doi:10.25143/prom-rsu_2022-13_dt

RĪGA STRADIŅŠ UNIVERSITY

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Association of Epithelial Mesenchymal Transition, Stemness and Inflammation in Colorectal Carcinoma

Doctoral Thesis for obtaining a doctoral degree
"Doctor of Science (*PhD*)"

Sector – Basic Sciences of Medicine, including Pharmacy
Sub-Sector – Pathology

Riga, 2022

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Riga, 2022

Annotation

Colorectal cancer (CRC) and its treatment is one of the hot topics in medicine, as there is a still a high number of patients with CRC and deaths from CRC. In the era of personalized medicine, a huge field of research is devoted to inflammation, a process that plays an important dual role in carcinogenesis. Survival studies have highlighted the prognostic significance of peritumoural inflammation in CRC. The current theoretical background allows us to link inflammation, the epithelial-mesenchymal transition (EMT), and the closely associated stem cell differentiation in CRC. However, there is scarce direct morphological evidence.

The **aim** of this work was to investigate the role of inflammation in cancer growth and invasion by analyzing the association between inflammation and known morphological prognostic features of CRC, EMT, and mismatch repair (MMR) protein expression.

Materials and methods: This retrospective, morphological, and immunohistochemical investigation involved 553 consecutive cases of surgically treated CRC. Besides histopathological CRC assessment, peritumoural inflammation, EMT-characterizing molecular parameters, and MMR protein immunohistochemical detection within CRC tissue was performed. Descriptive statistical analysis was done. The research was carried out in accordance with the Declaration of Helsinki and received approval from the Committee of Ethics of Riga Stradins University [No E-9 (2), 04.09.2014].

Results: The tumours of the 553 CRC cases were predominantly located in the left part of the bowel (70.9 %), and were significantly associated with an annular appearance and smaller size ($p < 0.05$). Mucinous and signet ring cell carcinomas detected within this study were associated with a larger tumour size and significant local structure involvement. Locally advanced tumours predominated within this study: pT3 comprised 49.6 % of cases, pT4 comprised 35.6 % of cases, and pN + comprised 40.5 % of cases. The extent of necrosis was significantly higher in tumours with higher pT, grade, and pN+. There were significant associations between high-grade inflammation and lower pT ($p = 0.002$), the absence of lymph node metastases ($p < 0.001$), and less frequent local structure involvement ($p < 0.05$). Expression of EMT markers and MMR proteins yielded no significant associations with peritumoural inflammation based on the Klintrup-Mäkinen score or the density of lymphoid follicles. Nevertheless, E-cadherin expression was significantly associated with the eosinophil density ($p = 0.007$), and lower N-cadherin expression was significantly associated with the presence of synchronous CRC. Lower MMR protein expression was associated with changes in E-cadherin and CD44 expression ($p < 0.05$). Lower MLH1 expression was associated with a lower neutrophil density within the CRC ($p = 0.02$).

Conclusion: High-grade peritumoural inflammation is associated with beneficial morphologic CRC features, including less frequent invasion, and is not secondary to tissue damage and necrosis. Crohn's disease-like lymphoid reaction (CLR) is not associated with cancer spread by pTN; this finding indirectly suggests an independent role of CLR in carcinogenesis. Further, inflammation based on the Klintrup-Mäkinen score and CLR are not dependent on EMT and stem cell differentiation. MMR protein expression is a marker for EMT within CRC, indicating the need to perform these tests on routine examination to detect patients who might need more advance treatment. MLH1 expression within CRC affects the density of neutrophils within peritumoural inflammation, showing a potential role for the MMR status in anti-tumour immunity. This research highlights the complex associations between inflammation, tumour morphology, EMT, and MMR protein expression in human CRC tissues.

Keywords: colorectal carcinoma, inflammation, EMT, immunohistochemistry, mismatch repair proteins.

Anotācija

Kolorektālais vēzis (KRV) joprojām ir viena no aktuālākajām problēmām medicīnā, jo novērojama tā augsta izplatība un augsts nāves gadījumu skaits. Personalizētās medicīnas laikmetā arvien lielāku nozīmi pievēršot imūnajai atbildei KRV gadījumā, liela nozīme ir arī iekaisuma izpētei. Peritumorozs iekaisums, lai arī tam ir divējāda loma kanceroģenēzē, ir pierādīts kā viens no KRV prognozē nozīmīgajiem faktoriem. Teorētiskajos pētījumos ir raksturota saistība starp iekaisumu, epiteliāli mezenhimālo transformāciju un cilmes šūnu diferenciāciju KRV, bet šobrīd tam ir maz tiešu morfoloģisku pierādījumu.

Šī pētījuma **mērķis** bija raksturot iekaisuma nozīmi kolorektālā vēzī, analizējot tā saistību ar KRV morfoloģiskajiem prognostiskajiem faktoriem, epiteliāli mezenhimālo transformāciju (EMT) un DNS labotājgēnu proteīnu ekspresiju.

Materiāli un metodes. Retrospektīvā pētījumā tika izvērtēti 553 secīgi ķirurģiski operēti KRV gadījumi. Tika veikta morfoloģiska un imūnhistoķīmiska izmeklēšana, nosakot KRV morfoloģiskos parametrus, kā audzēja izplatības dziļums (pT), metastātisku limfmezglu klātesamība (pN +), invāzija lokālajās struktūrās, nekrozes plašums. Tika novērtēts peritumorozs iekaisums, kā arī EMT un DNS labotājgēnu kodējošo proteīnu raksturojošo IHĶ marķieru ekspresija KRV audos. Tika veikta deskriptīva statistiska analīze. Pētījums veikts saskaņā ar Helsinku deklarāciju un ir saņēmis Rīgas Stradiņa universitātes Ētikas komitejas atļauju (izsniegta 04.09.2014., E-9 (2)).

Rezultāti. Pētījumā tika iekļauti 553 secīgi KRV gadījumi. Lielākā daļa audzēju bija lokalizēti kreisajā zarnu pusē (70,9 %), un tos raksturoja cirkulārs (gredzenveida) izskats, kā arī mazs audzēja izmērs (< 0,05). Pētījumā iekļautajiem KRV gadījumiem ar mucinozu un gredzenšūnu morfoloģiju bija raksturīgs liels audzēja izmērs un statistiski nozīmīga lokālo struktūru iesaiste. Pētījumā dominēja KRV gadījumi ar augstu lokālu izplatību: pT3 audzēji veidoja 49,6 %, pT4 35,6 % un pN + 40,5 % no pētāmās grupas. Audzējos ar augstu pT, pN un zemu diferenciācijas pakāpi tika novērota statistiski nozīmīgi plašāka nekrozes izplatība. Tika atrasta statistiski nozīmīga asociācija ar izteiktu lokālu iekaisumu un zemāku pT ($p = 0,002$), pN0 ($p < 0,001$), kā arī retākām lokālo struktūru invāzijām ($p < 0,05$). EMT (CD44, -katenīna, N-kadherīna un E-kadherīna), kā arī DNS labotājgēnu raksturojošo marķieru (MSH2, MSH6, PMS2 un MLH1) IHĶ ekspresija statistiski nozīmīgi neatšķīrās audzējos ar dažādas intensitātes peritumorozu iekaisumu un Krona slimībai līdzīgo limfocitāro reakciju. Tomēr, analizējot iekaisuma šūnu subpopulācijas, tika atrasta statistiski nozīmīga atšķirība E-kadherīna izteiktākā ekspresijā pie palielināta eozinofilo leikocītu daudzuma ($p = 0,007$). Zemāka N-kadherīna ekspresija bija raksturīga multifokāliem KRV gadījumiem. DNS labotājgēnu ekspresija statistiski ticami atšķīrās audzējos ar izmaiņām E-kadherīna un CD44 ekspresijā ($p < 0,05$).

MLH1 proteīna zemāka ekspresija statistiski ticami saistījās ar zemāku neitrofilo leikocītu klātbūtni KRV ($p = 0,02$).

Secinājumi. Audzējiem ar izteiktu peritumorozo iekaisumu ir raksturīga mazāka izplatība un lokālo struktūru invāzija. Krona slimībai līdzīgā limfocitārā reakcija neietekmē audzēja izplatību (pTN), tas netieši norāda uz šīs reakcijas neatkarīgu lomu kanceroģenēzē. Iekaisums, noteikts pēc Klintrupa–Makinena skalas (*Klintrup-Makinen score*), un Krona slimībai līdzīgā limfoīdā reakcija nav atkarīgi no EMT un cilmes šūnu diferenciācijas KRV. DNS labotājgēnu (MSH2, MSH6, PMS2 un MLH1) samazināta ekspresija KRV ir norāde par EMT procesu, kas potenciāli var ietekmēt terapijas izvēli KRV. MLH1 ekspresija KRV ietekmē neitrofilo leikocītu daudzumu peritumorozā iekaisumā, netieši norādot potenciālo DNS labotājgēnu nozīmi audzēja imunitātē. Šajā pētījumā raksturota iekaisuma, KRV morfoloģijas, EMT un DNS labotājgēnu mijiedarbība.

Atslēgvārdi: kolorektāls vēzis, iekaisums, epiteliāli mezenhimāla transformācija, imūnhistoķīmija, DNS labotājgēni.

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Abbreviations used in the Thesis

AAMs	alternatively activated macrophages
AGR2	anterior gradient 2
AJCC	American Joint Committee on Cancer
APC	adenomatous polyposis coli gene
BMP4	bone morphogenetic protein 4
BRAF	B-Raf proto-oncogene
CAF	cancer associated fibroblasts
CD	cluster of differentiation
CEA	carcinoembryonic antigen
CG	cytosine-guanine dinucleotides
CI	confidence interval
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
CLR	Crohn's disease like lymphoid reaction
PTEN	phosphatase and tensin homolog
COX	cyclooxygenase
CRC	colorectal cancer
CSC	cancer stem cells
DNA	deoxyribonucleic acid
EMT	epithelial-mesenchymal transition
FAP	familial adenomatous polyposis
gFOBT	guaiac faecal occult blood test
gFOBT-TC	guaiac faecal occult blood test-total colonoscopy
HE	haematoxylin and eosin
HNPCC	hereditary non-polyposis colorectal cancer
HR	hazard ration
IBD	inflammatory bowel disease
IHC	immunohistochemistry
IL	interleukin
IQR	interquartile range
KRAS	Kirsten Ras oncogene
LGR5	leucine-rich repeat containing G protein-coupled receptor 5
MAPK	mitogen activated protein kinase
mCRC	metachronous colorectal carcinoma
miRNA	micro RNA

MLH1	mutL homolog 1 gene / protein
MMAH	monoclonal mouse antibody against human antigen
MMR	mismatch repair gene / proteins
MRAH	monoclonal rabbit antibody against human antigen
MSH2	MutS homolog 2 protein
MSH6	MutS homolog 6 protein
MSI	microsatellite instability
MSS	microsatellite stabile
MUTYH	mutY DNA glycosylase
NA	not applicable
NF-κB	nuclear factor kappa B
NICE	National Institute for Health and Care Excellence
NSAID	nonsteroidal anti-inflammatory drugs
OM	original magnification
OR	odds ratio
OS	overall survival
PD-L1	programmed cell death- ligand 1
pG1-4	characteristics of tumour differentiation
PGE2	prostaglandin E2
pM0-1	designation of the presence or absence of distant metastasis in a patient affected by a malignant tumour based on pathological examination
PMS2	PMS1 homolog 2
pN1-2	characteristics of regional lymph node status regarding metastases of a malignant tumour based on pathological examination
pR0-2	resection margin status regarding the presence or absence of a malignant tumour based on pathological examination
pT1-4	characteristics of local tumour spread based on pathological examination
pTis	*in situ* carcinoma
ROC	receiver-operating characteristic
RSPO	R-spondin
SCN4B	sodium voltage-gated channel β subunit 4
sCRC	synchronous colorectal carcinoma
SD	standard deviation
SDF-1	stromal cell derived factor-1
Slug	*SNAI2* gene
SMAD4	SMAD family member 4 gene
Snail	*SNAI1* gene

SPKC	Centre for Disease Prevention and Control of Latvia
SSL	sessile serrated lesions
STAT3	signal transducer and activator of transcription 3 gene
TAM	tumour-associated macrophages
TCF	T cell factor
TFs	transcription factors
TGFα	transforming growth factor α
TGFβ	transforming growth factor β
Th	T helper
TMA	tissue microarray
TNFα	tumour necrosis factor α
TNM	tumour, node, metastasis: classification of a malignant tumour by the anatomic disease extent reflecting the local spread of the tumour (T), the status of regional lymph nodes regarding tumour metastases (N), and the presence of distant metastasis (M)
TWIST1	Twist family BHLH transcription factor 1
UICC	Union for International Cancer Control
USA	United States of America
vs	versus
WHO	World Health Organization
x	x axis
y	y axis
z	z axis
ZEB1	zinc finger E-box binding homeobox 1 gene
ZEB2	zinc finger E-box binding homeobox 2 gene
π	an irrational number, with a value of 3.141592653589793238

Introduction

Colorectal cancer (CRC) has been the focus of myriad scientific studies, and it remains a hot research topic in oncology. Nevertheless, CRC still represents one of deadliest tumours worldwide. According to global cancer statistics (GLOBOCAN), 19.3 million new CRC cases were diagnosed and 0.94 million CRC-related deaths occurred in 2020 (Xi & Xu, 2021). Although CRC is known to occur after 50 years of age, in last decade the number of young patients has increased significantly worldwide (Stoffel & Murphy, 2020), marking the need to establish and / or modify screening programs for CRC and to identify factors leading to CRC progression.

One of the factors affecting CRC carcinogenesis and progression is inflammation. Certain inflammatory diseases, such as Crohn's disease and ulcerative colitis, increase the risk of CRC. In contrast, in epidemiological, observational, and clinical studies, nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin and selective inhibitors of cyclooxygenase-2 (COX-2), have been demonstrated as effective tools to decrease the risk of CRC, the recurrence of adenomas, and the development of new tumours. Regression of the existing adenomas has also been reported (Kraus, Sion, & Arber, 2015). Inflammation is known to affect development of other malignancies as well (Okada, 2014). In an already established tumour, an inflammatory reaction can either promote or suppress tumour progression. Inflammatory cells are able to produce growth factors that stimulate the proliferation of neoplastic cells, to enhance angiogenesis or to degrade the connective tissue matrix that in turn facilitates invasion. Consequently, inflammation might create a microenvironment that is beneficial for tumour development. On the contrary, inflammation can induce cancer cell death (Galdiero, Garlanda, Jaillon, Marone, & Mantovani, 2013; Hanahan & Coussens, 2012). One of the main factors involved in tumour progression is the pro-inflammatory cytokine tumour necrosis factor α (TNFα), produced by macrophages. TNFα also has a crucial role in epithelial-mesenchymal transition (EMT) as it induces overexpression of the transcription factor (TF) Snail, leading to downregulation of E-cadherin and upregulation of N-cadherin (H. Wang et al., 2013; Z. Zhang et al., 2019), which are among the key molecules involved in EMT. Another key molecule involved with EMT is β-catenin; its expression is also known to be affected by inflammatory cytokines and to affect the development of CRC (Keerthivasan et al., 2014). Stem cell differentiation by CD44 expression in CRC (Mukohyama, Shimono, Minami, Kakeji, & Suzuki, 2017) is strongly associated with EMT (Fabregat, Malfettone, & Soukupova, 2016) and currently represents an attractive treatment target (Xu et al., 2019). However, the relationship between CD44, E-cadherin, N-cadherin, and β-catenin expression, and local inflammation has not been studied extensively.

The response to treatment can depend on the degree of tumour heterogeneity. It is described not only in CRC (Molinari et al., 2018; Zlatian et al., 2015), but also in other carcinomas (Prince et al., 2007). Regarding CRC, the heterogeneity is more frequent in tumours exhibiting microsatellite instability (MSI) than in microsatellite-stable (MSS) tumours (De Smedt et al., 2015; McCarthy et al., 2019). Changes in mismatch repair (MMR) protein expression detected via immunohistochemistry (IHC) could be a hallmark for further DNA sequencing and personalized treatment choice.

Studies observing local inflammation and CRC histopathology have shown significant associations between inflammation and CRC survival, as patients with intense, high-grade peritumoural inflammation have shown better survival rates than those who have low-grade inflammation (Klintrup et al., 2005; Shibutani et al., 2017). Some studies have revealed links between decreased inflammation and the development of EMT-related tissue changes (Witschen et al., 2020). However, the results are controversial. Considering these prognostic trends, associations between the listed factors might be hypothesized, but few researchers have tried to directly assess the mutual relationships between inflammation, EMT, stem cell differentiation, and the expression of MMR proteins in the same cohort of CRC cases.

The **aim** of this work was to investigate the association between peritumoural inflammation and known adverse morphological features of CRC, including local spread (pT), the involvement of regional lymph nodes (pN), and manifestations of invasive growth as well as with the molecular landscape of EMT, cancer stem cell (CSC) differentiation, and expression of MMR proteins in Latvian patients.

To achieve this aim, the following tasks were conducted:

1. characterize demographic data, and gross and microscopic morphological features of CRC by pTNM, grade, manifestations of invasive growth, synchronous colorectal carcinoma (sCRC), and tumour volume.
2. Assess the molecular profile of CRC by immunohistochemical evaluation, analyzing the expression intensity of markers showing mesenchymal and stem cell differentiation in CRC.
3. Evaluate peritumoural inflammation and Crohn's-like inflammatory reaction (CLR), as well immunohistochemical CD8+ T cell presence within CRC.
4. Evaluate MMR protein expression within CRC tissue by using IHC.
5. Analyse the interrelations of the clinical, morphological, and molecular parameters.

Hypotheses of the Thesis

1. Local inflammation shows a significant association with CRC progression.
2. The DNA MMR proteins MSH2, MSH6, MLH1 and PMS2 could potentially have a role in epithelial mesenchymal process.

Novelty of the Thesis

1. Immunohistochemical CD44 expression in study group in difference from cell culture studies does not show correlation to inflammation degree;
2. This study is one of the first complex studies, that describe associations between tumour stemness and inflammation in human colorectal carcinoma tissue, and points out immune cell, specifically, eosinophil leucocyte possible role in tumour protection;
3. Complex approach in tissue studies reveals significant associations in difference from linear cell culture studies.

Author's contribution

The author has performed all stages of the study, including the study design, selection of IHC markers, evaluation of IHC results, measurements, and statistical data analysis. The author also performed the IHC visualization and took all the gross and microscopical photographs presented in this work.

1 Literature review

1.1 Colorectal cancer: Epidemiology and risk factors

1.1.1 Epidemiology

CRC is one of the three most common malignant tumours in the Western population, ranking third by incidence (10.0 %) after breast cancer (11.7 %) and lung cancer (11.4 %). According to global cancer statistics (GLOBOCAN), 19.3 million new CRC cases were diagnosed and 0.94 million CRC-related deaths occurred in 2020 (Xi and Xu, 2021).

Data from the Centre for Disease Prevention and Control of Latvia (SPKC) show that in 2017 there were 1131 new CRC cases in Latvia, most of which were stage III and stage IV tumours (Figure 1.1) (SPKC, 2020).

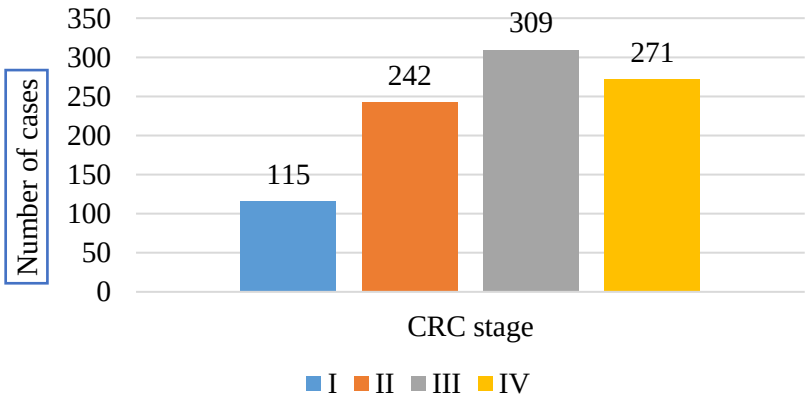

Figure 1.1 **Colorectal cancer diagnosis made in Latvia in 2017**

Mortality from CRC has increased over the past 5 years in Latvia. In 2013, mortality from CRC was 33.8 per 100,000 people, while in 2018 it was 34.4 per 100,000 people (Figure 1.2) (SPKC, 2020).

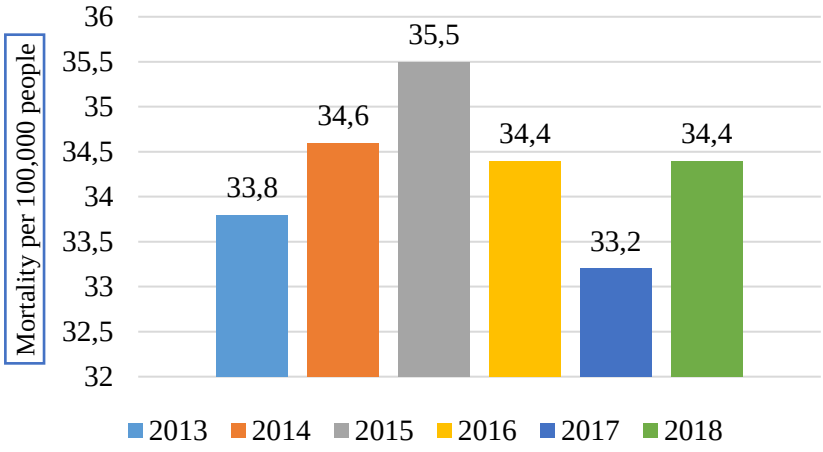

Figure 1.2 **Mortality from colorectal cancer (CRC) in Latvia from 2013 to 2018**

Regarding patient age, more than 80 % of CRC cases occur in patients older than 60 years. In the United States of America (USA), the median age of patients with rectal cancer is 63 years when considering both sexes, but 68 years in men and 72 years in women (Siegel, Miller, & Jemal, 2020). However, in recent years there has been a significant increase in CRC in younger patients (Glover, Mansoor, Panhwar, Parasa, & Cooper, 2019; Stoffel & Murphy, 2020). Studies have shown that young patients have a tendency for more advanced disease at the moment of diagnosis as well as a higher incidence of mucinous adenocarcinoma and signet ring cell carcinoma. However, due to early treatment they have slightly better cancer-specific survival than older patients (Kocián et al., 2019; Willauer et al., 2019). In patients younger than 40 years, CRC is usually associated with genetic predisposition, but there are also variations (Willauer et al., 2019).

1.1.2 Risk factors and prognostic factors of colorectal carcinoma

Although most of the colon cancer cases are sporadic, about 20–25 % of all patients with CRC have a close relative who has been diagnosed with the disease (Aaltonen, Johns, Järvinen, Mecklin, & Houlston, 2007; Butterworth, Higgins, & Pharoah, 2006). Recently, in a cohort of unselected patients with CRC, pathogenic germline variants were identified in 9.9 % of patients, showing changes in genes not previously associated with CRC risk (Yurgelun et al., 2017). About 2–5 % of CRC cases occur due to inherited syndromes, like Lynch syndrome (also known as hereditary non-polyposis colorectal cancer [HNPCC]), familial adenomatous polyposis syndrome (FAP), Peutz-Jeghers syndrome, mutY DNA glycosylase (MUTYH)-associated polyposis, juvenile polyposis, and Cowden / phosphatase and tensin homolog (PTEN) hamartoma syndrome (Kidambi, Kohli, Samadder, & Singh, 2019; H. Ma et al., 2018).

Nevertheless, the main risk factors for CRC are age, lifestyle (diet, lack of physical activity), inflammatory bowel diseases (IBD), and simultaneous benign tumours (Hamoya et al., 2016; Imperiale et al., 2014). There are also differences in geography: CRC is more common in Western countries.

The role of obesity in the context of carcinoma development has been debated, as adipocytes release different immune system mediators that have a significant role in providing low-grade inflammation and activation of different immune cells, including macrophages and CD8+ T cells (Kolb, Sutterwala, & Zhang, 2016). In patients with obesity, macrophages infiltrate adipose tissue and release interleukin-6 (IL-6) and TNFα as a response to mechanical damage of adipocytes (Kern et al., 2019). The levels of these two inflammatory mediators are known to be increased in patients with IBD, so chronic inflammation could be a major link between obesity and the tumour microenvironment in CRC (Kern et al., 2019;

Ye, Xi, Huang, & Xu, 2020). Overall, studies have shown that obesity, both general and central, increases the risk for colon cancer and could be associated with an early onset of CRC (P. H. Liu et al., 2019; Y. Ma et al., 2013).

There have been suggestions that high amounts of animal protein in the diet, especially red meat consumption, could be a risk factor for CRC, but the data are controversial (Alexander, Weed, Miller, & Mohamed, 2015). The risk for CRC development decreases if there is high amount of fruit and vegetables in diet (van Duijnhoven et al., 2009). Moreover, NSAIDs use is associated with decreased CRC incidence and mortality (Hamoya et al., 2016), as NSAIDs could affect growth and apoptosis in colonic mucosa (Martin et al., 2002). (Sada, Ahmed, Jeldo, & Shafi, 2020) showed that the risk of any adenoma among frequent NSAID users was lower (26.8 %) than among placebo subjects (39.9 %). Studies have also shown that NSAID use is associated with decreased risk of CRC recurrence and significantly improves the CRC outcome (Johnson, Jankowski, Rolnick, Yood, & Alford, 2017).

IBD is the third leading condition with a high risk for CRC development; it ranks after two genetic conditions, namely FAP and HNPCC (Kulaylat & Dayton, 2010). The risk factors for CRC development in patients with IBD include prolonged disease duration (> 8 to 10 years), extensive mucosal involvement (pan-colitis), and a family history of colorectal dysplasia (Friedman et al., 2001). Dysplasia risk is estimated to be 5–20 % after 10 years of disease (Friedman et al., 2001; E. R. Kim & Chang, 2014). A history of IBD in patients with CRC may be associated with increased mortality (Ording et al., 2013). Patients with early-onset CRC and IBD tend to have mucinous or signet ring histology and are less likely have adenomatous polyposis coli (*APC*) mutations compared with patients with early-onset CRC without predisposing conditions (Willauer et al., 2019).

Unfavourable prognostic factors for CRC include a large tumour size, macroscopic type of growth – circular ulcer, local spread of the process, metastases in the lymph nodes, high-grade differentiation, the presence of perforation, the presence of residual adenoma, and sCRC – as well as involvement of the perineural space and blood and lymphatic vessels (Caliskan et al., 2010; Compton et al., 2000; Grin et al., 2013; Hosseini et al., 2017; Huh, Lee, Kim, & Kim, 2013; Yang, Jy, & Chen, 2011). The prognostic importance of the extent of cancer necrosis has also been discussed (S. A. Vayrynen et al., 2016). With more distinct knowledge of the molecular mechanisms involved in CRC development, the MSI status is considered to be a meaningful prognostic criterion in CRC, as it also significantly affects the treatment choice (Deng et al., 2020).

sCRC is found in about 3–6 % of all CRC cases; it is defined as more than one primary CRC discovered in a single patient at the time of initial presentation. Neoplasms diagnosed sometime after resection and / or diagnosis of the first lesion are called metachronous colorectal carcinoma (mCRC) (Chin, Kuo, & Chiang, 2019; De Raffele et al., 2018; B. C. Lee et al., 2017; van Leersum et al., 2014). The presence of sCRC requires a complicated diagnostic algorithm, and delayed diagnosis of its presence could require additional operative treatment, prolonging hospitalization time (W. He et al., 2019; Yang et al., 2011). The status of MMR protein expression does not significantly differ in patients with sCRC and mCRC, although as shown by (C. Zhang et al., 2021), the prevalence of MMR-deficient tumours is slightly higher in patients with mCRC (36.4 % vs. 20.0 %, $p = 0.289$). Overall, the prognosis of patients with sCRC is significantly worse through TNM stages I to III than in solitary CRC (TNM stage I hazard ratio [HR] 1.86, $p < 0.001$; stage II HR 1.65, $p < 0.001$; and stage III HR 1.40, $p < 0.001$) (Chin et al., 2019). The presence of sCRC has been associated with habitual behaviours such as alcohol consumption and smoking; however, there are independent risk factors for sCRC, such as age and male sex, while coffee consumption could be a protective factor for lowering sCRC risk (Kuo, Hung, You, Chiang, & Chin, 2019; Yang et al., 2011).

In the colon, a mass raised above the colon mucosa is called a polyp, and advanced adenomatous as well as large serrated polyps within colon are associated with an increased risk for CRC (X. He et al., 2020). Serrated polyps are characterized by genetic alterations (B-Raf proto-oncogene [*BRAF*] or Kirsten Ras oncogene [*KRAS*] mutations) and epigenetic (CpG island methylator phenotype [CIMP]) that cooperate to initiate and drive malignant transformation from normal colon mucosa to polyps, and then to CRC (De Palma et al., 2019; Snover, Jass, Fenoglio-Preiser, & Batts, 2005).

Conventional colorectal adenomas are benign CRC precursor lesions with cellular dysplasia. These lesions are classified morphologically based on the World Health Organization (WHO) classification as tubular low / high grade, tubulovillous low / high grade, and villous low / high grade adenomas based on the presence of intraepithelial dysplasia (neoplasia) (Lokuhetty et al., 2019). The development of CRC from conventional adenomas is mostly associated with a chromosomal instability (CIN) pathway, although other pathways also could lead to CRC development (Armaghany, Wilson, Chu, & Mills, 2012). In adenomas with a mainly villous architectural configuration and high-grade dysplasia, the risk of transformation into cancer rises to 50 % (Burt et al., 2010); however, increased risk of CRC also remains after adenoma removal, especially in cases with high-grade dysplasia, multiple adenomas, and large adenomas (Cross et al., 2020; Euscher, Niemann, Lucas, Kurokawa, & Frankel, 2001). (Moon

et al., 2010) showed that patients with synchronous advanced adenomas in CRC have a higher risk for metachronous neoplasia in a follow-up period. Overall detection of adenomas and early-stage CRC via screening colonoscopy, as well as faecal tests, have shown decreased mortality rates in countries with long-standing screening programs (Cardoso et al., 2021).

1.2 Molecular pathway of colorectal carcinoma

CRC is associated with at least three genetic and epigenetic pathways: MSI, CIN, and CIMP (Sieber, Heinimann, & Tomlinson, 2003). The MSI pathway is characterized by alterations in the length of microsatellite that mostly is caused by loss of DNA MMR genes (Yurgelun et al., 2012). It is mostly associated with hereditary nonpolyposis CRC, although this pathway appears in up to 15 % of sporadic CRC cases (Markowitz & Bertagnolli, 2009; Pawlik, Raut, & Rodriguez-Bigas, 2004). Physiologically, MMR maintains correct DNA replication and high fidelity by repairing DNA base mismatches, which allows for genomic stability and reduces spontaneous mutations, but in the case of mutations this mechanism is altered (S. K. H. Li & Martin, 2016). The CIN pathway is characterized by abnormalities in chromosomes – their copy numbers and structure, the result of defective mitotic process (Bakhoum et al., 2014). This pathway is the most common one mutated in sporadic CRC cases, as it is found in about 85 % of cases, namely alterations in the *APC*, *TP53*, and *SMAD4* genes (Markowitz & Bertagnolli, 2009). CIMP refers to hyper-methylation at CG dinucleotides (CpG islands) in tumour suppressor gene promoter regions, especially the *MLH1* gene, causing silencing of these genes (Markowitz & Bertagnolli, 2009; Nazemalhosseini Mojarad, Kuppen, Aghdaei, & Zali, 2013). However, many sporadic MSI-high CRC cases are also CIMP positive, because CpG island hyper-methylation leads to inactivation of MMR genes, causing the MSI level to rise (Funkhouser et al., 2012; Markowitz & Bertagnolli, 2009).

Most CRC develop through the classic adenoma-carcinoma sequence, which is thought to be the most common pathogenic pathway in CRC development and is associated with CIN (Markowitz & Bertagnolli, 2009). This classic pathway describes how accumulation of genetic and epigenetic alterations leads to transformation of a normal colon epithelium into adenoma, and further accumulation of mutations favours progression into invasive carcinoma. Inactivation of the tumour suppressor gene *APC* results in overactivation of the Wnt/β-catenin signalling pathway, which results in increased cellular proliferation leading to the rise of an adenoma (Dow et al., 2015; Grady & Carethers, 2008), followed by *KRAS* oncogene mutations and tumour suppressor gene *TP53* inactivation (Dow et al., 2015). Overall, *KRAS* oncogene mutations are more common in MSS CRC and are associated with the presence of villous

adenomas in colon (Zauber, Marotta, & Sabbath-Solitare, 2013). Moreover, overall progression into invasive carcinoma is higher for these adenomas (Cross et al., 2020).

Another CRC development pathway is the serrated pathway, which involves increased expression of microRNA (miRNAs) in sessile serrated lesions (SSLs); it appears in about 15–30 % of CRC cases (Peruhova et al., 2020). In SSLs, most mutations are in the *BRAF* gene; other CRC-characterizing mutations in *KRAS* are less common, and *APC* mutations are rare (Travaglino et al., 2019). The serrated pathway is divided into two subtypes: (1) the traditional serrated pathway, where *BRAF* or *KRAS* are mutated in normal colon due to alterations in methylation, leading to the development of traditional serrated adenoma and serrated adenocarcinoma; (2) the sessile serrated pathway, where *BRAF* mutations lead to alterations in MLH1 isoforms (De Palma et al., 2019). *KRAS* and *BRAF* mutations result in activation of the mitogen-activated protein kinase (MAPK) pathway, which controls cell proliferation, survival, and differentiation (Yamane, Scapulatempo-Neto, Reis, & Guimarães, 2014).

The third proposed CRC development pathway is inflammation. In patients with long-standing IBD, the cumulative risk for CRC development increases from 2 % after 10 years of the disease to 18 % after 30 years of disease (Stidham & Higgins, 2018). Differently from the adenoma-carcinoma sequence pathway, mutations in these patients arise early and are connected to *TP53*, and mutations in *APC* appear later or not at all (Itzkowitz & Yio, 2004). IBD-related carcinogenesis has been associated with cellular communication via exosomes, which are cell-derived extracellular vesicles that promote cell-cell communication and immunoregulatory functions. They shuttle various molecules, including miRNAs, to recipient cells; inappropriate release of miRNAs from exosomes may cause significant alterations in biological pathways (Hu, Drescher, & Chen, 2012). Recent research has shown that in CRC cells, exosomal WNT1 increases proliferation and migration of CRC cells, but miR-424-5p promotes CRC proliferation and metastasis by directly inhibiting the tumour suppressor gene *SCN4B* (Khalyfa, Punatar, Aslam, & Yarbrough, 2021). Various immune signalling pathways, such as nuclear factor kappa B (NF-κB), prostaglandin E2 (PGE2)/COX-2, IL-6/signal transducer and activator of transcription 3 (STAT3), and IL-23/T helper 17 cell (Th17) are involved in CRC associated with IBD (T. Hirano et al., 2020).

1.3 Epithelial mesenchymal transition in colorectal carcinoma

EMT is one of the processes what characterizes tumour growth and spread, as during EMT cells acquire properties that allow them to invade surrounding tissues and move, leading to tumour growth and development of metastasis (Fabregat et al., 2016; C. Hogan et al., 2009). Most of the tumours within the gastrointestinal tract have an epithelial origin, and EMT is associated with downregulation of epithelial markers, such as E-cadherin, and aberrant upregulation of mesenchymal markers, such as vimentin and N-cadherin (Kalluri & Weinberg, 2009; Ryu, Park, Kim, Kim, & Lee, 2012; H. Wang et al., 2013). EMT in cancer is triggered by complex signalling pathways that include upregulation of EMT TFs and / or miRNAs (Nieto, Huang, Jackson, & Thiery, 2016). EMT TFs include Snail (also known as SNAIL1), Slug (also known as SNAIL2), twist-related protein 1 (TWIST1), zinc-finger E-box-binding homeobox 1 (ZEB1), and ZEB2 (M. A.-O. Stemmler, Eccles, Brabletz, & Brabletz, 2019). Overexpression of EMT TFs leads to downregulation of E-cadherin and upregulation of N-cadherin. Loss of intercellular junction proteins, especially E-cadherin, leads to an event cascade that results in translocation of β-catenin into the nucleus and activation of the Wnt signalling pathway (Mitselou et al., 2016). It appears due to upregulation of NF-κB signalling pathway that is upregulated by TNFα, a pro-inflammatory cytokine that is produced by macrophages and CD4+ T lymphocytes (H. Wang et al., 2013; Z. Zhang et al., 2019). TNFα and another pro-inflammatory cytokine, transforming growth factor β (TGF-β), which is produced by activated lymphocytes, stimulate cell self-renewal (Cabarcas, Mathews, & Farrar, 2011; Smith, Robin, & Ford, 2012), and are also involved in tissue renewal after injurious stimuli and organ fibrosis (Kalluri & Weinberg, 2009). TGF-β activates NF-κB to induce EMT process and enhance stemness by CD44 expression in CRC (Buhrmann et al., 2018). Overexpression of SNAIL has been associated with decreased expression of E-cadherin, leading to enhanced cell migration and invasion ($p < 0.002$ vs. control) and significantly increased expression of the CSC markers CD133 and CD44 (Fan et al., 2012).

