed.

In case of tumor location at the left transverse colon near the left colonic flexure, also the middle colic artery and lymph nodes were dissected, thereby performing an extended left hemicolectomy. ***(Fig. 7)*** In both techniques colorectal anastomosis was performed by transanally applied circular staplers. [35]

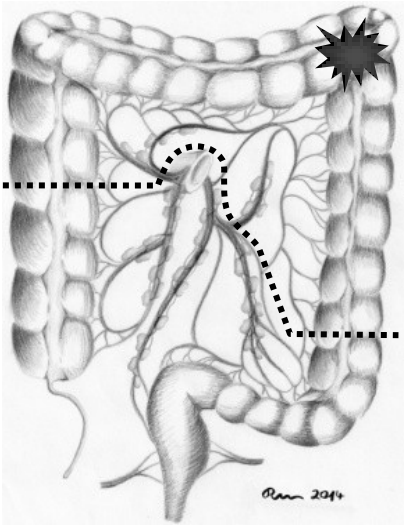

Figure 7 Resection line adjusted for tumors of the left colonic flexure

Anterior rectum resection was chosen in case of tumors located at the rectum or rectosigmoid junction. Tumors located in the middle or lower rectal third were resected by a total mesorectal excision (TME) and high ligation of the inferior mesenteric artery. For tumors located at the upper rectal third or at the rectosigmoid junction a partial mesorectal excision with a distal resection margin of 5 cm surrounded by mesorectal tissue was performed. Protective ileostomy or transversostomy was fashioned in case of coloanal anastomosis or in selected patients at the discretion of the surgeon. A colonic J-pouch was constructed in selected patients following TME. [35]

In case of very low tumor location or invasion of the levator- or sphincter muscle, abdominoperineal excision with creation of terminal colostomy was chosen as surgical technique. [35]

2.2.3 Study population

Several criteria were defined that disqualified 27 patients from the study population. Patients suffering from rectal cancer who besides from surgical treatment underwent neoadjuvant chemoradiation had to be excluded (n=24). This treatment is known to influence lymph node yield as well as lymph node size. [8] In fact, radiotherapy is suspected to cause apoptosis and nodal involution. [36,37] Further patients with multiple tumors in one resection specimen were excluded (n=3). In these cases tumor characteristics and lymph node parameters cannot be correlated. If tumors occur in two separate resection specimens of the same patient those lesions were called synchronous and were treated as individual specimen. [38] The final cohort contains 148 patients ***(Fig. 8)***, including 58 females and 90 males, with a ratio of 1:1.6 females to males ***(Fig. 9)***. Patients' age ranges from 36 to 92 years with am mean age of 68 ± 11.73 and a median age of 69 years ***(Fig. 10)***.

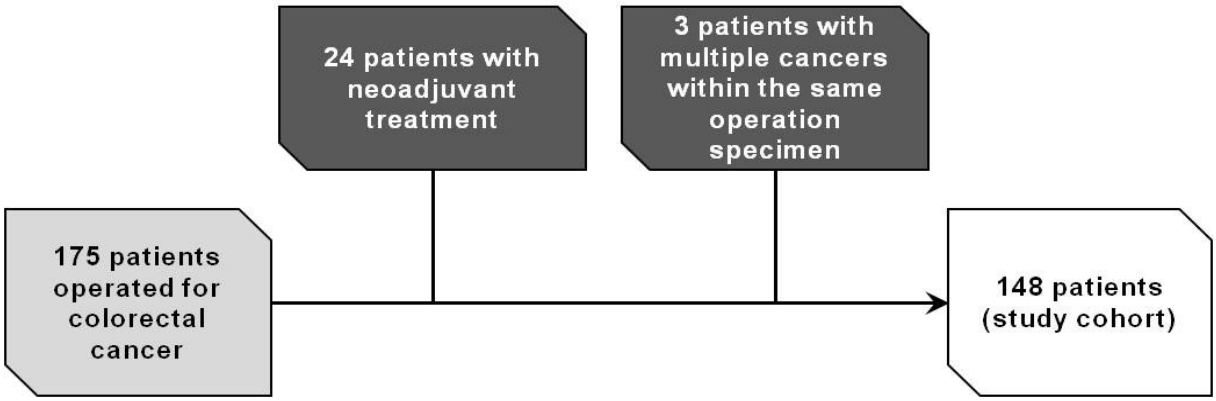

Figure 8 Graphic visualizing the patient selection process

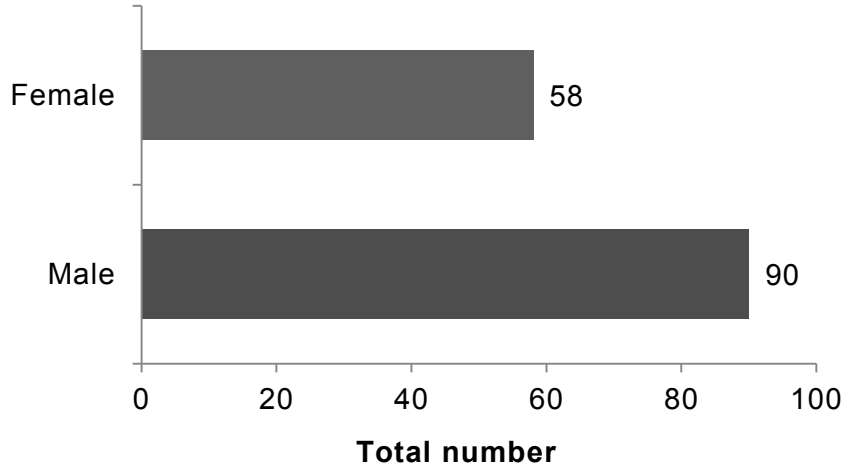

Figure 9 Distribution between the sexes

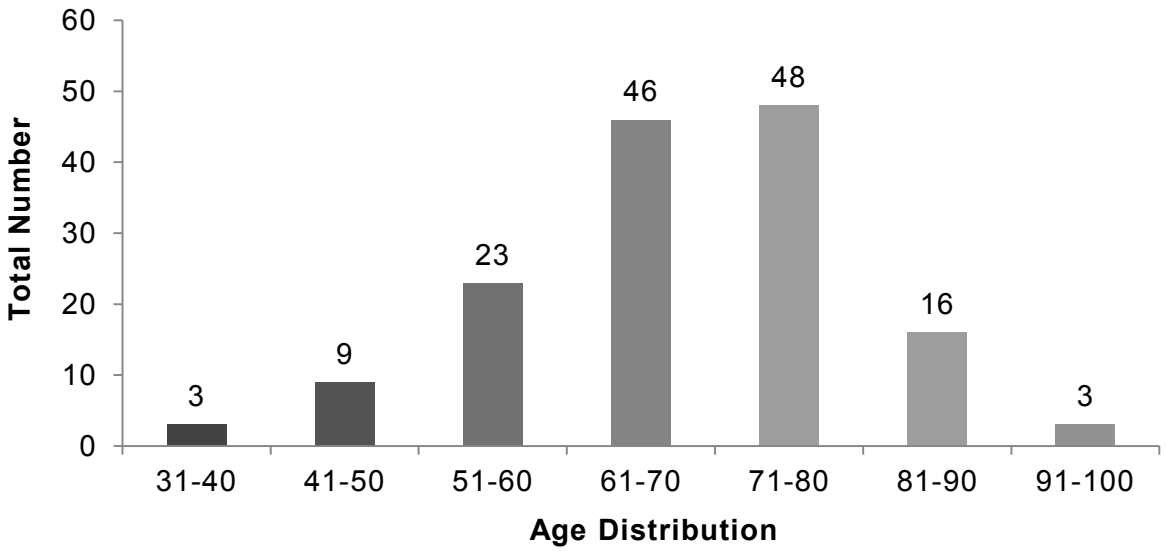

Figure 10 Distribution of age

2.3 Macroscopic evaluation of resection specimens

The resected specimen were opened along the antimesenteric border immediately after surgery, then submitted to 10% neutral buffered formalin and underwent fixation for a minimum of 24 hours. This guarantees optimal preparation for macroscopic evaluation, which was performed by a single pathologist Univ.-Doz. Dr.med. Cord Langner in 127 (86%) of the cases. All remaining specimen (n=21) underwent examination by different, well-experienced board-certified pathologists. This practice was chosen to control the parameters that might affect the quality of pathological lymph node yield.

2.3.1 Primary tumor characteristics

The primary tumor's maximum diameter was measured after fixation. Depth of penetration into the bowel wall was recorded. Thereby special attention was paid according to the coherence to the adjacent serosal surface. ***(Fig. 11)*** All of the primary tumors were consistently sliced into parallel section. Those sections were perpendicularly cut to the luminal surface. All tumors were embedded completely in order to leave no tumor tissue behind.

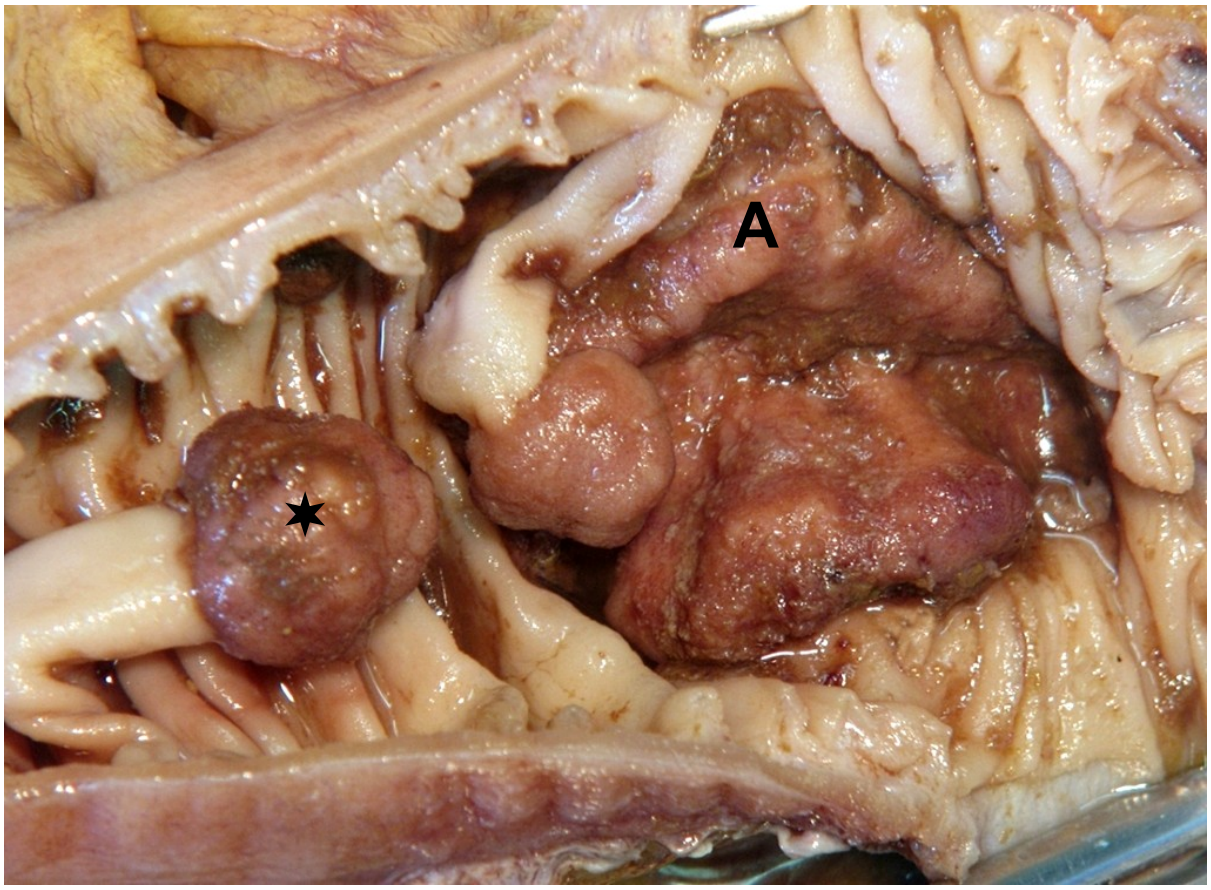

Figure 11 Resection specimen of a colorectal tumor

Specimen is opened, the primary tumor (A) and a socialized polyp (✶) can be seen.

Assessment of the primary tumors' location was conducted as follows: cancer located from cecum to transverse colon were defined as right-sided tumors, and cancer specimens located form the left colonic flexure to the rectosigmoid junction were defined as left-sided cancers. All cancers located within 16 cm distal of the anal verge were defined as rectal tumors. ***(Fig 12)***

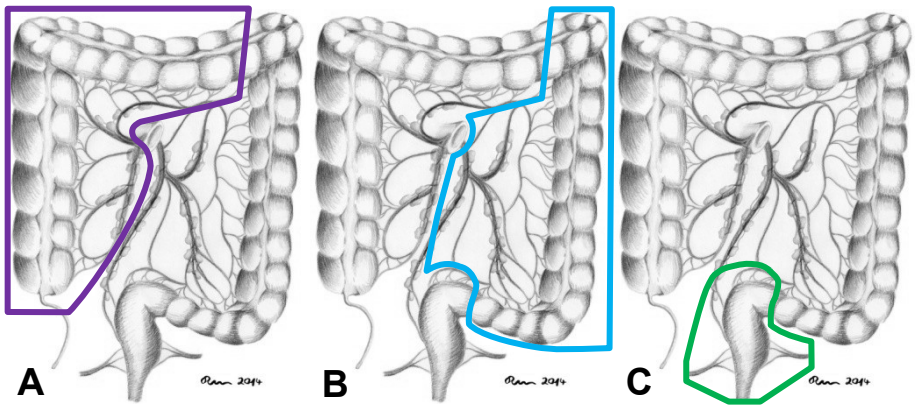

Figure 12 Scheme illustrating the definition of right sided, left sided and rectal tumors

A: Violet line: Right-sided tumors; B: Blue line: left-sided tumors; C: Green line: rectal tumors.

2.3.2 Lymph node assessment

A standardized protocol was used for manual dissection of the lymph nodes. More precisely, lymph node search was conducted by palpitation following the major vessels. In a next step, the mesenteric fatty tissue was sectioned transversely, visually evaluated and carefully palpated in order to detect further lymph nodes. ***(Fig. 13)*** It was decided not to use ancillary techniques in this study. These techniques included methylene blue injection, as well as fat clearing methods like acetone elution of the tissue with our without fat compression. [8]

Figure 13 Graphic shows the manual dissection technique of the fatty tissue Transverse sectioning of the fatty tissue in order to detect lymph nodes

2.4 Histology

For histological examination, paraffin embedded tumors and lymph node tissue were cut into 2 µm thick sections. Then they were stained with hematoxylin and eosin (H&E) for histological differentiation of the various cell types. ***(Fig. 14)***

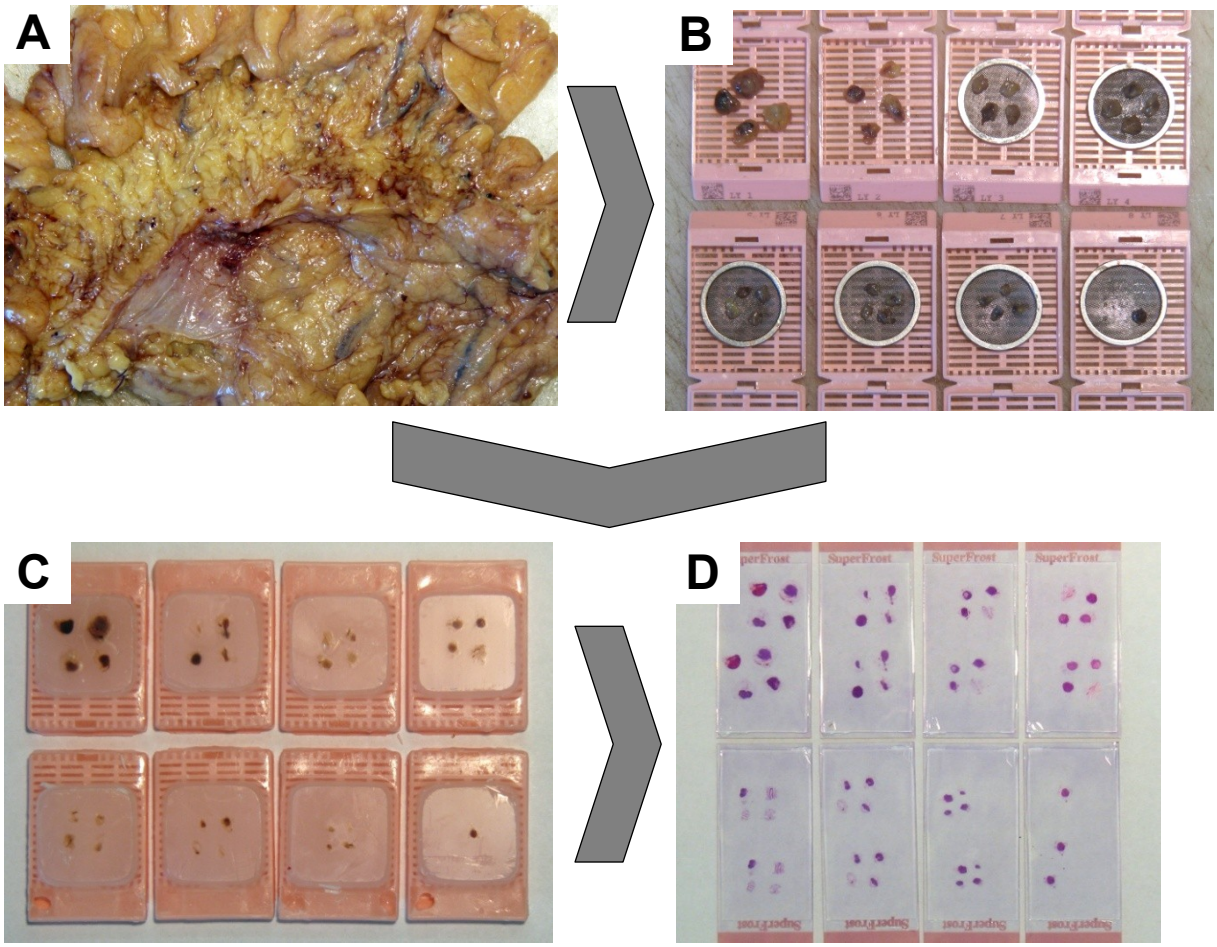

Figure 14 Graphic visualizing the process of lymph node examination

A: Mesenteric fatty tissue, lymph nodes are extracted from. B: Lymph node samples encapsulate in order to get embedded. C: Lymph nodes embedded in paraffin, blocks are prepared to get sliced. D: Sliced and H&E stained lymph node samples.

2.4.1 Lymph node characteristics

The maximum lymph node diameter was measured for each node from the original histological slides. Measurement was performed with a ruler and size of the nodes was noted in millimeters, expressed to the nearest 5 mm, proceeding similarly to the method of Murphy and Scott [21,36]. To reduce observational error, the measurement was carried out by one single investigator, Cand.med. Ortrun Rössler, only.

Microscopic analysis for metastatic infiltration of all nodes was performed by Univ.-Doz. Dr.med. Cord Langner, and included assessment of at least two lymph node levels. Assessment of N-status was applied according to the 7th edition of the AJCC/UICC TNM classification [9]. Following this classification system N0 stage is classified as no metastatic cancer spread can be found in regional lymph nodes. N1a and N1b stage is classified as metastatic cancer spread occurring in one regional lymph node and two to three regional lymph nodes, respectively. ***(Fig 15)*** N1c stage is classified as tumor cell satellites occurring in the surrounding tissue of the colon, but not appearing as lymph nodes (no capsule, no sinus, and no residual lymphoid tissue). Thus, this stage is defined lymph node negative. N2a and N2b stage is classified as metastatic cancer spread occurring in four to six and seven or more lymph nodes, respectively.

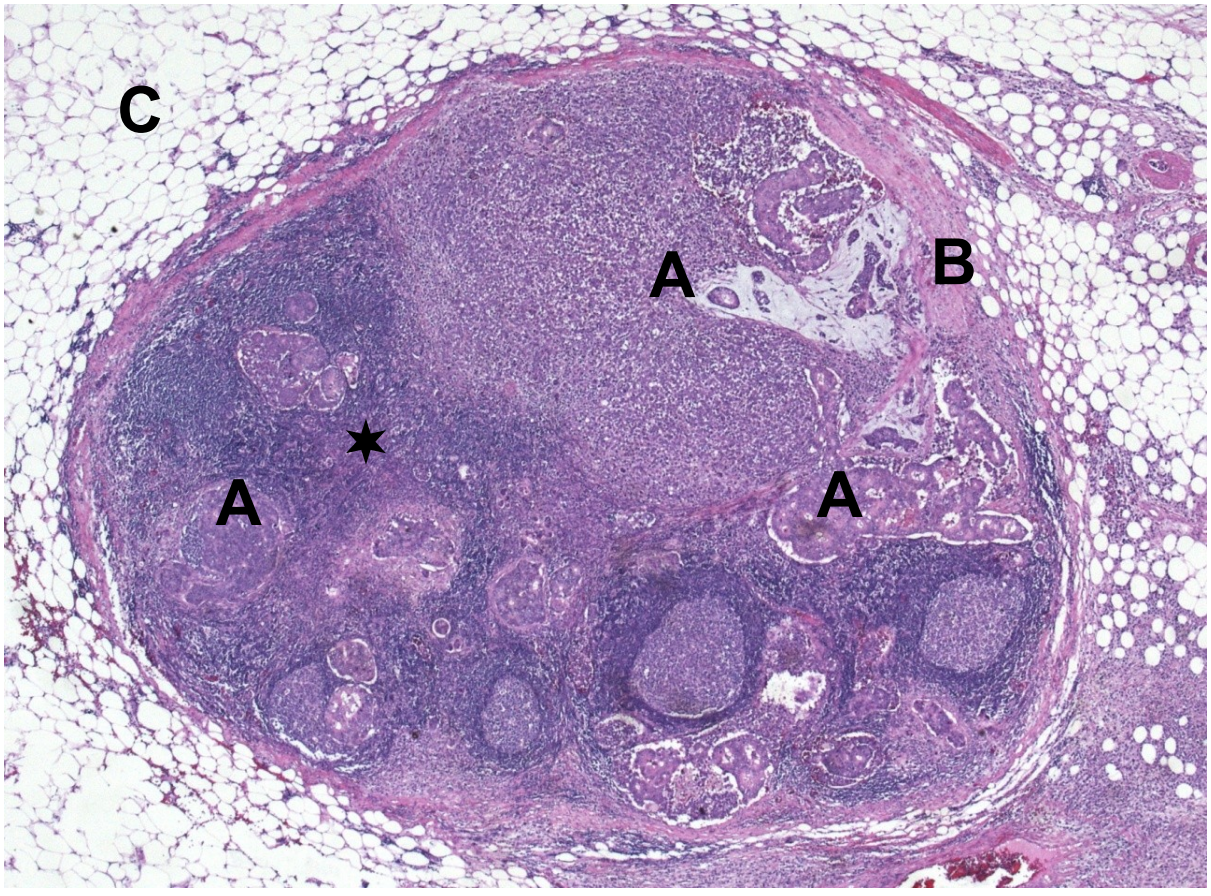

Figure 15 Graphic illustrating a metastatic infiltrated lymph node

A: Metastatic cancer spread replacing the normal lymph node tissue (✶ dark blue spots, formed up by lymphocytic cells). B: Lymph node capsule; the sinus beyond the capsule cannot be seen. C: Surrounding fatty tissue.