The APC/Wnt/β-catenin signalling pathway is widely described in association with EMT in CRC and is mentioned as a main target of SNAIL overexpression products (Stemmer, de Craene, Berx, & Behrens, 2008). This pathway participates in the cell cycle of undifferentiated stem cells located at the base of the colonic crypts, allowing survival of both normal cells and CSCs. Dysfunction in this pathway has been found in hereditary as well as sporadic CRC cases (Baeg et al., 1995; Christie et al., 2013). *APC* gene mutation leads to cascade of events that result with enhanced Wnt signalling by β-catenin, which stimulates transcription of Wnt target genes, like c-myc and cyclin D1, and enhances T cell factor (TCF)

targets. The overall effects are increased cell growth, differentiation, spread, and adhesion of colorectal cells (Lecarpentier, Schussler, Hébert, & Vallée, 2019; Najdi, Holcombe, & Waterman, 2011; Schneikert & Behrens, 2007). The multiprotein destruction complex, containing binding domains for β-catenin, like axin / conductin, is responsible for destruction of unstable phosphorylated β-catenin. However, as a consequence of *APC* mutations, this complex does not form, and cytoplasmic β-catenin levels increase (Najdi et al., 2011; Schneikert & Behrens, 2007). β-catenin is also involved in cell junctions via connection to E-cadherin and the actin cytoskeleton via tyrosine phosphorylation changes in β-catenin. Phosphorylation of Tyr142 promotes binding of β-catenin to the nuclear cofactor B cell lymphoma 9-2 (Bcl9-2), thereby contributing to enhanced transcription of Wnt target genes (Schneikert & Behrens, 2007). One of the Wnt target genes is leucine-rich repeat-containing G-protein coupled receptor 5 (*LGR5*), a multipotent stem cell marker in different malignancies, including CRC (B. G. Jang et al., 2018; Morgan, Mortensson, & Williams, 2018; X. Wang et al., 2018). LGR5 is a ligand of R-spondin (RSPO), which, when bound, acts in cooperation with Wnt receptors (Frizzled and LRP5/6) to potentiate Wnt/β-catenin signalling. This LGR5/RSPO complex promotes Wnt signalling through the neutralization of two transmembrane E3 ligases, RNF43, and ZNRF3 (Morgan et al., 2018).

Of note, studies about Wnt signalling and proteins affecting this pathway are controversial. In CRC, a decrease in the intra- and extracellular protein anterior gradient 2 (AGR2), which is encoded by the *AGR2* gene, is associated with a higher tumour grade and tumour localization in the left-sided colon (Riener et al., 2014). However, Dahal et al. in 2018, reported a positive correlation between AGR2 and the Wnt/β-catenin signalling pathway in colon CSCs, as high levels of β-catenin and AGR2 protein was found in the presence of active Wnt signalling (Dahal Lamichane et al., 2018).

1.4 Histological characteristics and WHO classification of colorectal carcinomas

1.4.1 Gross description

Grossly, CRC can have different shapes: polypoid, exophytic / fungus-like, ulcerating, annular, and diffusely infiltrating, among others. Tumours located in the proximal colon (caecum, colon ascendens, colon transversum) tend to be larger and more likely to obstruct the bowel, but distal colon cancer (rectum, colon sigmoideum, colon descendens) tend to have an annular (Figure 1.3) or polypoid shape (G. H. Lee et al., 2015). It has been suggested that the tumour appearance could also have prognostic significance, because associations with CRC metastatic potential have been found in tumours with a circumferential (annular) appearance

(Kamocki, Wodyńska, Żurawska, & Zaręba, 2017). Non-polypoid early stage carcinomas (pT1) tend to infiltrate the submucosa with more distinct involvement of lymphatic vessels and veins than polypoid carcinomas (K. Hirano et al., 2012). Right-sided or proximal CRC is associated with more advanced disease stage and liver metastasis at diagnosis (Nawa et al., 2008; Treska et al., 2020). In addition, the clinical manifestation of disease is different between right- and left-sided tumours. In right-sided CRC, there are often signs and symptoms like microcytic anaemia and weight loss, but in case of the left-sided CRC bleeding and alterations in defecation are more common (G. H. Lee et al., 2015).

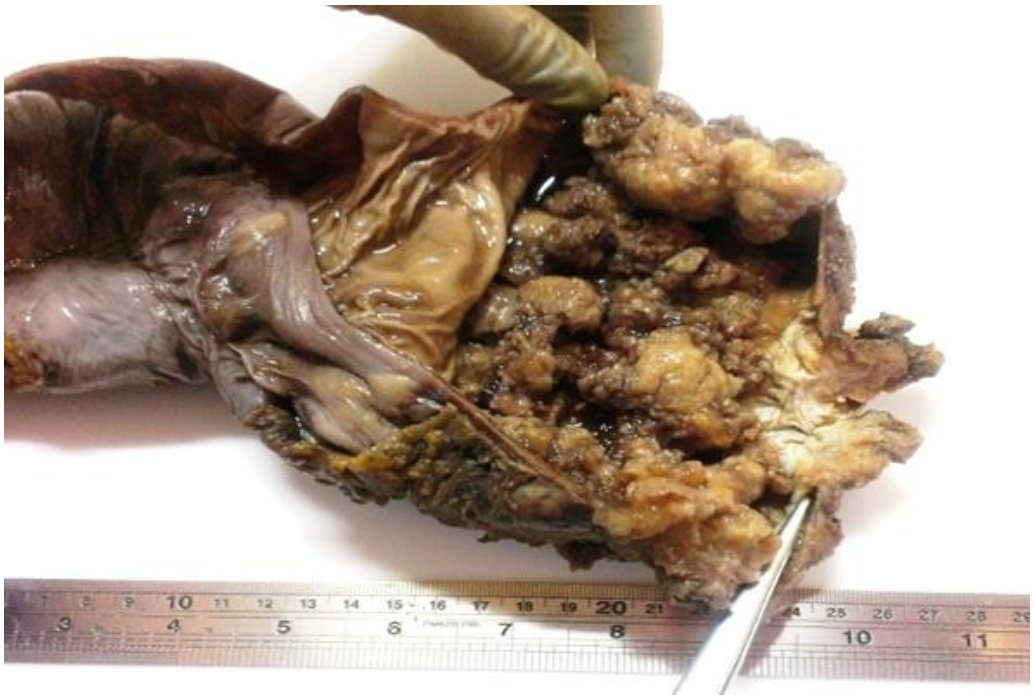

Figure 1.3 **Rectal carcinoma with annular appearance in a gross examination of bowel**

Photo by Inese Briede.

1.4.2 Morphology of colorectal carcinoma

The most common histological subtypes of CRC are adenocarcinoma, which occurs in more than 90 % of cases, mucinous adenocarcinoma, and signet ring cell carcinoma. However, there are other subtypes of CRC.

Adenocarcinoma is characterized by a glandular structure that will decrease in the case of anaplastic growth. Highly differentiated adenocarcinoma is characterized by a well-expressed glandular architecture and a low anaplastic degree (Figure 1.4A); > 95 % of the tumour consists of glands. In moderate differentiated adenocarcinoma there is a decrease in the stroma, but the glandular structure is mostly preserved. By contrast, in poorly differentiated adenocarcinoma (Figure 1.4), the cancer architecture is almost undistinguishable, and the

glandular structures comprise < 50 % of cancer (Fleming, Ravula, Tatishchev, & Wang, 2012). So, the diagnosis must usually be made with IHC tests that are specifically for colorectal neoplasia, such as cytokeratin 20, CDX-2, and carcinoembryonic antigen (CEA).

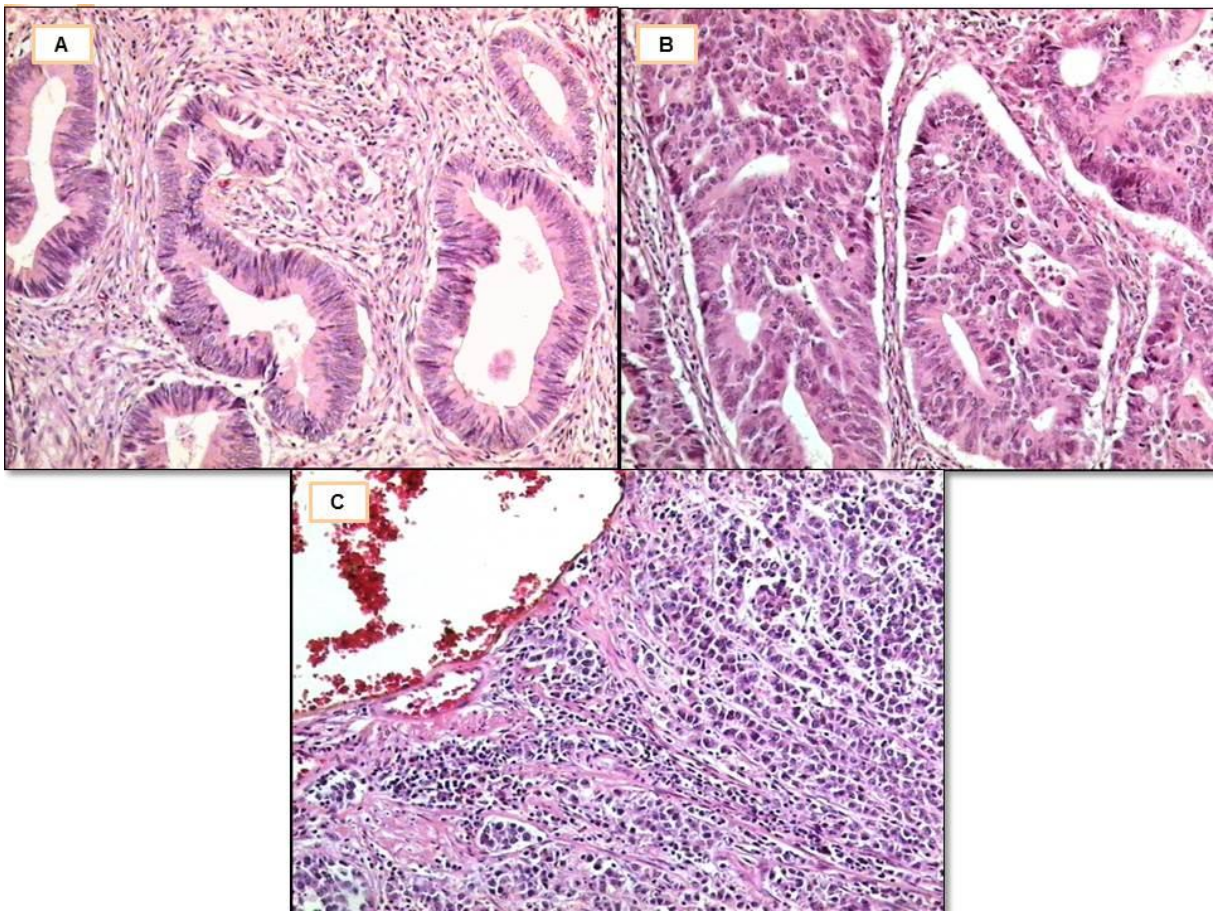

Figure 1.4 **Colorectal adenocarcinoma**

A – highly differentiated (grade 1) carcinoma, haematoxylin and eosin (HE) staining, original magnification (OM) 100×; B – moderately differentiated (grade 2) tumour, HE staining, OM 100×;
C – low differentiation tumour, HE staining, OM 40×

Images taken by Inese Briede.

In **mucinous adenocarcinoma**, more than 50 % of the tumour mass is composed of pools of extracellular mucin that contain malignant epithelial cells as acinar structures or separate tumour cells, and the stroma is almost absent (Fleming et al., 2012; Lokuhetty et al., 2019) (Figure 1.5).

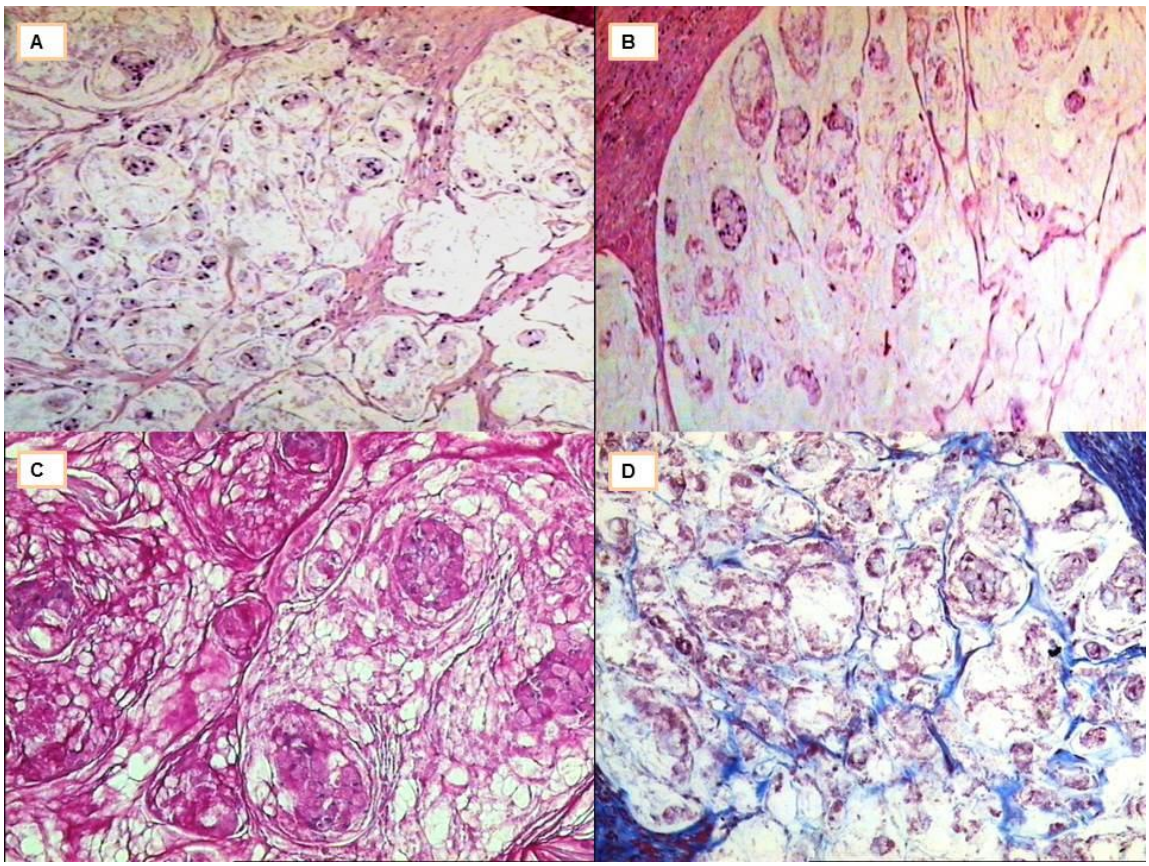

Figure 1.5 **Mucinous colorectal carcinoma**

A, B × malignant cells in mucin pools, haematoxylin and eosin (HE) straining, original magnification (OM) 40×, 100×; C – mucin component stained with periodic-acid Schiff staining, OM 40×; D – scant amount of stroma in mucinous carcinoma, Masson's trichrome staining, OM 40×.

Images taken by Inese Briede.

Mucinous histology is associated with more frequent alterations in BRAF, PIK3CA, and the TGF-β pathway compared with non-mucinous carcinomas (J. Shia et al., 2017). Overall, mucinous carcinomas tend to be located more frequently on the right side of the bowel and they tend to have a higher stage and lower differentiation than adenocarcinomas, but compared with non-mucinous adenocarcinomas these tumours have lower rates of vessel invasions (Nitsche et al., 2013; J. S. Park et al., 2015). Compared with signet ring cell carcinoma, mucinous carcinoma appear in older patients and less frequently show lymph node metastasis (Sung et al., 2008).

Signet ring cell carcinoma is characterized by the presence of intracytoplasmic inclusions of mucin inside the tumour cells in more than 50 % of cells, with displacement and moulding of the nucleus (Lokuhetty et al., 2019) (Figure 1.6). Signet ring cell morphology in CRC has been associated with worse prognosis, higher recurrence rates, younger age at presentation, involvement of lymph nodes, as well as more frequent presence of synchronous peritoneal metastasis in a moment of diagnosis (Lurvink et al., 2021; Nitsche et al., 2013).

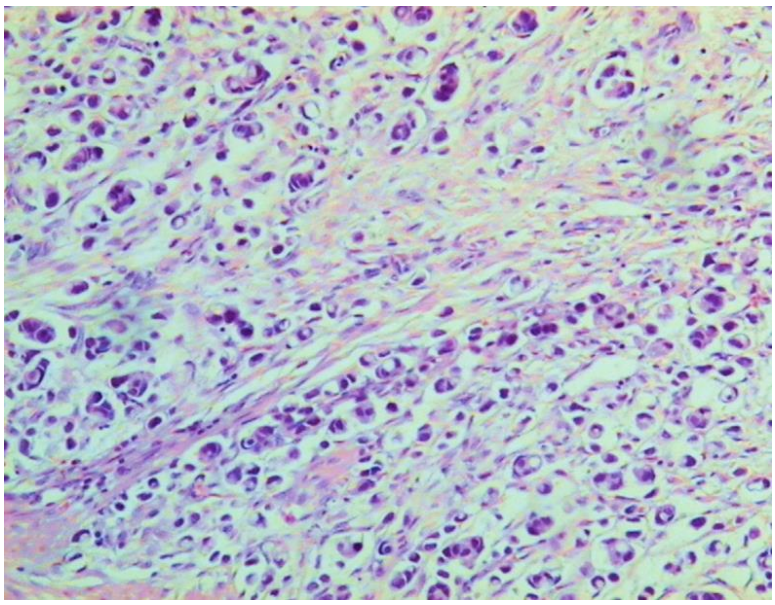

Figure 1.6 **Signet ring cell carcinoma, haematoxylin and eosin staining**

Original magnification 100×. Image taken by Inese Briede.

Medullary cancer (Figure 1.7) in the colon is rare; it is characterized by sheets of malignant cells with vesicular nuclei, prominent nucleoli, and abundant pink cytoplasm exhibiting prominent infiltration by lymphocytes and neutrophils (Lokuhetty et al., 2019). This CRC subtype is MMR deficient, as more than 80 % of medullary carcinomas have lost MSH2 (*PMS2*) and *MLH1* (Lin et al., 2014), and *BRAF* is most frequently mutated gene (Knox et al., 2015). Although it is a poorly differentiated tumour, it has a good prognosis (Puerta Vicente, Vilar Tabanera, & García Pérez, 2020).

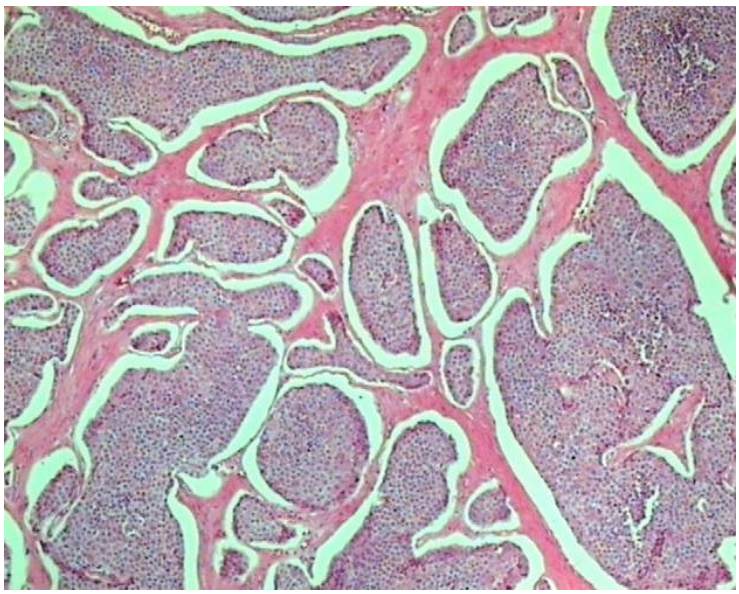

Figure 1.7 **Medullary carcinoma with a scant amount of stroma, haematoxylin and eosin staining**

Original magnification 40×. Image taken by Inese Briede.

In the latest WHO classification, other subtypes of CRC include serrated adenocarcinoma, micropapillary adenocarcinoma, and adenoma-like adenocarcinoma (Lokuhetty et al., 2019). However, their incidence is low compared with classical colorectal adenocarcinoma.

There are also other subtypes of CRC, such as adenosquamous carcinoma, which is a rare tumour in the colon with an incidence of less than 1 % (Toumi et al., 2018). Adenosquamous carcinoma has features of both adenocarcinoma and squamous cell carcinoma. This tumour has low differentiation, and patients with this CRC subtype have low overall survival rates and present at later stages (Nasseri et al., 2021). Within the colon there are also small number of carcinomas with sarcomatoid components or rhabdoid features (Lokuhetty et al., 2019), as well as undifferentiated carcinomas that lack morphological, immunohistochemical, and molecular evidence of differentiation beyond that of an epithelial tumour. These carcinomas differ from medullary carcinomas because they lack pushing borders, a syncytial growth pattern, and lymphoplasmacytic infiltrates (Lokuhetty et al., 2019).

1.5 pTNM classification and staging of colorectal carcinoma

The pTNM classification is based on the tumour anatomical spread, taking into account the following parameters: local spread of the primary tumour, characterized by the size and specified local invasion (pT parameter); regional lymph node status regarding metastases (pN parameter); and the presence or absence of distant metastasis which represents pM parameter (Brierley, Gospodarowicz, & Wittekind, 2016).

Although pT1 CRC could potentially be removed completely endoscopically with endoscopic submucosal dissection or endoscopic full thickness resection, studies have shown that in up to 10 % of cases, pT1 stage CRC can metastasize in regional lymph nodes and could develop synchronous distant metastasis (Ichimasa et al., 2021; Q. Li, Wang, Luo, Li, & Chen, 2021). Progression of pT1 CRC with the development of lymph node metastasis has been found in association with tumour invasion into lymphatic vessels as well as tumour budding (Wada et al., 2015), and in a recent study the authors found an association with a polypoid shape, poor differentiation, and rectal location (Ebbehøj, Jørgensen, Krarup, & Smith, 2021).

Studies have shown that as CRC invades the muscle layer (pT2), the chance for development of lymph node metastasis increases from up to 12 % in tumours with moderate to well differentiation, to up to 41.5 % in low-grade tumours (Fields et al., 2021). pT2 CRC has been associated with hematogenous metastasis development in patients with rectal tumours and positive lymph nodes (Tong et al., 2011).

Tumour budding has proven to be significantly associated with a higher pT stage, as its amount significantly increases in pT3 tumours (Meşină et al., 2019). Recurrence-free survival of 16 months could be used to distinguish between early and late CRC recurrence based on overall survival ($p < 0.001$), as was defined by Wiesmueller et al.(Wiesmueller et al., 2021). They found that in patients with advanced CRC (pT3 and pT4), even after they received treatment (ypT3 and ypT4), early tumour recurrence was significantly higher than in tumours with lower pT.

Lymph node metastasis (Figure 1.8) is known to affect overall prognosis in CRC. Patients without lymph node involvement (pN0) have a better prognosis than patients with pN+ (Y. Li et al., 2022; Priolli et al., 2009). As only 10 % of patients with early CRC develop lymph node metastasis, it is crucial to identify factors that could affect metastatic potential of these carcinomas. Among early carcinomas, pN+ findings are associated with cancer invasion into lymphatic vessels, neutrophil infiltration within cancer complexes, the depth of cancer invasion, and tumour budding (Akishima-Fukasawa et al., 2011). In the pathological investigation, accurate identification of lymph nodes has a significant role in the prognosis. In a recent study by Zhang et al., survival analysis showed significant differences in patients with pN0 and pN+, the 5-year survival rate for pN0 tumours was 75.2 %, while in patients with small amount of retrieved lymph nodes and pN+ it was 17.7 % (C.-H. Zhang et al., 2018).

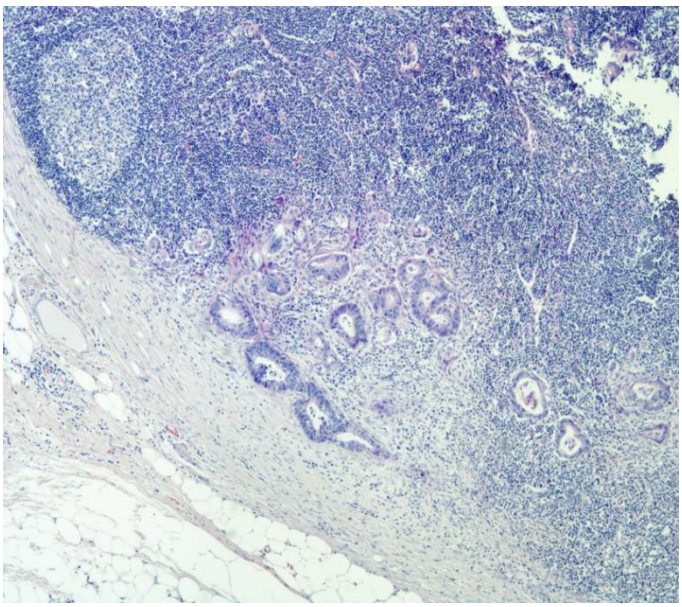

Figure 1.8 **Metastasis of moderately differentiated adenocarcinoma in lymph node, with invasion into the lymph node capsule, haematoxylin and eosin staining**

Original magnification 40×. Image taken by Inese Briede.

The details of pTNM staging of CRC, according to the 8th edition of TNM classification (2017), is described in Chapter 2, page 36.

1.6 Inflammation in a colorectal carcinoma

Inflammation plays a prominent role in CRC progression, especially in patients with an underlying chronic illness, such as Crohn's disease or ulcerative colitis. In addition, the risk of death from CRC in patients with IBD is higher than in reference individuals diagnosed with CRC (Olén et al., 2020).

Quantitative assessment of inflammatory reaction and its effect on a patient's survival rate and overall CRC characteristics have been described by researchers with development of two scoring systems. (J. P. Vayrynen et al., 2014) defined CLR in CRC as lymphoid structures surrounding the primary tumours, not associated with mucosa-associated lymphoid tissue or pre-existing lymph nodes (Figure 1.9). The number of CLR follicles was counted and the length of the invasive front was measured, and further CLR density was calculated (J. P. Vayrynen et al., 2014). High CLR density in CRC was associated with better survival, lower tumour stage, and deficient MMR enzyme expression (J. P. Vayrynen et al., 2014).

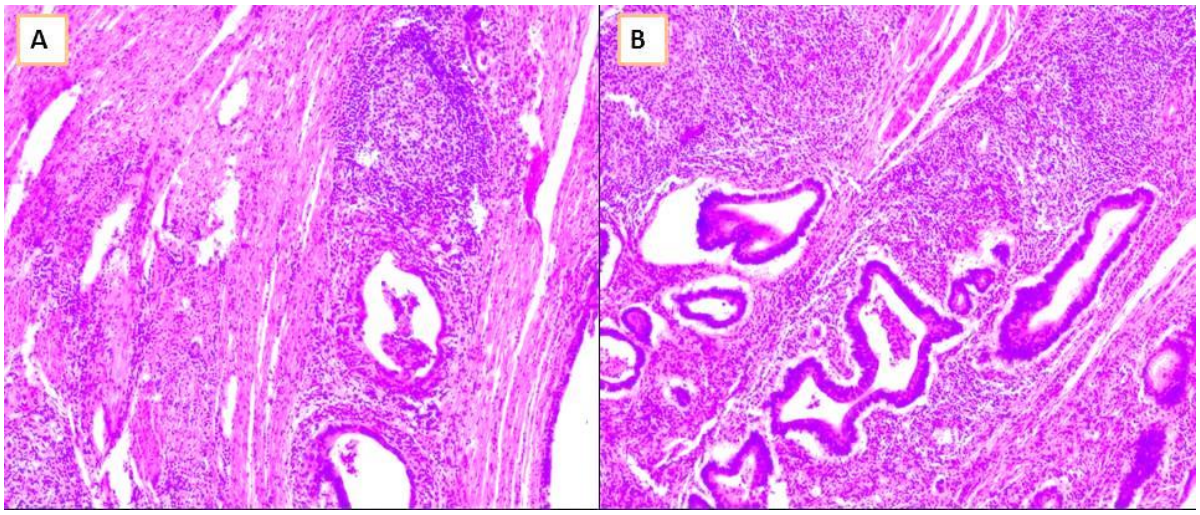

Figure 1.9 **Crohn's like lymphoid reaction within colorectal cancer**

A – mild reaction, haematoxylin and eosin (HE) staining, original magnification (OM) 40×;
B – moderate reaction, HE staining, OM 40×.

Images taken by Inese Briede.

Another scoring system involves evaluation of peritumoural inflammation, because patients featuring intense, high-grade peritumoural inflammation have better survival rates than those who have low-grade inflammation (Shibutani et al., 2017). The most common assessment method for peritumoural inflammation is the Klintrup-Mäkinen inflammation score, where the peritumoural inflammatory reaction and the number of inflammatory cell subpopulations are described based on four degrees: absent (0) reaction; mild (1) or weak; moderate (2) amount of cells / inflammation and severe (3) inflammation. In this scoring system, a score of 0 is given

if there is no increase in inflammatory cells, a score 1 is given in patchy increase of inflammatory cells, a score of 2 is given in the presence of band-like infiltrate, and a score of 3 is given in case of prominent inflammatory reaction (Klintrup et al., 2005). The Klintrup-Mäkinen score has also been modified and re-distributed into two classes: low-grade (absent or mild inflammation) versus high-grade (moderate or severe) inflammation (Klintrup et al., 2005). A low Klintrup-Mäkinen score and a low T cell count have been associated with a higher tumour recurrence rate (Väyrynen et al., 2013).

The cellular composition in CRC consists of multiple stromal and immune cell types, including CAFs, endothelial cells, monocytes / macrophages, granulocytes, B cells, and T cells (Koi & Carethers, 2017; Uhlitz et al., 2020). An increased lymphocyte count and an increased number and density of CD8+ T cells have been associated with improved survival in CRC; however, a high number of mast cells, and a low number of lymphocytes, CD8+ T cells, and CAFs have been associated with poor survival in CRC (Koi & Carethers, 2017). Although it is not always possible to take informative samples from tumour, it is suggested to perform pre-operative screening of peripheral blood, as indicated in published research. The peripheral monocyte count resembles the density of the tumour-associated macrophages (TAMs), which creates a microenvironment favourable for cancer development and is associated with a poor prognosis in CRC (Shibutani et al., 2017). Nevertheless, other immune cell types have demonstrated a potential role in CRC progression. In 2012, (H. Rao et al., 2012) showed that a high number of intratumoural neutrophils is an independent factor for poor prognosis of patients with CRC. They observed a high number of CD66b+ neutrophils in CRC tissue (45.4 %) compared with adjacent mucosal tissues (12.7 %) and a high intratumoural neutrophil count positively correlated with pT status, pM status, and clinical stage ($p < 0.05$). In CRC, increased peri- and intratumoural eosinophil counts are significantly associated with lower T and N stages, higher tumour grade, absence of vascular invasion, as well as improved progression-free and cancer-specific survival (Harbaum et al., 2015).

The authors of several studies exploring inflammatory pathways have identified mechanisms of CD44 upregulation by inflammatory cells and / or the relevant mediators. TNFβ, produced by activated lymphocytes, induces NF-κB activation and EMT (including upregulation of vimentin and downregulation of E-cadherin), and enhances stemness by CD44 expression in CRC (Buhrmann et al., 2018). In mouse studies, researchers have identified loss of CD44 expression and as a consequence delayed progression of breast cancer. It happened in the presence of decreased CD206+ macrophages, which are usually associated with tumour growth promotion (Witschen et al., 2020). In another mouse study, overexpression of the CSC marker CXCR4 was associated with colitis associated and *APC*-mutation driven

tumourigenesis, as there was an increase in macrophages within colon tissues and EMT-related tissue changes (Yu et al., 2019). One of the EMT signs in CRC is the presence of tumour buds, and high tumour budding has been associated with a worse prognosis (Grigore, Jolly, Jia, Farach-Carson, & Levine, 2016). Studies have shown that inflammation could affect tumour budding and overall survival, because high tumour budding has been detected in CRC cases with low-grade peritumoural inflammation, while high-grade inflammation has been found in a cases with fewer tumour buds (Max et al., 2016).

1.7 Immunohistochemical markers used in this study and their role in colorectal cancer

1.7.1 CD8

CD8 is a transmembrane glycoprotein expressed on cytotoxic T cells. CD8+ lymphocytes are known to limit neoplastic cell growth, to suppress tumour infiltration, and to induce elimination of tumours (Steele et al., 2018). In CRC, the presence of CD8+ lymphocytes is associated with high MSI, although data about overall survival is controversial (Lea et al., 2021; Millen et al., 2020; Sudoyo et al., 2019). A high CD8+ T cell count has been associated with stage I-II CRC ($p = 0.017$), and the CD8+ T cell count is significantly higher in the tumour centre and invasive margin in MSI tumours compared with MSS tumours (Lea et al., 2021). Researchers have also suggested that a combined score of CD8+ T cells and tumour buds in CRC could be a better prediction marker for nodal metastases and survival (Dawson et al., 2020).

1.7.2 Epithelial mesenchymal transition markers

1.7.3 CD44

CD44 is a transmembrane glycoprotein that normally participates in cell-cell interactions, adhesion of the cytoskeleton to the extracellular matrix, and cell migration. In cancers, CD44 is associated with clonality, metastatic spreading ability, resistance to chemotherapy and radiotherapy, as well as lower overall survival (Mare et al., 2021; Simtniece et al., 2015; C. Wang et al., 2012; Z. Wang et al., 2019). CD44 along other CRC cell surface antigens like CD133, CD24, ALDH1, CXCR4, and LGR5 are considered to be CSC markers (Fedyanin, Popova, Polyanskaya, & Tjulandin, 2017; Manhas et al., 2016; Wu et al., 2016). CD44 upregulation is associated with a worse prognosis in different malignancies (Abugomaa, Elbadawy, Yamawaki, Usui, & Sasaki, 2020; Hirata et al., 2013).

1.7.4 E-cadherin

E-cadherin is a calcium-dependent epithelial transmembrane glycoprotein that supports epithelial layer integrity and polarity (Simtniece et al., 2015). In CRC, loss of E-cadherin expression has been associated with the presence of lymphogenous metastasis and lower disease-free survival and overall survival (Choi et al., 2017; Elzagheid et al., 2012; S. A. Kim et al., 2016). In the cell membrane, E-cadherin is connected with various proteins, including catenins, to maintain cell membrane integrity (Hayashida et al., 2005). At its cytoplasmic tail region, E-cadherin is connected to β-catenin and γ-catenin, but α-catenin links the bound between β-catenin and actin filaments in the cytoskeleton (Buda & Pignatelli, 2011). Decreased E-cadherin expression and increased β-catenin expression have been found in CRC cells in infiltrative areas, supporting the hypotheses and findings about mesenchymal transition in carcinoma cells (Hayashida et al., 2005). Downregulation of E-cadherin expression has been found in various malignancies (Burandt et al., 2021; Sommariva & Gagliano, 2020), and decrease E-cadherin expression causes an incomplete EMT (Aban et al., 2021).

1.7.5 N-cadherin

N-cadherin is a calcium-dependent single-chain transmembrane glycoprotein that mediates homotypic and heterotypic cell-cell adhesion. Its presence has a crucial role in the developmental and functional regulation of the nervous system, brain, heart, skeletal muscles, blood vessels, and the hematopoietic microenvironment (Cao, Wang, & Leng, 2019; M. P. Stemmler, 2008). Aberrant N-cadherin expression has been found in lung cancer, breast cancer, prostate cancer, and squamous cell carcinoma, and high levels have been associated with a large tumour size, high grade, and tumour necrosis (N. R. Jang, Choi, & Gu, 2021; M. P. Stemmler, 2008). Abnormal N-cadherin expression is closely related to aspects of malignant tumour progression in humans, such as transformation, adhesion, apoptosis, angiogenesis, invasion, and metastasis, suggesting that N-cadherin could be a therapeutic target for tumour invasion and metastasis (Blaschuk, 2015; Gheldof & Berx, 2013; Labernadie et al., 2017; Pal, Bhattacharya, Kalyan, & Hazra, 2018; Radice, 2013). In colon carcinoma, increased N-cadherin expression is associated with disease progression and indicates a poor prognosis (Xuebing Yan et al., 2015).

1.7.6 Vimentin

Vimentin is a type III intermediate filament characteristically found in cells of mesenchymal origin, like fibroblasts, macrophages, and endothelial cells (Ngan et al., 2007). Its overexpression has been detected in various carcinomas as a consequence of EMT, where

cells gain the mesenchymal phenotype and invasive properties (Korsching et al., 2005; Serrano-Gomez, Maziveyi, & Alahari, 2016; Yin, Chen, & Yang, 2018). However, the results are controversial given that some studies have not identified such vimentin expression changes (Vermani et al., 2020). Vimentin overexpression in combination with E-cadherin loss has been found in patients with pancreatic neuroendocrine tumours, where it was associated with lymph node metastasis, the presence of distant metastasis, and disease progression (Zhou et al., 2021). In gastric carcinomas, overexpression of vimentin is associated with poor overall survival (Yin et al., 2018). In CRC, stromal overexpression of vimentin has been associated with tumour progression and higher recurrence rates (Ngan et al., 2007), and its stromal overexpression could indicate lower response to standard adjuvant chemotherapy (L.-G. Liu, Yan, Xie, Jin, & Yang, 2017).

1.7.7 β-catenin

β-catenin is an intracellular protein encoded by the *CTNNB1* gene. It interacts with adhesion molecules such as cadherins, transmembrane-type mucins, signalling regulators like APC, and epigenetic or transcriptional regulators (M. Katoh, 2018; Masuko Katoh & Katoh, 2017). It is a key protein in the Wnt signalling pathway, and deregulation of this pathway is a major contributor to hereditary and sporadic CRC (Christie et al., 2013). The canonical Wnt/β-catenin pathway is mutated in approximately 90 % of CRC cases, and signalling activation leads to β-catenin accumulation in the nucleus, where it induces transcription of Wnt target genes (Lecarpentier et al., 2019; Pronobis, Rusan, & Peifer, 2015). As a part of EMT, the Wnt/β-catenin pathway also has a role in other malignancies. High cytoplasmic and membranous β-catenin levels lead to a poor outcome in patients with breast carcinoma (López-Knowles et al., 2010), while high nuclear β-catenin levels have been correlated with a poor prognosis in patients with CRC (Baldus et al., 2004).

1.7.8 Mismatch repair proteins

The MMR pathway corrects inappropriate nucleotide insertions, deletions, and single nucleotide mismatched incorporations. Aberrations in MMR genes (*MSH2*, *MSH6*, *PMS2*, and *MLH1*) are closely linked to the development of malignancies, as inactivating mutations and hyper-methylation in MMR genes lead to the development of Lynch syndrome and MSI, increasing the risk of developing CRC or other cancers (Doghri et al., 2019; Salem et al., 2020; Sehgal et al., 2014). Approximately 50 % of MMR-deficient sporadic CRC cases are connected with *BRAF* oncogene mutations as a consequence of inactivation of the *MLH1* gene and CIMP

(Kawakami, Zaanan, & Sinicrope, 2015). Immunohistochemical expression of MMR proteins is suggested as a screening tool to identify MSI tumours (Doghri et al., 2019). In 2017, the United Kingdom National Institute for Health and Care Excellence (NICE) published recommendations to screen all patients with CRC at the moment of diagnosis. Screening involves either IHC for four MMR proteins or MSI testing, followed by *BRAF* mutation and *MLH1* promoter methylation analysis as appropriate in cases with *MLH1* loss/MSI (Westwood et al., 2019). Loss of *MLH1* and *MSH2* has been identified in 90.9 % of MSI-high CRCs (Lanza et al., 2002), but there are also cases when MSI cannot be detected by IHC due to missense mutations (Gelsomino, Barbolini, Spallanzani, Pugliese, & Cascinu, 2016). Heterogeneous MMR protein expression could indicate cases of MSI-high CRC (McCarthy et al., 2019). Loss of MMR protein detection is important because it has been associated with tumour resistance to chemotherapy (Kawakami et al., 2015; Malik et al., 2019). Moreover, MMR-deficient carcinomas are characterized by more frequent appearance of synchronous carcinomas in gastrointestinal tract not only in a case of Lynch syndrome, but also in sporadic tumour cases (Latham et al., 2021; Vyas et al., 2021).

1.7.9 MSH2

DNA mismatch repair protein MSH2, or MutS homolog 2, is a protein encoded by the *MSH2* gene, located on chromosome 2. Alterations in this gene are mostly found in patients with HNPCC. Mutations in the *MSH2* and *MLH1* genes have been found in about 56 % of CRC cases (Mangold et al., 2005).

Loss of *MSH2* has also been found in other malignancies. In prostate carcinoma, *MSH2* loss has been associated with a high tumour-infiltrating lymphocyte count and a high tumour grade (Guedes et al., 2017). The role of *MSH2* has also been considered in breast carcinoma progression (Malik et al., 2021).

Compared with MSH2- and MLH1-positive cases, MSH2- and MLH1-deficient colorectal adenocarcinomas have been associated with a younger age (38 vs. 43 years, $p = 0.0224$), a larger tumour size (60 ± 6 vs. 46 ± 2 mm, $p = 0.0291$), a higher number of tumour-infiltrating lymphocytes assessed by CD3 immunostaining (202 ± 48 vs. 33 ± 4 CD3+ lymphocytes/10 high-power fields, $p = 0.0039$), and a grade 2 CLR (70 % vs. 9 %, $p = 0.0037$) (Paraf et al., 2001). These findings suggest that MLH1- and MSH2-deficient CRC in young patients could exhibit pathological and molecular features similar to HNPCC.

1.7.10 MSH6

DNA mismatch repair protein MSH6, or MutS homolog 6, is encoded by the *MSH6* gene. Mutations in this gene appear in 10–20 % of Lynch syndrome cases (Terui et al., 2013). It was first described in the budding yeast *Saccharomyces cerevisiae* (Fishel & Kolodner, 1995). MSH6 is unstable unless MSH2 is present (Edelbrock, Kaliyaperumal, & Williams, 2013), so it has been noted that MSH6 detection in immunohistochemical investigation is not always informative, because polymorphisms in it have been detected (Okkels et al., 2012). However, some researchers support detection of only two IHC markers–including MSH6–to prove MSI (Hall et al., 2010; Jinru Shia et al., 2009). Researchers recently reported a pattern of MSH6 expression, as heterogeneous staining does not indicate germline mutation in MSH6, but it is more likely associated with alterations in another MMR gene (W. Chen et al., 2020). In the Japanese population, diagnosis of most patients with CRC and *MSH6* mutation was made after age 50 and was associated with a distal tumour (Terui et al., 2013).

1.7.11 MLH1

MLH1, or MutL homolog1, is a protein encoded by the *MLH1* gene, located on chromosome 3; its presence has also been described in *S. cerevisiae* (Fishel & Kolodner, 1995). Mutations in *MLH1* have been widely described in HNPCC (Cohen et al., 2017; Tarancón-Diez, Büttner, & Friedrichs, 2020). Immunohistochemical expression of MLH1 tends to show variable results. In one study, MSI-high MLH1- and MSH2-positive CRC tended to be located more often in the distal colon and tended to exhibit greater differentiation than CRC without MLH1 and MSH2 expression (Lanza et al., 2002). In a more recent study, however, MLH1- and MSH2-positive tumours were more frequently located in the proximal colon and tended to have poor differentiation (S.-M. Wang et al., 2019). The immunohistochemical pattern of MLH1 staining could help to indicate sporadic MLH1-/PMS2-deficient CRC cases prior to methylation analysis (Tarancón-Diez et al., 2020).

1.7.12 PMS2

PMS2, or PMS1 homolog 2, is encoded by the *PMS2* gene located on chromosome 7. It is a crucial component of the MMR system as it forms MutLα heterodimer together with MLH1 and possesses endonucleolytic activity responsible for removal of mismatched DNA (Nakagawa et al., 2004). Similarly to other MMR gene mutations, *PMS2* mutations are also found in patients with Lynch syndrome, although mutations in this gene are associated with older age of presentation and weak family history (Gulati, Gustafson, & Daw, 2011). PMS2 co-expression with MLH1 is widely described and is associated with sporadic CRC as well in changes of MLH1 expression (Tarancón-Diez et al., 2020). In CRC, isolated loss of PMS2 expression has been found in younger patients and has been associated with more aggressive behaviour than other MSI tumours (Alpert et al., 2018).

2 Materials and methods

2.1 Study model and ethical principle

The study was designed as a retrospective morphological and immunohistochemical investigation of a representative group of consecutive, surgically treated CRC cases. It was carried out in accordance with the Declaration of Helsinki, and it received approval from the Committee of Ethics of Riga Stradins University (No E-9 (2), 04.09.2014).

2.2 Patient identification and cohort selection

The study included 553 consecutive, potentially radically operated CRC cases, which were identified by archive search in a single university hospital from January 2011 to December 2014. The inclusion criteria were potentially radical surgery and diagnosis of CRC (adenocarcinoma, mucinous cancer, medullary cancer, undifferentiated cancer, and signet ring cell cancer). Patients who had a colorectal tumour of a different histogenesis (neuroendocrine tumour, squamous cell cancer, etc.) or other tumour metastasis, as well as those patients who did not undergo potentially radical surgery (had a biopsy) or did not have an invasive tumour (pTis), were excluded from this study.

Demographic information, such as age at the time of cancer diagnosis, gender, the presence of perforation, synchronous CRC presence, the type of operation, and the operated side, were collected from the medical histories and surgical reports, submitted to the Department of Pathology by Department of Surgery.

The location of the tumour within the bowel and the type of surgery were verified with the surgical reports, obtained from the Department of Surgery. Morphological data about tumour grossing (appearance, size) were obtained from pathological reports. Microscopic re-assessment was done for to verify the diagnosis and to perform measurements.

2.2.1 Gross examination data

The gross examination data included the data about submitted organs, tumour and the lymph nodes. It proceeded based on the following scheme.

1. First, surgical material was described and measured in three dimensions for each of submitted organs (large and small bowels, mesenterial fat tissue, omentum, anus, and other if applicable).

2. Second, the tumour(s) was(were) evaluated and described in detail, including:
    - distance from the closest resection margin;
    - tumour size in three dimensions;
    - assessment of tumour appearance (circular vs. half-circular; bowl shaped vs. ulcer; mushroom or node-like).
3. Third, lymph nodes were assessed in mesenterial fat tissue; if no lymph nodes were grossly detected, mesenterial fat tissue was collected for further investigation.

The gross examination data served as the basis for further evaluation of the tumour, including tumour volume, origin, and assessment of pTN, as well as for identification of the tumour localization within bowel.

2.2.2 Tissue processing and microscopic examination

The tissue samples that were dissected during grossing were fixed in 10 % neutral-buffered formalin (Sigma-Aldrich, Saint Louis, MO, UA) and processed by increasing grades of 2-propanol (Sigma-Aldrich), followed by incubation in histograde xylene (J. T. Baker, Deventer, the Netherlands) and paraplast (Diapath S.r.l., Belgamo, Italy) in the Tissue-Tek® VIP™ 5 vacuum infiltration processor (Sakura Seiki Co., Ltd., Nagano, Japan). The processed tissue was then embedded in paraplast (Diapath S.r.l.) using the TES 99 tissue embedding system (Medite GmbH, Burgdorf, Germany). After embedding, 4-μm sections were cut from the paraffin blocks with a Microm HM 360 microtome (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and placed on glass slides (Menzel-Glaser, Braunschweig, Germany). The slides were stained with haematoxylin and eosin (HE) by using a TST 44 automated tissue stainer (Medite Medizintechnik, Burgdorf, Germany) and covered with cover glass (Biosigma, Cona, Italy) using an automated cover slipper (Dako, Glostrup, Denmark) and Pertex covering medium (Histolab, Gothenburg, Sweden).

Standard slides, stained with haematoxylin and eosin, were examined under a light microscope to obtain the following data:

- characteristics of the primary CRC;
- lymph node status, including the presence of CRC metastasis, other malignant tumour, or reactive changes in lymph nodes;
- presence of perforation within the tumour;
- synchronous adenoma type, if applicable;
- synchronous malignancy type and characteristics, if applicable.

The following CRC parameters were evaluated by using the obtained data: histological type of tumour, tumour characteristics by pTNM classification (pT for local tumour invasion – T1–T4; pN for lymph node metastasis – N0, N1a, N1b, N1c, N2, Nx; pM for distant metastasis – M0, M1, if applicable), tumour biological potential by differentiation grade, status of resection margins (R0, R1, R2), intravascular (separately for venous and arterial) invasion, peri- and intraneural invasion, lymphatic vessel invasion, tumour necrosis, stromal desmoplasia, peritumoural inflammation, and Crohn's lymphoid-like inflammation.

Primary tumour, as well as synchronous tumour and synchronous adenoma morphology and pTNM parameters, were evaluated according to the classifications and criteria as defined by the WHO and the American Joint Committee on Cancer (AJCC) (Brierley et al., 2016; Lokuhetty et al., 2019). Detailed pTNM staging for CRC is described below:

pT parameter

- pTx not assigned (cannot be determined based on available pathological information);
- pT0: no evidence of primary tumour;
- pTis: carcinoma *in situ*, intramucosal carcinoma (involvement of lamina propria with no extension through muscularis mucosae);
- pT1: tumour invades the submucosa (through the muscularis mucosa but not into the muscularis propria);
- pT2: tumour invades the muscularis propria;
- pT3: tumour invades through the muscularis propria into pericolorectal tissues;
- pT4: tumour directly invades other organs or structures and / or perforates visceral peritoneum;
    - pT4a: tumour perforates visceral peritoneum (including gross perforation of the bowel through tumour and continuous invasion of tumour through areas of inflammation to the surface of the visceral peritoneum);
    - pT4b: tumour directly invades adjacent organs or structures.

pN parameter

- pNx: regional lymph nodes cannot be assessed;
- pN0: no regional lymph node metastasis;
- pN1: one to three regional lymph nodes are positive (tumour in lymph nodes measuring greater than or equal to 0.2 mm), or any number of tumour deposits are present and all identifiable lymph nodes are negative;

    - pN1a: one regional lymph node is positive;
    - pN1b: two or three regional lymph nodes are positive;
    - pN1c: no regional lymph nodes are positive, but there are tumour deposits in the subserosa, mesentery, non-peritonealized pericolic or perirectal / mesorectal tissues;
- pN2: four or more regional nodes are positive;
    - pN2a: four to six regional lymph nodes are positive;
    - pN2b: seven or more regional lymph nodes are positive.

pM parameter

- pM0: no distant metastasis;
- pM1: metastasis to one or more distant sites or organs or peritoneal metastasis is identified;
    - pM1a: metastasis to one site or organ is identified without peritoneal metastasis;
    - pM1b: metastasis to two or more sites or organs is identified without peritoneal metastasis;
    - pM1c: metastasis to the peritoneal surface is identified alone or with other site or organ metastases.

Regarding biological potential, CRC cases were classified into four groups as described in the WHO classification: G1, well differentiated; G2, moderately differentiated; G3, low differentiated; and G4, undifferentiated (Lokuhetty et al., 2019; J. Shia et al., 2017).

Synchronous adenomas were grouped into two groups, as described in the WHO classification, namely tubular, tubulovillous and villous adenomas (Lokuhetty et al., 2019).

The following resection margins were identified grossly and were examined grossly and microscopically for tumour invasion: proximal and distal resection margin of the bowel, resection margin of the mesenterial fat tissue and / or adventitial fat tissue if part of the bowel was involved. The status of resection margins was classified as R0, R1, R2, or Rx by the following criteria:

- R0, negative resection margins – based on gross and microscopic evidence, no cancer cells were found in resection margin;

- R1, microscopic residual tumour in the resection margin – any resection line is microscopically positive for cancer presence despite negative gross appearance;
- R2, grossly positive resection line – any of the resection lines contain grossly evident tumour mass and the cancer presence in resection line is confirmed by microscopic assessment;

The peri-and intraneural invasion as well as intravascular (venous and arterial) and lymphatic vessel invasion were assessed as a categorical variable: present or absent. The spread of tumour necrosis was measured as the relative area of tumour occupied by necrosis in all tumour slides, as demonstrated by (S. A. Vayrynen et al., 2016). Additional presence of necrosis was categorized into three groups: focal, moderate, or extensive.

The tumour volume was assessed using ellipsoid formula (Tirumani et al., 2016):

$$V= 4/3*\pi*x*y*z /8$$

π- an irrational number, with a value of 3.141592653589793238;

x- Diameter in cm in the x axis;

y- Diameter in cm in the y axis;

z- Diameter in cm in the z axis.

Peritumoural inflammation was assessed according to the Klintrup-Mäkinnen inflammation score, by identifying four groups: no inflammation, mild inflammation, moderate inflammation, and severe inflammations. They were then distributed into two classes: low-grade (no or mild inflammation) versus high-grade (moderate or severe) inflammation (Klintrup et al., 2005). CLR was assessed according to the Vayrynen criteria, by counting CLR follicles in the invasive front, and it was defined as CLR density (Väyrynen et al., 2013).

The complete scheme of microscopical investigation and evaluated parameters in this study is depicted in Figure 2.1.

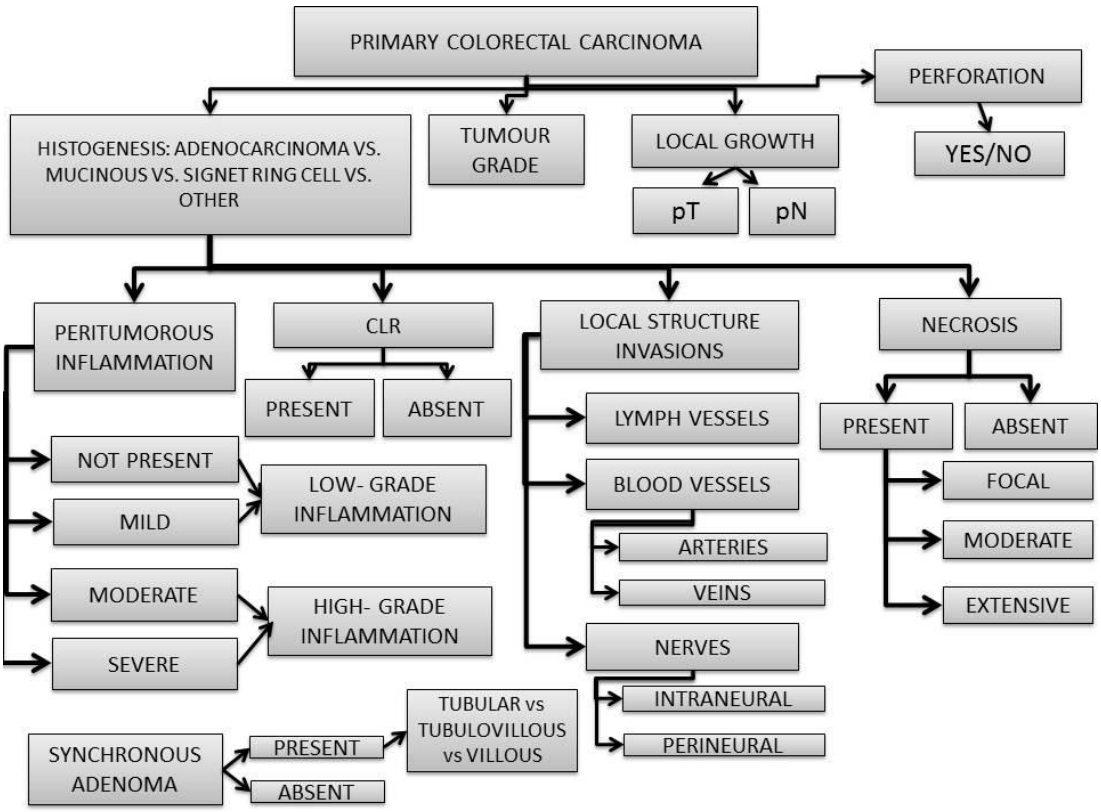

Figure 2.1 **Parameters in a microscopical evaluation in a study group**

Prepared by Inese Briede.

The gross photographs were taken by the author. The morphological and immunohistochemical images were taken by the DeltaPix Insight image base program (DeltaPix, Smorum, Denmark) using a Nikon-Eclipse Ci-S microscope (Nikon Corporation, Tokyo, Japan) as the optical system and Invenio 5SIII camera (DeltaPix).

2.3 Immunohistochemical visualization and assessment

Immunohistochemical visualization of the researched antigens was performed on formalin-fixed paraffin-embedded CRC and non-neoplastic control tissues. For IHC, 124 consecutive cases were selected. From each case, a representative block of formalin-fixed paraffin-embedded tumour tissue was selected for immunohistochemical visualization. Colorectal tissue from tumour-free regions was used to detect the immunophenotype of non-neoplastic control tissues.

For IHC, 3-µm-thick sections were cut on electrostatic glass slides (Histobond, Marienfeld, Germany). After deparaffinization and rehydration, antigen retrieval was performed in a microwave oven (3 × 5 min) using a basic TEG (pH 9.0) buffer, followed by blocking of endogenous peroxidase (Sigma-Aldrich). The sections were incubated with primary antibodies (full antibody characteristics and dilutions is described in Table 2.1) at room temperature.

Bound antibodies were detected by the enzyme-conjugated polymeric visualization system EnVision, linked with horseradish peroxidase using 3,3′-diaminobenzidine as the chromogen. The stained slides were covered by cover glass (Biosigma). All IHC reagents were produced by DakoPositive and negative quality controls were invariably performed and reacted appropriately.

Table 2.1

Characteristics of primary antibodies

Antigen	Antibody characteristics	Clone	Dilution	Incubation min.	Expression pattern
E- cadherin	MMAH	NCH-38	1:50	60	Membranous
Vimentin	MMAH	Vim 3B4	1:200	60	Membranous
CD44	MMAH	DF1485	1:50	60	Membranous
β-catenin	MRAH	β-catenin-1	1:50	60	Membranous
N-cadherin	MMAH	6G11	1:50	60	Membranous
CD8	MRAH	C8/144B	1:50	60	Nuclear
MMR proteins					
MSH2	MMAH	FE11	1:100	20	Nuclear
MSH6	MMAH	EP49	1:100	20	Nuclear
MLH1	MMAH	ES05	1:50	20	Nuclear
PMS2	MMAH	EP51	1:40	30	Nuclear

Abbreviations: MMAH, monoclonal mouse antibody against human antigen; MRAH, monoclonal rabbit antibody against human antigen

Expression of immunohistochemical markers was evaluated by light microscopy using high-power total magnification of 400× (i.e., 40× objective and 10× ocular lenses; 0.65 mm^2 per field). The expression of IHC markers – E-cadherin, vimentin, N-cadherin, β-catenin, and CD44 – and MMR proteins was assessed based on the intensity and the extent. The expression intensity was evaluated on a scale ranging from 0 to 3 as follows: 0, no expression; 1 weak expression; 2, moderate expression; and 3, strong expression (Figure 2.2) The relative extent (%) was measured as the fraction of cancer cells expressing the given marker at the given intensity. The final IHC score was calculated as the sum of the mathematical products of intensity and the relative extent. Subsequently, the MMR protein expression was reclassified as low versus high by using the median value as the cut-off threshold: 1.39 for MSH2, 1.9 for MSH6, 1.8 for PMS2, and 1.43 for MLH1. Additionally, the expression of MMR proteins was evaluated as a binary variable (complete loss vs. presence).

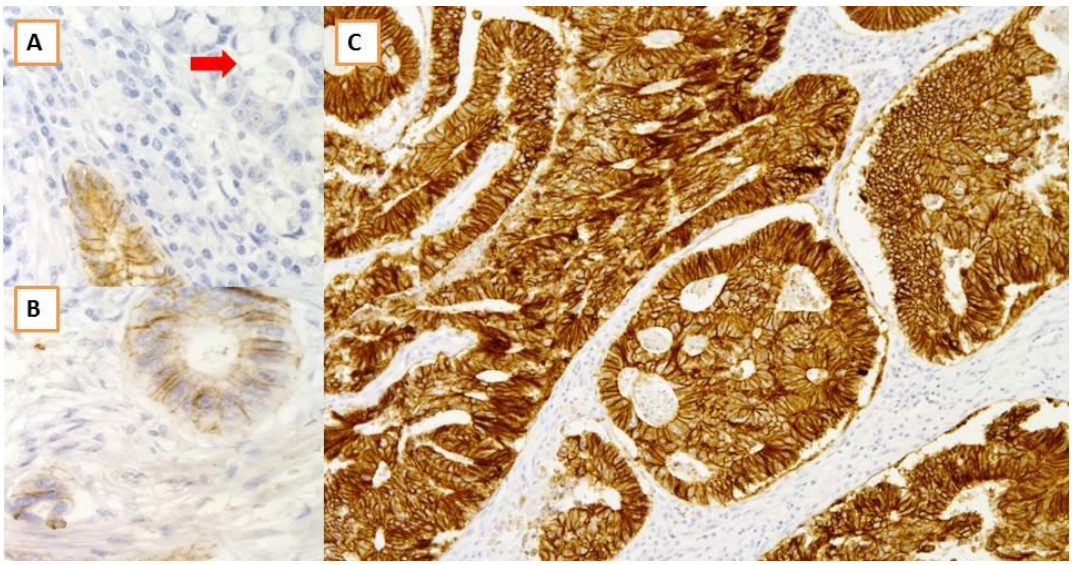

Figure 2.2 **Immunohistochemical marker expression evaluation**

A – total loss of E-cadherin expression in colorectal cancer (red arrow), original magnification (OM) 400×; B – weak E-cadherin expression in colorectal cancer cells, OM 400×;
C – moderate and strong E-cadherin expression in colorectal cancer, OM 100×.

Images taken by Inese Briede.

For CD8+ tumour-infiltrating lymphocytes, the cell number per mm^2 was counted via Diapath in the tumour centre and the invasive margin, as described in previous studies (Lea et al., 2021; Ueno et al., 2014).

2.4 Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics Version 22.0 (IBM Corp., Armonk, NY, USA). The normality of the data was checked with the Shapiro-Wilk test. The descriptive data are expressed as the mean ± standard deviation (SD), the median with the interquartile range (IQR), or the relative frequency with the 95 % confidence interval (CI). Categorical data are presented as frequencies. For mean values and frequencies, 95 % CI were calculated. Because the data were not normally distributed, non-parametric methods, including the Mann-Whitney test, Spearman's rank correlation, and Pearson’s chi-square were used for analytical statistics. The Kruskal-Wallis one-way analysis of variance by ranks followed by the post hoc analysis with Bonferroni correction was applied to determine differences between three or more groups. A two-tailed $p < 0.05$ was considered statistically significant. For data with significant differences, receiver-operating characteristic (ROC) line analysis was used to determine the impact of the independent variable.

3 Results

3.1 Patients and surgical approach

3.1.1 Study group characteristics

The study included 553 consecutive primary CRC cases collected from pathology examination reports from 2011 to 2014. There were 258 male patients (46.6 %, 95 % CI 42.5–50.8 %) and 295 female patients (53.4 %, 95 % CI 49.2–57.5 %). The patient age ranged from 33 to 97 years. For the entire study group, the mean ± SD age was 68.8 ± 10.8 years (95 % CI 67.9–69.7), with a median age of 71 years (IQR 15). For the women, the mean ± SD age was 69.5 ± 11.1 years (95 % CI 68.2–70.8), with a median age of 71 years (IQR 15). For the men, the mean ± SD age was 68.0 ± 10.4 years (95 % CI 66.7–69.3), with a median age of 70 years (IQR 16) years. Most of the patients were elderly: 93.0 % (95% CI 90.5–94.8 %) were older than 50 years. Only 39/553 cases were patients younger than 50 years, including 18/39 men (46.2 %, 95 % CI 31.6–61.4 %) and 21/39 women (53.8 %, 95 % CI 38.6–68.4 %). The age and sex distribution of study group is presented in the Figure 3.1.

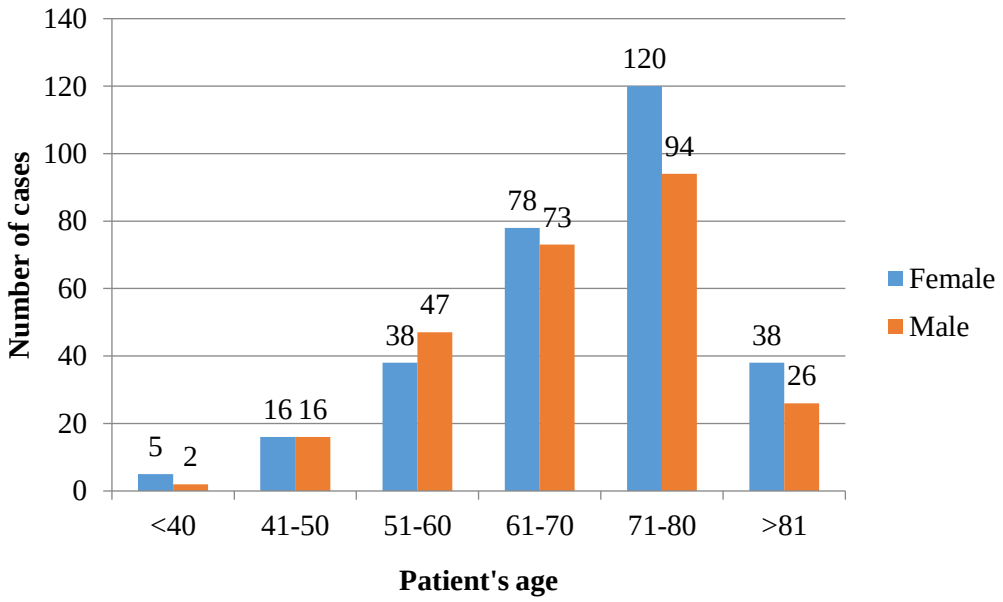

Figure 3.1 **The age and sex distribution of study group**

3.1.2 Gross findings

The tumour location distribution is presented in the Figure 3.2. The majority of the tumours (278/392, 70.9 %, 95 % CI 67.0–74.5 %) were located in the left part of large bowel. In patients younger than 50 years, left-sided tumours were present in 31/39 cases

(79.5 %, 95 % CI 64.5–89.2 %). Total colectomy was performed in one case (1/553), due to FAP, but the invasive tumour was proven to be located on the left side, within the rectum.

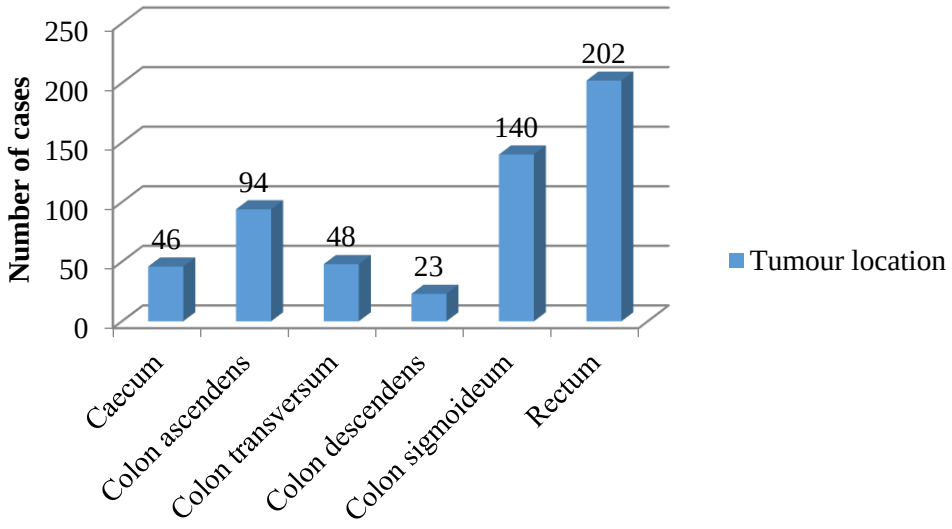

Figure 3.2 **Tumour location area in a large bowel**

In 23/553 cases (4.2 %, 95 % CI 2.8–6.2 %), simultaneous CRC was found nearby, and it was found significantly ($p < 0.01$) more often with left-sided tumours (18/23 cases, 78.3 %, 95 % CI 58.1–90.3 %). The presence of simultaneous CRC differed significantly based on age ($p = 0.04$): In patients younger than 50 years, simultaneous CRC was found 4/39 cases (10.3 %, 95 % CI 4.1–23.6 %), while in patients older than 50 years it was present in 19/495 cases (3.7 %, 95 % CI 2.4–5.7 %).

In 155/553 cases (28.0 %, 95 % CI 24.4–31.9 %), synchronous adenoma (Figures 3.3 and 3.4) was found within the operation material. Histologically, most of synchronous adenomas comprised tubular morphology, appearing in 82/155 cases (52.9 %, 95 % CI 45.1–60.6 %), while villous adenomas were found in 26/155 cases (16.8 %, 95 % CI 11.7–23.4 %) and tubulovillous adenoma was found in 46/155 cases (29.7 %, 95 % CI 23.1–37.3 %).

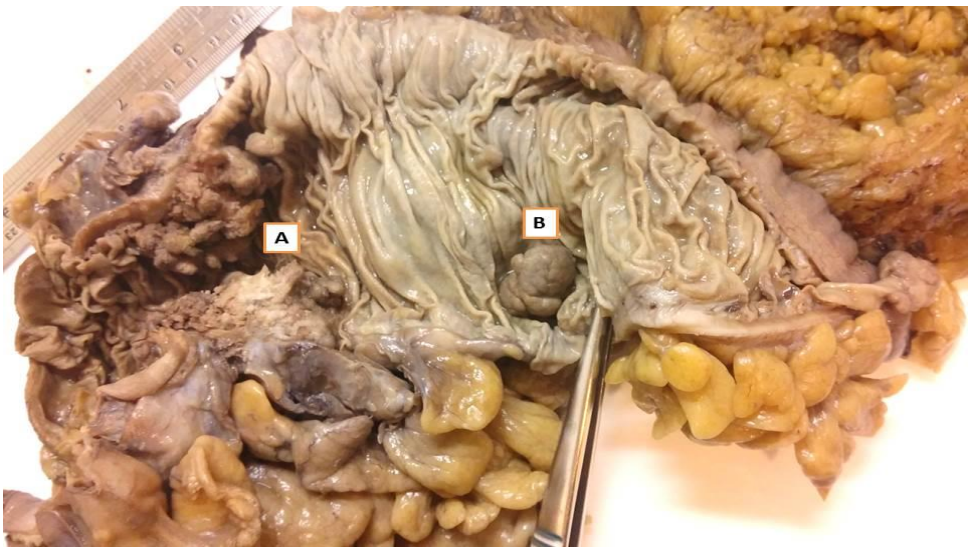

Figure 3.3 **Colorectal carcinoma with synchronous adenoma in a nearby colon**

A – primary tumour; B – synchronous adenoma.

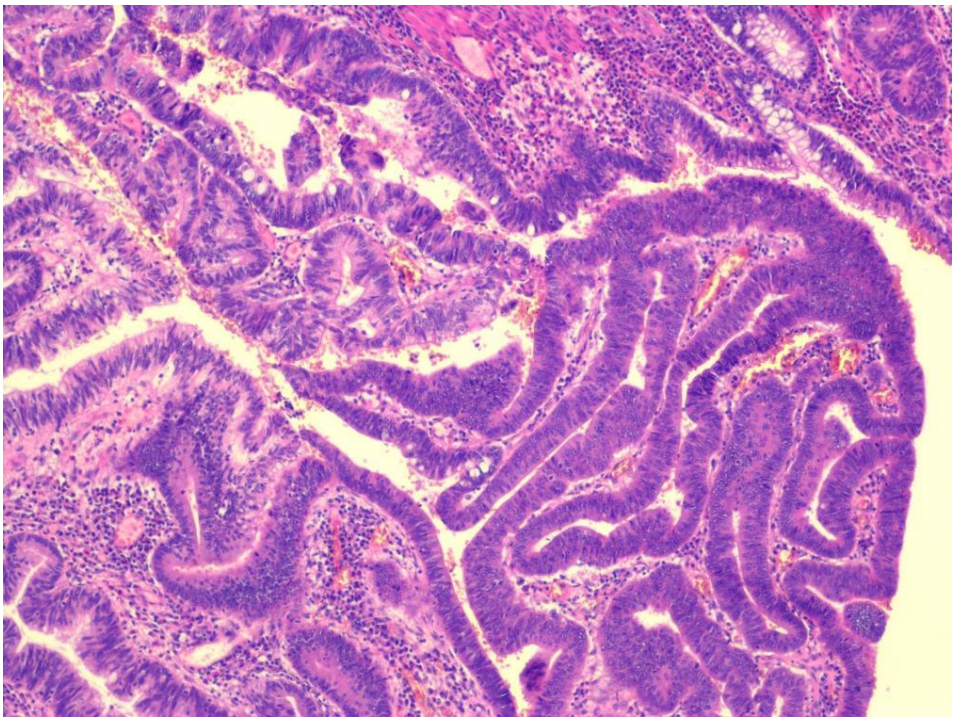

Figure 3.4 **Tubulovillous adenoma with severe dysplasia, haematoxylin and eosin staining**

Original magnification 100×.