2.4.2 Primary tumor characteristics

All primary tumors were histologically evaluated by one single examiner, Univ.-Doz. Dr.med. Cord Langner, as a board certified pathologist and experienced expert in the field of gastrointestinal pathology. The examiner was blinded to lymph node size.

2.4.2.a Tumor stage

Assessment of tumor stage was performed according to the 7th edition of the AJCC/UICC TNM classification. [9] Following this classification system T1 stage is classified as the tumor invading the submucosa. T2 stage is classified as the tumor invading the muscularis propria. T3 stage is classified as the tumor invading the subserosa or beyond (e.g. tissues surrounding the colon or the rectum) but without involvement of other surrounding organs. T4 stage is classified as the tumor perforating the peritoneum (T4a) or invading the adjacent organs (T4b).

After classification of the T and the N status, the collected parameters were combined to form the AJCC-stage [10]. In the study population, there was no patient with tumor stage IV according to AJCC/UICC. The detailed classification of the AJCC/UICC stages is shown in Table 1.

Table 1 Comparison of the AJCC/ UICC Stage [10] with the TNM Stage [9]

AJCC stage	TNM stage
Stage 0	Tis N0 M0
Stage I	T1 N0 M0
Stage I	T2 N0 M0
Stage II-A	T3 N0 M0
Stage II-B	T4 N0 M0
Stage III-A	T1-2 N1 M0
Stage III-B	T3-4 N1 M0
Stage III-C	any T, N2 M0
Stage IV	any T, any N, M1

2.4.2.b Tumor grade

All tumors were graded according to the current WHO guidelines [10,39]. Therefore, assessment of the extent of the tumor tissue's glandular appearance was applied. Following these guidelines grade 1 (well differentiated) is given when glandular structures in more than 95% of the tumor are exhibited ***(Fig 16)***. Grade 2 (moderately differentiated) is given when glandular structures in 50% - 95% of the tumor are exhibited ***(Fig 17a)***. Grade 3 (poorly differentiated) is given when glandular structures in 5% - 50% of the tumor are exhibited ***(Fig 17b)***. Grade 4 (undifferentiated) is given when glandular structures in less than 5% of the tumor are exhibited (not shown).

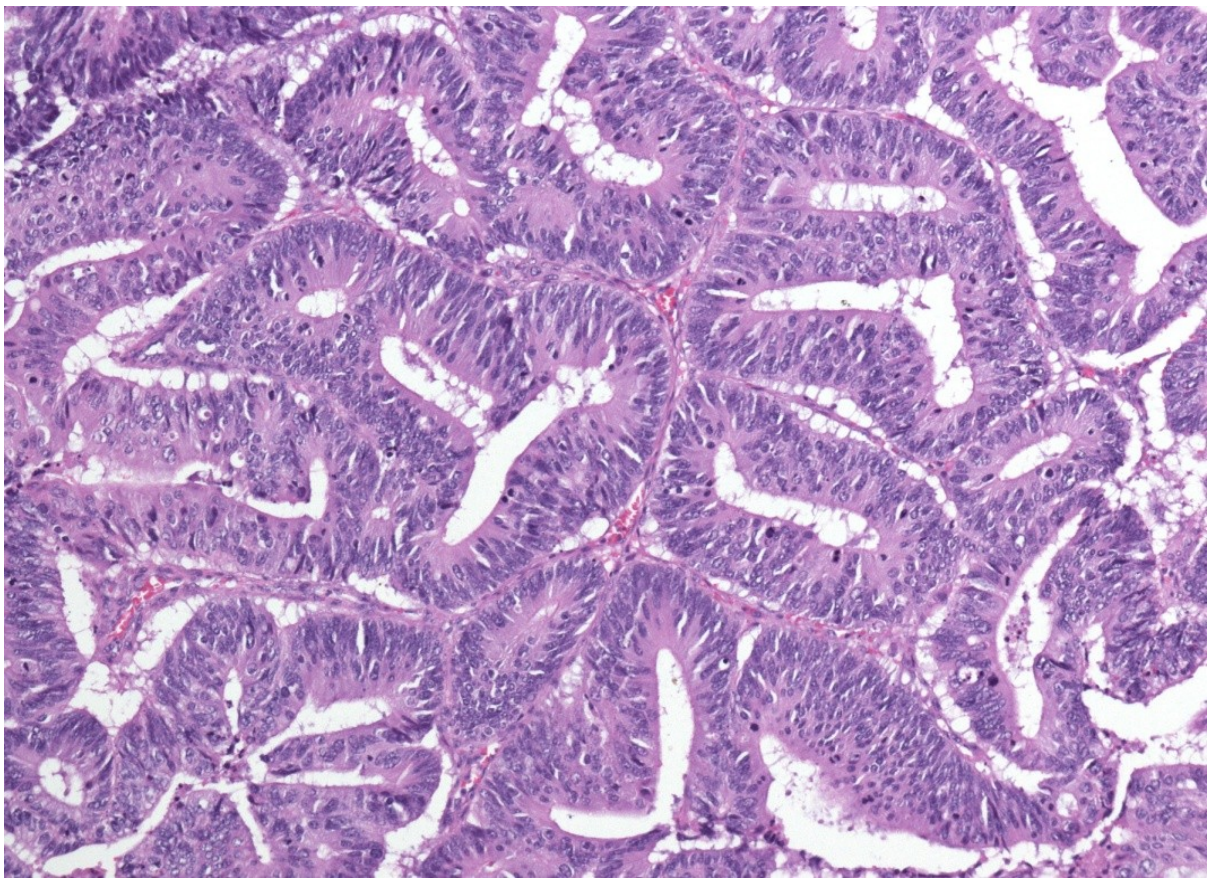

Figure 16 Graphic describing the G1 tumor grade according to the WHO classification [10,39]

More than 95% glandular structures are exhibited; the tumor is classified as well differentiated.

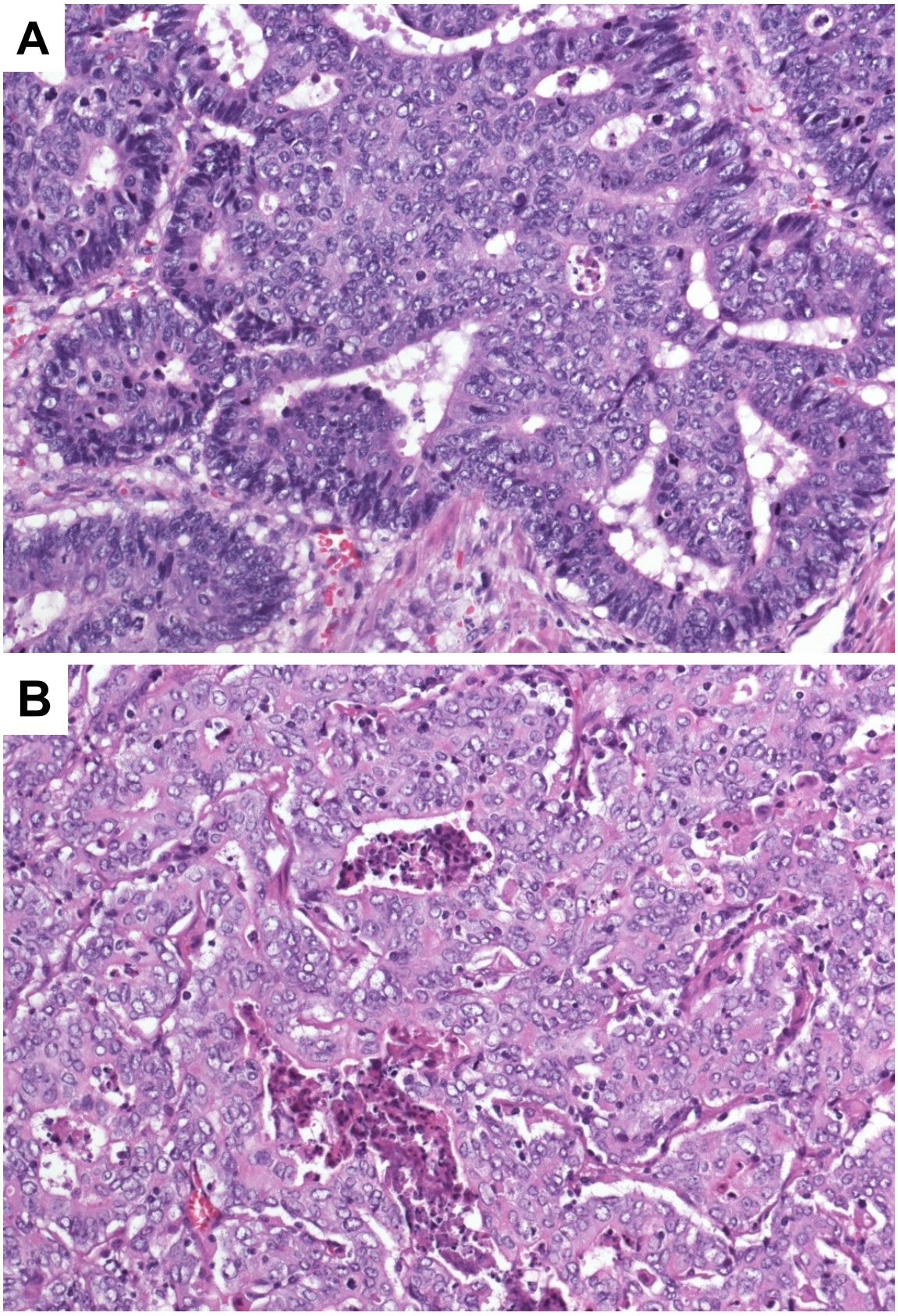

Figure 17 Graphic describing the G2 and G3 tumor grade according to the WHO classification [10,39]

A: Between 50% - 95% glandular structures are exhibited; the tumor is classified as moderately differentiated. B: Between 5% - 50% glandular structures are exhibited; the tumor is classified as poorly differentiated.

2.4.2.c Tumor necrosis

The extent of histological tumor necrosis was evaluated. Therefore the method recently introduced by Pollheimer et al. [40] was used. The assessment of extend of tumor necrosis was performed semiquantitatively at low magnification (x40). ***(Fig 18)*** The scoring system was based on the histological evaluation of every available tumor block. Tumor necrosis was recorded as absent (score 0) when no necrosis was identified, focal (score 1) when less than 10% of the tumor area was necrotic, moderate (score 2) when 10-30% of the tumor area can be identified as necrotic, or extensive (score 3) when more than 30% of the tumor area can be described as necrotic.