Histological description of synchronous adenomas according to their location within the bowel is presented in Figure 3.5.

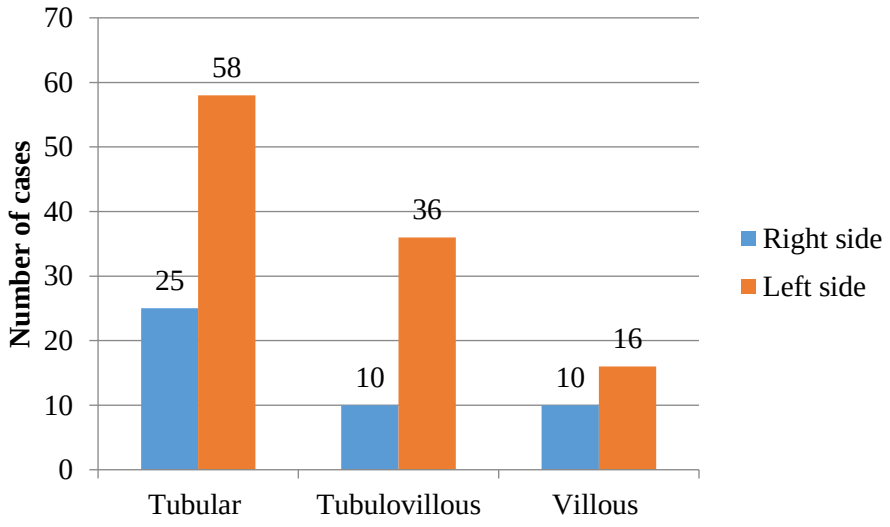

Figure 3.5 **Morphology of synchronous adenomas and their location in the left or right large bowel**

In elderly patients, synchronous adenomas were found in 147/514 cases (28.6 %, 95 % CI 24.8–32.6 %), while in patients younger than 50 years synchronous adenomas were present in 8/39 cases (20.5 %, 95 % CI 10.8–35.5 %).

The tumour volume was calculated by using the given 3D tumour measurements. Overall, precise measurements for tumours were present in 525/553 cases (94.9 %, 95 % CI 92.8–96.5 %), with an average size of 23.8 (95 % CI 19.7–27.8) cm^3, most of the cases (155/525, 29.5 %, 95 % CI 25.8–33.6 %) where in tumour volume group I (tumour volume < 5 cm^3). The tumour volume group distribution according to the operated side is presented in Figure 3.6.

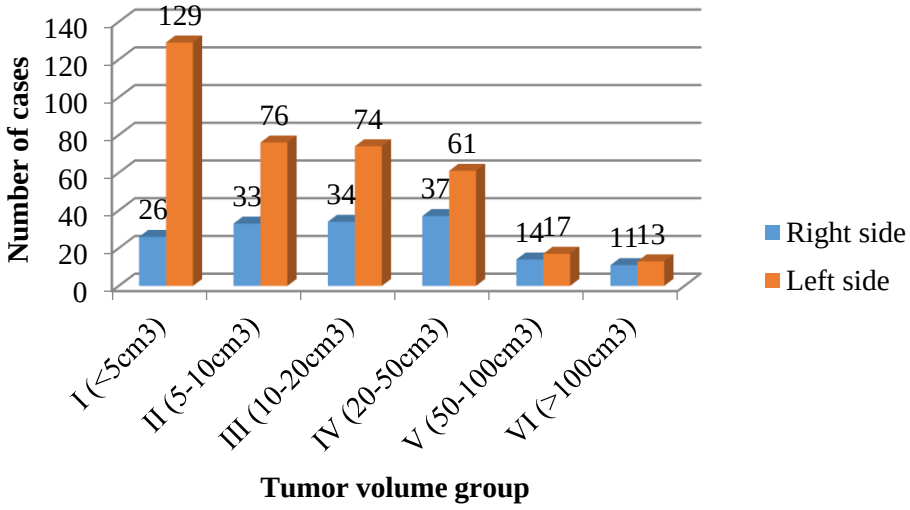

Figure 3.6 **Tumour volume groups and their relation to the right or left bowel side**

Right-sided tumours (29.4, 95 % CI 23.0–35.9 cm^3) were significantly larger than left-sided tumours (21.4, 95 % CI 16.3–26.5 cm^3) ($p < 0.001$). There were no significant differences in the tumours volume according to gender ($p = 0.41$) – males, 24.7 (95 % CI18.5–31.0) cm^3, and females 22.9 (95 % CI 17.7–28.2) cm^3 – or age ($p = 0.75$) – young patients, 26.2 (95 % CI 15.6–36.7) cm^3, and patients older than 50 years, 23.6 (95 % CI 19.3–27.9) cm^3.

Gross description of CRC revealed the presence of circular tumour (Figure 3.7) in 256/524 cases (48.8 %, 95 % CI 44.6–53.1 %) and half-circular tumour (Figure 3.8) in 268/524 cases (51.2 %, 95 % CI 46.9–55.4 %). There was ulcer in 222/313 cases (70.9 %, 95 % CI 65.7–75.7 %) and node-like in 91/313 cases (29.1 %, 95 % CI 24.3–34.3 %).

For circular tumours, there was a significant difference in the circumference ($p < 0.05$) between right- and the left-sided tumours. Out of the circular tumours, 192 (75.0 %, 95 % CI 69.3–79.9 %) were found in the left side and 64 (25.0 %, 95 % CI 20.1–30.7 %) were found on the right side.

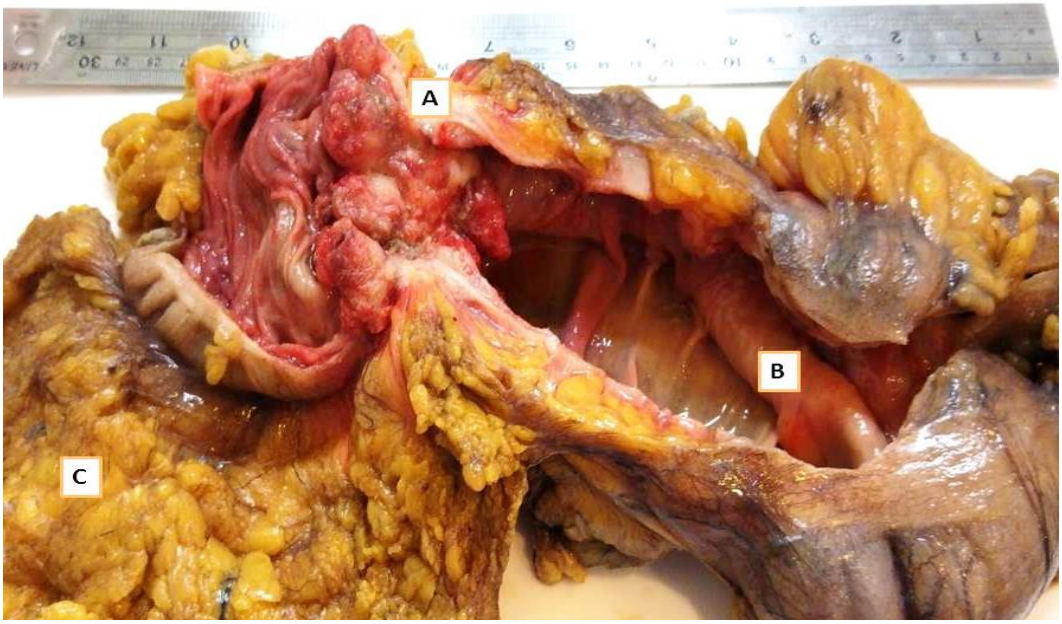

Figure 3.7 **Circular colorectal carcinoma, with bowel dilatation proximal to the disease**

A – tumour; B – dilatated bowel; C – omentum.

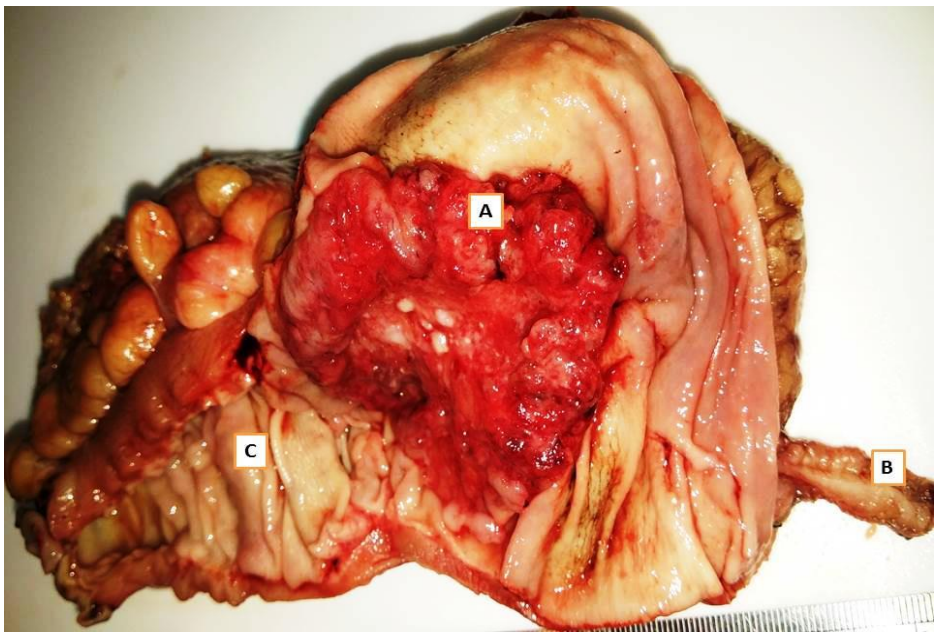

Figure3.8 **Half-circular colorectal carcinoma in the caecum region**

A – tumour; B – appendix; C – colon ascendens.

Left-sided tumours were significantly more likely to have an ulcer than right-sided tumours ($p < 0.01$) (Figure 3.9). Ulcer was present in 173/222 left-sided tumours but only 49/222 right-sided tumours.

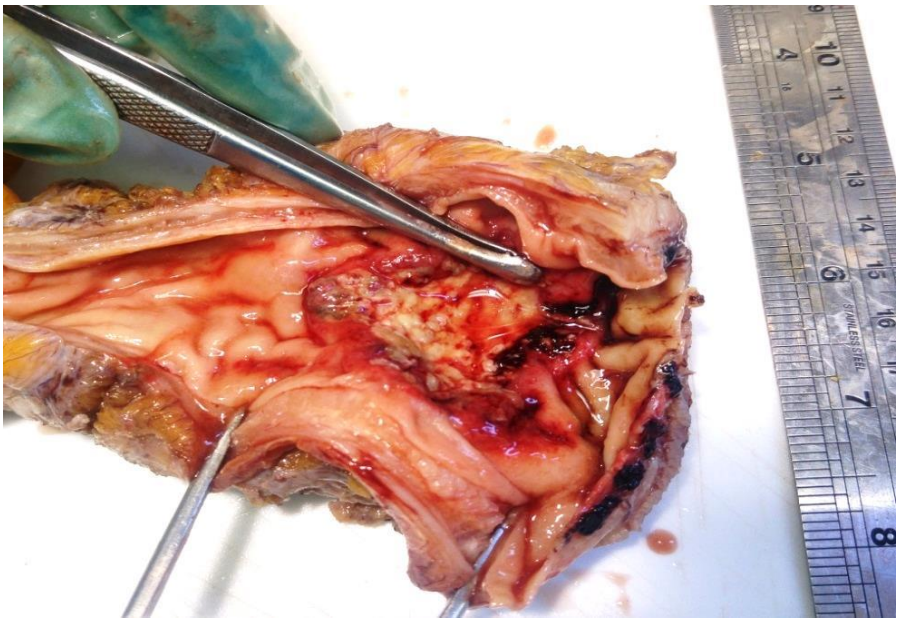

Figure 3.9 **Ulcer-like colorectal carcinoma with a fibrinous cap in gross examination of the rectum**

Perforation was present in 38/553 cases (6.9 %, 95 % CI 5.0–9.3 %) cases, and it was significantly more common in left-sided tumours ($p < 0.01$), where it was present in 29/38 cases (76.3 %, 95 % CI 60.8–87.0 %). In patients younger than 50 years, perforation was present in 4/39 cases (10.3 %, 95 % CI 4.1–23.6 %).

3.2 Morphology

3.2.1 Characteristics of primary colorectal carcinoma

Regarding the morphology, colorectal adenocarcinoma (Figure 3.10A) was found in 491/553 cases (88.8 %, 95 % CI 85.9–91.2 %), mucinous adenocarcinoma (Figure 3.10B) in 53/553 cases (9.6 %, 95 % CI 7.4–12.3 %), and primary colorectal signet ring cell carcinoma (Figure 3.10C) in only 7/553 cases (1.2 %, 95 % CI 0.6–2.6 %). There was a single case (0.2 %, 95 % CI 0.0–1.0 %) of medullary and undifferentiated carcinoma. A summary of frequency of different histological types is presented in Table 3.1.

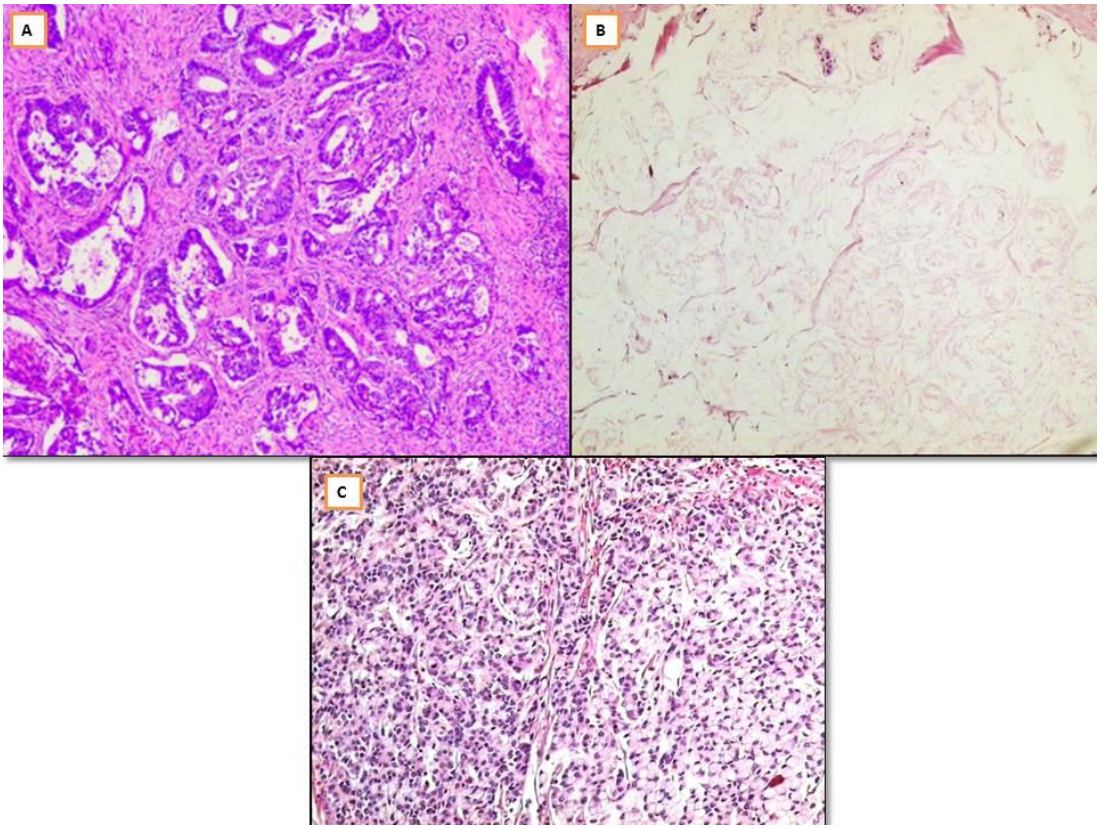

Figure 3.10 **Morphology of colorectal carcinoma in the study group**

A – moderately differentiated colorectal adenocarcinoma, haematoxylin and eosin staining, original magnification 40×; B – mucinous carcinoma; C – signet ring cell carcinoma.

Table 3.1

Morphological profile of a study group

WHO histological type	Count	Proportion, %	95 % confidence interval
Adenocarcinoma	491	88.8	85.9–91.2
Mucinous carcinoma	53	9.6	7.4–12.3
Signet-ring cell cancer	7	1.2	0.6–2.6
Medullary carcinoma	1	0.2	0.0–1.0
Undifferentiated carcinoma	1	0.2	0.0–1.0

There were no differences in the tumour morphology distribution between genders: Its overall distribution corresponded to mean results of study group. In patients younger than 50 years, only adenocarcinomas and mucinous carcinomas were detected, in 33/39 cases (84.6 %, 95 % CI 70.3–92.7 %) and 6/39 cases (15.4 %, 95 % CI 7.3–29.7 %), respectively. In patients older than 50 years, adenocarcinoma was present in 458/514 cases (89.1 %, 95 % CI 86.1–91.5 %), while mucinous carcinoma (47/514 cases, 9.1 %, 95 % CI 6.9–11.9 %) and signet ring cell carcinoma (7/514 cases, 1.4 %, 95 % CI 0.6–2.8 %) were much less common. There were also a single medullary and undifferentiated carcinoma cases in this age group.

The tumour volume was significantly different depending on the CRC histology ($p < 0.01$). The mean tumour volume was 21.0 (95 % CI 17.4–24.6) cm^3 for adenocarcinoma, 45.3 (95 % CI 20.9–69.6) cm^3 for mucinous carcinoma, and 26.8 (95 % CI 4.2–49.4) cm^3 for signet ring cell carcinoma.

Evaluating the T parameter, locally advanced tumours predominated: pT3 carcinoma (Figure 3.11C) represented 274/553 cases (49.6 %, 95 % CI 45.4–53.7 %), pT4 represented 197/553 cases (35.6 %, 95 % CI 31.7–39.7 %), and pT2 represented 66/553 cases (11.9 %, 95 % CI 9.5–14.9 %). By grade, moderately differentiated (G2) cancers constituted 354/553 cases (64.0 %, 95 % CI 59.9–67.9 %), and high-grade (G3) carcinoma was found in 142/553 cases (25.7 %, 95 % CI 22.2–29.5 %).

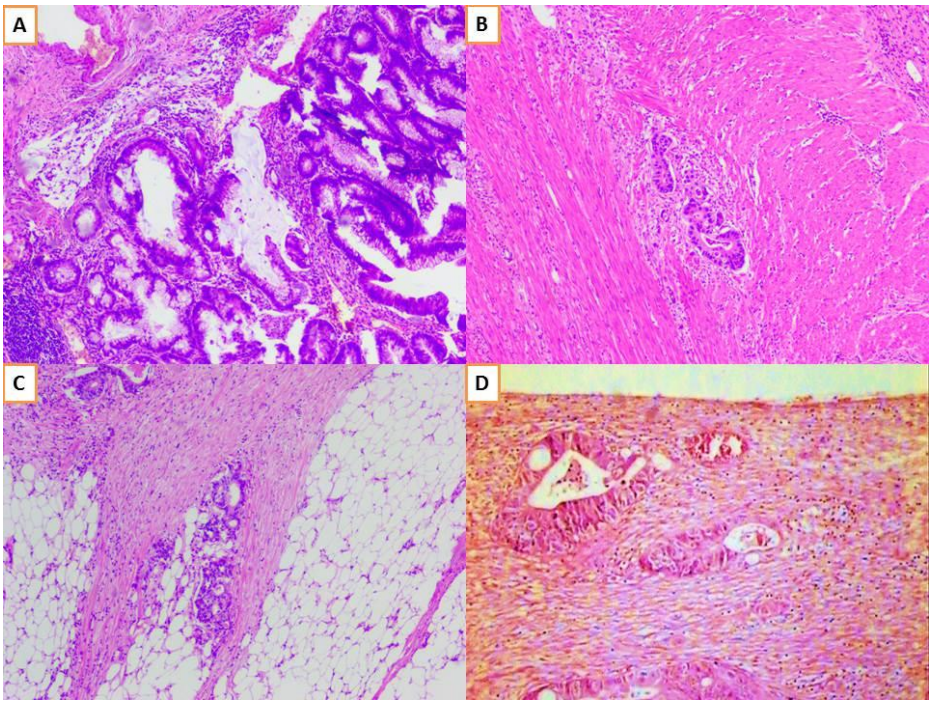

Figure 3.11 **pT in the study group**

A – pT1, haematoxylin and eosin (HE) staining, original magnification (OM) 40×; B – pT2, carcinoma complexes in muscle tissue, HE staining, OM 40×; C – pT3, colorectal carcinoma growth into mesenterial fat tissue, HE staining, OM 40×; D – pT4a, tumour complexes near the serosal surface, HE staining, OM 100×.

Regarding lymph node involvement, metastases in lymph nodes (pN+) (Figure 3.12) were found in 224/533 cases (40.5 %, 95 % CI 36.5–44.6 %). In 56/533 cases (10.1 %, 95 % CI 7.9–12.9 %) only tumour deposits (pN1c) were found within pericolonic or perirectal adipose tissue (Figure 3.13). The median number of retrieved lymph nodes was 11 (IQR 8). pTN and the tumour grade are summarized in Table 3.2. In patients younger than 50 years, tumours with high pT dominated: There was 18/39 cases of pT3 (46.2 %, 95 % CI 31.6–61.4 %) and 18/39 cases of pT4 (46.2 %, 95 % CI 31.6–61.4 %).

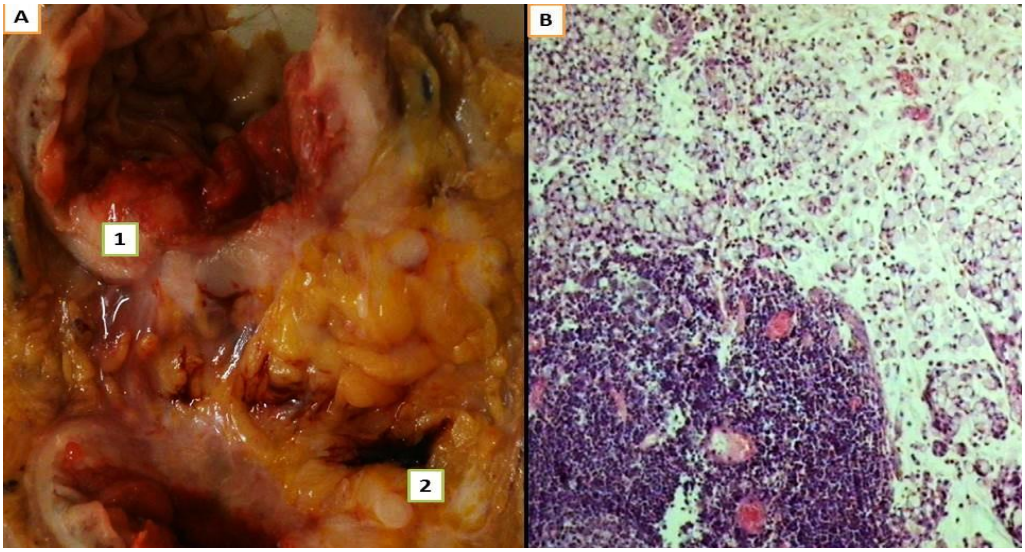

Figure 3.12 **Lymph node metastasis in colorectal carcinoma**

A – gross examination of colorectal carcinoma showing primary tumour (1) and lymph nodes with metastasis (2); B – microscopic view of signet ring cell cancer metastasis in a lymph node, haematoxylin and eosin staining, original magnification 100×.

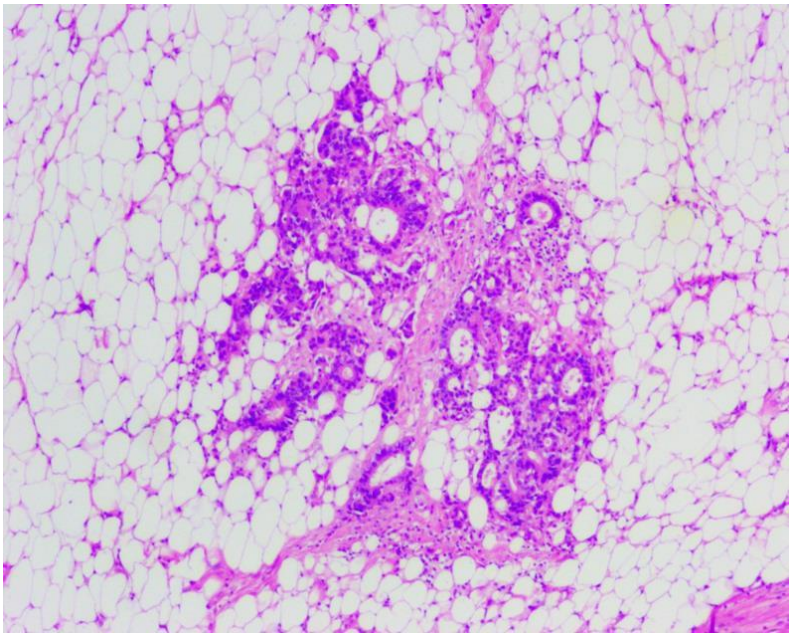

Figure 3.13 **Colorectal carcinoma deposit in mesenterial fat tissue, without the presence of lymphoid tissue (pN1c), haematoxylin and eosin staining**

Original magnification 40×.

Table 3.2

Characteristics of pTN, pG, and lymph node status in a study group

Variable	Count	Proportion, %	95 % confidence interval
pT			
pT1	16	2.9	1.8–4.6
pT2	66	11.9	9.5–14.9
pT3	274	49.6	45.4–53.7
pT4	197	35.6	31.7–39.7
pN			
pN0	273	49.4	45.2–53.5
pN1	156	28.2	24.6–32.1
pN1c	56	10.1	7.9–12.9
pN2	68	12.3	9.8–15.3
Grade			
G1	56	10.1	7.9–12.9
G2	354	64.0	59.9–67.9
G3	142	25.7	22.2–29.5
G4	1	0.2	0.0–1.0

Tumours with low differentiation (pG3) showed a significant association with the tumour volume, compared with moderate and well differentiated tumours (Kruskal-Wallis test, $p < 0.01$). The results are summarized in Table 3.3.

Table 3.3

pG and CRC volume differences

pG	Mean tumour volume, cm^3	95 % CI	p value
G1	14.6	7.4–21.8	**0.004**
G2	20.1	16.1–24.2	
G3	36.8	25.0–48.6	

There was also a significant difference according to tumour size and pT for left-sided tumours: pT3 and pT4 tumours had a greater volume ($p < 0.01$). The results are summarized in Table 3.4.

Table 3.4

CRC volume differences according tumour side and pT

pT	Mean tumour volume, cm^3; 95 % CI			
	Right side	p value	Left side	p value
pT1	39.82 [0.0–120.47]	0.051	11.11 [0.0–23.24]	**0.006**
pT2	11.93 [4.03–19.8]		8.76 [6.12–11.39]	
pT3	30.56 [20.68–40.44]		23.35 [15.27–31.43]	
pT4	29.41 [21.74–37.07]		24.42 [15.32–33.52]	

Colorectal adenocarcinoma was mostly detected at pT3 (249/491 cases, 50.7 %, 95 % CI 46.3–55.1 %) and pT4 (166/491 cases, 33.8 %, 95 % CI 29.7–38.1 %). No cases of mucinous carcinoma and signet ring cell carcinoma were found at pT1. Signet ring cell

carcinoma was mostly detected at pT4 (6/7 cases, 85.7 %, 95 % CI 48.7–97.4 %), indicating its advanced growth. The pT and pN distribution for different tumour histology is summarized in Table 3.5.

Table 3.5

Tumour spread according to its histology in a study group

Parameter	Adenocarcinoma			Mucinous carcinoma		Signet ring cell carcinoma	
	–	Count	% [95 % CI]	Count	% [95 % CI]	Count	% [95 % CI]
pT	pT1	16	3.3 [2.0–5.2]	0	0 [0.0–6.8]	0	0 [0.0–35.4]
	pT2	60	12.2 [9.6–15.4]	6	11.3 [5.3–22.6]	0	0 [0.0–35.4]
	pT3	249	50.7 [46.3–55.1]	23	43.4 [30.9–56.7]	1	14.3 [2.6–51.3]
	pT4	166	33.8 [29.7–38.1]	24	45.3 [32.7–58.5]	6	85.7 [48.7–97.4]
pN	pN0	251	51.1 [46.7–55.5]	21	39.6 [27.6–53.1]	1	14.3 [2.6–51.3]
	pN+	240	48.9 [44.5–53.3]	32	60.4 [46.9–72.4]	6	85.7 [48.7–97.4]

In the whole study group, tumour invasion in lymphatic vessels (Figure 3.14A) was found in 352/553 cases (63.6 %, 95 % CI 59.6–67.5 %). Perineural growth (Figure 3.14C) occurred in 277/553 cases (50.1 %, 95 % CI 45.9–54.2 %), while intraneural growth (Figure 3.14. B) occurred in 172/553 cases (31.1 %, 95 % CI 27.4–35.1 %).

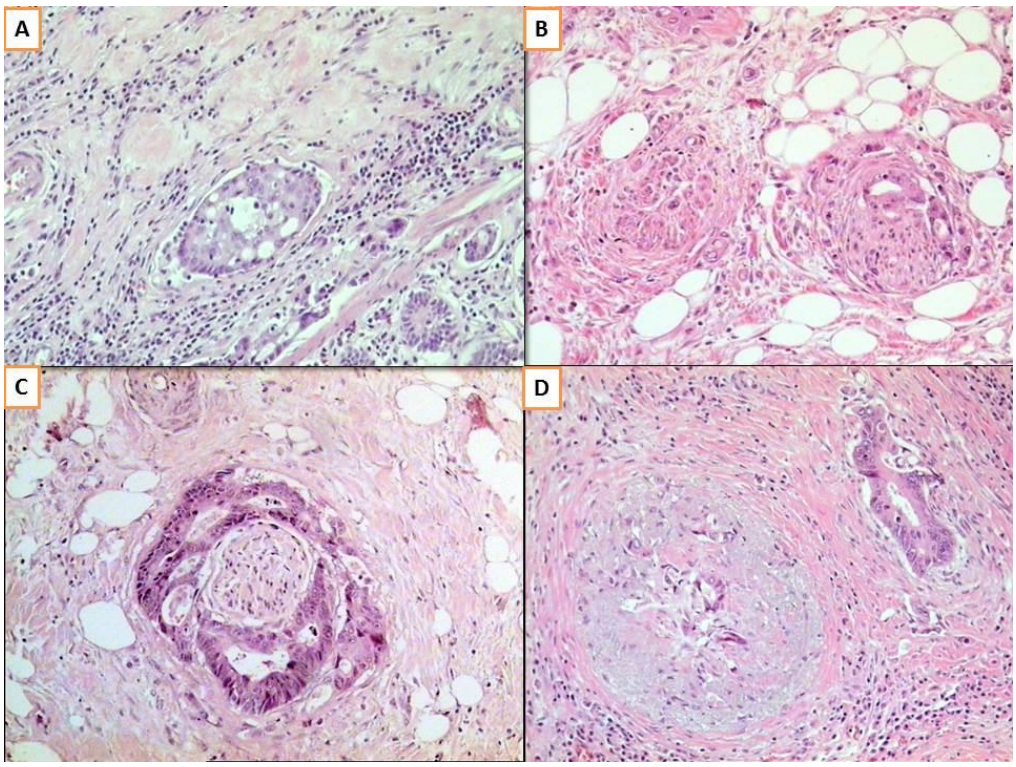

Figure 3.14 **Local structure involvement in colorectal carcinoma**

A – invasion into lymphatic vessels, haematoxylin and eosin (HE) staining, original magnification (OM) 100×; B – intraneural tumour growth, HE staining, OM 100×; C – perineural growth, HE staining, OM 100×; D – intraarterial growth, HE staining, OM 100×.

Blood vessel involvement (Figure 3.14D) was described separately. Invasion into veins occurred in 127/533 cases (23.0 %, 95 % CI 19.6–26.6 %), while invasion in arteries was less frequent (19/533 cases, 3.4 %, 95 % CI 2.2–5.3 %). Figure 3.15 provides a summary of tumour invasion into local structures. Overall, there was no significant difference in local structure involvement between right- and left-sided tumours ($p > 0.05$).

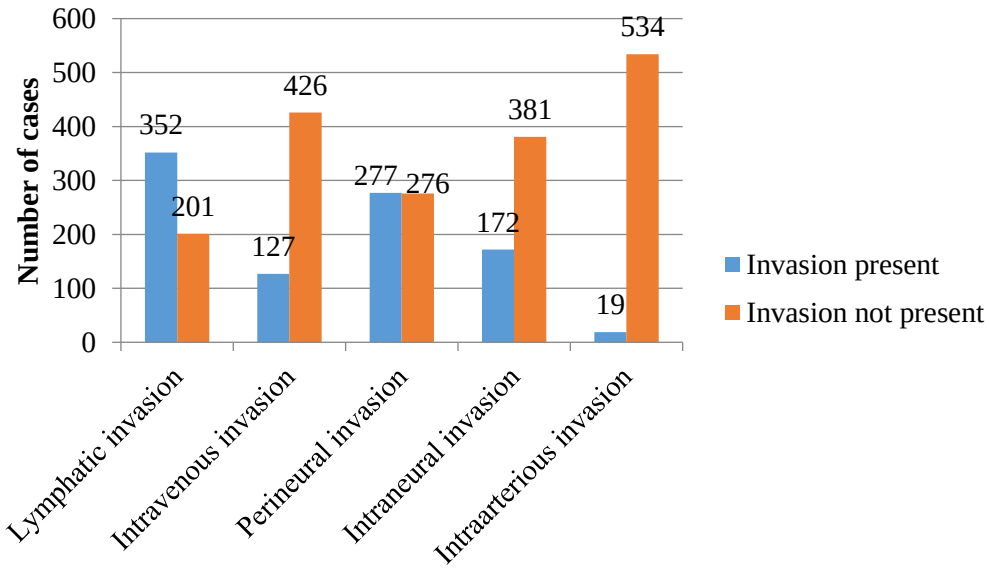

Figure 3.15 **CRC invasion into local structures**

There were no significant differences in lymphatic, intravenous, perineural, and intraneural invasion according to tumour histology ($p > 0.05$). Nevertheless, there were significant differences according to tumour histology and intraarterial growth, as signet ring cell morphology had significantly higher rates of intraarterial growth. Table 3.6 shows a summary of local structure involvement and tumour histology relationships.

Table 3.6

Local structure involvement according to tumour histology in a study group

Type of invasion	Adenocarcinoma		Mucinous carcinoma		Signet ring cell carcinoma		p value
	Count	% [95 % CI]	Count	% [95 % CI]	Count	% [95 % CI]	
Lymphatic invasion							
Present	309	62.9 [58.6–67.1]	35	66.0 [52.6–77.3]	6	85.7 [48.7–97.4]	0.14
Absent	182	37.1 [32.9–41.4]	18	34.0 [22.7–47.4]	1	14.3 [2.6–51.3]	
Intravenous invasion							
Present	112	22.8 [19.3–26.7]	10	18.9 [10.6–31.4]	4	57.1 [25.1–84.2]	0.23
Absent	379	77.2 [73.3–80.7]	43	81.1 [68.6–89.4]	3	42.9 [15.8–74.9]	
Intraarterial invasion							
Present	13	2.6 [1.5–4.5]	3	5.7 [1.9–15.4]	2	28.6 [8.2–64.1]	**0.000**
Absent	478	97.4 [95.5–98.5]	50	94.3 [84.6–98.1]	5	71.4 [35.9–91.2]	
Perineural invasion							
Present	248	50.5 [46.1–54.9]	23	43.4 [30.9–56.7]	5	71.4 [35.9–91.2]	0.99
Absent	243	49.5 [45.1–53.9]	30	56.6 [43.3–69.1]	2	28.6 [8.2–64.1]	
Intraneural invasion							
Present	154	31.4 [27.4–35.6]	12	22.6 [13.4–35.5]	5	71.4 [35.9–91.2]	0.51
Absent	337	68.6 [64.4–72.6]	41	77.4 [64.5–86.6]	2	28.6 [8.2–64.1]	

Overall, tumour necrosis was present in 295 cases, with most of the carcinomas comprising a moderate amount of necrosis, which was present in 146/295 cases (49.5 %, 95 % CI 43.8–55.2 %), while extensive amount of necrosis was observed only in 45/295 cases (15.3 %, 95% CI 11.6–19.8 %). The mean extent of necrosis was significantly different in terms of pT ($p = 0.04$), pN ($p = 0.02$), and pG ($p < 0.01$), as it was more extensive in tumours with a higher stage and lower differentiation. The full results are described in Table 3.7. Perforation within the tumour showed no association with extent or presence of necrosis ($p = 0.33$). There were also no significant differences in the extent of necrosis and histological

type of CRC. In patients younger than 50 years, necrosis was present in 21/39 cases (53.8 %, 95 % CI 38.6–68.4 %) cases, with no significant difference in the extent of necrosis (p > 0.05).