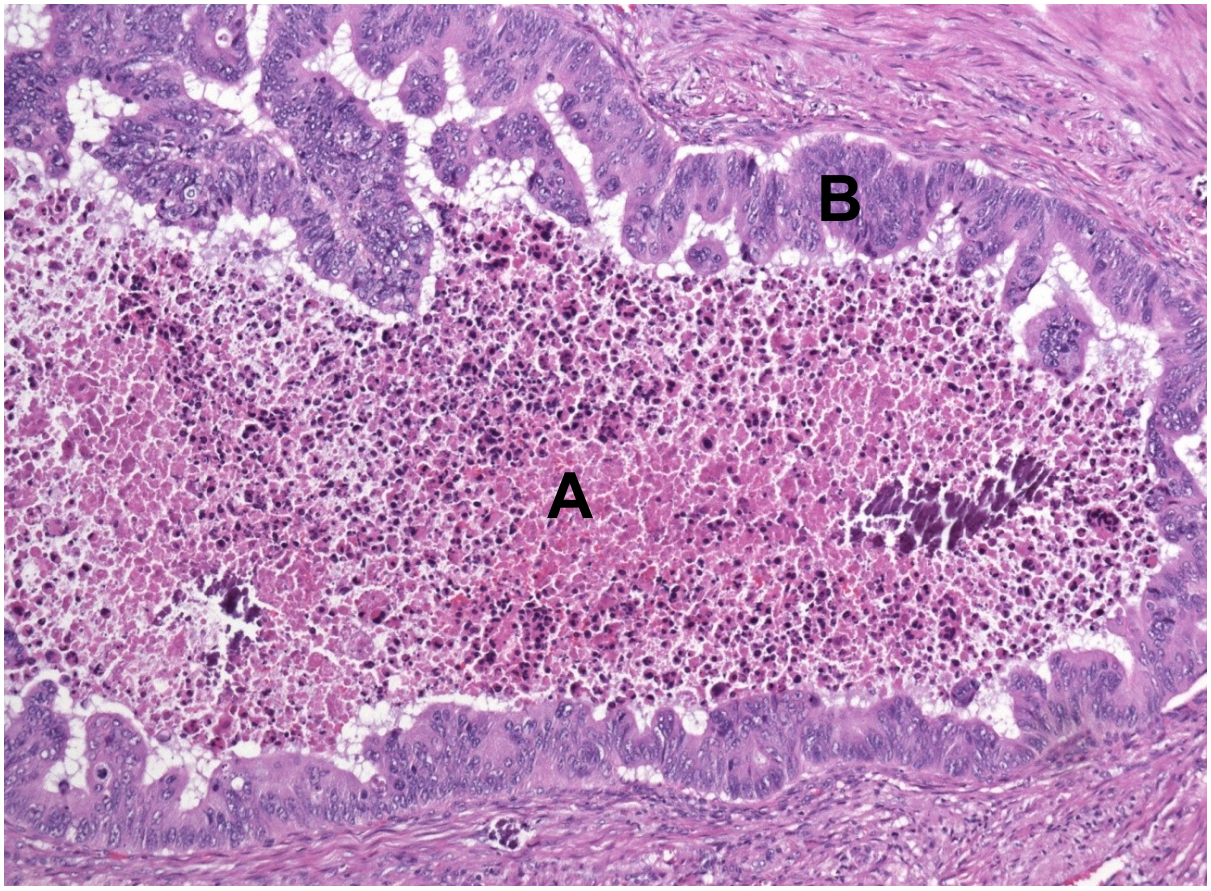

Figure 18 Graphic describing tumor necrosis

Tumor necrosis was defined according to Pollheimer et al. [40] A: Tumor necrosis: detritus (pink) and neutrophilic granulocytes (dark blue dots within the pink areas) can be seen. B: Tumor tissue (violet) is surrounding the necrotic area.

2.4.2.d Inflammatory cell reaction

Special attention was paid to the inflammatory cell reaction of each tumor. Assessment of the reaction was performed by looking at the invasive margin of the tumor and the central area. Estimation of the reaction was performed by using the four-degree scale which was introduced by Klintrup et al. [41] They gave a score of 0 when no increase of inflammatory cells was seen in the tumor. A score of 1 was denoted when they detected a mild and patchy increase of the inflammatory cells at the invasive margin without destruction of invading cancer cell islets by the inflammatory cells. A score of 2 was given when the inflammatory cells formed a band-like structure at the invasive margin, thereby destroying some of the cancer cell islets; a score of 3 was given by detection of a very prominent inflammatory reaction, building cup-like zones at the invasive margin with frequent and invariably present destruction of cancer cell islets. ***(Fig 19)***

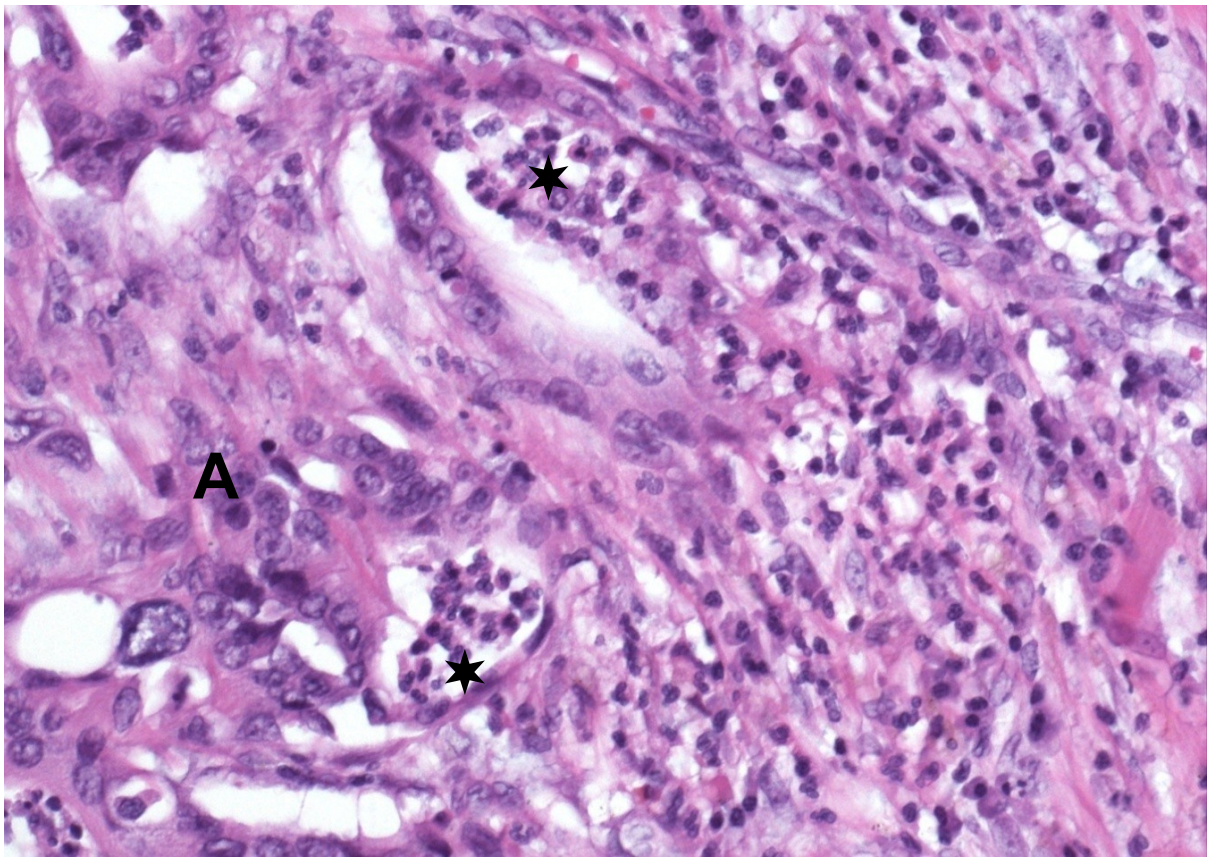

Figure 19 Graphic illustrating the granulocytic inflammatory cell reaction in tumor tissue

Cell reaction was defined according to Klintrup et al. [41]; inflammatory cell reaction was classified as neutrophilic lymphocytes (✶) invading the tumor tissue (A).

2.4.2.e Lymphocytic anti-tumor reaction

In addition, the lymphocytic anti-tumor reaction ***(Fig 21)*** was investigated more detailed by sub classifying the anti-tumor reaction into four components. Therefore the method according to Ogino et al. [42] was applied by paying special attention to Crohn's-like lymphoid reaction, peritumoral lymphocytic reaction, intratumoral periglandular reaction, and tumor-infiltrating lymphocytes (TIL). This scoring system was performed on every tumor. Each of the reaction components was scored as 0 (=absent), 1+ (=mild), 2+ (=moderate), or 3+ (=marked). A total reaction score was achieved by summing up the scores given to each compartment, thereby reaching a score between 0-12 points for each tumor. Similar to Ogino et al. [42] the data showed a skewed distribution of the lymphocytic scores ***(Fig. 20)***. Further, the majority of cases laid in the categories of scores 0 to 2 with the majority of cases achieving a score of 2. Confronted with this problem, creating the score categories was conducted as introduced by Ogino et al. [42] Thus, the reference group (i.e., low score) was set as those with scores 0 to 2, so providing a robust reference group. In addition, non-low-score cases were divided into two groups because of a wide range of scores from 3 to 12. The cutoff between middle and high groups was set as ≤6 versus ≥7 because this lay in the middle of the total score of 12.

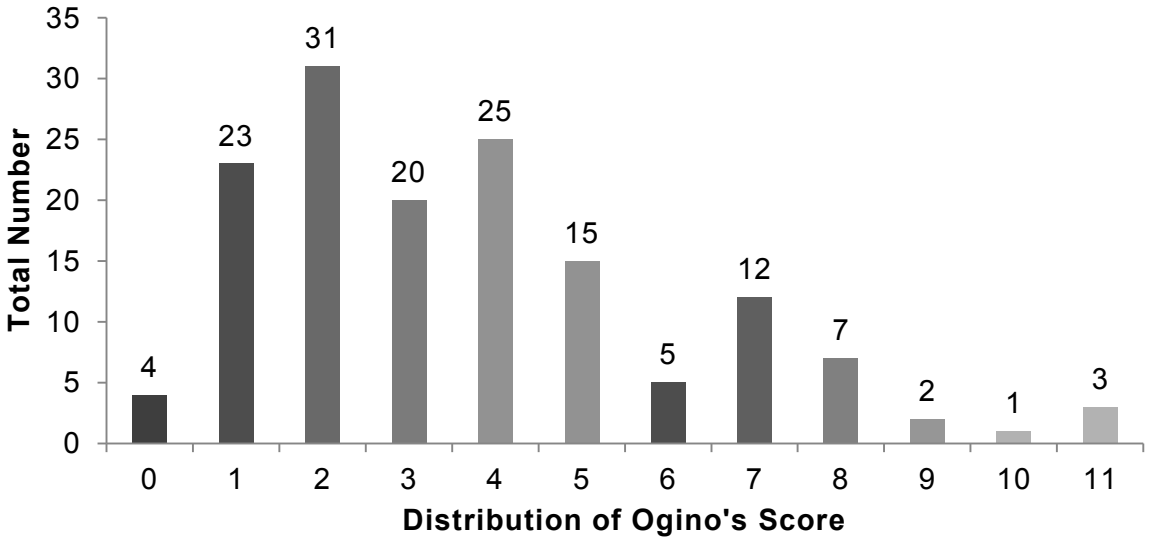

Figure 20 Distribution of Ogino's score

Within the categories 0-2 in all 58 cases were described. None of the examined tumors achieved a full score of 12 in this study cohort.

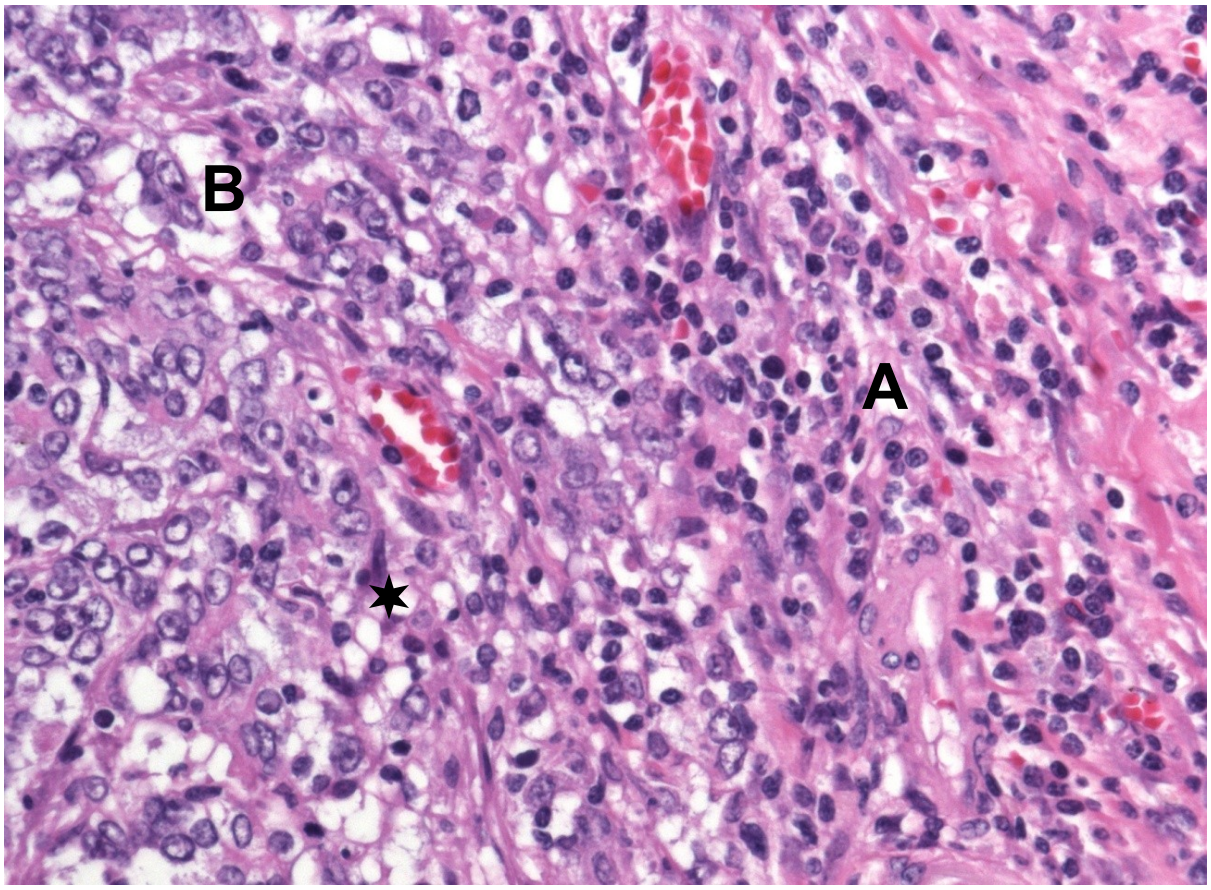

Figure 21 Graphic illustrating lymphocytic anti tumor reaction

Lymphocytic anti tumor reaction was defined according to Ogino et al. [42] Lymphocytes (A) are infiltrating the tumor tissue (B) from its border. Tumor-infiltrating lymphocytes (✶) and peritumoral lymphocytic reaction (A) can be seen in this picture.

2.4.2.f Tumor budding

A closer look was also given to tumor budding as it is known to be frequently accompanied by dense peritumoral lymphocytic inflammation. [43] Occurrence of one single tumor cell or a tumor cell cluster up to 5 cells at the invasive front of the colorectal cancer is defined as tumor budding. ***(Fig 22)*** Tumor budding clusters were assessed on H&E slides. Tumor budding was classified as either low or high. Low tumor budding was classified as ≤ 6 tumor budding loci, high tumor budding was classified as > 6 tumor budding loci.

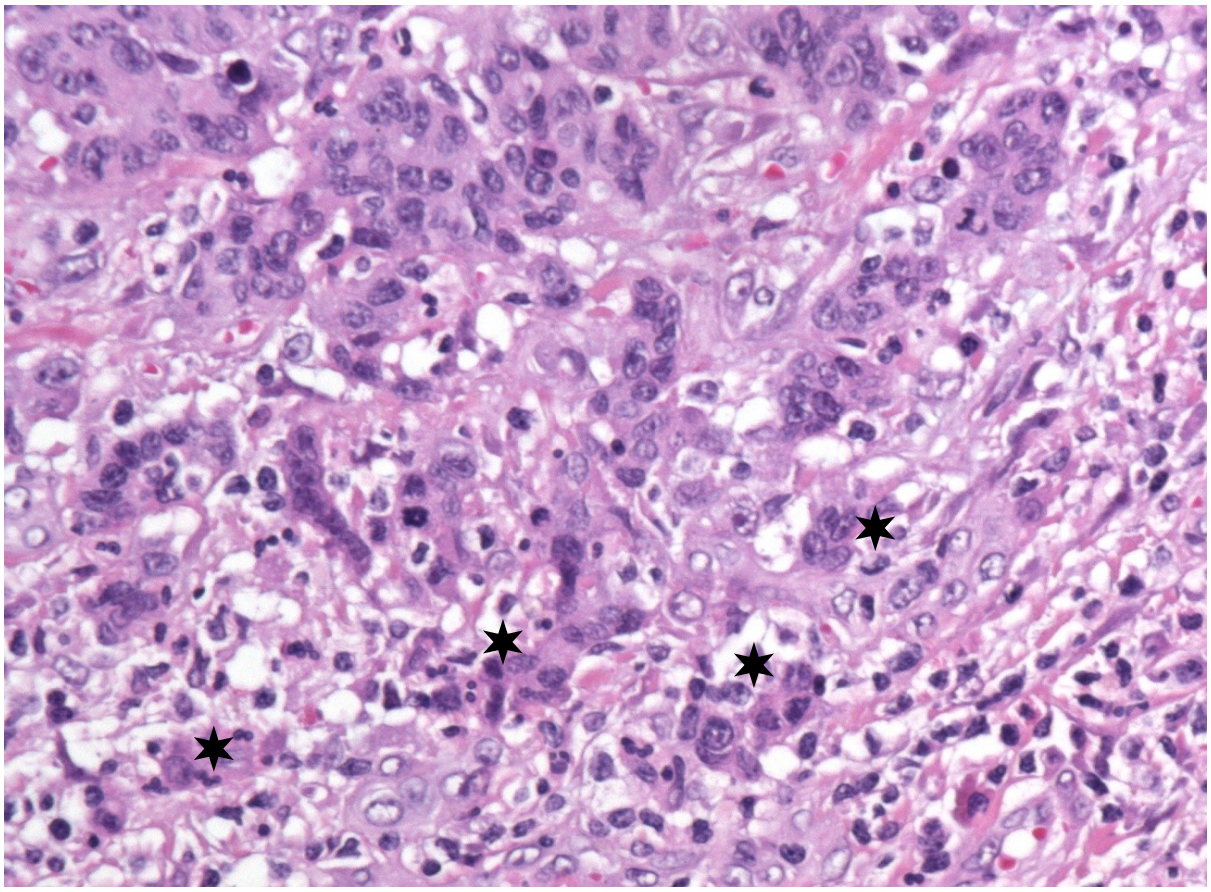

Figure 22 Graphic illustrating tumor budding

✶Several tumor budding foci can be seen at the invasive front of the tumor.

2.5 Immunohistochemistry

For immunohistochemical analysis, four different monoclonal antibodies were used in this study. All of them responded to the presence of several DNA mismatch repair (MMR) proteins. The antibodies were directed to MLH1, MSH2, MSH6 and PMS2 (for details see Table 2).

Table 2 List of used antibodies, dilution factors and manufacturing companies for detecting MMR in tumors

Mismatch repair protein	Monoclonal antibody type	Dilution factor	Manufacturing company
MLH1	clone G168-15	1:50	Biocare, Concorde, CA, USA
MSH2	clone G219-1129	ready to use	Ventana, Tucson, AZ, USA
MSH6	clone BC-44	1:50	Biocare, Concorde, CA, USA
PMS2	clone MRQ-28	1:50	Cell Marque, Rocklin, CA, USA

Assessment of primary antibody binding was performed either by the Dako EnVision+ (Dako, Glostrup, Denmark) or the UltraView DAB 760-500 (Ventana) detection kit. Diaminobenzidine (DAB) was used to act as chromogen. As positive control, intratumoral lymphocytes were used. For negative controls, the incubation with antibody diluents and the primary antibodies were omitted.

A tumor was only denominated as MMR-proficient when immunoreactivity of all four MMR (MLH1, MSH2, MSH6, and PSM2) was detected. Loss of immunoreactivity of a single MMR characterized the tumor as MMR-deficient ***(Fig. 22)***. In all MMR-deficient cancer specimens, molecular tests were performed to detect microsatellite instability (MSI).

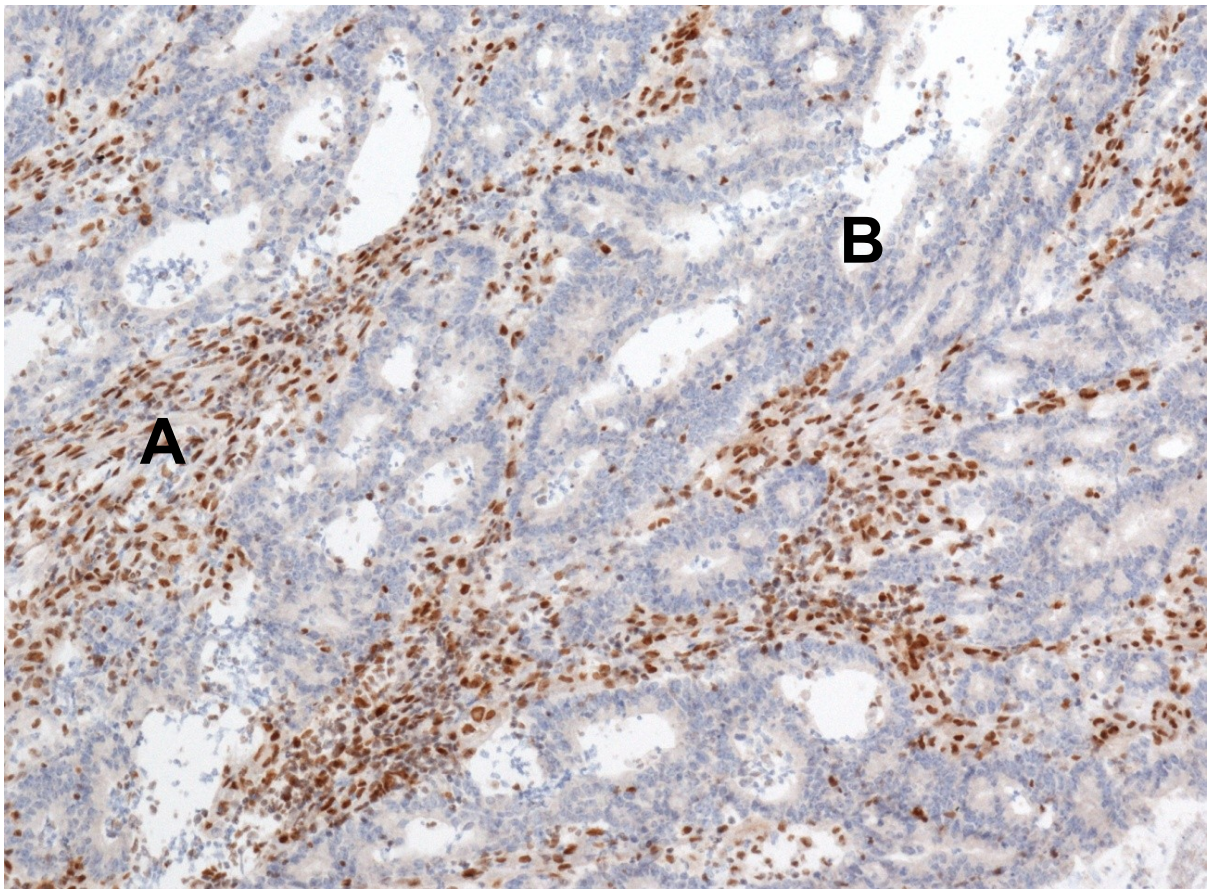

Figure 23 Graphic visualizing the loss of MLH1 MMR protein.