Table 3.7

Extent of necrosis (mean %) in CRC according to pT, pN and tumour grade

Parameter	%	95 % CI	p
pT			
pT1	12.5	0.0–107.8	**0.04**
pT2	9.6	6.1–13.1	
pT3	14.5	12.5–16.4	
pT4	16.6	13.9–19.4	
pN			
pN0	12.8	10.7–14.8	**0.02**
pN+	16.7	14.2–19.2	
pG			
pG1	9.3	5.2–13.3	**0.004**
pG2	13.4	11.8–15.0	
pG3	19.9	16.1–23.7	

3.2.2 Characteristics of peritumoural inflammation

Analysis of an inflammatory reaction within peritumoural tissue showed weak inflammation in 240/553 cases and complete absence of inflammation in 52/553 cases. After re-distribution based on the Klintrup-Mäkinen score, 292/533 cases (52.8 %, 95 % CI 48.6–56.9 %) had low-grade inflammation (Figure 3.16).

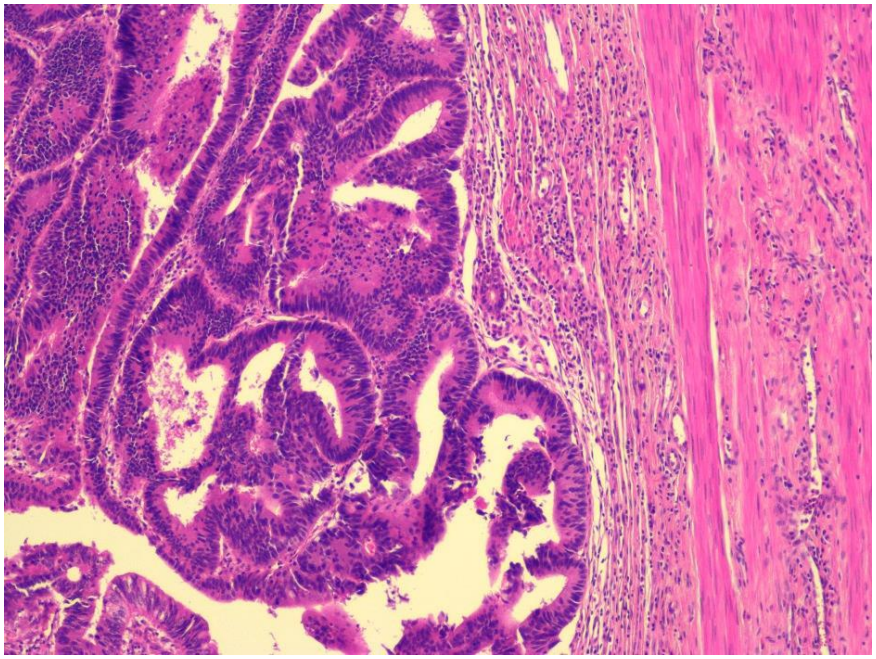

Figure 3.16 **Low-grade peritumoural inflammation in colorectal carcinoma, haematoxylin and eosin staining**

Original magnification 100×.

High-grade inflammation (including 204 cases of moderate inflammation and 57 cases of severe inflammation) was observed in total in 261/533 cases (47.2 %, 95 % CI 43.1–51.4 %) (Figure 3.17). These groups showed no statistically significant age differences ($p = 0.13$). The mean age of patients presenting low-grade inflammation was 68.1 ± 10.6 years (95 % CI 66.9–69.3), contrasting with 69.6 ± 11.0 years (95 % CI 68.2–70.9) in those with high-grade inflammation.

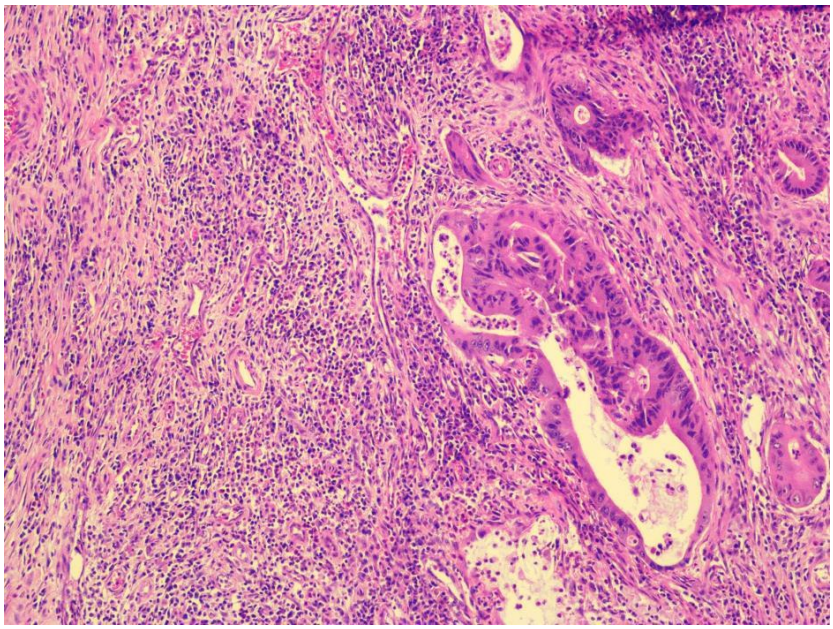

Figure 3.17 **Colorectal carcinoma with a moderate peritumoural inflammation, haematoxylin and eosin staining**

Original magnification 100×.

Regarding tumour volume, there was no significant difference ($p = 0.46$) between tumours with high-grade inflammation (23.6, 95 % CI 18.3–28.8) cm^3) and low-grade inflammation (23.9, 95 % CI 17.9–30.0) cm^3). There were significant differences (Table 3.8) regarding pT distribution ($p = 0.002$) and status of regional lymph nodes, reflected by pN ($p < 0.001$) in relation to low- and high-grade inflammation. However, there was no significant difference between the degree of inflammation and pG (p=0.07).

Table 3.8

Characteristics of pT, pN and pG in a study group

Parameter	Low-grade inflammation		High-grade inflammation		p
	Count	F, % [95 % CI]	Count	F, % [95 % CI]	
pT					
pT1	6	2.1 [0.9–4.4]	10	3.8 [2.1–6.9]	**0.002**
pT2	29	9.9 [7.0–13.9]	37	14.2 [10.5–18.9]	
pT3	132	45.2 [39.6–50.9]	142	54.4 [48.3–60.3]	
pT4	125	42.8 [37.3–48.5]	72	27.6 [22.5–33.3]	

Table 3.8 continued

Parameter	Low-grade inflammation		High-grade inflammation		p
	Count	F, % [95 % CI]	Count	F, % [95 % CI]	
pN					
pN0	118	40.4 [34.9–46.1]	155	59.4 [53.3–65.2]	**< 0.001**
pN+	174	59.6 [53.9–65.1]	106	40.6 [34.8–46.7]	
Grade of the carcinoma (G)					
G1	26	8.9 [6.1–12.8]	30	10.3 [7.3–14.3]	0.07
G2	178	61.0 [55.3–66.4]	176	60.3 [54.6–65.7]	
G3	87	29.8 [24.8–35.3]	55	18.8 [14.8–23.7]	
G4	1	0.3 [0.0–2.1]	0	0.0 [0.0–1.6]	

Assessing the morphological manifestations of the invasive growth, tumours surrounded by low-grade peritumoural inflammation significantly more frequently featured invasion in lymphatic vessels (p = 0.003), as well as intraarterial (p = 0.012), intravenous (p = 0.017), perineural (p = 0.001), and intraneural (p = 0.01) growth (Figures 3.18 and 3.19).

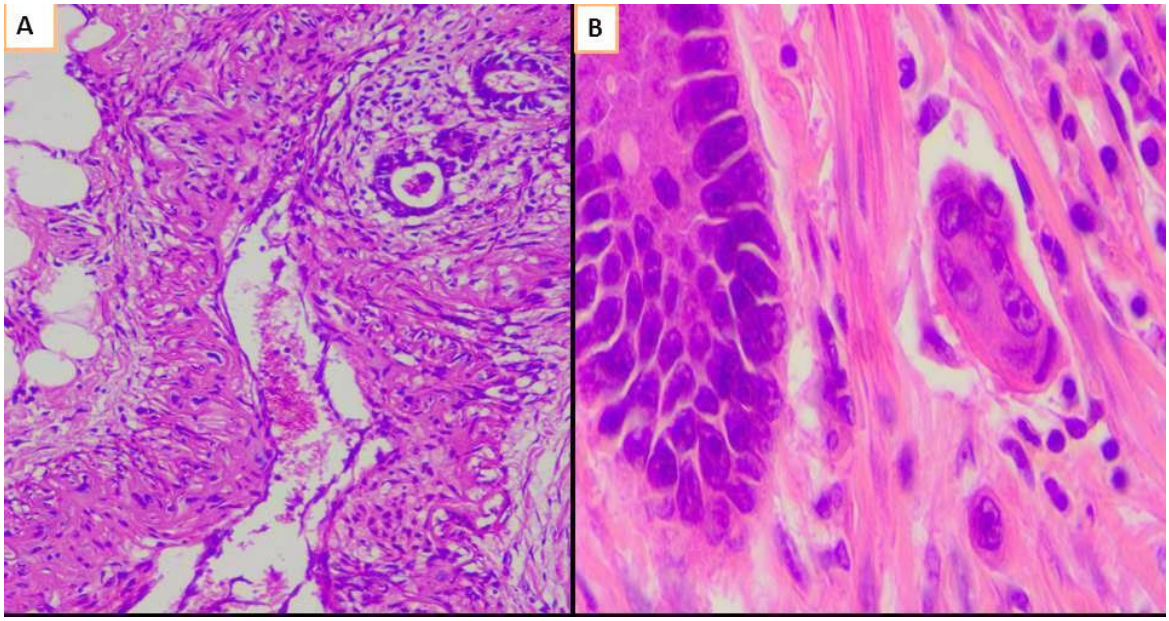

Figure 3.18 **Colorectal carcinoma invasion into local structures in tumours with low-grade inflammation**

A – invasion into an artery wall, with no colorectal carcinoma in its lumen, haematoxylin and eosin (HE) staining, original magnification (OM) 100×; B – colorectal carcinoma complex in a lymphatic vessel, HE staining, OM 400×.

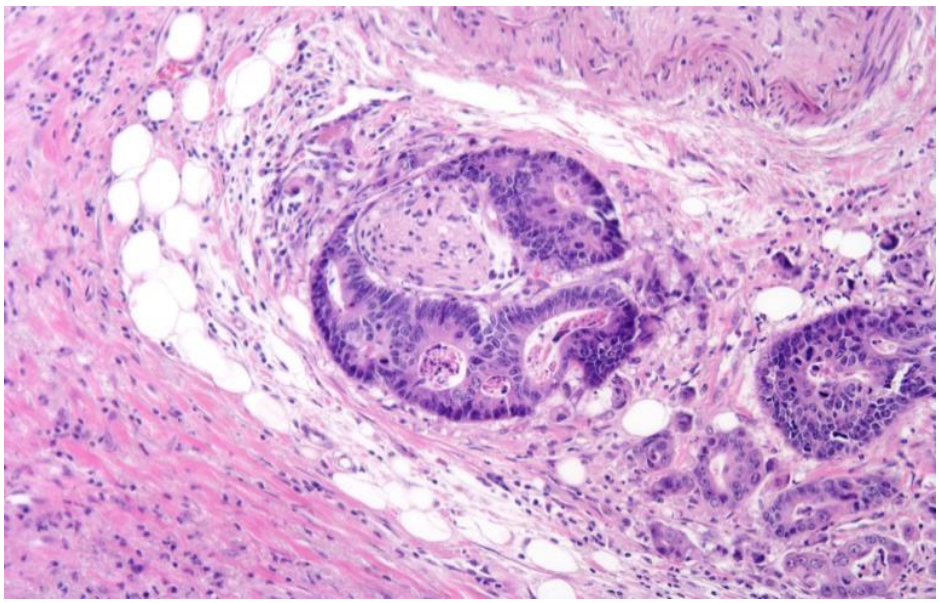

Figure 3.19 **Perineural invasion in presence of low-grade inflammation in colorectal carcinoma, haematoxylin and eosin staining**

Original magnification 40×.

The degree of inflammation was not significantly different between right- and left-sided tumours (p = 0.18) or by the presence (p = 0.63) or extent (p = 0.11) of tumour necrosis. The relations between peritumoural inflammation and tumour growth into a local structure, tumour localization, and the extent of necrosis is described in Table 3.9.

Table 3.9

Characteristics of a CRC local invasion, localisation and extent of necrosis.

Parameter	Low-grade inflammation		High-grade inflammation		p
	Count	F, % [95 % CI]	Count	F, % [95 % CI]	
Tumour localisation					
Left side	77	26.4 [68.3–78.3]	81	31.0 [25.7–36.9]	0.18
Right side	215	73.6 [21.7–31.7]	180	69.0 [63.1–74.3]	
Invasion					
Perineural	167	57.2 [51.5–62.7]	110	42.1 [36.3–48.2]	**0.001**
Intraneural	103	35.3 [30.0–40.9]	69	26.4 [21.5–32.1]	**0.01**
Lymphatic	204	69.9 [64.4–74.8]	148	56.7 [50.6–62.6]	**0.003**
Into veins	77	26.4 [21.6–31.7]	50	19.2 [14.8–24.4]	**0.017**
Into arteries	15	5.1 [3.1–8.3]	4	1.5 [0.6–3.9]	**0.012**
Necrosis					
Present	153	52.4 [46.7–58.1]	142	54.4 [48.3–60.3]	0.63
Absent	139	47.6 [41.9–53.3]	119	45.6 [39.7–51.7]	
Focal	58	37.9 [30.6–45.8]	46	32.4 [25.2–40.5]	0.11
Moderate	78	51.0 [43.1–58.8]	68	46.6 [38.7–54.6]	
Extensive	17	11.1 [7.0–17.1]	28	19.7 [14.0–27.0]	

CLR (Figure 3.20) was found in 193/553 cases (34.9 %, 95 % CI 31.0–39.0 %) cases. Most of these tumours (165/193, 85.5 %, 95 % CI 79.8–89.7 %) showed a low density of lymphoid follicles.

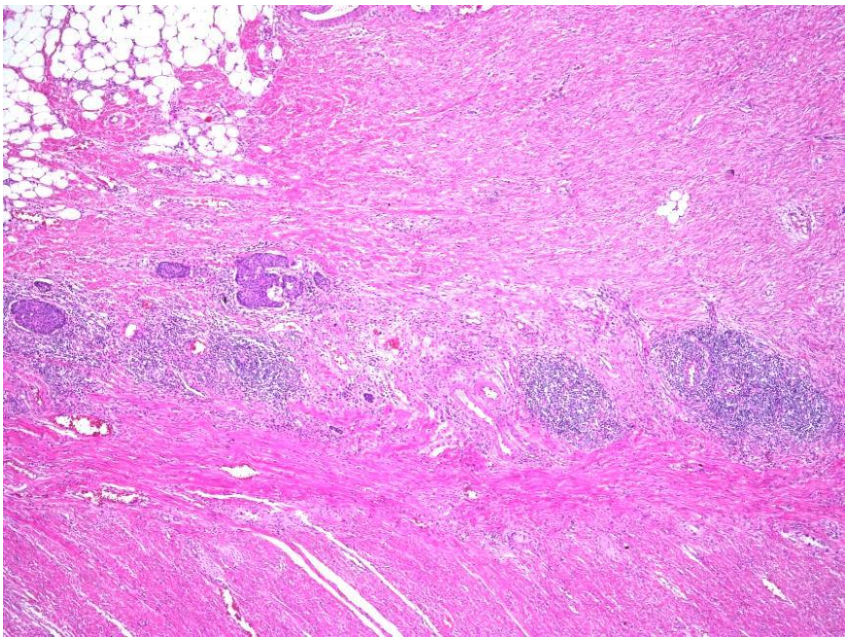

Figure 3.20 **Crohn's-like lymphoid reaction in colorectal carcinoma, haematoxylin and eosin staining**

Original magnification 40×.

There were no significant differences in the distribution of CLR density by cancer location (right versus left side of the large bowel), pT, pN, grade, tumour invasion into surrounding structures (blood or lymphatic vessels, nerves), or necrosis (Table 3.10).

Table 3.10

CLR characteristics in a study group

Parameter	CLR low density		CLR high density		p
	Count	F, % [95 % CI]	Count	F, % [95 % CI]	
Tumour localization					
Right side	57	29.5 [23.5–36.3]	12	6.2 [3.6–10.5]	0.39
Left side	108	55.0 [48.9–62.8]	16	8.3 [5.2–13.0]	
pT					
pT1	3	1.5 [0.5–4.5]	0	0 [0.0–1.9]	0.86
pT2	16	8.3 [5.2–13.0]	3	1.5 [0.5–4.5]	
pT3	103	53.4 [46.3–60.3]	19	9.8 [6.4–14.9]	
pT4	43	22.3 [17.0–28.7]	6	3.1 [1.4–6.6]	
pN					
pN0	87	45.1 [38.2–52.1]	19	9.8 [6.4–14.9]	0.97
pN+	64	33.2 [26.9–40.1]	7	3.6 [1.8–7.3]	
pN1c	14	7.3 [4.4–11.8]	2	1.0 [0.3–3.7]	
pG					
pG1	15	7.8 [4.8–12.4]	6	3.1 [1.4–6.6]	0.95
pG2	116	60.1 [53.1–66.7]	16	8.3 [5.2–13.0]	
pG3	34	17.6 [12.9–23.6]	6	3.1 [1.4–6.6]	

Table 3.10 continued

Parameter	CLR low density			CLR high density		p
	Count	F, % [95 % CI]		Count	F, % [95 % CI]	
Necrosis						
Focal	36	18.6 [13.8–24.7]		9	4.6 [2.5–8.6]	0.81
Moderate	40	20.7 [15.6–26.9]		4	2.1 [0.8–5.2]	
Extensive	14	7.2 [4.4–11.8]		3	1.5 [0.5–4.5]	
Absent	75	38.9 [32.3–45.9]		12	6.2 [3.6–10.5]	
Invasions						
Lymphatic invasion	Present	95	49.2 [42.2–56.2]	15	7.8 [4.8–12.4]	0.69
	Absent	70	36.3 [29.8–43.3]	13	6.7 [4.0–11.2]	
Intravenous	Present	28	14.5 [10.2–20.2]	3	1.5 [0.5–4.5]	0.40
	Absent	137	71.0 [64.2–76.9]	25	12.9 [8.9–18.4]	
Perineural	Present	76	39.4 [32.8–46.4]	9	4.6 [2.5–8.6]	0.17
	Absent	89	46.1 [39.2–53.2]	19	9.8 [6.4–14.9]	
Intraneural	Present	43	22.3 [17.0–28.7]	4	2.1 [0.8–5.2]	0.18
	Absent	122	63.2 [56.2–69.7]	24	12.4 [8.5–17.8]	

In contrast, there were significant differences between the presence and absence of CLR regarding the manifestations of invasive growth (Table 3.11).

Table 3.11

Association between presence of CLR and manifestations of invasive growth of colorectal carcinoma

Type of invasion	Number of cases; frequency, % [95 % CI]		p
	CLR present	CLR absent	
Lymphatic invasion	110 56.9 [49.9–63.8]	242 67.2 [62.2–71.9]	**< 0.001**
Perineural invasion	85 44.0 [37.2–51.1]	192 53.3 [48.2–58.4]	**< 0.001**
Intraneural invasion	47 27.3 [21.2–34.3]	125 72.7 [65.6–78.8]	**< 0.001**
Intravenous invasion	31 24.4 [17.8–32.6]	96 75.6 [67.4–82.2]	**< 0.001**

Inflammatory cell subpopulation analysis (Figure 3.21) highlighted several significant associations between the density of certain tumour-infiltrating cells and the invasive capacity of the carcinoma, reflected by the manifestation of invasive growth (present vs. absent). There were significant differences regarding the number of neutrophils in cases with perineural ($p = 0.04$) and lymphatic ($p = 0.01$) invasion (Table 3.12). In addition, the number of lymphocytes significantly differed in tumours with perineural ($p < 0.01$) and lymphatic ($p = 0.03$) invasion. Eosinophil absence was significantly associated with perineural invasion ($p = 0.01$) and invasion into lymphatic vessels ($p < 0.01$).

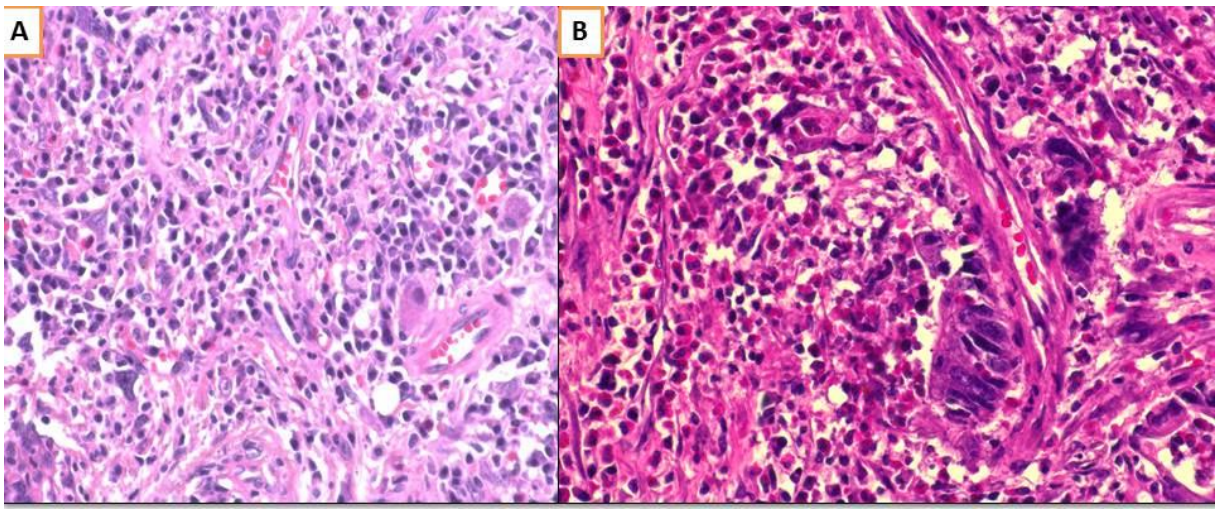

Figure 3.21 **Inflammatory cell composition in the peritumoural area**

A – Inflammatory cell composition in the peritumoural area, haematoxylin and eosin (HE) staining, original magnification (OM) 100×; B – predominance of eosinophilic leukocytes in the peritumoural area, HE staining, OM 400×.

Table 3.12

Inflammatory cell subpopulation analysis: neutrophil leucocytes

Type of identified invasion	Density of inflammatory cells: number of cases; proportion, % [95 % CI]				
	Absent	**Mild**	**Moderate**	**High**	**p value**
Neutrophilic leukocytes					
Perineural	202; 72.9 [67.4 – 77.8]	52; 18.8 [14.6–23.8]	17; 6.1 [3.8–9.7]	6; 2.2 [0.9–4.8]	**0.04**
Intraneural	126; 73.3 [66.2–79.3]	32; 18.6 [13.5–25.1]	12; 7.0 [3.9–11.9]	2; 1.2 [0.1–4.4]	0.13
Lymphatic	265; 75.3 [70.5–79.5]	57; 16.2 [12.7–20.4]	23; 6.5 [4.4–9.7]	7; 2.0 [0.9–4.1]	**0.01**
Intravenous	103; 81.1 [73.4–87.0]	16; 12.6 [7.8–19.6]	4; 3.2 [1.0–8.1]	4; 3.2 [1.0–8.1]	0.27
Intraarterial	16; 84.2 [61.6–95.3]	2; 10.5 [1.7–32.6]	0; 0.0 [0.0–19.8]	1; 5.3 [0.0–26.5]	0.48

Lymphocyte density differed in tumours with perineural ($p < 0.01$) and lymphatic ($p = 0.03$) invasion, but showed no significant difference in terms of vascular invasion and intraneural tumour growth. Table 3.13. shows a summary of lymphocyte density in a presence of invasions.

Table 3.13

Inflammatory cell subpopulation analysis: lymphocytes

Type of identified invasion	Density of inflammatory cells: number of cases; proportion, % [95 % CI]				
	Absent	**Mild**	**Moderate**	**High**	**p value**
Lymphocytes					
Perineural	32; 11.6 [8.3–15.9]	147; 53.1 [47.2–58.9]	87; 31.4 [26.2–37.1]	11; 4.0 [2.2–7.1]	**0.003**
Intraneural	20; 11.6 [7.6–17.4]	92; 53.5 [46.0–60.8]	53; 30.8 [24.4–38.1]	7; 4.1 [1.8–8.3]	0.052
Lymphatic	39; 11.1 [8.2–14.8]	184; 52.3 [47.1–57.4]	115; 32.7 [28.0–37.7]	14; 4.0 [2.3–6.6]	**0.03**
Intravenous	14; 11.0 [6.6–17.8]	66; 52.0 [43.4–60.5]	43; 33.9 [26.2–42.5]	4; 3.2 [1.0–8.1]	0.88
Intraarterial	3; 15.8 [4.7–38.4]	13; 68.4 [45.8–84.8]	3; 15.8 [4.7–38.4]	0; 0.0 [0.0–19.8]	0.09

Eosinophil absence was significantly associated with perineural invasion ($p = 0.01$) and invasion into lymphatic vessels ($p < 0.01$). However, no other local structure invasion was associated with an increase or decrease in the eosinophil density. Table 3.14 provides a full summary of the eosinophil density relationship to local structure invasion.

Table 3.14.

Inflammatory cell subpopulation analysis: eosinophilic leucocytes

Type of identified invasion	Density of inflammatory cells: number of cases; proportion, % [95 % CI]				
	Absent	**Mild**	**Moderate**	**High**	**p value**
Eosinophilic leukocytes					
Perineural	207; 74.7 [69.3–79.5]	64; 23.1 [18.5–28.4]	6; 2.2 [0.9–4.8]	0; 0.0 [0.0–1.7]	**0.01**
Intraneural	128; 74.4 [67.4–80.4]	39; 22.7 [17.0–29.5]	5; 2.9 [1.1–6.8]	0; 0.0 [0.0–2.6]	0.31
Lymphatic	264; 75.0 [70.2–79.3]	82; 23.3 [19.2–28.0]	6; 1.7 [0.7–3.8]	0; 0.0 [0.0–1.3]	**0.008**
Intravenous	92; 72.4 [64.1–79.5]	32; 25.2 [18.4–33.4]	3; 2.4 [0.5–7.0]	0; 0.0 [0.0–3.5]	0.21
Intraarterial	17; 89.5 [67.4–98.3]	2; 10.5 [1.7–32.6]	0; 0.0 [0.0–19.8]	0; 0.0 [0.0–19.8]	0.36

3.3 Characteristics of immunohistochemistry markers in colorectal carcinoma

Immunohistochemistry was performed to 124 consecutive CRC cases, from year 2011, to illustrate the presence of CD8+ cytotoxic cells, epithelial mesenchymal transition and MMR proteins within study group. In these 124 cases, overall distribution corresponded to mean results of study group ($p>0.05$). There were 46.8 % [38.2–55.5] male and 53.2 % [44.5–61.8] female patients, with mean age 68.9 ±11.09 [67.0–70.9], median 71 (IQR=15). Patients younger than 50 years were found in 6.4 % [3.3–12.2] of cases. By morphology, colorectal adenocarcinoma was presented in 91.1 % [84.8–94.9], mucinous carcinoma in 7.3 % [3.9–13.2] of cases. Overall, pT3 CRC was found in 50.0 % [41.3–58.7] and pT4 in 33.4 % [26.1–42.6] of cases within this group and pN0 CRC were present in 48.4 % [39.8–57.1] and pN+ tumours within 51.6 % [42.9–60.2] of selected cases.

3.3.1 Cytotoxic T cells (CD8)

There were significantly more CD8+ T cells ($p < 0.01$) in the invasive margin or peritumoural area compared with the tumour centre or intratumoural region. Within the peritumoural area, there was an average of 148 CD8+ T cells (range 4–417) (Figure 3.22), but within the intratumoural region, there was an average of 18 CD8+ T cells (range 0–120) (Figure 3.23).

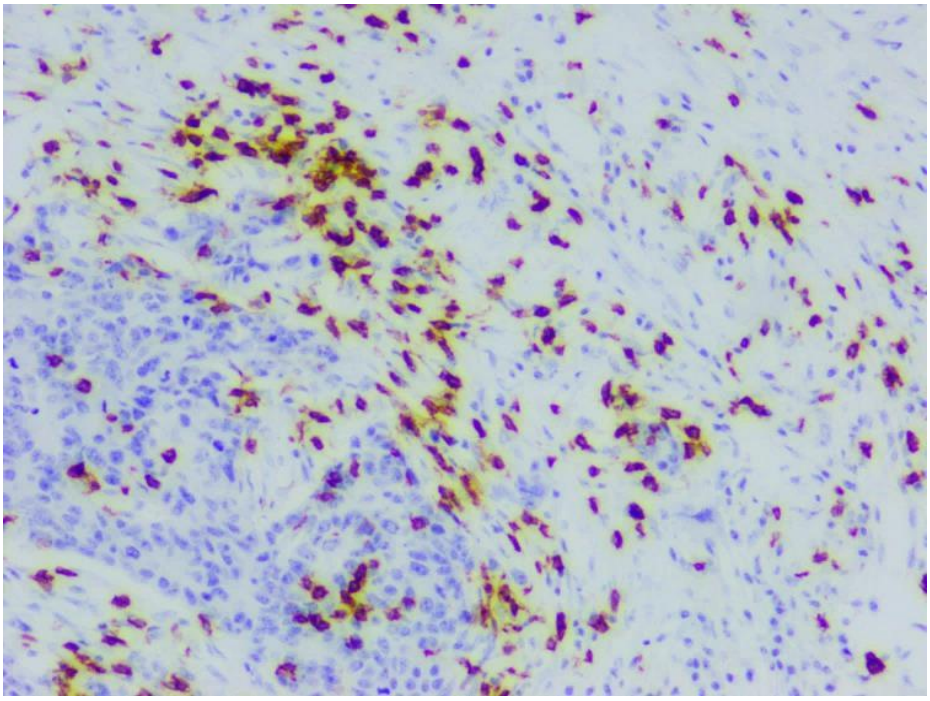

Figure 3.22 **CD8+ T cells in the peritumoural area, immunoperoxidase staining**

Original magnification 100×.

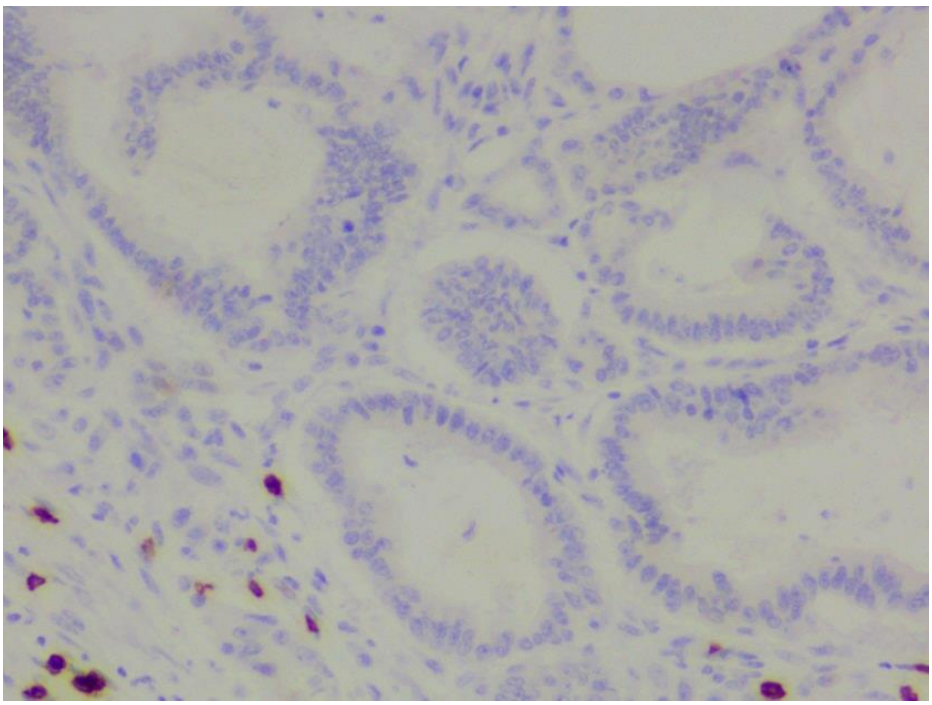

Figure 3.23 **Colorectal carcinoma with a low number of intratumoural CD8+ T cells, immunoperoxidase staining**

Original magnification 100×.

Regarding tumour histology, there were no significant differences in the number of CD8+ T cells amount in the peritumoural (p = 0.23) or intratumoural (p=0.41) areas. The mean number of peritumoural CD8 + T cells was 149.5 (95 % CI 133.0–165.9) in adenocarcinomas, 147.0 (95 % CI 89.0–205.0) in mucinous carcinomas, and 49.0 (95 % CI 0.0–620.8) in signet ring cell carcinomas. The mean number of intratumoural CD8+ T cells was 18.3 (95 % CI 13.9–22.7) in adenocarcinomas, 11.7 (95 % CI 4.0–19.5) in mucinous carcinomas, and 5.0 (95 % CI 0.0–30.0) in signet ring cell carcinomas.

There was a significant correlation between the peritumoural CD8+ T cell count and pT (r = -0.2, p = 0.03), but no significant correlation between the number of intratumoural CD8+ T cells and pT (p = 0.13). There was a significant difference between the number of peritumoural CD8+ T cells in tumours without lymph node metastasis (pN0) and in tumours with metastasis in lymph nodes (pN+) or separate tumour nodes in fat tissue (p = 0.05). There was no significant difference between the number of peritumoural and intratumoural CD8+ T cells according to the tumour grade (p = 0.93 and p = 0.10, respectively). Table 3.15 presents the CD8+ T cell relations to pT, pN, and tumour grade.

Table 3.15

Peritumourous and intratumourous CD8+ cytotoxic cell amount in relation to pT, pN and tumour grade

Parameter	PT CD8+	p value	IT CD8+	p value
pT				
pT1	198.5 [82.6–314.4]	**0.03**	21.5 [0.0–129.5]	0.13
pT2	153 [111.6–193.6]		26.0 [9.7–42.2]	
pT3	163 [141.5–185.0]		18.4 [12.7–24.1]	
pT4	121 [95.7–146.9]		12.9 [7.0–18.9]	
pN				
pN0	163.2 [140.2–186.2]	**0.05**	20.5 [13.9–27.1]	0.16
pN+	136.6 [113.4–160.0]		15.5 [9.5–21.6]	
pN1c	120.0 [65.8–174.3]		12.4 [5.1–19.7]	
pG				
pG1	100.0 [14.4–186.2]	0.93	12.0 [0.0–50.8]	0.10
pG2	151.6 [132.0–171.2]		15.6 [11.2–19.9]	
pG3	142.3 [114.9–169.8]		22.8 [13.2–32.4]	

Regarding the cytotoxic T cell distribution, there was a significant difference in the number of peritumoural CD8 cells in CRC with and without perineural ($p = 0.02$) and intraneural ($p = 0.02$) invasions, but there was no significant correlation with other local structure involvement (Table 3.16). The number of intratumoural CD8+ T cells did not show any significant correlation with local structure invasions or other parameters.

Table 3.16

Local structure involvement and CD8+ cell amount

Parameter		PT CD8+	p value	IT CD8+	p value
Lymphatic invasion	Present	136.2 [112.4–159.9]	0.21	17.3 [10.2–24.5]	0.39
	Absent	155.9 [135.1–176.6]		17.8 [12.9–22.6]	
Intravenous invasion	Present	151.3 [77.5–225.1]	0.81	23.4 [0.0–52.4]	0.78
	Absent	147.2 [131.2–163.3]		17.1 [13.2–21.0]	
Perineural invasion	Present	125.2 [103.1–147.3]	**0.02**	14.7 [7.8–21.5]	0.10
	Absent	160.2 [139.6–180.8]		19.2 [14.1–24.4]	
Intraneural invasion	Present	108.6 [69.8–147.3]	**0.02**	19.9 [3.2–36.7]	0.66
	Absent	153.8 [137.0–170.6]		17.2 [13.2–21.2]	

3.3.2 Epithelial-mesenchymal transition characteristics in the study group

E-cadherin, N-cadherin, β-catenin, vimentin, and CD44 were detected with IHC to describe EMT (Figure 3.24). The overall CD44 score was 1.34 (95 % CI 1.21–1.47), reaching 1.28 (95 % CI 1.15–1.41) in adenocarcinomas and 2.00 (95 % CI 1.61–2.38) in mucinous carcinomas ($p = 0.02$). The overall E-cadherin score was 1.86 (95 % CI 1.78–1.94): 1.91 (95 % CI 1.83–1.98) in adenocarcinomas and 1.48 (95 % CI 1.23–1.73) in mucinous carcinomas ($p = 0.001$). The overall β-catenin score was 2.59 (95 % CI 2.50–2.67): 2.60 (95 % CI 2.50–2.69) in adenocarcinomas and 2.69 (95 % CI 2.47–2.92) in mucinous

carcinomas (p = 0.41). The N-cadherin score was 1.74 (95 % CI 1.53–1.92), reaching 1.78 (95% CI 1.58–1.98) in adenocarcinomas and 1.49 (95 % CI 0.55–2.43) in mucinous carcinomas (p = 0.37).

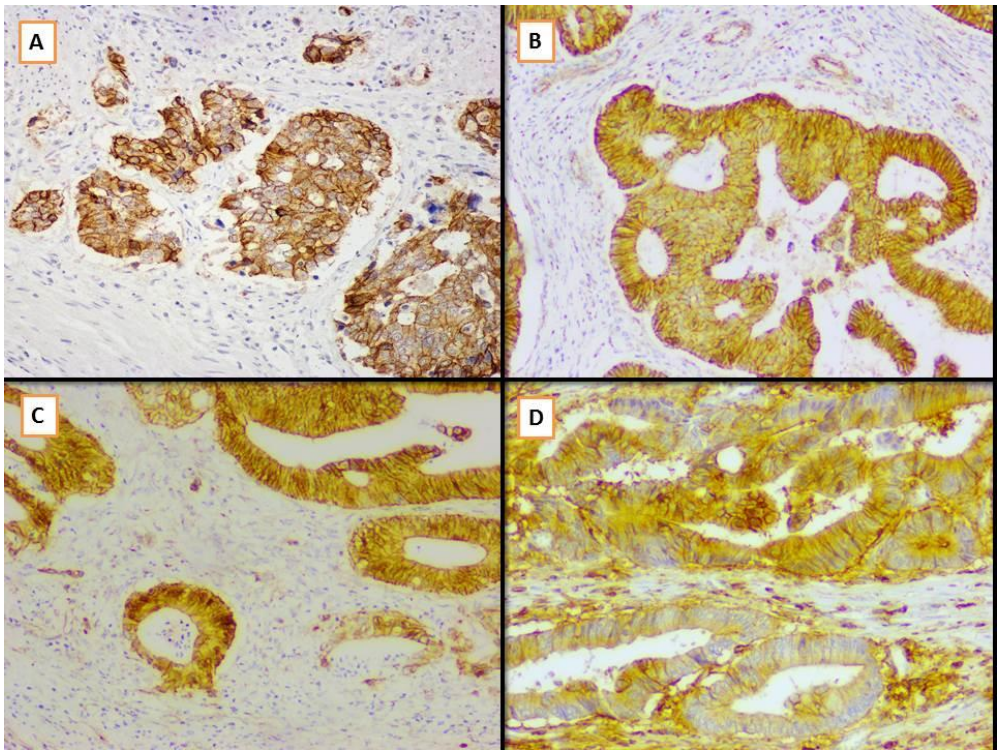

Figure 3.24 **Epithelial-mesenchymal transition marker immunohistochemistry in colorectal carcinoma**

A – E-cadherin expression, immunoperoxidase staining, original magnification (OM) 40×; B – N-cadherin expression, immunoperoxidase staining, OM 40×; C – heterogenous β-catenin expression, immunoperoxidase staining, OM 100×; D – heterogenous CD44 expression, immunoperoxidase staining, OM 100×.

Immunohistochemically, CD44 expression showed significant differences regarding tumour side (p < 0.01). Moreover, CD44 expression was significantly higher in pN0 tumours than in pN+ tumours (p = 0.02). In contrast, CD44 levels did not differ based on the presence of sCRC, pT, grade, or manifestations of invasive growth. There were also no differences in CD44 expression between the inflammation grades. Table 3.17 presents the CD44 expression characteristics.

Table 3.17

CD44 expression characteristics in a study group

Parameter	**CD44**		
	Score	**95 % CI**	**p**
Tumour location			
Left side	1.21	1.07–1.36	**0.002**
Right side	1.62	1.38–1.87	
sCRC			
Present	1.53	1.05–2.01	0.40
Absent	1.32	1.19–1.45	

Table 3.17 continued

Parameter	CD44		
	Score	95 % CI	p
pT			
pT1	2.22	0.63–3.81	0.23
pT2	1.28	0.89–1.67	
pT3	1.27	1.08–1.46	
pT4	1.39	1.19–1.59	
pN			
pN0	1.46	1.28–1.64	**0.02**
pN+	1.18	0.96–1.39	
pG			
G1	2.05	1.40–2.69	0.07
G2	1.24	1.09–1.38	
G3	1.47	1.22–1.72	
Invasions			
Perineural			
Present	1.22	1.02–1.42	0.28
Absent	1.38	1.23–1.55	
Intraneural			
Present	1.35	1.03–1.67	0.93
Absent	1.33	1.19–1.47	
Lymphatic			
Present	1.21	1.01–1.41	0.09
Absent	1.41	1.25–1.57	
Into veins			
Present	1.55	1.08–2.02	0.39
Absent	1.31	1.18–1.44	
Inflammation			
Low-grade	1.23	1.06–1.40	0.23
High-grade	1.42	1.23–1.61	
Neutrophilic leukocytes			
Low density	1.30	1.17–1.44	0.14
High density	1.55	1.23–1.88	
Eosinophilic leukocytes			
Low density	1.31	1.18–1.44	0.33
High density	1.58	1.05–2.10	
Lymphocytes			
Low density	1.26	1.09–1.42	0.33
High density	1.40	1.21–1.60	
Macrophages			
Low density	1.31	1.14–1.47	0.64
High density	1.38	1.19–1.57	
Crohn's like lymphoid reaction (CLR) by density of lymphoid follicles per mm			
Low density	1.46	1.25–1.67	0.93
High density	1.42	0.55–2.29	

β-catenin expression was significantly different in relation to pT, showing higher expression in more advanced tumours ($p = 0.007$). However, there were no expression differences regarding the presence of sCRC, local structure invasion, or involvement of lymph nodes. Interestingly, β-catenin expression levels were affected by the number of neutrophils,

with lower β-catenin expression in cases with a low neutrophil count (p = 0.001). Table 3.18 illustrates β-catenin expression in the study group.

Table 3.18

β-catenin expression characteristics in a study group

Parameter	β-catenin		
	Score	**95 % CI**	**p**
Tumour location			
Left side	2.69	2.63–2.75	0.11
Right side	2.38	2.11–2.65	
sCRC			
Present	2.58	2.14–3.03	0.58
Absent	2.59	2.50–2.68	
pT			
pT1	2.92	2.61–3.24	**0.007**
pT2	2.64	2.47–2.81	
pT3	2.65	2.50–2.80	
pT4	2.47	2.34–2.60	
pN			
pN0	2.59	2.43–2.75	0.42
pN+	2.55	2.44–2.66	
pG			
G1	2.73	1.80–3.67	0.30
G2	2.63	2.54–2.73	
G3	2.50	2.29–2.70	
Invasions			
Perineural			
Present	2.63	2.52–2.73	0.70
Absent	2.58	2.45–2.70	
Intraneural			
Present	2.61	2.41–2.81	0.90
Absent	2.59	2.49–2.69	
Lymphatic			
Present	2.57	2.46–2.68	0.16
Absent	2.61	2.48–2.74	
Into veins			
Present	2.58	2.30–2.85	0.60
Absent	2.60	2.50–2.69	
Inflammation			
Low-grade	2.65	2.56 –2.74	0.88
High-grade	2.54	2.39–2.69	
Neutrophilic leukocytes			
Low density	2.56	2.46–2.66	**0.001**
High density	2.88	2.81–2.94	
Eosinophilic leukocytes			
Low density	2.59	2.49–2.68	0.34
High density	2.71	2.42–2.99	
Lymphocytes			
Low density	2.64	2.55–2.73	0.68
High density	2.54	2.39–2.70	

Table 3.18 continued

Parameter	β-catenin		
	Score	95 % CI	p
Macrophages			
Low density	2.60	2.50–2.70	0.51
High density	2.59	2.40–2.77	
Crohn's like lymphoid reaction (CLR) by density of lymphoid			
Low density	2.57	2.42–2.72	0.06
High density	2.84	2.71–2.97	

N-cadherin expression differed significantly only in tumours with a synchronous lesion (p = 0.03), and based on pT (p = 0.03) and the eosinophil density (p = 0.02). Table 3.19 summarizes the N-cadherin expression levels.

Table 3.19

N cadherin expression characteristics in a study group

Parameter	N-cadherin		
	Score	95 % CI	p
Tumour location			
Left side	1.85	1.62–2.07	0.06
Right side	1.54	1.17–1.91	
sCRC			
Present	0.80	0.0–2.10	**0.03**
Absent	1.80	1.61–1.99	
pT			
pT1	1.10	0.00–15.1	**0.03**
pT2	1.87	1.28–2.45	
pT3	1.97	1.71–2.23	
pT4	1.45	1.12–1.78	
pN			
pN0	1.85	1.58–2.13	0.31
pN+	1.73	1.42–2.03	
pG			
G1	1.95	0.00–3.95	0.85
G2	1.76	1.52–1.99	
G3	1.75	1.37–2.12	
Invasions			
Perineural			
Present	1.68	1.35–2.01	0.39
Absent	1.80	1.55–2.04	
Intraneural			
Present	1.79	1.25–2.32	0.98
Absent	1.75	1.54–1.96	
Lymphatic			
Present	1.70	1.39–2.00	0.79
Absent	1.80	1.54–2.05	
Into veins			
Present	1.61	0.99–2.22	0.24
Absent	1.77	1.57–1.98	

Table 3.19 continued

Parameter	N-cadherin		
	Score	95 % CI	p
Inflammation			
Low-grade	1.83	1.56–1.97	0.57
High-grade	1.69	1.40–1.98	
Neutrophilic leukocytes			
Low density	1.77	1.56–1.97	0.72
High density	1.68	1.03–2.32	
Eosinophilic leucocytes			
Low density	1.84	1.65–2.03	**0.02**
High density	0.66	0.00–1.67	
Lymphocytes			
Low density	1.84	1.58–2.09	0.56
High density	1.67	1.37–1.97	
Macrophages			
Low density	1.82	1.60–2.05	0.51
High density	1.62	1.24–2.00	
Crohn's like lymphoid reaction (CLR) by density of lymphoid follicles per mm			
Low density	1.70	1.39–2.02	0.16
High density	2.40	2.08–2.71	

E-cadherin expression differed significantly based on grade (p = 0.001) and tumour side (p = 0.02), but not by pT, pN, manifestations of invasive growth, or presence of sCRC. There was also a significant difference in E-cadherin expression and eosinophil density: E-cadherin was upregulated when there was a high density of eosinophils (p = 0.007). Table 3.20 provides a summary of E-cadherin expression.

Table 3.20

E-cadherin expression characteristics in a study group

Parameter	E-cadherin		
	Score	95 % CI	p
Tumour location			
Left side	1.91	1.82–1.99	**0.02**
Right side	1.72	1.56–1.89	
Other CRC			
Present	1.62	1.14–2.09	0.18
Absent	1.87	1.79–1.95	
pT			
pT1	1.91	0.00–5.48	0.07
pT2	2.04	1.77–2.31	
pT3	1.90	1.81–1.99	
pT4	1.72	1.59–1.85	
pN			
pN0	1.88	1.77–1.99	0.16
pN+	1.80	1.69–1.92	
pG			
G1	1.81	0.00–3.79	**0.001**
G2	1.95	1.88–2.04	
G3	1.64	1.49–1.79	

Table 3.20 continued

Parameter	E-cadherin		
	Score	95 % CI	p
Invasions			
Perineural			
Present	1.78	1.67–1.90	0.30
Absent	1.90	1.80–1.99	
Intraneural			
Present	1.67	1.43–1.91	0.13
Absent	1.89	1.81–1.97	
Lymphatic			
Present	1.79	1.67–1.91	0.22
Absent	1.91	1.81–2.00	
Into veins			
Present	1.84	1.53–2.15	0.85
Absent	1.86	1.78–1.94	
Inflammation			
Low-grade	1.87	1.76–1.97	0.90
High-grade	1.85	1.74–1.96	
Neutrophilic leucocytes			
Low density	1.86	1.77–1.94	0.95
High density	1.88	0.66–3.11	
Eosinophilic leucocytes			
Low density	1.82	1.75–1.90	**0.007**
High density	2.31	1.88–2.10	
Lymphocytes			
Low density	1.85	1.75–1.96	0.85
High density	1.87	1.75–1.98	
Macrophages			
Low density	1.87	1.78–1.96	0.97
High density	1.84	1.70–1.98	
Crohn's like lymphoid reaction (CLR) by density of lymphoid follicles per mm			
Low density	1.84	1.75–1.94	0.38
High density	1.77	1.38–2.15	

Vimentin expression (Figure 3.25) was detected in 5/124 samples (4.0 %, 95 % CI 1.7–9.1 %). There were significant differences in vimentin expression regarding tumour histology ($p = 0.01$) and grade ($p = 0.001$), as all vimentin-expressing tumours were grade 3 carcinomas. There were no significant differences in vimentin expression regarding pT ($p = 0.91$), pN ($p = 0.92$), tumour side ($p = 0.13$), invasion into local structures ($p > 0.05$), density of inflammation or inflammatory cell subgroups ($p > 0.05$), or CLR presence ($p = 0.60$).

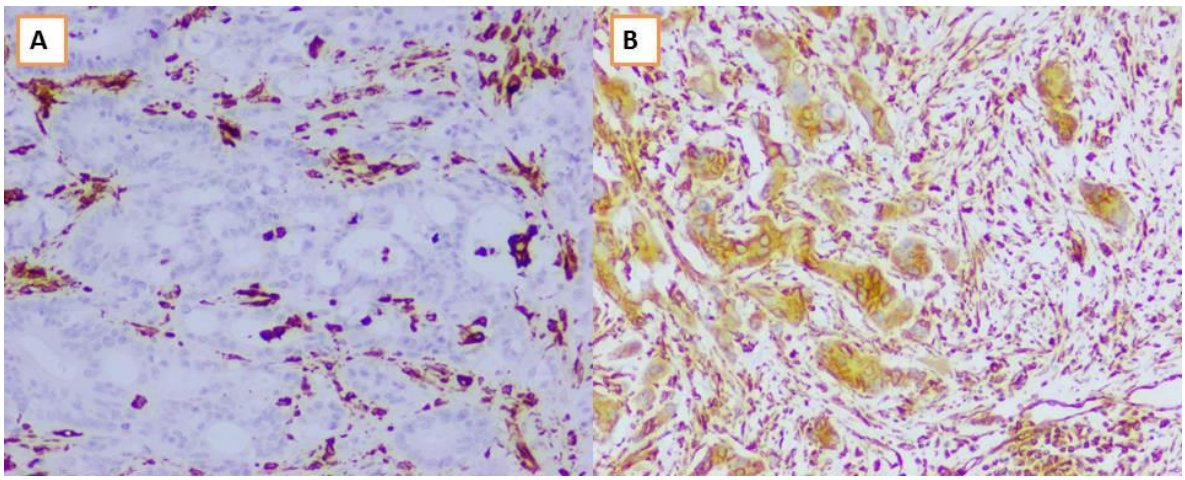

Figure 3.25 **Vimentin expression in colorectal carcinoma**

A – no expression within adenocarcinoma, immunoperoxidase staining, original magnification (OM) 100×;
B – distinct vimentin expression in cancer cells within colorectal carcinoma,
immunoperoxidase staining, OM 100×.

Analysis of EMT marker expression cross-correlation showed significant differences in β-catenin and E-cadherin expression ($p = 0.018$) and between β-catenin and N-cadherin expression ($p = 0.000$). There were no other significant correlations (Table 3.21).

Table 3.21

EMT marker expression relationships by r/rs and p values

Variable	β-catenin	E-cadherin	N-cadherin	CD44
β-catenin	NA	**0.219** **0.018**	**0.354** **0.000**	−0.024 0.798
E-cadherin	**0.219** **0.018**	NA	0.049 0.598	−0.073 0.425
N-cadherin	**0.354** **0.000**	0.049 0.598	NA	−0.180 0.053
CD44	−0.024 0.798	−0.073 0.425	−0.180 0.053	NA

In low-grade inflammation, there was a significant correlation between β-catenin and E-cadherin expression ($p < 0.05$) and between β-catenin and N-cadherin expression ($p < 0.01$). There were no other significant correlations for low grade inflammation. Interestingly, there were no significant correlations between EMT markers in high-grade inflammation (Table 3.22).

Table 3.22

Correlations between EMT markers in high- and low-grade inflammation by r/rs and p values

	β-catenin	**E-cadherin**	**N-cadherin**	**CD44**
Low-grade inflammation				
β-catenin	N/A	**0.290** **0.03**	**0.491** **0.00**	−0.106 0.44
E-cadherin	**0.290** **0.03**	N/A	0.232 0.08	−0.134 0.32
N-cadherin	**0.491** **0.00**	0.232 0.08	N/A	−0.107 0.43
CD44	−0.106 0.44	−0.134 0.32	−0.107 0.43	N/A
High grade inflammation				
β-catenin	N/A	0.145 0.265	0.235 0.07	0.024 0.85
E-cadherin	0.145 0.265	N/A	−0.092 0.48	−0.031 0.81
N-cadherin	0.235 0.07	−0.092 0.48	N/A	−0.21 0.10
CD44	0.024 0.85	−0.031 0.81	−0.21 0.10	N/A

3.3.3 Mismatch repair protein expression in the study group

MMR protein expression was detected in 118/124 cases (95.2 %, 95 % CI 89.8–97.8 %); 6/124 cases (4.8 %, 95 % CI 2.2–10.2 %) had total MMR protein loss. The mean ± SD expression was 1.43 ± 0.06 for MSH2, 1.79 ± 0.07 for MSH6, 1.46 ± 0.08 for PMS2, and 1.31 ± 0.07 for MLH1. The MMR protein expression was reclassified as low versus high by using the median value as the cut-off threshold: 1.39 for MSH2, 1.9 for MSH6, 1.8 for PMS2, and 1.43 for MLH1.

MMR protein expression was not significantly different in tumours with sCRC ($p > 0.05$). However, there were significant differences in MSH6 ($p = 0.03$) and PMS2 ($p < 0.01$) expression based on the tumour histological type (Table 3.23).

Table 3.23

Mismatch repair protein expression and colorectal carcinoma histogenesis

Histological type	**MSH2 Score [95% CI]**	**p value**	**MSH6 Score [95% CI]**	**p value**	**PMS2 Score [95% CI]**	**p value**	**MLH1 Score [95% CI]**	**p value**
Adenocarcinoma	1.48 [1.35–1.60]	0.06	1.84 [1.69–1.98]	**0.03**	1.54 [1.39–1.70]	**0.002**	1.36 [1.21–1.50]	0.13
Mucinous carcinoma	1.14 [0.47–1.80]		1.65 [0.97–2.33]		0.72 [0.15–1.30]		0.87 [0.17–1.57]	
Signet ring cell carcinoma	0.14 [0.0–1.53]		0.07 [0.0–0.39]		0.02 [0.0–0.21]		–	

MSH2 expression (Figure 3.26) was significantly different based on tumour grade and operated side (Table 3.24), with higher MSH2 expression in left-sided tumours and in tumours with higher differentiation. There were no significant changes in MSH2 expression in terms of pT and pN.

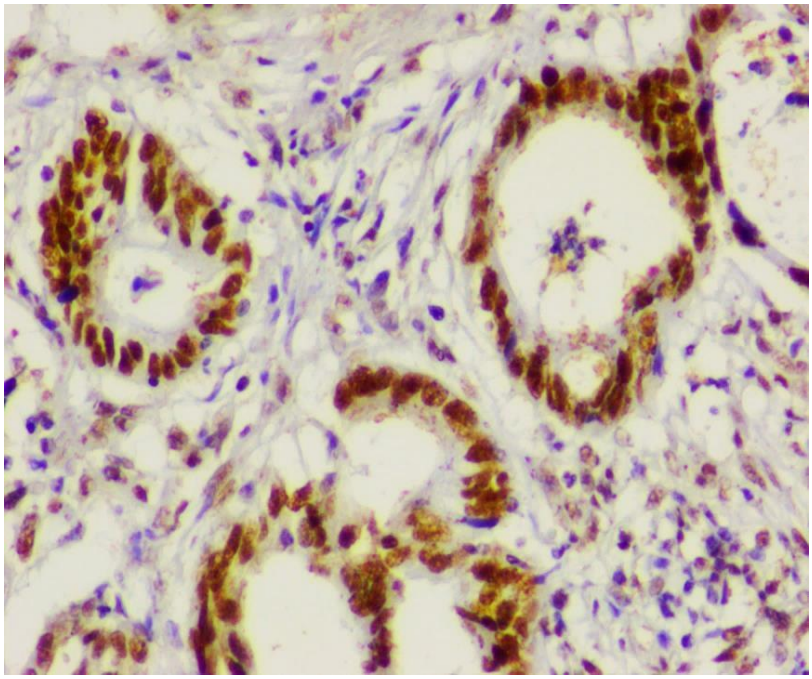

Figure 3.26 **MSH2 strong expression in CRC, immunoperoxidase**

OM 200×.

Table 3.24

MSH2 expression characteristics in the study group

Parameter	MSH2		
	Score	**95 % CI**	**p**
Tumour location			
Left side	1.58	1.44–1.72	**0.001**
Right side	1.07	0.81–1.32	
pT			
pT1	1.92	0.0–10.5	0.58
pT2	1.46	1.05–1.87	
pT3	1.47	1.28–1.66	
pT4	1.33	1.13–1.53	
pN			
pN0	1.50	1.31–1.69	0.53
pN+	1.34	1.14–1.54	
pN1c	1.45	0.99–1.90	
pG			
G1	1.30	0.48–2.11	**0.02**
G2	1.55	1.41–1.70	
G3	1.17	0.91–1.43	

MSH6 expression (Figure 3.27) was significantly different (p = 0.01) according to tumour side: Left-sided tumours showed intense expression, while right-sided tumours showed slightly weaker expression. There was no significant difference in MSH6 expression in terms of pT, pN, and tumour grade (Table 3.25).

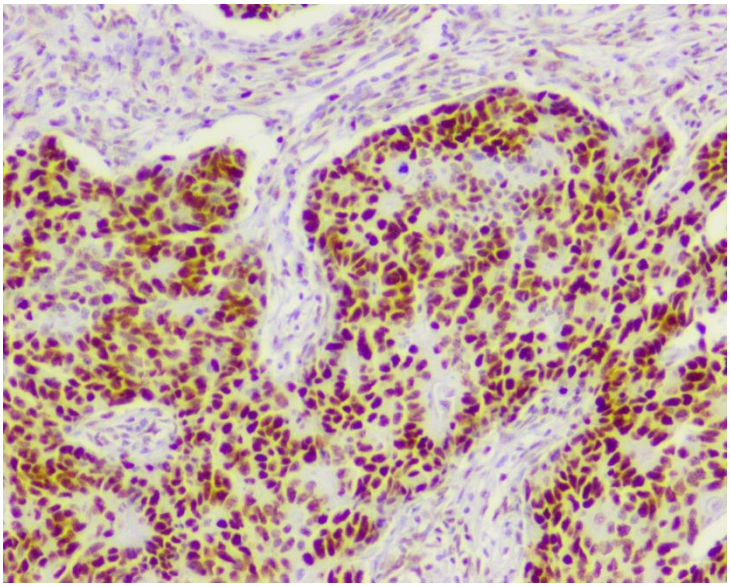

Figure 3.27 **MSH6 strong expression in CRC, immunoperoxidase**

OM 100×.

Table 3.25

MSH6 expression characteristics in the study group

Parameter	MSH6		
	Score	**95% CI**	**p**
Tumour location			
Left side	1.90	1.75–2.06	**0.01**
Right side	1.52	1.22–1.81	
pT			
pT1	2.72	1.77–3.68	0.11
pT2	1.92	1.59–2.26	
pT3	1.84	1.63–2.04	
pT4	1.63	1.38–1.88	
pN			
pN0	1.89	1.69–2.10	0.29
pN+	1.72	1.50–1.94	
pN1c	1.61	1.13–2.08	
pG			
G1	2.10	0.73–3.47	0.62
G2	1.82	1.66–1.99	
G3	1.69	1.41–1.98	

Similarly to the MSH2 expression pattern, PMS2 expression (Figure 3.28) was significantly different depending on tumour side and pG (Table 3.26). PMS2 expression also showed weaker expression in right-sided tumours and in tumours with low differentiation.

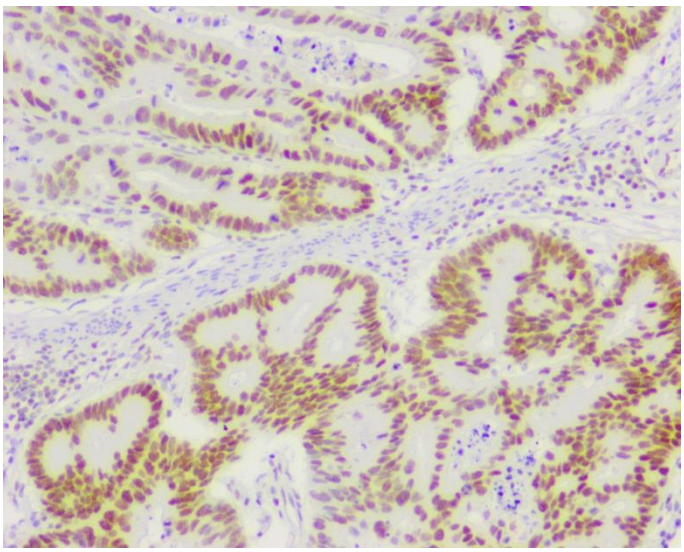

Figure 3.28 **PMS2 moderate expression in CRC, immunoperoxidase**

OM 100×.

Table 3.26

PMS2 expression characteristics in the study group

Parameter	PMS2		
	Score	**95 % CI**	**p**
Tumour location			
Left side	1.70	1.55–1.85	**< 0.001**
Right side	0.84	0.53–1.15	
pT			
pT1	2.17	0.0–7.58	0.68
pT2	1.51	1.04–1.99	
pT3	1.47	1.26–1.68	
pT4	1.37	1.09–1.65	
pN			
pN0	1.52	1.29–1.75	0.53
pN+	1.34	1.09–1.59	
pN1c	1.62	1.19–2.06	
pG			
G1	1.06	0.0–2.60	**< 0.001**
G2	1.69	1.52–1.86	
G3	1.00	0.70–1.29	

MLH1 (Figure 3.29) expression was significantly different ($p < 0.001$) depending on tumour side (Table 3.27), with weak expression associated with right-sided tumours. MLH1 showed no expression changes according to tumour differentiation.

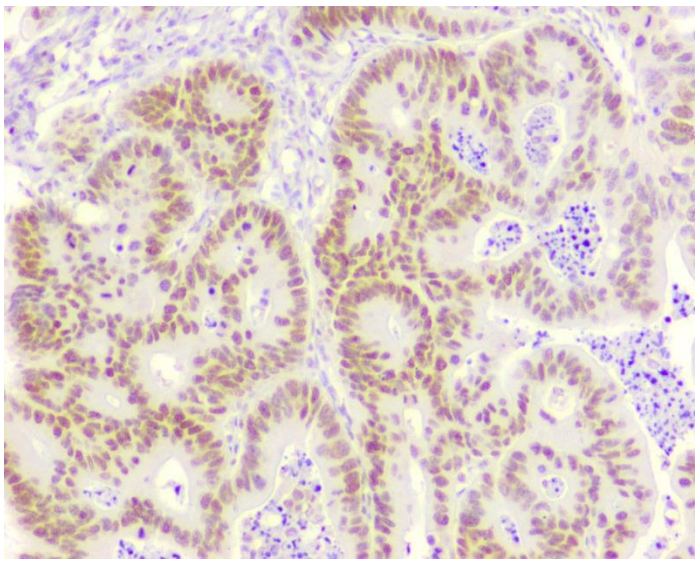

Figure 3.29 **MLH1 moderate expression in CRC, immunoperoxidase**

OM 200×.

Table 3.27

MLH1 expression characteristics in the study group

Parameter	MLH1		
	Score	95 % CI	p
Tumour location			
Left side	1.53	1.38–1.68	**< 0.001**
Right side	0.79	0.54–1.04	
pT			
pT1	1.90	0.0–5.7	0.28
pT2	1.13	0.71–1.56	
pT3	1.41	1.22–1.61	
pT4	1.23	0.97–1.48	
pN			
pN0	1.38	1.16–1.59	0.59
pN+	1.23	1.01–1.45	
pN1c	1.35	0.92–1.78	
pG			
G1	1.55	0.04–3.06	0.09
G2	1.42	1.25–1.59	
G3	1.07	0.80–1.34	

In cases with invasion into local structures, only MSH6 expression showed significant differences, specifically for intraneural invasion (p = 0.05). Invasion of arteries was present in only one case, with MSH2 expression at 0.07, MSH6 expression at 0.2, PMS2 expression at 0.35, and MLH1 expression at 1.6. All MMR expression differences are described in Table 3.28. When stratifying the cases into high versus low MMR protein expression, there was only a significant difference for MLH1 in terms of lymphatic invasion (r = 0.19, p = 0.04).

Table 3.28

Mismatch repair protein expression in relation to a local structure invasion

	MSH2 Score [95 % CI]	p value	MSH6 Score [95 % CI]	p value	PMS2 Score [95 % CI]	p value	MLH1 Score [95 % CI]	p value
Lymphatic invasion								
Present	1.33 [1.12–1.53]	0.21	1.69 [1.47–1.91]	0.20	1.36 [1.11–1.61]	0.40	1.18 [0.98–1.39]	0.06
Absent	1.50 [1.34–1.66]		1.87 [1.68–2.05]		1.52 [1.32–1.73]		1.41 [1.21–1.60]	
Perineural invasion								
Present	1.37 [1.17–1.58]	0.44	1.63 [1.38–1.87]	0.07	1.48 [1.23–1.73]	0.82	1.30 [1.09–1.52]	0.81
Absent	1.46 [1.29–1.63]		1.89 [1.72–2.06]		1.44 [1.24–1.64]		1.32 [1.13–1.51]	
Intraneural invasion								
Present	1.23 [0.79–1.66]	0.19	1.38 [0.91–1.84]	**0.05**	1.19 [0.69–1.68]	0.24	1.23 [0.84–1.62]	0.59
Absent	1.46 [1.33–1.59]		1.86 [1.72–2.01]		1.50 [1.34–1.66]		1.33 [1.17–1.48]	
Intravenous invasion								
Present	1.53 [0.94–2.12]	0.63	1.88 [1.07–2.67]	0.42	1.63 [1.03–2.24]	0.56	1.58 [1.06–2.11]	0.37
Absent	1.42 [1.29–1.55]		1.79 [1.64–1.93]		1.44 [1.28–1.60]		1.29 [1.14–1.44]	

After stratifying the cases into high versus low MMR protein expression, there was a significant difference (Mann-Whitney test) in E-cadherin expression relative to MSH2 and PMS2 expression; N-cadherin and β-catenin showed no significant differences in relation to MMR protein expression. CD44 only showed a significant difference with PMS2 expression. Table 3.29 summarizes the EMT protein expression relative to the degree of MMR protein expression.

Table 3.29

Epithelial-mesenchymal transition marker levels relative to mismatch repair protein expression in colorectal carcinoma

MMR proteins	E-cadherin: mean IHC score [95 % CI]			CD44: mean IHC score [95 % CI]		
	Low MMR	High MMR	p value	Low MMR	High MMR	p value
MSH2	1.72 [1.61–1.82]	1.91 [1.81–2.01]	**0.008**	1.39 [1.17–1.60]	1.29 [1.15–1.43]	0.35
MSH6	1.79 [1.69–1.90]	1.81 [1.70–1.93]	0.54	1.22 [1.04–1.41]	1.38 [1.21–1.57]	0.27
PMS2	1.71 [1.60–1.82]	1.95 [1.85–2.05]	**0.013**	1.43 [1.24–1.62]	1.17 [1.00–1.34]	**0.05**
MLH1	1.83 [1.72–1.95]	1.87 [1.77–1.96]	0.97	1.33 [1.13–1.53]	1.33 [1.16–1.50]	0.80
MSH2	1.92 [1.66–2.17]	1.60 [1.29–1.90]	0.39	2.50 [2.33–2.67]	2.68 [2.61–2.75]	0.65

Table 3.29 continued

MMR proteins	E-cadherin: mean IHC score [95 % CI]			CD44: mean IHC score [95 % CI]		
	Low MMR	High MMR	p value	Low MMR	High MMR	p value
MSH6	1.83 [1.56–2.09]	1.69 [1.40–1.98]	0.93	2.55 [2.40–2.70]	2.66 [2.59–2.74]	0.87
PMS2	1.77 [1.49–2.05]	1.76 [1.48–2.05]	0.86	2.48 [2.31–2.64]	2.69 [2.62–2.76]	0.18
MLH1	1.67 [1.37–1.97]	1.83 [1.57–2.09]	0.74	2.49 [2.33–2.66]	2.69 [2.63–2.77]	0.27

There was no significant association between the grade of peritumoural inflammation according to the Klintrup-Mäkinen score and MMR protein expression (Table 3.30).

Table 3.30

Peritumoural inflammation and mismatch repair protein expression

Mismatch repair proteins	Low-grade inflammation	High-grade inflammation	p value
	Mean IHC score [95% CI]		
MSH2	1.36 [1.18–1.55]	1.49 [1.32–1.66]	0.42
MSH6	1.66 [1.45–1.87]	1.92 [1.74–2.10]	0.09
PMS2	1.52 [1.31–1.73]	1.40 [1.18–1.62]	0.51
MLH1	1.41 [1.21–1.60]	1.23 [1.03–1.43]	0.26

Individual cell composition analysis of peritumoural inflammatory infiltrate showed no significant differences for any peritumoural inflammatory cell and MSH2 expression. Full MSH2 protein expression and its association with peritumoural cell counts and CLR is described in Table 3.31.

Table 3.31

MSH2 expression and the peritumoural inflammatory cell composition

Parameter	MSH2		
	Score	95 % CI	p
Neutrophilic leucocytes			
Low density	1.39	1.26–1.53	0.13
High density	1.71	1.39–2.03	
Eosinophilic leucocytes			
Low density	1.41	1.29–1.54	0.29
High density	1.64	0.76–2.52	
Lymphocytes			
Low density	1.38	1.21–1.56	0.53
High density	1.48	1.28–1.67	
Macrophages			
Low density	1.39	1.24–1.54	0.27
High density	1.52	1.26–1.77	
Crohn's like lymphoid reaction (CLR) by density of lymphoid follicles per mm			
Low density	1.34	1.14–1.54	0.71
High density	1.40	0.51–2.28	

Although there was slightly higher MSH6 expression with a high lymphocyte density, the difference was not significant. Moreover, the density of other cells did not affect MSH6 expression. Full MSH6 protein expression and its association with inflammatory cell density and CLR is described in Table 3.32.

Table 3.32

MSH6 expression and the peritumoural inflammatory cell composition

Parameter	MSH6		
	Score	95 % CI	p
Neutrophilic leucocytes			
Low density	1.76	1.61–1.91	0.34
High density	2.04	1.73–2.36	
Eosinophilic leucocytes			
Low density	1.78	1.63–1.92	0.43
High density	2.02	1.39–2.64	
Lymphocytes			
Low density	1.67	1.47–1.87	0.07
High density	1.93	1.73–2.13	
Macrophages			
Low density	1.77	1.60–1.94	0.71
High density	1.84	1.59–2.10	
Crohn's like lymphoid reaction (CLR) by density of lymphoid follicles per mm			
Low density	1.66	1.44–1.89	0.75
High density	1.58	0.80–2.36	

PMS2 expression was not significantly different in relation to lymphocyte, eosinophil, neutrophil, and macrophage density. In addition, there were no significant changes in its expression in terms of CLR density. Full PMS2 protein expression and its association with inflammatory cell density and CLR is described in Table 3.33.