A: Expression of MLH1 MMR Protein in the extratumoral tissue. B: Loss of nuclear mismatch repair protein expression in the tumor tissue (inflammatory cells serving as internal positive control).

2.6 Molecular analysis

Extraction of DNA was conducted from formalin-fixed and paraffin-embedded tissue. Therefore, the Qiagen QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) was used. Investigation of MSI was performed by use of the Promega Microsatellite Analysis System version 1.2 (Promega, Mannheim, Germany). This PCR multiplex system uses five mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24, MONO-27) for MSI determination. For internal control, two pentanucleotide markers (Penta C and Penta D) were used. Separation of polymerase chain reaction (PCR) products was performed by capillary electrophoresis. Therefore, the ABI Prism 3100 genetic analyzer (Applied Biosystems, Vienna, Austria) was used. In case, MSI was found at two mononucleotide loci the tumor was counted as MSI-high. If MSI was found at only one locus, the tumor was counted as MSI-low. When not instability was found at any locus at all, the tumor was counted as microsatellite stable (MSS).

2.7 Statistical analysis

Categorical variables are reported by absolute and relative frequencies, numerical variables by means +/- standard deviation, medians and ranges. For the examination of differences in the categorical variables Fisher's exact test or the χ^2 test, as appropriate, were used. Examination of differences in numerical variables was performed using a Student's t-test. Identification of the threshold providing the most precise discrimination between lymph node positive and negative cancer specimen, receiver-operator characteristic (ROC) curve was conducted. Discrimination referred to highest sum of sensitivity and specificity. Primary tumor characteristics and their influence on lymph node size were assessed by binary logistic regression. Assessment of goodness of fit of the model was controlled by Hosmer-Lemeshow-test. These results are reported as 95% confidence intervals and adjusted hazard rations. SPSS statistics 20 (IBM, Armonk, New York, USA) was used to perform statistical analyses. A two-sided significance level of $\alpha=0.05$ was used for all statistical analyses. Statistical analysis was conducted by Dr.med.univ. Lars Harbaum.

3 Results

3.1 Tumor characteristics

Tumor location was distributed as follows: 18 (12%) tumors were found in the cecum, 24 (16%) tumors appeared in the ascending colon, 14 (9%) tumors were collected from the hepatic flexure, 6 (4%) tumors were found in the transverse colon, 3 (2%) tumors appeared at the splenic flexure, 6 (4%) tumors were collected from the descending colon, 42 (28%) tumors were found in the sigmoid colon, 11 (7%) tumors appeared at the rectosigmoid junction, and 24 (16%) tumors were collected from the rectum. To summarize these findings by using the formerly introduced definition, 62 (42%) right-sided cancer specimens, 62 (42%) left-sided cancer specimens, as well as 24 (16%) rectal cancer specimens were collected. Those data are summarized in Table 3.

Table 3 Distribution of tumor appearance along the large intestine

Location	Total Number	Category Percentage	Category	Overall Percentage
Cecum	18	12%	Right sided (n=62)	42%
Ascending Colon	24	16%		
Hepatic flexure	14	9%		
transverse Colon	6	4%		
Splenic flexure	3	2%	Left sided (n=42)	42%
Descending Colon	6	4%		
Sigmoid colon	42	28%		
Rectosigmoid junction	11	7%		
Rectum	24	16%	Rectum (n=24)	16%

The mean tumor size was 3.8 ± 2.09 cm, with a median tumor size of 4 cm and a range from 0.5 to 17 cm.

Overall, four different histological subtypes were found in this study. The majority of the tumors were classified as adenocarcinomas (n=127). In 27 cases, the tumors were classified as mucinous adenocarcinomas. At least three tumors were classified as signet-ring cell carcinoma and even two carcinomas received the classification of a micropapillary adenocarcinoma. Data are presented in Figure 24.

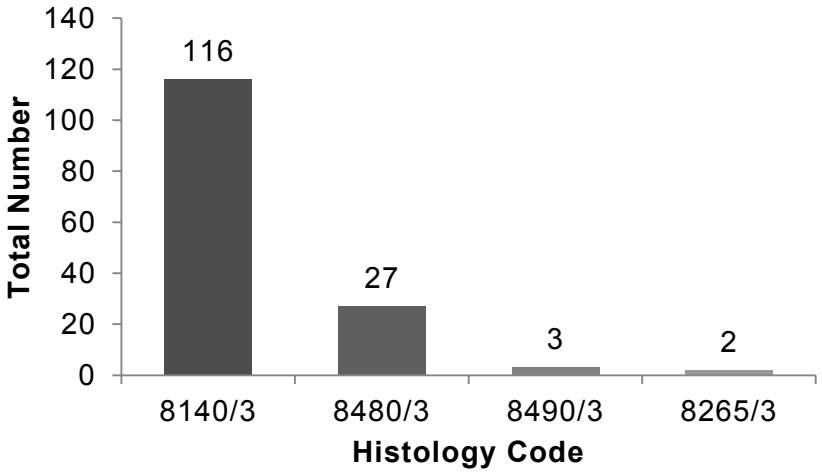

Figure 24 Distribution of the histological subtypes detected in this study

Translation of the codes:

- **8140/3 - adenocarcinoma**
- **8480/3 - mucinous adenocarcinoma**
- **8490/3 - signet ring cell carcinoma**
- **8265/3 - micropapillary adenocarcinoma**

T category was distributed as follows: T1 classification contains 15 (10%) tumors, T2 classification was applied to 24 (16%) tumors, T3 classification was given to 68 (46%) tumors, and T4 classification was used for 41 (28%) tumors. For details, see Table 4.

Tumor grades were distributed as follows: 38 (26%) of all tumors were recorded as G1, 60 (41%) of the tumors were recorded as G2 and 50 (34%) of all cases were recorded as G3. ***(Fig. 25)***

Table 4 Detailed distribution of all T categories

T Classification	Subcategory Number	Total Number	Total Percentage
T1	15	15	10%
T2	24	24	16%
T3a	26	68	46%
T3b	29		
T3c	11		
T3d	2		
T4a	31	41	28%
T4b	10		

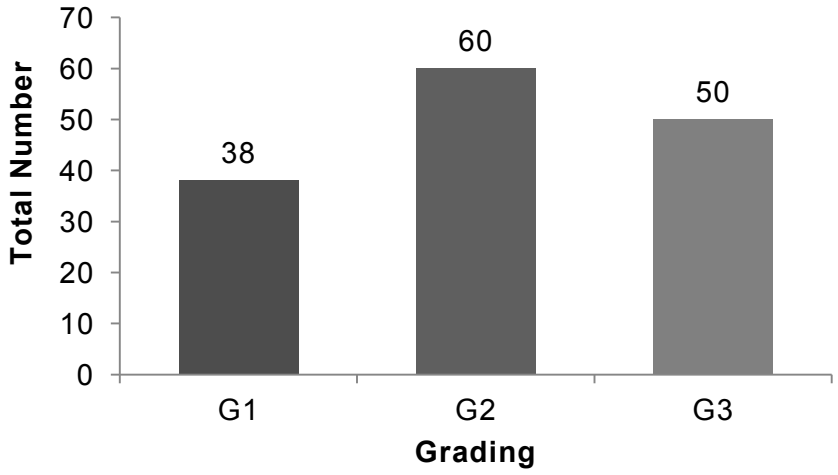

Figure 25 Distribution of tumor grading

Tumor budding was distributed as follows: 88 (59%) of all tumors were classified as low budding and the remaining 60 (41%) tumors were classified as high budding. Data are presented in Figure 26.

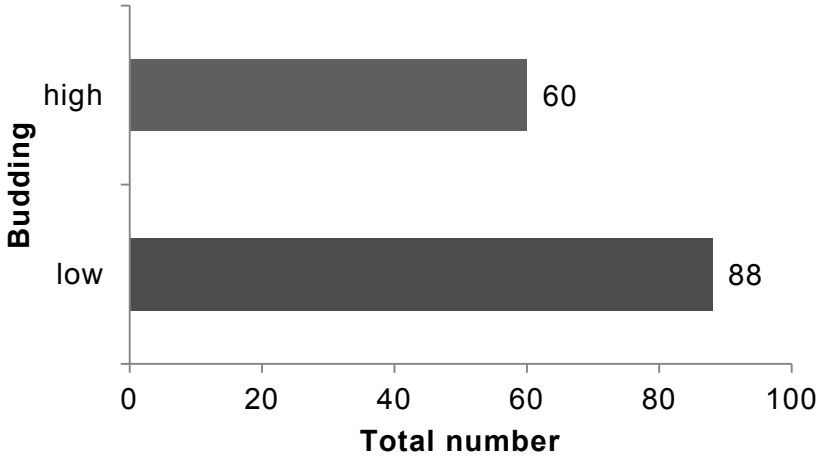

Figure 26 Distribution of tumor budding

In 61 (41%) of all cases metastatic cancer spread in lymph nodes was detected. Distribution of the N1 and N2 category was carried out as follows: in 15 (10%) cases, lymph nodes were classified as N1a or N1b. In 13 (9%) cases, lymph nodes were classified as N2a or N2b. For details, see Table 5. The mean number of positive nodes was 5.3 ± 5.05, with a median number of 3 and a range from 1 to 32 cm.

Table 5 Distribution of the N categories

	N-Status	Category Number	Total Number	Overall Percentage
Counted as nodal negative	N0	86	87	58%
	N1c	1		
Counted as nodal positive	N1a	18	62	42%
	N1b	15		
	N2a	15		
	N2b	13		

3.2 Lymph node size is related to presence of lymph node metastasis

The dedicated lymph node search generated 4167 lymph nodes in 148 cases. This leads to a mean lymph node count of 28.2 ± 11.06 nodes per case, with a median count of 26 and a range between 9 - 67 nodes. Recovery of 12 or more nodes was achieved in 145 (98%) cases. The mean size of all lymph nodes was 3.79 ± 0.84 mm, with a median size of 3 and a range between 1 - 24 millimeters. Details are presented in Figure 27.