Table 3.33

PMS2 expression and the peritumoural inflammatory cell composition

Parameter	PMS2		
	Score	95 % CI	p
Neutrophilic leucocytes			
Low density	1.41	1.24–1.57	0.06
High density	1.86	1.51–2.20	
Eosinophilic leucocytes			
Low density	1.43	1.27–1.59	0.12
High density	1.74	0.87–2.61	
Lymphocytes			
Low density	1.44	1.23–1.65	0.78
High density	1.47	1.24–1.71	
Macrophages			
Low density	1.48	1.29–1.67	0.53
High density	1.39	1.12–1.67	
Crohn's like lymphoid reaction (CLR) by density of lymphoid follicles per mm			
Low density	1.25	0.99–1.49	0.73
High density	1.11	0.34–1.88	

Further analysis showed that an increased neutrophil density was associated with increased MLH1 expression, ranging from 1.26 (95 % CI 1.11–1.41) in cases with low-grade infiltration to 1.75 (95 % CI 1.46–2.04) in carcinomas showing a moderate to high neutrophil count. There were no other significant differences in MLH1 expression in relation to other inflammatory cells. The full MLH1 protein expression and its association with inflammatory cell density and CLR is described in Table 3.34.

Table 3.34

MLH1 expression and the peritumoural inflammatory cell composition

Parameter	MLH1		
	Score	**95 % CI**	**p**
Neutrophilic leucocytes			
Low density	1.26	1.11–1.41	**0.02**
High density	1.75	1.46–2.04	
Eosinophilic leucocytes			
Low density	1.31	1.16–1.45	0.70
High density	1.41	0.66–2.16	
Lymphocytes			
Low density	1.35	1.16–1.55	0.58
High density	1.27	1.06–1.48	
Macrophages			
Low density	1.35	1.18–1.52	0.36
High density	1.23	0.95–1.50	
Crohn's like lymphoid reaction (CLR) by density of lymphoid follicles per mm			
Low density	1.26	1.02–1.49	0.73
High density	1.15	0.44–1.87	

The number of peritumoural and intratumoural CD8+ cytotoxic T cells was not significantly different between the low and high MMR protein expression groups or complete loss of MMR (Table 3.35).

Table 3.35

MMR protein expression and CD8+ T cell counts in colorectal carcinoma

Parameter	Peritumoural CD8+, mean count [95 % CI]	p value	Intratumoural CD8+, mean count [95 % CI]	p value
MSH2				
Low MMR	140.9 [116.9–165.0]	0.31	14.8 [10.3–19.3]	0.25
High MMR	153.6 [132.2–175.1]		19.2 [13.0–25.6]	
MSH6				
Low MMR	134.1 [111.5–156.6]	0.17	16.7 [11.1–22.3]	0.63
High MMR	159.0 [137.0–181.0]		18.7 [12.7–24.7]	
PMS2				
Low MMR	145.9 [122.4–169.3]	0.68	17.6 [11.8–23.4]	0.94
High MMR	151.2 [129.6–172.8]		18.0 [12.0–24.0]	
MLH1				
Low MMR	151.5 [128.1–174.9]	0.95	15.9 [10.4–21.4]	0.19
High MMR	148.5 [127.0–170.0]		19.9 [13.6–26.1]	

Parameter	Peritumoural CD8+, mean count [95 % CI]	p value	Intratumoural CD8+, mean count [95 % CI]	p value
Loss of MMR				
Present	104.8 [11.6–198.0]	0.27	23.0 [0.0–76.5]	0.23
Absent	149.5 [133.6–165.3]		17.3 [13.4–21.3]	

Table 3.36 presents the full results of immunohistochemical marker correlations in this study.

Table 3.36

Correlations between immunohistochemical markers by r/rs and p values

Variable	β-catenin	E-cadherin	N-cadherin	CD44	CD8 PT	CD8 IT	MSH2	MSH6	PMS2	MLH1
β-catenin	NA	**0.219** **0.018**	**0.354** **0.000**	−0.024 0.798	0.099 0.299	0.142 0.134	0.097 0.306	0.054 0.568	**0.200** **0.03**	0.117 0.212
E-cadherin	**0.219** **0.018**	NA	0.049 0.598	−0.073 0.425	0.110 0.244	−0.118 0.210	0.170 0.068	0.104 0.265	**0.226** **0.014**	0.132 0.157
N-cadherin	**0.354** **0.000**	0.049 0.598	NA	−0.180 0.053	0.020 0.831	**0.192** **0.043**	−0.097 0.302	−0.052 0.584	0.032 0.733	0.051 0.586
CD44	−0.024 0.798	−0.073 0.425	−0.180 0.053	NA	**0.206** **0.029**	0.158 0.095	0.001 0.992	0.125 0.180	−0.132 0.158	0.032 0.729
CD8 PT	0.099 0.299	0.110 0.244	0.020 0.831	**0.206** **0.029**	NA	**0.408** **0.000**	0.147 0.121	**0.203** **0.031**	0.035 0.708	0.053 0.580
CD8 IT	0.142 0.134	−0.118 0.210	**0.192** **0.043**	0.158 0.095	**0.408** **0.000**	NA	**0.201** **0.033**	0.123 0.194	0.057 0.546	0.075 0.427
MSH2	0.097 0.306	0.170 0.068	−0.097 0.302	0.001 0.992	0.147 0.121	**0.201** **0.033**	NA	**0.542** **0.000**	**0.575** **0.000**	**0.498** **0.000**
MSH6	0.054 0.568	0.104 0.265	−0.052 0.584	0.125 0.180	**0.203** **0.031**	0.123 0.194	**0.542** **0.000**	NA	**0.397** **0.000**	**0.431** **0.000**
PMS2	**0.200** **0.03**	**0.226** **0.014**	0.032 0.733	−0.132 0.158	0.035 0.708	0.057 0.546	**0.575** **0.000**	**0.397** **0.000**	NA	**0.702** **0.000**
MLH1	0.117 0.212	0.132 0.157	0.051 0.586	0.032 0.729	0.053 0.580	0.075 0.427	**0.498** **0.000**	**0.431** **0.000**	**0.702** **0.000**	NA

4 Discussion

CRC remains one of the malignancies with the highest incidence as well as mortality (Siegel et al., 2020). Although there are prognostic factors of this malignancy that we cannot change, like patient age and tumour location, several factors (EMT, stemness, immune response) can be affected and altered with treatment. Recently, discussion about CRC treatment and immunotherapy role in it has become a "hot topic" (Golshani & Zhang, 2020), as it has proven its role in other malignancies like melanoma (Lugowska, Teterycz, & Rutkowski, 2018), breast cancer (Bayraktar, Batoo, Okuno, & Glück, 2019), and lung cancer (Corrales et al., 2018). It could be a potential treatment option especially in advanced CRC cases. Nevertheless, it is still unclear what leads to such a high number of advanced CRC cases despite development of screening programs and preventive measures. Factors driving the metastatic potential of CRC are thought to be EMT and CSCs (Fabregat et al., 2016; Fedyanin et al., 2017), but their potential interaction with each other as well other tumour-driving factors like inflammation is unclear.

The goal of this study was to determine the molecular mechanisms involved in CRC development and their connection with histopathological findings, EMT, and the inflammatory reaction.

4.1 Clinical and morphological findings

Gender. In some Western countries, the CRC incidence does not differ between female and male patients (Abotchie, Vernon, & Du, 2012; Ghebrial et al., 2021). The current study showed slight predominance of female patients, who constituted 53.4 % (95 % CI 49.2–57.5 %) of the study group. However, in other Western countries, the incidence is higher in male patients. In the United Kingdom, within the age group of 70–74 years, the male-to-female ratio is 1.7:1 (White et al., 2018). The differences between these studies could be explained by the study design, as this study did not include patients who had not received surgical treatment because of advanced tumour spread or significant comorbidities (e.g., cardiovascular). It is known that in men, cardiovascular diseases appear more often and that could be a contraindication to surgery (Khan, Andrews, Jennings, Sampson, & Chin-Dusting, 2019). In addition, women show a slightly higher response to screening invites (White et al., 2018), which could explain why male patients tend to have more advanced tumours.

Age. CRC is known to appear mostly after the age of 50 years, although lately there has been an increased tendency to identify CRC in patients younger than 50 years and a decrease in the number of elderly patients, especially in Western countries (Vuik et al., 2019;

Wong et al., 2021). In the current study, 93 % (95 % CI 90.5–94.8 %) of the study group was over 50 years old, with a median age of 71 (IQR 15) years. In Sweden, the median age for male patients with CRC is 70 years, but for females it is 69 years (Ghazi et al., 2012). Although other studies indicate that most patients with CRC are elderly, the average age of diagnosis in other studies varies up to the age of 70 years (S. Li et al., 2019; Lim, Kuk, Kim, & Shin, 2017). The number of young patients with CRC varies from 0.8 % to 15 % depending on the population, reaching even 36 % in Iran, and there is a slight tendency for younger patients to have more advanced tumours (Campos, Figueiredo, Monteiro, Nahas, & Cecconello, 2017; Dolatkhah et al., 2015; Virostko, Capasso, Yankeelov, & Goodgame, 2019). Age-related differences have also been described in patients with CRC according to the operated side, as younger patients tend to have left-sided bowel tumours more often (66.6 ± 11.4 years vs. 69.7 ± 11.8 years, $p < 0.01$) (Nawa et al., 2008). In the current study, in the young patients (7 % of the study group), left-sided CRC was more frequent than right-sided CRC, being present in 79.5 % of cases. Moreover, the majority of patients within this age group had pT3 and pT4 tumours, a finding similar to other studies.

Operated side. Studies indicate that most tumours within the colon are located in the left part of the bowel. In a recent study done by Kuan et al., where stage II CRC was analysed, only 29.1 % of cases where located in right part of the bowel (Kuan et al., 2019). Vayrynen et al. in 2016, reported similar results: 33.3 % of their study cohort patients had a tumour on the right side of the bowel (S. A. Vayrynen et al., 2016). In the current study, left-sided CRC was more common, occurring in 70.9 % of cases. This result is similar to a recent study done in Italy where right-sided tumours occurred in 30.1 % of cases in a study group comprising more than 29.000 patients with CRC (Mangone et al., 2021).

Simultaneous tumours. The current study indicated that patients with left-sided CRC more often tend to have sCRC, as their presence in the left part of the bowel was observed in 78.3 % of patients with sCRC, and overall sCRC occurred in 4.2 % of all cases. Lee et al., also found that sCRC was more frequent on the left side, although the overall incidence of sCRC was lower, only 2.6 % in their study group, and overall only 33.1 % of tumours were located on the right side (B. C. Lee et al., 2017).

In the current study, patients younger than 50 years tended to have sCRC more often (10.3 % vs. 3.7 %), a finding different from another study where the mean ± SD age in patients with single CRC was lower (59.8 ± 11.1 years) than in patients with sCRC (62.3 ± 10.4 years) (B. C. Lee et al., 2017). In a Swedish study, patients aged < 60 years had fewer synchronous tumours than elderly patients (Ghazi et al., 2012). Recent study by He et al. also showed that presence of sCRC in difference from solitary CRC cases is associated

with a higher amount of polyps within bowel, mucinous histology, older patients age and male sex (W. He et al., 2019). Although the prevalence of sCRC in young patients could be explained by the presence of risk factors, no clinical information regarding meaningful risk factors was analysed within this study. sCRC has been associated with FAP, ulcerative colitis, and hyperplastic polyposis; however, these were only present in 10 % of sCRC cases (Lam et al., 2011). Recently, molecular analysis of 47 tumours in 23 patients revealed a different mutation status in simultaneous lesions, as most tumour pairs did not show similar changes in genes, indicating heterogeneity of tumours that could complicate the treatment choice (Hänninen et al., 2019). The authors also evaluated Immunoscore differences in sCRC: Only 16% of synchronous tumours had an equal Immunoscore, and the overall immune response was more significant in MSI tumours (Hänninen et al., 2019). These results indicate that multiple factors are involved in the development of sCRC, and this could be future research path in the current study group and in Latvia in general.

In the current study, 155/553 cases (28 %) presented synchronous adenomas. This result is very similar to other countries – the range of synchronous adenomas in patients with CRC varies from 25 % to 39 % (S. Li et al., 2019; Sun et al., 2020). The morphology of synchronous adenomas in the current study was slightly different from other studies. Tubular adenoma was detected in 52.9 % of cases. On the contrary, Sun et al. reported that out of 123 CRC cases with synchronous adenoma, the majority (84.8 %) were tubular adenomas, while villous adenomas were detected in only 4 % of cases (Sun et al., 2020).

For the villous morphology, large size (> 2 cm), multiple lesions, and a high degree of dysplasia within lesions are features of high-risk adenomas (Cross et al., 2020). The extent of high-risk adenomas varies, as in one study large size 20 % of cases were villous adenomas (S. Li et al., 2019). In a recent study, although endoscopic removal of colorectal adenomas was associated with a lower risk for CRC development, in patients who had ≥ 5 adenomas, there was higher chance for development of mCRC in follow-up endoscopy (odds ratio [OR] 2.575, $p = 0.049$) (H. Yoon, Shin, Park, Kim, & Lee, 2022). The current study had no follow-up data regarding development of mCRC in patients or metachronous adenomas. These factors should be evaluated in the future.

Tumour size, perforation, and gross appearance. The significance of tumour size in CRC has been widely disputed. In a recent study, patients with stage T1/2 N0 rectal tumours with a larger volume had a lower disease-free survival rate than patients with smaller volume rectal tumours (80.4 % vs. 89.6 %, $p = 0.042$) (Jiang et al., 2018). Moreover, larger tumours showed significantly higher local recurrence rates than smaller tumours (10.5 % vs. 3.0 %, $p = 0.03$) (Jiang et al., 2018). The methods for tumour volume assessment mostly involve

radiographic image analysis with measurements of the x-, y-, and z-axes of a tumour (Tirumani et al., 2016). However, there could be significant differences between radiographic and pathological volumes, as shown previously in breast cancer (Hamza et al., 2018) and other tumours (Cortadellas et al., 2017; Jeffery, Douek, Guo, & Patel, 2011). Considering that the radiographic tumour size data were not available in the current study, pathological assessment was performed and the tumour volume was calculated based on a previously described method (Tirumani et al., 2016).

There was a significant difference in the tumour volume depending on its location: left-sided tumours were smaller than right-sided tumours (21.4 [95 % CI 16.3-26.5] cm^3 vs. 29.4 [95 % CI 23.0–35.9] cm^3, respectively). In other studies, left-sided tumours also tended to be smaller. In a large retrospective studies done in Korea, the mean ± SD side of a left-sided CRC was 4.3 ± 2.2 cm and 4.4 ± 2.3 cm, compared with 5.1 ± 2.7 cm and 5.6 ± 3.1 cm for right-sided CRC (H. D. Kwak et al., 2021; Lim et al., 2017). In a study done in Romania, left-sided tumours also tended to be smaller than right-sided tumours (Mogoantă et al., 2014). This phenomenon could be explained by the fact that right-sided CRC have a less specific clinical presentation like microcytic anaemia and weight loss; hence, the tumour could grow for a longer time before detection (Hussain et al., 2016; G. H. Lee et al., 2015). On the other hand, left-sided tumours tend to obstruct bowel and perforate, leading to an increased need for urgent surgery compared with right-sided tumours (17.0 % vs. 8.5 %) (Mik, Berut, Dziki, Trzcinski, & Dziki, 2017). Bowel obstruction mostly appears because of the circular tumour growth, and the current study showed that left-sided CRC tended to grow annularly more frequently than right-sided CRC, as circular CRC was present in 75.0 % of cases in the left part of the bowel. Perforation was also more frequent for left-sided tumours, occurring in 76.3 % of left-sided CRC cases.

Morphology. In current study, colorectal adenocarcinoma was found in 88.8 % of cases. This finding is consistent with other studies, namely that it is the most common morphological type of CRC. In other studies, colorectal adenocarcinomas has varied from 80 % to 90 % (Betge et al., 2012). The prevalence of mucinous carcinomas (9.6 % of cases) is lower than in studies done in Romania (19.3 % of cases) (Mogoantă, Vasile et al., 2014) and Austria (11 % of cases) (Betge et al., 2012; Mogoantă et al., 2014). The prevalence of other histological types was slightly lower than in other studies. For example, signet ring cell carcinoma was found in 1.2 % of cases compared with 3.4 % in a study done in Romania (Mogoantă et al., 2014).

Similarly to a previous study, mucinous tumours tended to be larger than non-mucinous carcinomas, with a mean tumour volume of 45.3 (95 % CI 20.9–69.6) cm^3. In comparison, the mean non-mucinous adenocarcinoma volume in this study was 21.0 (95 % CI 17.4–24.6) cm^3. In a recent meta-analysis, mucinous carcinomas larger than 5 cm were seen in 51.1 % of cases while non-mucinous tumours larger than 5 cm were seen in only 31.6 % of cases (Z.-P. Li et al., 2020).

pT. The study cohort showed a remarkable predominance of locally advanced tumours: pT3 carcinoma represented 49.6 % (95 % CI 45.4–53.7 %) of cases and pT4 represented 35.6 % (95 % CI 31.7–39.7 %) of cases, but pT2 only represented 11.9 % (95 % CI 9.5–14.9 %) of cases. In other countries, researchers have reported fewer pT4 tumours; this difference could be explained by delayed diagnosis of CRC, given that in Latvia the response to screening is < 12 % (Senore et al., 2019). Betge et al. reported that pT4 comprised only 17.0% of cases; the majority of the cases they included in their study were pT3 (57.2 %), and the percentages of pT1 and pT2 cases were higher than in the current study (Betge et al., 2012).

Regarding histology, in the current study most colorectal adenocarcinoma cases (50.7 %) were detected at pT3 and pT4 (33.8 %), while only 3.3 % and 12.2 % of cases were pT1 and pT2, respectively. Mucinous carcinomas were found equally at the pT3 and pT4 stages (43.4 % and 45.3 %, respectively). However, signet ring cell carcinoma was mostly detected at pT4 (85.7 %), indicating its aggressive growth.

Previous studies have shown very similar percentages of mucinous carcinoma cases in the colon. Park et al. reported that 90.9 % of mucinous carcinoma was pT3 and pT4 (J. S. Park et al., 2015). Moreover, they observed clinical signs of bowel obstruction in only 13.9 % of cases and bowel perforation in 1.5 % of cases. These findings indicate the problem of mucinous tumour detection, as there are no symptoms in most of the cases.

There have been variable results regarding the distribution and stage of signet ring cell carcinoma. In most of the research, signet ring cell carcinoma has been associated with the presence of vascular invasion and pN+; however, the number of pT4 tumours is lower than the current study. Sung et al. found only 22 % of signet ring cell carcinoma at pT4, a much lower percentage than the current study (Sung et al., 2008). This difference could be explained by the small number of signet ring cell cases in the current study (7 cases, 1.2 %). Nevertheless, Nitsche et al. included only 0.9 % of signet ring cell carcinoma cases and also showed a lower number of pT4 cases (3 0%) compared with the current study (Nitsche et al., 2013).

There was a higher percentage of advanced adenocarcinoma in the current study compared with previous studies. More than half of the patients (50.7 %) had pT3 tumours and 33.8 % had pT4 tumours. Nitsche et al. also observed a high percentage of pT3 cases (54 %),

but a markedly lower percentage of pT4 cases (16 %) (Nitsche et al., 2013). In another study, pT4 colorectal adenocarcinoma comprised only 8% of cases, while pT3 was found in 71 % of cases (Hosseini et al., 2017).

The abundance of advanced CRC cases in the current study could be explained by the low response to screening in Latvia, as an overall coverage examination indicated that only 11.1 % of invited people responded to the screening invite in 2014 (Senore et al., 2019). Screening coverage varies widely in other European countries: 77–80 % in Germany where guaiac faecal occult blood test–total colonoscopy (gFOBT-TC) is used (Guo et al., 2020), to 4.5–66.6 % in countries like Latvia where guaiac faecal occult blood test (gFOBT) screening programs are used (Senore et al., 2019).

Another factor that could have an effect on the prevalence of pT4 tumours is hyper-diagnosis. As mentioned previously, the diagnostic potential of a pT4 tumour could be affected by the presence of heavy inflammation within CRC tissues as well as mesothelial reaction (Stewart, Hillery, Platell, & Puppa, 2011; Swamy, 2010). To exclude this factor from this research, all CRC cases included within this study was re-examined independently from the previous diagnosis.

pN. In the current study, the median number of retrieved lymph nodes was 11 (IQR 8). Although the well-established and accepted guidelines for CRC investigation suggest that at least 12 lymph nodes must be retrieved, there are variations among countries (Compton et al., 2000; Kuijpers et al., 2013). Researchers in U.S. and European clinics even suggest that it is not always possible to reach the target number of 12 retrieved lymph nodes (Li Destri, Di Carlo, Scilletta, Scilletta, & Puleo, 2014). In a Turkish study, the number of retrieved lymph nodes was independently associated with CRC depth of invasion (pT), tumour size, AJCC/Union for International Cancer Control (UICC) stage, right-sided tumour location, and preoperative therapy, as lymph node yield significantly decreased in patients who had received treatment before surgery (Altintas & Bayrak, 2019). The highest lymph node yield retrieved so far has been reported in right-sided tumours and has been associated with CRC cases in younger patients (Ahmadi, Stringer, Black, & McCall, 2015). The different make-up of the cohort included in this study – mainly left-sided CRC cases and elderly patients – could explain the lower number of retrieved lymph nodes. Italian researchers recently demonstrated that tumour side influences the number of retrieved lymph nodes. For left-sided CRC, 26.6 % of cases had less than 12 lymph nodes and 17.1 % of cases had more than 21 lymph nodes. In contrast, for right-sided CRC, 14.0 % of cases had less than 12 lymph nodes while 33 % of cases had more than 21 lymph nodes (Mangone et al., 2021). The result of the current study identifies a potential area for future improvement.

In the current study, 40.5 % of cases were pN+ and 49.4 % of cases were pN0. These percentages are different from patients in other studies, where pN0 was slightly more common (54.0–55.9 %) (Betge et al., 2012; Ptok, Meyer, Croner, Gastinger, & Garlipp, 2022). Although the number of pN0 patients is similar, the current study had a higher number of patients with advanced-stage disease.

Another indicator of more advanced tumours in the study group is the presence of tumour nodules in fat tissue (pN1c), identified in 10.1 % of cases. Research has shown that patients with pN1c CRC should be considered high risk and treated with adjuvant chemotherapy, as their presence is associated with higher pTNM stage, vascular emboli, and lower 3-year overall survival compared with pN1a CRC (81.8 % vs. 89.1 %) (Bouquot et al., 2018). Nevertheless, there are controversial data about how pN1c affects the prognosis in the presence of pN+. In their meta-analysis, Liu et al. found that when following the pTNM classification, pN1c in the presence of pN+ should be included as an additional pN category because it is associated with a worse prognosis (F. Liu et al., 2019). Hence, in the current study pN1c was included in the pN+ group.

In the study group, pN+ was present in 48.9 % of adenocarcinoma cases, 60.4 % of mucinous carcinoma cases, and 85.7 % of signet ring cell carcinoma cases. Previous studies have shown fewer pN+ mucinous carcinoma cases, again theoretically proving the relatively high number of advanced CRC cases in the current study population. Park et al. identified pN+ in 46.4 % of their 274 mucinous carcinomas (J. S. Park et al., 2015). Nitsche et al. observed pN+ in 51 % of mucinous carcinoma cases and in 83% of signet ring cell carcinoma cases, while only 44 % of non-mucinous adenocarcinoma cases were pN+ (Nitsche et al., 2013). Hosseini et al. reported that less than 40 % of the adenocarcinoma and mucinous carcinoma cases were pN+ (Hosseini, Bananzadeh et al., 2017.

pG. There were a relatively high number of tumours with low differentiation compared with other studies. Specifically, 64.0 % of cases were pG2 and 25.7 % were pG3. However, Vayrynen et al. found that only 12.9 % of their cases were pG3 tumours (S. A. Vayrynen et al., 2016). Lee et al. also identified fewer pG3 tumours: 80.6 % 5.4 % of single CRC cases were pG2 and pG3, respectively, and 85.7 % and 4.6 % of sCRC cases were pG2 and pG3, respectively (B. C. Lee et al., 2017). In a Romanian study group comprising 245 CRC adenocarcinomas, 51.83 % of the tumours were moderately differentiated, but only 12.65 % of cases were pG3 (Mogoantă et al., 2014).

Differentiation within malignancies has been widely studied. High differentiation – when a tumour acquires a similar cellular morphology to its origin – is mostly associated with a good prognosis. On the other hand, malignancies with low differentiation are aggressive and

show behaviour similar to stem cells. So, the theory of CSCs has been proposed in some malignancies (Cherciu, Barbalan, Pirici, Margaritescu, & Saftoiu, 2014; Jögi, Vaapil, Johansson, & Påhlman, 2012; Papi & Orlandi, 2016; Ricci-Vitiani, Fabrizi, Palio, & De Maria, 2009). The proposed theories regarding the development of CSCs are (1) oncogenic mutations accumulate within normal adult cells or embryonic stem cells, leading to uncontrolled proliferation (Ricci-Vitiani et al., 2009) or (2) CSCs are normal cells that dedifferentiate into a stem-like state (Kreso & Dick, 2014). Although CSC-targeting treatments are under development, CSCs exhibit heterogeneity that leads to cancer subtype switching, which affects treatment and prognosis (Abugomaa et al., 2020). Lower differentiation in CRC has been associated with stem cell properties, including surface expression of stem cell markers like CD44 and CD133 (Manhas et al., 2016). CRC cases showing properties of CSCs in a main tumour mass have a higher chance of lymph node and distant metastasis, and these cases have a worse overall prognosis (Fedyanin et al., 2017; C. Wang et al., 2012).

Invasions. Local structure invasions are one of the main prognostic factors in CRC. These factors have been widely studied, and their presence is associated with low survival rates in CRC (J. Hogan et al., 2015). Lymphovascular invasion is one of the main prognostic factors in CRC, and its presence has also been found in stage II tumours, where no regional lymph node metastasis are found (pN0) (Kuan et al., 2019).

In the current study lymphovascular invasion was detected in 63.6 % of cases, perineural invasion in 50.1 % of cases, and intraneural growth in 31.1% of cases. In other studies, lymphovascular invasion varies from 19.7 % to 27.8 % (B. C. Lee et al., 2017; J. S. Park et al., 2015), and perineural invasion varies from 5.8 % to 14.7% (B. C. Lee et al., 2017; J. S. Park et al., 2015). The incidence of reported venous invasion varies from 11 % to 89.5 % (Kojima et al., 2013). In a Swedish study, Ghazi et al. detected perineural invasion in 15.6 % of male and 16.8 % of female patients (Ghazi et al., 2012). These findings indicate high numbers of advanced-stage tumours in this study and could be explained by a late diagnosis of CRC.

Differences in local structure involvement according to tumour histology have also been reported, but the results are heterogenous. In a large study that involved 274 mucinous CRC cases and 6201 non-mucinous CRC cases, there were no significant differences between lymphovascular and perineural tumour growth ($p = 0.11$ and $p = 0.80$, respectively) (J. S. Park et al., 2015). In the current study, there was only a significant difference in terms of intraarterial growth: It was significantly higher in signet ring cell carcinoma. Previous studies have indicated signet ring cell carcinoma is an aggressive subtype of CRC with significantly greater involvement of blood vessels (Thota, Fang, & Subbiah, 2014).

In a recent paper from Korea, the authors analysed 966 CRC cases and found significant differences in local structure invasions according to tumour location (H. D. Kwak et al., 2021). Vascular and lymphatic invasion was associated with right-sided CRC, while neural invasion was associated with left-sided tumours (H. D. Kwak et al., 2021). Differently, in the current study there were no significant differences between local structure involvement and tumour side.

Tumour necrosis affects patient survival and tumour progression in CRC (S. A. Vayrynen et al., 2016) as well as other cancer types, including nasopharyngeal carcinoma (Liang et al., 2021) and lung carcinoma (Oiwa et al., 2021). In the current study, necrosis was present in 295 cases, presenting mostly a moderate amount of necrosis (49.5 %, 95 % CI 43.8–55.2 %). Its presence was significantly higher in pT4 tumours, where its mean extent was 16.6% of the tumour mass ($p = 0.04$), in pN+ tumours ($p = 0.02$) and in low-differentiated tumours ($p = 0.004$). These results are somewhat similar to other studies. Vayrynen et al. found that the necrotic area was associated with a higher pT stage and showed variations regarding lymph node involvement: There was a median of 25.0 % necrosis in pN2 tumours compared with 7.0 % in pN0 tumours (S. A. Vayrynen et al., 2016). These results are controversial – in another study, the researchers have shown that the extent of necrosis in CRC and its metastasis in the lungs is not affected by tumour size, microvascular density, and tumour cell proliferation (Suzuki et al., 2019).

4.2 Inflammation within colorectal carcinoma

The current study showed a slight predominance (52.8 %) of CRC cases with low-grade peritumoural inflammation. There was no significant difference ($p = 0.13$) between the mean ages of patients with low-grade (68.1, 95 % CI 66.9–69.3) and high-grade (69.6, 95 % CI 68.2–70.9) peritumoural inflammation. There were no significant differences regarding tumour volume and grade of local inflammation, indicating that the local immune response could potentially play a different role in CRC. Previous research has shown ambiguous results, as mostly systemic inflammation effects have been evaluated, namely C-reactive protein, fibrinogen, and other acute phase proteins (Koper-Lenkiewicz, Dymicka-Piekarska, Milewska, Zińczuk, & Kamińska, 2021; Rasic, Radovic, & Aksamija, 2016). The results of one of these studies showed that in patients with a tumour > 3 cm in size, the C-reactive protein concentration was about twice as high as in patients with a tumour ≤ 3 cm in size, indicating a potential role for systemic inflammation in tumour growth (Koper-Lenkiewicz et al., 2021).

There were also no significant differences between high- and low-grade inflammation in relation to presence ($p = 0.63$) or extent ($p = 0.11$) of necrosis. There have been different results in other studies. Richards et al. found that the extent of necrosis was significantly different ($p = 0.004$) between CRCs with low- and high-grade inflammation, but there was no difference ($p = 0.06$) in the extent of necrosis in pN0 CRC (Richards et al., 2012).

Peritumoural inflammation was significantly associated with the depth of local invasion as well as the regional lymph node status. Low-grade inflammation was frequently observed in pT4 cases (63.5 %, 95 % CI 56.5–69.9 %) and was significantly less common in pT3 cases (48.2 %, 95 % CI 42.3–54.1 %). An association between high-grade inflammation and T1-2 (opposed to T3-4) has been reported (Richards et al., 2012). Thus, the Klintrup-Mäkinen score has distinct associations with the full scope of pT, from the earliest pT parameters to advanced cases, which dominated in this study. Similarly, only 40.4 % (95 % CI 34.9–46.1 %) of patients with low-grade inflammation had pN0 CRC, in contrast to 59.4 % (95 % CI 53.3–65.2 %) in those presenting with high-grade peritumoural inflammation. These findings are consistent with Richards et al., who also reported a significant difference ($p = 0.0039$) between inflammation grade in pN0 versus pN+ (Richards et al., 2012). Given that pT and pN are the strongest prognostic factors, the significant association between high-intensity inflammation and less extensive cancer spread indirectly confirms the previously described association between high-grade inflammation based on the Klintrup-Mäkinen score and beneficial cancer-specific or overall survival. However, the tendency toward a lower intensity of inflammation in advanced cases also indicates that peritumoural inflammation is not a simple secondary phenomenon related to the extent of tissue damage and / or compromised intestinal motility.

There were significant differences between local structure involvement and the degree of peritumoural inflammation, as low-grade peritumoural inflammation more frequently featured invasion into lymphatic vessels ($p = 0.003$), arteries ($p = 0.012$), and veins ($p = 0.017$), as well as perineural ($p = 0.001$) and intraneural ($p = 0.01$) growth. These results are somewhat different from previous reports. In a study were the authors evaluated the MMR status in primary operable CRC in relation to local and systemic inflammation, there were no significant differences in local structure involvement when there was a high density of intratumoural lymphocytes (Park et al., 2016). However, in a recent study tumour-infiltrating lymphocytes were associated with the absence of venous, lymphatic, and perineural invasion (Jakubowska, Koda, Grudzińska, Lomperta, & Famulski, 2021). The differences in the results indicate complex mechanisms involved in the immune response, and this topic should be evaluated in greater depth.

Graham and Appelman first described the development of lymphoid aggregates along the invasive front of CRC (Graham & Appelman, 1990). Since that initial report, significant progress has been made in pathogenetic understanding and evaluation methods. The considered phenomenon was initially described as CLR. Later, Vayrynen et al. proceeded with a detailed immunohistochemical evaluation of the cellular composition of these lymphoid aggregates (J. P. Vayrynen et al., 2014). As there was no evidence of granulomas, representing the hallmark of Crohn's disease, the researchers suggested the term "colorectal cancer-associated lymphoid reaction" and used computer-assisted quantitative evaluation of this reaction.

In the current study, CLR was found in only 34.9 % of cases and mostly showed a low density of lymphoid follicles. There were no significant differences in CLR density based on tumour spread or local structure involvement, CRC side, grade, or the presence and extent of necrosis.

The association between CLR density and TNM parameters is still controversial. Initially, Graham and Appelman observed that CLR was associated with transmural invasion (in contrast to cancer limited to the intestinal wall), but a lower incidence of metastases in regional lymph nodes (Graham & Appelman, 1990). In addition, there was an association with a right-sided location in the context of transmural growth (Graham & Appelman, 1990), suggesting an impact of MMR-deficient tumours. A more recent study reported an association between higher CLR density and right-sided CRC (Hussain et al., 2016). Using a quantitative approach analogous to the current study, (J. P. Vayrynen et al., 2014) identified a significant association between the CLR count and pT in a retrospective cohort of 418 patients. There were no significant associations with pT and pN. The lack of association with grade and pN is consistent with previous studies (J. P. Vayrynen et al., 2014). This conclusion indirectly indicates that CLR density could be an independent variable to be included in morphological protocols and algorithms of computed-based whole slide analysis.

Interestingly, that presence or absence of CLR (Graham & Appelman, 1990) in the current study did show significant associations with the morphological manifestations of invasive growth. Cancers not surrounded by CLR significantly more frequently displayed lymphatic ($p < 0.001$), perineural ($p < 0.001$), intraneural ($p < 0.001$), and intravenous ($p < 0.001$) invasion. Thus, the prognostically important threshold, identified by Vayrynen et al by ROC analysis of survival, could be more related to the outcome of the disease than to particular morphological profile of the tumour (J. P. Vayrynen et al., 2014). This eventuality again indirectly suggests an independent protective role of immune and inflammatory reactions that is not simply proportional to the tumour burden.

The Immunoscore (Galon et al., 2012; Guo et al., 2020), which is calculated by using the densities of CD3-positive and CD8-positive lymphocytes, allows clinicians to evaluate the status of local immune system in CRC. However, the use of computer-based immunohistochemical assessment has been questioned (Richards et al., 2012; Väyrynen et al., 2013). There is strong trend to determine most of the tumour microenvironment characteristics on routine HE-stained slides (J. H. Park et al., 2015; Richards et al., 2012). Richards et al. reported the possibility to count cells reliably by using HE-stained slides (Richards et al., 2012). They reported reasonable inter-observer variability, reaching 0.92 for lymphocytes, 0.80 for plasmatic cells, and 0.92 for eosinophils. Therefore, in the current study the cellular composition of the inflammatory infiltrate was evaluated by HE staining. In addition to the classically tested relations with pTNM (Väyrynen et al., 2013), the association with the manifestations of invasive growth was evaluated.

The density of eosinophils ($p = 0.008$), neutrophils ($p = 0.01$), and lymphocytes ($p = 0.03$) was significantly associated with cancer invasion into lymphatic vessels. Perineural growth was significantly associated with the same cellular players: lymphocytes ($p = 0.003$), eosinophils ($p = 0.01$), and neutrophils ($p = 0.04$). Thus, the densities of particular inflammatory cells, evaluated in terms of the tumour growth pattern, define a distinct morphological syndrome of lymphatic and perineural invasion. These morphological manifestations of infiltrative growth are remarkable because they are the most sensitive markers of invasion due to the thin tissue layer subjected to damage. Moreover, they are also the most frequent among the morphological signs of cancer invasion, increasing the power of statistical analysis. The current study might have had a greater ability to detect the associations between the density of neutrophils, eosinophils, and lymphocytes and the pattern of tumour invasion because the cohort is characterized by predominance of advanced tumours and hence a high frequency of invasive growth. Although additional research is clearly necessary to confirm the reported findings and to determine the intra- and inter-observer variability, it is suggested that evaluation of the inflammatory infiltrate should be included in the routine diagnostic evaluation of CRC tissues, in order to gain experience and data for further computer-based analysis.

There are reports that a high number of stromal eosinophils in CRC are associated with lower tumour stage and better overall and cancer-specific 5-year survival, reflected by the HRs for death of 0.61 (95 % CI 0.36–1.02, $p = 0.02$) and 0.48 (95 % CI 0.24–0.93, $p = 0.01$), respectively (Prizment et al., 2016). Further, higher density of peritumoural eosinophils was significantly associated with lower pT, pN, and pG; absence of vascular invasion; and longer progression-free and cancer-specific survival (Harbaum et al., 2015).

The previous findings on neutrophils infiltrating CRC are more controversial (Mizuno et al., 2019). High intratumoural neutrophil counts correlated with higher pT, pM, and stage. Rao et al. showed that the presence of a high number of intratumoural neutrophils is an independent factor for poor prognosis of patients with CRC as high intratumoural neutrophil amount positively correlated with pT status, pM status, and clinical stage ($p < 0.05$) (H. Rao et al., 2012). Neutrophil infiltration in cancer cells is an independent predictor of lymph node metastasis (Akishima-Fukasawa et al., 2011). In contrast, a Swedish research team from Umea University found that neutrophil infiltration in the tumour front is a favourable prognostic factor in early CRC (Wikberg et al., 2017). These controversies might be explained by the duality of neutrophils, which comprise a tumour-suppressive N1 subpopulation as well as a tumour-supportive N2 population (Mizuno et al., 2019). The N1 versus N2 phenotype of tumour-infiltrating neutrophils depends on the signals encountered in the cancer microenvironment (Mizuno et al., 2019), which might be dependent on the stage. Recent research showed that high CD66b+ neutrophil count is associated with poor prognosis in CRC, and the number of neutrophils infiltrating CRC tissue is dependent on IL-37 levels (Zhu et al., 2018). Another recent study demonstrated the role of intrastromal neutrophils: Higher counts are associated with lymph node metastasis ($p = 0.04$) and the presence of tumour deposits ($p = 0.04$), but the intratumoural neutrophil count is associated with the number of resected lymph nodes (Jakubowska et al., 2022).

Changes in TAMs have been widely studied in CRC. In a study with 81 CRC cases, the researchers evaluated TAM relationships with EMT markers (E-cadherin and vimentin). TAM surface antigens CD68 and CD163 were mainly expressed at the tumour invasive front and stroma, with no to weak expression in the tumour nest. Near the tumour invasive front, high CD163 expression was associated with low E-cadherin and high vimentin expression, indicating EMT process. The authors performed univariate and multivariate analyses and showed that CD163 expression at the invasive front was an independent prognostic factor associated with poor relapse free survival (RFS) (HR 2.414, 95 % CI 1.016–4.523, $p = 0.045$) and overall survival (HR 3.234, 95 % CI 1.176–8.889, $p = 0.023$) (Wei et al., 2019). Macrophages are able to produce a wide spectrum of biologically active substances. *In vivo* and *in vitro* experiments show that TAMs secrete higher levels of osteopontin when cultivated together with CRC cells. By binding to its receptor (CD44), osteopontin activates c-jun-NH(2)-kinase signalling and promotes the clonogenicity of CRC cells (Rao et al., 2013).

CD8+ T cells have been known to have an effect on CRC patient survival for decades (Naito et al., 1998), and their presence within different malignancies has been associated with a better prognosis (Lalos et al., 2021; Piersma et al., 2007). T cells play one of main roles in

a tumour immunity, and enhancement of T cell cytotoxic activity is crucial for the development of cancer immunotherapy (Iwahori, 2020). In CRC, a low CD8+ T cell density has been associated with infiltrative tumour border, which is one of the indicators for poor prognosis in CRC (Lalos et al., 2021). The Immunoscore is considered a strong prognostic marker especially in advanced CRC (Kwak et al., 2016; Lea et al., 2021). However, recent research has indicated that a combined model measuring the density of CD3+ and CD8+ lymphocytes shows similar results to a research model with only the CD8+ T cell count determined in tissue microarray (TMA) cores (Glaire et al., 2019). Thus, in the current study only the number of CD8+ T cells was counted.

There were significant differences between the peritumoural CD8+ T cell count and pT ($p = 0.03$) and pN ($p = 0.05$). CRC with a higher pT in the peritumoural tissue had a lower CD8+ T cell count compared with CRC with low pT: 121 (95 % CI 95.7–146.9) in pT4 and 198.5 (95 % CI 82.6–314.4) in pT1. In relation to pN, there was a significant difference in the peritumoural CD8+ T cell count ($p = 0.05$). Interestingly, the peritumoural CD8+ T cell count showed significant differences in terms of perineural ($p = 0.02$) and intraneural ($p = 0.02$) invasion. Lea et al. reported very similar results: Perineural invasion was associated with a low Immunoscore ($p = 0.008$), but lymphatic invasion had no significant association with its changes ($p = 0.10$) (Lea et al., 2021). These results could indicate the potential role of CD8+ T cells in tumour metastasis potential and local structure involvement, as well as local tumour growth. In contrast, another study indicated that a low Immunoscore characterizes tumour potential to invade lymphatic and blood vessels (Ko & Pyo, 2019).

The current CRC treatment involves mostly surgery and chemotherapy and / or radiation therapy (Shinagawa et al., 2017). However, as in other malignancies the role of immunotherapy has expanded and has produced good results (Bayraktar et al., 2019; Lugowska et al., 2018). In CRC, immunotherapy targeted to programmed death-ligand 1/2 (PD-L1/2) has been that topic of conversation (Yaghoubi, Soltani, Ghazvini, Hassanian, & Hashemy, 2019). For now, immunotherapy of CRC is developing in several directions, including checkpoint blockade, adoptive cell transfer, and vaccination. Thus, anti-PD1-L1 treatment has been approved by the U.S. Food and Drug Administration for CRC with MSI. As most of the metastatic CRC cases are MSS and the possibilities of immunotherapy are still limited in such tumours, researchers have advised intensifying research of the complex tumour microenvironment in association with the microsatellite status (Kather, Halama, & Jaeger, 2018). A recent study published by researchers from France suggests that medication combination of atezolizumab and tiragolumab could restore T cell function in microsatellite-stable CRC (Thibaudin et al., 2022).

In CRC, a high CD8+ T cell count has been associated with high PD-L1 expression on tumour and immune cells; however, no association with the cell count and PD-L1 expression regards better survival has been found (Ko & Pyo, 2019). Moreover, activation of the PD-1 pathway has been associated with T cell exhaustion, as reviewed by Yaghoubi et al. (Yaghoubi et al., 2019), leading researchers to think about more molecular mechanisms involved in immune response in cancer. One of these possible mechanisms that affects T cell density within CRC could be stromal cell derived factor 1 (SDF-1), a chemokine that affects leukocyte migration (D'Apuzzo et al., 1997). High SDF-1 expression has been related to a high CD8+ T cell density in CRC and has been associated with independent favourable prognosis in univariate and multivariate Cox proportional hazards regression survival analysis (HR 0.34, 95 % CI 0.17–0.66, $p = 0.002$ and HR 0.45, 95 % CI 0.23–0.89, $p = 0.021$, respectively) (Lalos et al., 2021).

4.3 Epithelial mesenchymal transition

EMT is one of the key events in CRC pathogenesis that leads to tumour invasion and metastatic spread (Grigore et al., 2016). In CRC, EMT properties such as cytoskeletal deformability and motility and co-expression of EMT markers are evident in small cell clusters known as tumour buds (Grigore et al., 2016). Recently, Sato et al. analysed 32 CRC cases displaying tumour budding. They showed that cancer buds expressed various amounts of LGR5 and PD-L1 and suggested that patients with PD-L1-negative tumour buds should receive a different treatment that targets the CSC marker LGR5 (Sato et al., 2021). Thus, tumour budding could be closely associated with stemness. This linkage is not limited to CRC but is likely to reflect a general feature of carcinogenesis. One of the CRC stem cell property markers is CD44, and its upregulation within CRC tissues is associated with aggressive tumour behaviour (Mohamed et al., 2019) as well as resistance to chemotherapy (Pothuraju et al., 2020). Within breast cancer, CD44 expression changes have been associated with p53 mutation; however, within CRC this phenomenon has not been observed (Zeilstra et al., 2013).

In the current study, there were **CD44** expression changes in relation to CRC morphology, as its overexpression was seen in mucinous CRC (2.00, 95 % CI 1.61–2.38) compared with adenocarcinomas (1.28, 95 % CI 1.15–1.41). On the contrary, studies have shown loss of CD44 expression in mucinous carcinomas (Ismaiel et al., 2016). In the current study, CD44 overexpression was associated with right-sided tumours ($p = 0.002$), but there were no significant differences in CD44 expression in terms of pT, pG, manifestations of invasive growth, and inflammation. Interestingly, there was significantly higher CD44 expression in pN0 tumours. Such a phenomenon has also been described in mucinous ovarian

carcinomas, as borderline tumours have been found to express higher levels of CD44 than invasive carcinomas (Matuura et al., 2018). In CRC, this phenomenon could possibly be explained by CD44 isoform switch, as described previously (Mashita et al., 2014; Z. Wang et al., 2019).

In other studies, the authors have used different methods to describe the presence of CD44 in tissue. (Mohamed et al., 2019) found that CD44 expression correlates with a lower tumour grade ($p = 0.006$), as well as lymphovascular invasion (85.7 % of patients) and perineural invasion (91.7 % of patients). When there was no CD44 expression, lymphovascular and perineural invasion were much lower (14.3 % and 8.3 %, respectively). The authors also showed mild and moderate lymphocyte infiltration in the presence of CD44 expression, while no CD44 expression was mostly associated with marked lymphocytic infiltrate (Mohamed et al., 2019). Nevertheless, in their meta-analysis Wang et al. found significant associations with increased CD44 expression and pN+ (OR [CD44] 1.56, 95 % CI 1.01–2.41, $p = 0.044$; OR [CD44v6] 1.97, 95 % CI 1.19–3.26, $p = 0.008$; OR [total CD44 isoforms] = 1.57, 95 % CI 1.15–2.14, $p = 0.004$), and a trend for an association between a high level of total CD44 isoform expression and poor differentiation (OR 1.44, 95 % CI 1.00–2.08, $P = 0.051$) (Z. Wang et al., 2019).

The use of semi-quantitative methods – by evaluating the percentage of positive cells (H. Kim et al., 2019; Zlobec et al., 2009) – could be more informative, because CRC has great heterogeneity in terms of genomic instability and environmental factor effects (Molinari et al., 2018). The current study included mostly semi-quantitative methods for evaluation of included IHC marker expression.

E-cadherin expression also showed significant differences according to tumour morphology ($p = 0.001$), with a higher expression score in colorectal adenocarcinoma (1.91, 95 % CI 1.83–1.98) than in mucinous carcinoma (1.48, 95 % CI 1.23–1.73). Nevertheless, downregulation of E-cadherin was associated with lower differentiation ($p = 0.001$), as has also been described in other studies (Mogoantă et al., 2014; Sayar et al., 2015). There was lower E-cadherin expression in right-sided compared with left-sided tumours ($p = 0.02$). However, in a previous study E-cadherin expression did not differ according to tumour localization (Elzagheid et al., 2012). In the current study, E-cadherin expression did not differ according to pT ($p = 0.07$), pN ($p = 0.16$), and invasive growth ($p > 0.05$). These results are very similar to study done by Seo et al., who included 174 patients with CRC (Seo, Kim, & Kim, 2015). They found E-cadherin expression was significantly more frequent ($p = 0.007$) in G1-G2 carcinomas (78.2 %) than in G3 carcinomas (33.3 %). There was no association with pT ($p = 0.697$), pN ($p = 0.456$), and lymphatic invasion ($p = 0.710$) (Seo et al., 2015). Other studies have indicated

a role for E-cadherin expression loss in tumour progression, as it is considered to be marker for extranodal tumour extension, that is highly associated with vascular invasion and high pN stage in pT3 CRC (H. Kim et al., 2019).

β-catenin expression was not significantly different in relation to CRC morphology ($p = 0.41$), differentiation ($p = 0.30$), CRC side ($p = 0.11$), pN ($p = 0.42$,) and local structure invasions. However, there was a significant association between higher pT and lower β-catenin expression ($p = 0.007$).

Although most research has shown an unclear relevance of β-catenin accumulation to worse prognosis in CRC, some studies have described β-catenin expression differences within the tumour centre and invasive margin, pointing to the heterogeneity of CRC (Gao, Lu, Wang, Han, & Guo, 2014). There have been variable results regarding associations of β-catenin expression and pT and pG. Lee et al. showed that β-catenin expression was not different according to pT, as more advanced tumours (pT3 and pT4) showed similar β-catenin expression as pT1 and pT2 tumours ($p = 0.07$) (K. S. Lee et al., 2016). However, low-grade CRC was more likely to show positive β-catenin expression (62.5 % of cases) than high-grade CRC (38.9 % of cases) (K. S. Lee et al., 2016). Gao et al. found reduced membranous β-catenin expression levels at the tumour centre to be significantly associated with the occurrence of lymph node metastasis ($p = 0.002$) and the TNM stage ($p = 0.002$) (Gao et al., 2014). Similarly to the current study, they found no significant differences in β-catenin expression relative to local structure involvement, histology, and tumour location (Gao et al., 2014). Nevertheless, these results could indicate a future research direction, as β-catenin expression level differences in different CRC areas have shown controversial results regarding prognosis of patients with CRC (Gao et al., 2014; K. S. Lee et al., 2016; Wangefjord et al., 2013)

N-cadherin is one of the components of the EMT, and its overexpression has been associated with a worse prognosis in a various tumours (Noh et al., 2017). N-cadherin expression differed significantly with regard to pT ($p=0.03$): Higher N-cadherin expression was associated with pT3 tumours (1.97, 95 % CI 1.71–2.23), although slightly lower N-cadherin expression was observed for pT4 tumours (1.45, 95 % CI 1.12–1.78). In previous studies that have also used semi-quantitative evaluation of N-cadherin, researchers have noticed several significant associations in N-cadherin expression in terms of tumour differentiation, tumour size, invasion, and the presence of metastasis (Xuebing Yan et al., 2015). Interestingly, in the current study N-cadherin expression was significantly different in tumours with sCRC, showing lower expression in these tumours. N-cadherin downregulation is mostly associated with reduced migration and invasion in a various malignancies (K. Li, He, Lin, Wang, & Fan,

2010; X. F. Zhang, Zhang, Chang, Wu, & Guo, 2018). Future research could evaluate N-cadherin expression in sCRC.

Although **vimentin** is one of the most widely used markers to detect tissue of mesenchymal origin, its use in CRC is debatable. Nevertheless, it has been suggested that its expression in CRC could correlate with the stage of neoplastic progression (Lazarova & Bordonaro, 2016).

Vimentin expression was detected in only 4.0 % (95 % CI 1.7%9.1 %) of cases. Higher tumour grade as well as non-adenocarcinoma morphology, specifically signet ring cell carcinoma, were significantly associated with vimentin expression. However, there was no difference in vimentin expression in terms of pT, pN, tumour localization, local structure involvement, or inflammatory reaction. Other researchers have reported similar results. In a study comprising 202 CRC cases, vimentin expression was detected in 17.3 % of cases and was associated only with a higher tumour grade and the presence of distant metastasis, as well as short overall survival (log-rank 5.112, p = 0.024) and disease-free survival (log-rank 6.173, p = 0.013) (Al-Maghrabi, 2020). In a recent study including 142 CRC, aberrant vimentin expression was detected only in one CRC case with signet ring cell morphology and nodal as well distant metastasis (Dai et al., 2021). These results indicate that CRC can acquire mesenchymal properties, a phenomenon that leads to a poor prognosis.

4.4 Interactions between epithelial-mesenchymal transition and inflammation

Researchers have described relationships between EMT markers. There is extensive evidence on how downregulation of E-cadherin leads to upregulation of N-cadherin (Cao et al., 2019; N. R. Jang et al., 2021), and how levels of CD44 and β-catenin differ at various stages of CRC (Iseki et al., 2017; Masaki et al., 2001; Qu et al., 2017). However, the relationships between EMT markers and different degrees of inflammation has not been widely studied.

Iseki et al. showed that combined low expression of CD44 and E-cadherin within CRC is associated with poor survival (HR 15.118, 95 % CI 2.645–77.490, p = 0.0039) and is an independent risk factor for progression-free survival (HR 8.276, 95 % CI 1.383–43.311, p = 0.0227) (Iseki et al., 2017). In oral squamous cancer, reduced E-cadherin and β-catenin expression has been found in association with higher N-cadherin expression (Angadi et al., 2016), indicating the role of EMT in other carcinomas. In a recent CRC cell study, the authors found a role for miRNA in EMT. Specifically, miR-425-5p overexpression in CRC was

significantly associated with upregulation of fibronectin, N-cadherin, and vimentin expression, as well as downregulation of E-cadherin expression (D. Liu, Zhang, Cui, Chen, & Feng, 2020).

In the current study, there were sig nificant correlations between β-catenin and E-cadherin expression ($p = 0.018$) and between β-catenin and N-cadherin expression ($p < 0.001$), indicating cadherin and β-catenin interactions within CRC. There were no significant correlations between these markers and CD44 expression, indicating a more complex mechanism in EMT development.

Various cell studies have demonstrated the role of local inflammation in potential tumour spread. Indeed, cytokines such as IL-1β, IL-6, and TNFα stimulate CRC cell adhesion to the mesothelium (van Grevenstein et al., 2007), indicating their potential role in CRC spread. This theory was overlooked by scientists from Poland, who in *in vitro* and *in vivo* mouse studies showed that colorectal SW480 cell proliferation and invasion was stimulated by IL-6 and development of EMT by TGF-β1 released from mesothelial cell lines (Mikuła-Pietrasik J. et al., 2015). They also studied the effects of the EMT-driving TFs SMAD2/3 and Snail1, as inhibition of these TFs significantly decreased transformation of CRC cells into mesenchymal phenotype with decreased expression of E-cadherin and increased vimentin expression (Mikuła-Pietrasik J. et al., 2015). Nevertheless, IL-6 is also released from other cells of mesenchymal origin, including normal colon stromal cells, as well as CAFs (Nagasaki et al., 2014). In CRC, CAFs produce IL-6, which in turn upregulates the CSC markers ALDH1 and LGR5 via an IL-6-dependent mechanism. In addition, stromal cells, via IL-6, divert the inflammatory response toward a Th17-driven process favouring tumour growth (Huynh et al., 2016). However, CAFs possess both tumour-promoting and tumour-restraining functions. Selective depletion of α-smooth muscle-actin-positive CAFs increases tumour invasiveness and lymph node metastasis and reduces overall survival. The underlying mechanism includes lower production of bone morphogenetic protein 4 (BMP4) and increased TGF-β1 secretion from stromal cells, which in turn leads to upregulation of LGR5 CSCs and the development of an immunosuppressive microenvironment with increased frequency of Foxp3+ regulatory T cells and suppression of CD8+ T cells. Thus, inflammation and stemness are regulated via an intricate, balanced biological network of cells and mediators (McAndrews et al., 2021).

The role of inflammation has also been evaluated from the therapy point of view. NSAIDs suppress CD44-expressing stem cells of CRC via COX-2 inhibition. This effect was evident in cell culture as well as *in vivo* mouse xenograft model (Moon et al., 2014). In breast cancer cell cultures, combined doxorubicin and aspirin treatment significantly reduced the proportion of CSCs and the colony-forming ability. This treatment delayed the inhibition of

IL-6 secretion, which is mediated by both COX-dependent and COX-independent pathways (Khoo et al., 2019).

In the current study, there was a significant albeit weak correlation between low-grade inflammation and β-catenin and E-cadherin expression (r/rs = 0.290, p = 0.03). There was also a sig nificant, moderate correlation between β-catenin and N-cadherin expression (r/rs = 0.491, p = 0.00), but there were no other significant correlations between marker expression and degree of inflammation. These results could indicate the potential role of inflammation, but local inflammation within CRC might not be the only thing driving CRC development, as systemic inflammation has tumour-driving potential in several cancers, including CRC (J.-H. Chen et al., 2017).

Cell subpopulation analysis in the current study revealed a significant difference in β-catenin expression in terms of the neutrophil count: A high neutrophil count was associated with a hig her β-catenin expression (p = 0.001). Previous research has shown that one of the cytokines responsible for neutrophil recruitment (IL-37) is responsible for suppressing β-catenin expression within CRC (Xiaofei Yan, Zhao, & Zhang, 2017), showing the possible anti-tumoural activity of neutrophils. However, the results are not consistency. In a recent study, there were significantly higher IL-37 levels even with a low neutrophil count, and a significantly higher CD66b+ neutrophil count was associated with diminished overall survival (Zhu et al., 2018). The role of inflammation and β-catenin expression has been demonstrated in mouse models, but in association with T cell counts, as upregulation of β-catenin was noted in the CRC and ulcerative colitis (Keerthivasan et al., 2014). Also in this mouse study, β-catenin activation in T cells resulted in progressive leukocyte infiltration in colonic mucosa leading to ulcer, prolonged crypt, as well as crypt abscess formation in 8–10 weeks old mice, which was followed by development of polyps in more than a half of the mice when they were 4–8 months old (Keerthivasan et al., 2014). Although the current study did not show differences in β-catenin expression and the lymphocyte count (p = 0.68), the presence of neutrophils could be explained by a lymphocyte-related reaction as they promote neutrophil activation in a various directions (Oberg, Wesch, Kalyan, & Kabelitz, 2019). Upreg ulation of β-catenin in the presence of neutrophils supports previous research, where high a intratumoural neutrophil count in CRC was associated with poor prognosis (H. Rao et al., 2012).

Interestingly, analysis of inflammatory cell subpopulations showed significant results in terms of upregulation of E-cadherin and a high eosinophil density (p = 0.007). A protective association (p = 0.003) has been reported between increased blood count of eosinophils and decreased risk of CRC, with HRs of 1.0, 0.70 (95 % CI 0.50–0.98), and 0.58 (95 %

CI 0.40–0.83) across the tertiles of the absolute eosinophil count (Prizment, Anderson, Visvanathan, & Folsom, 2011). A protective role has also been ascribed to stromal eosinophils in CRC (Prizment et al., 2016). Thus, E-cadherin upregulation is associated with beneficial tumour features, possibly including recruitment of eosinophils as one of the mechanisms.

The inflammatory reaction did not have a significant effect on N-cadherin expression; however, there was lower N-cadherin expression when there was high eosinophil infiltration ($p = 0.02$). This result could support the idea of the protective role of eosinophils in CRC (Prizment et al., 2016).

A few studies have evaluated inflammatory cell subpopulation connections and density to EMT in pathologies other than cancer. Patients with long-term bronchial asthma develop sub-epithelial fibrosis and myoepithelial hyperplasia. Researchers used a mouse model of this condition and showed that the process is closely associated with increased eosinophil infiltration, as after instillation of eosinophils in bronchial mucosa the mice developed significant fibrosis with type I collagen deposition (Yasukawa et al., 2013). Mice that had received eosinophil instillation had significantly lower E-cadherin expression than control mice that had received saline injections (Yasukawa et al., 2013). The authors also showed that EMT in the bronchus was associated with increased TGF-β1 expression and Smad3 phosphorylation in bronchial epithelial cells (Yasukawa et al., 2013). IL-13 is produced by several immune cell types, including eosinophils, and its overproduction is found in variety of inflammatory conditions, including bronchial asthma (Rael & Lockey, 2011). Patients with long-term eosinophilic esophagitis also develop significant sub-epithelial fibrosis. Medication that affects IL-13 production significantly reduces the vimentin-positive cell count (by 0.94 % in the placebo group to 4.92 % in the high-dose group, $p = 0.032$). Moreover, IL-13 levels significantly correlated with increased E-cadherin expression within tissue (Gann et al., 2020). Hence, there could be role for immunotherapy in CRC, especially with distinct signs of EMT.

Inflammation drives EMT via a complex mechanism, as E-cadherin overexpression in macrophages could indicate potential immunosuppression of some subpopulation of macrophages. Researchers showed that alternatively activated macrophages (AAMs) expressing E-cadherin on their surface have an immune-suppressing role, as in AAMs lower production of inflammatory cytokines was observed (Van den Bossche et al., 2015). However, another mouse study showed that E-cadherin levels could be a potential indicator for inflammation severity, as mice with acute pancreatitis had elevated E-cadherin expression and statistical analysis showed a significant association with severe acute pancreatitis (Yuan et al., 2015). Nevertheless, some researchers have reported different associations between E-cadherin expression and inflammation. Canine kidney cells, presented reduced E-cadherin expression after stimulating them with TGF-α, TGF-β, and IL-6. Besides, 6 days after stimulation with inflammatory cytokines, characteristic EMT cell changes, including spindling and weakened cell-cell adhesions was found (Saito et al., 2014)..

These results indicate complex mechanisms involved in EMT: The network leading to EMT could be more complicated, and inflammation could play a significant role in this process as well.

4.5 Mismatch repair proteins

Detection of MMR gene aberrations (*MSH2*, *MSH6*, *PMS2*, and *MLH1*) is crucial in CRC, as loss of MMR protein production is an indicator for MSI in carcinomas. In the era of personalized medicine, precise diagnosis is crucial, as MSI tumours have shown resistance to chemotherapy (Aggarwal, Quaglia, McPhail, & Monahan, 2022; Jo & Carethers, 2006). MLH1 and MSH2 IHC has acceptable sensitivity and specificity for high-MSI status: 92.3 % and 100 %, respectively (Lindor et al., 2002). Therefore, this approach has been applied in other studies (Amira et al., 2014; Lanza et al., 2002), and IHC detection of MMR proteins is considered to be an appropriate alternative to molecular testing (Raffone et al., 2020). Moreover, IHC-based detection of MMR proteins is more cost-effective and not as time-consuming as other methods (Bai et al., 2019; Pérez-Carbonell et al., 2012).

Nevertheless, there have been heterogenous findings regarding immunohistochemical expression of MMR proteins (McCarthy et al., 2019). In CRC, heterogeneity is observed more frequently in tumours exhibiting MSI than in MSS tumours (De Smedt et al., 2015). The treatment response can depend on the degree of tumour heterogeneity. It has been described in CRC (Zlatian et al., 2015) and in other carcinomas (Prince et al., 2007) in relation to cancer stemness (Papi & Orlandi, 2016). Considering that MMR status has been detected by IHC and has been proven to verify the MMR status (S.-M. Wang et al., 2019), IHC was employed in the

current study. Besides MMR status detection, the heterogeneity of MMR protein expression was evaluated in relation to the clinicopathological aspects of CRC.

There were significant differences in MSH6 ($p = 0.03$) and PMS2 ($p = 0.002$) expression in relation to CRC histogenesis, as signet ring cell carcinoma showed markedly lower expression of MSH6 (0.07, 95 % CI 0.0–0.39) and PMS2 (0.02, 95 % CI 0.0–0.21) compared with adenocarcinoma (MSH6: 1.84, 95 % CI 1.69–1.98; PMS2: 1.54, 95 % CI 1.39–1.70). Previous studies have also reported loss of MMR protein expression via IHC in poorly differentiated CRC (S.-M. Wang et al., 2019). Researchers have observed that MSI mucinous carcinomas tend to be located in the proximal colon and tend to have distinct intratumoural and peritumoural inflammation when compared with MSS mucinous carcinomas (Arai et al., 2007). In a study that included 2025 patients, 202 (10 %) cases had MSI, and patients with mucinous differentiated tumours has a higher frequency of MSI compared with those with non-mucinous CRC ($p < 0.001$) (Y. S. Yoon et al., 2015). The authors also found that mucinous CRC with MSI showed a trend toward right colon predilection and infrequent lymph node metastasis compared with MSS CRC ($p = 0.005$ vs. 0.03) (Y. S. Yoon et al., 2015).

Although in the current research there were no significant differences in MMR protein expression in relation to the degree of inflammation, MSH2, MSH6, PMS2, and MLH1 expression was different according to tumour location, with significantly higher expression in left-sided tumours. MSH2 and PMS2 expression was different depending on pG, showing significantly lower expression within high- and low-grade tumours, while in the case of moderate differentiation the levels of both MMR proteins were higher. There were no differences in MMR protein expression based on local structure involvement except in case of intraneural invasion and MSH6 expression, as absence of invasion was characterized by higher MSH6 expression.

Researchers have also shown differences in MMR protein expression according to tumour location and pG. Wang et al. found that MLH1/MSH2-negative CRC occurred more frequently in the right than in the left colon (27.88 % vs. 17.86 %, $p = 0.029$), and MMR negativity was associated with poor differentiation and mucin production (S.-M. Wang et al., 2019). However, changes in MLH1/MSH2 expression were not associated with age, gender, tumour stage, tumour size, lymphocytic infiltration, or circumscribed margin ($P > 0.05$) (S.-M. Wang et al., 2019). Recently, researchers noted that about 25 % of patients with sCRC have intratumoural changes in MMR protein expression, suggesting that molecular mechanisms affecting development of sCRC varies and detection of changes in MMR expression is necessary in treatment decision (Vyas et al., 2021). In the current study, differences in MMR protein expression did not differ in sCRC cases compared with cases without sCRC ($p > 0.05$).

However, further investigation is needed given the small number of sCRC cases in the current study.

Certain MMR proteins showed associations with the molecular characteristics of CRC. A higher mean E-cadherin score was significantly associated with the presence of MSH2 ($p = 0.008$) and PMS2 ($p = 0.014$), while lower CD44 expression was associated with PMS2 ($p = 0.05$). There were no differences with regard to β-catenin and N-cadherin expression and MMR proteins. Only a few studies have analysed MMR status and molecular characteristics in CRC. A recent study demonstrated that only MMR-proficient CRC that lacked evidence of differentiation totally lost E-cadherin expression (Perna et al., 2021). Such a phenomenon has also been demonstrated in gastric carcinomas with MLH1 hyper-methylation (Moghbeli et al., 2014). In another study, MMR-proficient CRC had elevated nuclear β-catenin expression and loss of membranous E-cadherin, and these changes were independently associated with higher N stage ($p = 0.03$ and $p < 0.0001$), vascular invasion ($p < 0.01$ and $p < 0.001$), and worse survival ($p < 0.01$ and $p < 0.001$) (Lugli et al., 2007).

In the current study, the expression of MMR proteins did not differ depending on the intensity of inflammation (low and high, based on the Klintrup-Mäkinen score). There were no significant differences in MMR protein expression among the inflammatory cell subpopulation, except higher MLH1 expression in cases with a higher neutrophil density ($p = 0.02$). This is different from a recent study in which patients with neutrophil-rich CRC show higher rates of MMR-deficient CRC ($p < 0.01$). Nevertheless, those authors showed that the neutrophil count does not affect 5-year survival rates, as MMR-proficient and MMR-deficient CRC showed similar survival rates in neutrophil rich carcinomas (Rottmann et al., 2021).

In the current study, MSH6 levels showed a significant correlation with the peritumoural CD8+ T cell count ($r/rs = 0.203$, $p = 0.031$), indicating its possible role toward immune mechanism activation. However, there were no significant differences when analyzing low MMR versus high MMR protein expression, as well as presence / absence of MMR proteins and the peritumoural CD8+ T cell count. In a previous study, researchers described a correlation between CD8+ T cells and MSH2 expression in lung adenocarcinoma: High MSH2 expression correlated strongly with increased PD-L1 expression and CD8+ T cell infiltration. This finding led the authors to think about the potential for immunotherapy as detection of MMR proteins via ICH is relatively easy and fast (Jia, Yao, Yang, & Chi, 2020). Nevertheless, there are other factors that could affect the CD8+ T cell count, as in CRC immature desmoplastic stromal reaction have been associated with lower CD8+ T cell and macrophage counts, as well as normal MMR protein expression (Ueno et al., 2014).
It is possible that changes in MMR protein expression could first be detected with IHC. The

results could then be used to guide further DNA sequencing and to develop personalized treatment.

Conclusions

1. This study overall shows high distribution of locally advanced CRC in Latvia, presenting as pT4 and pN+.
2. Although the overall incidence of synchronous adenomas corresponds to other studies, villous adenomas were more frequent. The distribution of sCRC was higher in younger patients, indicating a possible role of inherited mutations within its development.
3. High-grade inflammation significantly less frequently features specific manifestations of the higher invasive capacity of the tumour, including perineural and intraneural growth and invasion into lymphatic vessels, veins, and arteries. Thus, high-grade peritumoural inflammation is associated with beneficial morphologic features of CRC and is not secondary to tissue damage and necrosis.
4. Low peritumourous and intratumourous lymphocytic, neutrophil and eosinophil density is associated with local structure involvement, and absence of CLR is associated with more advanced tumours in their local spread.
5. In CRC EMT is connected to histogenesis and differentiation of tumour: it is more common in low differentiated adenocarcinomas and tumours with non-adenocarcinoma morphology, as E-cadherin expression significantly decreases. EMT has a role in a tumour progression and it could partially be affected by inflammation.
6. CD44 expression depends on histogenesis of tumour; however, it does not depend on differentiation of tumour. Lower expression of CD44 within CRC could be a marker for lymphogenous metastasis.
7. β-catenin could be used as an indicator for advanced CRC, as its levels significantly increases in tumours with higher pT. Levels of β-catenin are associated with immune response.
8. N-cadherin levels significantly decreases and E-cadherin expression significantly increases in tumours with high amounts of eosinophils, indicating protective role of these immune cells. However, lower N-cadherin expression were seen in CRC cases with sCRC.
9. MMR protein expression significantly decreases in non-adenocarcinomas and right-side tumours; however, their expression is not significantly different in tumours with and without local structure involvement as well as in tumours with high- and low-grade

inflammation. MLH1 protein expression significantly differs in tumours according to neutrophilic leucocyte infiltration, potentially indicating molecular mechanism role in anti-tumour immunity.

10. MMR status potentially affects EMT, as in tumours with low MMR protein expression significantly decreases amounts of E-cadherin and increases CD44 expression.

Practical recommendations

1. The histopathology report for each CRC case should include evaluation of inflammation based on the Klintrup-Mäkinen score.
2. Immunohistochemical investigation should be performed in all CRC cases to evaluate stemness (CD44) and MMR protein (MSH2, MSH6, PMS2, MLH1) expression.

Publications and reports on topics of the Doctoral Thesis

Publications

1. **Briede, I.**, Strumfa, I., Vanags, A., Gardovskis, J. 2020. The Association Between Inflammation, Epithelial Mesenchymal Transition and Stemness in Colorectal Carcinoma. J Inflamm Res. 08.01.2020. 13:15–34. doi: 10.2147/JIR.S224441.
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Supplements

Veidlapa Nr. E-9 (2)

RSU ĒTIKAS KOMITEJAS LĒMUMS

Rīga, Dzirciema iela 16, LV-1007
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	Komitejas sastāvs	Kvalifikācija	Nodarbošanās
1.	Profesors Olafs Brūvers	Dr.theo.	teologs
2.	Profesore Vija Sīle	Dr.phil.	filozofs
3.	Asoc.prof. Santa Purviņa	Dr.med.	farmakologs
4.	Asoc.prof. Voldemārs Arnis	Dr.biol.	rehabilitologs
5.	Profesore Regīna Kleina	Dr.med.	patalogs
6.	Profesors Guntars Pupelis	Dr.med.	ķirurgs
7.	Asoc.prof. Viesturs Liguts	Dr.med.	toksikologs

Pieteikuma iesniedzējs: **Inese Driķe, 1. studiju gada rezidente**
Tālākizglītības fakultāte

Pētījuma nosaukums: „Kolorektāla vēža inovatīvu morfoloģisko un molekulāro parametru biežums un saistība ar audzēja progresiju".

Iesniegšanas datums: 28.07. 2014.

Pētījuma protokols: Izskatot augstāk minētā pētījuma pieteikuma materiālus (protokolu) ir redzams, ka pētījuma mērķis tiek sasniegts veicot pacientu audu materiāla un medicīniskās dokumentācijas datu izpēti, iegūto datu apstrādi un analīzi, kā arī izsakot priekšlikumus. Personu (pacientu, dalībnieku) datu aizsardzība un konfidencialitāte tiek nodrošināta. Līdz ar to pieteikums atbilst biomedicīniskā pētījuma ētikas prasībām.

Izskaidrošanas formulārs: nav nepieciešams

Piekrišana piedalīties pētījumā: nav nepieciešama

Komitejas lēmums: **piekrist** pētījumam

Komitejas priekšsēdētājs Olafs Brūvers Tituls: Dr. miss., prof.

Paraksts

ĒTIKAS KOMITEJA

Ētikas komitejas sēdes datums: 04.09.2014.