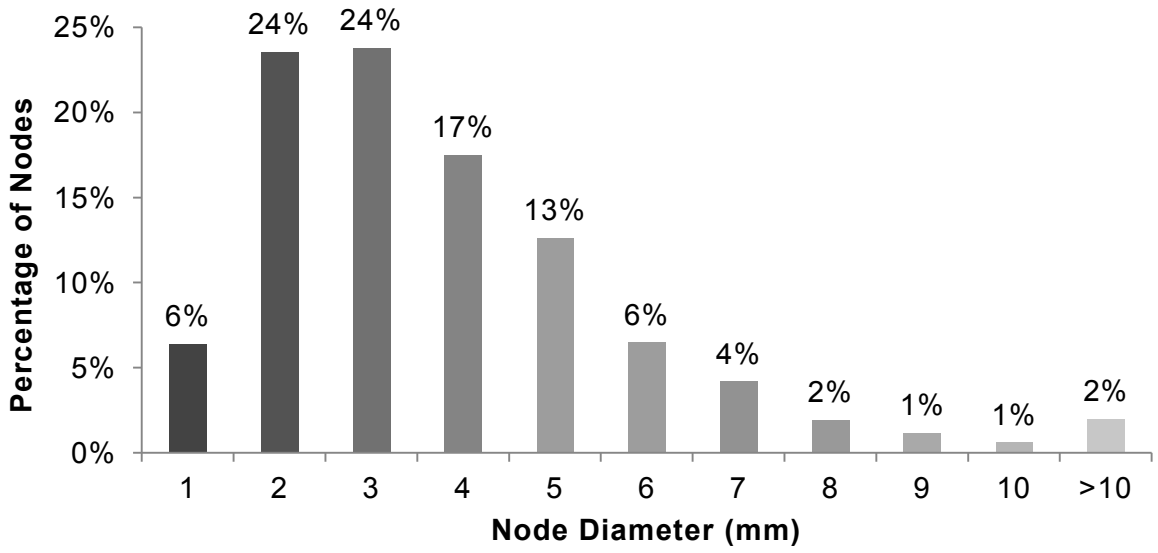

Figure 27 Size distribution of all collected lymph nodes

Interestingly, right-sided tumors tend to harbor larger lymph nodes than left-sided tumors with a mean lymph node size of 4.16 ± 0.76 mm to 3.53 ± 0.80 mm ($p<0.001$, t-test), respectively.

In 320 (8%) of all lymph nodes metastatic cancer spread was found. Positive nodes tend to be larger with a mean size of 5.63 ± 1.92 mm, whereas negative nodes showed a mean size of 3.6 ± 0.84 mm ($p<0.001$, t-test) ***(Fig. 28A)***. Furthermore, N-positive tumors showed larger lymph nodes than N-negative tumors, with a mean size of 4.01 ± 0.92 mm compared to 3.64 ± 0.75 mm ($p=0.008$, t-test), respectively ***(Fig. 28B)***.

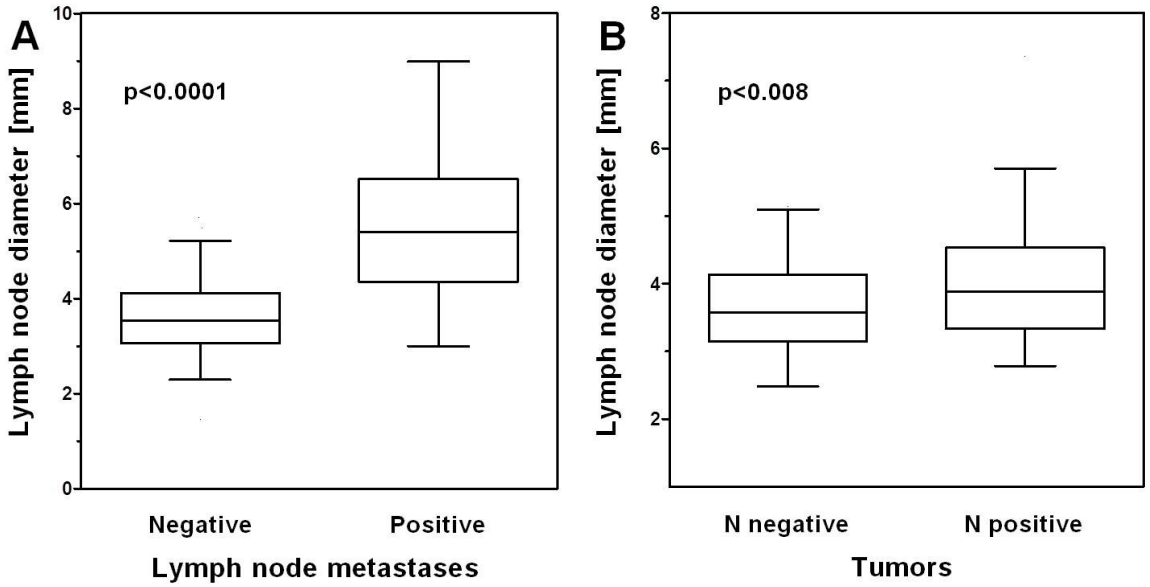

Figure 28 Lymph node size is related to presence of lymph node metastases

A: mean size of positive nodes is 5.63 ± 1.92 mm, compared to 3.6 ± 0.84 mm in negative nodes (p<0.001, t-test). B: mean node size in N-positive tumors is 4.01 ± 0.92 mm, compared to 3.64 ± 0.75 mm in N-negative tumors (p=0.008, t-test).

The relation between lymph node size and presence of lymph node metastases was more closely analyzed detecting that gain of lymph node size raises the risk for metastatic cancer spread. Remarkably, lymph node metastases can be detected in nodes as small as 1 mm. On the contrary, in 74% of all lymph nodes >10 mm no cancer spread can be detected at all. Details are presented in Table 6.

Altogether 18 cases with metastatic infiltrated nodes were collected, which showed positive nodes at the size of 1 or 2 mm. All of those cases had lymph node metastases in larger nodes too. In 6 (35%) cases, the identification of such small nodes (1 or 2 mm) changed the N classification and therefore caused upstaging. Furthermore, metastatic infiltrated nodes at the size of 3 mm were identified in 27 cases. An upstaging resulted from detection of these nodes in at least 13 (48%) of these cases. Three of these cases showed nodes measuring 3 mm as the only positive nodes. This caused a qualification of these cases for AJCC/UICC stage III disease.

Table 6 Metastatic cancer tissue is found predominantly, yet not exclusively in large lymph nodes

Lymph node size	n	Metastatic cancer spread	
		n	%
1 mm	266	1	0.4
2 mm	983	29	3
3 mm	993	38	4
4 mm	730	45	6
5 mm	526	59	11
6 mm	270	45	17
7 mm	174	41	24
8 mm	80	18	23
9 mm	48	14	30
10 mm	24	9	38
>10 mm	82	21	26
∑	**4176**	**320**	**8**

3.3 Primary tumor characteristics are related to the presence of lymph node metastasis

Presence of lymph node metastases was related to several characteristics of the primary tumor. Positive association was found between lymph node metastases and increasing T classification, enlargement of tumor size, worsening of tumor grade, high tumor budding, and presence as well as increase of tumor necrosis. Presence of infiltrated lymph nodes was negatively associated with increase of lymphocytic anti-tumor reaction. Details are presented in Table 7.

Table 7 Primary tumor characteristics are related to the presence of lymph node metastasis in univariable analysis

Category		N0 (n=87)	N1/N2 (n=61)	p-value
T classification	1 (n=15)	15 (100%)	0 (0%)	<0.001
	2 (n=24)	22 (92%)	2 (8%)	
	3 (n=68)	39 (57%)	29 (43%)	
	4 (n=41)	11 (27%)	30 (73%)	
Size	≤3.8 cm (n=76)	53 (70%)	23 (30%)	0.007
	>3.8 cm (n=72)	34 (47%)	38 (53%)	
Grade	1 (n=39)	35 (90%)	4 (10%)	<0.001
	2 (n=59)	38 (64%)	21 (36%)	
	3 (n=50)	14 (28%)	36 (72%)	
Tumor budding (ref. [43])	Low (n=88)	75 (85%)	13 (15%)	<0.001
	High (n=60)	12 (20%)	48 (80%)	
Location	Right (n=62)	41 (67%)	21 (33%)	0.12
	Left (n=62)	36 (58%)	26 (42%)	
	Rectum (n=24)	10 (42%)	14 (58%)	
Necrosis (ref. [40])	0 (n=28)	23 (82%)	5 (18%)	<0.001
	<10% (n=50)	34 (68%)	16 (32%)	
	10-30% (n=42)	23 (55%)	19 (45%)	
	>30% (n=28)	7 (25%)	21 (75%)	
Inflammation at the invasive margin (ref.[41])	0 (n=20)	13 (65%)	7 (35%)	0.18
	1 (n=35)	15 (43%)	20 (57%)	
	2 (n=61)	38 (62%)	23 (38%)	
	3 (n=32)	21 (65%)	11 (35%)	
Lymphocytic reaction (ref.[42])	0-2 (n=58)	29 (50%)	29 (50%)	0.004
	3-6 (n=65)	36 (55%)	29 (45%)	
	>6 (n=25)	22 (88%)	3 (12%)	

3.4 Primary tumor characteristics are related to the size of regional lymph nodes

To detect a reasonable cut-off value a ROC analysis was performed. The number of lymph nodes ≥ 5 mm was included. The calculated cut-off value was at least 5 lymph nodes (area under the curve of 0.642, 95% confidence interval (0.533-0.714, p=0.011). This cut-off represented the highest sensitivity and specificity for discriminating between node positive and negative cancer specimen. This value described lymph node enlargement and was applied for further analyses.

Relation of several characteristics of the primary tumor with the presence of at least 5 lymph nodes ≥ 5 mm was drawn in univariable analysis. Details are presented in Table 8.

All parameters that showed a statistically significant association got included in the multivariable analysis, which was conducted as logistic regression. Presence of at least 5 lymph nodes ≥ 5 mm was associated with high T classification (T > 2), presence of lymph node metastases, large tumor size (> 3.8 cm), right tumor location, moderate and extensive tumor necrosis (score 2/3) according to Pollheimer et al. [40], inflammation at the invasive margin (score 2/3) according to Klintrup et al. [41] and lymphocytic anti-tumor reaction (score > 6) according to Ogino et al. [42]

This analysis identified several independent parameters on lymph node enlargement such as high T-classification, right-sided tumor location, and large tumor size. Lymphocytic anti-tumor reaction slightly failed achieving statistically significance for association with lymph node size. Surprisingly no significant association between N classification and presence of at least 5 nodes ≥ 5 mm was drawn in multivariable analysis. Adequate fit of the model was proven by Hosmer-Lemeshow-test, revealing a p-value of 0.21 (χ^2 (8) = 10.859). Details are shown in Table 9.

Table 8 Primary tumor characteristics are related to regional lymph node size in univariable analysis

Category		Presence of 5 lymph nodes ≥ 5 mm		p-value
		no (n=37)	yes (n=111)	
N classification	0 (n=87)	28 (32%)	59 (68%)	0.044
	1 (n=33)	6 (18%)	27 (82%)	
	2 (n=28)	3 (11%)	25 (89%)	
T classification	1 (n=15)	8 (54%)	7 (46%)	0.001
	2 (n=24)	11 (45%)	13 (55%)	
	3 (n=68)	10 (14%)	58 (86%)	
	4 (n=41)	8 (20%)	33 (80%)	
Size	≤ 3.8 cm (n=76)	31 (41%)	45 (59%)	<0.001
	> 3.8 cm (n=72)	6 (8%)	66 (92%)	
Grade	1 (n=39)	14 (36%)	25 (64%)	0.098
	2 (n=59)	15 (26%)	44 (74%)	
	3 (n=50)	8 (16%)	42 (84%)	
Tumor budding (ref. [43])	Low (n=88)	25 (28%)	63 (72%)	0.33
	High (n=60)	12 (20%)	48 (80%)	
Location	Right (n=62)	6 (10%)	56 (90%)	<0.001
	Left (n=62)	25 (40%)	37 (60%)	
	Rectum (n=24)	6 (25%)	18 (75%)	
Necrosis (ref. [40])	0 (n=28)	12 (43%)	16 (57%)	0.022
	<10% (n=50)	12 (24%)	38 (76%)	
	10-30% (n=42)	11 (26%)	31 (74%)	
	>30% (n=28)	2 (7%)	26 (93%)	
Inflammation at the invasive margin (ref.[41])	0 (n=20)	7 (35%)	13 (65%)	0.044
	1 (n=35)	13 (38%)	22 (62%)	
	2 (n=61)	14 (23%)	47 (77%)	
	3 (n=32)	3 (9%)	29 (91%)	
Lymphocytic reaction (ref.[42])	0-2 (n=58)	18 (31%)	40 (69%)	0.027
	3-6 (n=65)	18 (28%)	47 (72%)	
	>6 (n=25)	1 (4%)	24 (96%)	

Table 9 Impact of primary tumor characteristics on presence of 5 lymph nodes ≥ 5 mm assessed by multivariable analysis (binary logistic regression)

	Presence of 5 lymph nodes ≥ 5 mm		
Variables	Adjusted Hazard Ratio	95% CI	p-value
T classification > 2	3.38	1.18-9.75	0.024
N classification > 0	1.32	0.41-4.30	0.64
Tumor size > 3.8 cm	7.61	2.41-24.08	0.001
Right tumor location	5.92	2.35-14.71	<0.001
Tumor necrosis (score 2/3) (ref. [40])	3.82	0.68-21.51	0.13
Inflammation at the invasive margin (score 2/3) (ref. [41])	1.97	0.43-9.16	0.39
Lymphocytic reaction (score > 6) (ref. [42])	8.87	0.97—80.81	0.053

It is known that MSI cancers show a marked intra- and peritumoral lymphocytic anti-tumor reaction. [44,45] So it was suspected, that nodes in MSI cancer might tend to be larger than nodes in MSS cancer specimens. In the study population, 20 (14%) of the tumors appeared to be MMR-deficient. Most often loss of MLH1 as well as PMS2 expression was found. Molecular analyses proved all MMR-deficient tumors as MSI-high. Of these, all tumors were right sided except of three cancers. Investigation of all 148 collected cancers showed that the mean lymph node size in MSI tumors was 4.19 ± 0.79 mm, whereas the mean lymph node size in MSS tumors was 3.73 ± 0.83 mm (p=0.023, t-test). Restriction of analyses to positive or negative nodes obtained similar findings (data not shown). Caution must be paid to the fact that MSI cancers predominantly occur on the right side, more precisely proximal to the splenic flexure [23,46]. In addition, larger nodes can be found more often in right-sided cancer specimen (see Table 9). Thus, restriction of comparison of MSI and MSS tumors to right-sided lesions was performed. No significant difference was observed in this subgroup analysis. Mean lymph node diameter of MSI cancers was 4.24 ± 0.74 mm in MSI cancers and 4.13 ± 0.78 mm in MSS cancers (p=0.62, t-test).

4 Discussion

The lymph node status in patients with colorectal cancer is of eminent clinical importance regarding prognosis and prediction of patients' outcome. Nodal positivity guides clinical decision-making by selection of AJCC/UICC stage III patients for adjuvant chemotherapy. [12,13] In contrast the situation is more complex in patients with rectal cancer. There decision-making and applying to neoadjuvant therapy is mainly based on depth of tumor penetration and less on lymph node size, due to unreliability of lymph node size as indicator of metastatic disease. [12,14,15]

Nevertheless, the investigation showed that lymph nodes harboring metastatic cancer spread were significantly larger than metastases free nodes (5.63 ± 1.92 mm vs. 3.6 ± 0.84 mm). In addition, other studies reported similar data. Mönig et al. [19] observed a mean diameter of 5.9 ± 3.4 mm for metastatic positive nodes compared to 3.8 ± 2.3 mm for metastatic negative nodes in 30 analyzed colorectal cancer patients. This study noted occurrence of metastases in 62 of 170 (36.5%) nodes measuring > 5 mm compared to 70 of 528 (13.3%) nodes measuring ≤ 5 mm.

The data revealed that the mean lymph node size in nodal positive cancers was significantly larger than in nodal negative cancer specimen. That confirms a previous study by Wong et al. [47]. They showed that the mean lymph node size in nodal positive cancers was 4.0 mm ± 3.0 mm compared to 3.6 mm ± 2.4 mm in nodal negative cancer specimens. However, Märkl et al. [20] compared nodal positive and nodal negative cancers, thereby revealing an almost identical size distribution in both subgroups.

In addition, a nearly linear relationship between lymph node size and presence of lymph node metastases was observed. Data are shown in Table 6. A similar relationship was noted by Murphy et al. [21,48]. They indentified metastatic cancer spread in 6.5% of lymph nodes measuring 1 mm (n=92), in 12.4% of lymph nodes measuring 2 mm (n=225), in 15.3% of lymph nodes measuring 3 mm (n=307), and in 34.4% of lymph nodes measuring ≥4 mm (n=873), respectively. Similar data were published in a study by Märkl et al.

[20]. They observed that the proportion of metastatic infiltrated nodes increased with increasing lymph node diameter. Overall 151 (49.5%) out of 305 cases tumor deposits were found in nodes smaller than and as small as 5 mm. The highest proportion of metastatic infiltrated nodes (27%) was found in nodes larger than 10 mm.

Of note, metastatic cancer spread can already be identified in very small nodes. In this study, 1 out of 266 (0.4%) nodes measuring 1 mm and 29 out of 983 (3%) nodes measuring 2 mm harbored metastatic cancer deposits. In the study by Märkl et al. [20] 2 out of 413 (0.5%) nodes in the size of 1 mm were involved by tumor deposits. Remarkably, in the present investigation, these small infiltrated lymph nodes caused upstaging in the N category in 6 cases. Notably, those nodes did not qualify patients as nodal positive alone, because all patients with tumor deposits in 1 or 2 mm lymph nodes also had positive larger nodes.

One might ask if this means that small nodes need not to be sampled because they seem not to effect decision making for patients' treatment. Yet, this question cannot be definitively answered. However, one can obtain a certain clue from other studies that investigate the potential benefit of techniques that increase lymph node yield in colorectal cancer resection specimen. These techniques include fat clearing methods, methylene blue-assisted lymph node dissection, as well as acetone elution with subsequent compression of adipose tissue (“acetone compression”) [8]. Applying these techniques has been proven to show a dramatically increase in lymph node harvest compared to conventional techniques. [49,50] These techniques are unarguably useful in some cases but are also known as time consuming as well as labor intensive. [25] In addition, the used chemicals are toxic and very expensive. [25] Furthermore, recently published studies are indicating that application of these techniques does not seem to show an association of an increased detection of lymph node metastases. [50,51]

The finding that only 21 out of 82 (26%) lymph nodes larger than 10mm harbored tumor deposits is well in the line with data previously published by Märkl et al. [20] They observed lymph node metastases in 27% of this

subgroup (>10 mm). This showed that the majority of large lymph nodes appeared uninvolved. However, neither the factors determining nodal size and lymph node enlargement in absence of metastatic cancer spread nor factors that lower the value of lymph node size as predictor of metastatic disease are yet well known.

The statistical analysis led to identification of large tumor size, deep tumor penetration (high T category), and right tumor location as independent predictors of large lymph node size in the resection specimen. Additionally intra- and peritumoral inflammation was investigated in this study. Thereby it showed that high lymphocytic anti-tumor reaction markedly increased the likelihood of lymph node enlargement (adjusted hazard ratio 8.869, 95% CI 0.97-80.81), but the analysis just missed statistical significance (p=0.053). Furthermore, presence of lymph node metastases was not identified as independent predictor.

Large tumor size and/or deep tumor penetration was expected to provide a more intense antigenic immune challenge to the local draining lymph nodes, which may result in hyperplasia and inflammation. [52,53] That might cause a reactive enlargement of regional lymph nodes [52,53]. As this study analyzed different types of intra- and peritumoral inflammation, this hypothesis is supported by the achieved results. Lymphocytic anti-tumor was assessed according to Ogino et al. [42]. It was identified as independent predictor of large regional lymph nodes in univariable, but not in multivariable analysis. However, the overall inflammatory cell reaction was assessed by Klintrup et al. [41] This inflammatory cell reaction was associated with lymph node size in univariable analysis only.

As tumor budding is known to be associated with occurrence of lymph node metastases and distant metastases as well as local recurrence [43] this study investigated the influence of tumor budding on the lymph nodes. Influence on occurrence of lymph node metastases was detected in the current study, but influence on the lymph node size failed statistical significance in the univariable analysis.

Dedicated search achieved only one study that systematically tested different tumor characteristics influencing the size of the recovered lymph nodes. Murphy et al. [21] identified stromal lymphoid infiltrate, peritumoral lymphoid aggregates (Crohn's like lymphoid reaction), as well as tumor growth pattern (infiltrative vs. circumscribed) as significant influencing parameters. Nevertheless, this study is hampered due to poor lymph node recovery with a mean lymph node count of 12.4 ± 6.2 nodes per case (median 11, range 1-33), retrospective nature, and lack of multivariable analysis.

Further attention has to be paid to the possible influence of intra- and peritumoral lymphocytic inflammation. In clinical practice, these four lymphocytic anti-tumor reaction components can easily be implemented as assessment of the components can be done on routine histopathologic examination. [42] Interestingly, marked intra- and peritumoral lymphocytic inflammation is known to be shown by MSI cancer specimens. [44,45] Thus, it was speculated that lymph nodes of MSI tumors might be larger than lymph nodes of MSS tumors. Notably, in AJCC/UICC stage I or II colorectal cancers appearance of a high number of lymph nodes showed a strong association with the MSI phenotype. [54] It was speculated, that this is may be caused by reactive enlargement of lymph node in the mesentery, maybe resulting in an easier discovery of those lymph nodes. [55]

The study showed that mean lymph node size of MSI cancer specimen was significantly larger than that of MSS cancer specimen (4.19 ± 0.79 vs. 3.73 ± 0.83 mm). MSI tumors are known to occur proximal of the splenic flexure in a large number of the cases [46,56]. Further, the number of retrieved lymph nodes is usually larger in resection specimen of right-sided colon cancers compared with those of left-sided colon cancers. [38,57] So, in a second step, analysis of lymph node size was restricted to right-sided tumors. However, there was no significant difference observed in this subset because lymph node size was 4.24 ± 0.74 mm in MSI cancers compared to 4.13 ± 0.78 mm in MSS cancers.

In conclusion, this study delivered further evidence for the fact that small lymph nodes, especially those measuring 1 or 2 mm, are less likely to harbor metastatic cancer tissue. Nevertheless, small lymph nodes shall not be ignored as such small nodes caused upstaging within the N category in one third of the cases where such small nodes had been discovered. Surprisingly, the majority of large nodes, especially those measuring > 10 mm, are not affected with metastatic cancer spread. That indicates that lymph node size is not a reliable factor for occurrence of lymph node metastases. Referring to the study data, within the resection specimens, lymph node enlargement is independently predicted by large tumor size, deep tumor penetration, and right-sided tumor location, whereas high lymphocytic anti-tumor reaction just missed statistical significance.

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